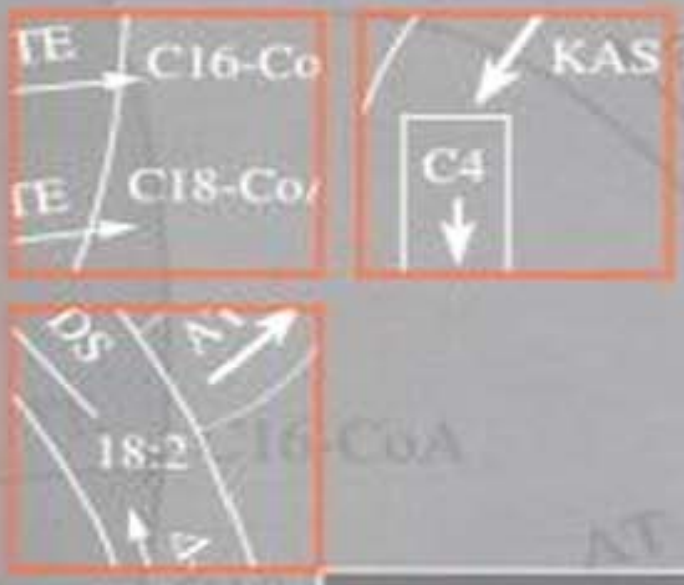
Lipid Biotechnology



edited by
Tsung Min Kuo
Harold W. Gardner

Lipid Biotechnology

edited by
Tsung Min Kuo
Harold W. Gardner
National Center for Agricultural Utilization Research
Agricultural Research Service
U.S. Department of Agriculture
Peoria, Illinois

Marcel Dekker, Inc. New York · Basel

ISBN: 0-8247-0619-6

This book is printed on acid-free paper.

Headquarters
Marcel Dekker, Inc.
270 Madison Avenue, New York, NY 10016
tel: 212-696-9000; fax: 212-685-4540

Eastern Hemisphere Distribution
Marcel Dekker AG
Hutgasse 4, Postfach 812, CH-4001 Basel, Switzerland
tel: 41-61-261-8482; fax: 41-61-261-8896

World Wide Web
http://www.dekker.com

The publisher offers discounts on this book when ordered in bulk quantities. For more information, write to Special Sales/Professional Marketing at the headquarters address above.

Current printing (last digit):
10 9 8 7 6 5 4 3 2 1

PRINTED IN THE UNITED STATES OF AMERICA

Preface

Lipid Biotechnology outlines current advances and provides an in-depth review of biotechnological principles and approaches employed in the production and use of lipids. In this book, ''lipids'' are broadly defined as any natural substance soluble in organic solvents and include fats, oils, phospholipids, glycolipids, terpenoids, steroids, and oxygenated fatty acids. Accordingly, the volume covers a wide spectrum of scientific disciplines that develop and use such biological systems as enzymes, microbes, and plant cells, as well as marine organisms, for improving the yield, quality, and usefulness of lipids and lipid-related materials. These disciplines include traditional fermentation, biocatalysis, enzymology, molecular biology, bioprocess engineering, and conventional crop-breeding techniques. Many lipid-active enzymes, such as lipase, phospholipase, lipoxygenase, desaturase, and a family of cytochrome P450s, are described in detail.

In recent years, biotechnological research on lipids has become dynamic and progressive; perhaps inevitably it has also become the responsibility of industry, academia, and government to deal with such important issues as conservation of natural resources, protection of a clean environment, quality of food products, and utilization of renewable feedstocks. Consequently, there has been a tremendous increase in the understanding of lipid metabolism and regulation, the advancement of gene-transfer techniques, and the development of effective bioprocesses. This has resulted in a large volume of literature concerning various biotechnological applications of lipid research, such as increasing biomass, improving traits, making cheaper industrial chemicals, and producing valuable pharmaceutical compounds and specialty foods.

The text is divided into four parts: Oilseed Quality Improvement; Oxylipins: Formation, Potential Uses, and Their Role in Defense; Lipases and Food Nutrition; and Biocatalysts in Nonfood Applications.

Part I deals with the current understanding of regulation of fatty acid metabolism and acylglycerol biosynthesis, key metabolic controls, and enzymes involved in both genetic engineering and conventional crop breeding. Specific goals are the improvement of yield and/or the quality of oil and lipid composition, enhancement of industrial development of oilseed crops to commercial production through traditional and modern biotechnology, and the use of tissue culture techniques in research. Formation of unusual fatty acids, such as short chain, epoxides, hydroxides, and those with unusual positioning of unsaturation, is discussed.

Part II details the formation and mechanism of action of naturally produced or pathogen-elicited oxygenated compounds (oxylipins) which are formed from fatty acids by reactions involving mono-oxygenase- or dioxygenase-catalyzed oxygenation by plants, fungi, and marine organisms. Oxylipins possess diverse biological activity and often are utilized by the organism as effective defense substances against pathogens. Biosynthetic pathways are described for cutin polymers, flavor volatiles, and terpenoid compounds, many of them also involved in the defense mechanism.

Part III provides basic mechanisms of action and general accounts of biotechnological applications using a wide range of lipases and phospholipases to produce structured lipids and specialty fats, such as infant formula (Betapol), modified milkfat, cocoa butter, margarine, low-calorie fat, and phospholipids enriched in beneficial long-chain polyunsaturated fatty acids, such as eicosapentaenoic acid and docosahexaenoic acid. Also discussed are reaction conditions, reactor design, solvent selection, immobilization technology, and enzyme sources paramount to optimal large-scale production.

Part IV describes a wide range of biotechnological applications using enzymes and microorganisms for producing value-added, nonfood products, such as chiral pharmaceutical intermediates, hormonal compounds, cosmetic substances, biofuels, biosurfactants, biological detergents, and antimicrobial agents. In addition, certain food uses are explored. Also discussed are the principles and applications of supercritical fluid technology, especially supercritical carbon dioxide, which is of tremendous interest to industry as an environmentally benign solvent for lipid analysis, fractionation, and enzymatic reactions.

Every effort has been made to involve internationally recognized experts from all sectors of academia, industry, and government research as contributors to this book. The basic information and theoretical considerations for a specific topic area or a specific biotechnological application are provided, and every effort has been made to include the most current information. It is therefore expected that this book will serve as a valuable reference for researchers in the field and as a complementary text for graduate-level reading and teaching. Finally, we would like to thank all the contributors for their cooperation and support, and the Marcel Dekker, Inc., editorial staff, especially Anita Lekhwani and Moraima Suarez, for their guidance and assistance.

Tsung Min Kuo
Harold W. Gardner

Contents

Contributors

Casimir C. Akoh, Ph.D. Department of Food Science and Technology, The University of Georgia, Athens, Georgia

Victor M. Balcão, Ph.D. College of Health Sciences, Universidade Fernando Pessoa, Porto, Portugal

Elizabeth Blée, Ph.D. Laboratory of Phytooxylipines, Institut de Biologie Moléculaire des Plantes, Centre National de la Recherche Scientifique, Strasbourg, France

Joseph W. Burton, Ph.D. Agricultural Research Service, U.S. Department of Agriculture, and Department of Crop Science, North Carolina State University, Raleigh, North Carolina

Franck E. Dayan, Ph.D. Natural Products Utilization Research Unit, Agricultural Research Service, U.S. Department of Agriculture, University, Mississippi

Ralph E. Dewey, Ph.D. Department of Crop Science, North Carolina State University, Raleigh, North Carolina

Stephen O. Duke, Ph.D. Natural Products Utilization Research Unit, Agricultural Research Service, U.S. Department of Agriculture, University, Mississippi

Maarten R. Egmond, Ph.D. Department of Membrane Enzymology, Center for Biomembranes and Lipid Enzymology, University of Utrecht, Utrecht, and Unilever Research, Vlaardingen, The Netherlands

Thomas A. Foglia, Ph.D. Hides, Lipids and Wool Research Unit, Eastern Regional Research Center, Agricultural Research Service, U.S. Department of Agriculture, Wyndmoor, Pennsylvania

Lydia B. Fomuso, M.S. Department of Food Science and Technology, The University of Georgia, Athens, Georgia

Harold W. Gardner, Ph.D.* Myotoxins Research Unit, National Center for Agricultural Utilization Research, Agricultural Research Service, U.S. Department of Agriculture, Peoria, Illinois

William H. Gerwick, Ph.D. College of Pharmacy, Oregon State University, Corvallis, Oregon

Alexander N. Grechkin, Ph.D. Kazan Institute of Biochemistry and Biophysics, Russian Academy of Sciences, Kazan, Russia

Michael J. Haas, Ph.D. Hides, Lipids and Wool Research Unit, Eastern Regional Research Center, Agricultural Research Service, U.S. Department of Agriculture, Wyndmoor, Pennsylvania

Tomoko Hatanaka, Ph.D. Department of Agronomy, University of Kentucky, Lexington, Kentucky

David C. Herman, Ph.D. Department of Civil and Environmental Engineering, University of Houston, Houston, Texas

David Hildebrand, Ph.D. Department of Agronomy, University of Kentucky, Lexington, Kentucky

Herbert L. Holland, Ph.D. Department of Chemistry, Brock University, St. Catharines, Ontario, Canada

Masashi Hosokawa, Ph.D. Graduate School of Fisheries Sciences, Hokkaido University, Hakodate, Hokkaido, Japan

Ching T. Hou, Ph.D. Microbial Genomics and Bioprocessing Research Unit, National Center for Agricultural Utilization Research, Agricultural Research Service, U.S. Department of Agriculture, Peoria, Illinois

Yugo Iwasaki, Ph.D. Graduate School of Bio- and Agro-Sciences, Nagoya University, Nagoya, Japan

---

* *Current affiliation*: Veteran's Administration Hospital and University of Wisconsin-Madison, Madison, Wisconsin.

Tsuneo Kaneshiro, Ph.D.* Oil Chemical Research Unit, National Center for Agricultural Utilization Research, Agricultural Research Service, U.S. Department of Agriculture, Peoria, Illinois

Jerry W. King, Ph.D. National Center for Agricultural Utilization Research, Agricultural Research Service, U.S. Department of Agriculture, Peoria, Illinois

Anthony J. Kinney, D.Phil. Crop Genetics, DuPont Experimental Station, Wilmington, Delaware

Mozaina Kobaisy, Ph.D. Agricultural Research Service, U.S. Department of Agriculture, Oxford, Mississippi

Tsung Min Kuo, Ph.D. Microbial Genomics and Bioprocessing Research Unit, National Center for Agricultural Utilization Research, Agricultural Research Service, U.S. Department of Agriculture, Peoria, Illinois

Jiann-Tsyh Lin, Ph.D. Western Regional Research Center, Agricultural Research Service, U.S. Department of Agriculture, Albany, California

Raina M. Maier, Ph.D. Department of Soil, Water, and Environmental Science, University of Arizona, Tucson, Arizona

F. Xavier Malcata, Ph.D. College of Biotechnology, Universidade Católica Portuguesa, Porto, Portugal

Alejandro G. Marangoni, Ph.D. Department of Food Science, University of Guelph, Guelph, Ontario, Canada

Thomas A. McKeon, Ph.D. Crop Improvement and Utilization, Western Regional Research Center, Agricultural Research Service, U.S. Department of Agriculture, Albany, California

Jerry F. Miller, Ph.D. Northern Crop Science Laboratory, Agricultural Research Service, U.S. Department of Agriculture, Fargo, North Dakota

Osato Miyawaki, Ph.D. Department of Applied Biological Chemistry, The University of Tokyo, Tokyo, Japan

Martin J. Mueller, Ph.D. Julius-von-Sachs-Institut für Biowissenschaften, Pharmazentische Biologie, University of Würzburg, Würzburg, Germany

Kumar D. Mukherjee, Dr. rer. nat. Institute for Biochemistry and Technology of Lipids, H. P. Kaufmann-Institute, Federal Center for Cereal, Potato and Lipid Research, Münster, Germany

* Retired.

Kozo Nakamura, Ph.D. Department of Applied Biological Chemistry, The University of Tokyo, Tokyo, Japan

Jun Ogawa, Ph.D. Division of Applied Life Sciences, Graduate School of Agriculture, Kyoto University, Kyoto, Japan

John B. Ohlrogge, Ph.D. Department of Plant Biology, Michigan State University, East Lansing, Michigan

Hiroyuki Ohta, Ph.D. Department of Biological Sciences, Graduate School of Bioscience and Biotechnology, Tokyo Institute of Technology, Midori-ku, Yokohama, Japan

Ernst H. Oliw, M.D., Ph.D. Department of Pharmaceutical Biosciences, Uppsala University, Uppsala, Sweden

Vincent R. Pantalone, Ph.D. Department of Plant and Soil Science, University of Tennessee, Knoxville, Tennessee

Ramesh N. Patel, Ph.D. Enzyme Technology, Process Research and Development, Bristol–Myers Squibb Pharmaceutical Research Institute, New Brunswick, New Jersey

George J. Piazza, Ph.D. Hides, Lipids and Wool Research, Eastern Regional Research Center, Agricultural Research Service, U.S. Department of Agriculture, Wyndmoor, Pennsylvania

Suryadevara Rao, Ph.D. Department of Agronomy, University of Kentucky, Lexington, Kentucky

Joanne G. Romagni, Ph.D. Department of Biology, University of St. Thomas, Houston, Texas

Eiji Sakuradani, Ph.D. Division of Applied Life Sciences, Graduate School of Agriculture, Kyoto University, Kyoto, Japan

Kevin K. Schrader, Ph.D. Natural Products Utilization Research Unit, Agricultural Research Service, U.S. Department of Agriculture, University, Mississippi

Peter Schreier, Prof. Dr. rer. nat. Department of Food Chemistry, University of Würzburg, Würzburg, Germany

David J. Schultz, Ph.D.* Department of Plant Biology, Michigan State University, East Lansing, Michigan

Wilfried Schwab, Ph.D. Department of Food Chemistry, University of Würzburg, Würzburg, Germany

---

* *Current affiliation*: Department of Biology, University of Louisville, Louisville, Kentucky.

Subramani Sellappan, Ph.D. Department of Food Science and Technology, The University of Georgia, Athens, Georgia

Jei-Fu Shaw, Ph.D. Institute of Botany, Academia Sinica, Taipei, Taiwan

Daisuke Shibata, Ph.D. Kazusa DNA Research Institute, Kisarazu, Chiba, Japan

Yuji Shimada, Ph.D. Osaka Municipal Technical Research Institute, Osaka, Japan

Sakayu Shimizu, Ph.D. Division of Applied Life Sciences, Graduate School of Agriculture, Kyoto University, Kyoto, Japan

Inder Pal Singh, Ph.D. College of Pharmacy, Oregon State University, Corvallis, Oregon

Chao Su, Ph.D. Department of Pharmaceutical Biosciences, Uppsala University, Uppsala, Sweden

Akio Sugihara, Ph.D. Osaka Municipal Technical Research Institute, Osaka, Japan

Koretaro Takahashi, Ph.D. Graduate School of Fisheries Sciences, Hokkaido University, Hakodate, Hokkaido, Japan

Mario R. Tellez, Ph.D. Natural Products Utilization Research Unit, Agricultural Research Service, U.S. Department of Agriculture, Oxford, Mississippi

Yoshio Tominaga, Ph.D. Osaka Municipal Technical Research Institute, Osaka, Japan

Brady A. Vick, Ph.D. Northern Crop Science Laboratory, Agricultural Research Service, U.S. Department of Agriculture, Fargo, North Dakota

Randall J. Weselake, Ph.D. Department of Chemistry and Biochemistry, University of Lethbridge, Lethbridge, Alberta, Canada

Richard F. Wilson, Ph.D. Agricultural Research Service, U.S. Department of Agriculture, and Department of Crop Science, North Carolina State University, Raleigh, North Carolina

Xuebing Xu, Ph.D. BioCentrum-DTU, Technical University of Denmark, Lyngby, Denmark

Tsuneo Yamane, Ph.D. Graduate School of Bio- and Agro-Sciences, Nagoya University, Nagoya, Japan

Vivienne V. Yankah, Ph.D. Department of Food Science and Technology, The University of Georgia, Athens, Georgia

1

Metabolic Engineering of Fatty Acid Biosynthesis

DAVID J. SCHULTZ* and JOHN B. OHLROGGE

Michigan State University, East Lansing, Michigan

1 INTRODUCTION

Production of vegetable oil exceeds 75 million metric tons (1996/1997) valued in excess of US$38 billion (U.S. Department of Agriculture, WASDE-345 and Oil Crops Update 1998). The majority of vegetable oils in common use are composed primarily of four fatty acids: palmitic (16:0), oleic ($18:1^{\Delta 9}$), linoleic ($18:2^{\omega 6,9}$), and linolenic ($18:3^{\omega 3,6,9}$). However, these fatty acids represent only a small fraction of the diverse structures of fatty acids produced by plants. When considering the known alterations in chain length, double-bond position, and number as well as oxygenated functional groups, the number of described plant fatty acids exceeds 200 [1,2]. If available at the low costs of current commodity vegetable oils, many of the less common fatty acid structures may have attractive applications as industrial feedstocks, polymer precursors, and other petrochemical replacements. Through gene transfers, the opportunity exists for a wide range of alterations in fatty acid biosynthesis in traditional oilseed crops. Certainly, oilseed lipid metabolism provides one of the best models for the cellular biochemistry involved in producing high-value products in plants. However, for metabolic engineering to be feasible and attractive, at least two prerequisites must be met. First, detailed information must be available (both biochemical and genetic) about the pathway(s) of interest and, second, the potential for economic returns of investments must exist. Hitz recently presented an excellent example of cost analysis used in evaluating the potential of transgenic crops [3]. In at least a few cases, metabolic engineering of oilseed lipid biosynthesis satisfies both criteria.

The biosynthesis of triacylglycerol (TAG) in plants is often considered to occur by

* *Current affiliation*: University of Louisville, Louisville, Kentucky.

the sequential acylation of glycerol-3-phosphate to form phosphatidic acid, followed by phosphatidate phosphatase action to yield diacylglycerol (DAG), which is then acylated to form TAG [4]. However, the production of vegetable oils is more complex when considering that it is divided into two distinct, spatially separated reactions, namely fatty acid biosynthesis in the plastids and lipid biosynthesis in the cytosol. In the past 10 years, almost all of the genes encoding enzymes of fatty acid biosynthesis and desaturation as well as genes involved in TAG production have been identified. Starting with the first committed step of fatty acid biosynthesis (acetyl-CoA carboxylase, ACCase) and ending with TAG production, more than 20 distinct biochemical steps are involved (Fig. 1). Within this pathway, the gene for only one reaction remains unidentified. Surveying all available sequence data in Genbank, including plant EST databases, Mekhedov et al. [5] identified gene sequences for all reactions of fatty acid biosynthesis and TAG production, with the exception of the cytosolic glycerol-3-phosphate acyltransferase (Fig. 1). In addition, genes for the production of numerous unusual fatty acids (e.g., alterations in chain length, double-bond placement, functional group attachment) have been identified in non-agricultural crops.

The vast information now available through both genetic and biochemical studies makes metabolic engineering of lipid production an attractive target. Metabolic engineering of TAG can be divided into three distinct target goals. The first goal is the increase in vegetable oil production in established oilseed crops. Based on production figures of 1996/1997, increasing world vegetable production by only 5% would translate into US $2 billion annually in new value. The second goal for metabolic engineering of oilseeds is the alteration of lipid composition to optimize for ''common'' fatty acids. To date, this area has shown the most success in metabolic engineering of lipid metabolism. Genetically engineered lines have been developed with reduced linolenic acid, high oleic acid, and reduced saturates. Although these oils will not increase total vegetable oil production, the value of the oil may be increased and, thus, these vegetable oils may garner a higher market price or at least an increased market share for their producers. Transgenic oilseeds with modifications of the ''common'' fatty acids are now in commercial production and several other lines are undergoing field trials. The final goal of lipid metabolic engineering is the alteration of lipid composition to optimize for ''uncommon'' fatty acids for production of ''specialty oils.'' The production of high-laurate canola is one of the most technically successful examples of this approach to oilseed metabolic engineering. The production of ''specialty oils'' in traditional oilseed crops may eventually have the highest economic impact on oilseed markets, as the production of new types of oils could broaden the vegetable oil market into new areas, increasing overall demand and thereby raising all vegetable oil prices. The importance of these specialty oils is, in part, because they would serve as renewable resources, replacing petroleum-derived products. In some cases, the markets can be quite large, as indicated in Table 1. The two examples given in this table indicate the potential crop area needed to produce the U.S. supply of ricinoleic or adipic acid. Clearly, producing the bulk of adipic acid for nylon manufacture from oilseed crops could have a major impact on the agricultural economy. For comparison, approximately 1 billion gallons of ethanol are produced from 2 million hectares of corn each year.

In this chapter, we review metabolic engineering of fatty acid biosynthesis, highlighting critical or target steps which will influence the three goals outlined. We will focus on distinct areas, using recent research projects to highlight the successes and unsolved problems of oilseed metabolic engineering. As most steps within the fatty acid biosynthesis pathway have been targeted in metabolic engineering of both the ''common'' and ''un-

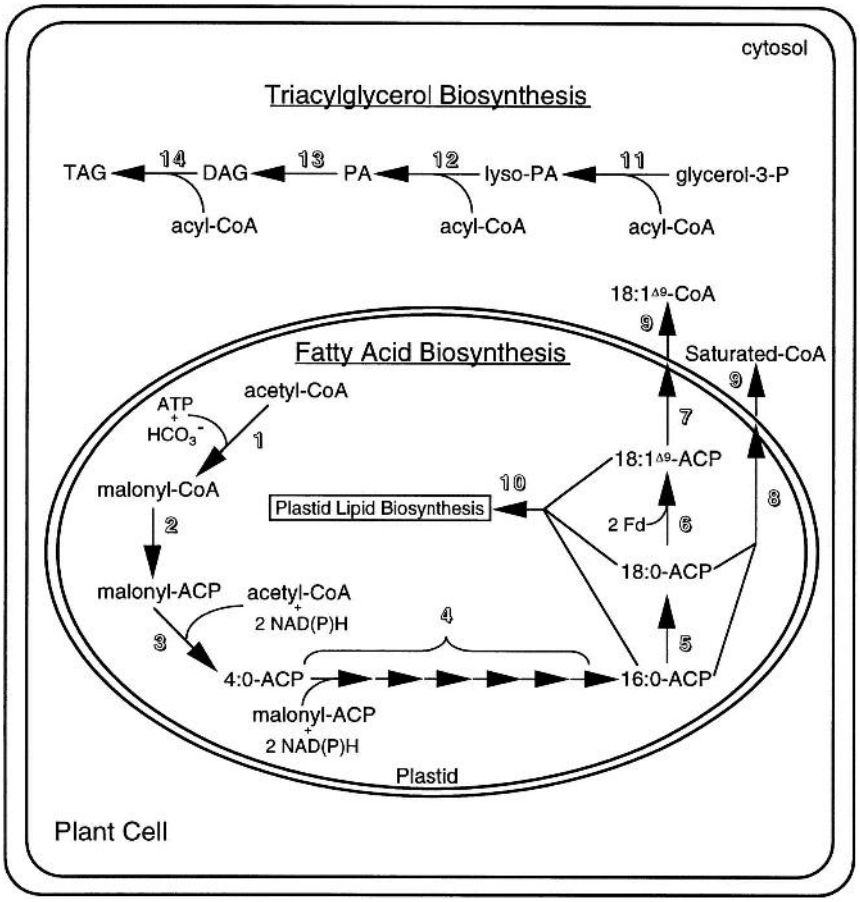


Figure 1 Simplified schematic showing the two spatially separate pathways that contribute to vegetable oil production. The first committed step of fatty acid biosynthesis is the production of malonyl-CoA. Fatty acids are synthesized in a stepwise manner by the addition of two carbons. Fatty acid biosynthesis is terminated at 16:0- or 18:0-ACP (acyl carrier protein) by hydrolysis and export from the plastid or by incorporation into the plastidal lipid biosynthesis pathway. Alternatively, 18:0-ACP may be desaturated prior to hydrolysis or incorporation into the plastid lipid biosynthesis pathway. Subsequent production of TAG results from the sequential acylation of glycerol-3-P: (1) acetyl-CoA carboxylase, (2) malonyl-CoA:ACP transacylase, (3) β-ketoacyl-acyl carrier protein synthase (KAS III), β-ketoacyl-ACP reductase, β-hydroxyacyl-ACP dehydrase, and enoyl-ACP reductase, (4) β-ketoacyl-acyl carrier protein synthase (KAS I), β-ketoacyl-ACP reductase, β-hydroxyacyl-ACP dehydrase, and enoyl-ACP reductase, (5) β-ketoacyl-acyl carrier protein synthase (KAS II), β-ketoacyl-ACP reductase, β-hydroxyacyl-ACP dehydrase, and enoyl-ACP reductase, (6) Δ9 stearoyl-ACP desaturase, (7) thioesterase (Fat A), (8) thioesterase (Fat B), (9) acyl-CoA synthetase, (10) plastidal glycerol-3-phosphate acyl transferase, (11) cytosolic glycerol-3-phosphate acyl transferase, (12) lyso-phosphatidic acid acyl transferase, (13) phosphatidic acid phosphatase, (14) diacylglycerol acyl transferase.

Table 1 Potential Impact of Agricultural Production of Industrial Chemicals

	Ricinoleic acid: a ''smaller market''	Adipic acid: a ''large market''
Approximate cost of chemical	$1–2/kg	$2/kg
Approximate U.S. market size	30 million kg; $60 million	1 billion kg; $2 billion
Estimated area needed to produce chemical in transgenic oilseed	0.3 million ha soybean; 0.1 million ha rapeseed	28 million ha soybean; 10 million ha rapeseed

common'' fatty acids, we briefly review this pathway as an introduction to the specific biochemical reactions which are potentially important for engineering oilseed crops. Within this framework, we will cover advances in engineering increased vegetable oil production, the alteration of lipid composition, the introduction and production of unusual fatty acids, and, finally, the potential use of new technologies to further advance these research projects.

2 FATTY ACID BIOSYNTHESIS PATHWAY

2.1 ACCase

In plants, fatty acid biosynthesis is catalyzed by a dissociable type II (or prokaryotic) fatty acid synthesis system. The first committed step to fatty acid biosynthesis is the production of malonyl-CoA by ACCase in plastids. This reaction catalyzes the production of malonyl-CoA from acetyl-CoA and bicarbonate (Fig. 1). ACCase occurs as two forms: one targeted to plastids and a second located in the cytosol. All plants contain two ACCase enzymes, but species differ in the type of ACCase found in the plastids. In dicots and probably in most monocots, two structurally distinct isoforms are present, whereas in Gramineae such as corn, two structurally similar isoforms are present [6–9]. In all cases, ACCase has three functional domains: biotin carboxylase (BC), biotin carboxyl carrier protein (BCCP), and carboxyltransferase (CT). The dicot plastidal ACCase form has a dissociable multisubunit structure similar to that found in *Escherichia coli* [10]. Because this ACCase is made up of multiple subunits, the term ''heteromeric ACCase'' has been adopted to differentiate it from the cytosolic ACCase holoenzyme (homomeric ACCase). In contrast, Gramineae do not have a heteromeric plastidal ACCase but, instead, contain a second homomeric plastidal ACCase. The plastidal ACCase provides malonyl-CoA to fatty acid biosynthesis, whereas the cytosolic ACCase provides malonyl-CoA for flavonoid and isoflavonoid biosynthesis as well as for elongation of long-chain fatty acids. The difference in plastidal ACCase types (heteromeric versus homomeric) provides a selective distinction between Gramineae and other plants, as two herbicide types (oxypropionic acid and cyclohexanedione) have been shown to inhibit the homomeric ACCase but are not effective at inhibiting the heteromeric ACCase [9].

The genes encoding each subunit (BC, BCCP, αCT, and βCT) of the heteromeric ACCase have all been identified [6,11–13]. In addition, genes encoding the homomeric ACCase has also been isolated [14,15]. Recently, an additional complexity to the ACCase story was uncovered when it was demonstrated that the homomeric ACCase occurs in at least two forms in *Brassica*. Surprisingly, this gene family contains a homomeric ACCase isoform which is also targeted to the plastid [16].

2.2 Acyl Carrier Protein

Acyl carrier protein (ACP) is a small acidic protein containing a phosphopantetheine prosthetic group which forms a thioester linkage to fatty acids. The involvement of ACP in fatty acid biosynthesis and localization of fatty acid biosynthesis was determined with immunological studies using spinach leaf protoplasts [17]. In addition to its role as a cofactor in the reactions of acyl-chain elongation, ACP is also an important cofactor in plastidal acyl-desaturation and acyl-transferase reactions [18,19]. Additional analysis of ACP in plants has revealed multiple distinct ACP isoforms [20–22]. Furthermore, in a recent survey of lipid biosynthesis genes, five plastidal ACP isoforms were identified in both *Arabidopsis* and rice [5]. To date, distinct functions of the multiple ACP isoforms in relation to fatty acid biosynthesis remain unresolved. However, studies of ACP have provided evidence that the isoforms are regulated both developmentally as well as at the level of tissue specificity [23–25]. Thus, it can be speculated that ACP may function, in part, to control fatty acid biosynthesis (also see Sec. 5.2.1).

2.3 Acyl-ACP Elongation

After the production of malonyl-CoA and the transfer of the malonyl group to ACP, the core reactions of fatty acid synthesis consist of acyl-ACP elongation reactions which occur by the successive two-carbon addition from malonyl-ACP (with the loss of CO_2) to acyl-ACP (with the loss of ACP) [26]. The condensation and elongation of acyl-ACP chains are catalyzed by a multicomponent system separable into distinct activities, including β-ketoacyl-ACP synthase (KAS), β-ketoacyl-ACP reductase, β-hydroxyacyl-ACP dehydrase, and enoyl-ACP reductase [27]. Furthermore, it was shown that multiple isoforms of the condensing enzyme (KAS) exist which catalyze the elongation at distinct steps [28]. The initial analysis of KAS activity suggested that the elongation of acetyl-ACP to palmitoyl-ACP was catalyzed by KAS I and the elongation of palmitoyl-ACP to stearoyl-ACP was catalyzed by KAS II. However, based on differential KAS sensitivity to cerulenin, Jaworski et al. [29] identified an additional condensing enzyme, KAS III, which is responsible for the production of short-chain acyl-ACPs (4:0- and 6:0-ACP). Thus, the emerging picture of fatty acid elongation has become a system in which KAS III primarily participates in production of short-chain acyl-ACPs, KAS I functions in elongation of short chain acyl-ACPs to 14:0- and 16:0-ACPs, and the final elongation to 18:0-ACP is catalyzed by KAS II. The genes encoding KAS I [30,31], KAS II [32], and KAS III [33–35] have now been cloned. In addition, expressed sequence tag (EST) or BAC clones have been identified for each KAS in numerous species [5].

2.4 Stearoyl-ACP Desaturase

Acyl-ACP desaturases are a class of soluble enzymes which function to add a double bond to an acyl group esterified to ACP. As such, this enzyme catalyzes the only plastid modification of a fatty acid prior to incorporation into glycerolipids. The stearoyl-ACP desaturase (Δ9 18:0-ACP desaturase) was first recognized and studied in *Euglena gracilis* [36] and, subsequently, was identified in higher plants (spinach, avocado, and safflower) [37]. The first stearoyl-ACP desaturase clones were reported for castor seed and cucumber by Shanklin and Somerville [38] and for safflower by Thompson et al. [39]. Further biochemical analyses defined the function of this desaturase, revealing that the positioning of the double bond is from the carboxyl end of stearoyl-ACP [40]. In addition, this study also

demonstrated that substrate specificity was 100-fold greater for 18:0-ACP compared to 16:0-ACP. This specificity results from the greater V_{max} with 18:0-ACP, as the K_m values between 16:0-ACP and 18:0-ACP were not significantly different. Further functional characterization of the stearoyl-ACP desaturase has led to a better understanding of the mechanism by which the enzyme recognizes its substrate and catalyzes double-bond formation. Stearoyl-ACP desaturase has been shown to have a diiron active site and is a member of the diiron-oxo protein family [41]. More recently, the castor Δ9 18:0-ACP desaturase has been crystallized and the structure resolved to 2.4 Å [42]. The crystal structure aided in the identification of the substrate binding channel, and when stearoyl-ACP is modeled into this binding channel, the 9 and 10 carbon units are placed in the vicinity of 1 iron atom from the diiron active site [43]. With the detailed knowledge of the stearoyl-ACP desaturase, manipulation of this reaction has become possible [44,45]. However, additional complexity in controlling this reaction arises because the stearoyl-ACP desaturase is encoded by a gene family. In *Thunbergia alata*, at least three distinct, presumably seed-specific stearoyl-ACP desaturase genes were identified [46], whereas in *Arabidopsis* and rice, at least four stearoyl-ACP desaturase genes have been identified [5]. Thus, control of stearoyl-ACP desaturase may involve multiple isoforms in ways not yet understood.

2.5 Acyl-ACP Thioesterase

In oilseeds, the major termination reaction of fatty acid biosynthesis is catalyzed by acyl-ACP thioesterases. This step is also an important determinant for the subcellular destination of fatty acids. Fatty acids not hydrolyzed by thioesterase enter the plastid lipid biosynthetic pathway by action of the plastidal glycerol-3-phosphate acyltransferase [18,47]. Fatty acids hydrolyzed by the thioesterase are transported through the plastid membrane by an uncharacterized mechanism, then likely converted to acyl-CoA derivatives by the action of an acyl-CoA synthetase associated with the outer plastid membrane. Acyl-CoA can then enter the glycerol lipid biosynthetic pathway. An initial partial purification and characterization of thioesterase activity in avocado mesocarp [48] revealed the activity of an oleoyl-ACP-specific thioesterase, not only in plants with lipids enriched in 18 carbon fatty acids (avocado) but also in plants with lipids enriched in medium-chain fatty acids (coconut and jojoba). Several later studies have now clarified that at least two classes of acyl-ACP thioesterases exist in most plants (reviewed by Voelker [49]). Because thioesterases are the final reaction in fatty acid biosynthesis prior to export to the cytoplasm, specificity of the thioesterases can in many cases have a major influence on the acyl profile of plant lipids. For example, at least two oleoyl-ACP thioesterase genes have been found in safflower and *Brassica napus* [50,51]. However, when the activities of the two safflower oleoyl-ACP thioesterases were compared [50], it was determined that they differed in activity by twofold, and the thioesterase with the lower $18:1^{\Delta 9}$ specificity showed a broader specificity with twofold to threefold higher activity with saturated 16 and 18 carbon fatty acids. Further clarification of the thioesterase activity came with the discovery that two evolutionarily distinct classes of thioesterase exist [52]. These classes were termed Fat A for oleoyl-ACP thioesterase and Fat B for thioesterase having specificities for saturated acyl-ACP.

3 POTENTIAL METABOLIC ENGINEERING TARGETS FOR INCREASING OIL YIELD

The production of vegetable oil requires the coordination of two distinct pathways: fatty acid biosynthesis and triacylglycerol biosynthesis. To increase total vegetable oil produc-

tion, it would be plausible that the output, or rate of either pathway, could limit total oil production. Recently, in studies of TAG production in *Cuphea* and elm species, it was determined that the supply of fatty acid was a likely limiting factor for TAG biosynthesis [53]. In addition, Harwood et al. [54] found that fatty acid synthesis accounted for 60% of the control of lipid synthesis compared with enzymes of the Kennedy pathway. It would, therefore, seem that the later enzymes (including acyltransferases) of TAG biosynthesis could accommodate higher levels of fatty acid production. Thus, a logical argument could be made that by increasing the rate of fatty acid biosynthesis, higher levels of oil accumulation can be obtained. A logic-based approach to increasing vegetable oil production requires that the potential limiting steps in the fatty acid biosynthesis pathway be identified. Carbon for the fatty acid biosynthesis pathway is supplied as acetyl-CoA. However, the source of acetyl-CoA for fatty acid biosynthesis remains incompletely understood, possibly supplied directly by plastidal pyruvate dehydrogenase or indirectly by nonplastidal reactions [55]. In some cases, a combination of reactions may supply the necessary acetyl-CoA. If the supply of acetyl-CoA is a limiting factor for fatty acid biosynthesis, a depletion of the acetyl-CoA pool would be expected when rates of fatty acid biosynthesis are high. However, the levels of acetyl-CoA (and acetyl-ACP) are found to be fairly consistent, regardless of the rate of fatty acid biosynthesis, both in leaves and developing castor seeds, and thus may not be a limiting factor to fatty acid biosynthesis [56].

Early investigations into the role of specific enzymes within the fatty acid synthesis system led to the suggestion that acetyl CoA : ACP transacylase is a potential limiting step to fatty acid biosynthesis [57]. However, results from more recent studies make this reaction unlikely as a regulatory point. Analysis of KAS III indicates the condensation of acetyl-CoA with malonyl-ACP occurs at a rate which is fivefold higher than the acetyl-CoA : ACP transacylase reactions [29]. Thus, the need for acetyl-ACP as a substrate for fatty acid biosynthesis is eliminated. Further clarification stems from two ''metabolic profiling'' studies which measured in vivo pools of acyl-CoAs and acyl-ACPs [56,58]. Analysis of these pool sizes is useful, as this approach provides direct in vivo information on fatty acid biosynthesis activity and its regulation. Pool sizes that change under higher rates of fatty acid biosynthesis indicate potential rate-limiting reactions. Analysis of the acetyl-ACP, acetyl-CoA, malonyl-CoA, and malonyl-ACP pools have led to the conclusion that, in leaves, the most likely candidate for metabolic control of fatty acid synthesis is ACCase [56,58]. This conclusion has been further supported by inhibitor studies on ACCase in maize and rice [59] and by analysis of acyl-ACP pools in tobacco suspension cultures [60]. Recently, the complexity of ACCase regulation has been revealed by studies indicating metabolic control by thioredoxin [61], phosphorylation [62], and by acetyl-CoA [63].

3.1 Will Alterations in Expression of ACCase Lead to Higher Levels of Fatty Acid Biosynthesis?

Based on in vitro as well as in vivo analyses, ACCase seems to be one likely rate-limiting step in fatty acid biosynthesis and, therefore, a potential target for controlling fatty acid biosynthesis. ACCase expression in plants is complex, due to the two independent ACCase activities (the single peptide homomeric form and the multiple subunit heteromeric form). This complexity leads to the question of the best approach to manipulate ACCase activity. Increased expression of ACCase in plastids could be achieved by overexpressing either the homomeric or heteromeric ACCase isoforms in plastids. Each approach presents distinct challenges. The homomeric isoform is a large peptide ($>$200 kDa), which, in dicots, would have to be targeted to the plastid by a foreign transit peptide. In contrast, the heteromeric

ACCase subunits are relatively small (30–80 kDa). All but one subunit (βCT) is encoded in the nuclear genome and thus have transit peptides. However, the complex regulation of this multisubunit complex is not well understood [63]. For example, it was not known if overexpression of a single subunit would increase total ACCase activity or if overexpressing all four subunits would be required to increase activity.

3.1.1 Increasing Oil Composition by Overexpression of Homomeric ACCase

The first approach to increasing oil content by increasing the activity of ACCase was through the overexpression of the *Arabidopsis* homomeric ACCase gene (ACC1) in *Brassica napus* seeds [64]. Approaching the overexpression of the homomeric ACCase in plastids of *Brassica* presented many questions. Due to its large size (>200 kDa), could the homomeric ACCase be correctly targeted and transported into the plastid? Would this ACCase be biotinylated and have enzymatic activity? Would expression of the homomeric ACCase in plastids of *Brassica* lead to an increase or alteration in oil content? The work by Roesler et al. [64] provided evidence that the homomeric ACCase could be targeted to the plastid in an active, stable form and that the expression of the homomeric ACCase could slightly increase oil composition. ACCase overexpression resulted in a onefold to twofold increase in enzyme activity, leading to an increase in oil content of approximately 5%. This work demonstrated the possibility of increasing the production of oil by overexpression of a potential limiting reaction in fatty acid biosynthesis.

3.1.2 Can the Heteromeric ACCase Be Manipulated to Increase Oil Content?

An additional aspect of controlling fatty acid biosynthesis through ACCase is to consider the influence of each type of ACCase. Although overexpression of the homomeric ACCase in *Brassica* led to a small increase in oil content, the question remained whether similar or higher increases could be achieved by manipulating the level of activity generated by the heteromeric ACCase? The expression of the heteromeric ACCase has been reported to be coordinately regulated in both castor and *Brassica* [65] as well as in *Arabidopsis* [66,67]. Because multisubunit complexes found in the plastids, such as rubisco, were already know to be coordinately controlled, Shintani et al. [68] investigated the coordinate expression of the heteromeric ACCase in response to alterations in the levels of BC in order to gain a more complete understanding of the control of heteromeric ACCase expression. The first half of the ACCase reaction involves the initial carboxylation reaction catalyzed by two closely associate subunits, BC and BCCP. In this study, the levels of BC were increased to 500% (relative to wild type) by overexpression or decreased to 26% (relative to wild type) by antisense expression. Despite the large alterations in BC levels, no major difference in the levels of BCCP were detected. In addition, the levels of BC had no influence on the αCT subunit from the second-half reaction of ACCase. Furthermore, this study revealed that BC is most likely in excess, as reduction of BC to 50% (relative to wild type) showed no phenotypic difference. Fatty acid content was not increased by overexpression of BC and was not significantly influenced by decreases of BC levels up to 50% (relative to wild type). However, in one line containing BC levels at 26% wild type, a stunted phenotype was observed. This line did show a small (8%) but significant decrease in fatty acid content. Thus, although the overexpression and antisense analysis of BC expression cannot rule out a coordinate transcriptional control, it does provide evidence that any coordinate regulation is not controlled posttranslationally. In a

similar study, Qian et al. [69] used antisense suppression of BCCP to analyze ACCase coordinate expression. These results indicated a correlation in BCCP and BC levels, but no correlation with αCT and βCT levels. Thus, to achieve control of fatty acid biosynthesis using overexpression of the heteromeric ACCase, multiple subunits will likely have to be targeted.

3.2 Additional Factors That May Limit Fatty Acid Biosynthesis

Although ACCase is likely one site of major control of fatty acid biosynthesis, the possibility of additional factors influencing fatty acid production must be considered. Most efforts to identify additional factors controlling fatty acid biosynthesis have focused on additional limiting reactions such as the condensing enzymes or on feedback regulation. Evidence for additional enzymatic reactions, which influence the rate of fatty acid biosynthesis, was uncovered during studies of the rate-limiting steps of fatty acid biosynthesis [56]. In this study, fatty acid biosynthesis was stimulated 30–80%, whereas the levels of malonyl-CoA and malonyl-ACP were increased fivefold. Post-Beittenmiller et al. [56] concluded that an additional factor (possibly fatty acid biosynthesis enzymes, substrates, or cofactors) is likely limiting during high rates of fatty acid biosynthesis. The potential target enzymes in fatty acid biosynthesis are the condensing enzymes. The intermediate pools of acyl-ACPs are relatively equal during fatty acid biosynthesis [58] and, thus, are in equilibrium. Therefore, an alteration in action of a single condensing enzyme may have a negligible impact on total fatty acid production. Using the *E. coli* fabH gene (encoding KAS III), Verwoert et al. [70] overexpressed KAS III in *Brassica* seed. This experiment indicated that KAS III has no direct influence of oil content. However, increasing KAS III did alter fatty acid composition, producing higher levels of polyunsaturated fatty acids and a lower oleic acid content. This result is possibly because increased flux through the condensation steps catalyzed by KAS III is limited by the activity of subsequent condensation or other enzymatic steps. Thus, to impact fatty acid production to a major extent, many enzymes may have to be increased.

An additional mode of control for fatty acid biosynthesis may be through feedback regulation. Feedback regulation was first analyzed using tobacco suspension cells [60]. Suspension cells supplied with exogenous lipid (in the form of oleic-Tween esters) and ^{14}C acetate were used to detect changes in acyl-ACPs. Alterations in specific acyl-ACP levels would indicate a feedback step. The major influence of the supplied lipid was a decrease in ^{14}C acetate into long-chain acyl-ACPs and an increase in acetyl-ACP, with the levels of medium-chain acyl-ACPs remaining unchanged. Based on ^{14}C-labeled acyl-ACP pool sizes, it appeared that no significant feedback regulation occurred at the condensation steps (KAS I, II, or III). Because no regulation of KAS III was likely, the increased levels of acetyl-ACP could only be explained by a regulation of ACCase. The mechanism of this inhibition is still not known, although direct influence on the levels of ACCase protein expression was ruled out by analyzing BC and BCCP levels over time when cells are supplied with oleoyl-Tween esters [60].

In vitro investigations into fatty acid biosynthesis in *Cuphea* seeds have also suggested a feedback-regulation system [71]. However, the target for inhibition in this system was concluded to be KAS III. This mechanism of inhibition was extended to include *Brassica* seeds and spinach leaves. The potential for acyl-ACP feedback regulation was determined when it was revealed that rates of fatty acid biosynthesis differed depending on the presence or absence of reducing cofactor in the reactions. Surprisingly, the activity

of KAS III was found to be lower when a reducing equivalent was added. The reaction product was a ketoacyl-ACP when no reducing equivalent was supplied, whereas the reaction proceeds to an acyl-ACP when reducing power was supplied. Fatty acid biosynthesis assays which included cerulenin to inactivate KAS I and KAS II were conducted when medium- and long-chain acyl-ACPs were supplied. The results of this study show the strongest level of inhibition in *Cuphea* occurs when medium-chain (8:0 and 10:0) acyl-ACPs were supplied. In *Cuphea* seeds, where the major acyl product is 10:0, this feedback regulation in addition to activity of the medium-chain thioesterase may provide a basis for tight control of 10:0-ACP production. However, this does not explain the higher level of 10:0-ACP inhibition in *Brassica* seeds and spinach leaves relative to 16:0- and 18:0-ACP inhibition.

Although it has been demonstrated that feedback inhibition potentially influences the activity of fatty acid biosynthesis in plants, the exact mechanism of this reaction still remains a mystery. As yet, it is not certain that the same system will function in leaves and seeds or which target enzyme(s) for feedback regulation play the largest in vivo role.

Targeting increased oil content clearly has a great economic potential. However, the complexity of the pathway's regulation has not yet been overcome to substantially increase oil in commercial production systems. Considering the potential regulatory mechanisms for the pathway (both input and output), it may be necessary to target both increasing flux through the fatty acid supply pathway as well as increasing the downstream acyl-chain metabolism. For example, expression of a yeast *sn*-2 acyltransferase gene in *Brassica* and *Arabidopsis* has lead to significant increases (8–48%) in seed oil content [72]. Perhaps, combinations of acyltransferase demand for fatty acids with ACCase driven increases in supply will provide the most effective and stable increases in oil content.

4 MANIPULATION OF "COMMON" FATTY ACID COMPOSITIONS

Creating tailored oil compositions is an attractive target for metabolic engineering of fatty acid biosynthesis. The major current market for vegetable oils is for edible uses and a number of improvements in fatty acid composition have been long sought to enhance the nutritional value, stability, and processing of edible oils. However, the complexity of plant lipid production (the balance between two distinct organellar pathways) makes this undertaking potentially difficult. The goal of modifying plant storage lipids is to create the desired composition in the storage lipid (TAG) while maintaining optimal membrane lipid composition critical for cell function. Adding to the potential difficulty is the relatively complex interchange between and within lipids of distinct pathways (plastidal and cytosolic pathways). Commercial targets for alteration in the levels of distinct fatty acids in edible oils revolve around the modifications in production of saturated fatty acids or the modifications in production of specific unsaturated fatty acids. The target steps for these modification are thioesterases and stearoyl-ACP desaturases in the plastid and membrane-associated lipid desaturases in the cytosol. Flux through any biochemical step is influenced by three factors: the pool of available substrates, the activity of the enzyme catalyzing the step, and the demand or sink for products. Thus, multiple targets are generally present and, sometimes, may need to be manipulated for each desired alteration.

4.1 Production of High-Stearate Oilseeds

High stearate content is desirable for increasing the melting point of oils, thereby providing margarine or shortening feedstocks which do not require hydrogenation. Approaches to

improve stearate composition have focused on modifying the activity of two separate enzymatic steps. By increasing the activity of 18:0-ACP thioesterase, the available pool of 18:0-ACP for stearoyl-ACP desaturase is diminished and, thus, should result in increased pools of stearate in TAG. Conversely, the approach of reducing the activity of stearoyl-ACP desaturase and thus increasing the pool of 18:0-ACP for the stearoyl-ACP thioesterase has also been taken. The first genetic engineering success in increasing stearate levels was through antisense expression of the stearoyl-ACP desaturase in *Brassica* [73]. Production of stearate was increased 10–20-fold in *Brassica napus* and *B. rapa* lines. The increased level of stearate was the result of nearly complete abolishment of stearoyl-ACP desaturase activity.

Recently, a thioesterase with altered activity toward 18:0-ACP was identified in *Garcinia mangostana* [74]. This enzyme is a Fat-A-type thioesterase and, hence, has highest preference for $18:1^{\Delta 9}$. However, activity of this thioesterase toward 18:0-ACP in mangosteen was threefold higher than with 16:0-ACP. As comparison, the Fat A thioesterase in safflower and *Brassica* shows equal activity for 16:0-ACP and 18:0-ACP. Subsequently, a clone encoding a mangosteen Fat A gene (*Garm*FatA2) was isolated and expressed in *B. napus*, resulting in increased stearate composition (10-fold higher). Further work optimizing the activity of *Garm*FatA2 by site-directed mutagenesis has lead to an increase in 18:0-ACP specificity (to 13-fold), which results in up to 30% higher stearate accumulation [75].

Thus, similar increases in stearate content have been achieved either by controlling the substrate pool available to stearoyl-ACP desaturase or by directly controlling the activity of the stearoyl-ACP desaturase. Interestingly, additional modifications in oil composition occurred in both approaches. Increases in stearate were combined with reduction of oleate levels and increases in very long-chain saturated fatty acids. Based on the results of these experiments, it now seems likely that further increases in stearate composition could be achieved using a dual approach where the 18:0-ACP pool is decreased using a thioesterase such as *Garm*FatA2 and the further utilization of the 18:0-ACP pool by stearoyl-ACP desaturase is diminished by antisense inhibition of the stearoyl-ACP desaturase.

4.2 Modification of Unsaturated Fatty Acid Composition

Reductions in dietary saturated fatty acid consumption is generally considered to reduce the incidence of a number of cardiovascular diseases and, therefore, has been a long-term goal of oilseed breeders and genetic engineers. Tailoring the unsaturated fatty acid composition to enhance oleic, linoleic, or linolenic acids can be achieved by control of the membrane-associated microsomal lipid desaturases (oleate or linoleate desaturases). By controlling the level of desaturases (by a combination of either overexpression or antisense suppression), it is possible to enrich the composition of oil for a specific unsaturated fatty acids. An increase in oleic acid would yield a more oxidatively stable oil with low saturates and, thus, would be ideal for food purposes. The approach to achieving high oleate lines is through the overexpression of the stearoyl-ACP desaturase or by suppression of the oleate desaturase. An increase in stearoyl-ACP desaturase would most likely result in an increased flux through oleic acid into linoleic and linolenic; thus, this approach would not likely be successful. However, blocking the oleate desaturation step has the potential of increasing the levels of oleate incorporated into TAG. This approach has been found to be useful in modifying oil composition in both soybean and canola [76]. Using

antisense or cosuppression of the oleate desaturase, the level of oleate was increased from 63% to 83% in canola and from 22% to 79% in soybean. A major added benefit of this single gene modification was a concomitant reduction in saturated (mainly palmitic) acid content. Thus, oils were produced which may have ideal nutritional properties via their low saturates and high oleic acid content. Similarly, production of high linoleic acid content with reduced linolenic acid content can be made by blocking the linoleic acid desaturase. In soybean, this approach has lead to a decrease in 18:3 from 9% to 1.4% with an increase in 18:2 content from 55% to 65% [76].

Because the production of 18:3 is a ''terminal'' step in terms of fatty acid modification of common fatty acids, increasing this fatty acid must be approached through modification of the input. By overexpressing the linoleate desaturase, it may be possible to increase the production of 18:3. Increased production of 18:3 (fourfold to fivefold) was observed in tobacco root when the linoleate desaturase was overexpressed [77]. However, leaf 18:3 content was not significantly affected, and it is still not known to what extent overexpression of the 18:2 desaturase may increase 18:3 in a seed oil.

5 METABOLIC ENGINEERING OF OILSEEDS FOR PRODUCTION OF UNUSUAL FATTY ACIDS

Although modifications which increase total oil content or increase oil composition of any one of the common fatty acids will be of economic importance, the largest gains in plant oil utilization may come from the production of new ''specialty'' oils. To date, lauric acid (12:0), petroselinic acid ($18:1^{\Delta 6}$), and hydroxy, epoxy, and acetylenic acids are examples that have been newly produced in transgenic oilseed plants.

Because plants produce an enormous diversity of fatty acids, reviewing the potential or attempted metabolic engineering projects targeting these modifications is not possible in this chapter. Thus, we will focus on two projects, laurate production and unusual monoenoic fatty acid production, to show the success and difficulties of metabolic engineering projects. It is hoped that information from these projects can serve as a guide for future lipid metabolic engineering work. Obstacles and limitations discovered through these largely ''applied science'' projects have also produced a wealth of new fundamental ''basic science'' information. When possible, we will highlight these new areas and their relation to metabolic engineering of plant lipids.

5.1 Laurate Overproduction

Laurate production in canola is one of the most technically successful demonstrations of metabolic engineering of lipid metabolism. Although high levels of laurate have been produced by adding a single enzymatic activity (12:0-ACP thioesterase), the accumulation of such medium-chain fatty acids in transgenic lines has proven to be complex. Additionally, new medium-chain-specific fatty acid biosynthesis enzyme isoforms are still being identified in the native species. These discoveries may lead to even further optimization of the medium-chain fatty acid production in canola. Early work using the California Bay plant rich in 10:0 and 12:0 led to the initial discovery of a thioesterase with a high preference for 12:0-ACP [78,79]. With the identification of the medium-chain-specific thioesterase, it was realized that fatty acid biosynthesis in oilseed plants could be ''short-circuited'' by expression of a 12:0-ACP thioesterase.

Subsequently, the gene encoding the lauroyl-ACP thioesterase (*UcFatB1*) was identified in the California Bay plant and overexpressed in *Arabidopsis*. The success of the thioesterase in terminating the endogenous fatty acid biosynthesis system in this heterologous system was confirmed with the detection of laurate at levels between 10% and 24% [80]. Production of laurate by the *UcFatB1* gene in rapeseed was found to be limited by additional factors [81]. Multiple lines were generated with single or tandem thioesterase constructs. Transgenics were then scored for number of insertion copies in the genome. The resulting lines were analyzed for laurate composition and found to have a wide distribution, from 0% to nearly 60%. Additionally, these lines were tested for total 12:0-ACP thioesterase activity. When laurate production was compared with in vitro 12:0-ACP activity, a linear relationship between activity and accumulation was found only up to about 30% laurate. For an additional 2-fold increase in laurate production, an approximately 20-fold increase in activity was necessary. Thus, it was apparent some additional factors were limiting the production of laurate.

5.1.1 Laurate Catabolism May Limit Production in Leaves and Seeds

Limitations to medium-chain fatty acid production could be envisioned at either input or output. The production of high amounts of an unusual fatty acid may present problems for the plant in maintaining normal membrane function. Thus, production may be limited by mechanisms which the plant uses to preserve normal membranes. Alternatively, additional components of the fatty acid biosynthesis pathway may be required to optimize the substrate pool for the thioesterase activity. Recently, both β-oxidation and additional fatty acid biosynthesis components have been analyzed and found to be potentially limiting for medium-chain fatty acid production. *Brassica napus* transformed with the *UcFatB1* thioesterase under the control of the *CaMV35S* promoter were found to accumulate laurate in seeds, but not in leaves [82]. To determine if the lack of laurate production was the result of differences in protein expression, thioesterase activity was analyzed in both leaves and seeds. The results indicated that 12:0-ACP thioesterase activity in leaves was comparable to the endogenous oleoyl-ACP thioesterase and, additionally, the leaf lauroyl-ACP thioesterase activity was greater than seed lauroyl-ACP activity, which resulted in significant seed laurate accumulation. Furthermore, ^{14}C acetate labeling of isolated leaf plastids revealed that a significant proportion of labeled lipids (34%) were laurate. Thus, the lauroyl-ACP thioesterase was active in leaves, but no accumulation of laurate was detected. Therefore, leaf production of laurate was hypothesized to be limited by degradation of the product. In support of this theory, β-oxidation was assessed using isocitrate lyase and malate synthase as marker enzymes of the glyoxylate pathway. The results of this analysis led to the conclusion that the breakdown, not the synthesis of laurate in leaves, is the limiting factor to laurate accumulation [82]. In a similar study, Hooks et al. [83] found that the lauroyl-ACP thioesterase expressed in *Arabidopsis* under the control of the 35S promoter also accumulated no laurate in leaves but did have laurate in seeds. Subsequently, laurate catabolism was analyzed by assessing the expression levels of enzymes involved in β-oxidation as well as the glyoxylate pathway. In contrast to the findings of Eccleston et al. [82], Hooks et al. [83] found no induction of β-oxidation or glyoxylate pathway enzymes. However, the endogenous β-oxidation pathway was still proposed to be sufficient to provide a mechanism for laurate degradation.

Because laurate production in the seed does not remain linear with increases in 12:0-ACP thioesterase activity, laurate catabolism was also analyzed as a possible limiting

factor to laurate accumulation in seeds [84]. This study indicated that in *Brassica* seed expressing the lauroyl-ACP thioesterase, an induction of three biochemical pathways (β-oxidation, glyoxylate, and fatty acid biosynthesis) occurred, creating a futile, recycling system for laurate production which may contribute to the limitations of laurate production. In lines expressing lauroyl-ACP thioesterase, β-oxidation degrades laurate, as evidenced by increased activity of lauroyl-CoA oxidase, but not palmitoyl-CoA oxidase. Subsequently, as with the early study of leaf laurate catabolism [82], the glyoxylate pathway enzymes, isocitrate lyase and malate synthase, are induced to higher activity levels. Surprisingly, although a major portion of fatty acids produced appeared to be degraded, the oil content of high lauric seeds was not reduced. The increase in β-oxidation and glyoxylate pathway in the seed could lead to acetyl-CoA production and, thus, the potential for reincorporation of carbon from lauric acid into the fatty acid biosynthesis pathway. Thus, the levels of key fatty acid biosynthesis enzymes were also analyzed and found to be expressed at higher activity levels in seed expressing the lauroyl-ACP thioesterase. It was concluded that in high-laurate *Brassica* seeds, a coordinate induction of the fatty acid biosynthesis pathway occurs to compensate for the lost acyl-composition due to the catabolism of laurate by β-oxidations [84]. Although it might be concluded that the oxidation pathway limits laurate accumulation, it is perhaps more likely that oxidation is a symptom rather than a cause of the limitation. Thus, the lack of incorporation of laurate at the *sn*-2 position of TAG may lead to the accumulation of lauroyl-CoA, which triggers the induction of the degradation pathway.

5.1.2 Additional Genes May Be Required for High Laurate Production

Further control of medium-chain fatty acid production may be achieved with additional components of the fatty acid biosynthesis system. Studying the production of caprate (30%) and laurate (54%) in *Cuphea wrightii*, Leonard et al. [85] have proposed that additional fatty acid biosynthesis components such as condensing enzymes may be specific for production of medium-chain fatty acids and, thus, required for optimal levels of production in transgenic plants. The role of condensing enzymes in the production of medium-chain fatty acids has recently been further investigated in *Cuphea* [86]. Using cerulenin sensitivity and a *Cuphea* mutation line (cpr-1), a KAS enzyme with novel characteristics was identified. In vitro assays pretreated with 10 μ*M* cerulenin (to inhibit KAS I), it was discovered that *Cuphea* was still capable of catalyzing condensations up to 10:0-ACP. In addition, the caprate-deficient *Cuphea* mutant cpr-1 was shown to be inhibited in the elongation of 8:0-ACP to 10:0-ACP. Taken together, these data indicate that an additional KAS, specific for elongation of medium-chain acyl-ACP, is present in *Cuphea*. Subsequently, two additional KAS genes, termed *CwKasA1* and *CwKas4*, have been independently identified in the *Cuphea wrightii* [87] as well as *C. pulcherrima* and *C. hookeriana* [88], respectively. Each *Cuphea* KAS was tested for activity in transgenic oilseed. *CwKasA1* was used to transform *Arabidopsis* and *CwKas4* was used to transform *Brassica* [87,88]. In both cases, no alteration of the fatty acid profile was detected when the new KAS was expressed. However, the lipid composition was enriched for medium-chain fatty acids relative to expression of the thioesterase alone when expressed in conjunction with a medium-chain-length-specific thioesterase. The likely mechanism for this reaction is that the medium-chain-length KAS enzyme creates an enriched pool of medium-chain-length acyl-ACPs which support the higher production of medium chain length lipids by the thioesterase.

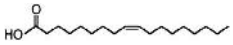
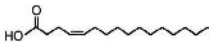
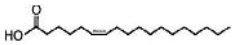
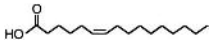
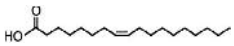
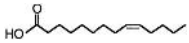
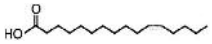
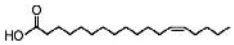
An additional factor that may influence the production of medium-chain-length fatty acids is ACP. In studies of *Cuphea lanceolata*, two ACP isoforms have been identified [89]. Expression analysis has shown that both ACP-1 and ACP-2 were expressed in *Cuphea* embryos. However, ACP-1, but not ACP-2, was also found to be highly expressed in the seed coat. Analyzing the developmental profile of caprate production showed that expression of ACP-2 corresponded to caprate production. In a continuation of this work, Schütt et al. [90] found ACP-1 and ACP-2 were able to support similar medium-chain-length-specific thioesterase activities. Thus, the function of ACP-2 during medium-chain fatty acid production was not likely to channel medium-chain fatty acids to the thioesterase, as was first proposed [89]. However, ACP-1 and ACP-2 do differ in their ability to support fatty acid biosynthesis activity. As measured by ^{14}C malonate incorporation, ACP-2 supported approximately twofold higher activity when compared with ACP-1 in *Cuphea*. These results, in combination with the medium-chain fatty-acid-specific condensing enzymes recently discovered, make it interesting to speculate on a role of ACP in acyl-ACP channeling. If the *Cuphea* ACP-2 is kinetically favored in the reaction with the medium-chain fatty acid condensing enzymes, the coordinate expression of ACP-2 with caprate production could provide for a tight control on medium-chain fatty acid production. Such control, as well as a better understanding and control of catabolic limitations, may be critical in attaining the highest possible levels of medium-chain fatty acid production.

5.2 Production of Unusual Monoenoic Fatty Acids

As a model system for understanding seed unusual fatty acid metabolism, our lab has focused on the production of unusual monoenoic fatty acids in transgenic oilseeds. Acyl-ACP desaturases are a class of soluble enzymes which function to add a double bond to an acyl group esterified to ACP. Since the discovery of the first stearoyl-ACP desaturase clones was reported [38,39], genes encoding various ''unusual'' acyl-ACP desaturases have been identified. These acyl-ACP desaturases have altered products and substrate specificity compared with the stearoyl-ACP desaturases. To date, ''unusual'' acyl-ACP desaturases have been isolated from coriander (*Coriandrum sativum*, Δ4 16:0-ACP desaturase), black-eyed susan vine (*Thunbergia alata*, Δ6 16:0-ACP), garden geranium (*Pelargonium* × *hortorum*, Δ9 14:0-ACP), milkweed (*Asclepias syriaca*, Δ9 16:0-ACP), and cats claw (*Doxantha unguis-cati*, Δ9 16:0-ACP desaturase) [44,91–94].

The discovery of the novel acyl-ACP desaturases has led to the realization of the potential application of these genes to modify plant oils for production of industrially useful monoenoic oils. One long-term goal of our project has been the development of oilseed varieties with tailored monoene composition. These ''specialty oil lines'' might eventually serve specific industrial or food applications (Table 2). However, production of the monoenes in transgenic plants has proven to be more complex than initially imagined. In all cases tested thus far, production of monoenes resulting from expression of the novel acyl-ACP desaturases has led to only low levels of the expected monoenes (Ref. 91 and Schultz et al., unpublished observations). To date, at least five unique fatty acid biosynthesis components have been identified which are likely specifically involved in monoene production in the native plant species. In addition to the complexity found in the fatty acid biosynthesis pathway, there will most likely be further surprises in mechanisms for incorporation of these unusual monoenes into TAG and/or exclusion from mem-

Table 2 Potential Food and Industrial Uses of Monoene ''Specialty Oils''

Desaturase	Monoene	Structure	m.p.[a]	Potential applications
Δ9 18:0-ACP	$18:1^{\Delta 9}$		12°C	Vegetable oil
Δ4 16:0-ACP	$16:1^{\Delta 4}$		?	?
	$18:1^{\Delta 6}$		33°C	Vegetable shortening/margarine; industrial polymers (nylon 6)
Δ6 16:0-ACP	$16:1^{\Delta 6}$		?	Industrial polymers (nylon 6)
	$18:1^{\Delta 8}$		23°C	Industrial polymers
Δ9 14:0-ACP	$14:1^{\Delta 9}$		4.5°C	Low-temperature lubricants
	$16:1^{\Delta 11}$		0–2°C	Low-temperature lubricants; industrial polymers (nylon 11)
	$18:1^{\Delta 13}$		27°C	Vegetable shortening/margarine; industrial polymers (nylon 13,13)

Note: Primary products of desaturase reaction may be further elongated so that multiple distinct monoenes can be obtained from a single desaturase reaction.
[a] m.p. = melting point.

brane lipids in oilseed crops. In this subsection, we have outlined the current status of monoene production in transgenic plants and have attempted to predict additional areas which may prove to be important to future work.

5.2.1 Can ACP Isoforms Serve as a Controlling Point in Fatty Acid Biosynthesis?

Acyl carrier protein is a small acidic protein that functions as a critical cofactor in plant fatty acid biosynthesis. An in-depth analysis of fatty acid biosynthesis genes in public databases has revealed the presence of at least five isoforms in *Arabidopsis* and rice [5]. Biochemical analyses have revealed the potential of ACP isoforms to differentially influence rates of key fatty acid biosynthetic steps. Initial studies of ACP isoforms in fatty acid biosynthesis revealed that ACP I and ACP II from spinach were not interchangeable in all reactions [95]. For oleoyl-ACP thioesterase and glycerol-3-phosphate acyltransferase reactions, the ACP isoforms had nearly identical V_{max} values in each reaction. However, ACP II was found to be a poor substrate in the thioesterase reaction, having a K_m value 10-fold higher than ACP I. In contrast, ACP I was found to be a poor substrate in the acyltransferase reaction, having a K_m value fivefold higher than ACP II. Both ACP I and ACP II were indistinguishable in malonyl-CoA:ACP transacylase reactions. These results implied the potential for ACP to at least partially mediate the flow of acyl groups within the fatty acid biosynthesis pathway.

As discussed earlier, ACP has also been found to potentially influence the activity of the condensing enzyme responsible for medium-chain fatty acid biosynthesis [90]. How-

ever, this study found that ACP had no influence on thioesterase activity. Recently, we have investigated the possible role of ACP in the production of novel monoenes in coriander and *Thunbergia alata* [96]. Competition assays were used to determine whether substrates generated with total seed ACP of either coriander or *T. alata* or substrates generated with *E. coli* ACP showed highest activity. In both systems, substrates prepared from the seed ACPs of the native species provided the highest activity of the unusual acyl-ACP desaturase. Subsequently, an ACP isoform (*Cs-ACP-1*) with predominantly seed-specific expression was isolated from coriander. Comparison of seed and leaf ACP isoforms to the Cs-ACP-1 isoform protein indicated that the predominate seed isoform and Cs-ACP-1 had the same mobility on sodium doceyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and may be the same protein. Further analysis revealed a nearly fivefold increase in Δ4 16:0-ACP desaturase activity when supplied with 16:0 substrate prepared with Cs-ACP-1 compared with spinach ACP I and nearly a 10-fold increase in activity when compared with *E. coli* ACP. Analysis of the coriander fatty acid biosynthesis system in malonyl-CoA labeling studies to assess the influence of Cs-ACP-1 in comparison with spinach ACP-1 and *E. coli* ACP on production of petroselinic acid ($18:1^{\Delta 6}$, the direct elongation product of $16:1^{\Delta 4}$) showed no differences in the ratios of $18:1^{\Delta 6}$ produced when compared with the activity of the Δ4 16:0-ACP desaturase with each ACP. This provides indirect evidence that Cs-ACP-1 does not act to influence the condensing enzyme responsible for the elongation of $16:1^{\Delta 4}$ to $18:1^{\Delta 6}$. In addition, thioesterase assays were conducted to determine the influence of CS-ACP-1. The results from this assay indicated no influence of CS-ACP-1 on thioesterase activity. Thus, the influence of Cs-ACP-1 is most likely a direct interaction with the desaturase, not with thioesterase or condensing enzymes.

5.2.2 Are Additional Components of the Fatty Acid Biosynthesis System Required for Production of Novel Monoenes?

The unusual monoenoic acyl-ACPs have distinct structures caused by the double-bond placement (Table 2). Thus, the ''common'' fatty acid biosynthesis enzymes may not function optimally when supplied with high levels of the unusual fatty acids. In addition, enzymes such as thioesterases and condensing enzymes could provide a partial mechanism for controlling production of the unusual monoenes in seeds. Dörmann et al. [97] were able to separate two distinct thioesterase activities in coriander and dill endosperm (both members of the Umbelliferae family, which produce petroselinic acid). One thioesterase showed higher activity with oleoyl-ACP and a second separable thioesterase showed highest activity with petroselinyl-ACP. Furthermore, the activity of both thioesterases was comparable. Additional research has revealed a condensing enzyme which is likely specific for the elongation of $16:1^{\Delta 4}$ to $18:1^{\Delta 6}$ exists in coriander endosperm. Assays in crude extracts of coriander endosperm have revealed the presence of a KAS specific for $16:1^{\Delta 4}$. Using a polymerase chain reaction (PCR) approach, a cDNA clone has been isolated and shown to be expressed predominantly in coriander endosperm [98]. Thus, key components of fatty acid biosynthesis, each with specificity for the unusual monoenes, may be needed for optimal production in transgenic oilseeds.

5.2.3 Ferredoxin Isoforms Further Influence the Rate of Acyl-ACP Desaturases

Initial characterization of the stearoyl-ACP desaturase revealed ferredoxin was required as an electron donor [36,99,100]. Furthermore, ferredoxin-reducing systems dependent on

either NADPH/ferredoxin $NADP^+$ reductase or on the photoreduction of ferredoxin when supplied with chloroplast lamellae functioned in the desaturase reaction [37]. However, ferredoxin from different sources did not support identical levels of desaturase activity. Studies by Nagai and Bloch [36,99] directly compared desaturase activity when supplied with spinach (*Spinacia oleracea*) and *Euglena* (*Euglena gracilis*) ferredoxins. In the presence of *Euglena* ferredoxin, the *Euglena* stearoyl-ACP desaturase was 10-fold more active than when supplied with the spinach ferredoxin. Furthermore, the spinach stearoyl-ACP desaturase was also found to be more active with *Euglena* ferredoxin than with spinach ferredoxin. In contrast, when the *Euglena* and spinach ferredoxins were tested in reactions as electron acceptors with *Euglena* NADPH oxidase and in reactions as electron donors in $NADP^+$ reduction by illuminated spinach chloroplasts, activities were found to be equivalent with both ferredoxins. Thus, the difference in stearoyl-ACP desaturase activity was not likely due to a general redox capacity of either ferredoxin, but was more likely due to specific interactions with the desaturase.

More recent work has shown the potential for ferredoxins to interact differentially with distinct acyl-ACP desaturases. In studies of petroselinic acid biosynthesis in coriander, Cahoon and Ohlrogge [101] found that the addition of spinach ferredoxin stimulated the activity of the Δ9 18:0-ACP desaturase (increased production of oleic acid by twofold), but it had no significant influence on the activity of the Δ4 16:0-ACP desaturase (production of petroselinic acid remained constant). In addition, when *Thunbergia* Δ9 18:0-ACP and Δ6 16:0-ACP desaturase assays were supplied with spinach ferredoxin, the activity of the Δ9 18:0-ACP desaturase was 1.7-fold higher than the Δ6 16:0-ACP desaturase [92]. This was surprising, as the seed composition of *Thunbergia* is greater than 80% [102] and, thus, the Δ6 16:0-ACP desaturase activity was expected to be higher than the Δ9 18:0-ACP desaturase. Recently, we have initiated research to determine the influence of ferredoxins on acyl-ACP desaturases and, more specifically, to find out if distinct ferredoxin isoforms are needed for optimal activity of the unusual acyl-ACP desaturases.

Ferredoxins occur in plants as one of two classes. Photosynthetic ferredoxins have been shown to be light regulated and restricted in distribution to photosynthetic tissues. In contrast, the nonphotosynthetic ferredoxins have been shown to be independent of light regulation and have a more ubiquitous tissue distribution [103,104]. Furthermore, ferredoxins of each class have been shown to support distinct levels of biochemical activity [105,106]. We have tested the activity of three ferredoxin types (photosynthetic, heterotrophic, and cyanobacterial) in *Thunbergia alata* acyl-ACP desaturase assays [107]. Using in vitro acyl-ACP desaturase assays to test the influence of ferredoxin source on Δ6 16:0-ACP and Δ9 18:0-ACP desaturase activities revealed the cyanobacterial and heterotrophic ferredoxins increased Δ6 16:0-ACP activity by up to 10-fold as compared to the photosynthetic ferredoxin. Most importantly, ferredoxin source influenced the relative activities of the desaturases. When supplied with photosynthetic ferredoxin, the Δ9 18:0-ACP desaturase was nearly twofold higher than the Δ6 16:0-ACP desaturase. In contrast, when supplied with either cyanobacterial or heterotrophic ferredoxins, the Δ6 16:0-ACP desaturases was found to be nearly twofold more active than the Δ9 18:0-ACP desaturase. Thus, a possible limitation to production of novel monoenes in transgenic oilseeds could be the type of ferredoxin available for acyl-ACP desaturation. We have initiated a project to isolate additional ferredoxin isoforms and testing the in vivo influence of ferredoxin isoforms on acyl-ACP desaturase activity.

5.2.4 Will TAG Biosynthesis by the Kennedy Pathway Function with Unusual Monoenes or Does an Alternative Pathway Including PC Exist?

Triacylglycerol production is covered in other chapters; thus, we will focus only on considerations pertinent to production of unusual monoenoic fatty acids. The biosynthesis of TAG in plants is often considered to occur by the sequential acylation of glycerol-3-phosphate to form phosphatidic acid followed by phosphatidate phosphatase action to yield diacylglycerol (DAG), which is acylated to form TAG [4]. However, to produce the common polyunsaturated fatty acids, $18:1^{\Delta 9}$ must be esterified to phosphatidylcholine (PC) for further desaturation prior to its incorporation into TAG [108]. Likewise, hydroxylated, epoxygenated, and acetylenic fatty acids also are synthesized while esterified to PC. In contrast, medium chain as well as the unusual monoenoic fatty acids require no further modification after their synthesis in the plastid and, thus, would not be expected to be incorporated into PC at appreciable levels. It has been speculated that a controlling point for exclusion of unusual fatty acids from membrane lipids resides in the specificities of diacylglycerol acyltransferase and choline phosphotransferase. However, Vogel and Browse [109] have analyzed the specificities of these enzymes and found that neither have sufficiently selective substrate specificities in vitro to account for the strong exclusion of unusual fatty acids (decanoic, ricinoleic, and erucic acid) from PC. In addition, studies of TAG biosynthesis in coriander and *Thunbergia alata* indicate a flux of unusual monoenes (most likely through the *sn*-2 position) during TAG biosynthesis [110,111]. Recently, an alternative mechanism for TAG biosynthesis has been proposed by Sten Stymne's group. This pathway involves the selective channeling of specific acyl groups through PC directly into TAG biosynthesis via a PC:DAG acyltransferase [112]. This work indicated that the PC channeling into TAG varies between distinct species. Thus, this newly discovered mechanism of TAG biosynthesis, in addition to the flux of unusual monoenoic fatty acids trough PC during TAG biosynthesis in *Thunbergia alata* and coriander [110,111], led to speculation that unusual monoenes may be targeted to TAG via this pathway. If this is the case, an additional limitation to production of unusual monoenes into TAG in transgenic oilseeds could be the correct targeting to TAG.

6 SUMMARY AND FUTURE TRENDS IN METABOLIC ENGINEERING PROJECTS: FUNCTIONAL GENOMICS

The identification of key genes as described earlier has occupied the resources of many academic and industrial laboratories for the last 5–10 years. However, it turns out that this may not be the most central problem in oilseed modifications. Table 3 indicates the limited success which has been achieved in plant metabolic engineering through the introduction of a single gene. In all cases where a newly identified gene has been transferred into rapeseed, soybean, or another species, the proportion of the desired product in the transgenic host has been considerably lower than in the wild species from which the gene was obtained. The activity of the introduced enzyme has generally not been limiting, so it is necessary to determine what other factors limit product accumulation. Research from several labs indicates that other enzymes involved in processing the new fatty acid and channeling it into the accumulated oil are important. It is also necessary to consider ways to prevent the selective breakdown of the new fatty acid or its accumulation in cellular membranes, which may impair the cell biology and physiology of the plant or seed.

Table 3 Challenge of Unusual Fatty Acid Production in Transgenic Oilseeds

Fatty acid	Level in native plant	Level in transgenic plant
$18:1^{\Delta 6}$ (petroselinic)	85%	<10%
$16:1^{\Delta 6}$	80%	<10%
$16:1^{\Delta 11}$ + $18:1^{\Delta 13}$	>80% derived products	<10%
Cyclopropane	40%	<5%
Ricinoleic	90%	17%
Acetylenic	70%	25%
Epoxy	60%	15%
Lauric (+ 10:0)	65% (+25%)	50–60%

Addressing these issues will require a far more complete knowledge of the cellular biochemistry in oil-accumulating tissues than is currently available. The goal of oilseed metabolic engineering research is shifting away from gene isolation toward the specific aim of increasing the yield of new fatty acid products in transgenic plants. Meeting this goal will necessarily require the application and integration of many biochemical and biological approaches. It is particularly urgent to develop a better understanding of the relationships among the subcellular organelles involved in oil synthesis and accumulation—particularly the plastids, endoplasmic reticulum, and the oil bodies in which triacylglycerols accumulate. This is a neglected field of research, in part because increased knowledge in this area has not seemed to promise improvement in existing oilseeds. However, for successfully producing unusual fatty acids in transgenic plants, these issues may have great importance. For example, it has been proposed that separate domains of the endoplasmic reticulum might be used for membrane phospholipid synthesis and for triacylglycerol synthesis. Evaluating this hypothesis is vital in deciding on strategies for ensuring that unusual fatty acids are channeled into the oil and excluded from the membrane lipids. Fortunately, new tools in cell biology, such as immuno-gold-labeled antibodies and tagged proteins, now permit these issues to be addressed and resolved.

6.1 Gene Discovery

The discovery of new genes and elucidation of their function is a major driving force of biological research today. The complete sequence of at least 30 organisms, including *Arabidopsis* and rice, is known today and others will be completely sequenced soon. However, these efforts will only begin to touch the immense biodiversity represented in the genomes of 400,000 plant species. Plants have evolved the biosynthetic reactions to produce over 25,000 different lipophilic structures. The combined germplasm within the plant kingdom represents a vast and 99% untapped reservoir of genes coding for biocatalysts that can produce many valuable chemicals. A major goal for the future will be to discover and exploit new biocatalysts. The advent of high-throughput automated DNA sequencing has created possibilities to rapidly discover new genes in a cost-effective manner. In particular, obtaining 1000–3000 partial cDNA sequences (expressed sequence tags, ESTs) from a tissue producing high levels of a desired compound has proven to be a very rich resource for the discovery of new genes. With current technology, one 96-capillary sequencer can

produce 5000 sequences of 600 bp within 10 days at a cost approximating a few months' salary of a technician. In the fatty acid field, this approach was successfully used to identify the fatty acid hydroxylase gene from castor seed [113], which produces the industrially valuable ricinoleic acid and the ''conjugase'' genes from *Momordica charantia* and *Impatiens balsamina*, which produce conjugated fatty acids such as eleostearic and parinaric acids [114].

6.2 DNA Microarray Discovery of Regulatory Genes

The general regulatory mechanisms which control how much protein, oil, or carbohydrate are produced in seeds are very poorly understood. As just one example, we do not understand the underlying mechanisms that result in soybean producing seeds with 40% protein and 20% oil, whereas canola produces seeds with 45% oil and 27% protein and maize produces seeds with 85% starch. As part of a functional genomic effort to better understand seed development, an effort is underway to produce a catalog of *Arabidopsis* genes expressed specifically in seeds. This will be accomplished with EST sequencing of seed cDNA libraries, DNA microarray technology, and the *Arabidopsis* genome sequence. Approximately 10,000 cDNA clones from a developing seed cDNA library have recently been partially sequenced and characterized [115] and microarray data are available describing the expression patterns of most of the unique sequences [116]. Bioinformatics analysis of sequences will identify candidate transcription factors and other regulatory proteins. DNA microarrays will then further be used to describe expression of candidates throughout seed development and in a battery of mutant and transgenic seeds altered in seed storage product accumulation. Insertional gene knockout collections of *Arabidopsis* will allow further characterizations of candidates.

6.3 Enzyme Engineering

Site-specific mutagenesis of polypeptides has already been successfully used by the Shanklin lab to change the substrate specificities of plant acyl-ACP desaturases and membrane fatty acid hydroxylases [43]. Analysis of sequence differences between native enzymes with differing substrate specificities was crucial in determining domain swaps and sites for site-specific mutagenesis in these endeavors. This technology, by changing enzyme specificity to produce a novel product or remove an unwanted side product, will complement the use of native genes in oilseed genetic engineering, and provide the ability to produce structures not present in nature.

7 SUMMARY

The Internet will play an increasingly important role in the future because the large datasets generated by functional genomics and other approaches cannot easily be presented in conventional publication formats. In fact, it is likely that >95% of microarray and large-scale DNA sequence information will only be available via specialized websites devoted to their collation. In Table 4, we have attempted to list a few of the Internet resources currently evolving in the field of oilseed metabolic engineering.

Metabolic engineering of fatty acid biosynthesis has led to promising initial advances in controlling lipid metabolism. Oil content has been increased, acyl composition has been tailored to meet specific needs, and production of unusual fatty acids has led to new products and has spurred additional areas of basic research. With the rapid expansion

Table 4 Internet Sites for Plant Lipid Biotechnology

	Internet resources related to oilseed metabolic engineering	
Organization	Website content	URL
National Plant Lipid Consortium (NPLC)	• Directory of scientists involved in plant lipid research • E-mail newsgroup for information on plant lipids • Abstracts of NPLC meetings	http://www.msu.edu/user/ohlrogge
Michigan State University	• Survey and catalog of genes for plant lipid metabolism	http://www.canr.msu.edu/lgc/index.html
	• Gene expression profiles based on seed microarrays	http://www.bpp.msu.edu/seed/seedarray.htm
Kathy Schmid, Butler University	• Links to many oilseed research labs and websites	http://trevor.butler.edu/~kschmid/lipids.html
Arabidopsis Functional Genomics Consortium (AFGC)	• Microarray and gene knockout facilities for *Arabidopsis*	http://afgc.stanford.edu
Benning and Ohlrogge Labs	• Database and analysis of >10,000 ESTs from developing *Arabidopsis* seeds	http://benningnt.bch.msu.edu
USDA Oilseed Database	• Data on fatty acid composition of seed of thousands of species	http://www.ncaur.usda.gov/nc/ncdb/search.html-ssi

of functional genomics, metabolic engineering projects may find the tools that will allow more complete control over the products produced in seeds. In addition, these techniques may provide the best and fastest alternatives to exploring underinvestigated areas such as regulatory factors which influence the partitioning of carbon between the major seed storage products.

REFERENCES

1. FJ van de Loo, BG Fox, C Somerville. In: TS Moore, Jr., ed. Lipid Metabolism in Plants. Boca Raton: CRC Press, 1993, pp 91–126.
2. GM Kishore, CR Somerville. Curr Opin Biotechnol 4:152–158, 1993.
3. B Hitz. Curr Opin Plant Biol 2:135–138, 1999.
4. EP Kennedy. Fed Proc Am Soc Exp Biol 3:2250–2254, 1961.
5. S Mekhedov, O Martinez de llráduya, JB Ohlrogge. Plant Physiol 122:389–401, 2000.
6. Y Sasaki, K Hakamada, Y Suama, Y Nagano, I Furusawa, R Matsuno. J Biol Chem 268: 25,118–25,123, 1993.
7. C Alban, P Baldet, R Douce. Biochem J 300:557–565, 1994.
8. MA Egli, BG Gengenbach, JW Gronwald, DA Somers, DL Wyse. Plant Physiol 101:499–506, 1993.
9. T Konishi, Y Sasaki. Proc Natl Acad Sci USA 91:3598–3601, 1994.
10. CG Kannangara, PK Stumpf. Arch Biochem Biophys 152:83–91, 1972.
11. BS Shorrosh, LJ Savage, J Soll, JB Ohlrogge. Plant J 10:261–268, 1996.
12. J-K Choi, F Yu, ES Wurtele, BJ Nikolau. Plant Physiol 109:619–625, 1995.
13. BS Shorrosh, KR Roesler, D Shintani, FJ van de Loo, J Ohlrogge. Plant Physiol 108:805–812, 1995.
14. BS Shorrosh, RA Dixon, JB Ohlrogge. Proc Natl Acad Sci USA 91:4323–4327, 1994.
15. KR Roesler, BS Shorrosh, JB Ohlrogge. Plant Physiol 105:611–617, 1994.
16. W Schulte, R Topfer, R Stracke, J Schell, N Martini. Proc Natl Acad Sci USA 94:3465–3470, 1997.
17. JB Ohlrogge, DN Kuhn, PK Stumpf. Proc Natl Acad Sci USA 76:1194–1198, 1979.
18. M Frentzen, E Heinz, TA McKeon, PK Stumpf. Eur J Biochem 129:629–636, 1983.
19. TA McKeon, PK Stumpf. J Biol Chem 257:12,141–12,147, 1982.
20. R Safford, JHC Windust, C Lucas, J de Silva, CM James, A Hellyer, CG Smith, AR Slabas, SG Hughes. Eur J Biochem 174:287–295, 1988.
21. L Hansen. Carls Res Communication 52:381–392, 1987.
22. JB Ohlrogge, TM Kuo. J Biol Chem 260:8032–8037, 1985.
23. A Hlousek-Radojcic, D Post-Beittenmiller, JB Ohlrogge. Plant Physiol 98:206–214, 1992.
24. KM Schmid, JB Ohlrogge. Plant Mol Biol 15:765–778, 1990.
25. M Voetz, B Klein, J Schell, R Topfer. Plant Physiol 106:785–786, 1994.
26. J Jaworski, D Post-Beittenmiller, J Ohlrogge, R Clough, A Matthis. In: N Murata, C Somerville, ed. Biochemistry and Molecular Biology of Membrane and Storage Lipids of Plants. Rockville, MD: American Society of Plant Physiologists, 1993, pp 96–101.
27. T Shimakata, PK Stumpf. Arch Biochem Biophys 217:144–154, 1982.
28. T Shimakata, PK Stumpf. Proc Natl Acad Sci USA 79:5808–5812, 1982.
29. JG Jaworski, RC Clough, SR Barnum. Plant Physiol 90:41–44, 1989.
30. M Siggaard-Andersen, S Kauppinen, P von Wettstein-Knowles. Proc Natl Acad Sci USA 88:4114–4118, 1991.
31. S Kauppinen. J Biol Chem 267:23,999–24,006, 1992.
32. M Wissenbach. Plant Physiol 106:1711–1712, 1994.
33. H Tai, JG Jaworski. Plant Physiol 103:1361–1367, 1993.
34. MB Slabaugh, H Tai, J Jaworski, SJ Knapp. Plant Physiol 108:443–444, 1995.

35. H Tai, D Post-Beittenmiller, JG Jaworski. Plant Physiol 106:801–802, 1994.
36. J Nagai, K Bloch. J Biol Chem 243:4626–4633, 1968.
37. BS Jacobson, JG Jaworski, PK Stumpf. Plant Physiol 54:484–486, 1974.
38. J Shanklin, C Somerville. Proc Natl Acad Sci USA 88:2510–2514, 1991.
39. GA Thompson, DE Scherer, S Foxall-Van Aken, JW Kenny, HL Young, DK Shintani, JC Kridl, VC Knauf. Proc Natl Acad Sci USA 88:2578–2582, 1991.
40. KJ Gibson. Biochim Biophys Acta 1169:231–235, 1993.
41. BG Fox, J Shanklin, C Somerville, E Münck. Proc Natl Acad Sci USA 90:2486–2490, 1993.
42. Y Lindqvist, W Huang, G Schneider, J Shanklin. EMBO J 15:4081–4092, 1996.
43. J Shanklin, EB Cahoon. Annu Rev Plant Physiol Plant Mol Biol 49:611–641, 1998.
44. EB Cahoon, S Shah, J Shanklin, J Browse. Plant Physiol 117:593–598, 1998.
45. EB Cahoon,Y Lindqvist, G Schneider, J Shanklin. Proc Natl Acad Sci USA 94:4872–4877, 1997.
46. EB Cahoon, CK Becker, J Shanklin, JB Ohlrogge. Plant Physiol 106:807–808, 1994.
47. L Löhden, M Frentzen. Planta 176:506–512, 1988.
48. JB Ohlrogge, WE Shine, PK Stumpf. Arch Biochem Biophys 189:382–391, 1978.
49. T Voelker. In: JK Setlow, ed. Genetic Engineering. New York: Plenum Press, 1996, pp 111–133.
50. DS Knutzon, JL Bleibaum, J Nelsen, JC Kridl, GA Thompson. Plant Physiol 100:1751–1758, 1992.
51. NM Loader, EM Woolner, A Hellyer, AR Slabas, R Safford. Plant Mol Biol 23:769–778, 1993.
52. A Jones, HM Davies, TA Voelker. Plant Cell 7:359–371, 1995.
53. X Bao, J Ohlrogge. Plant Physiol 120:1057–1062, 1999.
54. JL Harwood, US Ramli, RA Page, PA Quant. Biochem Soc Trans 27:285–289, 1999.
55. J Ohlrogge, J Browse. Plant Cell 7:957–970, 1995.
56. D Post-Beittenmiller, G Roughan, JB Ohlrogge. Plant Physiol 100:923–930, 1992.
57. T Shimakata, PK Stumpf. J Biol Chem 258:3592–3598, 1983.
58. D Post-Beittenmiller, JG Jaworski, JB Ohlrogge. J Biol Chem 266:1858–1865, 1991.
59. RA Page, S Okada, JL Harwood. Biochim Biophys Acta 1210:369–372, 1994.
60. DK Shintani, JB Ohlrogge. Plant J 7:577–587, 1995.
61. Y Sasaki, A Kozaki, M Hatano. Proc Natl Acad Sci USA 94:11,096–11,101, 1997.
62. LJ Savage, JB Ohlrogge. Plant J 18:521–527, 1999.
63. SC Hunter, JB Ohlrogge. Arch Biochem Biophys 359:170–178, 1998.
64. K Roesler, D Shintani, L Savage, S Boddupalli, J Ohlrogge. Plant Physiol 113:75–81, 1997.
65. KR Roesler, LJ Savage, DK Shintani, BS Shorrosh, JB Ohlrogge. Planta 198:517–525, 1996.
66. J Ke, JK Choi, M Smith, HT Horner, BJ Nikolau, ES Wurtele. Plant Physiol 113:357–365, 1997.
67. J Sun, J Ke, JL Johnson, BJ Nikolau, ES Wurtele. Plant Physiol 115:1371–1383, 1997.
68. D Shintani, K Roesler, B Shorrosh, L Savage, J Ohlrogge. Plant Physiol 114:881–886, 1997.
69. H-R Qian, B Fatland, BJ Nikolau, ES Wurtele. Altering the expression of the plastidic acetyl-CoA carboxylase with anti-sense RNA results in pleiotropic changes in Arabidopsis. National Plant Lipid Cooperative Meeting, South Lake Tahoe, 1999, p 14.
70. I Verwoert, KH van der Linden, MC Walsh, HJ Nijkamp, AR Stuitje. Plant Mol Biol 27: 875–886, 1995.
71. FM Brück, M Brummel, R Schuch, F Spener. Planta 198:271–278, 1996.
72. J Zou, V Katavic, EM Giblin, DL Barton, SL MacKenzie, WA Keller, X Hu, DC Taylor. Plant Cell 9:909–923, 1997.
73. DS Knutzon, GA Thompson, SE Radke, WB Johnson, VC Knauf, JC Kridl. Proc Natl Acad Sci USA 89:2624–2628, 1992.
74. DJ Hawkins, JC Kridl. Plant J 13:743–752, 1998.
75. MT Facciotti, PB Bertain, L Yuan. Nature Biotechnol 17:593–597, 1999.

76. WD Hitz, NS Yadav, RS Reiter, CJ Mauvais, AJ Kinney. In: J-C Kader, P Mazliak, eds. Plant Lipid Metabolism. Dordrecht: Kluwer Academic Publishers, 1995, pp 506–508.
77. Y Kang, B Min, H Park, K Lim, T Huh, S Lee. J Biochem Mol Biol 29:308–313, 1996.
78. MR Pollard, L Anderson, C Fan, DJ Hawkins, HM Davies. Arch Biochem Biophys 284: 306–312, 1991.
79. HM Davies, L Anderson, C Fan, DJ Hawkins. Arch Biochem Biophys 290:37–45, 1991.
80. TA Voelker, AC Worrell, L Anderson, J Bleibaum, C Fan, DJ Hawkins, SE Radke, HM Davies. Science 257:72–74, 1992.
81. TA Voelker, TR Hayes, AM Cranmer, JC Turner, HM Davies. Plant J 9:229–241, 1996.
82. VS Eccleston, AM Cranmer, TA Voelker, JB Ohlrogge. Planta 198:46–53, 1996.
83. MA Hooks, Y Fleming, TR Larson, IA Graham. Planta 207:385–392, 1999.
84. VS Eccleston, JB Ohlrogge. Plant Cell 10:613–621, 1998.
85. JM Leonard, MB Slabaugh, SJ Knapp. Plant Mol Biol 34:669–679, 1997.
86. MB Slabaugh, JM Leonard, SJ Knapp. Plant J 13:611–620, 1998.
87. JM Leonard, SJ Knapp, MB Slabaugh. Plant J 13:621–628, 1998.
88. K Dehesh, P Edwards, J Fillatti, M Slabaugh, J Byrne. Plant J 15:383–390, 1998.
89. J Kopka, M Robers, R Schuch, F Spener. Planta 191:102–111, 1993.
90. BS Schütt, M Brummel, R Schuch, F Spener. Planta 205:263–268, 1998.
91. EB Cahoon, J Shanklin, JB Ohlrogge. Proc Natl Acad Sci USA 89:11,184–11,188, 1992.
92. EB Cahoon, AM Cranmer, J Shanklin, JB Ohlrogge. J Biol Chem 269:27,519–27,526, 1994.
93. DJ Schultz, EB Cahoon, J Shanklin, R Craig, DL Cox-Foster, RO Mumma, JI Medford. Proc Natl Acad Sci USA 93:8771–8775, 1996.
94. EB Cahoon, SJ Coughlan, J Shanklin. Plant Mol Biol 33:1105–1110, 1997.
95. DJ Guerra, JB Ohlrogge, M Frentzen. Plant Physiol 82:448–453, 1986.
96. MC Suh, DJ Schultz, JB Ohlrogge. Plant J 17:679–688, 1999.
97. P Dörmann, M Frentzen, JB Ohlrogge. Plant Physiol 104:839–844, 1994.
98. S Mekhedov, E Cahoon, J Ohlrogge. Plant Mol Biol. In press.
99. J Nagai, K Bloch. J Biol Chem 241:1925–1927, 1966.
100. J Nagai, K Bloch. J Biol Chem 242:357–362, 1967.
101. EB Cahoon, JB Ohlrogge. Plant Physiol 104:827–837, 1994.
102. GF Spencer, R Kleiman, RW Miller, FR Earle. Lipids 6:712–714, 1971.
103. Y Kimata, T Hase. Plant Physiol 89:1193–1197, 1989.
104. T Hase, Y Kimata, K Yonekura, T Matsumura, H Sakakibara. Plant Physiol 96:77–83, 1991.
105. K Aoki, K Wada. Plant Physiol 112:651–657, 1996.
106. T Hase, S Mizutani, Y Mukohata. Plant Physiol 97:1395–1401, 1991.
107. DJ Schultz, MC Suh, JB Ohlrogge. Plant Physiol 124:681–692, 2000.
108. J Browse, C Somerville. Annu Rev Plant Physiol Plant Mol Biol 42:467–506, 1991.
109. G Vogel, J Browse. Plant Physiol 110:923–931, 1996.
110. EB Cahoon, JB Ohlrogge. Plant Physiol 104:845–855, 1994.
111. DJ Schultz, JB Ohlrogge. Plant Physiol Biochem 38:169–175, 2000.
112. A Dahlqvist, A Banas, S Stymne. In: J Sánchez, E Cerdá-Olmedo, E Martínez-Force, eds. Advances in Plant Lipid Research. Sevilla: Universidad de Sevilla, 1998, pp 211–214.
113. FJ van de Loo, P Broun, S Turner, C Somerville. Proc Natl Acad Sci USA 92:6743–6747, 1995.
114. EB Cahoon, TJ Carlson, KG Ripp, BJ Schweiger, GA Cook, SE Hall, AJ Kinney. Proc Natl Acad Sci USA 96:12,935–12,940, 1999.
115. JA White, J Todd, T Newman, N Focks, J Girke, O Martinez de Ilárduya, JG Jaworski, JB Ohlrogge, C Benning. Plant Physiol 124:1582–1594.
116. T Girke, J Todd, S Ruuska, J White, C Benning, J Ohlrogge. Plant Physiol 124:1570–1581, 2000.

2

Biochemistry and Biotechnology of Triacylgycerol Accumulation in Plants

RANDALL J. WESELAKE

University of Lethbridge, Lethbridge, Alberta, Canada

1 INTRODUCTION

A number of plants accumulate relatively large quantities of oil in their seeds or fruits. Plant oils are economically important in both food and industrial applications. For example, in Canada, 8.6 million metric ton of canola (consisting of cultivars of *Brassica napus* and *B. rapa*) were produced in 1999, which amounted to over 2 billion Canadian dollars at the farmgate. If canola seed oil were increased by 1%, it is estimated that this could result in an additional $25 million per year at Canada's farmgate [personal communication, D. Adolph, Canola Council of Canada, November 1999].

Triacylglycerols (TAGs) are the main component of seed oils. Recent developments in the biochemistry and molecular genetics of TAG biosynthesis in plants have begun to result in the implementation of molecular genetic strategies to increase seed oil content and to generate oils with desired fatty acid (FA) composition. Recent review articles have examined aspects of TAG biosynthesis and biotechnology [1–9]. This chapter focuses on recent advances in the enzymology and molecular genetics of TAG biosynthesis in the endoplasmic reticulum (ER) of developing oilseeds. Aspects of lipid biosynthesis in oleaginous plant cultures, leaves, and fruits will also be discussed because these investigations have advanced our understanding of seed oil biosynthesis. The chapter begins with a brief discussion of TAG accumulation in seeds and plant cultures. Characteristics of isoforms of lipid biosynthetic enzymes in the plastid are discussed. As well, the importance of cell and tissue culture in advancing the field will be emphasized, along with a discussion of TAG accumulation in developing pollen grains and environmental effects on TAG accumulation. Because the author is involved in research on TAG biosynthesis in oilseed rape, many of the examples in this chapter are based on recent research with this oilseed

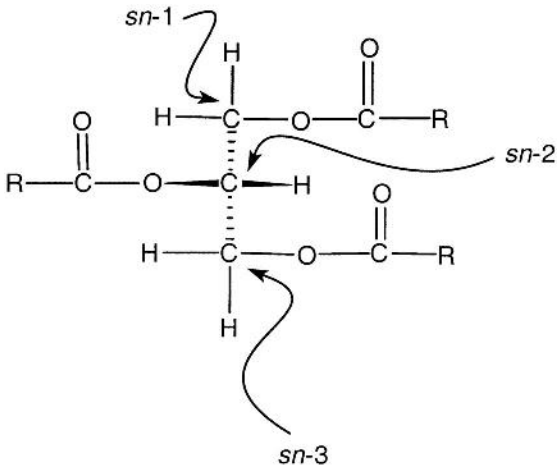


Figure 1 General structure of a triacylglycerol. R = fatty acyl chain minus the carboxyl group.

crop. The final section of the review will deal with recent advances in the genetic engineering of TAG accumulation in oilseed rape.

2 TRIACYLGLYCEROL STRUCTURE

The general structure of TAG is depicted in Figure 1. TAGs are composed of three FAs that are esterified to the hydroxyl groups of a glycerol backbone. Because glycerol lacks rotational symmetry, it is possible to distinguish the carbon atoms from each other [10]. A stereochemical numbering system is routinely used to identify the three positions on the glycerol derivative as *sn*-1, *sn*-2, and *sn*-3 from top to bottom with secondary hydroxyl to the left of the central carbon [11,12]. Methods are available for determining the positional distribution of FAs on the glycerol backbone [12,13]. Analyses of various seed oils have indicated that saturated FAs tend to occupy the *sn*-1 position, whereas unsaturated FAs are found at the *sn*-2 position [10,14]. More variable molecular species of FAs tend to be found at the *sn*-3 position.

3 TRIACYLGLYCEROL ACCUMULATION IN DEVELOPING SEEDS AND CULTURES

Biochemical studies with subcellular fractions from maturing seeds, such as safflower (*Carthamus tinctorius*), have increased our knowledge of plant lipid biosynthesis considerably [15]. Rapid TAG accumulation in developing seeds, however, occurs over a relatively narrow time frame compared to the total time required for plant development. Thus, the investigation of the biochemistry of TAG accumulation requires developing seeds obtained during the active phase of oil accumulation. Plants with developing seeds must be available at various stages of development in order to have a continuous supply of fresh tissue with high TAG biosynthetic capacity [10]. Depending on the study undertaken, it may be possible to freeze developing seeds for analyses in the future. TAG biosynthesis in seeds is not restricted to seed development but can also occur to some extent during seed germination [16]. In terms of genetic engineering of lipid biosynthesis in oilseed crops, species

such as *Brassica napus* can be readily transformed [17]. The use of seed-specific promoters, such as napin, has facilitated the expression of foreign cDNAs encoding lipid biosynthetic enzymes during seed maturation [18–20].

In contrast to developing seeds, cultures of oil-forming plants provide a more convenient and suitable source of tissue or cells for the study of lipid biosynthesis in plants. Extensive work on plant lipid metabolism has been conducted with cultures such as those derived from carrot (*Daucus carota*) [21–26], olive (*Olea europaea*) [27,28], and oilseed rape [29–32]. In most cases, however, plant cultures do not contain lipid levels that are comparable to those of oil-rich seeds and fruits [33–35]. As well, in the long term, plant cultures can become genetically unstable [36–38]. On the positive side, cell cultures are easy to maintain and TAG biosynthesis can be studied in the absence of complicating factors associated with cellular differentiation [9]. Techniques are available to obtain stable transformations of plant cell cultures [39,40] and, therefore, it should be possible to develop model systems to study genetically engineered alterations in lipid biosynthesis in plants.

Microspore-derived (MD) embryos of oilseed rape accumulate TAG levels that are comparable to the developing seed [9,41–45]. As well, the trend in lipid accumulation in MD embryos of oilseed rape is similar to zygotic embryos. The FA composition of zygotic and MD embryos are very similar at the very late-cotyledonary stage of development [42,46]. The proportion of TAG in total acyl lipid (TL) of late-stage MD embryos of *B. napus* L. cv Topas was shown to be about 90% [42], which was similar to that of the mature seed of a number of oilseed rape cultivars [47]. Because of the similarities in lipid accumulation characteristics to zygotic embryos, MD embryos have been used in numerous studies of the enzymology of TAG biosynthesis [1,9]. MD embryos of oilseed rape can be obtained on a continual basis and are relatively easy to maintain in culture. MD embryos are also potentially useful in breeding programs for screening FA composition of oilseed rape [43,46,48]. A portion of a single cotyledon from an MD embryo can be used to analyze FA composition with the remainder of the embryo available for regeneration of a mature plant [44,46,49]. The chromosome number of plantlets generated from MD embryos can be doubled by treatment of the roots with colchicine [50–52]. The resulting dihaploids can shorten the breeding cycle and are useful in detection of recessive mutations [43,53,54]. It is possible to transform MD embryos using both *Agrobacterium*-mediated DNA transfer [55,56] and microprojectile bombardment [57–59]. The reader is referred to recent reviews [1,9] which discuss in detail the characteristics of MD embryos and their use in studies of lipid biosynthesis.

4 TRIACYLGLYCEROL BIOSYNTHESIS

In developing oilseeds, TAG biosynthesis is catalyzed by membrane-bound enzymes of the ER [9,15,60]. A generalized scheme for TAG biosynthesis in developing seeds involving the Kennedy pathway [61] and other reactions is depicted in Figure 2. Most of the enzymes of TAG biosynthesis use activated FAs or fatty acyl-CoAs as cosubstrates for donation of the fatty acyl moieties to a glycerol backbone. Oleic (18:1) and palmitic (16:0) acid are produced from bicarbonate and acetyl-CoA via the catalytic action of prokaryotic acetyl-CoA carboxylase (ACCase, E.C. 6.4.1.2) and the FA synthase complex, which are localized to the plastid [66]. Biosynthesis of the FAs occurs while they are attached to acyl carrier protein (ACP) via a thioester linkage [4]. A soluble Δ^9-stearoyl-ACP desaturase (E.C. 1.14.99.6) that is dependent on reduced ferridoxin and molecular

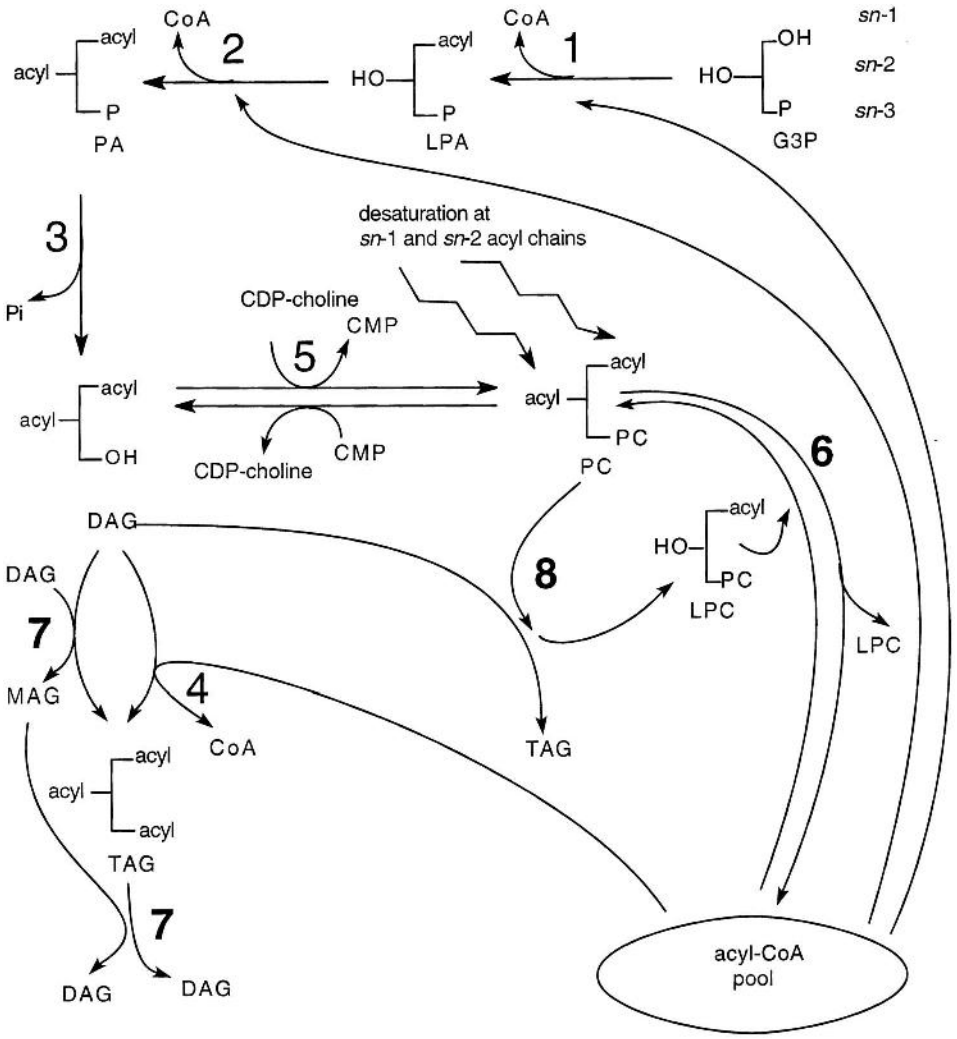


Figure 2 Generalized scheme for triacylglycerol biosynthesis in developing oleaginous seeds. Enzymes: (1) *sn*-glycerol-3-phosphate acyltransferase, (2) lysophosphatidate acyltransferase, (3) phosphatidate phosphatase, (4) diacylglycerol acyltransferase, (5) CDP-choline:1,2-diacylglycerol cholinephosphotransferase, (6) lysophosphatidylcholine acyltransferase, (7) diacylglycerol transacylase, (8) phospholipid:diacylglycerol acyltransferase. (Adapted from Refs. 15 and 62–65.)

oxygen catalyzes the introduction of the first double bond in FAs in plants [67]. Acyl-ACP hydrolase (E.C. 3.1.2.14) catalyzes the release of the FAs from ACP [5,68] and, thereafter, the FAs are exported from the plastid and then converted to fatty acyl-CoAs via the catalytic action of acyl-CoA synthetase (E.C. 6.2.1.3) [69,70]. In seeds producing very long-chain FAs, further elongation beyond 18 carbons involves the action of an extraplastidial elongation system [71–73]. A cytosolic form of ACCase (the eukaryotic form) appears to catalyze the formation of malonyl-CoA for use in FA elongation [4,74].

sn-Glycerol-3-phosphate (G3P), the glycerol backbone used in TAG biosynthesis in the ER, is mainly derived from dehydrogenation of dihydroxyacetone phosphate catalyzed by L-glycerol-3-phosphate:NAD^+ oxidoreductase (E.C. 1.1.1.8) [15,75]. The glycerol backbone of G3P undergoes three acylations catalyzed by acyltransferases [15] (Fig. 2). The final acylation of *sn*-1,2-diacylglycerol (DAG) occurs after removal of the phosphate group from the *sn*-3 position of the glycerol backbone. The DAG backbone component for some membrane lipids is also synthesized via this pathway. Phosphatidylcholine (PC) can be formed via donation of a phosphocholine group to the *sn*-3 position of *sn*-1,2-DAG [15]. FA groups on PC can undergo further desaturation and the FA group at the *sn*-2 position may undergo acyl-exchange with the fatty acyl-CoA pool in the cytosol, thereby increasing the proportion of unsaturated FAs in this pool [15]. FAs desaturated at the level of PC can also be introduced into TAG via removal of the phosphocholine group from PC to generate *sn*-1,2-DAG. Recent studies have shown that formation of TAG in developing oilseeds can also occur by transfer of an acyl moiety between two molecules of DAG [62,63] and from acyl moieties at the *sn*-2 position of PC [64,65]. It also appears to be possible to form two DAGs from TAG and monoacylglycerol (MAG), thereby providing an opportunity to remodel TAG [63]. Many of the studies on TAG biosynthesis have involved enzyme assays with particulate fractions from developing seeds and MD embryos. Other studies have involved feeding radiolabeled precursors to tissue slices, embryo homogenates, or particulate fractions to gain insight into the properties of one or more TAG biosynthetic enzymes (e.g., Refs. 15, 41, 62, 63, and 76–80).

4.1 *sn*-Glycerol-3-phosphate Acyltransferase

Microsomal *sn*-glycerol-3-phosphate acyltransferase (GPAT, E.C. 2.3.1.15) catalyzes the transfer of a fatty acyl moiety from an acyl-CoA to the *sn*-1 position of G3P to generate lysophosphatidate (LPA) (Fig. 2) [15,81]. Microsomal GPAT activity has been characterized in particulate fractions from various sources [81], including germinating castor bean (*Ricinus communis*) [82], germinating soybean (*Glycine max*) [83], spinach (*Spinacia oleracea*) [84], etiolated pea (*Pisum sativum*) seedlings [84], and various developing seeds, including safflower [85,86], *Cuphea* spp. [87,88], nasturtium (*Tropaeolum majus*) [89], meadowfoam (*Limnanthes* spp.) [89], and oilseed rape (*B. napus* and *B. rapa*) [86,90]. Acyl-CoA specificity and selectivity studies of the microsomal enzyme from a high-erucic-acid cultivar of *B. napus* showed similar activities with palmitoyl- and oleoyl-CoA but decreased activity with erucoyl-CoA [90]. The FA composition at the *sn*-1 position of TAG mainly depended on the acyl-CoA mixture presented to the enzyme.

Microsomal GPAT has been solubilized and partially purified from spinach leaves [84] and avocado (*Persea americana*) fruit mesocarp [91]. Effective solubilization of spinach microsomal GPAT was dependent on a combination of 3% (w/v) Tween-40 and 0.8 *M* NaCl [84]. Solubilization altered the positional specificity of spinach GPAT but not the acyl-CoA specificity. GPAT from avocado mesocarp was solubilized using 0.25% (w/v) CHAPS (3-[(3-cholamidopropyl)-dimethylammonio]-1-propane-sulfonate) and purified 150-fold by chromatography on immobilized glycerophosphoryl-ethanolamine [91]. The solubilized enzyme could not be frozen without loss of activity but was stable for at least 1 day at 4°C. Solubilization lowered the pH optimum of avocado GPAT and concentrations of oleoyl-CoA beyond 50 μ*M* resulted in substantial inhibition of the solubilized enzyme. Proteinase treatment of microsomal vesicles suggested that the active site of

avocado GPAT is located on the cytoplasmic side of the ER. The microsomal form of GPAT, however, has not been purified to homogeneity nor has its cDNA been cloned from any source.

4.2 Lysophosphatidate Acyltransferase

1-Acyl-*sn*-glycerol-3-phosphate acyltransferase or lysophosphatidate acyltransferase (LPAAT, E.C. 2.3.1.51) catalyzes the transfer of an FA from an acyl donor to LPA to generate phosphatidate (PA) [15]. Microsomal LPAAT also uses acyl-CoA as the FA donor [81]. LPAAT activity has been characterized in microsomal fractions from a number of sources [81], including germinating castor bean [82], spinach leaves [92], etiolated shoots of peas [93], and various developing seeds, including safflower [86,94], maize (*Zea mays*) [95], palm (*Butia capitata* and *Syagrus cocoides*) [95–97], oilseed rape [86,90,95,97–99], meadowfoam [89,96,99,100], nasturtium [89], *Cuphea* spp. [87,88], and soybean [101]. Microsomal LPAAT from oilseed rape has been shown to discriminate against erucoyl-CoA and lauroyl-CoA in in vitro assays [81,90,95]. The low specificity for erucoyl moieties is in agreement with the absence of erucic acid (22:1) at the *sn*-2 position of TAG in the seed of *B. napus* [14]. Certain varieties of *B. oleracea*, however, have been shown to have appreciable proportions of 22:1 at the *sn*-2 position of TAG [102]. The fatty acyl moiety at the *sn*-1 position of LPA has been shown to be important in determining the acyl preference of LPAAT [97]. Microsomal LPAAT from maize and oilseed rape exhibited a high specificity for LPA and acyl-CoA with 18:1 acyl chains compared to lauroyl (12:0) acyl chains.

1-Acyl-*sn*-glycerol-3-phosphate acyltransferase has been partially purified from microsomes of developing seeds [98] and MD embryos [99] of oilseed rape. The enzyme has also been partially purified from immature coconut (*Cocos nucifera*) endosperm [103]. High concentrations of NaCl in the presence of CHAPS had to be included in the solubilization buffer and chromatography buffers. As indicated in Section 3.1, a high salt concentration in the presence of detergent was also required for solubilization of spinach microsomal GPAT [84]. It was also necessary to reactivate coconut LPAAT with exogenous phospholipid following chromatography [103]. In a follow-up study, the enzyme was partially purified to the extent that the preparation was enriched with a 29-kDa polypeptide [104]. The polypeptide was electroblotted onto nitrocellulose for amino acid sequencing in order to develop a probe for use in cloning the first LPAAT.

A number of cDNA clones encoding plant LPAAT have been isolated from five plant species [104–109]. Six of the cDNA clones have been shown to encode a microsomal LPAAT. The clone identified as *pRAT1* encoded a microsomal LPAAT with a substrate preference that matched the microsomal enzyme from oilseed rape [105]. The deduced amino acid sequences of these plant LPAAT cDNA clones have been compared to those of other organisms, and alignments have indicated a conservation of two regions (Box1 and Box2) relative to their sequence and spacing [9,110]. The spacing between these two boxes was also highly conserved. Invariant amino acid residues within these boxes may be critical for acyltransferase activity. Site-directed mutagenesis experiments have been performed to identify specific residues involved in the catalytic mechanism of LPAAT [110]. For example, substitution of the glutamate residue in Box2 (FP/VEGTR) with glutamine, histidine, or alanine residues resulted in nonactive recombinant LPAAT. It was suggested that the invariant glutamate residue could be involved in the positioning and stabilization of the enzyme's substrates.

4.3 Phosphatidate Phosphatase

Phosphatidate phosphatase (also called PA phosphohydrolase) (E.C. 3.1.3.4) catalyzes the hydrolysis of PA to generate *sn*-1,2-DAG and P_i [15,111] (Fig. 2). Microsomal PA phosphatase has been characterized in extracts and subcellular fractions of developing peanuts (*Arachis hypogaea*) [112], safflower seeds [15,77,78,113,114], developing seeds and MD cultures of oilseed rape [111,115–117], and the mesocarp of avocado [118]. PA phosphatase may have a rate-limiting role in TAG biosynthesis [15,111].

Safflower PA phosphatase activity in microsomes has been shown to exhibit a broad selectivity for unsaturated PA species, suggesting that the enzyme reaction probably has little effect on the FA composition of TAG in the oil from this plant [77,114]. As well, safflower PA phosphatase has been shown to translocate from the soluble fraction to microsomes in response to increased oleate concentration, suggesting that TAG production may be regulated by a feedforward control mechanism involving this FA [119]. PA phosphatase was previously shown to translocate from the cytosolic to microsomal fraction in mammalian systems [120]. Microsomal PA phosphatase activity has been shown to be maximal during the active phase of TAG accumulation in developing seeds of groundnut [112] and safflower [119]. Total PA phosphatase activity (soluble and membrane bound) was also greatest at this time in safflower [119], but as oil levels reached a plateau, the amount of membrane-bound PA phosphatase activity was negligible. The pH optimum for the activity of microsomal PA phosphatase from safflower was 6.7, but the pH dependence of the soluble form of the enzyme was not reported [119]. Microsomal PA phosphatase from safflower exhibited an absolute requirement for Mg^{2+} [78,119].

Phosphatidate phosphatase activity has been shown to be associated with all fractions obtained following differential centrifugation of homogenates from both developing seeds and MD cultures of oilseed rape [115]. A relatively small proportion (10–20%) of the recovered enzyme activity was associated with the 10,000–100,000*g* particulate fraction (microsome) in each case. Because the ER was the major site of TAG biosynthesis, it was reasonable to assume that the PA phosphatase involved in this process was localized to the microsomal fraction. Microsomal PA phosphatase from developing seeds of oilseed rape (*B. napus* L. cv Westar) has been shown to have a pH optimum of 6–7 [115]. The soluble fraction contained 36% of the total PA phosphatase activity recovered, following differential centrifugation of homogenate of developing seeds. The soluble PA phosphatase exhibited a pH optimum of 5, suggesting that it was a different PA phosphatase or perhaps a nonspecific acid phosphatase. PA phosphatase activity in microsomes from MD embryos of *B. napus* L. cv Topas was inhibited 50% following preincubation with 0.5 m*M* *N*-ethylmaleimide (NEM), a thiol-reactive agent [111,116]. Microsomal PA phosphatase activity from MD embryos exhibited a partial dependence on Mg^{2+} [116]. PA phosphatase activity in microsomes from MD cell suspension cultures of *B. napus* L. cv. Jet Neuf was also partially inhibited following preincubation with NEM [116]. The responses of microsomal PA phosphatase activity of MD embryos to Mg^{2+} and NEM implicated similarities to mammalian systems. An isoform of the enzyme from mammals, known as PA phosphatase-1, is involved in glycerolipid synthesis. It is sensitive to NEM and dependent on Mg^{2+} for activity [111,120,121]. PA phosphatase-1 is believed to accumulate in the cytosol and become functionally active in glycerolipid synthesis following translocation to the ER [120]. FAs were involved in mediating this translocation in a fashion similar to that described for safflower PA phosphatase [119]. The isoform of the enzyme in mammals known as PA phosphatase-2 is associated with the plasma membrane, insensitive to NEM,

and independent of Mg^{2+} for activity [122]. The second isoform of the enzyme is associated with the plasma membrane and appears to play a role in signal transduction [111,122–124]. The observations on the properties of PA phosphatase activity in membrane fractions of MD cultures of oilseed rape suggested that this plant system may also contain two forms of the enzyme. It is also possible that microsomal PA phosphatase from oilseed rape is only partially inhibited by NEM.

Microsomal PA phosphatase has been solubilized from MD embryos of oilseed rape [115] and mesocarp of avocado [118]. The most effective solubilization of microsomal PA phosphatase from MD embryos of oilseed rape was achieved using Tween-20 at a concentration of 0.4% (w/v) at a detergent:protein ratio of 1:1 (w/w). Solubilized PA phosphatase from MD embryos displayed a pH optimum similar to the membrane-bound enzyme from developing seeds of oilseed rape [115]. The solubilized microsomal PA phosphatase lost about 60% of its activity following storage at 4°C for 24 hr [116]. Inclusion of bovine serum albumin (BSA) (10 mg/mL) in the storage buffer, however, enhanced the stability of the solubilized enzyme [116,117]. Solubilized microsomal PA phosphatase from MD embryos of *B. napus* L. cv Topas has been shown to catalyze the release of P_i from a number of phosphate-containing compounds [115]. The solubilized enzyme catalyzed the hydrolysis of dioleoyl-PA at twice the rate of dierucoyl-PA. The ability to utilize dierucoyl-PA suggested that genetic engineering experiments designed to enhance the 22:1 content in seed oil were not likely to be restricted by the substrate preference of endogenous PA phosphatase. Although solubilized PA phosphatase dephosphorylated a number of phosphate-containing compounds, its organization in the membrane relative to other TAG-biosynthetic enzymes may limit it to primarily accepting PA generated via the catalytic action of microsomal LPAAT [115]. Gel filtration chromatography on Superose 6 (HR 10/30 column, Pharmacia Biotechnology) of solubilized microsomal PA phosphatase from MD embryos resulted in the elution of enzyme activity throughout most of the sieving range of the gel [115]. The eluted PA phosphatase, however, displayed a minimum molecular mass of 40 kDa, which was similar to the native molecular mass of 45–55 kD reported for avocado PA phosphatase [118]. Anion-exchange chromatography on Macro Prep 50Q of solubilized PA phosphatase from MD embryos resulted in unbound enzyme activity and activity that was eluted with 0.4 *M* NaCl [116]. Anion-exchange chromatography did not resolve NEM-sensitive and NEM-insensitive forms of the enzyme.

Microsomes from maturing avocado mesocarp have been shown to contain only NEM-insensitive PA phosphatase that was not dependent on Mg^{2+} [118]. Although this implicated a PA phosphatase involved in signal transduction, the enzyme likely has a role in glycerolipid synthesis because avocado accumulates high levels of TAG. PA phosphatase from avocado has been purified 7000-fold from microsomes by solubilization with CHAPS, Q Sepharose chromatography, Affi-Gel Blue chromatography, ammonium sulfate fractionation, and Phenyl Superose chromatography [118]. The enzyme was solubilized from the membrane fraction sedimenting at 30,000–100,000*g* using 0.5% (w/v) CHAPS at a microsomal protein concentration of 5 mg/mL. The molecular mass of the purified avocado PA phosphatase was 49 kDa based on sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE). Gel filtration chromatography indicated that the native enzyme was a monomer. The surface dilution kinetic model previously applied to phospholipase A_2 [125] was used in kinetic analyses of the purified enzyme using PA and LPA in Triton X-100 mixed micelles [118]. The phospholipids *sn*-1-LPA, *sn*-2-LPA, and ceramide-1-phosphate were all utilized by the enzyme, and *sn*-1-LPA was a better substrate than PA. Similar to microsomal PA phosphatase from MD embryos of oilseed rape

[115], the avocado enzyme utilized phosphate-containing compounds other than PA. The investigators suggested that metabolic channeling for TAG biosynthesis may occur in the ER, thereby preventing LPA from interacting with PA phosphatase [118]. Sequencing of avocado PA phosphatase or production of anti-PA phosphatase antibodies should provide probes for use in obtaining the encoding cDNA.

4.4 Diacylglycerol Acyltransferase

Microsomal diacylglycerol acyltransferase (DGAT) (E.C. 2.3.1.20) catalyzes the transfer of a fatty acyl moiety from an acyl-CoA to the *sn*-3 position of *sn*-1,2-DAG to generate TAG [15] (Fig. 2). TAG accumulation and DGAT activity have been shown to occur in association with the ER [15,60,81,126–128]. The properties of microsomal DGAT have been examined in a number of plant systems [9,81], including developing seeds of safflower [129,130], oilseed rape [90,131,132], sunflower (*Helianthus annuus*) [133], castor bean [126,133], peanuts [112,126], soybean [126], maize [126,132], *Cuphea* spp. [132,133], and palm (*Elaeis guineenis*) [134]. DGAT activity has also been found in germinating soybean [127,135]. As well, the enzyme has been extensively characterized in MD cultures of oilseed rape [9,31,32,80,116,136–140].

Investigations with developing seeds [130,141–143] and mammalian systems [144,145] have suggested that the DGAT-catalyzed reaction may be rate limiting in TAG biosynthesis. For example, significant quantities of DAG are produced in developing seeds of *B. napus* during the active phase of oil accumulation, suggesting that the DGAT-catalyzed reaction may restrict the flow of carbon into TAG [141,142]. As well, a chemically induced mutant (AS11) of *Arabidopsis thaliana* has been identified with decreased DGAT activity and TAG accumulation and an increased DAG pool [146]. As indicated in Section 4.3, there is also some evidence that PA phosphatase may have a rate-limiting role in TAG accumulation [15,147]. A recent study with developing embryos of *Cuphea lanceolata*, *Ulmus carpinifolia*, and *Ulmus parvifolia* has shown that the supply of FAs can be a limiting factor in TAG accumulation [148]. A developmental profile for DGAT specific activity and activity per seed in *B. napus* L. cv Westar, as a function of days after flowering, is depicted in Figure 3 [31]. Enzyme activities are shown in relation to the lipid content and dry weight of seeds. DGAT activity increased substantially during the rapid phase of oil accumulation and then decreased markedly as the oil content of the seed reached a plateau. This observation was corroborated by another study which also demonstrated that LPAAT and GPAT activity displayed similar activity profiles during seed development in *B. napus* [149]. Microsomal DGAT and PA phosphatase also reached a maximum during the active phase of oil accumulation in developing peanuts [112]. Similar results were reported for microsomal PA phosphatase from developing safflower seeds [119]. In developing seeds of *B. napus*, the components of the FA synthase complex are largely induced before TAG production and their activities remain elevated when the rate of seed lipid accumulation decreases [150–152]. These observations imply that the TAG-biosynthetic pathway of the ER is more tightly regulated than the reactions of the FA synthase complex.

The substrate specificity/selectivity properties of microsomal DGAT have been examined in a number of developing seeds and cultures. DGAT is most reliably assayed using radiolabeled acyl-CoA as a substrate. Assays that are not based on the incorporation of radiolabeled acyl moieties, derived from acyl-CoA, into TAG may be subject to possible interference by transacylase activity [63]. Safflower DGAT has been shown to display a

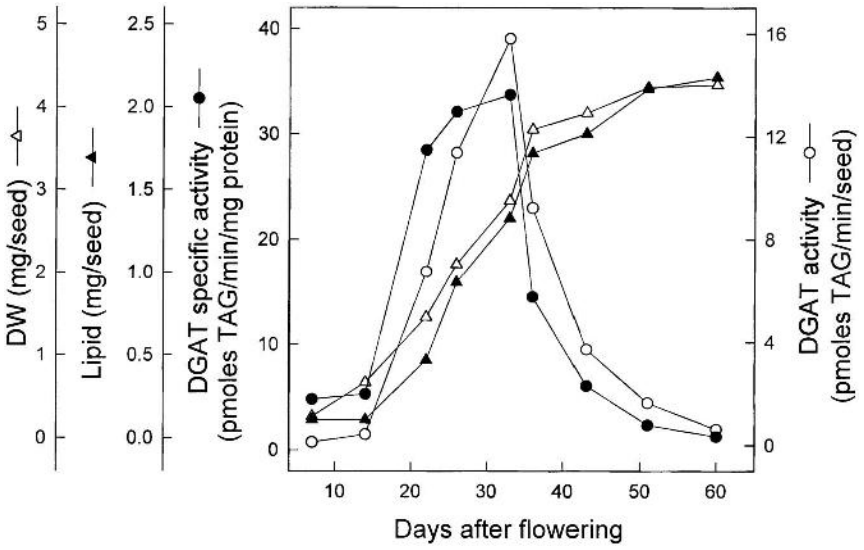


Figure 3 DGAT activity, lipid content, and dry weight (DW) of maturing seeds of oilseed rape (*B. napus* L. cv. Westar). Groups of 25 seeds, obtained from plants grown in the field at the Lethbridge Research Centre (Agriculture and Agri-Food Canada), were used for each type of analysis. Enzyme assays were conducted with 14 μ*M* [1-^{14}C]oleoyl-CoA as the donor substrate and 10 μL of seed homogenate. (From Ref. 31.)

broad specificity for *sn*-1,2-DAGs, including those containing saturated and unsaturated fatty acyl moieties [129]. Soluble *sn*-1,2-DAGs (diacetylglycerol and dibutyrylglycerol) and *sn*-1,2-distearoylglycerol, however, were ineffective as substrates. The safflower microsomal enzyme was also active with a range of saturated and unsaturated acyl-CoAs. The investigators suggested that the FA composition at the *sn*-3 position of TAG was dependent on the nature of the endogenous acyl-CoA pool. Cao and Huang [132] reported that microsomal DGAT from low- and high-erucic-acid cultivars of oilseed rape exhibited essentially identical acyl-CoA preference. The highest activities were found using lauroyl-CoA, and the two enzymes were more active with oleoyl-CoA than erucoyl-CoA at concentrations above 10 μ*M* acyl-CoA. The results implied that the gene encoding DGAT was not affected during the breeding process used to obtain cultivars that differed greatly in 22:1 content. Instead, the 22:1 content probably reflected the elongation capability of the cultivar. In contrast, microsomal DGAT from a cultivar of *B. napus* containing 54% 22:1 in the seed oil has been shown to exhibit enhanced specificity for erucoyl-CoA over oleoyl-CoA [90]. In the same study, however, a cultivar containing 35% of 22:1 in the seed oil showed no difference in preference for erucoyl-CoA or oleoyl-CoA. Microsomal DGATs from MD embryos of low- and high-erucic-acid cultivars of *B. napus* have both been shown to display enhanced activity with erucoyl-CoA over oleoyl-CoA at substrate concentrations greater than 5 μ*M* [136]. Microsomal DGAT from MD embryos of a high-erucic-acid cultivar of oilseed rape (*B. napus* L. cv Reston) could utilize *sn*-1,2-dierucin, indicating that the introduction of 22:1 into the *sn*-2 position of TAG in developing seeds via genetic engineering would provide a substrate that was usable by DGAT [137]. Specificity studies with microsomal DGAT and *sn*-1,2-DAG, however, are subject to possible interference by endogenous DAG. DGAT activity in microsomes from various plant sys-

tems has been shown to be dependent to varying extents on endogenous DAG [126,136–138,140,153]. Endogenous DAG has been shown to be present in solubilized preparations of DGAT [138,153], and the native enzyme may be dependent on DAG for stabilizing the activity [153].

The effect of a number of compounds on microsomal DGAT activity from MD cultures of oilseed rape has been investigated. Fluoride salts have been shown to enhance DGAT activity twofold to threefold in the 1500–100,000*g* particulate fraction from MD embryos of oilseed rape [138]. BSA at a concentration of 3–4 mg/mL reaction mixture has been shown to stimulate DGAT activity of microsomes of MD embryos by fourfold to fivefold [138]. BSA may prevent the formation of acyl-CoA micelles that are believed to be inhibitory [154] and/or bound acyl-CoAs may be better substrates for acyltransferases [155]. Microsomal DGAT activity from MD cell suspension cultures of oilseed rape (*B. napus* L. cv Jet Neuf) has been shown to be stimulated by inorganic divalent ions (e.g., Mg^{2+}), PA, CoA, ATP, and an unidentified organic factor(s) [140,156]. The stimulatory effects of CoA and ATP were decreased when $MgCl_2$ was present in the reaction mixture. PA enhanced DGAT activity by about twofold at a bulk concentration of 500 μ*M*. This was in agreement with the stimulation of DGAT from *Mortierella ramanniana* by PA [157]. Studies with mammalian DGAT have suggested that the enzyme is inhibited by phosphorylation involving the catalytic action of a cytosolic DGAT-kinase [158,159]. These observations prompted an investigation of a similar regulatory mechanism in the MD cell suspension culture [140]. Incubation of the cytosolic fraction (100,000*g* supernatant) and microsomes from the cell suspension culture in the presence of ATP and Mg^{2+} did not result in a decrease in DGAT activity. Instead, the investigations led to the discovery of a small organic molecule(s) that stimulated the enzyme.

The DGATs have proven to be very difficult to purify. The first report on the solublization of a plant DGAT was in 1986 [135]. The enzyme was solubilized from microsomes prepared from germinating soybean cotyledons using CHAPS and was further purified by gel filtration chromatography and agarose gel electrophoresis [135]. Later, DGAT was solubilized from the 1500–100,000*g* particulate fraction from MD cultures of oilseed rape using a combination of 1% (w/v) octanoyl-*N*-methylglucamide (MEGA-8) and 2 *M* NaCl at a detergent:protein ratio of 2:1 (w/w) [138]. As indicated previously, a high salt concentration combined with detergent was required to solubilize GPAT from spinach leaves [84] and LPAAT from immature coconut endosperm [103]. Gel filtration chromatography of DGAT solubilized from MD embryos of oilseed rape on Superose 6, in the presence of 0.1% MEGA-8 and 2 *M* NaCl, resulted in an enzyme of increased specific activity eluting with a molecular mass of about 2×10^6 Da [138]. DGAT may have associated with itself and other hydrophobic proteins to form a large complex. Solubilized DGAT from germinating soybean has been shown to display a similar high molecular mass during gel filtration chromatography [135]. Both membrane-bound and solubilized forms of DGAT from MD embryos have been shown to lose about 25–30% of the initial activity following storage at 4°C for 3 days [116,139]. Manipulation of the enzyme by chromatography, however, resulted in a more rapid deactivation. Incorporation of 10% (w/v) BSA into storage solutions enhanced the stability of solubilized DGAT [117]. Both membrane-bound and solubilized DGAT from MD embryos exhibited a neutral pH optimum [136,138]. The solubilized enzyme had a greater preference for oleoyl-CoA and palmitoyl-CoA over stearoyl-CoA at thioester concentrations above 2 μ*M* [138].

Kamisaka et al. [160] have purified a DGAT to apparent homogeneity from the lipid body fraction of the oleaginous fungus, *Mortierella ramanniana* var. *angulispora*. The

enzyme exhibited a molecular mass of 53 kDa based on SDS-PAGE. In addition to *sn*-1,2-DAG, the enzyme could utilize *sn*-2-monooleoylglycerol as a substrate. Recently, Lardizabal et al. [161] purified two other isoforms of DGAT from *Mortierella ramanniana* and the encoding cDNAs were identified [161]. These DGATs [161] were not related to the enzyme purified by Kamisaka et al. [160].

In 1998, Cases et al. [162] reported the isolation and characterization of a cDNA encoding a mouse DGAT (GenBank Accession No. AF078752) which shared some homology with acyl-CoA:cholesterol acyltransferase (ACAT). In the same year, Oelkers et al. [163] speculated that a cDNA encoding a ACAT-related protein (Accession No. AF059202) may, in fact, encode DGAT. The speculation was based on the presence of a DAG-binding motif in the predicted amino acid sequence. The cDNAs encoding the DGATs purified by Lardizabal et al. [161] from *Mortierella ramanniana* were not related to the mouse cDNA encoding DGAT. The information contained in the sequence of the cDNA encoding mouse DGAT and the predicted amino acid sequence quickly led to the cloning of the first cDNAs encoding DGATs from *A. thaliana* (GeneBank Accession Nos. AJ131831 and AJ238008) [164,165] and *B. napus* (Accession Nos. AF155224 and AF164434) [166,167]. The cDNA encoding mouse DGAT shared significant homology to an *A. thaliana* expressed sequence tag clone (E6B2T7) [164]. The sequence information was used to clone the first cDNA encoding *A. thaliana* DGAT [164]. The gene encoding *A. thaliana* DGAT, identified as *TAG1*, contained 16 exons and 15 introns over a sequence of about 3.4 kbp [164,165] and was located on the upper arm of chromosome II [164]. Analysis of *A. thaliana* genomic DNA by Southern blotting indicated the presence of one *DGAT* gene [164]. The altered lipid phenotype of *A. thaliana* mutant AS11 [146] was caused by a 147-bp insertion located in the central region of intron 2 of the *A. thaliana DGAT* gene [165]. *A. thaliana* DGAT shared 38% amino acid sequence identity to mouse DGAT and had a predicted molecular mass of 59 kDa, which was 6 kDa larger than the mouse enzyme [162,164]. Northern blots revealed that the transcript of the homolog of *A. thaliana* DGAT in *B. napus* was mainly expressed in developing embryos, flower petals, and developing flower buds with lower levels of expression in leaf and stem tissue [164]. The deduced amino acid sequences existing between mouse DGAT and two putative *A. thaliana* ACATs (GenBank Accession Nos. AC003058 and AC005917) were used in a cloning strategy involving rapid amplification of cDNA ends to obtain cDNAs *BnDGAT1* [167] and *BnDGAT2* [166] from MD cell suspension cultures of *B. napus* L. cv Jet Neuf. The predicted molecular masses of the isoforms BnDGAT1 and BnDGAT2 were 56.9 and 39.5 kDa, respectively. The predicted amino acid sequence of BnDGAT1 exhibited 84% identity and 92% similarity to the *A. thaliana* enzyme but was 17 residues shorter in length [9,167]. BnDGAT2 displayed 90% identity and 92% similarity in amino acid sequence to *A. thaliana* DGAT [166]. Interestingly, BnDGAT2 was similar in predicted molecular mass to a polypeptide of 39 kDA previously identified through photoaffinity labeling of a solubilized fraction from MD embryos of oilseed rape [168]. The hydropathy plots of *A. thaliana* DGAT and of *B. napus* DGAT isoforms suggest that these enzymes have a number of membrane-spanning segments, a characteristic typical of integral membrane proteins [164–167]. BnDGAT2, however, lacks a relatively hydrophilic N-terminal segment that is present in *A. thaliana* DGAT and BnDGAT1. Functional expression of cDNA encoding *A. thaliana* DGAT has been achieved in insect cell cultures [164] and the yeast line YMN5 [*slc1 Δ2::LEU2ura3*] [165], whereas functional expression of *BnDGAT1* and *BnDGAT2* was attained in *Pichia pastoris* (Nykiforuk et al., unpublished data).

4.5 Cholinephosphotransferase

The transfer of phosphocholine from cytidine diphosphate (CDP)-choline to generate PC and cytidine monophosphate (CMP) is catalyzed by CDP-choline:1,2-diacylglycerol cholinephosphotransferase (CPT, E.C. 2.7.8.2) (Fig. 2) [15,169]. Studies with microsomal preparations from developing safflower seeds have revealed that the reaction is reversible, leading to the formation of *sn*-1,2-DAG from PC [170–172]. The reverse reaction provides a means of providing DAGs with increased unsaturation as substrates for DGAT and transacylases (see Sec. 4.8). Maximum CPT activity has been shown to be associated with the greatest rates of PC and TAG production in maturing safflower seeds [171]. CPT activity decreased to about half-maximum by the time seed lipid content reached a plateau [171]. CPT activity in microsomes from oilseed rape (*B. napus* L. cv Indore) has been shown to display little or no preference for various molecular forms of *sn*-1,2-DAG [131]. As well, lipid-precursor feeding studies with extracts of MD embryos have shown that G3P stimulates conversion of [^{3}H]PC to [^{3}H]*sn*-1,2-DAG, suggesting regulation of CPT by G3P [80].

Cholinephosphotransferase has not been purified from any source [169]. cDNAs encoding proteins with CPT activity, however, have been obtained for yeast (cDNAs *EPT1* and *CPT1*) [169,173,174] and developing soybean seeds (cDNA *AAPT1*) [169,175]. The cDNA encoding the soybean enzyme was isolated by complementation of a CPT-deficient mutant of yeast. Seven membrane-spanning segments were apparent in the predicted amino acid sequences for both forms of yeast CPT and soybean CPT [169,173–175]. The amino acid sequence, predicted from soybean *AAPT1*, was 32–33% identical to the amino acid sequences predicted using yeast *EPT1* and *CPT1* [169,175].

4.6 Desaturation of Membrane Lipids

In addition to desaturation of acyl-ACP [67], FA desaturation also occurs on the fatty acyl moieties of the membrane lipids of plastids and the ER [176] (Fig. 2). Modeling of TAG involves desaturase action at the level of PC because of the reversibility of the CPT-catalyzed reaction that facilitates re-entry of *sn*-1,2-DAG into the mainstream of TAG biosynthesis. Due to problems with the purification of membrane-bound PC desaturases [177], most of these genes have been isolated via molecular genetic approaches with *A. thaliana* [4,176–181]. The production of microsomal polyunsaturated phospholipids occurs mainly through the catalytic action of oleoyl-PC desaturase, which is encoded by *FAD2* [182]. The resulting 18:2 also serves as a substrate for further desaturation to α-18:3 catalyzed by linoleoyl-PC desaturase, which is encoded by *FAD3* [4]. Linoleic acid and α-18:3 both play a critical role in the bioassembly of plant cellular membranes [180]. The identification of lesions in mutants of *A. thaliana* generated by T-DNA insertion mutagenesis [183] led to the isolation of the *FAD2* and *FAD3* [4,180]. *A. thaliana* mutants displaying altered lipid phenotypes have proven useful in gaining insights into regulation of desaturation in plants [177,181].

4.7 Lysophosphatidylcholine Acyltransferase

Lysophosphatidylcholine acyltransferase (LPCAT, E.C. 2.3.1.23) catalyzes the acyl-CoA-dependent acylation of lysophosphatidylcholine (LPC) to generate PC and CoA [15,184] (Fig. 2). The LPCAT-catalyzed reaction, however, appears to be freely reversible and facilitates the entry of polyunsaturated fatty acids into the acyl-CoA pool, thereby creating an opportunity for these acyl moieties to be incorporated into TAG [15,77,185–187].

Incorporation of oleoyl moieties in the *sn*-2 position of PC also results in additional opportunities for desaturation and further modification of the DAG component of PC [15,79]. Most of the information on the characteristics of plant LPCAT and the reversibility of the reaction has been based on studies with microsomes of developing safflower and sunflower seeds [15,79,184–188]. Microsomal LPCAT, however, has also been investigated in other tissues, including soybean [189,190] and developing pea leaves [191]. Ichihara et al. [188] conducted specificity and selectivity studies using the 3000–20,000*g* particulate fraction from maturing safflower seeds. When assayed in the direction of PC formation, the relative order of preference of LPCAT for native acyl-CoAs was 18:2-CoA > 18:1-CoA > 18:0-CoA = 16:0-CoA, with 12:0-CoA and 22:1-CoA being ineffective. Molecular species of LPC containing 16:0, 18:0, 18:1, and 18:2 moieties were all effective as acceptor substrates. Erucoyl-CoA has also been shown to be a poor substrate of microsomal LPCAT from developing seeds of high-erucic-acid oilseed rape (*B. napus* L. var. Lenora × CHR 1775/82) [90]. LPCAT from developing soybean catalyzed the acylation of LPC at 40 times the rate of *lyso*-phosphatidylethanolamine [189]. The enzyme in microsomes from germinating soybean has been shown to nonselectively acylate *sn*-2-acyl-LPC and *sn*-1-acyl-LPC [190]. LPCAT has been solubilized from safflower microsomes using octylglucoside and the solubilization process appeared to alter some properties of the enzyme [184]. Photoaffinity labeling of membrane fractions from developing castor bean has been used in the identification of a 52-kDa polypeptide that might represent LPCAT or one of its subunits [192]. The enzyme has not been purified from any source and a cDNA encoding the enzyme has not been identified [193].

4.8 Transacylases

Recent studies with developing oilseeds have identified non-acyl-CoA-dependent enzyme activities that catalyze the transfer of acyl moieties between glycerolipids, resulting in other routes for the production of TAG (Fig. 2). Diacylglycerol transacylase (DGTA) catalyzes the formation of TAG and MAG from two molecules of DAG [194]. The enzyme was first purified from the microsomes of rat intestinal villus cells and characterized by Lehner and Kuksis [194]. Recent investigations have indicated that DGTA is present in developing safflower seed and castor endosperm and that the reverse reaction may have a role in remodeling TAG [62,63]. It has also been reported that microsomes from developing castor endosperm catalyze the transfer of hydroxylated and epoxidated acyl groups from the *sn*-2 position of PC to DAG [64]. A gene encoding a phospholipid:diacylglycerol acyltransferase has been identified in yeast [65].

4.9 Incorporation of Unusual Fatty Acids into Triacylglycerol

Triacylglycerols generally contain a wider assortment of FAs than are found in membranes [4]. Unusual FAs, including erucic, lauric, petroselinic, and ricinoleic acids, do not accumulate in membranes [4,80,195]. Studies with developing seeds and MD embryos of high-erucic-acid cultivars of oilseed rape have indicated that erucic acid is primarily incorporated at the *sn*-3 position of TAG [80,90,137]. Unusual FAs, however, appear to be transiently incorporated into PC before eventual accumulation in TAG [80,137,196,197]. The exclusion of unusual FAs from membranes may involve factors such as phospholipase action [196,198,199], selectivity of DGAT [133], and spatial separation of TAG biosynthesis from membrane biosynthesis [128,131,195,200]. The concept of spatial separation of TAG biosynthesis was supported by analyses of lipid biosynthetic enzymes

in membrane fractions obtained following sucrose density-gradient centrifugation of microsomes from zygotic embryos of oilseed rape [128]. The study demonstrated that enzymes involved in the mainstream of TAG biosynthesis were enriched in a low-density membrane fraction, whereas enzymes involved in membrane formation were enriched in a high-density membrane fraction.

4.10 Formation of Oil Bodies

Oil bodies or lipid droplets 0.2–2.5 μm in diameter, containing TAG, accumulate in the cytosol of maturing seeds [201,202]. A major theory has proposed that oil bodies form through accumulation of TAG between the phospholipid layers of the ER [203,204]. Eventually, the oil bodies bud from the ER with a single layer of phospholipid encapsulating them. The existence of this single layer of phospholipid or half-unit membrane has been supported by electron microscopy and biochemical analyses of lipid bodies from peanuts [205,206]. Oleosins are 15–26-kDa proteins which are embedded in the half-unit membrane surrounding oil bodies [201,202,207]. A long internal hydrophobic segment of the oleosin polypeptide serves to harness them to oil bodies [207]. The oleosins shield adjacent oil bodies from each other and prevent them from coalescing, and they provide more surface area for mobilization of lipid reserves [207]. Oleosins might also contain sequences for binding of lipase during the germination process [207]. In studies with developing seeds of oilseed rape, there has been some controversy as to whether or not TAG accumulation and oleosin synthesis occur at the same time [149,202,208–210]. A conserved ''proline knot'' sequence has been localized to the central hydrophobic domain of oleosins [201]. Mutations in this sequence have been shown to block the accumulation of the mutated oleosin polypeptide on oil bodies, although the modified protein was still incoporated into the ER [211]. Recently, in studies with developing sunflower, Lacey et al. [212] provided biophysical evidence to demonstrate deposition of oil droplets within the lipid bilayer of the ER [212]. In the same study, an immunochemical assessment of the oleosin content of membrane fractions indicated that oleosins were targeted to and/or accumulated in distinct regions of ER where TAG biosynthesis and deposition occurred. In another recent study with the sunflower system, Beaudoin et al. [213] demonstrated that oleosin mRNA was associated with the rough ER and that the oil body protein was synthesized on bound ribosomes and cotranslationally inserted into the microsomal membrane.

5 ACYLTRANSFERASES AND PHOSPHATIDATE PHOSPHATASE IN PLASTIDS

Enzymes involved in glycerolipid and TAG biosynthesis in the ER are also present in the plastid. Acyl-ACPs (16:0 and 18:1) serve as in vivo substrates for these acyltransferases in the formation of plastidial glycerolipids via the prokaryotic pathway [4,214]. The plastidial enzymes can also utilize acyl-CoAs [81].

The properties of plastidial GPAT have been studied in various plant species [81,215], including spinach leaves [216–219], pea leaves [217–219], and squash (*Cucurbita moschata*) [220]. Unlike microsomal GPAT, the plastidial enzyme is soluble in the chloroplast stroma [81]. The enzyme in pea and spinach has been shown to have an absolute specificity for G3P [217]. Plastidial GPAT has been purified to homogeneity [218,221] and a number of cDNAs encoding the enzyme have been isolated from various sources [105,215,222,223]. The purified enzyme was selective for oleoyl moieties over palmitoyl

moieties even when high ratios of palmitoyl-CoA/oleoyl-CoA were present in the reaction mixture [218]. The selectivity for oleoyl moieties was enhanced when acyl-ACP forms were used in the enzyme reaction [218]. GPAT and acyl-ACP hydrolase may have a role in channeling the acyl flux through the prokaryotic and eukaryotic pathways [224]. Closely related plant species appear to have similar GPAT sequences, but GPATs from related plant species do not necessarily have similar enzymatic properties, and FA selectivity appears to be governed by a few critical amino acid residues [105]. Plastidial GPAT is encoded by a nuclear gene [105]. The preprotein formed in the cytosol enters the plastid via a cleavable N-terminal transit peptide [105]. The specificity and selectivity properties of chloroplast GPAT have indicated that the enzyme has a role in regulating the level of unsaturated FAs in phosphatidylglycerol [216,217,220]. A recent study with chimeric gene products of plastidial GPAT from spinach and squash has indicated that the central regions of these two enzymes are important in the determination of FA specificity [225].

Acyl-ACP:LPAAT is bound to the envelope of chloroplasts [81]. Plastidal LPAAT has been shown to discriminate against oleoyl-ACP and direct palmitoyl moieties to the *sn*-2 position of LPA [105,217]. This selectivity agrees with the role of the enzyme in the prokaryotic pathway where 16:0 is predominantly esterified at the *sn*-2 position of plastidial glycerolipids [4,214]. A cDNA (*BAT2*) encoding plastidial LPAAT has been isolated by functional complementation of the *Escherichia coli* strain JC201 with an immature embryo cDNA library of *B. napus* [226]. Although the predicted molecular mass of the protein was 38 kDa, chloroplast-import studies indicated that a precursor polypeptide underwent posttranslational processing to form a mature protein of 32 kDa localized to the chloroplast membrane fraction. When expressed in bacteria, the plant LPAAT displayed a preference for palmitoyl-CoA over oleoyl-CoA, which was characteristic of the plastidial enzyme. The deduced amino acid sequence of the plastidial LPAAT shared a strong identity to microsomal LPAATs of seeds.

Phosphatidate phosphatase has been extensively studied in the chloroplast of spinach leaves. The enzyme is associated with the inner envelope of the plastid, where it plays an important role in the regulation of glycerolipid biosythesis [111,227–230]. PA can be converted to phosphatidylglycerol via CDP–DAG and DAG [231,232]. In turn, DAG can serve as a precursor in the synthesis of galactolipids and sulfolipids [231,232]. Plastidial PA phosphatase has been shown to exhibit optimal activity at pH 9.0 [227,228]. Various molecular species of *sn*-1,2-DAG were powerful inhibitors of the enzyme, with *sn*-1,3-DAG showing very little inhibition [229]. Plastidial PA phosphatase has been solubilized using 6 m*M* CHAPS and partially purified using hydroxyapatite chromatography [230].

Triacylglycerol has been shown to accumulate in spinach leaf plastids [233–235], phototrophic cell cultures of soybean [236], mesophyll protoplasts from *A. thaliana* leaves [237], and pea plastids [238]. Spinach leaf plastids have been shown to contain DGAT activity in assays using acyl-CoA and *sn*-1,2-DAG [233]. Recently, Northern blotting experiments with molecular genetic probes encoding *A. thaliana* DGAT have revealed transcripts in leaves of *A. thaliana* [165] and *B. napus* [164], which may encode a plastidial form of the enzyme.

6 TRIACYLGLYCEROL ACCUMULATION IN DEVELOPING POLLEN GRAINS

Developing pollen grains also accumulate TAG and other storage lipids [239]. Immature pollen grains or microspores are produced via meosis in the immature anther [240]. The

microspore divides to form two identical daughter cells that, in turn, lead to the development of a pollen grain [53,241–243]. Recent advances in understanding lipid accumulation in pollen have been based on studies with oilseed rape. Mature pollen grains of *B. napus* have been shown to contain about one-third lipid on a dry-weight basis [244–246]. The majority of this lipid is associated with the internal region of the pollen grain [244] and may play a role in the development of the pollen tube [239,247]. The developing microspore is nourished by a layer of cells known as the tapetum [248]. During pollen development, the cells of the tapetum rupture to discharge additional lipids and proteins onto the pollen grain surface to form the tryphine [239]. The outer lipid provides the pollen grain with adhesion characteristics and appears to play a role in attracting insect pollinators [207,248,249].

Triacylglycerol has been shown to account for almost 40% of the total lipids in the internal region of the pollen grain of *B. napus* [250]. In contrast, the tryphine has been shown to contain very little TAG but has higher proportions of neutral esters and free FAs [250]. The tapetum cells of developing pollen from *B. napus* contain specialized structures referred to as tapetosomes, which consist of patches of TAG distributed among vesicles [251–253]. Other lipids of the tapetum cells are located in plastids that contain globuli mainly composed of neutral esters containing FAs and sterols [251,254,255]. The FA composition of TAGs from both the pollen internal region and the tryphine have been shown to be very similar at maturity [250]. Linolenic acid (α-18:3) has been shown to account for about 63% and 65% of the FAs in TAG in the internal region and the tryphine of *B. napus* L. cv Topas pollen, respectively, with 18:1 representing only a minor constituent of the TAG [250]. In contrast, seeds of the same cultivar contain 18:1 as the predominant FA [9,42]. During pollen development, the proportion of α-18:3 in both TAG and polar lipids has been shown to increase as the proportions of both 18:1 and 18:2 decrease, suggesting an increase in desaturase activity [250].

Gene expression in the internal region of developing pollen grains is based on a haploid genome [256–258], whereas expression in the tapetum is based on a diploid genome [248,249]. Bud length has been used as an indicator of pollen maturity in studies of lipid biosynthesis and associated gene expression [250,257]. Most of the storage lipid in maturing pollen grains of oilseed rape has been shown to accumulate during the late-vacuolate and early-maturation stages of development, at bud lengths ranging from 3.4 to 4.3 mm [257]. Lipid accumulation in the tapetum, however, occurred at an earlier stage of development, corresponding to a 2.7-mm bud length. Maximal expression of Δ^9-stearoyl-ACP desaturase and enoyl-ACP reductase genes in the internal region of pollen have also been shown to occur at a somewhat later stage of bud development than for expression of these genes in the tapetum [250]. Thus, FA biosynthesis in both the internal region and tapetum appeared to involve the same genes that were regulated differentially [250].

Northern blotting with a molecular genetic probe, encoding *A. thaliana* DGAT, has indicated that buds of *B. napus* express high levels of mRNA encoding DGAT, which is in agreement with the high levels of TAG in developing pollen grains [164]. The structural similarities between pollen DGAT(s) and the form associated with developing seeds of *A. thaliana* remain to be determined. In general, the enzymology of lipid biosynthesis in developing pollen grains is in need of further investigation.

Most of the research on the molecular biology of oil formation in pollen grains has involved studies of the expression, targeting, and subcellular localization of oleosinlike proteins [207,255,258–264], which share some homology with seed oleosins [207,262].

The oleosinlike proteins of the internal region of the pollen grain probably have a role(s) similar to the seed oleosins [207], whereas the role(s) of the oleosinlike proteins in the tapetum is not as clear [251]. Following rupture of the tapetum cells, the oleosinlike proteins and lipids of the tapetum have been shown to undergo degradation [251,262,265,266].

7 EFFECT OF ENVIRONMENT ON TAG ACCUMULATION

The manner in which developing plants respond to environmental conditions can provide important insights into the regulation of TAG accumulation. Plant cultures, such as MD embryos and cell suspension cultures, have been very useful in examining the effect of environment on TAG biosynthesis [9]. Factors, including temperature, nutrient content, light intensity, osmotic potential, and concentrations of exogenous hormones, and various other additives, can be carefully controlled. As well, the cultures can be sampled at regular intervals.

Decreasing temperature has been shown to increase the proportion of polyunsaturated FAs in both zygotic embryos [267,268] and MD embryos [269] of oilseed rape. Decreasing growth temperature has also been shown to result in an increase in the proportion of 22:1 formed in both zygotic embryos [267] and MD embryos [270] of certain high-erucic-acid cultivars of oilseed rape. The temperature effect in MD embryos of *B. napus* L. cv Reston appeared to be linked to an increase in the quantity of elongase enzyme [147]. Decreasing temperature during seed development in *B. napus* L. cv Nugget was previously reported to lead to increased total seed oil accumulation in oilseed rape [267]. Studies with MD embryos of oilseed rape have revealed genotypic differences on the effect of temperature on FA composition and oil content [270]. When MD embryos of *B. napus* L. cv Reston were grown under various conditions, including different temperatures, the total oil produced varied more than the total acyltransferase activity as assayed by production of TAG from [U-^{14}C]G3P and oleoyl-CoA [147]. The bulk of the radioactivity was incorporated into PA followed by DAG, suggesting that PA phosphatase may have been rate limiting in TAG production, possibly sharing some control with DGAT.

The hormone abscisic acid (ABA) has been shown to stimulate a variety of plant responses that are also affected by water stress or increased osmoticum [271,272]. As well, ABA may have an important role in the maturation and dessication of seeds [273]. ABA has been shown to induce TAG accumulation in MD embryos of oilseed rape [274–276]. Also, application of the hormone has been shown to result in elevated levels of 22:1 in zygotic embryos [271] and MD embryos [46,269,274–276] of high-erucic-acid cultivars of oilseed rape. Similar to the effect of decreasing temperature, studies with MD embryos have indicated that the increase in the proportion of 22:1 may be attributable to an increase in the components of the elongation system [147,275]. ABA has also been shown to increase the level of FA unsaturation in both high- [269] and low- [269,276] erucic-acid cultivars of MD embryos of oilseed rape.

Microspore-derived embryos of *B. napus* have been particularly useful in assessing the effects of growth regulators and osmoticum on the biosynthesis of lipids, oleosins, and storage proteins [9]. Treatment of MD embryos of oilseed rape with ABA, jasmonic acid (JA), or sorbitol has also been shown to stimulate production of transcripts encoding oleosins [277] and oleosin polypeptides [275,278]. JA is a metabolite of α-18:3 that naturally occurs in *Brassica* embryos [279]. The growth regulator has been implicated in stress-related plant responses [280]. MD embryos appeared to use endogenous JA to modulate

the effect of ABA on expression of oleosin [281]. Application of (+)-ABA or its metabolite, 8′-OH-ABA, has been shown to induce the production of transcripts encoding oleosin and $\Delta^{15}(\omega^3)$ desaturase genes [282]. In addition, this type of treatment resulted in the accumulation of very long-chain monounsaturated FAs.

High osmoticum in the form of increasing concentrations of mannitol (in the presence of about 1% sucrose) has been shown to induce TAG accumulation and associated DGAT activity in immature maize embryos [283]. Another study with peanut somatic embyros, however, demonstrated that sucrose at a concentration of about 0.6 *M* had more of an effect on TAG accumulation than the osmoticum provided by mannitol and sorbitol at 0.2 *M* and 0.6 *M*, respectively [284]. Increasing the sucrose concentration in the growth medium of MD cell suspension cultures of oilseed rape (*B. napus* L. cv. Jet Neuf) has been shown to result in increased TAG accumulation [32] (Table 1). TAG content on a dry-weight (DW) basis increased by twofold, when comparing cells cultured for two weeks in 22% (w/v) sucrose to cells cultured in 2% sucrose. On a fresh-weight (FW) basis, TAG content increased by nearly fivefold over this sucrose concentration range. There was also a substantial enrichment in the proportion of TAG in TL in cells cultured at concentrations of sucrose >2%. DGAT activity and abundance of oleosin transcript increased as the sucrose concentration was raised from 2% to 14% [32]. More recent studies have shown that ABA (16 μ*M*) enhances TAG accumulation in this system as sucrose concentration increases (Hodges et al., unpublished results). Increasing concentrations of sorbitol in the presence of 1% sucrose, however, had little effect on TAG production on a FW basis in this system. The observations with sorbitol suggested that sucrose-induced TAG accumulation may have been caused by increased carbon supply from sucrose and/or another sucrose-mediated effect. The time course of TAG accumulation in these cells has been examined following transfer from lower to higher sucrose concentrations (2–14% sucrose and 6–14% sucrose) (Ref. 285; Nykiforuk et al., unpublished results). TAG content and DGAT activity increased and then declined rapidly within the first 24 hr of transfer. The decline in DGAT activity was associated with an increase in TAG lipase activity. The short-term elevation in TAG content and DGAT activity was followed by a rapid decline in TAG lipase activity and then a more gradual increase in TAG content and DGAT

Table 1 Effect of Sucrose Concentration on DW, TL, and TAG Content of MD Cell Suspension Cultures

Sucrose conc.	DW/FW[a] (%)	TL/DW[a] (%)	TAG/TL[a] (%)	TAG/DW (%)
2%	7.6	10.5	42.5	4.5
6%	12.8	9.4	67.4	6.3
10%	14.5	9.6	66.7	6.4
14%	17.0	8.8	60.0	5.3
18%	17.7	11.9	63.7	7.6
22%	18.1	12.7	71.8	9.1

Note: Isolation of the TAG fraction and subsequent methylation of TL and TAG was performed according to Weselake et al. [32]. Fatty acid methyl esters were determined by gas–liquid chromatography (GLC) and values for TL and TAG were based on the total integrated area of the chromatogram representing each sample. Lipid preparations from three independent cultures were analyzed by GLC and the values were averaged.

[a] DW = dry weight, FW = fresh weight, TAG = triacylglycerol, TL = total acyl lipid.

Source: From Ref. 32.

activity. Short-term exposure to a higher sucrose concentration somehow programmed the cells to respond in this manner. Further investigation of this sucrose-induced effect could provide insights into the regulation of TAG accumulation and mobilization.

8 GENETIC ENGINEERING OF TRIACYLGLYCEROL ACCUMULATION

Advances in the genetic engineering of oil accumulation have been based on the availability of cDNAs encoding lipid biosynthetic enzymes and the extensive body of knowledge generated in the area of plant lipid biosynthesis. Many of these advances have been made using *B. napus* as a target crop for modification.

In an early report on genetic manipulation, the level of chilling sensitivity of tobacco (*Nicotiana tabacum*) plants was altered through expression of cDNAs encoding plastidial GPAT from squash and *A. thaliana* [286]. Plants, such as squash, contain relatively low levels of *cis*-unsaturated FAs in their chloroplast membranes, which imparts chilling sensitivity [286]. The selectivity properties of squash GPAT account for the high proportion of saturated FAs at the *sn*-1 position of plastidial phosphatidylglycerol [220]. Subsequently, expression of squash *GPAT* cDNA in tobacco led to increased chilling sensitivity. In contrast, *A. thaliana* contains relatively high levels of *cis*-unsaturated FAs that impart resistance to chilling and, thus, introduction of the *A. thaliana* enzyme into tobacco decreased chilling sensitivity [286].

Genetic engineering of *B. napus* to produce seed containing high levels of lauric acid could lead to new applications for the crop as a source of structured TAGs for the production of cocoa-butter-like oils [287]. The California Bay (*Umbellularia californica*) plant has been shown to contain an acyl-ACP hydrolase that is specific for medium-chain ACP thioesters [288]. Expression of the cDNA encoding this enzyme in developing seeds of *A. thaliana* resulted in the accumulation of medium-chain FAs in the resulting transgenic seed [18]. Based on these results, a genetically engineered line of *B. napus* was developed that expressed the California Bay acyl-ACP hydrolase [289]. The transgenic seed contained high levels of 12:0 (up to 50%) but only accumulated small quantities of this FA at the *sn*-2 position, providing in vivo evidence to support earlier biochemical results where *B. napus* LPAAT was shown to discriminate against lauryl-CoA [95]. Eccleston and Ohlrogge [290] reported that expression of lauroyl-ACP hydrolase in these seeds induced FA β-oxidation and the glyoxylate cycle [290]. The FA synthesis pathway was also induced, presumably to replace the 12:0 lost via β-oxidation. The investigators suggested that metabolic regulation of β-oxidation should be an important consideration in genetic engineering of oilseed crops.

A number of genetic engineering studies of oilseeds have focused on the LPAAT of the ER. Recently, *B. napus* expressing the cDNA encoding California Bay medium-chain acyl-ACP hydrolase was crossed with *B. napus* expressing the cDNA encoding LPAAT from coconut endosperm [291]. The LPAAT of coconut was previously shown to be specific for medium-chain FAs [103]. The resulting plants incorporated 12:0 at the *sn*-2 position of the glycerol backbone to produce trilaurin and produced higher levels of 12:0 in their seed oil than plants expressing only the medium-chain acyl-ACP hydrolase [291].

The theoretical breeding limit for incorporation of 22:1 in *B. napus* seed oils is about 66 mol% [99,292]. As in the case of lauroyl-CoA, this has been shown to be attributable to the inability of *B. napus* LPAAT to effectively utilize erucoyl-CoA as a substrate [90,95].

High-erucic-acid oils are used as starting material in the production of high-temperature lubricants, nylon, and plasticizers [293]. Introduction of 22:1 at the *sn*-2 position of the TAG of high-erucic-acid oilseed rape should, theoretically, result in the production of trierucin. Developing meadowfoam seeds contain LPAAT in the ER that has been shown to catalyze the incorporation of 22:1 into the *sn*-2 position of LPA [89,96,100]. LPAAT cDNAs from meadowfoam expressed in high-erucic-acid oilseed rape have been shown to result in transgenic seeds that produced small proportions of trierucin [20,106].

Expression of a mutated yeast *sn*-2 acyltransferase gene (*SLC1-1*) [294] in developing seeds of oilseed rape (*B. napus* L. cv Hero) has been shown to increase seed oil content [295]. The resulting transgenic seeds also had increased incorporation of very long-chain FAs at the *sn*-2 position of TAG, as well as increases in both 22:1 content and average seed weight [295]. The *SLC1-1* gene was shown to encode a LPAAT that could accept a range of acyl-CoAs, including erucoyl-CoA. This study indicates that the LPAAT-catalyzed reaction can affect the flow of carbon into TAG. The *SLC1-1* gene and/or its gene product (LPAAT) may be free of regulation or regulated in a different way than the plant (meadowfoam) transgenes. Also, introduction of the mutated yeast enzyme into developing seeds may exert other effects in the ER that affect TAG accumulation.

The seed oil content in *B. napus* has also been increased by genetic manipulation of FA biosynthesis. The cytosolic ACCase of *A. thaliana* has been directed to the plastid using a chimeric DNA construct encoding ACCase along with a transit peptide and napin promoter [19]. Genetically engineered plants displayed an increase in seed oil content of 5%, demonstrating that FA supply during seed maturation was increased through the action of the cytosolic enzyme in the plastid. Cytosolic ACCase may have been more effective than plastidial ACCase in promoting TAG accumulation because of differences in the regulatory properties.

Genetic manipulation of the desaturation of fatty acyl groups on PC is an important facet in the modeling of TAG because *sn*-1,2-DAG derived from membrane can re-enter the mainstream of TAG biosynthesis (Fig. 2). Lipid mutants of *A. thaliana* have provided valuable information for genetic engineering of desaturation in plants [177,296]. For example, overexpression of *FAD2* and silencing of *FAD3* in oilseed rape and soybean, respectively, have produced seed lines with increased levels of 18:2. Silencing of both the *FAD2* genes (*BnFAD2-1* and *BnFAD2-2*) in oilseed rape, in a seed-specific manner, resulted in an 18:1 content of about 85% in the seed oil [7].

9 CONCLUSIONS AND FUTURE PERSPECTIVES

Studies with homogenates and subcellular fractions from developing seeds and plant cultures have provided us with an enormous wealth of information on TAG biosynthesis. Purification studies of TAG biosynthetic enzymes have provided important insights into their properties. Plant cell and tissue cultures offer the advantage of being able to study lipid biosynthesis and associated gene expression under carefully controlled conditions. Nondifferentiating cell cultures offer the further advantage of being able to study these processes in the absence of the metabolic and genetic complications associated with cellular differentiation. The response of oleaginous plant systems, especially cell cultures, to environmental changes has increased our understanding of the regulation of TAG biosynthesis. In many cases, information on the specificity/selectivity of acyltransfeases have proven useful in designing genetic engineering strategies to produce crops with novel seed oils. Enzymes involved in TAG bioassembly and modification in the ER have proven

difficult to purify. In recent years, cDNAs and genes encoding many of these enzymes have been identified via a combination of protein purification and molecular genetic approaches. The availability of these nucleic acid sequences has resulted in a number of advances in the genetic engineering of both FA composition and seed oil content. Many genetic engineering approaches to seed oil modification have involved studies with *B. napus* and have relied on expressing TAG biosynthetic enzymes from other sources in this crop. The approach wherein a lipid biosynthetic enzyme from one subcellular compartment is expressed in another part of the cell, as in the case of ACCase [19], is one that might be further explored for other enzymes such as the acyltransferases. Lipid biosynthetic enzymes from developing pollen grains may also have unique properties that may be valuable in the genetic modification of seed oil. A detailed understanding of the structure–function relationships of LPAAT and DGAT will set the stage for site-directed mutagenesis of the native enzymes in order to modify their performance. It is also possible to create new enzymes by engineering chimeric cDNAs that encode segments of enzymes catalyzing the same reaction from different subcellular compartments or sources. Characterization of possible allosteric sites or sites of covalent modification on TAG-biosynthetic enzymes might provide other opportunities for genetic engineering of seed oil biosynthesis. For example, site-directed mutagenesis of potential allosteric sites could lead to desensitization of acyltransferases to possible negative effectors. It may also be possible to alter pathways that produce modulators of TAG biosynthesis. Given these capabilities, the greatest challenge in genetic engineering of crops is to convince consumers that products, such as oils from transgenic seeds, will be safe and that plants producing these seeds will not result in adverse effects to the ecosystem.

ACKNOWLEDGMENTS

Preparation of this review was supported in part by a Research Grant to the author from the Natural Sciences and Engineering Research Council (NSERC) of Canada. The author thanks NSERC, the Alberta Agricultural Research Institute, and the Saskatchewan Agriculture Development Fund for supporting his research on TAG biosynthesis in oilseed rape and acknowledges the support of the many students, research assistants, postdoctoral researchers, and other collaborators who contributed to the research. The author also thanks Dr. J. Boothe and Dr. L. A. Holbrook for their critical evaluation of this chapter.

REFERENCES

1. RJ Weselake. In Vitro Cell Dev Biol. Plant 36:338–348, 2000.
2. DJ Murphy. Prog Lipid Res 33:71–85, 1994.
3. JB Ohlrogge. Plant Physiol 104:821–826, 1994.
4. JB Ohlrogge, J Browse. Plant Cell 7:957–970, 1995.
5. R Töpfer, N Martini, J Schell. Science 268:681–686, 1995.
6. GJ Budziszewski, KPC Croft, DF Hildebrand. Lipids 31:557–569, 1996.
7. AJ Kinney. In: JK Setlow, ed. Genetic Engineering, New York: Plenum Press, 1997, pp 149–166.
8. JB Ohlrogge, JG Jaworski. Annu Rev Plant Physiol Plant Mol Biol 48:109–136, 1997.
9. RJ Weselake, DC Taylor. Prog Lipid Res 38:401–460, 1999.
10. MI Gurr. In: PK Stumpf, ed. The Biochemistry of Plants, Vol. 4. New York: Academic Press, 1980, pp 205–248.

11. IUPAC–IUB Commission on Biochemical Nomenclature (CBN). Biochem J 105:897–902, 1967.
12. H Brockerhoff. Lipids 6:942–956, 1971.
13. JJ Myher, A Kuksis, K Geher, PW Park, DA Diersen-Schade. Lipids 31:207–215, 1996.
14. G Norton, JF Harris. Phytochemistry 22:2703–2707, 1983.
15. S Stymne, AK Stobart. In: PK Stumpf, ed. The Biochemistry of Plants, Vol. 9, Lipids: Structure and Function. New York: Academic Press, 1987, pp 175–214.
16. RF Wilson, P Kwanyuen. Biochim Biophys Acta 877:231–237, 1986.
17. MM Moloney, JM Walker, KK Sharma. Plant Cell Rep 8:238–242, 1989.
18. TA Voelker, AC Worrell, L Anderson, J Bleibaum, C Fan, DJ Hawkins J, SE Radke, HM Davies. Science 257:72–74, 1992.
19. K Roesler, D Shintani, L Savage, S Boddupalli, J Ohlrogge. Plant Physiol 113:75–81, 1997.
20. CL Brough, JM Coventry, WW Christie, JTM Kroon, AP Brown, TL Barsby, AR Slabas. Mol Breed 2:133–142, 1996.
21. H Kleinig, K Cornelia. Planta 139:61–65, 1978.
22. H Kleinig, C Steinki, C Kopp, K Zaar. Planta 140:233–237, 1978.
23. GS Warren, MW Fowler. Planta 144:451–454, 1979.
24. PC Dutta, L-Å Appelqvist. Plant Sci 64:167–177, 1989.
25. PC Dutta L-Å Appelqvist. Plant Sci 75:177–183, 1991.
26. JHE Ross, DJ Murphy. Plant Sci 88:1–11, 1993.
27. M Williams, J Sanchez, AC Hann, JL Harwood. J Exp Bot 44:1717–1723, 1993.
28. AJ Rutter, J Sanchez, JL Harwood. Phytochemistry 46:265–272, 1997.
29. SS Radwan, S Grosse-Oetringhaus, HK Mangold. Chem Phys Lipids 22:177–184, 1978.
30. M Williams, D Francis, AC Hann, JL Harwood. J Exp Bot 42:1551–1556, 1991.
31. RJ Weselake, MK Pomeroy, TL Furukawa, JL Golden JL, DB Little, A Laroche. Plant Physiol 102:565–571, 1993.
32. RJ Weselake, SD Byers, JM Davoren JM, A Laroche, DM Hodges, MK Pomeroy, TL Furukawa-Stoffer. J Exp Bot 49:33–39, 1998.
33. N Weber, DC Taylor, EW Underhill. In: A Fiechter, ed. Advances in Biochemical Engineering/Biotechnology, Vol. 45. Berlin: Springer-Verlag, 1992, pp 99–131.
34. SS Radwan, HK Mangold. Adv Lipid Res 14:171–211, 1976.
35. SS Radwan, F Spener, HK Mangold. Chem Phys Lipids 14:72–80, 1975.
36. K Norstog, WE Wall, GP Howland. Bot Gaz 130:83–86, 1969.
37. KM Tran Thanh Van. Annu Rev Plant Physiol 32:291–311, 1981.
38. EDP Whelan. Genome 33:260–266, 1990.
39. M Joersho, J Brunstedt. Physiol Plant 81:256–264, 1991.
40. C Forreiter, M Kirschner, L Nover. Plant Cell 9:2171–2181, 1997.
41. DC Taylor, N Weber, EW Underill, MK Pomeroy, WA Keller, WR Scowcroft, RW Wilen, MM Moloney, LA Holbrook. Planta 181:18–26, 1990.
42. MK Pomeroy, JKG Kramer, DJ Hunt, WA Keller. Physiol Plant 81:447–454, 1991.
43. E Wiberg, L Råhlen, M Hellman, E Tillberg, K Glimelius, S Stymne. Theor Appl Genet 82: 515–520, 1991.
44. JL Chen, WD Beversdorf. Euphytica 58:145–155, 1991.
45. DC Taylor, N Weber. Fat Sci Technol 96:228–235, 1994.
46. S Albrecht, C Möllers, GJ Röbbelen. J Plant Physiol 143:526–529, 1994.
47. R Zadernowski, F Sosulski. J Am Oil Chem Soc 56:1004–1007, 1979.
48. C Möllers, S Albrecht. Plant Physiol 144:376–384, 1994.
49. S Albrecht, C Möllers, G Röbbelen. Plant Breed 114:210–214, 1995.
50. L Polsoni, LS Kott, WD Beversdorf. Can J Bot 66:1681–1685, 1988.
51. EB Swanson, MJ Herrgesell, M Arnoldo, DW Sippell, RSC Wong. Theor Appl Genet 78: 525–530, 1989.
52. AMR Baillie, DJ Epp, D Hutcheson, WA Keller. Plant Cell Rep 11:234–237, 1992.

53. SS Bhojwani, MK Razdan. In: Plant Tissue Culture: Theory and Practice, A Revised Edition. New York: Elsevier, 1996, pp 167–213.
54. RA Morrison, DA Evans. Biotechnology 6:684–690, 1988.
55. P Pechan. Plant Cell Rep 8:387–390, 1989.
56. EB Swanson, LR Erickson. Theor Appl Genet 78:831–835, 1989.
57. JL Chen, WD Beversdorf. Theor Appl Genet 88:187–192, 1994.
58. H Fukuoka, T Ogawa, M Matsuoka, Y Ohkawa, H Yano. Plant Cell Rep 17:323–328, 1998.
59. L Nehlin. The Use of Rapeseed (*Brassica napus* L.) Microspores as a Tool for Biotechnological Applications. PhD dissertation, Swedish University of Agricultural Sciences, Uppsala, 1999.
60. AK Stobart, S Stymne, S Höglund. Planta 169:33–37, 1986.
61. EP Kennedy. Fed Proc Am Soc Exp Biol 20:934–940, 1961.
62. M Mancha, S Stymne. Planta 203:51–57, 1997.
63. K Stobart, M Mancha, M Lenman, A Dahlqvist, S Stymne. Planta 203:58–66, 1997.
64. A Dahlqvist, A Banas, S Stymne. In: J Sánchez, E Cerdá-Olmedo, E Martínez-Force, eds. Advances in Plant Lipid Research. Sevilla: Secretariado de Publicaciones, Universidad de Sevilla, 1998, pp 211–214.
65. A Dahlqvist, U Ståhl, M Lenman, A Banas, M Lee, L Sandager, H Ronne, S Stymne. Proc Natl Acad Sci USA 97:6487–6492, 2000.
66. JL Harwood. Annu Rev Plant Physiol Mol Biol 39:101–138, 1988.
67. J Shanklin, EB Cahoon. Annu Rev Plant Physiol Mol Biol 49:611–641, 1988.
68. WE Shine, M Mancha, PK Stumpf. Arch Biochem Biophys 172:110–116, 1976.
69. J Andrews, K Keegstra. Plant Physiol 72:735–740, 1983.
70. K Ichihara, M Nakagawa, K Tanaka. Plant Cell Physiol 34:557–566, 1993.
71. RK Downey, BM Craig. J Am Oil Chem Soc 41:475–478, 1964.
72. E Fehling, KD Mukherjee. Biochim Biophys Acta 1082:239–246, 1991.
73. HV Whitfield, DJ Murphy, MJ Hills. Phytochemistry 32:255–258, 1993.
74. X Bao, M Pollard, J Ohlrogge. Plant Physiol 118:183–190, 1998.
75. SA Finlayson, DT Dennis. Arch Biochim Biophys 199:179–185, 1980.
76. DJ Murphy, KD Mukherjee. Lipids 22:293–298, 1987.
77. S Stymne, AK Stobart, G Glad. Biochim Biophys Acta 752:198–208, 1983.
78. G Griffiths, AK Stobart, S Stymne. Biochem J 230:379–388, 1985.
79. G Griffiths, S Stymne, AK Stobart. Planta 173:309–316, 1988.
80. DC Taylor, N Weber, DL Barton, EW Underhill, LR Hogge, RJ Weselake, MK Pomeroy. Plant Physiol 97:65–79, 1991.
81. M Frentzen. In: TS Moore Jr., ed. Lipid Metabolism in Plants. Ann Arbor, MI: CRC Press, 1993, pp 195–230.
82. B Vick, H Beevers. Plant Physiol 59:459–463, 1977.
83. GM Carman, GF Harrington, R Amegah. J Food Biochem 5:185–195, 1981.
84. M Frentzen. Plant Sci 69:39–48, 1990.
85. K Ichihara. Arch Biochem Biophys 232:685–698, 1984.
86. M Bafor, AK Stobart, S Stymne. J Am Oil Chem Soc 67:217–225, 1990.
87. M Bafor, L Jonsson, AK Stobart, S Stymne. Biochem J 272:31–38, 1990.
88. M Bafor, S Stymne. Phytochemistry 31:2973–2976, 1992.
89. I Löhden, M Frentzen. Planta 188:215–224, 1992.
90. R Bernerth, M Frentzen. Plant Sci 67:21–28, 1990.
91. VS Eccleston, JL Harwood. Biochim Biophys Acta 1257:1–10, 1995.
92. W Hares, M Frentzen. J Plant Physiol 131:49–59, 1987.
93. W Hares, M Frentzen. Planta 185, 124–131, 1991.
94. K Ichihara, T Asahi, S Fujii. Eur J Biochem 167:339–347, 1987.
95. C Sun C, Y-Z Cao, AHC Huang. Plant Physiol 88:56–60, 1988.
96. P Laurent, AHC Huang. Plant Physiol 99:1711–1715, 1992.

97. K-C Oo, AHC Huang. Plant Physiol 91:1288–1295, 1989.
98. EW Edwards, DJ Murphy. Biochem Soc Trans 17:684–685, 1989.
99. DC Taylor, JR Magus, R Bhella, J Zou, SL MacKenzie, EM Giblin, EW Pass, WL Crosby. In: SL MacKenzie, DC Taylor, eds. Seed Oils for the Future. Champaign, IL: AOCS Press, 1993, pp 77–102.
100. Y-Z Cao, K-C Oo, AHC Huang. Plant Physiol 94:1199–1206, 1990.
101. R Rajasekharan, V Nachiappan. Arch Biochem Biophys 311:389–394, 1994.
102. DC Taylor, SL MacKenzie, AR McCurdy, PBE McVetty, EM Giblin, EW Pass, SJ Stone, R Scarth, SR Rimmer, MD Pickard. J Am Oil Chem Soc 71:163–167, 1994.
103. MH Davies, DJ Hawkins, JS Nelsen. Phytochemistry 39:989–996, 1995.
104. DS Knutzon, KD Lardizabal, JS Nelsen, JL Bleibaum, HM Davies, JG Metz. Plant Physiol 109:999–1006, 1995.
105. M Frentzen, F-P Wolter. In: JL Harwood, ed., Plant Lipid Biosynthesis: Fundamentals and Agricultural Applications. Society of Experimental Biology Seminar Series Vol 67. New York: Cambridge University Press, 1998, pp 247–272.
106. MW Lassner, CK Levering, HM Davies, DS Knutzon. Plant Physiol 109:1389–1394, 1995.
107. AP Brown, J Coleman, AM Tommey, MD Watson, AR Slabas. Plant Mol Biol 26:211–223, 1994.
108. AP Brown, CL Brough, JTM Kroon, AR Slabas. Plant Mol Biol 29:267–278, 1995.
109. C Hanke, F-P Wolter, J Coleman, G Peterek, M Frentzen. Eur J Biochem 232:806–810, 1995.
110. D Weier, AF zu Münster, F-P Wolter, M Frentzen. Site directed mutagenesis of lysophosphatidic acid acyltransferases. Proceedings of the National Plant Lipid Cooperative 1999 Biochemistry and Molecular Biology of Plant Fatty Acids and Glycerolipids Symposium, South Lake Tahoe, CA, 1999, p 37.
111. MG Kocsis, RJ Weselake. Lipids 31:785–802, 1996.
112. V Sukumar, PS Sastry. Biochem Int 14:1153–1158, 1987.
113. K Ichihara, S Norikura, S Fujii S. Plant Physiol 90:413–419, 1989.
114. K Ichihara. Planta 183:353–358, 1991.
115. MG Kocsis, RJ Weselake, JA Eng, TL Furukawa-Stoffer, MK Pomeroy. Phytochemistry 41: 353–363, 1996.
116. TL Furukawa-Stoffer, SD Byers, DM Hodges, A Laroche, RJ Weselake. Plant Sci 131:139–147, 1998.
117. TL Furukawa-Stoffer. Characterization of Triacylglycerol Biosynthetic Enzymes from Microspore-Derived Cultures of Oilseed Rape. MSc thesis, University of Lethbridge, Lethbridge, 1996.
118. ML Pearce, AR Slabas. Plant J 14:555–564, 1998.
119. K Ichihara, N Murota, S Fujii. Biochim Biophys Acta 1043:227–234, 1990.
120. DN Brindley. Prog Lipid Res 23:115–133, 1984.
121. A Martin, A Gómez-Munõz, Z Jamal, DN Brindley. Methods Enzymol 197:553–563, 1991.
122. Z Jamal, A Martin, A Gómez-Munõz, DN Brindley. J Biol Chem 266:2988–2996, 1991.
123. DW Waggoner, A Martin, J Dewald, A Gómez-Munõz, DN Brindley. J Biol Chem 270: 19,422–19,429, 1995.
124. DW Waggoner, A Gómez-Munõz, J Dewald, DN Brindley. J Biol Chem 271:16,506–16,509, 1996.
125. RA Deems, BR Eaton, EA Dennis. J Biol Chem 250:9013–9020, 1975.
126. Y-Z Cao, AHC Huang. Plant Physiol 82:813–820, 1986.
127. SH Settlage, RF Wilson, P Kwanyuen. Plant Physiol Biochem 33:399–407, 1995.
128. DJ Lacey, MJ Hills. Planta 199:545–551, 1996.
129. K Ichihara, M Noda. Phytochemistry 21:1895–1901, 1982.
130. K Ichihara, T Takahashi, S Fujii. Biochim Biophys Acta 958:125–129, 1988.
131. G Vogel, J Browse. Plant Physiol 110:923–931, 1996.

132. Y-Z Cao, AHC Huang. Plant Physiol 84:762–765, 1987.
133. E Wiberg, E Tillberg, S Stymne. Phytochemistry 36:573–577, 1994.
134. KC Oo, Y-H Chew. Plant Cell Physiol 33:189–195, 1992.
135. P Kwanyuen, RF Wilson. Biochim Biophys Acta 877:238–245, 1986.
136. RJ Weselake, DC Taylor, MK Pomeroy, SL Lawson, EW Underhill. Phytochemistry 30: 3533–3538, 1991.
137. DC Taylor, N Weber, LR Hogge, EW Underhill, MK Pomeroy. J Am Oil Chem Soc 69: 355–358, 1992.
138. D Little, R Weselake, K Pomeroy, T Furukawa-Stoffer, J Bagu. Biochem J 304:951–958, 1994.
139. RJ Weselake, MK Pomeroy, TL Furukawa, RL Oishi. In: SL MacKenzie, DC Taylor, eds. Seed Oils for the Future. Champaign, IL: AOCS Press, 1993, pp 103–115.
140. SD Byers, A Laroche, KC Smith, RJ Weselake. Lipids 34:1143–1149, 1999.
141. HJ Perry, JL Harwood. Phytochemistry 32:1411–1415, 1993.
142. HJ Perry, JL Harwood. Phytochemistry 33:329–333, 1993.
143. SB Settlage, P Kwanyuen, RF Wilson. J Am Oil Chem Soc 75:775–781, 1998.
144. N Mayorek, I Grinstein, J Bar-Tana. Eur J Biochem 182:395–400, 1989.
145. LBM Tijburg, MJH Geelen, LMG van Golde. Biochim Biophys Acta 1004:1–19, 1989.
146. V Katavic, DW Reed, DC Taylor, EM Giblin, DL Barton, J Zou, SL MacKenzie, PS Covello, L Kunst. Plant Physiol 108:399–409, 1995.
147. JA Wilmer, R Lessire, JPFG Helsper, LHW van der Plas. Physiol Plant 102:185–191, 1998.
148. X Bao, J Ohlrogge. Plant Physiol 120:1057–1062, 1999.
149. JTC Tzen, Y-Z Cao, P Laurent, C Ratnayake, AHC Huang. Plant Physiol 101:267–276, 1993.
150. AR Slabas, CM Sidebottom, A Hellyer, RMJ Kessell, MP Tombs. Biochim Biophys Acta 877:271–280, 1986.
151. AR Slabas, J Harding, A Hellyer, P Roberts, HE Bambridge. Biochim Biophys Acta 921: 50–59, 1987.
152. RW MacKintosh, DG Hardie, AR Slabas. Biochim Biophys Acta 1002:114–124, 1989.
153. L Valencia-Turcotte, R Rodríguez-Sotres. In: J Sánchez, E Cerdá-Olmedo, E Martínez-Force, eds. Advances in Plant Lipid Research, Sevilla: Secretariado de Publicaciones, Universidad de Sevilla, 1998, pp 184–186.
154. AK Stobart, S Stymne. In: JL Harwood, JR Bowyer, eds. Methods in Plant Biochemistry, Vol. 4. London: Academic Press, 1990, pp 19–46.
155. S Hershenson, M Ernst-Fonberg. Biochim Biophys Acta 751:412–421, 1983.
156. SD Byers, A Laroche, KC Smith, RJ Weselake. In: J Sánchez, E Cerdá-Olmedo, E Martínez-Force, eds. Advances in Plant Lipid Research. Sevilla: Secretariado de Publicaciones, Universidad de Sevilla, 1998, pp 191–193.
157. Y Kamisaka, T Nakahara. J Biochem 119:520–523, 1996.
158. MA Rodriguez, C Dias, TE Lau. Lipids 27:577–581, 1992.
159. A Baldo, N Dardano, A Sniderman, K Cianflone. McGill J Med 2:12–19, 1996.
160. Y Kamisaka, S Mishra, T Nakahara. J Biochem 121:1107–1114, 1997.
161. K Lardizabal, D Hawkins, J Mai, N Wagner. Identification of a new diacylglycerol acyltransferase gene family. Proceedings of the National Plant Lipid Cooperative 1999 Biochemistry and Molecular Biology of Plant Fatty Acids and Glycerolipids Symposium, South Lake Tahoe, CA, 1999, p 20.
162. S Cases, SJ Smith, Y-W Zheng, HM Myers, SR Lear, E Sande, S Novak, C Collins, CB Welch, AJ Lusis, SK Erickson, RV Farese Jr. Proc Natl Acad Sci USA 95:13,018–13,023, 1998.
163. P Oelkers, A Behari, D Cromely, JT Billheimer, SL Sturley. J Biol Chem 273:26,765–26,771, 1998.
164. DH Hobbs, C Lu, MJ Hills. FEBS Lett 452:145–149, 1999.
165. J Zou, Y Wei, C Jako, A Kumar, G Selvaraj, DC Taylor. Plant J 19:645–653, 1999.

166. CL Nykiforuk, A Laroche, RJ Weselake. Plant Physiol 120:1207, 1999.
167. CL Nykiforuk, A Laroche, RJ Weselake. Plant Physiol 121:1053, 1999.
168. R Weselake, K Pomeroy, T Furukawa-Stoffer, D Little, R Rajasekharan. In: J-C Kader, P Mazliak, eds. Plant Lipid Metabolism. Boston: Kluwer Academic Publishers, 1995, pp 518–520.
169. CR McMaster, RM Bell. Biochim Biophys Acta 1348:100–110, 1997.
170. CR Slack, LC Campbell, JA Browse, PG Roughan. Biochim Biophys Acta 754:10–20, 1983.
171. CR Slack, PG Roughan, JA Browse, SE Gardiner. Biochim Biophys Acta 833:438–448, 1985.
172. AK Stobart, S Stymne. Biochem J 232:217–221, 1985.
173. RH Hjelmstad, RM Bell. J Biol Chem 265:1755–1764, 1990.
174. RH Hjelmstad, RM Bell. J Biol Chem 266:5094–5103, 1991.
175. RE Dewey, RF Wilson, WP Novitzky, JH Goode. Plant Cell 6:1495–1507, 1994.
176. DR Tocher, MJ Leaver, PA Hodgson. Prog Lipid Res 37:73–117, 1998.
177. S Gibson, DL Falcone, J Browse, C Somerville. Plant Cell Environ 17:627–637, 1994.
178. C Somerville, J Browse. Science 252:80–87, 1991.
179. NS Yadav, A Wierzbicki, M Aegerter, CS Caster, L Pérez-Grau, AJ Kinney, WD Hitz, R Booth Jr, B Schweiger, KL Stecca, SM Allen, M Blackwell, RS Reiter, TJ Carslon, SH Russell, KA Feldmann, J Pierce, J Browse. Plant Physiol 103:467–476, 1993.
180. J Okuley, J Lightner, K Feldmann, N Yadav, E Lark, J Browse J. Plant Cell 6:147–158, 1994.
181. M Miquel, J Browse. Plant Physiol Biochem 36:187–197, 1998.
182. M Miquel, J Browse. J Biol Chem 267:1502–1509, 1992.
183. KA Feldmann, MD Marks, ML Christianson, RS Quatrano. Science 243:1351–1354, 1989.
184. RA Moreau, PK Stumpf. Plant Physiol 69:1293–1297, 1982.
185. S Stymne, AK Stobart. Biochem J 223:305–314, 1984.
186. S Stymne, G Glad. Lipids 16:298–305, 1981.
187. S Stymne, AK Stobart. Biochem J 220:481–488, 1984.
188. K Ichihara, K Mae, Y Sano, K Tanaka. Planta 196:551–557, 1995.
189. BA Martin, RW Rinne. Plant Physiol 81:41–44, 1986.
190. C Demandre, J Bahl, H Serghini, MJ Alpha, P Mazliak. Phytochemistry 35:1171–1175, 1994.
191. DJ Murphy, KD Mukherjee, E Latzko. Planta 161:249–254, 1984.
192. R Rajasekharan, H Roychowdhury. In: J-C Kader, P Mazliak, eds. Plant Lipid Metabolism. Boston: Kluwer Academic Publishers, 1995, pp 525–527.
193. MG Richard, CR McMaster. Lipids 33:1229–1234, 1998.
194. R Lehner, A Kuksis. J Biol Chem 268:8781–8786, 1993.
195. S Stymne. In: N Murata, CR Somerville, eds. Biochemistry and Molecular Biology of Membrane and Storage Lipids of Plants. Current Topics in Plant Physiology Vol 9. Rockville, MD: The American Society of Plant Physiologists, 1993, pp 150–158.
196. M Bafor, MA Smith, L Jonsson, K Stobart, S Stymne. Biochem J 280:507–514, 1991.
197. EB Cahoon, JB Ohlrogge. Plant Physiol 104:845–855, 1994.
198. A Banaś, I Johansson, S Stymne. Plant Sci 84:137–144, 1992.
199. U Ståhl, A Banaś, S Stymne. Plant Physiol 107:953–962, 1995.
200. H Aach, F Hornig, K-P Heise. J Plant Physiol 151:323–328, 1997.
201. AHC Huang. Annu Rev Plant Plant Physiol Mol Biol 43:177–200, 1992.
202. DJ Murphy. Prog Lipid Res 32:247–280, 1993.
203. G Wanner, RR Theimer. Planta 140:163–169, 1978.
204. G Wanner, H Formanek, RR Theimer. Planta 151:109–123, 1981.
205. LY Yatsu, TJ Jacks. Plant Physiol 49:937–943, 1972.
206. TJ Jacks, TP Hensarling, JN Neucere, LY Yatsu, RH Barker. J Am Oil Chem Soc 67:353–361, 1990.

207. AHC Huang. Plant Physiol 110:1055–1061, 1996.
208. DJ Murphy, I Cummins, AS Kang. Biochem J 258:285–293, 1989.
209. I Cummins, MJ Hills, JHE Ross, DH Hobbs, MD Watson, DJ Murphy. Plant Mol Biol 23: 1015–1027, 1993.
210. JA Napier, AK Stobart, PR Shewry. Plant Mol Biol 31:945–956, 1996.
211. BM Abell, LA Holbrook, M Abenes, DJ Murphy, MJ Hills, MM Moloney. Plant Cell 9: 1481–1493, 1997.
212. DJ Lacey, F Beaudoin, CE Dempsey, PR Shewry, JA Napier. Plant J 17:397–405, 1999.
213. F Beaudoin, DJ Lacey, JA Napier. Plant Physiol Biochem 37:481–490, 1999.
214. J Browse, C Somerville. Annu Rev Plant Physiol Mol Biol 42:467–506, 1991.
215. N Murata, Y Tasaka. Biochim Biophys Acta 1348:10–16, 1997.
216. M Bertrams, E Heinz. Plant Physiol 68:653–657, 1981.
217. M Frentzen, E Heinz, T McKeon, PK Stumpf. Eur J Biochem 729:629–636, 1983.
218. D Douady, J-P Dubacq. Biochim Biophys Acta 921:615–619, 1987.
219. D Douady, C Passaquet, J-P Dubacq. Plant Sci 66:65–72, 1990.
220. M Frentzen, I Nishida, N Murata. Plant Cell Physiol 28:1195–1201, 1987.
221. I Nishida, M Frentzen, O Ishizaki, N Murata. Plant Cell Physiol 28:1071–1079, 1987.
222. O Ishizaki, I Nishida, K Agata, G Eguchi, N Murata. FEBS Lett 238:424–430, 1988.
223. S Weber, F-P Wölter, F Buck, M Frentzen, E Heinz. Plant Mol Biol 17:1067–1076, 1991.
224. I Löhden, M Frentzen. Planta 176, 506–512, 1988.
225. SR Ferri, T Toquri. Arch Biochem Biophys 337:202–208, 1997.
226. F Bourgis, J-C Kader, P Barret, M Renard, D Robinson, C Robinson, M Delseny, TJ Roscoe. Plant Physiol 120:913–922, 1999.
227. J Joyard, R Douce. FEBS Lett 102:147–150, 1979.
228. MA Block, A-J Dorne, J Joyard, R Douce. FEBS Lett 164:111–115, 1983.
229. A Malherbe, MA Block, J Joyard, R Douce. J Biol Chem 267:23,546–23,553, 1992.
230. A Malherbe, MA Block, R Douce, J Joyard. Plant Physiol Biochem 33:149–161, 1995.
231. J Joyard, MA Block, A Malherbe, F Maréchal. In: TS Moore Jr, ed. Lipid Metabolism in Plants. Boca Raton, FL: CRC Press, 1993, pp 231–258.
232. J Joyard, E Maréchal, A Malherbe, MA Block. Prog Lipid Res 33:105–118, 1994.
233. BA Martin, RF Wilson. Lipids 19:117–121, 1984.
234. T Sakaki, K Saito, A Kawaguchi, N Kondo, M Yamada. Plant Physiol 94:766–772, 1990.
235. T Sakaki, N Kondo, M Yamada. Plant Physiol 94:773–780, 1990.
236. BA Martin, ME Horn, JM Widholm, RW Rinne. Biochim Biophys Acta 796:146–154, 1984.
237. J Browse, CR Somerville, CR Slack. Plant Sci 56:15–20, 1988.
238. L Xue, M McCune, KF Kleppinger-Sparace, MJ Brown, MK Pomeroy, SA Sparace. Plant Physiol 113:549–557, 1997.
239. B Piffanelli JHE Ross, DJ Murphy. Sex Plant Reprod 11:65–80, 1998.
240. V Raghavan. Cell Differ 21:213–226, 1987.
241. Z Fan, KC Armstrong, WA Keller. Protoplasma 147:191–199, 1988.
242. MAM Zaki, HG Dickinson. Sex Plant Reprod 4:48–55, 1991.
243. TL Reynolds. Plant Mol Biol 33:1–10, 1997.
244. DE Evans, NE Rothnie, MV Palmer, DG Burke, JP Sang, RB Knox, EG Williams, EP Hilliard, PA Salisbury. Phytochemisty 26:1895–1897, 1987.
245. DE Evans, NE Rothnie, JP Sang, MV Palmer, DL Mulchy, MB Singh, RB Knox. Theor Appl Genet 76:411–419, 1998.
246. DE Evans, PE Taylor, MB Singh, RB Knox. Plant Sci 73:117–126, 1991.
247. M Wolters-Arts, WM Lush, C Mariani. Nature 392:818–821, 1998.
248. E Pacini, GG Franchi, M Hesse. Plant Syst Evol 149:155–185, 1985.
249. J Heslop-Harrison. New Phytol 67:779–786, 1968.
250. P Piffanelli, JHE Ross, DJ Murphy. Plant J 11:549–562, 1997.

251. SSH Wu, KA Platt, C Ratnayake, T-W Wang, JTL Ting, AHC Huang. Proc Natl Acad Sci USA 94:12,711–12,716, 1997.
252. KA Platt, AHC Huang, WW Thomson. Int J Plant Sci 159:724–737, 1998.
253. AHC Huang. In: J Sánchez, E Cerdá-Olmedo, E Martínez-Force, eds. Advances in Plant Lipid Research. Sevilla: Secretariado de Publicaciones, Universidad de Sevilla, 1998, pp 200–203.
254. SSH Wu, RA Moreau, BD Whitaker, AHC Huang. Lipids 34:517–523, 1999.
255. I Hernández-Pinzón, JHE Ross, KA Barnes, AP Damant, DJ Murphy. Planta 208:588–598, 1999.
256. D Preuss. Trends Genet 11:147–153, 1995.
257. DE Evans, PE Taylor, MB Singh, RB Knox. Planta 186:343–354, 1992.
258. MR Roberts, R Hodge, JHE Ross, A Sorensen, DJ Murphy, J Draper, R Scott. Plant J 3: 629–636, 1993.
259. K Lee, FY Bih, GH Learn, JTL Ting, C Sellers, AHC Huang. Planta 193:461–469, 1994.
260. LS Robert, J Gerster, S Allard, L Cass, J Simmonds. Plant J 6:927–933, 1994.
261. MR Roberts, R Hodge, R Scott. Planta 195:469–470, 1995.
262. JHE Ross, DJ Murphy. Plant J 9:625–637, 1996.
263. RK Ruiter, GJ Van Eldik, RMA van Herpen, JAM Schrauwen, GJ Wullems. Plant Cell 9: 1621–1631, 1997.
264. MA Ferreira, J de Engler, FC Miguens, M van Montagu, G Engler, DE de Oliveira. Plant Physiol Biochem 35:729–739, 1997.
265. DJ Murphy, JHE Ross. Plant J 13:1–16, 1998.
266. T-W Wang, RA Balsamo, C Ratnayake, KA Platt, JTL Ting, AHC Huang. Plant J 11:475–487, 1997.
267. DT Canvin. Can J Bot 43:63–69, 1965.
268. H Trémolières, A Trémolières, P Mazliak. Phytochemistry 17:685–687, 1978.
269. C Möllers, S Albrecht, G Röbbelen. J Plant Physiol 143:530–533, 1994.
270. JA Wilmer, JPFG Helsper, LHW van der Plas. J Plant Physiol 147:486–492, 1996.
271. R Finkelstein, C Somerville. Plant Sci 61:213–217, 1989.
272. RR Finkelstein, ML Crouch. Plant Physiol 81:907–912, 1986.
273. RR Finkelstein, CR Somerville. Plant Physiol 94:1172–1179, 1990.
274. JA Wilmer, JPEG Helsper, LHW van der Plas. J Plant Physiol 150:414–419, 1997.
275. LA Holbrook, JR Magus, DC Taylor. Plant Sci 84:99–115, 1992.
276. MK Pomeroy, DCW Brown, Y Takahata. In Vitro Cell Dev Biol 30P:196–203, 1994.
277. GJH van Rooijen, RW Wilen, LA Holbrook, MM Moloney. Can J Bot 70:503–508, 1992.
278. LA Holbrook, GJH van Rooijen, RW Wilen, MM Moloney. Plant Physiol 97:1051–1058, 1991.
279. RW Wilen, GJH van Rooijen, DW Pierce, RP Pharis, LA Holbrook, MM Moloney. Plant Physiol 95:399–405, 1991.
280. B Parthier. J Plant Growth Reg 9:57–63, 1990.
281. DB Hays, RB Wilen, C Sheng, MM Moloney, RP Pharis. Plant Physiol 119:1065–1072, 1999.
282. J Zou, GD Abrams, DL Barton, DC Taylor, MK Pomeroy, SR Abrams. Plant Physiol 108: 563–571, 1995.
283. F Pacheco-Moisés, L Valencia-Turcotte, M Altuzar-Martínez, R Rodríguez-Sotres. Plant Physiol 114:1095–1101, 1997.
284. VB Mhaske, K Chengalrayan, S Hazra. Plant Cell Rep 17:742–746, 1998.
285. CL Nykiforuk, P Huff, TL Furukawa-Stoffer, DC Taylor, J-T Zou, A Laroche, RJ Weselake. In: J Sánchez, E Cerdá-Olmedo, E Martínez-Force E, eds. Advances in Plant Lipid Research. Sevilla: Secretariado de Publicaciones, Universidad de Sevilla, 1998, pp 225–227.
286. N Murata, O Ishizaki-Nishizawa, S Higashi, H Hayashi, Y Tasaka, I Nishida. Nature 356: 710–713, 1992.

287. AJD Del Vecchio. INFORM 7:230–242, 1996.
288. MR Pollard, L Anderson, C Fan, DJ Hawkins, HM Davies. Arch Biochem Biophys 284: 306–312, 1991.
289. TA Voelker, TR Hayes, AC Cranmer, HM Davies. Plant J 9:229–241, 1996.
290. VS Eccleston, JB Ohlrogge. Plant Cell 10:613–621, 1998.
291. DS Knutzon, TR Hayes, A Wyrick, H Xiong, HM Davies, TA Voelker. Plant Physiol 120: 739–746, 1999.
292. RK Downey. In: JKG Kramer, FD Saver, WJ Pidgen, eds. High and Low Erucic Acid Rapeseed Oils: Production, Usage, Chemistry and Toxicological Evaluation. New York: Academic Press, 1983, pp 1–20.
293. NOV Sonntag. INFORM 2:449–463, 1991.
294. MM Nagiec, GB Wells, RL Lester, RC Dickson. J Biol Chem 268:22,156–22,163, 1993.
295. J Zou, V Katavic, EM Giblin, DS Barton, SL MacKenzie, WA Keller, X Hu, DC Taylor. Plant Cell 9:909–923, 1997.
296. S Shah, Z Xin, J Browse. Plant Physiol 114:1533–1539, 1997.

3

Redirecting Lipid Metabolism in Plants

DAVID HILDEBRAND, SURYADEVARA RAO, and TOMOKO HATANAKA

University of Kentucky, Lexington, Kentucky

1 INTRODUCTION

Advances in understanding lipid biosynthesis has facilitated the genetic engineering of oilseeds as a supplement to breeding oilseeds for improved seed oil composition. Although traditional breeding has been very successful in improving the composition of many major oilseed oils, new molecular genetic tools have accelerated this progress. This success has been greatest with *Brassica napus* and *B. rapa*, the source of canola and rapeseed oil. Although genetic improvement of soybean oil has been more difficult, considerable progress has been made with soybean oil due to the large effort devoted to this most important oil crop. This has included molecular genetic improvement of soybean oil composition despite the continuing recalcitrance of soybean transformation and regeneration perhaps best exemplified by DuPont's high-oleic-acid soybean. It was stated in 1992 [1] that the ''most important concern . . . is whether consumers will accept genetically engineered foods.'' This remains the most important concern today, but it is expected to eventually dissipate once consumers begin to understand that foods improved via recombinant DNA techniques can be every bit as safe (sometimes safer) as traditional foods and can offer a number of health, environmental, and economic benefits.

Alteration of lipid metabolism in plants is predicated on an understanding of plant lipid biosynthesis. Major lipids in plant tissues include the glycerolipids, fatty acid (FA) derivatives, isoprenoids, and some phenylpropanoids. Triacylglycerols (TAGs) are the principal storage lipids, whereas diacylglycerols (DAGs) constitute the main membrane lipids. For most commercially important oil plants, lipid accumulates in the seed in the form of TAGs. Oil biosynthesis occurs during the second stage of seed maturation [2], at which time the relevant biosynthetic enzymes are expressed at high activity. The major FAs of plants (and most other eukaryotic organisms) have a chain length of 16 or 18

carbons and contain from zero to three cis double bonds. Five FAs (18:1, 18:2, 18:3, 16:0, and, in some species, 16:3) make up over 90% of acyl chains of structural glycerolipids of almost all plant membranes [3]. The nature of the acyl composition of the TAG is dependent on the availability of the FAs from the acyl-CoA substrate pool as well as the selectivity of the acyltransferases of the Kennedy pathway [4] and, possibly, transacylases.

This chapter will review the progress in using modern genetic techniques to change plant fatty acid metabolism to improve seed oils for edible and industrial uses, with emphasis on desaturase and related enzyme manipulations and application to soybean oil. The first part of this chapter will review biosynthesis of the main unsaturated fatty acids in plants and their accumulation in TAG. The second part will cover the synthesis and metabolism of some unusual unsaturated fatty acid derivatives, with emphasis on oxygenated fatty acids; the last part will review soybean transformation and regeneration as used in genetic improvement of soybean oil quality.

2 FATTY ACIDS: FOOD AND NONFOOD USES

Altering the FA composition in the TAGs can increase the demand for plant oils for both edible and industrial uses, whereas reducing the saturated FA levels of plant oil will improve its application in several edible uses. On the other hand, increasing the saturated FA content will enhance oxidative stability of the vegetable oil, thereby eliminating the need for postharvest modifications like hydrogenation. Recently, there is considerable interest in increasing the palmitoleic acid content in diets for health purposes [5,6]. This is the basis of the interest and the current very high price for macadamia nut oil. Linoleic acid (18:2) has nutritional importance, as it increases the low-density to high-density lipoproteins ratio. Increasing the tri-unsaturated (α-linolenic acid) content will make such oil more attractive as a drying oil for paints, inks, and other industrial products. α-Linolenic acid is the precursor of the nutritionally important ω-3 FAs, eicosapentaenoic acid (EPA, 20:5), docosapentaenoic acid (DPA, 22:5), docosahexaenoic acid (DHA, 22:6), certain prostaglandins, and other oxylipins or eicosanoids derived from these long-chain FAs. The term ''oxylipin'' was introduced by Gerwick et al. [7] ''as an encompassing term for oxygenated compounds which are formed from FAs by reaction(s) involving at least one step of mono- or dioxygenase-catalysized oxygenation. As such, this term includes the eicosanoids as well as the biosynthetically related compounds of longer and shorter chain length.'' The various edible and industrial applications of different FAs are summarized in Table 1.

3 FATTY ACID SYNTHESIS

Overall, FA synthesis (Fig. 1) and, consequently, its regulation apparently are more complicated in plants than in any other organism. Unlike in other organisms, plant FA synthesis is not localized within the cytosol but occurs mostly in an organelle, the plastid. Although a portion of the newly synthesized acyl chain is then used for lipid synthesis within plastids (the prokaryotic pathway), the major portion is exported into the cytosol for glycerolipid assembly at the endoplasmic reticulum (ER) or other sites (the eukaryotic pathway) [8]. The plastidial pathway of FA biosynthesis is catalyzed by the two enzyme systems: acetyl-CoA carboxylase (ACCase) and FA synthase (FAS). ACCase catalyzes the extension of the growing acyl chains with malonyl-ACP (acyl carrier protein). The final products of FA synthesis are usually 16:0 and 18:0-acyl carrier protein, and the final FA composition of a plant is determined by activities of several enzymes that use these acyl-ACPs as the

Table 1 Examples of Present and Potential Uses for Fatty Acids and Their Derivatives

Fatty acid or lipid type	Food uses	Nonfood uses
C8 to C10	Dietary margarine	Soaps, detergents, cosmetics
C12 to C14	Confectionery, synthetic creams	Detergents, laundries, cosmetics
C16	Shortenings	Soaps, candles, lubricant grease
C18:0	Confectionery	Cosmetics, pharmaceuticals, candles
Δ6 C18:1	Shortenings	Detergents, polymers, cosmetics, pharmaceuticals
Δ9 C18:1	Margarine, frying oil, salad oil	Soaps, detergents, coatings, plasticizers, cosmetics, pharmaceuticals, polymers
Δ9,12 C18:2[a]	Salad oil, margarine	Coatings, drying oils
Δ9,12,15 C18:3[a]	Salad oil, margarine	Varnishes, paints, coatings, linoleum, drying oil
Hydroxy	N/A[b]	Lubricants, plasticizers, coatings, pharmaceuticals, cosmetics, printing inks, textile dyes, leather manufacture, medicinal applications
Long chain	N/A	Lubricants, antislip agents, polymers, cosmetics, plasticizers, surfactants, detergents, pharmaceuticals
Epoxy	N/A	Plasticizers, coatings, adhesives, resins, paints, composites
Wax esters	N/A	Lubricants, cosmetics

[a] Essential fatty acids in human diets.
[b] Not applicable.
Source: From Ref. 196, and adapted from Refs. 197 and 198.

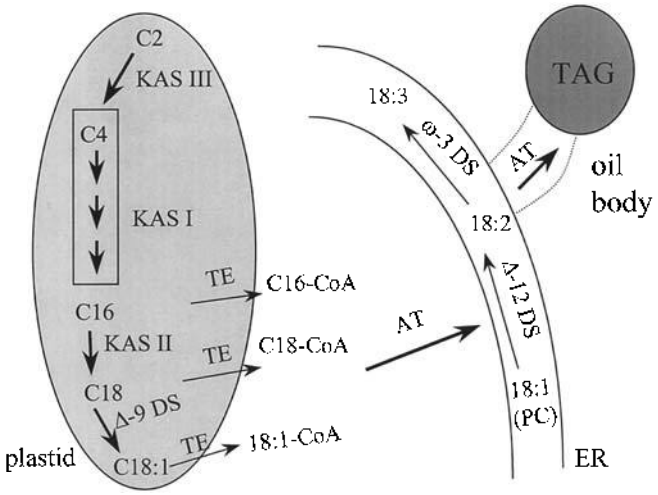


Figure 1 A simplified representation of fatty acid and triacylglycerol biosynthesis in plant cells from maturing oilseed tissue. Cxx:x = fatty acid of xx carbon length and x double bonds, KAS = 3-ketoacyl-ACP synthase, DS = desaturase, TE = thioesterase, AT = acyltransferase, PC = phosphatidylcholine, ER = endoplasmic reticulum.

termination phase of FA synthesis and of enzymes that cleave the thioester bonds of acyl-ACP. The relative activities of these enzymes therefore regulate the products of FA synthesis.

Reactions of FA synthesis are terminated by hydrolysis or transfer of the acyl chains from the ACP by ACP-hydrolase or an acyltransferase consecutively. The ''competition'' for the substrate is thus a competition between the termination of synthesis, a function of thioesterase and transferase activity, and extension, a function of β-ketoacyl-ACP synthase (KAS) I and KAS II isoforms [9,10]. ACP-thioesterases are one of two main types [11]. One thioesterase is relatively specific for 18:1 ACP, encoded by *Fat A*, and a second is more specific for saturated acyl-ACPs, encoded by *Fat B*. Short-chain FAs of California bay are produced by a thioesterase that shows maximal hydrolytic activity on 12C-ACP thioesters. The medium-chain thioesterase thus diverts a portion of FA from the elongation pathway and forms lauric acid [12]. FA molecules formed in the chloroplast stroma are released from ACPs by thioesterases and cross the membrane by an unknown mechanism. After the FAs cross the membrane, they are converted to acyl-CoA esters through the catalytic activity of an acyl-CoA synthetase located on the outer membrane. In the cytoplasm, the fatty acyl-CoA forms a pool for further FA elongation and glycerolipid synthesis. California bay's TAG profile in seeds may consist of up to 70% lauric acid (12:0).

4 DESATURASES

The FA biosynthesis pathway produces saturated FAs, but in most plant tissues, over 75% of the FAs are unsaturated. Two types of desaturase have been identified, one soluble and the other membrane bound, that have different consensus motifs. Database searching for these motifs reveals that these enzymes belong to two distinct multifunctional classes, each of which includes desaturases, hydrolases, and epoxygenases that act on FA or other substrates [13]. Free FA are not thought to be desaturated in vivo, rather they are esterified to ACP for the soluble plastid desaturase or to CoA or to phospholipids for integral membrane desaturases.

4.1 Δ-9 Desaturases

The first double bond in unsaturated FAs is introduced by a soluble enzyme, stearoyl-ACP desaturase [12,13]. Plant stearoyl-ACP desaturase cDNAs have been isolated from potato [14], flax [15], safflower [16], castor [17], and rice [18] plus >20 additional plant Δ-9 desaturases (GenBank). Desaturases that convert saturated FA to mono-unsaturated FA, share several common characteristics. They perform stereospecific Δ-9 desaturation of an 18:0/16:0 substrate with the removal of the 9-D and 10-D hydrogens [19,20]. Protein crystallographic studies on the purified desaturase from castor bean have shown that it contains a diiron cluster [21]. The protein is active as a homodimer and consists of a single domain of 11 helices. This diiron center is the active site of the desaturase [22]. This is the only detailed study about the structure of any desaturase in plants. Such studies are required because they can provide clues for designer proteins that can be used for transforming crop plants for better quality traits. Like the soluble stearate desaturase, the Δ-4 palmitate desaturase from coriander [23], the Δ-6 palmitate desaturase from *Thunbergia* [24], and the Δ-4 palmitate/myristate desaturase from milkweed (*Asclepias syriaca*) [25] all use acyl-ACP substrates and are localized in the stromal fraction of plastids in developing seeds [26].

Expression and regulation of Δ-9 desaturase in plants have been studied extensively [27,28]. The expression of the promoter of the *B. napus* stearoyl desaturase gene in tobacco was found to be temporally regulated in developing seed tissues. However, the promoter was also particularly active in other oleogenic tissues such as tapetum and pollen grains, raising the interesting question of whether seed expressed lipid synthesis genes are regulated by separate tissue-specific determinants or by a single factor common to all oleogenic tissues [28]. The temporal and tissue-specific regulation of two stearoyl-ACP desaturase isoenzymes was analyzed in developing seed of sesame. The Northern blot analysis of the transcripts showed that levels initiated at 7 days after anthesis (DAA) peaked at 21 DAA and reduced rapidly after 35 DAA. Both the cDNAs were seed-specific, however, one of the cDNA messages accumulated at a low level in young leaves in addition to seeds [29]. Two stearoyl-ACP desaturase cDNAs from sunflower were expressed strongly in developing seeds, moderately expressed in leaves and flowers and weakly expressed in cotyledons, roots, and stems [30]. In *Saccharomyces cerevisiae*, the addition of saturated FAs induced Δ-9 FA desaturase mRNA (*Ole1* mRNA) by 1.6-fold, whereas a large family of unsaturated FAs repress *Ole1* transcription by 60-fold. A 111-bp FA regulation region (FAR) approximately 580 bp upstream of the start codon that is essential for the transcription activation and unsaturated FA repression was identified [31]. Not only the transcriptional regulation but also unsaturated FAs mediated changes in the half-life of the *Ole1* mRNA [32].

Alteration of Δ-9 desaturase levels by genetic manipulation led to the changes in the composition of saturated (18:0, 16:0) and mono-unsaturated FA (16:1, 18:1) levels. A mutation at the *FAB2* locus of *Arabidopsis* caused increased levels of 18:0, where an 80% increase of stearate was observed in the leaf tissue. However, the plants produced had an extremely altered phenotype, with the plants being miniature under normal growth conditions. This mutation, however, did not affect 18:1, 18:2, and 18:3 amounts, indicating that the mutation affected the stearoyl–acyl carrier protein desaturase gene [33]. The amount of stearate content increase varied considerably among different lipids, from a high of 85% in phosphatidate (PC) to a low of 29% in monogalactosyl diacylglycerol (MGDG).

Seed-specific antisense gene constructs of *B. napus* stearoyl-ACP desaturase have been used to reduce the protein concentration and enzyme activity of stearoyl-ACP desaturase [34]. The expression of the *Brassica rapa* stearoyl-ACP desaturase was detected at 19 DAA, was maximal at 25 DAA and was not detected at 35 DAA. In contrast, the expression of napin storage Bc Na1 in *B. rapa* seeds is first detected at 17 DAA and then reaches very high levels between 21 and 30 DAA. Antisense gene constructs utilizing the napin and ACP cassettes were combined in one binary vector for plant transformation. Of the 22 transgenics studied, 20 produced seeds with stearate levels varying between 3% and 32%, with the highest stearate levels observed in the 3–9% range. The increased stearate levels were accompanied by decreased oleic acid. Levels of palmitate, the precursor to stearate, were not significantly altered in the transformants. High-stearate seeds (32% stearate-containing seeds) also have an increased percentage of 18:3 and low but increased levels of long-chain FAs. High-stearate seeds have a much reduced oil content (39 ± 17 μg of FA per seed) in comparison to normal-stearate plants (721 ± 26 μg of FA per seed). Enzyme-activity assays and Western blot analysis showed no detectable enzyme activity and reduced levels of stearoyl-ACP desaturase protein. Many high-stearate seeds with low oil content did not germinate. Seeds with relatively normal oil content and high stearate content (39.8%) germinated, however, indicating the difference

in germination could be attributed to oil content of the seeds. Because the TAGs in canola oil contained almost exclusively unsaturated FAs in the *sn*-2 position, the low amount of oil production could be due to the specificity of *sn*-2 acyltransferase [34].

A multimer hammerhead ribozyme linked to a selectable maker gene was used [35] to downregulate the maize Δ-9 desaturase gene. Two out of 13 transgenic maize lines had twofold to fourfold increases in leaf stearic acid. There was a concomitant decrease in Δ-9 desaturase mRNA and protein and the plants expressed ribozyme RNA. Despite the alteration in leaf FA profiles, there was no modification of FA profiles for seed embryos. Merlo et al. [35] concluded that to downregulate Δ-9 desaturase activity in embryos, an increase in ribozyme activity comparable with the increase of Δ-9 desaturase mRNA in the embryos would be required.

Using a rat liver Δ-9 desaturase, somatic embryos of soybeans were successfully transformed [36]. The transformed embryos had 16:1 levels ranging from 0% to over 10% of total FAs, whereas the levels of 16:0 dropped from 25% to approximately 5% of total FAs. However, due to the infertility of the R0 plants, the zygote seeds were not evaluated. Expression of a yeast Δ-9 desaturase under the control of CaMV 35S promoter resulted in significantly increased levels of palmitoleic acid in the leaf tissues of *Petunia hybrida*. Smaller compensatory changes were observed in the level of 16:0 and 18:0 in transformed plants. No obvious phenotypic differences were apparent, however, in the transformed plants [37]. Localization of the yeast gene in the chloroplasts was ruled out because the region comprising the first 35 amino acids of the desaturase is strongly acidic and does not contain the extremely high levels of serine or threonine residues found in chloroplast transit peptides. When a yeast Δ-9 desaturase gene was expressed in tobacco under the CaMV 35S promoter with a polyadenylation signal, a 10-, 11-, and 6-fold increase of 16:1 was observed in leaf, stem, and root tissues, respectively, in comparison to wild-type plants which have less than 2% palmitoleic acid. A modest increase (twofold to threefold) in the seeds was also observed. These results indicated that the yeast Δ-9 desaturase functioned in plants, presumably by using a cytochrome b_5-mediated electron-transport system. Positional analysis showed that 16:1 is found at the *sn*-2 position of most major polar lipids, including the cytoplasmically produced ''eukaryotic'' fraction of galactolipids except phosphatidylglycerol (PG), which is considered to be produced almost exclusively in chloroplasts. Positional analysis indicated that the *sn*-2 acyltransferases are selective for unsaturated FAs rather than chain length [38,39]. Smaller, compensatory changes were observed in other FAs, including decreases in 16:0 and 18:0 levels of expressed plants. Although there appeared to be minor increases in 18:1 levels in expressing plants, levels of 18:2 and 18:3 were found to be identical in wild-type controls. In this case, the transformed plants did not show any abnormal phenotypes.

4.2 Δ-12 Desaturases

Plant Δ-12 desaturases are plastidial membrane-bound or ER membrane-bound enzymes that catalyze the introduction of the second double bond in the biosynthesis of 18:2 FAs. cDNA encoding *Arabidopsis* plastidal Δ-12 desaturases have been isolated using degenerate oligonucleotides based on amino acid sequences conserved between plant and cyanobacterial desaturases [38]. The *Arabidopsis* Δ-12 desaturase was also used to screen rape and soybean cDNA libraries and the homologous sequences isolated [40]. These plant chloroplast Δ-12 desaturases all show a high degree of similarity (around 50%)

with cyanobacterial Δ-12 desaturases, but less with cyanobacterial and plant ω-3 desaturases.

The Δ-12 desaturase is particularly active in microsomal preparations from developing seed cotyledons of some oilseed species where it is associated with the biosynthesis of TAGs [41]. The microsomal Δ-12 desaturase requires NAD(P)H as reductant and molecular oxygen, and is inhibited by cyanide but not carbon monoxide, suggesting that cytochrome P-450 is not involved in the electron-transport chain [42].

More than 10 plant microsomal and a similar number of plastidial Δ-12 cDNAs and genes have been isolated and reported to date. *Arabidopsis* mutants lacking both microsomal Δ-12 (*fad2*) and ω-3 desaturases (*fad3*) have been isolated [43,44]. Mutants at the *Fad2* locus of *Arabidopsis* that are deficient in the major and, perhaps, only Δ-12 desaturase of the eukaryotic pathway have been isolated and characterized. It was shown that the *Arabidopsis fad2* mutants had similar growth characteristics to wild type at 22°C, but their growth was greatly impaired at 12°C and the mutants died at 6°C [45]. This experiment showed that *Arabidopsis* requires polyunsaturated FAs for low-temperature survival [46]. Subsequently, Okuley et al. [47] isolated the entire *Arabidopsis fad2* cDNA sequence with a T-DNA-tagged line with higher 18:1 content in seeds, roots, and leaves than the wild-type line.

After screening soybean libraries with the *Arabidopsis fad2* cDNA, two different Δ-12 desaturase cDNAs, *FAD2-1* and *FAD2-2*, were isolated [48]. *FAD2-1* was expressed in developing seeds, whereas *FAD2-2* was expressed in several tissues (leaves, roots, and stems) in addition to developing seeds.

The microsomal Δ-12 desaturase in parsley (*Petroselinium crispum*) is rapidly and transiently induced by fungal infection. Kirsch et al. [49] have shown that treatment of cultured parsley cells with the pathogenic fungal peptide elicitor (Pep25) caused rapid and large changes in polyunsaturated FA composition. The Δ-12 FA desaturase mRNA accumulated rapidly and transiently in elicitor-treated parsley cells, protoplasts, and leaves. This study suggested that FA desaturation and its transcriptional regulation might be an important component of the pathogen response in plants [49].

Comparison of available sequence information has shown that there is a high degree of similarity between the same class of membrane-bound desaturases in different plant species, but much less similarity between different classes of desaturases, even in the same species [26]. Membrane-bound enzymes most likely contain similar diiron complexes [21]. The most strictly conserved feature is the presence of eight histidines in three separate clusters. These clusters are held in position by a different ligation sphere, which may involve the three histidine boxes [50] that are characteristic for this group of enzymes [HX3-4H, HX2-3HH, (H/Q)X2HH]. This motif was also found in the Δ-12 oleate hydroxylases from castor bean and *Lesquerella fendleri* [51,52], epoxygenase from *Vernonia galamensis* [53], acetylenase and epoxygenase from *Crepis* spp. [54], and the Δ-6 linoleate desaturase from borage [55]. Recently, cDNAs encoding FA-modifying enzymes forming the conjugated double bond from *Impatiens balsamina* and *Momordica charantia* have been isolated and found to be Δ-12-desaturase-type enzymes [56]. These enzymes were referred to as ‘‘conjugases.’’ This highlights the close similarity of these enzyme types and implies that these hydroxylases, acetylenases, epoxygenases, and conjugases may be derived from modified microsomal Δ-12 desaturases (see Sec. 7).

Fatty acid desaturases in all organisms are subject to several different types of regulation, depending on their localization and function. Those desaturases involved in mem-

brane lipid biosynthesis have important ''housekeeping'' functions and are therefore constitutively regulated [26].

Some evidence that housekeeping-type desaturases, such as the soluble Δ-9 and ER-bound Δ-12 enzymes, have central roles in plant growth and development comes from hormonal studies. For example, the involvement of auxin in the upregulation of the ER-bound Δ-12 desaturase mRNA levels suggests an intriguing causal link between the correct level of membrane desaturation and cell elongation [57,58].

A cold-inducible plastidial ω-3 desaturase gene has been isolated from *Arabidopsis* [59] and there are several other reports that are consistent with the presence of cold-inducible ω-3 and Δ-12 desaturase genes in soybeans [60,61]. However, there are other reports of the isolation of *Arabidopsis* and soybean Δ-12 desaturase genes that are not regulated by low temperature [47,48]. Because multigene families encode many desaturases, it is possible that some plant species may have both cold-inducible and non-cold-inducible forms of the same class of desaturase enzyme and/or gene [26].

4.3 ω-3 Desaturases

The ω-3 desaturases catalyze the introduction of the third double bond in the biosynthesis 18:3 FAs (which are important constituents of plant membranes). In most species, the FAs present in the galactolipids of the chloroplast membrane are ~70–80% trienoic FAs. In leaf tissue, there are two distinct pathways for polyunsaturated FA biosynthesis: one located in the microsomes and the other located in the plastidial membranes. In nongreen tissues and developing seeds, the microsomal pathway predominates. Cytosolic and plastidial ω-3 desaturations that result in the production of triene FAs are controlled by *FAD3*, *FAD7*, and *FAD8* loci [62,63]. It has been postulated that these loci correspond to the structural genes for the ω-3 desaturase enzymes. For *FAD3*, proof of this postulate has come from cloning of a cDNA encoding a desaturase corresponding to the *FAD3* locus [64,65]. The *FAD3*, *FAD7*, and *FAD8* genes of *Arabidopsis* also were isolated and characterized [43,62,66]. Since *FAD3, FAD7,* and *FAD8,* and the first ω-3 desaturase gene from cyanobacteria [67], more than 20 other ω-3 desaturase genes have been cloned.

Much of our knowledge of the regulation of ω-3 desaturase function has come from the characterization of *Arabidopsis* mutants which lack one or more ω-3 desaturase activities [43,64,68]. The expression of the ω-3 desaturase gene is strongly regulated by a series of developmental and environmental stimuli. For example, there is a general inverse relationship between growth temperature and the polyunsaturated content of plant membranes. Such responses to temperature plus the response to salinity and drought stress are part of the homoeviscous adaptation of the membranes that is required to maintain biological activity [26]. The chloroplastic ω-3 desaturase confers cold tolerance in transgenic tobacco [69–71]. Differential expression of maize *FAD7* and *FAD8* to temperature was reported by Berberich et al. [72]. In the maize plants, overall expressions of the *FAD7* and *FAD8* transcripts were elevated upon exposure to low temperatures. The maize *FAD7* and *FAD8* transcripts were also elevated due to high-salt treatment. The spatial expression pattern of the *FAD7* genes increased rapidly after local wounding treatments [73]. When the *Arabidopsis FAD7* promoter fused to the GUS or the luciferase reporter gene expressed in tobacco plants, the promoter conferred tissue-specific and light-responsive expression. *Arabidopsis FAD3* and *FAD7* genes were expressed constitutively at all temperatures tested, whereas the *FAD8* gene was expressed at temperatures below

20°C [59,68]. The tobacco plastidial ω-3 desaturase gene was also responsive to wounding [74]. Martin et al. [75] used a potato homolog of the tobacco *fad7* in the antisense direction to reduce the 18:3 levels. Successful transformants have been studied for wound responses. In antisense lines, 50% lower levels of 18:3 were observed in both leaves and tubers. Transgenic lines also had a lower level of 16:3 and a corresponding increase in 16:2. They observed that reduction of 18:3 in the transgenic lines lowered production of the 18:3-derived regulatory molecule, jasmonic acid, in wounded plants. When the cultivated parsley cells were treated with a peptide elicitor (Pep25) of fungal origin, it caused a rapid increase in the chloroplast ω-3 desaturase mRNA and linolenic acid accumulation [49].

The illumination of etiolated leaves induced a rapid differentiation of etioplasts into chloroplast accompanied by enhanced production of 18:3, both in *Avena* [76] and in cucumber [77]. The plastidial ω-3 desaturase is considered to be the principal enzyme for production of trienoic FAs in at least some leaf tissue [43]. Short-term labeling studies had shown that the activity of plastidial ω-3 desaturase was considerably lower than those of Δ-9 and microsomal Δ-12 desaturases in *B. napus*, suggesting that the plastid ω-3 desaturase was rate limiting for the production of linolenic acid [78]. The *FAD7* mutant of *A. thaliana* has been characterized as being deficient in the activity of plastid ω-3 FA desaturase [43]. A corresponding gene for the enzyme *FAD7* was isolated from *A. thaliana* and was shown to complement the *FAD7* mutation [68]. The mRNA level of *Arabidopsis thaliana* (At) *FAD7* in light-grown wheat leaves was much higher than that in dark-grown leaves. During the greening of etiolated leaves, the level of At *FAD7* mRNA increased significantly and was accompanied by an increase of the 18:3 level in total FA. This suggested that the *FAD7* gene is involved in the chloroplast development. Expression studies of wheat *FAD3* and *FAD7* genes suggested a spatial distribution pattern. At *FAD3* expression was linked with the active membrane biogenesis required for cell division and the At *FAD7* expression was linked with the development of thylakoid systems in plants [79]. The *Arabidopsis FAD3* and *FAD7* genes were expressed constitutively at all temperatures tested, whereas the *FAD8* gene was expressed at temperatures below 20°C [59,68]. In *Zea mays*, the Zm *FAD7* and Zm *FAD8* were differentially expressed in response to temperature. The level of Zm *FAD7* transcript was downregulated by low temperatures, whereas the Zm *FAD8* transcript level increased at low temperatures [72]. The rice *FAD3* gene was expressed preferentially at moderately low temperatures. The transcript level decreased considerably at chilling temperatures in root tissues but was unaffected in leaf tissues. It appeared that there was no obvious difference in the transcriptional response of the Oryza sativa (Os) *FAD3* gene to low temperatures between the chilling tolerant cultivars [80]. *In situ* hybridization of *Olea eusopea* L. *FAD7* showed particularly prominent expression in the palisade and vascular tissues of young leaves, the embryo sac, transmitting tissue of carpal, tapetum pollen grains, and vascular tissue of anthers [81]. The distinctive spatial, temporal, and environmental regulation of the putative *FAD7* gene was consistent with major roles not only in thylakoid membrane formation but also in the provision of α-linolenate-derived signaling molecules that were particularly important in transportation and reproduction. Although many attempts to find a causal relationship between an increase in polyunsaturated FA content and chilling or freezing acclimation have been inconclusive, the acclimation to low temperatures in plants was also associated with alteration in other biochemical metabolic processes such as changes in lipid composition [82]. Recently, Hamada et al. [83] studied the chilling tolerance of tobacco plants

transformed with microsomal ω-3 desaturase gene under the control of a highly efficient promoter containing the E12Ω sequence. The freezing tolerance as assessed by electrolyte leakage was almost the same between the transformed and control plants, indicating that the increase in the 18:3 level in phospholipids was not directly involved in compensation for the reduction in growth on membrane properties. Dehydration, salt stress, and exogenous abscisic acic (ABA) induced some of the cold-induced genes. Among the desaturase encoding genes, the rapeseed *FAD3* gene in microspore-derived embryos was induced by ABA. Neither exogenous ABA application nor drought stress had an effect on maize Zm *FAD7* and Zm *FAD8* transcript levels. Cycloheximide was reported to induce the accumulation of Zm *FAD7* transcript by an unknown mechanism [72].

Microsomal ω-3 desaturases are responsible for the production of extraplastidial 18:3. This enzyme accounts for over 80% of the 18:3 in *Arabidopsis* root tissues. *Arabidopsis FAD3* mutants were characterized by reduced levels of 18:3 and corresponding increased 18:2 levels. However, studies with the *Arabidopsis FAD3* mutants revealed that exchange of lipid between chloroplast and ER allows the chloroplast desaturase to provide highly unsaturated lipid to the extrachloroplast membranes of leaf cells [44]. Soybean genotypes A5 and A23 had reduced linolenic acid contents when compared with current cultivars. The DNA polymorphism results showed that the reduced linolenic acid concentration in A5 was at least partially the result of partial or full deletion of a microsomal ω-3 desaturase gene. On the basis of hybridization and polymerase chain reaction (PCR) results, the investigators suggested that at least two copies of microsomal ω-3 desaturase gene might be present in A5, which could explain why seeds of this line still made 34 g linolenic acid/kg oil [84].

The *Arabidopsis* microsomal ω-3 desaturase gene was used to manipulate the levels of 18:3 in transgenic plant tissues such as carrot hairy roots. The 18:3 amount varied in the different transformed lines with a high content of 62% to a low of 7.6%. The average content of 18:3 in control roots was 9.2%. There was no significant change in FAs other than 18:2 and 18:3. When expressed under a seed-specific promoter, the *FAD3* gene of *Arabidopsis* increased 18:3 content by 12-fold in R2 seeds of a transgenic *FAD3* mutant and 2-fold in a transgenic control (wild type) [65]. Overexpression of the rapeseed enzyme in *Arabidopsis* roots resulted in a 1.6-fold increase in 18:3 [64]. Transgenic tobacco plants were produced by Hamada et al. [85] that expressed the transcripts of a tobacco microsomal *FAD3* gene (Nt *FAD3*) in antisense and sense orientation under the control of cauliflower mosaic virus 35S promoter. The antisense transformants showed decreases of the steady-state Nt *FAD3* mRNA level up to 30% and the 18:3 content decreased to about 80% in root tissues and to about 70–80% in leaf tissues when compared with control plants. Interestingly, the 16:3 content decreased by 6% in some transformants, suggesting the inhibition of *FAD7* mRNA accumulation, which was responsible for conversion of 16:2 to 16:3. The investigators suggested that the region of the *FAD3* gene, which they used in the antisense direction, had a highly conserved nucleotide sequence between *FAD3* and *FAD7*. In one of the sense transformants, Nt *FAD3* mRNA levels increased by 8-fold and 18:3 content increased by 1.5-fold in root tissues and about 1.1-fold in leaf tissues compared to control plants. This indicates that the regulation of the *FAD3* transcript level can be useful in modifying the 18:3 content in the vegetative tissues of higher plants. The investigators identified the 3′ noncoding region as a specific target sequence for specific antisense suppression because there was no similarity between the 3′ noncoding regions of *FAD3* and *FAD7*.

5 ACYLTRANSFERASES

Triacylglycerols are synthesized by the two acylations of *sn*-glycerol-3-phosphate to produce phosphatidic acid (PA), catalyzed by the enzymes glycerol-3-phosphate acyltransferase (GPAT) and lysophatidic acid acyltransferase (LPAAT), respectively. These enzymes usually display selectivity for different acyl-CoAs that are to be incorporated into the glycerol backbone [86]. The PA formed is subsequently dephosphorylated to diacylglycerol (DAG), which then serves as a precursor for TAG. The third acylation step is catalyzed by DAG acyltransferase (DGAT). In oilseeds, PC has been identified as an intermediate in oil biosynthesis and plays a central role in the production of polyunsaturated FAs by serving as a substrate for Δ-6, Δ-9, Δ-12, and Δ-15 desaturases [87]. Studies with rapeseed and sunflower LPAAT and DGAT showed that LPAAT from both of the plant species was more specific toward different acyl moieties tested than the DGAT. TAG biosynthesis studies using radiolabeled (^{14}C) acetate in the filamentous fungus, *Mucor circinilloide*, revealed that LPAAT activity was particularly high, which might have accounted for PA accumulation in the membranes. Unlike the oilseed enzymes, LPAAT and GPAT were nonspecific for a range of saturated and unsaturated species of acyl-CoA, although GPAT showed selectivity for palmitoyl-CoA and the LPAAT for oleoyl- and linoleoyl-CoA [87]. In order to induce changes in oil composition, LPAAT was considered a target enzyme whose activity has to be altered because of its selective discrimination ability [88]. Rapeseed (*B. napus*) and meadowfoam (*Limnanthes*) have 60% and 90% erucic acid in their TAGs, respectively. In rapeseed, erucic acid is excluded from the *sn*-2 position, whereas in meadowfoam, it was present in the *sn*-2 position of TAGs. This difference was attributed to the substrate specificity of LPAAT enzyme in both species [89]. To alter the stereochemical composition of rapeseed oil, a cDNA encoding *Limnanthes* seed-specific LPAAT was expressed in *B. napus* plants using a napin expression cassette. In the transgenic plants, 22.3% erucic acid was present at the *sn*-2 position, leading to the production of trierucin. However, alteration of erucic acid at the *sn*-2 position did not affect the total erucic acid content. It may be that the meadowfoam LPAAT may not increase the erucic acid content of rapeseed [90] because of the limited pool size of 22:1-CoA in the maturing embryos of *B. napus*. The metabolism of laurate was found to be different in transgenic *B. napus* lines (transformed with a California bay lauroyl-ACP thioesterase cDNA driven by napin promoter) and the natural laurate accumulators coconut, oil palm, and *Cuphea wrightii* [89]. When tested at the mid-stage of embryo development, the PC had up to 26 mol% of laurate in the transgenic rapeseed high-laurate line, whereas for other species, it ranged between 1 and 4 mol%. The laurate in the *Brassica* TAG was almost totally confined to the outer *sn*-1 and *sn*-3 positions, whereas the laurate in coconut and *Cuphea* was highest in the *sn*-2 position. Very low amounts of laurate were found in the *sn*-2 position in DAG and PC of the rapeseed lipids, indicating that no arrangement of laurate between the outer and *sn*-2 positions occurred in any of the lipids. There was an enhanced activity of lauroyl-PC metabolizing enzymes in the laurate, producing rapeseed when the embryos were fed with radiolabeled ^{14}C-lauroyl-PC and ^{14}C-palmitoyl-PC. The data indicated that DAG was preferentially utilized from natural laurate accumulators like oil palm, coconut, and *Cuphea* [91]. Transgenic rapeseed oil expressing California bay thioesterase produced 60% saturated FA with laurate alone comprising 48%. In these plants, laurate was presented at *sn*-1 and *sn*-3 positions only. When these plants were crossed with transgenic lines expressing coconut LPAAT in the resulting hybrids, laurate was present in all three

positions of the glycerol backbone. An overall increase in the oil content was also observed.

When a yeast LPAAT gene *SLC1* and *SLC1-1* (mutated LPAAT) genes of yeast were expressed in *B. napus* and *Arabidopsis* under the CaMV35S promoter, both TAG and very long-chain fatty acid (VLCFA) contents were increased by 56% and 80%, respectively [92]. In the transgenic plants, seed weight increased, indicating at least a partial contribution from enhanced oil content. In the total oil content, 60–75% consisted of VLCFAs and 40% that of non-VLCFAs such as palmitate, oleate, linoleate, and linolenate. No increase in total oil content was reported in coconut or meadowfoam transformed rapeseed. This could be due to different regulatory properties of the plant and yeast LPAAT enzymes. The plant LPAAT genes have 62% amino acid identity among themselves, whereas the yeast gene had 24% homology. In transgenic plants, the high expression of the *SLC1-1* gene did not correlate with high oil content, indicating that even small levels of expression were sufficient to overcome the PA limitations during TAG biosynthesis. Although *SLC1-1* levels were stronger in leaves than in seeds, no significant changes were observed in the FA composition in leaves, indicating that the pools of available LPA and/or acyl-CoAs may be more tightly regulated in leaves (source) than in seeds (sink).

Recently, a LPAAT cDNA that uses a palmitoyl-ACP has been isolated from *B. napus* [90]. The homology studies showed strong similarities with yeast and plant genes. The isolated cDNA has a chloroplast target sequence and a high homology with a possible LPAAT sequence in *Synechocystis* spp. This gene has high selectivity for palmitoyl-CoA and is implicated in the prokaryotic-type FA synthesis. If this plastidial gene can be redirected to the ER, it may be possible to genetically engineer high palmitic acid producing plants.

Diacylglycerol acyltransferase has been purified to apparent homogeneity from lipid body fractions of an oleaginous fungi, *Mortierella ramanniana*, which accumulated γ-linolenic acid in its TAG [93]. The purified DGAT utilized a broad range of molecular species of both DAG and acyl-CoA as substrates, as was the case with mammalian cells [94] and higher plants [95]. The fungal DGAT had highest selectivity toward lauroyl-CoA in spite of the absence of lauric acid in this fungus, indicating the potential use of this fungus to produce medium-chain FA by genetic manipulation. The first plant DGAT was cloned recently from *Arabidopsis* [96]. The amino acid sequence shared 38% identity and 59% similarity with the mouse DGAT, the first one cloned [144]. Analysis revealed potential membrane-spanning helices and also a 14-kDa hydrophilic domain at the N-terminus. It had no significant sequence homology with plant GPAT and LPAAT genes. The DGAT gene was highly expressed in *Arabidopsis* inflorescences and much lower concentrations in leaves. Studies on expression of the homolog in *B. napus* showed that the DGAT mRNA was present in the highest concentrations in developing embryos, petals of flowers, and developing flower buds, but in very low amounts in leaf and stem tissues.

Apart from the activities of the FA biosynthetic enzymes and acyltransferases, transacylase action also appears to affect the composition of TAGs. It was shown that oleate in the preformed TAGs can be desaturated to linoleate [97] at low temperatures and within time periods in which *de novo* FA synthesis was negligible. Under intense desaturation conditions, the mass of TAG decreased and that of PC increased. However, this acyl exchange was specific in that only oleate can move in both directions, whereas linoleate transfer to TAG was irreversible. Initially, it was suggested that DGAT was involved in

this process, as the acyl transfer required the involvement of DGAT. The other possibility that could explain the metabolism of preformed TAG was through the reversible transacylation between two DAGs yielding one TAG and one monoacylglycerol (MAG) as suggested by Stobart et al. [98]. Radiolabeled studies with ^{14}C 18:1 showed 60% of radioactivity remaining in MAG after 1 hr, 17% was converted to DAG, and 23% to FFA. Neither PC nor TAG was labeled. After a 3-hr incubation period, 40% of the radioactivity was in MAG, 25% in DAG, and 35% in FFA [97]. However, after a 15-hr incubation, 36% of the label was found in TAG, whereas DAG, PC, FFA, other phospholipids, and MAG had 20%, 16%, 15%, 10%, and 2% radioactivity, respectively. These experiments suggest that DGAT is not the only enzyme that can form TAG in plants.

6 FACTORS AFFECTING TAG ACCUMULATION

Triacylglycerols are actively synthesized and accumulated in specific types of cell such as hepatocytes, adipocytes, plant seeds, and oleaginous fungi [99,100]. When oil palm cultures were treated with radiolabeled 18:1 along with radiolabeled (^{14}C) glycerol, the cultures resulted in almost a twofold increase in TAG [101], presumably by providing extra substrate for the acyltransferases of the Kennedy pathway. From these studies, the investigators concluded that FA synthesis exerts more control over carbon flux than the Kennedy pathway (58% of the control resides in *de novo* FA synthesis versus 42% in the Kennedy pathway). Studies with *C. lanceolata, U. carpinifolia*, and *U. parvifolia* showed that acyltransferases activities and glycerol-3-phosphate levels were found in excess for in vivo TAG biosynthesis and the amount of oil produced in these oilseeds is determined by the FA synthesis in the chloroplasts [102]. However, when a yeast LPAAT gene was expressed in *Brassica* plants, TAGs were increased by 56% and the VLCFA content by 80% [84]. Keeping these two results in view, it is difficult to come to a conclusion on which pathway is more important in increasing carbon flux toward TAG formation. In mammalian tissue and also in yeast, PA phosphohydrolase appears to be the rate-limiting step in TAG synthesis [87]. Another reaction that appears to be involved in TAG accumulation is the reversible conversion of PC into DAG in the presence of CDP-choline phosphotransferase. Slack et al. [103] gave indirect evidence for the reversibility of PC by labeling studies in vivo with linseed cotyledons and in vitro with safflower cotyledons. When sunflower microsomes were incubated with radiolabeled PC, the radioactivity was progressively incorporated into DAG. When the concentration of the microsomal protein was increased, the activity also increased, indicating the reversible reaction of choline phosphotransferase in sunflower [104].

Changes to lipid bioassembly via the Kennedy pathway can result in the changes in extraplastidial FAs in *A. thaliana*, as shown through studies with the *Arabidopsis* seed mutant AS11. This mutant had reduced DGAT activity, reduced TAG content, and the repression of VLCFA biosynthesis with an accumulation of 18:3 in TAGs. These results suggest that the overexpression of DGAT activity in the wild-type plants might have provided a means for channeling more carbon into VLCFAs and, ultimately, into TAGs. One interesting finding from this study was the dangers in characterizing complex seed lipid mutants based only on FA composition obtained by simple gas chromatographic (GC) analysis [105]. The mutant gene in the *A. thaliana* AS11 mutant is designated *THG1*. The *THG1* mutation was mapped to chromosome 2 between the phenotypic markers cp2 and st1. A more recent study has shown that the reduced TAG biosynthetic capacity of AS11

may be attributable to a 147-bp insertion in the central region of intron 2 of the DGAT gene [106].

In a novel mutant locus, a wrinkled seed mutant (*wri*1) of *Arabidopsis* causes an 80% reduction in the seed oil content [106]. Developing homozygous *wri*1 seeds are impaired in the incorporation of sucrose and glucose into TAGs but incorporate pyruvate and acetate at an increased rate. The activities of several glycolytic enzymes (in particular, hexokinase) was reduced, suggesting that *wri*1 is involved in the developmental regulation of carbohydrate metabolism during seed filling, thereby affecting carbon flux into oil.

7 UNUSUAL FATTY ACIDS

There is a wide and diverse range of unusual FAs in the seed oil of many wild plants. The source of plant species that accumulate unusual FAs is often unsuitable for modern agricultural practices. They could be of use if the genes encoding the key biosynthetic enzymes were transferred into oilseed species.

Extensive surveys of the FA composition of seed oils from different species of higher plants have resulted in the identification of more than 210 naturally occurring FAs which can be broadly classified into 1 of 18 structural classes [107]. The number and arrangement of double or triple bonds and various functional groups, such as hydroxyls, ketones, epoxys, cyclopentenyl or cyclopropyl groups, furans, or halogens, define the classes. Some unusual FAs might be expected to disrupt membrane structure and function if incorporated into membrane-forming lipids.

Results of studies with *Escherichia coli* show that branched-chain, brominated, and trans unsaturated FAs could support growth, as could cyclopropanoid acids, which are, in fact, ''usual'' FAs for this organism. These studies show that at least some unusual FAs can exist in some functional membranes, but it remains quite possible that their inclusion in normal plant membranes would be deleterious [107–109].

The biological role of most of these ''unusual'' FAs are not known, but it is thought that many or most of them may have a role in defense against pests, as do many secondary products in plants [10].

7.1 γ-Linolenic Acid and Octadecatetraenoic Acids

γ-Linolenic acid ($\Delta^{6,9,12}$-18:3, GLA) and octadecatetraenoic acid ($\Delta^{6,9,12,15}$-18:4) are synthesized from linoleic acid ($\Delta^{9,12}$-18:2) and α-linolenic acid ($\Delta^{9,12,15}$-18:3), respectively. Both FAs are of importance as pharmaceuticals and general health supplements, and they occur in several plant families, including members of the *Boraginaceae* and *Onagraceae* [110]. Microsomes prepared from developing borage cotyledons actively desaturated [^{14}C]linoleate to γ-linolenate and [^{14}C]γ-linolenate to octadecatetraenoate. This activity was dependent on NADH, and the substrate appears to be esterified to the *sn*-2 position of phosphatidylcholine [41,111,112]. Recently, a cDNA from borage which encodes a Δ^6-fatty acid desaturase was identified [55,113]. The amino acid sequence of this desaturase was distinct from that of other higher plant desaturases in that it had an N-terminal extension that contained a cytochrome b_5-like domain, complete with a diagnostic heme-binding motif.

Beremand et al. [55] developed transgenic tobacco with the borage Δ^6-desaturase gene under the control of seed-specific promoters. They stated that this has resulted in the production of seed oils with significant levels of GLA, but the definite level was not reported. Napier et al. [114] tried to introduce the borage Δ^6-desaturase into tobacco and

Arabidopsis. When the Δ^6-desaturase was expressed in developing seeds of tobacco, high levels (>20%) of GLA were observed in early stages of seed development. However, these levels declined significantly, to <2% by seed maturity. Similar results were also obtained in *Arabidopsis*. These results may indicate the requirement for additional factors to be considered in the engineering of high GLA-containing oilseeds.

7.2 Conjugated Fatty Acids

Several conjugated isomers of α-linolenic acid occur in a limited number of plant families and sometimes become a major constituent of the seed oil. α-Eleostearic acid (α-ESA; *cis*-9,*trans*-11,*trans*-13-octadecatrienoic acid) constitutes 77–86% of the FAs of the seed oil of *Aleurites fordii*, which is the source of tung oil that is widely used in drying oils [115].

A number of chemical mechanisms have been proposed to describe the biological origins of conjugated trienoic acids. These include the stereospecific isomerization of α-linolenic acid, the formation of a radical of linoleic acid due to a lipoxygenase-type reaction, or the formation of an epoxy derivative of linoleic acid.

Pulse-chase experiments with ^{14}C-labeled acetate or linoleate in developing seed of the bitter gourd (*Momordica charantia*) indicated that α-ESA is synthesized from linoleate and that this conversion occurs while these acyl moieties are esterified to the phospholipid PC [116].

7.3 Epoxy and Acetylenic Fatty Acids

Epoxy FAs have many industrial applications. Certain genotypes of several plant species accumulate high levels of epoxy FAs in the seed oil. Epoxy FAs, like vernolic (*E*-12,13-epoxyoctadeca-*E*-9-enoic) and coronaric (*E*-9,10-epoxyoctadeca-*E*-12-enoic) acids, have been found as a component of the seed oil of species represented by a number of plant families, such as Asteraceae, Euphorbiacea, Pnagraceae, Dipsacaceae, and Valerianacea [117]. The highest known natural accumulators of vernolic acid are *Vernonia galamensis* and *Euphorbia lagascae* in which vernolic acid constitutes 80% and 60%, respectively, of TAG FAs [118–121].

Many plants are known to possess enzymes which transform unsaturated FAs into epoxy FAs [122,123]. Although major oilseeds (such as soybeans) clearly have the enzymes (such as lipoxygenase and peroxygenase) required to produce epoxy FAs, the process by which the seeds of certain species of plants (such as *Vernonia* and *Euphorbia*) accumulate epoxy TAGs appears to be due to an enzyme not present in major commercial oilseeds. The production of epoxy FAs and derivatives in defense responses appears to be widespread in plants, but the accumulation in storage oil of certain seeds, such as *Vernonia* and *Euphorbia*, appears to be due to a different, much less common mechanism [124]. Biochemical studies by Bafor et al. [120] indicate that developing seeds of these plants contain an enzyme known as an epoxygenase that converts linoleic acid into vernolic acid in a one-step reaction. In some cases, epoxygenation reactions in biological systems appear to be catalyzed by cytochrome P-450 monooxygenase enzymes. The genes of arachidonic acid epoxygenase from human, mouse, and rat are identified [125–127].

Stymne's group [120] provided good evidence that a P-450 monooxygenase enzyme catalyzes vernolic acid biosynthesis in *Euphorbia lagascae*. The *Euphorbia* enzyme utilized linoleoyl PC and other phospholipids as the substrate producing vernoleoyl PC.

The vernoleate did not accumulate in the PC, only in TAGs. Preliminary results from our lab indicated that vernoleate biosynthesis in developing *Vernonia* seeds was generally similar to that in *Euphorbia*, including the CO inhibition of the activity (Seither and Hildebrand, unpublished results). We established that in *Vernonia*, as in *Euphorbia*, the biosynthesis of vernolic acid involves a microsomally located epoxygenase that converts linoleoyl PC to vernoleoyl PC. However, further studies with an improved assay with *Vernonia* extracts indicated some fundamental differences (Ref. 128 and Seither and Hildebrand, unpublished results). Our results with *Vernonia* microsome assays show the following: (1) CO apparently inhibited the activity but less so than the *Euphorbia* enzyme; (2) both NADH and NADPH were necessary for activity and both supported the activity to about the same extent; (3) the activity was inhibited by cyanide; (4) the activity is not inhibited by anticytochrome P-450 reductase antibodies; (5) the activity was inhibited by anticytochrome b_5 antibodies. Results 1 and 5 are generally consistent with Stymne's findings with *Euphorbia*, but Results 2–4 are not. We repeated the assays with *Euphorbia* microsomes and found it to be a typical P-450 mono-oxygenase as was reported by Bafor et al. [120]. These results confirm that the *Vernonia epoxygenase* is distinctly different from both usual P-450 mono-oxygenases and from *Euphorbia* epoxygenase. This suggests that the ability to synthesize vernolic acid has arisen independently throughout evolution. Data presented by Lee et al. [54] support this hypothesis.

Peroxygenase is another possible enzyme catalyzing epoxide formation. This enzyme is a CO-binding hemoprotein distinct from classical P-450s based on its spectral properties [122]. In contrast to classical cytochrome P-450, this enzyme did not carry out oxidative reactions in the presence of NADPH and O_2. Blee and Schuber [129] showed that this enzyme was able to epoxidize double bonds of unsaturated FAs. Hamberg and Hamberg [130] confirmed the presence of such an epoxygenase in the bean *Vicia faba*. Blee et al. [131] also demonstrated some activities (i.e., hydroxylation, sulfoxidation, and epoxidation) result from co-oxidative processes catalyzed by a single enzyme or closely related isoenzymes.

Acetylenic bonds are present in more than 600 naturally occurring compounds [132]. The seed oil of *Crepis alpina* contains about 70% of the acetylenic FA, crepenynic acid (9-octadecen-12-ynoic acid) [54]. It might be speculated that the acetylenic FAs could be derived from epoxy FA, followed by two dehydration steps, yielding first the enol and then the acetylenic FA [54].

Lee et al. [54] indicated that Δ-12 epoxygenation and Δ-12 acetylenation enzymes of *Crepis* had biochemical characteristics typical of FA desaturases, and they isolated an epoxygenase gene of *C. palaestina* and an acetylenase gene of *C. alpina* using a Δ-12 desaturase-like sequence as a probe. Both enzymes have characteristics similar to the membrane proteins containing nonheme iron that have histidine-rich motifs.

Recently, an epoxygenase encoding cDNA from *Vernonia galamensis* was cloned [53]. In addition, we cloned an epoxygenaselike gene from *Stokesia laevis*. The deduced amino acid sequences of these genes have high similarities to Δ-12 desaturases of many other plant species. Hydroxylases of *Ricinus communis* [51] and *Lesquerella fendleri* [52] also have very similar amino aid sequences. It could be thought that they all have evolved from one ancestor, namely Δ-12 desaturases. The diverse reactions that these enzymes catalyze probably use a common reactive center [107]. Histidine-rich motifs are thought to form part of the diiron center, where oxygen activation and substrate oxidation occur [133,134]. At least four reactions (desaturation, hydroxylation, epoxygenation, and acetylenation) are catalyzed by Δ-12 desaturase-like plant enzymes.

The amino acid sequence of an *S. laevis* epoxygenase-like gene is more homologous to that of *V. galamensis* than *C. palaesitina*. They all belong to *Asteraceae*, but *Crepis* is in a different tribe than *Vernonia* and *Stokesia*. Lenman et al. [135] reported a partial sequence of an epoxygenaselike gene of *V. galamensis*. It was quite different from *Vernonia* epoxygenase cloned by Hitz [53]. It may be that *Vernonia* has multiple isozymes of epoxygenase.

7.4 Metabolism of Unusual Fatty Acids

Data of Liu et al. [116] indicated that α-ESA is first synthesized on PC and that it is quickly mobilized from this phospholipid and incorporated into the neutral lipids DAG and TAG, where it accumulates. In addition to the structural role of this phospholipid in membranes, PC has been shown to be intimately involved in the biogenesis of polyunsaturated FAs (linoleate and α-linolenate), the biogenesis of the unusual FAs, ricinoleate [120,136,137], and vernoleate [120].

In plants, accumulating high amount of medium-chain hydroxy-, epoxy-, and acetylenic-FAs in the seed TAGs, these unusual FAs are virtually absent from the membranes. Despite the fact that nearly 90% of the FAs in the TAG can be made up by the unusual FA in plants that naturally accumulate such FAs, the main phospholipid, PC, has only a few percent of these unusual FAs maximally [138]. High amounts of these unusual FAs in membrane lipids could be expected to lead to impaired membrane function in cells. Therefore, the plant might keep these unusual FAs out of its membrane lipids. For example, although the content of these unusual FAs can reach levels in the range of 60–80% in the seeds of wild species, such as *C. palaestina* and *Euphorbia lagascae* for the epoxy FA and *Ricinus communis* for the hydroxy FA, there is only a few percent of these unusual FAs in the membrane lipids of these plants [139]. It is probable that the high accumulation of these FAs in transgenic plants will most likely necessitate an efficient channeling of these uncommon FAs from the membrane lipids into TAG.

Kinney et al. [140] developed a transgenic soybean expressing *Vernonia* epoxygenase, but the content of epoxy FA in seed oil was only about 8%. This suggests that a high concentration of epoxy FAs might be toxic for soybean.

Stymne et al. [138] suggested that the diacylglycerol acyltransferase (DGAT) might be an enzyme involved in the channeling of unusual FAs from PC to TAG. The DGAT in *R. communis* microsomes, *C. palaestina*, and *E. lagascae* utilized DAGs with ricinoleoyl or vernoleoyl groups more efficiently than oleoyl-DAGs. Hence, in *E. lagascae*, the channeling of vernolic acid into TAG might involve acyl-CoA-dependent acyltransferases in the reacylation of the released vernolic acid. However, this difference in activity was only two to four times higher, which is probably insufficient to account for the very efficient removal of these acids from the membrane lipids. However, the utilization of caproyl-DAGs by the DGAT in microsomal preparations from *Cuphea procumbens* seeds was 16–50-fold greater than that of the oleoyl-DAGs [141]. Dahlqvist et al. [139,142] suggested that *R. communis* and *C. palaestina* have an efficient channeling of the unusual FAs, such as ricinoleic acid and vernolic acid, from PC to TAG. This reaction apparently did not involve acyl-CoA and was, instead, suggested to involve a transacylase. It has also been suggested [98] that transacylation of DAG molecules could result in the synthesis of TAG and monoacylglycerol. Transacylation between lipid molecules may be a key step in the specific channeling of ricinoleic and vernolic acid from the site of synthesis on PC to a TAG pool of *R. communis* and *C. palaestina*, respectively. These results indicate that

the substrate specificity of crucial enzymes involved in the channeling of FAs from PC to TAG, as measured in vitro, differ among different plants. It is possible that every species which is accumulating unusual FAs has its own mechanism to remove unusual FAs from membrane lipids and that several enzyme mechanisms are responsible for this reaction. Among the enzymes responsible for these mechanisms are phospholipases, transacylases, and acyltransferases.

Trierucin biosynthesis has been achieved in transgenic high-erucic rapeseed (HEAR) lines expressing the 22:1-CoA specific lysoPA acyltransferase (LPAAT) from *Limnanthes* species [90,143,144]. Munster et al. [145] developed double constructs for the transformation of HEAR lines containing the rapeseed LPAAT sequences in the antisense and the 22:1-CoA-specific LPAAT from *Limnanthes douglasii* [146] in the sense orientation. Their method appeared to be suited for optimizing the channeling of 22:1 into the *sn*-2 position of transgenic rapeseed oil. This is the most successful example of a transgenic plant producing an unusual FA.

Recently, cDNAs encoding DGAT from mouse [147], *Arabidopsis thaliana* [96], and *Brassica napus* [148–150] were cloned. Their amino acid sequences are highly conserved. Cloned cDNAs encoding DGAT from plant species accumulating unusual FAs might be applied to increasing unusual FAs contents in transgenic oil crops such as HEAR lines.

8 SOYBEAN TRANSFORMATION AND REGENERATION

Many phytochemicals, which can be very useful for industrial and edible purposes, are not present in significant amounts in important crop plants. Introducing genes that regulate the production of such useful compounds into crop plants could provide an avenue for their economical production. Soybean is the most important commodity crop among the grain legumes and oilseed crops. Soybean represents the most important source of vegetable oil and protein in the world [151]. Soybean oil is more valuable than protein on a unit weight basis. Thus, increasing the oil content and/or changing the oil quality ideally without reducing the remaining protein level would improve the profitability of soybean production.

Regular unhydrogenated soy oil contains about 11% palmitic acid, 4% stearic acid, 23% oleic acid, 54% linoleic acid, and 8% linolenic acid [152]. The development of an efficient method for genetic transformation will be quite valuable for the improvement of soybean oil content and composition. Although many foreign genes have been expressed in different crop plants starting with tobacco in 1984 [153], soybean transformation is still far from routine [154]. Since the first reports of soybean transformation [155,156], only a few reports concerning transgenic soybean plants have been published [157–162]. Several private companies, such as Monsanto, Du Pont (see Chap. 4) and Pioneer, have been very active in the soybean transformation field. Monsanto (1995–1996) and Pioneer (1996–1997) have released Roundup® herbicide resistance soybean cultivars [153].

8.1 Transformation/Regeneration Systems

Both *Agrobacterium*-mediated and particle bombardment have been used for soybean transformation. The use of *Agrobacterium* to introduce DNA into soybean has been difficult, because soybean is not generally considered to be a good host for *Agrobacterium*

infection [163]. Further, soybean susceptibility to *Agrobacterium* can be genotype-specific [164–166]. Modified methods to introduce *Agrobacterium* into soybeans and improved vectors with overexpressing *vir* genes have increased the efficiency of soybean transformation [167–169]. Trick et al. [154] reported sonication-assisted *Agrobacterium* transformation (SAAT) of soybean embryogenic suspension cultures. The SAAT procedure has shown promise for increasing the transformation efficiency of many crops recalcitrant to *Agrobacterium*-mediated transformation. The enhanced transformation rates using SAAT seems to be due to microwounding the target tissue [170]. The optimal sonication time for immature cotyledons is 2 sec, with longer time periods causing extensive damage to the tissue. An advantage with *Agrobacterium* is that low copy numbers of DNA are delivered into the target [153]. Large plasmids may be subjected to fragmentation when delivered directly. Cotransformation of 12 plasmids into soybeans by particle bombardment, however, showed the presence of all the cotransformed plasmids in most of the transgenic clones and there was no preferential uptake of any of the plasmids [171].

At least two systems have been used successfully for soybean transformation. One, designated the somatic embryogenesis system [36,172,173], relies on particle delivery of the DNA. The surface origin of some of the somatic embryos facilitates targeting morphogenic cells. A second system, known as the cotyledon node transformation system [174], uses *Agrobacterium* to deliver the DNA into shoot meristematic cells such as cells of cotyledon nodes. With this second shoot morphogenetic system, plant recovery is faster. This method avoids the maintenance of donor plants and long-term cultures, but the transformed plants using this method are frequently chimeric. However, with proper selection in the second generation, recovery of uniformly transgenic soybeans is possible [154,175]. The somatic embryogenesis system appears advantageous with seed-specific genes because expression can be assessed in an individual transgenic line prior to regeneration (see below).

Christou et al. [176] were the first to bombard immature cotyledons with DNA-coated gold particles. Their results demonstrated the stable transformation of the tissues and expression of heterologous genes in the intact soybean tissue. When soybean shoot tips and embryogenic suspension cultures were transformed by particle bombardment [177], shoot tips produced chimeric transgenic shoot primordia and plantlets, whereas embryogenic suspensions produced nonchimeric, glucuronidase (GUS)-positive regenerated plants. Target cells for the particles are more than two cell layers deep for the shoot tips, and in the case of embryogenic tissue, the epidermal layer is the target. Sato et al. [177] found that embryogenic tissue is more efficient in comparison to shoot apices as a target tissue for particle bombardment.

8.2 Somatic Embryogenesis

Somatic embryos can be induced from immature cotyledons by placing explants on high levels of 2,4-D (2,4-dichlorophenoxyacetic acid) (e.g., 40 mg/L). Globular somatic embryos can be observed 4 weeks after initiation (Fig. 2). Genotype has a large effect on the ability of immature cotyledons to undergo auxin-stimulated somatic embryogenesis. It was found that two ancestral genotypes, Manchu or A. K. Harrow, are in the pedigrees of 33 highly regenerative soybean cell lines. When highly regenerative Manchu was crossed with poorly regenerative Shiro, F1 hybrid cotyledons showed intermediate regenerative capacity [173]. Similar results were reported by different groups [165,178,179].

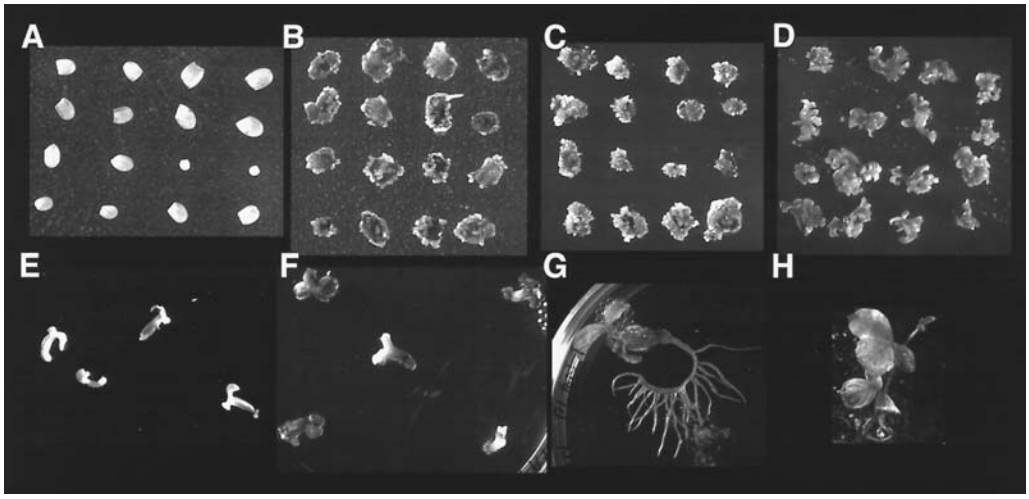


Figure 2 Regeneration of soybean plants via somatic embryogenesis. (A) Immature zygotic embryo cotyledon explants on medium containing 40 mg/L 2,4-D (D40 medium); (B) somatic embryo induction on D40 medium; (C) somatic embryo proliferation on D20 medium; (D) matured cotyledonary-stage embryos on medium containing 6% maltose (MSM6); (E) desiccation of matured embryos; (F) germination on medium containing 3% sucrose (MSO3); (G) germinated somatic embryo with well-defined root and shoot system; (H) regenerated soybean plant transferred to soil.

Several studies were conducted to improve soybean somatic embryogenesis, including the effect of ethylene inhibitors [180], use of spermine and solid/liquid/solid culture [181], effect of explant orientation, wounding, and so forth [170].

Somatic embryos can be maintained in a continuous and self-replicating state by subculturing on solid D20 (e.g., 20 mg/L 2,4-D) or, alternatively, in a liquid medium with lower levels of 2,4-D. Secondary somatic embryos are formed either singly or in clusters from the apical or terminal portions of the primary somatic embryos (Fig. 3). Light microscopy revealed the surface origin of the secondary somatic embryos [182]. Keeping in view the importance of a carbohydrate source, the ammonium-to-nitrate ratio and auxin type [183], Samoylov et al. [184] developed an optimal liquid medium for embryo proliferation and referred to it as FN lite, indicating a low sucrose and nitrogen content.

When the amount of exogenous auxin levels are reduced, the globular embryos undergo histodifferentiation to reach the cotyledonary stage. Globular embryos either produce enough auxin or they adsorb a sufficient amount from the D20 medium [173] to reach this stage. Like zygotic embryos, mature somatic embryos have hypocotyls, roots, and shoot axes, but they typically do not have cotyledons as large as zygotic embryos. Neither maturation of somatic embryos nor their germination into whole plants requires exogenous hormones. This makes it necessary to use different media for soybean regeneration, making the whole process time-consuming and labor intensive. Samoylov et al. [184] used a modified liquid-medium protocol that reduced the time for transgenic seed harvesting to 9 months from the usual 13-month period when solid-medium-based protocols are used. Embryo maturation in MS medium with maltose and without hormones, or the standard solid maturation medium, or FN-lite-based liquid medium without hormones supple-

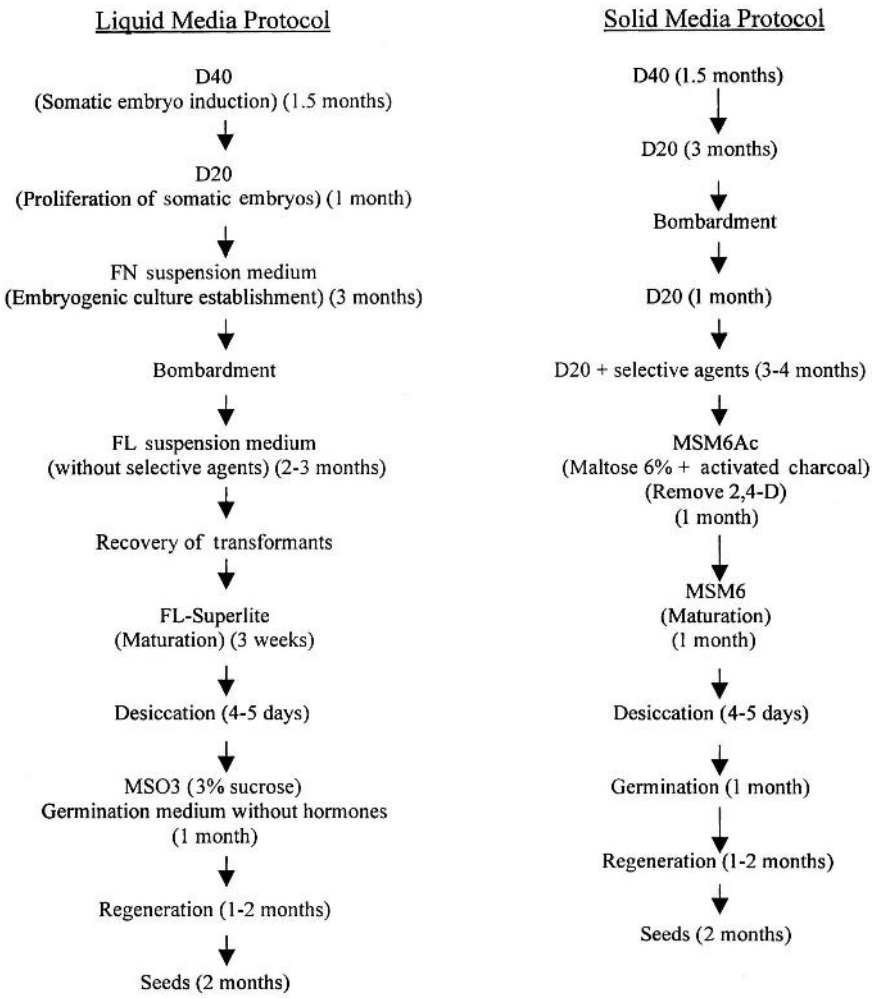


Figure 3 Transformation of soybean by particle bombardment using somatic embryos. Once embryogenic cultures are established, one can get plenty of material with a liquid-medium-based protocol for transformation. However, with older cultures, the regenerated plants are mostly sterile [199]. With solid-medium-based protocols, somatic embryos need to be induced regularly. The maturation of somatic embryos on MSM6 medium takes much longer than with liquid-medium-based protocols with FL-Superlite [184]. (From Ref. 154.)

mented with equimolar amounts of sucrose and maltose showed that sucrose promoted faster embryo maturation. However, the germination rate with this protocol was lower [184].

Although soybean somatic embryos are morphologically similar to zygotic embryos, the germination frequencies of somatic embryos is very low [36,185]. Desiccation (or partial drying) of soybean somatic embryos under either controlled [186] or uncontrolled [173,187] humidity regimes has resulted in a dramatic increase in the frequency of germination. Desiccation might decrease the ABA content [186], leading to the germination. However, Liu and Hildebrand [188] concluded that not only was there a decrease in the

ABA content but also other proteins associated with germination like the Mat1 protein were expressed along with other proteins with similar molecular masses, such as LEA or late embryogenesis abundant proteins, increased, perhaps leading to the complete maturation of the somatic embryos. Sugars seem to play an important role in soybean somatic embryo maturation. The raffinose type of sugars, which are thought to help zygotic embryos withstand desiccation [189,190], are found in lesser amounts in somatic embryos and may be an important factor in the lower regeneration rates of somatic embryos [191].

''Somatic embryo cycling'' is a system in which somatic embryogenic cultures are used to initiate new globular somatic embryos [185]. This is a modified but more efficient method of soybean somatic embryo transformation (Fig. 4). The explant used for transformation by particle bombardment is hypocotyl tissue of mature somatic embryos. When placed on D40 medium, the hypocotyl tissue initiates new somatic embryos at 10 times the frequency of zygotic cotyledons and essentially all the globular embryos originate from single epidermal cells [185]. The zygotic cotyledon is not an ideal target for particle bombardment due to the low frequency of globular embryo initiation [156]. Cycling also offers a convenient source of primary explants for somatic embryo initiation and somatic embryos of uniform age in greater quantities than the zygotic cotyledons. Somatic embryo cycling requires no alternative techniques, equipment, or material in a program already using established techniques of somatic embryogenesis and particle-delivery-mediated transformation [36].

Realizing the time and labor invested in soybean transformation and regeneration, it is always wise to check the functionality of a gene before expending such an effort [192]. Highly proliferative somatic embryo culture lines like Chapman 90 are better suited for this purpose (Finer, personal communication). Chapman 90 and similar continuously proliferating somatic embryogenic cultures usually produce sterile plants when regenerated but seed-specific genes are expressed in the matured somatic embryos [36,192]. Somatic embryos also accumulate storage oil and protein similar to zygotic embryos [193–195]. Using such cultures, it is easier and much faster for testing gene function prior to transformation of cultivars like Jack followed by regeneration into fertile plants.

The highly transformable and proliferative embryogenic Chapman 90 cultures are maintained as liquid suspension cultures. The cultures, which look like globular clumps,

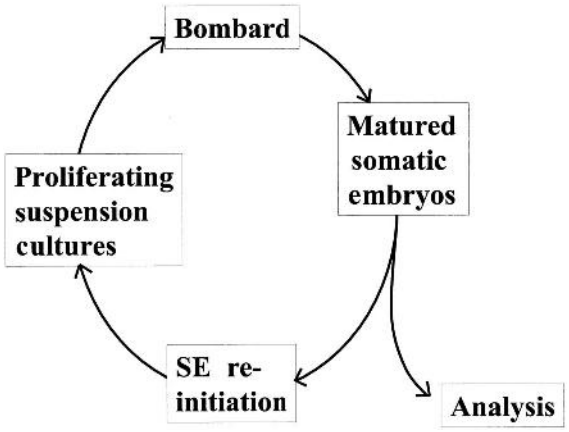


Figure 4 Somatic embryos (SE) as a test system for evaluating expression of seed-specific genes.

are transferred to fresh medium every 15 days. Two days prior to bombardment with plasmids using a particle-delivery system, the cultures are transferred to fresh liquid medium. After maintaining them in the liquid suspension medium without any selective agents for ~10 days, these globular clusters are dispersed by pressing gently with a spatula and transferred to a liquid suspension medium with selective agents. Actively growing cultures are transferred to maturation medium and allowed to mature in the presence of selective agents for a month. Small portions of the matured embryos are used for analysis. The remaining portion can be left in the medium to form globular embryogenic suspension cultures. Confirmed transgenic embryogenic clumps can be maintained in the liquid medium and used for biochemical studies. The whole process for testing seed-specific gene constructs using Chapman 90 or similar rapidly proliferating cultures takes 2–2.5 months.

9 CONCLUSIONS

Our understanding of FA biosynthesis in plants and advances in transformation and regeneration make it possible to directly manipulate FA biosynthesis and alter FA composition of seed oils such as soybean oil. For example, the genes encoding the three enzymes involved in normal unsaturated fatty acid biosynthesis, Δ-9, Δ-12, and ω-desaturases, have been cloned and molecular genetic manipulation of these three steps has been accomplished. The list of genes involved in unusual FA biosynthesis that have been isolated continues to grow. Four different unusual FAs have now been shown to be synthesized by diiron-oxo enzymes apparently evolved from microsomal Δ-12 desaturases. For the precise tailoring of oil composition, our understanding of the metabolic control of FA incorporation into TAG is still insufficient. Also, what determines the final TAG content of tissues, such as plant seeds, is not clear, but enzymes involved in fatty acid synthesis and TAG synthesis, such as DGAT, appear to be involved. As well, scientists involved in crop improvement may need to better educate the public on genetics before full acceptance of genetically modified crops is achieved.

REFERENCES

1. DF Hildebrand. Food Technol 46:71–74, 1992.
2. JL Harwood, RA Page. In: DJ Murphy, ed. Designer Oil Crops. Weinheim: VCH, 1994, pp 165–194.
3. JB Ohlrogge, J Browse. Plant Cell 7:957–970, 1995.
4. J Harwood. In: JL Harwood, ed. Plant Lipid Biosynthesis Fundamentals and Agricultural Applications. New York: Cambridge University Press, 1998, pp 1–26.
5. H Ako, D Okuda, D Gray. Nutrition. 11:286–288, 1995.
6. J Curb, G Wergowske, J Dobbs, R Abbott, B Huang. Arch Intern Med 160:1154–1158, 2000.
7. WH Gerwick, M Moghaddam, M Hamberg. Arch Biochem Biophys 290:436–444, 1991.
8. JB Ohlrogge, JG Jawaorski. Annu Rev Plant Physiol Plant Mol Biol 48:109–136, 1997.
9. TA Voelker, AC Worell, L Anderson, J Bleiaum, C Fan, DJ Hawkins, SE Radke, HM Davies. Science 257:72–74, 1992.
10. G Budziszewski, KPC Croft, DF Hildebrand. Lipids 31:557–569, 1996.
11. P Dormann, M Frentzen, JB Ohlrogge. Plant Physiol 104:839–844, 1994.
12. MR Pollard, L Anderson, C Fan, DJ Hawkins, HM Davies. Arch Biochem Biophys 284: 306–312, 1991.
13. J Shanklin, BE Cahoon. Mol Biol 49:109–136, 1998.
14. MA Taylor, SB Smith, VD Howard, LR Burch. Plant Physiol 100:533–534, 1992.

15. S Singh, S McKinney, A Green. Plant Physiol 104:1075, 1994.
16. GA Thompson, DE Scherer, S Foxall-Van Aken, JW Kenny, HL Young, DK Shintani, JC Kridl, VC Knauf. Proc Natl Acad Sci USA 88:2578–2582, 1991.
17. J Shanklin, C Somerville. Proc Natl Acad Sci USA 88:2510–2514, 1991.
18. H Akagi, T Baba, H Shimada, T Fujimura. Plant Physiol 108:845–846, 1995.
19. DK Bloomfield, K Bloch. J Biol Chem 235:337–345, 1960.
20. JB Mudd, PK Stumpf. J Biol Chem 236:2602–2609, 1961.
21. B Fox, J Shanklin, C Somerville, E Munck. Proc Natl Acad Sci USA 90:2486–2490, 1993.
22. Y Lindqvist, W Huang, G Schneider, J Shanklin. EMBO J 15:4081–4092, 1996.
23. E Cahoon, P Dormann, J Ohlrogge. Prog Lipid Res 33:155–163, 1994.
24. E Cahoon, C Becker, J Shanklin, J Ohlrogge. Plant Physiol 106:807–808, 1994.
25. E Cahoon, S Coughlan, J Shanklin. Plant Mol Biol 33:1105–1110, 1997.
26. DJ Murphy, P Piffanelli. In: JL Harwood, ed. Plant Lipid Biosynthesis Fundamentals and Agricultural Applications. New York: Cambridge University Press, 1998, pp 1–26.
27. T Fawcett, WJ Simon, R Swinhoe, J Shanklin, I Nishida, WW Christie, AR Slabas. Plant Mol Biol 26:155–163, 1994.
28. SP Slocombe, P Piffanelli, D Fairbairn, S Bowra, P Hatzopoulos, M Tsiantis, DJ Murphy. Plant Physiol 104:1167–1176, 1994.
29. Y Yukawa, F Takaiwa, K Shoji, K Masuda, K Yamada. Plant Cell Physiol 37:201–205, 1996.
30. V Hongtrakul, BB Slabaugh, SJ Knapp. Mol Breed 4:195–203, 1998.
31. JY Choi, J Stuky, SY Hwang, CE Martin. J Biol Chem 271:3581–3589, 1996.
32. CI Gonzalez, CE Martin. J Biol Chem 271:25,801–25,809, 1996.
33. J Lightner, J Wu, J Browse. Plant Physiol 106:1443–1451, 1994.
34. DS Knutzon, GA Thompson, SE Radke, WB Johnson, VC Knauf, JC Kridl. Proc Natl Acad Sci USA 89:2624–2628, 1992.
35. AO Merlo, N Cowen, T Delate, B Edington, O Folkerts, N Hopkins, C Lemieux, TT Skokut, K Smith, A Woosley, Y Yang, S Young, M Zwick. Plant Cell 10:1603–1622, 1998.
36. W Liu, RS Torisky, KP McAllister, S Avdiushko, D Hildebrand, GB Collins. Plant Cell Tissue Organ Cult 47:33–42, 1996.
37. ML Choudhary, C Chin, JJ Polashock, CC Martin. Plant Growth Regul 15:113–116, 1994.
38. JJ Polashock, CK Chin, CE Martin. Plant Physiol 100:894–901, 1992.
39. H Moon, J Hazebroek, D Hildebrand. Lipids. 35:471–479, 2000.
40. D Falcone, S Gibson, B Lemieux, C Somerville. Plant Physiol 106:1453–1459, 1994.
41. G Griffiths, AK Stobart, Biochem J 252:641–647, 1988.
42. I Vijay, PK Stumpf. J Biol Chem 247:360–366, 1972.
43. J Browse, N Warwick, C Somerville, C Slack. Biochem J 235:25–31, 1986.
44. J Browse, M McConn, DJ James, M Miquel. J Biol Chem 268:16,345–16,351, 1993.
45. M Miquel, D James, H Dooner, J Browse. Proc Natl Acad Sci USA 90:6208–6212, 1993.
46. DR Tocher, MJ Leaver, PA Hodgson. Prog Lipid Res 37:73–117, 1998.
47. J Okuley, J Lightner, K Feldmann, N Yadav, E Lark, J Browse. Plant Cell 6:146–158, 1994.
48. EP Heppard, AJ Kinney, KL Stecca, GH Miao. Plant Physiol 110:311–319, 1996.
49. C Kirsch, M Takamiya-Wik, S Reinold, K Hahlbrock, IE Somssich, DS Knutzon, TR Hayes, A Wyrick, H Xiong. Proc Natl Acad Sci USA 94:2079–2084, 1997.
50. J Shanklin, E Whittle, BG Fox. Biochemistry 33:12,787–12,794, 1994.
51. FJ van de Loo, P Broun, S Turner, C Somerville. Proc Natl Acad Sci USA 92:6743–6747, 1995.
52. P Broun, N Hauker, CR Somerville. In: JP Williams, MU Khan, NW Lem, eds. Physiology, Biochemistry and Molecular Biology of Plant Lipids. Dordrecht: Kluwer, 1997, pp 342–344.
53. WD Hitz. US Patent 5,846,784, 1998.
54. M Lee, M Lenman, A Banas, M Bafor, S Singh, M Schweizer, R Nilsson, C Liljenberg, A Dahlqvist, P-O Gummeson, S Sjodahl, A Green, S Stymne. Science 280:915–918, 1998.

55. PD Beremand, AN Nunberg, AS Reddy, T Thomas. In: JP Williams, MU Khan, NW Lem, eds. Physiology, Biochemistry and Molecular Biology of Plant Lipids. Dordrecht: Kluwer Academic, 1997, pp 351–353.
56. EB Cahoon, TJ Carlson, KG Ripp, BJ Schweiger, GA Cook, SE Hall, AJ Kinney. Proc Natl Acad Sci USA 96:12,935–12,940, 1999.
57. KT Yamamoto. Plant Physiol 103:467–476, 1994.
58. J Lightner, DW James Jr, HK Dooner, J Browse. Plant J 6:401–412, 1994.
59. S Gibson, V Arondel, K Iba, C Somerville. Plant Physiol 106:1615–1621, 1994.
60. AJ Kinney. Curr Opin Biotechnol 5:144–151, 1994.
61. BD Rennie, JW Tanner. J Am Oil Chem Soc 86:1622–1624, 1989.
62. BMM Lemieux, C Somerville, J Browse. Theor Appl Genet 80:234–240, 1990.
63. J Browse, CR Somerville. Annu Rev Plant Physiol Plant Mol Biol 42:467–506, 1991.
64. V Arondel, B Lemieux, I Hwang, S Gibson, H Goodman, C Somerville. Science 258:1353–1355, 1992.
65. NS Yadav, A Wierzbicki, M Aegerter, CS Caster, L Perez-Grau, AJ Kinney, WD Hitz, JR Booth Jr, B Schweiger, KL Stecca. Plant Physiol 103:467–476, 1993.
66. M McConn, S Hugly, J Browse, C Somerville. Plant Physiol 106:1609–1614, 1994.
67. H Wada, Z Gombos, N Murata. Nature 347:200–203, 1990.
68. K Iba, S Gibson, T Nishiuchi, T Fuse, M Nishimura, V Arondel, S Hugly, CR Somerville. J Biol Chem 268:24,099–24,105, 1993.
69. H Kodama, T Hamada, G Horiguchi, M Nishimura, K Iba. Plant Physiol 105:601–605, 1994.
70. H Kodama, G Horiguchi, T Nishiuchi, M Nishimura, K Iba. Plant Physiol 107:117–118, 1994.
71. I Nishida, N Murata. Annu Rev Plant Physiol Plant Mol Biol 47:541–568, 1996.
72. T Berberich, M Harada, K Sugawara, H Kodama, K Iba, T Kusano. Plant Mol Biol 36:297–306, 1998.
73. T Nishiuchi, T Hamada, H Kodama, K Iba. Plant Cell 9:1701–1712, 1997.
74. T Hamada, T Nishiuchi, H Kodama, M Nishimura, K Iba. Plant Cell Physiol 37:606–611, 1996.
75. M Martin, J Leon, C Dammann, JP Albar, G Griffiths, JJ Sanchez-Serrano. In: J Sánchez, E Cerdá-Olmedo, E Martínez-Force, eds. Advances in Plant Lipid Research. Sevilla: Secretariado de Publicaciones, Universidad de Sevilla, 1998, pp 500–502.
76. J Ohnishi, M Yamada. Plant Cell Physiol 21:1595–1606, 1980.
77. DJ Murphy, PK Stumpf. Plant Physiol 66:666–671, 1980.
78. JP Williams, MU Khan, D Wong. Plant Physiol 101:977–983, 1993.
79. G Horiguchi, N Kawakami, K Kusumi, H Kodama, K Iba. Plant Cell Physiol 39:540–544, 1998.
80. H Kodama, H Akagi, K Kusumi, T Fujimura, K Iba. Plant Mol Biol 33:493–502, 1997.
81. ZP Poghosyan, K Haralampids, AI Martsinkovskaya, DJ Murphy, P Hatzopoulos. Plant Physiol Biochem 37:109–119, 1999.
82. CL Guy, KJ Niemi, R Brambl. Proc Natl Acad Sci USA 82:3673–3677, 1985.
83. T Hamada, H Kodama, K Takeshita, H Utsumi, K Iba. Plant Physiol 118:591–598, 1998.
84. JR Byrum, AJ Kinney, KL Stecca, DJ Grace, BW Diers. Theor Appl Genet 94:356–359, 1997.
85. T Hamada, H Kodama, M Nishimura, K Iba. Transgen Res 5:115–121, 1996.
86. G Griffiths, A Stobart, S Stymne. Biochem J 230:378–388, 1985.
87. FM Jackson, L Michaelson, TCM Fraser, AK Stobart, G Griffiths. Microbiology 144:2639–2645, 1998.
88. G Franzosi, E Battistel, M Santoro, R Iannacone. In: J Sánchez, E Cerdá-Olmedo, E Martínez-Force, eds. Advances in Plant Lipid Research, Sevilla: Universidad de Sevilla, 1998, pp 679–682.
89. Y Cao, KC Oo, AHC Huang, Plant Physiol 9:1199–1206, 1990.
90. MW Lassner, CK Levering, HM Davies, DS Knutzon. Plant Physiol 109:1389–1394, 1995.
91. E Wiberg, A Banas, S Stymne. Planta 203:341–348, 1997.

92. J Zou, V Katavic, EM Giblin, DL Barton, SL MacKenzie, WA Keller, X Hu, DC Taylor. Plant Cell 9:909–923, 1997.
93. Y Kamisaka, S Mishra, T Nakahara. J Biochem 121:1107–1114, 1997.
94. R Coleman, R Bell. J Biol Chem 251:4537–4543, 1976.
95. G Vogel, J Browse. Plant Physiol 110:923–931, 1996.
96. DH Hobbs, C Lu, M Hills. FEBS Lett 452:145–149, 1999.
97. R Garces, C Sarmiento, M Mancha. Planta 193:473–477, 1994.
98. K Stobart, M Mancha, M Lenman, A Dahlqvist, S Stymne. Planta 203:58–66, 1997.
99. LB Tijburg, MJ Geelen, LM van Golde. Biochim Biophys Acta 1004:1–19, 1989.
100. C Ratledge. Biochem Soc Trans 17:1139–1141, 1989.
101. U Ramli, PA Quant, JL Harwood. In: J Sanchez, E Cerda-Olmedo, E Martinez-Force, eds. Advances in Plant Lipid Research. Sevilla: University of Sevilla, 1998, pp 219–221.
102. X Bao, J Ohlrogge. Plant Physiol 120:1057–1062, 1999.
103. CR Slack, PG Roughan, JA Browse, SE Gardiner. Biochim Biophys Acta 833:438–448, 1985.
104. S Triki, J Ben Hamida, P Mazliak. In: J Sanchez, E Cerda-Olmedo, E Martinze-Force, eds. Advances in Plant Lipid Research. Sevilla: University of Sevilla, 1998, pp 236–239.
105. V Katavic, DW Reed, DC Taylor, EM Giblin, DL Barton, J Zou, SL Mackenzie, PS Covello, L Kunst. Plant Physiol 108:399–409, 1995.
106. J Zou, Y Wei, DC Taylor. Plant J 19:645–654, 1999.
107. FJ van de Loo, BG Fox, C Somerville. Lipid Metabolism in Plants. Boca Raton, FL: CRC Press, 1993, pp 91–126.
108. DF Silbert, RC Ladenson, JL Honegger. Biochem Biophys Acta 311:349–361, 1973.
109. NA Machtiger, CF Fox. Annu Rev Biochem 42:575, 1973.
110. DF Horrobin. Prog Lipid Res 40:1234–1241, 1992.
111. S Stymne, AK Stobart. Biochem J 240:385–393, 1986.
112. G Griffiths, EY Brechany, WW Christie, S Stymne, K Stobart. In: PA Biacs, T Kremmer, eds. Biological Role of Plant Lipids. New York: Plenum Press, 1989, p 151.
113. O Sayanova, MA Smith, P Lapinskas, AK Stobart, G Dobson, WW Christie, PR Shewry, JA Napier. Proc Natl Acad Sci USA 94:4211–4216, 1997.
114. JA Napier, B Libish, AK Stobart, PR Shewry, O Sayanova. In: J Sanchez, E Martinez-Force, eds. Advances in Plant Lipid Research. Sevilla: Universidad de Sevilla, 1998, pp 641–643.
115. EG Hammond. In: P Wan, ed. Fats and Oils Processing. Champaign, IL: American Oil Chemists Society, 1991, pp 1–15.
116. L Liu, EG Hammond, BJ Nikolau. Plant Physiol 113:1343–1349, 1997.
117. CJ Smith. In: FD Gunstones, ed. Topics in Lipid Chemistry. London: Logos Press, 1970, pp 277–368.
118. RE Perdue. Agric Eng 70:11–13, 1989.
119. MJ Pascal, E Correal. Crop Sci 32:95–98, 1992.
120. M Bafor, MA Smith, L Jonsson, K Stobart, S Stymne. Arch Biochem Biophys 303:145–151, 1993.
121. A Thompson, DA Dierig, K Kleiman. Ind Crops 3:175–183, 1994.
122. E Blee. Prog Lipid Res 37:33–72, 1998.
123. HW Gardner. Biochem Biophys Acta 1084:221–239, 1991.
124. E Blee, U Stahl, S Stymne. Biochem Biophys Res Commun 197:778–784, 1993.
125. S Wu, CR Moonmaw, KB Tomer, JR Falck, DC Zeldin. GenBank U37143. 1996.
126. S Wu, E Murphy, S Gabel, W Chen, KB Tomer, JR Foley, C Steebergen, JR Falck, CR Noomaw, DC Zeldin. GenBank U39943. 1996.
127. A Karata, K Makita, HR Jacobson, JR Folk, PF Guengerich, RN DuBois, J Capdevila. GenBank U04733. 1994.
128. C Seither, S Avdiushko, DF Hildebrand. In: JP Williams, MU Khan, NW Lem, eds. Physiology, Biochemistry and Molecular Biology of Plant Lipids. Dordrecht: Kluwer Academic, 1997, pp 389–391.

129. E Blee, F Schuber. J Biol Chem 265:12,887–12,894, 1990.
130. M Hamberg, G Hamberg. Arch Biochem Biophys 283:409–416, 1990.
131. E Blee, A Wilcox, LJ Marnett, F Schuber. J Biol Chem 268:1708–1715, 1993.
132. RC Badami, KB Patil. Prog Lipid Res 19:119–153, 1981.
133. J Shanklin, J Achim, H Schmidt, BG Fox, E Munck. Proc Natl Acad Sci USA 94:2981–2986, 1997.
134. L Shu, JC Nesheim, K Kauffmann, E Munck, JD Lipscomb, L Que Jr. Science 275:515–518, 1997.
135. M Lenman, M Lee, A Banas, M Bafor, S Singh, M Schweizer, R Nilsson, C Liljenberg, A Dahlqvist, PO Gummeson, S Sjodahl, A Green, S Stymne. In: J Sanchez, E Martinez-Force, eds. Advances in Plant Lipid Research. Sevilla: Universidad de Sevilla, 1998, pp 657–660.
136. RA Moreau, PK Stumpf. Plant Physiol 67:672–676, 1981.
137. M Bafor, MA Smith, L Jonsson, K Stobart, S Stymne. Biochem J 280:507–514, 1991.
138. S Stymne, M Bafor, A Banas, A Dahlqvist, P-O Gummeson, M Lee, M Lenman, U Stahl, E Wiberg. In: J Sanchez, E Martinez-Force, eds. Advances in Plant Lipid Research. Sevilla: Universidad de Sevilla, 1998, pp 204–210.
139. A Dahlqvist, A Banas, S Stymne. In: J Sanchez, E Martinez-Force, eds. Advances in Plant Lipid Research. Sevilla: Universidad de Sevilla, 1998, pp 211–214.
140. A Kinney, WD Hiz, S Knowlton, E Cahoon. In: J Sanchez, E Cerda-Olmedo, E Martinez-Force, eds. Advances in Plant Lipid Research. Sevilla: Universidad de Sevilla, 1998, pp 623–628.
141. E Wiberg, E Tillberg, S Stymne. Phytochemistry 36:573–577, 1994.
142. A Dahlqvist, U Stahl, M Lenman, A Banas, M Lee, L Sandager, H Ronne, S Stymne. Proc Natl Acad Sci USA 97:6487–6492, 2000.
143. C Brough, JM Coventry, WW Christie, JTM Kroon, AP Brown, TL Barsby, A Slabas. Mol Breed 2:133–142, 1996.
144. D Weier, C Hanke, A Eickelkamp, W Luhs, J Dettendorfer, E Schaffert, C Mollers, W Friedt, FP Wolter, M Frentzen. Fett/Lipid 99:160–165, 1997.
145. A Munster, W Luhs, DS Borchardt, FP Wolter, M Frentzen. In: J Sanchez, E Martinez-Force, eds. Advances in Plant Lipid Research. Sevilla: Universidad de Sevilla, 1998, pp 671–674.
146. C Hanke, FP Wolter, J Coleman, G Peterek, M Frentzen. Eur J Biochem 233:806–810, 1995.
147. S Cases, SJ Smith, Y-M Zheng, HM Myers, SR Lear, E Sande, S Novak, C Collins, CB Welch, AJ Lusis, SK Erickson, RV Farese Jr. Proc Natl Acad Sci USA 95:13,018–13,023, 1998.
148. C Nykiforuk, A Laroche, R Weselake. GenBank AF155224, AF164434, 1999.
149. C Nykiforuk, A Laroche, R Weselake. Plant Physiol 120:1207, 1999.
150. C Nykiforuk, A Laroche, R Weselake. Plant Physiol 121:1053, 1999.
151. P Christou. Genetic Engineering and In Vitro Culture of Crop Legumes. Lancaster, PA: Technomic Publishing, 1992, pp 37–127.
152. K Liu. INFORM 10:868–878, 1999.
153. R Birch. Annu Rev Plant Physiol Plant Mol Biol 48:109–136, 1997.
154. HN Trick, RD Dinkins, ER Santarem, R Di, VM Samoylov, C Meurer, D Walker, WA Parrott, JJ Finer, GB Collins. Plant Tissue Cult Biotechnol 3:9–26, 1997.
155. DE McCabe, WF Swain, BJ Martinell, P Christou. Bio/Technology 6:923–926, 1988.
156. WA Parrott, LM Hoffman, DF Hildebrand, EG Williams, GB Collins. Plant Cell Rep 7:615–617, 1989.
157. R Di, V Purcell, GB Collins, SA Ghabrial. Plant Cell Rep 15:746–750, 1996.
158. WA Parrott, JN All, MJ Adang, MA Bailey, HR Boerma, JCN Stewart. In Vitro Cell Dev Biol 30:144–149, 1994.
159. CN Stewart Jr, MJ Adang, JN All, HR Boerma, G Cardineau, D Tucker, WA Parrott. Plant Physiol 112:121–129, 1996.

160. JA Nordlee, SL Taylor, JA Townsend, LA Thomas, RK Bush. N Engl J Med 334:688–692, 1996.
161. DM Denbow, EA Grabau, GH Lacy, ET Kornegay, DR Russell, PF Umbeck. Poult Sci 77: 878–881, 1998.
162. PJ Maughan, R Philip, M-J Cho, JM Widholm, LO Vodkin. In Vitro Cell Dev Biol 35:334–349, 1999.
163. AG Matthysse, RHG Gurlitz. Physiol Plant Pathol 21:381–387, 1982.
164. LD Owens, DE Cress. Plant Physiol 77:87–89, 1985.
165. M Bailey, H Boerma, W Parrot. Crop Sci 34:514–519, 1994.
166. AO Mauro, TW Pfeiffer, GB Collins. Crop Sci 35:1152–1156,1995.
167. G Hansen, M Larribe, D Vaubert, J Tempe, BJ Biermann, AL Montoya, MD Chilton, J Brevet. Proc Natl Acad Sci USA 88:7763–7767, 1991.
168. Y Ishida, H Saito, S Ohta, Y Hiei, T Komari, T Kumashiro, FM Jackson, L Michaelson, TC Fraser, AK Stobart. Nat Biotechnol 14:745–750, 1996.
169. RS Torisky, L Kovacs, S Avdiushko, JD Newman, AG Hunt, GB Collins. Plant Cell Rep 17:102–108, 1997.
170. ER Santarém, HN Trick, JS Essig, JJ Finer. Plant Cell Rep 17:752–759, 1998.
171. MZ Hadi, MD McMullen, JJ Finer. Plant Cell Rep 15:500–505, 1996.
172. PA Lazzeri, DF Hildebrand, GB Collins. Plant Mol Biol Rep 3:160–167, 1985.
173. WA Parrott, EG Williams, DF Hildebrand, GB Collins. IAPTC Newslett 54:10–19, 1988.
174. MAW Hinchee, DV Connor-Ward, CA Newell, RE McDonnell, SJ Sato, CS Gasser, DA Fischhoff, DB Re, RT Fraley, RB Horsch. Bio/Technology 6:915–922, 1988.
175. CA Meurer, RD Dinkins, GB Collins. Plant Cell Rep 18:180–186, 1998.
176. P Christou, DE McCabe, WF Swain. Plant Physiol 87:671–674, 1988.
177. S Sato, C Newell, K Kolacz, L Tredo, J Finer, M Hinchee. Plant Cell Rep 12:408–413, 1993.
178. LN Tian, DCW Brown, H Voldeng, J Webb. Plant Cell Tissue Organ Cult 36:269–273, 1994.
179. J Li, AE Grabau. Plant Cell Tissue Organ Cult 44:87–89, 1996.
180. KGB Santos, E Mundstock, MHB Zanettini. Plant Cell Rep 16:859–864, 1997.
181. AN Orczyk, W Orczyk, Euphytica 80:137–143, 1994.
182. JJ Finer, A Nagasawa. Plant Cell Tissue Organ Cult 15:125–136, 1988.
183. SA Merkle, WA Parrott, BS Flinn. In: TA Thorpe, ed. In Vitro Embryogenesis in Plants. Dordrecht: Kluwer Academic, 1995, pp 155–203.
184. VM Samoylov, DM Tucker, WA Parrott. Plant Cell Rep 18:49–54, 1998.
185. W Liu, PJ Moore, GB Collins. In Vitro Cell Dev Biol 28:153–160, 1992.
186. JA Buchheim, SM Colburn, JP Ranch. Plant Physiol 89:768–775, 1989.
187. N Hammat, MR Davey. J Plant Physiol 128:219–226, 1987.
188. W Liu, DF Hildebrand, PJ Moore, GB Collins. Planta 194:69–76, 1994.
189. RL Obendorf, M Horbowicz, AM Dickerman, P Brenac, ME Smith. Crop Sci 38:78–84, 1998.
190. RL Obendorf, AM Dickerman. TM Pflum, MA Kacalanos, ME Smith. Plant Sci 132:1–12, 1998.
191. S Chanprame, TM Kuo, JM Widholm. In Vitro Cell Dev Biol 34:64–68, 1998.
192. B Mazur, E Krebbers, S Tingey. Science 285:372–375, 1999.
193. ML Dahmer, GB Collins, DF Hildebrand. Crop Sci 31:741–746, 1991.
194. ML Dahmer, DF Hildebrand, GB Collins. In Vitro Cell Dev Biol 28P:106–114, 1992.
195. AJ Kinney. J Food Lipids 3:273–292, 1996.
196. H Moon. Effects of a Mammalian Stearoyl-CoA Desaturase on Plant Tissue Lipids and Transformation of Soybean via Particle Bombardment of Somatic Embryos. PhD thesis, University of Kentucky, 1998.
197. JB Ohlrogge. Plant Physiol 104:821–826, 1994.
198. R Topfer, N Martini, J Schell. Science 268:681–686, 1995.
199. CB Hazel, TM Klein, M Anis, HD Wilde, WA Parrott. Plant Cell Rep 17:765–772, 1998.

4

Perspectives on the Production of Industrial Oils in Genetically Engineered Oilseeds

ANTHONY J. KINNEY

DuPont Experimental Station, Wilmington, Delaware

1 INTRODUCTION

Plant oils were once the main source of aliphatic carbon compounds for industrial purposes. The advent of the petroleum industry and development of fatty acid hydrogenation during the first part of this century resulted in the rapid growth of plant oils for food use and the decline of these oils for nonfood applications. Before hydrogenation, most plant oils were previously too oxidatively unstable for food applications [1]. The advent of hydrogenation also resulted in the development of vegetable-oil-based margarine and shortenings as butter and lard replacements [2]. Thus, soybeans alone now provide about one-third of the world's supply of plant oil, only about 2% of which is used for purposes other than food and cooking. Most nonfood soybean oil is also chemically modified (e.g., by epoxidation) for specific industrial uses such as poly(vinyl chloride) (PVC) plasticizers.

The success of replacing chemically modified food oils by genetically modified food oils, such as oxidatively stable soybean and corn oils [3], has led to attempts at replacing chemically modified nonfood oils with genetically modified oils having novel functionality.

In addition to simply replacing existing markets for chemically modified plant oils, it may also be possible to use fatty acid metabolism in seeds to produce ingredients for materials whose feedstocks are currently made in steel plants rather than green plants. By extension it may also be possible to produce starting materials for entirely new end prod-

ucts. This would place plant-derived products in competition with petroleum-derived feedstocks. Although this competition may currently be economically unfavorable, in the long term the ability to produce industrial ingredients from renewable resources may make this an economically and ecologically profitable enterprise.

2 INDUSTRIAL USES OF EDIBLE OILS FROM TRANSGENIC OILSEEDS

Using the information obtained from transgene constructs tested in soybean somatic embryos ([4,5]; see also Chap. 3), it has been possible to produce a number of different soybean and corn lines that have oils with modified fatty acid profiles. In terms of replacing chemical hydrogenation, oils with a total polyunsaturated content of less than 3% and oleic acid content of 87% have been produced by suppressing the *Fad2-1*gene [6]. The new oils have an oxidative stability of more than 10 times that of refined, bleached, and deodorized (RBD) soybean oil and thus provide a suitable substitute for chemically hydrogenated oils for food uses.

Vegetable oils, including soybean oil, also have very good lubricating properties and, thus, can make good industrial lubricants [7]. This is especially true in applications where biodegradability is important, because vegetable oils can be highly biodegradable when compared with mineral oils. High-oleic-acid soybean oil is a low-viscosity liquid oil with good oxidative stability and, thus, has been found to be a good industrial lubricant. When heated, the accumulation of polymer oxidation products in the high-oleic-acid soybean oil is far less than that of normal soybean oil [8] and, with the addition of some food-quality antioxidants, performs almost as well as commercial mineral oil lubricants in machine-wear lubrication tests [8].

Because the high-oleic-acid oils consist of triacylglycerol with a maximum of only three double bonds per molecule, one per fatty acid, they are also an attractive feedstock for other industrial applications such as PVC epoxy plasticizer or resin transfer molded composites [8]. For example, to prevent cross-linking and other reactions with the polymer chains, epoxidized oil with a maximum of one epoxy group per fatty acid molecule is desirable for many PVC plasticizer applications [9,10] and chemical epoxidation of high-oleic-acid soybean results in such an oil.

It is now possible, however, to produce epoxy oils themselves directly in a commercial crop such as soybean [3,11,12]. This oil could provide a renewable alternative to petrochemical-derived plasticizers such as dioctyl phthalates. In addition, because epoxy oils act as PVC stabilizers and do not migrate from the polymer matrix, these new epoxy oils would be functionally superior to dioctyl phthalates and result in less atmospheric pollution [10].

There are many other potential uses for a naturally epoxidized vegetable oil. It has been demonstrated that fatty acids derived from *Vernonia galamensis* oil (which contains about 80% 12-epoxy fatty acids) can be used to make 11-aminoundecanoic and 12-aminododecanoic acids [13]. These can then be used as monomers for nylon 11 and nylon 12 synthesis (see Fig. 1b). *Vernonia* seed oil has also been used as a source of other industrial aliphatics such as hydroxy alkoxy fatty esters [14]. All of these uses would be commercially viable if that oil were reasonably priced and easy to produce, which is why producing epoxy oil in soy or corn is an attractive proposition.

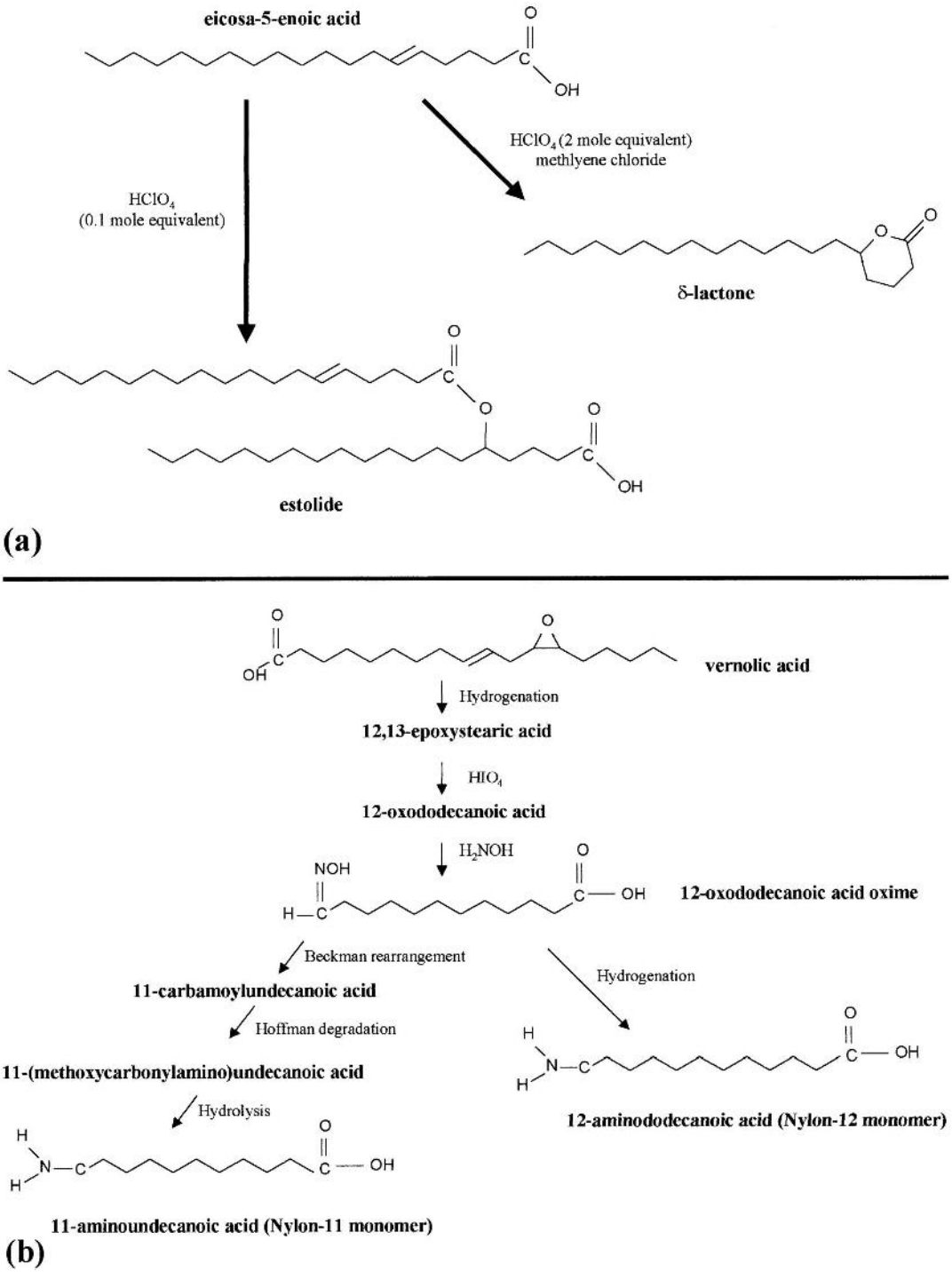


Figure 1 Functionalized fatty acids as feedstocks. (a) Δ^5 Fatty acids from *Limnanthes* oil can be used to produce estolides and δ-lactones (From Refs. 36 and 37.) (b) 12-Epoxy fatty acids from *Vernonia* oil can be used to produce precursors of nylon 11 and nylon 12. (From Ref. 13.)

3 POTENTIAL FOR REPLACEMENT OF CHEMICALLY MODIFIED INDUSTRIAL OILS AND EXOTIC PLANT OILS WITH GENETICALLY MODIFIED OILS

The enzyme that catalyzes the formation of epoxidized fatty acids in *Vernonia* [11,15] and the similar enzyme from *Crepis palaestina* [16] are members of the omega-6 phospholipid desaturase family. The genes encoding these epoxygenases are therefore members of the *Fad2* gene family. Epoxidized soy oil was developed by expressing this *Vernonia*-diverged *Fad2* in soybean seeds [11,12]. Currently, soybean lines with as much as 8% 12-epoxy fatty acids have been identified and a key issue in commercial development of these lines is increasing this concentration to 70–80% (see Sec. 7).

In addition to epoxy acids, there have been hundreds of different fatty acids identified in the oils of exotic oilseed species [17]. These potentially useful fatty acids include hydroxy, keto, ethylenic, acetylenic, cyclopropanoid and even fluoro fatty acids (see Fig. 2 for some examples). A successful approach to isolating these genes has been by the use of high-throughput screening of cDNA libraries or automated express sequence tag (EST) sequencing. Automated sequencing of cDNA libraries made from unusual oilseeds has allowed the identification of useful genes by their homology to known genes [11,18]. Additionally, cloned genes have been used as probes to isolate related genes from cDNA libraries of exotic species [19–21] and the sequences of cloned genes have been used to design polymerase chain reaction (PCR) primers to isolate genes from genomic DNA [16]. Once the potential gene was identified by sequence homology, its identity was confirmed by expressing them in plants or in microbes.

In addition to identifying the *Vernonia* and *Crepis* fatty acid epoxygenases, these methods have proved successful for the identification of fatty acid hydroxylases from

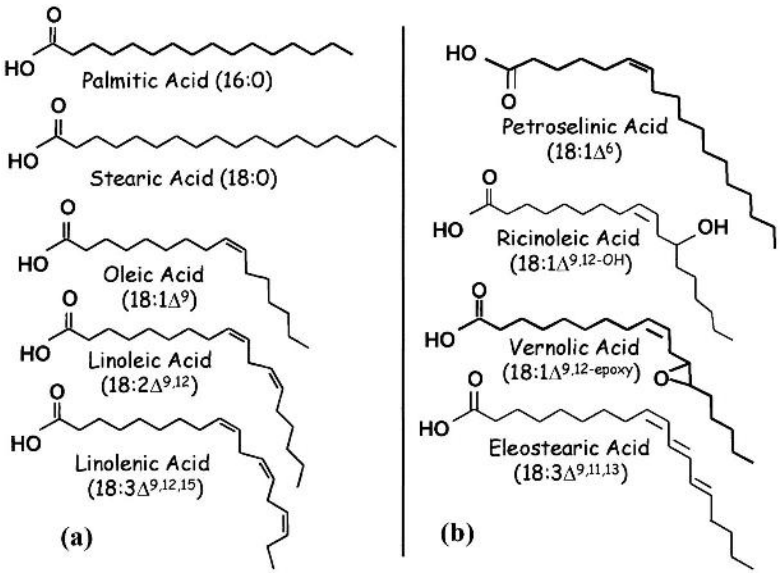


Figure 2 Fatty acids found in the oils of (a) common oilseed crops such as soybean and (b) exotic oilseed plant species such as *Umbelliferae* (petroselinic acid), *Ricinus* (ricinoleic acid), *Vernonia* (vernolic acid), and *Momordica* (eleostearic acid).

Ricinus and *Lesquerella* [20–22], acetylenases [16], and fatty acid conjugases [18]. All of these enzymes, like the epoxygenases, are encoded by diverged *Fad2*-like desaturases [11,12,18,22], which allowed for their relatively easy identification.

In the case of conjugases, it was not obvious that this would have been so. Although the biosynthetic origin of conjugated double bonds in plants was previously examined by radiolabeling of seeds [23], the results of these experiments did not reveal the underlying enzymatic nature of the conjugated fatty acid pathway. However, expression of diverged *Fad2* cDNAs from *Momordica* and *Impatiens* in soybean was sufficient for the formation of the conjugated fatty acids α-eleostearic acid and parinaric acid. Expression of genes encoding additional proteins with novel lipoxygenase, isomerase, or cytochrome P-450 activities, which have previously been suggested to be involved in conjugation mechanisms [23], was not necessary [18].

Vegetable oils that are enriched in fatty acids with conjugated double bonds, such as α-eleostearic acid, are used commercially in coating materials. They are obtained primarily from seeds of the Tung tree (*Aleurites fordii*), whose cultivation is limited to subtropical climates [24]. These seed oils have drying properties that are superior to those that contain polyunsaturated fatty acids with methylene-interrupted double bonds, but their use is limited by the restricted world supply of the oil and its cost [25]. Identification of conjugase genes and their expression in soybean suggests that it is possible to extend the usage of conjugated oils by producing them in domesticated oilseed crops.

4 NOVEL FATTY ACIDS AS INDUSTRIAL FEEDSTOCKS

Most of the above examples have as their initial target markets applications that are currently filled by exotic plant oils or chemically modified soybean oil. The possible production of the Δ^6 monounsaturated fatty acid petroselinic acid in oilseed crop plants is an example of the use of a novel fatty acid to supply an industrial feedstock currently obtained from petrochemicals. The transfer at least three genes from coriander under the control of seed-specific promoters to soybean could introduce the synthesis of petroselenic acid into a domesticated oilseed. A key enzyme in petroselenic acid production is a soluble acyl-ACP desaturase (ACP is acyl carrier protein), the Δ^4 palmitate desaturase, the gene for which was cloned from coriander seeds and introduced into transgenic plants a few years ago [26,27]. Work from this same group has indicated that a specific condensing enzyme, to elongate the Δ^4 palmitate to petroselenic acid, and a specific acyl-ACP thioesterase, to make the petroselenic acid available for incorporation into triglyceride, are also necessary to produce reasonable amounts of petroselenic acid [28,29].

Recent reports suggest that other genes may be required to produce petroselenic acid at a commercially viable concentration [30]. The effort may be worth it, however, because if purified petroselenic acid could be oxidatively split at the double bond on a commercial scale, as is done on a laboratory scale by ozonolysis, the resulting products would be the adipic acid and lauric acid. The 6-carbon diacid adipic acid is an intermediate in the production of nylon 6,6, and a very large market exists for it at a weight unit price about twice the average price of most vegetable oils. Because the yield of adipic acid from the conversion is less than one-half of the petroselenic acid starting material, the market for lauric acid and glycerol by-products would also be very important in making this economically feasible.

Genes for other novel acyl-ACP desaturases have recently been cloned, suggesting there may be other approaches to producing industrial useful diacids in plant oils. These

soluble desaturases include a Δ^9 desaturase from *Thunbergia*, which also has activity toward palmitoyl-ACP and hence the potential for producing Δ^6 double bonds [31,32], and a Δ^4 palmitate/myristate desaturase from milkweed, *Asclepias syriaca* [33].

5 PRODUCTION OF OILS AND FATTY ACIDS WITH NEW INDUSTRIAL USES

The seed oil of several *Limnanthes* species is distinct from that of other plants because of its high content of long-chain (C_{20} and C_{22}) fatty acids with a Δ^5 double bond. The most abundant component of the seed oil of these plants is Δ^5-eicosenoic acid ($20:1\Delta^5$), which accounts for 60% of the total fatty acids [34]. The proximity of the double bond in this fatty acid to the carboxyl terminus results in chemical and physical properties that are not found in the monounsaturated fatty acids described earlier. As a result of these properties, Δ^5 fatty acids can serve as precursors for the synthesis of industrial compounds such as δ-lactones and estolides as shown in Figure 1a [35–37]. In addition, the novel properties associated with $20:1\Delta^5$ make the actual seed oil of *Limnanthes* species useful in the manufacture of cosmetics, surfactants, and lubricants [38–40]. Indeed, because of some of these seed oil properties, meadowfoam (*Limnanthes alba*) has been grown in the Pacific Northwest for several years although, as with all new oilseed crops, considerable technical challenges have been encountered in attempting to produce the oil on a commercial scale [39,41].

In a recent study [42], random sequencing of a cDNA library prepared from developing *Limnanthes douglasii* seeds resulted in the identification of a new class of plant desaturases. The novel cDNA encoded a homolog of the acyl-CoA desaturases found in animals, fungi, and cyanobacteria [43]. When this cDNA was expressed in somatic soybean embryos, the seed oil of these embryos accumulated a Δ^5-hexadecenoic acid ($16:1\Delta^5$) to amounts of 2–3% (w/w) of the total fatty acids of the embryo oil. Two other unsaturated fatty acids ($18:1\Delta^5$ and $20:1\Delta^5$) were also observed at a lesser concentration [$<$1% (w/w) each of the total fatty acids]. When this cDNA was coexpressed with another *Limnanthes* cDNA, which encoded a homolog of a plant fatty acid elongase 1 (FAE1), transgenic embryos produced $20:1\Delta^5$ and $22:1\Delta^5$ fatty acids at concentrations up to 15% of the total fatty acids in the embryo oil [42].

These experiments showed it is possible to partially reconstruct the biosynthetic pathway of $20:1\Delta^5$ in a domesticated oilseed plant. It is believed that by coexpressing a third cDNA in soybean embryos, encoding a palmitoyl-ACP thioesterase, will result in even greater amounts of $20:1\Delta^5$ fatty acid in the embryo oil because this third enzyme will provide a larger substrate pool for the FAE1 16:0-CoA elongase.

6 CURRENT LIMITATIONS TO THE PRODUCTION OF NOVEL FATTY ACIDS IN TRANSGENIC PLANTS

In all of the above examples where fatty acid metabolism has been manipulated in transgenic plants, the resulting novel fatty acid content of the seed oil has been considerably less than that of exotic plants, which produce similar fatty acids in their seed oil. The key to commercial viability for any of the above novel fatty acids is the ability to produce them at reasonable concentrations (i.e., 70–90% of the total fatty acids of the seed oil). It is fair to say that how this may be achieved and if one or many extra genes will be required is not yet fully understood.

The attachment of fatty acids to the glycerol backbone is catalyzed by enzymes called acyltransferases (see Chap. 2). There is some evidence that acyltransferases may have different acyl specificities in oilseeds which make unusual fatty acids when compared with acyltransferases from domesticated oilseeds [44–46]. This would suggest that genes for acyltransferases from exotic oilseeds will be required, in addition to genes for fatty-acid-modifying enzymes, to efficiently incorporate the novel fatty acid into triacylglycerol.

Furthermore, transgenic plants which accumulate unusual fatty acids also tend to accumulate oleic acid in increased concentrations [19–21,42]. It is thought that most, if not all, monounsaturated fatty acids cycle through membrane phospholipids [29,47,48]. Even products of diverged soluble desaturases, such as petroselinic acid, cycle through phosphatidylcholine before incorporation into triacylglycerol [47]. The increased oleate content seen in almost all transgenic plants making unusual fatty acids may be an indication that this cycling process is not functioning efficiently.

Finally, it is known that membrane phosphatidylcholine is the substrate for many desaturase-related fatty acid modifications such as hydroxylation and epoxidation [49,50]. However, oxygenated fatty acids, such as ricinoleic and vernolic acids, are not normally found in the phospholipids of plants which contain these fatty acids in their oils [51]. Transgenic plants expressing hydroxylases and epoxygenases, however, tend to accumulate these fatty acids in phospholipid fractions during seed development ([19,20]; Calhoon, unpublished data).

Thus, there is evidence to suggest that the inefficient synthesis in transgenics of oils containing novel fatty acids may be caused by one or more of a number of factors (see discussion in Ref. 52). These may include inefficient incorporation of unusual fatty acids into triacylglycerol, reduced flux through membrane lipids of the precursors to unusual lipids, and/or some unusual fatty acids getting ''stuck'' in membrane lipids. Whatever the cause, the consequence of an unusual acyl group of not being incorporated into storage oil appears to be degradation. The end result for the oilseed is an increase in fatty acid turnover rate, observed as a coordinate increase in both fatty acid synthesis and fatty acid β-oxidation, in order to maintain a constant rate of oil biosynthesis [53].

The recent discovery of a phospholipid:diacylcerol acyltransferase in plants [54], which appears to have specificity for vernolic acyl-CoAs when isolated from *Vernonia*, ricinoleic acyl-CoAs when isolated from *Ricinus*, and neither when isolated from sunflower, may at least provide a tool to begin to address this problem.

7 PROTEIN ENGINEERING TO PRODUCE HIGHLY SPECIALIZED INDUSTRIAL FATTY ACIDS

Structural and functional studies of soluble and membrane-bound desaturases have made it possible to consider designing new enzymes which will put functional groups at new positions on a fatty acid molecule [55,56]. Thus, it may be possible to design enzymes which will put a functional group at exactly the position that an industrial chemist would like it for a particular application.

Furthermore, comparison of the primary amino acid sequences of fatty acid desaturases and desaturase-related genes make it possible to identify the specific amino acids which determine what functional group is inserted into the fatty acid [19].

Recently, the technique of directed evolution [57], when coupled with the extensive knowledge being accumulated of gene families involved in unusual fatty acid biosynthesis,

may considerably speed up the process of producing enzymes catalyzing the insertion of novel functional groups at useful positions on the carbon chain.

Using the above techniques, oils from transgenic oilseeds will eventually be tailored to meet many different industrial applications and fatty acids produced to supply many different feedstock applications. The widespread production of industrial aliphatics in plants may help bring a renaissance in the use of plant-derived chemicals in the place of petrochemicals and help meet the increasing desire to find renewable sources for our material needs.

REFERENCES

1. EN Frankel. Prog Lipid Res 19:1–22, 1980.
2. NS Yadav. In: DPS Verma, RC Shoemaker, eds. Soybean: Genetics, Molecular Biology and Biotechnology. Wallingford, UK: CAB International, 1996, pp 165–188.
3. AJ Kinney. Fett/Lipid 100:173–176, 1998.
4. JJ Finer, MD McMullen. In Vitro Cell Dev Biol 27:175–182, 1991.
5. AJ Kinney. J Food Lipids 3:273–292, 1996.
6. AJ Kinney, S Knowlton. In: S Roller, S Harlander, eds. Genetic Modification in the Food Industry. London: Blackie, 1998, pp 193–213.
7. J Flider. INFORM 6:1031–1035, 1995.
8. AJ Kinney. In: JL Harwood, ed. Plant Lipid Biosynthesis: Fundamentals and Agricultural Applications. Cambridge: Cambridge University Press, 1998, pp 273–286.
9. JP McGuigan. J Paint Technol Eng 34:939–952, 1962.
10. JK Sears, JR Darby. The Technology of Plasticizers. New York: Wiley, 1982.
11. AJ Kinney, WD Hitz, S Knowlton, EB Cahoon. In: J Sánchez, E Cerdá-Olmedo, E Martinez-Force, eds. Advances in Plant Lipid Research. Sevilla: Universidad de Sevilla Press, 1998, pp 623–628.
12. AJ Kinney. Lipid Technol Newsett 5:36–39, 1999.
13. FO Ayorinde, EY Nana, PD Nicely, AS Woods, EO Price, CP Nwaonicha. J Am Oil Chem Soc 74:531–538, 1997.
14. KAA Bryant, CP Nwaonicha, M Hassan, MA Anderson, FO Ayroinde. J Am Oil Chem Soc 70:457–460, 1993.
15. WD Hitz. United States Patent 5,846,784. 1998.
16. M Lee, M Lenman, A Banas, M Bafor, S Singh, M Schweizer, R Nilsson, R Liljenberg, A Dahlqvist, P-O Gummeson, S Sjodahl, A Green, S Stymne. Science 280:915–918, 1998.
17. RC Badami, KB Patil. Prog Lipid Res 19:119–153, 1981.
18. EB Cahoon, TJ Carlson, KG Ripp, BJ Schweiger, GA Cook, SE Hall, AJ Kinney. Proc Natl Acad Sci USA 96:12,395–12,940, 1999.
19. P Broun, J Shanklin, E Whittle, C Somerville. Science 282:1315–1317, 1998.
20. P Broun, S Boddupalli, C Somerville. Plant J 13:201–210, 1998.
21. FN van de Loo, P Broun, S Turner, CR Somerville. Proc Natl Acad Sci USA 92:6743–6747, 1995.
22. AJ Kinney. Genet Eng 19:149–166, 1997.
23. L Liu, EG Hammond, BJ Nikolau. Plant Physiol 113:1343–1349, 1997.
24. EG Hammond. In: P Wan, ed. Fats and Oils Processing. Champaign, IL: American Oil Chemists Society, 1991, pp 1–15.
25. MW Formo. In: D Swern, ed. Bailey's Industrial Oil and Fat Products. New York: Wiley, 1979, pp 708–709.
26. EB Cahoon, JB Ohlrogge. Plant Physiol 104:827–837, 1994.
27. EB Cahoon, P Dormann, JB Ohlrogge. Prog Lipid Res 33:155–163, 1994.
28. P Dormann, M Frentzen, JB Ohlrogge. Plant Physiol 104:839–844, 1994.

29. J Ohlrogge. Plant Physiol 104:821–826, 1994.
30. J Shanklin, EB Cahoon, E Whittle. In: J Sánchez, E Cerdá-Olmedo, E Martinez-Force, eds. Advances in Plant Lipid Research. Sevilla: University of Seville Press, 1998, pp 115–117.
31. EB Cahoon, CK Becker, J Shanklin, JB Ohlrogge. Plant Physiol 106:807–808, 1994.
32. EB Cahoon, AM Cramner, J Shanklin, JB Ohlrogge. J Biol Chem 269:27519–27526, 1994.
33. EB Cahoon, S Coughlan, J Shanklin. Plant Mol Biol 33:1105–1110, 1997.
34. RW Miller, ME Daxenbichler, FR Earle, HS Gentry. J Am Oil Chem Soc 41:167–169, 1964.
35. SM Erhan, R Kleiman, TA Isbell. J Am Oil Chem Soc 70:460–465, 1993.
36. TA Isbell, R Kleiman. J Am Oil Chem Soc 73:1097–1107, 1996.
37. TA Isbell, RD Plattner. J Am Oil Chem Soc 74:153–158, 1997.
38. DA Burg, R Kleiman. J Am Oil Chem Soc 68:190–192, 1991.
39. F Hirsinger. In: G Röbbelen, RK Downey, A Ashri, eds. Oil Crops of the World. New York: McGraw-Hill, 1989, pp 518–532.
40. TA Isbell, TA Abbott, KD Carlson. Ind Crops Prod 9:15–123, 1999.
41. C Watkins. INFORM 10:828–836, 1999.
42. EB Cahoon, E-F Marillia, KL Stecca, SE Hall, DT Taylor, AJ Kinney. Plant Physiol 124:1–9, 2000.
43. T Girke, P Sperling, E Heinz. In: J Sánchez, E Cerdá-Olmedo, E Martinez-Force, eds. Advances in Plant Lipid Research. Sevilla: Universidad de Sevilla Press, 1998, pp 103–109.
44. M Frentzen. Fett/Lipid 100:161–166, 1998.
45. AA Millar, MA Smith, L Kunst. Trends in Plant Sci 5:95–101, 2000.
46. J Ohlrogge, J Browse. Plant Cell 7:957–970, 1995.
47. EB Cahoon, JB Ohlrogge. Plant Physiol 104:845–855, 1994.
48. DJ Schultz, JB Ohlrogge. Plant Physiol Biochem 38:169–175, 2000.
49. M Bafor, MA Smith, L Jonsson, K Stobart, S Stymne. Biochem J 280:507–514, 1991.
50. M Bafor, MA Smith, L Jonsson, K Stobart, S Stymne. Arch Biochem Biophys 303:145–151, 1993.
51. FB Padley, FD Gunstone, JL Harwood. In: FD Gunstone, JL Harwood, FB Padley, eds. The Lipid Handbook. London: Chapman & Hall, 1994, pp 49–170.
52. T Voelker, AJ Kinney. Annu Rev Plant Physiol Plant Mol Biol 52:335–361, 2001.
53. VS Eccleston, JB Ohlrogge. Plant Cell 10:613–622, 1998.
54. A Dahlqvist, U Ståhl, M Lenman, A Banas, M Lee, L Sandager, H Ronne, S Stymne. Proc Natl Acad Sci USA 97:6487–6492, 2000.
55. EB Cahoon, Y Lindqvist, G Schneider, J Shanklin. Proc Natl Acad Sci USA 94:4872–4877, 1997.
56. J Shanklin, EB Cahoon. Annu Rev Plant Physiol Plant Mol Biol 49:611–641, 1998.
57. J Minshull, WP Stemmer. Curr Opin Chem Biol 3:284–290, 1999.

5

New Gene Combinations Governing Saturated and Unsaturated Fatty Acid Composition in Soybean

RICHARD F. WILSON and JOSEPH W. BURTON

Agricultural Research Service, U.S. Department of Agriculture, and North Carolina State University, Raleigh, North Carolina

VINCENT R. PANTALONE

University of Tennessee, Knoxville, Tennessee

RALPH E. DEWEY

North Carolina State University, Raleigh, North Carolina

1 INTRODUCTION

Advances in genetic technology have greatly improved knowledge of biological systems, especially those related to the genetic alteration of soybean oil composition. A great deal more remains to be discovered and will be discovered in a timely manner because the science of improving soybean oil quality and utility is a dynamic continuum, rather than a linear process. This continuum involves many scientific disciplines, where discoveries in one area of science lead to breakthroughs in another. Sometimes, the immediate value of a discovery may be unclear, and the findings must be held in reserve until needed to address a particular problem in the oilseed industry. To be certain, the priorities of the oilseed industry do change with time. Consequently, research priorities also must be adapted to address those current needs.

The ability and flexibility to respond to new priorities, without loss of momentum, is the primary strength of a dynamic system, compared to a one-dimensional research process. With each collective step upward on the learning curve, a point is reached periodically when practical application of the knowledge gained becomes a necessity. This junc-

ture in the technological spiral usually leads full circle to the initial field of science from which the continuum originated. In this case, the original concepts underlying the state of the art of genetic modification of soybean oil composition trace directly to traditional plant breeding.

We now approach a time when the cumulative knowledge of the genetic regulation of lipid composition in soybean, gained from breeding, biochemistry, and molecular genetic approaches, must be applied in commercial products that help the oilseed industry respond to critical issues. The most pressing problems of today are the need for a soybean oil with lower saturated fat content, the need to produce food products with lower levels of trans isomers of unsaturated fatty acids, and the need to develop these traits in nontransgenic commercial cultivars. Of course, other related issues are beginning to surface gradually, but the knowledge and arsenal of genetic materials amassed to date will enable a timely response to current and future problems. In that regard, an attempt is made here to document the genetic resources that are available at this time and to demonstrate how they may be used to enhance the competitive position of soybean in various oilseed markets. The first example of these resources relates to the initial efforts in the area of soybean oil modification, the genetic reduction of linolenic acid concentration.

2 GENETIC MODIFICATION OF UNSATURATED FATTY ACID COMPOSITION IN SOYBEAN OIL

2.1 Reduction of Linolenic Acid Concentration

Each revolution in this technology cycle closes the gap to some tangible goal, which usually is defined by a major problem in the oilseed industry. About 30 years ago, the United States experienced an energy crisis. Hence, the cost of hydrogenating vegetable oil became a major issue. Although this was a temporal problem, it prompted Howell, Rinne (at the University of Illinois), and Brim (USDA–ARS at North Carolina State University) to propose that the cost of hydrogenation might be reduced if cultivated soybeans (*Glycine max* L. Merr.) contained lower levels of linolenic acid (18:3) [1]. Furthermore, they resolved to achieve this goal in a novel manner, through natural gene recombination. At the time, virtually nothing was known about the genetic or biochemical regulation of the trait, except that 18:3 synthesis in soybean probably was mediated by two enzymes that catalyzed sequential desaturation of oleic acid (18:1) to 18:3 and that the inheritance of 18:1 concentration was negatively correlated with linoleic (18:2) and 18:3 concentration. Even though genetic variation for 18:3 concentration practically was nonexistent in the USDA Soybean Germplasm Collection, Brim devised a recurrent mass and within half-sib family breeding method for selection of germplasm with higher 18:1 to indirectly reduce 18:3 [2,3]. After several selection cycles, 18:1 was increased from 22% to 42%, and 18:3 was reduced from 9% to 6% of crude oil. These traits were captured in the germplasm line, N78-2245 [4], which was the first soybean intentionally bred for lower 18:3 concentration (Table 1).

Other breeding programs soon followed with low-18:3 germplasm developed through chemical mutagenesis. Wilcox (USDA–ARS at Purdue University) and coworkers mutagenized the cultivar Century with ethylmethanesulfonate (EMS) and selected a line, C1640, that contained about 3.5% 18:3 [5]. Inheritance studies revealed that this trait was controlled by a single recessive allele, designated *fan* [6]. Fehr (Iowa State Uni-

Table 1 Soybean Genetic Resources with Mutations Governing Unsaturated Fatty Acid Composition

Trait	Line	Fatty acid[a] 16:0	18:0	18:1	18:2	18:3	Putative genotype	Ref.
Normal	N83-375	12	4	21	55	9	*FadFad FanFan*	13
Low 18:3	N78-2245	10	4	42	39	6	*fadfad FanFan*	4
	PI-123440	10	4	30	51	5	*FadFad fanfan*	1
	PI-361088B	12	4	19	61	4	*FadFad fanfan*	9
	C1640	10	4	25	57	4	*FadFad fanfan*	5
	A5	9	4	47	36	3	*FadFad* fan_1fan_1	7
	N85-2176	10	3	44	40	3	*fadfad fanfan*	12
	N87-2120-3	6	4	39	48	3	*FadFad fanfan*	16
	N87-2122-4	5	3	48	39	5	*fadfad FanFan*	16
High 18:3	PI-424031	15	4	11	52	19	Fad_3Fad_3 Fan_3Fan_3	16
	PI-342434	11	3	14	55	15	Fad_2Fad_2 Fan_2Fan_2	16
High 18:1	DuPont[b]	9	3	79	3	6	Anti-*FAD2*	18
	N97-3363-4	8	4	60	26	2	*fadfad* fad_1fad_1 *fanfan*	19

[a] All data are reported as crude oil and given in percent fatty acid.
[b] Transgenic.

versity) and co-workers mutagenized the line FA9525 and selected a line, A5, which contained about 4% 18:3 [7]. The single recessive allele in A5 was designated, *fan*$_1$. Subsequently, Fehr and Hammond described two additional mutations, *fan*$_2$ and *fan*$_3$, at *Fan* loci. When combined (*fan*$_1$ *fan*$_1$ *fan*$_2$ *fan*$_2$ *fan*$_3$ *fan*$_3$) in the germplasm line A29, these alleles reportedly produce soybean oil with 1.1% 18:3 [8]. In addition, two low-18:3 plant introductions from the USDA Soybean Collection (PI-123440, identified by Brim [1], and PI-361088B, identified by Rennie et al. [9] at the University of Guelph) contained natural mutations at *Fan* loci that were shown to be either allelic or identical to the original *fan* allele in C1640 [10].

All of these respective *fan* alleles represent mutations in different genes or different mutations in the same gene. All are natural mutations, created by acceptable breeding techniques or spontaneous events through natural gene recombination. However, one must keep in mind that these descriptors are based on an altered oil phenotype, not a gene sequence. In practice, putative alleles are distinguished by classical Mendelian genetic studies and the statistical probability that progeny will segregate into discrete phenotypic classes in sufficient frequency to fit a predicted gene inheritance model. Such information provides evidence that a given allele may represent an independent genetic event or a different mutation at arbitrary gene loci. It does not reveal what gene the allele represents or how the apparent mutations alter enzyme activities in the lipid synthetic pathway. Knowing which gene has a mutation that affects a change in fatty acid composition, what the mutation is, where it occurs in the genome, and what the gene encodes is the key to solving current and future oilseed industry issues.

Early attempts to associate alleles governing 18:3 concentration with specific genes and altered enzyme activities suggested that the mutation resident in N78-2245 (*fadfad FanFan*) affected ω-6 desaturase activity, and the mutation in PI-123440 (*FadFad fanfan*) affected ω-3 desaturase activity. Evidence in support of this theory was obtained from a mating of N78-2245 × PI-123440, where the frequency distribution for 18:3 concentration among four phenotypic classes in the F_3 generation fit a model for two independently segregating gene loci [11]. An inbred line, N85-2176, derived from this population contained 3.5% 18:3 and represents the only known soybean having recessive mutations affecting both ω-6 desaturase and ω-3 desaturase activity [12]. This was confirmed by statistical analysis of the genotypic frequency distributions for ω-6 desaturase and ω-3 desaturase activity among F_4 progenies from N83-375 (*FadFad FanFan*) × N85-2176 (*fadfad fanfan*) [13]. Then, more recently, soybean cDNA for FAD2 (ω-6 desaturase) and FAD3 (ω-3 desaturase) has been used to probe mRNA and DNA from inbred lines representing the major phenotypic and predicted genotypic classes from F_4 progenies of N83-375 × N85-2176. These data further document the premise that mutations at *Fad* loci determine desaturation of 18:1 to 18:2 and mutations at *Fan* loci determine conversion of 18:2 to 18:3. Furthermore, a separate investigation has shown that the *fan*$_1$ allele in A5 germplasm is associated with the FAD3 gene [14].

2.2 Elevation of Linolenic Acid Concentration

Genetic control of polyunsaturated fatty acid synthesis also may lead to new high-18:3 industrial oils. In that regard, overexpression of the FAD2-2 gene is reported to affect elevated 18:3 [15]. However, another approach toward a more complete understanding of the genetic regulation of 18:3 synthesis may involve the wild ancestor of cultivated soybean, *Glycine soja* Sieb. and Zucc. Wild soybean accessions in the USDA–ARS Soy-

bean Germplasm Collection may contain as much as 23% 18:3. Although little is known of the genetic mechanisms that govern 18:3 in wild soybean, it is proposed that a different complement of genes may condition expression of the very high-18:3 trait. To test that hypothesis, inheritance of 18:3 was investigated among inbred progeny of four *G. max* × *G. soja* populations [16].

Interspecific hybridization of N87-2120-3 (*G. max*, *FadFad fanfan*) × PI-342434 (*G. soja*, Fad_2Fad_2 Fan_2Fan_2) or PI424031 (*G. soja*, Fad_3Fad_3 Fan_3Fan_3) revealed a wide range of segregation for ω-6 desaturation among F_3 progeny of these populations. These populations exhibited a skewed frequency distribution for 18:1-desaturation that is typical of *G. max* lines that segregate for ω-6 desaturase activity. However, the frequency distribution in the respective interspecific populations segregating for *Fad* Fad_2 and *Fad* Fad_3 were distinct or independent from each other. This observation suggested that the Fad_2 and Fad_3 alleles represented different gene loci. Transgressive segregants in both populations, where ω-6 desaturation exceeded the *G. soja* parents, also indicated that the parents carried different *Fad* alleles. In addition, chi-square analyses of these data showed the progeny class frequencies from the *FadFad* × Fad_2_ and the *FadFad* × Fad_3_ populations each fit the phenotypic distribution expected for a single gene in the F_3 generation. This is further evidence suggesting that PI-342434 and PI-424031 did carry different alternative *Fad* alleles governing ω-6 desaturase activity.

Interspecific hybridization of N87-2122-4 (*G. max*, *fadfad FanFan*) with PI-342434 or PI-424031 exhibited a wide range of segregation for ω-3 desaturation among *G. max* × *G. soja* progeny in the F_3 generation. Each population gave a normal distribution without transgressive segregants. However, these segregation patterns failed to overlap, as would be expected if the putative Fan_2 and Fan_3 alleles were identical to each other. Thus, dissimilar distributions between these datasets again indicated that different *Fan* alleles were present. Chi-square analyses of these data confirmed this assumption, by showing that the progeny class frequencies from the *FanFan* × Fan_2_ and the *FanFan* × Fan_3_ populations each fit the phenotypic distribution expected for a single gene in the F_3 generation. This suggested that PI-342434 and PI-424031 also carried different alternative *Fan* alleles governing ω-3 desaturase activity.

To gain a better understanding of the inheritance of alternative desaturase genes in wild soybeans, their mode of gene interaction was evaluated in F_2 populations. Comparison of the F_2 population mean with mid-parent values showed no maternal or cytoplasmic effects on unsaturated fatty acid composition. A comparison of 18:1 concentration with ω-6 desaturase activity among F_2 progeny, however, revealed a significant number of transgressive segregants for 18:1 concentration, both above and below the respective parents. Surprisingly, this frequency distribution was heavily skewed toward higher 18:1, in a 13:3 phenotypic ratio (where Fad_2_Fad_3_ was the minority phenotypic class). Such a phenotypic ratio was consistent with a model for epistatic gene interaction.

Considering the putative alternative *Fan* alleles, few transgressive segregants were found in the comparison of 18:3 concentration to ω-3 desaturase activity among F_2 progeny from PI-342434 × PI-424031. This suggested that the putative Fan_2 and Fan_3 genes were allelic to each other (i.e., represented different mutations in the same gene). This frequency distribution was heavily skewed toward lower 18:3, also in a 13:3 phenotypic ratio (where Fan_3Fan_3 was the minority phenotypic class). Statistical tests supported the interpretation that this pattern also fits a model for epistatic gene interaction. Although low in frequency, inbred lines with a Fad_2_Fad_3_Fan_3Fan_3 exhibited the highest levels of 18:3. Therefore, as compared to *G. max*, it appeared that at least three alternative

desaturases were responsible for the elevated polyunsaturated fatty acid levels in these wild soybeans.

Overall, these experiments suggested that wild soybeans contained desaturase genes that may have been lost in the domestication of cultivated soybean. However, when recombined with *G. max*, these genes acted in an additive genetic manner, to effect a higher 18:3 concentration. Thus, transfer of the wild soybean desaturase genes to *G. max* germplasm could establish the foundation for development of highly polyunsaturated soybean oils that have application in the manufacture of lubricants and drying oils (Table 1).

2.3 Elevation of Oleic Acid

Soybeans typically contain about 20% 18:1 [17]. As mentioned earlier, the germplasm N78-2245 was perhaps the first soybean developed with higher levels (about 42%) of 18:1. We now know that this line contains a natural mutation in the FAD2 gene, and the FAD2 encodes the predominant ω-6 desaturase activity in soybean seed. When a normal FAD2 gene is expressed in antisense orientation (or by cosuppression) in transgenic soybeans, the seed oil may contain up to 80% 18:1 [18]. Therefore, it may be presumed that natural mutations at *Fad* gene loci determine the high-18:1 trait in nontransgenic soybeans. Until recently, transgenic events appeared to be the only feasible approach to achieve soybean oil with exceptionally high levels of 18:1. However, through natural gene recombination, Burton (USDA–ARS at Raleigh, NC) has developed a population with segregants that range from 45% to 70% 18:1. An experimental inbred line [19], N97-3363-4, containing about 60% 18:1 has been selected from this population (Table 1). It is believed that this line contains mutations in two different alleles ($fadfad\,fad_1fad_1$) at *Fad* loci, possibly in different FAD2 genes. Undoubtedly, this discovery will expand efforts toward production of soybean oils with superior oxidative stability without hydrogenation.

3 GENETIC MODIFICATION OF SATURATED FATTY ACID COMPOSITION IN SOYBEAN OIL

3.1 Elevation of Palmitic Acid Concentration

Palmitic acid (16:0) concentration in soybean typically averages about 11% of crude oil [17]. However, this phenotypic trait may be genetically altered by certain mutations at gene loci designated *Fap*. Nearly all of the described mutations at *Fap* have been induced by chemical mutagenesis. Germplasm with about 150% or more of 16:0 levels in normal soybean oil may carry homozygous recessive fap_2 (C1727 [20]), fap_{2b} (A21 [21]), fap_4 (A24 [21]), or fap_5 (A27 [22]) alleles. Various combinations of these alleles may elevate 16:0 concentration up to 35% of crude oil [22]. However, classical Mendelian genetic studies provide the only evidence that fap_2, fap_{2b}, fap_4, and fap_5 might represent independent genetic events or different mutations at *Fap* loci. The gene products (enzymes) of these alleles also are unknown. As more high-16:0 alleles are described, it becomes a point of order to determine their function (Table 2).

Mutations at *Fap* loci could affect the activity of a number of enzymes within the fatty acid synthetic pathway [23]. The possibilities include at least eight enzymes [3-keto-acyl-ACP synthetase III (KAS-III), 3-keto-acyl-ACP synthetase II (KAS-II), 18:0-ACP desaturase (Δ9DES), 16:0-ACP thioesterase (16:0-ACP TE), 18:1-ACP thioesterase (18:1-ACP TE), glycerol-3-phosphate acyltransferase (G3PAT), lysophosphatidic acid

Table 2 Soybean Genetic Resources with Mutations Governing Saturated Fatty Acid Composition

Trait	Line	Fatty acid[a] 16:0	18:0	18:1	18:2	18:3	Putative genotype	Ref.
Normal	N83-375	12	4	21	55	9	*FapFap FasFas*	13
Low 16:0	C1726	8	3	26	58	5	fap_1fap_1 *FasFas*	20
	N79-2077-12	6	4	39	44	7	$fap_{nc}fap_{nc}$ *FasFas*	25
	A22	4	3	27	58	9	fap_3fap_3 *FasFas*	21
	ELLP2	6	3	22	61	8	*fap*fap* FasFas*	33
	N94-2575	4	3	23	62	8	fap_1fap_1 $fap_{nc}fap_{nc}$	36
	C1943	4	4	31	55	6	fap_1fap_1 $fap_{nc}fap_{nc}$	36
High 16:0	C1727	18	3	16	55	8	fap_2fap_2 *FasFas*	20
	A21	16	4	21	52	7	$fap_{2b}fap_{2b}$ *FasFas*	21
	A24	15	4	25	48	8	fap_4fap_4 *FasFas*	21
	A27	17	4	26	46	7	fap_5fap_5 *FasFas*	22
High 18:0	A6	8	28	20	38	7	*FapFap* fas^afas^a	40
	FA41545	8	15	23	45	9	*FapFap* fas^bfas^b	41
	A81606085	7	19	20	45	9	*FapFap fasfas*	41
	FAM94-41	10	9	25	51	5	*FapFap* $fas_{nc}fas_{nc}$	39
	KK-2	11	7	25	50	7	*FapFap* st_1st_1	42
	M-25	9	20	18	45	8	*FapFap* st_2st_2	42

[a] All data are reported as crude oil, unless identified as refined.

acyltransferase (LPAAT), and diacylglycerol acyltransferase (DGAT)]. In addition, any of these enzyme activities may be influenced by modifier genes (genes of minor effect) that are associated with major effects of *fap* loci [24].

Only a few investigations have explored the potential metabolic targets for *fap* and related modifier gene action in soybean [25,26]. That work tested the hypothesis that fap_2 alleles altered the activity of glycerolipid acyltransferase enzymes. However, based on a comparison of in vivo saturation kinetics for triacylglycerol (TAG) synthesis from exogenous acetate and 16:0-CoA, it was apparent that fap_2 alleles did not contribute major genetic effects to the activities of G3PAT, LPAAT, DGAT, or any event downstream of acyl-CoA formation. Rather, higher 16:0 concentration appeared to be a function of endogenous 16:0-CoA synthesis or supply from plastids of developing soybean cotyledons. This finding focused attention on KAS-II, Δ9DES, 16:0-ACP TE, and 18:1-ACP TE as the prime candidates for the fap_2 gene product. KAS-II catalyzes the elongation of 16:0-ACP to 18:0-ACP, Δ9DES desaturates 18:0-ACP to 18:1-ACP, 16:0-ACP TE initiates conversion of 16:0-ACP or 18:1-ACP (with little activity on 18:0-ACP) to their respective acyl-CoA derivatives, and 18:1-ACP TE is reported to have a preference for 18:1-ACP [23]. Based on reports from cottonseed [27] and high-16:0 sunflower germplasm [28], 16:0-ACP TE activity may be primarily responsible for genetically enhanced 16:0 content of vegetable oils (albeit lower KAS-II activity also may be involved). In addition, seeds of transgenic canola expressing a 16:0-ACP TE gene from *Cuphea* are reported to have 35% 16:0 [29], and similar transgenic events appear to increase 16:0 levels in *Arabidopsis* [30]. In transgenic soybean embryos [17], it is observed that increased 16:0-ACP TE activity leads to elevated esterification of glycerolipids with 16:0 and decreased export of 18:1-CoA from plastids. Conversely, antisense constructs or sense suppression of the 16:0-ACP TE gene produces the opposite effect on the levels of 16:0-CoA and 18:1-CoA that are available for glycerolipid synthesis. In comparison, overexpression of the KAS-II or the Δ9DES gene had little effect on the 16:0 concentration of soybean oil. Therefore, it would appear that genetic manipulation of 16:0-ACP TE activity is the most effective way to increase the 16:0 concentration of soybean oil.

However, in developing seed of the high-16:0 soybean germplasm, C1727, acetate saturation kinetics also showed a significantly lower V_{max} for 18:0 synthesis and a considerably longer half-life ($t_{1/2}$) for 18:0 incorporation into total phospholipids (TPL) [31]. This observation suggested that the fap_2fap_2 genotype might mediate an increase in 16:0 synthesis through gene action that reduces KAS-II activity rather than or in addition to increased 16:0-ACP TE activity. Furthermore, pulse-chase experiments with exogenous acetate removed the possibility that C1727 exhibited a greater rate of 18:0 metabolism or transfer from TPL toward final deposition in TAG. The $t_{1/2}$ for 18:0 decay from pulse-chase experiments was not significantly different among genotypes, averaging 8.7 ± 0.9 hr. In contrast, significant genotypic differences were found in the first-order decay rates for 16:0. The $t_{1/2}$ for 16:0 turnover averaged about 18.5 ± 4.5 hr, and a strong negative correlation ($r^2 = 0.94$) was found between the V_{max} for 16:0 synthesis (from acetate saturation kinetics) and the $t_{1/2}$ for 16:0 turnover (from acetate pulse-chase experiments). These findings supported previous work showing an apparent lack of major genetic effects of fap_2 alleles on glycerolipid acyltransferase activities or any event downstream of acyl-CoA formation [26]. Thus, the amount of 16:0 that entered and passed through TPL was a function of the 16:0-CoA produced by plastids. However, the fact that fap_2 is a recessive trait in soybean makes it difficult to envision how a mutation in the FAT B gene (which encodes 16:0-ACP TE) might affect a high-16:0 phenotype. Hence, the role of reduced

KAS-II activity cannot be discounted in the case of C1727. Additional research is needed to determine whether fap_2 represents a mutation in the FAT B gene or if elevated 16:0-ACP TE activity is a direct or indirect function of a reduction in KAS-II activity.

3.2 Reduction of Palmitic Acid Concentration

N79-2077-12 was the first soybean germplasm developed with lower 16:0 concentration [25,32] and remains the only germplasm that carries a serendipitous natural mutation, designated as the recessive fap_{nc} allele. Subsequently, other soybean germplasm exhibiting about 50% of 16:0 levels in normal soybean oil have been developed (Table 2). These germplasm may carry homozygous recessive fap_1 (C1726 [20]), fap_3 (A22 [21]), or the temporary designation *fap** (ELLP2 [33]) alleles. Combinations of homozygous fap_1 and fap_3 [34], or fap_1 and fap_{nc} [35], or fap_1 and *fap** [33] alleles constitute transgressive segregates, from matings of the respective parental lines, that exhibit less than 4.5% 16:0. The inbred lines C1943 (with northern maturity) and N94-2575 (with southern maturity) are examples of selections in which fap_1 and fap_{nc} are combined [36]. Based on this information, it is highly probable that the mutations represented by the fap_3, fap_{nc} and *fap** descriptors are different and distinct from fap_1. However, it is not known whether fap_3, fap_{nc}, and *fap** are independent or allelic to each other.

Given that fap_{nc} is independent of fap_1, efforts have been made to identify the enzyme(s) that are subject to the major genetic effects exerted by these two alleles. By implication, it is speculated that reduced 16:0-ACP TE activity determines a low-16:0 phenotype in soybean. However, such assumptions are in lieu of direct evidence that the fap_1, fap_2, and fap_{nc} alleles in soybean represent natural or chemical mutations in the FAT B gene, or that an association exists between these alleles and expression of lower 16:0-ACP TE activity. Furthermore, these two alleles are known to segregate as independent loci. Similar claims are made for five other *fap* alleles. Thus, it is possible that some of these mutations may actually occur in genes other than those that govern 16:0-ACP TE activity.

Assessing genotypic differences in 16:0-ACP TE activity with purified protein is difficult because most plants contain very low levels of this enzyme [37]. However, evaluation of in vivo 16:0 metabolism shows that combinations of homozygous recessive or dominant fap_1 and fap_{nc} alleles produce significant effects on the 16:0 concentration of TPL in developing seed. At 35 days after flowering (DAF), the stage of reproductive growth when peak accumulation or synthesis of 16:0 occurs in soybean seed [26], 16:0 concentration in TPL of *fap* genotypes is correlated strongly and positively ($r^2 = 0.94$) with the 16:0 concentration in total lipid (TL) at seed maturity. A similar report supports this observation [18] and indicates that TPL composition at 35 DAF is predictive of the fatty acid composition at seed maturity. Also, the lack of a correlation ($r^2 = 0.03$) between the observed 16:0 and stearic acid (18:0) concentration among these genotypes is consistent with postulates that genetic control of 18:0 concentration in soybean oil is independent of these two *fap* alleles [24].

The TPL also has been shown to be the most metabolically active glycerolipid fraction in developing soybean cotyledons [38]; thereby, genotypic differences in endogenous 16:0 incorporation in TPL at 35 DAF should provide metabolic indicators of *fap* allele function. In vivo saturation kinetics of exogenous acetate metabolism in developing seed at 35 DAF [31] showed that over all genotypes, TPL at 35 DAF accounted for 72.7 $\pm$ 1.9% of the total lipid radioactivity in 16:0 synthesized from acetate, but there were

genotypic differences in the rate of 16:0 incorporation in TPL; where Dare (Fap_1Fap_1 $Fap_{nc}Fap_{nc}$) > C1726 (fap_1fap_1 $Fap_{nc}Fap_{nc}$) = N79-2077-12 (Fap_1Fap_1 $fap_{nc}fap_{nc}$) > N94-2575 (fap_1fap_1 $fap_{nc}fap_{nc}$). Estimates of V_{max} for 16:0 synthesis derived from these data reflected major genetic effects of *fap* alleles that were positively correlated (r^2 = 0.80) with 16:0 concentration in TPL at 35 DAF. In addition, significant genotypic differences also were found in the estimated first-order rate constant for 16:0 synthesis from acetate, which may be used to calculate the approximate $t_{1/2}$ or time required for esterification of 50% of the 16:0-CoA exported from plastids into phospholipid. Based on these calculations, N94-2575 (fap_1fap_1 $fap_{nc}fap_{nc}$) exhibited the lowest capacity for *de novo* 16:0 synthesis among these genotypes.

Comparison of the estimated V_{max} for 16:0, 18:0, and total 18:1 synthesis with the concentration of 16:0 in TPL at 35 DAF (from lines having Fap_1Fap_1 $Fap_{nc}Fap_{nc}$, fap_1fap_1 $Fap_{nc}Fap_{nc}$, Fap_1Fap_1 $fap_{nc}fap_{nc}$, or fap_1fap_1 $fap_{nc}fap_{nc}$ genotypes) showed that the rate of 16:0 synthesis was inversely proportional to the rate of 18:1 synthesis. No relation (r^2 = 0.04) was found between V_{max} for 18:0 and 16:0 concentration. Thus, this information helped to narrow the identification of fap_1 and fap_{nc} allele function to the genes that encode Δ9DES, 16:0-ACP TE or 18:1-ACP TE.

Assuming that alterations in 9DES, 16:0-ACP TE or 18:1-ACP TE activity might be apparent at the transcriptional level, mRNA purified from cotyledons at 25, 35, and 45 DAF has been hybridized with soybean cDNA clones of genes that encode the 16:0-ACP thioesterase (FAT B), 18:0-ACP thioesterase (FAT A), and plastidial Δ-9 desaturase (PDS1). The steady-state levels of transcripts followed the trend in incremental rates of oil accumulation during seed development [31]. At 35 DAF, when the peak rate of oil accumulation occurred, no genotypic differences in steady-state transcripts were observed with the FAT A or PDS1 probes. However, transcription of FAT B was directly related to the 16:0 concentration exhibited by the Fap_1Fap_1 $Fap_{nc}Fap_{nc}$, fap_1fap_1 $Fap_{nc}Fap_{nc}$, Fap_1Fap_1 $fap_{nc}fap_{nc}$, or fap_1fap_1 $fap_{nc}fap_{nc}$ genotypes. Therefore, it is probable that major genetic effects of the fap_1 and fap_{nc} alleles are the result of mutations in two different genes that encode the 16:0-ACP TE in soybean.

3.3 Elevation of Stearic Acid Concentration

Stearic acid (18:0) concentration in soybean typically averages about 3% of crude oil [17]. However, this phenotypic trait may be genetically altered by certain mutations at gene loci designated *Fas*. The described mutations at *Fas* typically result in elevated 18:0 concentration (Table 2). Nearly all of these variants have been induced by chemical or x-ray mutagenesis, with the exception of a newly developed line, FAM94-41 [39]. FAM94-41 (9% 18:0) is the only known soybean germplasm that carries a serendipitous natural mutation, presumably at *Fas*, which is designated as the recessive fas_{nc} allele. Prior to the discovery of fas_{nc}, five germplasm lines were reported to carry homozygous recessive *fas* alleles {fas^a (A6 [40]), fas^b (FA41545 [41]), *fas* (A81-606085 [41]), st_1 (KK-2 [42]), or st_2 (M25 [42])}. As reported [41], fas^a (30% 18:0), fas^b (15% 18:0), and *fas* (19% 18:0) are allelic to each other and presumably represent different mutations in the same gene. In an F_3 population segregating for st_1 and st_2, the observation of progeny with 18:0 concentration above and below the respective parental means provides evidence for two independently inherited genes governing higher 18:0 concentration in soybean. The proposed st_1st_1 st_2st_2 genotype may elevate 18:0 concentration beyond 35% of crude oil [42]. However, it is unknown whether st_1 or st_2 are allelic to fas^a, fas^b, or *fas*. Mendelian genetic studies for

uniqueness and allelism among these *fas* and *st* alleles have not been conducted. Still, other evidence supports the hypothesis that genes at different *Fas* loci may contribute to a very high-18:0 phenotype. In the mating of A6 and ST2 (another line derived from chemical mutagenesis), the F_1 progeny mean was greater than either parent [43]. Based on this information, it is probable that mutations in at least two independent genes determine the activity of one or more enzymes involved in 18:0 synthesis in soybean.

In other plants, two additive mutant alleles designated fas_2 and fas_x determine lower-18:0 concentration in sunflower [44]. Also, two mutant alleles in *Arabidopsis* elevate 18:0 concentration in both seed and leaf tissue [45]. The designations $fab_{2\text{-}1}$ (20% 18:0) and $fab_{2\text{-}2}$ (6% 18:0) represent mutations at *Fab* loci in *Arabidopsis*. However, both $fab_{2\text{-}1}$ and $fab_{2\text{-}2}$ cause severe reduction in *Arabidopsis* plant size. This observation may be analogous to reports that the yielding ability of soybean is severely depressed by fas^a, fas^b, or *fas* alleles [46,47]. Poor yielding ability attributed to these *fas* alleles has been a significant obstacle to the development of acceptable commercial soybean varieties with higher-18:0 content. However, fas_{nc} in FAM94-41 may be an exception to this problem. About 80% of the pedigree of FAM94-41 comes from the high-yielding maturity group VI cultivar Brim [48]. Thus, FAM94-41 displays agronomic attributes that may be used to overcome the apparent yield depression intrinsic to material developed with the fas^a, fas^b, or *fas* alleles.

Both A6 and FAM94-41 have been mated to determine whether the inheritance of fas_{nc} is independent or allelic to fas^a [39]. Analysis of seed from FAM94-41 × A6 showed that the F_2 progeny mean for each of the measured traits was not significantly different from the respective calculated mid-parent values. This suggested that maternal and cytoplasmic effects were not important factors in the genetic control exerted by fas_{nc} or fas^a on 18:0 concentration in soybean. However, the frequency distribution for 18:0 concentration among F_2 progeny segregating for fas_{nc} and fas^a showed a wide range of values from 6% to 26% 18:0. Within statistical limits of certainty, the variation in 18:0 concentration among all of these progeny fell between the two parents. A lack of transgressive segregants (progeny with 18:0 concentration above the high-parent value or below the low-parent value) in this population removed the possibility that this phenotypic variation arose from the combination of complementary or independently inherited genes. Furthermore, chi-square analyses did not support the inheritance of two different genes, but did support a 1:2:1 progeny ratio that fits a single gene model for differing alleles between FAM94-41 and A6. Therefore, fas_{nc} and fas^a were determined to be allelic to each other and, as such, these alleles represented different mutations in the same gene locus.

An analogous conclusion was reported from the inheritance pattern for fas^a, fas^b, and *fas* alleles [41] where the fas^a allele was dominant over the *fas* allele, but not dominant over fas^b. The genetic analysis of A6 × FAM94-41 progeny suggested that the fas_{nc} allele is different and partially dominant over the fas^a allele in A6. Thus, all five of these *fas* alleles probably contributed major genetic effects to the activity of the same enzyme in the fatty acid or glycerolipid synthetic pathway.

Although the gene product (enzyme) of these *fas* alleles is unknown, further interpretation of the population segregating for fas_{nc} and fas^a provides useful information regarding the nature of these genetic effects. As an example, a strong negative correlation (r^2 = 0.96) was found between 18:1 and 18:0 concentration [39]. In fact, the decline in 18:1 accounted for 89% of the increase in 18:0 within the F_2 progeny of A6 × FAM94-41. Only a weak negative correlation (r^2 = 0.28) existed between 16:0 and 18:0. Among all enzymes in the fatty acid and glycerolipid pathways, the enzymes KAS-II, Δ9DES,

16:0-ACP TE, and 18:1-ACP TE have the highest probability of effecting change in 18:0 concentration in soybean oil. In view of the weak relation between 16:0 and 18:0 in this population, it was deemed unlikely that the high-18:0 trait arose from gene action that increased conversion of 16:0 to 18:0, as may be envisioned by greater KAS-II or reduced 16:0-ACP TE activities. However, it could be that the mutations resident in fas_{nc} and fas^{a} effect either reduced Δ9DES or perhaps reduced 18:1-ACP TE activity. Although this is still under investigation, other lines of evidence have established precedents. As an example, the high-18:0 mutation at the fab_2 locus of *Arabidopsis* effected reduced Δ9DES activity [49], and 30% 18:0 was achieved in *Brassica* seed oil with antisense expression of Δ9DES [50]. Yet, there also is at least one report that a FAT A class acyl-ACP thioesterase from mangosteen (*Garcinia mangostana*) shows relatively high substrate preference for 18:0-ACP [51]. When expressed in transgenic canola, the gene encoding this enzyme enabled production of 22% 18:0 in seed oil.

4 COMBINING ALLELES TO IMPROVE THE UTILITY OF SOYBEAN OIL IN SPECIFIC MARKETS

4.1 Current Oilseed Industry Issues

The most pressing problems U.S. oilseed processors and food manufacturers acknowledge today are the need for a soybean oil with lower saturated fat content, the need to produce food products with lower levels of trans isomers of unsaturated fatty acids, and the need to develop these traits in nontransgenic commercial cultivars. These issues affect about 96% of the domestic market for soybean oil that includes cooking and liquid oil applications (about 44% of domestic soybean oil consumption) and shortening and frying fats (about 52% of domestic soybean oil usage). Both of these product areas are subject to various U.S. Food and Drug Administration (FDA) guidelines related to the health claims that may be printed on food product labels. To qualify for a low-saturated-fat food, manufacturers must ensure their products contain less than 7% total saturated fatty acids. At this time, soybean oil contains about 15% total saturated fat. This is one reason why soybean oil is losing significant market share to canola and sunflower oils. In addition, the FDA recently announced that the amount of trans isomers (a product of hydrogenation) must not only be listed on food product labels, but also may be included in the estimate of total saturated fat. This brings the dilemma into full view. The industry must balance the need to meet consumer demand for low-saturated-fat foods, with the need for functional oils with high oxidative stability to maintain product characteristics and quality. The science of product formulation may alleviate this problem in the short term, but the only long-term solution is to use vegetable oils with a more favorable composition. Thus, to remain competitive in specific markets, fundamental genetic changes are required in soybean oil composition. Yet, the manner in which genetic change is achieved will be critical to consumer acceptance and commercial success. Public views of transgenically modified crops are well known and have crippled the U.S. soybean export market. Hence, the industry must give their customers what they want. This means that genetically modified soybean oils should be accomplished with natural gene mutations to be economically viable in commercial use. The genetic resources documented earlier represent a positive step toward resolving all three of the main current industry issues. A new type of soybean oil may have to be created for each of the major domestic markets to ensure greatest impact. These proposed changes in soybean oil composition are shown in Table 3. The

Table 3 Target Compositions for Genetically Modified Soybean Oil

		Products[a]		
Fatty acid	Normal oil[a]	Cooking oil	Margarine	Paints
Saturated	15	7	42	11
Oleic	23	60	19	12
Linoleic	53	31	37	55
Linolenic	9	2	2	22

Note: All targets may be achieved by natural gene selection and are not genetically modified oils.
[a] Data given as percent crude soybean oil.

first priority for variety development will be a soybean oil with less than 7% total saturated fat, 50–55% 18:1, plus less than 3% 18:3. Research will continue toward the other two goals, but it is most likely that advances toward those objectives will be held in reserve until such time as the industry calls for them. Nevertheless, the following subsections give a prelude to the future and provide examples of how various genes (alleles) may be combined to accomplish a given target oil composition.

4.2 Combination of Genetic Traits for Improved General-Purpose Applications of Soybean Oil

Research at Pioneer Hi-Bred International has demonstrated that various combinations of natural alleles may be used to create a wide range of phenotypic variations of soybean oil [52]. These experimental phenotypes include low 16:0 plus low 18:3, low 16:0 plus high 18:3, high 16:0 plus low 18:3, high 16:0 plus high 18:3, or high 16:0 plus high 18:0. At this time, it appears that the low-16:0 plus low-18:3 phenotype will be acceptable to the oilseed industry, as a means to strengthen the position of soybean oil in domestic markets. Since 1990, the United Soybean Board (a U.S. farmer-directed organization that administers the National Soybean CheckOff program, under the oversight of the U.S. Secretary of Agriculture) has invested about US$7 million in public research to breed soybeans with oils having lower saturated fat content plus improved oxidative stability. This work is coordinated by USDA–ARS scientists at Raleigh, NC, and involves nine breeding programs in the states of Minnesota, Maryland, Indiana, Virginia, Missouri, Tennessee, North Carolina, South Carolina, and Georgia. Within the next 3 years, this collaborative group will release a number of agronomic low-16:0 plus low-18:3 soybean varieties that are adapted to respective areas of the entire U.S. soybean production region (Maturity Groups I through VIII). The first of these new varieties has been released from the program in North Carolina (Table 4). The public release, N93-132, is a high-yielding low-18:3 variety and has been given the commercial name Soyola. N97-3524 is a high yielding low-16:0 plus low-18:3 variety with the $fap_1 fap_1 fap_{nc} fap_{nc}$ *FadFad fanfan* genotype. This variety will be named Satellite.

Work already is under way to develop the next improvement on this theme, the replacement of *FadFad* alleles with *fadfad* alleles in varieties like Satellite. The initial source of the recessive *fad* alleles will be the germplasm line N97-3363-4, which exhibits higher 18:1 concentration. This germplasm is unique in several ways. It represents the only known mid-18:1 soybean in the public sector, and the trait is more stable to environmental factors (such as growth temperature) than earlier versions of relatively high-

Table 4 New Soybean Varieties with Genetically Modified Oil Composition

Line	Phenotype	16:0	18:0	18:1	18:2	18:3	Yield[a] (bushels/acre)
Brim	Normal	11	4	23	53	9	47
N93-132	Low 18:3	11	4	29	52	4	48
N97-3525	Low (16:0 + 18:3)	3	3	37	54	3	51
N97-3363-4	Mid-18:1	8	4	60	26	2	ND
$LSD_{0.05}$		4	0	15	13	3	2

Note: Compositions are given as percent crude oil.
[a] Yielding ability: 3-year average over four locations in North Carolina: Maturity Group VI; N97-3363-4 is a germplasm line; yielding ability ND, not determined.

18:1 germplasm. As an example, the composition of N97-3363-4 seed oil ranged from 58% to 64% 18:1, when plants were grown under controlled environments (daily mean temperatures from 19°C to 28°C) continuously throughout the reproductive growth stage. Under the same conditions, seed oil from N78-2245, the first relatively high-18:1 soybean germplasm, exhibited about 22% 18:1 in the lowest-temperature treatment and 65% 18:1 in the highest-temperature treatment (Wilson, unpublished). However, N97-3363-4 is not impervious to genotype × environmental variation affecting 18:1 concentration. In the summer of 1999, N97-3363-4 was grown under field conditions in Mexico. The oil from these soybeans contained almost 78% 18:1 (Fig. 1).

4.3 Combination of Genetic Traits for Low-trans Isomer Margarine

Although soybean oil with higher saturated fat content is not a high priority for the U.S. oilseed industry at this time, such oil may become an important ingredient of trans-free

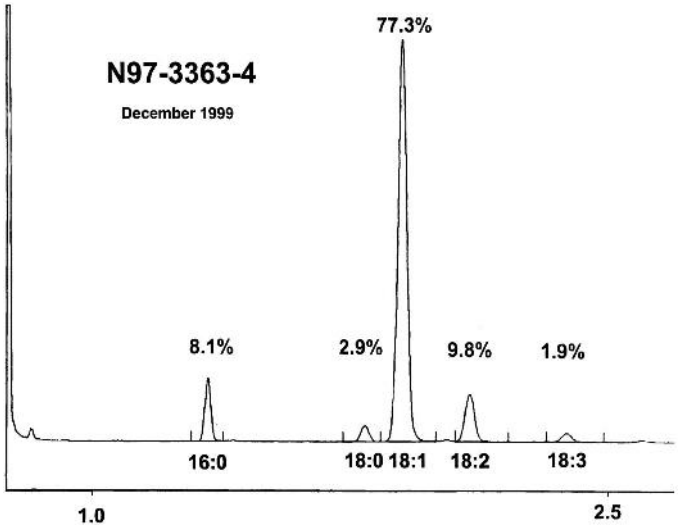


Figure 1 Effect of high growth temperature on 18:1 concentration in N97-3363-4. Gas chromatogram of fatty acid methyl esters from oil samples of N97-3363-4 seed grown in Mexico during the summer of 1999.

margarine in the future. Several studies have shown that experimental high-16:0 plus high-18:0 soybean oils have good potential for margarine production [53]. However, a great deal of work remains to correct deficiencies for the yielding ability in high-saturated fatty acid variety and to improve the frequency of high-16:0 plus high-18:0 progeny in populations segregating for *fap* and *fas* alleles. This latter problem may be a function of the enzyme activities these alleles govern. A population (FAM94-41 × C1727) was created to test this theory [39]. C1727 is distinguished by a high-16:0 phenotype that is determined by a homozygous recessive mutation (fap_2) at gene loci designated as *Fap* [20]. The fap_2 allele affects greater 16:0-ACP TE activity, possibly due to reduced KAS-II activity [31]. It then may be reasoned that if fas_{nc} encodes greater KAS-II or reduced 16:0-ACP TE activity, a population segregating for fas_{nc} and fap_2 should produce no progeny with 16:0 plus 18:0 concentration greater than the original parents. Assuming the complementation of opposing genetic effects on KAS-II or 16:0-ACP TE activity, the variation in total saturated fatty acid (TS) concentration among segregants should fit an epistatic gene action model for overdominance toward a normal saturated fatty acid composition for soybean oil. Conversely, if fas_{nc} mediates reduced Δ9DES or perhaps reduced 18:1-ACP TE activity, a small proportion of the progeny from this population should represent transgressive segregants with a high-16:0 plus high-18:0 phenotype. Following this premise, some progeny may emulate, as an example, the combination of greater 16:0-ACP TE activity plus reduced Δ9DES.

Initial information on the combined inheritance of fas_{nc} and fap_2 showed no maternal and cytoplasmic effects on 18:0 and 16:0 levels in soybean. The frequency distribution for 18:0 concentration among F_2 progeny segregating for fas_{nc} and fap_2 (ranging from 3% to 10.5% 18:0) was within the parental range. A chi-square analysis of these data supported a 1:2:1 progeny ratio for a single fas_{nc} gene model. In addition, the frequency distribution for 16:0 concentration in this population (ranging from 10% to 17.9% 16:0) also fell between the parental values and fit a 1:2:1 progeny ratio for a single fap_2 gene model. Hence, fas_{nc} and fap_2 represented independent genes at different gene loci.

Genetic effects of the segregation of fas_{nc} and fap_2 on TS concentration ranged from 13.8% to 23.2% TS. However, the pattern of variation in TS concentration demonstrated that transgressive segregants occurred above the high parent value and below the low parent value in this population. This type of phenotypic variation was consistent with the combination of independently inherited genes. Furthermore, these data did not fit an epistatic model for overdominance (where TS levels in all progeny segregate 15:1 for lower TS than the low parent value and produce a normal TS phenotype for soybean oil). Rather, 81% of the progeny exhibited a TS concentration that was significantly greater than the standard cultivar (Dare), whereas only 19% of the progeny have a TS concentration that was equal to or greater than the calculated mid-parent values. Chi-square analyses showed that these data fit a 9:3:3:1 ratio for a two-gene model. Thus, recombinants of fas_{nc} and fap_2 may be represented by the following phenotypic classes: $Fap_2\_Fas_{nc}\_$, $Fap_2\_fas_{nc}$ fas_{nc}, fap_2fap_2 $Fas_{nc}Fas_{nc}$, and $fap_2fap_2fas_{nc}fas_{nc}$. Based on this interpretation, the elevated TS phenotype in FAM94-41 and A6 is governed by two different genes, and it is unlikely that fas_{nc} encodes greater KAS-II or reduced 16:0-ACP TE activity.

Although additional evidence is needed to postulate that fas_{nc} mediated reduced Δ9DES or perhaps reduced 18:1-ACP TE activity, the possibility that major genetic effects of fas_{nc} and fap_2 alleles were complementary and might produce a normal phenotype could be discounted. Genetic regulation of 16:0 and 18:0 accumulation in soybeans segregating for these alleles was competitive in nature. In other words, full expression of altered

activities of the enzymes encoded by fas_{nc} and fap_2 was interdependent on the level of substrate produced by each enzyme. As an example, the relation between 16:0 and 18:0 concentration in progeny from FAM94-41 × C1727 reveals a wide crescent-shaped pattern (Fig. 2). Generally, progeny segregating for fap_2 occurred within a broad range for 16:0 (10–18%) concentration, but were confined to a narrow range of 18:0 (3–4.8%) concentration. Conversely, a majority of progeny that segregated for fas_{nc} occurred in a narrow range of 16:0 (10–13%) concentration. As a result, transgressive segregates for TS in this population rarely achieved or exceeded the combined parental phenotype (18% 16:0 plus 9% 18:0). Most of these progeny exhibited either very high-16:0 plus moderately high 18:0 concentration, the converse composition, or an intermediate

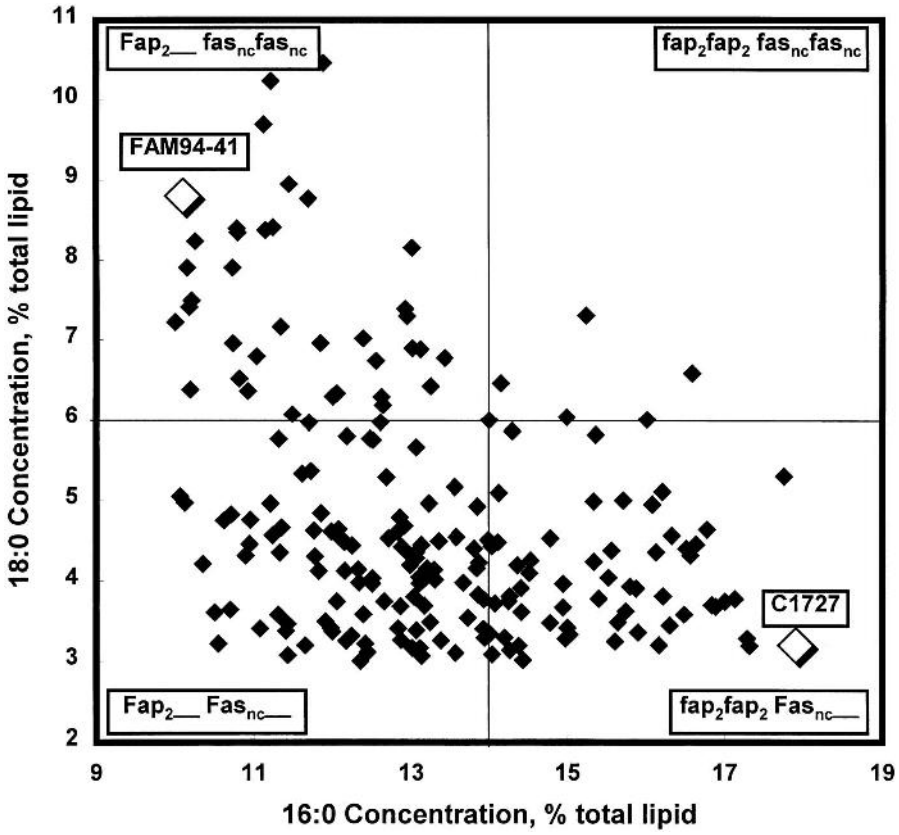


Figure 2 Relation of 18:0 and 16:0 concentration in oil of mature seed from an F_2 population of FAM94-41 × C1727. Parental lines were the high-18:0 line FAM94-41 ($fas_{nc}fas_{nc}$) and the high-16:0 line C1727 (fap_2fap_2). Independently, the fas_{nc} allele segregated 3:1 for low-18:0 concentration, and the fap_2 allele segregated 3:1 for low-16:0 concentration. Quadrants representing the respective genotypes were formed about the mid-parent values for 16:0 and 18:0 concentration. About 62% of the progeny segregating for fap_2 occurred in a narrow range between 3% and 4.5% 18:0. About 68% of the progeny segregating for fap_{nc} occurred in a range between 10% and 13% 16:0. Only 1% of these progeny exhibited the highest level of total saturated fatty acid concentration in the population (16.6% 16:0 plus 6.6% 18:0, and 15.2% 16:0 plus 7.3% 18:0). (Adapted from Ref. 39.)

combination of high-16:0 and high-18:0 concentration. Nevertheless, those individuals with higher than normal 18:0 plus higher than normal 16:0 concentration demonstrated that fas_{nc} and fap_2 may be combined to effect increased TS. Albeit mutations in these alleles probably target adjacent enzymes in the fatty acid synthetic pathway, other mutations may be found in the future that enable the full potential of fas_{nc} or fap_2 recombinants to elevate TS levels in soybean oil.

4.4 Combination of Genetic Traits for Improved Industrial Applications of Soybean Oil

Although industrial applications, such as inks, resins, and coatings only account for about 4% of the domestic market for soybean oil, representatives of this industry could make greater use of a more reactive soybean product. Perhaps the most direct approach toward increasing polyunsaturated fatty acid levels for that purpose would be the introduction of alternative desaturase genes from wild soybean through interspecific hybridization with *Glycine max* varieties. Significant gains in 18:3 concentration have been observed with the addition of the putative Fad_2, Fad_3, Fan_2, and Fan_3 alleles to cultivated soybeans. However, even greater polyunsaturate levels might be achieved if the *G. max* parent exhibits a fap_1fap_1 $fap_{nc}fap_{nc}$ genotype for lower 16:0. The 16:0 is often regarded as a waste material in the manufacture of industrial products. Therefore, without loss of oil content, the low-16:0 trait not only would further enhance total polyunsaturate levels but also provide an economic advantage. The segregating population shown in Figure 3 gives an idea of how easily this concept may be applied. In this case, the mean of the population is shifted significantly toward higher polyunsaturates. Thus, it would appear that inbred lines could be selected with greater than 85% 18:2 plus 18:3. Current soybean oil typically contains less than 65% total polyunsaturates.

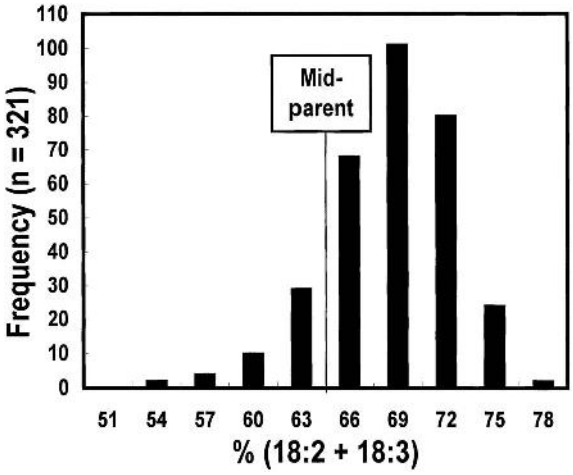


Figure 3 Frequency distribution of polyunsaturated fatty acid concentration in oil of mature seed from an F_2 population of N94-2575 × PI424031. Parental lines were the very low-16:0 *Glycine max* line N94-2575 (fap_1fap_1 $fap_{nc}fap_{nc}$ *FadFad FanFan*) and the high-18:3 *Glycine soja* line PI424031 (Fap_1Fap_1 $Fap_{nc}Fap_{nc}$ Fad_3Fad_3 Fan_3Fan_3). In addition to the alternative desaturase genes from wild soybean, the low-16:0 trait further increased the frequency of very high-polyunsaturated phenotypes in cultivated soybean oil.

5 OUTLOOK

We must always look forward and anticipate the challenges that lay ahead. Each step upward on the learning curve improves our vision. We know that the resources to solve current and foreseeable problems are at hand and await deployment; but what of the future? An obvious hurdle will be public perception of the use of transgenic plants in commercial foods and feeds. The solution to this dilemma most likely will come as a drastic change in biotechnological methods. As an example, the concept of creating nontransgenic mutations that affect the level of a gene product or creating discrete nontransgenic mutations at specific sites in specific genes to directly alter the activity of a key metabolic enzyme is an emerging field of science. These technologies could supplant transgenic technology and alleviate dependence on chance discovery of desired genetic events. Native gene surgery represents another juncture in the technological spiral. This technology has the potential to be a unifying force for breeders, biochemists, and molecular geneticists as the circle turns full and the science of genetic modification of soybean oil composition continues.

ACKNOWLEDGMENTS

The authors wish to thank Mr. William P. Novitzky and Mr. David Haak for invaluable technical expertise and dedication to the advancement of knowledge in the area of genetic modification of soybean oil. The authors also are grateful for the financial assistance received from the United Soybean Board that enabled this work under project number 8205.

REFERENCES

1. RW Howell, CA Brim, RW Rinne. J Am Oil Chem Soc 49:30–32, 1972.
2. RF Wilson, RW Rinne, CA Brim. J Am Oil Chem Soc 53:595–597, 1976.
3. RF Wilson, JW Burton, CA Brim. Crop Sci 21:788–791, 1981.
4. JW Burton, RF Wilson, CA Brim, RW Rinne. Crop Sci 29:1583, 1989.
5. JR Wilcox, JR Cavins, NC Nielsen. J Am Oil Chem Soc 61:97–100, 1984.
6. JR Wilcox, JR Cavins. J Hered 78:410, 1987.
7. SE Hawkins, WR Fehr, EG Hammond, SR de Cianzio. Crop Sci 23:897–899, 1983.
8. WR Fehr, EG Hammond. US Patent 5986118, 1999.
9. BD Rennie, J Zilba, MM Cramber, WD Beversdorf. Crop Sci 28:655–657, 1988.
10. BD Rennie, JW Tanner. Crop Sci 29:1081–1084, 1989.
11. RF Wilson, JW Burton. In: AR Baldwin, ed. Proceedings of the World Conference on Emerging Technologies in the Fats and Oils Industry. Champaign, IL: American Oil Chemists Society, 1986, pp 386–391.
12. RF Wilson. In: JR Wilcox, ed. Soybeans: Improvement, Production and Uses. 2nd ed. Madison, WI: American Society of Agronomy 1987, pp 643–686.
13. RF Wilson, JW Burton, P Kwanyuen. In: D Erickson, ed. Proceedings of the World Conference Edible Oils and Fats Processing. Champaign, IL: American Oil Chemists Society 1990, pp 355–359.
14. JR Byrum, AJ Kinney, KL Stecca, DJ Grace, BW Diers. Theor Appl Genet 94:356–359, 1997.
15. WD Hitz, N Yadav, RS Reiter, CJ Mauvais, AJ Kinney. In: P Mazliak, ed. Plant Lipid Metabolism. Amsterdam: Academic Press, 1994, pp 506–508.
16. VR Pantalone, GJ Rebetzke, JW Burton, RF Wilson. J Am Oil Chem Soc 74:159–163, 1997.
17. USDA, ARS, National Genetics Resources Program. Germplasm resources information network online database, National Germplasm Resources Laboratory, Beltsville MD, 1999. Available at www.ars-grin.gov/cgi-bin/npgs/html.

18. AJ Kinney. Curr Opin Biotech 5:144–151, 1994.
19. RF Wilson. Lipid Technol 11:107–110, 1999.
20. EA Erickson, JR Wilcox, JF Cavins. J Hered 79:465–468, 1988.
21. SR Schnebly, WR Fehr, GA Welke, EG Hammond, DN Duvick. Crop Sci 34:829–833, 1994.
22. WR Fehr, EG Hammond. US Patent 5750846, 1998.
23. J Ohlrogge, J Browse. Plant Cell 7:957–970, 1995.
24. GJ Rebetzke, JW Burton, TE Carter, Jr, RF Wilson. Crop Sci 38:303–308, 1998.
25. RF Wilson, P Kwanyuen, JW Burton. In: TH Applewhite, ed. Proceedings of the World Conference on Biotechnology for the Fats and Oils Industry, Champaign, IL: American Oil Chemists Society, 1988, pp 290–293.
26. RF Wilson, TC Marquardt, WP Novitzky, JW Burton, JR Wilcox, RE Dewey. J Am Oil Chem Soc 78:329–334, 2001.
27. RM Pirtle, DW Yoder, TT Huynh, M Nampaisasuk, IL Pirtle, KD Chapman. Plant Cell Physiol 40:155–163, 1999.
28. E Martinez-Force, R Alvarez-Ortega, R Graces. Planta 207:533–538, 1999.
29. A Jones, H Maelor Davies, TA Voelker. Plant Cell 7:359–371, 1995.
30. JM Leonard, SJ Knapp, MB Slabaugh. Plant J 13:621–628, 1998.
31. RF Wilson, TC Marquardt, WP Novitzky, JW Burton, JR Wilcox, AJ Kinney, RE Dewey. J Am Oil Chem Soc 78:335–340, 2001.
32. JW Burton, RF Wilson, CA Brim. Crop Sci 34:313, 1994.
33. D Stojsin, GR Ablett, BM Luzzi, JW Tanner. Crop Sci 38:1437–1441, 1998.
34. TF Horejsi, WR Fehr, GA Welke, DN Duvick, EG Hammond, SR Cianzio. Crop Sci 34:331–334, 1994.
35. JR Wilcox, JW Burton, GJ Rebetzke, RF Wilson. Crop Sci 34:1248–1250, 1994.
36. JW Burton, JR Wilcox, RF Wilson, WP Novitzky, GJ Rebetzke. Crop Sci 38:1407, 1998.
37. P Dormann, M Frentzen, JB Ohlrogge. Plant Physiol 104:839–844, 1994.
38. RF Wilson, HH Weissinger, JA Buck, GD Faulkner. Plant Physiol 66:545–549, 1980.
39. VR Pantalone, RF Wilson, WP Novitzky, JW Burton. J Am Oil Chem Soc. In press.
40. EG Hammond, WR Fehr. Crop Sci 23:192–193, 1983.
41. GL Graef, WR Fehr, EG Hammond. Crop Sci 25:1076–1079, 1985.
42. SM Rahman, Y Takagi, T Kinoshita. Theor Appl Genet 95:772–776, 1997.
43. DM Bubeck, WR Fehr, EG Hammond. Crop Sci 29:652–656, 1989.
44. JF Miller, BA Vick. Crop Sci 39:364–367, 1999.
45. J Lightner, E Lark, D James, J Browse. Theor Appl Genet 94:975–981, 1997.
46. PO Lundeen, WR Fehr, EG Hammond, SR Cianzio. Crop Sci 27:1102–1105, 1987.
47. RB Hartmann, WR Fehr, GA Welke, EG Hammond, DN Duvick, SR Cianzio. Crop Sci 37: 124–127, 1997.
48. JW Burton, TE Carter, Jr, EB Huie. Crop Sci 34:301, 1994.
49. J Lightner, J Wu, J Browse. Plant Physiol 106:1443–1451, 1994.
50. DS Knutzon, GA Thompson, SE Radke, WB Johnson, VC Knauf, JC Kridl. Proc Natl Acad Sci USA 89:2624–2628, 1992.
51. J Hawkins, JC Kridl. Plant J 13:743–752, 1998.
52. J Reske, J Siebrecht, J Hazebroek. J Am Oil Chem Soc 74:989–998, 1997.
53. GR List, TL Mounts, F Orthoefer, WE Neff. J Am Oil Chem Soc 73:729–732, 1996.

6

Strategies for the Development of NuSun Sunflower Hybrids

BRADY A. VICK and JERRY F. MILLER

Northern Crop Science Laboratory, Agricultural Research Service, U.S. Department of Agriculture, Fargo, North Dakota

1 INTRODUCTION

Radiant and dazzling, the common sunflower has pleased and fascinated mankind for centuries. From the early Hopi Indians who adorned their hair during ceremonies with showy wild blossoms, to the Spanish explorers who introduced the colorful North American flower into European botanical gardens, to the contemporary family with a splashy yellow bouquet on the kitchen table, all are bonded across time and cultures in their passion for the ''flower that turns toward the sun.'' The sunflower continues to captivate the admiration of artists and gardeners, poets and photographers, the old and the young, for its extraordinary beauty and appeal. However, beyond its pure visual delight, there has also existed, for millennia, a life-sustaining role of the sunflower as a healthful, nourishing food source for humankind.

The story of the sunflower and its passage from a simple wild prairie flower to a major oilseed commodity on the world market is marked by several milestones of human ingenuity. For this discussion, we will note four significant events that enabled this transition. A fifth event, which is the subject of this chapter, is currently in progress. The first of these landmarks was the domestication and cultivation of the wild sunflower by native North American tribes. Second, the introduction of the sunflower to Europe and the subsequent recognition, primarily in Russia, that the seed was a good source of quality cooking oil not prohibited under church laws led to wide acceptance by the populace of eastern Europe. A third turning point, occurring in the twentieth century, was the doubling of the

seed oil concentration in sunflower varieties developed by Russian plant breeders, which made large-scale cultivation of the crop economically practical. The fourth historic achievement in the development of the cultivated sunflower crop was the discovery of cytoplasmic male sterility in the sunflower and the resultant utilization of this trait to produce a hybrid sunflower with enhanced yields.

The North American sunflower industry is presently engaged in a fifth venture that promises to be of no less significance than earlier milestones. In response to requests from users of sunflower oil in the food industry, commercial hybrid sunflowers are now being bred to produce a precise composition of fatty acids that has optimal frying performance. In this chapter, we describe the genetic strategy used to produce a new type of commercial sunflower oil, NuSun™, which is distinguished by the moderately high level of oleic acid (55 to 75%) in its triacylglycerols.

2 LEGACY OF THE SUNFLOWER

2.1 Sunflower Among Native North American Tribes

Heiser [1] suggests that the annual sunflower (*Helianthus annuus* L.) was domesticated to produce a large seed by Native Americans in the central United States, even though native tribes in the southwestern United States are generally credited with the earliest development of refined agricultural practices in North America. Archeological evidence of the domesticated sunflower in the southwest United States is lacking, and tribes in this region likely gathered the seed of wild sunflowers for food. Carbon-14 dating evidence indicates that Native Americans were using the sunflower at sites in the Mississippi–Missouri basin nearly 3000 years ago [2].

As time passed, the geographic range of the cultivated sunflower broadened, spanning the breadth of the Great Plains from Texas to the Dakotas, and eastward to Ontario and the east coast of the United States. Apparently, only some tribes learned to cultivate the sunflower, whereas others relied on gathering wild sunflowers. By the time European explorers arrived in North America, the domesticated sunflower was cultivated by Native Americans in widely scattered locations across the continent. Accounts from early explorers and descriptions by sixteenth-century herbalists reveal that Native Americans had developed a tall (>2 m) sunflower with a single large head [1].

For some tribes, the colors extracted from the plant may have been of more importance than as a food. The Hopi tribe in the southwestern United States used the purple achenes of some varieties to produce purple dyes used in coloring baskets and the ray petals were used to produce a yellow dye. They also made a cooked wafer made from the crushed seed. The Mandan tribe of North Dakota parched the seeds in a clay pot, then pounded the seeds into a meal which was combined with squash, beans, and corn to produce a dish called ''four-vegetables-mixed.'' Iroquois tribe members pounded the seeds, heated them, and then put them in boiling water until the oil separated. The skimmed oil was used as a food flavoring, for hair care, and as a base for pigments painted on the body [3,4].

2.2 Introduction of the Sunflower to Europe

The sunflower was probably first brought to Europe from the southern United States or Mexico by Spanish explorers, perhaps as early as 1510, and later by English and French explorers. The first published description of the sunflower appeared in 1568. From Spain,

the sunflower was introduced into Italy and France, and by the end of the sixteenth century, it was grown as an ornamental plant in gardens of most of the western European countries. Although it spread rapidly throughout Europe, the sunflower did not have spectacular success until it reached Russia, where it was probably introduced by Peter the Great after a trip to western Europe in 1697. In Russia, the sunflower was quickly adopted as a source of oil, largely because of religious laws that prevented the consumption of foods rich in oil during the days preceding Christmas and during the season of Lent preceding Easter. Because the sunflower had only recently been introduced into Russia, it was not on the list of forbidden foods and could be consumed without infringement of religious law. As a result, the popularity of the sunflower soared.

By the 1850s, dozens of sunflower crushing mills were operating in central European Russia. Many of the peasants from that region later migrated to the Ukraine and southern Russia, bringing their seeds with them. At this time, the oil content of the seed was about 25%, but breeding efforts beginning in the 1920s at institutes in Russia resulted in significant increases in oil percentage. The most successful of these programs was developed by Pustovoit at Krasnodar near the Black Sea. By 1965, Pustovoit was testing lines with more than 50% oil while maintaining or increasing total yield [3].

2.3 Sunflower Returns to North America

North American farmers began trying to grow sunflowers imported from Russia by the early 1900s. Until the 1940s, much of the sunflowers grown in North America was probably derived from seed that Mennonite settlers brought to the Prairie Provinces of Canada when they emigrated from Russia in the late 1800s. Most of the sunflowers grown during this period was used for silage rather than for the oil. However, two advances eventually led to the establishment of the sunflower as a major oilseed crop in North America. The first advance was the introduction in 1964 into Canada of the cultivar Peredovik, a high-oil sunflower developed by Pustovoit. This increased the oil percentage of cultivated sunflowers in North America from about 33% to approximately 44%. The second advance was the discovery of cytoplasmic male sterility by Leclercq in France in 1968 [5] and the identification of fertility restoration genes by Kinman in the United States in 1970 [6]. With these discoveries, a convenient system for the production of hybrid seed was possible, and the heterotic effects of hybrid seed significantly increased the yield of the sunflower. The first hybrids became available for commercial production in the United States in 1972, and, today, virtually all production of sunflowers in the United States uses hybrid seed.

3 TRADITIONAL SUNFLOWER OIL

Sunflower oil traditionally has been considered a highly polyunsaturated oil because of the substantial content of linoleic acid in seed produced in the major northern production areas. However, the amount of linoleic acid present in the oil is very sensitive to environmental conditions. In the northern hemisphere, seeds produced in cool northern climates, such as in North Dakota, normally contain 68% or more linoleic acid, whereas seeds produced in southern latitudes, such asin Texas, may have as low as 25% linoleic acid [7]. Sunflowers grown in Kansas usually contain about 60% linoleic acid. Even within a region, the actual weather conditions during seed development will increase or decrease the linoleic acid percentage.

A typical bottle of sunflower oil purchased from the supermarket shelf in the United States contains four principal fatty acid components in the oil triacylglycerols. The major saturated fatty acids consist of approximately 6% palmitic acid (16:0) and 5% stearic acid (18:0), whereas oleic acid (18:1) varies from 14% to 19% and linoleic acid (18:2) ranges from 67% to 72%. Other minor fatty acids include <0.3% linolenic acid (18:3), <0.3% arachidic acid (20:0), <0.3% gondoic acid (20:1), <1% behenic acid (22:0), and <0.3% nervonic acid (24:0). Traditional sunflower oil is characterized by a light color and a bland taste, two important qualities preferred by consumers. The highly polyunsaturated nature of sunflower oil has made it an attractive product for health conscious consumers who desire a diet with a moderate intake of fat possessing a high polyunsaturate/saturate ratio, which is considered beneficial in reducing the risk of cardiovascular disease [8–10].

4 INDUSTRY INITIATIVE TO REDESIGN SUNFLOWER OIL

Although traditional high-linoleic sunflower oil was viewed for decades as a healthful vegetable oil with desirable flavor, several factors emerged in the United States during the 1990s to prompt a reconsideration of the most desirable fatty acid composition for sunflower oil. Gupta [11] has reviewed the issues which led to discussions among the snack food industry, oilseed processors, seed companies, USDA Agricultural Research Service researchers, and the National Sunflower Association concerning the feasibility and the economic viability of transforming the U.S. oilseed sunflower industry from a high-linoleic to a mid-oleic sunflower oil.

One factor in the decision to explore an alternative composition for sunflower oil was strong competition with several other vegetable oils, including both the bottled oil on the U.S. supermarket shelf and bulk oil for frying by the snack food, restaurant, and food service industries. Although sunflower oil is perceived by most U.S. consumers to be a healthful oil, similar wholesome images are evoked by some competing vegetable oils. Most northern Europeans were introduced to sunflower oil as a cooking and salad oil during the 1960s, when the Soviet Union exported large amounts of inexpensive sunflower oil to this area as a source of hard currency. Many consumers in Europe appreciated the quality of the sunflower oil for its high polyunsaturation and bland taste and were willing to pay a premium price above the cost of many other vegetable oils. South American consumers also developed a preference for sunflower oil, and as a result, sunflower oil commanded a higher price on the world market.

This favorable situation changed in the late 1980s when Europe introduced lucrative production subsidy programs for oilseed production, including sunflower. The Europeans no longer depended on sunflower imports and actually began exporting sunflower oil. Argentina dramatically increased its sunflower production, resulting in additional surpluses. In the meantime, structural changes were occurring in several major importing countries, such as Mexico and Egypt. These countries shifted from a central governmental buying agency for food crops to an open-market system. The buying agencies had been highly subsidized and their large purchases of certain types of vegetable oil supported price premiums for sunflower oil. In contrast, the open-market system, combined with accelerated production in Europe and Argentina, contributed to lower world sunflower prices.

During the 1970s, the fledgling U.S. sunflower industry emerged with high expectations that U.S. consumers also would embrace sunflower oil with the same passion as

their European and South American counterparts. However, this did not happen. Other highly polyunsaturated oils, such as corn oil, were already well established on the North American supermarket shelf. Soybean oil was widely accepted by the U.S. food industry despite some inherent quality problems. The U.S. industry learned how to use the product because it was readily available and cost less. Later, as canola production surged in Canada, this oil also became a major competitor to sunflower oil. The low saturated fatty acid content of canola oil (6% compared to 12% in sunflower oil) was exploited by the canola industry in advertising campaigns that were successful in attracting a significant share of the vegetable oil market. Thus, despite the premium price commanded by sunflower oil on the international market, bottled sunflower oil on the U.S. supermarket shelf was forced to compete vigorously with other oils and did not attract a higher price. For industrial frying, at least two factors worked against the use of sunflower oil. One drawback was the higher market price normally commanded by sunflower oil and the other was the reduced frying stability due to high-linoleic-acid content and the decreased shelf life of fried food because of low content of γ-tocopherol antioxidants. Although sunflower oil has a high content of α-tocopherol (vitamin E), it is lower than soybean oil in γ-tocopherol content, which imparts greater oxidative stability to the oil than α-tocopherol [12]. Because most frying oils are hydrogenated for industrial frying uses, the food industry preferred the least expensive and most available oil for hydrogenation, and that was soybean oil.

Because of aggressive competition with other U.S. vegetable oils in the domestic market, the sunflower industry came to rely heavily on unpredictable export markets. Curiously, whereas about 80% of sunflower oil was exported because of insufficient domestic demand, U.S. sunflower processors concurrently imported other oilseeds, such as canola and flaxseed that were destined largely for the domestic market, to satisfy the capacity of the crushing plants. Clearly, there was a need to increase domestic demand for sunflower oil to keep the sunflower processing plants at a high capacity and reduce the amount of imported oilseeds.

Another element which prompted the initiative to redesign sunflower oil was a new perception of the effects of monounsaturated fatty acids on cardiovascular disease. In 1988, the American Heart Association's Dietary Guidelines for Healthy American Adults was revised. Previous guidelines recommended limitation of total fat intake to 30 energy %, with 10% derived from saturated fatty acids, 10% from monounsaturated fatty acids, and 10% from polyunsaturated fatty acids. The new guidelines retained the same 30 energy % maximum intake, but altered the recommendation in favor of an increase of up to 15% for monounsaturated fatty acids while limiting polyunsaturated fatty acids to no more than 10% and saturated fatty acids to less than 10% [13]. The impetus to increase the proportion of monounsaturated fatty acids in the diet was derived in part from demographic observations that populations which consume a Mediterranean-style diet, in which highly monounsaturated olive oil is the major vegetable oil, have a low incidence of heart and cardiovascular disease compared to those who consume the typical North American diet.

Some researchers believe that the favorable effect of monounsaturated fatty acids on risk reduction for cardiovascular heart disease is simply due to displacement of saturated fatty acids from the diet [14]. Other evidence suggests direct beneficial effects of monounsaturated fatty acids, such as their ability to reduce the plasma concentration of plasminogen activator inhibitor type-1, which can retard the breakdown of blood clots and increase the probability of stroke [15]. An analysis from the Nurses' Health Study that assessed dietary fat intake revealed that higher intakes of both monounsaturated and

polyunsaturated fatty acids were associated with reduced risk of cardiovascular heart disease [16]. Other research has suggested that diets high in oleic acid can reduce the plasma level of oxidized low-density lipoproteins, which are proinflammatory and promote atherogenesis [17]. In summary, an increase in dietary oleic acid is desirable because it has similar health advantages to increasing linoleic acid intake and is, possibly, more beneficial because of the decreased tendency for oxidation of low-density lipoproteins containing oleic acid as compared to linoleic acid.

Other considerations also entered into the proposal to increase the oleic acid content of sunflower oil. A longer shelf life and increased stability during frying were predicted benefits of an oil with a low level of polyunsaturated fatty acids, which are more susceptible to oxidation. Because there is negligible linolenic acid in sunflower oil, there is the major advantage of eliminating hydrogenation during processing. Hydrogenation results in the formation of trans fatty acids, which are associated with increased risk of cardiovascular heart disease. Thus, the prospect of improving the health benefits of sunflower oil, prolonging the shelf life, and enhancing frying properties by genetic manipulation of seed oil deposition, while avoiding chemical hydrogenation, was an attractive proposal. Consumer groups had been lobbying the U.S. Food and Drug Administration (FDA) to require labeling for trans fatty acids on foods, and the sunflower industry was aware that the FDA was considering a decision regarding this labeling. If labeling of trans fatty acids were to become a requirement in the United States, then food companies were expected to make every effort to eliminate trans fatty acids from their products. Mid-oleic sunflower oil is one of the few oil choices that does not require hydrogenation in most industrial frying applications and could produce a trans-free food product.

The idea of producing sunflower oil with an increased oleic acid content was not new. A high-oleic sunflower line was reported in 1976 by Soldatov [18] and was distributed to researchers around the world. In 1986, a patent [19] was awarded in the United States for the high-oleic trait ($>$80%), and the resulting exclusivity and high cost of the oil effectively precluded its widespread use in the snack and restaurant food industries. However, desirable frying properties were not restricted to the high-oleic ($>$80%) type of sunflower oil. Consumer taste panel evaluations showed that corn and potato chips fried in either high-oleic or mid-oleic sunflower oil scored equally well with chips fried in cottonseed oil, the industry standard. This result was not surprising because previous research by Warner [20] had shown that frying oils should have moderate levels of oleic acid and moderately low levels of linoleic acid to produce the best flavors. The requirement for a modest amount of linoleic acid can be explained by the fact that linoleic acid oxidizes more readily than oleic acid to produce important flavor and aroma compounds, and 25–30% was determined to be a desirable amount in a frying oil.

Therefore, under the leadership of the National Sunflower Association, a partnership of customers, processors, and public scientists, concluded that a sunflower oil with a target oleic acid content of 65% would be a high-quality product that could compete more effectively in the marketplace and not infringe on existing patents. This task force predicted that sunflower oil with 65% oleic acid would offer desirable frying and flavor characteristics, increase the lifetime of the heated oil, and confer a healthful fatty acid composition free of trans fatty acids. In addition, increasing the oleic acid composition would have the added benefit of slightly lowering the saturated fatty acid content. The only question remaining was whether, in fact, it was possible to produce a sunflower whose oil was intermediate in oleic acid composition. In the next section, we describe the genetic studies and tools used to accomplish the transformation of traditional sunflower oil to NuSun.

5 GENETICS OF OLEIC ACID CONTENT IN SUNFLOWER

5.1 Background

The development of a sunflower with a high oleic acid content was reported by Soldatov in 1976 [18]. Seed of the variety VNIIMK 8931 was treated with a 0.5% solution of dimethyl sulfate, a chemical mutagen. Seed was advanced to the M_3 generation and screened for differing levels of oleic acid. The individual seeds that were selected contained about 50% oleic acid. Using individual selection, evaluation of the progeny, and subsequent cross-pollination of the best plants, the oleic acid content of oil from the bulk of plants was increased to 67%. It was found that the high oleic content was stable and well maintained in further generations, indicated by a close relationship of plants with high oleic content and their progeny ($r = 0.67$). By bulking the superior plants with high oleic content, the Pervenets variety was created and released to producers in Russia by the VNIIMK research center [21]. The Pervenets variety had an oleic acid content of approximately 70–75%, whereas the oleic acid content of the original VNIIMK 8931 was 30–35%. In 1978, Russian scientists reported that they had created breeding lines with oleic contents as high as 89.5% [21].

Seed of the open-pollinated Pervenets (PI 483077) was planted in the 1982 spring greenhouse of the USDA–ARS Sunflower Research Unit at Fargo, ND, and plants were self-pollinated. Plants were harvested and the fatty acid composition of the self-pollinated seed was determined by capillary gas chromatography. Ten selections were made, each having an oleic acid content greater than 85%. Self-pollinated seed was planted in the field in 1982 and the oleic acid content was determined after harvest. Four of the selections had oleic acid contents exceeding 85%, and analysis of single seeds of these plants confirmed that they were homogeneous for oleic acid content. One of these plants, designated Pervenets 306, was utilized to determine the genetic control of high oleic acid content in sunflower.

5.2 Genetic Studies

Plants of the inbred line, HA 89, were hand-emasculated and pollinated with the Pervenets 306 selection [22]. HA 89 is a traditional high-linoleic-acid line with approximately 11% oleic acid. The F_1 crossed seeds were planted in the field in 1983 and were self-pollinated to produce F_2 seed. Single seeds were used in the fatty acid analysis of the F_2 populations and segregation ratios were tested by a chi-square goodness-of-fit and heterogeneity analysis.

Analysis of F_2 seed showed trimodal distributions for oleic acid content. An intermediate class was clearly evident, ranging from 48% to 72% in oleic content. The high-oleic class ranged from 82% to 92%, whereas the low oleic class was similar to HA 89 and ranged from 11% to 18%. The number of seeds in the intermediate class was too large to support a single, dominant gene theory. However, this study did confirm the presence of a major gene with partially dominant gene action, as reported by Fick [23] and Urie [24], and designated *Ol*. This gene produced seed with oleic composition levels of 60–75%. A second gene, designated *Ml*, appears to modify the oleic content. When in the recessive form, *mlml*, and combined with the gene *Ol*, oleic levels in seed were 82% or higher. HA 89 appeared to possess dominant alleles of the modifier gene. A theoretical genotype *Ol_Ml_*would produce intermediate oleic contents, whereas the *Ol_mlml* genotypes would produce high oleic contents.

The realization that lines and hybrids could be developed with the genotype *Ol_Ml_* stimulated interest by the sunflower industry in the mid-oleic (intermediate) level of oleic acid in sunflower seed oil. The existence of a single, partially dominant gene *Ol* ensured that hybrids could be bred to produce oil that is in the desired 55–70% range of oleic acid. The presence of a modifier gene in the dominant form prevents oleic acid from reaching the 85–90% level. In the time since these initial genetic studies were conducted, the combined experience of public and private sunflower researchers has led them to suspect that several modifier genes are present in lines of sunflower, each having an effect on the intermediate level of oleic acid in hybrids. However, with rapid testing of lines and their hybrids, the sunflower industry has now produced several excellent hybrids which have a high yield, high oil, desired agronomic and morphological traits, and desired disease resistance and possess the mid-oleic level of oleic acid.

5.3 Breeding Strategies to Produce Mid-Level Oleic Acid in Sunflower Hybrids

There are three basic breeding strategies, based on genetic inheritance factors, to produce hybrids with a mid-oleic level of oleic acid. At present, most industry companies are utilizing the first strategy, with a few companies applying the third strategy.

5.3.1 High × Low Crosses or Low × High Crosses

The crossing of a high-oleic female parent (*OlOlmlml*) with a low-oleic male parent (traditional linoleic) (*ololMlMl*) would produce hybrid seed with the genotype *OlolMlml*. Planting of seed with this genotype has produced a sunflower crop which generally averages 60–70% oleic acid. The crossing of a low-oleic female parent (*ololMlMl*) with a high-oleic male parent (*OlOlmlml*) would also produce hybrid seed with the genotype *OlolMlml*. However, planting of seed with this genotype has typically produced a sunflower crop that averages 50–65% oleic acid. This result is due to maternal influence, or the influence of the genotype of the maternal parent on the resulting oleic content of seed produced on that plant [22]. It appears that the high-oleic female parent continues to influence the oleic content of seed produced on that plant.

5.3.2 Mid-Oleic × Mid-Oleic Crosses

The crossing of a true-breeding mid-oleic female parent (*OlOlMlMl*) with a true-breeding mid-oleic male parent (*OlOlMlMl*) would produce a hybrid with the mid-oleic trait because the genotype of the hybrid would also be *OlOlMlMl*. Seed from this plant should be more stable than high × low or low × high crosses. However, the breeding effort appears much more involved and it is difficult to create these genotypes. The obstacle may lie in the number of modifier genes having their effect on the oleic content. Hybrids must be tested extensively to be sure specific modifier genes are the same in both genotypes, ensuring that the correct mid-oleic level will be produced by the hybrid. An industry breeder must have two programs, both male and female, for the selection of specific mid-oleic genotypes.

5.3.3 High-Oleic × High-Oleic Crosses with Differing Numbers of Modifier Genes

The crossing of a high-oleic female parent having 80–82% oleic acid and possessing a modifier gene with a slight effect on oleic content (*OlOlmlmlM1M1ml2ml2*) with a

high-oleic male parent possessing a different modifier gene, also with a slight effect on oleic content (*OlOlmlmlm1ml1Ml2Ml2*), would produce a genetically complex hybrid (*OlOlmlmlMl1ml1Ml2ml2*). Planting of seed with this genotype has produced a sunflower crop which generally averages 75% oleic acid. This result is due primarily to the small effect each different modifier gene has on the *Ol* gene effect itself, lowering the oleic content from the potentially high level. Several hybrids of this type were produced by industry and USDA–ARS when the female line HA 341 was used as a female parent in experimental hybrids.

6 THE TRANSITION

6.1 The Decision to Convert the Industry

The initial idea to redesign traditional sunflower oil to contain a mid-level oleic acid content was first suggested by representatives from the snack food and oil-processing industry. In early 1995, they approached the National Sunflower Association and proposed that an industry conversion to mid-oleic sunflower oil was worth exploring. During the 1995 summer meeting of the National Sunflower Association, they presented the recommendation to a broad spectrum of the sunflower industry. In their proposal, they advocated that a mid-oleic hybrid be produced on a commercial scale and that the seeds be grown, traded, and processed through traditional commercial channels. They reasoned that it was important to produce and distribute the new oil as a commodity, rather than as a specialty oil, to keep the price competitive with other oils. If the new oil were to become a specialty oil, and by definition a higher-priced oil, there would be little incentive for the snack food or restaurant industries to choose sunflower oil over established oils with proven customer acceptance. The companies would have to recover the increased costs through higher prices or suffer a reduced profit margin, neither of which was acceptable.

Under the leadership of the National Sunflower Association, a consensus was reached among the sunflower industry sectors to aggressively explore the potential for conversion of the entire oilseed sunflower commodity to a mid-oleic sunflower. This required a plan to produce a large enough quantity of the new oil for research and testing in order to assure that products fried in mid-oleic sunflower oil would have high consumer acceptability. A committee was formed with representatives from industrial oil users, oil refiners, crushers, seed company breeders, and growers (see also Sec. 7). During the summer and fall of 1996, an oilseed company contributed mid-oleic hybrid seed, several growers grew the crop, an oilseed processor bought the seed and extracted the oil, the National Sunflower Association paid to have the oil refined, and a large snack food company used the oil to prepare potato chips in its large-scale pilot plant. The snack food company consumer-tested the product and reported the results to the National Sunflower Association. Their evaluations showed parity with cottonseed oil, the traditional standard of high acceptance for potato chip frying, from the standpoint of oxidative and flavor stability over the shelf life of the product.

6.2 Flavor Characteristics

In addition to the preliminary ''in-house'' acceptability tests of potato chips by a snack food company, Warner [25] independently reported the results of flavor tests of tortilla chips fried in NuSun oil obtained from the 1996 mid-oleic sunflower crop. The results demonstrated that tortilla chips fried in NuSun oil had significantly greater flavor stability

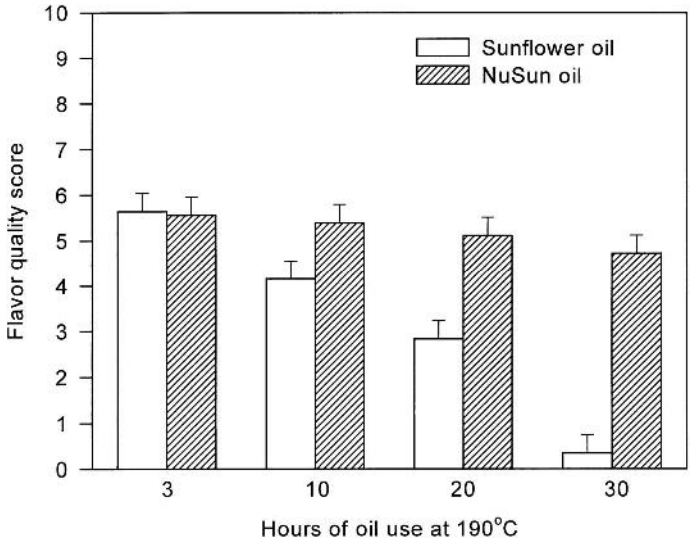


Figure 1 Overall flavor quality scores for tortilla chips fried in traditional sunflower oil or NuSun oil. Chips were fried in oil that had been used for 3, 10, 20, or 30 hr, and then aged for 4 months at 25°C prior to flavor evaluation. Score: 10 = excellent; 1 = bad. (K. Warner, personal communication, 1998.)

after aging for 4 months at 25°C than chips prepared in traditional sunflower oil (Fig. 1). As the hours of frying increased, the stability difference between the two oils became apparent, with NuSun oil demonstrating higher stability after long frying times. The flavor quality scores correlated inversely with the amount of polar compounds present in the oils. Polar compounds are oxygenated fatty acids that result from oxidation of the oil during frying. Traditional sunflower oil, with its higher-linoleic-acid composition, had a significantly higher content of polar compounds as compared to NuSun oil and had the highest intensity of off-odors in room odor tests, including acrid and burnt odors. Thus,

Table 1 Fresh Oil Analysis of Peanut, Soybean, Canola, and NuSun Oils Used in Frying of Frozen, Water-Blanched Diced Potatoes

Analysis	Peanut	Soybean	Canola	NuSun
Peroxide value	0.8	0.5	0.8	0.6
Free fatty acids (% oleic)	0.04	0.04	0.05	0.05
Iodine value	97.9	133.5	115.4	101.9
Fatty acid composition				
Palmitic	10.7	10.3	4.2	4.6
Stearic	2.7	4.1	2.0	4.2
Oleic	51.3	23.2	61.5	61.3
Linoleic	31.1	53.8	20.1	27.3
Linolenic	—	7.8	10.6	0.6
Oxidative stability index at 110°C	38.3	23.8	39.0	33.6

Source: Adapted from Ref. 26.

NuSun oil outperformed traditional sunflower oil in both the flavor of the chips and the odor quality of the heated oil.

6.3 Frying Characteristics

Campbell et al. [26] compared the frying stability of NuSun oil (from the 1997 crop) with several commercial food service frying oils including peanut, soybean, and canola. Table 1 shows the fresh oil analysis of each oil used in the experiment. In their experiments, the oil temperature was maintained at 175°C over an 8-day period, with batches of frozen, water-blanched diced potatoes fried daily. NuSun oil compared favorably with the other oils in showing low hydrolysis to free fatty acids over the 8-day testing period (Fig. 2A). The *para*-anisidine value (Fig. 2B), an indicator of oxidative deterioration, demonstrated that the stability of NuSun oil was superior to that of soybean and canola oils and equiva-

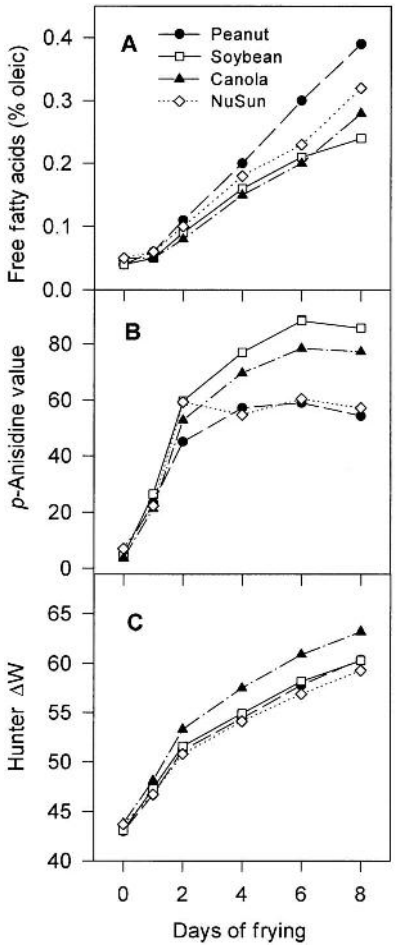


Figure 2 Quality characteristics of peanut, soybean, canola, and NuSun oils after 8 days continuously at 175°C, with daily frying of batches of frozen, water-blanched diced potatoes: (A) free fatty acid content, (B) *p*-anisidine value, and (C) Hunter ΔW (difference to a white plate) value.

lent to the stability of peanut oil. The Hunter ΔW (difference to a white plate) test (Fig. 2C), which measures color change of the oil during frying, showed that NuSun oil outperformed the other oils in color stability. NuSun oil also had similar frying performance and heat stability when compared to partially hydrogenated soybean oil, which is commonly used in restaurant frying operations. NuSun oil, however, with less than 10% saturated fatty acids, had a nutritional advantage over partially hydrogenated oil, which contained 17% saturated fatty acids and 15% trans fatty acids. These researchers concluded that NuSun sunflower oil can be successfully used as a food service frying medium, offering good frying stability while producing fried food products with good taste and good nutritional characteristics.

7 COMMERCIAL SEED PRODUCTION SCALE-UP

In 1996, hybrid seed of the type described in Section 5.3.3 was crushed, and over 2000 L of mid-oleic oil were utilized for initial testing of frying stability, flavor characteristics, and shelf-life acceptability. Industry seed companies were informed of the mid-oleic concept in meetings held by the National Sunflower Association, which led to the formation of a Mid-Oleic Sunflower Task Force. The NSA Mid-Oleic Sunflower Task Force adopted a standard four-step testing procedure used to determine the quality of a food product fried in oil. Step 1 required approximately 75–110 L of deodorized oil to prepare the food product for laboratory screening with taste testing of the product after 1 week. Step 2 was a pilot-plant test that used about 1800 kg of oil. The product was tested for shelf-life stability and consumer acceptability, a process that took about 2 months. Step 3 was a trial in which the product was prepared in a snack food company's frying line. The test required 80,000 kg of oil (produced from about 145 ha of mid-oleic sunflower) to test shelf stability and consumer acceptability. The testing took about 3 months to complete. Step 4 was a final test that required 450,000 kg of oil (produced from 800 ha of mid-oleic sunflower) for a 12-week processing plant run. Fully refined sunflower oil was required, and shelf stability and consumer acceptability were tested. The objective of the NSA Mid-Oleic Task Force was to stimulate enough interest in the mid-oleic concept to create 80,000 kg of oil by 1998 for the Step 3 testing.

However, because industry seed companies already possessed the male and female genetic resources to create mid-oleic hybrid seed and were eager to participate in the venture and because processors were willing to buy, segregate, and crush mid-oleic sunflower seed as an identity-preserved oil, the 1998 production of mid-oleic hybrids was approximately 40,000 ha, far exceeding industry expectations. This allowed Step 3 and Step 4 testing to progress much more rapidly, with the final test being highly successful. Enough oil was produced for distribution to restaurants in the Midwest for evaluation in actual batch frying. In addition, oil was distributed to the Food Quality and Safety Research Group and the USDA National Center for Agricultural Utilization Research. The 1999 sunflower harvest crop produced approximately 1.36×10^8 kg of NuSun sunflower oil, and the year 2000 harvest was forecast to yield over 4.5×10^8 kg of NuSun oil, with nearly complete conversion of the sunflower industry to NuSun oil shortly thereafter.

8 FUTURE OUTLOOK

During the first 3 years of commercial pilot testing or actual use, there were no major criticisms received about the quality of NuSun oil. It appears that NuSun oil is living up

to the expectations that were advanced by its proponents at the outset of the industry transition. The initial results have been promising, and the restaurant and snack food industries continue to show considerable interest in NuSun oil.

8.1 Challenges

There are, to be sure, challenges that must be met for this venture to be successful. To ensure a continuing strong demand for NuSun oil, the sunflower production industry will be compelled to produce a consistent, adequate supply of the oil. Oilseed processors and the food industry need the assurance of a constant supply of seed and oil to avoid costly processing changes or potential variations in product quality if the seed or oil supply should be interrupted. The sunflower industry must also guarantee a consistent quality of NuSun oil to its end-user customers. Seed companies will need to provide sunflower hybrids whose seed will maintain the designated 55–75% oleic acid content to qualify as NuSun oil, despite the vagaries of weather, which influences the oleic/linoleic ratio. Although initial indications are favorable, the NuSun hybrids presently in production are unproven over the long term and under different environmental conditions. Although technically within the defined oleic acid range for NuSun hybrids, some commercial hybrids still need genetic adjustment to reach the stable target level of 65% oleic acid.

8.2 Opportunities

NuSun oil will enjoy a significant advantage over several other popular vegetable oils, such as soybean and canola, because it does not have to be hydrogenated and, therefore, has negligible trans fatty acids. This has become even more important since the 1999 FDA proposal to require the labeling of trans fatty acids on food products. As consumers continue to become more educated about the negative health aspects reported for trans fatty acids in the diet, they are likely to demand food products in which trans fatty acids are low or absent. In addition to altering the content of oleic acid, sunflower breeders are also presently engaged in an effort to lower the saturated fatty acid content of NuSun oil even further. It is likely that NuSun oil could be lowered to 6–7% total saturated fatty acid levels in the future. The consumer trend toward a preference for low saturated fatty acids and zero trans fatty acids in their vegetable oils resonates well for the future of NuSun oil.

NuSun oil is derived from a nongenetically modified organism (non-GMO). For the near future, this could be the most important favorable economic advantage for NuSun oil. European distrust of the introduction of GMO foods during the late 1990s also aroused misgivings in North America and Asia about food derived from transgenic plants. Although the time frame for acceptance of GMO foods worldwide is unpredictable (and they may never be accepted), U.S.-produced NuSun oil will undoubtedly benefit from the controversy, as sunflower is not predicted to become a GMO crop in the United States for many years, if ever, because of the unresolved risks of outcrossing of transgenic sunflower with native populations of wild sunflowers. Thus, the near-term future of NuSun oil in the U.S. market appears very favorable, and the economic experiment to convert the U.S. sunflower industry from traditional sunflower oil to NuSun oil is likely to succeed.

ACKNOWLEDGMENTS

The authors are indebted to Ed Campbell, Monoj Gupta, Larry Kleingartner, and Kathleen Warner for their critical reviews of the manuscript and helpful suggestions.

REFERENCES

1. CB Heiser Jr. In: JF Carter, ed. Sunflower Science and Technology. Madison, WI: American Society of Agronomy, Crop Science Society of America, and Soil Science Society of America, 1978, pp 31–53.
2. CB Lees. Sunflower Hortic 43:24–25, 1965.
3. CB Heiser Jr. The Sunflower. Norman, OK: University of Oklahoma Press, 1976, pp 29–46.
4. ED Putt. In: JF Carter, ed. Sunflower Science and Technology. Madison, WI: American Society of Agronomy, Crop Science Society of America, and Soil Science Society of America, 1978, pp 1–29.
5. P Leclercq. Ann Amelior Plant 19:99–106, 1969.
6. ML Kinman. New developments in the USDA and state experiment station sunflower breeding programs. Proceedings of the 4th International Sunflower Conference, Memphis, TN, 1970, pp 181–183.
7. PW Unger, TE Thompson. Agron J 74:389–395, 1982.
8. F ten-Hoor. Nutr Metab 24(suppl 1):162–180, 1980.
9. P Hoffmann. Prostagland Leukotr Med 21:113–147, 1986.
10. WS Shrapnel, GD Calvert, PJ Nestel, AS Truswell. Med J Aust 156(suppl):S9–16, 1992.
11. M Gupta. INFORM 9:1150–1154, 1998.
12. K Warner. Tocopherols in sunflower oil and soybean oil. Proceedings of the 21st Sunflower Research Workshop, Fargo, ND, 1999, pp 74–76.
13. American Heart Association. Circulation 77:721A–724A, 1988.
14. S Heyden. Ann Nutr Metab 38:117–122, 1994.
15. F Lopez-Segura, F Velasco, J Lopez-Miranda, P Castro, R Lopez-Pedrera, A Blanco, J Jimenez-Pereperez, A Torres, J Trujillo, JM Ordovas, F Perez-Jimenez. Arterioscler Thromb Vasc Biol 16:82–88, 1996.
16. FB Hu, MJ Stampfer, E Rimm, A Ascherio, BA Rosner, D Spiegelman, WC Willett. Am J Epidemiol 149:531–540, 1999.
17. S Tsimikas, A Philis-Tsimikas, S Alexopoulos, F Sigari, C Lee, PD Reaven. Arterioscler Thromb Vasc Biol 19:122–130, 1999.
18. KI Soldatov. Chemical mutagenesis in sunflower breeding. Proceedings of the 7th International Sunflower Conference, Krasnodar, Russia, 1976, pp 352–357.
19. GN Fick. US Patent No. 4,627,192, 1986.
20. K Warner, P Orr, M Glynn. J Am Oil Chem Soc 74:983–988, 1997.
21. AV Pukhalsky, NI Dvoryadkin. Achievements of sunflower breeding in the USSR. Proceedings of the 8th International Sunflower Conference, Minneapolis, MN, 1978, pp 48–55.
22. JF Miller, DC Zimmerman, BA Vick. Crop Sci 27:923–926, 1987.
23. GN Fick. Inheritance of high oleic acid in the seed oil of sunflower. Proceedings of the 6th Sunflower Research Workshop, Bismarck, ND, 1984, p 9.
24. AL Urie. Crop Sci 25:986–989, 1985.
25. K Warner, L Kleingartner. Frying quality and stability of mid-oleic level sunflower oil. Abstracts of the Annual Meeting of the American Oils Chemists' Society, Chicago, IL, 1998, p 4.
26. EC Campbell, JA Gerdes, TR Tiffany. NuSun frying stability. Proceedings of the 21st Sunflower Research Workshop, Fargo, ND, 1999, pp 65–73.

7

Biosynthesis of Ricinoleic Acid for Castor-Oil Production

THOMAS A. McKEON and JIANN-TSYH LIN

Western Regional Research Center, Agricultural Research Service, U.S. Department of Agriculture, Albany, California

1 INTRODUCTION

Vegetable oils can function as a source of food and also provide an important feedstock for the chemical industry. Most oilseed crops grown in temperate regions, such as the United States, produce vegetable oils that contain the same five fatty acids in varying proportions. These oils contain the saturated fatty acids palmitate and stearate, along with the unsaturated fatty acids oleate, linoleate, and α-linolenate. Vegetable oils are vital components of a balanced diet, as they provide caloric value and the essential polyunsaturated fatty acids. Moreover, there are numerous industrial uses for such oils, depending on their composition. Oils with higher saturated and oleate content are oxidatively stable and are used as sources of fatty acids, esters, and alcohols for surfactants and hair care products [1]. Oils with high linoleate composition, such as soybean oil, may be used in printing inks [2] or as epoxidized oils for use as plasticizers or in coatings [3]. Oils with high linolenate content, such as linseed oil, are especially useful as drying oils and are an important component of coatings and other applications where its drying properties are an asset [4]. Important advantages in the use of vegetable oils for chemical and other industrial purposes are their availability as a renewable resource and their biodegradability. However, there are limits to their usefulness.

The presence of only five major fatty acids in most domestically produced vegetable oils represents a significant genetic limitation to their uses and available applications. The usefulness of these oils is impeded as a result of the limited functionality available in the

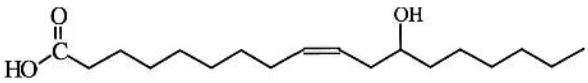


Figure 1 Ricinoleic acid.

oils. However, there are numerous plants that produce oils containing fatty acids with additional functional groups which enhance their value [5]. One such oil is castor oil, derived from the bean produced by the castor plant *Ricinus communis* L. Castor oil is composed of up to 90% ricinoleic acid (Fig. 1). The presence of the midchain hydroxy group makes ricinoleate an excellent source of greases and other lubricants. Furthermore, the reactivity of the fatty acid resulting from the hydroxy group enables it to be used as a source of plastics, directly or after conversion to undecanoate derivatives, and as a drying oil [6,7]. There are up to 300 patented uses of castor oil and ricinoleate. Many of these inventions have not been developed as products as a result of the limited availability of the oil [6].

The castor bean contains the protein toxin ricin, toxic alkaloids, and potent allergens that result in sensitization of workers and development of severe bronchial asthma [7,8]. Although formerly processed in the United States, the expense of detoxification and deallergenization of the castor bean seed meal became impractical due to the energy expense. The United States imports 1.1×10^8 lbs. of castor oil per year [9], and the availability of an alternate source would supplement imports and expand the use of castor oil. One approach to developing an alternative source of castor oil is to genetically engineer soybean to produce an oil comparable in composition to castor oil.

Annual U.S production of soybean oil is approaching 2×10^{10} lbs., nearly 40% of the worldwide total [9], and approximately 1.5×10^9 lbs. is carried over annually (Fig. 2). One approach to reducing this surplus of soy oil is to expand the range of products for which it can be used. We are using the strategy of altering the fatty acid composition of soybean oil to broaden its uses. Development of a soybean that produced high levels of ricinoleate would have a significant effect on the surplus of soy oil (Fig. 2). Such a ricinoleate-rich soybean would approach 10% of the surplus soybean oil carryover at our current castor-oil import level and ricinoleate use would increase considerably more through additional uses if sufficient quantities were to become reliably available [6]. This chapter, then, will describe our efforts to elucidate the biochemistry of castor-oil production by the castor bean, so that we can transfer the appropriate genes to develop transgenic soy that produces a suitable alternative to castor oil.

2 BIOSYNTHESIS OF RICINOLEATE

The key reaction in ricinoleate production is the hydroxylation of oleate to ricinoleate (Fig. 3). Studies on the enzyme involved in hydroxylation were initiated over 30 years ago by Yamada and Stumpf [10] in the United States and James et al. [11] in the United Kingdom. To date, the oleoyl-12-hydroxylase has not been purified, although it has been extensively characterized and its cDNA has been cloned.

The earliest research efforts on the hydroxylase demonstrated that it was associated with cell membranes [10] and was shown shortly thereafter to be associated with the microsomal fraction of the developing castor bean endosperm [12]. The enzyme requires its putative substrate oleoyl-CoA, either NADH or NADPH, and oxygen for activity. The

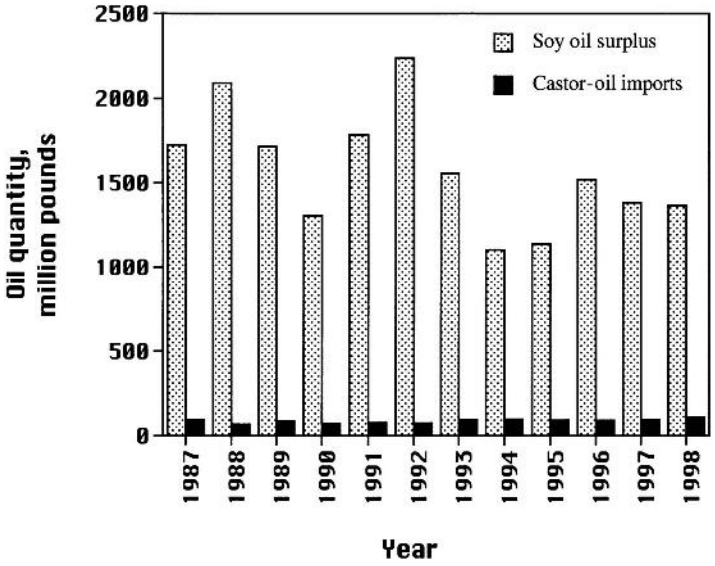


Figure 2 Annual soybean oil surplus compared to castor-oil imports.

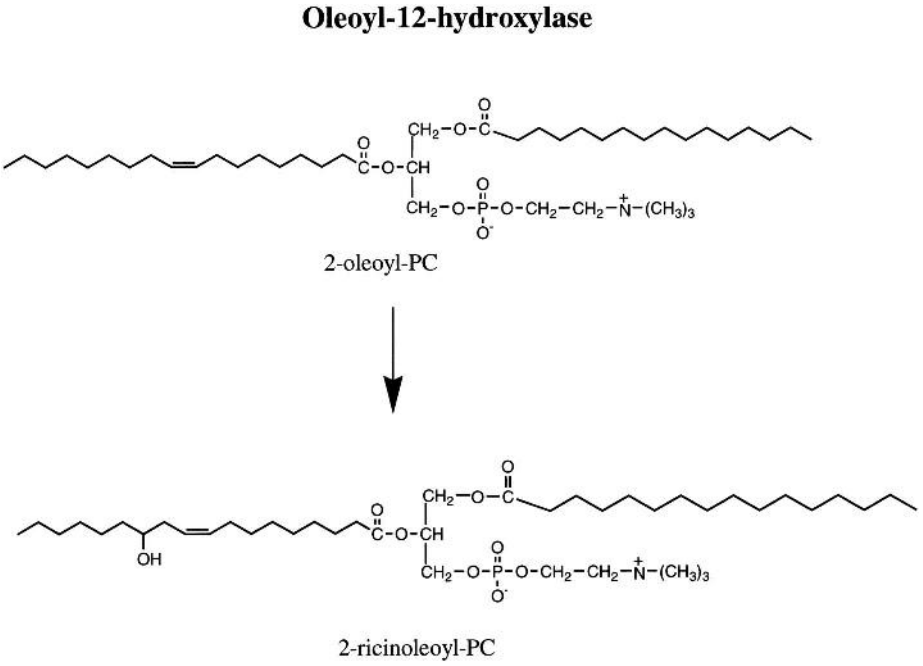


Figure 3 Hydroxylase reaction.

observation by Moreau and Stumpf [13] that the oleate from oleoyl-CoA is rapidly incorporated into phosphatidylcholine (PC), led to the conclusion that oleoyl-PC is actually the substrate for the hydroxylase. This conclusion was later corroborated [14] by thin-layer chromatographic (TLC) analysis of microsomal incubations using oleoyl-CoA as the oleate donor. We have used oleoyl-PC directly as a substrate and demonstrated conversion of oleate in the *sn*-2 position to ricinoleoyl-PC with the ricinoleate in the *sn*-2 position [15]. Our recent research demonstrates that the related phosphatidyl ethanolamine (PE), oleoyl-2-PE, is not a direct substrate for the hydroxylase. However, the oleoyl-2-PE is converted by castor microsomes to oleoyl-2-PC and this substrate is then used by the hydroxylase [16].

The association of the hydroxylase with membrane fractions is an important factor in elucidating hydroxylase activity and in vivo function. Because of similarities between the hydroxylase reaction and the stearoyl-CoA desaturase reaction [17], Moreau and Stumpf suggested that the hydroxylase used a membrane-bound reducing protein, such as the cytochrome b_5 used by the mammalian desaturase [13]. It was later shown that oleate desaturation in plants does use cytochrome b_5 [18] based on inhibition of desaturation by antibodies to plant cytochrome b_5. Based on these results, it was then demonstrated that antibodies to cytochrome b_5 blocked hydroxylation of oleate when NADH was used as the reductant [19]. Thus, NADH reduces cytochrome b_5 via cytochrome b_5 reductase-dependent reduction and the reduced cytochrome b_5 is required for hydroxylase activity [19]. The NADPH-dependent hydroxylation of oleate is approximately 70% of the NADH-dependent activity and results from the cytochrome P450 reductase-dependent reduction of cytochrome b_5 [19].

The membrane association of the hydroxylase has been the main obstacle in attempts to purify the enzyme to homogeneity. Differential centrifugation for the preparation of microsomes does provide some purification [12,13]. Because the microsomal fraction is a fairly crude preparation composed of a collection of plant cell membranes, there have been several attempts to purify specific membrane fractions. It has proven difficult to retain hydroxylase activity in these preparations due to the instability of the enzyme. However, there has been one successful attempt which separated mitochondrial, peroxisomal, and endoplasmic reticulum membranes and demonstrated that hydroxylase activity resides in the endoplasmic reticulum [20].

Further purification efforts have been confounded due to the difficulty of solubilizing the hydroxylase activity from the membrane fraction. The addition of detergent inactivated the enzyme [13], and crude microsomal preparations of the hydroxylase had been reported to be unstable, even to storage at −80°C [14]. It has not always been clear how much of the hydroxylase instability is a result of its inactivation and how much is due to the loss or release of a membrane-associated enzyme upon which hydroxylase activity is dependent. We therefore took steps to reduce losses of hydroxylase activity. We eliminated the extensive washing of microsomes previously reported [14], added catalase as suggested [13,14], and added antipain, an inhibitor of papain-like proteases. The application of these steps has contributed greatly to the stabilization of hydroxylase in microsomal preparations, and such preparations remain active for prolonged periods of storage at −80°C [21]; microsomal suspensions stored for up to 4 years retain over 50% of their activity. The stability of these microsomal preparations has allowed us to make progress in characterizing the hydroxylase and in characterizing the incorporation of ricinoleate into triacylglycerol [15,22]. Based on the amounts of microsomal protein needed for activity, these preparations are more active, using 10–20 μg of protein for short (10 min) incubation times

versus the 300 to >1000 μg of protein for incubation times up to 1 hr or more reported previously. The use of such small amounts of protein has facilitated reconstitution experiments, as well. We have shown that the hydroxylase can be solubilized by 0.5% Triton X-100, based on complete loss of activity after ultracentrifugation of microsomes treated with the detergent. Upon dilution into reaction mix, the activity returns, thus demonstrating the ability to reconstitute the hydroxylase with the other essential constituents, a key step recovering activity after solubilizing the enzyme for purification [21].

The assays used to detect hydroxylase activity measure the conversion of ^{14}C-oleate to ^{14}C-ricinoleate. Previous researchers have used gas chromatography (GC) or TLC to separate the labeled oleate and ricinoleate and then determined the extent of label by scintillation counting, scanning radiometry, or gas flowthrough radiometry. We have developed a rapid quantitation technique based on high-performance liquid chromatographic (HPLC) separation of the fatty acid methyl esters on a 5-cm C_{18} reversed-phase column eluted with aqueous methanol [21,22]. The esters are separated within 10 min and the radiolabel is counted by radioflow detection of the column eluent. One advantage of this system is that it allows simultaneous detection of hydroxylation and desaturation. Highest levels of activity are observed when oleoyl-CoA is used as the substrate, although the label is rapidly incorporated into PC [13]. The hydroxylase displays less overall activity when the true substrate, 2-oleoyl-PC, is used; however, we have been successful in optimizing the measurable activity on 2-oleoyl-PC [22]. Using this optimized assay, we have been able to identify the initial product of the hydroxylation reaction, 2-ricinoleoyl-PC [15]. Moreover, we have demonstrated that the substrate analog oleoyl-oxyethyl-phosphocholine is an effective inhibitor of hydroxylation, using either oleoyl-CoA or oleoyl-PC as substrates [15,21].

Because the purification of oleoyl-12-hydroxylase appeared intractable, an alternate approach was used by the Somerville group to clone the gene for the hydroxylase [23]. Operating on the premise that the hydroxylation reaction is carried out by an enzyme similar to the oleoyl-12-desaturase, they sequenced a library of expressed sequences generated from developing castor bean and screened them for the presence of histidine clusters characteristic of plant oleoyl and linoleoyl desaturases. Full-length clones of promising sequences were used to transform *Nicotiana*, and a transgenic plant producing minor amounts of ricinoleate was detected. This clone was further confirmed as coding for the hydroxylase by its high degree of homology to a similar enzyme in *Lesquerella*, another plant that produces an oil containing hydroxy fatty acids [23]. The hydroxylase gene has been expressed in a number of plants and has produced up to 20% of hydroxy fatty acid in the oil [24,25]. Interestingly, not only are the hydroxylase and desaturase homologous, they also share a high degree of homology with enzymes that add an epoxy or add a triple bond to the 12,13-position of oleate [26,27].

3 ALTERED FATTY ACID COMPOSITION IN TRANSGENIC PLANTS

Genetic engineering has been an extremely effective tool in modifying fatty acid composition [28]. Commercial successes include laurate canola and high-oleic soybean [29], with other transgenic oilseed crops expected soon. The early commercial successes were brought about by modification of a single enzyme in the fatty acid biosynthetic pathway (increased or decreased expression of the enzyme activity) or by introduction of a new enzyme activity that alters the fatty acid biosynthetic pathway. However, it also became apparent that there are limitations on the amount and types of fatty acid that can be incorpo-

rated into the seed oil. Canola modified to produce oil containing laurate is unable to incorporate it into the *sn*-2 position of triacylglycerol, and laurate canola was limited to a content of 40% laurate by weight. By including the gene for lysophosphatidic acid acyltransferase (LPAAT) from coconut, which can incorporate laurate into the *sn*-2 position of the triacylglycerol, levels of laurate are increased to 55% [30]. It has thus become clear that the development of commercially desired oils in transgenic plants may require expression of several additional genes.

Additionally, other processes may interfere with the accumulation of desired fatty acids. Again, in the case of laurate production, it is found that more laurate is synthesized than is being incorporated into triacylglycerol [31]. The unincorporated laurate is β-oxidized, as the lauroyl-CoA formed in the course of oil production is also an intermediate in β-oxidation. This process may serve as a protective mechanism for the plant membranes, as medium-chain acyl-CoA is an excellent detergent and could disrupt membrane function. Similarly, hydroxylase activity can be detected in vegetative tissue of transgenic plants when the gene is driven by a constitutive promoter, yet there is no detectable hydroxy fatty acid accumulated by such tissue [32]. Because the substrate and product of the oleoyl-hydroxylase are PCs, the presence of the hydroxy fatty acid in phospholipid could have a deleterious effect on membrane function if the hydroxy fatty acyl phospholipid were incorporated into the membrane. It is thus thought that membranes have an ''editing'' function that eliminates fatty acids that disrupt membrane function [33]. The presence of fatty acids with midchain polar groups would result in hydrogen-bonding in the nonpolar region of the lipid bilayer and disrupt its fluidity. This dual role of phospholipids, as components of the cellular membranes and as substrates for triacylglycerol synthesis, is an important factor in attempts to produce an uncommon fatty acid or a greater amount of a common fatty acid in the seed oil. Because the fatty acid will be incorporated in phospholipid during triacylglycerol biosynthesis, the change in fatty acid composition could disrupt proper membrane function. Plants that produce these fatty acids clearly have mechanisms that prevent the accumulation of the fatty acid in the membrane while incorporating large amounts of it into oil. Early work on transgenic production of hydroxy fatty acids in *Nicotiana* found only 0.1% ricinoleate made [23], whereas castor produces an oil with up to 90% ricinoleate. To date, the highest levels of hydroxy fatty acid produced are 20%, in the model plant system *Arabidopsis thaliana* [24]. Therefore, we have analyzed castor-oil biosynthesis in extracts of castor bean in order to identify enzymatic activities that lead to high-ricinoleate production and incorporation into castor oil while maintaining oleate in phospholipid form for conversion to ricinoleate [15].

4 CASTOR-OIL BIOSYNTHESIS

Castor oil contains up to 90% ricinoleate, approximately 4% oleate, 4% linoleate, and small amounts of palmitate and linolenate [6,7]. Therefore, the conversion of oleate to ricinoleate in castor is highly efficient and competition for oleate as a substrate for desaturation or inclusion in triacylglycerol is very limited. Clearly, oleoyl-12-hydroxylase, the enzyme that converts oleate to ricinoleate, is crucial to the castor-oil biosynthetic pathway. Figure 4 illustrates the overwhelming activity of oleoyl hydroxylase compared to desaturase, with the hydroxylase activity reaching and maintaining a high level to nearly full maturity, whereas desaturase activity is low and diminishes well before maturity. This difference is an underlying reason for the high level of ricinoleate produced in the castor bean [21]. Yet, when transgenic rapeseed expressed this cDNA, the level of hydroxy fatty acids produced was limited to 17%, composed of a mixture of 18-, 20-, and 22-carbon-

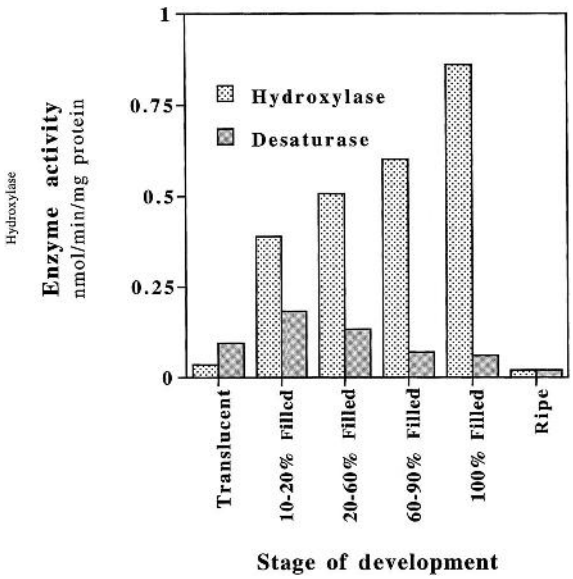


Figure 4 Hydroxylase and desaturase activities as a function of maturity.

chain fatty acids [25]. The castor bean has evolved the enzymatic systems necessary to produce high levels of ricinoleate. To achieve further progress in developing a castor-oil replacement, we examined the metabolism of oleate, 2-oleoyl PC, and ricinoleate in isolated castor microsomes [15]. This research demonstrated the rapid incorporation of oleate into lyso-PC to form the substrate for the hydroxylase, the limited release of oleate from added 2-oleoyl PC, and the rapid release of ricinoleate from 2-ricinoleoyl PC formed in situ or added to incubations. These results pointed to the lyso-PC acyltransferase (LPCAT) as one of the key enzymes in maintaining oleate as a substrate for hydroxylation. Phospholipase A_2 served the important function as the enzyme that removes ricinoleate from phospholipid, presumably as the membrane ''editing'' function. The final enzyme identified as underlying high ricinoleate incorporation is the diacylglycerol acyltransferase (DAGAT), based on the selective incorporation of ricinoleate to yield triacylglycerols containing two or three ricinoleoyl residues and the very low incorporation of oleate. The pathway to triricinolein is depicted in Figure 5, and the key steps in high ricinoleate production and incorporation into triacylglycerol are summarized:

1. Lyso-PC acyltransferase transfers oleate from oleoyl CoA into the *sn*-2 position of PC to serve as the hydroxylation substrate.
2. The oleoyl-12-hydroxylase hydroxylates the *sn*-2 oleoyl-PC.
3. Phospholipase A_2 preferentially removes ricinoleate from the *sn*-2 position and releases lyso-PC for reincorporation of oleate by LPCAT.
4. The free ricinoleate is preferentially incorporated into ricinoleoyl-containing diacylglycerol by DAGAT, which preferentially incorporates ricinoleate to form diricinoleins and triricinolein.

Previous investigations of ricinoleate biosynthesis used oleoyl-CoA as the substrate donor. As a result, the LPCAT is the first essential step in the production of ricinoleate, generating the PC substrate for the hydroxylase reaction. Moreau and Stumpf [13] first

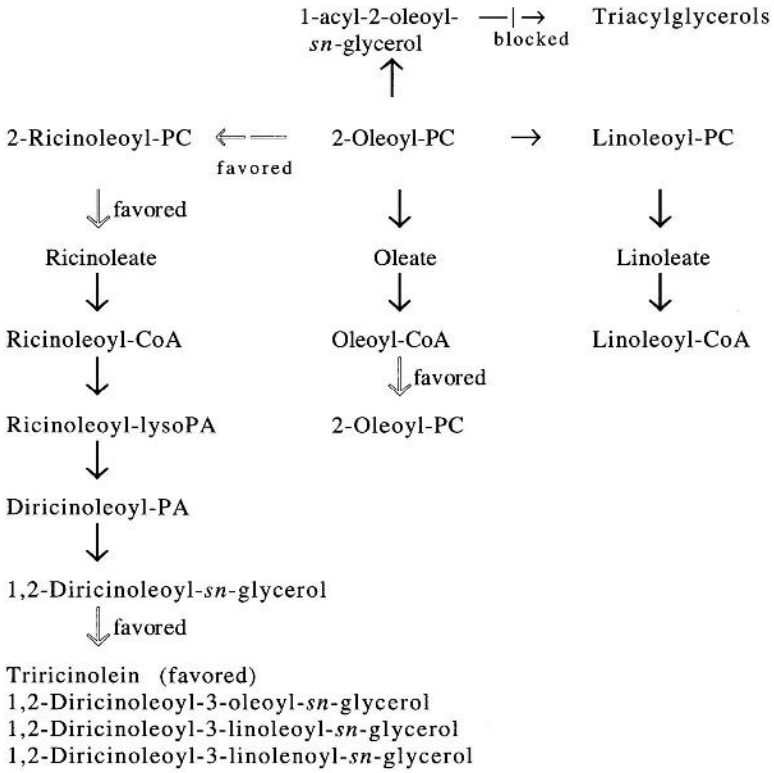


Figure 5 Pathway for castor-oil biosynthesis.

recognized oleoyl-PC as the likely substrate when they found that most of the labeled oleate from ^{14}C-oleoyl-CoA is rapidly incorporated into the PC fraction after addition to the microsomal reaction mix. Support for oleoyl-PC as the substrate grew as other research groups added increasing levels of evidence leading to this same conclusion [14]. We have since isolated the labeled product *sn*-2-ricinoleoyl-PC from microsomal incubations of *sn*-2-oleoyl-PC. We have also demonstrated that when labeled ricinoleate is added to microsomes, only small amounts are incorporated into PC. We have thus demonstrated that LPCAT is the first essential step leading to ricinoleate production. By producing *sn*-2-oleoyl-PC, LPCAT maintains oleate as part of the substrate pool for eventual conversion to ricinoleate and prevents a buildup of ricinoleoyl-phospholipid by its low activity for incorporating ricinoleate [15]. Although the enzyme has not been purified from any plant or animal source, Moreau and Stumpf have successfully solubilized the enzyme using several different detergent treatments [34].

The hydroxylation of the *sn*-2-oleate after incorporation in PC is the next key step in castor-oil biosynthesis and was described earlier in this chapter.

After hydroxylation, the ricinoleate is released from PC, reducing the level of available phospholipid containing the hydroxy fatty acid and avoiding the possibility of its incorporation in the membranes, where it could have detrimental effects. It has been previously shown that the castor microsomes have a strong preference for release of ricinoleate versus oleate [14]. Moreover, this specificity for phospholipids containing oxygenated

fatty acids is common among phospholipases A_2, even from oilseeds that do not make oxygenated fatty acids in their oils [33]. As mentioned previously, this activity is thought to be part of a membrane editing system that removes such fatty acids from PC to prevent their incorporation into membrane. We have demonstrated that free ricinoleate is one of the labeled products detected in microsomal incubations of *sn*-2-^{14}C-oleoyl PC and that it is derived from the *sn*-2-^{14}C-ricinoleoyl PC produced by hydroxylase action. When *sn*-2-^{14}C-ricinoleoyl-PC, the putative endogenous substrate of phospholipase A_2, is added to microsomal incubations, ^{14}C-ricinoleate is rapidly released [15]. The phospholipase A_2 from castor has not yet been isolated; however, one has been purified from developing seed of elm [35], rice seedlings [36], as well as two others cloned from rice [36] and one from carnation [37]. These are homologous to the secretory phospholipase A_2 from animals, which have numerous functions, including signaling. Like the animal enzymes, the plant phospholipases A_2 are dependent on the calcium ion.

Diacylglycerol acyltransferase catalyzes the final step in triacylglycerol biosynthesis, transferring the acyl group from acyl-CoA to the *sn*-3 position of *sn*-1,2-diacylglycerol. In microsomal incubations with ^{14}C-ricinoleate, the chromatographic peaks identified as triacylglycerols containing two or three ricinoleoyl groups contained almost all of the label incorporated into the triacylglycerol fraction. In contrast, the oleate from *sn*-2-^{14}C-oleoyl-PC was converted to ricinoleate prior to incorporation into the triacylglycerol fraction, because the principal labeled triacyglycerols contained labeled ricinoleate. The only peak significantly labeled with ^{14}C-oleate was diricinoleoyl-oleoyl-glycerol [15]. This distribution pattern of label indicates a strong preference for ricinoleate incorporation into triacylglycerol already acylated with one or two ricinoleates, given that most of the label is present as ^{14}C-oleate, with only small amounts converted to ^{14}C-ricinoleate in such microsomal incubations. Previous work by Vogel and Browse [38] on the activity of castor DAGAT indicated at least a threefold preference for transferring oleate from oleoyl-CoA into diricinoleoyl-glycerol over other diacylglycerols, as measured by DAGAT assay. The DAGAT from castor has not yet been isolated or further characterized. However, based on homology to other acyltransferases, the DAGAT from mouse has been cloned [39] and, as a result, progress has been made in cloning plant DAGAT. An *Arabidopsis* mutant defective in DAGAT activity was isolated and characterized as having reduced levels of triacylglycerol (TAG), increased levels of diacylglycerol (DAG) in the seed oil, an altered fatty acid composition, and a decreased level of DAGAT activity [40]. The mutation in this strain was found to reside in the gene corresponding to DAGAT based on its homology to the mammalian DAGAT gene and detection of DAGAT activity upon expression of the gene in yeast [41]. The lower level of oil produced by the mutant lends support to the generally held view that the DAGAT step is a rate-controlling step in oil biosynthesis, leading to the possibility of higher oil yield if DAGAT is overexpressed.

It was once accepted dogma that triacylglycerols, after being formed, were deposited in oil bodies and not subject to modification until seed germination. It has since been demonstrated that acyl-exchange reactions do occur in castor [42], so that even after biosynthesis of the triacyglycerol, there is still an opportunity to adjust their composition. Acyl-exchange reactions allow transfer of acyl groups among triacylglycerols and other lipids, as well as from triacylglycerols to monoacylglycerols and diacylglycerols. Using castor microsomes labeled with ^{14}C-glycerol lipids or ^{14}C-ricinoleoyl lipids, Mancha and Stymne demonstrated that acyl groups are actively exchanged, with ricinoleoyl groups tending to transfer from diricinolein or triricinolein to monoricinolein or diricinolein. Furthermore, they demonstrated that there are two pools of triacylglycerol in castor endosperm—the

microsomal pool, where acyl exchange is active, and the oil bodies, which are rich in triricinolein and not active in exchange reactions. Their findings suggest that until it is incorporated in the oil body, the triacylglycerol can be actively tailored in the microsomes. Incorporation of the triacylglycerol in the oil body is the committed step in determining fatty acid composition. In castor, this acyl exchange would allow for the selection of triricinolein and would also maintain oleate as a substrate for conversion to ricinoleate. The question that arises from these observations, then, is what determines that a given triacylglycerol will then be incorporated into the oil body? This final regulatory step may be another essential component in controlling the fatty acid composition of an oil.

We are testing our conclusions on the identification of the key enzymes in castor-oil biosynthesis by developing transgenic plants that express the castor enzymes that lead to high-ricinoleate production. Such plants should be able to produce the high levels of ricinoleate needed to develop a transgenic crop that replaces castor. A transgenic soybean that produces ''castor'' oil could expand the industry dependent on ricinoleate, because the oil could be provided in greater quantity without the hazards involved in castor cultivation.

5 CONCLUSIONS

Commercial and industrial uses of vegetable oils are dependent on fatty acid composition. Most U.S.-produced vegetable oils are composed of the same five fatty acids, constituting a genetically imposed limitation on potential uses for such oils. Incorporation of other fatty acids into commercial oilseed production will expand uses for the oils and represent a major contribution to the development of a bio-based economy. One commercially important fatty acid is ricinoleic acid (12-hydroxy-octadec-9-enoic acid), the principal constituent of castor oil. We have identified the enzymes that are involved in the high-level production and incorporation of ricinoleate into castor oil. Genes for the enzymes that we have identified will be used in strategies to develop transgenic soybeans, which will produce commercially useful levels of ricinoleate. Such transgenic plants will be value-added crops and renewable resources that reduce the need for imports and enhance exports.

NOTE ADDED IN PROOF

We have also found that an additional activity, the phosphatidylcholine diacylglycerol acyltransferase (PDAT), plays a key role in castor-oil biosynthesis.

REFERENCES

1. G Bognolo. In: FD Gunstone, FB Padley, eds. Lipid Technologies and Applications. New York: Marcel Dekker, 1997, pp 633–694.
2. SZ Erhan, MO Bagby. Ind Crops Prod 3:237–246, 1995.
3. FD Gunstone. In: FD Gunstone, FB Padley, eds. Lipid Technologies and Applications. New York: Marcel Dekker, 1997, pp 759–769.
4. J Bentley. In: FD Gunstone, FB Padley, eds. Lipid Technologies and Applications. New York: Marcel Dekker, 1997, pp 711–736.
5. JB Ohlrogge. Plant Physiol 104:821–826, 1994.
6. Caupin, HJ. In: FD Gunstone, FB Padley, eds. Lipid Technologies and Applications. New York: Marcel Dekker, 1997, pp 787–795.

7. D Atsmon. In: G Röbbeln, RK Downey, A Ashri, eds. Oil Crops of the World. New York: McGraw-Hill, 1989, pp 438–447.
8. SC Thorpe, DM Kemeny, RC Panzani, B McGurl, M Lord. J Allergy Clin Immunol 82:67–72, 1988.
9. Agricultural Statistics. Washington, DC: United States Government Printing Office, 1999, pp III-1–III-30.
10. M Yamada, PK Stumpf. Biochem Biophys Res Commun 14:165–171, 1964.
11. At James, HC Hadaway, JPW Webb. Biochem J 95:448–452, 1965.
12. T Galliard, PK Stumpf. J Biol Chem 241:5806–5812, 1966.
13. RA Moreau, PK Stumpf. Plant Physiol 67:672–676, 1981.
14. M Bafor, MA Smith, L Jonsson, K Stobart, S Stymne. Biochem J 280:507–514, 1991.
15. JT Lin, CL Woodruff, OJ Lagouche, TA McKeon, AE Stafford, M Goodrich-Tanrikulu, JA Singleton, CA Haney. Lipids 33:59–69, 1998.
16. JT Lin, KM Lew, JM Chen, Y Iwasaki, TA McKeon. Lipids 35:481–486, 2000.
17. P Strittmater, L Spatz, D Corcoran, MJ Rogers, B Setlow, R Redline. Proc Natl Acad Sci USA 71:4565–4569, 1974.
18. EV Kearns, S Hugly, CR Somerville. Arch Biochem Biophys 284:431–436, 1991.
19. MA Smith, L Jonsson, S Stymne, K Stobart. Biochem J 287:141–144, 1992.
20. DE Richards, RD Taylor, DJ Murphy. Plant Physiol Biochem 31:89–94, 1993.
21. TA McKeon, JT Lin, M Goodrich-Tanrikulu, AE Stafford. Ind Crops Prod 6:383–389, 1997.
22. JT Lin, TA McKeon, M Goodrich-Tanrikulu, AE Stafford. Lipids 31:571–577, 1996.
23. FJ van de Loo, P Broun, S Turner, C Somerville. Proc Natl Acad Sci USA 92:6743–6747, 1995.
24. P Broun, N Hawker, CR Somerville. In: JP Williams, MU Khan, NW Lem, eds. Physiology, Biochemistry and Molecular Biology of Plant Lipids. Dordrecht: Kluwer Academic Publishers, 1997, pp 342–344.
25. P. Broun, C Somerville. Plant Physiol 113:933–942, 1997.
26. P Broun, J Shanklin, E Whittle, C Somerville. Science 282:1315–1317, 1998.
27. M Lee, M Lenman, A Banas, M Bafor, S Singh, M Schweizer, R Nilsson, C Liljenberg, A Dahlqvist, PO Gummeson, S Sjodahl, A Green, S Stymne. Science 280:915–918, 1998.
28. TA McKeon, JT Lin, M Goodrich-Tanrikulu, AE Stafford. In: G Fuller, TA McKeon, D Bills, eds. Agricultural Materials as Renewable Resources: Non-food and Industrial Applications. Washington, DC: ACS Books, 1996, pp 158–178.
29. U Bornscheuer. INFORM 9:485–488, 1998.
30. DS Knutzon, KD Lardizabal, JS Nelsen, JL Bleibaum, HM Davies, JG Metz. Plant Physiol 109:999–1006, 1995.
31. Y Poirier, G Ventre, D Calelari. Plant Physiol 121:1359–1366, 1999.
32. P Broun, S Boddupalli, C Somerville. Plant J 13:201–210, 1998.
33. U Stahl, A Banas, S Stymne. Plant Physiol 107:953–962, 1995.
34. RA Moreau, PK Stumpf. Plant Physiol 69:1293–1297, 1982.
35. U Stahl, B Ek, S Stymne. Plant Physiol 117:197–205, 1998.
36. U Stahl, M Lee, S Sjodahl, D Archer, F Cellini, B Ek, R Iannacone, D MacKenzie, L Semeraro, E Tramontano, S Stymne. Plant Mol Biol 41:481–490, 1999.
37. JY Kim, YS Chung, SH Ok, SG Lee, WI Chung, IY Kim, JS Shin. Biochim Biophys Acta 1489:389–392, 1999.
38. G Vogel, J Browse. Plant Physiol 110:923–931, 1996.
39. S Cases, SJ Smith, YW Zheng, HM Myers, SR Lear, E Sande, S Novak, C Collins, CB Welch, AJ Lusis, SK Erickson, RV Farese. Proc Natl Acad Sci USA 95:13,018–13,023, 1998.
40. V Katavic, DW Reed, DC Taylor, EM Giblin, DL Barton, JT Zou, SL MacKenzie, PS Covello, L Kunst. Plant Physiol 108:399–409, 1995.
41. J Zou, Y Wei, C Jako, A Kumar, G Selvaraj, DC Taylor. Plant J 19:645–653, 1999.
42. M Mancha, S Stymne. Planta 203:51–57, 1997.

8

Enzymatic Transformation of Lipids as a Plant Defense Mechanism

DAISUKE SHIBATA

Kazusa DNA Research Institute, Kisarazu, Chiba, Japan

HIROYUKI OHTA

Tokyo Institute of Technology, Nagatsuta, Midori-ku, Yokohama, Japan

1 INTRODUCTION

Several lines of accumulated evidence show that oxidized derivatives of lipids are involved in plant defense mechanisms, functioning as toxic compounds effective against pathogens, or as signaling molecules (jasmonate and its derivatives) for activation of defense genes. Such oxidized lipids (oxylipins) are synthesized via the lipoxygenase pathway (or the oxylipin pathway) (Fig. 1). Lipoxygenases (linoleate: oxygen oxidoreductase, E.C. 1.13.11.12) initiate the peroxidation of fatty acids that contain a (*Z*,*Z*)-1,4-pentadiene moiety. In higher plants, linolenic acid and linoleic acid, which are common in plasma membrane phospholipids and thylakoid membrane glycolipids, are substrates for lipoxygenases. Fatty acid hydroperoxides generated by lipoxygenation are substrates for lyase, peroxygenase, or allene oxide synthase. These metabolites are further transformed into various oxylipins as shown in Figure 1. Various oxylipins directly inhibit the growth of pathogens when exogenously applied. This suggests defensive roles of these oxylipins, such as hydroperoxide lyase products described in Chapter 9. Roles of the peroxygenase cascade, which leads to products relevant to plant defense mechanisms, are described in Chapter 12. Among the oxylipins, jasmonate and methyl jasmonate have been investigated mostly in terms of their role in plant defense, especially against insect attack or wounding,

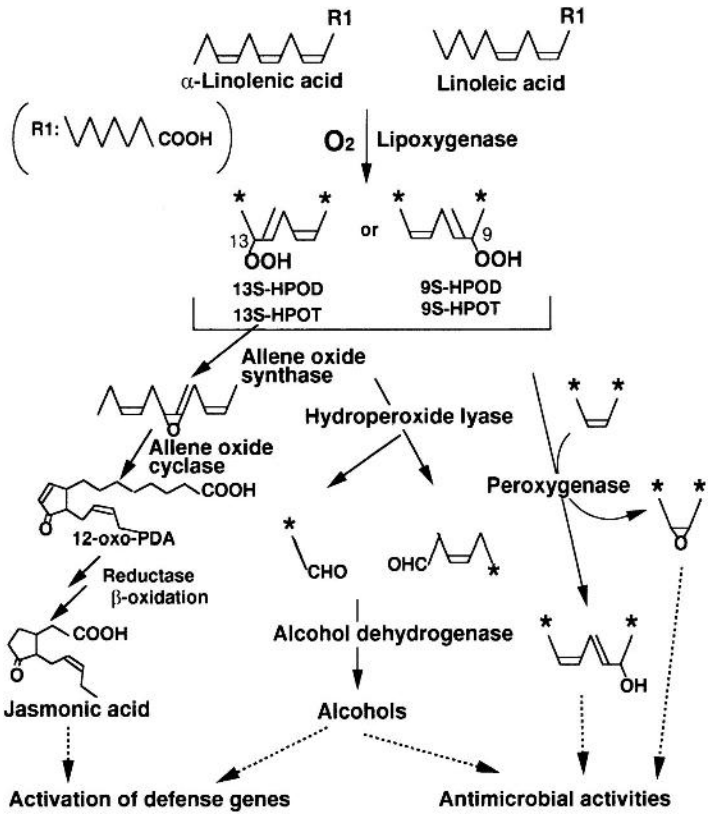


Figure 1 Lipid transformation via the lipoxygenase pathway as a defense mechanism. For simplicity, side structures of reaction products are represented by an asterisk.

as described in Chapter 11. This chapter deals with plant defense mechanisms against pathogens, as related to lipoxygenase function.

2 EVIDENCE FOR THE INVOLVEMENT OF THE LIPOXYGENASE PATHWAY IN PLANT DEFENSE AGAINST PATHOGENS

There have been a number of reports indicating that increases in lipoxygenase activity occur when plants are confronted with pathogens. This has been demonstrated in several plant species, including oats [1], wheat [2,3], rice ([4,5]; see also Sec. 3.2), cucumber [6], tomato [7], bean [8,9], *Arabidopsis* [10], and tobacco [11]. In general, lipoxygenase activity increases more quickly and to a higher level in resistant plants than in susceptible ones, when compatible and incompatible interactions are compared. These studies examined the possible involvement of lipid transformation via lipoxygenases in defensive responses. An excellent review on pathogenesis-related lipoxygenases is available [12].

Molecular genetic approaches provide direct evidence for involvement of the lipoxygenase pathway enzymes and their metabolic products in plant defense against pathogens, as well as in wounding responses. An *Arabidopsis* triple mutant *fad3*-2 *fad7*-2 *fad8* deficient in linolenic acid biosynthesis, which thus cannot accumulate jasmonate, has been

isolated [13]. The mutant was found to be extremely susceptible to root rot caused by the fungal root pathogen *Pythium mastophorum* (Drechs.), a soil fungus, and application of exogenous methyl jasmonate substantially protected the mutant plants [14]. These results indicate that jasmonate is essential for plant defense against *P. mastophorum* in *Arabidopsis*. Mutants of *Arabidopsis* defective in the jasmonate response allele *jar1* are insensitive to jasmonic acid during seedling root growth and are susceptible to *P. irregulare* [15], similar to the *fad3*-2 *fad7*-2 *fad8* triple mutant. An *Arabidopsis* mutant, *coi1*, with a defect affecting the jasmonate-response pathway, shows enhanced susceptibility to infection by fungal pathogens [16]. Transgenic tobacco plants expressing antisense lipoxygenase sequences show suppression of the incompatible interaction with *Phytophthora parasitica* var. *nicotianae* race 0 [17]. These transgenic tobacco plants also show enhanced susceptibility toward the compatible fungus *Rhizoctonia solani*. These lines of evidence convince us of the direct involvement of the lipoxygenase pathway, especially jasmonate signaling, in plant defense mechanisms against pathogens in these plant species.

However, we also should note that resistance to infection by pathogens in barley [18] and rice [19] is not associated with enhanced levels of endogenous jasmonates (see Sec. 3.3). It is also reported that divinyl ether oxylipins, colneleic and colnelenic acids, which are inhibitory to the late-blight pathogen *Phytophthora infestans*, are accumulated in potato leaves infected with *P. infestans*, but the levels of members of the jasmonic acid family do not change significantly during pathogenesis [20].

3 RICE LIPOXYGENASE PATHWAY ENZYMES INVOLVED IN PLANT DEFENSE

3.1 Antifungal Oxylipins from Rice

A major group of antifungal compounds isolated from rice leaves turned out to be oxylipins (Fig. 2). In studies aimed at elucidating the mechanisms of plant defense against the

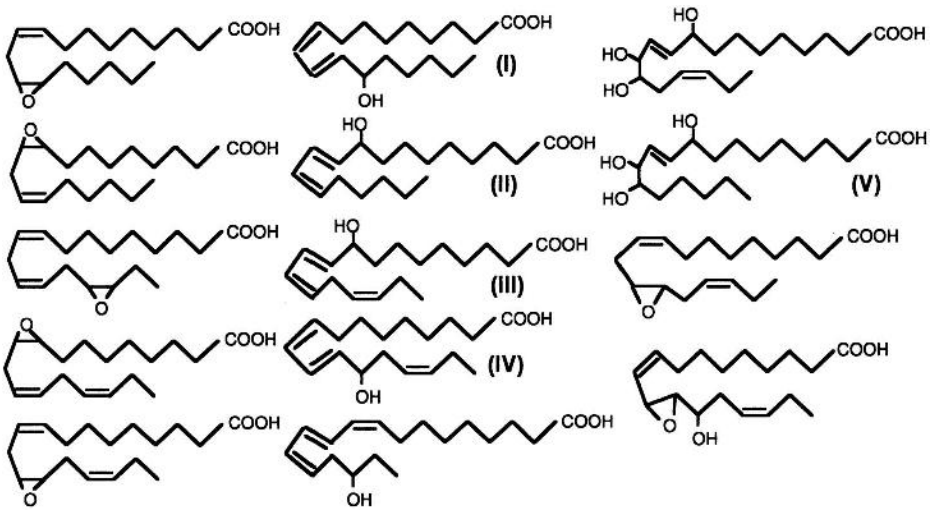


Figure 2 Structures of antifungal oxylipins isolated from rice leaves. The compounds described in the text are numbered. (From Refs. 24–27.)

rice blast fungus *Magnaporthe grisea* (*Pyricularia oryzae*), several research groups have identified terpenoid phytoalexins, oryzalexins [21] and momilactones [22], and a flavonoid, sakuranetin [23]. On the other hand, Kato and co-workers searched for antifungal compounds that were distinct from these and found various oxylipins that exhibited antifungal activity [24–26]. They extracted compounds with acetone from rice leaves of three varieties that exhibit different responses to *M. grisea* race 033 and, after sequential extraction with several solvents, obtained four fractions designated A, NA, N, and NN, respectively. These fractions were examined for antifungal activity by determining whether the substance(s) in each fraction was effective in inhibiting germination of the fungus [27]. They found that the antifungal activity of each fraction varied among the three varieties. None of the fractions obtained from extracts of the variety Sasanishiki, which is susceptible to race 033, exhibited an inhibitory effect on fungal germination. However, upon challenge of the fungus, fractions A and NA isolated from the leaves were found to inhibit germination. Interestingly, fractions A and NA extracted from healthy leaves of two other varieties, Fukuyuki and Fukunishiki, which exhibit resistance to this race were found to be inhibitory. From these fractions, they isolated hydroxides and epoxides of 18-carbon fatty acids (Fig. 2).

Another group has also identified defensive oxylipins from rice leaves in which systemic acquired resistance (SAR) was induced. Probenazole (3-allyloxy-1,2-benzisothiazole-1,1-dioxide) has been used widely as an agent to control rice blast and rice bacterial leaf blight [28]. The chemical has no direct toxicity to these pathogens, but rather enhances resistance of the host plant even when inoculated with compatible races of the pathogens [29], indicating that the chemical induces SAR. Probenazole was applied to rice leaves and the plant was subsequently challenged with *Pyricularia oryzae* (*M. grisea*). Thereafter, anticonidial germination compounds effective against the blast fungus were isolated from the leaves [30]. Through chemical analysis, the compounds were identified as 13-hydroxy-9(*Z*),11(*E*),15(*Z*)-octadecatrienoic acid (13-HOTE) (Fig. 2, compound IV) and 9-hydroxy-10(*E*),12(*Z*),15(*Z*)-octadecatrienoic acid (9-HOTE) (Fig. 2, compound III) [31]. These compounds were also found to suppress the growth of a wide variety of phytopathogenic fungi and bacteria. In addition, it was shown that α-linolenic acid itself exhibited anticonidial germination activity against the blast fungus. These results imply that the SAR induced by probenazole involves the function of the lipoxygenase pathway, for producing antifungal oxylipins. Recently, a probenazole-inducible gene, designated *PBZ1*, was cloned from rice [32]. The PBZ1 protein shares homology with intracellular pathogenesis-related (IPR) proteins. These data suggest some role of the protein in the disease-resistance response against the rice blast fungus. However, no relationship between this protein and the lipoxygenase pathway has been noted.

Li et al. [33] demonstrated that rapid accumulation of antifungal oxylipins, 13-hydroperoxy-9(*Z*),11(*E*)-octadecadienoic acid (13-HPODE) and 13-hydroxy-9(*Z*),11(*E*)-octadecadienoic acid (13-HODE) (Fig. 2, compound I), occurs after inoculation of rice plants with the blast fungus. The levels of these compounds increased rapidly to a plateau within 24 hr postinoculation and production of the diterpenoid phytoalexin momilactone A began 24 hr postinoculation. Interestingly, they also showed that exogenous application of linoleic or linolenic acid derivatives stimulated accumulation of momilactone A in rice leaves. These data imply that some oxylipins other than jasmonate function as signaling molecules to activate phytoalexin accumulation. This possibility has not been elucidated further in this case. Recently, Bate and Rothstein [34] showed that C6 volatiles derived from the lipoxygenase pathway induce a subset of defense-related genes in *Arabidopsis.*

Jasmonic acid has also been identified as an antifungal substance from wild rice, *Oryza officinalis* [35]. It was reported that jasmonic acid inhibited germination of 100% of the spores of the blast fungus at 250 ppm, 86% at 200 ppm, and 10% at 100 ppm, and that appressorium formation among germinated spores was inhibited by 30% at 100 ppm. Whether it actually functions as an antifungal substance in vivo is questionable (see Sec. 3.3).

These lines of evidence suggest that oxylipins play a role in suppressing the growth of the blast fungus in rice leaves as defensive compounds and/or as signaling compounds which promote further accumulation of other phytoalexins. Whether these oxylipins function in plant defense in vivo, however, remains to be tested.

3.2 Mechanisms of Antifungal Oxylipin Synthesis

A pathway of biosynthesis of some oxylipins, especially hydroperoxides and epoxides of unsaturated fatty acids, has been proposed. Ohta and co-workers [36] proposed that lipid hydroperoxides are produced from either linoleic or linolenic acid through the action of lipoxygenase and then converted enzymatically to hydroxy derivatives, and a search for lipid hydroperoxide-decomposing activity in rice seeds was conducted. When 9-hydroperoxy-10(*E*),12(*Z*)-octadecadienoic acid (9-HPODE) was used as the substrate, two distinct fractions obtained following ion-exchange chromatography were found to exhibit such activity. The enzymes in these fractions transformed the substrate to 9-hydroxy-10(*E*),12(*Z*)-octadecadienoic acid (9-HODE) (Fig. 2 compound II) and 9,12,13-trihydroxy-10-octadecenoic acid (Fig. 2, compound V), which are antifungal oxylipins among those identified by Kato and co-workers [24–26]. As pointed out by Hamberg and Fahlstadius [37], the enzyme responsible for such a transformation must be a peroxygenase. In fact, Ohta et al. identified a rice seed peroxygenase that is responsible for the lipid-decomposing activity (unpublished data). The proposed mechanism of synthesis of 9-HODE and 9,12,13-trihydroxy-10-octadecenoic acid is shown in Figure 3. The proposed

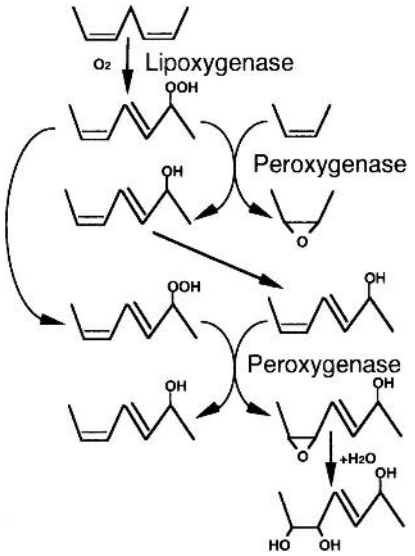


Figure 3 Proposed mechanism of synthesis of antifungal oxylipin by rice peroxygenase.

scheme explains the means of biosynthesis of only some of the antifungal oxylipins found by Kato and co-workers [24–26]. Further studies should be done using various peroxygenase substrates.

Although these experiments were carried out using rice seed embryos, the results obtained led to a search for lipoxygenase and peroxygenase activities in leaves relevant to defense mechanisms, as described below.

3.3 Lipoxygenase Pathway Enzymes in Defense Mechanisms

3.3.1 Induction of Lipoxygenase and Peroxygenase Activities by Fungal Infection

The level of lipoxygenase activity in rice leaves has been found to increase in the case of an incompatible combination, but not in the case of a compatible combination of host and blast fungus [38]. The fifth leaves of rice plants of the variety Aichiasahi that is resistant to race 131 of the rice blast fungus (*M. grisea*) were inoculated with this race (Fig 4a). An increase in lipoxygenase activity to a significantly high level was observed, whereas a much weaker increase in lipoxygenase activity was seen in the leaves when inoculated with a compatible race, 007 (Fig. 4b). An obvious increase in the level of a lipoxygenase called leaf LOX-3 was observed following fractionation by means of an ion-exchange column. Lipoxygenases found in other fractions also showed slight increases in activity. Enhanced lipoxygenase activity upon infection with the blast fungus in rice cultivars carrying either of the blast fungus resistance genes, *Pi-a* or *Pi-i*, has also been reported by Namai et al. [4]. As they did not take into account the nonlinearity of the lipoxygenase reaction [39] in their activity measurement, quantitative discussion must be limited to a certain degree.

The leaf LOX-3 preferentially produced 13-HPODE rather than 9-HPODE. The optimum pH for activity was pH 5.0. The enzyme was found to prefer free fatty acids and linoleic and linolenic acid over methylated ones as substrates.

Peroxygenase activity measured using 13-HPODE as substrate also increased significantly in the case of an incompatible combination of host and pathogen, but moderately in the case of a compatible combination (Fig. 4c).

Taken together with the results of Li et al. [33] and Ohta et al. [38], it is plausible that the coordinate increase in lipoxygenase and peroxygenase activities upon infection with an incompatible race of the blast fungus involves the synthesis of antifungal oxylipins, resulting in suppression of fungal growth by oxylipins at sites of ingress.

3.3.2 Involvement of Chloroplasts in Oxylipin Synthesis

To examine the molecular basis of antifungal oxylipin synthesis, the gene coding for a pathogen-inducible lipoxygenase has been cloned [40]. Young leaves of rice plants of the variety Aichiasahi were infected with *M. grisea* race 131, which is incompatible with this rice plant, and RNA extracted from the leaves was then used to produce cDNA clones. Using a mixed oligonucleotide probe, prepared based on a peptide sequence conserved in plant and animal lipoxygenases, a lipoxygenase cDNA was isolated. Finally, a full-length cDNA clone was isolated and sequenced. The gene was found to encode a novel lipoxygenase that has a putative transit peptide for targeting to chloroplasts. Targeting of the lipoxygenase to chloroplasts was demonstrated by in vitro experiments, in which the S^{35}-labeled precursor and isolated spinach chloroplasts were mixed (Shirano and Shibata,

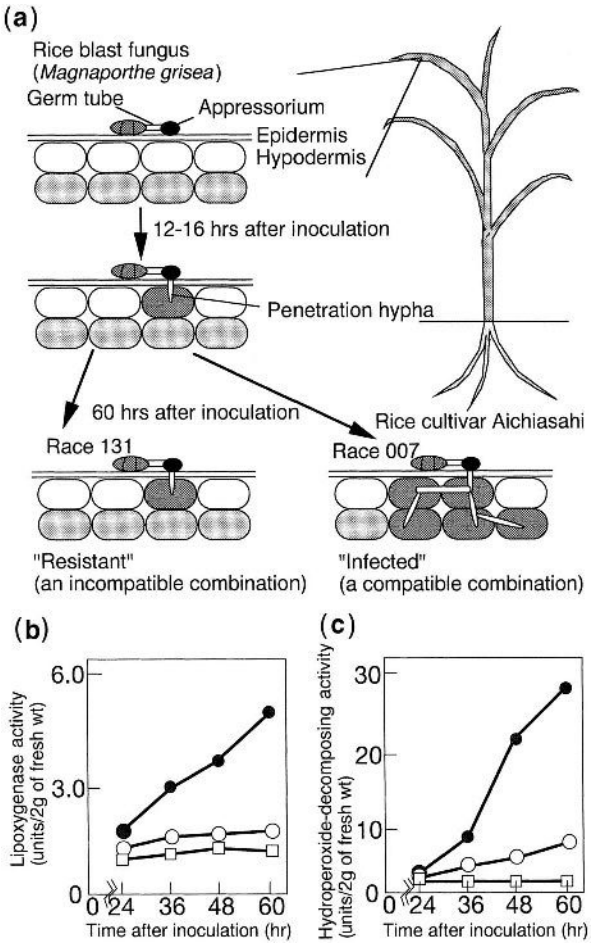


Figure 4 Induction of lipoxygenase and peroxygenase activities upon inoculation of rice seedlings with the rice blast fungus. Ingress of the rice blast fungus (race 131) into a rice leaf (Aichiasahi variety) is illustrated schematically (a). The young leaves of rice seedlings were inoculated with race 131 (incompatible) (closed circle), race 007 (compatible) (open circle), or mock inoculated (open square). Lipoxygenase activity (b) and peroxygenase activity (c) were measured. (From Ref. 38.)

unpublished data). The gene was designated *Lox2:Os:1* according to the proposed rule for lipoxygenase nomenclature [41].

The enzymatic characteristics of the chloroplast lipoxygenase are consistent with those of leaf LOX-3 identified in a previous study by Ohta et al. [38]. The protein was expressed in *Escherichia coli* in an active form by the low-temperature cultivation method [42]. The enzyme introduces molecular oxygen into the 13-position of either linoleic or linolenic acid. The regiospecificity of the lipoxygenase has been re-examined in detail [43].

The lipoxygenase gene is not expressed in young healthy leaves at all, but is induced upon infection with an incompatible race of the blast fungus. Compatible infection also

induces expression of the gene, but to a much lower degree. The expression profile is consistent with the appearance of the protein and the enzyme activity, indicating that expression of the lipoxygenase is controlled at the level of transcription.

The preferential incorporation of oxygen into the 13-position of linolenic acid by Lox2:Os:1 may suggest that the lipoxygenase is involved in synthesis of jasmonate, as 13-HPOTE is the precursor of this oxylipin (Fig. 1). This view may be supported by the finding that there are high amounts of linolenic acid in glycolipids of the thylakoid membrane and the finding that stress-dependent fatty acid release occurs in spinach chloroplasts, as shown by Sakaki et al. [44]. Transgenic *Arabidopsis* plants expressing antisense sequences of a chloroplastic lipoxygenase gene, *AtLOX2*, have been shown to exhibit lower levels of production of jasmonic acid [45]. However, the competition among enzymes that use lipoxygenase reaction products as substrates (i.e., hydroperoxide lyase, peroxygenase, and allene oxide synthase) should be taken into account for the production of various oxylipins. These enzymes are known to be located in chloroplasts in several plant species [46].

3.3.3 Does Jasmonate Play a Role in Phytoalexin Synthesis in Rice?

To elucidate the role of jasmonate in phytoalexin synthesis, the effects of jasmonate and an elicitor on production of the phytoalexin momilactone A were examined in suspension-cultured rice cells [47]. Exogenous application of jasmonate induced the production of momilactone A. Upon addition of the elicitor *N*-acetylchitoheptaose, the rice cells were found to accumulate endogenous jasmonate prior to accumulation of momilactone A. A synergistic effect of exogenously applied jasmonate on the elicitor-mediated accumulation of this phytoalexin was also observed. Jasmonate and its amino acid conjugates also induce the flavonoid phytoalexin sakuranetin in rice leaves [48]. This phytoalexin exhibits strong toxicity against the rice blast fungus; the ED_{50} for inhibition of spore germination of the fungus is 15 ppm and that for inhibition of germ tube growth is 5 ppm. Although sakuranetin is absent from healthy leaves, it was detected in a leaf extract upon infection with the fungus. The content in a resistant cultivar after infection was much higher than that in a susceptible cultivar.

LOX2:Os:1 may not be involved in the jasmonate production elicited by *N*-acetylchitoheptaose in suspension-cultured cells, because the gene is not activated in suspension-cultured cell lines by *N*-acetylchitoheptaose or other elicitors (Shirano and Shibata, unpublished data).

In contrast to the results obtained using suspension-cultured cells, Schweizer et al. [19] demonstrated that rice plants attacked by the rice blast fungus did not accumulate endogenous jasmonate. However, jasmonate-inducible pathogenesis-related (PR) gene transcripts were accumulated. These results suggest that accumulation of jasmonate is not necessary for induction of PR genes and that jasmonate does not orchestrate localized defense responses in pathogen-attacked rice. They speculate that jasmonate functions in a signaling network embedded in another pathogen-induced pathway(s) and may be required at a certain minimal level for induction of some PR genes. Healthy leaves contain several lipoxygenase species. Therefore, it may not be surprising that these lipoxygenases are involved in jasmonate synthesis to a certain low extent immediately after pathogen attack. The concomitant increase in peroxygenase activity with that of *LOX2:Os:1* as described earlier may compete with the jasmonate synthesis pathway, resulting in low-level production of jasmonate. Hause et al. [18] also showed that in barley leaf cells,

jasmonates do not act as a signal during compatible or incompatible interactions with the powdery mildew fungus (*Erysiphe graminist* f. sp *hordei*).

To elucidate the role of lipoxygenase functions in defensive mechanisms, characterization of all lipoxygenases at the molecular level is crucial. At least three lipoxygenases, L-1, L-2, and L-3, have been found in rice seedlings and characterized [49,50], and the L-2 gene (*Loxl:Os:2*) has been cloned [51]. Differential induction of lipoxygenase isoforms is seen in wheat upon treatment with a rust fungus elicitor such as chitin oligosaccharides, chitosan, or methyl jasmonate [3]. Perhaps a molecular genetic approach, such as antisense-gene suppression or gene knockout might serve as critical tests of lipoxygenase functions in host–pathogen interactions.

4 MULTIPLICITY OF PLANT LIPOXYGENASE FUNCTIONS IN DEFENSE

A multiplicity of plant lipoxygenases, as well as divergent functions of the lipoxygenase pathway in plant physiology, makes interpretation of their functions in plant defense elusive. In this section, we describe the multiplicity of soybean lipoxygenases and refer to the discussion by Slusarenko [12] of bean lipoxygenase functions in plant defense. We also describe a photosynthetic cultured soybean cell line useful as a tool for studying plant defense mechanisms, including light-dependent jasmonate-inducible gene function. The use of this cell line may simplify the elucidation of lipoxygenase-related phenomena.

4.1 Multiple Lipoxygenases in Defense

Among plant and mammalian lipoxygenases, soybean lipoxygenase L-1, found in mature seeds, has been most extensively investigated biochemically [52,53]. In contrast, less attention had been paid to lipoxygenases in vegetative tissues. Kato et al. [54] demonstrated the appearance of new lipoxygenases in soybean cotyledons after germination, which were distinct from the seed lipoxygenases, L-1, L-2, and L-3. These enzymes were designated L-4, L-5, and L-6. The differential expression of wound-inducible lipoxygenase genes, *LOX7* and *LOX8*, in soybean leaves has also been reported [55]. Except for the L-5 gene, all of the other lipoxygenase genes have been cloned [56–60].

Expression of the lipoxygenase L-4 gene is induced in leaves when methyl jasmonate is exogenously applied [61]. Jasmonate-mediated induction of lipoxygenases is also reported to occur in soybean [62], *Arabidopsis* [63,64], tobacco [65], tomato [66,67], maize [68], and barley [69,70]. Are these lipoxygenases involved in further synthesis of jasmonate? What is the role of these lipoxygenases? It has been shown that L-4 is a one of the vegetative storage proteins (VSPs) [61,71]. As the K_m values for L-4 acting on linolenic acid (the jasmonate precursor) or linoleic acid as substrate are 10 m*M* and 0.79 m*M*, respectively, it is unlikely that L-4 actually functions in the peroxidation of free fatty acids. Most of the jasmonate-inducible lipoxygenases of other plant species have not been characterized in terms of substrate specificity and enzymological properties. Therefore, it is difficult to assess their physiological roles. Recently, Wang et al. [72] showed that lipoxygenases L-1 to L-6 are not involved in lipid metabolism by demonstrating the absence of these proteins in lipid bodies.

The inducibility of the soybean lipoxygenase L-4 gene by methyl jasmonate is suggestive of its involvement in plant defense. Hildebrand et al. [73,74] showed that a large

increase in lipoxygenase activity occurs upon wounding of soybean leaves and variable increases occur due to feeding by the two-spotted spider mite. Although this study did not examine the molecular species of lipoxygenases in soybean leaves, it seems likely that wounding and mite infestation result in induction of the L-4 gene, probably via jasmonate signaling.

To our knowledge, analysis of the expression of soybean lipoxygenase genes in actual host–pathogen systems has not been reported. However, Slusarenko's laboratory has worked on the role of bean (*Phaseolus vulgaris*) lipoxygenases in plant defense mechanisms [9,12]. Bean also shows a multiplicity of lipoxygenases [75]. In the leaves of bean, the spatial and temporal accumulation of defense gene transcripts, including lipoxygenase transcripts, has been examined in relation to bacterial-induced hypersensitive cell death [9]. Bean leaves were inoculated with virulent or avirulent isolates of *Pseudomonas syringae* pv. *Phaseolicola*, or saprophytic *P. fluorescens*. Accumulation of phenylalanine ammonia-lyase, chalcone synthase, and chitinase transcripts was found to be more rapid and generally of greater magnitude in the case of incompatible interaction as compared to compatible interaction and was observed essentially only in the inoculated zone of the leaf. In contrast, lipoxygenase transcripts did not accumulate at the inoculation site itself in the incompatible interaction compared to the relatively strong induction observed in the case of the compatible interaction. Extensive accumulation of lipoxygenase transcripts was seen in the case of both the compatible and incompatible interactions, in areas other than the inoculation site, starting about 14 hr postinoculation. The authors noted that the pattern of accumulation of lipoxygenase transcripts observed in the hypersensitive response has features in common which might be expected for a VSP form of the enzyme. In response to pathogen stress, a VSP form of lipoxygenase might be induced to accumulate, except in tissues where cells are fully switched over to defense metabolism in the incompatible interaction [9].

Slusarenko [12] argues that the legume system with its multiplicity of lipoxygenase genes and recalcitrance to genetic transformation is not ideally suited to study the regulation of the lipoxygenase response to pathogens at the molecular level. Transformation of legume plants including *Medicago sativa* [76] and *Lotus japonica* [77], however, was reported. These plants may be useful for investigating the role of the legume lipoxygenase pathway in plant defense, even if these plants also show a multiplicity of lipoxygenases. Gene isolation and establishment of host–pathogen combinations will be needed for further studies.

4.2 Photosynthetic Cultured Soybean Cells as a Tool for Investigating Plant Defense

Nonphotosynthetic cultured cells of soybean have been used for investigating plant defense mechanisms [78]. However, these cells cannot be used for examining light-dependent gene expression, whereas light is required for induction of plant defense in some pathosystems [79] and for activation of some jasmonate-inducible genes [80]. Here, we describe a photosynthetic cultured cell line, SB-P, as a useful tool for analysis of light-dependent defense mechanisms and the link between the lipoxygenase pathway and synthesis of a major phytoalexin, glyceollin.

The photosynthetic cultured soybean cell line, SB-P, was established by Horn et al. [81]. The cells develop chloroplasts well under light illumination conditions and lose chlorophyll during dark adaptation by dedifferentiation to amyloplasts [82]. Therefore,

the SB-P cells are useful for dissecting the mechanisms involved in jasmonate-inducible gene expression, especially the light-dependent aspects.

A jasmonate-inducible gene, *cyp93A1*, was isolated from the SB-P cells by the differential display method [83]. It encodes a novel member of the cytochrome P-450 family that shares relatively low homology with previously identified plant cytochrome P-450s. The expression of this gene in the soybean photosynthetic cultured cell line SB-P has been studied in comparison with that of the lipoxygenase L-4 gene [84]. For high-level induction of *cyp93A1*, 30 μ*M* methyl jasmonate was required, whereas 3 μ*M* was sufficient for induction of lipoxygenase L-4 transcripts. The induction of *cyp93A1* was slower than that of the L-4 gene (Fig. 5b).

Elicitors were found to induce expression of the *cyp93A1* gene, but not the L-4 gene [84]. These elicitors include yeast extracts and fungal elicitors isolated from carbohydrates of the cell-wall fraction from a fungal pathogen, *Phytophthora megasperma* f. sp. *Glycinea*. These elicitors are known to induce soybean phytoalexins [85]. Elicitor-induced cytochrome P-450s were isolated from soybean also by the differential display method [86]. The authors indicated that CYP93Al is a dihydroxypterocarpan 6a-hydroxylase (D6aH) involved in the biosynthesis of soybean phytoalexins, the glyceollins [87] (Fig. 5a).

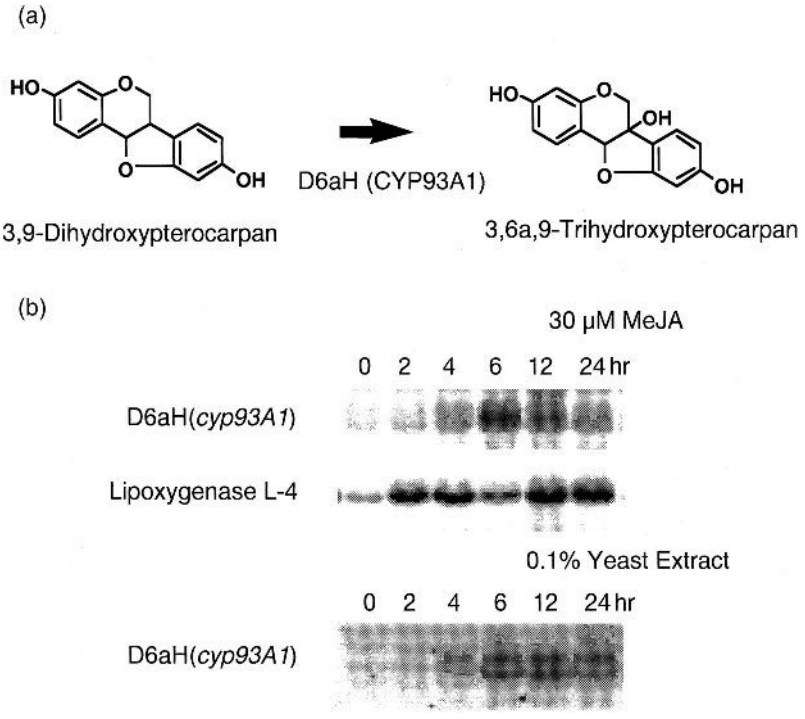


Figure 5 Reaction catalyzed by the cytochrome P-450 gene *cyp93A1* and the induction by methyl jasmonate and elicitor. The soybean phytoalexins, glyceollins, are synthesized via the phenylpropanoid pathway. CYP93A1 (dihydroxypterocarpan 6a-hydroxylase, D6aH) catalyzes the almost final step of glyceollin synthesis (a). The P-450 gene *cyp93A1* is activated by either methyl jasmonate (MeJA) or elicitor (b). (From Ref. 84.)

A characteristic feature of the methyl jasmonate-mediated expression of the P-450 gene *cyp93A1* is its light dependence [84]. For methyl jasmonate-mediated expression of *cyp93A1*, irradiation of the photosynthetic cells with light is required. Trifluoperazine, an inhibitor of electron transport in photosystem II, and 3-(3,4-dichlorophenyl)-1, 1-dimethyl urea (DCMU), another inhibitor specific to chloroplast electron transport, were shown to suppress induction of the gene by methyl jasmonate. In contrast, the L-4 gene was induced by methyl jasmonate in the darkness and its induction was not inhibited by these chemicals. *Atvsp*, an *Arabidopsis* homolog of soybean *Vsp* also requires light illumination for jasmonate-mediated gene expression [88]. These results show that there are two distinct signaling pathways for jasmonate-inducible gene expression: a light-independent pathway for the lipoxygenase L-4 gene and a light-dependent pathway for *cyp93A1*. Moreover, elicitor-induced expression of the P-450 gene is not inhibited by an electron-transport inhibitor, showing that the pathways for jasmonate- and elicitor-induced expression of the *cyp93A1* gene are also different. These results indicate that jasmonate can partly mimic the elicitor-induced resistance response of plants, but they do not share the same pathway.

Use of the photosynthetic suspension cultured cell line, SB-P, might be a powerful approach for dissecting the multiplicity of the lipoxygenase pathway, especially the aspects involving light-dependent gene expression. If particle bombardment for gene transfer can be used in the case of SB-P cells, this may serve as a good system for studies of the lipoxygenase pathway using molecular genetic approaches. Gene transfer into nonphotosynthetic calli of soybean by bombardment is reported to be successful [89].

5 CONCLUSIONS

Much information has been accumulated concerning mechanisms of plant defense against the rice blast fungus *M. grisea* as described in this chapter. Upon inoculation of rice plants with this fungus, a series of resistance responses is seen, including activation of lipoxygenase and peroxygenase [38,40], and accumulation of antifungal phytoalexins [21–23], including oxylipins [24–28]. These observations support the view that the lipoxygenase pathway plays a role in orchestrating the production of antifungal oxylipins. However, the involvement of jasmonates in the mechanisms of defense against the blast fungus remains elusive [19]. The possible involvement of certain oxylipins other than jasmonates in the production of phytoalexins, as suggested by Li et al. [33], needs to be proven. Crucial tests must be conducted to test these hypotheses. Molecular genetic approaches will be useful for these tests.

Rice is an ideal monocot plant for investigation of host–pathogen interactions at the molecular level. Exceptional among cereal crops, rice is easily transformed by *Agrobacterium*-mediated protocols [90]. Recently, transposon-mediated knockout has become feasible [91]. An expansion of expressed sequence tag (EST) databases of rice [92] may allow us to easily identify plant defense gene homologs isolated in other pathosystems. Definitely, the rice genome sequencing project currently ongoing [93] will also help such approaches. In addition to these advantages, well-established host–pathogen systems for compatible and incompatible interactions are also available in rice.

The multiplicity of lipoxygenase and also the divergent functions of the lipoxygenase pathway from development to plant defense must be the cause of the difficulty encountered in interpretation of defense responses and other physiological phenomena observed. The major lipoxygenase L-4 in vegetative tissues is induced by methyl jasmonate, and it has a role as a VSP [61]. As pointed out by Slusarenko [12], a VSP form of bean

lipoxygenase might be induced in response to pathogen stress. Photosynthetic cultured cells such as the SB-P line might be useful for dissecting the multiplicity of the functions of the lipoxygenase pathway.

Many questions concerning the actual functions of the various oxylipins in plant defense still remain to be answered. In addition to those described in this chapter, other relevant topics include direct membrane oxidation during hypersensitive responses [9,94] and release of lipoxygenase substrates by lipases [95]. Recent progress in plant functional genomics provides a means of analysis of genomewide gene expression (i.e., DNA microarray technology). This will be useful for elucidating the function of the lipoxygenase pathway under various defense conditions.

REFERENCES

1. H Yamamoto, T Tani. J Phytopathol 116:329–337, 1986.
2. CA Ocampo, B Moerschbacher, HJ Grambow. Z Naturforsch 41:559–563, 1986.
3. C Bohland, T Balkenhohl, G Loers, I Feussner, HJ Grambow. Plant Physiol 114:679–685, 1997.
4. T Namai, T Kato, Y Yamaguchi, Y Togashi. Ann Phytopathol Soc Japan 56:26–32, 1990.
5. H Ohta, K Shida, Y-L Peng, I Furusawa, J Shishiyama, S Aibara, Y Morita. Plant Physiol 97:94–98, 1991.
6. SA Avdiushko, XS Ye, DF Hildebrand, J Kuc. Physiol Mol Plant Pathol 42:83–95, 1993.
7. E Koch, BM Meier, H-G Eiben, A Slusarenko. Plant Physiol 99:571–576, 1992.
8. KP Croft, CR Voisey, AJ Slusarenko. Physiol Mol Plant Pathol 36:49–62, 1990.
9. BM Meier, N Shaw, AJ Slusarenko. Mol Plant–Microbe Interact 6:453–466, 1993.
10. MA Melan, X Dong, KR Davis, FM Ausubel, TK Peterman. Plant Physiol 101:441–450, 1993.
11. C Véronési, M Rickauer, J Fournier, ML Pouénat, MT Esquerré-Tugayé. Plant Physiol 112: 997–1004, 1996.
12. AJ Slusarenko. In: G Piazza, ed. Lipoxygenase and Lipoxygenase Pathway Enzymes. Champaign, IL: AOCS Press, 1996, pp 176–197.
13. M McConn, J Browse. Plant Cell, 8:403–416, 1996.
14. P Vijayan, J Shockey, CA Levesque, RJ Cook, J Browse. Proc Natl Acad Sci USA 95:7209–7214, 1998.
15. PE Staswick, GY Yuen, CC Lehman. Plant J 15:747–754, 1998.
16. BPHJ Thomma, K Eggermont, IAMA Penninckx, B Mauch-Mani, R Vogelsang, BPA Cammue, WF Broekaert. Proc Natl Acad Sci USA 95:15,107–15,111, 1998.
17. I Rance, J Fournier, MT Esquerre-Tugaye. Proc Natl Acad Sci USA 95:6554–6559, 1998.
18. B Hause, KH Kogel, B Parthier, C Wasternack. J Plant Physiol 150:127–132, 1997.
19. P Schweizer, A Buchala, P Silberman, M Seskar, I Raskin, J-P Métraux. Plant Physiol 114: 79–88, 1997.
20. H Weber, A Chetelat, D Caldelari, EE Farmer. Plant Cell 11:485–494, 1999.
21. H Sekido, T Endo, R Suga, O Kodama, T Akatsuka, Y Kono, S Takeuchi. J Pestcide Sci 11: 369–372, 1983.
22. D Cartwright, P Langcake, RJ Pryce, DP Leworthy. Nature 267:511–513, 1977.
23. O Kodama, J Miyakawa, T Akatsuka, S Kiyosawa. Phytochemistry 31:3807–3809, 1992.
24. T Kato, Y Yamaguchi, T Uyehara, T Yokoyama, T Namai, S Yamanaka. Tetrahedron Lett 24:4715–4718, 1983.
25. T Kato, Y Yamaguchi, T Hirano, T Yokoyama, T Uyehara, T Namai, S Yamanaka, N Harada. Chem Lett 409–412, 1884.
26. T Kato, Y Yamaguchi, N Abe, T Uyehara, T Nakai, M Kodama, Y Shiobara. Tetrahedron Lett 26:2357–2360, 1985.
27. T Kato, Y Yamaguchi, T Namai, T Hirukawa. Biosci Biotechnol Biochem 57:283–287, 1993.

28. T Watanabe, H Igarashi, K Matsumoto, S Seki, S Mase, Y Sekizawa. J Pestcide Sci 2:291–296, 1977.
29. M Iwata, Y Suzuki, S Watanabe, S Mase, Y Sekizawa. Ann Phytopathol Soc Japan 46:297–306, 1980.
30. M Shimura, M Iwata, N Tashiro, Y Sekizawa, Y Suzuki, S Mase, T Watanabe. Agric Biol Chem 45:1431–1435, 1981.
31. M Shimura, S Mase, M Iwata, A Suzuki, T Watanabe, Y Sekizawa, T Sasaki, K Furihata, H Seto, N Otake. Agric Biol Chem 47:1983–1989, 1983.
32. N Midoh, M Iwata. Plant Cell Physiol 37:9–18, 1996.
33. WX Li, O Kodama, T Akatsuka. Agri Biol Chem 55:1041–1047, 1991.
34. NJ Bate, SJ Rothstein. Plant J 16:561–569, 1998.
35. GC Neto, Y Kono, H Hyakutake, M Watanabe, Y Suzuki, A Sakurai. Agric Biol Chem 55: 3097–3098, 1991.
36. H Ohta, K Shida, Y-L Peng, I Furusawa, J Shishiyama, S Aibara, Y Morita. Plant Cell Physiol 31:1117–1122, 1990.
37. M Hamberg, P Fahlstadius. Plant Physiol 99:987–995, 1992.
38. H Ohta, K Shida, Y-L Peng, I Furusawa, J Shishiyama, S Aibara, Y Morita. Plant Physiol 97:94–98, 1991.
39. B Axelrod, TM Cheesbrough, S Laakso. Methods Enzymol 71:441–451, 1981.
40. Y-L Peng, Y Shirano, H Ohta, T Hibino, K Tanaka, D Shibata. J Biol Chem 269:3755–3761, 1994.
41. D Shibata, A Slusarenko, R Casey, D Hildebrand, E Bell. Plant Mol Biol Rep 12:S41–S42, 1994.
42. Y Shirano, D Shibata. FEBS Lett 271:128–130, 1990.
43. L-Y Zhang, M Hamberg. Lipids 31:803–809, 1996
44. T Sakaki, K Saito, A Kawaguchi, N Kondo, M Yamada. Plant Physiol 94:766–772, 1990.
45. E Bell, RA Creelman, JE Mullet. Proc Natl Acad Sci USA 92:8675–8679, 1995.
46. E Blée, J Joyard. Plant Physiol 110:445–454, 1996.
47. H Nojiri, M Sugimori, H Yamane, Y Nishimura, A Yamada, N Shibuya, O Kodama, N Murofushi, T. Omori. Plant Physiol 110:387–392, 1996.
48. S Tamogami, R Rakwal, O Kodama. FEBS Lett 20:239–242, 1997.
49. H Ohta, S Ida, B Mikami, Y Morita. Plant Cell Physiol 27:911–918, 1986.
50. H Ohta, S Ida, B Mikami, Y Morita. Agric Biol Chem 50:3165–3171, 1986.
51. H Ohta, Y Shirano, K Tanaka, Y Morita, D Shibata. Eur J Biochem 206:331–336, 1992.
52. D Shibata. In: G Piazza, ed. Lipoxygenase and Lipoxygenase Pathway Enzymes. Champaign, IL: AOCS Press, 1996, pp 39–55.
53. D Shibata, B Axelrod. J Lipid Mediators Cell Signal 12:213–228, 1995.
54. T Kato, H Ohta, K Tanaka, D Shibata. Plant Physiol 98:324–330, 1992.
55. DM Saravitz, JN Siedow. Plant Physiol 110:287–299, 1996.
56. D Shibata, J Steczko, JE Dixon, M Hermodoson, R Yazdanparast, B Axelrod. J Biol Chem 262:10,080–10,085, 1987.
57. D Shibata, J Steczko, JE Dixon, PC Andrews, M Hermodoson, B Axelrod. J Biol Chem 263: 6816–6821, 1988.
58. RL Yenofsky, M Fine, C Liu. Mol Gen Genet 211:215–222, 1988.
59. D Shibata, T Kato, K Tanaka. Plant Mol Biol 16:353–359, 1991.
60. T Kato, J Terao, D Shibata. Biosci Biotechnol Biochem 56:1344, 1992.
61. T Kato, Y Shirano, H Iwamoto, D Shibata. Plant Cell Physiol 34:1063–1072, 1993.
62. TK Park, MA Holland, JG Laskey, JC Polacco. Plant Sci 96:109–117, 1994.
63. E Bell, JE Mullet. Mol Gen Genet 230:456–462, 1991.
64. E Bell, JE Mullet. Plant Physiol 103:1133–1137, 1993.
65. S Avdiushko, KP Croft, GC Brown, DM Jackson, TR Hamilton-Kemp, D Hildebrand. Plant Physiol 109:1227–1230, 1995.

66. T Heitz, DR Bergey, CA Ryan. Plant Physiol 114:1085–1093, 1997.
67. N Beaudoin, SJ Rothstein. Plant Mol Biol 33:835–846, 1997.
68. AB Jensen, E Poca, M Rigaud, G Freyssinet, M Pages. Plant Mol Biol 33:605–614, 1997.
69. J Rouster, R Leah, J Mundy, V Cameron-Mills. Plant J 11:513–523, 1997.
70. K Voros, I Feussner, H Kühn, J Lee, M Lobler, B Parthier, C Wasternack. Eur J Biochem 251:36–44, 1998.
71. TJ Tranbarger, VR Franceschi, DF Hildebrand, HD Grimes. Plant Cell 3:973–987, 1991.
72. C Wang, KPC Croft, U Järlfors, DF Hildebrand. Plant Physiol 120:227–235, 1999.
73. DF Hildebrand, JG Rodriguez, CS Legg, GC Brown, G Bookjans. Z Naturforsch 44:655–659, 1989.
74. DF Hildebrand, JG Rodriguez, GC Brown, KT Luu, CS Volden. J Econ Entomol 79:1459–1465, 1986.
75. HG Eiben, AJ Slusarenko. Plant J 5:123–135, 1994.
76. S Austin, ET Bingham, RG Koegel, DE Mathews, MN Shahan, RJ Straub, RR Burgess. Ann NY Acad Sci 721:234–244, 1994.
77. P Oger, A Petit, Y Dessaux. Plant Sci 116:159–168, 1996.
78. VK Rajasekhar, C Lamb, RA Dixon. Plant Physiol 120:1137–1146, 1999.
79. A Guo, PJ Reimers, JE Leach. Physiol Mol Plant Pathol 42:413–425, 1993.
80. A Sadka, DB DeWald, GD May, WD Park, JE Mullet. Plant Cell 6:737–749, 1994.
81. ME Horn, JH Sherrard, JM Widholm. Plant Physiol 72:426–429, 1983.
82. MA Gillott, G Erdös, DE Buetow. Plant Physiol 96:962–970, 1991.
83. G Suzuki, H Ohta, T Kato, T Igarashi, F Sakai, D Shibata, A Takano, T Masuda, Y Shioi, K Takamiya. FEBS Lett 383:83–86, 1996.
84. H Ohta, G Suzuki, K Awai, T Masuda, T Kato, D Shibata, K Takamiya. Physiol Plant 100: 647–652, 1997.
85. M Yoshikawa. Nature 275:546–547, 1978.
86. CR Schopfer, J Ebel. Mol Gen Genet 258:315–322, 1998.
87. CR Schopfer, G Kochs, F Lottspeich, J Ebel. FEBS Lett 432:182–186, 1998.
88. S Berger, E Bell, A Sadka, JE Mullet. Plant Mol Biol 27:933–942, 1995.
89. MZ Hadi, MD McMullen, JJ Finer. Plant Cell Rep 15:500–505, 1996.
90. Y Hiei, T Komari, T Kubo. Plant Mol Biol 35:205–218, 1997.
91. H Hirochika. Plant Mol Biol 35:231–240, 1997.
92. K Yamamoto, T Sasaki. Plant Mol Biol 35:135–144, 1997.
93. T Sasaki. Proc Natl Acad Sci USA 95:2027–2028, 1998.
94. Y Kondo, T Miyazawa, J Mizutani. Biochim Biophys Acta 1127:227–232, 1992.
95. S Roy, ML Pouénat, C Caumont, C Cariven, MC Prévost, MT Esquerré-Tugayé. Plant Sci 107:17–25, 1995.

9

Biocatalysis by the Plant Lipoxygenase Pathway

Oxygenated Fatty Acid Production and Hydroperoxide Lyases

HAROLD W. GARDNER*

National Center for Agricultural Utilization Research, Agricultural Research Service, U.S. Department of Agriculture, Peoria, Illinois

ALEXANDER N. GRECHKIN

Kazan Institute of Biochemistry and Biophysics, Russian Academy of Sciences, Kazan, Russia

1 INTRODUCTION

Aspects of the lipoxygenase (LOX) pathway are reviewed with regard to the production of a variety of oxygenated fatty acids. A number of LOX isozymes can catalyze the formation of diverse regiospecific and stereospecific hydroperoxides from naturally occurring polyunsaturated fatty acids. LOX will also oxidize glyceride polyunsaturated fatty acids, as well as several ''contrived'' substrates that mimic essential features of the natural substrate. Such oxidations expand the possibilities for useful biocatalysis.

Oxidation by LOX is only the first step in the plant ''LOX cascade.'' By using LOX-produced hydroperoxide fatty acids as substrates, several hydroperoxide-metabolizing enzymes can be utilized to form a considerable array of polyoxygenated fatty acids and aldehyde cleavage products. The first of these hydroperoxide reactions discussed in this chapter are carbon-chain cleavage reactions. One type of chain cleavage gives products resembling those obtained from homolytic β-scission of alkoxyl radicals. According to current theory, this type of reaction is catalyzed by either (1) LOX itself under conditions of oxygen deprivation or (2) a specific ''homolytic'' hydroperoxide lyase (HOMLS).

* *Current affiliation*: Veteran's Administration Hospital and University of Wisconsin-Madison, Madison, Wisconsin.

Another type of cleavage reaction is termed ''heterolytic'' hydroperoxide lyase (HETLS) because it affords chain cleavage that resembles Hock–Criegee acid cleavage of hydroperoxides. This latter enzyme is widely distributed in the plant kingdom and its action on fatty acid hydroperoxides gives the characteristic green-grassy and cucumberlike odors of wounded plant tissue.

In the following chapter, several other hydroperoxide-metabolizing enzymes are reviewed. One of these enzymes, allene oxide synthase (AOS), forms an unstable allene oxide fatty acid. This enzyme has great potential because the reaction conditions can be manipulated to give diverse products from subsequent nonenzymic reactions of the unstable allene oxide. In addition, the large turnover number and stability of AOS gives tremendous catalytic advantage. Another hydroperoxide-degrading enzyme, divinyl ether synthase, cleaves the carbon chain while inserting an ether bond across the two carbons that were cleaved. Formerly, it was thought that divinyl ether synthase was found only in potato tubers, but there is increasing evidence of its existence in other plants. Peroxygenase and epoxy alcohol synthase convert fatty acid hydroperoxides into their corresponding epoxyhydroxy derivatives; however, their mechanisms of action are quite different. Peroxygenase causes both intramolecular and intermolecular transfer of the distal hydroperoxide oxygen to form epoxides from double bonds while the hydroperoxide is reduced to a hydroxide. In this way, the double bonds of either hydroperoxide fatty acid or ordinary unsaturated fatty acids, like oleic acid, are epoxidized. On the other hand, epoxy alcohol synthase catalyzes only the intramolecular transfer of distal hydroperoxide oxygen to epoxidize the double bond of hydroperoxide fatty acid. In addition, co-oxidation reactions catalyzed by free-radical-initiated reactions of LOX are reviewed in the next chapter.

Finally, in the following chapter, we discuss the various chemical methods utilized to produce a number of oxygenated fatty acids from hydroperoxide fatty acids. The inherent reactivity of the hydroperoxy-diene provides advantage for facile conversions through (1) acid catalysis, (2) base catalysis, (3) alkoxyl-radical-catalyzed rearrangement, and (4) Sharpless-type epoxidation of double bonds.

2 LIPOXYGENASE

2.1 General Properties of LOX

Lipoxygenase is a nonheme iron protein that catalyzes the dioxygenation of methylene-interrupted pentadiene fatty acids to furnish conjugated hydroperoxydiene fatty acids. A large number of recent publications review various aspects of LOX and enzymes that metabolize hydroperoxydiene fatty acid products [1–14]. With LOXs of plant origin, the preferred substrates are mainly the C-18 fatty acids, linoleic acid, or linolenic acid, and usually oxidation occurs with (*S*)-stereospecificity at either C-13 or C-9. For example, linoleic acid can be oxidized to either 13(*S*)-hydroperoxy-9(*Z*),11(*E*)-octadecadienoic acid [13(*S*)-HPODE], 9(*S*)-hydroperoxy-10(*E*),12(*Z*)-octadecadienoic acid [9(*S*)-HPODE], or both. Although plant LOXs do not normally encounter C-20 polyunsaturated fatty acids, they are capable of oxidizing these substrates. Depending on the oxidation specificity of the plant LOXs, oxidation of C-20 fatty acids usually occurs at either C-15, C-8, or C-5, but occasionally either C-11 or C-12 is oxidized. The rate-limiting step is hydrogen removal from the bis-allylic methylene on the opposite side of the fatty acid from O_2 insertion (Fig. 1).

Many plant LOXs have now been cloned and sequenced, and such data have been conveniently tabulated in a recent review [2].

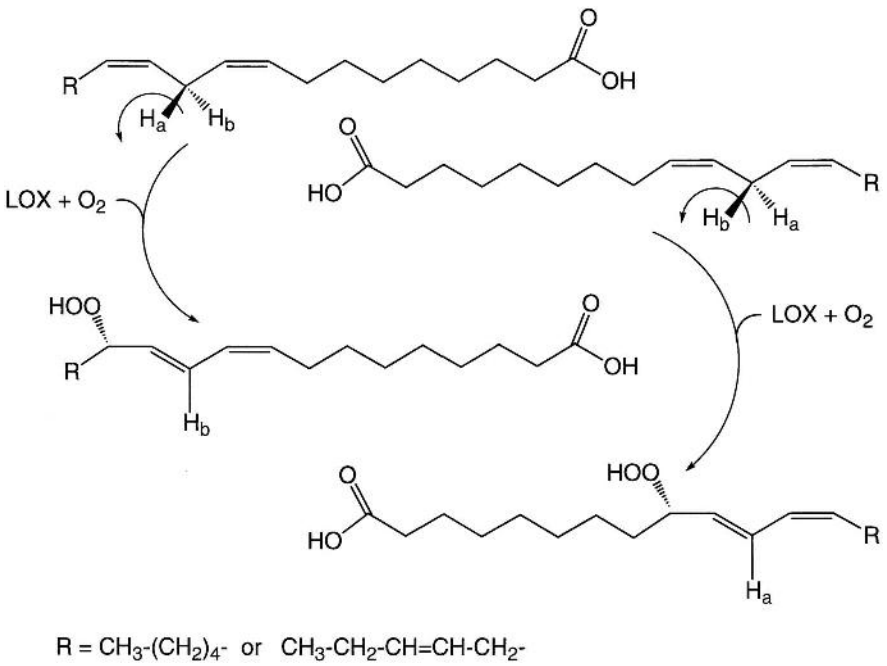


Figure 1 Oxidation of either linoleic (18:2) or linolenic acid (18:3) by LOXs from plants. Oxidation is preceded by stereospecific H removal followed by placement of O_2 on the opposite side of the fatty acid, compared to H removal. Note that reverse orientation (head-to-tail) gives the same relative spatial relationship affording the 13(*S*)- or 9(*S*)-HPODE/HPOTE as shown.

2.2 Factors Affecting Oxidation Specificity

In order to fully understand regiospecificity and stereospecificity of LOX, it is important to be aware of the ''reverse orientation'' phenomenon of LOX (see review in Ref. 15). With 13(*S*)-specific LOXs, H removal and O_2 insertion occurs on opposite sides of the molecule [16]. In the case of 9(*S*)-specific LOXs, H removal and O_2 insertion is completely the reverse of the 13(*S*)-specific LOX [17]; that is, for 9(*S*)-oxidation, the substrate must be inserted into the active site in a reverse orientation by a head-to-tail flip [18–20]. After a reverse flip, H removal and O_2 insertion of the 9(*S*)-specific LOX become spatially identical to the 13(*S*)-specific LOX (Fig. 1). There are numerous reports of dual 9(*S*)- and 13(*S*)-hydroperoxidation by the same LOX isozyme, implying that reverse orientation does occur. LOX-1 of soybean has a high pH optimum at about pH 9–10. At its optimum, virtually all the oxidation is 13(*S*)-specific. As the pH is lowered, the share of 9(*S*)-oxidation increased in parallel with the titration curve of linoleic acid up to a maximum of 25%, until the enzyme became inactive at about pH 6.5 [19]. This implied that the proximal-carboxylate anion of linoleic acid could not enter the active site in the reverse orientation. In the oxidation of arachidonic acid, the rice isozyme, LOX RSL-2, displayed the same pH-dependent behavior favoring 5(*S*)-oxidation at low pH and 15(*S*)-oxidation at high pH [21]. It may be because of this pH-dependent phenomenon that most LOXs with neutral or acidic pH optima oxidize linoleic acid to either 9(*S*)-HPODE or a mixture of 9(*S*)- and 13(*S*)-HPODE, whereas those with basic pH optima yield largely 13(*S*)-HPODE.

Similarly, substrates blocked at the proximal-carboxylic acid end of the molecule, like phospholipids [22,23], usually afford ester-bound 13(*S*)-HPODE exclusively.

Additional control of regio-configuration of the hydroperoxide regards the size of the hydrophobic cavity of the particular LOX adding yet another very important variable to product specificity; thus, changes in pH are not a predictable modifier of regio-specificity. Site-directed mutagenesis proved that modification of certain amino acid residues causes the LOX to change its regio-specificity of oxidation (see review in Ref. 24).

2.3 LOX Products with Polyunsaturated Fatty Acid Substrates

Table 1 summarizes the diverse products possible using LOXs from different sources. Twenty-four different products are listed, which does not include various dimers and other free-radical products obtained from the reaction under oxygen deficiency. With some exceptions, most plant LOXs afford 9(*S*)- and 13(*S*)-hydroperoxides. However, some LOXs, like soybean seed LOX-3, are known to have considerable peroxidative character [40]. As a result, the products of LOX-3 are fairly racemic [41] by virtue of the intermediacy of peroxyl radicals known to produce hydroperoxide racemates. As seen in Table 1, the fungal LOXs (or dioxygenases) give very unique hydroperoxide products (see also Chap. 14).

2.4 Multiple Dioxygenations of Polyenoic Fatty Acids

Some oxylipins—in particular, hydro(pero)xy fatty acids having methylene-interrupted double bonds—can serve as LOX substrates. Bild et al. [42,43] found that oxidations of arachidonate and γ-linolenate led to formation of polar conjugated trienes, identified as 8,15-dihydroperoxy-5,9,11,13-eicosatetraenoic (8,15-diHPETE) and 6,13-dihydroperoxy-7,9,11-octadecatrienoic acids (6,13-diHPOTE), respectively (i.e., products of double dioxygenation).

Van Os et al. [37] showed that soybean LOX-1 converts arachidonate into 60% of (5*Z*,9*E*,11*Z*,13*E*)-8,15-diHPETE (60%) and 40% of 5,15-dihydroperoxy-6(*E*),8(*Z*),11(*Z*),13(*E*)-eicosatetraenoic acid (5,15-diHPETE) through intermediate 15(*S*)-hydroperoxy-5(*Z*),8(*Z*),11(*Z*),13(*E*)-eicosatetraenoic acid [15(*S*)-HPETE]. The resulting double hydroperoxides had stereo configurations 8(*S*),15(*S*)-diHPETE and 5(*S*),15(*S*)-diHPETE. Seed LOX of *Lupinus albus* also produced 8,15-diHPETE along with 15-HPETE [44,45]. Wheat seed LOX [46] and rice seed LOX-2 [21] converted arachidonic acid into a mixture of 5,15- and 8,15-diHPETE.

Double dioxygenation is often accompanied by another conversion, involving an epoxide intermediate like leukotriene A_4. Shimizu et al. [47] demonstrated conversion of exogenous arachidonic acid into 6(*E*)-leukotriene B_4 and 12-epi-6(*E*)-leukotriene B_4 in in vitro experiments with potato tuber LOX. The same products were detected by van Aarle et al. [48] in their experiments with arachidonic acid and barley seed LOX. Dihomo-γ-linolenic acid was converted by potato tuber LOX into 8,9-leukotriene A_3 and a mixture of 8(*S*),15(*S*) and 8(*S*),15(*R*) double hydroperoxides [49]. Soybean LOX-1 was able to biosynthesize lipoxins A and B from arachidonate via 5,15-diHPETE [50].

In higher plants, α-linolenate is the only endogenous precursor for the double dioxygenation pathway. Feiters et al. [51] detected double dioxygenation of α-linolenate by soybean LOX-2. Sok and Kim [52] reported their observations on double dioxygenation of α-linolenate by crude soybean LOX, as well as LOX-1. Sok and Kim initially performed an incubation at acidic conditions (pH 6.5) to increase 9-hydroperoxy-10(*E*),12(*Z*),15(*Z*)-

Table 1 Products from LOX Using Various Substrates and Conditions

Substrate(s)	Product(s)[a]	Source	pH	O_2	Ref.
(9,12)-18:2	13(*S*)-HPODE	Soy LOX-1	9–10	+	16, 19
(9,12)-18:2	9(*S*)-HPODE	Tomato, grain, potato	5.5–7	+	25–29
(9,12)-18:2	8(*R*)-HPODE	Fungus	7.2	+	30
(9,12)-18:2	11(*S*)-HPODE, 13(*R*)-HPODE	Fungus/Mn-LOX	7.4	+	31
(13,16)-18:2	17(*S*)-HPODE	Soy LOX-1	9	+	32
(9,12,15)-18:3	13(*S*)-HPOTE	Soy LOX-1	10	+	33
(9,12,15)-18:3	9(*S*)-HPOTE, 9(*S*),16-diHPOTE	Potato	6.5	+	34
(5,8,11,14)-20:4	15(*S*)-HPETE	Soy LOX-1	9–10	+	16
(5,8,11,14)-20:4	8(*S*)-HPETE, 12(*S*)-HPETE, 15(*S*)-HPETE	Cucumber	8	+	35
(5,8,11,14)-20:4	5(*S*)-HPETE	Barley seed	7	+	28
(5,8,11,14)-20:4	5(*S*)-HPETE, 8(*S*)-HPETE, 11(*S*)-HPETE	Tomato	6.8	+	36
(5,8,11,14)-20:4	5(*S*),15(*S*)-diHPETE, 8(*S*),15(*S*)-diHPETE	Soy LOX-1	8.7	+	37
(9,12)-18:2, 13(*S*)-HPODE	13-oxo-(9,11)-18:2, 13-oxo-(9,11)-13:2, pentane	Soy LOX-1		−[b]	38
(9,12)-18:2, 13(*S*)-HPOTE	13-oxo-(9,11)-13:2, 2-penten-1-ol, 1-penten-3-ol	Soy LOX-1		−[b]	39

[a] Abbreviations: 13(*S*)-hydroperoxy-9(*Z*),11(*E*)-octadecadienoic acid, 13(*S*)-HPODE; 9(*S*)-hydroperoxy-10(*E*),12(*Z*)-octadecadienoic acid, 9(*S*)-HPODE; 8(*R*)-hydroperoxy-9(*Z*),12(*Z*)-octadecadienoic acid, 8(*R*)-HPODE; 11(*S*)-hydroperoxy-9(*Z*),12(*Z*)-octadecadienoic acid, 11(*S*)-HPODE; 13(*R*)-hydroperoxy-9(*Z*),11(*E*)-octadecadienoic acid, 13(*R*)-HPODE; 17(*S*)-hydroperoxy-13(*Z*),15(*E*)-octadecadienoic acid, 17(*S*)-HPODE; 13(*S*)-hydroperoxy-9(*Z*),11(*E*),15(*Z*)-octadecatrienoic acid, 13(*S*)-HPOTE; 9(*S*)-hydroperoxy-10(*E*),12(*Z*),15(*Z*)-octadecatrienoic acid, 9(*S*)-HPOTE; 9(*S*),16-dihydroperoxy-10(*E*),12(*Z*),14(*E*)-octadecatrienoic acid, 9(*S*),16-diHPOTE; 15(*S*)-hydroperoxy-5(*Z*),8(*Z*),11(*Z*),13(*E*)-eicosatetraenoic acid, 15(*S*)-HPETE; 8(*S*)-hydroperoxy-5(*Z*),9(*E*),11(*Z*),14(*Z*)-eicosatetraenoic acid, 8(*S*)-HPETE; 12(*S*)-hydroperoxy-5(*Z*),8(*Z*),10(*E*),14(*Z*)-eicosatetraenoic acid, 12(*S*)-HPETE; 5(*S*)-hydroperoxy-6(*E*),8(*Z*),11(*Z*),14(*Z*)-eicosatetraenoic acid, 5(*S*)-HPETE; 11(*S*)-hydroperoxy-5(*Z*),8(*Z*),12(*E*),14(*Z*)-eicosatetraenoic acid, 11(*S*)-HPETE; 5(*S*),15(*S*)-dihydroperoxy-6(*E*),8(*Z*),11(*Z*),13(*E*)-eicosatetraenoic acid, 5(*S*),15(*S*)-diHPETE; 8(*S*),15(*S*)-dihydroperoxy-5(*Z*),9(*E*),11(*Z*),13(*E*)-eicosatetraenoic acid, 8(*S*),15(*S*)-diHPETE.

[b] Conditions of oxygen deficiency with fatty acid alone (18:2 or 18:3), or anaerobic conditions in the presence of both hydroperoxide and fatty acid (18:2 or 18:3).

octadecatrienoic acid (9-HPOTE) formation. Next they incubated the 9-HPOTE-enriched preparation with a larger amount of enzyme at pH 9.0. The sodium borohydride reduction products afforded four isomeric 9,16-dihydroxy-10,12,14-octadecatrienoic acids (9,16-diHPOTE) [52]. Two of them (including the predominant one) were proposed to be the products of double dioxygenation. Two others (hypothetically identified as all-trans isomers) are formed from 9-HPOTE in the absence of oxygen. Their formation was attributed to hydrolysis of a 9,10-epoxy intermediate (see Fig. 2) [52,53].

Potato tuber LOX under an oxygen atmosphere converted α-linolenate via 9(*S*)-HPOTE into linolenic acid with an undetermined mixture of 9- and 16-hydroperoxide/hydroxide groups (10*E*,12*Z*,14*E*)-9,16-diH(P)OTE with a large (up to 80%) yield [34,54,55]. Some additional LOXs, excepting soybean LOX 2 and potato tuber LOX, were shown to perform the double dioxygenation of α-linolenate (Fig. 2). These were barley [56], wheat, and rice (Grechkin and Kuramshim, unpublished results).

Along with 9,16-diH(P)OTE (ultraviolet absorbance maximum at 267 nm), potato LOX converted α-linolenate into unprecedented metabolites, having oxotriene conjugation (maximum at 309 nm). These compounds were identified as 9-hydro(pero)xy-16-oxo-10(*E*),12(*Z*),14(*E*)-octadecatrienoic and 9-oxo-16-hydro(pero)xy-10(*E*),12(*Z*),14(*E*)-octadecatrienoic acids [54,55]. Similar compounds were detected then in marine green alga *Acrosiphonia coalita* [57]. Conjugated trienals from *A. coalita* possess antimicrobial properties [57]. Although 9-LOXs are widespread within higher plant species, the physiological

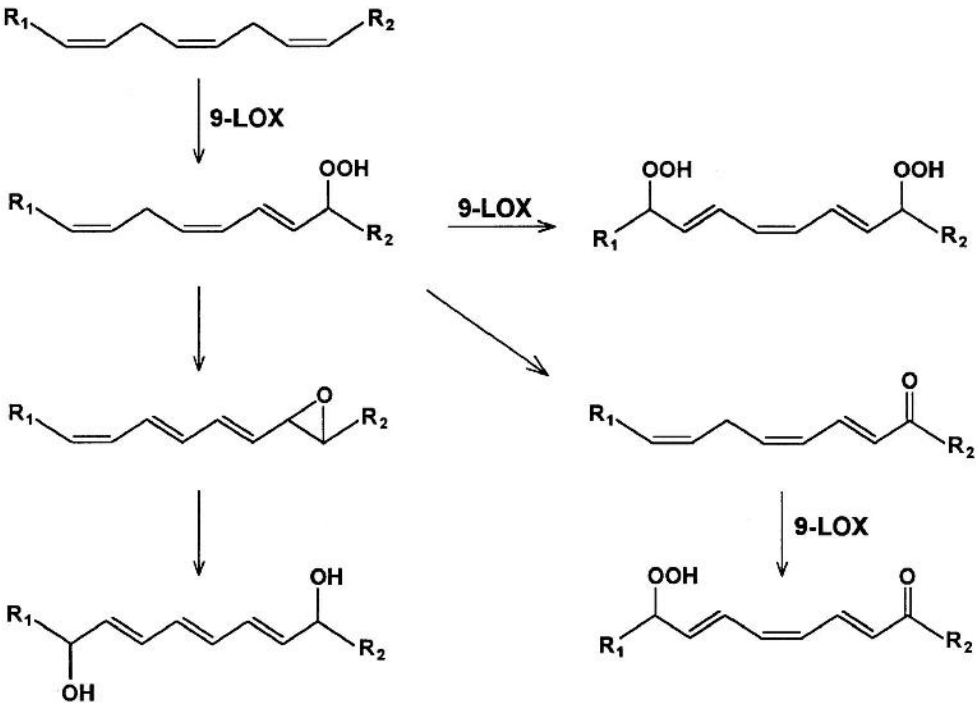


Figure 2 Double dioxygenation and related conversions of α-linolenic acid catalyzed by 9-LOXs. $R_1 = CH_3—CH_2—$ and $R_2 = —(CH_2)_7COOH$.

properties of 9,16-diHPOTE and related products still remain insufficiently studied. In view of the close similarity of these compounds with such potent bioregulators as leukotrienes, one can propose that 9,16-diHPOTE and related conjugated trienes and oxotrienes are signaling compounds in many plant tissues possessing 9-LOX activity.

One more type of oxidation related to double dioxygenation involves formation of endoperoxide intermediates. Bild et al. observed formation of prostaglandin $F_{2\alpha}$ during the incubation of arachidonic acid with soybean LOX-2 [58]. Recently, the similar conversion of α-linolenate into dinor isoprostanes E_1 by soybean LOX at a very small yield was reported [59]. However, the presence of these oxylipins in plants in vivo as well as their physiological importance remains to be answered.

2.5 LOX Oxygenation of β,γ-Unsaturated Carbonyl Compounds

As is well known, the ordinary catalytic function of LOX is the dioxygenation of the 1(*Z*),4(*Z*)-pentadienyl moiety of fatty acids. As shown recently, the requirement for 1,4-pentadiene fragment is not absolute. For instance, β, γ-unsaturated ketones and aldehydes can act as LOX substrates.

Three investigations related to the oxidation of β, γ-unsaturated ketones were independently published by different research groups in 1991 [60–62]. Abián et al. [60] found that maize embryos pretreated with abscisic acid produced elevated amounts of α- and γ-ketols in vitro, as well as a pair of polar products, identified as ketodiols 9,13-dihydroxy-10-oxo-11(*E*)-octadecenoic and 9,13-dihydroxy-12-oxo-10(*E*)-octadecenoic acids. The biosynthetic pathway to ketodiols was not clear from their data [60]. As described below, independent results [62] revealed that the biosynthetic pathway to these products was undoubtedly through LOX oxidation of the 9- and 13-HPODE-derived α-ketols, 9-hydroxy-10-oxo-12(*Z*)-octadecenoic acid and 13-hydroxy-12-oxo-9(*Z*)-octadecenoic acid. The same type of LOX oxidation of a different β, γ-unsaturated ketone was described by Kühn et al. [61].

Grechkin et al. [62] identified 9-hydroperoxy-12-oxo-13-hydroxy-10(*E*),15(*Z*)-octadecadienoic acid (α-ketol hydroperoxide) as the product of in vitro 13-hydroperoxy-9(*Z*),11(*E*),15(*Z*)-octadecatrienoic acid (13-HPOTE) metabolism in wheat seedlings. The authors observed formation of the same product during the incubation of pure 12-oxo-13-hydroxy-9(*Z*),15(*Z*)-octadecadienoic acid (α-ketol) preparation with potato tuber LOX (see Fig. 3). An isomeric α-ketol hydroperoxide, 9-hydroxy-10-oxo-13-hydroperoxy-11(*E*),15(*Z*)-octadecadienoic acid, was isolated and identified after 9-HPOTE incubation with flaxseed AOS preparation. The obtained nuclear magnetic resonance (NMR) data unequivocally suggested that the primary oxidation products are hydroperoxides [62].

Kühn et al. [61] observed the oxidation of 12-oxo-9(*Z*)-octadecenoic acid during its incubation with soybean LOX-1. The predominant product was identified as 9,12-dioxo-10(*E*)-octadecenoic acid. Labeling with $^{18}O_2$ revealed that oxygen of the carbonyl group at C-9 comes from the atmosphere. Thus, formation of the 9,12-dioxo compound via the LOX-catalyzed dioxygenation was proposed. The detection of diketone instead of hydroperoxide by Kühn et al. is probably explained by either oxygen deficiency during the incubation or an increased susceptibility of the hydroperoxide to degradation by the Fe^{2+} form of LOX. As described on page 165, this phenomenon of ketone formation was also observed with the LOX oxidation of β,γ-unsaturated aldehydes; however, addition of catalytic amounts of 13(*S*)-HPODE promoted formation of good yields of hydroperoxyalkenals [63].

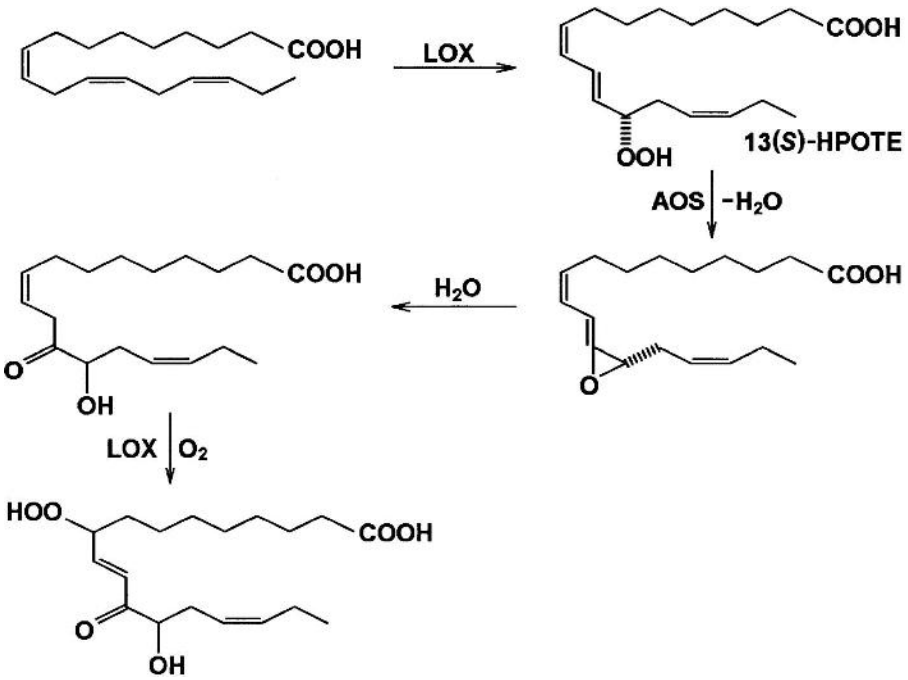


Figure 3 Biosynthesis of α-ketol hydroperoxides via the dioxygenation of α-ketol by LOX.

The above data [61,62] demonstrate that β,γ-unsaturated ketones can serve as nonclassical LOX substrates. The target of LOX attack in these substrates is 3(*Z*)-butene-1-one residue instead of ordinary 1(*Z*),4(*Z*)-pentadiene system. The methylene function between the carbonyl and the double bond loses one hydrogen atom and the resulting radical after allylic rearrangement binds oxygen to form hydroperoxide (Fig. 3). As found recently (Grechkin and Hamberg, unpublished data), α-ketol hydroperoxide prepared from the α-ketol, 13-hydroxy-12-oxo-9(*Z*)-octadecenoic acid, by oxidation with potato tuber LOX possesses partial chirality at C-9, which is represented by 67% (*S*) and 33% (*R*) epimers. Thus, potato LOX possesses partial stereospecificity during the oxidation of α-ketol.

β,γ-Unsaturated ketones, such as α-ketols, are also oxidized nonenzymatically [64]. This conversion proceeds under strong alkaline or acidic conditions in the presence of oxidizing reagents like 2,6-dichlorophenolindophenol or copper(II) acetate. The rate-limiting stage of conversion is keto-enol tautomerism. In the presence of oxygen the reaction affords the same α-ketol hydroperoxides which are formed during the LOX oxidation of α-ketols.

A related enzyme, oxygenating 3(*Z*)-nonenal and 3(*Z*)-hexenal, was detected in the high-speed particle fraction from broad beans (*Vicia faba* L.) and soybeans [65,66]. The enzyme converts 3(*Z*)-nonenal into 4-hydroxy-2(*E*)-nonenal as the principal product. In the same manner, 3(*Z*)-hexenal was oxidized into 4-hydroxy-2(*E*)-hexenal. The primary product of 3(*Z*)-nonenal oxidation was the hydroperoxide, 4-hydroperoxy-2(*E*)-nonenal, which is then reduced into 4-hydroxy-2(*E*)-nonenal. The oxidation products were racemic 4(*R*,*S*)-hydro(pero)xy derivatives [65,66]. Gardner and Hamberg [65] named the enzyme

''3(*Z*)-alkenal oxygenase.'' Although the 3(*Z*)-alkenal oxygenase resembled LOX in its action, it was not inhibited by the LOX inhibitors 5,8,11,14-eicosatetraynoic acid and nordihydroguaiaretic acid. However, recently, Gardner and Grove [63] identified a 3(*Z*)-alkenal oxygenase from soybeans as being due to LOX-1. They found that 4-hydroperoxy-2(*E*)-nonenal formed by LOX-1 oxygenation of 3(*Z*)-nonenal is 83% pure 4(*S*)-hydroperoxide. Initially, 4-oxo-2(*E*)-nonenal was found to be the main product of LOX action; however, the addition of a catalytic amount of 13(*S*)-HPODE greatly increased the yield of 4(*S*)-hydroperoxy-2(*E*)-nonenal at the expense of 4-oxo-2(*E*)-nonenal. Presumably, the 13(*S*)-HPODE contributed to maintaining LOX in the active Fe^{3+} state of the enzyme.

Recently, a new oxylipin, 9-hydroxy-12-oxo-10(*E*)-dodecenoic acid, named 9-hydroxy-traumatin, was detected as the product of 13-HPODE conversion by enzyme preparations from soybean and alfalfa seedlings [67]. It has been proposed that 9-hydroxy-traumatin is the product of LOX oxygenation of 9(*Z*)-traumatin, 12-oxo-9(*Z*)-dodecenoic acid.

2.6 LOX Oxidation of Complex Lipids and Biomembranes

It is well known that storage triacylglycerols in seeds of many plant species contain oxygenated fatty acid residues [68]. The biogenetic origin of such ''complex oxylipins'' was not known until recently. There is increasing evidence that different LOXs can oxidize the acyl moieties within glycerolipids. Yamauchi et al. [69] described the oxygenation of dilinolenoyl monogalactosyldiacylglycerol (MGD) in dipalmitoylphosphatidylcholine liposomes by crude soybean LOX preparation. The resulting oxygenated MGD species contained residues of 9-, 12-, 13-, and 16-hydroperoxy, 9,16-dihydroperoxy, and some cyclic endoperoxy linolenate derivatives.

The literature fully documents LOX oxygenation of fatty acid moieties within phospholipids. Soybean LOX-1 oxidized the fatty acyl residues within phosphatidylcholine [22,23], phosphatidylethanolamine, and phosphatidylinositol [22]. Arachidonyl and linoleoyl moieties in the phosphatidylcholine were converted exclusively to the 15(*S*)-hydroperoxides [22] and 13(*S*)-hydroperoxides [22,23], respectively. Kondo et al. [70] incubated 1-palmitoyl-2-linoleoyl-*sn*-glycero-3-phosphatidylcholine with a LOX preparation extracted from elicited soybeans. Linoleoyl residues were specifically oxidized into 13(*S*)-hydroperoxy derivatives [70]. Cucumber root LOX was also reported to oxidize phosphatidylcholine [71], but regiospecificity and stereospecificity of oxidation were not revealed.

Increasing literature data suggest the ability of LOXs to oxygenate biomembranes. Soybean LOX-2 exhibits more stereospecificity upon oxidizing fatty acyl moieties in membrane lipids than during oxygenation of free nonmembranous fatty acids [72]. The role of soybean LOX-2 in in vivo remodeling of biomembranes was proposed [72]. LOX oxygenation of membrane lipids was also detected in plants under water stress [73].

It was demonstrated that specific LOX genes are expressed at the early stages of seed germination [74,75]. Appearance of LOX at the lipid body membrane initiated the oxidation of storage lipids in cucumber seeds [76]. As a result, triacylglycerols accumulated molecular species, possessing one, two, and three 13(*S*)-HPODE residues esterified to glycerol [77]. In vitro, the recombinant lipid body LOX was capable of oxidizing all three acyl residues within trilinoleoylglycerol [78]. Cucumber cotyledon LOX oxygenated the linoleoyl residues within trilinoleoylglycerol at the lipid–water interface, being nonactive toward free linoleate in water solution [79].

2.7 Modified Fatty Acid Substrates and ''Tailored'' Oxidations

As discussed in Section 2.6, polyunsaturated fatty acids modified at the carboxylic acid end of the chain are substrates for LOX. Those substrates having bulky groups at the carboxylic acid end of the fatty acid, like glycerides, generally have reduced activity compared with free fatty acids, and these substrates often require the addition of substances that are thought to modify the physical state of the substrate [22,23,71,72,77–82]. Obviously, a bulky group at the proximal-carboxylic end would seem to prevent the substrate from entering the active site in reverse orientation, as discussed earlier. This would seem to be the case with soybean LOX-1, as exclusive 13(*S*)-oxidation specificity has been shown with phosphatidylcholine [22], even at neutral pH [23]. Lipid body cucumber LOX also gave 13(*S*)-oxidation specificity with triacylglycerols [77]; however, this LOX, including cDNA-expressed cucumber LOX, did afford 13–16% 9-HPODE with substantial *S*-enantiomeric excess [78]. This oxidation specificity presents a theoretical mechanistic dilemma for cucumber LOX, as a ''head-to-tail'' reverse orientation would not seem possible; that is, production of both 9(*S*)- and 13(*S*)-HPODE would require H removal and oxidation on opposite sides of the substrate.

Substrates with relatively small substituent or modified groups on the proximal end of the fatty chain usually are nearly as active as the natural substrate, linoleic acid. Linoleyl sulfate being more soluble over a wider pH range gave a broader pH optimum for LOX [83]. Although the oxidation specificity of this substrate is not known, prior knowledge with other substrates suggests that the highly polar proximal end would direct oxidation to 13(*S*)-hydroperoxides. Various *N*-linoleoyl amides (amide, ethanolamide, methylamide, and dimethylamide) were shown to be fairly effective substrates for soybean LOX-1, affording the 13(*S*)-hydroperoxide in each case [84]. Arachidonylethanolamide (also known as anandamide, a ligand for cannabinoid receptors) was oxidized by soybean LOX-1 to products similar to those obtained from arachidonic acid (Table 1) [i.e., mainly 15(*S*)-hydroperoxide and doubly oxygenated 5,15- and 8,15-dihydroperoxides] [85]. However, these workers showed that tomato and barley LOX acted anomalously to give 11(*S*)-hydroperoxide with arachidonylethanolamide, compared to 5(*S*)-HPETE as the usual tomato/barley LOX product of arachidonic acid. They surmized that the change in size of the carboxylate terminus by the ethanolamide group affected the regiospecificity. Presumably, this would occur by the reverse orientation of substrate or ethanolamide-first presentation to barley/tomato LOXs. Various [ω-6(*Z*), ω-9(*Z*)] dien-1-ols from C-12 to C-20 were tested with soybean LOX, and the most activity obtained with the 15:2 dien-1-ol was almost comparable to linoleic acid [86]. Interestingly, the products were in a 1:2 ratio of the 10(*S*)-hydroperoxide (the expected ω-6 oxidation) and 6-hydroperoxide (ω-6 oxidation in reverse orientation); however, the 6-hydroperoxide was of low stereospecificity with slight (*R*) excess. However, in another study using synthetic substrates with a proximal alcohol group, the reverse orientation product (hydroperoxidation of the diene carbon nearest to the alcohol) was of 96% (*S*) stereoconfiguration [87].

The methylene-interrupted (*Z*,*Z*)-pentadiene structure is another well-known feature necessary for LOX activity, and several modifications of this have been tested, including a number of suicide inhibitors that will not be discussed here. As discussed in more detail earlier, ketone [61,62] or aldehyde [63] can serve as a ''double bond'' replacement for one of the double bonds of the (*Z*,*Z*)-pentadiene moiety, but with the aldehyde, 3(*Z*)-nonenal, the activity was much reduced compared to linoleic acid [63]. The position of the (*Z*,*Z*)-pentadiene moiety in the C-18 fatty acid chain was shown to be important [88];

activity steadily increased from none, using a substrate with the C-4 and C-7 position of the double bonds, up to a maximum at C-9 and C-12. Activity then decreased steadily until a substrate with C-12 and C-15 unsaturation was tested, where activity was about 10–20% of the maximum, and then again increased to about 40–50% of the maximum at C-13 and C-16. There was negligible activity with C-14 and C-17 double bonds. The substrate showing the second peak of activity with unsaturation at C-13 and C-16 gave 17(*S*)-hydroperoxy-15(*E*),13(*Z*)-octadecadienoic acid as a product (Table 1).

A strategy was developed to synthesize a number of artificial substrates for soybean LOX-1, and such work provided information on the influence of the residues attached to the methylene-interrupted (*Z*,*Z*)-pentadiene. A number of pentadiene-1-ols was synthesized with various distal substitutions [e.g., 3(*Z*),6(*Z*)-dodecadien-1-ol having a distal $CH_3(CH_2)_4$—, plus several others], and the alcohol was esterified with adipate to provide the proximal carboxylate [89]. By reduction of the product and removal of the adipate, a chiral secondary alcohol was obtained, providing a number of potentially useful products [e.g., 7(*S*)-hydroxy-3(*Z*),5(*E*)-dodecadien-1-ol]. In a subsequent study, where the chain length and/or type of substituent were varied for both the proximal and distal end of the synthetic substrate, it was noted that the regiospecificity of hydroperoxidation of the pentadiene toward the distal side versus the proximal side of the substrate was dependent on the relative hydrophobicity of the two ends [90]; that is, when the distal group was increased from the optimum of $CH_3(CH_2)_4$—, the regiospecificity of hydroperoxidation shifted toward the proximal end of the diene and, conversely, an increase of hydrophobicity of the proximal end led to preference of oxidation toward the distal end of the diene. Other workers [91] showed a strong correlation of regioselectivity with hydrophobicity differences between the proximal and distal ends of the substrate. Novak [87] observed that a ''pocket fit'' may be more relevant to the differences in regiospecificity of the synthetic substrates rather than hydrophobicity per se, a possibility also discussed by Datcheva et al. [90]. Thus, it would appear that reverse orientation may be a factor involved in the regiospecificity; however, the stereospecificity of hydroperoxidation toward the proximal group was found to be racemic compared with the predominantly (*S*)-stereospecificity of hydroperoxidation of the diene toward the distal end [91]. This result could be due to the use of a pH 9 buffer in the incubation, which would prevent the carboxylate anion from reverse orientation in the active site. Nonetheless, the mechanism of this apparent nonenzymic oxidation toward the proximal residue remains unanswered.

As discussed earlier, there are opportunities to tailor various synthetic substrates for LOX action. Such methods can be utilized for chemoenzymatic synthesis. For example, methyl 15(*S*)-HETE was synthesized by reduction of 15(*S*)-HPETE produced by soybean LOX-1 oxidation of arachidonic acid. The 15(*S*)-hydroxyl was then esterified with succinic acid in order to provide a substrate with a distal recognition site for LOX resulting in a 5(*S*)-hydroperoxidation. Reduction of the 5(*S*)-hydroperoxide and hydrolysis of the succinate afforded 5(*S*),15(*S*)-diHETE [92].

2.8 Maximizing Yields of Hydroperoxides

In our laboratory, 250–300 mg of either 13(*S*)-HPODE or 13(*S*)-HPOTE with 99% purity are routinely prepared by a relatively simple procedure using commercial soybean LOX followed by chromatographic purification [5]. We have found by experience that sufficient O_2 availability and high pH (pH 10) are among the most important factors in obtaining high yields. The importance of oxygen sufficiency in obtaining high regiospecificity (and

by inference, high stereospecificity) was studied by Berry et al. [93], and the same group [94] found that excess substrate inhibition is avoided at high oxygen concentration. Thus, we use pure O_2, low temperatures (to improve O_2 solubility), optimum LOX concentration (too much LOX causes the reaction to become anaerobic), and high pH [to prevent formation of 9(*S*)-HPODE by inverse substrate orientation]. For example, one preparative method for production of 13(*S*)-HPODE from linoleic acid utilized 4 atm of O_2, aqueous buffer (pH 9) at 0–4°C with stirring [95]. Other factors to consider are the presence of inhibitory nonionic detergents [96,97] and alcohols [98]; however, these reagents are often used for the convenience of dispersing fatty acid substrate. Inhibition by nonionic detergents was found to be primarily due to sequestering the substrate within detergent micelles, and the reaction with LOX occurs when the substrate is released as a monomer in equilibrium with micelles [96].

A number of workers have researched methods of producing hydroperoxides in large-batch incubations. Martini et al. [99] have optimized the formation of 13(*S*)-HPODE in a relatively large quantity for the purpose of preparing 13(*S*)-HODE by triphenylphosphine reduction of the hydroperoxide. The optimal parameters adopted by them included pure O_2 at 2.5 atm, temperature of 5°C, pH 11 buffer, 0.1 *M* linoleic acid, and LOX (Fluka) at 4 mg/mL. A method was developed to produce 13(*S*)-HPOTE from either linolenic acid or hydrolyzed flaxseed oil using soybean LOX, a constant flow of O_2, temperature of 2°C, pH 9.5 buffer, and rapid stirring at 800 rpm [100]. Production of 13(*S*)-HPOTE was as high as 60 g/L/hr in a 10-L reactor. Preliminary work by Elshof et al. [101] using oxygen in air for soybean LOX oxidation of linoleic acid from hydrolyzed safflower oil led to the development of a more refined procedure by them that utilized air under partial pressure with agitation in pH 10 borate buffer [102]. In this latter procedure, substrate and soybean LOX were fed incrementally into the bioreactor to prevent depletion of O_2 and product inhibition (final added substrate was 80g in 2 L). The addition of cysteine at the start of reaction enhanced the yield of product and additionally reduced the hydroperoxide to 13(*S*)-HODE. This method of reduction is more convenient than the usual reductants, $NaBH_4$ and triphenylphosphine. $NaBH_4$ reduction contributes to the formation of racemic HODEs by reducing the by-product 13-oxo-9,11-octadecadienoic acid. Triphenylphosphine and its oxide can be inconvenient because they are solvent extracted as by-products along with desired 13(*S*)-HODE.

Soybean LOX-1 in a biphasic system of octane and borate buffer was found to be more efficient than aqueous buffer alone in the production of 13(*S*)-HPODE from linoleic acid in concentrations of 20–40 g/L [103]. A biphasic octane/borate buffer containing both soybean LOX-1 and lipase was used to transform trilinolein or sunflower oil into 13(*S*)-HPODE [104]. The triglyceride, dissolved in octane, was lipase-hydrolyzed to linoleic acid, which was oxidized subsequently by LOX-1 in the aqueous pH 9 buffer to afford 13(*S*)-HPODE. Separation of 13(*S*)-HPODE (aqueous) from unreacted triolein (octane) was achieved simply by phase separation. One disadvantage may be the large amount of enzyme required (12 g/L lipase and 25 g/L LOX-1, both from commercial sources).

An older published method used crude soybean LOX to oxidize 0.014–0.36 *M* linoleic acid concentrations in either 10% ethanol or 20% dimethylsulfoxide [105]. In this procedure, pure O_2 was used before adding the LOX and then closing the system for manometric measurement. There was no indication of the amount of headspace O_2. It is possible that enhanced O_2 solubility in ethanol and dimethylsulfoxide permitted the reaction to proceed with good yields. It is also noted that $NaBH_4$ was used to reduce the product to HODE, which would also reduce oxooctadecadienoic acid to HODE. Substan-

tial amounts of oxooctadecadienoic acid are produced in the oxygen-deficient reaction of LOX (see Sec. 4). No data were given on the ragioselectivity and stereoselectivity of the products.

2.9 Immobilization of LOX

Several methods of immobilizing LOX have been developed. The advantage of immobilization is facile recovery of the biocatalyst and its retention of activity over long periods of time, permitting multiple uses in reactions. However, there is a lack of information regarding scale-up, thus immobilized LOX systems tested thus far afford up to about 100 μmol of HPODE at best. The following systems have been successful in immobilizing LOX for use in either aqueous or organic solvent reaction systems: agarose [106], polyacrylamide gel derivatized with glutaraldehyde [107], cross-linked phyllosilicates [108,109], carbonyldiimidazole-activated support (Reacti-gel™) [110,111], talc adsorption [112–114], oxirane acrylic beads [115], and alginate–silicate sol–gel matrix [116].

3 LOX EXPRESSION AND PHYSIOLOGICAL EFFECTS

3.1 Factors Affecting LOX Activity and LOX Gene Expression

It is known from recent work that different stress actions, including wounding [117–122], mechanical treatment [117], water deficit [73,122], infection [123–128], thermal (heat and cold) injury [129], ozone stress [129,130], ultraviolet irradiation [131], activate LOXs and induce the expression of LOX genes. Fatty acid hydroperoxides and other oxylipins derived from the LOX pathway are involved with stress signaling and responsive reactions. In this connection, data on LOX oxidation of polar lipids and biomembranes are of great interest. To reveal the general patterns of signaling through the plant LOX cascade, it is important to know what happens at the initiating stage—that is, hydrolysis or oxygenation of membrane lipids? It is known that stress action induces rapid liberation of free linoleic and linolenic acids [132]. At the same time, some workers recently presented evidence for direct oxygenation of membrane lipids under stress conditions. For instance, water stress causes the expression of LOX-1 and LOX-2 genes and oxidation of membrane lipids resulting in the accumulation of the esterified fatty acid hydroperoxides within complex lipids in soybeans [73].

It has been shown that the specific LOX genes are induced at the early stages of barley [133,134], cucumber [35,74,135–137], and soybean [74] seed germination. Such a specific LOX isoenzyme was localized in lipid bodies in cucumber and soybean seeds [74]. In the course of seed germination, this LOX became the main protein within the lipid body membrane. Triacylglycerols of lipid bodies contained partly oxygenated molecular species, having one, two, or three 13(*S*)-HPODE residues [136]. The amount of oxygenated lipids strongly increased during germination [77]. Lipid body LOX initiated the mobilization of storage lipids through their oxidation and hydrolysis [76,135,136].

3.2 Physiological Activity of Fatty Acid Hydroperoxides and Hydroxy Derivatives

Primary products of LOX activity, fatty acid hydroperoxides and related hydroxy derivatives, possess physiological activity. As mentioned earlier, different stress signals activate LOXs and lead to the expression of LOX genes. Hydroperoxides and hydroxy derivatives

of linoleic and linolenic acids, formed in infected plants, possess potent antimicrobial activity toward a number of pathogenic fungi species [123–125]. As found earlier, 13(*S*)-HPOTE along with jasmonate initiates the synthesis of proteinase inhibitors [138]. These results and others demonstrated the involvement of 13-HPOTE in plant signaling systems (see Chapter 11).

Increasing literature data demonstrate the physiological importance of hydroperoxy and hydroxy derivatives of linoleate and α-linolenate in mammals. 9(*S*)-HODE was found to be a strong stimulator of the adenylate cyclase activity in human platelets [139]. Accumulation of 9-HODE in low-density lipoproteins is correlated with the development of atherosclerosis [140,141]. 13(*S*)-HODE acted also as an adenylate cyclase inducer in platelets and endothelial cells [142] and inhibitor of protein kinase C [143]. 13(*S*)-HODE is known as the potent antithrombotic agent [144], inhibitor of platelet adhesion to blood vessel walls [142–147], and a modulator of degranulation of human polymorphonuclear leukocytes [148]. 13(*S*)-HPODE selectively inhibited platelet cyclooxygenase [149]. In view of important regulatory roles of HPODEs and HODEs in mammals, one can propose that the current level of knowledge only partly reflects their physiological importance in plants.

4 OXYGEN-DEFICIENT LOX AND "HOMOLYTIC" HYDROPEROXIDE LYASE

It may be appropriate to review both the HOMLS and the O_2-deficient reaction of LOX in the same section, because the basic mechanism appears to be identical; that is, both appear to convert fatty acid hydroperoxides into alkoxyl radicals. For example, under O_2 deficiency, LOX catalyzes the production of alkoxyl radicals from product hydroperoxides (Fig. 4). The alkoxyl radicals react according to the rules of free-radical chemistry. By the process of β-scission, a percentage of the alkoxyl radicals cleave into an aldehyde and a hydrocarbon or alcohol [152]. β-Scission of the 13-HPODE alkoxyl radical would afford mainly pentane and 13-oxo-9,11-tridecadienoic acid, and the 9-HPODE alkoxyl radical would cleave into 2,4-decadienal and octanoic acid. Similarly, HOMLS affords products that indicate that this enzyme is also functioning through the production of alkoxyl radicals from substrate fatty acid hydroperoxide. For this reason, the enzyme catalyzing the reaction could be easily misidentified. One should collect data to specifically prove whether the cleavage activity is due to HOMLS or O_2-deficient LOX activity.

It has been known for many years that the O_2-deficient or anaerobic reaction of LOX leads to an aberrant functioning of LOX, whereby linoleic acid pentadienyl radicals and alkoxyl radicals originating from linoleic acid and its hydroperoxide are released from the enzyme [150]. This reaction of LOX can be catalyzed either by anaerobic conditions in the presence of both linoleic acid and 13-HPODE, or by low O_2 concentrations. Linoleic acid serves to reduce the ferric form of the enzyme, in turn giving a linoleic acid pentadienyl radical, whereas, 13-HPODE oxidizes the ferrous form of LOX, giving rise to the alkoxyl radical of 13-HPODE (Fig. 4). From the alkoxyl radical, one obtains, by the process of β-scission, 13-oxo-9,11-tridecadienoic acid and pentane [38]. 13-Oxo-9,11-octadecadienoic acid also is formed [38], presumably by H abstraction or Fe^{3+} LOX oxidation of the alkoxyl radical. Also, the alkoxyl radical has a propensity to become rearranged into a 12,13-epoxyoctadecenoic acid with a hybridized allylic radical between C-9 and C-11 (see review in Ref. 152). This epoxyallylic radical reacts with the linoleic acid penta-

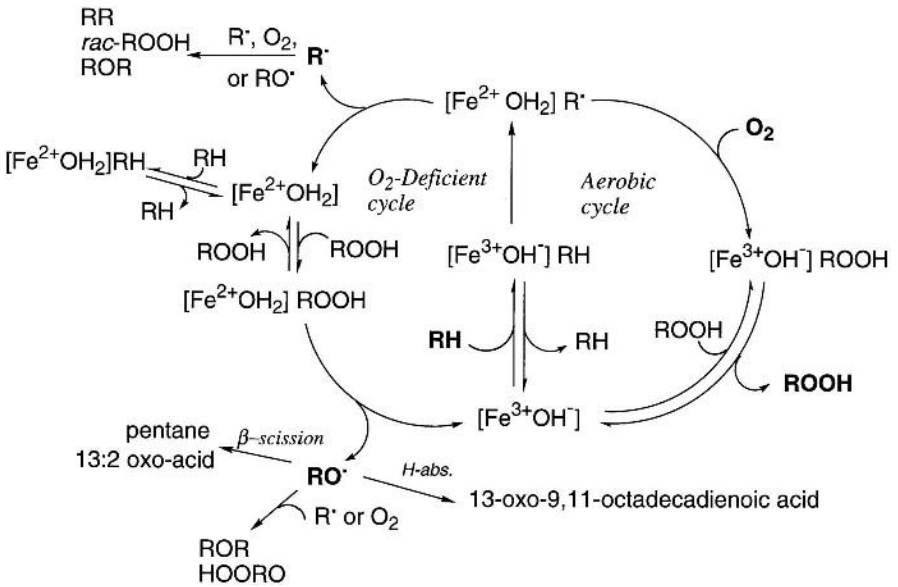


Figure 4 The iron redox cycle of the LOX active site shows the reaction of linoleic acid with O_2 under conditions of adequate aeration, as well as an aberrant reaction with linoleic acid hydroperoxide under O_2-starved conditions. Shown in bold are the entry of linoleic acid (RH) and O_2 into the cycle and products initially released from the active site—that is, linoleic acid hydroperoxide (ROOH), the alkoxyl radical of linoleic acid hydroperoxide ($RO^{\bullet}$), and the linoleic acid pentadienyl radical ($R^{\bullet}$). The alkoxyl radical ($RO^{\bullet}$) often rearranges into an epoxyallylic carbon radical which reacts with O_2 to form epoxyhydroperoxyoctadecenoic acid (HOORO), leading to epoxyhydroxyoctadecenoic acid. ROR represents dimers of epoxyoctadecenoic acid with the conjugated diene of linoleic acid. RR represents conjugated linoleic acid dimers, and *rac*-ROOH signifies racemic hydroperoxides formed by nonenzymic reaction of O_2 with the pentadienyl radical of linoleic acid. (Adapted from Refs. 14, 150, and 151.)

dienyl radical to give dimers, and the pentadienyl radicals react with one another to give linoleic acid dimers, most of which are conjugated dienes [153].

Because the 13(*S*)-HPOTE alkoxyl radical is more susceptible to β-scission than the alkoxyl radical from 13(*S*)-HPODE, the O_2-deficient LOX reaction with 13(*S*)-HPOTE forms cleavage products comparatively readily, giving mainly 13-oxo-9,11-tridecadienoic acid, 2-penten-1-ol, 1-penten-3-ol, and pentene dimers [39]. The increased susceptibility of the alkoxyl radical of 13(*S*)-HPOTE to β-scission relates to the allylic 15,16-double bond which affords a resonance stabilized radical as found for facile β-scission of the 10-hydroperoxy-8,12-octadecadienoic acid alkoxyl radical [154].

There are only a few examples of the isolation of an apparent authentic HOMLS (i.e., an enzyme with substantial difference in molecular size compared to the 90–100 kDa of LOX). Thus, the HOMLS from *Chlorella pyrenoidosa* at a size of 48 kDa [155] and *Oscillatoria* sp. at 56 kDa [156] would seem to be authentic; however, further work in characterizing these enzymes would be desirable. *C. pyrenoidosa* cleaved 13(*S*)-HPODE into 13-oxo-9(*Z*),11(*E*)-tridecadienoic acid and pentane [155], and *Oscillatoria* afforded the same reaction, except pentan-1-ol was produced instead of pentane [156]. In another report, three LOX isozymes of *Vicia sativa* were found to possess HOMLS activity

in addition to LOX activity [157]. In this case, it would appear that the O_2-deficient action of LOX was involved; however, all three LOX isozymes had a higher than usual molecular size of 237 kDa, and only 9-HPODE, not 13-HPODE, was susceptible to cleavage, affording 2(*E*),4(*E*)-decadienal. Also, a 9-HPODE-forming LOX and a 2,4-decadienal HOMLS was purified from *Pisum sativum* [114]. Further work in characterizing these enzymes should be completed.

Linoleic acid is the precursor of the mushroom odor, 1-octen-3-ol, as well as 10-oxo-8(*E*)-decenoic acid in *Psalliota bispora* [158]. Inasmuch as this reaction afforded the typical mushroom odor substance, a teleological argument could be made for the authenticity of the mushroom enzyme. Additional work showed strong evidence for specific enzymic action. Although the intermediate hydroperoxide was not detected, only the (*S*)-isomer, 10(*S*)-hydroperoxy-8(*E*),12(*Z*)-octadecadienoic acid [10(*S*)-HPODE], was utilized by mushroom homogenate when racemic 10(*R*,*S*)-HPODE was used as a substrate. This reaction gave the same (3*R*)-1-octen-3-ol stereoisomer as obtained from natural mushroom odor [159]. Similarly, the 10(*S*)-hydroperoxy-8(*E*),12(*Z*),15(*Z*)-octadecatrienoic acid [10(*S*)-HPOTE] was utilized from a racemate to afford (3*R*)-1,5(*Z*)-octadien-3-ol, 2(*Z*),5(*Z*)-octadien-1-ol, and 10-oxo-8(*E*)-decenoic acid [160]. Using labeled $^{18}O_2$ and linoleic acid, both products, 1-octen-3-ol and 10-oxo-8(*E*)-decenoic acid, contained ^{18}O-label [161], leading these investigators to suggest a concerted homolytic mechanism where both hydroperoxide oxygens are retained in the products [160]. However, one could just as easily suggest an enzyme-directed homolysis followed by $^{18}O_2$ hydroperoxidation of the resonance-stabilized octene radical and then hydroperoxide reduction. Extracts of another mushroom, *Pleurotus pulmonarius*, also oxidized linoleic acid into 1-octen-3-ol, and mild heat treatment of the extract led to termination of HOMLS activity and accumulation of an unidentified HPODE [162].

Although a number of products typical of homolytic scission have been found in plant and animal tissues or extracts (see review in Ref. 3), the specific enzyme responsible for the activity usually had not been determined. For example, a report of HOMLS activity in soybeans that cleaved 13(*S*)-HPOTE [163] is suspect, because this activity was found to exactly coelute chromatographically with two isozymes of LOX [39]. Further work with soybeans deficient in one of three wild-type isozymes showed the absence of both HOMLS and LOX activity at the expected elution time of the appropriate LOX isozyme absent in the mutant soybean (Gardner and Salch, unpublished work). It must be concluded that caution is advised in interpretation of data based only on product analysis.

5 "HETEROLYTIC" HYDROPEROXIDE LYASE PATHWAY

5.1 General

Tressl and Drawert [164] first reported HETLS in apple and banana. HETLS is widely distributed in a variety of plant tissues, especially leaves [165]. The term ''heterolytic'' derives from the mechanism, which resembles an acid-catalyzed Hock–Criegee heterolytic cleavage [166]. In actuality, the mechanism apparently is a heterolytic mimic, because the cytochrome P-450, subfamily CYP74B [167], is thought to catalyze homolytic hydroperoxide cleavage followed by a loss of an electron (Fig. 5). A number of reviews are available that discuss HETLS in more detail [3,4,7,169–173].

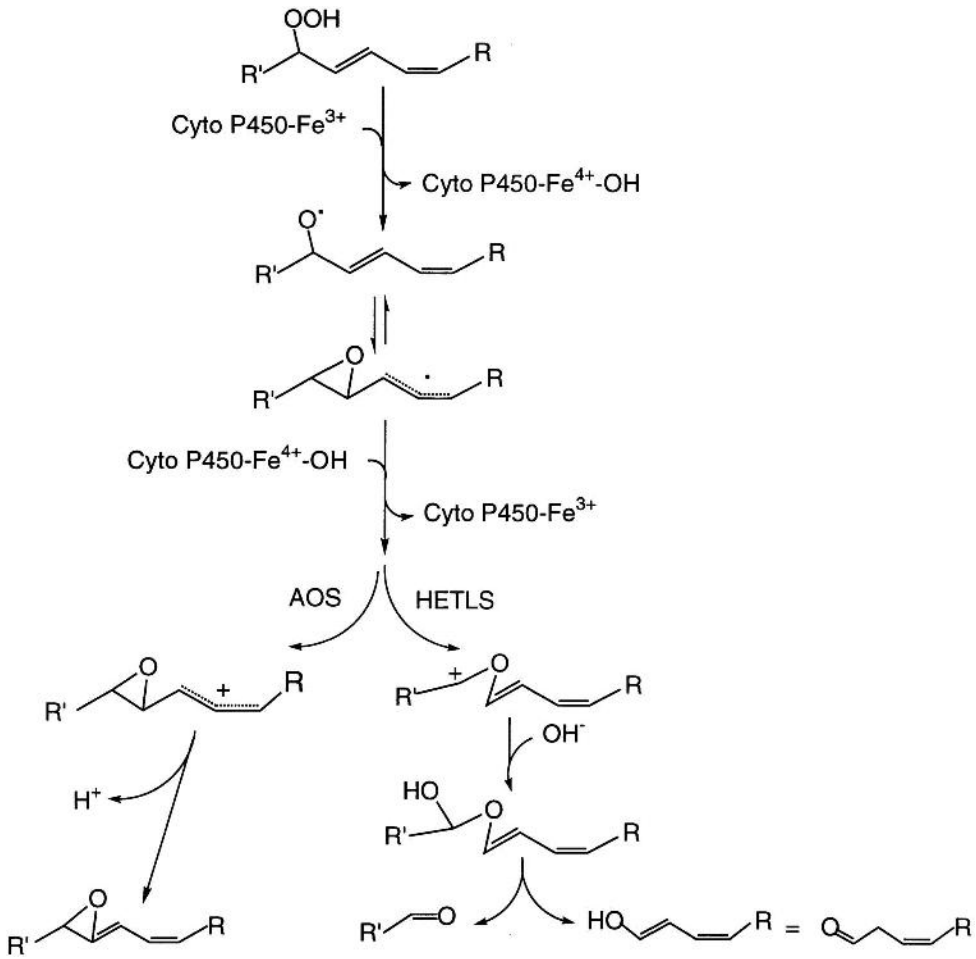


Figure 5 The mechanism of action of HETLS and AOS, both cytochrome P-450s, on substrate hydroperoxide of linoleic acid. The figure is not meant to imply that HETLS and AOS are identical enzymes, but rather their mechanism of action may be very similar (see Refs. 4 and 168). In regard to substrate 13-hydroperoxy-9(*Z*),11(*E*)-octadecadienoic acid, R′ = $CH_3(CH_2)_4$— and R = —$(CH_2)_7COOH$.

5.2 Aldehyde Products

Heterolytic hydroperoxide lyase cleaves 13-hydroperoxides, 13(*S*)-HPODE and 13(*S*)-HPOTE, into 12-oxo-9(*Z*)-dodecenoic acid and either hexanal [from 13(*S*)-HPODE] or 3(*Z*)-hexenal [from 13(*S*)-HPOTE]. The odor of hexanal is rancid/green, and 3(*Z*)-hexenal gives a strong note of freshly mowed grass. The 9-hydroperoxides, 9(*S*)-HPODE and 9(*S*)-HPOTE, cleave into two nine-carbon fragments, 9-oxononanoic acid and either 3(*Z*)-nonenal [from 9(*S*)-HPODE] or 3(*Z*),6(*Z*)-nonadienal [from 9(*S*)-HPOTE]. The latter two aldehydes have characteristic melon/cucumberlike odors.

The aldehydes are susceptible to further enzymic change (see reviews in Refs. 169 and 171–173). Reduction by alcohol dehydrogenase to their corresponding alcohols occurs. The odors of the alcohols are more subdued, but the overall flavor notes are usually similar to the corresponding aldehyde. Additionally, 3(*Z*)-hexenal, 3(*Z*)-nonenal, 3(*Z*),6(*Z*)-nondienal, and 12-oxo-9(*Z*)-dodecenoic acid are transformed into 2(*E*)-alkenals and 12-oxo-10(*E*)-dodecenoic acid by either isomerase(s) or nonenzymic transformation and, consequently, modify the odors. For a discussion of flavor notes obtained from the products of the HETLS pathway, a review by Hatanaka [169] should be consulted. In certain plants, the 3(*Z*)-alkenals are also converted into nonvolatile 4-hydroxy-2(*E*)-alkenals by LOX and hydroperoxide peroxygenase [63]. Likewise, it is thought that 9-hydroxy-12-oxo-10(*E*)-dodecenoic acid originates by a similar oxidative mechanism from 12-oxo-9(*Z*)-dodecenoic acid [67].

The entire pathway initiated by HETLS is summarized in Figure 6.

5.3 Isozymes of HETLS

Heterolytic hydroperoxide lyase was found to be difficult to isolate because of binding to membranes, often chloroplasts (e.g., Ref. 174) or microsomal membranes [175], requiring detergent solubilization. Additionally, HETLS is usually not very stable, and substrate hydroperoxides cause irreversible inactivation [176]. The enzyme usually exists as a trimer, tetramer, or multimer of the monomeric cytochrome P-450. Partial purification of HETLS by early workers indicated a molecular size ranging from 200 to 2000 kDa [177–181]. After purification of tea leaf HETLS to homogeneity, sodium dodecyl sulfate (SDS)-electrophoresis demonstrated a monomeric size of two isozymes to be 53 and 55 kDa in size [175]. Now, HETLS has been purified to homogeneity from sunflower hypocotyls [182], cucumbers [183], tomato [176], and bell pepper [184]. Also, HETLS has been cloned and sequenced from bell pepper [167] and *Arabidopsis* [185].

5.4 Substrate Specificity

Current knowledge indicates that HETLSs are specific for either of the 9(*S*)- or 13(*S*)-hydroperoxides of linoleic and linolenic acid, or both. Evidently, only the (*S*)-enantiomer serves as a substrate for HETLS [33,186]. There are many examples of narrow substrate specificity. Two separate isozymes from cucumber gave different substrate specificities, one specific for 13(*S*)-HPODE and one acting only on 9(*S*)-HPODE [187]. Pear fruit HETLS is another example of a HETLS specific for only 9(*S*)-hydroperoxides [179]. Two isozymes purified from bell pepper cleaved 13(*S*)-HPODE and 13(*S*)-HPOTE, but not the 9(*S*)-HPODE nor the 9(*E*),11(*E*)-diene isomer of the 13-hydroperoxide [184]. Many HETLS from various sources, mainly in tissues containing chloroplasts, have been reported to have about 10-fold more activity with 13(*S*)-HPOTE compared to 13(*S*)-HPODE [33,175,182,184,185]. Cucumber fruit mesocarp contained a HETLS isozyme that cleaved all four isomers, 9(*S*)-HPODE, 9(*S*)-HPOTE, 13(*S*)-HPODE, and 13(*S*)-HPOTE [183], but 9(*S*)-hydroperoxides are not metabolized by HETLS of sunflower hypocotyl [182], tomato leaf [176], bell pepper fruit [184], and tomato fruit [188]. Although soybean seed HETLS cleaves 9(*S*)-HPODE, 13(*S*)-HPODE, and 13(*S*)-HPOTE, only the 13(*S*)-isomers are substrates for soybean leaf [33]. From the work of Zhuang et al. [189], hydroperoxides of diacylglycerols and triacylglycerols are not substrates of HETLS.

Hatanaka et al. [190] examined a number of ''unnatural'' substrates for activity with HETLS, all were ω6-(*S*)-hydroperoxy-dienoic and ω6-(*S*)-hydroperoxytrienoic acids

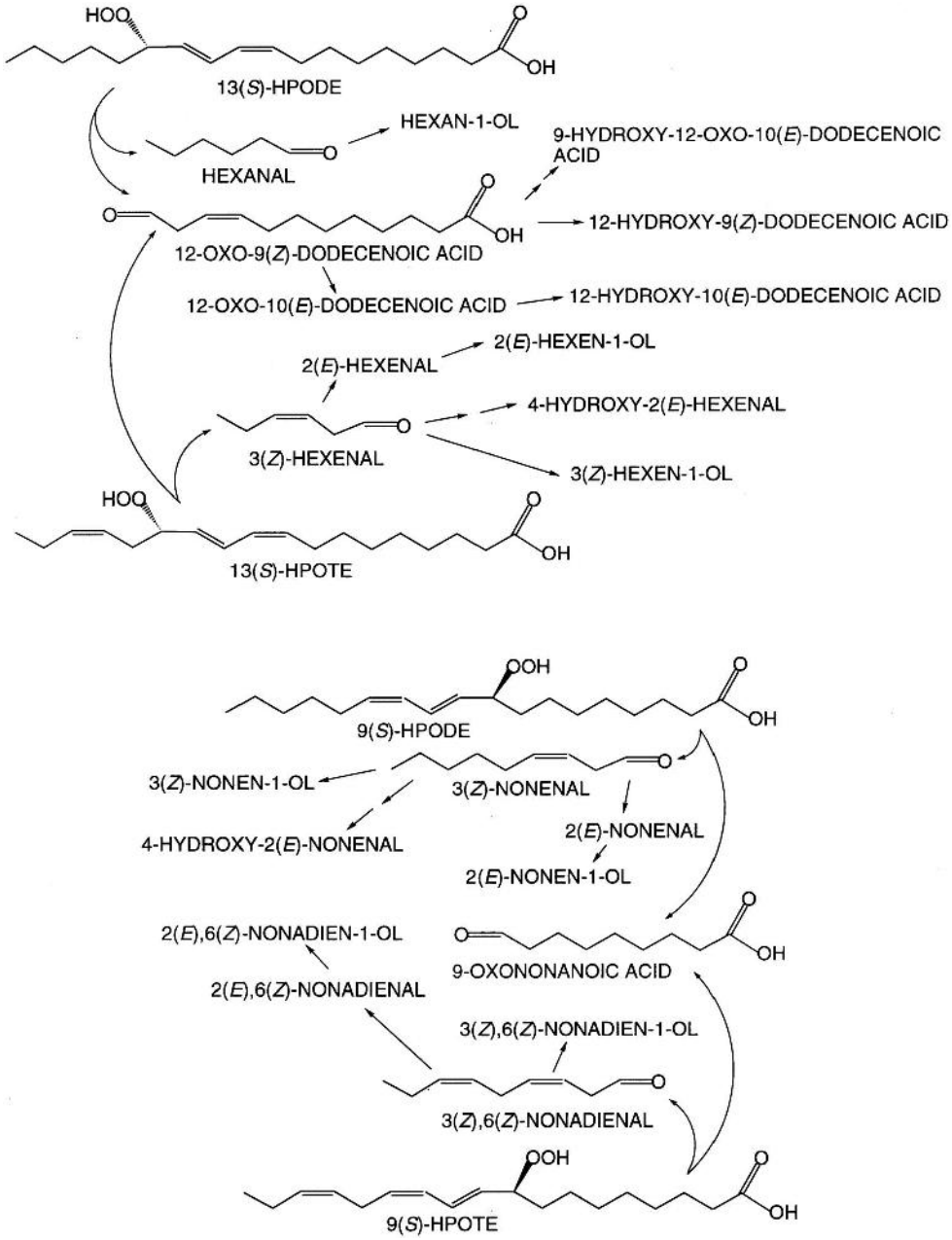


Figure 6 The HETLS pathway starting from 13(*S*)-HPODE/13(*S*)-HPOTE (top) and 9(*S*)-HPODE/9(*S*)-HPOTE (bottom).

ranging in size from C-14 to C-24. Although there was little activity from C-14 to C-17, surprisingly the activity increased substantially up to a maximum at C-22. 13-Hydroperoxy-linoleyl alcohol also serves as a substrate for tea HETLS [166].

5.5 Potential of HETLS as a Biocatalyst

As discussed earlier, HETLS has the potential of providing industry with flavors known to provide ''fresh'' notes associated with unprocessed fruit and vegetables. Consequently, fairly large quantities of aldehydes and alcohols from the HETLS pathway are produced by industry [175]. Interest in enzymic production is promoted by consumer preference and less stringent regulations necessary to produce biochemicals by enzymic methods.

Many difficulties are encountered with obtaining satisfactory HETLS activity. However, workers at Firmenich [191] have surmounted a number of pitfalls by utilizing an active-yeast sugar fermentation in conjunction with crude HETLS action on 13(*S*)-HPOTE substrates. The yeast fermentation acts to reduce HETLS-produced aldehydes to their corresponding alcohols in situ, thereby converting 3(*Z*)-hexenal to more chemically stable 3(*Z*)-hexen-1-ol. By delaying the addition of fermenting yeast, the majority of 3(*Z*)-hexenal was converted to 2(*E*)-hexenal, permitting the Firmenich workers to selectively produce 2(*E*)-hexen-1-ol, instead of 3(*Z*)-hexen-1-ol obtained in continuous fermentation.

Some of the problems encountered with plant HETLS have been alleviated by expression of recombinant HETLS in *Escherichia coli* [167,170]. It is likely that future innovation will concentrate on efforts at overexpression of HETLS in cloned systems, like *E. coli*.

5.6 Physiological Significance of HETLS

Since the first publication of fungal growth inhibition by products of HETLS [192], many reports have followed, including several from our lab. In general, alkenals, especially 2-alkenals, are more effective fungal growth inhibitors and inhibitors of fungal spore germination than alkanals. In addition, 12-oxo-10(*E*)-dodecenoic acid, called traumatin, was observed to cause wound repair in cut bean pod sections [193], but there have been very few related reports since then.

The physiological relevance of HETLS is rather enigmatic because there are only a few investigations that show a direct physiological consequence to plants on a molecular basis. In one such study [194], the HETLS products of isomeric hexenals and hexen-1-ols were measured after infection of bean plants with *Pseudomonas syringae* pv *phaseolicola*. An avirulent strain (incompatible combination) caused extensive hypersensitive cell collapse and production of HETLS volatiles, whereas the virulent strain (compatible combination) did not. The formation of HETLS volatiles were produced in high enough concentrations to inhibit the growth of the *P. syringae*, and their formation preceded phytoalexin elicitation giving an early defense against the microorganism. It is of interest that another member of the LOX pathway, methyl jasmonate, caused increased expression of LOX and HETLS activity [195,196]. Although the jasmonates have been shown to cause expression of a plethora of enzymes and proteins, evidence for similar gene expression caused by the HETLS products is surprisingly lacking, with the exception of a recent report showing that 2(*E*)-hexenal induced the expression of several genes similar to the ones induced by methyl jasmonate [197]. Potential for probing the physiological function

of HETLS also lies in future experiments that will upregulate or downregulate HETLS expression.

REFERENCES

1. GJ Piazza, ed. Lipoxygenase and Lipoxygenase Pathway Enzymes. Champaign, IL: AOCS Press, 1996.
2. GA Veldink, MP Hilbers, WF Nieuwenhuizen, JFG Vliegenthart. In: AF Rowley, H Kühn, T Schewe, eds. Eicosanoids and Related Compounds in Plants and Animals. London: Portland Press, 1998, pp 69–96.
3. A Grechkin. Prog Lipid Res 37:317–352, 1998.
4. E Blée. Prog Lipid Res 37:33–72, 1998.
5. HW Gardner. In: WW Christie, ed. Advances in Lipid Methodology—Four. Dundee, Scotland: The Oily Press, 1997, pp 1–43.
6. S Yamamoto. Biochim Biophys Acta 1128:117–131, 1992.
7. BA Vick. In: TS Moore, Jr, ed. Lipid Metabolism in Plants. Boca Raton, FL: CRC Press, 1993 pp 167–191.
8. HW Gardner. Biochim Biophys Acta 1084:221–239, 1991.
9. JN Siedow. Annu Rev Plant Physiol Plant Mol Biol 42:145–188, 1991.
10. D Shibata, B Axelrod. J Lipid Mediators Cell Signal 12:213–228, 1995.
11. BJ Gaffney. Annu Rev Biophys Biomol Struct 25:431–459, 1996.
12. HW Gardner. HortScience 30:197–205, 1995.
13. WH Gerwick. Biochim Biophys Acta 1211:243–255, 1994.
14. EI Solomon, J Zhou, F Neese, EG Pavel. Chem Biol 4:795–808, 1997.
15. WD Lehmann. Free Radical Biol Med 16:241–253, 1994.
16. M Hamberg, B Samuelsson. J Biol Chem 242:5329–5335, 1967.
17. MR Egmond, JFG Vliegenthart, J Boldingh. Biochem Biophys Res Commun 48:1055–1060, 1972.
18. MO Funk, Jr, JC Andre, T Otsuki. Biochemistry 26:6880–6884, 1987.
19. HW Gardner. Biochim Biophys Acta 1001:274–281, 1989.
20. K Schwarz, S Borngräber, M Anton, H Kühn. Biochemistry 37:15,327–15,335, 1998.
21. L-Y Zhang, M Hamberg. Lipids 31:803–809, 1996.
22. AR Brash, CD Ingram, TM Harris. Biochemistry 26:5465–5471, 1987.
23. M Pérez-Gilabert, GA Veldink, JFG Vliegenthart. Arch Biochem Biophys 354:18–23, 1998.
24. DL Sloane. In: GJ Piazza, ed. Lipoxygenase and Lipoxygenase Pathway Enzymes. Champaign, IL: AOCS Press, 1996, pp 57–79.
25. JA Matthew, HW-S Chan, T Galliard. Lipids 12:324–326, 1977.
26. HW Gardner, D Weisleder. Lipids 5:678–683, 1970.
27. T Galliard, DR Phillips. Biochem J 124:431–438, 1971.
28. PGM van Aarle, MMJ de Barse, GA Veldink, JFG Vliegenthart. FEBS Lett 280:159–162, 1991.
29. H Kühn, R Weisner, VZ Lankin, A Nekrasov, L Alder, T Schewe. Anal Biochem 160:24–34, 1987.
30. M Hamberg, L-Y Zhang, ID Brodowsky, EH Oliw. Arch Biochem Biophys 309:77–80, 1994.
31. M Hamberg, C Su, E Oliw. J Biol Chem 273:13,080–13,088, 1998.
32. MR Egmond, GA Veldink, JFG Vliegenthart, J Boldingh. Biochim Biophys Acta 409:399–401, 1975.
33. HW Gardner, D Weisleder, RD Plattner. Plant Physiol 97:1059–1072, 1991.
34. AN Grechkin, RA Kuramshin, EY Safonova, YJ Yefremov, SK Latypov, AV Ilyasov, IA Tarchevsky. Biochim Biophys Acta 1081:79–84, 1991.

35. I Feussner, H. Kühn. FEBS Lett 367:12–14, 1995.
36. D Regdel, H Kühn, T Schewe. Biochim Biophys Acta 1210:297–302, 1994.
37. CPA van Os, GPM Rijke-Schilder, H van Halbeek, J Verhagen, JFG Vliegenthart. Biochim Biophys Acta 663:177–193, 1981.
38. GJ Garssen, JFG Vliegenthart, J Boldingh. Biochem J 122:327–332, 1971.
39. YP Salch, MJ Grove, H Takamura, HW Gardner. Plant Physiol 108:1211–1218, 1995.
40. JR Kanofsky, B Axelrod. J Biol Chem 261:1099–1104, 1986.
41. HW Gardner, H Takamura, DF Hildebrand, KPC Croft, TD Simpson, YP Salch. In: GJ Piazza, ed. Lipoxygenase and Lipoxygenase Pathway Enzymes. Champaign, IL: AOCS Press, 1996, pp 162–175.
42. GS Bild, CS Ramadoss, S Lim, B Axelrod. Biochem Biophys Res Commun 74:949–954, 1977.
43. GS Bild, CS Ramadoss, B Axelrod. Arch Biochem Biophys 184:36–41, 1977.
44. RH Andrianarison, M Tixier, JL Beneytout. Biochem Biophys Res Commun 172:985–992, 1990.
45. RH Andrianarison, M Tixier, JL Beneytout. Plant Sci 75:215–220, 1991.
46. D Heydeck, R Wiesner, H Kühn, T Schewe. Biomed Biochim Acta 50:11–15, 1991.
47. T Shimizu, O Rådmark, B Samuelsson. Proc Natl Acad Sci USA 81:689–693, 1984.
48. PGM van Aarle, MMJ de Barse, GA Veldink, JFG Vliegenthart. In: C Abdelkader, ed. Metabolism, Structure and Utilization of Plant Lipids. Tunis: CNP, 1992, pp 216–219.
49. CC Reddy, C Bertler, S Hammarström. Arch Biochem Biophys 279:211–217, 1990.
50. D-E Sok, TS Phi, CH Jung, YS Chung, JB Kang. Biochem Biophys Res Commun 153:840–847, 1988.
51. MC Feiters, GA Veldink, JFG Vliegenthart. In: G Rotilio, JV Bannister, eds. Oxidative Damage and Related Enzymes. Life Chemistry Reports, Supplement Series; EMBO Workshop, Rome, Italy, 1983. London: Harwood Academy Publishers, 1984, pp 132–138.
52. D-E Sok, MR Kim. Arch Biochem Biophys 277:86–93, 1990.
53. D-E Sok, MR Kim. J Agric Food Chem 42:2703–2708, 1994.
54. AN Grechkin, RA Kuramshin, YJ Yefremov, SK Latypov, EY Safonova, AV Ilyasov. Dokl AN SSSR (Proc Acad Sci USSR) 314:1247–1249, 1990.
55. I Tarchevsky, R Kuramshin, A Grechkin. In: PJ Quinn, JL Harwood, eds. Plant Lipid Biochemistry, Structure and Utilization. London: Portland Press, 1990, pp 298–300.
56. MMJ de Barse, PGM van Aarle, GA Veldink, JFG Vliegenthart. In: C Abdelkader, ed. Metabolism, Structure and Utilization of Plant Lipids. Tunis: CNP, 1992, pp 212–215.
57. MW Bernart, GG Whatley, WH Gerwick. J Nat Prod 56:245–259, 1993.
58. GS Bild, SG Bhat, CS Ramadoss, B Axelrod. J Biol Chem 253:21–23, 1978.
59. S Parchmann, MJ Mueller. J Biol Chem 273:32,650–32,655, 1998.
60. J Abián, E Gelpi, M Pagès. Plant Physiol 95:1277–1283, 1991.
61. H Kühn, L Eggert, OA Zabolotsky, GI Myagkova, T Schewe. Biochemistry 30:10,269–10,273, 1991.
62. AN Grechkin, RA Kuramshin, SK Latypov, EY Safonova, TE Gafarova, AV Ilyasov. Eur J Biochem 199:451–457, 1991.
63. HW Gardner, MJ Grove. Plant Physiol 116:1359–1366, 1998.
64. AN Grechkin, RA Kuramshin, EY Safonova, LS Mukhtarova, SK Latypov, AV Ilyasov. Chem Phys Lipids 66:199–208, 1993.
65. HW Gardner, M Hamberg. J Biol Chem 268:6971–6977, 1993.
66. H Takamura, HW Gardner. Biochim Biophys Acta 1303:83–91, 1996.
67. HW Gardner. Lipids 33:745–749, 1998.
68. CR Smith. Prog Chem Fats Other Lipids 11:139–177, 1979.
69. R Yamauchi, M Kojima, K Kato, Y Ueno. Agric Biol Chem 49:2475–2477, 1985.
70. Y Kondo, Y Kawai, T Hayashi, M Ohnishi, T Miyazawa, S Itoh, J Mizutani. Biochim Biophys Acta 1170:301–306, 1993.

71. K Matsui, M Nishioka, M Ikeyoshi, Y Matsumura, T Mori, T Kajiwara. Biochim Biophys Acta 1390:8–20, 1998.
72. M Maccarrone, PGM van Aarle, GA Veldink, JFG Vliegenthart. Biochim Biophys Acta 1190:164–169, 1994.
73. M Maccarrone, GA Veldink, A Finazzi Agrò, JFG Vliegenthart. FEBS Lett 371:223–226, 1995.
74. I Feussner, H Kindl. FEBS Lett 298:223–225, 1992.
75. T Kato, H Ohta, K Tanaka, D Shibata. Plant Physiol 98:324–330, 1992.
76. I Feussner, C Wasternack, H Kindl, H Kühn. Proc Natl Acad Sci USA 92:11,849–11,853, 1995.
77. I Feussner, TJ Balkenhohl, A Porzel, H Kühn, C Wasternack. J Biol Chem 272:21,635–21,641, 1997.
78. I Feussner, A Bachmann, M Höhne, H Kindl. FEBS Lett 431:433–436, 1998.
79. K Matsui, T Kajiwara. Lipids 30:733–738, 1995.
80. MP Luquet, C Pourplanche, M Podevin, G Thompson, V Larreta-Garde. Enzyme Microb Technol 15:842–848, 1993.
81. GJ Piazza, A Nuñez. J Am Oil Chem Soc 72:463–466, 1995.
82. GJ Piazza, TA Foglia, A Nuñez. J Am Oil Chem Soc 73:1045–1049, 1996.
83. JC Allen. J Chem Soc Chem Commun 906–907, 1969.
84. M van der Stelt, WF Nieuwenhuizen, GA Veldink, JFG Vliegenthart. FEBS Lett 411:287–290, 1997.
85. G van Zadelhoff, GA Veldink, JFG Vliegenthart. Biochem Biophys Res Commun 248:33–38, 1998.
86. A Hatanaka, T Kajiwara, K Matsui, M Yamaguchi. Z Naturforsch 44c:64–70, 1989.
87. MJ Novak. Bioorg Med Chem Lett 9:31–34, 1999.
88. RT Holman, PO Egwim, WW Christie. J Biol Chem 244:1149–1151, 1989.
89. P Zhang, KS Kyler. J Am Chem Soc 111:9241–9242, 1989.
90. VK Datcheva, K Kiss, L Solomon, KS Kyler. J Am Chem Soc 113:270–274, 1991.
91. G Scheller, E Jäger, B Hoffmann, M Schmitt, P Schreier. J Agric Food Chem 43:1768–1774, 1995.
92. D Martini, G Buono, G Iacazio. J Org Chem 61:9062–9064, 1996.
93. H Berry, H Débat, VL Garde. J Biol Chem 273:2769–2776, 1998.
94. H Berry, H Débat, VL Garde. FEBS Lett 408:324–326, 1997.
95. G Iacazio, G Langrand, J Baratti, G Buono, C Triantaphylides. J Org Chem 55:1690–1691, 1990.
96. MJ Schilstra, GA Veldink, JFG Vliegenthart. Lipids 29:225–231, 1994.
97. S Srinivasulu, AGA Rao. J Agric Food Chem 41:366–371, 1993.
98. S Srinivasulu, AGA Rao. J Agric Food Chem 43:562–567, 1995.
99. D Martini, G Iacazio, D Ferrand, G Buono, C Trianthaphylides. Biocatalysis 11:47–63, 1994.
100. M-L Fauconnier, M Marlier. Biotechnol Tech 10:839–844, 1996.
101. MBW Elshof, M Janssen, GA Veldink, JFG Vliegenthart. Rec Trav Chim Pays-Bas 115: 499–504, 1996.
102. MB Elshof, GA Veldink, JFG Vliegenthart. Fett/Lipid 100:246–251, 1998.
103. P Drouet, D Thomas, MD Legoy. Tetrahedron Lett 35:3923–3926, 1994.
104. M Gargouri, MD Legoy. J Am Oil Chem Soc 74:641–645, 1997.
105. EA Emken, HJ Dutton. J Am Oil Chem Soc 48:324–329, 1971.
106. S Grossman, M Trop, P Budowski, M Perl, A Pinsky. Biochem J 127:909–910, 1972.
107. MC Pinto, P Macías. Appl Biochem Biotechnol 59:309–318, 1996.
108. S Shen, A-F Hsu, TA Foglia, S-I Tu. Appl Biochem Biotechnol 69:79–90, 1998.
109. A-F Hsu, S Shen, E Wu, TA Foglia. Biotechnol Appl Biochem 28:55–59, 1998.
110. D Parra-Diaz, DP Brower, MB Medina, GJ Piazza. Biotechnol Appl Biochem 18:359–367, 1993.

111. GJ Piazza, DP Brower, D Parra-Diaz. Biotechnol Appl Biochem 19:243–252, 1994.
112. S Battu, J Cook-Moreau, JL Beneytout. Biochim Biophys Acta 1211:270–276, 1994.
113. S Battu, H Rabinovitch-Chable, J-L Beneytout. J Agric Food Chem 42:2115–2119, 1994.
114. B Liagre, S Battu, M Rigaud, JL Beneytout. J Agric Food Chem 44:2057–2062, 1996.
115. MC Pinto, JL Gata, P Macías. Biotechnol Prog 13:394–398, 1997.
116. A-F Hsu, TA Foglia, GJ Piazza. Biotechnol Lett 19:71–74, 1997.
117. F Mauch, A Kmecl, U Schaffrath, S Volrath, J Görlach, E Ward, J Ryals, R Dudler. Plant Physiol 114:1561–1566, 1997.
118. J Royo, G Vancanneyt, AG Pérez, C Sanz, K Störmann, S Rosahl, JJ Sánchez-Serrano. J Biol Chem 271:21,012–21,019, 1996.
119. A Geerts, D Feltkamp, S Rosahl. Plant Physiol 105:269–277, 1994.
120. DM Saravitz, JN Siedow. Plant Physiol 110:287–299, 1996.
121. T Heitz, DR Bergey, CA Ryan. Plant Physiol 114:1085–1093, 1997.
122. E Bell, JE Mullet. Mol Gen Genet 230:456–462, 1991.
123. T Kato, Y Maeda, T Hirukawa, T Namai, N Yoshioka. Biosci Biotech Biochem 56:373–375, 1992.
124. T Kato, Y Yamaguchi, T Namai, T Hirukawa. Biosci Biotech Biochem 57:283–287, 1993.
125. T Namai, T Kato, Y Yamaguchi, T Hirukawa. Biosci Biotech Biochem 57:611–613, 1993.
126. MA Melan, X Dong, ME Endara, KR Davis, FM Ausubel, TK Peterman. Plant Physiol 101: 441–450, 1993.
127. Y-L Peng, Y Shirano, H Ohta, T Hibino, K Tanaka, D Shibata, J Biol Chem 269:3755–3761, 1994.
128. C Véronési, M Rickauer, J Fournier, ML Pouénat, M-T Esquerré-Tugayé. Plant Physiol 112: 997–1004, 1996.
129. M Maccarrone, GA Veldink, JFG Vliegenthart. FEBS Lett 309:225–230, 1992.
130. M Maccarrone, GA Veldink, JFG Vliegenthart, A Finazzi Agrò. FEBS Lett 408:241–244, 1997.
131. A Conconi, MJ Smerdon, GA Howe, CA Ryan. Nature 383:826–829, 1996.
132. A Conconi, M Miquel, JA Browse, CA Ryan. Plant Physiol 111:797–803, 1996.
133. WL Holtman, G van Duijn, NJA Sedee, AC Douma. Plant Physiol 111:569–576, 1996.
134. WL Holtman, JC Vredenbregt-Heistek, NF Schmitt, I Feussner. Eur J Biochem 248:452–458, 1997.
135. M Höhne, A Nellen, K Schwennesen, H Kindl. Eur J Biochem 241:6–11, 1996.
136. I Feussner, H Kühn, C Wasternack. FEBS Lett 406:1–5, 1997.
137. K Matsui, K Hijiya, Y Tabuchi, T Kajiwara. Plant Physiol 119:1279–1287, 1999.
138. EE Farmer, CA Ryan. Plant Cell 4:129–134, 1992.
139. DY Henry, F Gueritte-Voegelein, PA Insel, N Ferry, J Bouguet, P Potier, T Sevenet, J Hanoune. Eur J Biochem 170:389–394, 1987.
140. W Jira, G Spiteller, A Schramm. Chem Phys Lipids 84:165–173, 1996.
141. W Jira, G Spiteller, W Carson, A Schramm. Chem Phys Lipids 91:1–11, 1998.
142. TA Haas, MC Bertomeu, E Bastida, MR Buchanan. Biochim Biophys Acta 1051:174–178, 1990.
143. J Pongracz, JM Lord. Biochem Biophys Res Commun 256:269–272, 1999.
144. A Truitt, G McNeill, JY Vanderhoek. Biochim Biophys Acta 1438:239–246, 1999.
145. MA Tloti, DG Moon, LK Weston, JE Kaplan. Thromb Res 62:305–317, 1991.
146. SA Spindler, KS Clark, DM Callewaert, RG Reddy. Biochem Biophys Res Commun 218: 187–191, 1996.
147. MJ van de Velde, F Engels, PAJ Henricks, PGM Bloemen, FP Nijkamp. FEBS Lett 369: 301–304, 1995.
148. Y Fujimoto, T Sumiya, M Tsunomori, H Nishida, S Sakuma, T Fujita. Int J Biochem 26: 127–131, 1994.

149. D Moch, T Schewe, H Kühn, D Schmidt, P Buntrock. Biomed Biochim Acta 49:201–207, 1990.
150. JJMC de Groot, GA Veldink, JFG Vliegenthart, J Boldingh, R Wever, BF van Gelder. Biochim Biophys Acta 377:71–79, 1975.
151. MA Schilstra, GA Veldink, JFG Vliegenthart. Biochemistry 32:7686–7691, 1993.
152. HW Gardner. Free Radical Biol Med 7:65–86, 1989.
153. GJ Garssen, JFG Vliegenthart, J Boldingh. Biochem J 130:435–442, 1972.
154. R Labeque, LJ Marnett. J Am Chem Soc 109:2828–2829, 1987.
155. BA Vick, DC Zimmerman. Plant Physiol 90:125–132, 1989.
156. R-H Andrianarison, J-L Beneytout, M Tixier. Plant Physiol 91:1280–1287, 1989.
157. RH Andrianarison, JL Beneytout. Plant Sci 87:143–150, 1992.
158. M Wurzenberger, W Grosch. Z Lebensm Unters Forsch 175:186–190, 1982.
159. M Wurzenberger, W Grosch. Biochim Biophys Acta 795:163–165, 1984.
160. M Wurzenberger, W Grosch. Lipids 21:261–266, 1986.
161. M Wurzenberger, W Grosch. Biochim Biophys Acta 794:18–24, 1984.
162. S Assaf, Y Hadar, CG Dosoretz. Enzyme Microb Technol 21:484–490, 1997.
163. Y Kondo, Y Hashidoko, J Mizutani. Biochim Biophys Acta 1255:9–15, 1995.
164. R Tressl, F Drawert. J Agric Food Chem 21:560–565, 1973.
165. J Sekiya, T Kajiwara, K Munechika, A Hatanaka. Phytochemistry 22:1867–1869, 1983.
166. A Hatanaka, T Kajiwara, J Sekiya, H Toyota. Z Naturforsch 41c:359–362, 1986.
167. K Matsui, M Shibutani, T Hase, T Kajiwara. FEBS Lett 394:21–24, 1996.
168. W-C Song, SW Baertschi, WE Boeglin, TM Harris, AR Brash. J Biol Chem 268:6293–6298, 1993.
169. A Hatanaka. Phytochemistry 34:1201–1218, 1993.
170. K Matsui. Belg J Bot 131:50–62, 1998.
171. HW Gardner. In: DB Min, TH Smouse, eds. Flavor Chemistry of Lipid Foods. Champaign, IL: American, Oil Chemists Society, 1989, pp 98–112.
172. HW Gardner. In: DB Min, TH Smouse, eds. Flavor Chemistry of Fats and Oils. Champaign, IL: American Oil Chemists Society, 1985, pp 189–206.
173. A Hatanaka, T Kajiwara, K Matsui. Z Naturforsch 50c:467–472, 1995.
174. E Blée, J Joyard. Plant Physiol 110:445–454, 1996.
175. K Matsui, H Toyota, T Kajiwara, T Kakuno, A Hatanaka. Phytochemistry 30:2109–2113, 1991.
176. M-L Fauconnier, AG Perez, C Sanz, M Marlier. J Agric Food Chem 45:4232–4236, 1997.
177. P Schreier, G Lorenz. Z Naturforsch 37c:165–173, 1981.
178. JM Olías, JJ Rios, M Valle, R Zamora, LC Sanz, B Axelrod. J Agric Food Chem 38:624–630, 1990.
179. I-S Kim, W Grosch. J Agric Food Chem 29:1220–1225, 1981.
180. BA Vick, DC Zimmerman. Plant Physiol 57:780–788, 1976.
181. BA Vick, DC Zimmerman. Plant Physiol 85:1073–1078, 1987.
182. A Itoh, BA Vick. Biochim Biophys Acta 1436:531–540, 1999.
183. AR Hornostaj, DS Robinson. Food Chem 66:173–180, 1999.
184. Y Shibata, K Matsui, T Kajiwara, A Hatanaka. Plant Cell Physiol 36:147–156, 1995.
185. NJ Bate, S Sivasankar, C Moxon, JMC Riley, JE Thompson, SJ Rothstein. Plant Physiol 117:1393–1400, 1998.
186. T Kajiwara, J Sekiya, M Asano, A Hatanaka. Agric Biol Chem 46:3087–3088, 1982.
187. K Matsui, Y Shibata, T Kajiwara, A Hatanaka. Z Naturforsch 44c:883–885, 1989.
188. T Galliard, JA Matthew. Phytochemistry 16:339–343, 1977.
189. H Zhuang, DF Hildebrand, RA Andersen, TR Hamilton-Kemp. J Agric Food Chem 39:1357–1364, 1991.
190. A Hatanaka, T Kajiwara, K Matsui, H Toyota. Z Naturforsch 47c:677–679, 1992.
191. IM Whitehead, BL Muller, C Dean. Cereal Foods World 40:193–197, 1995.

192. RT Major, P Marchini and T Sproston, J Biol Chem 235:3298–3299, 1960.
193. DC Zimmerman, CA Coudron. Plant Physiol 63:536–541, 1979.
194. KPC Croft, F Jüttner, AJ Slusarenko. Plant Physiol 101:13–24, 1993.
195. S Avdiushko, KPC Croft, GC Brown, DM Jackson, TR Hamilton-Kemp, D Hildebrand. Plant Physiol 109:1227–1230, 1995.
196. M Kohlmann, A Bachmann, H Weichert, A Kolbe, T Balkenhohl, C Wasternack, I Fuessner. Eur J Biochem 260:885–895, 1999.
197. NJ Bate, SJ Rothstein. Plant J 16:561–569, 1998.

10

Biocatalysis by the Plant Lipoxygenase Pathway

Hydroperoxide-Metabolizing Enzymes

ALEXANDER N. GRECHKIN

Kazan Institute of Biochemistry and Biophysics, Russian Academy of Sciences, Kazan, Russia

HAROLD W. GARDNER*

National Center for Agricultural Utilization Research, Agricultural Research Service, U.S. Department of Agriculture, Peoria, Illinois

1 ALLENE OXIDE SYNTHASE

1.1 General Properties

Allene oxide synthase (AOS) transforms conjugated hydroperoxydiene fatty acids from lipoxygenase (LOX) action into allene oxides, such as the conversion of 13(*S*)-hydroperoxy-9(*Z*),11(*E*)-octadecadienoic acid [13(*S*)-HPODE] into 12,13(*S*)-epoxy-9(*Z*),11-octadecadienoic acid. A unique feature of AOS is its unusually high turnover of about 1000/sec [1]. This feature combined with the lack of requirements for cofactors, O_2, or other special conditions, make AOS an attractive possibility for use as a biocatalyst. As discussed later, diverse product fatty acids can be derived from the allene oxide.

Zimmerman [2] first reported AOS activity in flaxseed. Because the allene oxide generated from 13(*S*)-HPODE was unstable, he actually observed a hydrolysis product, the α-ketol fatty acid, 12-oxo-13-hydroxy-9(*Z*)-octadecenoic acid. For this reason, AOS was initially incorrectly named hydroperoxide isomerase. An authentic hydroperoxide isomerase (recently renamed epoxy alcohol synthase; see Sec. 3) was later found in the fungus *Saprolegnia parasitica*; additionally, this enzyme is described in Chapter 14. AOS was

* *Current affiliation*: Veteran's Administration Hospital and University of Wisconsin-Madison, Madison, Wisconsin.

also named hydroperoxide cyclase, hydroperoxide dehydrase, and hydroperoxide dehydratase before the current AOS designation became accepted. Hamberg [3] first observed the unstable allene oxide intermediate 12,13(*S*)-epoxy-9(*Z*),11-octadecadienoic acid, which has a half-life of only 33 sec at 0°C. Brash et al. [4] found that the allene oxide could be isolated for spectral measurements, provided low temperatures and precautions are used to prevent solvolysis.

Allene oxide synthase is an unusual cytochrome P-450 of 53–59 kDa size. Because of its weak CO binding [1] and other catalytic features, AOS has been designated in the CYP74 family of cytochrome P-450s [5]. Evidence suggests that AOS catalytically converts hydroperoxide into allene oxide by a two-electron process [6] that has features similar to the mechanism suggested for transforming hydroperoxides by another cytochrome P-450 heterolytic hydroperoxide lyase (HETLS) specialized for cleaving the carbon chain of fatty acid hydroperoxides (see Fig. 5 of Chap. 9). AOS has been cloned and sequenced from flaxseed [5], guayule [7], and *Arabidopsis* [8]. Usually, the enzyme has been found to be membrane bound, especially to chloroplast membranes and, consequently, the flax and *Arabidopsis* AOS was found to have a transit sequence resembling a chloroplast transit peptide. However, the AOS found in guayule rubber particles is uniquely soluble because of the absence of this peptide.

An AOS from the animal kingdom (coral) has been cloned and sequenced [9]. Interestingly, it was found to be unlike plant AOS, having homology with catalase, and, additionally, it existed as a fusion protein with the coral LOX that forms the coral AOS substrate, the 8(*R*)-hydroperoxide of arachidonic acid.

1.2 Noncyclic Fatty Acid and Macrolactone Products

With allene oxide derived from hydroperoxydiene fatty acids, such as 13(*S*)-HPODE and 9(*S*)-hydroperoxy-10(*E*),12(*Z*)-octadecadienoic acid [9(*S*)-HPODE], a number of fatty acids have been formed from substitution with various nucleophiles. In aqueous buffers, the most usual products obtained are α-ketols [2] and γ-ketols [10] from hydrolysis of the allene oxide through a delocalized carbocation. In addition to hydroxyl anion, a large number of other nucleophiles have been known to substitute at the allene oxide (Fig. 1), such as the anions of fatty acids [10,11], sulfhydryls [11], and methanol [3,11]. Substitution by the methoxyl group was accomplished in two ways: by reacting AOS with hydroperoxide in 20% methanol [11] or quenching with methanol within 10–90 sec after rapidly converting the hydroperoxide to the allene oxide on ice [3]. Similarly, after a short 10-sec reaction of AOS with 13(*S*)-HPODE and quenching with non-nucleophilic solvents, such as acetonitrile, the allene oxide undergoes an intramolecular attack by the carboxylic acid moiety to form macrolactones [13].

Along with nucleophilic (S_N2 or S_N1) mechanisms, allene oxides are hydrolyzed through the electrophilic (acid dependent) mechanism [14]. Acidic conditions are strongly favorable for γ-ketol formation and unfavorable for α-ketol formation [14]. Formation of a third minor ketol, 11-hydroxy-12-oxo-9(*Z*),15(*Z*)-octadecadienoic acid, was observed during 13-hydroperoxy-9(*Z*),11(*E*),15(*Z*)-octadecatrienoic acid (13-HPOTE) incubation with flax AOS under the acidic conditions [14,15].

A number of ''unnatural'' substrates of AOS were tested by Schneider and Schreier [16]. They found that hydroperoxides of oleic acid and 12-hydroperoxy-9(*Z*),13(*E*)-octadecadienoic acid were not active, but both (*R*)- and (*S*)-enantiomers of 13-HPODE and 13-HPOTE were substrates for AOS. Additionally, they found that synthetic substrates

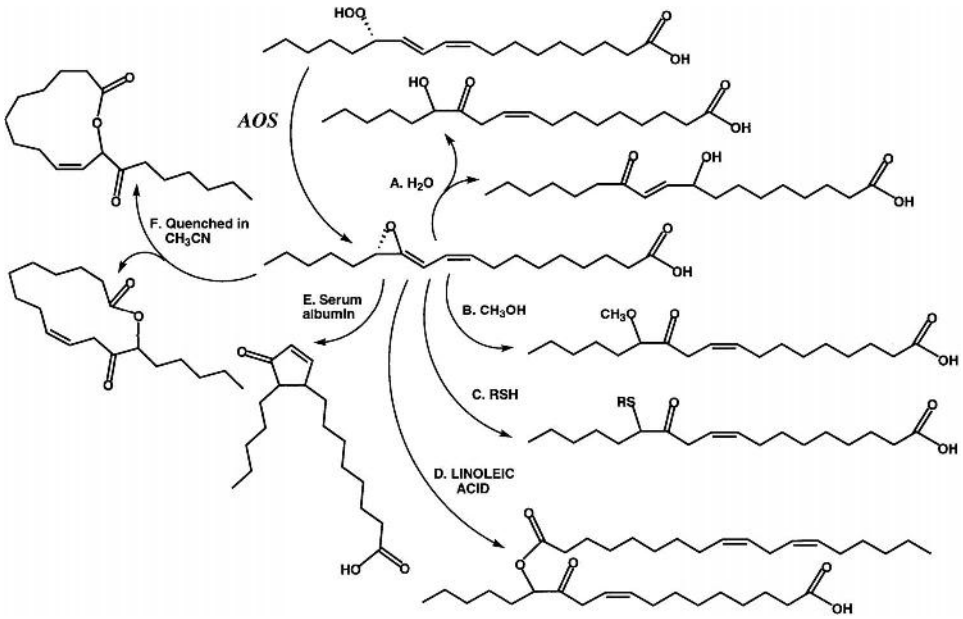


Figure 1 The diverse products obtained by action of AOS on 13(*S*)-HPODE. The allene oxide formed (middle) reacts with (A) water, forming α- and γ-ketols [2,10], (B) methanol, giving methoxy-oxo fatty acids [3,11], (C) ethylthiol [11], (D) linoleic acid, forming an estolide [10,11], (E) serum albumin, which promotes cyclization [12], (F) and quenching in aprotic solvent (acetonitrile), promoting the formation of macrolactones [13].

structurally resembling the natural substrate 13-HPODE [adipates of 7-hydroperoxy-3(*Z*),5(*E*)-dodecadien-1-ol and 10-cyclohexyl-7-hydroperoxy-3(*Z*),5(*E*)-decadien-1-ol] were also active substrates.

1.3 Cyclic Fatty Acid Products

Unlike the conversion of hydroperoxydiene fatty acids described earlier, the hydroperoxide of linolenic acid, 13(*S*)-HPOTE, spontaneously cyclizes into racemic 12-oxo-phytodienoic acid to the extent of about 12% with the remainder being α- and γ-ketols [17]. It is this conversion that is of considerable interest to plant physiologists because 12-oxo-phytodienoic acid serves as a precursor to formation of the jasmonate family of phytohormones. Although the extra 15(*Z*) double bond provided by 13(*S*)-HPOTE does not participate directly in cyclization, it determines the capability to undergo the cyclization [18]. Spontaneous cyclization affords 12-oxo-phytodienoic acid with cis side chains of racemic configuration [9(*S*),13(*S*)- and 9(*R*),13(*R*)-]. The 12-oxo-phytodienoic acid with 9(*S*),13(*S*) side chains is the ''natural'' product of the plant oxylipin pathway, and this isomer is the precursor of (+)-(3*R*,7*S*)-7-iso-jasmonic acid [19]. In some applications, (3*R*,7*S*)-7-iso-jasmonic acid has greater biological activity than jasmonic acid [20]. The 9(*S*),13(*S*)-12-oxo-phytodienoic acid isomer was specifically produced in the presence of both AOS and allene oxide cyclase [17]. Allene oxide cyclase has been recently purified from maize seeds [21]. With sufficient allene oxide cyclase activity, the yield of 12-oxo-phytodienoic acid increased greatly at the expense of hydrolysis to ketols. However, in order for allene

oxide cyclase to function optimally, the concentration of substrate must be very low (3 μ*M*), making it impractical to form 9(*S*),13(*S*)-12-oxo-phytodienoic acid isomer in quantity [17]. A suggested method of surmounting the problem could be by the ''natural'' method employed by wounded plants; that is, lipase could provide controlled release of linolenic acid from glyceride lipid to feed a sequential enzyme system comprised of a 13-oxidizing LOX, AOS, and allene oxide cyclase.

After it is formed, the 9(*S*),13(*S*)-12-oxo-phytodienoic acid isomer is further susceptible to chemical isomerization because of the 12-oxo-11-ene structure. A portion of the 13(*S*) side chain can isomerize into the (9*S*,13*R*) isomer [19,22], giving the configuration needed for the biosynthesis of ''natural'' (−)-(3*R*,7*R*)-jasmonic acid. In addition, the ring double bond shifts to a lesser extent [22].

Unlike the facile cyclization of 13(*S*)-HPOTE, spontaneous cyclization of 13(*S*)-HPODE into 12-oxo-10-phytoenoic acid (also known as 15,16-dihydro-12-oxo-phytodienoic acid) furnished very small yields. Typically, the resulting cyclopentenone/α-ketol ratio is 1 : 100 or less (e.g., Refs. 5 and 18). Such a metabolite would be a possible precursor of the occasionally observed 9,10-dihydro-jasmonic acid [20]. Furthermore, yields of 12-oxo-10-phytoenoic acid were not improved by the addition of purified allene oxide cyclase [21]. However, Hamberg and Hughes [12] showed that serum albumin promoted cyclization of 13(*S*)-HPODE into a racemic mixture of 12-oxo-10-phytoenoic acid (up to 24% yield) with side chains predominantly in a trans configuration. Additionally, they found that serum albumin greatly prolonged the half-life of the allene oxide as long as 14 min at 0°C, which possibly contributed to the cyclization. Gundlach and Zenk [23] utilized the serum albumin method to obtain 12-oxo-10-phytoenoic acid for physiological testing. 12-Oxo-10-phytoenoic acid was determined to have lower but significant activity, compared to 12-oxo-phytodienoic acid. Although Gundlach and Zenk detected 12-oxo-10-phytoenoic acid from a reaction of flaxseed AOS in the absence of serum albumin, they did not report its relative yield [23]. Other claims for an ''linoleic acid'' pathway have not yet demonstrated a significant yield of 12-oxo-10-phytoenoic acid either in vivo or in vitro. For example, in unpublished work (see review in Ref. 24) from Gardner's lab, 12-oxo-10-phytoenoic acid was obtained from 13(*S*)-HPODE reaction with corn AOS as demonstrated by gas chromatography–mass spectrometry (GC–MS) analysis, but the yield was not determined. This isolate separated into three isomers usually obtained by GC of 12-oxo-phytodienoic acid [22] (i.e., cis side chains, trans side chains, and a ring double-bond shift). Grechkin and Hamberg in a new work (unpublished data) obtained further evidence that allene oxides generated from 13- and 9-HPODEs afford extremely poor yields of cyclopentenones in the absence of albumin. Thus, this area of research could use more work to determine if there is much significance to the ''linoleic acid'' pathway of lipid signaling.

1.4 Occurrence

Because AOS is the first enzyme in a series of transformations that leads to biosynthesis of the phytohormone jasmonic acid, it is likely that AOS exists in all plants. The biological activity of the jasmonic acid family of phytohormones is discussed by Mueller in Chapter 11.

According to Lau et al. [25], AOS was the predominant cytochrome P-450 in many plants, but the tissue in which AOS expressed is not always predictable. AOS often com-

petes with other hydroperoxide-metabolizing enzymes, often being completely overwhelmed by the competing enzymes. For example, AOS was found in abundance in flaxseed [2] and the embryo of maize seed [10], but it was undetectable in mature soybean seed [26]. In soybean, the activity was mainly localized in immature seed coats and the pericarp. Methods for detection, assay, and isolation of AOS have been outlined by Brash and Song [27].

2 DIVINYL ETHER SYNTHASE

2.1 Occurrence of Divinyl Ethers

Divinyl ethers are unusual oxylipins, having an ether oxygen within their hydrocarbon chains. Up to date divinyl ethers were detected only within the plant kingdom. First, divinyl ethers, 9-[1′(*E*),3′(*Z*)-nonadienoxy]-8(*E*)-nonenoic (colneleic) and 9-[1′(*E*),3′(*Z*),6′(*Z*)-nonatrienoxy]-8(*E*)-nonenoic (colnelenic) acids (Fig. 2, structures **I** and **II**, respectively), were discovered by Galliard with collaborators in their in vitro studies with potato tubers [28–32]. Until recently, divinyl ethers were considered as the unique constituents of potato tubers, not being detected in any other plant or animal species. However in the last years, divinyl ethers were detected in a number of plants. Three new divinyl ethers were isolated from brown alga *Laminaria sinclairii* [33]. These were 12-[1′(*Z*),3′(*Z*)-hexadienyloxy]-6(*Z*),9(*Z*),11(*E*)-dodecatrienoic, 12-[1′(*Z*),3′(*Z*)-hexadienyloxy]-9(*Z*),11(*E*)-dodecadienoic and 14-[1′(*Z*),3′(*Z*)-hexadienyloxy]-5(*Z*),8(*Z*),11(*Z*),13(*E*)-tetradecatetraenoic acids (Fig. 2, structures **III–V**, respectively). One more new eicosanoid divinyl ether, 8-[1′(*Z*),3′(*Z*),6′(*Z*)-dodecatrienyl-1′-oxy]-5(*Z*),7(*E*)-octadienoic acid (Fig. 2, structure **VI**), was found in red alga *Polyneura latissima* [34]. Recent biosynthetic experiments in vitro with garlic bulbs [35] have led to detection of novel divinyl ethers 12-[1′(*E*)-hexenyloxy]-9(*Z*),11(*E*)-dodecadienoic (etheroleic) and 12-[1′(*E*),3′(*Z*)-hexadienyloxy]-9(*Z*),11(*E*)-dodecadienoic (etherolenic) acids (Fig. 2, structures **VII** and **VIII**, respectively). These oxylipins are formed by garlic enzyme, specifically utilizing 13-HPODE and 13-HPOTE [35–38].

Divinyl ethers have been isolated from some higher plant species in the last 2 years. Divinyl ether synthase (DES), efficiently synthesizing colneleate and colnelenate, was detected in the root tissues from tomato plants [39]. Hamberg detected strong DES activity in leaves of several *Ranunculus* species [40]. The enzyme of *Ranunculus* converts 13-HPOTE (preferential substrate) into compound **IV**. As mentioned earlier, this compound was detected also in *Laminaria sinclairii* [33]. Compound **IV** and etherolenic acid (**VIII**) differ only in geometrical isomerism of a double bond in position 1′,2′. Two more divinyl ethers described so far (**XI** and **XII**) are artificial products prepared by incubations of garlic DES with 15-hydroperoxy-5(*Z*),8(*Z*),11(*Z*),13(*E*)-eicosatetraenoic acid (15-HPETE) [38] and 15-hydroperoxy-8(*Z*),11(*Z*),13(*E*)-eicosatrienoic acid [41], respectively.

Recently, Weber et al. [41] demonstrated specific accumulation of colneleic and colnelenic acids in potato leaves infected by pathogen *Pytophthora infestans* and tobacco leaves infected by the tobacco mosaic virus. Weber et al. also demonstrated that colneleate and colnelenate inhibit the growth of pathogenic fungus *Phytophthora infestans*. The role for DES and divinyl ether oxylipins in plant defense is proposed [41].

It is noticeable that plant species, possessing divinyl ethers, are phylogenetically distant. The occurrence of divinyl ethers in red and brown alga suggests that this pathway appeared at the early stages of plant evolution.

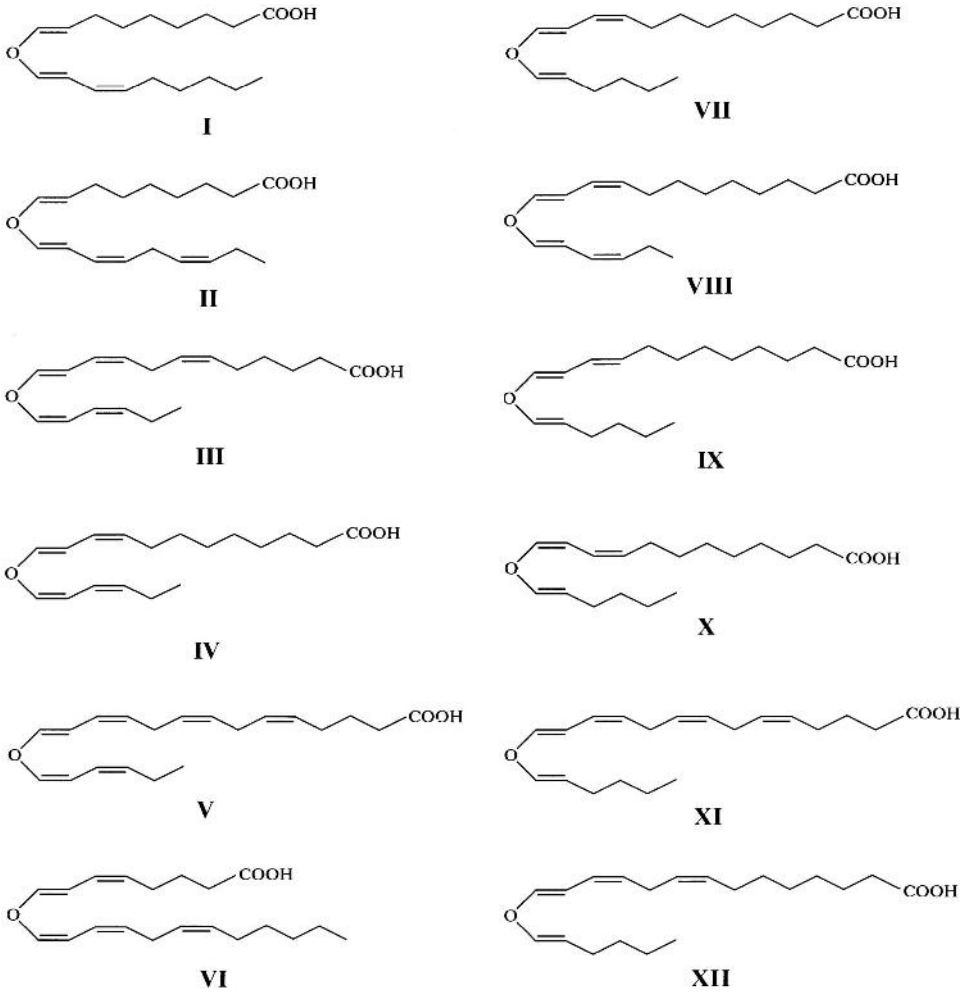


Figure 2 Divinyl ether oxylipins detected in plant species: colneleic acid (**I**), colnelenic acid (**II**), etherolenic acid (**VIII**), etheroleic acid (**VII**), and geometrical isomers of the latter, **IX** and **X**. Compounds **III–V** were isolated from brown alga *Laminaria sinclarii* and compound **VI** was isolated from red alga *Polyneura latissima*. Compounds **XI** and **XII** were biosynthesized through 15-HPETE and 15-HPETrE incubation with garlic divinyl ether synthase. (See text for the references.)

2.2 Biosynthesis of Divinyl Ethers

Galliard [28–32] and other researchers [42–44] in their works on colneleic acid biosynthesis used no name for the potato enzyme. Potato, garlic, tomato, and *Ranunculus* enzymes catalyze dehydration of fatty acid hydroperoxides. Thus, they could be described as hydroperoxide dehydrase in accordance with their catalytic function. However, there are a few different enzymes, performing dehydration of fatty acid hydroperoxides [45]. Moreover, the name ''hydroperoxide dehydrase'' has been previously used for another enzyme,

which is now called ''allene oxide synthase'' [20]. To prevent misunderstandings, the name ''divinyl ether synthase'' (DES) has been suggested for enzymes converting fatty acid hydroperoxides into divinyl ether oxylipins [35].

As found by Galliard and co-workers, the potato enzyme, synthesizing colneleic and colnelenic acids, specifically utilizes 9-HPODE and 9-hydroperoxy-10(*E*), 12(*Z*),15(*Z*)-octadecatrienoic acid (9-HPOTE) as substrates, being nonactive toward 13-hydroperoxides [31]. In contradiction, garlic and *Ranunculus* DESs use preferentially 13-HPODE and 13-HPOTE [35,36,38]. The most efficient substrates of garlic and *Ranunculus* enzymes are 13-HPODE and 13-HPOTE, respectively [35,40]. Biosynthesis of divinyl ethers in marine algae was not studied. The position of an ether function at C-8 in divinyl ether from *Polyneura latissima* and its co-occurrence with 9(*S*)-hydroxy-5(*Z*),7(*E*),11(*Z*), 14(*Z*)-eicosatetraenoic acid allowed Jiang and Gerwick to propose the precursory role of 9(*S*)-hydroperoxy-5(*Z*),7(*E*),11(*Z*),14(*Z*)-eicosatetraenoic acid [35]. Divinyl ethers of *L. sinclairii* co-occur with 13(*S*)- and 15(*S*)-hydroxy derivatives of C-18 and C-20 polyenoic acids [33]. This fact, along with structural peculiarities of *L. sinclairii* divinyl ethers (location of an ether bridge at C-12 or C-14), suggests that these oxylipins can be biosynthesized by similar enzyme, utilizing 13(*S*)- and 15(*S*)-hydroperoxides.

Both DESs from potato tubers [44] and garlic [36] bulbs were found to be localized in microsomal fractions. Recent studies revealed that the garlic enzyme possesses partial stereoselectivity. The remainder of unreacted racemic hydroperoxide 13(*R*,*S*)-HPODE after incubation with enzyme was composed of up to 94% 13(*R*)-HPODE enantiomer. Thus, 13(*R*)-HPODE is utilized much slower than 13(*S*)-HPODE. At the same time, 13(*R*,*S*)-HPODE is utilized almost completely when it is incubated with excess enzyme. Thus, this enzyme utilizes 13(*S*)-HPODE preferentially, but its stereoselectivity is not absolute. HETLS from tea leaves [45] and flaxseed AOS [15] exhibit similar limited preference toward 13(*S*)-HPODE. Unlike these enzymes, maize seed AOS specifically utilizes only 13(*S*)-HPODE from racemic 13(*R*,*S*)-HPODE, being not able to transform 13(*R*)-HPODE (Hamberg, personal communication).

The origin of oxygen at the ether function is an important question, related to the mechanism of enzymatic divinyl ether synthesis. In their early work, Galliard and Matthew [31] studied colneleic acid biosynthesis from [$^{18}O_2$-hydroperoxy]9-HPODE and found no incorporation of ^{18}O into divinyl ether. Thus, Galliard and Matthew proposed that ether oxygen of colneleic acid comes from water [31]. This proposal was not supported by later reinvestigation by Crombie et al. [43,46], who found a high extent of ^{18}O incorporation from [$^{18}O_2$-hydroperoxy]9-HPODE into the ether function of colneleic acid. Recently, the biosynthesis of etheroleic acid from [$^{18}O_2$-hydroperoxy]13(*S*)-HPODE by garlic DES was studied [38]. The authors found that the incorporation of ^{18}O into the ether bridge of etheroleic acid was essentially complete. These results clearly demonstrated that the ether oxygen of divinyl ethers comes from the hydroperoxy group of precursors.

Experiments on [9,10,12,13-$^2H_4$]9-HPODE conversion by potato enzyme revealed the incorporation of all four deuterium atoms into the colneleic acid [46,47]. These results along with data of experiments with ^{18}O allowed Crombie et al. to propose a mechanism of divinly ether synthesis involving the epoxyallylic carbocation intermediate [43,46,47]. The same mechanism was independently proposed by Corey et al. [42] on the basis of biomimetic synthesis of colneleic acid. The mechanism proposed by Crombie et al. [43,46,47] and Corey et al. [42] includes the first-stage protonation-dehydration of 9(*S*)-HPODE, which affords an epoxyallylic carbocation. Enzyme-assisted deprotonation of

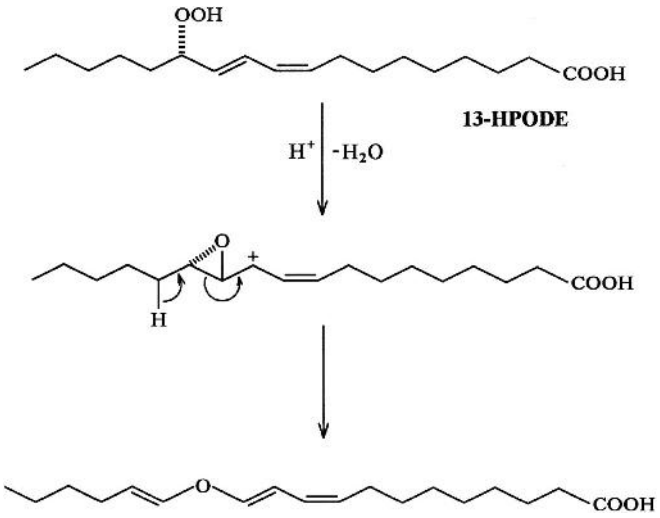


Figure 3 The proposed mechanism of divinyl ether biosynthesis.

epoxyallylic carbocation at C-8, accompanied by C—C bond cleavage of oxirane, affords colneleic acid (Fig. 3). An identical mechanism was proposed recently for etheroleic acid formation [38].

Hamberg and Fahlstadius [44] stereospecifically incubated deuterated [(8*R*)-^{2}H] 9-HPODE and [(8*S*)-^{2}H]9-HPODE with potato DES. These experiments reveal that deprotonation of an epoxyallylic cation intermediate possesses strong stereoselectivity: Pro-*R* hydrogen at C-8 is removed.

2.3 Geometrical Isomerism of Divinyl Ethers

It is interesting to note that all divinyl ethers isolated from higher plants, including colneleic and etheroleic acid (predominant isomer), have trans double bonds at both sides, in addition to the ether bridge. On the other hand, all divinyl ethers isolated from marine algae *L. sinclairii* [33] and *P. latissima* [34] and *Ranunculus* species [40] have a cis double bond at one side. Thus, potato and garlic DES, on the one hand, and algae and *Ranunculus* DES, on the other hand, probably possess the opposite stereospecificities of deprotonation of epoxyallylic carbocation. This may have relation to phylogenetic position of *Ranunculaceae*, which belong to the primitive Angiosperms.

As found recently, etheroleic acid is accompanied by two minor geometrical isomers [38], having (9*E*, 11*E*,1′*E*) and (9*Z*,11*Z*,1′*E*) double-bond geometry (Fig. 2, structures **IX** and **X**, respectively). Previously, Crombie et al. [46] detected minor peaks, having identical mass spectra with methyl colneleate during its GC–MS analysis. Appearance of a minor isomer was ascribed to cis–trans isomerization at C-3′ during gas–liquid chromatography (GLC) analysis [46]. However, minor isomers of etheroleic acid were detected and isolated during the high-performance liquid chromatography (HPLC) analysis after the mild extraction. Moreover, purified (9*Z*, 11*E*,1′*E*)-etheroleic acid underwent isomerization to all-trans isomer during analysis by GLC to only a very small extent. Grechkin et al. [38] concluded that two minor geometrical isomers are not the products of thermal isomerization, but predominantly the kinetically controlled by-products of etheroleic acid

formation. Formation of (9*Z*,11*Z*,1′*E*) and all-(*E*) isomers of etheroleic acid was attributed to deprotonation of resonance form and rotation conformer of the epoxyallylic cation, respectively. Nonphysiological substrates of garlic DES like 13(*R,S*)-HPODE and 15(*S*)-HPETE afford more minor geometrical isomers than ordinary physiological substrates like 13(*S*)-HPODE [38]. These observations suggest that deprotonation of epoxyallylic carbocation may present the rate-limiting stage of enzymatic conversion. The minor geometrical isomers of other divinyl ether oxylipins, excepting the etheroleic acid, have not been purified yet.

2.4 Physiological Significance of Divinyl Ethers

The physiological significance of divinyl ethers is still not fully studied. Previously, Corey et al. have found that colneleic acid is a potent inhibitor of 9-LOX [42]. Etheroleic acid possesses similar activity, inhibiting 13-LOX (Grechkin and Fazliyev, unpublished results). Recent data by Weber et al. [41] demonstrate the important role of DES activity and divinyl ethers in plant defense toward pathogens. The rapidly increasing number of publications on divinyl ethers and their occurrence in phylogenetically distant species allow one to propose that DES is one more widespread enzyme of the plant LOX pathway along with AOS and HETLS.

Metabolic pathways, controlled by DES and HETLS, possess an obvious similarity. Both enzymatic reactions include C—C bond cleavage. Galliard and Wardale presented some evidence for the formation of C_9-aldehydes through the enzymatic hydrolysis of colneleic and colnelenic acids [30]. Formation of C_{12} aldoacid was observed *in situ* during the incubation of 13(*S*)-HPOTE with preparation of garlic enzyme [35]. However, in later experiments, the same group failed to observe the hydrolysis of exogenous etheroleic and etherolenic acids in garlic extract [36].

3 EPOXY ALCOHOL SYNTHASE

Epoxy alcohol synthase (EAS), an enzyme from a parasitic fungus *Saprolegnia parasitica* that is known to convert fatty acid hydroperoxides into epoxy alcohol fatty acids, was first studied with the 15(*S*)-hydroperoxide of arachidonic acid as a substrate [48,49]. EAS was also found to utilize linoleic acid hydroperoxides [50]; that is, 13(*S*)-HPODE was converted into both 11(*R*), 12(*R*)-epoxy-13(*S*)-hydroxy-9(*Z*)-octadecenoic acid and 9(*S*), 10(*R*)-epoxy-13(*S*)-hydroxy-11(*E*)-octadecenoic acid, and 9(*S*)-HPODE was transformed in an analogous manner into both 10(*R*),11(*R*)-epoxy-9(*S*)-hydroxy-12(*Z*)-octadecenoic acid and 12(*R*),13(*S*)-epoxy-9(*S*)-hydroxy-10(*E*)-octadecenoic acid.

Because the fatty acid hydroperoxide was transformed into epoxy alcohols by intramolecular transfer of hydroperoxide–oxygen, EAS was initially named ''hydroperoxide isomerase'' [49]. However, the ''hydroperoxide isomerase'' nomenclature was unsuitable for several reasons. For example, hydroperoxide isomerase was the initial name given to AOS by Zimmerman [2] before the true mechanism of action of AOS was known. Then, other isomerases were discovered in both the fungus *Gaeumannomyces graminis* and red alga *Gracilariopsis lemaneiformis* that transformed fatty acid hydroperoxides into *vicinal* diols with retention of both hydroperoxide oxygens. With the enzyme from *Gaeumannomyces graminis*, 8(*R*)-hydroperoxy-9(*Z*),12(*Z*)-octadecadienoic acid was converted into 7(*S*),8(*S*)-dihydroxy-9(*Z*),12(*Z*)-octadecadienoic acid [51–53], whereas, with the enzyme from *Gracilariopsis lemaneiformis*, 13(*S*)-HPODE was isomerized into 13(*R*),14(*S*)-dihy-

droxy-9(*Z*),11(*E*)-octadecadienoic acid [54]. These inconsistencies in reaction types may be the reason Hamberg renamed the enzyme ''epoxy alcohol synthase,'' from *Saprolegnia parasitica* and the isomerases from *Gaeumannomyces graminis* and *Gracilariopsis lemaneiformis* were redesignated as vicinal diol synthases [55].

Until recently, the various isomerases described earlier have been restricted to lower plant forms, the fungi and algae. EAS has now been discovered in higher plants (potato leaves) by Hamberg [56]. In this interesting work, it was found that a preparation from potato leaves catalyzed the oxidation of linoleic acid into 9(*S*)-HPODE and 13(*S*)-HPODE in a 95:5 ratio. The 13(*S*)-HPODE was selectively metabolized by AOS into α- and γ-ketols, but 9(*S*)-HPODE was utilized by EAS to form both 10(*S*),11(*S*)-epoxy-9(*S*)-hydroxy-12(*Z*)-octadecenoic acid and 12(*R*),13(*S*)-epoxy-9(*S*)-hydroxy-10(*E*)-octadecenoic acid (Fig. 4). The absence of peroxygenase (POX), an enzyme that gives related products, was shown by the lack of double-bond epoxidizing activity in the presence of H_2O_2. It is noteworthy that the epoxide group of the former epoxy alcohol had the opposite stereoconfiguration compared the product obtained from 9(*S*)-HPODE using *Saprolegnia parasitica* EAS, whereas the latter epoxy alcohol had the same stereoconfiguration. Because potato leaves possessed epoxide hydrolase activity, the two epoxides each hydrolyzed selectivity into one isomer (Fig. 4). The 10,11-epoxy-9-hydroxide hydrolyzed into 9(*S*),10(*S*),11(*R*)-trihydroxy-12(*Z*)-octadecenoic acid, and 12,13-epoxy-9-hydroxide was transformed into 9(*S*),12(*S*),13(*S*)-trihydroxy-10(*E*)-octadecenoic acid. Ordinary acid-

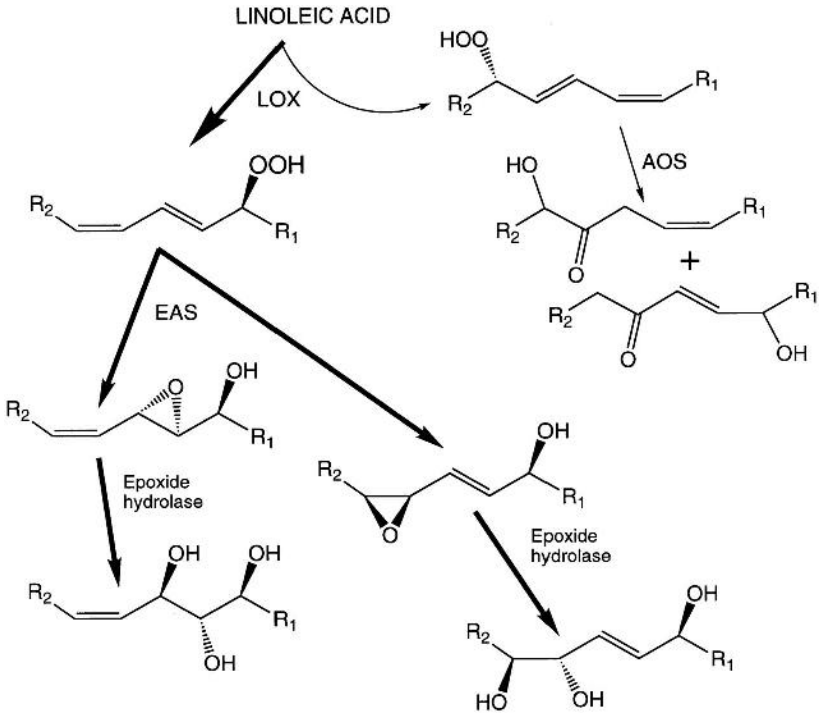


Figure 4 Metabolism of linoleic acid hydroperoxides by potato leaves through selective catalysis by EAS and AOS. The epoxy alcohols formed by EAS are then hydrolyzed to trihydroxy fatty acids by the action of epoxide hydrolase. (From Ref. 56.)

catalyzed hydrolysis of these allylic epoxides affords four isomers instead of one. Due to the intermediacy of an allylic carbocation distributed among three carbons caused by acid, one epoxy alcohol isomer affords two positional isomers, both with (*R,S*)-stereoconfiguration at the carbon substituted with water [57].

4 PEROXYGENASE PATHWAY AND OTHER CO-OXIDATIONS INVOLVING FATTY ACID HYDROPEROXIDES

4.1 Peroxygenase Pathway

The peroxygenase (POX) pathway is one of the most recently characterized routes of the plant LOX pathway. POX is a hemoprotein, which uses fatty acid hydroperoxides as oxygen donors for intermolecular or intramolecular double-bond epoxidation (Fig. 5). POX was detected in microsomers from soybean and broad beans [58–63] and cereal [64] seeds. The POX pathway is reviewed here only briefly, because it has been described in some recent reviews [59–62].

As seen in Figure 5, fatty acid hydroperoxide, being an oxygen donor, is reduced to hydroxy diene by POX. An oxygen atom lost by hydroperoxide is used for double-bond epoxidation of unsaturated fatty acids, which are POX cosubstrates. POX possesses partial regiospecificity. For example, the broad bean (*Vicia faba* L.) POX preferentially epoxidizes the 9,10-double bond within linoleic acid [63]. The oxidation product 9,10-epoxy-12(*Z*)-octadecenoic (coronaric) acid is represented by 83–87% (9*R*,10*S*) and 13–17% (9*S*,10*R*) enantiomers [63]. Thus, the stereospecificity of POX action corresponds to the stereoconfiguration of naturally occurring coronaric acid [65].

Hydro(pero)xy fatty acids also can play a role of POX substrates. For instance, 13(*S*)-HODE, being incubated with POX, is transformed into 9,10-epoxy-13-hydroxy-11-octadecenoic acid, having mostly (9*S*,10*R*,13*S*) stereoconfiguration (see Fig. 5) [63]. Another type of reaction occurs when 13(*S*)-HPOTE is incubated with POX [63,64]. The enzyme performs the intramolecular oxygen transfer and double-bond epoxidation. The product of this isomerization is 13(*S*)-hydroxy-15,16-epoxy-9(*Z*),11(*E*)-octadecadienoic acid. The stereoconfiguration of the epoxide ring in the product is not revealed.

Epoxides like coronaric and (9*Z*)-12,13-epoxy-9-octadecenoic (vernolic) acid are formed in two presently known ways. Excepting POX, cytochrome P-450 catalyses the

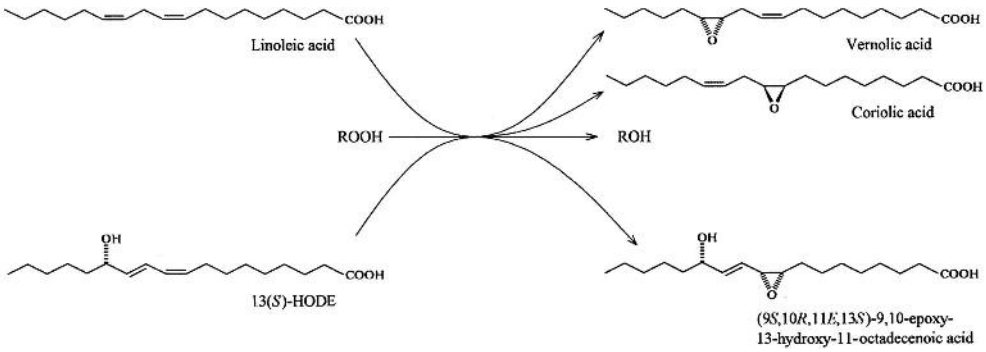


Figure 5 Metabolism of linoleic acid through the peroxygenase pathway. ROOH-fatty acid hydroperoxide; ROH-hydroxy fatty acid.

epoxy fatty acid biosynthesis [66]. Cytochrome P-450 oxidation is not observed in germinating seeds, but it is active in the developing *Euphorbia lagascae* seeds [66,67]. The main product of linoleate epoxidation with cytochrome P-450 is (12*S*,13*R*)-vernolate, which is known as a storage oil constituent in seeds of Euphorbiaceae and Valerianaceae species [66,67].

Epoxy fatty acids are the substrates of epoxide hydrolase, detected in many plant species [68–70]. Epoxides are transformed enzymatically into vicinal diols. Epoxide hydrolase exhibits unique stereospecificity. The enzyme transforms both chiral and racemic coronaric and vernolic acids into (*R,R*)-vicinal diols [70].

Epoxy fatty acids are known as physiologically active compounds. Coronaric and vernolic acids are plant defensive compounds against pathogenic fungi [71,72]. Coronaric acid (also named ''leukotoxin'') possesses also cytotoxicity in humans [73]. Epoxides, like 9,10-epoxystearate, as well as the corresponding vicinal diols and their ω-hydroxy derivatives are cutin monomers (see Chapter 12). During the plant–pathogen interaction, these monomers are liberated by cutinase and may take part in antimicrobial response.

4.2 Oxidation of Furan Fatty Acids

Furan fatty acids are widely occurring oxylipins, minor constituents of animal and plant lipids. Boyer et al. [74,75] reported that furan fatty acids can serve as unusual LOX substrates. They found that a synthetic furan 10,13-epoxy-10,12-octadecadienoic acid is oxidized by soybean LOX-1 into the corresponding α,β-unsaturated γ-diketone 10,13-dioxo-11-octadecenoic acid. The rate of furan oxidation increased in the presence of linoleic acid [75]. However, this dependence on linoleic acid was not absolute.

Spiteller and his colleagues incubated the sodium salt of the same furan 10,13-epoxy-10,12-octadecadienoic acid with soybean LOX-1 in the presence of linoleic acid and thiols [76]. The authors observed conversion of this furan into dioxoene and thiol ethers [76].

Zabolotsky et al. [77] reported that they did not observe any extent of oxidation during the incubation of a similar furan 9,12-epoxyoctadeca-9,11-dienoic acid with soybean LOX. Zabolotsky et al. concluded that LOX in disagreement with the previous observations [74–76] is not able to attack furan fatty acids [77]. After Zabolotsky's publication [77], Batna and Spiteller performed a reinvestigation of furan fatty acid incubations with soybean LOX-1 [78]. They used 9,12-epoxy-10,12-octadecadienoic acid and its 10,11-dimethyl and 10-methyl derivatives as substrates and found no oxidation in the absence of linoleic acid. At the same time, furans are oxidized in the presence of LOX-1 and linoleic acid. Moreover, 13-HPODE addition caused furan oxidation in the absence of LOX. Thus, results of Batna and Spiteller demonstrate that conversion of furans to dioxoenes in the presence of LOX is the co-oxidation reaction, where fatty acid hydroperoxides act as oxygen donors.

The same authors also tested phosphatidylcholines containing furan acids located at the *sn*-2 as soybean LOX-1 substrates [79]. They found that furan residues within phosphatidylcholine, containing linoleic acid in the *sn*-1 position, were effectively oxidized by LOX-1 without additional cosubstrates. Batna and Spiteller proposed that such an oxidation pathway may operate in plants under stress conditions [79].

4.3 Oxidation of Carotenoids

It is known that LOXs induce carotenoid bleaching. Mixed soybean isoenzymes bleach carotenoids actively, whereas separate purified isoenzymes possessed low activity [80].

There is increasing evidence that in vivo abscisic acid is formed through the oxidative cleavage of carotenoids [81,82]. According to some old literature data, LOX-catalyzed co-oxidation of violaxanthin in presence of linoleate leads to xanthoxin, an abscisic acid precursor [83].

Recently, a new abscisic-acid-deficient mutant of maize has been identified and the corresponding gene, *Vp14*, has been cloned [82]. The recombinant VP14 protein catalyzes the cleavage of 9-*cis*-epoxy-carotenoids to form C-25 apo-aldehydes and xanthoxin. Thus, a specific oxygenase is involved in oxidative cleavage. In disagreement with prior literature [83], data on VP14 suggest that oxygenase probably uses molecular oxygen as a cosubstrate [82] without involvement of LOX and fatty acid hydroperoxides.

5 CHEMICAL TRANSFORMATIONS OF FATTY ACID HYDROPEROXIDES

Often LOX-produced hydroperoxides can be chemically transformed into the same or similar products obtained by hydroperoxide-utilizing enzymes. Usually the advantages are (1) more efficient transformation, (2) ease of product recovery, (3) avoidance of substrate inactivation of enzymes, and (4) no need to purify enzymes.

5.1 Acid Catalysis

Acid catalysis of conjugated hydroperoxydiene fatty acids in protic solvent largely leads to the formation of epoxyallylic carbocations. Due to the diene conjugation allylic to the hydroperoxide carbon, a Hock–Criegee transition tends to rearrange into a more stable epoxyallylic carbocation, which reacts with protic solvent [84,85]. Figure 6 summarizes these acid-catalyzed reactions of 13-HPODE. Depending on the chemical nature of the protic solvent HX, there is potential for production of a variety of fatty epoxide derivatives with X substitution. In acid, the epoxides tend to solvolyze, especially the epoxides allylic to a double bond.

Interestingly, the epoxyallylic carbocation appears to be the conceptual intermediate in the biogenesis of many oxylipins, such as divinyl ethers, 12-oxo-phytodienoic acid, epoxyalcohols, and aldehydes [86,87]. It is apparent that these enzymic reactions mimic the chemistry because the cytochrome P-450 reactions involved cause a two-step electron transfer; that is, hydroperoxides are reduced to give the alkoxyl radical, which, in turn, rearranges into the epoxyallylic carbon radical, and this is followed by oxidation of the epoxyallylic carbon radical to furnish the epoxyallylic carbocation (see Fig. 5 of Chap. 9).

The expected products of acid catalysis by the Hock–Criegee mechanism are shorter-chain aldehydes from cleavage, and this reaction is a mimic for biocatalysis by the cytochrome P-450, HETLS. For example, in the presence of acid, 13-HPODE should afford hexanal and 12-oxo-10(*E*)-dodecenoic acid; the latter would arise from acid rearrangement of the theoretical product, 12-oxo-9(*Z*)-dodecenoic acid. Although the cleavage to aldehydes is relatively minor in protic solvent, nonprotic acids, such as BF_3-ether, lead to the expected cleavage into aldehydes [88].

5.2 Base Catalysis

Catalysis by base constitutes a facile method of producing the corresponding hydroxy fatty acids from hydroperoxides. O'Brien [89] noted that a variety of nucleophiles ''reduced'' linoleic acid hydroperoxides to the corresponding hydroxy fatty acids, and alkali

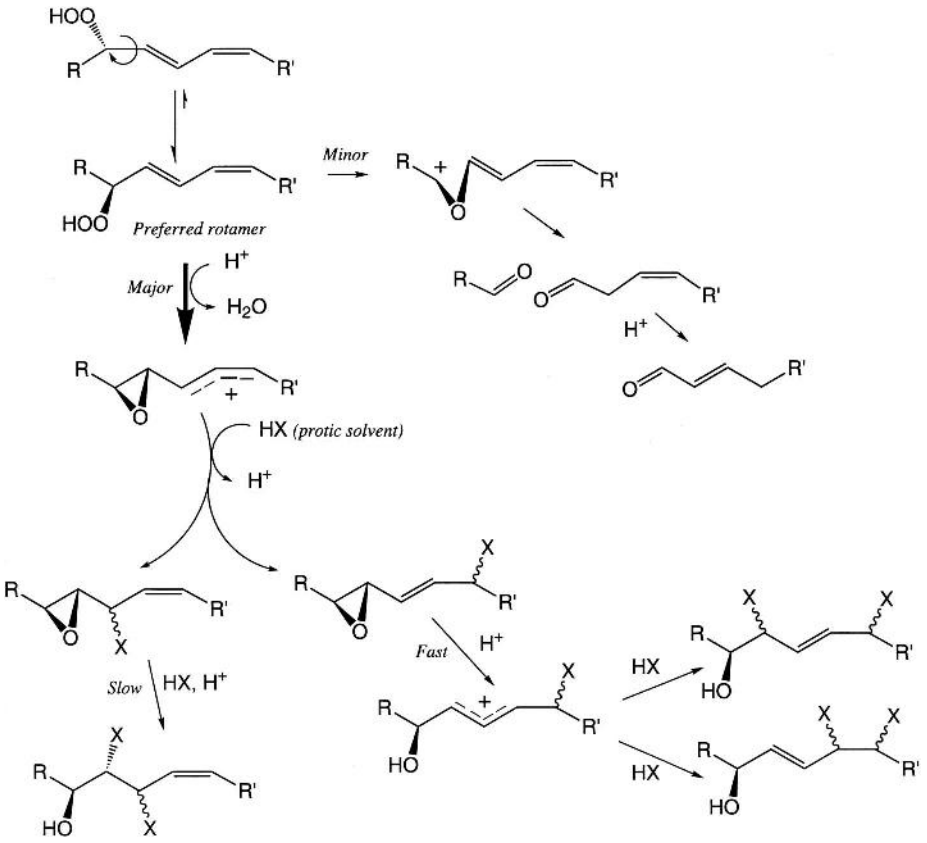


Figure 6 Acid catalysis of linoleic acid hydroperoxide in protic solvent (HX) yields mainly polysubstituted fatty acid isomers, and minor products arise from Hock–Criegee cleavage to aldehydes. (From Refs. 84 and 85.)

was reported to cause the same reaction [90]. This reaction should be considered when saponifying hydroperoxidized glyceride lipid. In theory, if the nucleophile had attacked carbon, the stereoconfiguration would be either inverted or scrambled, but loss of the distal oxygen of the hydroperoxide would leave the stereoconfiguration intact. It was found that treatment of fatty acid hydroperoxides, such as 13(*S*)-HPODE and 13(*S*)-HPOTE, with a strong base (1–5 *M* KOH) furnishes the hydroxy fatty acid in about 75–80% yield without loss of stereoconfiguration [91–93] showing that nucleophilic displacement did not occur at carbon. The remaining 20–25% of products are mainly from Favorskii rearrangement of an early intermediate by-product, 13-oxo-trans-11,12-epoxy-9(*Z*)-octadecenoic acid [92,93]. The mechanism of base conversion of 13(*S*)-HPODE appeared to be (1) formation of a 13(*S*)-HPODE hydroperoxyl anion, (2) loss of the hydroxyl anion from a small portion of the 13(*S*)-HPODE hydroperoxyl anion to form 13-oxo-9,11-octadecadienoic acid, and (3) conversion of the 13(*S*)-HPODE hydroperoxyl anion to the 13(*S*)-hydroxy-9(*Z*),11(*E*)-octadecadienoic acid [13(*S*)-HODE] hydroxyl anion with concomitant epoxidation of the 11,12-double bond of 13-oxo-9,11-octadecadienoic acid by the distal oxygen of the hydroperoxyl anion, affording 13-oxo-trans-11,12-epoxy-9(*Z*)-octadecenoic acid [93]. This

base-catalyzed mechanism is reminiscent of that of the enzyme POX described earlier. Although the proposed mechanism was consistent with the data, it did not completely explain why the yield of 13(*S*)-HODE was as high as 75–80%. Because 13(*S*)-HODE was inert under the reaction conditions, the loss of a good percentage of oxidation equivalents was unexplained. Base treatment offers an alternative method of ''reducing'' hydroperoxides other than the traditional methods of reducing reagents, like triphenylphosphine, $SnCl_2$, KI, and $NaBH_4$. $NaBH_4$ has the disadvantage of reducing traces of oxooctadecadienoic acid that may be present as by-products of LOX, thereby contaminating the preparation of stereospecific HODEs with undefined amounts of racemic HODEs.

5.3 Alkoxyl Radical Rearrangement

A number of catalysts are efficient in generating alkoxyl radicals from hydroperoxides. With hydroperoxydiene fatty acids, like 13-HPODE and 9-HPODE, a small portion of the alkoxyl radicals undergo β-scission to form a hydrocarbon and an aldehyde (10–20%), and a small portion is converted to oxooctadecadienoic acid. The majority of the alkoxyl radical rearranges into an epoxyallylic radical [94–98]; for example, 13-HPODE is converted into the 9,11-allylic carbon radical of 12,13-epoxyoctadecenoic acid. The epoxyallylic radical combines with the dominant oxygen radical that it encounters, which is usually O_2 under aerobic conditions. 12,13-Epoxy-9(11)-hydroperoxy-11(9)-octadecenoic acids are often the result (Fig. 7). Epoxyhydroperoxyoctadecenoic acids, in turn, degrade into epoxyhydroxyoctadecenoic acid and, with certain catalysts, epoxyoxooctadecenoic acid. Often these rearrangement products are observed in biological systems, and this

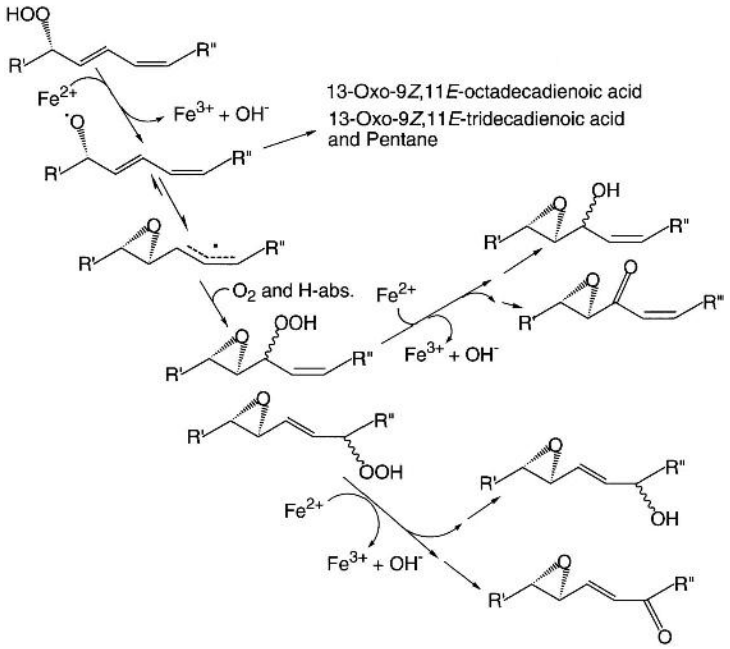


Figure 7 Homolytic cleavage of the hydroperoxide by various catalysts, such as Fe^{2+} shown here, leads to an alkoxyl radical, which rearranges to ultimately afford epoxyoxooctadecenoic acid and/or epoxyhydroxyoctadecenoic acid isomers. (From Ref. 95.)

alkoxyl-radical reaction (or the acid-catalyzed heterolysis, discussed earlier) can be identified by the position of the epoxide versus the position of precursor hydroperoxide; that is, 13-HPODE affords 12,13-epoxides, whereas EAS and POX furnish 9,10- and 11,12-epoxides, respectively.

An iron-cysteine catalyst at mildly acidic pHs is an efficient one-electron redox couple acting in concert with 13-HPODE [94]. In the presence of O_2, this catalyst produced both epoxyhydroxyoctadecenoic and epoxyoxooctadecenoic acids [94], but in the absence of O_2, epoxyoctadecenoic/cysteine adducts are formed [99]. In the presence of O_2, catalysis by ultraviolet (UV) photolysis [100], hematin [101], or hemoglobin [102] transformed 13-HPODE into largely epoxyhydroxyoctadecenoic acid as well as other alkoxyl radical-derived products. Under anaerobic conditions, UV photolysis resulted in combination of the epoxyallylic radical with the radicals $^{\bullet}OH$ and $^{\bullet}CH_2OH$ from methanol solvent [103].

Little is known of the alkoxyl-radical-generated products of 13-HPOTE, but theory predicts efficient β-scission into 13-oxo-9,11-tridecadienoic acid, 1-penten-3-ol, and 2-penten-1-ol. This assessment is based on the 92% efficiency of β-scission of a similarly configured hydroperoxide, 10-HPODE, where β-scission gives a stabilized allylic radical [104].

5.4 Double-Bond Epoxidation by Transition Metal Catalysis

Transition metals have been utilized to convert linoleic acid hydroperoxide to a specific regioisomeric epoxyhydroxyoctadecenoic acid, a topic recently reviewed by Piazza [105]. For example, methyl 13(*S*)-HPODE was converted into methyl 12,13-epoxy-11-hydroxy-9(*Z*)-octadecenoate by either vanadyl acetylacetonate, titanium(IV)isopropoxide, or niobium(V)ethoxide. Differences in product stereoisomers were obtained with different catalysts.

In one such study, Hamberg [57] explored, in depth, the stereochemistry of 13(*S*)-HPODE (methyl ester) transformation by a vanadium oxyacetylacetonate catalyst. This catalyst afforded two isomeric products, methyl 11(*R*),12(*R*)-epoxy-13(*S*)-hydroxy-9(*Z*)-octadecenoate and methyl 11(*S*),12(*S*)-epoxy-13(*S*)-hydroxy-9(*Z*)-octadecenoate. Both products were *trans*-epoxides having opposite stereoconfigurations. Hamberg showed that the epoxides were formed by an intermolecular transfer of oxygen from hydroperoxide to the *trans*-11,12-double bond. Because both epoxides were allylic, they were susceptible to hydrolysis into isomeric methyl 11,12,13-trihydroxy-9(*Z*)-octadecenoate and methyl 9,12,13-trihydroxy-11(*E*)-octadecenoate, particularly in the presence of weak acids. Because the intermediate during epoxide hydrolysis was undoubtedly a carbocation with the charge distributed between C-9 and C-11, *racemic* hydroxyl substitution from water occurred at both C-9 and C-11. Acid hydrolysis did not affect the stereoconfiguration of C-12 and -13.

6 CONCLUSION

The literature data reviewed in the present chapter, and Chapter 9 demonstrate that the lipoxygenase pathway and related fatty acid hydroperoxide conversions through enzymatic and spontaneous mechanisms are the source of great diversity of oxygenated compounds called oxylipins. Some of these products [e.g., 13(*S*)-HODE (coriolic acid)] are known as natural compounds for many years. Some others have been detected only recently. Our knowledge in this field is still expanding. Many of oxylipins have importance as

physiologically active compounds. In this respect, the most intensively studied compounds are eicosanoids in mammals and other animals. Increasing data demonstrate that octadecanoic oxylipins have physiological importance in mammals along with eicosanoids. Jasmonates are a newly acknowledged group of plant hormones. Jasmonates and other oxylipins play important roles in plant signaling and plant defense. Oxylipins biosynthesizing through the lipoxygenase pathway present applied interest as regulators of cell cycle, antimicrobial and antifungal compounds, regulators of cardiovascular functions in mammals, and some of them have importance for the perfume and food industry as fragrant compounds.

REFERENCES

1. W-C Song, AR Brash. Science 253:781–784, 1991.
2. DC Zimmerman. Biochem Biophys Res Commun 23:398–402, 1966.
3. M Hamberg. Biochim Biophys Acta 920:76–84, 1987.
4. AR Brash, SW Baertschi, CD Ingram, TM Harris. Proc Natl Acad Sci USA 85:3382–3386, 1988.
5. W-C Song, CD Funk, AR Brash. Proc Natl Acad Sci USA 90:8519–8523, 1993.
6. W-C Song, SW Baertschi, WE Boeglin, TM Harris, AR Brash. J Biol Chem 268:6293–6298, 1993.
7. Z Pan, F Durst, D Werck-Reichhart, HW Gardner, B Camara, K Cornish, RA Backhaus. J Biol Chem 270:8487–8494, 1995.
8. D Laudert, U Pfannschmidt, F Lottspeich, H Holländer-Czytko, EW Weiler. Plant Mol Biol 31:323–335, 1996.
9. R Koljak, O Boutaud, B-H Shieh, N Samel, AR Brash. Science 277:1994–1996, 1997.
10. HW Gardner. J Lipid Res 11:311–321, 1970.
11. DD Christianson, HW Gardner. Lipids 10:448–453, 1975.
12. M Hamberg, MA Hughes. Lipids 23:469–475, 1988.
13. M Hamberg. Chem Phys Lipids 46:235–243, 1988.
14. AN Grechkin, RA Kuramshin, EY Safonova, SK Latypov, AV Ilyasov. Biochim Biophys Acta 1086:317–325, 1991.
15. AN Grechkin, RA Kuramshin, IA Tarchevsky. Bioorg Khim 17:997–998, 1991.
16. C Schneider, P Schreier. Lipids 33:191–196, 1998.
17. M Hamberg, P Fahlstadius. Arch Biochem Biophys 276:518–526, 1990.
18. AN Grechkin. Biochim Biophys Acta 1213:199–206, 1994.
19. M Hamberg, O Miersch, G Sembdner. Lipids 23:521–524, 1988.
20. M Hamberg, HW Gardner. Biochim Biophys Acta 1165:1–18, 1992.
21. J Ziegler, M Hamberg, O Miersch, B Parthier. Plant Physiol 114:565–573, 1997.
22. BA Vick, DC Zimmerman, D Weisleder. Lipids 14:734–740, 1979.
23. H Gundlach, MH Zenk. Phytochemistry 47:527–537, 1998.
24. HW Gardner. In: WW Christie, ed. Advances in Lipid Methodology—Four. Dundee, Scotland: The Oily Press, 1997, pp 1–43.
25. S-MC Lau, PA Harder, DP O'Keefe. Biochemistry 32:1945–1950, 1993.
26. TD Simpson, HW Gardner. Plant Physiol 108:199–202, 1995.
27. AR Brash, W Song. Methods Enzymol 272:250–259, 1996.
28. T Galliard, DR Phillips. Biochem J 129:743–753, 1972.
29. T Galliard, DR Phillips. Chem Phys Lipids 11:173–180, 1973.
30. T Galliard, DA Wardale. JA Matthew. Biochem J 138:23–31, 1974.
31. T Galliard, JA Matthew. Biochim Biophys Acta 398:1–9, 1975.
32. T Galliard, HW-S Chan. In: PK Stumpf, EE Conn, eds. The Biochemistry of Plants: A Com-

prehensive Treatise, Vol. 4, Lipids: Structure and Function. New York: Academic Press, 1980, pp 131–161.
33. PJ Proteau, WH Gerwick. Lipids 28:783–787, 1993.
34. ZD Jiang, WH Gerwick. Lipids 32:231–235, 1997.
35. AN Grechkin, FN Fazliev, LS Mukhtarova. FEBS Lett 371:159–162, 1995.
36. AN Grechkin, M Hamberg. FEBS Lett 388:112–114, 1996.
37. AN Grechkin, M Hamberg. Bioorg Khim 22:944–945, 1996.
38. AN Grechkin, AV Ilyasov, M Hamberg. Eur J Biochem 245:137–142, 1997.
39. D Caldelari, EE Farmer. Phytochemistry 47:599–604, 1998.
40. M Hamberg. Lipids 33:1061–1071, 1998.
41. H Weber, A Chetalat, D Caldelari, EE Farmer. Plant Cell 11:485–493, 1999.
42. EJ Corey, R Nagata, SW Wright. Tetrahedron Lett 28:4917–4920, 1987.
43. L Crombie, DO Morgan, EH Smith. J Chem Soc Chem Commun 502–503, 1987.
44. M Hamberg, P Fahlstadius. J Chem Soc Perkin Trans 1 2027–2030, 1990.
45. HW Gardner. Biochim Biophys Acta 1084:221–239, 1991.
46. L Crombie, DO Morgan, EH Smith. J Chem Soc Perkin Trans 1 567–575, 1991.
47. L Crombie, DO Morgan. J Chem Soc Chem Commun 503–504, 1987.
48. M Hamberg, RP Herman, U Jacobsson. Biochim Biophys Acta 879:410–418, 1986.
49. M Hamberg, CA Herman, RP Herman. Biochim Biophys Acta 877:447–457, 1986.
50. M Hamberg. Lipids 24:249–250, 1989.
51. ID Brodowsky, M Hamberg, EH Oliw. J Biol Chem 267:14738–14745, 1992.
52. M Hamberg, L-Y Zhang, ID Brodowsky, EH Oliw. Arch Biochem Biophys 309:77–80, 1994.
53. C Su, M Sahlin, EH Oliw. J Biol Chem 273:20,744–20,751, 1998.
54. WH Gerwick, M Moghaddam, M Hamberg. Arch Biochem Biophys 290:436–444, 1991.
55. M Hamberg. Acta Chem Scand 50:219–224, 1996.
56. M Hamberg. Lipids 34:1131–1142, 1999.
57. M Hamberg. Chem Phys Lipids 43:55–67, 1987.
58. AP Kulkarni, DC Cook. Biochem Biophys Res Commun 155:1075–1081, 1988.
59. M Hamberg. J Lipid Mediat 6:375–384, 1993.
60. E Blée. In: G Piazza, ed. Lipoxygenase and Lipoxygenase Pathway Enzymes. Champaign, IL: AOCS Press, 1996, pp 138–161.
61. E Blée, F Schuber, F. In: JC Kader, P Mazliak, eds. Plant Lipid Metabolism. Dordrecht: Kluwer, 1995, pp 262–264.
62. E Blée. INFORM 6:852–861, 1995.
63. M Hamberg, P Fahlstadius. Plant Physiol 99:987–995, 1992.
64. M Hamberg, G Hamberg. Plant Physiol 110:807–815, 1996.
65. CR Smith. Prog Chem Fats Other Lipids 11:139–177, 1979.
66. E Blée, U Stahl, F Schuber, S Stymne. Biochem Biophys Res Commun 197:778–784, 1993.
67. M Bafor, MA Smith, L Jonsson, K Stobart, S Stymne. Arch Biochem Biophys 303:145–151, 1993.
68. AN Grechkin, NV Kukhtina, RA Kuramshin, EY Safonova, YJ Yefremov, IA Tarchevsky. Bioorg Khim 16:413–418, 1990.
69. A Stark, AK Lundholm, J Meijer. Phytochemistry 38:31–33, 1995.
70. E Blée, F Schuber. Eur J Biochem 230:229–234, 1995.
71. T Namai, T Kato, Y Yamaguchi, T Hirukawa. Biosci Biotechnol Biochem 57:611–613, 1993.
72. T Kato, Y Yamaguchi, T Uyehara, T Yokoyama, T Namai, S Yamanaka. Tetrahedron Lett 24:4715–4718, 1983.
73. H Iwase, T Takatori, H Niijima, M Nagao, T Amano, K Iwadate, Y Matsuda, M Nakajima, M Kobayashi. Biochim Biophys Acta 1345:27–34, 1997.
74. RF Boyer, C Lindstrom, B Darby, M Hylarides. Tetrahedron Lett 47:4111–4114, 1975.
75. RF Boyer, D Litts, J Kostishak, RC Wijesundera, FD Gunstone. Chem Phys Lipids 25:237–246, 1979.

76. J Jandke, J Schmidt, G Spiteller. Liebigs Ann Chem 29–34, 1988.
77. DA Zabolotsky, P Ludwig, H Kühn, T Schewe, GI Myagkova. Biokhimiya 57:46–54, 1992.
78. A Batna, G Spiteller. Chem Phys Lipids 70:179–185, 1994.
79. A Batna, G Spiteller. Lipids 29:397–403, 1994.
80. CS Ramadoss, EK Pistorius, B Axelrod. Arch Biochem Biophys 190:549–552, 1978.
81. BC Tan, SH Schwartz, JAD Zeevaart, DR McCarty. Proc Natl Acad Sci USA 94:12,235–12,240, 1997.
82. SH Schwartz, BC Tan, DA Gage, JAD Zeevaart, DR McCarty. Science 276:1872–1874, 1997.
83. BA Vick, DC Zimmerman. In: PK Stumpf, ed. The Biochemistry of Plants. A Comprehensive Treatise. Vol. 9. Lipids: Structure and Function. Orlando, FL: Academic Press, 1987, pp 53–90.
84. HW Gardner, D Weisleder, EC Nelson. J Org Chem 49:508–515, 1984.
85. HW Gardner, EC Nelson, LW Tjarks, RE England. Chem Phys Lipids 35:87–101, 1984.
86. L Crombie, DO Morgan. J Chem Soc Perkin 1 581–587, 1991.
87. WH Gerwick. Lipids 31:1215–1231, 1996.
88. HW Gardner, RD Plattner. Lipids 19:289–293, 1984.
89. PJ O'Brien. Can J Biochem 47:485–492, 1969.
90. EN Frankel, CD Evans, DG McConnell, EP Jones. J Am Oil Chem Soc 38:134–137, 1961.
91. TD Simpson, HW Gardner. Lipids 28:325–330, 1993.
92. HW Gardner, TD Simpson, M Hamberg. Lipids 28:487–495, 1993.
93. HW Gardner, TD Simpson, M Hamberg. Lipids 31:1023–1028, 1996.
94. HW Gardner, PA Jursinic. Biochim Biophys Acta 665:100–112, 1981.
95. HW Gardner, R Kleiman. Biochim Biophys Acta 665:113–125, 1981.
96. AL Wilcox, LJ Marnett. Chem Res Chem Toxicol 6:413–416, 1993.
97. R Yamauchi, N Yamamoto, K Kato. Lipids 30:395–404, 1995.
98. R Yamauchi, K Goto, K Kato. Lipids 33:77–85, 1998.
99. HW Gardner, RD Plattner, D Weisleder. Biochim Biophys Acta 834:65–74, 1985.
100. P Schieberle, Y Trebert, J Firl, W Grosch. Chem Phys Lipids 41:101–116, 1986.
101. TA Dix, LJ Marnett. J Biol Chem 260:5351–5357, 1985.
102. M Hamberg. Lipids 10:87–92, 1975.
103. P Schieberle, Y Trebert, J Firl, W Grosch. Chem Phys Lipids 37:99–114, 1985.
104. R Labeque, LJ Marnett. J Am Chem Soc 109:2828–2829, 1987.
105. GJ Piazza. In: G Knothe, JTP Derksen, eds. Recent Developments in the Synthesis of Fatty Acid Derivatives. Champaign, IL: AOCS Press, 1999, pp 182–195.

11

Prostaglandin-like Oxylipins in Plants

Biosynthesis, Molecular Signaling, and Response to Environment

MARTIN J. MUELLER

Julius-von-Sachs-Institut für Biowissenschaften, Pharmazentische Biologie, University of Würzburg, Würzburg, Germany

1 INTRODUCTION

In eukaryotic organisms, polyunsaturated fatty acids have four main functions. They serve as structural components of the membrane, as energy stores, and as precursors for the biosynthesis of antimicrobial compounds and signaling molecules. In animals and plants, lipid signals are synthesized de novo from fatty acids that are released from membranes after a severe physical trauma (such as wounding or UV light) or a bacterial, viral, or parasitic infection. Among the fatty acid derived signals, cyclopentanoids represent key players in defense programs. The best-studied defense signals in plants are C-12, C-16, and C-18 cyclopentanoids derived from allene oxide intermediates via the allene oxide synthase pathway, collectively termed ''jasmonates.'' Jasmonates include the C-18 compound 12-oxo-phytodienoic acid (12-oxo-PDA) and the C-16 compound dinor-oxo-phytodienoic acid (dinor-oxo-PDA) as well as all metabolites derived thereof, such as jasmonic acid (JA), JA methyl ester (MeJA), oxygenated derivatives of JA, as well as amino acid and sugar conjugates. Jasmonates show remarkable structural and biosynthetic similarities to prostaglandins (PGs) in animals (Fig. 1). PGs are, by definition, C-20 cyclopentanoids that are derived from bicyclo endoperoxide intermediates formed by a cyclooxygenase enzyme activity. As discussed in Section 2, the wide distribution of jasmonates and PGs in plants and mammals, respectively, is reflected by at least two common essential functions of these compounds, namely in the defense system and reproduction.

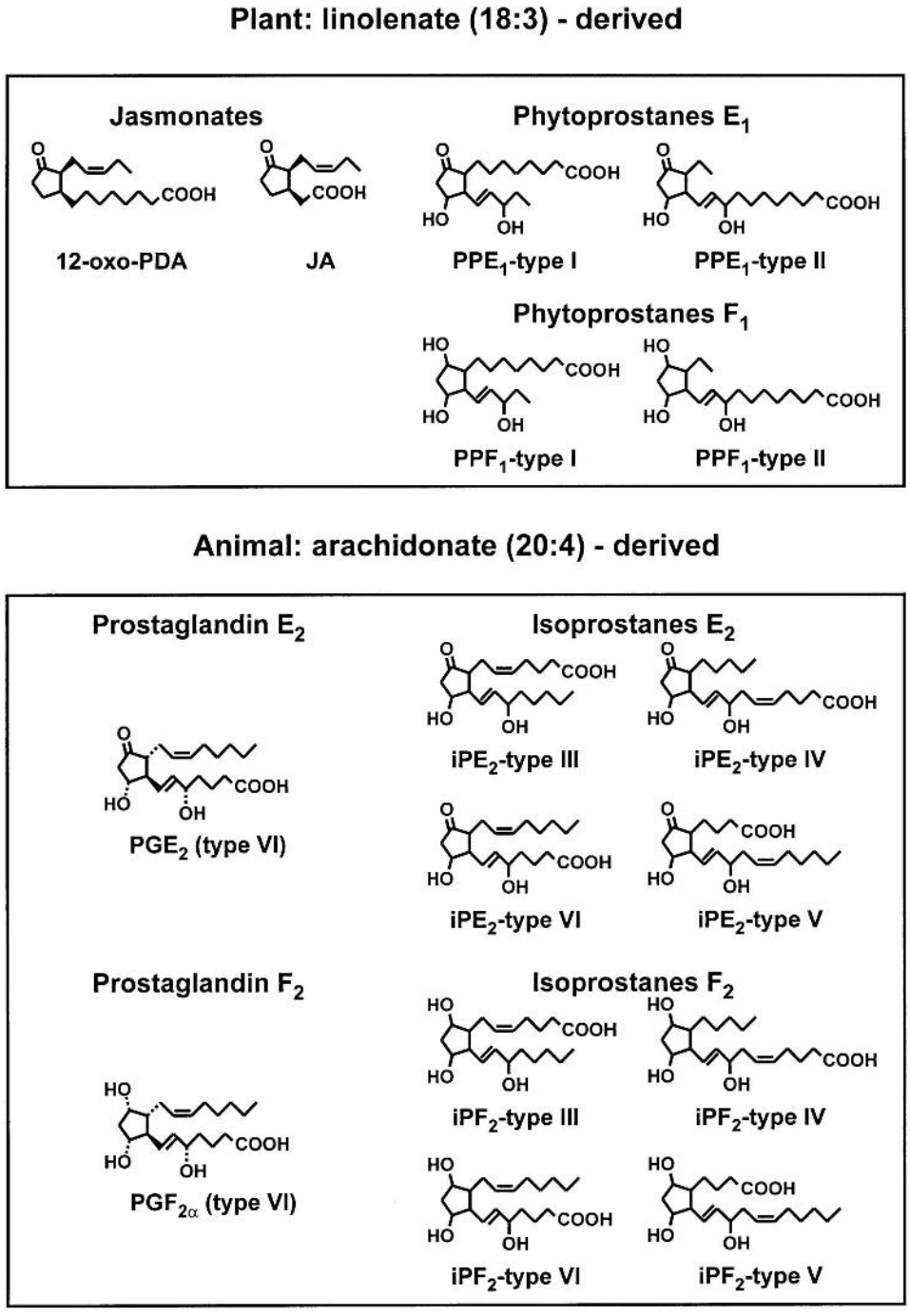


Figure 1 Representative cyclopentanoids in plants and animals. Linolenate is a precursor of non-enzymatically formed C-18 isoprostanoids (phytoprostanes, PP) and enzymatically generated jasmonates in plants. In animals, arachidonate can be converted via a free-radical-catalyzed pathway to C-20 isoprostanoids (isoprostanes, iP) that are isomers of the enzymatically formed prostaglandins.

In addition, plants and animals may form racemic bicyclo endoperoxides from polyunsaturated fatty acids by a free-radical catalyzed mechanism without the involvement of cyclooxygenase that was previously thought to be obligatory for prostanoid synthesis in vivo [1,2]. Because the products of the nonenzymatic bicyclo endoperoxide pathway derived from C-20 fatty acids represent isomers of the enzymatically synthesized PGs, the C-20 PG isomers are termed isoprostanes [3]. Compounds derived from bicyclo endoperoxides of various chain length have collectively been termed isoprostanoids throughout this chapter. Notably, isoprostanoid synthesis in vivo is only limited by the availability of suitable fatty acid precursors and the ability of aerobic cells to suppress endogenous free-radical formation. However, free-radical damage will always occur to some extent in aerobic organisms and, thus, isoprostanoids can presumably be found ubiquitously in cells utilizing polyunsaturated fatty acids. The isoprostanoid pathway has attracted considerable attention since isoprostanes have been shown to represent biologically highly active compounds for which a role as mediators of oxidative stress in mammals has been postulated [4]. However, the biological significance of the recently discovered C-18 isoprostanoids (phytoprostanes) in plants [5] remains to be established.

For mechanistic reasons discussed below, only fatty acids containing at least three double bonds can be converted to cyclopentanoids. In animals, arachidonic acid (C20:4) and to a lesser extend eicosapentaenoic acid (C20:5) and eicosatrienoic acid (C20:3) are precursors for PG [6] and isoprostane synthesis [7]. PGs are widely distributed in the animal kingdom and are found in frogs, fish, insects, birds, and mammals [6]. PGs are also found in some bacteria, fungi, and algae, where the mode of biosynthesis still remains to be established and their function is unknown [8,9].

Animals do not form jasmonates, as they are unable to synthesize α-linolenic acid (C18:3) de novo; they accumulate only low levels of linolenic acid (of dietary origin) and lack the enzymatic capacity (allene oxide synthase) to form jasmonates.

In contrast, higher plants generally do not accumulate fatty acids longer than C-18 [except as constituents of the surface coverings (wax, cutin, and suberin) or in the storage oil of certain seeds] and introduce no more than three double bonds [10]. Thus, trienoic fatty acids such as α-linolenic acid (C18:3) and, to a lesser extent, hexadecatrienoic acid (C16:3) may serve as precursors for the synthesis of jasmonates [11,12] and isoprostanoids [5,13].

Hence, the α-linolenic acid cascade can be regarded as the plant's counterpart to the mammalian arachidonate cascade.

2 JASMONATES

2.1 Occurrence

Jasmonate is the first cyclic fatty acid metabolite identified from higher plants with regulatory properties. In evolutionary terms, the most ancient and the only prokaryotic organism known to synthesize JA from endogenous α-linolenic acid is the cyanobacterium *Spirulina* [14]. Thus, linolenate biosynthesis and biosynthetic capacity for the synthesis of jasmonates may have evolved very early in evolution. Cyanobacteria are believed to have given rise through symbiosis to at least some eukaryotic chloroplasts. Interestingly, JA biosynthesis has been found to occur apparently ubiquitously in organisms that have plastids. JA has been identified in members of green protista (*Euglena* [15]), algae (*Gelidium* [16]), *Chlorella* [14]), mosses [17], ferns [17], and higher plants (reviewed in Ref. 18). In fact,

several of the key enzymes of linolenic acid and JA biosynthesis are localized in plastids. Notably, as an exception to this rule, some fungi, such as *Botryodiplodia* [19], *Gibberella* [20], and *Fusarium* [21], are capable of synthesizing JA. However, nothing is known about the biosynthetic enzymes and the intracellular localization of the jasmonate pathway in fungi.

2.2 Biosynthesis and Metabolism

2.2.1 Triggers of JA Synthesis

Jasmonate synthesis and concomitant JA-mediated defense gene activation can be triggered by stimuli that interact with the plant cell membrane through receptors by the systemic wound signal peptide systemin [22] and various elicitors generated by plant–pathogen interactions. Crude fungal and bacterial cell-wall preparations have been shown to be strong inducers of JA biosynthesis and defense gene activation [23,24]. Fragments of microbial cell walls such as oligosaccharides (glucan fragments, chitin oligomers), lipochitooligosaccharides, and glycoproteins also act as elicitors. In addition, microorganisms may secrete proteins that activate the jasmonate cascade, such as elicitins, xylanase, cellulase, harpins, and various polypeptides [25]. Furthermore, some microbial lipids act as elicitors in specific hosts such as arachidonic acid (produced by *Phytophtora infestans*) in potato tuber tissue, syringolides (produced by *Pseudomonas syringae*) in soybean, and ergosterol in tomato (reviewed in Ref. 25). In addition to these exogenous elicitors, endogenous elicitors may be formed by the activity of polygalacturonidase on plant cell-wall pectins. Polygalacturonidase was previously thought to be secreted by pathogens only, but it has recently been found as a systemin-, wound-, and oligosaccharide-inducible enzyme in plants [26]. Pectic fragments with a degree of polymerization of 10–15 generated by the enzyme are active elicitors in vivo [27] that trigger the jasmonate cascade [28]. In addition, a variety of stimuli for JA synthesis has been identified for which the molecular mechanisms of how cells sense these triggers are not clearly defined, such as local damage [29], ultraviolet (UV) light [30], mechanical stimulation [31], heavy metals [32], drought [33], and others [34].

2.2.2 Early Events at the Cell Membrane

Immediately downstream of the initial elicitor/systemin recognition site, changes of the activity of membrane-bound proteins take place (Fig. 2). A variety of evidence suggests the involvement of heterotrimeric G proteins in the initiation of subsequent events; however, most of the evidence is correlative and has been based on the use of nonhydrolyzable GTP analogs, toxins such as CTX, mastorpan, and recombinant G proteins (reviewed in Ref. 35).

The biochemical responses initiated by elicitors and the systemic wound signal systemin directly correlates with an increase in cytosolic free Ca^{2+} concentration [36]. Measurements of external Ca^{2+} with ion-selective electrodes and of Ca^{2+} fluxes using radiometric techniques have revealed a large and transient Ca^{2+} influx via plasma membrane Ca^{2+} channels [37,38]. Omission of Ca^{2+} from the culture medium of plant suspension cultures or inhibition of elicitor stimulated ion fluxes have been repeatedly reported to block defense gene activation [35]. In addition, it has been shown that the application of calcium ionophores may be sufficient to bypass receptor-mediated defense gene activation in soybean [39] and carrot [40]. However, at least in two well-studied species (parsley, tomato),

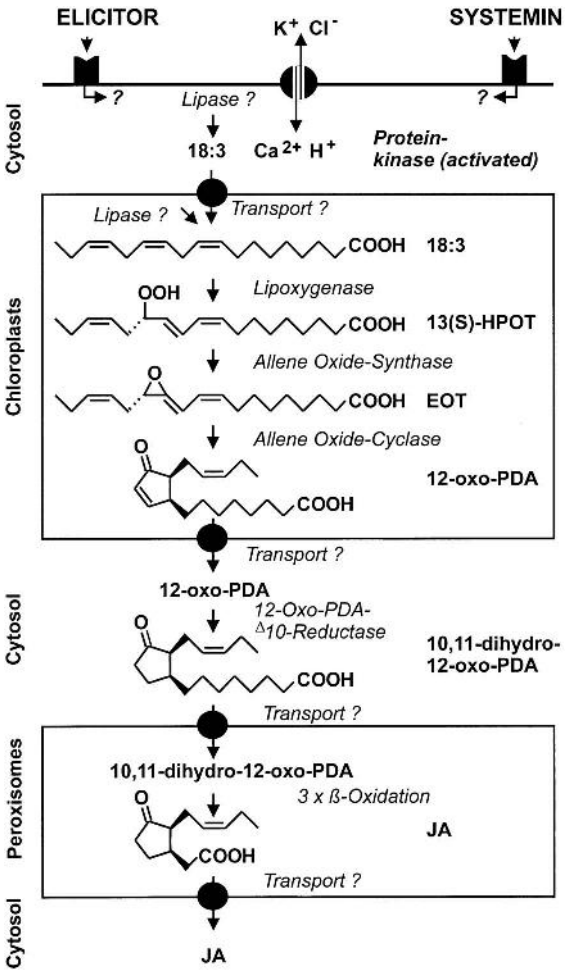


Figure 2 Biosynthesis of JA. The biosynthetic pathway of JA from α-linolenic acid (18:3) proceeds via 13(*S*)-hydroperoxy-9(*Z*),11(*E*),15(*Z*)-octadecatrienoic acid [13(*S*)-HPOTE], 12,13(*S*)-epoxy-9(*Z*),11,15(*Z*)-octadecatrienoic acid (EOT), 12-oxo-10,15(*Z*)-phytodienoic acid (12-oxo-PDA), and 10,11-dihydro-12-oxo-PDA.

ionophor-triggered Ca^{2+} influx is not sufficient (but necessary) for defense gene activation [40,41]. In addition to the increase of cytosolic calcium levels, loss of K^+ and Cl^- as well as influx of H^+ are the earliest events that can be observed after elicitation or wounding [40]. After activation of calcium channels, phosphorylation of proteins is another common early event, which may be either triggered by a kinase domain of a putative receptor or through second messengers such as Ca^{2+}. Wound- and elicitor-responsive mitogen-activated protein (MAP) kinases have been shown to be involved in transcriptional activation of defense genes [42–46].

Activation of protein kinases, in turn, leads to phosphorylation of several proteins. Among them appears to be the plasma membrane H^+-ATPase, which is inhibited by phos-

phorylation [47]. Commonly, inhibition of H^+-ATPase and a concomitant decrease of the cytosolic pH and alkalization of the extracellular milieu are observed after wounding and elicitor treatment [36,48,49]. In some cases, however, treatment with elicitors results in activation of the H^+-ATPase, increase of cytosolic pH, acidification of the extracellular milieu and hyperpolarization of the membrane potential [50]. It has been proposed that the differential effect of elicitors is in response to the difference in specific and nonspecific elicitors; that is, elicitors prepared from avirulent pathogen may activate H^+-ATPase, whereas elicitors from virulent pathogen inhibit the proton pump [35,41,50]. However, this hypothesis remains to be proven definitively. Whether or not proton fluxes at the plasma membrane play a role in signal transduction is a matter of controversy [41,51].

2.2.3 Initiation of JA Biosynthesis

The JA pathway (Fig. 2), except for the allene oxide intermediate, has principally been established by the work of Vick, Feng, and Zimmerman between 1966 and 1984 [52–56]. The crucial intermediate of the pathway, the highly unstable allene oxide, was identified by Hamberg [57] and Brash and co-workers [58] in 1987 and 1988, respectively. In higher plants, JA is biosynthesized from α-linolenate through allene oxide and 12-oxo-phytodienoic acid (12-oxo-PDA) by a biosynthetic machinery that is constitutively present at a low level in probably all plant cells and even undifferentiated cultured plant cells. The availability of linolenic acid at the site of biosynthesis appears to be a rate-limiting step, because JA is only found in low concentrations in unstimulated cells, but an immediate and dramatic increase of JA levels can be observed after exogenous application of linolenate [59]. With respect to the oxidative fatty acid metabolism in mammals, a lipase-releasing unsaturated fatty acids from membrane stores has been postulated to initiate JA biosynthesis [29]. The rapid linolenate release and JA synthesis observed after elicitation and wounding suggests that a lipase is indeed activated and may play a regulatory role for the JA pathway [24,60]. There is evidence that several lipases are activated after cell activation, such as a monogalactosyldiglyceride-hydrolyzing activity in chloroplasts or phospholipase A-type enzymes (reviewed in Refs. 11 and 61). However, it is not known if activation of lipases and concomitant release of linolenic acid is causally coupled with jasmonate synthesis. Neither the lipase nor the subcellular location of the postulated enzyme is known.

It has recently been shown that hexadecatrienoic acid (C16:3) is a precursor of dinor-12-oxo-PDA, which, in turn, may be metabolized to JA in *Arabidopsis*. In contrast to linolenate, which can be either derived from desaturation of linoleate in the ER membrane (prokaryotic pathway) or in plastids (eukaryotic pathway), C16:3 can only be synthesized via the prokaryotic pathway in plastids and is predominately localized in plastidic monogalactosylglycerols [10]. Mutant *Arabidopsis* plants lacking a plastidic desaturase (FAD5) failed to accumulate dinor-12-oxo-PDA and showed dramatically reduced levels of 12-oxo-PDA in uninjured plants [12]. Levels of 12-oxo-PDA in wounded leaves of the mutant are, however, similar to those in wild-type plants, suggesting that jasmonate levels are regulated differently in wounded and unwounded plants.

In addition, ω-3 fatty acid desaturase has been shown to accumulate around plant infection sites and may replenish or raise the levels of linolenate by desaturation of linoleic acid in order to feed octadecanoid synthesis [62].

Because levels of free linolenate in untreated cells appear to be orders of magnitude higher than required for synthesis of few nanograms of a signal molecule [11], it remains

an open question whether a lipase or a 13-lipoxygenase is the initial step of JA biosynthesis.

2.2.4 First Steps of JA Biosynthesis Occur in Plastids

Lipoxygenase

Unesterified linolenic acid is rapidly incorporated into membrane lipids when applied exogenously [24]. In addition, linolenic acid may be attacked by lipoxygenase (LOX), the first (or second) enzyme in the JA pathway. LOXs are nonheme iron-containing enzymes that catalyze dioxygenation of polyunsaturated fatty acids containing a 1(*Z*),4(*Z*)-pentadiene system, forming an enantiomeric pure (*S*)-hydroperoxy fatty acid. LOXs occur ubiquitously in higher eukaryotes and are abundant proteins in all plant tissues [63]. The most abundant LOX substrates in plants are linolenic, linoleic, and hexadecatrienoic acids. According to their positional specificity of oxygen insertion into C-18 unsaturated fatty acids, they are termed 9-LOX or 13-LOX, respectively. Among the possible LOX products, only 13(*S*)-hydroperoxy-9(*Z*),11(*E*)-15(*Z*)-octadecatrienoic acid (13-HPOTE) and 11(*S*)-hydroperoxy-7(*Z*),9(*E*),13(*Z*)-hexadecatrienoic acid can be converted to jasmonates. Hydroperoxy fatty acids formed by LOX may also be reduced to the corresponding alcohols or metabolized via the hydroperoxide lyase, the peroxygenase, or other pathways (reviewed in Ref. 64). Multiple isozymes of LOX that are located in plastids, in cytosol, or at the plasma membrane have been described. Most of the plant LOX genes isolated so far belong to so-called LOX1-type enzymes, based on their overall sequence similarity. Recently, LOX cDNAs from *Arabidopsis*, potato, rice, and barley with moderate overall sequence homology among each other were identified that encode a putative transit peptide sequence characteristic for chloroplasts. Based on this amino terminal extension, these LOXs were designated as LOX2-type enzymes [65]. In *Arabidopsis*, two isoforms, LOX1 and LOX2, have been cloned. Cosuppression resulting in specific elimination of the chloroplastidic LOX2 prevented wound-induced accumulation of JA in leaves but did not have an effect on basal JA levels [66]. Thus, stimulus-induced JA synthesis appears to be dependent on a constitutive plastidic LOX isoform, whereas basal JA synthesis may be maintained by other LOX isozymes.

Jasmonate, in turn, triggers the activation of genes involved in its own biosynthetic pathway, such as chloroplastidic LOXs [67], allene oxide synthase [68], and the systemic wound signal systemin [69]. Upon wounding, mRNA levels of these enzymes increase within 0.5 hr, maximize at 2–3 hr, and decrease thereafter. In contrast, transcripts of defense genes often begin to accumulate within 3–4 hr and peak at 5–8 hr [36]. This suggests that jasmonate synthesis can be reinforced in an autocrine fashion. Indeed, JA accumulation in elicitor-treated or wounded plant cells is biphasic in many plant species [23,24,70] and exogenously applied JA increases the levels of the JA precursor 12-oxo-PDA [71]. An immediate and transient accumulation of JA is often observed within 1 hr, followed by a strong second increase of JA after 1–2 hr, leading to an elevated JA level that is sustained for several hours [24,70]. Because JA can be secreted by plant cells in substantial amounts, JA may also act as a local alarm signal that triggers the jasmonate cascade in unchallenged neighboring cells [72]. This may rapidly induce endogenous defense mechanisms before an infection can spread.

However, the subcellular localization of jasmonate-induced LOX is not restricted to the chloroplast [66,73,74]. Inducible LOX isoforms are also found in the vacuole [75] or associated with the plasma membrane [76], suggesting different physiological functions of LOX isozymes in plant defense.

Allene Oxide Synthase

As mentioned, the LOX product 13-HPOTE may be channeled into competing metabolic pathways. The allene oxide synthase pathway that leads to 12-oxo-PDA and its metabolite JA (Fig. 2) is only a minor route of 13-HPOTE consumption in many plant tissues. In *Arabidopsis* leaves, allene oxide synthase activity represents only 3.5% of the 13-HPOTE-consuming enzymatic activity [68]. Often a major route of 13-HPOTE metabolism is the formation of antimicrobial aldehydes via hydroperoxide lyase [77]. Hydroperoxide lyase and allene oxide synthase are structurally related wound-inducible cytochrome P-450 enzymes that are located in plastids [78,79]. However, allene oxide synthase from guayule plants is unusual in that it lacks the N-terminal chloroplast targeting sequence and is located within discrete organelles called rubber particles [80]. Allene oxide synthase produces unstable allene oxides from 9(*S*)- and 13(*S*)-hydroperoxides of linoleic and linolenic acid, from 15(*S*)-hydroperoxide of C-20 acids and the 11(*S*)-hydroperoxide from 7(*Z*),10(*Z*),13(*Z*)-hexadecatrienoic acid [78,81–83]. However, in higher plants, only the allene oxides of 13(*S*)-hydroperoxide of linolenic acid and the 11(*S*)-hydroperoxide of hexadecatrienoic acid can be cyclized to jasmonates [11,12]. Allene oxide synthases belong to the CYP74 subfamily of cytochrome P-450 enzymes and are characterized by their lack of transmembrane segments and low affinity for CO. Allene oxide synthases do not bind/require oxygen and NADPH, as they use fatty acid hydroperoxides as substrates [78,84,85]. Because allene oxide synthase channels fatty acid hydroperoxides into the jasmonate pathway, it has been postulated that the enzyme represents a regulatory site in jasmonate biosynthesis [68]. This appears to be the case because enzyme activity, mRNA, and polypeptide levels as well as jasmonate concentrations are increased in wounded leaves (locally and systematically) and are also induced by jasmonates and other plant signals such as salicylic acid and ethylene [68]. There are conflicting reports on the effect of salicylic acid on allene oxide synthase. Salicylic acid is believed to block JA signaling in tomato leaves at a step prior to JA formation [86] as well as downstream of JA biosynthesis [87]. Moreover, recombinant allene oxide synthase from guayule plants [88] was competitively inhibited by salicylic acid (K_i of 238 μM), whereas recombinant enzyme from *Arabidopsis* was not inhibited by salicylic acid up to a concentration of 1 m*M* [68].

The allene oxide synthase promotor has been shown to be activated both locally as well as systemically upon wounding. Although 12-oxo-PDA and the pathway's end product JA may be transported from leaves to roots [89] and strongly activate enzyme transcription, the possibility that they may act as systemic signals has been ruled out, at least for *Arabidopsis* and *Nicotiana* species [90].

Allene Oxide Cyclase

12,13(*S*)-Epoxy-9(*Z*),11,15(*Z*)-octadecatrienoic acid resulting from the action of allene oxide synthase on 13-HPOTE is converted by allene oxide cyclase to 9(*S*),13(*S*)-12-oxo-PDA, which has the side chains at the cyclopentenone ring in cis configuration (Fig. 2). Notably, the allene oxide has a half-life of less than 30 sec in water and may cyclize spontaneously to 12-oxo-PDA with cis side chains (15–20% yield) or hydrolyze to the corresponding α- and γ-ketols (85–80% yield) [58]. Nonenzymatic cyclization, however, will afford racemic, cis-configured 12-oxo-PDA [i.e., a 1:1 mixture of 9(*S*),13(*S*)- and 9(*R*),13(*R*)-12-oxo-PDA]. Interestingly, allene oxide cyclase is more selective than allene oxide synthase and does not accept the allene oxide derived from the linoleic acid analog 13(*S*)-hydroperoxy-9(*Z*),11(*E*)-octadecadienoic acid, which also does not cyclize nonenzymatically to any significant extent [78,91]. Therefore, a third double bond at position

15 (in linolenate) is essential for efficient cyclization. Grechkin and Hamberg [91a] proposed a mechanism for the chemical cyclization whereby the 15(*Z*) double bond facilitates the opening of the oxirane ring of the allene oxide. The resulting C-13 carbocation is stabilized by the π-electrons of the 15(*Z*) double bond, and the cyclization is accomplished by ring closure involving the carbanion at C-9 and the carbocation at C-13. This mechanism could also apply to the enzymatic cyclization.

Allene oxide cyclase is a soluble enzyme that forms a dimer with a molecular mass of 47 kDa. The enzyme is not inhibited by a variety of nonsteroidal anti-inflammatory drugs, including salicylic acid and aspirin up to a concentration of 1 m*M* [91]. The subcellular location of allene oxide cyclase is not known. However, because of the chemical instability of the allene oxide substrate, it has been suggested that allene oxide synthase and cyclase may operate highly coordinated in plastids [61]. In fact, 9(*S*),13(*S*)-12-oxo-PDA formation appears to be a highly efficient process in vivo, because the 9(*R*),13(*R*)-enantiomer as well as α- and γ-ketols derived from nonenzymatic cyclization or hydrolysis of the allene oxide, respectively, are not detectable in plant tissues [92].

12-Oxo-PDA and Dinor-12-oxo-PDA Are Released from Plastids

Metabolism of fatty acid hydroperoxides in plastids affords two cyclic fatty acids, 12-oxo-PDA (from C18:3 precursor) and the dinor-12-oxo-PDA (from C16:3 precursor) [12]. Both compounds are thought to be exported by a putative transporter from plastids to the cytosol, where they act as signal in its own right. In addition, 12-oxo-PDA serves as a substrate for 12-oxo-PDA reductase (OPR). The enzyme is soluble and not associated with either plastids or microbodies and is thus probably cytosolic [93]. OPR catalyzes the reduction of the double bond in the cyclopentenone ring to afford 10,11-dihydro-12-oxo-PDA (from 12-oxo-PDA). OPR is active as a monomer of 41 kDa apparent molecular mass and depends on NADPH as a reductant. Two closely related OPR genes, termed *OPR1* and *OPR2*, are known. The first isozyme, OPR1, has been cloned from *Arabidopsis* [94] and utilizes 9(*R*),13(*R*)-12-oxo-PDA $\gg$ 9(*S*),13(*R*)-12-oxo-PDA, whereas 13(*S*)-configured isomers are not accepted as substrates [95]. In other words, the enzyme does not convert the natural 9(*S*),13(*S*)-12-oxo-PDA and the unnatural 9(*R*),13(*S*)-12-oxo-PDA isomers. Interestingly, OPR1 shows structural and functional similarities to yeast Old Yellow Enzyme. Previously, the yeast enzyme was known to reduce a wide variety of α,β-unsaturated carbonyls and now has been established to function as OPR [94]. However, the physiological role of OPR1 remains to be shown.

The other enzyme, OPR2, efficiently reduced all four 12-oxo-PDA isomers, including the natural 9(*S*),13(*S*)-12-oxo-PDA [96]. Expression analysis of OPR genes in RNA blot experiments demonstrated that OPR mRNA levels transiently increased as early as 15 min after wounding at the local wound site and returned to baseline levels 12 hr after wounding. However, the assay could not differentiate between *OPR1* and *OPR2* expression; thus, it could not be established if both or only one OPR gene had been induced. In systemic leaves, a moderate two-phase induction was seen [96]. However, wounding did not translate into a significant increase of OPR protein levels, which are constitutively high. Consequently, OPR probably has no regulatory function in the jasmonate pathway. The different jasmonate profiles (i.e., the ratios of 12-oxo-PDA to JA found in different plants) may thus be regulated by a putative 12-oxo-PDA transporter that makes 12-oxo-PDA available for subsequent metabolism to JA. Although it has not yet been shown, it is likely that *cis*-dinor-12-oxo-PDA is also a substrate for one or both of the OPR enzymes. (See Note Added in Proof at the end of this chapter.)

Uptake of 10,11-Dihydro-12-oxo-PDA into Peroxisomes and β-Oxidation

10,11-Dihydro-12-oxo-PDA is hypothesized to undergo three rounds of β-oxidation of the carboxyl side chain to yield JA. The putative dihydro derivative of dinor-12-oxo-PDA may undergo two rounds of β-oxidation to yield JA also. Intermediates shortened by one or two C-2 moieties are generally not detectable in plants [72] unless the pathway is pushed to its capacity limit by feeding precursors [55,56]. β-Oxidation appears to be a rather unspecific process because saturation of the pentenyl side chain as well as the stereochemistry at carbon-13 is not important [56]. In total, conventional β-oxidation of the C-18 precursor to JA would involve 15 steps, including the initial activation of the carboxyl group and the final release of JA by a thioesterase activity. The subcellular localization of the β-oxidizing enzymes is not known, but it is likely that plant peroxisomes (or glyoxysomes) catalyze β-oxidation of 10,11-dihydro-12-oxo-PDA. Whether or not higher plant mitochondria perform fatty acid degradation is a matter of controversy [97]. Finally, in order to function as a signal, JA has to be transported/released into the cytosol.

JA Is Metabolized via Four Main Routes

The common end product of fatty acid hydroperoxide metabolism via the allene oxide synthase/cyclase pathway is the side-chain cis-configured 3(*R*),7(*S*)-JA (also termed 7-*iso*-JA). When the 7-*iso*-JA nomenclature is used, JA is specifically identified as the trans isomer, 3(*R*),7(*R*)-(−)-JA. Cis-substituted cyclopentane derivatives such as 9(*S*),13(*S*)-12-oxo-PDA and 3(*R*),7(*S*)-JA are thermodynamically unstable and may readily epimerize via enolization to the energetically more favorable trans isomers 9(*S*),13(*R*)-12-oxo-PDA and 3(*R*),7(*R*)-JA, respectively. Thermodynamic equilibrium gives a cis/trans ratio of 1:9. Epimerization is a relatively slow process in the physiological pH range with a half-life of the cis epimer 3(*R*),7(*S*)-JA of more than 3 days at pH 7 and 25°C [70]. De novo synthesized 12-oxo-PDA and JA in stimulated plant cells appears to be the cis isomer (for technical reasons, accurate determinations of cis/trans ratios are difficult), whereas in unstimulated cells 12-oxo-PDA is predominately found as the cis-form, 9(*S*),13(*S*)-12-oxo-PDA, and JA at thermodynamic equilibrium [70,98]. Possibly, 12-oxo-PDA is generally turned over quickly, whereas JA is slowly metabolized in resting cells but rapidly modified/inactivated in activated cells.

Metabolism of 3(*R*),7(*S*)-JA and 3(*R*),7(*R*)-JA is characterized by four major routes (Fig. 3). Such reactions include hydroxylation at C-11 or C-12 as a primary step, reduction of the C-6 keto group, either with hydroxylated or nonhydroxylated side chains, followed by O-glucosylation of the hydroxyl groups or the carboxy group. Alternatively, the carboxy group of JA or its hydroxylated side-chain derivatives may be conjugated with hydrophobic amino acids (reviewed in Ref. 99).

2.3 Jasmonate Signaling

Jasmonates are gene activators that induce a broad spectrum of proteins that can be grouped in four larger groups:

1. Defense proteins such as proteinase inhibitors (proteins that inhibit the digestive tract of insects [100]), defensins [101], and thionins [102]. In addition, enzymes are induced that are involved in the synthesis of antimicrobial secondary metabolites termed phytoalexins [23]. In a screening program, induction of biosynthetic enzymes by 12-oxo-PDA and JA led to accumulation of secondary metab-

olites in 84% out of 165 different cell cultures assayed by high-performance liquid chromatography (HPLC) [103].
2. Proteins and polypeptides that play a role in jasmonate signal transduction such as LOXs, allene oxide synthase, prosystemin, and others, as discussed earlier.
3. Proteolysis-associated proteins such as ubiquitin-like protein and various proteinases [36].
4. Proteins functioning in plant development and proteins with unknown functions such as threonine deaminase, acyl CoA binding protein, vegetative storage proteins, and others [104].

The jasmonate signal transduction pathway is largely unknown. It is presumed that jasmonates interact with receptors in the cell that activate a signaling pathway, which ultimately results in changes in transcription, translation, and other responses. However, no such receptors have been identified yet. Jasmonate receptors and other components of the signal transduction pathway are likely to be discovered through analysis of mutants that are insensitive to jasmonates. Four classes of such mutants have been identified: *coi1*, *jar1*, *jin1*, and *jin4* [105–107]. The later three mutants were identified by their insensitivity to 1–10 m*M* JA in a root-growth bioassay, whereas *coi1* mutants did not develop chlorosis after application of coronatine, a toxin that is structurally and functionally similar to 12-oxo-PDA [108]. The *coi1* gene product contains 16 leucine-rich repeats (believed to be involved in protein–protein interactions) and an F-box motif which appears to tag repressor proteins of JA responses by ubiquitination for proteolytic removal [109–111].

2.4 Jasmonates Are Essential for Plant Development and Defense

2.4.1 Plant Development

Mutant plants that were deficient in the jasmonate biosynthesis or signaling have been used to unravel the biological function of jasmonates. An *Arabidopsis* triple mutant lacking two chloroplastidic desaturases (FAD7, FAD8) and one ER-localized desaturase (FAD3) was shown to be completely deficient in C16:3 and C18:3 fatty acids [112]. Mutant plants display a remarkable normal vegetative phenotype and apparently differed from wild-type plants only in that they produce no mature pollen and thus are male sterile. Exogenous addition of α-linolenate or JA restored fertility [112]. Hence, the only essential requirement for linolenate in the plant life cycle appears to be as a substrate for jasmonates. Other plant developmental processes that are triggered by jasmonates (12-oxo-PDA or JA) include induction of vegetative storage proteins [113] and fruit ripening [33]. Vegetative storage proteins do not accumulate in the flowers of *coi1* mutant plants although their level are high in flowers of wild-type plants [105,106]. Furthermore, in *coi1*, *jin1*, and *jin4* plant leaves, vegetative storage proteins are not inducible by MeJA.

Another well-studied effect mediated by jasmonates is coiling of *Bryonia dioica*, and *Phaseolus vulgaris* tendrils. 12-oxo-PDA rather than JA appears to be the signal in mechanosignal transduction [98]. Higher levels of exogenous jasmonates induce senescence/loss of chlorophyll as well as tuberization and inhibit seed as well as root growth (reviewed in Refs. 34 and 114). However, it has been questioned if these processes are mediated by jasmonates in vivo [61,115].

2.4.2 Plant Defense

The (*fad3-2fad7-2fad8*) triple mutant of *Arabidopsis* deficient in linolenate and jasmonate biosynthesis showed extremely high mortality (about 80%) from attack by insect larvae

of a common saprophagous fungal gnat, *Bradysia impatiens*, or the fungal root pathogen *Pythium mastophorum*, even though neighboring wild-type plants were unaffected [116,117]. Application of exogenous MeJA substantially protected the mutant plants and dramatically reduced mortality. A tomato mutant, termed *defenseless1* (*def1*), has been identified by Ryan and co-workers that has a defect in the jasmonate pathway between 13-HPOTE and 12-oxo-PDA [118]. This mutant fails to form JA and to accumulate proteinase inhibitors systemically in response to systemin and oligosaccharide elicitors (chitosan and polygalacturonide). The impaired response is associated with a dramatic reduction in resistance toward tobacco hornworm larvae (*Manduca sexta*). An interesting aspect of the *def1* wound response is that significant amounts of proteinase inhibitors accumulate in wounded leaves but not, as commonly observed, in systemic leaves. Possibly, a redundant pathway for octadecanoid metabolism exists that relies on severe membrane degradation [118]. In *Arabidopsis*, mutants that are either blocked in their response to jasmonate (*coi1*) or ethylene (*ein2*), systemic expression of the defensin gene was suppressed, suggesting that *Arabidopsis* requires components of signaling pathways of both jasmonate and ethylene [101], which appear to be activated in parallel in this species [119]. Unlike *Arabidopsis*, JA application in tomato plants results in a rapid but transient increase in ethylene emission, suggesting a sequential activation of both pathways. Inhibition of ethylene biosynthesis or ethylene perception abolished the responsiveness of wound-responsive proteinase inhibitor *pin2* gene in tomato [120]. However, JA but not ethylene alone triggered activation of the *pin2* gene in tomato [120]. Hence, the ethylene signal pathway cooperates somehow with the JA response pathway to effect expression of stress-related genes.

The structural similarity of 12-oxo-PDA and JA to mammalian PGs, as well as similarities in the mode of biosynthesis, have been recognized early [55]. Interestingly, recent research has revealed that there is also a functional parallel although the molecular mechanisms involved are completely different [121]. Like jasmonates, PGs are essential for—however animal-specific—developmental and reproductive processes such as kidney development [122] and birth [123,124] as has been established with knockout mice that lack prostaglandin (PG) synthase 1 or $PGF_{2\alpha}$ and E_2 receptors. In addition, prostanoids are also essential for defense responses such as wound healing mediated by thromboxane A_2 [125], sensing pain triggered by PGE_2 and prostacyclin [126], as well as pyrogen-induced fever mediated by PGE_2 [127].

Thus, prostanoids are not only involved in defense but also in wound healing and tissue regeneration. In contrast, jasmonates appear to be primarily for defense against invaders, and wound-healing genes have not been identified [36].

2.5 Jasmonates: Active Compounds

The question of active compounds in the jasmonate pathway other than JA/MeJA has now partially been answered. It has been noted that plants not only accumulate JA but also 12-oxo-PDA, the first cyclopentanoid of the pathway, and metabolites/conjugates of JA (Fig. 3). 12-oxo-PDA, JA, and amino acid conjugates of JA have been shown to transiently increase within 1 hr after elicitation, wounding, or mechanical stimulation [72,98,128]. These compounds as well as a variety of synthetic derivatives and analogs have been shown to exert potent biological activity, which may differ somewhat in different bioassays as well as in the profile of induced proteins [104,129]. Therefore, it has been postulated that the jasmonate signal has to be attributed to a continuum of jasmonates generated via the allene oxide synthase pathway [12].

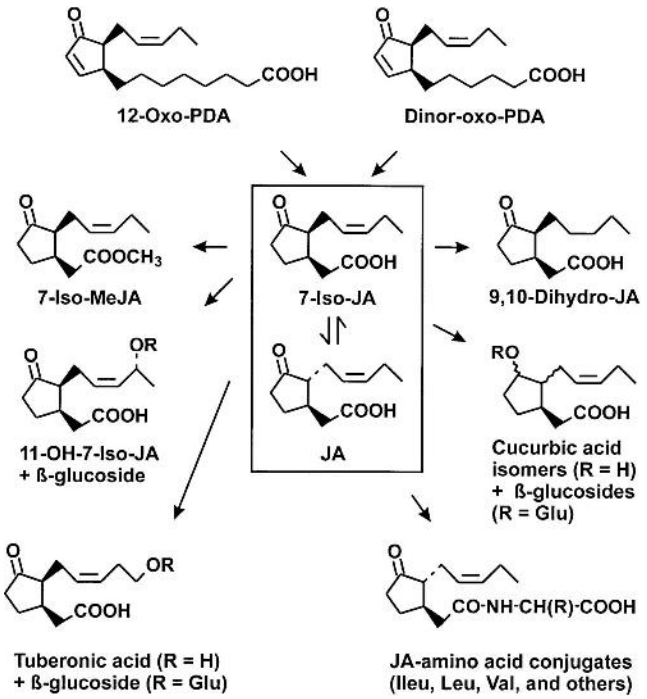


Figure 3 Main pathways of JA metabolism in plants.

However, 12-oxo-PDA appears to be the critical signal in mechanotransduction and elicitation, because it (1) is induced in many plants much earlier than other members of the jasmonate pathway [72,98], (2) accumulates in most species to higher levels than JA, which may not even accumulate [72,98], and (3) is much more active than JA in the *Bryonia* tendril coiling assay [31], as well as in induction of the secondary metabolites in most plant cell cultures [103]. Blechert et al. showed that methyl trihomojasmonate that cannot be metabolized via β-oxidation to JA is still active [130]. In addition, 12-oxo-PDA and JA have been shown to induce a different pattern of volatiles in lima bean [131] and proteins in tomato [132]. However, the spectrum of induced metabolites is generally the same for both jasmonates, as revealed by the analysis of 139 cell cultures [103]. Thus, it has been well established that 12-oxo-PDA is a signal in its own right, often acting as the primary signal that does not need to be metabolized to JA to become active [133]. In contrast to these findings, a series of 12-oxo-PDA derivatives with varying lengths of the carboxyl side chain have been synthesized and tested for their ability to induce proteins in tomato. Interestingly, derivatives with an even number of carbon atoms in the side chain (eight, six, and four carbons) expected to undergo β-oxidation to yield JA were active, whereas derivatives with an odd-numbered side chain containing seven, five, three, and one carbons, which cannot be metabolized to JA, were inactive. This suggested that β-oxidation is a requirement for 12-oxo-PDA to become active [104]. However, in the tendril-coiling assay, the derivative with seven carbons was active, whereas the putative β-oxidation products of this analog were inactive [133]. Notably, some plant species respond preferentially with the accumulation of JA rather than with 12-oxo-PDA [72,98]. Hence, the relevance of different jasmonate profiles is not clearly established. Final clarification

awaits the analysis of genetically modified plants that lack 12-oxo-PDA reductase (and, thus, cannot form JA) and a better understanding of the full spectrum of biological activities of jasmonates in different plant species.

Among the JA metabolites, exogenously applied tuberonic acid and tuberonic acid β-glucoside have been shown to be inducers of tuberization in potato [134,135]. However, the question of whether jasmonates are involved in tuber induction in vivo is a matter of controversy [115,136].

Jasmonate amino acid conjugates have also been shown to be potent gene activators when applied exogenously [137,138]. Among the JA conjugates, the JA form found predominately in plants is more active than the 7-*iso*-JA-form [137,139]. In addition, the natural L-amino acid conjugates are active, whereas D-amino acid conjugates were inactive or only active at high concentrations [137]. In wounded rice leaves, the leucine and valine conjugates were transiently induced and exhibited a similar kinetic as JA. Levels of conjugates and free JA were found to be in the same range [128]. At present, it is not known whether amino acid conjugates are active per se or only after hydrolysis by an as yet unknown amidase. An amidase or hydrolysis of amino acid conjugates has so far only been identified in a fungus that does not accumulate amino acid conjugates of JA [140].

2.6 Conclusion

The jasmonic acid pathway that has been elucidated in the 1970s and 1980s has been thought to play a role in plant growth regulation and senescence throughout the 1980s. During the 1990s, detailed functional studies of jasmonates have led to the appreciation of jasmonates as a group of diverse signal transducers in plant defense and development. Although the enzymology of JA has been largely established and many of the genes involved in JA synthesis have been cloned, little is known about the sequence of events that initiate jasmonate synthesis in plastids and the sequence of events that translate the jasmonate signal into gene activation. Rather than focusing on JA alone, it has become clear that different compounds (comprising 12-oxo-PDA, dinor-12-oxo-PDA, amino acid conjugates, tuberonic acid, and, probably, as yet unknown compounds) bear biological activity and contribute to the ''jasmonate'' signal. Furthermore, the spectrum of biological activity is different among various jasmonates so that it might be possible that different jasmonate signatures, found in taxonomically distant species, translate into somewhat different effects. However, because conversion of the early jasmonates into later jasmonates occurs (and even some interconversion may be possible), it is difficult to separate the effects of single members of the jasmonate family. The complexity of the picture is further increased by the fact that a variety of other plant signal cascades may be activated in parallel or sequentially. The analysis of mutant plants that lack critical components of the JA and other signal transduction pathways has just begun and will help to understand how jasmonate biosynthesis is regulated and how different signal transduction pathways work in consort in plant defense and development.

3 ISOPROSTANOIDS

3.1 Discovery of Isoprostanoids

In the presence of oxygen, arachidonic, linolenic acid, and other polyunsaturated fatty acids are prone to undergo autoxidation to yield cyclic oxylipins, which have the same cyclopentane ring system and oxygen functionalities as prostaglandins (PGs). As for PGs, the letter behind the isoprostanoid (isoprostane, phytoprostane) indicates the type of the

ring system and the numerical subscript denotes the number of double bonds in the side chains (Fig. 1).

The formation of PG-like compounds from unsaturated fatty acids was first described in 1967. Nugteren and co-workers autoxidized 8,11,14-eicosatrienoic acid (C20:3) to a PGE_1-like compound [141]. These studies were continued 9 years later by Pryor's group, which oxidized a C18:3 fatty acid, methyl linolenate, with ozone and obtained a mixture of isomeric bicyclic endoperoxides with a PGG_1 ring system [142]. They also formulated a general free-radical-catalyzed reaction mechanism that can be applied to fatty acids containing at least three double bonds (see Fig. 4). Because in the case of linolenate, theoretically 32 isomeric, highly unstable bicyclic endoperoxides can be formed from linolenic acid via the 9-, 12-, 13-, and 16-peroxylinolenate radicals, no stereochemical pure compounds were obtained. In order to reduce the number of stereomers, Porter and co-workers oxidized a single hydroperoxide, 9(*S*)-hydroperoxy-γ-linolenic acid, with di-*tert*-butylperoxyoxalate and obtained mass-spectroscopic evidence for the formation of C-18 analogs of PGF_1 that are derived from the unstable bicyclic endoperoxides by nonenzymatic reduction. However, the mixture of stereomers obtained from one linolenate hydroperoxide still contained 16 stereoisomers, which could not be resolved. In the early 1980s, Connor et al. were the first to succeed in the separation and analysis of the highly

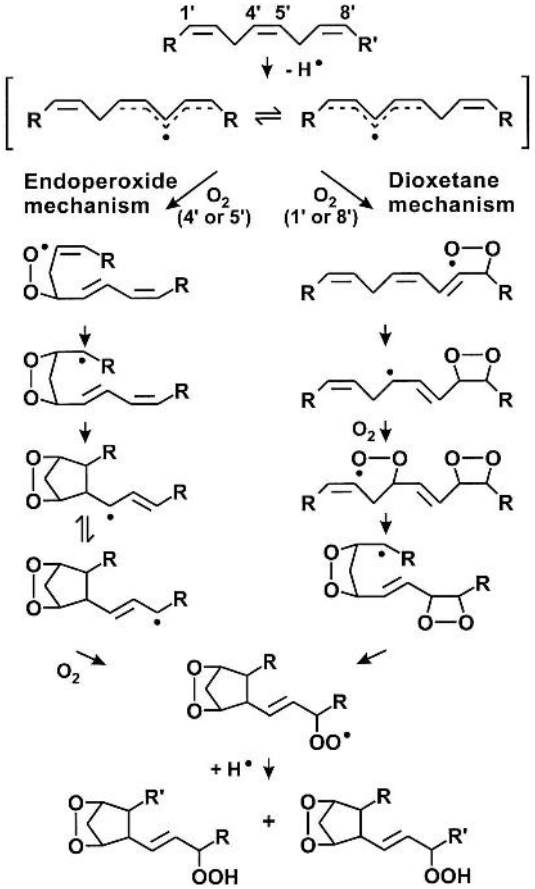


Figure 4 Free-radical-catalyzed mechanisms of isoprostanoid formation.

unstable bicyclic endoperoxides prepared by autoxidation of 13-HPOTE methyl ester [143,144].

However, for more than 20 years, these interesting observations were never carried beyond the realm of an in vitro chemical curiosity of fatty acid autoxidation, nor was it ever explored whether such products could be generated in vivo.

In 1990, Morrow and Roberts showed in a series of elegant studies that a series of PG-isomers termed isoprostanes are produced in vivo in humans independent of the cyclooxygenase enzyme by free-radical-catalyzed oxidation of arachidonic acid [145]. Not only was it demonstrated that isoprostanes occur in all mammalian tissues and body fluids at a concentration that exceeds the levels of PGs by at least an order of magnitude but also that isoprostane levels were dramatically induced during enhanced free-radical formation in vivo [2]. Furthermore, isoprostanes exert potent receptor-mediated biological activity [146]. Therefore, it has been suggested that isoprostanes may represent mediators of oxidative stress in mammals (see Sec. 3.2).

As arachidonic acid cannot be synthesized in higher plants, PGs and isoprostanes are generally absent. However, α-linolenic acid is the major unsaturated fatty acid in plants and has recently been shown to readily autoxidize to isoprostane E_1-like compounds in vitro and in vivo [5].

3.2 Biosynthesis and Nomenclature of Isoprostanoids

3.2.1 Formation of the PGG-Ring System

The ring system of PGG can be formed from any fatty acid containing one or more triene units (structural element that contains three double bonds interrupted by two methylene groups), as shown in Figure 4. Hydrogen abstraction from one of the two bisallylic methylene groups of a 1′,4′,7′ heptatriene unit yields one of two possible pentadienyl radicals. The 1′,4′ pentadienyl radical may be oxygenated in positions 1′ or 5′ whereas the 4′,7′ pentadienyl radical may be attacked by oxygen in positions 4′ or 8′. Two mechanisms have been proposed for the formation of G-ring compounds from the four racemic peroxyl radicals. One mechanism, the so-called endoperoxide mechanism, starts from the 4′- or 5′-peroxyl radical that has a cis-double bond homoallylic to the peroxyl group permitting its facile 1,3-cyclization by intramolecular radical addition to the double bond. Thereby, a new radical is generated that may cyclize to a bicyclic endoperoxyl radical which, subsequently, can trap an oxygen molecule, yielding a bicylic endoperoxy peroxyl radical [142]. The other mechanism, the dioxetane mechanism proposed by Corey and co-workers, starts from a 1′- or 8′-peroxyl radical that undergoes 1,2-cyclization to yield a dioxetane radical intermediate. The radical may trap another oxygen molecule, undergo 1,3-cyclization, and cyclize to the same bicylic endoperoxy peroxyl radical as obtained via the endoperoxide mechanism [147]. Because the methyl and the carboxyl terminus at a triene system of a fatty acid (R and R′ in Fig. 4, respectively) are mechanistically equivalent, two completely racemic regioisomeric radicals can be formed. These radicals may abstract a hydrogen atom from a fatty acid yielding PGG-like bicyclic endoperoxy hydroperoxides.

However, dioxetanes have never been isolated so far although fatty acid oxidation has been investigated extensively [148,149]. Therefore, an alternative mechanism could be that the 8′-peroxyl radical undergoes β-scission to afford pentadienyl radicals and oxygen (Gardner, personal communication). Oxygen could recombine as a 4′-peroxyl radical with 5(*E*),7(*E*)-conjugated double bonds. This mechanism has been well studied and it has

been established that β-scission of O_2 from peroxyl radicals and subsequent reoxygenation may change both the position of oxidation and the geometry of the diene conjugation [150–153]. Furthermore, oxygen involved in this type of reaction has been shown to exchange with gaseous O_2 [150]. Analogously, the 1′-peroxyl radical may isomerize to the 5′-peroxyl radical. Notably, the 4′- and 5′-peroxyl radicals generated by isomerization of the outer 8′- and 1′-peroxyl radicals, respectively, differ from the 4′- and 5′-peroxyl radicals formed by direct oxygenation by the double-bond geometry at positions 7′ and 1′, respectively; that is, the geometry would be predominately trans in the isomerization products and cis in the direct oxygenation products. Thus, it is clear that the inner peroxyl radicals formed by isomerization of the outer peroxyls may undergo the endoperoxide mechanism, yielding bicyclic endoperoxides. However, the stereochemical preferences are different in the endoperoxide and the putative dioxetane mechanism. The endoperoxide mechanism will almost exclusively yield racemic products with the side chains at the cyclopentane ring with cis substitution [144], whereas the alternative mechanism (the ''dioxetane'' mechanism) will lead to racemic products with a cis/trans ratio of 1:3 [147]. Thus, ring closure by the endoperoxide mechanism, yielding cis-configured products, is highly favored as predicted by the Woodward–Hoffman rules. Notably, cis-configured cyclopentanes are also formed by nonenzymatic as well as enzymatic ring closure of allene oxides, however, not in the case of enzymatically formed PGG, where a single trans-configured isomer, PGG, is the main product of the cyclooxygenase activity.

Because two double bonds of the precursor fatty acid are consumed during cyclization, products of trienoic fatty acids (linolenate, eicosatrienoic acid) are of the 1 series, whereas that of tetraenoic acids (arachidonate) are of the 2 series (subscript 1 and 2, respectively).

Two regioisomeric isoprostanoids can be generated from trienoic fatty acids (Figs. 4 and 5). In tetraenoic fatty acids, two triene units can be distinguished and, thus, four regioisomers can be formed. The regioisomers are best classified according to a systematic nomenclature system (Fig. 5) [3,13], which overcomes some important limitations of the previous nomenclature [154]. Because each regioisomer comprises theoretically 16 stereomers, trienoic, tetraenoic and pentaenoic acids will form 32, 64, and 124 isomers of each ring system, respectively.

3.2.2 PGG-like Compounds Are Precursors of a Variety of Prostanoids/Isoprostanoids

The highly unstable PGG that has been formed by the cyclooxygenase activity of PGH synthase is rapidly converted to PGH by the peroxidase activity of the bifunctional PGH synthase. PGH, in turn, is efficiently converted into PGD, PGE, PGF, and PGI as well as thromboxanes by cell-specific enzymes. Thus, the unique PG pattern formed from different cell types is dependent on their enzymatic capability and is tightly regulated [6,155].

In contrast, unstable PGG-like compounds formed by autoxidation (half-life of less than 5 min in aqueous medium) are nonenzymatically degraded to a variety of isoprostanoids. At higher temperatures or at low pH, isoprostanoids G represent a major source of malondialdehyde (Fig. 6), which can be detected by the popular thiobarbituric-acid-based lipid peroxidation assay [142,156,157]. However, in buffered solutions at ambient temperature, the initially formed G-ring isoprostanoids (Fig. 7) are isomerized predominately to a series of compounds with the PGE and PGD ring system (89% and 6% yield, respectively). Malondialdehyde (0.8%) and F-ring compounds (0.8%) are only minor products [158].

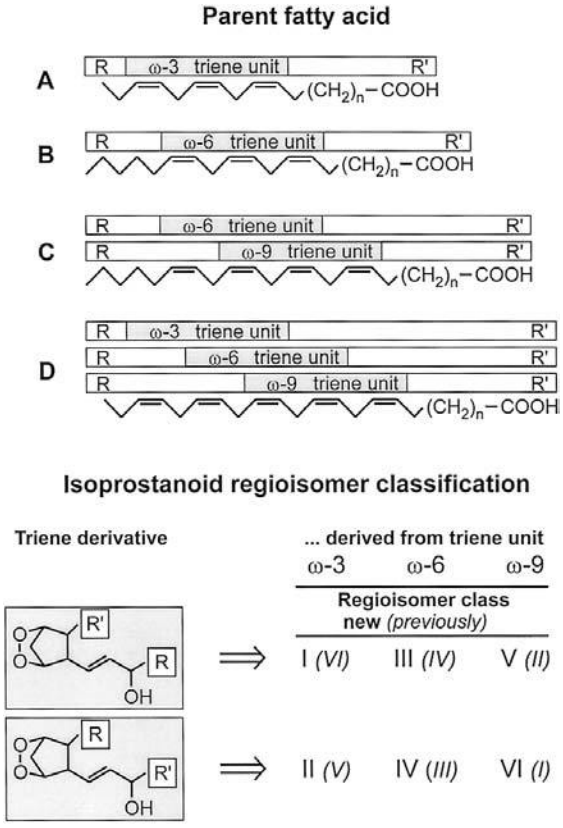


Figure 5 Architecture and nomenclature of isoprostane regioisomers. A triene unit is the principal building block for all (iso)prostanoid ring systems [prostaglandin H (PGH) ring system in this example]. The substitutes at the triene unit represent the methyl and the carboxyl terminus of the fatty acid. The methyl-terminal chain at each distinct triene unit may occupy positions R and R′ of the cyclopentanoid ring system, respectively. Thus, two types of regioisomers can be produced per triene unit. As shown, trienoic, tetraenoic, and pentaenoic fatty acids will form two, three, and six different regioisomers, respectively. Representative examples are (A) α-linolenate ($n = 6$), (B) γ-linolenate ($n = 3$), (C) arachidonate ($n = 2$), and (D) eicosapentaenate ($n = 2$). (Adapted from Ref 13.)

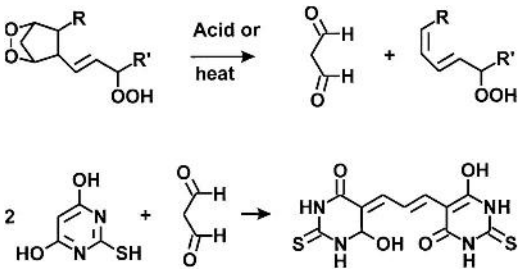


Figure 6 Formation of malondialdehyde and the thiobarbituric acid–malondialdehyde adduct from prostaglandin G-like endoperoxides under the conditions of the thiobarbituric acid test.

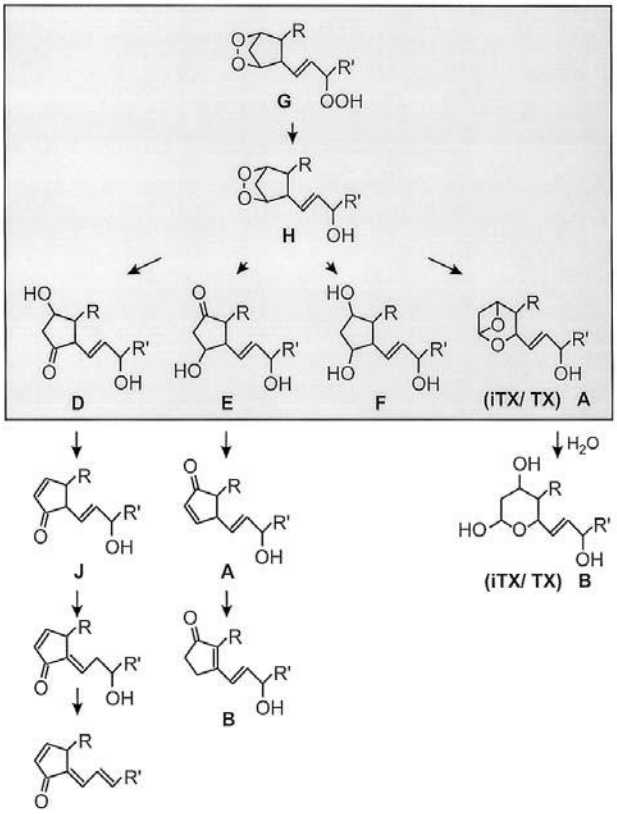


Figure 7 Enzymatic and nonenzymatic metabolism of G-ring prostaglandins/isoprostanoids. The primary enzymatic G-ring metabolites are shown in the box. All these products may also be formed nonenzymatically, however. Structures shown outside the box are derived from primary prostaglandins, thromboxanes (TX), isoprostanes, and isothromboxanes (iTX) by nonenzymatic reactions only.

However, in the presence of a natural reductant, such as glutathione or unsaturated fatty acids, G-ring compounds can readily be reduced to F-ring compounds; thus, F-ring structures may represent the main decomposition products depending on the environment [159]. Furthermore, the thromboxane A as well as the prostacyclin ring system can be formed in the presence of a metal catalyst from G-ring compounds—however, with poor yields (well below 2%) in vitro [158]. Thus, all natural PG skeletons can be formed nonenzymatically from arachidonic acid, and analogous families of compounds can be generated from other polyunsaturated fatty acids as well. In addition, these ''primary'' prostanoids may be nonenzymatically degraded to ''secondary'' prostanoids. For instance, thromboxane A-like and prostacyclin-like compounds do not accumulate because they are rapidly hydrolyzed by water, affording the thromboxane B-ring and 6-keto-PGF_1 (from PGH_2, not shown), respectively [158,160]. The D- and E-ring compounds are moderately stable under physiological conditions and dehydrate/isomerize via enolenolate intermediates slowly to A-, B-, and J-ring compounds [161]. In addition, J-ring structures are prone to undergo further degradation reactions [162]. Among the products of the PG/isoprostanoid pathways, F-ring compounds are the most stable compounds, which are, under physiological conditions, not chemically degraded to any significant extent [161].

3.3 Occurrence of Isoprostanoids

3.3.1 Mammalian Isoprostanoids

In mammals, arachidonic acid (C20:4) is the predominant fatty acid that can be converted to isoprostanes. In addition, eicosatrienoic acid (C20:3) and docosapentaenoic acid (C22:5) yield cyclic fatty acids too [141,163]. Because the term ''isoprostane'' refers to C-20 compounds, C-22 isoprostane homologs have been termed ''neuroprostanes'' because the C22:5 precursor fatty acid and neuroprostanes are especially enriched in the nervous system [163]. Linolenic acid (C18:3) is only a minor fatty acid in mammals and, consequently, isoprostanoid derivatives have not yet been detected in mammals.

Because fatty acids are found almost exclusively esterified in membrane lipids in mammals and plants (typically more than 99% [60,164]), isoprostane compounds are formed predominately in membranes from which they may be released by phospholipase A_2 [1]. Re-esterification of free prostanoids is not thought to occur. Once isoprostanes have been released from membranes, they are rapidly exported from the producer cells. The half-life of isoprostanes in plasma is short due to rapid uptake catalyzed by a PG transport protein that accepts a broad spectrum of prostanoids in mammals [165], followed by β-oxidation and urinary excretion [166,167].

For instance, isoprostanes F_2 have been found esterified in rat liver phospholipids (6 ng/g liver) and unesterified in fresh human plasma (35 pg/mL) and human urine (1.6 ng/mg creatinine) [1]. The major isoprostane F_2 metabolites identified in urine were in the same range as the unmetabolized compound [167]. In contrast, levels of isoprostanes D_2/E_2, which are major products of the isoprostane pathway in vitro, were not detectable in free form in plasma but were found esterified in rat liver phospholipids (0.9 ng/g liver) [168]. The discrepancy between the ratio of D/E-ring compounds to F-ring compounds found in vitro (favoring D/E-ring formation) and in vivo (favoring the F-ring formation) is thought to be due to the presence of natural reductants in vivo [159]. Degradation products of D_2/E_2 isoprostanes such as A_2/J_2 isoprostanes were not detectable in plasma but occur esterified in rat liver (5.1 ng/g liver) [169]. As mentioned earlier, isothromboxanes rapidly hydrolyze to isothromboxanes B_2. Levels of B_2 isothromboxanes were undetectable in plasma, whereas levels of the compounds esterified in rat liver were about 2.5 ng/g liver [160]. However, in animal models of lipid peroxidation (orogastric administration of CCl_4), levels of all aforementioned isoprostanes increased dramatically (up to 200-fold) in vivo and they were clearly detectable in plasma. Isoprostacyclins have thus far not been identified in vivo.

3.3.2 Plant Isoprostanoids

In plants, linolenic acid (predominately) and hexadecatrienoic acid are potential precursors of isoprostanoids. Formation of C-18 isoprostane E_1 analogs from linolenate has recently been reported [5]. Because they are two methylene groups shorter than the C-20 isoprostanes, they have previously been termed ''dinor isoprostanes'' [3,5]. It has been suggested to rename the plant derived dinor isoprostanes to phytoprostanes (PP) because β-oxidation of isoprostanes will also lead to C-18 dinor isoprostanes [169a]. The later group of compounds differs, however, from the phytoprostanes in the length of their side chains. The C-16 tetranor isoprostanes have been detected as β-oxidation metabolites of isoprostanes [167] but have not yet been found in plants (where they potentially could either be derived from β-oxidation of phytoprostanes or by cyclization of hexadecatrienoic acid).

In plants, high levels of phytoprostanes F_1 and E_1 (as compared to mammalian iso-

prostanes) were found in leaves, flowers, and roots of taxonomically distinct species, ranging from 43 to 1380 ng/g and from 37 to 935 ng/g of dry weight, respectively [169a]. In addition, esterified phytoprostanes F_1 were found in 10–150-fold higher levels than free phytoprostanes F_1 in all plant parts and species so far analyzed. Notably, extremely high concentrations of phytoprostanes E_1 and F_1 (14,363 and 32,440 ng/g of dry weight, respectively) were quantitated in fresh birch pollen [169a]. Thus, analogous pathways in animals and plants exist, each leading to a unique family of isoprostanoids.

3.4 Significance of Isoprostanoids

At present, the interest in mammalian isoprostanes encompasses two general areas: as markers of oxidative stress in vivo and as potential mediators of oxidant injury. Isoprostanes F_2 are chemically stable end products of lipid peroxidation and have been shown in an increasing number of studies to represent extremely sensitive and reliable markers of oxidative stress in vivo, correlating well with other classical markers of oxidative stress indices (reviewed in Refs. 4 and 7). Increased plasma and urinary isoprostane levels have been measured after intoxication and in several syndromes suspected of being associated with excessive generation of free radicals, including poisoning with paraquat [170] and CCl_4 [2], smoking [171], hepatic cirrhosis [172], coronary and liver reperfusion after ischemia [173,174], and atherosclerosis [175]. Elevated levels were also found in patients with hepatorenal syndrome [176], sleroderma [177], hypercholesteremia [178], Alzheimer's disease [179], diabetes [180], and others. In addition, isoprostane analysis greatly exceeds the value of other standard approaches to assess the effectiveness of antioxidants in vitro and in vivo and has been used to establish reliable dose-response relationships of antioxidant treatments in clinical trials (reviewed in Refs. 4, 7, and 13).

The second area of interest is concerned about the biological activity and the putative function of isoprostanes as mediators of oxidative damage. Thus far, however, only a few isoprostanes are available by total synthesis and have been investigated. For instance, isoprostane $F_{2\alpha}$ (8-*iso*-$PGF_{2\alpha}$) and isoprostane E_2 (8-*iso*-PGE_2) are extremely potent vasoconstrictors in the kidney [146,181], leading to a drop in glomerular capillary pressure and filtration rate in the low nanomolar range. These findings are surprising because $PGF_{2\alpha}$ and PGE_2, the cognate ligands of the PGF and PGE receptors, respectively, have opposite effects on the vasculature (i.e., PGF_2 is a vasoconstrictor and PGE_2 a vasodilator). 8-*Iso*-$PGF_{2\alpha}$ is also a potent vasoconstrictor in the liver, heart, lung, and eye retina (reviewed in Ref. 13) and a potent constrictor of peripheral lymphatics [182] and the airway [183].

Some isoprostanes appear to react incidentally with PG receptors (often in the micromolar range), but there is evidence that some isoprostanes might interact at low concentrations (nanomolar range) with specific isoprostane receptor(s) [184–186]. The postulated isoprostane receptor is assumed to display some similarity to thromboxane receptors, because isoprostane bioactivity can often be blocked by thromboxane receptor antagonists. However, an isoprostane receptor has yet to be cloned.

The finding that phytoprostanes are formed in plants at much higher levels than isoprostanes in mammals raises exciting questions about their possible function. Are polyunsaturated fatty acids evolutionary ancient sensors of oxidative stress that are converted to a mixture of isoprostanoids which mediate the stress signal and translate it into a cellular response? Although there is evidence that mammalian isoprostanes might have such a function, nothing is yet known about the biological activity and function of the recently discovered phytoprostanes in plants. On one hand, it will be interesting to see if phytopros-

tanes may induce antioxidative defense mechanisms or exert jasmonate-like effects in plants, as they are structurally related to 12-oxo-PDA. On the other hand, phytoprostanes may be biologically active in humans. Because they occur in high concentrations in plant pollen, phytoprostanes may readily come into contact with human bronchi and eyes. As phytoprostanes are close structural congeners of mammalian isoprostanes, they may exert biological activity similar to their C-20 homologs and provoke bronchoconstriction and tissue irritation.

Another area of interest would be their potential use as markers of oxidative stress in plants in vivo. Similar to isoprostanes, phytoprostanes represent stable end products of lipid peroxidation formed from a specific precursor, namely linolenic acid, which occurs ubiquitously in all plants. As common markers of lipid peroxidation, such as malondialdehyde, lipid hydroperoxides, alkanes, aldehydes and others, have often been found unreliable [187,188], phytoprostane levels may represent an ideal index of oxidative deterioration of plant products such as fatty oils and plant drugs.

ACKNOWLEDGMENT

This study was supported by the SFB 369 of the Deutsche Forschungsgemeinschaft, Bonn.

NOTE ADDED IN PROOF

During the preparation of this book considerable advances have been made in the understanding of 12-oxophytodienoate reductases and the regulation of 12-oxo-phytodienoate levels in vivo. A third isozyme of OPR (OPR3) has been cloned from *Arabidopsis* that is involved in jasmonate biosynthesis. OPR3 mRNA levels are upregulated by a variety of stimuli such as touch, wind, wounding, UV-light, and also by brassinosteroids. However, it is still unknown whether or not protein and enzyme activity levels actually change in response to that stimuli [189].

Recently, 12-oxophytodienoate has been found to be present in the monogalactosyl diglycerol fraction of chloroplast lipids from which 12-oxophytodienoate may be released by a lipase in vivo [190].

REFERENCES

1. JD Morrow, JA Awad, HJ Boss, IA Blair, LJ Roberts. Proc Natl Acad Sci USA 89:10,721–10,725, 1992.
2. JD Morrow, JA Awad, T Kato, K Takahashi, KF Badr, LJ Roberts, RF Burk. J Clin Invest 90:2502–2507, 1992.
3. J Rokach, SP Khanapure, S-W Hwang, M Adiyaman, JA Lawson, GA FitzGerald. Prostaglandins 54:853–873, 1997.
4. LJ Roberts, JD Morrow. Biochim Biophys Acta 1345:121–135, 1997.
5. S Parchmann, MJ Mueller. J Biol Chem 273:32,650–32,655, 1998.
6. WL Smith, LM Marnett. Biochim Biophys Acta 1083:1–17, 1991.
7. JD Morrow, J Chen, CJ Brame, J Yang, SC Sanchez, J Xu, WE Zackert, JA Awad, LJ Roberts. Drug Metab Rev 31:117–139, 1999.
8. M Lamacka, J Sajbidor. Prostaglandins Leukot Essential Fatty Acids 52:357–364, 1995.
9. M Lamacka, J Sajbidor. Lett Appl Microbiol 26:224–226, 1998.
10. JL Harwood. Biochim Biophys Acta 1301:7–56, 1996.

11. MJ Mueller. Physiol Plant 100:653–663, 1997.
12. H Weber, BA Vick, EE Farmer. Proc Natl Acad Sci USA 94:10,473–10,478, 1997.
13. MJ Mueller. Chem Biol 5:323–333, 1998.
14. J Ueda, K Miyamoto, M Aoki, T Hirata, T Sato, Y Momotani. Bull Univ Osaka Prefect Ser B 23:103–108, 1991.
15. J Ueda, K Miyamoto, T Sato, Y Momotani. Agric Biol Cem 55:275–276, 1991.
16. MV Krupina, W Dathe. Z Naturforsch 44c:1127–1129, 1991.
17. G Sembdner, D Gross. In: M Bopp, ed. Plant Growth Substances. Heidelberg: Springer-Verlag, 1986, pp 139–147.
18. M Hamberg, HW Gardner. Biochim Biophys Acta 1165:1–18, 1992.
19. O Miersch, A Preiss, G Sembdner, K Schreiber. Phytochemistry 26:1037–1039, 1987.
20. O Miersch, B Brückner, J Schmidt, G Sembdner. Phytochemistry 31:3835–3837, 1992.
21. O Miersch, H Bohlmann, C Wasternack. Phytochemistry 50:517–523, 1999.
22. JM Scheer, CA Ryan. Plant Cell 11:1525–1535, 1999.
23. H Gundlach, MJ Müller, TM Kutchan, MH Zenk. Proc Natl Acad Sci USA 89:2389–2393, 1992.
24. MJ Mueller, W Brodschelm, E Spannagl, MH Zenk. Proc Natl Acad Sci USA 90:7490–7494, 1993.
25. T Boller. Annu Rev Plant Physiol Plant Mol Biol 46:189–214, 1995.
26. DR Bergey, M Orozco-Cardenas, DS de Moura, CA Ryan. Proc Natl Acad Sci USA 96: 1756–1760, 1999.
27. P Reymond, S Grunberger, K Paul, M Muller, EE Farmer. Proc Natl Acad Sci USA 92: 4145–4149, 1995.
28. SH Doares, T Syrovets, EW Weiler, CA Ryan. Proc Natl Acad Sci USA 92:4095–4098, 1995.
29. EE Farmer, CA Ryan. Trends Cell Biol 2:236–241, 1992.
30. A Conconi, MJ Smerdon, GA Howe, CA Ryan. Nature 383:826–829, 1996.
31. EW Weiler, T Albrecht, B Groth, Z-Q Xia, M Luxem, H Liß, L Andert, P Spengler. Phytochemistry 32:591–600, 1993.
32. R Rakwal, S Tamogami, O Kodama. Biosci Biotechnol Biochem 60:1046–1048, 1996.
33. RA Creelman, JE Mullet. Proc Natl Acad Sci USA 92:4114–4119, 1995.
34. C Wasternack, B Parthier. Trends Plant Sci 2:302–307, 1997.
35. E Blumwald, GS Aharon, BC-H Lam. Trends Plant Sci 3:342–346, 1998.
36. CA Ryan, G Pearce. Annu Rev Cell Dev Biol 14:1–17, 1998.
37. S Zimmermann, T Nuernberger, JM Frachisse, W Wirtz, J Guern, R Hedrich, D Scheel. Proc Natl Acad Sci USA 94:2751–2755, 1997.
38. A Gelli, VJ Higgins, E Blumwald. Plant Physiol 113:269–279, 1997.
39. MR Stäb. Arch Biochem Biophys 257:416–423, 1987.
40. T Jabs, M Tschöpe, C Colling, K Hahlbrock, D Scheel. Proc Natl Acad Sci USA 94:4800–4805, 1997.
41. A Schaller, C Oecking. Plant Cell 11:263–272, 1999.
42. S Usami, H Banno, Y Ito, R Nishihama, Y Machida. Proc Natl Acad Sci USA 92:8660–8664, 1991.
43. S Seo, M Okamoto, H Seto, K Ishisuka, H Sano, Y Ohashi. Science 270:1988–1992, 1995.
44. AL Adam, S Pike, ME Joyos, JM Stone, JC Walker, A Novacacky. Plant Physiol 115:853–861, 1997.
45. JW Stratmann, CA Ryan. Proc Natl Acad Sci USA 94:11,085–11,089, 1997.
46. W Ligterink, T Kroj, U zur Nieden, H Hirt, D Scheel. Science 276:2054–2057, 1997.
47. B Lino, VM Baizabal-Aguirre, LE Gonzales de la Vara. Planta 204:352–359, 1998.
48. T Nürnberger, D Nennstiel, T Jabs, WR Sacks, K Hahlbrock, D Scheel. Cell 78:449–460, 1994.
49. Y Mathieu, D Lapous, S Thomine, C Lauriere, J Guern. Planta 199:416–424, 1996.

50. R Vera-Estrella, BJ Barkla, VJ Higgins, E Blumwald. Plant Physiol 104:209–215, 1994.
51. W Roos, S Evers, M Hieke, M Tschöpe, B Schuhmann. Plant Physiol 118:349–364, 1998.
52. DC Zimmerman. Biochem Biophys Res Comm 23:398–402, 1966.
53. DC Zimmerman, BA Vick. Plant Physiol 46:445–453, 1970.
54. DC Zimmerman, P Feng. Lipids 13:313–316, 1978.
55. BA Vick, DC Zimmerman. Biochem Biophys Res Commun 111:470–477, 1983.
56. BA Vick, DC Zimmerman. Plant Physiol 75:458–461, 1984.
57. M Hamberg. Biochim Biophys Acta 920:76–84, 1987.
58. AR Brash, SW Baertschi, CD Ingram, TM Harris. Proc Natl Acad Sci USA 85:3382–3386, 1988.
59. EE Farmer, CA Ryan. Plant Cell 4:129–134, 1992.
60. A Conconi, M Miquel, JA Browse, CA Ryan. Plant Physiol 111:797–803, 1996.
61. EW Weiler, D Laudert, F Schaller, B Stelmach, P Hennig. In: E Romeo, ed. Phytochemical Signals and Plant–Microbe Interactions. New York: Plenum Press, 1998,pp 179–205.
62. C Kirsch, M Takamiya-Wik, S Reinhold, K Hahlbrock, IE Somssich. Proc Natl Acad Sci USA 94:2079–2084, 1997.
63. JN Siedow. Annu Rev Plant Physiol Plant Mol Biol 42:145–188, 1991.
64. I Feussner, C Wasternack. Fett/Lipid 100:146–152, 1998.
65. D Shibata, A Slusarenko, R Casey, D Hildebrand, E Bell. Plant Mol Biol Rep 12:S41–S42, 1994.
66. E Bell, RA Creelman, JE Mullet. Proc Natl Acad Sci USA 92:8675–8679, 1995.
67. T Heitz, DR Bergey, CA Ryan. Plant Physiol 114:1985–1093, 1997.
68. D Laudert, EW Weiler. Plant J 15:675–684, 1998.
69. B McGurl, M Orozco-Cardenas, G Pearce, CA Ryan. Proc Natl Acad Sci USA 91:9799–9802, 1994.
70. MJ Mueller, W Brodschelm. Anal Biochem 218:425–435, 1994.
71. BA Stelmach, A Müller, EW Weiler. Phytochemistry 51:187–192, 1999.
72. S Parchmann, H Gundlach, MJ Mueller. Plant Physiol 115:1057–1064, 1997.
73. J Royo, G Vancanney, AG Perez, C Sanz, K Störmann, S Rosahl, JJ Sanchez-Serrano. J Biol Chem 271:21,012–21,019, 1996.
74. K Voros, I Feussner, H Kuhn, J Lee, A Graner, M Lobler, B Parthier, C Wasternack. Eur J Biochem 251:36–44, 1998.
75. TK Park, MA Holland, JG Laskey, JC Polacco. Plant Sci 96:109–117, 1994.
76. A Nellen, B Rojahn, H Kindl. Z Naturforsch 50c:29–36, 1995.
77. M Kohlmann, A Bachmann, H Weichert, A Kolbe, T Balkemhohl, C Wasternack, I Feussner. Eur J Biochem 260:885–895, 1999.
78. D Laudert, U Pfannschmidt, F Lottspeich, H Holländer-Czytko, EW Weiler. Plant Mol Biol 31:323–335, 1996.
79. NJ Bate, S Sivasankar, C Moxon, JMC Riley, JE Thompson, SJ Rothstein. Plant Physiol 117:1393–1400, 1998.
80. Z Pan, F Durst, D Werck-Reichhart, HW Gardner, B Camara, K Cornish, RA Backhaus. J Biol Chem 270:8487–8494, 1995.
81. BA Vick, DC Zimmerman. Plant Physiol 63:490–494, 1979.
82. P Feng, BA Vick, DC Zimmerman. Lipids 16:377–379, 1981.
83. W-C Song, AR Brash. Science 253:781–784, 1991.
84. WC Song, CD Funk, AR Brash. Proc Natl Acad Sci USA 90:8519–8523, 1993.
85. S-MC Lau, PA Harder, DP O'Keefe. Biochemistry 32:1945–1950, 1993.
86. H Pena-Cortes, T Albrecht, S Prat, EW Weiler, L Willmitzer. Planta 191:123–128, 1993.
87. SH Doares, J Narvaez-Vasquez, A Conconi, CA Ryan. Plant Physiol 108:1741–1746, 1995.
88. Z Pan, B Camara, HW Gardner, RA Backhaus. J Biol Chem 273:18,139–18,145, 1998.
89. Z-P Zhang, IT Baldwin. Planta 203:436–441, 1997.

90. I Kubigsteltig, D Laudert, EW Weiler. Planta 208:463–471, 1999.
91. J Ziegler, M Hamberg, O Miersch, B Parthier. Plant Physiol 114:565–573, 1997.
91a. AN Grechkin, M Hamberg. Febs Lett 466:63–66, 2000.
92. D Laudert, P Hennig, BA Stelmach, A Müller, L Andert, EW Weiler. Anal Biochem 246: 211–217, 1997.
93. F Schaller, EW Weiler. Eur J Biochem 245:294–299, 1997.
94. F Schaller, EW Weiler. J Biol Chem 272:28,066–28,672, 1997.
95. F Schaller, P Hennig, EW Weiler. Plant Physiol 118:1345–1351, 1998.
96. C Biesgen, EW Weiler. Planta 208:155–165, 1999.
97. H Kindl. In: PK Stumpf, ed. The Biochemistry of Plants. New York: Academic Press, 1987, Vol 9, pp 31–52.
98. BA Stelmach, A Müller, P Hennig, D Laudert, L Andert, EW Weiler. Phytochemistry 47: 539–546, 1998.
99. G Sembdner, B Parthier. Annu Rev Plant Mol Biol 44:569–589, 1993.
100. EE Farmer, CA Ryan. Proc Natl Acad Sci USA 87:7713–7716, 1990.
101. IA Penninckx, K Eggermont, FR Terras, BP Thomma, GW De Samblanx, A Buchala, JP Metraux, JM Manners, WF Broekaert. Plant Cell 8:2309–2323, 1996.
102. P Epple, K Apel, H Bohlmann. Plant Physiol 109:813–820, 1995.
103. H Gundlach, MH Zenk. Phytochemistry 47:527–537, 1998.
104. C Wasternack, O Miersch, R Kramell, B Hause, J Ward, M Beale, W Boland, B Parthier, I Feussner. Fett/Lipid 100:139–146, 1998.
105. CE Benedetti, DX Xie, JG Turner. Plant Physiol 109:567–572, 1995.
106. S Berger, E Bell, JE Mullet. Plant Physiol 111:525–531, 1996.
107. PE Staswick, W Su, SH Howell. Proc Natl Acad Sci USA 89:6837–6840, 1992.
108. EW Weiler, TM Kutchan, T Gorba, W Brodschelm, U Niesel, F Bublitz. FEBS Lett 345: 9–13, 1994.
109. D-X Xie, BF Feys, S James, M Nieto-Rostro, JG Turner. Science 280:1091–1094, 1998.
110. JG Turner. Trends Plant Sci 3:368–369, 1998.
111. RA Creelman. Trends Plant Sci 3:367–368, 1998.
112. M McConn, J Browse. Plant Cell 8:403–416, 1996.
113. HS Mason, JE Mullet. Plant Cell 2:569–579, 1990.
114. RA Creelman, JE Mullet. Annu Rev Plant Physiol Plant Mol Biol 48:355–381, 1997.
115. SD Jackson. Plant Physiol 119:1–8, 1999.
116. M McConn, RA Creelman, E Bell, JE Mullet, J Browse. Proc Natl Acad Sci USA 94:5473–5477, 1997.
117. P Vijayan, J Shockey, CA Levesque, RJ Cook, J Browse. Proc Natl Acad Sci USA 95:7209–7214, 1998.
118. GA Howe, J Lightner, J Browse, CA Ryan. Plant Cell 8:2067–2077, 1996.
119. IAMA Penninckx, BPHJ Thomma, A Buchala, J-P Metraux, WF Broekaert. Plant Cell 10: 2103–2113, 1998.
120. PJ O'Donnell, C Calvert, R Atzorn, C Wasternack, HMO Leyser, DJ Bowles. Science 274: 1914–1917, 1996.
121. DR Bergey, GA Howe, CA Ryan. Proc Natl Acad Sci USA 93:12,053–12,058, 1996.
122. SG Morham, R Langenbach, CD Loftin, HF Tiano, N Vouloumanos, JC Jennette, JF Mahler, KD Kluckman, A Ledford, CA Lee, O Smithies. Cell 83:473–482, 1995.
123. Y Sugimoto, A Yamasaki, E Segi, K Tsuboi, Y Aze, T Nishimura, H Oida, N Yoshida, T Tanaka, M Katsuyama, K-Y Hasumoto, T Murata, M Hirata, F Ushikubi, M Negeshi, A Ichikawa, S Narumiya. Science 277:681–683, 1997.
124. M Nguyen, T Camenisch, JN Snouwaert, E Hicks, TM Coffman, PAW Anderson, NN Malouf, BH Koller. Nature 390:78–81, 1997.
125. WL Smith, FA Fitzpatrick. In: DE Vance, JE Vance, eds. Biochemistry of Lipids, Lipoproteins and Membranes. New York: Elsevier Science, 1996, pp 283–307.

126. KR Bley, JC Hunter, RM Eglen, JAM Smith. Trends Pharmacol Sci 19:141–147, 1998.
127. F Ushikubi, E Segi, Y Sugimoto, T Murata, T Matsuoka, T Kobayashi, H Hizaki, K Tuboi, M Katsuyama, A Ichikawa, T Tanaka, N Yoshida, S Narumiya. Nature 395:281–284, 1998.
128. S Tamogami, O Kodoma. J Chromatgr A 822:310–315, 1998.
129. S Blechert, C Bockelmann, O Brümmer, M Füßlein, H Gundlach, G Haider, S Hölder, TM Kutchan, EW Weiler, MH Zenk. J Chem Soc, Perkin Trans 1, 3549–3559, 1997.
130. S Blechert, W Brodschelm, S Hölder, L Kammerer, TM Kutchan, Z-Q Xia, MH Zenk. Proc Natl Acad Sci USA 92:4099–4105, 1995.
131. T Koch, T Krumm, V Jung, J Engelberth, W Boland. Plant Physiol 121:153–162, 1999.
132. C Wasternack, B Ortel, O Miersch, R Kramell, M Beale, F Greulich, I Feussner, B Hause, T Krumm, W Boland, B Parthier. J Plant Physiol 152:345–352, 1998.
133. S Blechert, C Bockelmann, M Füßlein, TV Schrader, B Stelmach, U Niesel, EW Weiler. Planta 207:470–479, 1999.
134. Y Koda. Int Rev Cytol 135:155–198, 1992.
135. T Yoshikara, E-SA Omer, H Koshino, S Sakamura, Y Kikuta, Y Koda. Agric Biol Chem 53:2835–2873, 1989.
136. Y Koda. Physiol Plant 100:639–646, 1997.
137. R Kramell, O Miersch, B Hause, B Ortel, B Parthier, C Wasternack. FEBS Lett 414:197–202, 1997.
138. S Tamogami, R Rakwal, O Kodama. FEBS Lett 401:239–242, 1997.
139. T Krumm, K Bandemer, W Boland. FEBS Lett 377:523–529, 1995.
140. SC Hertel, H-D Knöfel, R Kramell, O Miersch. FEBS Lett 407:105–110, 1997.
141. DH Nugteren, H Vonkeman, DA Van Dorp. Recl Trav Chim Pays-Bas 86:1237–1245, 1967.
142. WA Pryor, JP Stanley, E Blair. Lipids 11:370–379, 1976.
143. DE O'Connor, ED Mihelich, MC Coleman. J Am Chem Soc 103:223–224, 1981.
144. DE O'Connor, ED Mihelich, MC Coleman. J Am Chem Soc 106:3577–3584, 1984.
145. JD Morrow, TM Harris, LJ Roberts. Anal Biochem 184:1–10, 1990.
146. K Takabashi, TM Nammour, M Fukunaga, J Ebert, JD Morrow, LJ Roberts, RL Hoover, KF Badr. J Clin Invest 90:136–141, 1992.
147. EJ Corey, Z Wang. Tetrahedron Lett 35:539–542, 1994.
148. HW Gardner. Free Radical Biol Med 7:65–86, 1989.
149. EN Frankel. Lipid Oxidation. Dundee: The Oily Press, 1998.
150. HW-S Chan, G Levett, JA Matthew. J Chem Soc Chem Commun 1978:756–757, 1978.
151. NA Porter, BA Weber, BA Weenen, JA Khan. J Am Chem Soc 102:5597–5601, 1980.
152. NA Porter, LS Lehman, BA Weber, KJ Smith. J Am Chem Soc 103:6447–6445, 1981.
153. NA Porter, DG Wujek. J Am Chem Soc 106:2626–2629, 1984.
154. DF Taber, JD Morrow, JL Roberts II. Prostaglandins 53:63–67, 1997.
155. WL Smith. Am J Physiol 263:F181–F191, 1992.
156. EN Frankel, WE Neff. Biochim Biophys Acta 754:264–270, 1983.
157. H Esterbauer, RJ Schaur, H Zollner. Free Radical Biol Med 11:81–128, 1991.
158. M Hecker, V Ulrich. J Biol Chem 264:141–150, 1989.
159. JD Morrow, LJ Roberts, VC Daniel, JA Awad, O Mirochnitchenko, LL Swift, RF Burk. Arch Biochem Biophys 353:160–171, 1998.
160. JD Morrow, JA Awad, A Wu, WE Zackert, VC Daniel, JL Roberts. J Biol Chem 271:23185–23190, 1996.
161. RG Stehle. Methods Enzymol 86:436–458, 1982.
162. FA Fitzpatrick, MA Wynalda. J Biol Chem 258:11,713–11,718, 1983.
163. LJ Roberts, TJ Montine, WR Markesbery, AR Tapper, P Hardy, S Chemtob, WD Dettbarn, JD Morrow. J Biol Chem 273:13,605–13,612, 1998.
164. FH Chilton, AN Fonteh, ME Surette, M Triggiani, JD Winkler. Biochim Biophys Acta 1299: 1–15, 1996.
165. VL Schuster. Annu Rev Physiol 60:221–242, 1998.

166. LJ Roberts, KP Moore, WE Zackert, JA Oates, JD Morrow. J Biol Chem 271:20,617–20,620, 1996.
167. C Chiabrando, A Valagussa, C Rivalta, T Durand, A Guy, E Zuccato, P Villa, J-C Rossi, R Fanelli. J Biol Chem 274:1313–1319, 1999.
168. JD Morrow, TA Minton, CR Mukundan, MD Campbell, WE Zackert, VC Daniel, KF Badr, IA Blair, LJ Roberts. J Biol Chem 269:4317–4326, 1994.
169. Y Chen, JD Morrow, LJ Roberts II. J Biol Chem 274:10,863–10,868, 1999.
169a. R Imbusch, MJ Mueller. Free Rad Biol Med 28:720–726, 2000.
170. RF Burk, KE Hill, JA Awad, JD Morrow, T Kato, KA Cockell, PR Lyons. Hepatology 21: 561–569, 1995.
171. JD Morrow, B Frei, AW Longmire, JM Gaziano, SN Lynch, Y Shyr, WE Strauss, JA Oates, LJ Roberts. N Engl J Med 332:1198–1203, 1995.
172. D Pratico, L Iuliano, S Basili, D Ferro, C Camastra, C Cordova, GA FitzGerald, F Violi. J Invest Med 46:51–57, 1998.
173. N Delanty, MP Reilly, D Pratico, JA Lawson, JF McCarthy, AE Wood, ST Ohnishi, DJ Fitzgerald, GA FitzGerald. Circulation 95:2492–2499, 1997.
174. WR Mathews, DM Guido, MA Fisher, H Jaeschke. Free Radical Biol Med 16:763–770, 1994.
175. C Gniwotta, JD Morrow, LJ Roberts, H Kühn. Arteroscler Thromb Vasc Biol 17:3236–3241, 1997.
176. JD Morrow, KP Moore, JA Awad, MD Ravenscraft, G Marini, KF Badr, R Williams, LJ Roberts. J Lipid Mediat 6:417–420, 1993.
177. CM Stein, SB Tanner, JA Awad, LJ Roberts, JD Morrow. Arthritis Rheum 39:1146–1150, 1996.
178. G Davi, P Alessandrini, A Mezzetti, G Minotti, T Bucciarelli, F Costantini, F Cipollone, GB Bon, G Ciabattoni, C Patrono. Aterioscler Thromb Vasc Biol 17:3230–3235, 1997.
179. D Pratico, V MY Lee, JQ Trojanowski, J Rokach, GA Fitzgerald. FASEB J 12:1777–1783, 1998.
180. G Davi, G Ciabattoni, A Consoli, A Mezzetti, A Falco, S Santarone, E Pennese, E Vitacolonna, T Bucciarelli, F Costantini, F Capani, C Patrono. Circulation 99:224–229, 1999.
181. M Fukunaga, K Takahashi, KF Badr. Biochem Biophys Res Commun 195:507–515, 1993.
182. H Sinzinger, A Oguogho, J Kaliman. Lymphology 30:155–159, 1997.
183. A Okazawa, I Kawikova, ZH Cui, BE Skoogh, J Lotvall. Am J Respir Crit Care Med 155: 436–441, 1997.
184. M Fukunaga, N Makita, LJ Roberts, JD Morrow, K Takahashi, KF Badr. Am J Physiol 264: C1619–C1624, 1993.
185. M Fukunaga, T Yura, KF Badr. J Cardiovasc Pharmacol 26:S51–S52, 1995.
186. M Fukunaga, T Yura, R Grygorczyk, KF Badr. Am J Physiol Renal Physiol 41:F477–F483, 1997.
187. JMC Gutteridge, B Halliwell. Trends Biol Sci 15:129–135, 1990.
188. LL De Zwart, JHN Meerman, JNM Commandeur, NPE Vermeulen. Free Radical Biol Med 26:202–226, 1999.
189. F Schaller, C Biesgen, C Müssig, T Altmann, EW Weiler. Planta 210:979–984, 2000.
190. BA Stelmach, A Müller, P Hennig, S Gebhard, M Schubert-Zsilavecz, EW Weiler. J Biol Chem 276:12832–12838, 2001.

12

Cutin Monomers
Biosynthesis and Plant Defense

ELIZABETH BLÉE

Institut de Biologie Moléculaire des Plantes, Centre National de la Recherche Scientifique, Strasbourg, France

1 INTRODUCTION

The plant cuticle, which covers virtually all the aerial surfaces of vascular plants, is chemically heterogeneous in nature, because it is generally composed of a mixture of insoluble polymers and waxes, which are deposited on the outer surface (epicuticular waxes) and embedded in the matrix (intracuticular waxes). The soluble cuticular waxes consist of monoesters of very long chains of fatty acids (C-18–C-22), alcohols (C-22–C-26), and a wide variety of other aliphatic and aromatic molecules [1–4]. The insoluble matrix is constituted by (1) cutin, a three-dimensional polymer network of esterified fatty acids [1,5] and (2) cutan, a nondegradable biopolymer made of aliphatic chains presumably cross-linked by ether bonds [6]. In addition, the cuticular layers also contain nonlipid components such as polysaccharides, phenols, and flavonoids. Large variations have been found in cuticular fine structure, wax load, or cutin composition. Indeed, the structure of the cuticle varies considerably depending on the species and on the age of the plant, but also between leaves, fruits, and stems of a single species and even between the upper and lower surfaces of individual leaves [7]. This complexity may explain the wide diversity of functions fulfilled by the cuticular layer. As an interface between the plant and its environment, the cuticle plays a key role in providing protection from mechanical damage, ultraviolet (UV) radiation [8,9], or penetration by fungal hyphae and insect mouthparts [10]. It also constitutes the main barrier limiting the transport across the plant–atmosphere exchange zone, impeding the foliar uptake of xenobiotics but also reducing the uncon-

trolled loss of water and apoplastic solutes from plant tissues [11,12]. It seems that waxes are largely responsible for the low permeability of cuticles, causing a lognormal distribution of transport parameters [13] and therefore contributing for example to the regulation of the cuticular transpiration. Permeability of cuticles differs greatly among plant species and it has been argued that these differences are more likely due to the physical arrangement of waxes, rather than their amounts or composition per se [14]. On the other hand, if the constitutive waxes markedly inhibit sorption of a wide range of organic compounds, they may not always form the main barrier to the penetration of chemicals through plant cuticles [15]. They do favor the penetration of biologically active compounds by acting as a compartment in which lipophilic compounds can accumulate [16]. Moreover, these lipids reduce water retention on the plant surface, which bears important consequences on germination of spores, survival of microorganisms, or deposition of dust, pollen, and so forth. Additionally, the chemical composition of epicuticular waxes may influence the interaction of herbivorous insects, with their plant hosts [17,18] acting as well in tritrophic interactions, by affecting the settling and oviposition behavior of some predators and parasitoids of these herbivores [18]. It was also reported that waxes found in the tryphine layer of pollen grains are essential for proper pollen-stigma signaling required for fertilization [19].

It is generally admitted that the biosynthesis of epicuticular waxes proceeds via multienzymatic complexes; both the proposed metabolic pathways by which wax constituents are synthesized and the genetic studies of mutants were reviewed in the past few years [20–22]. In sharp contrast with the mass of information which became recently available for the cuticular waxes, very little work has been devoted to the cutin matrix since the pioneering work of the groups of Kollatukudy and Holloway. The view of the cutin matrix was classically restricted to an inert scaffolding of the cuticle, but it appears that its components may act more actively as participants for signaling across the divider during the pathogen attack. The aim of this chapter is to summarize the most recent data concerning this quite new role for cutin monomers in plant defense, in addition to the latest insights in their biosynthetic pathways.

2 BIOSYNTHESIS OF CUTIN MONOMERS

2.1 Composition of the Cutin Biopolymer

If the investigations of waxes have been facilitated by their ease of extraction with organic solvents, cutin composition analysis was initially hampered by the difficulty encountered in the depolymerization of this biopolymer, which required quite strong chemical procedures. The first clue to the chemical nature of cutin components emerged from the investigations of Matic [23], who isolated hydroxylated fatty acids from the ether-soluble fraction obtained by extraction of hydrolysis products of the leaf cuticle of *Agave americana*. The hydroxy fatty acids that were characterized included 9,10,18-trihydroxystearate, 10,18-dihydroxystearic acid, 18-hydroxystearic acid, and 10,16-dihydroxypalmitic acids. The trihydroxystearic acid was found at that time to be predominant in most of the mixtures of cutin hydrolysates. Later, the use of milder degradative chemical methods (i.e., alcoholysis) confirmed 9,10-epoxy-18-hydroxyoctadecanoic acid as a common cutin constituent [24]. Since then, partial depolymerization of the cutin polyester has been accomplished using various chemical and enzymatic methods, including hydrogenolysis with $LiAlH_4$, hydrolysis with alcoholic KOH or HCl, and breakdown with cutinase [25]. Cutin

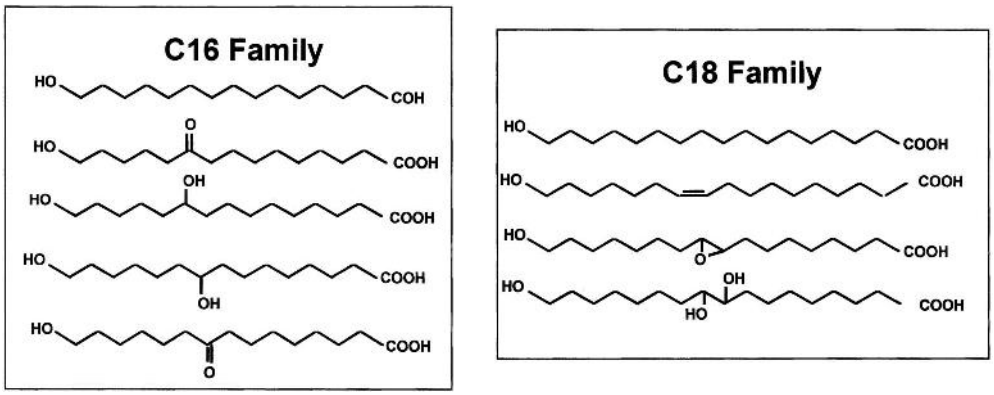


Figure 1 Structure of the major cutin monomers.

analysis of a large number of plants have established that plant cutins are built from a sole series of substituted C-16 and C-18 alkanoic acids (Fig 1). Species differ from one another primarily in the relative proportions of these two groups. For example, the cutin of bean leaves contains mostly C-16, in contrast to Poacae leaf cutin, which contain predominantly C-18 components. Commonly, cutin includes monomers of the two groups.

Although, from early investigations [26], cutin was described as an esterlike material, the details of how the monomers are linked together are still elusive. Clearly, the structural framework of cutin will depend on the chain lengths and on the number and positions of esterifiable groups in the monomers. Elongation of this biopolymer likely involves primary hydroxyl groups, whereas its reticulation comprises the secondary ones.

Such a network of hydroxy fatty acids linked by primary and secondary alcohols derived ester bonds has recently been confirmed in the case of lime cuticle (composed essentially of monomers of the C-16 family), observed by electron-impact mass spectrometry supported by solution-state nuclear magnetic resonance (NMR) spectroscopy [27]. These newly accessible analytical tools should permit a better understanding of the intermolecular structures of cutin biopolymers.

It was reported that the entire cutin biopolymer could not be depolymerized by treatment with BF_3-methanol, which cleaves ester linkages [28]. This was attributed to the presence of large amounts of epoxy fatty acids in the cuticles, which could form ether bonds. The formation and the complexity of the cross-linkages in cutin was found to increase with the age of the leaves [29], possibly reducing their water-permeability capacity.

2.2 Biosynthesis of the C-16 Cutin Monomer Family

Research on the biosynthesis of cutin components has been neglected during the past two decades. Until a few years ago, nearly all of our knowledge about cutin biosynthesis was based on the initial work of the group of Kolattukudy in the 1970s. Using labeled acetate, palmitate, and 16-hydroxypalmitate, these researchers proposed a major biosynthetic pathway for the C-16 cutin monomers, starting from palmitic acid and involving first the hydroxylation of this unsaturated fatty acid at the ω-position, followed by its in-chain (on carbon-9 or carbon-10) oxidation [30]. However, the recently identified 10-hydroxyhexa-

decanoic acid in lime fruit cutin indicates that ω-hydroxylation of palmitic acid is not a requisite, as hydroxylation of palmitic acid may also take place at other chain positions during formation of the polyester matrix [31].

Oxidation of the C-16 extremity, studied in *Vicia faba* microsomal fractions, exhibited features of cytochrome P-450-dependent catalysis (i.e., dependence on molecular oxygen, requirement of NADPH as cosubstrate, inhibition by CO) although the reversion of this inhibition by light was not obtained [32]. It should be noted that such a reversion by photons of the binding of CO to the ferryl-oxo complex can be difficult to achieve (Werck-Reichhart, personal communication). At this time, purification of the enzyme responsible for ω-hydroxylation of palmitic acid was not reported. Recently, *Arabidopsis thaliana* express sequence tag (EST) databases were screened with consensus motifs derived from families of P-450 catalyzing the ω-hydroxylation of fatty acids and alkanes in *Candida* and in mammals. Hereby, a gene, CYP86A1, encoding a similar enzyme was obtained [33]. Except for stearic acid, which was not metabolized, lauric, (C-12) myristic (C-14), and palmitic (C-16) acids were efficiently oxidized at their terminal position, the latter being the best substrate. However, no physiological role has yet been attributed to this new plant P-450 family. In parallel, another plant cytochrome P-450-dependent fatty acid ω-hydrolylase (CYP94A1) was cloned and expressed in *Saccharomyces cerevisae* overproducing a reductase from *Arabidopsis thaliana* [34]. This cytochrome P-450-dependent enzyme was tagged in *Vicia faba* microsomes, with a radiolabeled suicide inhibitor allowing its isolation and cloning. As expected, lauric acid was shown to be the best substrate for the expressed CYP94A1, but this enzyme is also able to oxidize other saturated fatty acids ranging from C-10 to C-16. The K_m value for palmitate was lower than that measured for the other substrates, and its was suggested that CYP94A1 could be involved in the biosynthesis of cutin monomer [34]. Unfortunately, no experimental data were provided in this work, confirming the claimed accumulation of CPY94A1 transcripts during plant development or wounding. This could have constituted a first indication of the implication of such a cytochrome P-450 in cutin formation or cuticle repair.

In addition to 16-hydroxypalmitic acid, 9,16- and 10,16-hydroxypalmitic acids are also present in plant cutins. It has been suggested that in-chain hydroxylation reactions result from cytochrome P-450 catalysis. In this regard, the first indication of a P-450-dependent in-chain hydroxylase was reported recently in *Helianthus tuberosus* tuber [35]. Such P-450, designed CPY81B1, catalyzed the hydroxylation of medium-chain saturated fatty acids, the major site of attack being at carbon-9 (capric and lauric acids) or carbon-10 (myristic acid), depending on the length of the aliphatic chains. In addition, a complex mixture of in-chain monohydroxylated derivatives was generated by this enzyme, denoting that saturated medium-chain fatty acids are probably not the physiological substrates. No longer-chain fatty acids such as palmitic or oleic acids were substrates for CYP81B1, ruling out the participation of this enzyme in cutin biosynthesis.

Thus, at present, data on the involvement of the already cloned cytochrome P-450-dependent hydroxylases in C-16 cutin monomer formation are not conclusive, but, hopefully, the systematic cloning of such plant enzymes will lead to better candidates in the future.

It should also be emphasized that in addition to cytochrome P-450, nonheme hydroxylases have been characterized in recent years. For example, ricinoleic acid is synthesized by direct hydroxy substitution at the 12-position of oleic acid by a diiron protein, related to a fatty acid desaturase family, which requires oxygen, NADH, and cytochrome b_5 for its activity [36]. Such a 12-hydroxylase can produce lesquerolic acid, a 14-hydroxylated

eicosanoic acid, but the possibility that this oxidase may catalyze in-chain hydroxylations at other positions with appropriate substrates is far from proven.

2.3 Biosynthesis of the C-18 Cutin Monomer Family

2.3.1 Epoxidation Step Catalyzed by Cytochrome P-450s?

In the early seventies, Kolattukudy and colleagues made the seminal finding that the biosynthesis of the C-18 family of cutin monomers proceeds through formation of epoxy derivatives [37]. [1-^{14}C] Oleic acid was rapidly incorporated into 9,10-epoxy-18-hydroxystearic acid, as well as 18-hydroxystearic and 9,10,18-trihydroxystearic acids by skin slices of young apples, grape and berry skins, and apple leaves. Likewise, 18-hydroxyoleic acid was transformed into its corresponding epoxy and dihydroxy derivatives leading to the conclusion that the sequence yielding the major C-18 cutin monomers was ω-hydroxylation, epoxidation of the double bond, and hydration of the epoxide. Trichloropropene oxide (TCPO), a noncompetitive inhibitor of microsomal epoxide hydrolase [38], was found to reduce incorporation of [1-^{14}C] oleic acid into 9,10,18-trihydroxystearic acid but also, surprisingly, into the epoxide [39]. A preparation from spinach leaves centrifuged at low speed (3000*g* and therefore probably enriched with nucleus, chloroplast fragments, unbroken cells, pieces of tissues, as well as cell walls and cutin biopolymer) was able to epoxidize the double bond of 18-hydroxyoleic acid. In contrast, oleic acid was a very poor substrate [40]. This epoxidase required molecular oxygen and NADPH for its activity, and it was inhibited by CO (this inhibition was completely reversed by light), Consequently, it was identified as a cytochrome P-450-dependent oxidase. Because CoA and ATP were needed for this catalysis, it was proposed that the CoA ester was the physiological substrate for this epoxidase [41]. Since this initial study, purification and further characterization of this protein have, however, not been accomplished.

This last decade, several oxidases have been demonstrated to be capable of oxidizing double bonds of unsaturated long-chain fatty acids. In particular, linoleate, linked to phosphatidylcholine in microsomes from developing seeds of *Euphorbia lagascae*, was epoxidized into vernolate in the presence of NADPH [42]. The formation of this derivative exhibited all of the characteristics of a cytochrome P-450-catalyzed reaction, including its inhibition by an anti-NADPH cytochrome P-450 reductase antibody. This cytochrome P-450-dependent epoxidation was characterized by a remarkable regioselectivity and enantioselectivity, [i.e., only the 12(*S*), 13(*R*)-enantiomer was formed in the endosperm of the seeds] [43]. Moreover, no compounds derived from 9,10-epoxy-12-octadecenoic acid were found to accumulate, no 9-epoxystearate was formed from [^{14}C] oleate, and only one of the three double bonds of linolenate was epoxidized, indicating that this epoxidase was specific for the C-12,13-position [42]. Because the biosynthesis of C-18 cutin monomers starts from 9-unsaturated octadecanoic acid, this cytochrome P-450 appears as an unlikely candidate for the formation of cutin epoxides.

2.3.2 Epoxidation Step Catalyzed by Diiron Proteins?

Another oxidase was recently shown to catalyze the epoxidation of the C-12,13 bond of linoleic acid. This epoxygenase, isolated from *Crepis palaestina* (related to the castor bean), required NADH or NADPH for its activity but was unaffected by carbon monoxide or antibodies to cytochrome P-450 reductase [44]. The gene encoding this epoxygenase was cloned and the deduced amino acid sequence was similar to that of other nonheme diiron proteins, such as the Δ12-hydroxylase (mentioned in Sec. 2.2), Δ12-desaturase,

and Δ12-acetylenase, all of them being integral membrane proteins. It has been shown that minor changes (as few as six amino acids) resulted in the conversion of an oleate hydroxylase to a desaturase [45], and it can be speculated that few changes will be needed for the recognition of an oleate instead of a linoleate substrate. Nevertheless, it seems that such enzymes are restricted to certain classes of higher plants such as *Euphorbeacae*, because neither vernolic acid nor ricinoleic acid is, for example, ever present in soybean or *Arabidopsis* seeds [46].

2.3.3 Epoxidation Step Catalyzed by Peroxygenases?

A third enzyme, a membrane-bound hemoprotein, has been reported likewise to catalyze the epoxidation of the C-12,13 double bond of linoleic acid. Characterized first as a hydroxylase [47] and then as a sulfoxidase [48], the peroxygenase was shown to actively catalyze, in the presence of alkylhydroperoxides as cosubstrates, the epoxidation of mono unsaturated and polyunsaturated fatty acids such as oleic acid or linoleic acid. The epoxidase activity was first discovered in soybean seedlings [49] and then in broad beans [50], and similar results were obtained later with another bean species [51]. However, this enzyme seems ubiquitous and is not only confined to the Leguminosae. Octadecenoic acids were found to be better substrates than shorter monounsaturated fatty acids (C-16 or C-14), but the position of the double bond (at position 6, 9, or 11) had little effect on the rates of oxidation. Only cis unsaturations were oxidized with retention of configuration resulting in *cis*-epoxide. Linolenic acid was oxidized with important regiofacial and enantiofacial selectivities [52] into the two positional monoepoxides and, as a minor product, the diepoxide. Analysis of the composition of various cutins had revealed that they contain epoxy and hydroxy derivatives at C-9 but also at C-12, yielding pentahydroxy acids. For example, 9,10,18-trihydroxy-12,13-epoxystearate and 9,10,12,13,18-pentahydroxystearate were found in *Rosamarinus officinalis*, probably arising by epoxidation and subsequent hydration of both the C-9,10 and C-12,13 epoxides of ω-hydroxylinoleic acid [53], although the order of the sequence of the reactions could differ. Because the peroxygenase did not present a strict regioselectivity, it should therefore be a favorable candidate for epoxidizing both oleic and linoleic acids into the precursors of cutin monomers. Using soybean microsomes, we have demonstrated that peroxygenase associated with a membrane-bound epoxide hydrolase and a cytochrome P-450 can catalyze, in vitro, the formation of the major cutin monomers of the C-18 family [54], but we did not establish real biological connections to cutin formation. Now we can provide (Blée et al., unpublished data) unequivocal proof for the involvement, in vivo, of plant peroxygenase in cutin biosynthesis. We have specifically altered peroxygenase activity via a mechanism-based inhibitor. No effect of this compound on other enzymes potentially involved in the formation of cuticle was noticed, such as membrane-bound and soluble lipoxygenases, epoxide hydrolases, cytochrome P-450, or fatty acid elongases. The specific inhibition, *in planta*, of the peroxygenase led to a dramatic decrease of content of cuticular epoxides from maize leaves, which could be vizualized by using a specific ultrahistochemical reaction [55]. This effect was restricted to plants, which are coated with a cutin rich in C-18 monomers. Accordingly, cuticles of treated plants which possess a majority of cuticular C-16 acids, such as pea, soybean, or vetch, remained unchanged. Moreover, we have established the existence of a strict relationship between the inhibition of the peroxygenase activity and the modification of the cuticle that is triggered by different molecules closely related to the inhibitor initially used. Altogether, these results constitute the first evidence of the physiological implication of possible biosynthetic enzymes in cutin formation but, most

importantly, confirm the plant peroxygenase as a key oxidase for the epoxidation of cutin monomers.

Consequently, because peroxygenase activity is dependent on alkyl-hydroperoxides, the biosynthesis of cutin monomers will be also under the control of the formation of such hydroperoxides. Fatty acid hydroperoxides are formed in the plant via chemical and/or enzymatic reactions; it is well known that oxidative stress due, for example, to injury can lead to chemical lipid peroxidation in membranes and that hydroperoxides are products of lipoxygenase-catalyzed oxidation of linoleic and linolenic acids. Heinen and Brand [56] had already reported stimulation of lipoxygenase activity during synthesis of cutin in wounded leaves. Moreover, an increasing number of publications deals with the induction of lipoxygenase activities during stress conditions, such as infection, injury, germination, and growth, some of which are known to lead to the biosynthesis of cutin. We have shown that a membrane-associated lipoxygenase was involved in the oxidative metabolism of oleic acid. However, we cannot exclude completely the participation of H_2O_2 in cutin formation. The level of this hydroperoxide is strongly enhanced by stress conditions, constituting with other activated oxygen radicals the so-called oxidative burst and may act as cosubstrate for peroxygenase during cutin repair. Nevertheless, it seems now that under conditions where alkyl-hydroperoxides are available, the biosynthetic pathways of cutin monomers involve peroxygenase activity as a potent epoxidase of unsaturated fatty acids. When hydroperoxides are limiting, one cannot a priori exclude a basal pathway involving also a cytochrome P-450-dependent epoxidase, as suggested previously by Croteau and Kolattukudy [39]. We and others [54,57] have, however, been unable to detect such an activity in bean-cell-free extracts. Related to this point, it is interesting to note that Croteau and Kolattukudy have observed incorporation of labeled fatty acid precursors into cutin only with rapidly growing tissues, such as young fruits or developing leaves i.e., precisely under conditions where high levels of lipoxygenase activity are classically found [57]. Curiously, it was reported that a spinach preparation capable of epoxidizing oleic acid via a cytochrome P-450-like activity showed no measurable ω-hydroxylase activity with this acid as the substrate [39]. This observation is in agreement with a subsequent report, which pointed out that in-chain and ω-hydroxylation P-450-dependent activities do not coexist in a single plant [58].

2.3.4 Epoxide Hydration Step

The presence of a vicinal diol in the C-18 cutin skeleton most likely results from hydration of the corresponding epoxide by an epoxide hydrolase. Among the first authors to suggest the presence of such an activity in plants were Croteau and Kolattukudy, who described the hydration of 9,10-epoxy-18-hydroxystearic acid to *threo*-9,10,18-trihydroxystearic acid by a particulate fraction prepared from the skin of young apples [59]. More recently, we have been able to purify and characterize a soluble form of an epoxide hydrolase from soybean that preferentially catalyzes the hydration of unsaturated fatty-acid-derived epoxides [60]. Furthermore, the isolation and expression of cDNAs encoding soluble epoxide hydrolases from soybean and two other plant species have been reported [61–63] and the enzymes tentatively ascribed as members of the HYL3 family [64]. It is clear now that multiple forms of epoxide hydrolase exist in plants, depending on the species and on the subcellular fractions examined. For example, besides the well-characterized cytosolic fatty acid epoxide hydrolase found in soybean seedlings, there also exists a membrane-bound isoform, which we have proposed to be involved in cutin synthesis [54]. This latter isoform hydrated preferentially the epoxide enantiomers formed prevalently by peroxygenase whereas 9,10-

epoxy-18-hydroxystearic acid seemed to be a poor substrate for this enzyme. Immunolocalization and *in situ* hybridization experiments revealed that the epoxide hydrolase is localized mainly in the epidermis of young leaves, substantiating their significant involvement in the formation of hydroxy cutin acids (Blée et al, unpublished data).

2.3.5 ω-Hydroxylation Step

Hydroxylation of oleic acid and of its epoxy and dihydroxy derivatives were catalyzed by a cytochrome P-450 [54,57]. Recently, it was reported that CYP94A1, initially proposed to be involved in C-16 cutin monomer formation (*vide supra*), oxidized 9,10 epoxy-stearate very efficiently with a K_m value of 1 μM, thus in the physiological range [65]. Interestingly, this cytochrome P-450 prevalently ω-hydroxylated the 9(*R*),10(*S*) enantiomer [65] (i.e., the isomer preferentially formed by peroxygenase) [49].

2.4 Biosynthetic Schemes

The biosynthetic scheme that we propose (Fig. 2) involves the peroxygenase pathway and includes most of the results related to this subject, as well as earlier reports which showed that cutin monomers could derive from reactions catalyzed by ''fatty acid oxidases'' and ''lipoxidase'' [56].

At the onset, oleic acid could be a substrate for ω-hydroxylation by a cytochrome P-450 and an epoxidation by a peroxygenase. The latter enzyme seems to be constitutively expressed; in contrast, cytochrome P-450 isoenzymes generally need to be induced, their basal activity being particularly low in plants. It follows that in the presence of endogenous fatty acid hydroperoxides, peroxygenase, an oxidase which compared with cytochrome

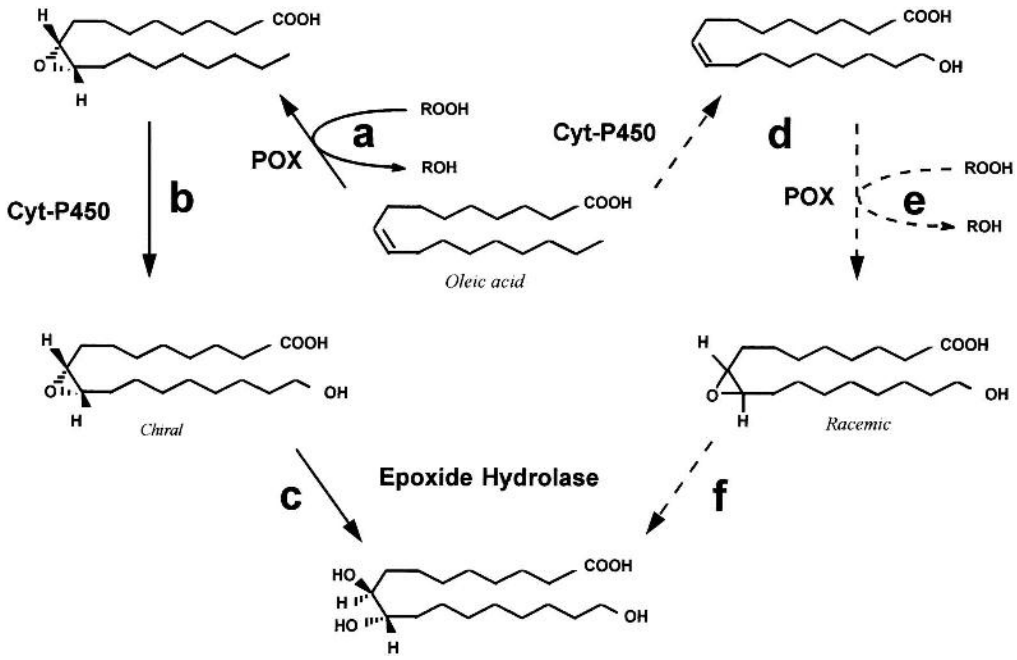


Figure 2 Biosynthetic scheme proposed for C-18 cutin monomers.

P-450-dependent enzymes are characterized by a high turnover number [52], should actively metabolize oleic acid (Fig. 2a). By reasoning on the specificity expressed by both the peroxygenase and the ω-hydroxylase [51,65], it appears that the favored route to 9,10-epoxy-18-hydroxy stearate from oleic acid would be pathway a–b. In comparison, pathway d–e involves a succession of least preferred substrates [54]. We also hypothesized that the first pathway, a–b, will yield to chiral 9,10-epoxy-18-hydroxystearate, in line with the strong stereoselectivity of both peroxygenase and ω-hydroxylase, which lead to such compounds in vitro. In contrast, pathway d–e will give a racemic epoxy–hydroxy derivative. From our scheme (Fig. 2), we also predict that the stereochemistry of the cutin monomers should predominantly be determined by the stereoselectivity of the peroxygenase and the epoxide hydrolase, and thus a single stereoisomer [i.e., 9(*R*), 10(*R*), 18-trihydroxystearic acid] should be formed. This stereochemistry corresponds to the one reported for a compound already described in *Chamaepeuce* seed oil [66], but, unfortunately, the absolute stereochemistry of most cutin components remains unknown to date.

It should be emphasized that the biosynthesis and composition of cutin will not only depend on the presence of specific substrates but also on the relative expression, activities, and compartmentation of the biosynthetic enzymes at a given time of development. For example, 9,10,18-trihydroxystearate was found in older leaves rather than in young ones [7]; this observation is in agreement with the detection of gene expression for soluble epoxide hydrolase (*Arabidopsis thaliana*) in aged leaves and stems [62]. Likewise, a particularly low amount of this trihydroxy derivative in spinach [5] could be correlated with a very low level of fatty acid epoxide hydrolase in this plant [60].

In summary, the scheme given in Figure 3 represents a *dynamic* combination of possible pathways which are modulated by the presence and levels of the involved biosyn-

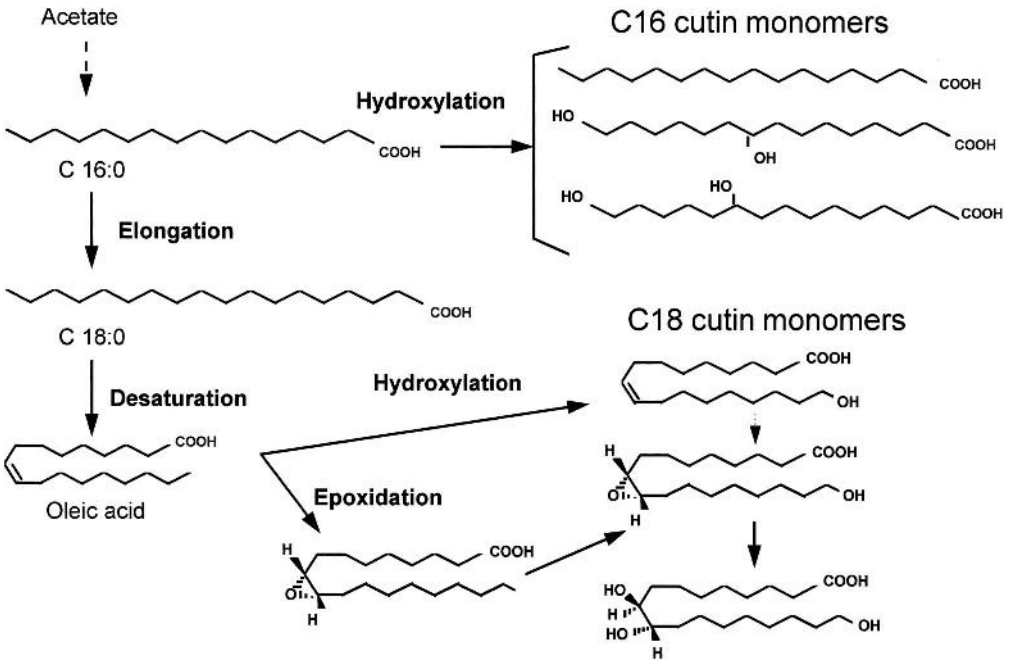


Figure 3 Hypothetical scheme for the biosynthesis of cutin monomers.

thetic enzymes reflecting the diverse composition of cutins between plants grown under a variety of environmental conditions. It should be emphasized that some of the crucial enzymes involved in the biosynthesis of C-18 cutin monomers, such as peroxygenase, ω-hydroxylase, or epoxide hydrolase, appeared to be constitutively present in plants that are coated with cuticles poor in these C-18 monomers, such as soybean [54]. This raises the question of the regulation of the biosynthetic scheme (Fig. 3) in such plants. Besides compartmentation of such enzymes, one could imagine that the bottlenecks of the cutin biosynthetic steps are specific elongases or desaturases, which could be absent or inactive (inhibitors or regulatory elements) in epidermal cells, rendering plants unable to synthesize C-18 precursors of cutin, thus yielding peroxygenase and ω-hydroxylase functionally orphaned. At present, we have no experimental evidence for this attractive idea.

2.5 Formation of Cuticle

Little is known about the biochemical mechanisms underlying the polymerization steps involved in the synthesis of cutin and the transport of the monomers to the surface of the cells. Whereas epicuticular waxes may crystallize spontaneously at the surface of the leaves or self-assemble into lamellae [67], polymerization of cutin requires enzymatic catalysis. The unique work in this field comes from the group of Kollatukudy, which showed that particulate enzyme preparations from two species were able to catalyze the incorporation of labeled C-16 cutin monomers into cutins, with ATP and CoA as the required cofactors. Such incorporation was stimulated by increasing the number of free hydroxyl groups in the cutin primer. Consequently, an extracellular transacylase was proposed to transfer CoA esters of the incoming monomers to the hydroxyl groups of the growing polymer [68]. No further characterization of this acyl-CoA–cutin transacylase was accomplished.

Hydrophobic cutin monomers and waxes undoubtedly must cross the aqueous environment of the apoplast from the epidermal cells, where they are synthesized, to reach the site of cutin synthesis and wax assembly in and outside of the cell wall. Lipid transfer proteins (LTPs) were recently suggested to facilitate the transfer of these cuticular components. Such proteins were initially characterized by their ability to catalyze the exchange of a large number of lipid molecules between natural and artificial membranes in vitro [69,70]. They also exhibit antibacterial and antifungal properties [71] and may, likewise, act in defense against pathogens in accordance with their high concentrations in waxes of some leaves [72], making them the major proteins in surface wax of some plants [73]. Their extracellular location [74], their capacity to bind palmitic and oleic acids [75], as well as their acyl-CoA derivatives [76], and the expression pattern of their encoding genes [74,77–79] led to the proposal that LTPs could play a role in the transport of cutin monomers through the extracellular matrix to sites of cutin synthesis. The fact that heavy metals, such as cadmium, triggered expression of barley LTP genes, followed by a thicker cuticle wax layer, adds circumstantial evidence to the hypothesis that LTP could function in the transfer of wax/cutin monomers from the site of their synthesis to the cuticle [80]. The binding mode of these proteins is still not fully understood. It appears that even if sequences and three-dimensional structures are very similar among plant LTPs, the shape and the volume of their internal cavity could vary considerably. For example, barley LTP was able to expand its hydrophobic cavity significantly upon binding of a ligand [81], whereas LTP from maize could bind easily only fatty acids of 16–19 atoms of carbon [76].

Moreover, although palmitate could bind to both barley and maize LTPs, the orientation of this fatty acid into the two proteins was exactly opposite [82]. Thus, the mechanism in situ for the transport of cutin monomers has yet to be assessed.

The compartment where cutin monomers are synthesized within the cells and the mechanism of their assembly at the surface of, for example, the leaves with the other cuticular components are also still unknown. Likewise, the regulation of the biosynthesis of cutin and waxes during growth or membrane repair is an open question. We have observed a decrease in the global cuticular thickness, paralleled with a fading of the epoxides in plants treated with an inhibitor of the peroxygenase, suggesting that regulation of the cotransport and/or the association of cutin monomers to waxes may occur. Finally, the nature of the attachment of cutins to cell walls remains unknown, probably the cutins are covalently bound to polysaccharides of the cell walls [83].

3 CUTIN MONOMERS AND PLANT DEFENSE

When intact, the cuticle will play its role of a protective layer against mechanical damage by covering all the aerial parts of a plant. Wounds, cracks, and breachs represent sites where cutin components will be released and act as a signal used both by the microbe to facilitate its penetration and by the plant to resist pathogen aggression.

3.1 Cutin as Structural Component of Cuticle

Cutin is known to be the chief structural component of the cuticle and is regarded as its strengthening constituent. One of the scarce reports on mechanical cuticular properties confirmed that cuticles are of importance for plants as reinforcing elements, especially in the case of organs which contain little fibers or those with a cuticle thicker than the epidermal cell wall [84]. The thickness of cuticles could be one of the factors causing leaves to be better protected against all kinds of mechanical injury because they will be harder to pierce, tougher to tear, and, therefore, difficult to consume [85]. However, no correlation between thickness and breaking stress has been found for fruit cuticles.

Cuticle is elastic and can be under tension and, therefore, may be part of the growth-limiting structures, which include epidermal cell walls, contributing to the restriction of cell expansion [86]. Its viscoelasticity is mainly due to its cutin matrix, whereas waxes confer its rigidity [87]. The toughness of the cuticle is therefore likely to be dependent on the distribution and composition of cutin, but it also depends on the nature of its association with other cuticle components.

3.2 Cutin Monomers as Signal Molecules Used by Pathogens

The cuticle acts as a prime mechanical barrier through which pathogenic fungi and insect mouthparts must breach for direct penetration of the epidermis, even if they may invade plants through natural openings such as stomata or through wounds [88]. Direct penetration through the cuticle could occur via two mechanisms. One implies the physical force of the growing hyphal tip due to increasing hydrostatic pressure by melanin in appressoria of certain pathogens such as *Magnaporthe grisea* [89,90]. The second involves the weakening or the desintegration of the protective barrier, resulting from the hydrolysis of the esters bonds present in the cutin matrix by cutinases. Fungal pathogens typically produce

such enzymes, and the first cutinase purified came from *Fusarium solani*, the asexual form of *Nectria haematococca*, saprophytically grown on cutin as the sole carbon source [91]. A cutinase was detected immunologically at the site of penetration of the fungus into the host *Pisum sativum* [92]. In parallel, the structure of the cuticle was modified after the contact with the fungus, probably as the result of enzyme activity [93]. Because expression and release of cutinase were triggered by monomers of cutin, it was postulated that conidia of virulent fungi can sense the contact with plants via cutin monomers that are released by the small amounts of cutinase carried on the conidia. These cutin monomers then amplify the production of cutinase in the germinating spores to assist the penetration into the host [94]. Among the cutin components, 10,16-dihydroxypalmitic and 9,10,18-trihydroxystearic acids were found to be the best inducers of cutinase transcription, which is believed to be due to the phosphorylation of cutinase transcription factor(s) [95–98]. Several lines of evidence supported this scenario. Disrupted cutinase genes as well as inhibitors and antibodies toward cutinase prevented fungal infection [94,99–101]. Mutants with poor content of cutinase presented low virulence, which could be restored by addition of exogenous cutinase [102–104]. Pathogens unable to infect plants devoid of wounds or breaches could penetrate intact surfaces of papaya fruits once a cutinase gene from a virulent fungus has been inserted by genetic engineering [105]. Nonetheless, the requirement of cutinase in pathogenic processes, and therefore the involvement of cutin monomers, has been a matter of debate over these last years [106–110]. Concerning this point, the findings that distinct classes of cutinases function either during the saprophytic mobilization of cutin or during early stages of plant infection may reconciliate the different theories [111]. Until now, disruption or mutation of cutinase genes were mainly applied to genes encoding for enzymes predominantly expressed during saprophytic stages of the respective pathogens, leaving intact significant amounts of cutinases which may play decisive roles in pathogenicity [112]. Accordingly, different cutinases–esterases have recently been shown to be induced in fungi and therefore be implicated in the initiation of the infection process [113–116]. For example, it was shown that a serine esterase, related to a lipase, was secreted by *Botrytis cinerea*, and it possessed strong cutinolytic activity [117]. Antibodies raised against this protein inhibited its catalytic activity but also prevented the infection of tomato leaves by conidia of this fungus. Because the enzyme was stimulated by cuticle components, it was postulated that it was probably involved in the penetration of the host surface during plant infection [118].

In addition to their general controversial role in cuticle penetration, involvement of cutinases in other steps of the infection process has been suggested. For example, it was proposed that these hydrolytic enzymes contribute to the adhesion of spores to host surfaces [115,119] (i.e., a process often associated with enzymatic modification of the host cuticle [120]). Moreover, release of cutin monomers may affect germination of fungal spores because it was shown that they could act as signals to trigger appressorium formation [121]. Among the major cutin monomers, 9,10-epoxy-18-hydroxy-octadecanoic acid was, by far, the most effective inducer of this infection structure [122].

3.3 Cutin Monomers as Signal Molecules Used by Plants

When a plant is challenged by an aggressor, a series of complex events are triggered upon perception of the transduced signals. This includes production of active oxygen species (‘‘oxidative burst’’), ion fluxes, changes in extracellular pH and in membrane potentials,

and activation of several phosphorylase and kinase cascades, all these events ultimately lead to various defense responses. The mechanism by which a plant perceives an aggression has been a challenging question these last years. Fungal pathogens that attempt to penetrate into leaf surfaces have to cope with the plant cuticle. Therefore, it has been considered that this first frontier could play a significant role in signaling. Indeed, evidence has been presented that free cutin monomers can be perceived by cultured potato cells and act as endogenous signal molecules [123]. They were found to trigger the alkalinization of the medium, this effect being paralleled by changes in the phosphorylation state of specific proteins. Moreover, this treatment also stimulated the production of the plant hormone ethylene and activated defense-related genes at the mRNA level [123]. In such experiments, cutin monomers of C-16 and C-18 families varied considerably in their potential to induce the alkalinization. For example, the major constituents of the potato leaf cutin *n*-16-dihydroxypalmitic acid ($n = 8$, 9, or 10) are less active than 9,10-epoxy-18-hydroxystearic acid. Furthermore, hydroxylation at the ω-position seemed to be an absolute requisite for this eliciting activity when the molecule possessed an epoxy function (such as 9,10-epoxy stearate versus 9,10-epoxy-18 hydroxy stearate), but not when it contained a vicinal diol. Thus, 9,10-dihydroxystearic acid is more active than 9,10,18-trihydroxystearic acid. Altogether, it clearly appears that structural features of cutin monomers are required for biological activity of such phytooxylipins, suggesting their specific interaction with one or several perceptive structures, such as receptor molecules, rather than unspecific binding.

In addition, cutin monomers were found to enhance elicitation of H_2O_2, the most thermodynamically stable state of active oxygen species [124]. Hydroxy and epoxy groups seemed important features for this eliciting potential. A recently characterized cutin monomer from cucumber, namely dodecan-1-ol, was also active in eliciting H_2O_2 via both a constitutive and an inducible generating system, this latter involving protein phosphorylation, Ca^{2+} influx, and NAD(P)H oxidase [125]. Furthermore, topical application of free cutin monomers partially protected the treated leaves from pathogen attack [126,127]. Because the cutin monomers exhibited no apparent fungicidal effect, the observed protection was suggested to be due to acquired resistance by the plants. Here, also, specific structural requirements were needed for the prevention of infection. Interestingly, the *cis*-epoxy or the *threo*-hydroxy group(s) in the middle of the C-18 molecules was necessary for protection, and replacement by a cis double bond yielded a completely inactive molecule. Strikingly, 9,10-epoxy-18-hydroxystearate and 9,10,18-trihydroxystearate, which are among the major C-18 cutin monomers, exhibited the strongest effect in eliciting defense mechanisms and protection against fungal aggression, whereas 16-hydroxy palmitic acid, which is present in low amounts in barley cuticle (Blée, unpublished result), showed no protective effect. This argues against a foliar surface modification provoked by the coating of the leaf with oxygenated fatty acids and is more likely in favor of a specific effect of cutin monomers. In this context, it was reported recently that cutinase and other lipolytic esterases protected bean leaves from fungal infection through an unknown mechanism not likely to involve the released cutin monomers [128]. However, before excluding any action of cuticular oxygenated fatty acids in the protective effect of hydrolases, the notion of threshhold for eliciting effects should be pointed out. Clearly, a dose of cutin monomers unable to trigger late defense responses, such as accumulation of antimicrobial proteins (i.e., ''pathogenesis-related proteins'') and tissue necrosis, could perfectly enhance the rapid onset of primary defense mechanisms [129].

4 CONCLUSION

For a long time, cutin was regarded only as an inactive reticulated framework filled up with waxes and other components of the cuticle. This limited-role conception has now evolved with the astonishing finding that cutin monomers could act as primary signals at the site of aggression. The identification of the components involved in signaling is just starting. Clearly, a good knowledge of their biosynthesis will be indispensable for biotechnological applications. Only recently, some of the enzymes involved in the biosynthetic pathway of the major cutin components have been characterized at the molecular level. For most of them, many issues remain to be solved such as tissue and cellular localizations and changes in expression levels under biotic or abiotic stress. Additionally, we ignore most of the enzymes responsible for the biosynthesis of minor cutin components, which, despite being present in low amounts, could prove to be very important from a physiological point of view.

The molecular structure of the precursors of the cutin monomers conditions the nature of the cross-linkage between these different constituents. The cutin network is primarily established via ester bonds which are susceptible to the enzymatic action of hydrolases (i.e., cutinases, esterases, lipases) secreted from some fungi. The presence in cuticles of linkages other than ester bonds may, therefore, protect plants from invasion by certain pathogens. For example, ether bonds, due to the reaction between epoxy groups and hydroxy substituents of adjacent monomers, have been shown to contribute to the reticulation of cutin. One can envision that the number of such ether bonds, which are resistant to the action of cutinase, could be increased by suppression of the hydrolysis of the epoxy groups present in the cutin monomers. Such a result could be attained by inhibition of the epoxide hydrolase, which also would decrease the amount of mid-chain hydroxy functions susceptible to form ester bonds. These last years, cDNAs coding for epoxide hydrolases of *Arabidopsis* and potato were obtained; however, these enzymes are soluble and may not participate in cuticle formation. Obviously, further work is needed to clone the membrane-bound epoxide hydrolase in order to construct transgenic plants expressing antisense RNA to this hydrolase.

Agrochemical molecules, which are very active in vitro, sometimes are useless under field conditions because they cannot cross the leaf cuticle. Therefore, modifying the composition of cutin may change the physical properties of this layer and especially its permeability. Such goals could be achieved by inhibition, for instance, of peroxygenase, one of the key enzymes in the biosynthetic pathway of cutin monomers. Specific mechanism-based inhibitors of this enzyme are presently under investigation in our laboratory to test their influence on the permeability of the cuticle to xenobiotics and to pathogens.

In addition, recent evidence has shown that epoxy and hydroxy derivatives of fatty acids produced by the peroxygenase pathway play a primary role in plant disease resistance. First, it was reported that two cutin monomers, which lack fungitoxic activity, act as endogenous inducers of acquired resistance in cereals, possibly through a mechanism involving transcription in the host. Second, epoxides formed from linoleic and linolenic acids were shown to induce resistance of rice to infection by *Magnaporthe grisea*. Moreover, infection of rice by the fungus triggers the activation of the peroxygenase pathway in plants. Thus, peroxygenase appears to be a key enzyme in such resistance mechanisms, and one could expect that overexpression of this enzyme could result in a better defense of the transformed plant against fungal attack. Presumably, researchers will clone this enzyme in the near future and establish its location in plant tissues.

Undoubtedly, our vision of cutin monomers is far from being complete: It is just beginning.

ACKNOWLEDGMENTS

Many thanks to Rachel Kahn and Francis Schuber for critically reading this manuscript.

REFERENCES

1. PE Kollatukudy. In: PK Stumpf, ed. The Biochemistry of Plants: Lipids. Structure and function, Cutin, Suberin and Waxes. New York: Academic Press, 1980, pp 571–646.
2. EA Baker. In: DF Cutter, KL Alvin and CE Price, eds. The Plant Cuticle: Chemistry and Morphology of Plant Epicuticular Waxes. London: Academic Press, 1982, pp 136–166.
3. TJ Walton. In: JL Harwood and JR Bowyer, eds. Lipids, Membranes and Aspect of Photobiology: Waxes, Cutin and Suberin. London: Academic Press, 1990, pp 105–158.
4. G Bianchi. In: RJ Hamilton, ed. Plant Waxes: Waxes: Chemistry, Molecular Biology and Functions. Dundee: The Oily Press, 1995, pp 177–222.
5. PJ Holloway. In: DF Cutler, KL Alvin, CE Price, eds. The Plant Cuticle: The Chemical Constitution of Plant Cutins. London, New York: Academic Press, 1982, pp 45–82.
6. JF Villena, E Dominguez, D Stewart, A Heredia. Planta 208:181–187, 1999.
7. M Riederer, J Schönherr. Planta 174:121–138, 1988.
8. JB Clarck, GR Lister. Plant Physiol 55:407–413, 1975.
9. DA Reicosky, JW Hanover. Plant Physiol 62:101–104, 1978.
10. MA Jenks, RJ Joly, PJ Peters, PJ Rich, JD Axtell, EA Ashworth. Plant Physiol 105:1239–1245, 1994.
11. P Luque, S Bruque, AA Heredia. Arch Biochem Biophys 317:417–422, 1995.
12. J Schönherr. Planta 131:159–164, 1976.
13. P Baur. Plant Cell Environ 20:167–177, 1997.
14. L Schreiber, J Riederer. Plant Cell Environ 19:1075–1082, 1996.
15. S Santier, A Chamel. Plant Physiol Biochem 36:225–231, 1998.
16. MJ Bukovac, PD Petracek, RG Fader, RD Morse. 38:289–298, 1990.
17. KE Espelie, EA Bernays, JJ Brown. Arch Insect Biochem Physiol 17:223–233, 1991.
18. SD Eigenbrode, KE Espelie. 40:171–194, 1995.
19. D Preuss, B Lemieux, G Yen, BW Davis. Genes Dev 7:974–985, 1993.
20. P von Wettstein-Knowles. In: RJ Hamilton, ed. Waxes: Chemistry, Molecular Biology and Functions: Biosynthesis and Genetic of Waxes. Dundee: The Oily Press, 1995, pp 91–129.
21. D Post-Beittenmiller. Ann Rev Plant Physiol Plant Mol Biol 47:405–430, 1996.
22. B Lemieux. Trends Plant Sci 1:312–318, 1996.
23. M Matic. Biochem J 63:168–176, 1956.
24. PJ Holloway, AHB Deas. Phytochemistry 12:1721–1735, 1973.
25. HC Gerard, WF Fett, SF Osman, RA Moreau. 17:181–189, 1993.
26. EA Baker, JT Martin. Nature 199:1268–1270, 1963.
27. A Ray, RE Stark. Phytochemistry 48:1313–1320, 1998.
28. HW Schmidt, J Schönherr. Planta 156:380–384, 1982.
29. CE Crisp. The Biopolymer Cutin. Ph. D. dissertation, University of California, 1965.
30. PE Kolattukudy, TJ Walton. Biochemistry 11:1897–1907, 1972.
31. AK Ray, YY Lin, HC Gerard, ZJ Chen, SF Osman, WF Fett, RA Moreau, RE Stark. Phytochemistry 38:1361–1369, 1995.
32. CI Soliday, PE Kolattukudy. Plant Physiol 59:1116–1121, 1977.
33. I Benveniste, N Tijet, F Adas, G Phillips, J-P Salaun, F Durst. Biochem Biophys Res Commun 243:688–693, 1998.

34. N Tijet, C Helvig, F Pinot, R Le Bouquin, A Lesot, F Durst, J-P Salaun, I Benveniste. Biochem J 332:583–589, 1998.
35. F Cabello-Hurtado, Y Batard, J-P Salaun, F Durst, F Pinot, D Werck-Reichhart. J Biol Chem 273:7260–7267, 1998.
36. FJ van de Loo, P Broun, S Turner, C Sommerville. Proc Natl Acad Sci USA 92:6743–6747, 1995.
37. PE Kollatukudy, TJ Walton, RP Kuswaha. Biochemistry 12:4488–4498, 1973.
38. F Oesch, N Kaubisch, DM Jerina, JW Daly. Biochemistry 10:4858–4866, 1971.
39. R Croteau, PE Kollatukudy. Arch Biochem Biophys 162:471–480, 1974.
40. R Croteau, PE Kollatukudy. Arch Biochem Biophys 170:61–72, 1975.
41. PE Kollatukudy. In: G Kerstein, ed. Plant Cuticles, an Integrated Functional Approach: Biosynthetic Pathways of Cutin and Waxes, and Their Sensitivity to Environmental Stress. Oxford: BIOS Scientific Publishers, 1996, pp 83–108.
42. M Bafor, MA Smith, L Jonsson, K Stobart, S Stymne. Arch Biochem Biophys 303:145–151, 1993.
43. E Blée, U Stahl, F Schuber, S Stymne. Biochem Biophys Res Commun 197:778–784, 1993.
44. M Lee, M Lenman, A Banas, M Bafor, S Singh, M Schweizer, M Nilsson, C Lijenberg, A Dahlqvist, PO Gummeson, S Sjodahl, A Green, S Stymne. Science 280:915–918, 1998.
45. P Broun, J Shanklin, E Whittle, C Somerville. Science 282:1315–1317, 1998.
46. B Mazur, E Krebbers, S Tingey. Science 285:372–375, 1999.
47. A Ishimaru, I Yamazaki. J Biol Chem 252:6118–6124, 1977.
48. E Blée, F Schuber. Biochemistry 28:4962–4967, 1989.
49. E Blée, F Schuber. J Biol Chem 265:12667–12894, 1990.
50. M Hamberg, G Hamberg. Arch Biochem Biophys 283:409–416, 1990.
51. F Pinot, J-P Salaun, H Bosch, A Lesot, C Mioskowski, F Durst. Biochem Biophys Res Commun 164:183–193, 1992.
52. E Blée, F Schuber. Biochem Biophys Res Commun 173:1354–1360, 1993.
53. R Croteau, PE Kollatukudy. Arch Biochem Biophys 162:458–470, 1974.
54. E Blée, F Schuber. Plant J 4:113–123, 1993.
55. PJ Holloway, GA Brown, J Wattendorff. J Exp Bot 32:1051–1066, 1991.
56. W Heinen, IVD Brand. Z Naturforsch 18b:67–79, 1963.
57. JN Siedow. Annu Rev Plant Physiol Plant Mol Biol 42:145–188, 1991.
58. J-P Salaun, C Helvig. Drug Metabolism Drug Interact 12:261–283, 1995.
59. R Croteau, PE Kollatukudy. Arch Biochem Biophys 170:73–81, 1975.
60. E Blée, F Schuber. Biochem J 282:711–714, 1992.
61. T Kiyosue, JK Beetham, F Pinot, BD Hammock. Plant J 6:259–269, 1994.
62. A Stapleton, JK Beetham, F Pinot, JE Garbarino, DR Rockhold, M Friedman, BD Hammock, WR Belknap. Plant J 6:251–258, 1994.
63. T Katsube, M Adachi, N Maruyama, K Ichise, Y Takenada, S Utsumi. Plant Physiol 109: 722–723, 1995.
64. JK Bettham, D Grant, M Arand, J Garbarino, T Kiyosue, F Pinot, F Oesch, WR Belknap, K Shinozaki, BD Hammock. DNA Cell Biol 14:61–71, 1995.
65. F Pinot, I Benveniste, J-P Salaun, O loreau, J-P Noel, L Schreiber, F Durst. Biochem J 342: 27–32, 1999.
66. LJ Morris, MI Crouchman. Lipids 7:372–379, 1972.
67. E Dominguez, A Heredia. Z Naturforsch 54c:141–143, 1999.
68. PE Kollatukudy. Annu Rev Plant Physiol 32:539–567, 1981.
69. DG Rueckert, K Schmidt. Chem Phys Lipids 56:1–20, 1990.
70. JC Kader. Subcell Biochem 16:69–110, 1990.
71. A Molina, A Segura, F Garcia-Olmedo. FEBS Lett 316:119–122, 1993.
72. J Pyee, PE Kollatukudy. Plant J 7:49–59, 1995.
73. J Pyee, H Yu, PE Kollatukudy. Arch Biochem Biophys 311:460–468, 1994.

74. P Sterk, H Booij, GA Schellekens, A van Kammen, S de Vries. Plant Cell 3:907–921, 1991.
75. EA Meijer, SC de Vries, P Sterk, DWJ Gadelle, KWA Wirtz, T Hendriks. Mol Cell Biochem 123:159–166, 1993.
76. A Zachowki, F Guerbette, M Grosbois, A Jolliot-Croquin, JC Kader. Eur J Biochem 257: 443–448, 1998.
77. L Sossountzov, I Ruiz Avila, E Vignols, A Jolliot, V Arondel, F Tchang, M Grosbois, F Guerbette, E Miginiac, M Delseny, P Puigdomenach, J-C Kader. Plant Cell 3:925–933, 1991.
78. S Thoma, Y Kanedo, C Sommerville. Plant J 3:427–436, 1993.
79. S Thoma, U Hecht, A Kippers, J Botella, S de Vries, C Sommerville. Plant Physiol 105: 35–45, 1994.
80. B Hollenbach, L Schreiber, W Hartung, KJ Dietz. Planta 203:9–19, 1997.
81. MH Lerche, BB Krageland, LM Bech, FM Poulsen. Structure 5:291–306, 1997.
82. MH Lerche, FM Poulsen. Protein Sci 7:2490–2498, 1998.
83. PJ Holloway. In:E Percy, ed. Air Pollutants and the Leaf Cuticle: Plant Cuticles, Physicochemical and Biosynthesis. Berlin: Springer-Verlag, 1994, pp 1–13.
84. P Wiedeman, C Neinhuis. Bot Acta 111:28–34, 1998.
85. G Kerstein. Trends Plant Sci 1:125–129, 1996.
86. S Hoffman-Benning, H Kende. Plant Physiol 104:719–723, 1994.
87. PD Petracek, MJ Bukowac. Plant Physiol 109:675–679, 1995.
88. RJ Howard, B Valent. Annu Rev Microbiol 50:491–512, 1996.
89. B Valent, FG Chumley. Annu Rev Phytopathol 29:443–467, 1991.
90. RJ Howard, MA Ferrari. Exp Mycol 13:403–418, 1989.
91. RE Purdy, PE Kollatukudy. J Biol Chem 270:11,753–11,756, 1995.
92. M Shaykh, C Soliday, PE Kollatukudy. Plant Physiol 60:170–172, 1977.
93. H Xu, K Mendgen. Mol Plant Microbe Interact 10:87–94, 1997.
94. PE Kollatukudy. Annu Rev Phytopathol 23:223–250, 1985.
95. CP Woloshuk, PE Kollatukudy. Proc Natl Acad Sci USA 83:1704–1708, 1986.
96. GK Podilla, MB Dickman, PE Kollatukudy. Science 242:922–925, 1988.
97. A Bajar, MB Dickman, PE Kollatukudy. Proc Natl Acad Sci USA 88:8208–8212, 1991.
98. D Li, PE Kollatukudy. J Biol Chem 270:11,753–11,756, 1995.
99. W Köller, CR Adam, PE Kollatukudy. Phytopathology 81:1375–1379, 1991.
100. LM Rogers, MA Flaishman, PE Kollatukudy. Plant Cell 6:935–945, 1994.
101. K Tanabe, S Nishimura, K Kohmato. Ann Phytopathol Soc Japan 54:483–492, 1988.
102. W Köller, DM Parker, CM Becker, Phyopathology 81:1375–1379, 1991.
103. AH Danzig, SH Zuckerman, MM Andonov-Roland. J Bacteriol 168:911–916, 1986.
104. MB Dickman, SS Patil. Physiol Mol Plant Pathol 28:235–242, 1986.
105. MB Dickman, GK Podila, PE Kollatukudy. Nature 342:446–448, 1989.
106. DJ Stahl, W Schäfer. Plant Cell 4:621–629, 1992.
107. B Oeser, OC Yoder. Mol Plant Microbe Interact 7:282–288, 1993.
108. DJ Stahl, A Theuerkauf, R Heitefuss, W Schäfer. Mol Plant Microbe Interact 6:713–725, 1994.
109. JAL van Kan, JW Klooster, CAM Wagenmakers, DCT Dees, CJB van der Vlugt-Bergmans. Mol Plant Microbe Interact 10:30–38, 1997.
110. RN Crowhurst, SJ Binnie, JK Bowen, BT Hawthorne, KM Plummer, J Rees-Gearge, EHA Rikkerink, MD Templeton. Mol Plant Microbe Interact 10:355–368, 1997.
111. C Yoa, W Köller. Mol Plant Microbe Interact 8:122–130, 1995.
112. JA Sweigard, FG Chumley, B Valent. Mol Gen Genet 232:174–182, 1992.
113. RL Nicholson, H Yoshida, N Yamaoka, H Kunoh. Exp Mycol 12:336–349, 1988.
114. SF Pascholati, H Yoshioka, H Kunoh, RL Nicholson. Physiol Mol Plant Pathol 41:53–59, 1992.
115. SF Pascholati, H Deisig, B Leite, D Anderson, RL Nicholson. Physiol Mol Plant Pathol 42: 37–51, 1993.

116. JA Suggi, SF Pascholati, H Kunoh, RJ Howard, RL Nicholson. Physiol Mol Plant Pathol 52:213–221, 1998.
117. P Comménil, L Belingheri, B Dehorter. Physiol Mol Plant Pathol 52:1–14, 1998.
118. P Comménil L, Belingheri, G Bauw, B Dehorter. Physiol Mol Plant Pathol 55:37–43, 1999.
119. H Delsing, RL Nicholson, M Haug, RJ Howard, K Mendgen. Plant Cell 4:1101–1111, 1992.
120. RL Nicholson, L Epstein. In: GT Cole, HC Hoch, eds. The Fungal Spore and Disease Initiation in Plants and Animals. New York: Plenum Press, 1991, pp 3–23.
121. SA Francis, FM Dewey, SJ Gurr. Physiol Mol Plant Pathol 49:201–211, 1996.
122. RD Gilbert, AM Johnson, RA Dean. Physiol Mol Plant Pathol 48:335–346, 1996.
123. P Schweizer, G Felix, A Buchala, C Muller, J-P Métraux. Plant J 10:331–341, 1996.
124. M Fauth, P Schweizer, A Buchala, C Markstader, M Reiderer, T Kato, H Kauss. Plant Physiol 117:1373–1380, 1998.
125. H Kauss, M Fauth, A Merten, W Jeblick. Plant Physiol 120:1175–1182, 1999.
126. P Schweizer, A Jeanguenat, E Mösinger, J-P Métraux. In: MJ Danils, JA Downie, AE Osbourn, eds. Advance in Molecular Genetic of Plant–Microbe Interactions. Dordrecht: Klewer Academic, 1994, pp 371–374.
127. P Schweizer, A Jeanguenat, D Whitacre, J-P Métraux, E Mösinger. Physiol Mol Plant Pathol 49:103–120, 1996.
128. DM Parker, W Köller. Mol Plant Microbe Interact 11:514–522, 1998.
129. L Costet, S Cordelier, S Dorey, F Bailleuil, F Fritig, S Kauffman. Mol Plant Microbe Interact 12:655–662, 1999.

13

Structural Diversity of Marine Oxylipins

WILLIAM H. GERWICK and INDER PAL SINGH

College of Pharmacy, Oregon State University, Corvallis, Oregon

1 INTRODUCTION

Marine organisms are the source of an absolutely fantastic diversity of oxylipin structures, even greater than that found in their terrestrial counterparts. Over the last 30 years, research discoveries made with marine oxylipins have played many key roles in the development of eicosanoid research. Indeed, even the term ''oxylipin,'' which is now broadly utililized to describe the enzymatically oxidized lipids of higher plants and animals, was first proposed in the description of marine algal substances [1]. However, the marine dimension of oxylipin chemistry is not as well recognized as other areas within this broad field, perhaps because these organisms have relatively little societal impact and are sometimes difficult to access. The overwhelming majority of marine oxylipins derive from lipoxygenase (LOX) metabolism of polyunsaturated fatty acid (PUFA) precursors of a variety of carbon lengths (C-16 to C-22) and unsaturation patterns. Additional marine compounds of the oxylipin class derive from the metabolism of PUFA precursors by unique oxidative enzymes, and in a few cases, prostaglandin H synthase metabolism is implicated. However, it is the broad range of LOX metabolites produced by marine creatures, which is truly outstanding. These metabolites are notable both in the diversity of the substrates utilized, the site specificity of marine LOXs, and the secondary biosynthetic manipulations of the initially formed hydroperoxides, which, in turn, produce an enormous number of uniquely functionalized and cyclized structures. For example, naturally occurring halogenated (chloro-, bromo-, and iodo-) prostaglandin analogs, which derive from LOX metabolism, have been reported from marine corals and brown algae [2–5].

Several reviews focus on various dimensions of oxylipin production by marine creatures, including that of the Rhodophyta [red algae [6]], marine algae in general [7], marine invertebrates [8], biosynthetic and structural highlights [9], intermediacy of epoxyallylic cations in the biosynthesis of many marine oxylipins [10], carbocyclic marine oxylipins [11], and a recent semicomprehensive treatment [12]. Nevertheless, it appears that this dimension of oxylipin metabolism is less well recognized than several other more classical aspects of this field, and there is generally little recognition of the many unique biosynthetic precedents that are available in these marine examples [13]. Hence, the focus of this chapter is to attempt to provide the reader with a broad sense of the biosynthetic and chemical themes present in marine oxylipins, with a particular emphasis on those that are of particular structural complexity and uniqueness.

2 OVERVIEW CONSIDERATIONS

2.1 Overview of Marine LOX Products

Lipoxygenase-derived metabolites are extraordinarily rich in marine life, with nearly all conceivable LOX positional specificities being illustrated in one or another marine creature. As such, marine life provides a fertile ground for the discovery and description of LOX chemistry, enzymology, molecular genetics, physiology, and chemical ecology. Therefore, the major objective of this chapter is to provide overview perspectives and insights into this class of secondary metabolite in marine organisms, as well as give an important access point to the abundant literature on this subject.

Because in marine organisms substrates of a variety of chain lengths (C-16 to C-22) and unsaturation patterns (ω3, ω6, ω9) are utilized in these LOX-initiated pathways of metabolism, selecting a convenient yet precise nomenclature for the LOX enzyme regiospecificity presents several problems. Some LOXs appear to recognize fatty acid substrates relative to the carboxyl terminus, introducing molecular oxygen at similar positions in various chain lengths of PUFAs. Other LOXs consistently introduce oxygen relative to the methyl terminus; for example, from C-18 substrates (e.g., linoleic acid), the initially formed product is 13-hydroperoxyoctadecadienoic acid, whereas from C-20 substrates [e.g., arachidonic acid (AA)], the comparable product is 15-hydroperoxyeicosatetraenoic acid (15-HPETE). Therefore, in this chapter, the following new nomenclatural standard is proposed; for describing the LOX metabolism of C-18 substrates, the LOX specificity is identified as an ''octadecanoid LOX'' or ''OLOX,'' and for C-20 substrates, it is identified as an ''eicosanoid LOX'' or ''ELOX.'' Similarly, positional specificity relative to hexadecanoid (HLOX) or dodecanoid (DLOX) substrates can be easily specified.

Metabolites deriving from the 5-ELOX pathway are relatively rare in marine life, with principal examples being from red algae and diatoms. These are relatively recent findings and illustrate both oxylipin metabolic pathways similar to those in mammalian systems [5-hydroxyeicosatetraenoic acid (5-HETE) and 5(*R*),6(*S*)-dihydroxyeicosatetraenoic acid [5(*R*),6(*S*)-diHETE]] as well as those that are apparently unique to a marine system [e.g., bacillariolide (**1**), Fig. 1] [14].

The 8-ELOX pathway is broadly utilized by marine life forms in the production of oxylipins. For example, the red alga *Agardhiella subulata* produces a tricyclic oxylipin, agardhilactone (Fig. 1, **2**), which is best rationalized as deriving from an initial 8*R*-ELOX pathway [15]. Marine corals, ascidians, arthropods, and echinoderms broadly possess an 8*R*-ELOX, which leads to the production of structurally simple [8(*R*)-HETE, **3**] as well

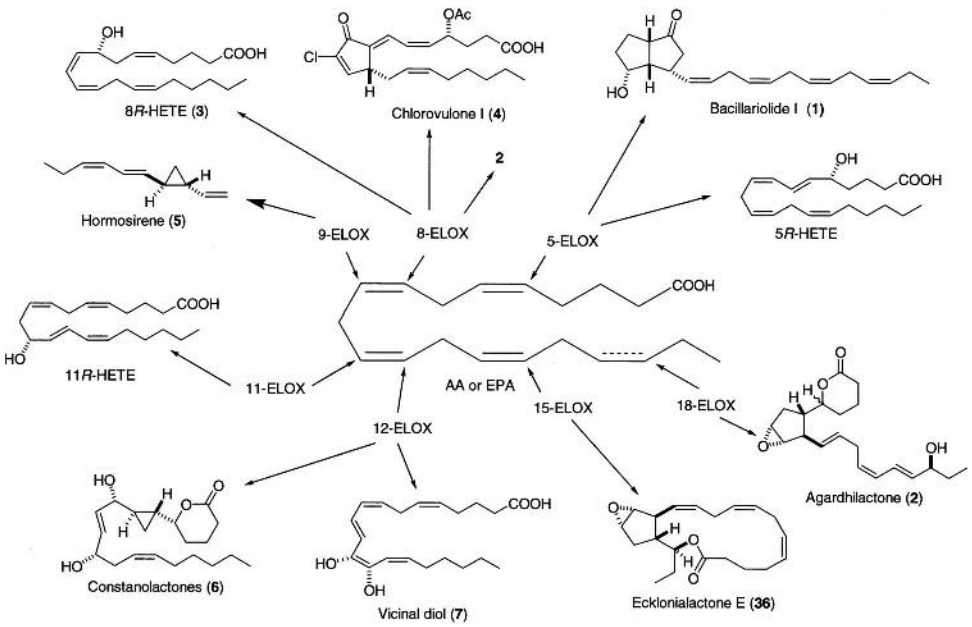


Figure 1 Depiction of various sites of lipoxygenation in marine fatty acids.

as complex (chlorovulone I, **4**) oxylipins [2]. In the case of corals, this metabolic pathway is also implicated in the formation of prostaglandins of similar or identical structure to those of mammalian occurrence. Moreover, the 8*R*-ELOX from *Plexaura homomalla* has been cloned and sequenced, and features of its stereospecificity have been examined [16].

Although the 9-ELOX pathway is not well represented in marine systems, clear examples exist in the red alga *Polyneura latissima* and in the formation of several brown algal pheromones. In the former, the products deriving from the initially formed hydroperoxide include simple dienyl alcohols, epoxy alcohols, and divinyl ethers, a spectrum very similar to what is seen in some higher plants (e.g., potato and various members of the Gramineae, including rice and oats) [17]. In brown algal pheromone formation, a 9-ELOX is implicated in the pathway that subsequently leads to carbon-chain scission and formation of volatile hydrocarbons (**5**) [18].

The 11-ELOX pathway is best illustrated in marine life forms by invertebrates, including corals, barnacles, and sea urchins. In the coral *P. homomalla*, the co-occurrence of 11(*R*)-HETE along with the rich assortment of prostaglandins typical of this species led to a hypothesis that the coral 11*R*-ELOX initiates or participates in the biosyntheses of prostaglandins [19]. In sea urchins and barnacles, the simple dienyl alcohols [e.g., 11(*R*)-HETE] are implicated in controlling reproductive events, including prevention of polyspermic fertilizations [20].

Probably the most widely present LOX pathway in marine life is the 12-ELOX pathway. Numerous red algae utilize this pathway to make simple hydroxy acids such as 12(*S*)-HETE and 12(*S*)-hydroxyeicosapentaenoic acid [12(*S*)-HEPE] (e.g., *Murrayella periclados* and *Lithothamnion corallioides*) [21,22] as well as more complex substances as exemplified by the constanolactones (**6**) [23] and *Gracilariopsis lemaneiformis* vicinal diols (**7**) [24]. A broad range of marine animals use LOXs with regiospecificity for the

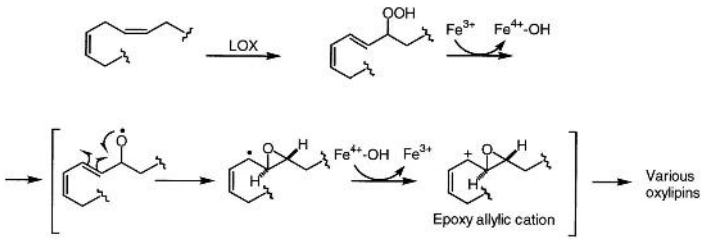


Figure 2 Intermediacy of epoxy allylic carbocations in the formation of oxylipins.

Δ12 position; however, in some cases with opposite stereospecificity [e.g. both 12(*S*)- and 12(*R*)-HETE is produced]. In most marine animals, investigations to date have identified 12-HETE of unknown or 12(*R*) stereochemistry as the primary product, and these are implicated with a variety of biological activities, including reproductive events (barnacles, sea urchins, and starfish) [25], neurochemical signaling (*Aplysia californica*) [26], and morphogenesis (*Hydra vulgaris*) [27].

Metabolites of the 13-OLOX or 15-ELOX pathway are principally expressed in brown algae. Both 18-carbon and 20-carbon substrates are commonly utilized by brown algae to produce oxylipins; therefore, in 18-carbon substrates, oxygen is introduced at C-13, whereas in 20-carbon substrates, oxygen is added at C-15. Examples exist in brown algae of simple reduction of the intermediate, 13(*S*)-hydroperoxide (in 18-carbon substrates) or 15(*S*)-hydroperoxide (in 20-carbon substrates), to dienyl alcohols. Also, the hydroperoxide is rearranged by pathways analogous to those in higher plants, leading to divinyl ethers, and the manipulation of these reactive intermediates by novel pathways to make unique substances (e.g., ecklonialactones and egregiachlorides) [5,28]. Some of these substances may function in the chemical defense of these palatable life forms [28,29].

A few examples exist wherein marine metabolites are observed with an ω3 alcohol in either 18-carbon (16-OLOX) or 20-carbon (18-ELOX) chains—for instance, in several metabolites of the green alga *Acrosiphonia coalita* [30] and in agardhilactone (**2**) from the red alga *Agardhiella subulata* [15].

Various other oxidative routes, which initiate the metabolism of PUFA, occur in marine life. For example, there is evidence for the functioning of unusual oxidases in red algae, which are able to make such molecular entities such as 13-HEPE [22]. Additionally, metabolites biosynthetically consistent with a cytochrome P-450 origin have been obtained from diverse sources, perhaps best exemplified by the brown alga *Notheia anomala* [31].

One of the more intriguing aspects of the enormous structural diversity of marine oxylipins is that many reasonably derive from a similar LOX-formed hydroperoxide, which is then transformed into an epoxy allylic carbocation (Fig. 2) [10]. Although this hypothesis for a common biosynthetic pathway has been recently applied to a broad assortment of marine-derived oxylipins [10,32], the mechanistic insight for this transformation was first envisioned by Gardner in his studies of corn-germ-derived oxylipins [33].

2.2 Overview of Types of Fatty Acid Substrates Used to Form Marine Oxylipins

A wide variety of chain lengths and unsaturation patterns are present in the fatty acid precursors to marine oxylipins. However, the dominant unique theme is the abundance

of ω3 fatty acids, typically α-linolenic and stearidonic acid in the 18-carbon class, 5(*Z*), 8(*Z*), 11(*Z*), 14(*Z*), 17(*Z*)-eicosapentaenoic acid (EPA) in the 20-carbon class, and docosahexaenoic acid in the 22-carbon class. Table 1 provides general themes on the utilization of various fatty acid precursors to form oxylipins by different phyla of marine life.

The fatty acid compositions of algae have been characterized in considerable detail and summarized in a number of places [34,35]. Curiously, in several cases, the abundance of PUFA substrates does not closely parallel their utilization in oxylipin biosynthetic pathways. For example, the brown algae (Phaeopyceae) are relatively depauperate in 18-carbon PUFAs [34]; however, they commonly utilize this substrate in LOX-initiated biosynthetic pathways. Cyanobacteria possess a rich assortment of 14-carbon to 18-carbon substrates, but are only known to metabolize this latter chain length to oxylipin structures. Although the Rhodophyta seem to fully utilize their complement of 18-carbon and 20-carbon substrates to produce these structures, green algae (Chlorophyta), like higher plants, mainly oxidize 18-carbon substrates. A striking trend evident in Table 1 is that marine animals almost exclusively metabolize 20-carbon substrates to oxylipins.

2.3 Overview of Carbocyclic Rings in Marine Oxylipins

The metabolism of initially formed dienyl hydroperoxides to carbocyclic oxylipins of various ring size and location within the fatty acid chain is a source of great structural diversity. The diverse oxylipin carbon skeleton types found in marine life have previously been reviewed [11]. As noted earlier, it has been proposed that the structures of many of these can be biosynthetically rationalized as deriving via intermediate formation of an epoxy allylic carbocation and that this transiently produced functionality is manipulated by secondary enzymes into a surprisingly diverse number of compounds (Fig. 2). In some cases, this results in the production of carbocyclic oxylipins with cyclopropyl rings, cyclopentyl rings, or, in some cases, in bicarbocyclic oxylipins. However, other biosynthetic mechanisms are also responsible for producing carbocyclic oxylipins.

Cyclopropyl rings have been found at the Δ10 position in a series of 28-carbon compounds, and at the Δ6-, Δ9-, Δ13-, and Δ16-positions of 20-carbon chains. Cyclopentyl ring locations show even greater diversity having been observed at the Δ9-position in 22-carbon compounds, Δ2-, Δ6-, Δ8-, and Δ10-positions in 20-carbon compounds, and at Δ3-, Δ8-, Δ11-, Δ12-, and Δ14-positions of chains from 12 carbon to 18 carbon in length (Fig. 3). In total, 17 unique carbocyclic carbon skeletons have been described among the nearly 200 known marine oxylipin metabolites.

2.4 Overview of Functional Group Types Observed in Marine Oxylipins

A final dimension of marine-derived oxylipins that warrants special mention is the uniqueness of some of the functional groups that are produced. For example, it is only in the marine environment that naturally occurring halogenated oxylipins have been encountered. These include vinyl-halogen-containing coral oxylipins {chlorovulones and related compounds from *Clavularia viridis* (chloro-, bromo-, iodo-) [2,4] and chlorinated punaglandins from *Telesto riisei* [3]} and the brown algal metabolite egregiachloride [5]. In this latter case, the halide ion is biogenetically proposed to add to a carbocation intermediate.

A wide diversity of oxygen-containing functional groups has been observed in marine oxylipins, including macrolactones, epoxides, ethers, esters, allene oxides, cyclic peroxides, hydroperoxides, as well as the more commonplace ketones, acids, and alcohols

Table 1 Utilization of Various PUFA Substrates to Form Oxylipins in Different Classes of Marine Life

Phylum	18:2	α18:3	γ18:3	18:4	20:4	20:5	22:6	Other
Cyanobacteria	−	++++	−	−	−	−	−	
Rhodophyta	++	+++	++	++	++++	++++	−	
Chlorophyta	+	++++	−	+++	+	+	+	C-22 oxylipin of unknown origin
Phaeophyceae	+	+	+	++++	++	++++	−	
Bacillariophyceae	++	?	?	−	−	++++	−	
Porifera (sponges)	−	−	−	−	++++	++		20–26 carbons with unusual unsaturation
Echinodermata	−	−	−	−	++++	++	−	
Cnidaria (corals)	−	−	−	−	++++	++	−	22:5
Mollusca	−	−	−	−	++++	++++	−	
Arthropoda	−	−	−	−	++++	+++	−	
Urochordata (tunicates)	−	−	−	−	++++	++	−	
Fish	+	+	+	−	++++	++	++	

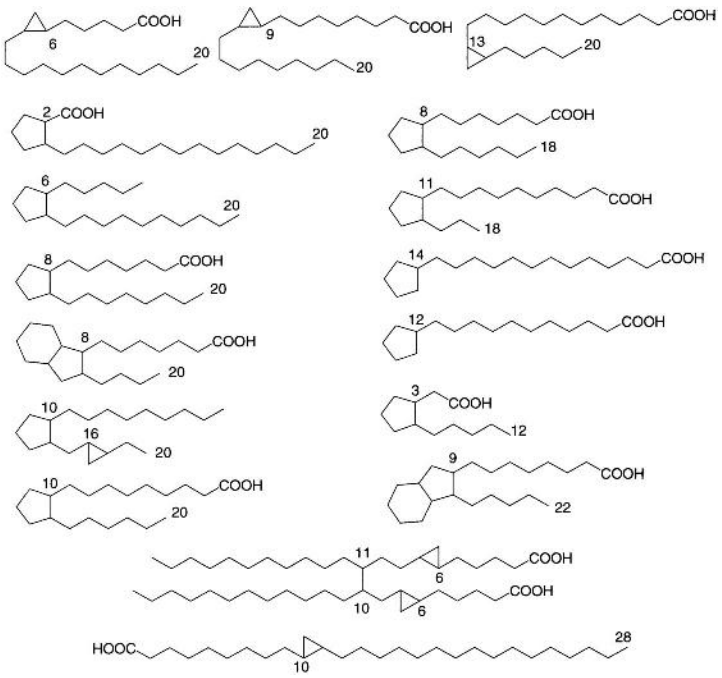


Figure 3 Variety of carbon skeletons found in marine oxylipins.

typical of oxylipin compounds. Conjugated olefins unlike those observed in terrestrially derived oxylipins are another unique modification of marine metabolism and include conjugated trienes without adjacent oxygen, cross-conjugated ketones, conjugated trienals, furan rings, and bis-allylic alcohols. Only a couple of marine examples of nitrogen-containing oxylipins exist; these are restricted to metabolites of the tunicate *Polyandrocarpa* sp. wherein both tertiary amides and guanido groups are observed [36].

3 EXAMPLES FROM MARINE PLANTS

3.1 Rhodophyta (Red Algae)

The Rhodophyta (red algae) have been the most prolific source of oxylipins among the different algae. Most of these unique oxylipins arise from 12-ELOX-(or 13-OLOX) initiated oxidation and subsequent reaction or rearrangement of the resultant dienyl hydroperoxide, thereby producing a variety of structures. Oxylipins resulting from 5-ELOX, 8-ELOX, or 9-ELOX pathways have also been encountered in the red algae. Moreover, red algae are a rich source of polyenoic fatty acids that serve as precursors to these various classes of oxylipins. The oxylipin chemistry of the Rhodophyta has been previously reviewed [6,7].

3.1.1 Polyenes from *Ptilota filicina*

The lipid extract of red alga *Ptilota filicina* from the Oregon coast showed strong antimicrobial activity against several Gram-positive and Gram-negative bacteria and, subsequently, yielded three unique metabolites. These were isolated as their semisynthetic

methyl ester derivatives and identified utilizing a number of spectroscopic techniques including two-dimensional (2D) nuclear magnetic resonance (NMR) as 5(*Z*),7(*E*),9(*E*), 14(*Z*),17(*Z*)-eicosapentaenoic acid (**8**), its C-5 geometrical isomer (**9**), and ptilodene (**10**) (see Fig. 4) [37,38]. The geometries of the double bonds in compounds **8** and **9** were deduced from coupling constant analysis and ultraviolet (UV) spectral features. Ptilodene (**10**) was shown to contain a unique cross-conjugated dienone moiety with all conjugated double bonds in the trans configuration and all nonconjugated ones in the cis configuration (Fig. 4).

The discovery of 5(*Z*),7(*E*),9(*E*),14(*Z*),17(*Z*)-eicosapentaenoic acid (**8**) and ptilodene (**10**) aroused considerable interest into the biochemical pathway of their formation. A crude tissue homogenate from *P. filicina* possessed isomerase activity that converted EPA to the corresponding conjugated triene **8** (Fig. 4). This enzyme, named polyenoic fatty acid isomerase (PFI), was highly purified using standard techniques and characterized as a 125-kDa homodimeric enzyme. Studies showed EPA to be the most rapidly turned over substrate, followed by AA and other C-22 and C-18 substrates. The biosynthesis of conjugated triene **8** in *P. filicina* was studied using both AA and a variety of ^{2}H-labeled linolenic acids [39]. Incubation of AA with PFI in the presence of deuterium oxide showed that the new hydrogen, which is added to C-12, derives from the solvent. The regiospecificity and stereospecificity of hydrogen transfers involved in the conversion of γ-linolenate to its corresponding conjugated triene, 6(*Z*),8(*E*),10(*E*)-octadecatrienoic acid (**11**), was studied by following the fate of deuterium ions in the PFI metabolism of (11*S*)-γ-[11-^{2}H] linolenate, (11*R*)-γ-[11-^{2}H] linolenate, (8*S*)-γ-[11-^{2}H] linolenate, and (8*R*)-γ-[11-^{2}H] linolenate. These experiments identified that the *pro*-(*R*) hydrogen at C-8 in linolenate is lost to the solvent, whereas the *pro*-(*S*) hydrogen at C-11 is intramolecularly transferred to the C-13 position (Scheme 1). The PFI enzyme was also able to convert anandamide to a conjugated triene derivative, which retained high-affinity binding characteristics to the cannabinoid receptor [40].

3.1.2 Polycyclic or Halogenated Oxylipins

Hybridalactone from Laurencia hybrida

Hybridalactone (Scheme 2, **12**) is a novel oxylipin originally isolated from the red alga *Laurencia hybrida* collected from Great Britain. The unique structural elements of this

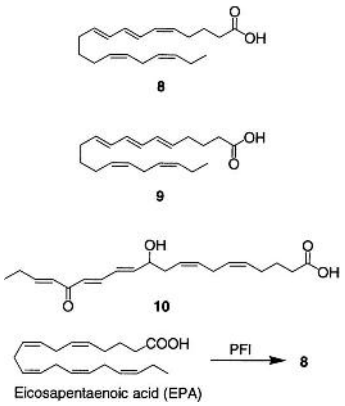
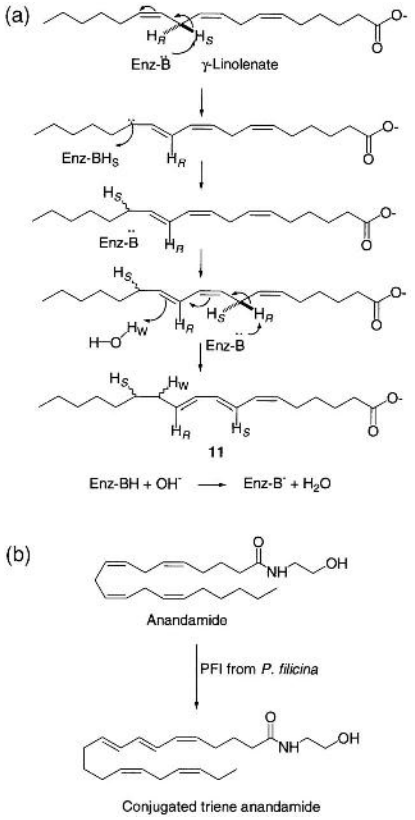


Figure 4 Structures of conjugated polyenes from the red alga *Ptilota filicina*.

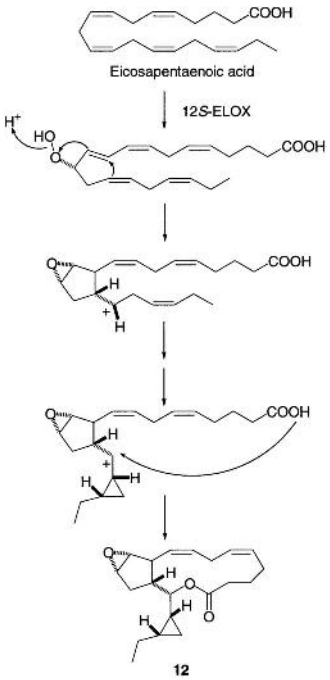


Scheme 1 Biosynthesis of (a) conjugated triene from the red alga *Ptilota filicina* and (b) conjugated triene anandamide using a purified enzyme from *P. filicina*.

compound are cyclopropyl, cyclopentyl, epoxide, and macrolactone rings. The structure of hybridalactone was established from spectral data and the relative stereochemistry deduced by analysis of ^{1}H-NMR coupling constant data [41]. Although superficially resembling a mammalian prostanoid, the position of the rings and other functional groups clearly indicated that metabolite **12** had a different biosynthetic origin than the mammalian substances. An insightful biosynthetic proposal by Corey and co-workers (Scheme 2) envisioned 12-ELOX-initiated oxidation of EPA, followed by cationic formation of the epoxide and subsequent carbocyclization to a cyclopentyl allylic cation. This allylic carbocation enters a cyclopropyl–cyclobutyl–cyclopropyl manifold to give the C-15 cation, which is then quenched by lactonization [42]. This biogenetic proposal predicts, beginning with the 12(*S*)-hydroperoxide, a 10(*S*),11(*R*),12(*S*),14(*R*),15(*S*),16(*R*),17(*S*) absolute stereochemistry for hybridalactone. Subsequently, this stereochemical proposal was confirmed by a stereospecific synthesis of metabolite **12** [43].

Agardhilactone from Agardhiella subulata

Agardhilactone (Fig. 1 **2**), isolated from the Atlantic red alga *A. subulata*, is a unique oxylipin in that it possesses a cyclopentyl ring at an unprecedented position (C-6 to C-10) of the 20-carbon chain (compare with the C-10–C-14 connection in **12**). Moreover,

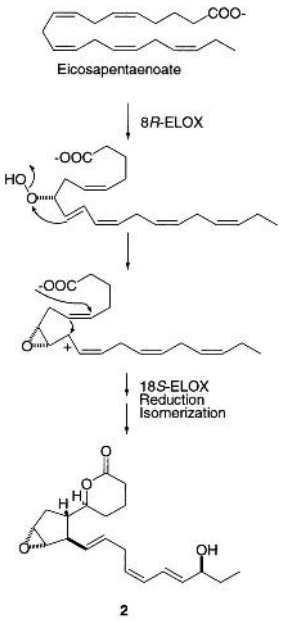


Scheme 2 Biogenesis of hybridalactone from the red alga *L. hybrida*.

its highly functionalized skeleton contains a cyclopentyl ring, an epoxide ring, a δ-lactone, a conjugated diene, and a secondary alcohol. The structure of **2** was largely established by NMR spectral studies of its acetate derivative, principally the double quantum filter (DQF) proton correlation spectroscopy (^{1}H-^{1}H-COSY) experiment which revealed a single extended spin system (H-2–H-20) and an isolated acetate group. The absolute stereochemistry of the C-18 hydroxyl was determined by gas chromatography–mass spectrometry (GC–MS) analysis of the fragment produced via formation of the (−)-menthoxycarbonyl derivative, ozonolysis of the C-16–C-17 olefinic bond, and methylation with CH_2N_2. The locations of the epoxide, cyclopentyl, and lactone rings suggested a biogenesis for agardhilactone involving the intermediacy of an epoxy allylic carbocation [15]. In this proposal, initial LOX oxidation occurs at C-8 of EPA with ensuing reaction with the C-9–C-10 olefin to form a C-8–C-9 epoxide and C-10 carbocation. In turn, under enzyme control, it is proposed to induce both cyclopentyl and lactone ring formation (Scheme 3). Introduction of the ω3 alcohol may occur via action of an 18*S*-ELOX; reduction of the hydroperoxide and isomerization of the C-11–C-12 double bond completes the biogenesis of agardhilactone (**2**).

3.1.3 Yendolipin, a Betaine from *Neodilsea yendoana*

Yendolipin (Fig. 5, **13**), a novel oxylipin isolated from the red alga *Neodilsea yendoana*, is an inhibitor of morphogenic restoration in the foliaceous green alga *Monostroma oxyspermum*. This latter alga is known to lose its typical foliaceous morphology under axenic conditions; this can be restored upon reinfection by suitable marine bacteria or the culture media of some species of macroalgae. Yendolipin was isolated in a bioassay directed



Scheme 3 Biogenesis of agardhilactone from the red alga *A. subulata*.

process from the growth media of *N. yendoana* cultures [44]. The ^{1}H-NMR of this highly polar lipid exhibited signals due to 16 olefinic protons, a trimethylamino group, and several envelopes of high-field aliphatic, allylic, and bis-allylic protons. The very polar nature of **13**, in combination with its lipid structural features and the presence of a trimethylammonium cation, suggested that yendolipin contained a betaine moiety. Methanolic hydrolysis of **13** produced EPA methyl ester (**14**), an amide (**15**) containing an N^5-trimethylornithine betaine moiety, 3-hydroxyeicosatrienoic acid methyl ester (**16**), and N^5-trimethylornithine betaine (**17**) (Fig. 5). EPA was apparently attached to the free hydroxy group in amide **15** as the only conceivable free site for esterification. The absolute stereochemistry at C-2 of the N^5-trimethylornithine betaine group was determined as (*S*) by a comparison of the optical rotation of **17** with a synthetic sample. The absolute configuration of the C-3′ hydroxy group, which was erroneously reported in an earlier article as (*S*), was finally established as (*R*) by NMR analysis of the Mosher's esters of **16**, as well as by comparison of the negative $[\alpha]_D$ of perhydro **16** with model compounds. The earlier erroneous assignment was attributed to the presence of an impurity of unknown structure.

3.1.4 Mammalian-Type Prostaglandins from *Gracilaria* sp.

Although prostaglandins have long been known to occur in animals, their presence in plants was only first detected in the red alga *Gracilaria lichenoides* in 1979. Prostaglandins PGE_2 (**18**) and $PGF_{2\alpha}$ (**19**) (Fig. 6) were isolated from aqueous extracts of *G. lichenoides* as antihypertensive compounds when administered intravenously to hypertensive rats [45]. However, the final purification of these two substances could only be achieved after esterification to methyl ester derivatives with diazomethane. That these two prostaglandins were actually produced by the alga was indicated by the absence of any visible symbionts or

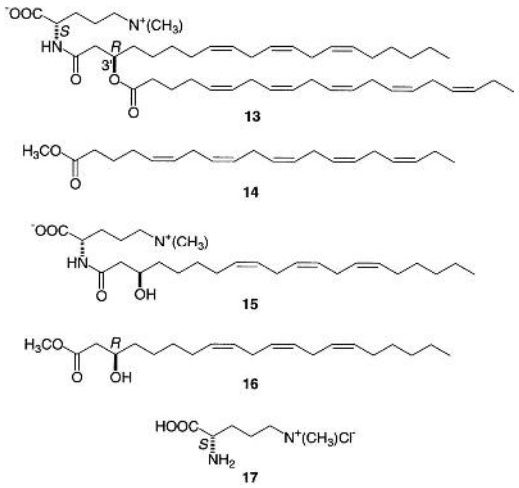


Figure 5 Structures of yendolipin and its degradation products from the red alga *Neodilsea yendoana*.

contaminants on the surface of the alga; it is conceivable that these substances serve adaptive roles by inhibiting the growth of potentially fouling or predatory organisms.

Prostaglandin **18** was also isolated from Japanese red alga *Gracilaria verrucosa* as a result of studies aimed at determining the cause of a human intoxication syndrome known as ''Ogonori poisoning'' [46]. Initially, high amounts of inorganic salts were cited as a reason for this poisoning following ingestion of *G. verrucosa*, but this was contradicted by the symptoms, which included severe diarrhea, and the fact that the algal specimens are typically soaked overnight in fresh water before ingestion. Prostaglandins A_2 (**20**) and **18** were subsequently isolated from the prepared algal specimens and identified as the causative chemicals using a mouse toxicity assay; prostaglandin **18** was the more potent toxin. Prostaglandin **18** is known to produce adverse physiological effects in mammals, some of which are similar to the symptomology of ''Ogonori poisoning'' at doses as low as 10^{-9} *M*. It is believed that soaking of the alga overnight, a process thought to

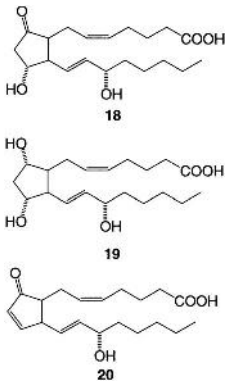


Figure 6 Structures of prostaglandins from the red algae *Gracilaria* spp.

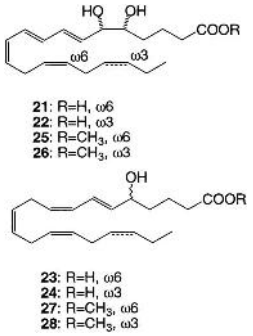


Figure 7 Structures of oxylipins from the red alga *Rhodymenia pertusa*.

remove naturally occurring prostaglandin synthesis inhibitors, is necessary to stimulate **18** production in the algal tissue. The identities of these putative prostaglandin biosynthesis inhibitors remain unknown, but could be a source of fruitful investigation in the future.

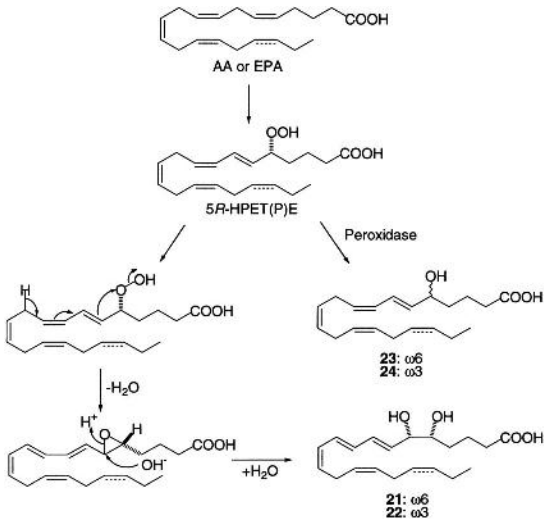
3.1.5 Oxylipins from *Rhodymenia pertusa*

Four oxylipins (Fig. 7, **21–24**) were recently isolated as their methyl esters (**25–28**) from the temperate marine red alga *Rhodymenia pertusa* [47]. The structure of derivative **25** was readily determined by ^{1}H-NMR because all of the connectivities of the C-2 to C-20 spin system were completely revealed by ^{1}H-^{1}H-COSY. From UV measurements (λ_{max} = 260, 271, 286 nm), derivative **25** contained a conjugated triene; all six associated protons were well resolved such that their coupling constants could be accurately measured, thus establishing a trans, trans, cis geometry for the conjugated triene system. The absolute configuration of the 5,6-diol in **21**, prepared by saponification of **25**, was determined as 5(*R*),6(*S*) by a comparison of its optical rotation with that of the four possible stereoisomers produced by chemical synthesis [48]. The structures of the three other *R. pertusa* metabolites were also established by NMR analysis and by comparison with derivative **25**. Although the stereochemistries of these related metabolites were not examined, it was assumed that these have the same stereochemistry at the corresponding carbon atoms based on their co-occurrence in this alga (Fig. 7).

Metabolites **21–24** represent two series of ω6/ω3 pairs, both of which can be biogenetically derived from a 5*R*-ELOX acting on AA or EPA to produce the 5(*R*)-hydroperoxides (Scheme 4). Simple peroxidase reduction of this hydroperoxide would give oxylipins **23** and **24**, whereas formation of the conjugated triene and C-5,C-6-epoxide followed by cationic opening of the epoxide ring would yield the diol oxylipins **21** and **22**. The formation of these 5*R*-ELOX-derived eicosanoids is intriguing because very few nonmammalian oxylipins are known to be formed by a 5-ELOX-initiated pathway (however, see Section 3.4.1 for another marine example). An interesting feature of the known marine algal 5-ELOX products is that they possess 5(*R*) stereochemistry, a chirality opposite that observed in products produced by mammalian [49] and some other higher-plant 5-ELOXs [50].

3.2 Chlorophyta

Most oxylipins described to date from the Chlorophyta arise from C-18 substrates, principally α-linolenic and stearidonic acids. In most cases, enzymatic oxidation occurs via a



Scheme 4 Biogenesis of oxylipins from the red alga *Rhodymenia pertusa*.

9-OLOX or 13-OLOX on these substrates (ω10- or ω6-positions), although other sites of oxidation have also been reported [9,34].

3.2.1 Dictyosphaerin from *Dictyosphaeria sericea*

Collections of the tropical green alga *Dictyosphaeria sericea* from several locations in Australia yielded a novel oxylipin, dictyosphaerin (Fig. 8, **29**), as an unstable lipid. Its structure was established from mass spectrometry and 2D-NMR spectroscopy, as well as chemical derivatization [51]. The unique structural features of metabolite **29** are the presence of fused five-membered and six-membered rings. Neither the relative nor absolute stereochemistry of dictyosphaerin were determined. The biogenesis of dictyosphaerin is not obvious; it may involve carbocyclization of γ-linolenic acid to a cyclopentadiene-containing octadecanoid. This then may react via a biological equivalent of the Diels–Alder reaction with a 1,4-butadiene-like material to form the fused cyclohexene ring. Alternately, a C-22 fatty acid of unusual unsaturation pattern could be precursor to metabolite **29**. The presence of a C-6-hydroxyl mechanistically implies a Δ6 lipid, typical of C-18 PUFAs but unusual in C-22 PUFAs, providing further support for a non-fatty-acid origin of the cyclohexene ring. (However, see the discussion of the sponge metabolite mucosin (**70**) in Sec. 5.1.)

3.3 Phaeophyceae (Brown Algae)

Phaeophyceae (brown algae) are also a rich source of structurally unique oxylipins, most of which result from enzymatic oxidation of C-18 and C-20 PUFA precursors by a 13-

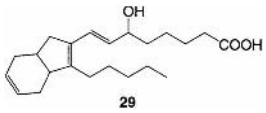


Figure 8 Structure of dictyosphaerin from the green alga *Dictyosphaeria sericea*.

OLOX/15-ELOX (ω6 LOX oxidation). The following examples illustrate this and subsequent biosynthetic rearrangements which give rise to various novel substances in the brown algae.

3.3.1 Ecklonialactones from *Ecklonia stolonifera* and Egregiachlorides from *Egregia menziesii*

The two related brown algae, *Ecklonia stolonifera* and *Egregia menziesii* from the Japanese and Oregon coasts, respectively, produce a number of similar oxylipin metabolites. Ecklonialactones A (**30**) and B (**31**) are C18 tricyclic compounds that were first isolated from *E. stolonifera* as metabolites with invertebrate antifeedant activity (Fig. 9). Their structures were established by spectral studies and x-ray diffraction analysis [28]. More recently, these same metabolites were isolated from *E. menziesii* and their absolute stereochemistry established by circular dichroic (CD) spectroscopy [52]. The epoxide ring of ecklonialactone A (**30**) was opened to a diol (**32**) from which a bis *p*-bromobenzoate derivative was prepared. Diols **32** and **33**, named ecklonialactone C and D, respectively, were also later isolated as natural products of *E. stolonifera* [29]. The CD spectrum of the benzoate derivative of **32** showed negative first and positive second Cotton effects, indicating that the two chromophores possessed a negative exciton chirality, establishing a 12(*R*),13(*R*) stereochemistry in diol **32** [correspondingly, epoxide **30** is of 12(*R*),13(*S*) stereochemistry]. The ecklonialactones exhibit an obvious structural similarity to hybridalactone (**12**), suggesting similar biogeneses. Ecklonialactone A is proposed to derive from action of an 13*S*-OLOX (ω6 position) on stearidonic acid (18:4ω3) which subsequently loses HO^- to produce the corresponding epoxy allylic carbocation. As for hybridalactone, this intermediate is proposed to induce carbocyclization from C-11–C-15, in turn generating a new carbocation at the ω3 position. This is quenched internally by the C-1 carboxylate to form the macrolactone ecklonialactone A (**30**), or in *Egregia menzeisii*, by Cl^- to form egregiachloride A (**34**) (Scheme 5). A similar sequence of reactions using α-linolenate (18:3ω3) as a substrate yields ecklonialactone B (**31**) or egregiachloride B (**35**), or using EPA and a 15*S*-ELOX, it yields ecklonialactone E (**36**) or egregiachloride C (**37**) [5,52].

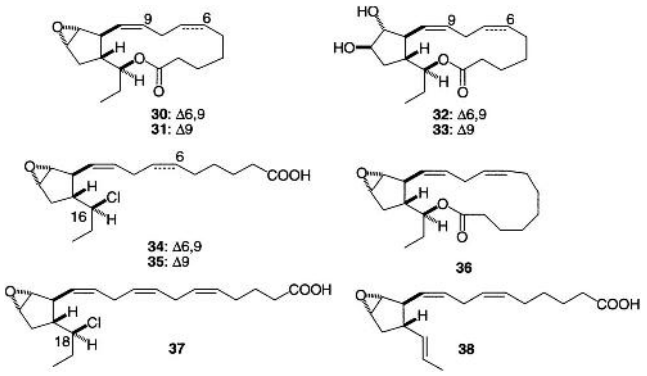
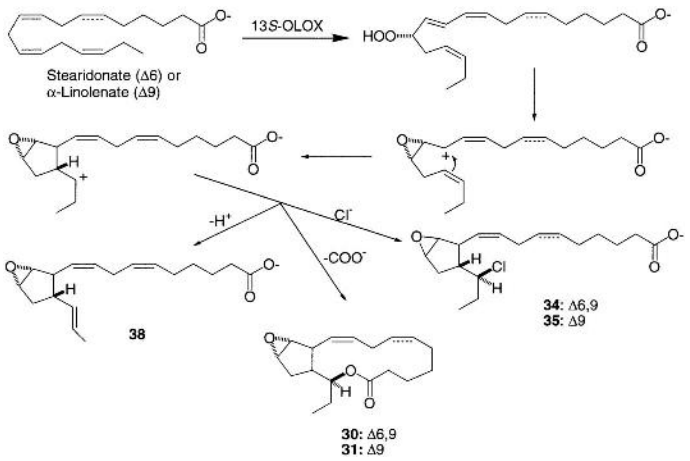


Figure 9 Structures of ecklonialactones and egregiachlorides from the brown algae *Ecklonia stolonifera* and *Egregia menziesii*, respectively.



Scheme 5 Biogenesis of ecklonialactones and egregiachlorides from the brown algae *Ecklonia stolonifera* and *Egregia menziessi*.

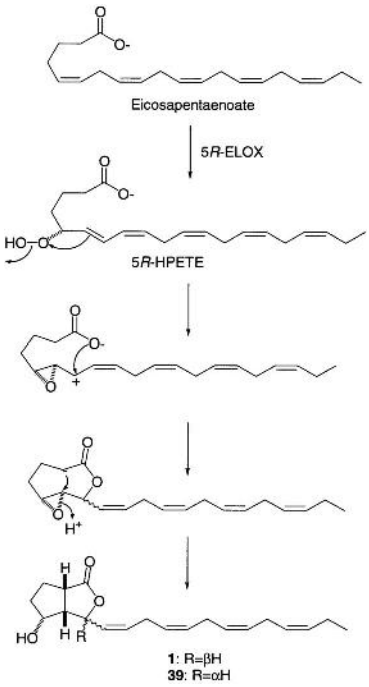
3.4 Microalgae

3.4.1 Bacillariolides from *Nitzschia pungens*

Bacillariolides I (**1**) and II (**39**) are novel oxylipins derived from the marine bacillariophyte *Nitzschia pungens* that contain two fused five-membered rings [14] (Fig. 1 and Scheme 6). This diatom species causes a human intoxication syndrome commonly known as amnesic shellfish poisoning (ASP) in which domoic acid, an unusual amino acid derivative, is the presumed toxin [53]. The structures of the bacillariolides were assembled from detailed NMR data, and their relative and absolute stereochemistries were deduced from an x-ray crystallographic study of a chiral derivative of **1** [54]. The bacillariolides are unique in the location of their cyclopentane ring (C-2 to C-6). The biogenesis of the bacillariolides has been proposed to be initiated by an eicosanoid 5*R*-ELOX, the product of which is transformed into the corresponding epoxy allylic carbocation (Scheme 6) [10]. The carbocation could be quenched by the carboxylate anion to form the lactone ring, and abstraction of an acidic C-2 hydrogen would yield a C-2 anion that could attack at C-6 of the epoxide to yield the bacillariolides. However, the order of these putative reactions is completely uncertain.

3.4.2 Biosynthesis of the Algal Pheromone Hormosirene

Simple C-11 hydrocarbons such as hormosirene (**5**), fanavarrene (**40**), multifidene (**41**), and ectocarpene (**42**) (Fig. 10) are used by marine brown algae and diatoms as male-attractant sex pheromones [55–57]. Interestingly, several of these same compounds are produced in the higher plant *Senecio isatidens*; however, in this plant, they are produced by a completely different biosynthetic route than that used by algae [58,59]. For example, the precursor of **40** and **42** in the higher plant is 3,6,9-dodecatrienoic acid that is subsequently decarboxylated, whereas in algae, the precursor is EPA that is cleaved by an intriguing hydroperoxide lyase reaction (Fig. 10).



Scheme 6 Biogenesis of bacillariolides from the diatom *Nitzschia pungens*.

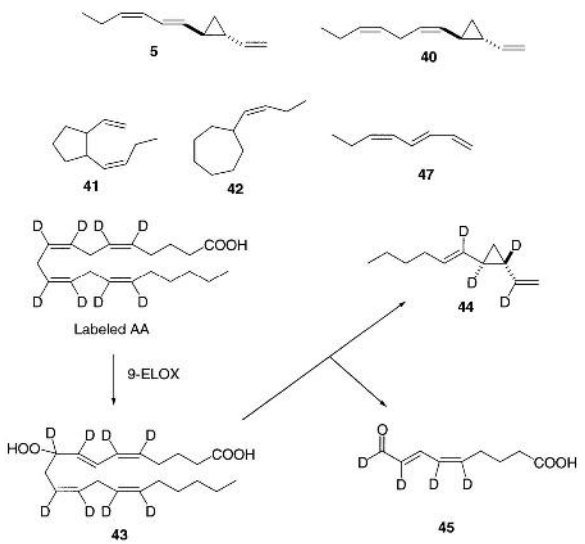
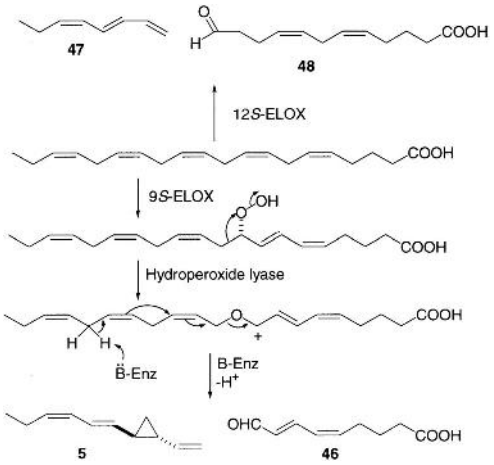


Figure 10 Structures of several algal pheromones.

The biosynthesis of algal pheromone **5** was studied in the freshwater diatom *Gomphonema parvulum* [18]. Treatment of a crude cell-free extract with deuterium labeled [5,6,8,9,11,12,14,15-^{2}H]-AA (**43**) yielded ^{2}H-labeled dictyopterene A (**44**) and 9-oxo-nona-5(*Z*),7(*E*)-dienoic acid (**45**). The presence of four deuterium atoms in **44** suggested that it was the C-10–C-20 fragment that was incorporated into dictyopterene. Through additional incorporation studies, it was proposed that EPA undergoes a 9*S*-ELOX-catalyzed peroxidation which is then speculated to undergo a hydroperoxide-lyase-induced Hock–Criegee rearrangement to generate an oxy-carbenium ion. Loss of a proton from C-16 and subsequent electon migrations results in hormosirene and the ω-oxoacid (**46**) (Scheme 7). Similar recent studies in the diatom *Asterionella formosa* have shown that a 12*S*-ELOX initiated the pathway by forming a 12(*S*)-hydroperoxide which was subsequently acted on by a hydroperoxide lyase to yield, following electron migrations, the algal pheromone fucoserratene (**47**) and oxoacid **48** [60].

3.4.3 Biosynthesis of Furan Fatty Acids

Furan fatty acids (F-acids) were first discovered in freshwater fish by Glass et al. [61] and later shown to be widely distributed in soft corals [62], crayfish [63], and many other marine fishes. F-Acids derive either from linoleic acid to yield a furan possessing a pentyl side chain or from linolenic acid, yielding a furan with a propyl side chain. It was reported that fish cannot synthesize F-acids de novo (no isotopic label was found in the furan ring or its alkyl substituents upon feeding labeled acetate) and that these acids are produced only in plants which are then subsequently incorporated into animals, including man, through nutritional routes [64–66]. Biosynthetic studies on F-acids in the diatoms *Isochrysis* sp. and *Phaeodactylum tricornutum* have shown that the first step in the pathway is the formation of 13-hydroperoxylinoleic acid by the action of a 13-OLOX (stereospecificity unknown) [67]. The resulting hydroperoxide cyclizes in a hydroperoxide dehydrase step, much like that seen in formation of allene oxides [50]; this is followed by double-bond migrations to yield a furan. Consistent with this proposal, the furan oxygen was shown to derive from atmospheric O_2, and the furan substituent methyl groups were shown to come from *S*-adenosylmethionine [68,69].



Scheme 7 Biosynthesis of the algal pheromones hormosirene and fucoserratene.

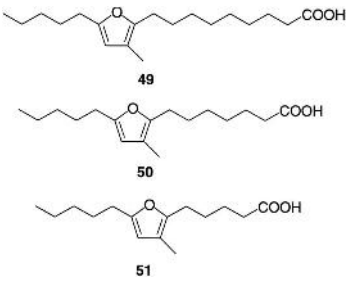


Figure 11 Structures of F-acids from marine bacteria.

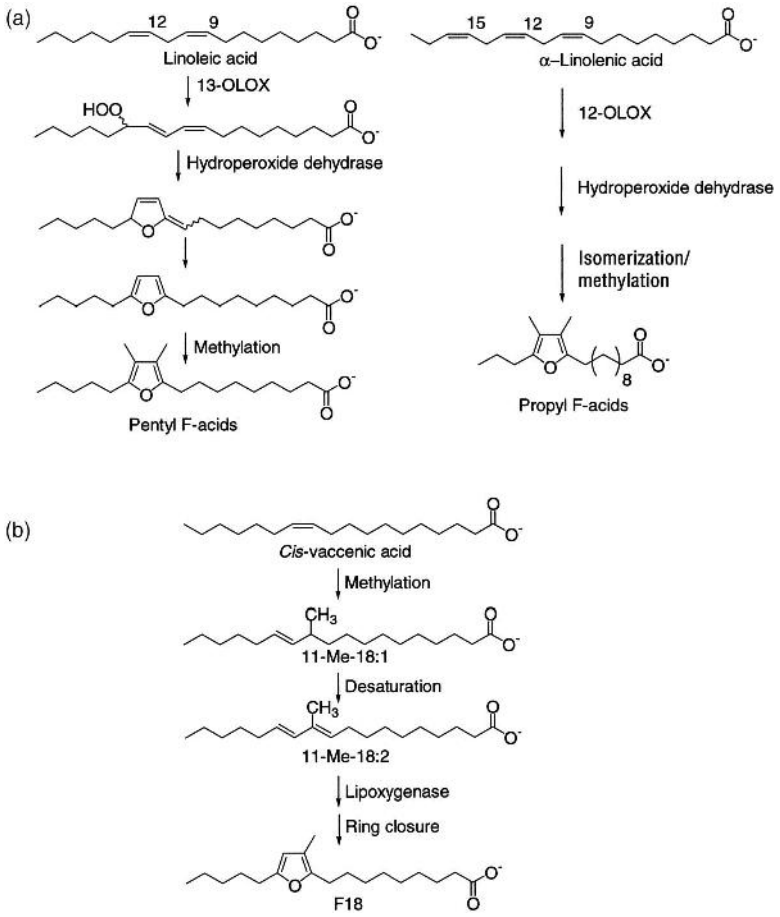
Recent detection of F-acids in different strains of marine bacteria isolated from fish has established that these oxylipins are produced not only by plants and algae but also by enteric bacteria [70]. Because most of the F-acid-producing bacterial strains do not produce linoleic acid, a different biosynthetic pathway has been envisaged for bacterial F-acids. A mutated form of the marine bacterium *Shewanella putrefaciens* 8CS7-4 was found to produce large quantities of 10,13-epoxy-11-methyloctadeca-10,12-dienoic acid (**49**) as a major F-acid, together with minor quantities of 8,11-epoxy-9-methylhexadeca-8,10-dienoic acid (**50**, F16), 6,9-epoxy-7-methyltetradeca-6,8-dienoic acid (**51**, F14), and the unsaturated fatty acids 11-me-12(*E*)-octadecenoic acid (11-Me-18:1) and 11-methyl-10(*E*), 12(*E*)-octadecadienoic acid (11-Me-18:2). Interestingly, these various unusual lipid species were predominately located at the *sn*-1 position of phospholipids (Fig. 11).

Changes in F-acid cellular levels gave some insight into their putative biosynthesis [71]. The amount of 11-Me-18:1 reached a maximum at 12 hr and then decreased rapidly and was followed by an increase in the amount of 11-Me-18:2, which reached a maximum after 24 hr. Following this, F-acid **49** increased, reaching a plateau at 36 hr, a point when neither 11-Me-18:1 nor 11-Me-18:2 were present in detectable levels. Based on metabolite profiles in wild and mutant strains of *S. putrefaciens*, it was deduced that transfer of a methyl group from *S*-adenosyl-L-methionine to the double bond of *cis*-vaccenic acid yields lactobacillic acid (cy19:0) and 11-Me-18:1. It was further postulated that 11-Me-18:1 is desaturated to 11-Me-18:2 and that this is then oxidatively transformed, possibly involving a 13-OLOX, into F-acid **49**. A comparison of biosynthetic pathways for F-acids in plants and marine bacteria is presented in Scheme 8. The important differences are (1) the starting substrate is linoleic acid in plants and *cis*-vaccenic acid in marine bacteria, (2) the oxygen incorporation is the first step and methylation occurs after ring closure in plants, whereas methylation is the first step in marine bacteria and is followed by oxygen incorporation and ring closure.

4 EXAMPLES FROM MARINE CORALS

4.1 Clavirins from *Clavularia virdis*

Two unique carbocyclic oxylipins were recently isolated, clavirins I (**52**) and II (**53**) (Fig. 12) from the extensively investigated Okinawan soft coral *Clavularia virdis* [72]. As noted in the chapter overview, this coral is an extremely rich source of halogenated and oxidized oxylipins [2,73–77]. The new compounds are geometrical isomers with a shortened α-chain possessing a terminal aldehyde group. Their overall structures were deduced from



Scheme 8 Biosynthesis of F-acids in (a) the diatoms *Isochrysis* sp. and *Phaeodactylum tricornutum* and (b) the marine bacterium *Shewanella putrefaciens*.

2D-NMR and absolute configuration at C-7 determined by stereoselective total synthesis (Fig. 12). The C-2–C-3 double-bond geometry was determined by evaluating ^{1}H-NMR chemical shift values for the C-1 and C-2 protons in the two isomers. The clavirins are of obvious structural and biogenetic relationship, including their 7(*S*) stereochemistry, to the clavulones (**54**–**56**) and claviridenone (**57**), prostanoids previously reported from this species with potent antitumor activities [78,79]. Biosynthetic studies on the clavulones in *C. viridis* have indicated that they arise from 8*R*-ELOX metabolism of AA to produce 8(*R*)-HPETE [80]. In turn, this hydroperoxide is transformed by the coral to a cyclopentanoid product, preclavulone-A (**58**), whose close structural similarity to LOX products from higher plants 12-oxophytodienoic acid (**59**) and isojasmonic acid (**60**) [81] suggested similar transformations in the coral [82,83]. Hence, it was envisioned that LOX-derived 8(*R*)-HPETE is first transformed to an allene oxide which then opens up to an oxidopentadienyl cation intermediate that finally cyclizes to preclavulone-A (**58**) [83]. Further oxidations and acetylations of **58** would subsequently generate a variety of the clavulones. Oxidative cleavage of the C-5–C-6 double bond of clavulone III (**56**) or claviridenone-a

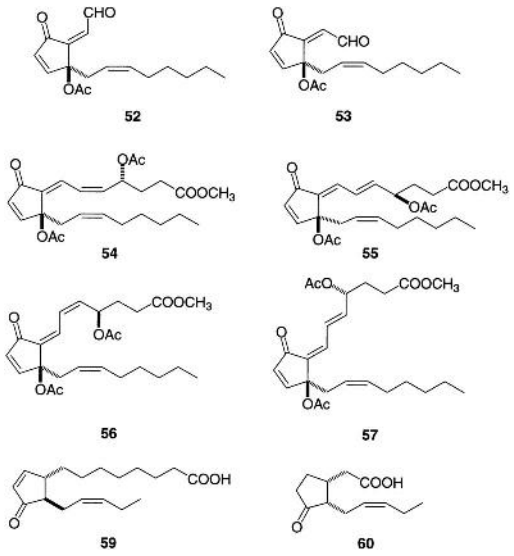
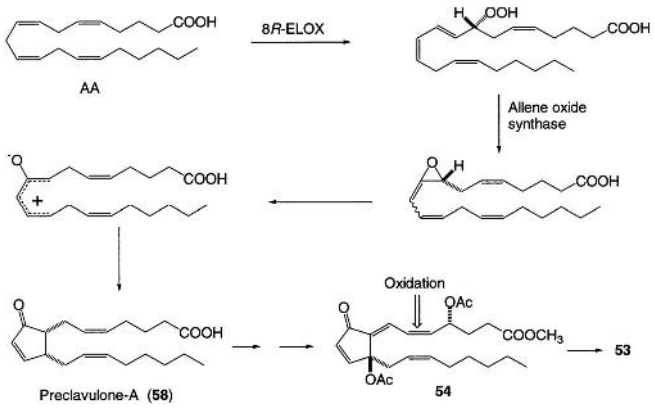


Figure 12 Structures of clavulones and related compounds from the marine coral *Clavularia virdis*.

(**57**) would generate clavirin I (**52**), whereas a similar cleavage of clavulone I or II (**54, 55**) would generate clavirin II (**53**) (Scheme 9).

4.2 Punaglandins from *Telesto riisei*

The punaglandins, related in structure to the clavulones, are biologically active oxylipins from the Hawaiian octocoral *Telesto riisei*. A total of 19 punaglandins have been isolated to date from this species, differing from each other in the oxygenation pattern at C-5, C-6, C-7, and C-12 and the geometry of the C-7–C-8 double bond [3]. Their structures (e.g., **61**–**66**) and relative stereoconfigurations were deduced from NMR data and absolute



Scheme 9 Biogenesis of the clavirins from the marine coral *Clavularia virdis*.

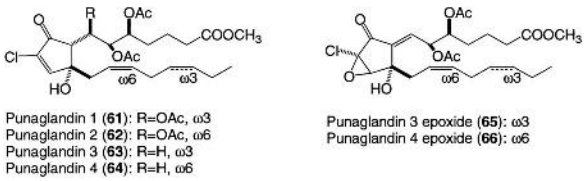
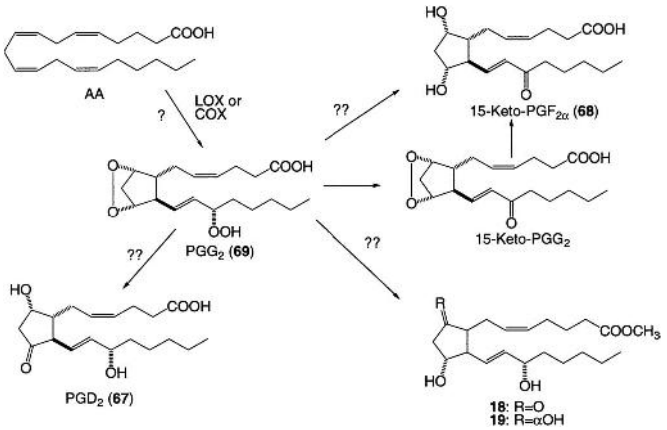


Figure 13 Structures of punaglandins from the marine coral *Telesto riisei*.

stereoconfigurations determined by synthetic studies (Fig. 13). The unique structural feature of these compounds is the presence of a chlorine substituent at C-10. Curiously, the absolute stereochemistry at C-12 of the punaglandins is opposite to that found in the clavulones at the same C-12 stereocenter. The punaglandins have shown a wide spectrum of biological activities, including potent inhibition of cultured L1210 mouse leukemia cells (punaglandin 3) and Ehrlich ascites cells, antitumor activity, and anti-inflammatory effects [79,84]. Their biogeneses are believed to be similar to that proposed for the clavulones and clavirins described in Sec. 4.1.

4.3 Prostaglandin Biosynthesis in *Gersemia fruticosa*

The biosynthesis of prostaglandins in the White Sea soft coral *Gersemia fruticosa* is exceptional compared to that of other corals in that it appears to involve a mammalian-type prostaglandin (PG)-endoperoxide intermediate [85]. An acetone powder preparation of *G. fruticosa* converted exogenous radioactive AA into chiral PGE_2 (**18**), $PGF_{2\alpha}$ (**19**), PGD_2 (**67**), and 15-keto-$PGF_{2\alpha}$ (**68**). Analysis of endogenous coral metabolites showed a substantial amount of 8(*R*)-HETE and 11(*R*)-HETE in addition to the above four prostaglandins. Subsequently, the prostaglandin endoperoxide PGG_2 (**69**) was identified as an unstable product of the coral acetone powder preparation, and this was then converted to chiral prostaglandins [86]. However, because this coral pathway was not inhibited by indomethacin, it was speculated that either a novel cyclooxygenase (COX) isozyme or a novel LOX pathway is responsible for PG biosynthesis in *G. fruticosa* (Scheme 10).



Scheme 10 Biosynthesis of prostaglandins from the soft coral *Gersemia fruiticosa*.

5 EXAMPLES FROM SPONGES

5.1 Mucosin from *Reniera mucosa*

A new bicyclic oxylipin, mucosin (**70**), was recently isolated from the polar fraction of an acetone extract of the Mediterranean sponge *Reniera mucosa* (Fig. 14) [87]. The presence of mucosin in the extract was revealed by a characteristic chromatic reaction on spraying the thin-layer chromatogram (TLC) plate with cerium sulfate. The structure of **70** was elucidated by a normal interplay of spectral features obtained for the methyl ester derivative of mucosin and is closely related to the green algal metabolite dictyosphaerin (**29**), isolated from *Dictyosphaeria sericea* [51] (see Sec. 3.2.1). The (*E*) geometry of the C-5–C-6 double bond was determined from the downfield ^{13}C-NMR resonance of C-4 and observation of the nuclear Overhauser effect (NOE) between H_2-7 and H-5. Nuclear Overhauser effect spectroscopy (NOESY) and rotating Overhauser effect spectroscopy (ROESY) data were used to establish the relative stereochemistry at C-8, C-9, C-14, and C-16. It has been proposed that mucosin may be formed from AA by intramolecular cycloaddition involving double bonds at C-8–C-9 and C-14–C-15, followed by isomerization of the C-5–C-6 double bond. However, it is unclear from this proposal how the C-8–C-16 bond forms. Alternatively, the biogenesis of mucosin might involve cycloaddition of a C-4 unit onto a carbocyclized C-16 fatty acid. The specific involvement of LOX or other oxidative enzymes in these processes is not apparent.

5.2 Amphimic Acid from *Amphimedon* sp.

Two novel unsaturated C-28 fatty acids, amphimic acid A (**71**) and B (**72**) were isolated from an Australian sponge *Amphimedon* sp. (Fig. 14). The structures of these unique cyclopropylidene-containing compounds were deduced from NMR analysis coupled with chemical degradation studies [88]. The geometry of the C-5 double bond was determined as (*Z*) from a 10.6-Hz coupling constant, whereas that of C-9 was found to be (*E*) from observation of a NOESY correlation between H-8 and H-11. The 12(*R*) absolute configuration was established by enantioselective synthesis of amphimic acid A (**71**). Both amphimic acid A and B showed inhibitory activity against DNA topoisomerase I (A: LC_{50} = 0.47 μ*M*; B: LC_{50} = 3.2 μ*M*).

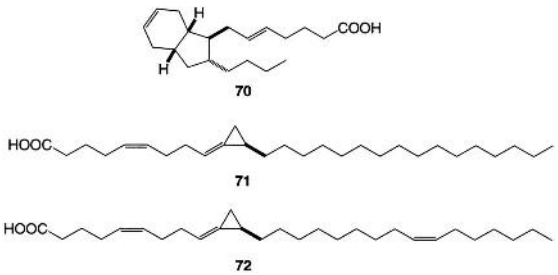
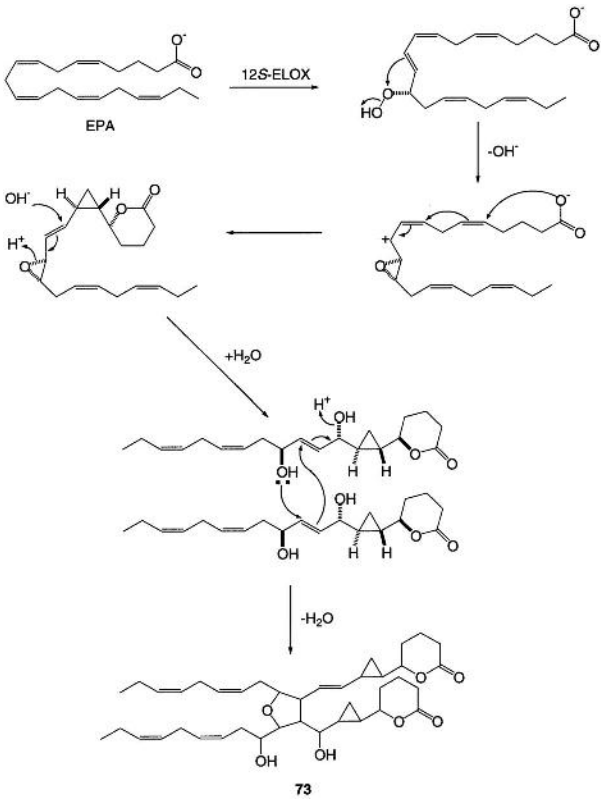


Figure 14 Structures of mucosin and amphimic acids from the sponges *Riniera mucosa* and *Amphimedon* sp.

6 EXAMPLES FROM MOLLUSCS

6.1 Aplydilactone from *Aplysia kurodai*

Aplydilactone (Scheme 11, **73**) is a highly unusual dimeric oxylipin isolated from the Japanese sea hare *Aplysia kurodai*. The gross structure of aplydilactone was assembled from several partial structures suggested by 2D-NMR spectral analysis and those obtained from chemical degradation studies [89]. The stereochemistry of both of the 1,2-disubstituted cyclopropane systems was deduced to be trans from coupling constant analysis. Because several other algal metabolites have previously been isolated from sea hares, it is conceivable that aplydilactone also has an algal origin. A biogenetic dissection of aplydilactone into two monomeric subunits yields structures that are very similar to the constanolactones (**6**) (Fig. 1), oxylipins previously isolated from the red alga *Constantinea simplex* [23]. Hence, aplydilactone may be formed by the unsymmetrical dimerization of two EPA-derived constanolactones, either enzymatically in the alga or the sea hare, or possibly nonenzymatically in the digestive gland of sea hare (Scheme 11). In turn, there is evidence that the constanolactones derive from the initiating action of a 12-ELOX on AA or EPA [23].



Scheme 11 Biogenesis of aplydilactone in the mollusc *Aplysia kurodai*.

7 CONCLUSIONS

The few examples given in Sections 4 and 5 of marine oxylipins with interesting structural features highlight the uniqueness of marine oxylipin metabolic pathways. Although reasonable biogenetic proposal can be put forth for some of these novel substances, this remains an area ripe for biosynthetic investigation, including mechanism, molecular genetics, and regulation.

However, the burning question in this field of marine oxylipin chemistry and biology is ''why''? Why are these substances being produced and why is the structural diversity so very great? Based on the striking structural diversity of marine oxylipins, it would seem reasonable that these compounds have adaptive functions wherein ''identity'' is a crucial attribute. In this regard, they may be produced as antipreditory compounds, or as chemical signals within a species, or perhaps between species. However, in only a few cases has a ecological purpose or function been ascribed to a marine oxylipin. For example, prostaglandin esters in the octacoral *Plexaura homomalla* appear to induce a ''learned adversion'' in potential predators by their emetic action [90]. In the hydrozoan *Hydra vulgaris*, hydroxy-containing oxylipins are believed to be involved in tentacle regeneration [27], control of metamorphosis [91], and feeding responses [92]. In some red algae, there is circumstantial evidence that oxylipins may be part of an osmoregulatory system [32].

Part of the problem is that the basic biology of many marine creatures is at a very primitive state such that we have problems working with these organisms, maintaining them in laboratory settings, or making critical insights into connections between their chemistry and biology. What is vitally needed in this field is for scientists with training in physiology and ecology to become aware of the extraordinary adaptation represented by these diverse marine oxylipins and, in concert with chemists and biochemists, to undertake the challenge to explore and define the ''raison d'être'' of marine oxylipin formation.

REFERENCES

1. WH Gerwick, M Moghaddam, M Hamberg. Arch Biochem Biophys 290:436–444, 1991.
2. K Iguchi, S Kaneta, K Mori, Y Yamada, A Honda, Y Mori. Tetrahedron Lett 26:5787–5790, 1985.
3. BJ Baker, PJ Scheuer. J Nat Prod 57:1346–1353, 1994.
4. K Iguchi, S Kaneta, K Mori, Y Yamada, A Honda, Y Mori. J Chem Soc Chem Commun 981–982, 1986.
5. JS Todd, PJ Proteau, WH Gerwick. Tetrahedron Lett 34:7689–7692, 1993.
6. WH Gerwick, MW Bernart, MF Moghaddam, ZD Jiang, ML Solem, DG Nagle. Hydrobiologia 204/205:621–628, 1990.
7. WH Gerwick, MW Bernart. In: DH Attaway, OR Zaborsky, eds. Marine Biotechnology. Volume 1, New York: Plenum Press, 1993, pp 101–152.
8. WH Gerwick, DG Nagle, PJ Proteau. Topics Curr Chem 167:117–180, 1993.
9. WH Gerwick. Biochim Biophys Acta 1211:243–255, 1994.
10. WH Gerwick. Lipids 31:1215–1231, 1996.
11. WH Gerwick. Chem Rev 93:1807–1823, 1993.
12. WH Gerwick. In: DHR Barton, K Nakanishi, O Meth-Cohn, eds. Comprehensive Natural Products Chemistry. Oxford: Elsevier, 1999, pp 207–254.
13. AF Rowley, H Kuhn, T Schewe. Eicosanoids and Related Compounds in Plants and Animals. Princeton, NJ: Princeton University Press, 1998.
14. R Wang, Y Shimizu. J Chem Soc Chem Commun 413–414, 1990.

15. MA Graber, WH Gerwick, DP Cheney. Tetrahedron Lett 37:4635–4638, 1996.
16. AR Brash, WE Boeglin, MS Chang, BH Shieh. J Biol Chem 271:20,949–20,957, 1996.
17. ZD Jiang, WH Gerwick. Lipids 32:231–235, 1997.
18. G Pohnert, W Boland. Tetrahedron 52:10,073–10,082, 1996.
19. AR Brash, SW Beartschi, CD Ingram, TM Harris. Adv Prostaglandin Thromb Leukotr Res 19:70–73, 1989.
20. DW Stanley-Samuelson. Am J Physiol 260:R849–R853, 1991.
21. MW Bernart, WH Gerwick. Tetrahedron Lett 29:2015–2018, 1998.
22. A Guerriero, MD' Ambrosio, F Pietra. Helv Chim Acta 73:2183–2189, 1990.
23. DG Nagle, WH Gerwick. J Org Chem 59:7227–7237, 1994.
24. ZD Jiang, WH Gerwick. Phytochemistry 30:1187–1190, 1991.
25. WC Song, DL Holland, KH Gibson, E Clayton, A Oldfield. Biochim Biophys Acta 1047: 239–246, 1990.
26. M Abe, M Klein, DJ Steel, A Thekkuveettil, E Shapiro, JH Schwartz, SJ Feinmark. Brain Res 683:200–208, 1995.
27. V DiMarzo, C Gianfrani, LD Petrocellis, A Milone, G Cimino. Biochem J 300:501–507, 1994.
28. K Kurata, K Taniguchi, K Shiraishi, N Hayama, I Tanaka, M Suzuki. Chem Lett 267–270, 1989.
29. K Kurata, T Taniguchi, K Shiraishi, M Suzuki. Phytochemistry 33:155–159, 1993.
30. MW Bernart, GG Whatley, WH Gerwick. J Nat Prod 56:245–259, 1993.
31. RA Barrow, RJ Capon. Aust J Chem 43:895–911, 1990.
32. WH Gerwick, MA Roberts, A Vulpanovici, DL Ballantine. In: S Nigam, P Asciak eds. Lipoxygenases and Their Metabolites. New York: Plenum Press, 1999, pp 211–218.
33. HW Gardner. Lipids 14:208–211, 1979.
34. WH Gerwick, PJ Proteau, DG Nagle, ML Wise, ZD Jiang, MW Bernart, M Hamberg. Hydrobiologia 260/261:653–665, 1993.
35. P Pohl, F Zurheide. In: HA Hoppe, T Levring, Y Tanaka, eds. Marine Algae in Pharmaceutical Science. Berlin: Walterole Gruyter, 1979, pp 473–523.
36. B Carte, DJ Faulkner. Tetrahedron Lett 23:3863–3866, 1982.
37. A Lopez, WH Gerwick. Lipids 22:190–194, 1987.
38. A Lopez, WH Gerwick. Tetrahedron Lett 29:1505–1506, 1988.
39. ML Wise, M Hamberg, WH Gerwick. Biochemistry 33:15,223–15,232, 1994.
40. ML Wise, K Soderstrom, TF Murray, WH Gerwick. Experientia 52:88–92, 1995.
41. MD Higgs, LJ Mulheirn. Tetrahedron 37:4259–4262, 1981.
42. EJ Corey, B De, JW Ponder, JM Berg. Tetrahedron Lett 25:1015–1018, 1984.
43. EJ Corey, BJ De. J Am Chem Soc 106:2735–2736, 1984.
44. Y Matsuo, R Ishida, T Matsumoto, M Tatewaki, M Suzuki. Tetrahedron 53:869–876, 1997.
45. RP Gregson, JF Marwood, RJ Quinn. Tetrahedron Lett 20:4505–4506, 1979.
46. N Fusetani, K Hashimoto. Bull Jpn Soc Sci Fish 50:465–469, 1984.
47. ZD Jiang, SO Ketchum, WH Gerwick. Phytochemistry 53:129–133, 2000.
48. C Kugel, JP Lellouche, JP Beaucourt, G Neil, JP Girard, JC Rossi. Tetrahedron Lett 30:4947–4950, 1989.
49. T Shimizu, O Rådmark, B Samuelsson. Proc Natl Acad Sci USA 81:689–693, 1984.
50. HW Gardner. Biochim Biophys Acta 1084:221–239, 1991.
51. SJ Rochfort, R Watson, RJ Capon. J Nat Prod 59:1154–1156, 1996.
52. JS Todd, PJ Proteau, WH Gerwick. J Nat Prod 57:171–174, 1994.
53. Atlantic Research Laboratory Technical Report 57 NRCC29086, Atlantic Research Laboratory, NRC, Halifax, Canada, 1988.
54. R Wang, Y Shimizu, JR Steiner, J Clardy. J Chem Soc Chem Commun 379–381, 1993.
55. I Maier, DG Muller. Biol Bull 170:145–175, 1986.
56. J Maier. Plant Cell Environm 16:891–907, 1993.
57. W Boland. Proc Natl Acad Sci USA 92:37–43, 1995.

58. W Boland, K Mertes. Eur J Biochem 117:83–91, 1985.
59. K Stratmann, W Boland, DG Muller. Angew Chem Intl Ed 31:1246–1248, 1992.
60. M Hombeck, W Boland. Tetrahedron 54:11033–11042, 1998.
61. RL Glass, TP Krick, AE Eckhardt. Lipids 9:1004–1008, 1974.
62. A Groweiss, Y Kashman. Experientia 34:299, 1978.
63. K Ishii, H Okajima, T Koyamatsu, Y. Okada, H Watanabe. Lipids 23:694–700, 1988.
64. DM Sand, RL Glass, DL Olson, HM Pike, H Schlenk. Biochim Biophys Acta 793:429–434, 1984.
65. CP Gorst-Allman, V Puchta, G Spiteller. Lipids 23:1032–1036, 1988.
66. DM Sand, H Schlenk, H Thoma, G Spiteller. Biochim Biophys Acta 751:455–461, 1983.
67. A Batna, J Scheinkonig, G Spiteller. Biochim Biophys Acta 1166:171–176, 1993.
68. A Batna, G Spiteller. Liebigs Ann Chem 9:861–863, 1991.
69. J Scheinkonig, G Spiteller. Liebigs Ann Chem 5:451–453, 1991.
70. N Shirasaka, K Nishi, S Shimizu. Biochim Biophys Acta 1258:225–227, 1995.
71. N Shirasaka, K Nishi, S Shimizu. Biochim Biophys Acta 1346:253–260, 1997.
72. M Iwashima, K Okamoto, K Iguchi. Tetrahedron Lett 40:6455–6459, 1999.
73. H Nagaoka, K Iguchi, T Miyakoshi, N Yamada, Y Yamada. Tetrahedron Lett 27:223–226, 1986.
74. H Kikuchi, Y Tsukitani, K Iguchi, Y Yamada. Tetrahedron Lett 23:5171–5174, 1982.
75. M Kobayashi, T Yasuzawa, M Yoshihara, H Akutsu, Y Kyogoku, I Kitagawa. Tetrahedron Lett 23:5331–5334, 1982.
76. M Kobayashi, T Yasuzawa, M Yoshihara, BW Son, Y Kyogaku, I Kitagawa. Chem Pharm Bull 31:1440–1443, 1983.
77. H Kikuchi, Y Tsukitani, K Iguchi, Y Yamada. Tetrahedron Lett 24:1549–1552, 1983.
78. A Honda, Y Yamamoto, Y Mori, Y Yamada, H Kikuchi. Biochim Biophys Res Commun 130: 515–523, 1985.
79. M Fukushima, T Kato, Y Yamada, I Kitagawa, S Kurozumi, PJ Scheuer. Proc Am Assoc Cancer Res 26:980, 1985.
80. EJ Corey, MD Alarcao, SPT Matsuda, PT Lansbury Jr, Y Yamada. J Am Chem Soc 109: 289–290, 1987.
81. M Hamberg, HW Gardner. Biochim Biophys Acta 1165:1–18, 1992.
82. BA Vick, DC Zimmerman. Biochem Biophys Res Commun 111:470–477, 1983.
83. AR Brash. J Am Chem Soc 111:1891–1892, 1989.
84. M Suzuki, Y Morita, A Yanagisawa, BJ Baker, PJ Scheuer, R Noyori. J Org Chem 53:286–295, 1988.
85. K Varvas, I Jarving, R Koljak, A Vehemets, T Pehk, AM Muurisepp, U Lille, N Samel. Tetrahedron Lett 34:3643–3646, 1993.
86. K Varvas, R Koljak, I Jarving, T Pehk, N Samel. Tetrahedron Lett 35:8267–8270, 1994.
87. A Casapullo, G Scognamiglio, G Cimino. Tetrahedron Lett 38:3643–3646, 1997.
88. T Nemoto, M Ojika, Y Sakagami. Tetrahedron Lett 38:5667–5670, 1997.
89. M Ojika, Y Yoshida, Y Nakayama, K Yamada. Tetrahedron Lett 31:4907–4910, 1990.
90. DJ Gerhart. Am J Physiol 260:R839–R843, 1991.
91. T Leitz, H Beck, M Stephan, WD Lehmann, D Petrocellis, V DiMarzo. J Exp Zool 269:422–431, 1994.
92. P Pierobon, LD Petrocellis, R Minei, V DiMarzo. Cell Mol Life Sci 53:61–68, 1997.

14

Formation of Oxygenated Fatty Acids by Fungal Enzymes

ERNST H. OLIW and CHAO SU

Uppsala University, Uppsala, Sweden

1 INTRODUCTION

It is well known that oxygenated derivatives of arachidonic, α-linolenic and linoleic acids, possess biological effects in mammals and plants [1–5]. These oxygenated fatty acids are formed by dioxygenases and by monooxygenases (cytochrome P-450s). Dioxygenases and monooxygenases are also present in fungi. The important role of oxygenated fatty acids in the biology of fungi has only emerged during the last two decades. Oxygenated fatty acids may affect fungal reproduction and pathogenicity, but their biological function is far from clear [6].

The fatty acid composition of fungi varies [7]. The C-18 and C-20 unsaturated fatty acids are of biological interest as precursors of signal molecules. Oxygenated arachidonic acid metabolites are important signaling molecules in mammals, and oxygenated linoleic and linolenic acids may serve a similar role in many fungi and in plants. Ascomycetes and basidiomycetes usually contain high levels of C-18 fatty acids and only low levels of C-20 fatty acids, but arachidonic and eicosapentaenoic acids occur in large amounts in oomycetes and in true fungi (see Ref. 7 for a review).

In this chapter, we will first describe some important fungal cytochrome P-450s. We will then discuss two groups of dioxygenases: lipoxygenases with a nonheme metal center and dioxygenases with heme. The latter appears to be evolutionary, related to important mammalian dioxygenases (cyclooxygenases). Only a selected number of fungi will be discussed, but it seems likely that many pathways may be found in other species. Fungal oxygenation of fatty acids may become important tools for biosynthesis of signal molecules. Stereospecific hydroxylation of progesterone by fungal enzymes was

developed in the 1950s by the pharmaceutical industry for cost-effective production of steroids [8].

2 FATTY ACID MONOOXYGENASES

2.1 Cytochrome P-450 and Fatty Acid ω-Hydroxylases

Cytochrome P-450 designates a group of heme-thiolate enzymes, which absorbs light at 450 nm after reduction and treatment with carbon monoxide. These enzymes occur in the endoplasmic reticulum and in the mitochondrion, but only the enzymes of the endoplasmic reticulum oxidize fatty acids and will be discussed here. The characteristic light-absorption property is due to the heme group with its ferric iron bound to a cysteine residue. Cytochrome P-450 is present in mammals, plants, fungi, and bacteria [9]. Partial or complete sequences of about 500 cytochrome P-450s have been published. All cytochrome P-450s seem to belong to the same gene superfamily, which obviously has a long history of evolution. The ancestral gene may have existed 3.5 billion years ago [9]. Recent information on the cytochrome P-450 gene family can be conveniently obtained from the Internet (e.g., http://www.icgeb.trieste.it/p450/or http://drnelseon.utmem.edu/homepage.html) or from excellent reviews (Ref. 9 and references therein). The fungal cytochrome P-450s (CYP) are found in the CYP51–CYP66 gene families. Two excellent reviews of fungal cytochrome P450s were published recently [10,11].

Oxygenation of fatty acids and other substrates occurs by cytochrome P-450 according to the formula

$$RH + O_2 + NADPH + H^+ \rightarrow ROH + H_2O + NADP^+$$

Electrons are transferred from NADPH (or NADH in a few cases) to cytochrome P-450s in the endoplasmic reticulum via a second enzyme, the cytochrome P-450 reductase. During the oxidation, one atom of oxygen will be incorporated in the substrate and the other reduced to water. The three-dimensional (3D) structures of four soluble prokaryotic enzymes have been reported: P-450cam (CYP101) of *Pseudomonas putida*, P450BM-3 (CYP102) of *Bacillus megaterium*, P450terp (CYP108) of a *Pseudomonas* species, and P450eryF of *Saccharopolyspora erythrea* [12–15]. It seems likely that eukaryotic cytochrome P-450s may have a similar 3D structure although their amino acid sequences are not well conserved [16]. In two cases, the fatty acid ω-hydroxylases of *Bacillus megaterium* (CYP102) and *Fusarium oxysporum*, the cytochrome P-450 and the cytochrome P-450 reductase have been fused into one protein. The fusion proteins have remarkably higher turnover numbers.

Cytochrome P-450s have traditionally often been studied with fatty acids as substrates. Verkade et al. [17] described ω-oxidation of saturated fatty acids in mammals in vivo in the 1930s. About 20 years later, liver microsomes were found to catalyze ω1- and ω2-hydroxylation of fatty acids in the presence of NADPH and molecular oxygen [18–23]. The rate-limiting step in hydroxylation of deuterium-labeled laurate was found to be hydrogen abstraction [24,25]. The position of the hydroxyl group was the same as that of the removed hydrogen. These observations indicated that a short-lived carbon-centered radical was formed during cytochrome P-450 catalysis.

Already in 1964, cytochrome P-450 was detected in baker's yeast (*Saccharomyces cerevisiae*) [26]. Now, when the entire genome of *S. cerevisiae* is sequenced, we know

that this organism contains only relatively few cytochrome P-450 genes and none of them are known to metabolize fatty acids.

Fungal oxygenation of arachidonic acid was reported a few years later [27]. Arachidonic acid was metabolized by a fungal root pathogen of wheat, the take-all fungus *Gaeumannomyces graminis*, to 18- and 19-hydroxyarachidonates, apparently by ω2- and ω3-hydroxylations [24,25].

Mammalian cytochrome P-450 can metabolize saturated fatty acids by terminal or subterminal ω-hydroxylations and unsaturated fatty acids (e.g., linoleic acid) in three additional ways [4,28]. Linoleic will thus be metabolized by the following:

1. Hydroxylation of carbons of the ω side of the fatty acid carbon chain to 18-hydroxylinoleic acid (18-HODE), 17-HODE, 16-HODE, and so forth
2. Epoxidation of double bonds to 9,10-epoxyoctadecenoic and 12,13-epoxyoctadecenoic acids
3. Hydroxylation of the bisallylic C-11 to 11-hydroxylinoleic acid (11-HODE)
4. Hydroxylation with double-bond migration to 9-hydroxy-10*E*, 12*Z*-octadecadienoic acid (9-HODE) and 13-hydroxy-9*Z*, 11*E*-octadecadienoic acid (13-HODE)

Fungal cytochrome P-450s have mainly been studied with saturated fatty acids and found to catalyze terminal or subterminal ω-hydroxylations. Linoleic acid may be metabolized at least by the above Reactions 1 and 2. We will describe in short the fatty acid ω-hydroxylases, which are induced by growing yeast on *n*-alkanes, and the fatty acid ω2- and ω3-hydroxylases of *Fusarium oxysporum* and *Gaeumannomyces graminis*.

2.2 Fatty Acid ω-Hydroxylases of *n*-Alkane–Assimilating Yeast

The cytochrome P-450 system of yeast grown on *n*-alkanes was first characterized in 1974 [29]. Biotechnological processes for biosynthesis of protein using yeasts grown on *n*-alkanes of petroleum as the only source of carbon and energy have been developed [30]. Alkane-grown cells will increase the P-450 content from 0.8% to 2.7–5.5% of the microsomal protein. *n*-Alkanes are converted to alcohols and fatty acids by this oxidation. The induced cytochrome P-450s belong to the CYP52 gene family [10,11]. Many CYP52 enzymes will also metabolize fatty acids by ω-oxidation. Induction of cytochrome P-450s by *n*-alkanes has been described in many yeasts (e.g., *Candida tropicalis*, *Candida maltosa*, *Candida apicola*, and *Saccharomycopsis lipolytica*).

The GenBank [31] lists over 20 members of the CYP52A gene family from several different fungi. The CYP52A subfamily of *C. maltosa* has been studied in detail. This subfamily comprises at least eight structurally related isoforms, which may have arisen by gene duplication events [32–34]. CYP52A3 oxidizes C-12–C-16 alkanes with preference, whereas CYP52A4, CYP52A9, CYP52A10, and CYP52A11 will catalyze ω-hydroxylation of lauric acid and, to some extent, palmitic acid. Thus the CYP52A subfamily prefers fatty acids and *n*-alkanes of medium chain length.

2.3 Fatty Acid ω1-, ω2-, ω3-, and ω4-Hydroxylases of *Fusarium oxysporum*

Shoun et al. [35,36] demonstrated that cell-free extracts of *Fusarium oxysporum* could oxidize lauric acid to 9-, 10-, and 11-hydroxydodecanoic acids. The enzyme was identified as a cytochrome P-450, which was present in both the soluble and the microsomal frac-

tions. Palmitic acid was also oxidized at the ω2, ω3, and ω4 carbons. In both cases, the two major products were formed by ω3- and ω4-hydroxylations.

Recently, a catalytic self-sufficient fatty acid ω1–ω3-hydroxylase was purified from *Fusarium oxysporum* MT-811 [37,38]. This enzyme was microsomal but showed many similarities with the soluble CYP102 of *B. megaterium* (e.g., the catalytic turnover, the regiospecificity of fatty acid hydroxylations, and the molecular weight). Most important, the enzyme appeared to be a fusion protein of the cytochrome P-450 and cytochrome P-450 reductase in analogy with CYP102. This interesting finding may have implications for the evolution of cytochrome P-450s. The amino acid sequence of the *F. oxysporum* enzyme is unknown and it will be of evolutionary interest to compare its sequence with CYP102.

2.4 Polyunsaturated Fatty Acid ω2- and ω3-Hydroxylase of *Gaeumannomyces graminis*

Sih et al. [27] reported that incubation of *Gaeumannomyces graminis* (formerly named *Ophiobolus graminis*) with arachidonic acid afforded 18- and 19-hydroxyarachidonates in large yields and small amounts of 17-hydroxyarachidonate. Later, 18- and 19-hydroxyarachidonates were identified as mammalian arachidonate metabolites in the liver, renal cortex, and seminal vesicles [4,28,39]. This prompted further investigation on the ω2- and ω3-hydroxylase of *G. graminis* in order to obtain suitable references for studies in mammals [39]. The hydroxyl groups of 18-hydroxyarachidonate and 19-hydroxyarachidonate, produced by *G. graminis*, were found to have the *R* configuration. Eicosapentaenoic acid was converted to 17,18-dihydroxyeicosatetraenoic acid, which presumably occurred via an ω-epoxide, 17,18-epoxyeicosatetraenoic acid, which seems likely because epoxide hydrolases are ubiquitous and have been described in fungi [40].

The enzyme activity was present in the cytosolic and microsomal fractions, strictly dependent on NADPH and inhibited by CO [41]. Linoleic acid was converted to 16-HODE and 17-HODE, whereas saturated fatty acids were not metabolized. This is an interesting difference between the subterminal ω-hydroxylases of *Fusarium oxysporum* and the ω1-hydroxylases of the CYP52 subfamily. The fatty acid hydroxylase of *Gaeumannomyces graminis* needs to be purified and further characterized. The most interesting feature of this fatty acid ω2- and ω3-hydroxylase is its specificity for unsaturated fatty acids.

3 FATTY ACID DIOXYGENASES

There are two classes of fungal fatty acid dioxygenases, namely those with and those without heme [6]. Lipoxygenases belong to the latter group and contain a mononuclear metal center with iron [42,43]. A few fungi have been found to contain lipoxygenases. Fatty acid dioxygenases with heme have only recently been demonstrated in fungi but have been studied extensively in man and animals. Prostaglandins (PGs) are formed by PGH synthases, which contain heme and catalyzes introduction of two oxygen molecules into the substrate (see Ref. 3 for a review). The first step in prostaglandin biosynthesis from arachidonic acid is abstraction of the pro-*S* hydrogen from C-13. This step is most likely catalyzed by a tyrosyl radical. Recent observations suggest that PGH synthases might be both structurally and mechanistically related to a fungal fatty acid heme dioxygenase, linoleate diol synthase (LDS) [44,45]. This enzyme also abstracts hydrogen from

the substrate and forms a tyrosyl radical during catalysis, but it forms metabolites other than prostaglandins, which have not been reported in fungi.

3.1 Lipoxygenases

Lipoxygenases catalyze the oxidation of polyunsaturated fatty acids to fatty acid hydroperoxides [1]. Lipoxygenases are widely distributed in animals and plants [46,47]. They abstract hydrogen from a bis-allylic methylene group and insert dioxygen antarafacially so that a 1,4(*Z*,*Z*)-pentadiene is converted to a 1-hydroperoxy-2,4(*E*,*Z*)-pentadiene. All sequenced lipoxygenases belong to the same gene family. A characteristic molecular and catalytic feature is nonheme iron, which is ligated to three conserved histidines, the carboxyl group of the C-terminal isoleucine, and, distantly, to asparagine or histidine [1,42]. According to the prevailing model of catalysis, lipoxygenation starts with oxidation of the ferrous iron to ferric by hydroperoxides, which is in agreement with the intrinsic lag time of catalysis. The ferric form of the enzyme then abstracts hydrogen from the bis-allylic carbon and is reduced to the ferrous state. The redox cycle Fe(II)/Fe(III) thus plays a key role in lipoxygenation [42,46,47].

Only a few reports of lipoxygenases in fungi have been published, but fungi may induce lipoxygenase enzymes in plants as a defense reaction. For example, the rice blast fungus, *Magnaporthe grisea*, will induce rice lipoxygenase as an early response of the host to the pathogen [48]. A lipoxygenase is also induced in the tobacco plant by *Phytophthora parasitica nicotianae* [49]. There are recent excellent reviews of this area [2,50,51], which is outside the scope of this chapter.

Lipoxygenase activity has been found in several fungi, but only a few enzymes have been purified and characterized. One fungal lipoxygenase contains manganese, which makes it unique among all described lipoxygenases (see the following subsections).

3.1.1 Lipoxygenase of Baker's Yeast

Shechter and Grossman [52] purified a lipoxygenase from baker's yeast, *Saccharomyces cerevisiae*. This lipoxygenase converted linoleic acid to a mixture of 9-HPODE (9-hydroperoxy-10*E*, 12*Z*-octadecadienoic acid) and 13-HPODE (13-hydroperoxy-9*Z*, 11*E*-octadecadienoic acid) with a K_m of 0.268 m*M*. The stereochemistry of the hydroperoxide groups was not determined and the nature of this lipoxygenase is obscure [1]. The genome of *S. cerevisiae* has been sequenced and GenBank [31] contains no reference to a yeast lipoxygenase.

3.1.2 Lipoxygenase Activity of *Saprolegnia parasitica* and *Pityrosporum orbiculare*

The oomycete *Saprolegnia parasitica* was found to convert arachidonic acid into 15*S*-hydroperoxy-5*Z*,8*Z*,11*Z*,13*E*-eicosatetraenoic acid, which could be isomerized into epoxy alcohols by an epoxy alcohol synthase [53–55]. The lipoxygenase enzyme was partially purified as a soluble protein, which retained the epoxy alcohol synthase activity. Therefore, it seems likely that the lipoxygenase and the epoxy alcohol synthase are present in the same protein or protein complex.

Pityriasis versicolor is a human skin disease with local depigmentation as the main symptom. The fungus *Pityrosporum orbiculare* causes this disease by damaging melanocytes. Lipoxygenase activity of *P. orbiculare* was detected in 1986 [56], but the enzyme has not been fully characterized. However, acetone powder of *P. orbiculare* can oxidize

linoleic acid into hydroperoxides, which indicates the presence of lipoxygenase activity [57]. It is an interesting possibility that *P. orbiculare* can oxidize unsaturated fatty acids of the skin surface and cause depigmentation by this mechanism. This interesting disorder warrants further studies.

3.1.3 Manganese Lipoxygenase of *Gaeumannomyces graminis*

Gaeumannomyces graminis causes take-all disease of wheat and other grasses. We recently found that *G. graminis* secretes a unique lipoxygenase. Unlike all previously described lipoxygenases, this enzyme contains manganese [58]. It was therefore designated manganese lipoxygenase (Mn-LO).

Purification of Mn-LO

The fungus *G. graminis* can secrete milligram amounts of Mn-LO per liter in a liquid culture in the course of a week [58]. The filtrate of the culture media is used as a starting material for the enzyme purification. Four chromatographic steps are usually adequate to purify Mn-LO to apparent homogeneity (Table 1). The purified enzyme had a yellow color and lacked heme.

Catalytic Properties of Mn-LO

Manganese lipoxygenase oxidizes α-linolenic, linoleic, and γ-linolenic acids, whereas arachidonic acid is not a substrate. α-Linolenic acid was oxidized twice as fast as linoleic acid, and γ-linolenic acid was a poor substrate [58,59]. Mn-LO oxidizes linoleic acid to 13*R*-HPODE and 11*S*-hydroperoxylinoleic acid (11*S*-HPODE). The enzyme converts the latter to 13*R*-HPODE, which is the end product [60]. Experiments with stereospecifically deuterium-labeled linoleic acids at C-11 showed a suprafacial relationship between hydrogen abstraction and oxygen insertion. There was also a marked isotope effect. The oxidation mechanism of Mn-LO is summarized in Figure 1.

Atomic Absorption and EPR Spectroscopy

The elemental analysis of the purified enzyme was performed by atomic absorption spectroscopy. A first estimate yielded 0.5–1 Mn atom per enzyme molecule [58]. The content of iron was insignificant. Based on total amino acid analyses for quantification of protein, the enzyme purification in Scheme B (Table 1), and atomic absorption spectroscopy, we obtained a more reliable value of 0.94 Mn atom per molecule (Fig. 2) [61]. Manganese appeared to be tightly bound to the protein and the enzyme activity was not increased by the addition of $MnCl_2$ [58].

Electron paramagnetic resonance (EPR) of native Mn-LO, which was purified from any contaminating Mn^{2+} in solution, only showed weak signals. These weak signals were consistent with octahedrally coordinate Mn(II) bound to the protein. Denaturation of Mn-

Table 1 Three Strategies for Isolation of Mn-LO from the Culture Medium of *G. graminis*

Scheme A	Scheme B	Scheme C
Phenyl Sepharose	Phenyl Sepharose	Phenyl Sepharose
CM Sepharose	CM Sepharose	Cu-chelating Sepharose
Mono S HR	Con A–Sepharose	CM Sepharose
Superdex 200 HR	Superdex 200 HR	Superdex 200 HR

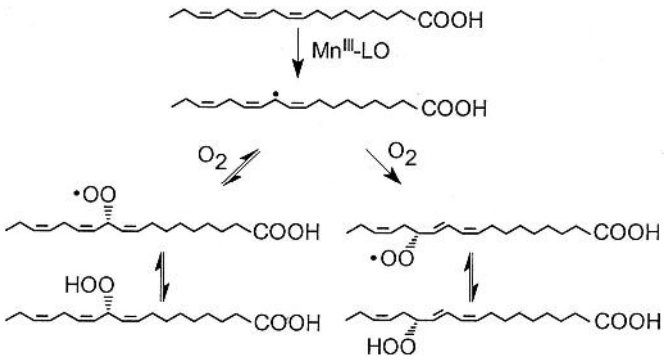


Figure 1 Oxidation of α-linolenic acid by Mn-LO. The enzyme is first activated by oxidation of Mn(II) to Mn(III). The first catalytic step is hydrogen abstraction at C-11. Dioxygen can then be reversibly inserted at C-11 and an 11-peroxyl radical is formed, which is then converted to a hydroperoxide (left panel). Dioxygen can also react irreversibly with C-13, leading to formation of the 13-hydroperoxide of α-linolenic acid (right panel).

LO with HNO_3 liberated manganese from the protein and yielded strong EPR signals. EPR showed the typical signals of octahedrally coordinate Mn(II). From the EPR analysis, the Mn content was estimated to be 20% lower than by atomic absorption spectroscopy, about 0.7 Mn atom per molecule [61]. The metal content and the EPR analysis suggest that Mn-LO contains a mononuclear rather than a binuclear metal center. Interestingly, incubation of Mn-LO with linoleate led to a decrease of these Mn(II) signals, possibly due to oxidation to Mn(III), which is EPR silent.

It might be added that other manganese-dioxygenases have been described. Extradiol catechol dioxygenases with either a manganese or an iron center have been found in bacte-

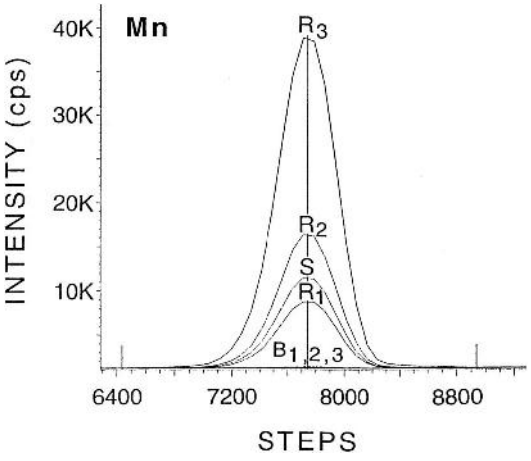


Figure 2 Elemental analysis of manganese in Mn-LO by atomic spectroscopy. R1, R2, and R3-manganese standards; S-sample with Mn-LO; B1, B2, and B3-buffer blanks. The instrument used was an inductively coupled plasma–emission spectrometer.

ria [62–64]. It is known that these enzymes appear to use identical coordinating residues for their iron- and manganese-dependent enzymes. It will be of interest to determine whether this applies to iron and manganese lipoxygenases. This seems most likely, as we have recently cloned and sequenced Mn-LO and found that it clearly belongs to the lipoxygenase gene family (L Hörnsten, C Su, and EH Oliw, unpublished observations).

Catalytic Mechanisms of Iron and Manganese Lipoxygenases

Iron lipoxygenases abstract hydrogen from a bis-allylic methylene group and insert dioxygen in an antarafacial way so that a 1,4(*Z*,*Z*)-pentadiene is converted to a 1-hydroperoxy-2,4(*E*,*Z*)-pentadiene [46,47]. According to the two current models of catalysis, lipoxygenation starts with activation of the lipoxygenase by oxidation of the ferrous iron to ferric by hydroperoxides. The ferric form of the lipoxygenase may then act as a base and abstract a proton from C-3 of the 1,4(*Z*,*Z*)-pentadiene. According to the first model, Fe(III) is reduced to Fe(II), concerted with the proton-transfer reaction, an alkyl (pentadienyl) free radical is formed, which then reacts with molecular oxygen at C-1. The peroxyl radical regains the lost proton, the hydroperoxide is formed, and Fe(II) is reoxidized to Fe(III) [46]. According to the alternative model, an electrophilic addition of Fe(III) to C-1 occurs with the formation of an organoiron intermediate concerted with the proton abstraction [65]. Molecular oxygen reacts with this allylic organoiron intermediate (by σ bond insertion) and 1-hydroperoxy-2,4(*E*,*Z*)-pentadiene and Fe(III)-LO are formed. In the last model, the regiospecificity and stereospecificity of dioxygen insertion are controlled by the Fe(III)—C-1 bond, and in the first model, they are controlled by steric factors caused by the binding of the alkyl radical to the enzyme and diffusion of dioxygen to accessible carbons of the pentadienyl radical.

Both models can explain many features of lipoxygenation. It has been experimentally difficult to determine whether lipoxygenation by iron lipoxygenases occurs by the first or second model. A key intermediate of the first model, the alkyl radical, has been detected by EPR [66], whereas the organoiron intermediate has not been directly demonstrated.

Does lipoxygenation by Mn-LO proceed via a free-radical mechanism or via organomanganese intermediates? Mn-LO contains a mononuclear metal center with Mn(II), which is likely converted to Mn(III) during catalysis. This is in analogy with the redox cycle of iron lipoxygenases. Dioxygen is bound reversibly to an alkyl radical [67], and the radical model might explain the isomerization of 11*S*-HPODE to 13*R*-HPODE [10,61]. An organomanganese model of Mn-LO is also consistent with some experimental observations. The organoiron model was based on oxygenation of substrate analogs of arachidonic acid at different oxygen pressures. It will be of interest to determine whether Mn-LO will oxidize the corresponding substrate analogs of linoleic acid in a similar way.

3.1.4 Lipoxygenase Activity of *Fusarium oxysporum*

Hemoproteins, like myoglobin, can catalyze lipoxygenaselike reactions in the presence of hydroperoxides, which leads to formation of *Z*,*E* conjugated hydroperoxy fatty acids with little regiospecificity and stereospecificity. The mechanisms has been studied in detail [68]. This enzyme activity may be referred to as pseudolipoxygenase activity and is apparently typical of many hemoproteins. It was therefore not surprising that a hemoprotein, which was purified from *Fusarium oxysporum*, was found to be associated with lipoxygenase activity [69]. This enzyme will need further characterization.

3.2 Fungal Fatty Acid Dioxygenases

Two fungi, *Gaeumannomyces graminis* and *Laetisaria arvalis*, have been found to contain fatty acid dioxygenases, which differ from lipoxygenases in forming 8*R*-hydroperoxylinoleate (8*R*-HPODE). It seems likely that a related enzyme also is present in certain strains of *Aspergillus nidulans*, but, so far, only the dioxygenase of *G. graminis* has been characterized in detail.

3.2.1 Linoleate 8*R*-Dioxygenase of *Aspergillus nidulans*

Champe and colleagues [70–74] studied sporulation in certain strains of *Aspergillus nidulans* and discovered and characterized a series of precocious sexual inducers. The most potent compounds were identified as metabolites of oleic and linoleic acids (viz. 5*S*,8*R*-dihydroxylinoleic acid and 5*S*,8*R*-dihydroxyoleic acid). In addition, 8*R*-hydroxylinoleic acid (8*R*-HODE) and 8*R*-hydroxyoleic acid were also demonstrated. With these articles, Champe and his colleagues have sparked interest in fungal oxylipins and their function. These reports are unique in defining biological function. As pointed out by Herman in an excellent review [6], there is too little information on structure, biosynthesis, and biological function of fungal oxylipins. The route of biosynthesis of these hormones has not been elucidated. It seems likely that an 8*R*-dioxygenase with hydroperoxide isomerase activity forms these oxylipins, but this hypothesis will need experimental verification, as other alternatives also are possible.

3.2.2 Linoleate 8*R*-Dioxygenase of *L. arvalis*

Bowers et al. [75] described biosynthesis of 8-HODE by *L. arvalis* in the very first report of this metabolite. 8-HODE was found to be fungicidal for some fungi. The mechanism of biosynthesis of 8-HODE was not elucidated until almost 10 years later [76]. High-speed supernatant of mycelia of *L. arvalis* was found to convert linoleic acid to 8*R*-HPODE, which was reduced to 8*R*-HODE. 8*R*-HPODE was thus formed by a dioxygenase and not by a monooxygenase. Attempts to purify this dioxygenase were not straightforward, indicating that this enzyme has different properties than linoleate diol synthase (LDS) described next (Su, unpublished observation).

3.2.3 Linoleate Diol Synthase of *G. graminis*

Catalytic Properties of LDS

Studies with crude preparations of LDS showed that it metabolized oleic, linoleic, and α-linolenic acids, whereas γ-linolenic and arachidonic acids were not substrates [41,77]. Main products were 8*R*-hydroperoxy fatty acids and 7*S*,8*S*-dihydroxy fatty acids. Experiments with deuterated linoleic acids at C-8 and C-7 and experiments under oxygen-18 showed that 8*R*-HPODE was formed by abstraction of the pro-*S* hydrogen and antarafacial insertion of dioxygen at C-8, whereas 7*S*,8*S*-dihydroxylinoleate was formed from the hydroperoxide by elimination of the pro-*S* hydrogen at C-7 and intramolecular oxygen transfer with suprafacial insertion [78]. LDS was strongly inhibited by the lipoxygenase inhibitor BW A4C [79].

Purification of LDS

Linoleate diol synthase was purified from the mycelia of *G. graminis* more than 1000-fold, up to a specific activity of 1.8 μmol/min/mg and to near homogeneity as a 130-kDa

protein band on sodium dodecyl sulfate–polyacrylamide electrophoresis (SDS–PAGE) [80]. The native enzyme was a homotetramer with a subunit size of 130 kDa. It showed a typical hemoprotein spectrum with at least 0.7 mol of heme per monomer. The purified hemoprotein showed both 8*R*-dioxygenase and hydroperoxide isomerase activities.

Detection of Ferryl Intermediates by Stopped Flow

Light spectroscopy showed that LDS contained primarily ferriheme in high spin (Table 2) [81]. Stopped flow showed that the Soret absorption at 406 nm decayed rapidly within 10–20 msec after mixing with 8*R*-HPODE, and then increased at a much lower rate. As the light absorption at 406 nm decreased, the light absorption increased at 421 nm after a lag time of 5 msec. These events can be attributed to a transient ferryl oxygen intermediate and a porphyrin π-cation radical (compound I of peroxidases). This intermediate is converted to a second intermediate, most likely a ferryl oxygen and a protein radical (compound II of peroxidases), after a time lag of about 5–10 msec. The formation of a protein radical could support this hypothesis.

A Tyrosyl Radical During LDS Catalysis

Electron paramagnetic resonance confirmed that heme of LDS was mainly in a ferric high-spin state [81]. A hyperfine splitting pattern of the EPR signal at $g = 2.005$ was recorded after rapid freeze-quenching of LDS with linoleic acid or with 8*R*-HPODE as the substrate. This transient EPR signal was likely due to a protein-derived tyrosyl radical. The relaxation properties of the radical during EPR indicated that it was located in the vicinity of the heme center, as previously observed for the tyrosyl radical of PGH synthase-1 [3].

The Gene and Deduced Protein Sequence of LDS

The gene of LDS was divided into four exons and three short introns. The primary sequence of LDS precursor was deduced from the open reading frame as a protein of 978 amino acids [45]. The deduced sequence of LDS showed similarity with PGH synthases.

Table 2 Light Absorption in the Soret Region of LDS and PGH Synthase-1 and Derivatives

	Soret absorption	
Derivative	LDS	PGH synthase-1
Ferrous		
$Na_2S_2O_4$	429 nm	424 nm
$Na_2S_2O_4$ + CO	421 nm	419 nm
Ferric		
Native enzyme	406 nm	411 nm
F^-	406 nm	410 nm
pH 6.5	406 nm	NA
pH 9.5	412 nm	NA
Ferryl		
Compound I ($Fe^{IV}O|.^+$)	~406 nm	410 nm
Compound II ($Fe^{IV}O|:$)	NA[a]	420 nm

[a] NA = data not available.

Source: Modified from Ref. 81.

The gapped BLAST algorithm search [31] aligned 391 amino acids of LDS with 367 amino acids of horse and rabbit PGH synthase-2, yielding 23–24% identity and 37% positives in a gap frequency of 24%. The homology was restricted to the catalytic domain of PGH synthases. The catalytically important tyrosine residue, the proximal heme ligand, and distal heme ligand of LDS could be tentatively located as Tyr-376, His-279, and His-203, respectively (with consensus to EFNxxx**Y**xW**H** and T**H**xxFxT, respectively). LDS also appeared to be distantly related to some peroxidases.

A Catalytic Model of LDS

Based on the apparent analogy with the prevailing reaction mechanism of PGH synthases [3,82–84], the following catalytic mechanism of LDS seems likely (Fig. 3). The initiating step of catalysis could be reduction of 8*R*-HPODE to 8*R*-HODE with formation of ferryl oxygen with a porphyrin π-cation (compound I). The latter is rapidly reduced to compound II with one electron, and a protein (tyrosyl) radical is formed. The protein (tyrosyl) radical

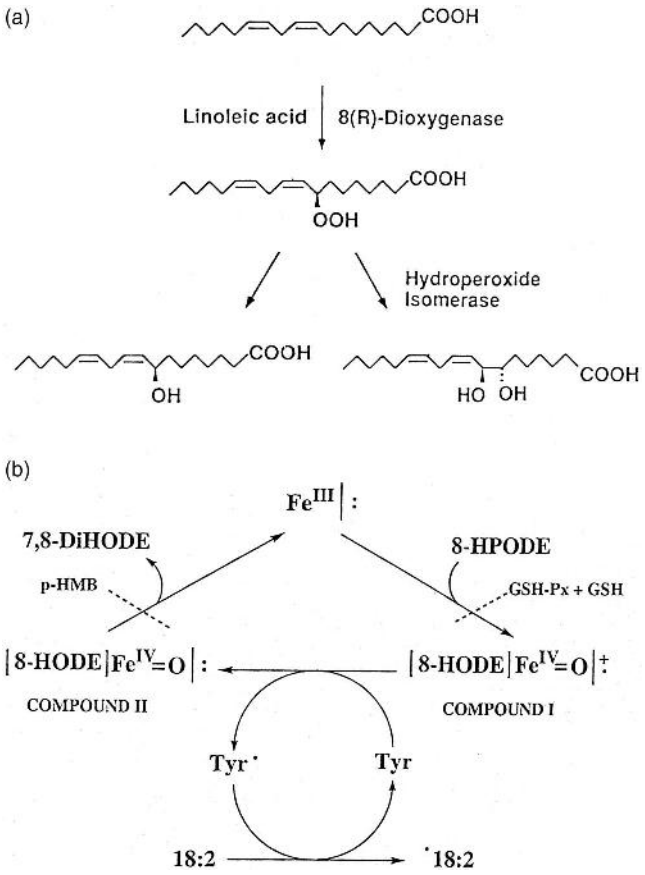


Figure 3 Oxidation of linoleic acid by linoleate diol synthase. (a) Overview of products formed; (b) oxidation mechanism of linoleate diol synthase. The first step is oxidation of Fe(III) of the enzyme to compound I by 8*R*-HPODE. Compound II and a tyrosyl radical are then formed. The tyrosyl radical initiates a new reaction (bottom), whereas compound II will form the diol by insertion of oxygen at C-7. (Modified from Ref. 44.)

could initiate the LDS cycle: (1) the pro-*S* hydrogen at C-8 of linoleate is eliminated by the tyrosyl radical; (2) dioxygen reacts with the alkyl radical; (3) 8*R*-peroxyl radical of linoleate is formed and it could form 8*R*-HPODE either by abstracting a hydrogen from the tyrosyl residue (and regenerating the tyrosyl radical) or by receiving an electron from other electron donors; (4) the hydroperoxide oxygen–oxygen bond is cleaved; (5) ferryl oxygen and 8*R*-HODE are formed; (6) the ferryl oxygen is inserted at C-7 of 8*R*-HODE, 7*S*,8*S*-DiHODE is released from LDS, and ferriheme is formed.

It should be emphasized that there are also obvious differences between LDS and PGH synthases. LDS has hydroperoxide isomerase, whereas PGH synthase-1 has peroxidase activity. The ferryl oxygen intermediates I and II of PGH synthase-1, which correspond to compounds I and II of peroxidases, are formed during the peroxidase-catalyzed reduction of PGG_2 to PGH_2 and during regeneration of ferric PGH synthase-1 with reducing equivalents. In contrast, the hydroperoxide isomerase of LDS does not need reducing equivalents.

4 MISCELLANEOUS

4.1 Oxygenase and Lyase Activities of Mushroom, *Psallioata bispora*

The characteristic flavor of mushroom is due to 1-octen-3-ol and 10-oxo-8*E*-decenoic acid. These compounds likely originate from oxidative cleavage of linoleic acid. Wurzenberger and Grosch [85,86] found that 10-hydroperoxylinoleic acid but no other linoleic acid hydroperoxides were converted to these two compounds by cell-free extracts of *Psallioata bispora*. It seems likely that 10-hydroperoxy-8*E*,12*Z*-ocadecadienoic acid is formed by mushrooms by a dioxygenase, but the enzyme has not been isolated.

4.2 Biosynthesis of 3-Hydroxyarachidonic Acid by the Yeast *Dipodascopsis uninucleata*

The yeast *Dipodascopsis uninucleata* transforms arachidonic acid to 3*R*-hydroxyarachidonic acid [87–89]. This metabolite was recently suggested to be an important regulator of the sexual phase of the yeast [90]. Little is known about the mechanism of biosynthesis of 3-hydroxyarachidonic acid and the relation to β-oxidation. A related 3-hydroxy metabolite of arachidonic acid with 14 carbons, 3-hydroxy-5,8-tetradecadienoic acid, was produced from arachidonic acid by the fungus *Mucor genevensis* [91], apparently by three rounds of β-oxidation.

5 SUMMARY AND PERSPECTIVES

The pharmaceutical industry has used cytochrome P-450 of fungi for the biosynthesis of steroid metabolites. *Mortierella alpina* may be used for the production of arachidonate [92]. Fungal cytochrome P-450s, which oxidize fatty acids, have not yet been fully explored for industrial use, although *n*-alkane–assimilating yeast was investigated in the 1970s and 1980s. However, the fungal cytochrome P-450s have provided unexpected results in basic research. The fact that *Fusarium oxysporum* contains a fusion protein between cytochrome P-450 and cytochrome P-450 reductase analogous to CYP102 with prominent fatty acid ω1–ω3-hydroxylase activity was unexpected. The heme-containing dioxygenases of fungi have not yet found any pharmaceutical applications but broadened

our understanding of these metalloenzymes. LDS is clearly related to PGH synthases and the quest for other members of the fatty acid heme dioxygenase family will continue. Recent additions to this family of enzymes are 8R-dioxygenase of *Leptomitus lacteus* [93] and the fatty acid α-dioxygenase of tobacco [94]. The discovery of the first manganese lipoxygenase will also be of interest for basic science, but this enzyme might find industrial application, as it is remarkably stable and the first lipoxygenase that is secreted. Soybean lipoxygenase has, for example, been used for the production of hydroxy-conjugated octadecadienoic acids, and since 1934, for the bleaching of wheat flour [95]. The biological function of the various oxylipins in fungal reproduction has only started to emerge due to important contributions of Champe and his associates. *G. graminis* causes take-all disease of wheat and other grasses and our knowledge of oxylipins and their b
might be used to combat this disease. With so much left to discover about the function of fungal oxylipins and with the ease of industrial production of fungal enzymes and metabolites, this area should be fruitful for further investigations.

REFERENCES

1. AR Brash. J Biol Chem 274:23,679–23,682, 1999.
2. HW Gardner. HortScience 30:197–205, 1995.
3. WL Smith, RM Garavito, DL DeWitt. J Biol Chem 271:33,157–33,160, 1966.
4. EH Oliw. Prog Lipid Res 33:329–354, 1994.
5. D Shibata, B Axelrod. J Lipid Mediat Cell Signal 12:213–228, 1995.
6. RP Herman. In: AF Rowley, H Kühn, T Schewe, eds. Eicosanoids and Related Compounds in Plants and Animals. London: Portland Press, 1998, pp 115–130.
7. JD Weete. Lipid Biochemistry of Fungi and Other Organisms. New York: Plenum Press, 1980, pp 49–95.
8. DH Peterson, HC Murray. J Am Chem Soc 74:1871–1872, 1952.
9. DR Nelson, L Koymans, T Kamataki, JJ Stegeman, R Feyereisen, DJ Waxman, MR Waterman, O Gotoh, MJ Coon, RW Estabrook, IC Gunsalus, DW Nebert. Pharmacogenetics 6:1–42, 1996.
10. H Vanden Bossche, L Koymans. Mycoses 41(suppl 1):32–38, 1998.
11. HJM van den Brink, RFM van Gorcom, CAMJJ van den Hondel, PJ Punt. Fungal Genet Biol 23:1–17, 1998.
12. TL Poulos, BC Finzel, IC Gunsalus, GC Wagner, J Kraut. J Biol Chem 260:16,122–16,130, 1985.
13. KG Ravichandran, SS Boddupalli, CA Hasemann, JA Peterson, J Deisenhofer. Science 261: 731–736, 1993.
14. CA Hasemann, KG Ravichandran, JA Peterson, J Deisenhofer, J Mol Biol 236:1169–1185, 1994.
15. JR Cupp-Vickery, TL Poulos. Nat Struct Biol 2:144–151, 1995.
16. PA Williams, J Cosme, V Sridhar, EF Johnson, DE McRee. J Inorg Biochem 81:183–190, 2000.
17. PE Verkade, M Elzas, J van der Lee, HH de Wolff, A Verkade-Sandbergen, D van der Sande. Proc Roy Acad Amsterdam 35:251–266, 1932.
18. K Robbins. Fed Proc 20:272, 1961.
19. K Wakabayashi, N Shimazono. Biochim Biophys Acta 48:615–617, 1961.
20. B Preiss, KJ Bloch. J Biol Chem 239:85–88, 1964.
21. M Kusunose, E Kusunose, MJ Coon. J Biol Chem 239:1374–1380, 1964.
22. T Omura, R Sato. J Biol Chem 239:2370–2378, 1964.
23. T Omura, R Sato. J Biol Chem 239:2379–2385, 1964.

24. M Hamberg, I Björkhem. J Biol Chem 246:7411–7416, 1971.
25. I Björkhem, M Hamberg. Biochem Biophys Res Commun 47:333–340, 1972.
26. A Lindenmayer, L Smith. Biochim Biophys Acta 93:445–461, 1964.
27. CJ Sih, G Ambrus, P Foss, CJ Lai. J Am Chem Soc 91:3685–3687, 1969.
28. EH Oliw, J Bylund, C Herman. Lipids 31:1003–1021, 1996.
29. W Duppel, J-M Lebeault, MJ Coon. Eur J Biochem 36:583–592, 1973.
30. H Honeck, W-H Schunck, H-G Müller. Pharmazie 40:221–227, 1985.
31. DA Benson, MS Boguski, DJ Lipman, J Ostell, BF Ouellette, BA Rapp, DL Wheeler. Nucleic Acids Res 27:12–17, 1999.
32. U Scheller, T Zimmer, E Kärgel, W-H Schunck. Arch Biochem Biophys 328:245–254, 1996.
33. T Zimmer, M Ohkuma, A Ohta, M Takagi, W-H Schunck. Biochem Biophys Res Commun 224:784–789, 1996.
34. T Zimmer, T Iida, W-H Schunck, Y Yoshida, A Ohta, M Takagi. Biochem Biophys Res Commun 251:244–247, 1998.
35. H Shoun, Y Sudo, Y Seto, T Beppu. J Biochem 94:1219–1229, 1983.
36. H Shoun, Y Sudo, T Beppu. J Biochem 97:755–763, 1985.
37. N Nakayama, H Shoun. Biochem Biophys Res Commun 202:589–590, 1994.
38. N Nakayama, A Takemae, H Shoun. J Biochem (Tokyo) 119:435–440, 1996.
39. EH Oliw. J Biol Chem 264:17,845–17,853, 1989.
40. PE Kolattukudy, L Brown. Arch Biochem Biophys 166:599–607, 1975.
41. ID Brodowsky, EH Oliw. Biochim Biophys Acta 1124:59–65, 1992.
42. ST Prigge, JC Boyington, BJ Gaffney, LM Amzel. Proteins: Struct Funct Genet 29:15–31, 1996.
43. SA Gillmor, A Villasenor, R Fletterick, E Sigal, MF Browner. Nature Struct Biol 4:1003–1009, 1997.
44. C Su, M Sahlin, EH Oliw. J Biol Chem 273:20744–20751, 1998.
45. L Hörnsten, C Su, AE Osbourn, P Garosi, U Hellman, C Wernstedt, EH Oliw. J Biol Chem 274:28,219–28,224, 1999.
46. GA Veldink, MP Hilbers, WF Nieuwenhuizen, JFG Vliegenthart. In: AF Rowley, H Kühn, T Schewe, eds. Eicosanoids and Related Compounds in Plants and Animals. London: Portland Press, 1998, pp 69–95.
47. N Ueda, H Suzuki, S Yamamoto. In: AF Rowley, H Kühn, T Schewe, eds. Eicosanoids and Related Compounds in Plants and Animals. London: Portland Press, 1998, pp 47–67.
48. YL Peng, Y Shirano, H Ohta, T Hibino, K Tanaka, D Shibata. J Biol Chem 269:3755–3761, 1994.
49. C Veronesi, M Rickauer, J Fournier, ML Pouenat, MT Esquerre-Tugaye. Plant Physiol 112: 997–1004, 1996.
50. EE Farmer, H Weber, S Vollenweider. Planta 206:167–174, 1998.
51. T Schewe, S Nigam. Adv Exp Med Biol 433:221–226, 1997.
52. G Shechter, S Grossman. Int J Biochem 15:1295–1304, 1983.
53. M Hamberg, RP Herman, U Jacobsson. Biochim Biophys Acta 879:410–418, 1986.
54. M Hamberg, CA Herman, RP Herman. Biochim Biophys Acta 877:447–457, 1986.
55. RP Herman, M Hamberg. Prostaglandins 34:129–139, 1987.
56. M Nazzaro-Porro, S Passi, M Picardo, R Mercantini, AS Breathnach. J Invest Dermatol 87: 108–112, 1986.
57. C De Luca, M Picardo, A Breathnach, S Passi. Exp Dermatol 5:49–56, 1996.
58. C Su, EH Oliw. J Biol Chem 273:13,072–13,079, 1998.
59. EH Oliw, C Su, T Skogström, G Benthin. Lipids 33:843–852, 1998.
60. M Hamberg, C Su, EH Oliw. J Biol Chem 273:13,080–13,088, 1998.
61. C Su, M Sahlin, EH Oliw. J Biol Chem 275:18830–18835, 2000.
62. L Que, J Widom, RL Crawford. J Biol Chem 256:10,941–10,944, 1981.
63. YR Boldt, MJ Sadowsky, LB Ellis, L Que Jr, LP Wackett. J Bacteriol 177:1225–1232, 1995.

64. YR Boldt, AK Whiting, ML Wagner, MJ Sadowsky, L Que Jr, LP Wackett. Biochemistry 36:2147–2153, 1997.
65. EJ Corey, R Nagata. J Am Chem Soc 109:8107–8108, 1987.
66. W Chamulitrat, RP Mason. J Biol Chem 264:20,968–20,973 1989.
67. HW-S Chan, G Levett, JA Matthew. Chem Phys Lipids 24:245–256, 1979.
68. SI Rao, A Wilks, M Hamberg, PR Ortiz de Montellano. J Biol Chem 269:7210–7216, 1994.
69. Y Matsuda, T Beppu, K Arima. Biochim Biophys Acta 530:439–450, 1978.
70. NZ Butnick, LN Yager, TE Hermann, M Berman Kurtz, SP Champe. J Bacteriol 160:533–540, 1984.
71. NZ Butnick, LN Yager, M Berman Kurtz, SP Champe. J Bacteriol 160:541–545, 1984.
72. SP Champe, AAE El-Zayat. J Bacteriol 171:3982–3988, 1989.
73. P Mazur, HV Meyers, K Nakanishi, AAE El-Zayat, SP Champe. Tetrahedron Lett 31:3837–3840, 1990.
74. P Mazur, K Nakanishi, AAE El-Zayat, SP Champe. J Chem Soc Chem Commun 20:1486–1487, 1991.
75. WS Bowers, HC Hoch, PH Evans, M Katayama. Science 232:105–106, 1986.
76. ID Brodowsky, EH Oliw. Biochim Biophys Acta 1168:68–72, 1993.
77. ID Brodowsky, M Hamberg, EH Oliw. J Biol Chem 267:14,738–14,745, 1992.
78. M Hamberg, L-Y Zhang, ID Brodowsky, EH Oliw. Arch Biochem Biophys 309:77–80, 1994.
79. ID Brodowsky, M Hamberg, EH Oliw. Eur J Pharmacol 254:43–47, 1994.
80. C Su, EH Oliw. J Biol Chem 271:14,112–14,118, 1996.
81. C Su, M Sahlin, EH Oliw. J Biol Chem 273:20,744–20,751, 1998.
82. R Karthein, R Dietz, W Nastainczyk, HH Ruf. Eur J Biochem 171:313–320, 1988.
83. R Dietz, W Nastainczyk, HH Ruf. Eur J Biochem 171:321–328, 1988.
84. WL Smith, DL DeWitt. Semin Nephrol 15:179–194, 1995.
85. M Wurzenberger, W Grosch. Biochim Biophys Acta 794:18–24, 1984.
86. M Wurzenberger, W Grosch. Biochim Biophys Acta 794:25–30, 1984.
87. MS van Dyk, JLF Kock, DJ Coetzee, OPH Augustyn, S Nigam. FEBS Lett 283:195–198, 1991.
88. A Akpinar, SR Fox, C Ratledge, J Friend. Adv Exp Med Biol 433:231–234, 1997.
89. P Venter, JL Kock, GS Kumar, A Botha, DJ Coetzee, PJ Botes, RK Bhatt, JR Falck, T Schewe, S Nigam. Lipids 32:1277–1283, 1997.
90. JL Kock, P Venter, D Linke, T Schewe, S Nigam. FEBS Lett 427:345–348, 1998.
91. CH Pohl, A Botha, JL Kock, DJ Coetzee, PJ Botes, T Schewe, S Nigam. Biochem Biophys Res Commun 253:703–706, 1998.
92. H Streekstra. J Biotechnol 56:153–165, 1997.
93. SR Fox, A Akpinar, AA Prabhune, J Friend, C Ratledge. Lipids 35:23–30, 2000.
94. A Sanz A, JI Moreno, C Castresana. Plant Cell 10:1523–1537, 1998.
95. EA Emken. J Am Oil Chem Soc 55:416–421, 1978.

15

Enzymic Formation of Flavor Volatiles from Lipids

WILFRIED SCHWAB and PETER SCHREIER

University of Würzburg, Würzburg, Germany

1 INTRODUCTION

Almost all types of cellular constituents can be metabolized and degraded to one or more types of flavor compounds. However, in addition to proteins and carbohydrates, lipids are the major source of flavor. For example, lipids are responsible for much of the desirable flavor of tangy cheeses, such as cheddar and Roquefort [1], for the flavor of fresh milk [2], and for the characteristic flavor of mushrooms [3], green beans [4], tomatoes [5], and cucumbers [6], as well as for much of the ripe flavors in fruits and berries [7].

In recent years, there has been a strong upsurge in the demand for natural products, including natural flavors [8]. The consumers believe that natural material including flavor are more healthy and safer than the synthetic counterpart. This has created a number of opportunities for biocatalysis to compete with traditional synthetic chemistry for the production of flavor volatiles (e.g., from lipids). Biocatalysts can be employed in a number of different ways, varying from the use of growing cells (fermentations), through the use of nongrowing cells as the biocatalyst (bioconversion), to the use of isolated enzymes [9].

The use of enzymes on an industrial scale is now common practice. However, whole microbial cell systems are often preferred over using isolated enzymes, especially in cases when a certain flavor substance is formed by many enzymes, which act in a multistep sequential manner. The use of isolated enzymes is beneficial if the biotransformation of interest involves a limited number of steps. Extremely pure products are obtained by using isolated enzymes, thus precluding the formation of side products. Enzymes operate under mild conditions at narrow temperature and pH ranges. They are required only in catalytic rather than stoichiometric amounts and their activity can be very selective for substrates and stereoselective in the catalyzed reactions [10].

Pure enzymes can be obtained from a number of sources using conventional methods of protein purification. However, large quantities of material are required to obtain a useful amount, as, in many cases, the enzyme content of the sources is very low. The frequently used lipase can be obtained from animal cells, but, again, one encounters difficulties with availability and cost. In general, microbial sources are not subjected to such limitations and therefore are much more suitable for enzyme production. Bacteria, yeast, and fungi are capable of producing a large variety of diverse enzymes that have the potential to be isolated and used in industrial processes for lipid degradation [11].

Generally, aroma compounds are formed from fatty acids by enzymatically catalyzed degradation processes. However, enzymic oxidative degradation of lipids is preceded by the action of acyl hydrolase, liberating the free fatty acids from acylgycerols. The most important degradation processes of fatty acids are the lipoxygenase reaction (in-chain oxidation), α-, β-, and ω-oxidation. The β-oxidation pathway comprising repetitive β-oxidations and several modifications of β-oxidation is the sole process currently known to lead to complete degradation of fatty acids. In contrast, α-oxidation appears to be restricted to long-chain fatty acids and to shorten them not beyond the C12 chain length. In-chain oxidation and ω-oxidation generate hydroxy, oxo, and epoxy fatty acids and are involved in the formation of polyfunctional fatty acids. Intermediates and products of in-chain oxidation, α-, β-, and ω-oxidation can be further metabolized to form volatile straight-chain alcohols, aldehydes, carboxylic acids, lactones, esters, ketones and miscellaneous compounds. As there are no known volatiles generated by ω-oxidation, this type of fatty acid degradation system is not included in this account.

The presentation of several important enzymatically catalyzed lipid bioconversions to flavor compounds will show the tremendous utility and potential of this approach for the production of natural flavors. However, the ratio of the number of enzymes used in commercial applications to the number of known enzymes is still very small. Lipase-type enzymes will not be discussed in detail, as part III of this volume is entirely devoted to this class of enzyme. However, lipases represent an important emerging bioprocessing area having many different applications also to production of flavors (see Sec. 4.1).

During the past 10 years, decisive advancements have been achieved for the elucidation of biosynthetic pathways of fatty-acid-derived aroma compounds. In this context, the application of modern molecular biology methods has been proved extremely useful, providing transgenic organisms with high synthetic capacities. They open the way for the future biotechnological production of natural aroma compounds from lipids.

2 VOLATILES FORMED BY LIPOXYGENASE (IN-CHAIN OXIDATION)

Lipoxygenases are essential components of the oxylipin pathway, converting unsaturated fatty acids into flavors, such as 3(*Z*)-hexen-1-ol, 2(*E*)-hexenal and 2(*E*),6(*Z*)-nonadienal (Fig. 1) [12–15]. Lipoxygenase (E.C. 1.13.11.12) is a nonheme, iron-containing dioxygenase that catalyzes the regioselective and enantioselective dioxygenation of unsaturated fatty acids containing one or more 1(*Z*),4(*Z*)-pentadienoic moieties. In plants, there exist two types of lipoxygenase: (1) one present in soybean seeds and tea leaves converts linoleic acid to 13(*S*)-hydroperoxy-9(*Z*),11(*E*)-octadecadienoic acid and (2) one occurring in potato tuber and tomato fruits oxygenates at C9 [16]. A free radical was proposed as intermediate in the lipoxygenase catalysis, which reacts directly with oxygen or an organoiron intermediate [17]. The three-dimensional protein structure of the native form of lipoxygenase isoenzyme L-1 from soybean has been elucidated [18,19]. In flavor biotechnology,

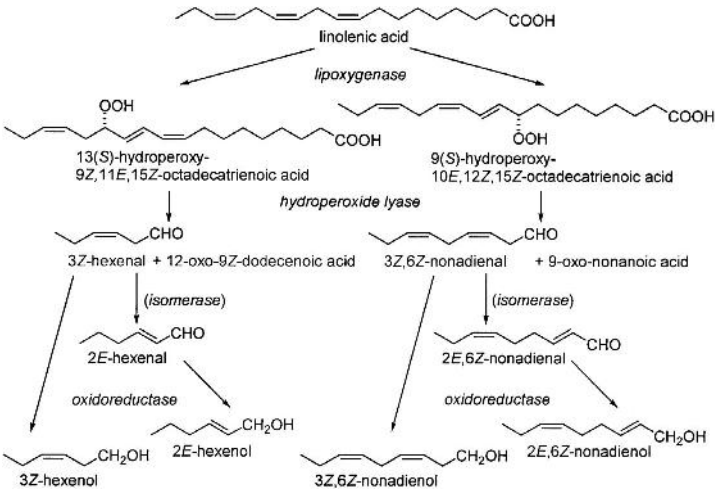


Figure 1 Summary of proposed pathways for the formation of C6- and C9-aldehydes and alcohols from linolenic acid. In a similiar reaction sequence, linoleic acid yields hexanal as well as 3(*Z*)- and 2(*E*)-nonenal. (Adapted from Ref. 12.)

lipoxygenase is an important enzyme for the industrial production of natural ''green note'' flavor compounds (i.e., the group of isomeric C6-aldehydes and alcohols) [16].

2.1 C6 Volatiles

The leaf aldehyde 2(*E*)-hexenal and the leaf alcohol 3(*Z*)-hexen-1-ol are responsible for the green flavors and aroma of fruits and vegetables [20]. 2(*E*)-Hexenal has a sharp, herbal-green aroma that also makes it desirable for use in perfumes and for providing a green nuance in fruity flavors. 3(*Z*)-Hexen-1-ol is used in perfumes and flavors to obtain green top notes [21]. Currently, synthetic compounds are used extensively. The natural compounds are obtained primarily from plant tissue that have been disrupted in some fashion.

In nature, the C6 compounds are formed in damaged plant tissues, such as leaves, fruits, or vegetables, in a cascade of enzymic reactions involving lipoxygenase, hydroperoxide lyase, and an oxidoreductase and/or double-bond isomerase. In general, the unsaturated fatty acids linoleic acid and linolenic acid are degraded via a lipoxygenase-catalyzed formation of hydroperoxides and a subsequent cleavage by a hydroperoxide lyase to form aliphatic C6 compounds such as 3(*Z*)-hexenal (Fig. 1). The aldehyde is further reduced by alcohol dehydrogenase to 3(*Z*)-hexen-1-ol or isomerized to 2(*E*)-hexenal and then reduced to the alcohol. Intact tissue contains only small amounts of C6 compounds as enzymes and substrates are localized in different cell compartments.

The source pools and subcellular location of the processes are unknown. A close relationship was found between the composition of polyunsaturated fatty acids and the composition of C6-aldehydes. In a current study, this relationship was tested using *Arabidopsis* polyunsaturated fatty acid mutant lines [22]. The differences in C6-aldehyde formation among the fatty acid mutants of *Arabidopsis* appeared not to result from alteration of lipoxygenase/hydroperoxide lyase pathway enzymes. Investigation of the fatty acid composition indicated that glycolipids of chloroplasts may be the major source of C6-aldehyde formation in *Arabidopsis* leaves.

Several approaches have been published describing the production of hexenal using either linolenic acid or its 13-hydroperoxide as substrate [23]. The formation of 3(*Z*)-/2(*E*)-hexenal by cell cultures of several fruits [24] or alfalfa [25] has been reported, but the yield was low (<0.18 mg/kg). Higher concentrations were obtained with quick grass (210 mg/kg) [26], apple peel (571 mg/kg) [27], or the leaves of various plants (450 mg/kg) [28]. A screening of vegetables and fruits resulted in a maximum of hexenals of a 370-mg/kg reaction mixture by green pepper [15]. Despite such high yields, the conversion rate was only moderate, especially when linolenic acid was applied as the substrate (5.9–16.1%).

Recently, an industrial process was proposed for the production of natural ''green notes'' following the natural pathways (Fig. 1). However, the patented application [29] of *Saccharomyces cerevisiae* for *in situ* reduction of enzymatically produced C6-aldehydes to the corresponding alcohols has been called into question, as the isomerization of 3(*Z*)- to 2(*E*)-hexenal is very rapid and the latter undergoes facile reduction to form 1-hexanol [30]. Additionally, *Saccharomyces cerevisiae* is able to add activated acetaldehyde to 2(*E*)-hexenal, thereby forming 4-octen-2,3-diol.

In view of the production of C6-aldehydes, two enzymes are crucial: (1) a lipoxygenase for the formation of the hydroperoxide and (2) a hydroperoxide lyase catalyzing the cleavage of the hydroperoxide. Much research has already been conducted on lipoxygenases, especially those from soybean. They catalyze the addition of molecular oxygen to the molecule at carbon-13. The resulting hydroperoxide is (*S*)-configured [16]. Lipoxygenases have also been detected in microorganisms and plant lipoxygenases have been expressed in host organisms. They are available for the biotechnological production of the 13-hydroperoxide. The other decisive enzyme is the hydroperoxide lyase. Due to the difficult isolation, biochemical information is rather sparse [12]. The most effective hydroperoxide lyase has been detected in alfalfa. However, 2(*E*)-hexenal was also generated with a high molar conversion rate by the incubation of a hydroperoxide-lyase–containing extract from mung bean seedlings and its substrate, 13(*S*)-hydroperoxy-9(*Z*),11(*E*),15(*Z*)-octadecatrienoic acid [31].

The construction of recombinant yeast cells containing the hydroperoxide lyase gene from banana fruit (*Musa* sp.) has been published [32]. The isolation and transfer of the hydroperoxide lyase gene from banana to yeast was successful. The yeast produced hexenals from 13-hydroperoxides. Thus, the way for the microbial production of hexenals from fatty acids is open as far as the respective host has been generated containing the lipoxygenase and the lyase genes.

Fatty acid hydroperoxide lyase from bell pepper fruits is a heme protein whose spectrophotometric properties greatly resemble a cytochrome P-450. The cDNA encoding fatty acid hydroperoxide lyase from bell pepper was expressed in *Escherichia coli*. The cDNA encodes 480 amino acids and shares homology with P-450s. The most closely related P-450 is allene oxide synthase [33].

A gene from *Arabidopsis* was characterized that is homologous to the cloned hydroperoxide lyase from green pepper (*Capsicum annuum*) [34]. The deduced protein sequence indicated that this gene encodes a cyctochrome P-450 with a structure similar to that of allene oxide synthase (see Chap. 11). The gene was cloned into an expression vector and expressed in *Escherichia coli* to demonstrate hydroperoxide lyase activity. Significant hydroperoxide lyase activity was evident when 13(*S*)-hydroperoxy-9(*Z*),11(*E*),15(*Z*)-octadecatrienoic acid was used as the substrate, whereas activity with 13(*S*)-hydroperoxy-9(*Z*),11(*E*)-octadecadienoic acid was approximately 10-fold lower. Analysis of headspace

volatiles, after the addition of the substrates to *Escherichia coli* extracts expressing the protein, confirmed enzyme-activity data, because 3(*Z*)-hexenal was produced by the enzymatic activity of the encoded protein. However, hexanal production was limited.

It is known that flaxseed produces high amounts of α-ketols from linoleic acid or linolenic acid via the highly unstable allene oxide (Fig. 2). No physiological role within the plant organism has been elucidated so far for this molecule. On the other hand, many soil bacteria are known to catalyze the Baeyer–Villiger oxidation of ketones. The Baeyer–Villiger oxidation is a reaction which inserts oxygen into a ketone to form an ester. Soil bacteria were screened for their ability to grow on 2-tridecanone as the sole source of carbon [35]. The procedure yielded a bacterial culture tentatively identified as *Ralstonia* sp. with abundant monooxygenase activity, which was used as a biological catalyst for a Baeyer–Villiger oxidation. The incubation of the α-ketol with the bacteria yielded hexanal and 3(*Z*)-dodecendioic acid, which can be transformed to traumatic acid, the well-known wound hormone (Fig. 2). Thus, the enzyme system from flaxseed and the monooxy-

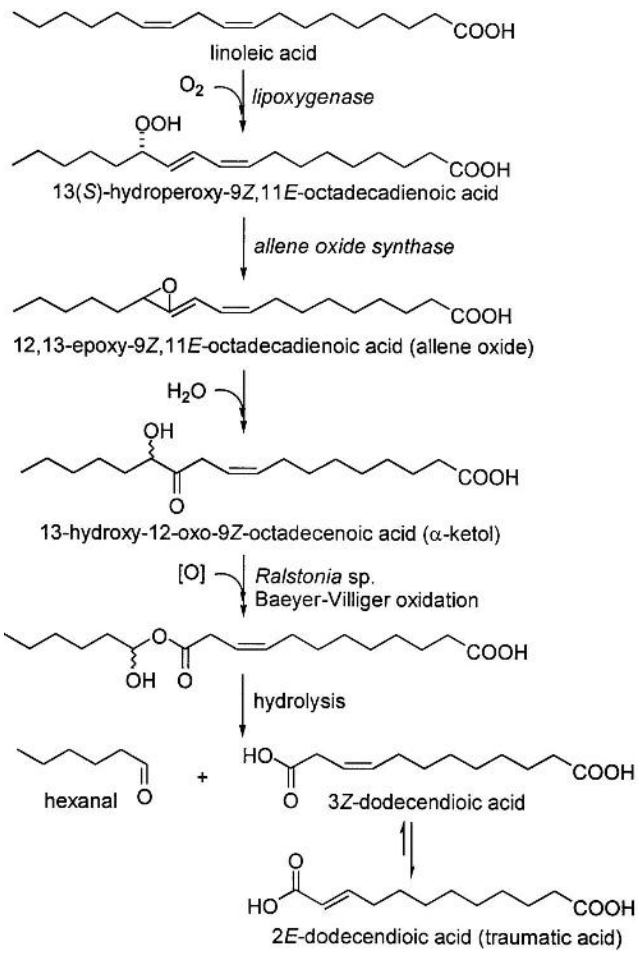


Figure 2 Formation of hexanal and 3(*Z*)-dodecenoic acid by a biological Baeyer–Villiger oxidation. (Adapted from Ref. 35.)

genase system from the microorganism represent a promising approach for the biotransformation of linolenic acid to natural hexenals.

Interconversion between C6-aldehydes and alcohols is thought to proceed through the action of oxidoreductase (Fig. 1). Using an alcohol dehydrogenase mutant of *Arabidopsis*, it has been shown that there were large quantitative and qualitative differences in the accumulation of C6 volatiles in the absence of alcohol dehydrogenase. The total quantity of lipoxygenase-derived volatiles was greater on a fresh weight basis in the alcohol dehydrogenase-deficient mutant [36]. Given the high value of natural leaf aldehydes and alcohols, further investigation into a successful biological system could be very profitable.

2.2 C9 Volatiles

Lipoxygenases and hydroperoxide lyases differ considerably in their enzymic characteristics, depending on their source (Fig 1). Lipoxygenases, such as those found in potato tuber and tomatoes, catalyze the production of 9-hydroperoxides from linoleic acid and linolenic acid [16]. These 9-hydroperoxides are, in turn, cleaved by hydroperoxide lyase to form the volatile products 3(*Z*)-nonenal, 2(*E*)-nonenal, 3(*Z*),6(*Z*)-nonadienal, and 2(*E*),6(*Z*)-nonadienal (violet leaf aldehyde), which are found in cucumber aroma. 2(*E*),6(*Z*)-Nonadienal is of commercial importance because it is one of the most potent fragrance and flavoring substance known. In cucumber fruits and pear fruits, a hydroperoxide lyase has been detected which subsequently cleaves the 9(*S*)-hydroperoxy-10(*E*),12(*Z*)-octadecadienoic acid into 9-oxononanoic acid and 3(*Z*)-nonenal [37]. Currently, there is no description of isolated enzymatic systems that could be utilized in the commercial production of the C9 components.

Recently, in work with soybean (*Glycine max*), it was reported that 3(*Z*)-nonenal was oxidized by lipoxygenase type I to 4(*S*)-hydroperoxy-2(*E*)-nonenal, which, in turn, was converted to 4-hydroxy-2(*E*)-nonenal by a hydroperoxide-dependent peroxygenase [38].

2.3 C8 Volatiles

A series of aliphatic compounds containing eight carbons have been reported as the main volatile contributors to the characteristic flavor of edible mushroom fruit bodies [39]. Among the volatile C8 compounds, including 1-octanol, 3(*R*)-octanol, 3-octanone, (*R*)-1-octen-3-ol, 2(*Z*)-octen-1-ol, and 1-octen-3-one [40], (*R*)-1-octen-3-ol, also known as mushroom alcohol, is the most important volatile associated with fresh mushrooms due to its low odor threshold of 0.01 ppm in water and in oil [10].

(*R*)-1-Octen-3-ol is efficiently formed from the enzymatic breakdown of linoleic acid [41,42], normally present in high levels in mushroom fruiting bodies. Two enzymes, lipoxygenase (linoleate:oxygen oxidoreductase, E.C. 1.13.11.12) and hydroperoxide lyase, are believed to be involved in the formation of (*R*)-1-octen-3-ol and 10-oxo-8(*E*)-decenoic acid [43] (Fig. 3). However, these enzymes have never been purified to homogeneity.

Fungal lipoxygenases exhibit different regioselectivity than lipoxygenase from plants because they catalyze the dioxygenation of linolenic acid to 10-monohydroperoxides [44]. The volatile impacts of mushroom aroma are generated by the activity of a hydroperoxide lyase and other subsequent enzymic steps in damaged fungal cells. Several suggestions have been made regarding the mechanism of the fragmentation reaction, but further work is required to fully characterize the entire sequence (Fig. 4). The investigation

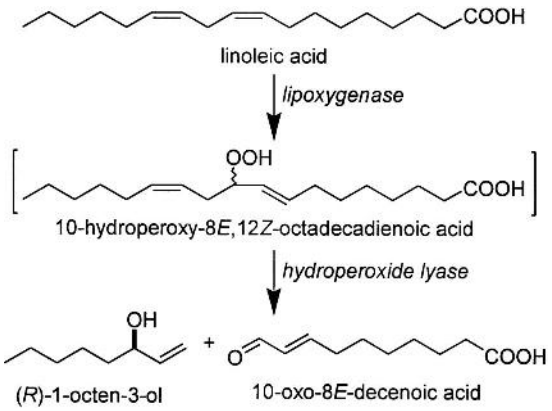


Figure 3 Postulated biosynthetic pathway for the formation of (*R*)-1-octen-3-ol from linoleic acid. (Adapted from Ref. 41.)

of the cleavage of linoleic acid by a mycelial-pellet homogenate of *Pleurotus pulmonarius* into (*R*)-1-octen-3-ol, 10-oxo-8(*E*)-decenoic acid, and 13-hydroperoxy-9(*Z*),11(*E*)-octadecadienoic acid showed that the 13-hydroperoxide of linoleic acid is not an intermediate of the (*R*)-1-octen-3-ol synthesis pathway [45].

Recently, 10-hydroperoxy-8(*E*),12(*Z*)-octadecadienoic acid was tentatively identified as an intermediate of the enzymic reaction [46]. In a similar way, 1,5(*Z*)-octadien-3-ol and 2(*Z*),5(*Z*)-octadien-1-ol are formed from linolenic acid [47]. The production of

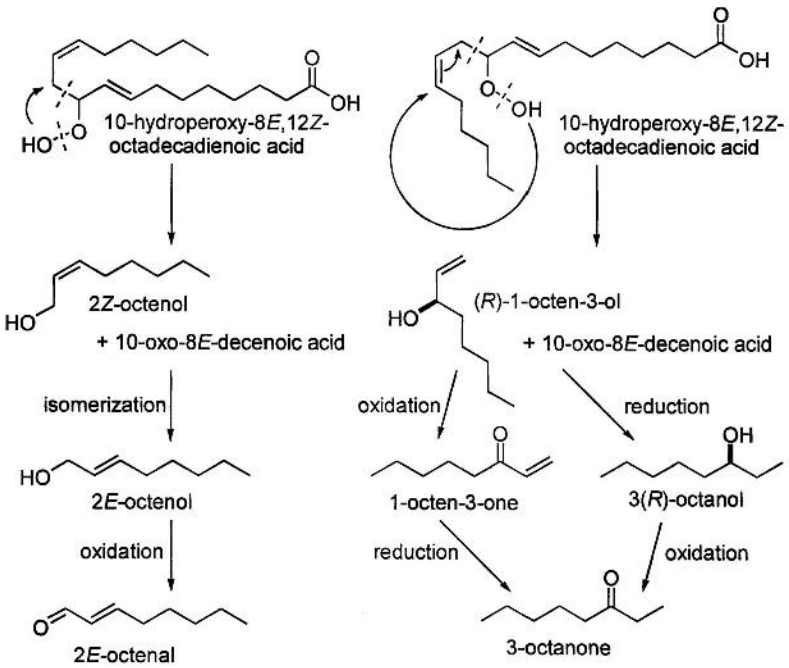


Figure 4 Formal derivation of C8 volatiles of mushroom. (Adapted from Ref. 44.)

enantiomerically pure (*R*)-1-octen-3-ol takes place in mushrooms, especially when the tissue of the fruiting bodies is damaged or disrupted [41,48]. 13-Hydroperoxy-9(*Z*),11(*E*)-octadecadienoic acid seems to inhibit the formation of (*R*)-1-octen-3-ol [45].

The ability to produce C8 alcohols is widespread among fungi. *Trichothecium roseum* has been reported to produce 3-octanol, (*R*)-1-octen-3-ol, (*R*)-1,5(*Z*)-octadien-3-ol, 1-octanol, and 2(*Z*)-octen-1-ol. Similarly, 12 other fragrance C8 alcohols have been identified from species of *Aspergillus*, *Penicillium*, and *Fungi imperfecti*, *Penicillium caseicolum*, and *Penicillium camemberti*. These species also produce (*R*)-1-octen-3-ol, (*R*)-1,5(*Z*)-octadien-3-ol, and 3(*R*)-octanol. However, all of these alcohols are believed to form the corresponding methyl ketones (see Sec. 3.4) by the reductive action of one or more dehydrogenases [49]. To produce these alcohols by biological processes, biosynthetic pathways would have to be manipulated to inhibit dehydrogenase activity.

(*R*)-1-Octen-3-ol has also been found in milk and butter following the oxidation of linoleic acid, and it has been found in large quantities in blue cheese, where it is a specific metabolite of *Penicillium roquefortii* [2]. In Camembert cheese, (*R*)-1-octen-3-ol is present at a concentration of 5–10 ppm, which is 5–10 times higher than in Roquefort. (*R*)-1-Octen-3-ol is a critical contribution to the distinctive flavor of Camembert; it also adds the pleasant moldy-type flavor of other fungal surface-ripened cheeses [50].

Cell-free preparations of *Pseudomonas oleovorans* have been found to convert the allylic alcohols (*R*)-1-octen-3-ol and 3(*R*)-octanol to 1-octen-3-one and 3-octanone, a flavor compound with a lavender note (Fig. 4) [51].

Recently, (*R*)-1-octen-3-ol has been found to attract tsetse flies [52,53]. Screening spike responses of olfactory cells showed that 40% of the olfactory cells of tsetse flies respond to (*R*)-1-octen-3-ol. Therefore, it has been suggested that (*R*)-1-octen-3-ol is involved in host detection from a distance. Furthermore, (*R*)-1-octen-3-ol, a component of the body odor of ruminants [53], was demonstrated to be an attractant for host-seeking hematophagous insects [54]. However, trap harvests with (*R*)-1-octen-3-ol as bait have been disappointing.

Investigations of the volatile compounds formed from mixed microbial cultures proved that bacteria are also capable of producing (*R*)-1-octen-3-ol [55]. Industrial processes have been developed to produce (*R*)-1-octen-3-ol, based on the feeding of mycelia with linoleic acid [10]. The effect of fatty-acid-rich substrates on the production of (*R*)-1-octen-3-ol by the edible fungus *Pleurotus pulmonarius* during growth was studied [56]. A clear relationship between the production of (*R*)-1-octen-3-ol and lipoxygenase activity was found. The results suggested that lipoxygenase activity and, subsequently, (*R*)-1-octen-3-ol biosynthesis in *Pleurotus pulmonarius* are enhanced by the presence of substrates containing fatty acids in the growth medium. Recently, agricultural treatments, such as the addition of calcium chloride to irrigation water, have been shown to improve lipoxygenase activity [3].

2.4 Jasmonic Acid Derivatives

(−)-Jasmonic acid and (+)-7-*iso*-jasmonic acid (epijasmonic acid) are important members of the family of jasmonates (Fig. 5). They are widely distributed in plants including lower eukaryotes. The methyl ester of (+)-7-iso-jasmonic acid contributes to the precious flavor of jasmine oil and also to *Rosmarinus*, *Gardenia*, *Artemisia*, and lemon oil and to black tea aroma [44]. Biogenetically, jasmonic acid derivatives originate from linolenic acid catalyzed by enzymes of the ''octadecanoid pathway,'' which contains 13(*S*)-hydroper-

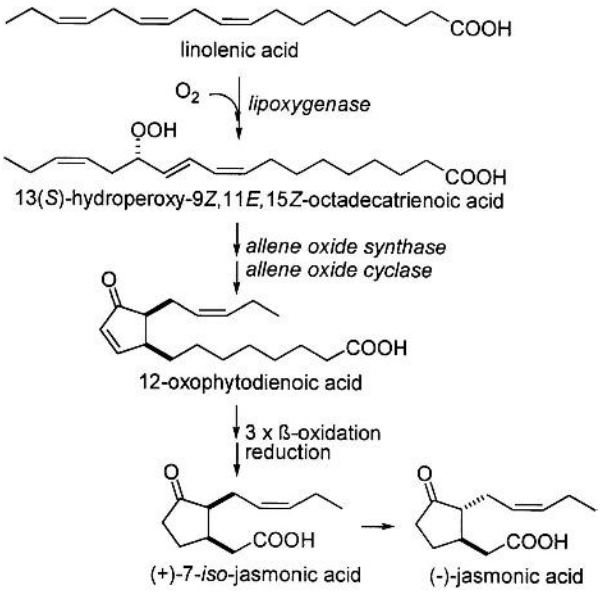


Figure 5 Formation of (−)-jasmonic acid and (+)-7-*iso*-jasmonic acid by enzymes of the ''octadecanoid pathway.'' (Adapted from Ref. 12.)

oxy-9(*Z*),11(*E*),15(*Z*)-octadecatrienoic acid and 12-oxophytodienoic acid as intermediate compounds (Fig. 5) [57]. Reduction of the double bond of the cyclopentanone ring and β-oxidation of the carboxylic acid side chain lead finally to the precursor acid of the odorous methyl 7-iso-jasmonate. Jasmonates, their precursors, and their metabolites are known to accumulate in higher plants and in fungi. For fungi, detailed analysis was performed on the occurrence of the various jasmonates in *Botryodiplodia theobromae*, *Gibberella fujikuroi*, and *Fusarium oxysporum*. Inspection of 46 fungal strains revealed (−)-jasmonic acid and (+)-7-*iso*-jasmonic acid as major constituents [58]. Some of the jasmonoid compounds regulate plant growth and senescence (see Chap. 11).

3 VOLATILES FORMED BY α- OR β-OXIDATION

Catabolism of straight-chain, common fatty acids by α- and β-oxidation is a major process for the production of flavor volatiles. The proposed fatty acid α-oxidation mechanism in plants involves free fatty acids (C12–C18) which are enzymatically degraded via one or two intermediates to C($n-1$) long-chain fatty aldehydes and CO_2 (Fig. 6) [59]. The β-oxidation results in successive removal of C2 units (acetyl-CoA) from the parent fatty acid. The detailed mechanisms of conventional β-oxidation are well established and will not be discussed here. Reviews on β-oxidation in mammalian cells and on the fatty acid degradation in plants have been published recently [60–63].

3.1 Carboxylic Acids

Straight-chain, saturated aliphatic acids are found in many essential oils and foods. These acids contribute to the aroma. In flavor compositions, aliphatic acids up to C10 are used

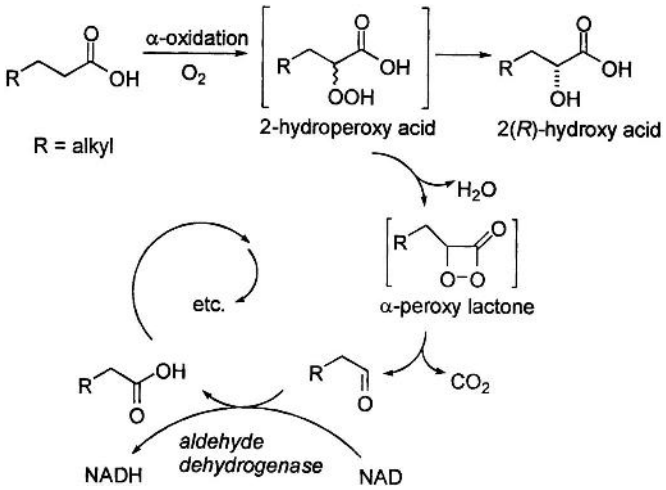


Figure 6 α-Oxidation of fatty acids in higher plants. (Adapted from Ref. 59.)

to accentuate certain aroma characteristics. Short- and medium-chain, linear carboxylic acids possess sharp, buttery, and cheeselike odors that change to fatty with increasing chain length. The common linear fatty acids are formed during repeated β-oxidative cycles upon lipolysis of regular triacylglycerols and are found, consequently, in many of the mixed food fermentations. The formation of short- and medium-chain carboxylic acids has been summarized [11,44,50].

Fatty acids can play a significant role in flavors, not only on their own, such as in dairy flavors, but also as substrates for other flavor biosyntheses. Specifically, flavor ester production using lipase-based conversions relies heavily upon an adequate natural source of inexpensive organic acids (see Sec. 4).

One of the first flavors produced on a large scale by means of enzymes was a product known as lipolyzed milk fat. The original process involved subjecting cream to a controlled enzymatic hydrolysis using lipases (see Sec. 4.1). Several lipases exhibit a high selectivity toward the flavor-active short-chain fatty acids, such as, for instance, the enzyme from *Mucor miehei* [10]. The fatty acids liberated in a lipase-catalyzed process can be isolated by steam distillation and the individual acids can then be further purified by fine distillation. In such a way, the short-chain fatty acids such as butanoic, hexanoic, octanoic, and decanoic can be obtained in pure form. As recently reported, lipolyzed milk fat products find wide applications (e.g., for the enhancement of cream/butterlike flavors) [10].

3.2 Lactones

A major group of fatty-acid-derived flavor compounds are lactones or alkanolides. The naturally occurring organoleptically important lactones generally have γ- (4-) or δ- (5-)-lactone structures and are straight-chained, and a few are even macrocyclic [64]. γ-Lactones are found primarily in plants and δ-lactones primarily in animal products. Lactone flavor substances play an important role in the overall aroma presentation of many of our foods and beverages. The chain length can be even or odd numbered. However, the even-numbered predominate. Sensory important lactones usually possess 8–12 carbon atoms

and some are very important flavor components for pineapples, apricots, strawberry, raspberry, mango, papaya, passion fruit, peach, plum, milk products, and fermented food. Due to their low odor threshold, they have a high flavor value in the fruits.

Lactones are produced in plants in minute amounts, by catabolic processes involving the structurally related fatty acids when the fruit ripens. However, the fact that both the optical purity and the absolute configuration can vary for identical lactones isolated from different sources supports the idea of the presence of different biosynthetic pathways. Thus, the biosynthesis of lactones in plants and microorganisms is complex and not that well understood. However, all lactones originate from their corresponding hydroxy carboxylic acids, which are formed by either of these systems:

1. Reduction of oxo acids by NAD-linked reductase
2. Hydration of unsaturated fatty acids
3. Epoxidation of unsaturated fatty acids (Fig. 7)
4. From hydroperoxides (Fig. 8)
5. From naturally occurring hydroxy fatty acids (Fig. 9)
6. Cleavage of hydroxylated long-chain fatty acids

Most of the work on the biosynthesis of lactones has been done using microorganisms [65,66]. However, recently, results were presented on the biotransformation of (*R*)-γ-dodecalactone in ripening strawberry fruits [67,68]. The 9,10-epoxyoctadecanoic acid, formed by epoxidation from oleic acid, was proposed as the precursor for (*R*)-γ-dodecalactone in

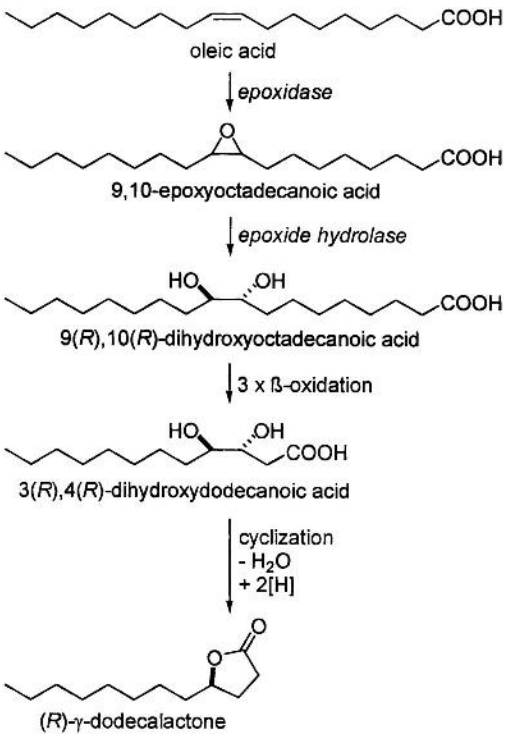


Figure 7 Biosynthesis of (*R*)-γ-dodecalactone in strawberry fruits. (Adapted from Ref. 68.)

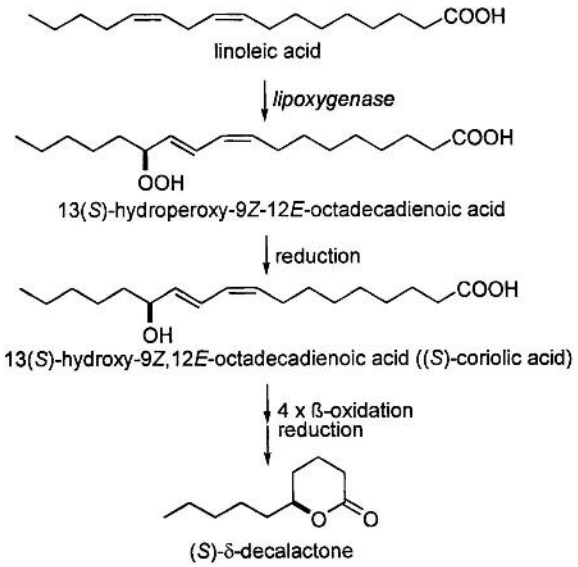


Figure 8 Conversion of (*S*)-coriolic acid to (*S*)-δ-decalactone by *Cladosporium suaveolens*. (Adapted from Ref. 78.)

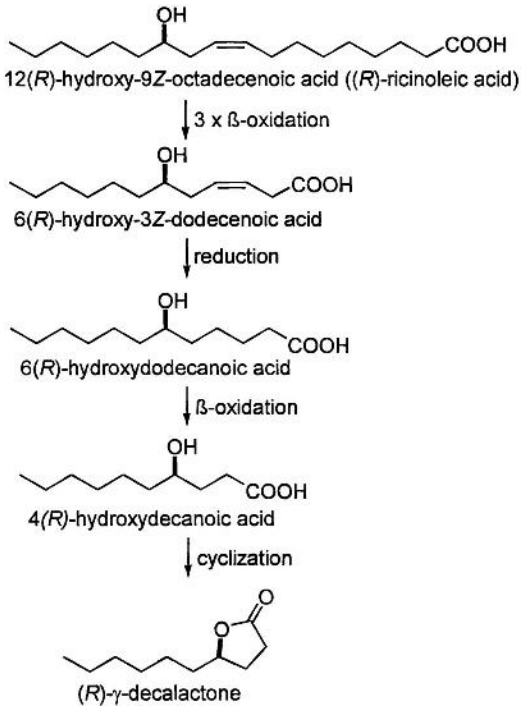


Figure 9 Proposed degradation of (*R*)-ricinoleic acid to (*R*)-γ-decalactone. (Adapted from Ref. 78.)

strawberry fruits (Fig. 7). Repeated β-oxidative cycles and cyclization leads to the lactone. The novel pathway relies on enzymatic reactions, which are ubiquitous in the plant kingdom and which are often involved in plant defense against microbial aggressors. A different degree of regioselectivity and enantioselectivity was obtained for strawberries and nectarines [68]. It has not been elucidated whether lactones are formed intracellularly and excreted, or if the hydroxy acids are released from the cell and then undergo spontaneous lactonization.

At present, lactones are made fairly expensively via chemical synthesis from oxo acids. On the other hand, microbially produced lactones have the advantage of being optically pure and natural.

There are numerous microorganisms that are known to synthesize lactones either by de novo synthesis, by β-oxidation from ricinoleic acid, free fatty acids, or hydroxy acids, or by reduction from unsaturated lactones or from cheese [49,69] (Fig. 10). The production of lactones and peroxisomal β-oxidation in yeast has been reviewed recently [70].

Although a variety of microorganisms can perform de novo lactone biosynthesis (Fig. 10), yields are, unfortunately, invariably very low and amount to only a few milligrams per liter or less. Such low yields usually preclude the use of such systems for

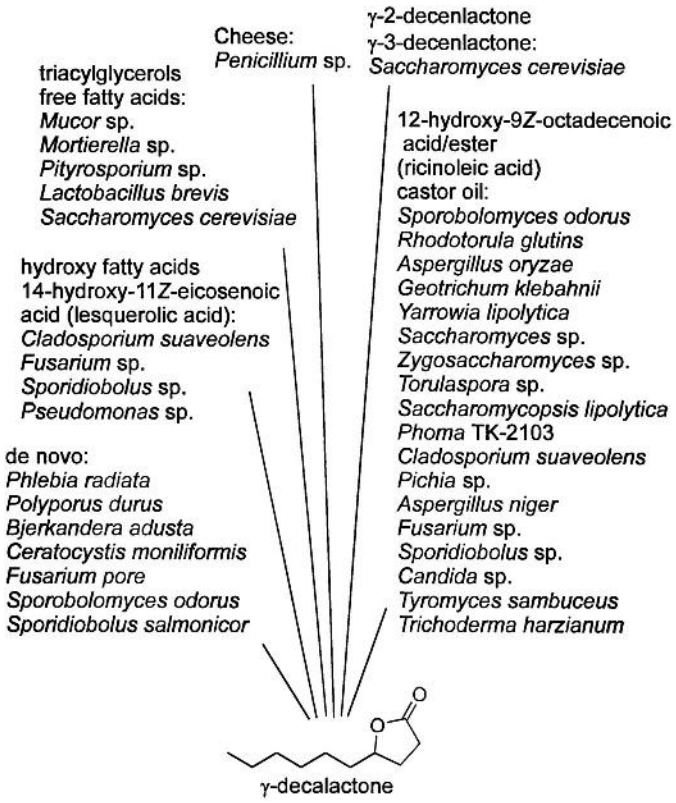


Figure 10 Metabolic pathways to γ-decalactone. (Adapted from Refs. 11 and 69.)

production purposes. Nonetheless, quite a number of systems have been described in which lactones are produced by using microorganisms.

The conversion of ricinoleic acid [12(*R*)-hydroxy-9(*Z*)-octadecenoic acid] into (*R*)-γ-decalactone by *Yarrowia lipolytica* and *Candida lipolytica* has been studied in some detail [71–74]. The experimental data indicated that ricinoleic acid first underwent three separate β-oxidation cycles to form 6(*R*)-hydroxy-3(*Z*)-dodecenoic acid before the carbon–carbon double bond was reduced by the yeast (Fig. 9). A fourth β-oxidation step then converted the so-formed 6(*R*)-hydroxydodecanoic acid into 4(*R*)-hydroxydecanoic acid producing (*R*)-γ-decalactone upon cyclization [75].

During this oxidation, the action of acyl-CoA oxidase is fundamental. In *Yarrowia lipolytica*, it was shown that five acyl-CoA-oxidase genes (ACO1 to ACO5) were present. In order to investigate the role of each ACO isoenzyme, mono-disrupted strains were constructed. The acyl-CoA activity was measured for each strain, showing that a long-chain oxidase was encoded by ACO2 and a short one by ACO3. Lactone production was increased in strains with disrupted ACO3 and to a lesser extent in strains with a nonfunctional ACO5. Lactone consumption was high for strains with disrupted ACO4 [76].

In *Pichia guilliermondii*, it has been shown that the metabolism responsible for the bioconversion of methyl ricinoleate was peroxisomal β-oxidation. It was suggested that the entry of fatty acids into the peroxisomes took place in a carnitine-dependent manner and this step might be a limiting step in the metabolism [77].

The successful biotransformation of ricinoleic acid via microbial β-oxidation has resulted in the search for sources of other suitably oxo-functionalized fatty acids, which can be converted into natural lactones. Such natural fatty acids can be found in, for example, sweet potatoes or Jalap resin containing 11-hydroxypalmitic acid and 3,11-dihydroxymyristic acid. *Saccharomyces cerevisiae* is capable of converting both these acids into the corresponding δ-lactones [78].

The formation of γ-dodecalactone through a two-step microbial conversion of oleic acid was suggested. Microorganisms were screened for production of 10-hydroxystearic acid from oleic acid, and a bacterium producing the hydroxy acid with a transformation yield of 62% was isolated. The hydroxy acid was found to be biotransformed to γ-dodecalactone by *Saccharomyces cerevisiae* [79].

The δ-decalactones and δ-dodecalactones can be produced using yeast or fungi to hydrogenate unsaturated lactones obtained from the bark oil of the Massoi tree [80]. Massoi bark oil is commercially available and, depending on the purity, may contain approximately 80% δ-2-decenlactone and 7% δ-2-dodecenlactone, the precursors of δ-decalactone and δ-dodecalactone, respectively.

In a similiar reaction, *Saccharomyces cerevisiae* is capable of reducing γ-3-decenlactone resulting in the formation of almost enantiomerically pure (+)-(*R*)-γ-decalactone. γ-3-Decenlactone is a by-product formed in the fermentative production of γ-decalactone from castor oil or ricinoleic acid [81].

Unfortunately, most of the organisms cited produce the compounds of interest only in trace amounts. Some of the studies mention the presence of lactones but fail to quantify them. However, from an industrial production viewpoint, there are several micro-organisms that are promising for lactone production; for example, *Trichoderma harzianum* was able to produce 260 mg/L of γ-decalactone after 7 days of culture in a stirred 14-L fermentor using castor oil as the sole carbon source [82].

As an alternative to whole-cell systems, there are a few enzyme systems that can be used to produce lactones; for example, a lipase-esterase preparation from *Mucor miehei*

was used in a nonaqueous environment to convert 4-hydroxybutyric acid to γ-butyrolactone [11]. Lactones are chiral molecules, and molecules extracted from fruit have a defined stereochemistry. Generally, the (*R*)-isomer predominates, but appreciable amounts of the (*S*)-isomer are found in some natural sources. Chemical synthesis of lactones only yields a racemic mixture. Therefore, microorganisms capable of forming optically active lactones were sought [83,84]. Enantiodifferentiation of γ-lactones produced during the bioconversion of soybean fatty acids by *Penicillium roquefortii* spore showed that γ-dodecalactone and γ-hexalactone were first produced with an enantiomeric excess (99%) in favor of the (*R*) isomer, whereas an enantiomeric excess in favor of the (*S*) form (94%) was found for γ-6(*Z*)-dodecenlactone, the major lactone produced by the fungus [85]. Alternatively, lipase-catalyzed stereoselective hydrolysis of saturated racemic γ- and δ-lactones leads to enantiomerically pure or enriched lactones [86].

In contrast to 4- and 5-hydroxy fatty acids, 3-hydroxy acids, the regular intermediates of the β-oxidation, do not form lactones. However, they are converted to the methyl- or ethyl-3-hydroxyester in plants (see Sec. 4.2) and contribute to the aroma of fruits such as pineapples, apples, tamarillos, and cajas [87–90].

3.3 Aldehydes

Long-chain aldehydes have been identified as volatile components in cucumber plants, germinated peanut cotyledons, pea leaf, potato tuber, rice seedlings, the marine green algae *Ulva pertusa*, and, very recently, the essential oil obtained from fresh green tobacco leaves [91,92]. The aldehydes are formed by an α-oxidation system from common fatty acids (Fig. 6). The mechanism of this biochemical reaction was worked out in the seventies [93]. Enzyme systems capable of fatty acid α-oxidation have been demonstrated in both plant and animal tissue. In plants, α-oxidation of fatty acids are responsible for the production of volatile compounds which may give rise to characteristic taste and flavor [94].

Oxidation in the α-position of the fatty acid carbon chain occurs in the formation and degradation of 2-hydroxy fatty acids, intermediates of peroxisomal catabolism of certain fatty acids. Following oxidation of 2-hydroxy fatty acids by 2-hydroxy acid oxidase, the 2-oxo acid formed undergoes oxidative decarboxylation leading back to the acyl-CoA track of β-oxidation [62].

However, the term ''α-oxidation'' actually refers to a different process located, presumably, at the endoplasmic reticulum of plant cells. α-Oxidation is known to act only on free fatty acids of C14–C18 chain length and yields a free fatty acid or aldehyde containing one carbon atom less than the parent fatty acid, CO_2, and/or 2(*R*)-hydroxy fatty acid (Fig. 6). According to the reaction mechanism proposed, the intermediate of this process is a 2(*R*)-hydroperoxy fatty acid, which is decarboxylated to fatty aldehyde and/or reduced to 2(*R*)-hydroxy fatty acid. The fatty aldehyde can be oxidized by a NAD-dependent fatty aldehyde dehydrogenase, yielding a free fatty acid [59] (Fig. 6). However, complete degradation of the fatty acid attacked by α-oxidation requires subsequent β-oxidation due to the restriction of α-oxidation to long-chain fatty acids. Long-chain fatty aldehydes synthesized by α-oxidation are volatile products formed by plants and serve different functions [95].

The universal nature of β-oxidation and its established role in certain plant tissues has possibly overshadowed the importance of α-oxidation in plants. It is possible that α-oxidation may play a further major role in metabolism of plants.

An enzyme with high fatty acid α-oxidation activity has been purified from cucum-

ber fruits (*Cucumis sativus*). The specific α-oxidation activity in the purified fraction was determined by the liberation of CO_2 from 1-^{14}C palmitic acid. The molecular weight of the native enzyme was 240,000 and that of the major subunit was 40,000, indicating an oligomeric structure [96]. Tetradecylthioacetic acid and tetradecylthiopropionic acid, two substrate analogs, could be α-oxidized in a cucumber α-oxidation system [97].

The substrate selectivities of the α-oxidation of saturated, unsaturated, and heteroatom-containing carboxylic acids by an enzyme extract of peas (*Pisum sativum*) indicated that this biotransformation proceeds highly enantioselectively [98,99].

α-Oxidation in animal systems was studied with 3-methyl-substituted fatty acids in rat liver using hepatocytes. The experiments revealed that the primary end product of α-oxidation in the rat liver is formic acid, which is then converted to CO_2. This mechanism is different from the plant system. It was suggested that α-oxidation, which is confined to peroxisomes, involves a fatty acid activation reaction and a dioxygenase reaction [100].

3.4 Methyl Ketones

Among the volatiles of *Penicillium*-ripened cheese, odd-numbered 2-alkanones (methyl ketones) from 5 to 11 carbons have received much attention [101]. They are generated during cheese ripening from lipid metabolism by the filamentous fungus [50]. The blue cheese taste is mainly obtained by the addition of methyl ketones, which are primarily recognized for their contribution to the flavor of blue-veined cheeses. The distinctive taste of Roquefort cheese is substantially due to 2-heptanone and 2-nonanone produced by *Penicillium roquefortii*, which also gives this cheese its blue veins [102]. Methyl ketones have also been detected in *Brassica napus* pods [103] and in the queen pheromone of stingless bees [104]. Also, 2-undecanone and 2-tridecanone have been found in leaf-surface exudates of *Lycopersicon hirsutum* f. *glabratum* accessions [105].

The need to produce methyl ketones that qualify for the natural label has prompted intensive studies, and several processes involving fatty acid breakdown have been proposed [101]. The details of the biochemical pathway, whereby the microorganism converts fatty acids into methyl ketones, have been elucidated and have been used as the basis for a large-scale fermentation process. The way microorganisms such as *Penicillium roquefortii* produce methyl ketones from medium-chain fatty acids is of considerable scientific interest, as it resembles conventional β-oxidation until the final step, at which time a decarboxylase produces the methyl ketone, replacing the thiolase found in β-oxidation (Fig. 11) [106]. The formation of methyl ketones from fatty acids is an aerobic process described by the following stoichiometric equation:

$$R{-}CH_2{-}CH_2{-}COOH + O_2 \rightarrow R{-}CO{-}CH_3 + CO_2 + H_2O$$

The bioformation results from an overflow of the β-oxidation cycle, where an excess of 3-oxoacyl-CoA ester is accumulated. The key component, 3-oxoacyl-CoA, can be converted either into methyl ketone by hydrolysis through thiohydrolase action followed by decarboxylation, or into CO_2 by thiolase followed by the Krebs cycle (Fig. 11). The last possibility corresponds to the complete β-oxidation of fatty acids. A part of the pool of 3-oxoacyl-CoA is not processed further to acetyl-CoA and acyl-CoA, but spontaneously hydrolyzed, followed by decarboxylation. A bioenergetic balance on all the reactions involved from fatty acids to methyl ketones shows that the reactions are energetically self-

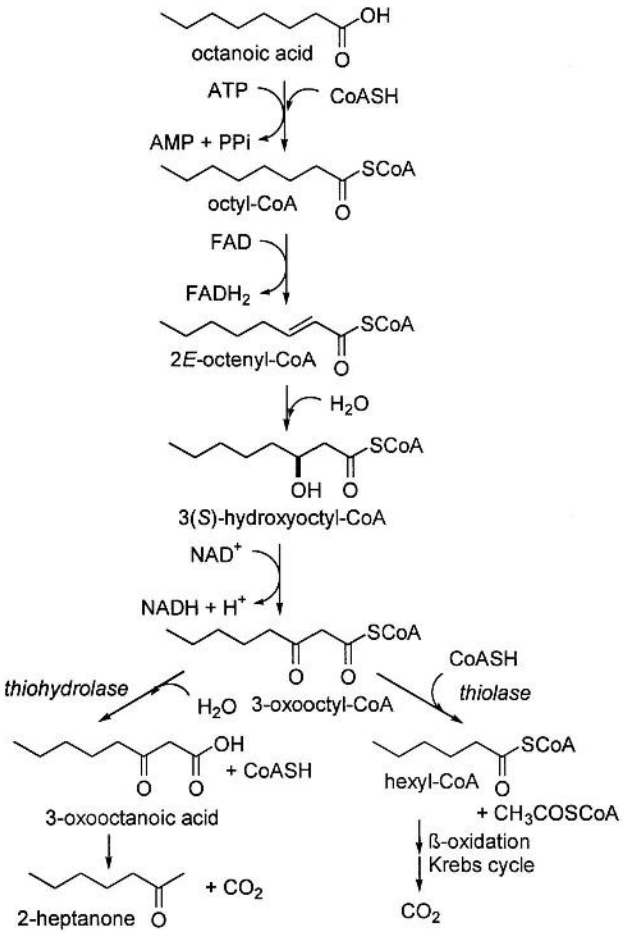


Figure 11 Biosynthesis of 2-heptanone from octanoic acid by *Penicillium roquefortii*. (Adapted from Ref. 101.)

sufficient and thus capable of functioning with a biotransformation yield equal to unity, without the need for an energetic substrate.

The biotechnological production of methyl ketones from lipids by microorganisms was recently reviewed [50]. Commercially successful microbial processes for making methyl ketones have been used for some time. In one process, spores of *Penicillium roquefortii* in a water–isoparaffin solvent, a two-phase system, are used in a fed-batch procedure carried out at constant substrate concentration for the production of C5–C9 methyl ketones from the corresponding C6–C10 fatty acids [101]. Spores are more active in producing methyl ketones than mycelium, and solid-state fermentations are sometimes used. Methyl ketones are also produced on a commercial scale, using spores of an *Aspergillus niger* strain. In this process, coconut fat, which has a high octanoic acid content, is mixed with cellulose powder and is converted into a mixture of methyl ketones by solid-state fermentation [101]. The octanoic acid is metabolized into 2-heptanone, which has a strong blue

cheese flavor [107]. In a similar process, octanoic acid was degraded to 2-heptanone by solid-state fermentation using the ascomycete *Monascus purpureus* [108].

4 FORMATION OF VOLATILE ESTERS

Aliphatic esters contribute to the aroma of nearly all fruits and many foods such as chocolate and fermented beverages. Some are responsible for a particular fruit aroma or for the smell of a particular flower; however, many of these esters possess a nonspecific fruity odor. As the number of carbon atoms increases, the odor changes to fatty-soapy and even metallic.

Important esters that can be found include amyl butyrate (strong etheral, fruity odor reminiscent of apricots, bananas, and pineapples), isobutyl butyrate (fruity odor suggestive of pears, pineapples, and bananas), and ethyl butyrate (pineapple note). Of particular commercial importance are ethyl propanoate, butanoate, laurate, and 2- and 3-methylbutyl acetates [21].

The straight-chain ester constituents are believed to be synthesized via β-oxidation of fatty acids to give acetic, butanoic, and hexanoic acids, which may then be reduced to the corresponding alcohols before transesterification. Traditionally, the esters are extracted directly from plant material. An alternative is the use of plant tissue culture; however, this is not cost-effective at this time, because of high processing costs from expensive media and dilute product levels.

Esters are usually prepared chemically by esterification of carboxylic acids with alcohols. The biological production of flavor esters can proceed via two routes: (1) esterification of an organic acid with an alcohol by lipases or (2) the alcoholysis of acyl-CoA compounds with alcohol acyl transferase (Fig. 12).

4.1 Lipases

Esters are formed in many fermentation processes, but they are not usually produced in high enough yields to make recovery attractive. Often, it is more economical to make the ester from the alcohol and the carboxylic acid using lipase as a natural catalyst.

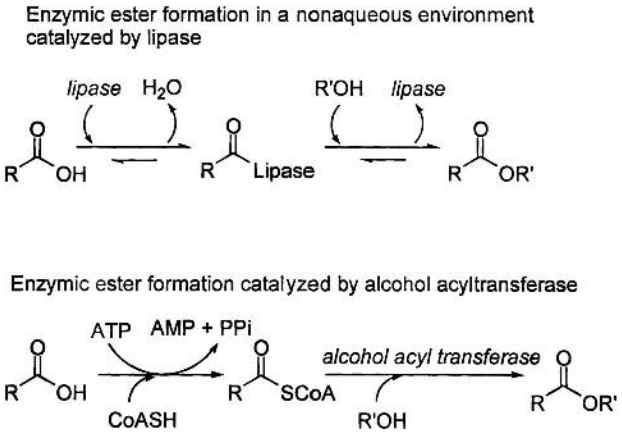


Figure 12 Lipase-mediated ester synthesis and ester formation catalyzed by alcohol acyltransferase. (Adapted from Refs. 11 and 115.)

Lipases (glycerol ester hydrolases, E.C. 3.1.1.3) comprise a group of enzymes of widespread occurrence in nature [11]. Lipase activity does not depend on cofactors. The chemical reagent is simply water. This feature simplifies process requirements and can have a significant impact on process economics. Lipases are active at the oil–water interface of heterogenous reaction systems.

Previously, it was believed that lipases contain a higher proportion of hydrophobic than hydrophilic amino acids, enabling closer interaction with hydrophobic substrates. However, a recent survey of the amino acid profiles of various lipases has indicated that, in fact, as a group, lipases are not more hydrophobic than other groups of enzymes [11]. Because lipases do not exhibit exceptionally high hydrophobicity, the strong interaction with hydrophobic substrates at an interface is most likely the result of hydrophobic patches on the surface of the enzyme. This is also the probable explanation of the strong self-association that is noted with lipases in aqueous solution.

In general, lipases are acidic glycoproteins of molecular weight ranging from 20,000 to 60,000, capable of catalyzing the partial or even complete hydrolysis of triacylglycerols to provide free fatty acids, diacylglycerols, monoacylglycerols, and glycerol. Examples of free fatty acids that are produced by this reaction are propionic and butyric acids for cheese-type flavors. These acids are now produced commercially in this manner (see Sec. 3.1).

However, it has been demonstrated that in organic solvents, due to low water activity, the equilibrium can be shifted so that a condensation-type reaction occurs. Two types of important reversible reactions are catalyzed by lipases: (1) interesterifications and (2) intraesterifications. Reactions favoring esterification can be encouraged by a relatively simple adjustment of the hydrophobic nature of the reaction solvent surrounding the enzyme; that is, the esterification reaction is favored if the water activity is low, and this can be very useful for production of flavor esters. In these nonaqueous systems, the bulk solvent is generally an apolar solvent. It is becoming increasingly clear that a small amount of water must be present in the apolar solvent to maintain conformational integrity and, hence, activity. The presence of this small amount of water allows the enzyme conformational changes required to accommodate substrate entry.

Esterification reactions can be conducted in organic solvents because lipases are stable and retain partial to full activity in such solvents [109]. Lipases are particularly well suited to this hydrophobic environment because they are often associated with the lipid membranes of cells. One advantage of using an organic solvent is that the reactants and products often have a much higher solubility in the solvent than in water. The increase in solubility leads to better enzyme–substrate interaction and, hence, better reaction efficiencies. Lipases are relatively stable but expensive, so there can be a greater incentive to use immobilization than with other enzymes. Crude enzyme preparations are much less expensive and are thus preferable in cases where the presence of side reactions catalyzed by contaminating enzymes can be tolerated.

The utility of using dried fungal mycelium from *Rhizopus arrhizus* in apolar solvents for production of esters has been demonstrated [11]. The approach is interesting, because it could eliminate the need for several preparatory steps, including enzyme solubilization, purification, and immobilization, which are sometimes required for enzyme-based processes. Additionally, the association of lipase with the cellular biomass could stabilize the endogenous enzyme system, as most enzymes are known to be more stable when associated with membranes and other biological structures.

The biocatalytic production of natural flavor esters was examined in both batch and

fed-batch immobilized enzyme reactors. Lipase from *Candida cylindracea*, immobilized on silica gel, was used as the catalyst, because it had been shown previously to possess broad substrate specificity. Significant levels of ethyl butyrate were produced by this system, along with various other important flavor esters [11].

Various other studies have examined lipase-mediated esterifications [49]. Experience has shown that conventional immobilization methods applied to lipase have resulted in preparations of low activity [110,111]. To date, lipases have shown potential for use in stereospecific and regiospecific hydrolysis and esterifications to yield pure, optically active aliphatic and aromatic esters. There is a possibility that lipases can be used to purify mixed enantiomeric compounds to yield optically active flavor esters. A list of microbial lipases commonly used in organic synthesis has been published [10]. However, careful selection of different lipases must be done, as they can have markedly different regiospecificities. It has been established that lipases, in general, have a catalytic site located on the bottom of a groove that is covered with a kind of lid [112]. The lid is important for the binding of the lipase to its substrate. In the groove between the lid and the catalytic site, there is a secondary binding site for the substrate. It may be possible to increase significantly the efficiency of lipase by modifying these regions around the active site. Recent advances in protein engineering should facilitate directed site modification [113,114].

An interesting example of how a bench-scale-level development can be made into a commercial reality by applying rDNA technology has been presented [112]. It was found that a lipase with suitable functional properties was produced by an unspecified organism isolated from the environment in a wide-ranging screening program. Through lipase gene transfer to a GRAS (Generally Recognized as Safe) host, a strain was produced suitable for industrial production. The fungus *Aspergillus oryzae*, which is used extensively for food enzyme production, was selected as the host. This example illustrates an approach that can be taken not only with lipase but with many other enzymes and opens the possibility of using further limited-availability enzymes for production of flavors (e.g., lyase enzymes for ''green'' alcohol/aldehydes) (see Sec. 2.1).

4.2 Alcohol Acyltransferase

Although volatile esters constitute one of the largest and main group of volatile compounds in fruits, there are very few results on the biochemical aspects of ester formation in fruits [115]. The mechanism of ester formation is better known in microorganisms [116–118]. It has been demonstrated that esterification of alcohols and acids is a coenzyme A-dependent reaction. First attempts for alcohol acyltransferase purification in banana, apple, and strawberry have been conducted [119–122].

Alcohol acyltransferase, a membrane-associated enzyme responsible for the transfer of alcohols to acyl-CoA, mediates the formation of esters. Normally, the levels of acyl-CoA, particularly acetyl-CoA, are regulated tightly, because acetyl-CoA is a major metabolite consumed by the Krebs cycle, but under some conditions, the intracellular pools can be increased significantly. Acyl and alkyl moieties can be prepared along common pathways such as the β-oxidation of fatty acids. The alkyl moieties are acylated by alcohol acyltransferase that transfers CoA-activated fatty acid moieties. The absolute amounts of the single ester compounds depend on the concentration and availability of the substrates and on the specificity of the transferase.

Recently, alcohol acyltransferase from strawberry fruits was studied. The enzyme was tested for its preference in using different acyl-CoAs and alcohols. Strawberry alcohol

acyltransferase was found to be active against acetyl-CoA (100%), butyl-CoA (70%), and propyl-CoA (20%), whereas hexan-1-ol was the preferred substrate among five alcohols tested. A clear correlation was observed between substrate preference and volatile esters present in strawberry var. Chandler [121]. Alcohol acyltransferase from banana proved to be a more selective enzyme forming acetate esters of butanol and only very low amounts of propionate and butyrate esters. In both cases, the results showed a clear correlation between substrate specificity and volatile esters present in each fruit aroma, suggesting a determining role for alcohol acyltransferase in flavor biogenesis [123].

The biosynthetic pathway of unsaturated C10-, C12-, and C14-esters has been investigated in Bartlett pears. The origin of the decadienoate esters can be explained by β-oxidation of linoleic acid followed by the action of an alcohol acyltransferase [4].

Very recently, biosynthesis of straight-chain ester volatiles was studied in apples using deuterium-labeled fatty acids [124]. Surprisingly, saturated and monounsaturated fatty acids were metabolized to hexyl, hexanoate, heptanoate, and octanoate esters, whereas linoleic acid produced only hexyl and hexanoate esters. Analysis of the mass spectra of the deuterated saturated esters revealed that they are derived from intermediates of β-oxidation. Unsaturated straight-chain ester constituents appear to arise only by the lipoxygenase pathway.

5 MISCELLANEOUS REACTIONS

5.1 Dehydrogenase

A number of aliphatic aldehydes can be produced using isolated, well-known enzymes such as alcohol oxidase and alcohol dehydrogenase. In this context, horse liver alcohol dehydrogenase has received more attention than any other alcohol dehydrogenase, as it can oxidize primary alcohols. Regarding flavor biotechnology, the recently described conversion of 3(*Z*)-hexen-1-ol to the corresponding aldehyde mediated by horse liver dehydrogenase is mentioned [10]. A maximum yield of 3(*Z*)-hexenal of 50–60% can be obtained in a biphasic system with propanol and an alcohol oxidase as the cofactor regenerating system.

5.2 1,3-Dioxanes

Recently, a novel group of volatiles was isolated, initially from apple cider and later also from apples and pears [125]. The basic structure of the new flavor volatiles is called 1,3-dioxane (Fig. 13). Generally, these compounds are formed by the reaction of aldehydes such as acetaldehyde, propanal, butanal, and hexanal with (*R*)-octan-1,3-diol, (*R*)-5(*Z*)-octen-1,3-diol [126], or 3(*R*),7(*S*)- and 3(*R*),7(*R*)-octan-1,3,7-triol [127–129]. Because the 1,3-dioxanes were initially isolated from apple cider, it was assumed that they have been synthesized from the 1,3-diols present in apples and acetaldehyde formed by the fermentation process. However, the 1,3-dioxanes were also found as naturally occurring components in mature, uninjured apple and pear fruits [130]. The odor impression of the 1,3-dioxanes has been described as green, mushroom, and fruity. The natural 1,3-dioxanes occur as a diastereomeric mixture of 2(*S*),4(*R*) and 2(*R*),4(*R*) with a ratio of 10:1 [125].

The biosynthesis of (*R*)-octan-1,3-diol and (*R*)-5(*Z*)-octen-1,3-diol was studied with isotopically labeled substrates [131]. In apples, the diols are synthesized from linoleic acid and linolenic acid, respectively. The 1,3-dioxanes are formed from aldehydes and the 1,3-diols. It is still unknown whether an enzyme is involved in the formation of the 1,3-

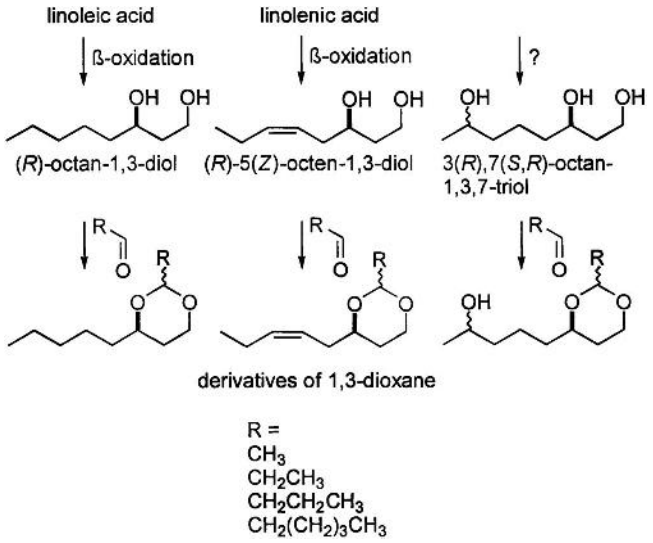


Figure 13 Naturally occurring 1,3-dioxanes and proposed biosynthetic pathway. (Adapted from Refs. 128 and 131.)

dioxane ring. 1,3-Dioxanes and 1,3-dioxolanes have also been identified as malodorous compounds at trace levels in river water, groundwater, and tap water [132].

6 CONCLUSION

Polyunsaturated fatty acids such as linoleic and linolenic acids are the main constituents of membrane glycerolipids and serve as major precursors for the generation of flavor volatiles. Enzymic oxidative degradation of lipids is preceded by the action of acylhydrolase, liberating the free fatty acids from acylgycerols. The lipoxygenase reaction (in-chain oxidation), α-oxidation, and β-oxidation play a significant role for the breakdown of fatty acids, whereas the ω-oxidation is of minor importance. The β-oxidation pathway is the sole process currently known to lead to complete degradation of fatty acids due to the liberation of acetyl-CoA units. In contrast, carbon dioxide is released from free fatty acids by α-oxidation. In-chain oxidation catalyzed mostly by lipoxygenase generates hydroxy, oxo, and epoxy fatty acids and are involved in the formation of polyfunctional fatty acids. Intermediates and/or products of these reaction types are further metabolized to form volatile straight-chain alcohols, aldehydes, carboxylic acids, lactones, esters, ketones, and miscellaneous compounds.

REFERENCES

1. A Gallois, D Langlois. Lait 70:89–106, 1990.
2. L Moio, J Dekimpe, P Etievant, F Addeo. J Dairy Res 60:199–213, 1993.
3. J-L Mau, RB Beelman, GR Ziegler. J Food Sci 58:331–334, 1993.
4. P Schreier. Chromatographic Studies of Biogenesis of Plant Volatiles. Heidelberg: Hüthig, 1984, pp 52–76.

5. G Hobson, D Grierson. In: GB Seymour, JE Taylor, GA Tucker, eds. Biochemistry of Fruit Ripening. London: Chapman & Hall, 1993, pp 405–442.
6. PW Goodenough. In: ID Morton, AJ MacLeod, eds. Developments in Food Science, Food Flavours, Part C, The Flavour of Fruits. Amsterdam: Elsevier, 1990, pp 327–350.
7. E Honkanen, T Hirvi. In: ID Morton, AJ MacLeod, eds. Developments in Food Science, Food Flavours, Part C, The Flavour of Fruits. Amsterdam: Elsevier, 1990, pp 125–193.
8. IM Whitehead. Food Technol 52:40–46, 1998.
9. PSJ Cheetham. In: T Scheper, RG Berger, eds. Advances in Biochemical Engineering Biotechnology, Biotechnology of Aroma Compounds. Berlin: Springer-Verlag, 1997, pp 1–49.
10. P Schreier. In: T Scheper, RG Berger, eds. Advances in Biochemical Engineering Biotechnology, Biotechnology of Aroma Compounds. Berlin: Springer-Verlag, 1997, pp 50–72.
11. DW Armstrong, LA Brown. In: A Gabelman, ed. Bioprocess Production of Flavor, Fragrance, and Color Ingredients. New York: Wiley, 1994, pp 41–94.
12. A Grechkin. Prog Lipid Res 37:317–352, 1998.
13. A Hatanaka. Phytochemistry 34:1201–1218, 1993.
14. Y Nishiba, S Furuta, M Hajiha, K Igita, I Suda. J Agric Food Chem 43:738–741, 1995.
15. IM Whitehead, BL Muller, C Dean. Cereal Foods World 40:193–197, 1995.
16. A Hatanaka. Food Rev Int 12:303–350, 1996.
17. EJ Corey. In: W Bartmann, KB Sharpless, ed. Stereochemistry of Organic and Bioorganic Transformations. Weinheim: VCH, 1987, pp 1–12.
18. JC Boyington, BJ Gaffney, M Amzel. Science 260:1482–1486, 1993.
19. W Minor, J Steczko, JT Bolin, Z Otwinowski, B Axelrod. Biochemistry 32:6320–6323, 1993.
20. NMM Paillard. In: ID Morton, AJ MacLeod, eds. Developments in Food Science, Food Flavours, Part C, The Flavour of Fruits. Amsterdam: Elsevier, 1990, pp 1–41.
21. K Bauer, D Garbe, H Surburg. Common Fragrance and Flavor Material. 2nd ed. Weinheim: VCH, 1990, pp 8–18.
22. H Zhuang, TR Hamilton-Kemp, RA Anderson, DF Hildebrand. Plant Physiol 111:805–812, 1996.
23. RG Berger. Aroma Biotechnology. Berlin: Springer-Verlag, 1995, pp 116–120.
24. RG Berger, A Kler, F Drawert. Plant Cell Tissue Org Culture 8:147–151, 1987.
25. S-R Chou, C-K Chin. In: C-T Ho, TG Hartmann, eds. Lipids in Food Flavors. Washington, DC: American Chemical Society, 1994, pp 282–291.
26. RG Berger, A Kler, F Drawert. Biochim Biophys Acta 883:523–530, 1986.
27. F Drawert, A Kler, RG Berger. Lebensm Wiss Technol 19:426–431, 1986.
28. E-M Götz-Schmidt, M Wenzel, P Schreier. Lebensm Wiss Technol 19:152–155, 1986.
29. P Brunerie. French Patent FR 2 652 587, 1989.
30. IL Gatfield. Perfumer Flavorist 20:5–14, 1995.
31. B Rehbock, D Ganßer, RG Berger. Food Chem 63:161–165, 1998.
32. A Häusler, B Schilling. In: HP Kruse, M Rothe, eds. Flavour Perception, Aroma Evaluation. Bergholz-Rehbrücke: Eigenverlag Universität Potsdam, 1997, pp 375–380.
33. K Matsui, M Shibutani, T Hase, T Kajiwara. FEBS Lett 394:21–24, 1996.
34. NJ Bate, S Sivasankar, C Moxon, JMC Riley, JE Thompson, SJ Rothenstein. Plant Physiol 117:1393–1400, 1998.
35. C Schneider, M Wein, D Harmsen, P Schreier. Biochem Biophys Res Commun 232:364–366, 1997.
36. NJ Bates, JCM Riley, JE Thompson, SJ Rothstein. Physiol Plant 104:97–104, 1998.
37. M Gargouri, M-D Legoy. Biotechnol Lett 20:23–26, 1998.
38. HW Gardner, MJ Grove. Plant Physiol 116:1359–1366, 1998.
39. Y Hadar, CG Dosoretz. Trends Food Sci Technol 2:214–218, 1991.
40. KH Fischer, W Grosch. Lebensm Wiss Technol 20:233–236, 1987.
41. M Wurzenberger, W Grosch. Z Lebensm Unters Forsch 175:186–190, 1982.
42. M Wurzenberger. Investigations on the Enzymatic Formation of the Flavor Compounds 1-

Octen-3-ol, (*Z*)-1,5-Octadien-3-ol and (*Z*,*Z*)-2,5-Octadien-1-ol in Mushroom (Psalliota bispora). PhD dissertation, Technical University of Munich, Munich, 1986.
43. M Wurzenberger, W Grosch. Biochim Biophys Acta 794:25–30, 1984.
44. RG Berger. Aroma Biotechnology. Berlin: Springer-Verlag, 1995, pp 56–62.
45. S Assaf, Y Hadar, CG Dosoretz. J Agric Food Chem 43:2173–2178, 1995.
46. S Assaf, Y Hadar, CG Dosoretz. Enzyme Microb Technol 21:484–490, 1997.
47. M Wurzenberger, W Grosch. Lipids 21:261–266, 1986
48. A Mosandl, G Heusinger, J Gessner. J Agric Food Chem 34:119–122, 1986.
49. FW Welsh, WD Murray, RE Williams. Crit Rev Biotechnol 9:105–169, 1989.
50. DC Eaton. In: A Gabelman, ed. Bioprocess Production of Flavor, Fragrance, and Color Ingredients. New York: Wiley, 1994, pp 169–203.
51. SW May, MS Steltenkamp, KR Borah, AG Katopodis, JR Thowsen. J Chem Soc Chem Commun 845–846, 1979.
52. WM Van Der Goes Van Naters, L Bootsma, CJ Den Otter, RG Belemtougri. J Chem Ecol 22:343–355, 1996.
53. DR Hall, PS Beevor, A Cork, BF Nesbitt, GA Vale. Insect Sci Appl 5:335–339, 1984.
54. A Blackwell, C Dyer, AJ Mordue, LJ Wadhams, W Mordue. Physiol Entomol 21:15–19, 1996.
55. A Korpi, A-L Pasanen, P Pasanen. Appl Environ Microbiol 64:2914–2919, 1998.
56. PA Belinky, S Masaphy, D Levanon, Y Hadar, CG Dosoretz. Appl Microbiol Biotechnol 40:629–633, 1994.
57. BA Vick, DC Zimmerman. Plant Physiol 75:458–461, 1984.
58. O Miersch, H Bohlmann, C Wasternack. Phytochemistry 50:517–523, 1999.
59. T Kajiwara, K Matsui, Y Akakabe. In: GR Takeoka, R Teranishi, PJ Williams, A Kobayashi, eds. Biotechnology for Improved Foods and Flavours. Washington, DC: American Chemical Society, 1996, pp 146–166.
60. H Schulz. Biochim Biophys Acta 1081:109–120, 1991
61. H Osmundson, J Bremer, JJ Pedersen. Biochim Biophys Acta 1085:141–158, 1991.
62. B Gerhardt. Prog Lipid Res 31:417–446, 1992.
63. PK Stumpf. Prog Lipid Res 33:1–8, 1994.
64. WS Leal, S Kuwahara, X Shi, H Higuchi, CEB Marino, M Ono, J Meinwald. J Chem Ecol 24:1817–1829, 1998.
65. W Albrecht, M Schwarz, J Heidlas, R Tressl. J Org Chem 57:1954–1956, 1992.
66. T Haffner, R Tressl. J Agric Food Chem 44:1218–1223, 1996.
67. M Schöttler, W Boland. Helv Chim Acta 78:847–856, 1995.
68. M Schöttler, W Boland. Helv Chim Acta 79:1488–1496, 1996.
69. U Krings, RG Berger. Appl Microbiol Biotechnol 49:1–8, 1998.
70. A Endrizzi, Y Pagot, A Le Clainche, J-M Nicaud, J-M Belin. Crit Rev Biotechnol 16:301–329, 1996.
71. IL Gatfield, M Güntert, H Sommer, P Werkhoff. Chem Mikrobiol Technol Lebensm 15: 165–170, 1993.
72. Y Pagot, A Endrizzi, J-M Nicaud, J-M Belin. Lett Appl Microbiol 25:113–116, 1997.
73. A Endrizzi, J-M Belin. J Basic Microbiol 35:285–292, 1995.
74. Y Pagot, A Le Clainche, J-M Nicaud, Y Waché, J-M Belin. Appl Microbiol Biotechnol 49: 295–300, 1998.
75. L Dufossé, G Féron, G Mauvais, P Bonnarme, A Durand, H-E Sinnler. J Ferm Bioeng 86: 169–173, 1998.
76. Y Waché, Y Pagot, J-M Nicaud, J-M Belin. J Mol Catal B: Enzymatic 5:165–169, 1998.
77. Y Pagot, J-M Belin. Appl Microbiol Biotechnol 45:349–354, 1996.
78. IL Gatfield. In: T Scheper, RG Berger, eds. Advances in Biochemical Engineering Biotechnology, Biotechnology of Aroma Compounds. Berlin: Springer-Verlag, 1997, pp 221–238.

79. S Gocho, N Tabogami, M Inagaki, C Kawabata, T Komai. Biosci Biotechnol Biochem 59: 1571–1572, 1995.
80. PH Van Der Schaft, N Ter Burg, S Van Den Bosch, AM Cohen. Appl Microbiol Biotechnol 36:712–716, 1992.
81. IL Gatfield, H Sommer. Adv Food Sci (CMTL) 19:12–14, 1997.
82. CL Serrano, C Flores, E Galindo. Biotechnol Prog 13:205–208, 1997.
83. L Dufossé, G Féron, A Latrasse, E Guichard, H-E Sinnler. Chirality 9:667–671, 1997.
84. A Latrasse, E Guichard, C Piffaut, N Fournier, L Dufossé. Chirality 5:379–384, 1993.
85. P Chalier, J Crouzet. Chirality 10:786–790, 1998.
86. MM Enzelberger, UT Bornscheuer, I Gatfield, RD Schmid. J Biotechnol 56:129–133, 1997.
87. K Umano, Y Hagi, K Nakahara, A Shoji, T Shibamoto. J Agric Food Chem 40:599–603, 1992.
88. H Kollmannsberger, RG Berger. Chem Mikrobiol Technol Lebensm 14:81–86, 1992.
89. A Torrado, M Suárez, C Duque, D Krajewski, W Neugebauer, P Schreier. Flav Fragr J 10: 349–354, 1995.
90. G Allegrone, M Barbeni. Flav Fragr J 7:337–342, 1992.
91. T Kajiwara, H Yoshikawa, K Matsui, A Hatanaka, T Kawai. Phytochemistry 28:407–409, 1989.
92. W Kawasaki, K Matsui, Y Akakabe, N Itai, T Kajiwara. Phytochemistry 49:1565–1568, 1998.
93. WE Shine, PK Stumpf. Arch Biochem Biophys 162:147–157, 1974.
94. P Baardseth, E Slinde, MS Thomassen. Biochim Biophys Acta 922:170–176, 1987.
95. B Gerhardt. In: TS Moore, ed. Lipid Metabolism in Plants. Boca Raton, FL: CRC Press, 1993, pp 527–565.
96. GIA Borge, E Slinde, A Nilsson. Biochim. Biophys Acta 1344:47–58, 1997
97. GIA Borge, E Slinde, A Nilson. Biochim Biophys Acta 1394:158–168, 1998.
98. W Adam, M Lazarus, CR Saha-Möller, P Schreier. Tetrahedron: Asymmetry 7:2287–2292, 1996.
99. W Adam, W Boland, J Hartmann-Schreier, H-U Humpf, M Lazarus, A Saffert, CR Saha-Möller, P Schreier. J Am Chem Soc 120:11,044–11,048, 1998.
100. K Croes, M Casteels, E De Hoffmann, GP Mannaerts, PP Van Veldhoeven. Eur J Biochem 240:674–683, 1996.
101. C Larroche, J-B Gros. In: T Scheper, RG Berger, eds. Advances in Biochemical Engineering Biotechnology, Biotechnology of Aroma Compounds. Berlin: Springer-Verlag, 1997, pp 179–220.
102. S Lubbers, N Cayot, C Taisant. Sci Alim 17:393–402, 1997.
103. I Richter, H Krain. Lipids 15:580–586, 1980.
104. E Engels, W Engels, G Lübke, W Schröder, W Francke. Apidologie 24:539–548, 1993.
105. SD Eigenbrode, JT Trumble. HortScience 28:932–934, 1993.
106. JE Kinsella, D Hwang. Biotechnol Bioeng 18:927–938, 1976.
107. A Humphrey. US Patent 5,185,252, 1993.
108. T Weinert, S Hausmanns, G Laufenberg, B Kunz. Med Fac Landbouww Univ Gent 61(4a): 1649–1650, 1996.
109. G Carrea. Trends Biotechnol 2:102–106, 1984.
110. J-F Shaw, R-C Chang, FF Wang, YJ Wang. Biotechnol Bioeng 35:132–137, 1990.
111. M Rucka, B Turkiewicz. Enzyme Microb Technol 12:52–55, 1990.
112. S Riisgaard. Genet Eng Biotechnol 10:11–14, 1990.
113. KE Jaeger, MT Reetz. Trends Biotechnol 16:396–403, 1998.
114. KE Jaeger, MT Reetz. Eur J Biochem 260:885–895, 1999.
115. JK Fellman, JP Mattheis. In: RL Rouseff, MM Leahy, eds. Fruit Flavors, Biogenesis, Characterization, and Authentication. Washington, DC: American Chemical Society, 1995, pp 149–162.

116. H Yamauchi, T Hasuo, T Amachi, O Akita, S Hara, K Yoshikawa. Agric Biol Chem 53: 1551–1556, 1989.
117. P Malcorps, JP Dufour. Eur J Biochem 210:1015–1022, 1992.
118. JC Mauricio, JJ Moreno, EM Valero, L Zea, M Medina. J Agric Food Chem 41:2086–2091, 1993.
119. M Harada, Y Ueda, T Iwata. Plant Cell Physiol 26:1067–1074, 1985.
120. JK Fellman, JP Mattheis, DS Matthinson, BC Bostick. HortScience 27:773–776, 1991.
121. AG Pérez, C Sanz, JM Olfas. J Agric Food Chem 41:1462–1466, 1993.
122. AG Pérez, C Sanz, R Olías, JJ Rios, JM Olías. J Agric Food Chem 44:3286–3290, 1996.
123. JM Olías, C Sanz, JJ Ríos, AG Pérez. In: RL Rouseff, ML Leahy, eds. Fruit Flavors, Biogenesis, Characterization, and Authentication. Washington, DC: American Chemical Society, 1995, pp 134–141.
124. DD Rowan, JM Allen, S Fielder, MB Hunt. J Agric Food Chem 47:2553–2562, 1999.
125. C Dietrich, T Beuerle, B Withopf, P Schreier, P Brunerie, C Bicchi, W Schwab. J Agric Food Chem 45:3178–3182, 1997.
126. T Beuerle, P Schreier, P Brunerie, C Bicchi, W Schwab. Phytochemistry 43:145–149, 1996.
127. T Beuerle, S Engelhard, C Bicchi, W Schwab. J Nat Prod 62:35–40, 1999.
128. P Brunerie. French Patent 2 751 329, 1998.
129. P Brunerie. French Patent 2 751 330, 1998.
130. T Beuerle, W Schwab. Z Lebensm Unters Forsch 205:215–217, 1997.
131. T Beuerle, W Schwab. Phytochemistry 45:1153–1155, 1997.
132. J Romero, F Ventura, J Caixach, J Rivera, LX Godé, JMA Niñerola. Environ Sci Technol 32:206–216, 1998.

16

Terpenoid-Based Defense in Plants and Other Organisms

MARIO R. TELLEZ and MOZAINA KOBAISY

Agricultural Research Service, U.S. Department of Agriculture, Oxford, Mississippi

STEPHEN O. DUKE, KEVIN K. SCHRADER, and FRANCK E. DAYAN

Agricultural Research Service, U.S. Department of Agriculture, University, Mississippi

JOANNE G. ROMAGNI

University of St. Thomas, Houston, Texas

1 TERPENOIDS AS LIPIDS

Lipids comprise the class of compounds extracted by nonpolar solvents (e.g., hexane, chloroform, diethyl ether) from the complex mixtures found in samples of biological origin. This is a physical separation based on solubility, and although the term ''lipid'' is commonly associated with triglycerides and fatty acids, it also includes terpenoids, waxes, phospholipids, prostaglandins, steroids, and other lipophilic and hydrophobic compounds. Triglycerides can also incorporate terpenoid fatty acid side chains (e.g., phytanic acid; Fig. 1), blurring even the boundaries between these classes of compounds.

Terpenoids comprise a large group of chemicals based on the 5-carbon (C5) isoprene unit (Fig. 2). Isoprene and other 5-carbon compounds derived from it are known as hemiterpenoids. Although hemiterpenoids, including isoprene itself, have been found as natural products, most terpenoid structures incorporate a combination of two or more isoprene units. Terpenoids with two such units (C_{10}) are referred to as monoterpenoids. Further incorporation of isoprene units leads to sesquiterpenoids (C_{15}), diterpenoids (C_{20}), sesterterpenoids (C_{25}), triterpenoids (C_{30}), tetraterpenoids or carotenoids (C_{40}), and on to polyterpenoids. These combinations can lead to cyclic, polycyclic, or acyclic compounds, saturated or with different degrees of unsaturation, and can incorporate a wide number of functionalities and stereocenters. Rearrangements and gain or loss of one or more carbons is also common. Steroids, gibberillic acid derivatives, abscisic acid derivatives, and meroterpen-

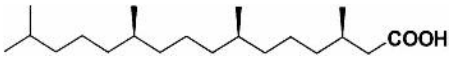


Figure 1 3,7,11,15-Tetramethylhexadecanoic acid (phytanic acid).

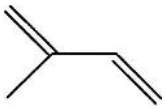


Figure 2 Isoprene.

oids (terpenoids incorporated into nonterpenoid groups), such as indole alkaloids, furanocoumarins, saponins, gibberillins, chlorophyll, ubiquinones, plastoquinones, prenylated proteins, prenylated pterocarpans, prenylated isoflavones, and certain anthraquinones, are also related to the terpenoids [1].

Bioactive terpenoids constitute an important part of the defensive mechanism of a large number of organisms and represent a fairly untapped source of active compounds of potential use in both agricultural (insecticides, fungicides, herbicides, etc.) and pharmaceutical (antimicrobials, antifungals, antimitotics, etc.) fields. With recent advances in chiral synthesis and genetic modification of organisms, these compounds, once identified, might be made in the laboratory, produced in high amounts by select plants/organisms, or incorporated into selected crops for added protection. Because of the extremely large variety in carbon skeletons, functionality, and stereochemistry, chances are very good that these active compounds will be unique in structure and possess new modes of action. Such parent compounds may be useful in the development of drugs and pesticides for use in the ongoing fight against resistance-developing organisms (pests). Terpenoids are already used for a wide range of purposes (Table 1), sometimes related, but often not, to their function as part of defensive mechanisms.

The remainder of this chapter will attempt to cover enough of the relevant recent work in terpenoid-based defensive mechanisms to give an entry point into the vast body of existing literature in the field. General reviews and reference sources are available [3–10]. Terpenoid nomenclature, which can become rather convoluted, as it tends to use both common names and/or CAS/IUPAC-approved nomenclature. Help with nomenclature can be obtained by checking the *Chemical Abstracts* nomenclature index, the introductory chapter to the *Dictionary of Terpenoids* [11], or the introductory chapters of the CRC terpenoid series [12]. Another good source for monoterpenoid and sesquiterpenoid (the main components of most essential oils) nomenclature is the *Identification of Essential Oil Components by Gas Chromatography* [13]. This last reference also goes a long way in standardizing the detection and identification of essential oil components by providing both mass spectral data (in hard-bound and computer-searchable database formats) and retention time information (in elution time and in Kovat's index formats) following easily duplicated laboratory conditions (e.g., Ref. 14).

2 BIOLOGICALLY ACTIVE TERPENOIDS: OCCURRENCE IN PLANT PARTS

Plants must protect themselves against microbial pathogens, nematodes, a wide range of herbivores, and other plant species that compete for resources. Secondary terpenoids are

Table 1 Selected Uses of Some Common Terpenoids

Tricyclene	Perfumes, plasticizers, and intermediates for drugs
α-Thujene	Synthesis of levocarvone
Sabinene	Synthesis of perfume components with cosmetic and medicinal uses
Sabinene hydrate	Effective insecticide for cloth
1,8-Cineole	Drug penetration enhancer; manufacture of drying solvents; manufacture of homopolymers; light-stabilizing agents to malt beverages; fragrance raw materials
α-Terpinene	Binder for the manufacture of thick-layer conductors; component of universal cleaning agent
(*E*)-β-Ocimene	Insect control; food additive
Terpinolene	Manufacture of coating materials; formulation of detergents; binder for conducting paste
Campholenal	Preparation of derivatives for use as detergent fragrances
Pinocarveol	Analeptic, bronchodilating, anti-inflammatory, and blood platelet antiaggregative activities; food additives; formulation of colognes, soaps, detergents, shampoos, deodorants; synthesis of phytotoxic β-dialkylaminoalkyl terpene ethers
Pinocarvone	Synthesis of perfume or aroma constituents; component of cleaners and disinfectants;
Borneol	Synthesis of pharmaceuticals; metal and plastic surface protection; deodorants; constituents of soothing aromatic ointments
Terpin-4-ol	Antimicrobial; insecticide for preserving clothes
p-Cymen-8-ol	Preparation of intermediates for musk fragrances; foaming agents for ore flotation
α-Terpineol	Food, flavor, and fragrance industry; manufacture of annealable printing pastes for printing on glass surfaces; manufacture of insect-repellent detergent compositions for use on solid surfaces
Myrtenal	Organoleptic characteristics of flavor materials; kairomones
Verbenone	Honeybee repellents, for repulsion from pesticide-treated areas; manufacture of insect attractants; therapeutical use
Carveol	Manufacture of liquid-crystal materials
Carvone	Synthesis of biologically active natural products; agriculture; perfumes or cosmetics
Carvacrol	Epoxy resin curing compositions
β-Caryophyllene	Pheromone in the management of beneficial insects; component of Vitex oil in the treatment of chronic tracheitis; suppression of gushing in beer; synthesis of caryophyllene alcohol
Farnesene	Inhibiting potato sprouting; aphid control; hormonal insecticide against aphids and acarids; augmenting or enhancing the aroma or taste of foodstuffs, chewing gums, medicinal products, and toothpastes
Germacrene D	Constituent in eye-disease pills; masking substance of attractants for the cerambycid beetle
Caryophyllene oxide	Antimutagenicity; fragrance raw material; food additive
α-Cadinol	Oral antibacterial, expectorant activity

Source: From Ref. 2.

a critical part of the chemical defense arsenal of many plant species. Indeed, some plant species accumulate over 10% of their dry weight as secondary terpenoids [15,16]. For maximum effect, the compounds must be synthesized and/or accumulated in the appropriate plant organ, tissue, cell type, and subcellular location at the appropriate time. Furthermore, many secondary terpenoids are phytotoxic [17,18], so the producing plant must sequester them to avoid autotoxicity. In some cases, disruption of secretory structures containing phytotoxic terpenoids can result in chemical injury to surrounding tissues (e.g., Refs. 19 and 20). Unlike phytotoxic phenolics, which are typically stored in esterified nonphytotoxic forms within plant vacuoles, the biologically active terpenoids are more commonly localized in fully phytotoxic forms outside of living cells, such as subcuticular spaces of secretory glands, lacticifers, resin canals/resin ducts, secretory cavities, nonliving bark cells, nonliving idioblast cells, or other locations that separate the compounds from living cytoplasm [21]. The plant expends considerable energy to sequester secondary terpenoids on complex, multicellular secretory structures [22]. We will concentrate on glandular trichomes as an example for which there is an abundance of literature, but we will mention some other examples.

The trichome is the exclusive site of accumulation of particular terpenoids of tobacco (*Nicotiana tobacum*), cotton (*Gossypium hirsutum*), and *Artemisia annua* [23–26]. Some studies have implied that when plants have glandular trichomes, the essential oil components are localized in these structures (e.g., Ref. 27). However, recent work has demonstrated that within the same plant, some essential oil components are localized and others are not [26]. In *A. annua*, for instance, the essential oil monoterpenoids are found almost exclusively in the trichome glands, whereas the essential oil sesquiterpenoids can be found outside of these glands [26].

Glandular trichomes consist of secretory cells located on top of a stalklike base. They appear globular due to expansion of the cuticle, which splits from the secretory cell walls of the apical cells (e.g., Refs. 27–30). As the subcuticular space fills with secretory products (primarily terpenoids), the appearance of the trichome changes from a stalk to a balloonlike structure. As the trichome matures, it often ruptures, spreading its nonvolatile contents over the epidermal surface of the plant to form a resinous coating (e.g., Refs. 30 and 31). Some nonpeltate, glandular trichomes exude or secrete secondary products onto the plant surface rather than storing them (e.g., the capitate glands of *Leonatis leonurus* described by Ascensão and Pais [32]).

Three methods have been used to selectively extract terpenoids from glandular trichomes: removal of the material from the glands in situ with a micropipette, isolation of the glands from the plant, and selective solvent extraction. The micropipette approach was used in cotton to compare the contents of the glandular trichomes with those of surrounding cells, revealing that cells surrounding glands were devoid of volatile terpenoids in which the glands were enriched [33]. Other examples of the use of microcapillaries to sample glandular trichomes for terpenoids involve *Mentha piperita* [34] and *Nepeta racemosa* [35]. Similar results have been obtained by removal of glandular trichomes from plant surfaces and subsequent chemical analysis (e.g., Refs. 36 and 37). Short rinses of glandular trichome-bearing plant tissue in organic solvents have been used to more selectively extract trichome contents [38]. Duke et al. [25] found that dipping *A. annua* tissues into chloroform for a few seconds selectively removed certain terpenoids from the plant's peltate glands. Further extraction of the entire plant tissues yielded only trace amounts of these products, indicating that they were localized exclusively in the glands. A mutant of *A. annua* lacking glandular trichomes provided further evidence that many terpenoids

accumulate only in the trichome [25,26]. This ''chemotype'' was discovered due to a lack of aroma and almost complete absence of the more volatile essential oil components. It has normal gross morphology and anatomy, but it lacks glandular trichomes and many terpenoids, including several sesquiterpenoids and most of the monoterpenoids found in the glanded wild type. Several of the compounds (e.g., artemisinin; Fig. 3) missing from the glandless mutant are potent phytotoxins. In addition to being the exclusive site of storage of most of the monoterpenoids and some of the sesquiterpenoids of this species, the glandular trichomes may be required to protect the plant from autotoxic effects when phytotoxic terpenoids are produced. Similarly, glandless varieties of cotton completely lack gossypol, hemigossypol, and related sesquiterpenoid and triterpenoid compounds [24]. Furthermore, most of the volatile terpenoids (mostly monoterpenoids) of cotton were not found or found in only trace amounts in glandless cotton varieties, compared to genetically similar glanded varieties [33].

Histochemical methods have also provided evidence for the exclusive localization of certain secondary compounds in glandular trichomes. For example, Cappelletti et al. [39] found that histochemical stains specific for sesquiterpenoid lactones stained the subcuticular space of peltate glands. After a chloroform wash of the plant surface to extract the subcuticular space, no histochemical staining of the tissues was observed.

Highly lipophilic terpenoid secretory products of trichomes or other storage or secretory structures are not likely to be imported from other plant tissues. Keene and Wagner [40] produced strong evidence that the glandular heads of tobacco trichomes were the only site of biosynthesis of duvatrienediol diterpenoids. Cells isolated from these glands were later shown to incorporate radiolabel from carbonate into complex diterpenoids [41]. Furthermore, biosynthesis of the diterpenoids labdenediol and sclareol from *trans*-geranylgeranyl pyrophosphate was observed in cell-free extracts prepared from leaf midvein epidermal peels of *Nicotiana glutinosa*; these bioactivities were localized exclusively in trichomes [42]. Isolated secretory cells from mint trichomes are very active in monoterpenoid biosynthesis and are good sources of key enzymes of monoterpenoid biosynthesis [43,44]. Isolated secretory cells of peppermint trichomes synthesize isoprenoids from the relatively simple glycolytic substrates, glucose-6-phosphate and pyruvic acid, incorporating them into monoterpenoids and sesquiterpenoids in a ratio closely resembling that observed in vivo [45]. Trichomes of some species appear to be photosynthetically competent (e.g., Ref. 29), whereas other species are without chloroplasts and apparently rely completely on underlying tissues as a carbon source (e.g., Ref. 45).

In the last decade, our understanding of the enzymes of secondary terpenoid biosynthesis and their subcellular location has advanced considerably [1,46]. Sesquiterpenoid and triterpenoids are thought to be synthesized in the cytoplasm, whereas monoterpenoids and diterpenoids appear to be made in plastids. In grand fir (*Abies grandis* Lindl.), terpenoid biosynthesis from isopentenyl diphosphate through dimethylallyl diphosphate, gera-

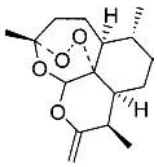


Figure 3 Artemisinin.

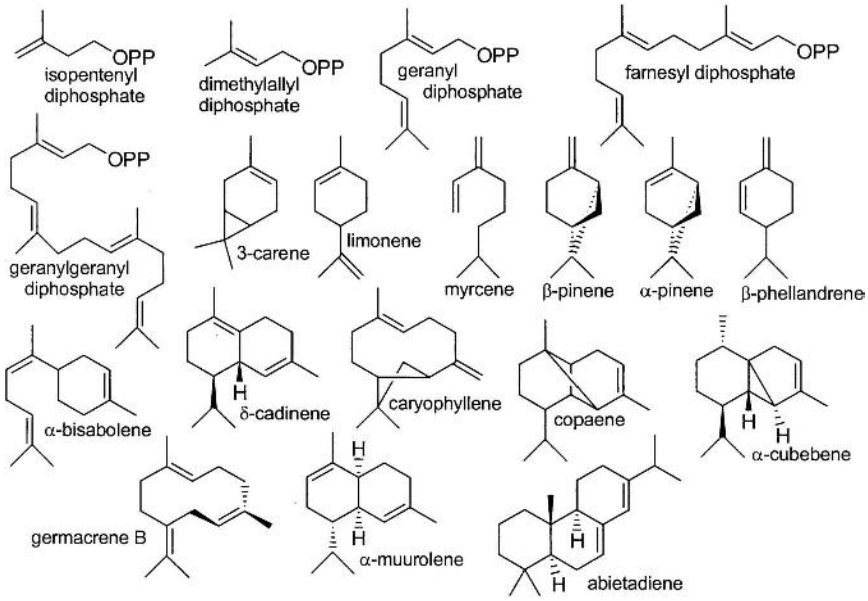


Figure 4 Some biosynthetic intermediates and selected terpenoid end products.

nyl diphosphate, farnesyl diphosphate, and geranylgeranyl diphosphate are catalyzed by monoterpenoid and diterpenoid synthases in the plastid and by sesquiterpenoid synthases in the cytoplasm [47]. Terpenoids thus produced in grand fir include monoterpenoids, sesquiterpenoids, and diterpenoids (Fig. 4). In another example, *Catharanthus roseus* cytochrome P-450 enzymes apparently participate in the biosynthesis of defense-related terpenoid indole alkaloids [48]. Plastids of the secretory cells of glands appear to be specialized, with a more amoeboid appearance and seldom containing starch [28,30,49]. The plastids of gland cells are often enveloped by tubules of endoplasmic reticulum, indicating active export of secretory products (e.g., Ref. 28). The synthesis and compartmentalization of isoprenoids in pine (*Pinus pinaster*) needles and in glandular trichomes of *Chrysanthemum morifolium* have been linked to the presence of leucoplasts that are in close association with smooth endoplasmic reticulum [50,51].

Carvone (Fig. 5) biosynthesis has been shown to take place exclusively in the glandular trichomes in spearmint (*Mentha spicata*) leaves [52]. In cotton, terpenoids are proposed to be synthesized in mesophyll pigment glands embedded in the leaves [53,54]. The sequestered terpenoids are then released upon damage caused by herbivore feeding [54].

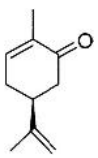


Figure 5 Carvone.

Terpenoids, like other chemical defensive mechanisms, are generally thought to be of highest benefit to seedlings or developing leaves than to mature plants/leaves where other defensive processes including physical ones (i.e., toughness) may dominate [55–60]. It has also been reasoned that those seedlings whose chemical defenses allow them to escape herbivory or other damage/death will be the ones to grow to reproduce, therefore favoring the expansion of plants with successful chemical defenses [55–60]. On the other hand, at least for the California bay tree (*Umbellularia californica*), the high levels of monoterpenoids that impart fully grown and sapling mature leaves with resistance against browsing by black-tailed deer appears to be absent in developing or seedling leaves which therefore undergo heavy browsing [60]. It has also been suggested [59,60] that defenses in seedlings coming from small seeds might be limited because of insufficient amounts of carbon available in the seed. The California bay tree, nevertheless, has large seeds and is still a poorly protected seedling, a fact which the authors attribute to the lack of development of secretory structures in the leaves rather than to lack of carbon availability in the seed [60].

Terpenoids localized in trichomes and other specialized storage structures are generally considered to be constitutive (Sec. 3.1). However, synthesis of further defensive terpenoids can be induced (Sec. 3.2) by stress (e.g., damage to the plant). This synthesis does not necessarily occur in the same tissues damaged by the pest. Much less is known of the tissue localization of these induced defensive terpenoids than of constitutive compounds. However, the fact that undifferentiated tissue cultures can be induced to synthesize terpenoid phytoalexins (e.g., Ref. 61) suggests that synthesis of induced defensive terpenoids may not be limited to a specific tissue.

3 DEFENSIVE MECHANISMS INVOLVING TERPENOIDS

3.1 Constitutive Terpenoids in Plants

Terpenoids can be part of the permanent makeup of a plant in which they may be involved in defense mechanisms against predators or disease [60,62]. These constitutive terpenoids, which are stored in glands or ducts (Sec. 2), are released immediately and at relatively constant levels upon damage, and return to preattack levels soon after injury is no longer being done to the plant [54]. Terpenoids can provide a competitive advantage to the producing plant by repelling or killing predators, disease, or other invading organisms, but they may also be counterproductive by functioning, for instance, as attractants [62] to the same or other deleterious organisms (Sec. 3.5). The effectiveness of these terpenoid defenses can be related to the total amount of terpenoids present, to the amount of a particular terpenoid present, or to a given ratio between two or more terpenoids.

Total terpenoid content has been associated with the ability of plants to defend themselves. Proof for this relationship is often lacking, requiring, for instance, the exhaustive determination that none of the components (even at trace levels) is responsible by itself (or in combination with one or two others) for the observed activity. Wink et al. reported that the only major barrier to feeding on plants by geese is the presence of essential oils, but plants containing alkaloids, amines, and sulfur compounds are fairly well tolerated [63,64]. The rubber rabbit brush, *Chrysothamnus nauseosus*, is also protected from herbivory by high amounts of terpenoids (80 μg/g dry wt) during the summer [65]. These terpenoids include α- and γ-muurolene, β-humulene, and (*E*)-β-farnesene (Fig. 6). During winter, the levels drop dramatically, allowing browsing by mule deer [64,65]. A

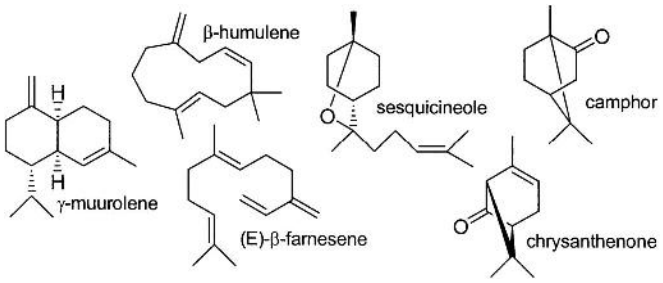


Figure 6 Selected monoterpenoids and sesquiterpenoids involved in constitutive plant defense.

similar compositae, *C. pulchellus* subsp. *pulchellus*, also has a high amount of constitutive terpenoids (900 μg/g leaf fresh weight) which include α- and γ-muurolene and (*E*)-β-farnesene, but with the monoterpenoid β-phellandrene and the sesquiterpenoid sesquicineole (Fig. 6) as the major components [66]. α-Pinene, limonene, chrysanthenone, and camphor are highly prevalent constitutive terpenoids in another half-shrub, *Dyssodia acerosa*, which is unpalatable to herbivores [67]. Total terpenoid content has been associated with the defense mechanism of several sagebrush (*Artemisia tridentata*) species against herbivory by deer [60,68–72].

Correlation of individual terpenoids, rather than total terpenoids, with defense-type activity is easier to accomplish, usually involving isolation of the terpenoid in question and setting up the appropriate bioassays. The use of an appropriate bioassay is a particularly important factor. A particular terpenoid may show high activity in vitro or under controlled laboratory conditions, whereas in the field, many factors come together which might lower or render the observed activity null. Such factors might involve volatility, decomposition, uptake, and/or biotransformation of the active terpenoids by incidental organisms, events which, in turn, might be influenced by a larger number of variables, including those found in highly variable soil, biota, and weather characteristics. The opposite is also possible: A given terpenoid proven to be biologically inactive in vitro under tightly controlled laboratory conditions might be rendered active when exposed to factors found in a field environment. It is therefore important to study the range of activities as found through assays ranging from in vitro laboratory conditions to those that attempt to find the actual events occurring in a relatively undisturbed natural ecosystem.

Many plants have been identified as having unique terpenoids associated with defense mechanisms. *Chrysanthemum cinerariaefolium*, a plant well known for its insecticidal properties, contains high amounts of insecticidal terpenoid pyrethroids (e.g., Fig. 7) stored in secretory canals and oil glands of flowers and in glandular trichomes of leaves [73,74]. Young mature-green lemons contain citral (Fig. 7), an antifungal monoterpenoid aldehyde [75]. The oat (*Avena*) saponin avenacin A-1 (Fig. 7), incorporating a triterpenoid as the sapogenin unit and found solely in the root epidermis, has shown strong in vitro activity against the fungal root pathogen *Gaeumannomyces graminis* var. *tritici* [76]. *Avena* species producing avenacin A-1 are resistant to some *G. graminis* varieties, but not to those able to enzymatically cleave sugar moieties off the saponin. *Avena* species that do not produce avenacin A-1 (either natural variants or sodium-azide-induced mutants) are more susceptible to *G. graminis* var. *tritici* [76]. Juvenile white spruce (*Picea glauca*) is protected by the monoterpenoid camphor against herbivory by snowshoe hares [77]. A single dominant gene can confer to some cucumber plants the ability to produce bitter

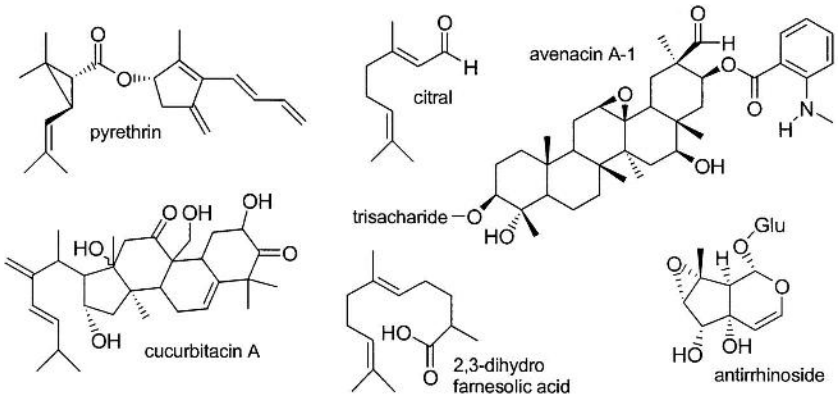


Figure 7 Selected bioactive terpenoids.

triterpenoids including cucurbitacin A (Fig. 7) that repel nematodes [78,79]. Trichomes of wild tomato, *Lycopersicon hirsutum*, contain the terpenoid 2,3-dihydrofarnesolic acid (Fig. 7), which protects the plant from arthropod herbivores [80]. Spraying this compound on leaves of domestic tomatoes protects the plants against the two-spotted spider mite, *Tetranychus urticae* [80]. At least 57 plant families contain constitutive iridoid glycoside terpenoids, which are extremely bitter and effective in defending plants against generalist insect herbivores, fungi, and other microbes [81]. One iridoid glycoside, antirrhinoside (Fig. 7), has been shown to be translocated throughout the plant through the phloem [81].

Finally, the effectiveness of terpenoid defenses has been correlated with ratios between two or more specific terpenoids. For example, colonization of the Jack pine *Pinus banksiana* by the bark beetle *Ips grandicollis* was negatively correlated to the ratio of α-pinene to β-pinene present in the phloem prior to attack [62]. Tree selection for nesting by squirrels has been correlated to the ratio of the monoterpenoid α-pinene in oleoresin composition [82].

Recent light-limiting experiments by Ralphs et al. [83] show that for tall larkspur (*Delphinium barbeyi*), carbon/nutrient balance theories [84], which predict a switch from synthesis of carbon-based to nitrogen-based defensive compounds on plant exposure to photosynthetic stress, do not hold true. In addition, growth/differentiation theories [85], which predict the synthesis of secondary compounds to take place in late growth stages, are not found to apply. Resources are not shunted from carbon-based compounds to nitrogen-based defensive compounds (e.g., larkspur's norditerpenoid alkaloids; Fig. 8) by photosynthate deficits, and these nitrogen-based defensive compounds are also made in early growth stages. They further conclude that these norditerpenoid alkaloids are not synthesized in the chloroplasts in the bud elongation stage.

3.2 Induced Terpenoids in Plants

Terpenoids produced by a plant in response to damage or stress are usually referred to as induced (e.g., Ref. 62). These responses can take place in a localized manner, around the site of invasion, or take place throughout the plant or even in parts remote from the injury or invasion site. Induction of terpenoid production may be nonspecific and occur in response to general stress [e.g., mechanical damage, water or nutrient deficit or over-

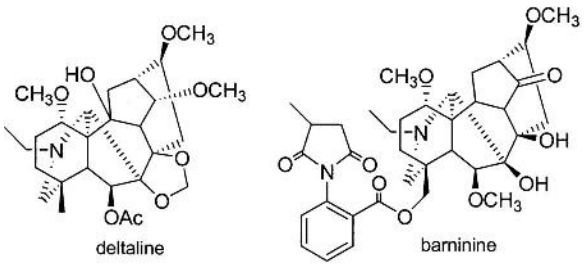


Figure 8 Selected methylenedioxyl- and *N*-(methylsuccinimido)anthranoylly-coctonine alkaloids.

abundance, temperature extremes, and/or amount of ultraviolet (UV) or light incidence] or may result from highly specific interactions between offending organisms and plants. Abiotic factors will not be considered in this chapter but should be kept in mind as possible variables in studies involving the induction of terpenoids as defensive mechanisms. A further factor is the diurnal periodicity of emission of induced terpenoids as exhibited by grape, *Vitis labrusca* [86], corn [87], and cotton [88].

Induced terpenoids can have positive and/or negative consequences for the producing plant. For instance, colonization of Jack pine, *Pinus banksiana*, by the bark beetle *Ips grandicollis* is negatively correlated to the ratio of α-pinene to β-pinene, but colonization is positively correlated to the presence of 3-carene, terpinene (Fig. 9), and myrcene [62]. These correlations are found, even though some of these compounds are deleterious to *Ips* species [89]. *Ips pini* is also able to differentiate between root-diseased and healthy pines by rapidly induced responses in *P. resinosa*, with extracts of the reactive tissue of healthy trees having a repellent effect [89].

Oleoresin is an approximately equal mixture of rosin (diterpenoid resin acids) and turpentine [mostly monoterpenoids (~85%) and sesquiterpenoids (~15%) [47]. Oleoresin is produced by many conifers as a response to bark beetle invasions [90–93] and serves to seal wounds as well as being toxic to invading insects and fungal pathogens [94–97]. Oleoresin is found as a constitutive component of grand fir (*Abies grandis*), but its production can also be induced by wounding and has been used as a model for induced oleoresin production in conifers. For conifers, it appears that induction of oleoresin is moderate in species with large amounts of constitutive oleoresins in duct storage systems (e.g., the pines), whereas induction of oleoresin can be significant in species, such as true firs, with low constitutive levels of oleoresin [98]. Studies of oleoresin induction at the biochemical and molecular levels indicate that wounding elicits synthase activity in the production of monoterpenoids, sesquiterpenoids, and diterpenoids with a shorter lag phase (as early as 2 hr after stem wounding) for monoterpenoids [47]. The monoterpenoids might serve a dual purpose, acting first as insect toxins and then as solvents for the delivery of wound-sealing diterpenoids [47]. The slower onset of sesquiterpenoid formation in grand fir,

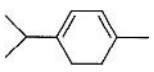


Figure 9 Terpinene.

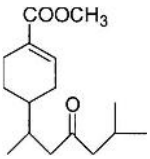


Figure 10 Juvabione.

including that of α-bisabolene [considered a precursor to juvabione (Fig. 10) and other juvenile hormone mimics] and δ-cadinene (precursor to several oxygenated phytoalexins in angiosperms [99,100]), has been proposed to be a defensive mechanism against secondary challenges by invading beetles, including the construction of egg galleries and spore generation of pathogenic fungi vectored by the beetles [47,96,101]. Two key sesquiterpenoid synthases, δ-selinene synthase and γ-humulene synthase, produce 34 and 52 different sesquiterpenoids, respectively, accounting for most of the constitutive sesquiterpenoids as well as the two induced sesquiterpenoids in grand fir oleoresin [102].

The role of terpenoids in the needles of conifers has also been explored. A study analyzing the distribution of terpenoids within needles and within canopy regions and their relationship to herbivory found that terpenoid distribution varies highly between the basal and distal parts of individual needles [103]. This distribution was associated with the almost exclusive feeding of basal parts of Jack pine (*Pinus banksiana* Lamb.) needles by Jack pine budworm (*Choristoneura pinus pinus* Freeman) [103]. The pine's chemical response [103] to defoliation by the budworm, however, takes place mostly in a different tissue, the phloem, and the changes are directly correlated with susceptibility to *Ips grandicollis* [62]. Tiger moth larvae (*Halisdota ingens* Hy. Edwards) eat ponderosa pine needles (*Pinus ponderosa* Lawson) beginning at the tip and proceeding down to about the middle of the needle before moving on to other needles [104]. Litvak and Monson [104] attributed this feeding pattern to the higher number and amounts of constitutive terpenoids and higher levels of inducible monoterpenoid cyclase activities in the lower part of the needles compared to the top. The activity of monoterpenoid cyclases [104] was significantly induced by simulated herbivory in the needles of ponderosa pine (*P. ponderosa*), lodgepole pine (*P. contorta* Douglas var. *latifolia* Engelmann), and white fir (*Abies concolor* Lindl. and Gordon), but not in Engelmann spruce [*Picea engelmanii* (Parry) Engelm.]. In *P. ponderosa*, the induction by real herbivory using tiger moth larvae was significantly higher than by simulated herbivory alone. Interestingly, no increase in total monoterpenoid pool size in ponderosa and lodgepole pines was detected even when wounding significantly increased the synthesis of monoterpenoids by needles, a fact the authors attribute to volatilization [104].

The production of cyclic terpenoids by resistant species of maple (*Acer rubrum* and *A. saccharinum*) in response to Japanese beetle (*Popillia japonica* Newman) feeding has been correlated to resistance [86]. Induced production of these cyclic compounds was much lower in susceptible species (*A. palmatum* and *A. platanoides*). Production of acyclic terpenoids, on the other hand, was high for both resistant and susceptible species [86].

Studies on the induced response of several resistant and sensitive rice genotypes to UV exposure and fungal attack have been performed [105]. The early production of the diterpenoid phytoalexin, momilactone A (Fig. 11), with an ED_{50} against *Pyricularia oryzae* of 31.4 ppm and the subsequent production of another phytoalexin diterpenoid, oryzalexin

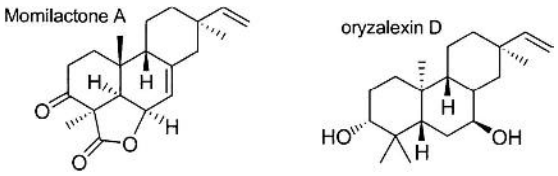


Figure 11 Selected diterpenoid phytoalexins.

D (Fig. 11), with an ED_{50} 30 ppm were determined to be part of a defensive response by the rice genotype Tetep to attack by the incompatible fungal pathogen *P. oryzae* [105].

Some pesticides may significantly influence the production of defensive terpenoids. The experimental fungicide WL 28325 (2,2-dichloro-3,3-dimethylcyclopropane carboxylic acid) induces production of the diterpenoids momilactone A and B [106,107]. The fungicide diniconazole has strong effects on terpenoid levels in sage (*Salvia officinalis* L.) and scotch spearmint (*Mentha cardiaca* L.) [108]. Another example is that of induced synthesis of sesquiterpenoid, hemigossypol in cotton by sublethal application of the herbicide acifluorfen [109], a compound that causes oxidative stress. The herbicide paraquat increases oleoresin levels in pine (*Pinus* spp.) [110]. This increase can result in increased resistance to insect attack [111,112].

Recent $^{13}CO_2$-labeling studies in cotton have revealed that several of the volatile terpenoids induced by herbivore insect damage are newly synthesized upon attack with little or no release of stored compounds [113,114]. Cotton's induced volatiles were produced, released, and incorporated ^{13}C in larger amounts upon beet armyworm feeding or upon exogenous application of beet armyworm oral secretion to mechanically damaged leaf tissue than upon mechanical damage of the leaf alone. Constitutive volatiles, on the other hand, did not incorporate much ^{13}C and release was not dependent on the leaf damage method [54]. Label incorporation was much higher on terpenoids emanated from top undamaged leaves kept under a $^{13}CO_2$-rich environment and with the bottom of the plant undergoing damage in a regular environment. Also, enriching only the bottom part's $^{13}CO_2$ atmosphere led to no differences in the volatiles emitted by the top part, clearly indicating that the terpenoids are not translocated from the damaged leaves to the undamaged (top) leaves but rather produced in situ. A signaling mechanism must therefore exist from the damaged parts of the plant to the distant undamaged ones [54].

Jasmonic acid and methyl jasmonate (Fig. 12) are known to be signal transducers in defensive responses of plants [115–117]. Kuroyonagi et al. [118] have recently shown that treatment of *Hyoscyamus albus* hairy roots with methyl jasmonate leads to the production of several phytoalexins (Fig. 12). Another known elicitor, volicitin, will be discussed in Section 3.4.1.

Cytological and histochemical studies by Daayf et al. [119] on the early vascular defense reaction of cotton roots against infection by a pathogenic strain of *Verticillium dahliae* showed ultrastructural modification of parenchyma cells of the vascular tissue associated with a strong production of terpenoids. This observation agrees with studies by Harrison and Beckman [120], in which successful defense (inhibition of fungal vascular ingress) was correlated with physical walling-off of infected vessels followed by production and release of terpenoid aldehydes into the lumen of infected vessels. Although early defense mechanisms in resistant cotton does not appear to stop infection of xylem vessels, Daayf et al. [119] proposed that early structural responses limit the lateral ingress of *V.*

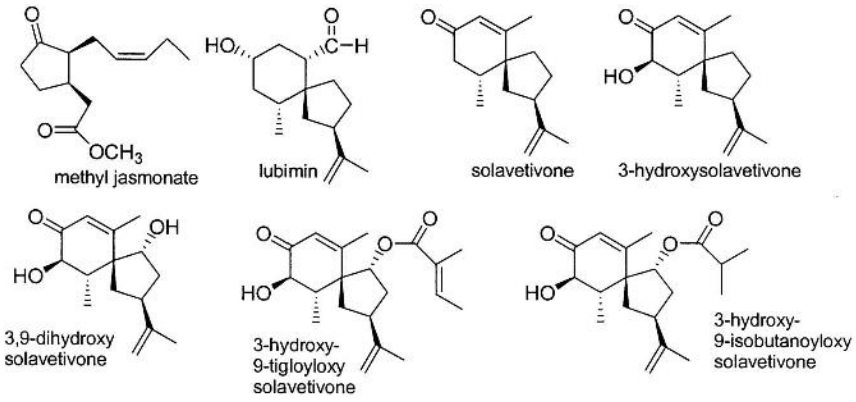


Figure 12 The signal transducer methyl jasmonate and selected phytoalexins.

dahliae within vascular tissues, favoring later contact between pathogen and the plant's fungal toxins. Susceptible cotton appears to be slower in the activation of these defenses, allowing for successful colonization by the infecting fungi.

3.3 Direct Mechanisms

3.3.1 Terpenoids Toxic to Pests

Many plant-produced terpenoid compounds act directly at the biochemical level on the species against which they defend. Little is known in many cases of the actual biochemical mechanisms of toxic action, with the possible exception of interactions of certain terpenoids with insects, for which several reviews exist (e.g., Refs. 10 and 121–123).

Exposure to atmospheres enriched with α-pinene, β-phellandrene, myrcene, limonene, or methyl chavicol (Fig. 13) is lethal, within 24 hr, to adult spruce beetles *Dendroctonus rufipennis* [124] and eastern larch beetles (*Dendroctonus simplex* Le Conte). Atmospheres with β-pinene, 3-carene, or camphene (Fig. 13) induced some degree of mortality over controls [124]. α-Pinene, β-pinene, limonene, and myrcene, all components of the oleoresin of the pines *Pinus resinosa* and *Pinus banksiana*, as well as terpinolene (Fig. 13), are toxic to the bark beetle *Ips pini* with LD_{50}'s (after 2 days of exposure) upon application of 1.4–6.3 mg/g monoterpenoid-free phloem substrate [96].

Pulegone, limonene, and linalool (Fig. 13) have insecticidal activity when applied to flies, cockroaches, and the western corn rootworm [125]. The LD_{50}'s for limonene, linalool, and pulegone (Fig. 13) were 5.2, 7.2, and 7.7 μg/female house fly, respectively, when topically applied (with piperonyl butoxide to inhibit oxidative inhibition); LC_{50}'s (ppm) were 19, 14, and 3.1, respectively, when fumigating rice weevil, and 23, 12, and 9.6, respectively, when fumigating german cockroach [125].

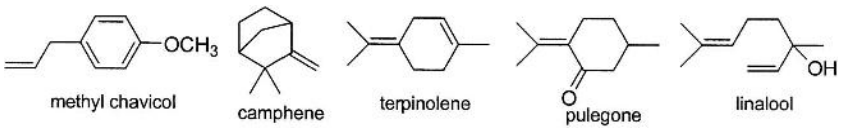


Figure 13 Selected insecticidal terpenoids.

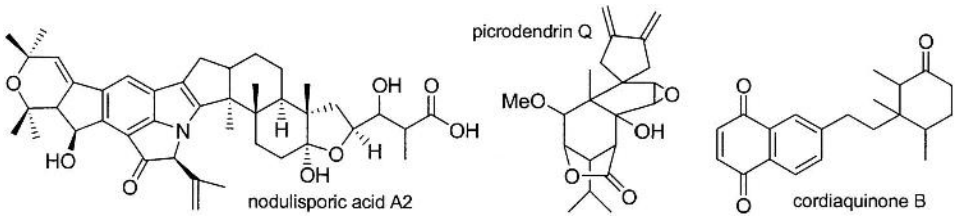


Figure 14 Selected nodulisporic acid indole, picrotoxane, and cordiaquinone terpenoids.

New indole terpenoid insecticides, nodulisporic acid A,A1, and A2 (Fig. 14), from *Nodulisporium* species have been reported with LD_{50}'s (μg/mL) of 0.3, 0.3–1.0, and 0.6–1.5, respectively, against the blowfly *Lucilia seracata* [126,127]. Ozoe et al. [128] evaluated the ability of 28 picrotoxane terpenoids from the Euphorbiaceae *Picrodendron baccatum* (L.) Krug and Urban to inhibit the noncompetitive antagonist of ionotropic GABA receptors, [3H] EBOB, to the housefly-head membranes. An IC_{50} of 22 n*M* was found for picrodendrin Q (Fig. 14). Quantitative structure–activity relationship studies revealed the importance of the degree of electronegativity at carbon-16 and of the presence or absence of hydroxyls at positions 4 and 8 for the inhibition potency of these norditerpenoids [128]. Ioset et al. [129] reported that cordiaquinone meroterpenoids (Fig. 14) were lethal, within 24 hr, to *Aedes aegypti* larvae at concentrations as low as 12.5 ppm. Antifungal activity against *Cladosporium cucumerinum* and *Candida albicans* for these compounds was reported with minimal inhibition concentrations in agar dilution assays ranging from 1 to 6 μg/mL [129].

For carveol, menthol, linalool, geraniol, carvacrol, and thymol, LD_{50}'s of 33–282 μg/housefly and an LC_{50} of 228 ppm for thujone's (Fig. 15) acute toxicity in mosquito larvae have been reported by Tsao and Coats [130]. These authors suggested that neurotoxic effects might be one of the mechanisms of action in acute toxicity with these monoterpenoids. They also suggested that the increased lipophilicity of esterified and etherified alcoholic and phenolic monoterpenoids leads to greater toxicity [130]. Bioactivity toward *Triatoma infestans* has also been reported for 63 essential oils isolated from Bolivian plants [131]. Oils from *Coriandrum sativum*, *Pimpinella anisum*, *Chenopodium ambrosioides*, *Tagetes pusilla*, *Menta arvensis*, and *Foeniculum vulgare* possessed larvicidal activity. Those from *Tagetes pusilla*, *Tagetes minuta*, *Mentha arvensis*, *Mynthostachys andina*, *Mynthostachys mollisi*, and *Foeniculum vulgare* had ovicidal activity [131]. The essential oil of *Callicarpa americana* exhibited selective activity against the cyanobacterium, *Oscillatoria perornata*, at 28.5 μg/mL, with little phytotoxic or antifungal activity [132]. The essential oil of two *Thymus vulgaris* clonal types have antifungal activity against *Botrytis cinerea* and *Rhizopus stolonifer*, with differences in activity attributed to differential accu-

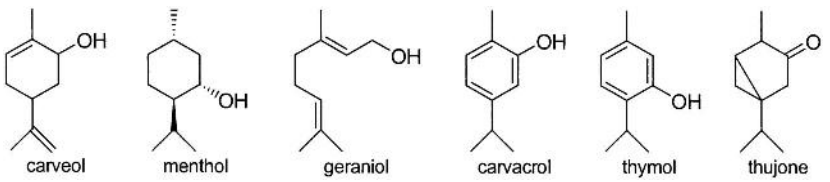


Figure 15 Selected monoterpenoids with insect toxicity.

mulation of individual components in the oil [133]. Prakash and Rao list over 20 terpenoids from natural sources which are either nematicidal, miticidal, or insecticidal [134].

The sesquiterpenoid phytoalexin rishitin is known to disrupt cell membranes of leaf tissues and tomato and potato protoplasts [135–137]. Fungitoxic activity of the sesquiterpenoids rishitin, phytotuberin, anhydro-β-rotunol (Fig. 16), and solavetinone also appears to take place by membrane lysis against *Phytophtora* species [138]. Lyon [136] concluded that rishitin was interacting with phospholipids and increased the permeability of a range of low-molecular compounds through liposomes. However, internal structure disorganization of motile zoospores was observed by electron microscope prior to destruction of the cell membrane [137]. Drimane sesquiterpenoids are known to have antimicrobial, molluscicidal, and phytotoxic activity [139]. Structure–activity relationship studies of drimane sesquiterpenoids (Fig. 16) from *Canella winterana* [139] showed that the dialdehyde moiety was important, whereas the presence of the hydroxyl geminal to the aldehyde was not important for phytotoxic activity [140].

A large number of plant terpenoids are known to be phytotoxic, and one of the issues already addressed in this chapter (Sec. 2) is the means taken by terpenoid-producing plants to either physically or physiologically compartmentalize these compounds to protect against autotoxicity. More difficult to elucidate is whether these toxins participate as a defensive mechanism in plant–plant interactions. Although the highest concentration of terpenoids is normally found in leaves, foliar injury associated with physical contact between foliage of adjacent plants remains anecdotal. Therefore, to be of ecological significance, terpenoids produced by one plant must be released (e.g., to the ground either by exudation or litter decomposition) and result in growth inhibition of another plant in close proximity. This adversary relationship is difficult to establish because many other factors may be involved. For example, strong inhibition of seed germination can be obtained by incorporating leaves or leaf extracts of annual wormwood (*Artemisia annua*) in soil [141]. Artemisinin (Fig. 3), a sesquiterpenoid endoperoxide lactone abundant in *Artemisia* species, is highly phytotoxic in the laboratory [142] and has been shown to inhibit root elongation by affecting mitosis [143]. However, little herbicidal activity can be observed when artemisinin is incorporated in soils, suggesting that the effect observed with *A. annua* leaves is not related to their artemisinin content alone [141].

The quassinoids ailanthone and chaparrinone (Fig. 17) are highly phytotoxic diterpenoids common in the Simaroubaceae plant family. These molecules have been shown to have both pre-emergence and postemergence activity in greenhouse tests [144], inhibiting monocots and dicots at rates as low as 0.125 kg/ha. The most active quassinoids are active at concentrations as low as 3 μ*M* in the laboratory [145]. This study also pointed out some of the structural requirements for herbicidal activity. In particular, the presence of the

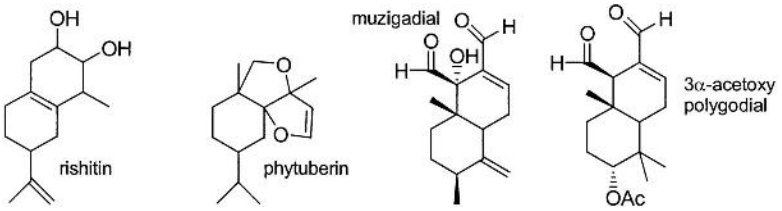


Figure 16 Selected fungitoxic sesquiterpenoids and two drimane terpenoids, muzigadial and 3α-acetoxypolygodial.

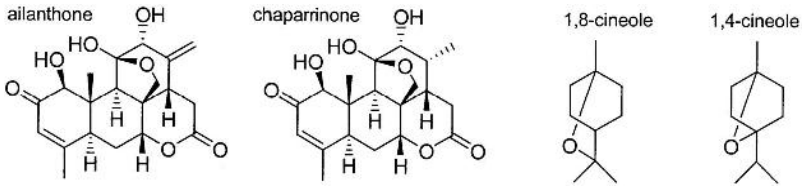


Figure 17 Two quassinoid and two cineole terpenoids.

oxymethylene bridge played a key role in activity, and molecules lacking this structural feature were totally inactive [145]. A recent study suggested that quassinoids have a novel mode of action by acting as potent inhibitors of NADH oxidase [146].

A particularly interesting aspect of plant–plant interaction involves volatile monoterpenoids of one plant repressing the growth of surrounding vegetation [147]. Again, conflicting observations have been reported with the effect of these compounds. Halligan [148] has argued that the effect of these volatile natural products may be insignificant in nature when compared to the overall competition for water, nutrient, and space occurring between the two plants. However, Romagni et al. [149] have recently demonstrated that the application of the monoterpenoid 1,8-cineole or 1,4-cineole (Fig. 17) caused significant growth reduction at levels as low as 10 μg/g soil. A comparison of the two compounds showed that these relatively similar molecules have different modes of action; that is, 1,8-cineole caused a strong inhibition of mitosis in roots, but 1,4-cineole did not.

In nonplant systems, stypoldione, derived from styprodiol (Fig. 18), is lethal to fish at a concentration of 1 mg/mL, is a potent inhibitor of cell cleavage in fertilized sea urchin eggs and of motility in urchin sperm, and inhibits both amino acid and nucleoside uptake [150–153]. Elatol (Fig. 18) is cytotoxic and inhibits 50% of the cell divisions in fertilized sea urchin eggs at concentrations of 7 μg/mL [154]. The antifungal and antibacterial activity of exocrine gland excretions from mustard leaf beetle larvae, *Phaedon cochleariae*, is due to its main component, the iridoid monoterpenoid epichrysomelidial (Fig. 18) [155]. Antifungal activity was not due to high efflux of potassium from treated cells, as is the mode of action with other common fungicides [155].

Recent studies by Guillet et al. [156] have shown that volatiles from glandular secretory cavities in leaves of the Asteraceae *Porophyllum gracile* and *Porophyllum ruderale* containing monoterpenoids and sesquiterpenoids are not insecticidal to third instar larvae of the lepidopteran *Ostrinia nubilalis*. These volatiles, however, appear to synergize the

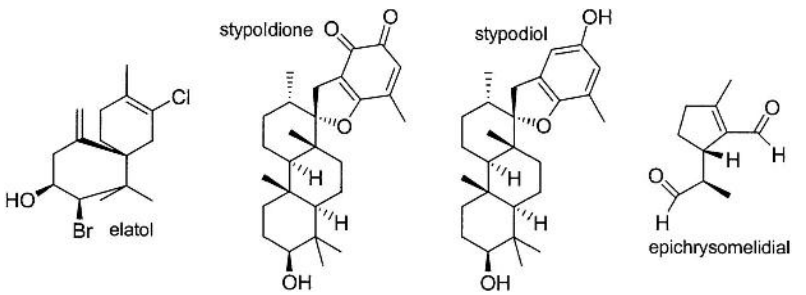


Figure 18 Selected marine and insect toxic terpenoids.

effects of the light-activated toxin α-terthienyl present in these two plants by enhancing its accumulation in the larvae. Although this constitutes, chemically speaking, an indirect mechanism by which terpenoids can act as defensive compounds, the term ''indirect defense'' seems to have been reserved in the literature for tritrophic interactions (*vide infra*).

3.3.2 Terpenoids Involved in Behavioral Changes

In many cases, terpenoids act defensively by altering the behavior of the deleterious organisms. This can include a whole range of mechanisms, including acting as repellents, feeding or oviposition deterrents, alarm signals, or interfering with communication signals (e.g., aggregation signals).

The attraction of *Dendroctonus* species to baited traps was inhibited by the addition of myrcene or methyl chavicol for *D. simplex* and by the addition of limonene, myrcene, β-phellandrene, or methyl chavicol for *D. rufipennis*. Interestingly, α-pinene, β-pinene, camphene, 3-carene, limonene, myrcene, methyl chavicol, or β-phellandrene, all directly toxic (see Sec. 3.3.1) to *D. rufipennis*, increased the attraction of traps to *D. rufipennis* compared to unbaited traps [124]. α-Pinene, β-pinene, camphene, 3-carene, limonene, myrcene, methyl chavicol, and β-phellandrene are all found in varying degrees in the oleoresins of white spruce [*Picea glauca* (Moench) Voss] and tamarack [*Latrix laricina* (DuRoi)], where they appear to form part of the defensive mechanism against attack by *Dendroctonus* beetle species [124].

Prakash and Rao [157] list four terpenoids, quadrangolide, 1-deoxy-8-epiivangustin, isoalantolactone, and diplophyllolide (Fig. 19), from the plant *Eupatorium quadrangularae* Linn., as having repellent activity to the leaf cutting ant *Atta cephalotes*. The monoterpenoid (*E*,*E*)-α-farnesene (Fig. 19) is suggested to act as an alarm signal in the Monarch butterfly caterpillar, *Danaus plexippus* [158]. Although this signal seems to be part of a defensive mechanism against parasitic wasps, it is not very specific, being activated by vibrations from bumblebees, human buzzing sounds, and even overhead jet aircraft noises [158].

The role of terpenoids in the defensive mechanism of *Eucalyptus* against herbivory by the common ringtail possum (*Pseudocheirus peregrinus*) and the common brushtail possum (*Trichosurus vulpecula*) has been investigated by Lawler et al. [159]. Meroterpenoid diformylphloroglucinols are the toxic constituents giving rise to the aversion of possum to feeding on leaves rich in these compounds. However, possums appear to key on volatile terpenoids, such as 1,8-cineole (Fig. 17), as a cue to the amounts of toxic nonvolatile meroterpenoids (i.e., jensenone; Fig. 20) present in *Eucalyptus* leaves [159].

Two strong antifeedant diterpenoids found in the yew tree (*Taxus baccata* L.), 10-deacetylbaccatin III and 10-deacetylbaccatin V (Fig. 20), are believed to be responsible for the strong resistance of *T. baccata* against insect pests [160]. Three isoryanodane (e.g.,

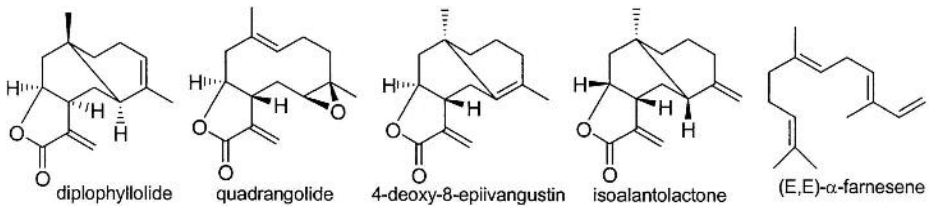


Figure 19 Selected behavior-modifying terpenoids.

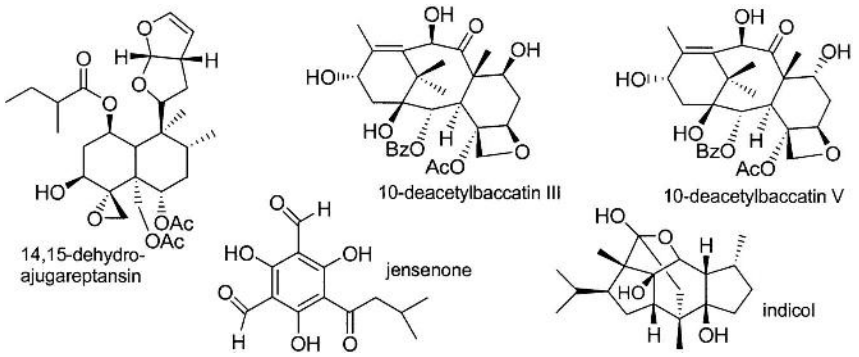


Figure 20 The meroterpenoid diformylphloroglucinol jensenone, isoryanadone diterpene indicol, and selected diterpenoid antifeedants.

indicol; Fig. 20) diterpenoids with antifeedant activity against the insect pest *Spodoptera litura* have been found in *Persea indica* [161]. Three neoclerodane diterpenoids have been isolated by Bremner et al. [162] from *Ajuga reptans*, and 14,15-dehydroajugareptansin (Fig. 20) was found to have significant antifeedant activity against *Spodoptera littoralis* larvae. Multiple other examples of antifeedant studies have been given by Simmonds [163].

3.4 Indirect Mechanisms of Defense

3.4.1 Attraction of Deleterious Organisms to Pests

Volatile terpenoids can also provide an alternate mechanism of defense for plants by attracting enemies of its pests. This mechanism can be especially effective when dealing with induced terpenoid formation because the plant is emanating signals as to the presence, both in time and space, of a particular offending organism such as an insect herbivore.

Regurgitate from several caterpillar species and from a grasshopper stimulates the release of *Zea mays'* defensive signaling terpenoids, attracting *Cotesis marginiventris* and *Microplitis croceipes* parasitic wasps [164]. More recently, *N*-(17-hydroxylinolenoyl)-(L)glutamine (volicitin; Fig. 21), either isolated from the beet armyworm caterpillar *Spodoptera exigua* Hübner or synthesized, was shown to elicit the systemic release of volatiles when applied to cut stems or mechanically damaged sites of corn seedlings at concentrations as low as 300 pmol [164,165]. Furthermore, ^{13}C-labeling studies have shown that this elicitor is synthesized by the insect from plant-derived fatty acid, which is hydroxylated and combined with glutamine by the caterpillar. In effect, this makes volicitin a very specific signaling mechanism that is only turned on when the insect is feeding on the plant, thereby signaling its presence to possible predators or parasites [166].

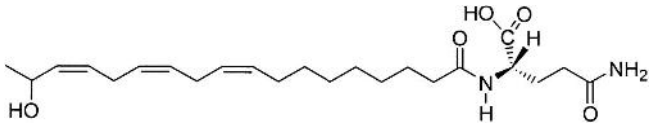


Figure 21 Volicitin.

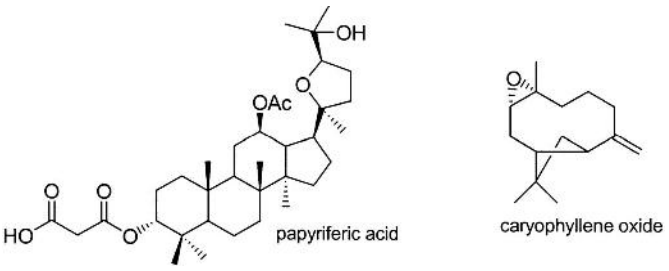


Figure 22 Papyriferic acid and caryophyllene oxide.

This same type of mechanism appears to be activated by beet armyworm larvae feeding on cotton plants (*Gossypium hirsutum*) [54].

Other recent reports of tritrophic interactions involving a plant, herbivore, and predator or parasitoid system include the following: (1) a lima bean (*Phaseolus lunatus*), herbivorous spider mite (*Tetranychus urticae*), and predatory mite (*Phytoseiulus persimilis*) system [167] (2) a Brussels sprout (*Brassica olerace* var. *gemmifera*), herbivorous caterpillar (*Pieris brassicae*), and parasitoid (*Cotesia glomerata*) system [168]. The biotic and abiotic factors affecting such defensive responses have also been reported [169].

3.4.2 Terpenoids Toxic to Organisms Beneficial to a Pest

Many terpenoids have recognized antimicrobial activity. This activity protects plants directly from a large number of pathogenic or herbivorous microorganisms. A second level of protection may be provided by the toxic effect of plant terpenoids on microbial organisms on which a potential herbivore depends for survival or well-being. The antibiotic effect of terpenoids, for instance, might negatively impact symbiotic micro-organisms that aid in the digestive process of herbivores. Symbiotic micro-organisms exist in the rumen of vertebrate herbivores and on the outside and inside (in extracellular and intracellular locations) of insect herbivores [163]. The Alaska paper birch (*Betula resinifera*) triterpenoid papyriferic acid (Fig. 22) is extremely toxic to elk (*Cervis canadensis*) rumen microbes [164] and might play a role in diet selection by elk. Papyriferic acid has also been correlated with reduced browsing of Alaska paper birch by herbivores including snowshoe hares (*Lepus americanus*) [164]. The antifungal properties of some monoterpenoids from grand fir (*Abies grandis*) on the symbiotic fungus (*Trichosporium symbioticum*) of the fir engraver beetle (*Scolytis ventralis*) might play an important role on whether the beetle is able to successfully establish a colony [47,163].

A similar mechanism occurs with leaf cutter ants (*Atta cephalotes* and *Acrmyrmex octospinosa*) which avoid harvesting leaves from tree species (e.g., *Hymenea courbaril*) with high levels of caryophyllene oxide (Fig. 22), a sesquiterpenoid highly toxic to the symbiotic fungi that they grow in their nests [4,165,166]. Another terpenoid, nerolidol, also inhibits fungal growth and is not toxic to adult ants at antifungal levels [4,167].

3.5 Pitfalls in the Use of Protective Terpenoids as Defensive Mechanisms

3.5.1 Use of Terpenoids by Pests to Locate the Producing Organisms

As has already been mentioned, terpenoids, at times work to the detriment of a terpenoid producing plant. This process is especially true of the more volatile components, including

monoterpenoids and sesquiterpenoids which, once emitted, might serve as signaling beacons to predators or parasitoids or might abet in the synthesis or effectiveness of aggregation signal processes of the herbivores or other deleterious organisms.

Much research exists on the use of chemical clues by herbivores to find their host plants [7]. Myrcene, a component of many plants, is one of the monoterpenoids known to increase *Ips* (bark beetle) aggregation, serving as synergist and/or precursor to their aggregation pheromones [62,168–177]. Metcalf et al. [178] detailed the importance of some volatiles, including terpenoids, in the attraction of several *Diabroticites* species to cucurbits and corn. In recent feeding studies involving the Japanese beetle (*Popillia japonica*), Loughrin et al. suggested that feeding-induced volatiles, including floral terpenoids, may serve as aggregation kairomones for the beetles by attracting them to host plants [86]. Additionally, although the induced volatile compounds may act as general feeding deterrents to herbivores, they might also act as phagostimulants on the Japanese beetle to the further detriment of the producing plant [86].

Although not directly used as a guiding mechanism, certain norsesterterpenoids and nortriterpenoids from soybean, the glycinoeclepins A, B, and C (Fig. 23), induce hatching of the nematode *Heterodera glycines*, in effect signaling the unhatched cyst nematode of the presence of available food. The nematodes then are able to use other chemical signals to locate the plant [179–182].

In a nonplant example, the use of (*E*)-β-farnesene as an alarm pheromone by aphids might also serve as an attractant to one of its predators, the lacewing *Chrysoperla carnea*. However, (*E*)-β-farnesene appears to have no effect on another lacewing, *C. cognata*, which seems to be attracted instead to the aphid's sex pheromones nepetalacotone and nepetalactol (Fig. 23) [183].

3.5.2 Use/Storage of Plant Constituents by Pests for Their Own Protection

Plant terpenoids can be incorporated by herbivores to form part of their own defensive mechanism against predators [184]. These tritrophic interactions usually involve the following: (1) a plant producing terpenoids; (2) a herbivore incorporating the terpenoids in their original form or as metabolites; and (3) a predator, parasitoid, or pathogen deterred by the terpenoids. The literature covering these interactions has been reviewed [184].

More recently, larval tortoise beetles (*Eurypedus nigrosignata*) were shown to use monoterpenoids and sesquiterpenoids taken directly from *Cordia curassavica* host plants (on which they feed) to defend themselves against predatory ants (*Myrmica rubra*) by incorporating these terpenoids into a fecal shield. Terpenoids in the shield are unmodified from the host plant. The plants' terpenoids (in the form of essential oil) was also repellent to the ants [185].

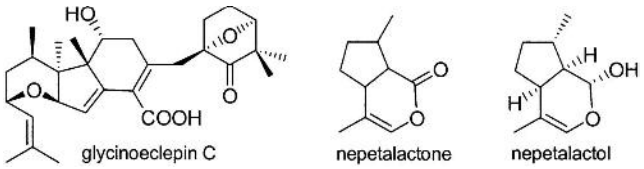


Figure 23 A nortriterpenoid glycinoeclepin and two aphid sex pheromones.

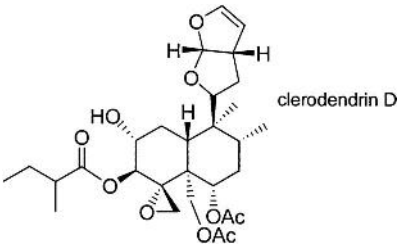


Figure 24 A neoclerodane diterpenoid.

Neoclerodane diterpenoids (Fig. 24) are bitter compounds with potent feeding-deterrent activity against phytophagous insects [186]. On the other hand, neoclerodane diterpenoids from *Clerodendron trichotum* Thunb. and *Ajuga decumbens* are not only strong feeding stimulants for adults of the turnip sawfly (*Athalia rosae ruficornis* Jakovlev) but are also incorporated in the tissues of this insect, where they protect the sawfly from predators and aid females in attracting males [187,188].

Larvae of the sawfly *Neodiprion sertifer* use resin sequestered from its Scotch pine (*Pinus sylvestris*) host, storing the resin in compressible pouches in the foregut to defend themselves against a number of predators, including ants and spiders [189]. The resin is kept sequestered and used in a defensive role by the larvae even after weeks of enclosure in their cocoons [189].

4 DEFENSIVE ROLE OF TERPENOIDS IN OTHER ORGANISMS

4.1 Terpenoid Defenses in Macrophytic Algae

In general, the defensive roles of terpenoids in marine species of algae have been studied and reported more than in freshwater species. The most common reasons given for terpenoid defenses in these types of organisms are as follows: (1) defense or deterrence against grazing by herbivores; (2) competitive advantages in terms of light, nutrients, and substrate for benthic species; and (3) inhibition of growth of pathogenic microorganisms. Terpenoids described as antifouling and/or allelopathic agents for the marine algae fall into the latter two categories of defense. Fouling organisms include algal spores and settling larval invertebrates, both of which can have adverse effects on marine algal growth.

The effects of the presence of terpenoids on the grazing behavior of herbivores are well documented. A reduction of palatability of the algae for the herbivore appears to be the most common function of terpenoid defense against grazing. Apparently, the high intensity of herbivory on coral reefs has led to the selection of algae that produce metabolites, such as terpenoids, that significantly deter consumption by a diverse group of reef herbivores [154]. Several terpenoids isolated from marine algae appear to also function as toxins [154]. Previous studies [190] found that terpenoids are contained in cytoplasmic vesicles located in cells near the photosynthetic organism's outer surface. Partitioned away from other important cellular compounds and processes but in an area of the algae that would initially be contacted by a herbivore, these terpenoids act as a feeding deterrent [190].

Species of red algae in the genus *Laurencia* (Rhodomelaceae) produce a variety of halogenated sesquiterpenoids and diterpenoids [191]. Halogenated sesquiterpenoids in *Laurencia* include a bromine atom-containing sesquiterpenoid in *Laurencia intermedia* Yamada [192], the brominated sesquiterpenoid alcohol β-snyderol (Fig. 25) in *Laurencia synderae* Dawson [190], and two halogenated sesquiterpenoid alcohols, each containing a bromine- and a chlorine-bearing carbon, produced by a *Laurencia* species from coastal Florida [191]. Bittner et al. [191] found significant ichthyotoxicities toward reef fish for the chamigrene sesquiternoid alcohols (e.g., the cyclohexaneoxabicycloheptanol in Fig. 25) they isolated. Hay et al. [154] reported that the terpenoids elatol (Fig. 18), from *Laurencia obtusa*, and isolaurinterol (Fig. 25), from several tropical and warm-temperate species of *Laurencia*, acted as feeding deterrents toward reef fishes and the herbivorous sea urchin *Diadema antillarum*. Elatol is a cytotoxin that may have more impact in reducing consumption rather than the probability of initial attack.

Genera of red algae in the families Plocamiaceae and Rhizophyllidaceae produce numerous halogenated monoterpenoids that have been found to have ichthyotoxicity, fish antifeedant, and antimicrobial activities [193]. Some of the terpenoids isolated include plocamine B (Fig. 25) from *Plocamium cartilagineum* [194], seven cyclic halogenated monoterpenoids (e.g., coccine; Fig. 25) from *P. coccineum* [195], and the halogenated monoterpenoid violacene (Fig. 25) from *P. violaceum* [193]. The presence and degree of halogenation in the terpenoid molecules may impart a disagreeable taste to the marine algae.

Among the brown algae, the family Dictyotaceae has the most abundant number of genera from which terpenoids have been isolated and identified. Hay et al. [154] found that the terpenoids stypotriol (Fig. 26), from the brown alga *Stypopodium zonale* [196], and pachydictyol-A (Fig. 26), produced by members in the genera of *Dictyota*, *Dilophus*, *Glossophora* [197], and *Pachydictyon* [198], exhibited antifeedant properties toward reef fishes and the sea urchin *Diadema antillarum*. Other terpenoids isolated from brown algae include the following:

1. Acetoxycrenulide (Fig. 26), a bicyclic cyclopropane-containing diterpenoid from *Dictyota crenulata* [199]
2. Dictyodial (Fig. 26) from *Dictyota crenulata* and *Dictyota flabellata* [200]
3. Dictyol H (Fig. 26), a tricyclic diterpenoid from *Dictyota dentata* [201]
4. Dictyol A, B, and E (Fig. 26) from *Dictyota dichotoma* [202,203]

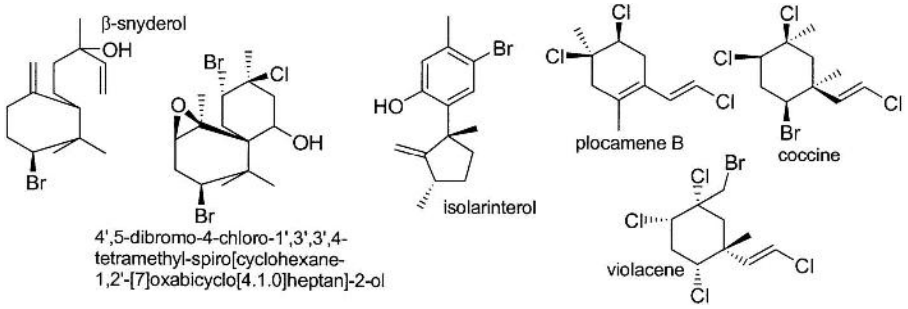


Figure 25 Selected marine halogenated sesquiterpenoid alcohols and halogenated monoterpenoids.

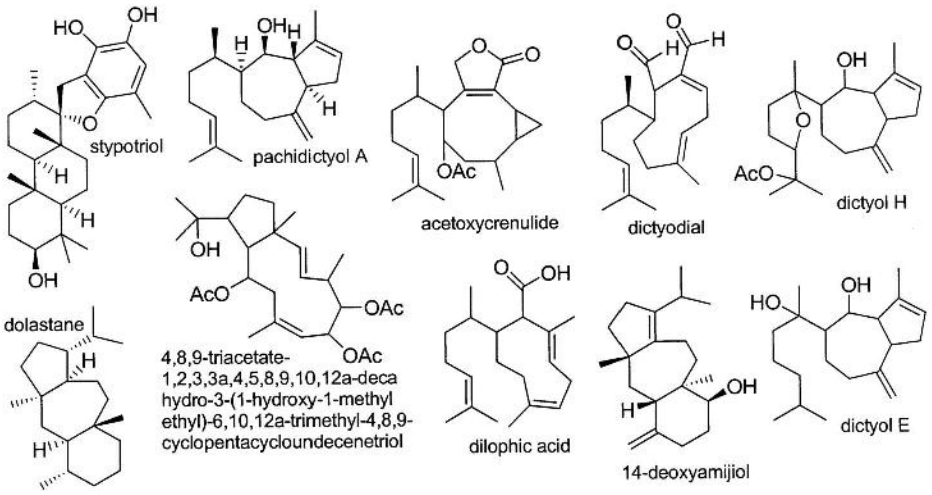


Figure 26 Selected brown algae terpenoids.

5. Dolastane (Fig. 26) diterpenoids from *Dictyota divaricata* and *Dictyota linearis* [204,205]
6. Several tetraoxygenated diterpenoids with a dolabellane skeleton (e.g., the cyclopentacycloundecenetriol derivative in Fig. 26) from *Dilophus fasciola* [206]
7. Dilophic acid (Fig. 26), a xenicane-type diterpenoid containing a carboxylic acid and isolated from *Dilophus guineensis* [207]
8. The diterpenoids amijiol, isoamijiol, and 14-deoxyamijiol (Fig. 26) from *Dilophus linearis* [208]
9. Hydroxydictyodial from *Dictyota spinulosa* [209].

The exact mechanism of antigrazing activity that each of these compounds infers to the producing species of algae is unknown; however, stypotriol does provide one example for the mode of action of an antigrazing terpenoid. Hay et al. [154] report that stypotriol undergoes rapid oxidation once *Stypopodium* is heavily damaged from herbivore feeding (i.e., chewing). The oxidation process probably occurs in the mouth and gut of the herbivore and results in the formation of orthoquinone stypoldione, a cytotoxin that can be lethal to fish at 1 μg/mL [154].

A variety of terpenoids have been isolated from marine green algae, especially from the genus *Caulerpa*. Examples include the following: (1) The diterpenoid alcohol caulerpol (Fig. 27) has been isolated from *C. brownii* [210]; (2) the acetylenic sesquiterpenoid caulerpenyne (Fig. 27) from *C. prolifera* (Forsk.) [211]; (3) the diterpenoid trifarin (Fig. 27) from *C. trifaria* [210]; (4) the acyclic sesquiterpenoid flexilin (Fig. 27) from *C. flexilis* [212]. Flexilin has also been isolated from the Caribbean green alga *Udotea conglutinata* [213]. Algae in the families Caulerpaceae and Udoteaceae are prolific producers of terpenoids and are not a preferred food source by most tropical herbivores [214]. Bioactive terpenoid compounds have been isolated from several species of *Udotea* and *Penicillus* [213], and the diterpenoid trialdehyde halimedatrial (Fig. 27) has been found in *Halimeda* spp. [215,216]. In addition, the linear sesquiterpenoids rhipocephalin and rhipocephenal

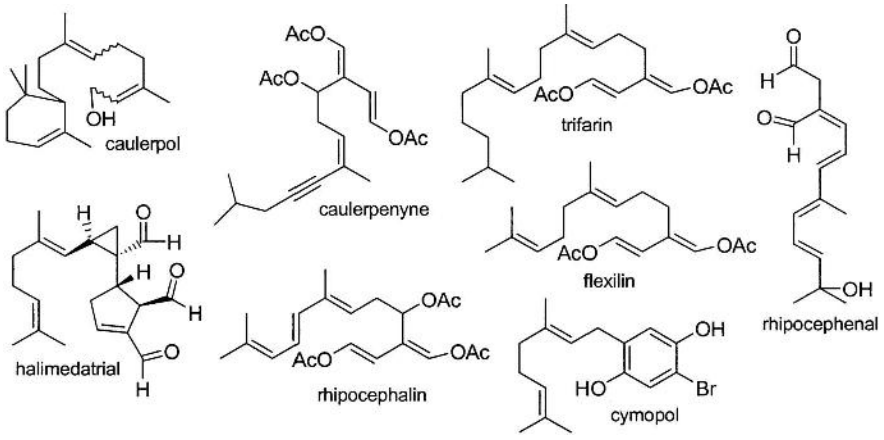


Figure 27 Selected green algae terpenoids.

(Fig. 27) have been isolated from the green alga *Rhipocephalus phoenix* (Codiaceae) [217], and cymopol (Fig. 27), a terpenoid bromohydroquinone, has been found in *Cymopolia barbata* (Dasycladaceae) [154].

Paul and Fenical [218,219] state that terpenoids found in green algae (order Caulerpales) can act as chemical defenses, serve as antifoulants, and act as protectants from marine pathogenic microorganisms. In addition, Paul et al. [220] hypothesize that antifeedant sesquiterpenoid aldehydes, such as those found in *Caulerpa ashmeadii*, are useful as chemical defenses due to the reaction of the aldehyde groups with herbivore proteins that result in adverse effects on protein and/or enzyme function in the herbivore. Paul et al. [221] report that the tropical green alga *Neomeris annulata* produces both brominated sesquiterpenoids as antifeedants and $CaCO_3$ in the form of aragonite as a structural defense, thereby combining chemical and structural defenses to allow the alga to protect against a variety of herbivores found on coral reefs.

Apparently, marine macrophytic algae can concentrate terpenoids in specific portions of their macrostructure. Young growing portions of the algae, such as blade tips, and reproductive structures have been found to contain higher concentrations of the terpenoids than mature algal tissues [218]. Also, tropical green algae subjected to the highest amount of herbivory produced the greatest concentrations and varieties of terpenoids [218].

Terpenoids can also undergo ''activation,'' whereby a less toxic and less deterrent form of the terpenoid molecule is converted to a more active deterrent [222]. Such transformations occur only after mechanical injury to the algae. Examples include the conversion of the less deterrent udoteal to the more deterrent petiodial (Fig. 28) in *Udotea flabellum* [223] and the conversion of the less deterrent halimeda tetraacetate (Fig. 28) to the more deterrent halimedatrial (Fig. 27) in *Halimeda* species [224].

Terpenoids also appear to play a role, either directly or indirectly, in protecting marine algae from microbial pathogens. A large number of reports exist concerning the antimicrobial activity of terpenoids isolated from marine algae. Examples include the following:

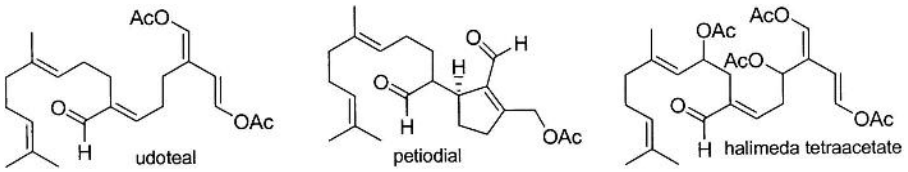


Figure 28 Selected activated-defense algal terpenoids.

1. Cycloeudesmol (Fig. 29) from *Chondria oppositiclada* [225]
2. Pre-pacifenol (Fig. 29) from *Laurencia filiformis* [225]
3. Laurinterol (Fig. 29) and debromolaurinterol from *Laurencia pacifica* [225]
4. Udoteal (Fig. 28) from *Udotea flabellum* [226]
5. Several halogenated alicyclic monoterpenoids from *Plocamium cartilagineum* [227]
6. Several polyhalogenated sesquiterpenoids from *Laurencia* species [227]
7. Dictyol F and epidictyol F (Fig. 29) from *Dictyota dichotoma* [228]
8. Greater antimicrobial activity appears to be related to higher halogen content [227].

There are several reports of marine mollusks concentrating terpenoids in certain areas of their bodies for protection. Most of these terpenoids appear to be obtained from algae that are consumed by the mollusks. An example linking diet contribution to terpenoid concentration in mollusks is the discovery of several cyclic polyhalogenated monoterpenoids in the digestive glands of the mollusk *Aplysia punctata*, with the origin of these terpenoids traced to the red alga *Plocamium coccineum*, a component of the diet of *A. punctata* [229]. The defensive secretions of *A. punctata* contain concentrations of these cyclic polyhalogenated monoterpenoids that are toxic to other marine animals [229]. Dictyol A, dictyol B, and pachydictyol A (Fig. 26) have also been isolated from the digestive glands of *Aplysia* spp., with the presence of dictyol A and B linked to the consumption of the alga *Dictyota dichotoma* and pachydictyol A to the consumption of the alga *Pachydicton coriaceum* [230]. In addition, Kelecom and Teixeira [231] hypothesize that the diterpenoids found in *Dictyota crenulata* may specifically attract predation by the mollusk *Aplysia vaccaria*, which is immune to the diterpenoids and actually concentrates them for use as a defensive measure to prevent predation by carnivores.

Other examples of terpenoid concentration in mollusks include the isolation of diterpenoids from digestive glands of the sea hare *Dollabella californica* Sterns [232], isolation of sesquiterpenoid esters from the digestive glands of the nudibranch *Dendrodoris limbata*

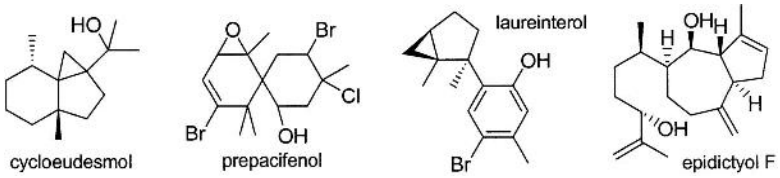


Figure 29 Antimicrobial terpenoids from algal sources.

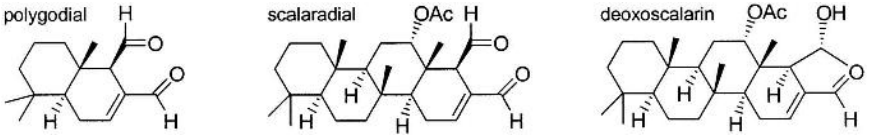


Figure 30 Selected mollusk terpenoids.

[233], and isolation of polygodial (Fig. 30), a sesquiterpenoid dialdehyde, from mantle (skin) extracts of *D. limbata* [234]. In the last case, polygodial secretion from skin glands into the mucus on the skin of the shell-less mollusks may deter consumption by carnivores. The nudibranch mollusk *Glossodoris pallida* contains the sesterterpenoids scalaradial, deacetylscalaradial, and deoxoscalarin (Fig. 30), which accumulate in the border of the mantle and in mucus secretions and serve as a defense mechanism against feeding by crabs and predatory reef fish [235]. Nudibranchs are known to obtain chemicals from bryozoans, cnidarians, tunicates, and sponges [236]. A recent review [237] discusses the transfer of secondary metabolites from sponges to opisthobranch predators. The reason for nudibranch accumulation of defensive compounds in the mantle's border is not clear, but it might be related to sequestration of toxic compounds away from internal organs (paralleling the sequestration of certain terpenoids in glands by plants) or simply placing them in a convenient place for incorporation into mucus secretions [235].

As chemical defenses, terpenoids do not exhibit broad-spectrum toxicity toward all types of herbivores that feed on marine algae. Metabolic transformations by mollusks of consumed terpenoids can occur and yield modified compounds that have reduced toxicity toward the mollusk [238]. Detoxification of terpenoids occurs in the gut of the mollusk. An example is the conversion, via acid catalysis, of isolaurinterol (Fig. 25) (found in *Laurencia* species) to the structurally similar compound aplysin (Fig. 31), a sesquiterpenoid ether [154]. Isolaurinterol is an antifeedant toward sea urchins and reef fishes, whereas aplysin does not exhibit any activity as a feeding deterrent [154]. The conversion occurs under very mild acid conditions and may be a mechanism by which the mollusk can safely ingest the algae without any deleterious side effects. Another study [239] indicates that defenses such as calcification and toughness of the algae may act as more of a defense mechanism against some types of herbivores than the presence of terpenoids.

4.2 Terpenoids in Lichens

Although not classified as plants, lichens are symbioses between a photosynthesizing partner and a fungal component. They are an important part of the ecosystem and are found as epiphytes on many higher plants. The fungal component of lichen produces a variety of unique secondary metabolites that are secreted to the surface of the hyphae rather than remaining within cells. The majority of these compounds are unique to lichens, with only

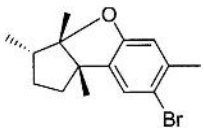


Figure 31 Aplysin.

a small number of these (~60) also occurring in other fungi or higher plants [240]. Ironically, the secondary chemistry of lichen compounds is better documented than any other phylogenetic group due to a long chemotaxonomic history. The physiological/ecological rationale for these compounds is largely undocumented, but protection from UV light, herbivory, and fungal pathogens are the most common explanations [241,242].

Secondary lichen compounds can comprise up to 25% of the thallus dry weight, although more typically they account for 5–10% of the dry weight [243]. The majority of these compounds are classified as depsides and depsidones, although other classes do exist [244]. Lawrey [245] has suggested that certain lichen acids (*O*-methylated) are more evolutionarily advanced and, therefore, more physiologically significant. Only a very few of the more than 20,000 known species of lichens have been tested and identified as containing biologically active secondary compounds.

Although many of the well-characterized toxic lichen compounds are not terpenoids, lichens do produce several terpenoids of unknown activity. Many of these compounds may have protective roles; however, most studies on lichen chemistry so far have been limited to chemotaxonomy. The high investment of resources into secondary products by an organism, which is only metabolically active when moist (when incorporating water to a weight equivalent of ~2 times the dry weight for green lichens, 3 times the dry weight for cyanobacterial-containing species, and ~10 times for gelatinous species), suggests that these compounds must provide some ecological/survival benefit. More than 50% of known lichen compounds have antimicrobial activity against human disease [242] and these antibiotics may have a role in the natural ecosystem as well. Under controlled conditions, aqueous lichen extracts are known to inhibit certain fungi and the growth of mycorrhizae. Reports have documented synergistic effects of usnic acid (Fig. 32), a nonterpenoid lichen secondary product, and crude extracts from *Cladonia cristatella*, which kill the growth of ectotrophic mycorrhizae [246].

Although there have been some laboratory studies [241] on the general allelopathic/phytotoxic properties of lichens, allelopathic interactions in ecosystems are not well understood. There are generally no differences in chemical defenses and amounts of secondary compounds between early and late lichen successional species. Those species without any recognizable secondary compounds are not eliminated [247].

In lichens, the majority of terpenoids isolated are triterpenoids [248]; however, these compounds do not occur as frequently in lichens as in higher plants. At present, approximately 70 lichen terpenoids have been isolated and identified [248]. Many terpenoids are restricted to specific family distributions. For example, the hopane triterpenoids [e.g., (−)-16α-hydroxykaurane; Fig. 32] appear to be distributed in the Lecanorineae (*Physcia* spp. and *Heterdermia* spp.), the Cladoniineae (some *Cladonia* spp.), and Peltigerineae (*Pseudocyphellaria* and *Peltigera*). However, sesterterpenoids (e.g., retigeranic acid; Fig. 32) are found only in the Peltigerineae (*Pseudocyphellaria*) and the dammarane triterpenoids [e.g., 25-acetoxy-20(*S*),24(*R*)-epoxydammarane-3β-ol; Fig. 32] in the Lecanorineae [240,249]. A specific example of a diterpenoid found in lichens, as well as in *Gibberella fujikuroi* and *Trachylobium verrucosum*, is (−)-16α-hydroxykaurane (Fig. 32), found in several *Ramalina* spp. [250].

Some species of lichens have cyanobacteria as their primary photobiont. These particular lichens generally do not produce large amounts of secondary products, and very few of them have been tested for phytotoxic or insecticidal properties. However, some cyanobacterial-containing species have been identified as producing unique triterpenoids, such as 7β-acetoxy-22-hydroxyhopane and 15α,22-dihydroxyhopane (Fig. 32) [251,252].

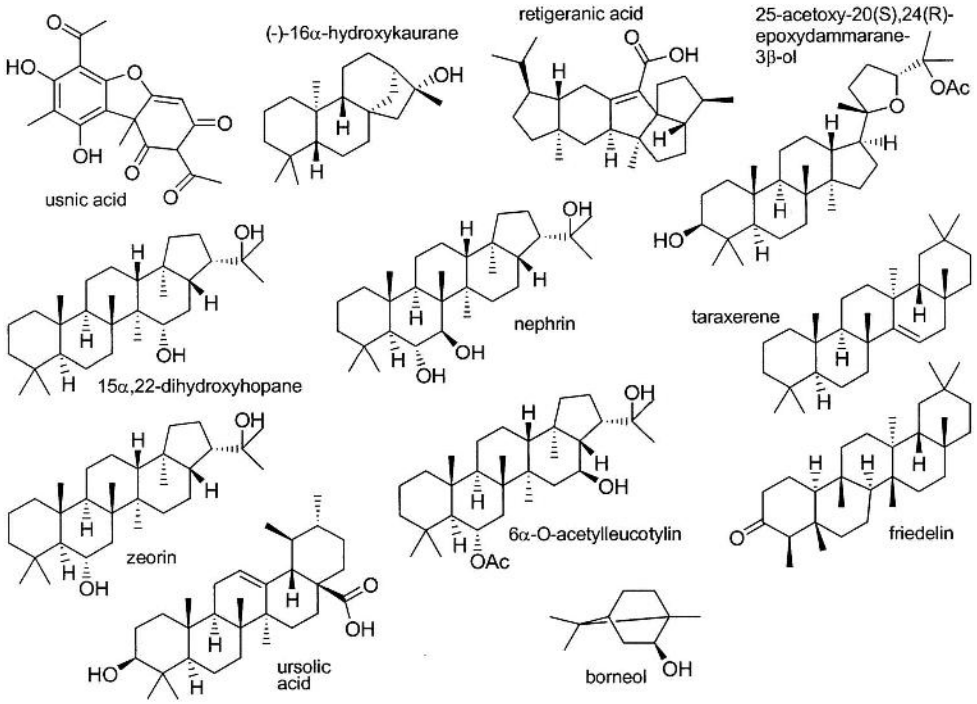


Figure 32 Selected lichen terpenoids.

Terpenoids which have been identified as unique to lichens include nephrin (Fig. 32) [253] found in the genus *Nephroma*, taraxerene (Fig. 32) from *Cladonia*, and zeorin (Fig. 32) found in several diverse lichen genera. One *Parmelia* species (*P. entotheiochroa*) contains several triterpenoids that appear to be unique to lichens [254] and includes several analogs of 6α-*O*-acetylleuoctylin (Fig. 32).

Terpenoids which are not unique to lichens include friedelin (Fig. 32), which is also found in nonlichen species, such as the tree species *Quercus suber*, *Salix japonica*, *Rhododendron westlandii*, and *Ceratopetalum trichotomum* [244], and ursolic acid (Fig. 32), a common compound in plants. Lichen monoterpenoids commonly found in many other organisms include borneol (Fig. 32), camphor, 1,8-cineole, limonene, linalool, and α- and β-pinene.

4.3 Terpenoid Defenses in Other Organisms

Bryophytes, nonvascular plants which include mosses, liverworts, and hornworts, have also been reported to produce terpenoids likely to be involved in defensive mechanisms. An example is the molluscicidal sesquiterpenoid ricciocarpin produced by the liverwort *Ricciocarpos natan* [255]. A recent comprehensive review covering the terpenoid contents of bryophytes as well as their antibacterial, antifungal, molluscicidal, insect antifeedant, plant growth regulatory, and piscicidal biological activities, among others, is available [256].

Fungi also produce terpenoid toxins, some of which are phytotoxic to the host plants, and some of which are toxic to other microbial forms. These latter toxins might be used

by the producing fungi as a competitive mechanism to prevent other fungi from colonizing the same food source. In this respect, Evidente et al. [257] recently found two more phytotoxic primarene diterpenoids from the phytopathogenic fungi *Sphaeropsis sapinea* F. sp. *cupressi* and *Diplodia mutila*. These sphaeropsidin phytotoxins led to dieback, browning, and necrosis of several species of cypress and oak as well as showing inhibitory effects on mycelial growth of seven fungi. Recent reviews [137,258] on phytochemical signals and on the biochemistry and cell biology of plant–microbe interactions are available.

A quick list of references cross-linking terpenoid-producing organisms with those they affect is presented in Table 2.

Table 2 Cross-reference of Organisms Producing, and Those Affected by, Bioactive Terpenoids

Terpenoid-producing organisms	Active against	Reference
Algae	Animals	
	Reef fish	154, 191, 196–198
	Urchin (*Diadema antillarum*)	154, 196–198
	Bacteria	218–220, 225–228
	Mycobacterium smegmatis	228
	Fungi	218–220, 228
Animals		
Insects	Bacteria	155
	Fungi	155, 259
	Insects	158, 184, 185, 189, 260
Mammal	Mammal (territorial defense)	261
Marine invertebrates	Bacteria	262
	Marine invertebrates (antifouling)	263
Bacteria	Bacteria	264
Fungi	Bacteria	265
	Plant	257
Plant	Animals	4, 5, 7, 8
	Cattle, sheep and goats	266
	Deer	60, 68–72
	Geese	63, 64
	Rodents	77
	Insects	5–8, 10, 121, 123
	Ant (*Atta cephalotes*)	157
	Beetles	99, 100, 124
	Cockroaches	125
	Flies	125
	Mites	80
	Mosquito larvae	129, 130
	Moth	104, 162
	Nematodes and worms	78, 79, 103, 125
	Termite	267–269
	Bacteria	139, 270–271
	Mycobacterium spp.	272
	Fungi	75, 105, 129, 133
	Plant	17, 18
	Protozoa (malaria)	273, 274

REFERENCES

1. AC Ramos-Valdivia, R van der Heijden, R Verpoorte. Nat Prod Rep 14:591–603, 1997.
2. SO Duke, C Canel, AM Rimando, MR Tellez, MV Duke, RN Paul. Adv Bot Res 31:121–151, 2000.
3. (a) CJW Brooks and DG Watson. Nat Prod Rep 8:367–389, 1991; (b) JD Connolly, RA Hill. Nat Prod Rep 16:221–240, 1999; (c) BM Fraga. Nat Prod Rep 16:21–38, 1999; (d) DH Grayson. Nat Prod Rep 15:439–475, 1998; (e) JR Hanson. Nat Prod Rep 16:209–219, 1999; (f) JR Hanson. Nat Prod Rep 13:529–535, 1996; (g) JR Hanson. Nat Prod Rep 12: 381–384, 1995; (h) JB Harborne. Nat Prod Rep 16:509–523, 1999; (i) J Leonard. Nat Prod Rep 16:319–338, 1999; (j) A Rahman, MI Choudhary. Nat Prod Rep 14:191–203, 1997; (k) JE Saxton. Nat Prod Rep 14:559–590, 1999.
4. JH Langenheim. J Chem Ecol 20:1223–1280, 1994.
5. A Prakash, J Rao. Botanical Pesticides in Agriculture. Boca Raton, FL: CRC Press, 1997, pp 1–462.
6. RA Hill. In: RH Thomson, ed. The Chemistry of Natural Products. Glasgow: Chapman Hall, 1993, pp 106–139.
7. R Teranishi, RG Buttery, H Sugisawa, eds. Bioactive Volatile Compounds from Plants. ACS Symposium Series 525. Washington, DC: American Chemical Society, 1993.
8. SO Duke, JJ Menn, JR Plimmer, eds. Pest Control with Enhanced Environmental Safety. ACS Symposium Series 524. Washington, DC: American Chemical Society, 1993.
9. WD Nes, EJ Parish, JM Trzaskos, eds. Regulation of Isopentenoid Metabolism. ACS Symposium Series 497. Washington, DC: American Chemical Society, 1992.
10. PA Hedin, ed. Naturally Occurring Pest Bioregulators. ACS Symposium Series 449. Washington, DC: American Chemical Society, 1993.
11. JD Connolly, RA Hill,eds. Dictionary of Terpenoids. New York: Chapman & Hall, 1991.
12. (a) S Dev, ed. CRC Handbook of Terpenoids: Monoterpenoids. Boca Raton, FL: CRC Press, 1982; (b) S Dev, ed. CRC Handbook of Terpenoids: Diterpenoids. Boca Raton, FL: CRC Press, 1985; (c) S Dev, ed. CRC Handbook of Terpenoids: Triterpenoids. Boca Raton, FL: CRC Press, 1989.
13. RP Adams. Identification of Essential Oil Components by Gas Chromatography/Mass Spectrometry. Carol Stream, IL: Allured Publishing, 1995.
14. MR Tellez, RE Estell, EL Fredrickson, KM Havstad. J Essent Oil Res 9:619–624, 1997.
15. PA Morrow, LR Fox. Oecologia 45:209–219, 1980.
16. PB Reichardt, JP Bryant, TP Clausen, GD Wieland. Oecologia 65:58–69, 1984.
17. SO Duke. In: RF Keeler, AT Tu, eds. Toxicology of Plant and Fungal Compounds. Handbook of Natural Toxins, Vol. 6. New York: Marcel Dekker, 1991, pp 269–296.
18. J Lydon, SO Duke. In: LE Craker, JE Simon, eds. Herbs, Spices, and Medicinal Plants. Recent Advances in Botany, Horticulture and Pharmacology, Vol. 4. Phoenix, AZ: Oryx Press, 1989, pp 1–41.
19. I Shomer, Y Erner. Bot Gaz 150:281–288, 1989.
20. BR Loveys, SP Robinson, JJ Brophy, EK Chacko. Aust J Plant Phyisol 19:449–457, 1992.
21. A Fahn. Secretory Tissues in Plants. New York: Academic Press, 1979, pp 1–302.
22. J Gershenzon. J Chem Ecol 20:1281–1328, 1994.
23. GJ Wagner. Plant Physiol 96:675–679, 1991.
24. AA Bell, RD Stipanovic, GW Elzen, HJ Williams. In: GR Waller, ed. Allelochemicals: Role in Agriculture and Forestry. ACS Symposium Series 330. Washington, DC: American Chemical Society, 1987, pp 477–470.
25. MV Duke, RN Paul, HK Elsohly, G Sturtz, SO Duke. Int J Plant Sci 155:365–373, 1994.
26. MR Tellez, C Canel, AM Rimando, SO Duke. Phytochemistry 52:1035–1040, 1999.
27. G Serrato-Valente, A Bisio, L Cornata, G Ciarallo. Ann Bot 79:329–336, 1997.
28. L Ascensão, N Marques, MS Pais. Int J Plant Sci 158:249–258, 1997.

29. TM Bourett, RJ Howard, DP O'Keefe, DL. Hallahan. Int J Plant Sci 155:623–632, 1994.
30. SO Duke, RN Paul. Int J Plant Sci 154:107–118, 1993.
31. E Werker, U Ravid, E Putievsky. Isr J Bot 34:31–45, 1985.
32. L Ascensão, MS Pais. Ann Bot 81:263–271, 1998.
33. GW Elzen, HJ Williams, AA Bell, RD Stipanovic, SB Vinson. J Agric Food Chem 33:1079–1082, 1985.
34. B Voirin, C Bayet, M Colson. Phytochemistry 34:85–87, 1993.
35. LJ Clark, JGC Hamilton, JV Chapman, MJC Rhodes, DL Hallahan. Plant J 11:1387–1393, 1997.
36. O Spring. In: NH Fischer, MB Isman, HA Stafford, eds. Modern Phytochemical Methods. Recent Advances in Phytochemistry, Vol. 25. Plenum Press: New York, 1991, pp 319–345.
37. K Schorr, FB Da Costa, R Zipper, O Spring. Boll Chim Farm 138:78, 1999.
38. E Rodriguez, MO Dillon, TJ Mabry, JC Mitchell, GHN Towers. Experientia 32:236–238, 1976.
39. EM Cappelletti, R Caniato, G Appendino. Biochem Sys Ecol 14:183–190, 1986.
40. CK Keene, GJ Wagner. Plant Physiol 79:1026–1032, 1985.
41. L Kandra, GJ Wagner. Arch Biochem Biophys 265:425–432, 1988.
42. Z Guo, GJ Wagner. Planta 197:627–632, 1995.
43. J Gershenzon, DG McCaskill, J Rajaonarivony, C Mihaliak, F Karp, R Croteau. In: NH Fischer, MB Isman, HA Stafford, eds. Modern Phytochemical Methods. Recent Advances in Phytochemistry, Vol. 25. New York: Plenum Press, 1991, pp. 347–370.
44. J Gershenzon, D McCaskill, JIM Rajaonarivony, C Mihaliak, F Karp, R Croteau. Anal Biochem 200:130–138, 1992.
45. D McCaskill, R. Croteau. Planta 197:49–56, 1995.
46. RJ Petroski, SP McCormick, eds. Secondary-Metabolite Biosynthesis and Metabolism. Environmental Science Research, Vol. 44. New York: Plenum Press, 1992.
47. CL Steele, S. Katoh, J Bohlmann, R. Croteau. Plant Physiol 116:1497–1504, 1998.
48. MI Lopes Cardoso, AH Meijer, S Rueb, J Queiroz Machado, J Memelink, JHC Hoge. Mol Gen Genet 256:674–681, 1997.
49. MT Neilson, CP Akers, UE Järlfors, GJ Wagner, GJS Berger. Bot Gaz 152:13–22, 1991.
50. JP Carde, C Bernard-Dagan, M Gleizes. In: P Mazliak, P Beneviste, C Costes, R Douce, eds. Biogenesis and Function of Plant Lipids. Amsterdam: Elsevier, 1980, pp 441–444.
51. J Vermeer, RL Peterson. Can J Bot 57:714–729, 1979.
52. J Gershenzon, M Maffei, R Croteau. Plant Physiol 89:1351–1357, 1989.
53. R Croteau, MA Johnson. In: E Rodriguez, PL Healey, I Metha, eds. Biology and Chemistry of Plant Trichomes. New York: Plenum Press, 1984, pp. 133–185.
54. PW Pare, JH Tumlinson. Phytochemistry 47:521–526, 1998.
55. JH Stoeckeler, RO Strothmann, LW Krefting. J Wildl Manag 21:75–80, 1957.
56. AF Hough. Ecology 46:370–373, 1965.
57. DR Klein. In: PA Jewell, S Holt, eds. Problems in Management of Locally Abundant Wild Mammals. New York: Academic Press, 1981, pp 119–127.
58. JL Harper. Oecologia 80:53–58, 1989.
59. JP Bryant, PB Reichardt, TP Clausen, FO Provenza, PJ Kuropat. In: GA Rosenthal, MR Berenbaum, eds. Herbivores. Their Interactions with Secondary Plant Metabolites. New York: Academic Press, 1992, pp 343–370.
60. RJL Goralka, JH Langenheim. Biochem Syst Ecol 24:13–23, 1996.
61. P Heinstein. J Nat Prod 48:907–915, 1985.
62. KF Wallin, KF Raffa. J Chem Ecol 25:861–880, 1999.
63. M Wink, A Hofer, M Bilfinger, E Englert, M Martin, D Schmeider. Chemoecology 4:93–107, 1993.
64. JB Harborne. Nat Prod Rep 14:83–98, 1997.
65. SC Halls, DR Gang, DS Weber. J Chem Ecol 20:2055–2063, 1994.

66. MR Tellez, RE Estell, EL Fredrickson, KM Havstad. J Essent Oil Res 10:201–204, 1998.
67. MR Tellez, RE Estell, EL Fredrickson, KM Havstad. J Agric Food Chem 45:3276–3278, 1997.
68. EJ Dimock, RR Silen, VE Allen. Forest Sci 35:106–121, 1976.
69. MA Radwan. Can J Forest Res 2:250–255, 1972.
70. S Elliot, A Loudon. J Chem Ecol 13:1343–1349, 1987.
71. TL Personious, CL Wambolt, JR Stophens, RG Kelsy. J Range Manag 40:84–88, 1987.
72. RO Bray, CL Wambolt, RG Kelsy. J Chem Ecol 17:2053–2062, 1991.
73. IT Baldwin, MJ Karb, P Callahan. J Chem Ecol 19:2075–2079, 1993.
74. M Matsui, I Yamamoto. In: M Jacobson, DG Crosby, eds. Naturally Occurring Insecticides. New York: Marcel Dekker, 1971, pp 4–70.
75. V Rodov, S Ben-Yehoshua, DQ Fang, JJ Kim, R Ashkenazi. J Agric Food Chem 43:1057–1061, 1995.
76. AE Osbourn, JP Wubben, RE Melton, JP Carter, MJ Daniels. In: JT Romeo, KR Downum, R Verpoort, eds. Phytochemical Signals and Plant–Microbe Interactions. Recent Advances in Phytochemistry, Vol. 32. New York: Plenum Press, 1998, pp 1–15.
77. ARE Sinclair, MK Jogia, RJ Anderson. J Chem Ecol 14:1505–1514, 1988.
78. RL Haynes, CM Jones. J Am Soc Hort Sci 101:422–424, 1976.
79. G Gheysen. In: JT Romeo, KR Downum, R Verpoort, eds. Phytochemical Signals and Plant–Microbe Interactions. Recent Advances in Phytochemistry, Vol. 32. New York: Plenum Press, 1998, pp 95–117.
80. JC Snyder, Z Guo, R Thacker, JP Goodman, JS Pyrek. J Chem Ecol 19:2981–2997, 1993.
81. E Gowan, BA Lewis, R Turgeon. J Chem Ecol 21:1781–1788, 1995.
82. MA Synder, YB Linhart. J Mammal 75:136–141, 1994.
83. MH Ralphs, GD Manners, DR Gardner. J Chem Ecol 24:167–182, 1998.
84. JP Bryant, FS Chapin, DR Klein. Oikos 40:357–368, 1983.
85. PL Lorio. Forest Ecol Manag 14:259–278, 1986.
86. JH Loughrin, DA Potter, TR Hamilton-Kemp, ME Byers. Phytochemistry 45:919–923, 1997.
87. TCJ Turlings, JH Loughrin, PJ McCall, USR Rose, WJ Lewis, JH Tumlinson. Proc Natl Acad Sci USA 92:4169–4174, 1995.
88. JH Loughrin, A Manukian, RR Heath, TCJ Turlings, JH Tumlinson. Proc Natl Acad Sci USA 91:11,836–11,840, 1994.
89. KD Klepzig, J Riba, EB Smalley, KF Raffa. J Chem Ecol 22:1367–1388, 1996.
90. S Katoh, R. Croteau. Phytochemistry 47:577–582, 1998.
91. M Johnson, R Croteau. In: G Fuller, WD Nes, eds. Ecology and Metabolism of Plant Lipids. ACS Symposium Series 325. Washington, DC: American Chemical Society, 1987, pp 76–91.
92. KF Raffa. In: DW Tallamy, MJ Raupp, eds. Phytochemical Induction by Herbivores. New York: Academic Press, 1991, pp 245–276.
93. TE Nebeker, JD Hodges, CA Blance. In: TD Schowalter, GM Filip, eds. Beetle–Pathogen Interactions in Conifer Forests. London: Academic Press, 1993, pp 158–173.
94. R Croteau, MA Johnson. In: T Higuchi, ed. Biosynthesis and Biodegradation of Wood Components. Orlando, FL: Academic Press, 1985, pp 379–439.
95. KF Raffa, AA Berryman, J Simasko, W Teal, BL Wong. Environ Entomol 14:552–556, 1985.
96. KF Raffa, EB Smalley. Oecologia 102:285–295, 1995.
97. MA Philips, RB Croteau. Trends Plant Sci 4:184–190, 1999.
98. CL Steele, E Lewinsohn, R Croteau. Proc Natl Acad Sci USA 92:4164–4168, 1995.
99. A Stoessl, JB Stothers, EB Ward. Phytochemistry 15:855–872, 1976.
100. DR Threlfall, IM Whitehead. In: JB Harborne, FA Tomas-Barberan, eds. Ecological Chemistry and Biochemistry of Plant Terpenoids. Oxford, UK: Clarendon Press, 1991, pp 159–208.
101. J Bohlman, J Crock, R Jetter, R Croteau. Proc Natl Acad Sci USA 95:6756–6761, 1998.

102. CL Steele, J Crock, J Bohlmann, R Croteau. J Biol Chem 273:2078–2089, 1998.
103. KF Wallin, KF Raffa. Can J Forest Res 28:228–233, 1998.
104. ME Litvak, RK Monson, Oecologia 114:531–540, 1998.
105. VM Dillon, J Overton, RJ Grayer, JB Harborne. Phytochemistry 44:599–603, 1997.
106. DW Cartwright, P Langcake, JP Ride. Physiol Plant Pathol 17:259–267, 1980.
107. P Langcake, SGA Wickins. Physiol Plant Pathol 7:113–126, 1975.
108. M Venkatramesh, RB Croteau. Pestic Biochem Physiol 34:32–38, 1989.
109. T Komives, JE Casida. J Agric Food Chem 31:751–755, 1983.
110. Scharwz, O.J. In: Nickell, L.G., ed. Plant Growth Regulating Chemicals, Vol. 2. Boca Raton, FL: CRC Press, pp 79–97, 1983.
111. TL Amburgey, FL Carter, DR Roberts. Wood Sci 10:187–192, 1978.
112. RH Beal, JD Bultman, JD Amburgey, DR Roberts. Forest Prod J 29:35–38, 1979.
113. PW Pare, JH Tumlinson. Plant Physiol 114:1161–1167, 1997.
114. PW Pare, JH Tumlinson. Nature 385:30–31, 1997.
115. EW Weiler, D Laudert, F Schaller, B Stelmach. In: JT Romeo, KR Downum, R Verpoort, eds. Phytochemical Signals and Plant–Microbe Interactions. Recent Advances in Phytochemistry, Vol. 32. New York: Plenum Press, 1998, pp 179–205.
116. H Nojiri, M Sugimori, H Yamane, Y Nishimura, A Yamada, N Shibuya, O Kodama, M Murofushu, T Omori. Plant Physiol 110:387–392, 1996.
117. EE Farmer, H Weber, S Vollenweider. Planta 206:167–174, 1998.
118. M Kuroyonagi, T Arakawa, Y Mikami, K Yoshida, N Kawahar, T Hayashi, H Ishimaru. J Nat Prod 61:1516–1519, 1998.
119. F Daayf, M Nicole, B Boher, A Pando, JP Geiger. Eur J Plant Pathol 103:125–136, 1997.
120. NA Harrison, CH Beckman. Physiol Plant Pathol 21:193–207, 1982.
121. JT Arnason, BJR Philogene, P Morand, eds. Insecticides of Plant Origin. ACS Symposium Series 387. Washington, DC: American Chemical Society, 1991.
122. E Hodgson, RJ Kuhr. Safer Insecticides; Development and Use. Drug and Chemical Toxicology, Vol. 7. New York: Marcel Dekker, 1990.
123. MB Green, PA Hedin, eds. Natural Resistance of Plants to Pests. ACS Symposium Series 296. Washington, DC: American Chemical Society, 1986, pp 118–176.
124. RA Werner. Environ Entomol 24:372–379, 1995.
125. JR Coats, LL Karr, CD Drewes. In: PA Hedin, ed. Naturally Occurring Pest Bioregulators. ACS Symposium Series 449. Washington, DC: American Chemical Society, 1991, pp 305–316.
126. OD Hensens, JG Ondeyka, AW Dombrowski, DA Ostlind, DL Zink. Tetrahedron Lett 40: 5455–5458, 1999.
127. JG Ondeyka, GL Helms, OD Hensens, MA Goetz, DL Zink, A Tsipouras, WL Shoop, L Slayton, AW Dombrowski, JD Polishook, DA Ostlind, NN Tsou, RG Ball, SB Singh. J Am Chem Soc 119:8809–8816, 1997.
128. Y Ozoe, M Akamatsu, T Higata, I Ikeda, K Mochida, K Koike, T Ohmoto, T Nikaido. Biorg Med Chem 6:481–492, 1998.
129. JR Ioset, A Marston, MP Gupta, K Hostettmann. Phytochemistry 47:729–734, 1998.
130. R Tsao, JR Coats. Chemtech 23–28, 1995.
131. D Laurent, LA Vilaseca, JM Chantraine, C Ballivian, G Saavedra, R Ibañez. Phytother Res 11:285–290, 1997.
132. MR Tellez, FE Dayan, KK Schrader, DE Wedge, SO Duke. 2nd Pan-Pacific Conference on Pesticide Science, 1999 (abstr P-53).
133. MV Bhaskara Reddy, P Angers, A Gosselin, J Arul. Phytochemistry 47:1515–1520, 1998.
134. A Prakash, J Rao. Botanical Pesticides in Agriculture. Boca Raton, FL: CRC Press, 1996, pp 355–367.
135. GD Lyon, MA Mayo. Phytopathol Z 92:294–304, 1978.
136. GD Lyon. J Exp Bot 31:957–966, 1980.

137. J Friend. Adv Plant Pathol 7:84–129, 1991.
138. JE Harris, C Dennis. Physiol Plant Pathol 11:163–169, 1977.
139. BP Ying, G Peiser, YY Ji, K Mathias, D Tutko, YS Hwang. Phytochemistry 38:909–915, 1995.
140. BP Ying, GD Peiser, YY Ji, KM Mathias, F Karasina, YS Hwang. J Agric Food Chem 43: 826–829, 1995.
141. J Lydon, JR Teasdale, PK Chen. Weed Sci 45:807–811, 1997.
142. SO Duke, KC Vaughn, EM Croom, HN Elsohly. Weed Sci 35:499–505, 1987.
143. FE Dayan, A Hernandez, SN Allen, RM Moraes, JA Vroman, MA Avery, SO Duke. Phytochemistry 50:607–614, 1999.
144. L-J Lin, G Peiser, B-P Ying, K Mathias, F Karasina, Z Wang, J Itatani, L Green, Y-S Hwang. J Agric Food Chem 43:1708–1711, 1995.
145. FE Dayan, SB Watson, JCG Galindo, A Hernandez, J Dou, JD McChesney, SO Duke. Pestic Biochem Physiol 65:15–24, 1999.
146. DJ Morre, PA Grieco, DM Morre. Life Sci 63:595–604, 1998.
147. CH Muller, WH Muller, BL Haines. Bot Gaz Sci 143:471–473, 1964.
148. JP Halligan. Ecology 56:999–1003, 1975.
149. JG Romagni, SO Duke, FE Dayan. J Chem Ecol 26:303–313, 2000.
150. JN Norris, W Fenical. In: K Rutzler, IG Macintyre, eds. Atlantic Barrier Reef Ecosystem, Carrie Bow Cay, Belize. 1. Structure and Communities. Smithsonian Contributions to the Marine Sciences, Vol. 12. Washington, DC: Smithsonian Institution Press, 1982, pp 417–431.
151. SJ White, RS Jacobs. Mol Pharmacol 24:500–508, 1983.
152. ET O'Brien, S White, RS Jacobs, GB Boder, L Wilson. Hydrobiology 116/117:141–147, 1984.
153. RS Jacobs, P Culver, R Langdon, T O'Brien, S White. Tetrahedron 41:981–984, 1985.
154. ME Hay, W Fenical, K Guftanson. Ecology 68:1581–1591, 1987.
155. J Gross, C Muller, A Vilcinskas, M Hilker. J Invert Pathol 72:296–303, 1998.
156. G Guillet, A Belanger, JT Arnason. Phytochemistry 49:423–429, 1998.
157. A Prakash, J Rao. Botanical Pesticides in Agriculture. Boca Raton, FL: CRC Press, 1996, p. 178.
158. M Rothschild, G Bergstrom. Phytochemistry 45:1139–1144, 1997.
159. IR Lawler, J Stapley, WJ Foley, BM Eschler. J Chem Ecol 25:401–415, 1999.
160. WM Daniewski, M Gumulka, W Anczewski, M Masnyk, E Bloszyk, KK Gupta. Phytochemistry 49:1279–1282, 1998.
161. BM Fraga, A Gonzalez-Coloma, C Gutierrez, D. Terrero. J Nat Prod 60:880–883, 1997.
162. PD Bremner, MSJ Simmonds, WM Blaney, NC Veitch. Phytochemistry 47:1227–1232, 1998.
163. MSJ Simmonds. Phytochemistry 49:1183–1190, 1998.
164. TCJ Turlings, PJ McCall, HT Alborn, JF Tumlinson. J Chem Ecol 19:411–425, 1993.
165. HT Alborn, TCJ Turlings, TH Jones, G Stenhagen, JH Loughrin, JH Tumlinson. Science 276:945–949, 1997.
166. PW Pare, HT Alborn, JH Tumlinson. Proc Natl Acad Sci USA 95:13,971–13,975, 1998.
167. M Dicke. J Plant Physiol 143:465–472, 1994.
168. L Mattiacci, M Dicke, MA Posthumus. J Chem Ecol 20:2229–2247, 1994.
169. J Takabayashi, M Dicke, MA Posthumus. J Chem Ecol 20:1329–1354, 1994.
170. MR Berenbaum. In: P Barbosa, DK Letourneau, eds. Novel Aspects of Insect–Plant Interactions. New York: Wiley, 1988, pp 97–123.
171. JP Bryant, FS Chapin. In: GA Cooper-Driver, T Swain, EE Conn, eds. Chemically Mediated Interactions Between Plants and Other Organisms. Recent Advances in Phytochemistry, Vol. 1. New York: Plenum Press, 1988, pp 219–237.
172. SP Hubell, DF Wiemer, A Adejore. Oecologia 60:321–327, 1983.

173. JJ Howard, TP Green, DF Wiemer. J Chem Ecol 15:2275–2288, 1989.
174. JJ Howard, J Coxin, DF Wiemer, J Chem Ecol 13:59–69, 1988.
175. JP Vite, A Bakke, JAA Renwick. Can Entomol 104:1967–1975, 1972.
176. PR Hughes. Z Angew Entomol 73:294–312, 1973.
177. SJ Seybold, T Ohtsuka, DL Wood, I Kubo. J Chem Ecol 21:995–1016, 1995.
178. RL Metcalf, RL Lampman, PA Lewis. J Econ Entomol 91:881–890, 1998.
179. T Masamune, M Anetai, M Takasugi, MN Katsui. Nature 297:495–496, 1982.
180. A Fukusawa, A Furuski, M Ikura, R Masamune. J Chem Soc 222–224, 1985.
181. A Fukusawa, H Matsue, M Ikura, R Masamune. Tetrahedron Lett 26:5539–5542, 1985.
182. SG Gheysen. In: JT Romeo, KR Downum, R Verpoort, eds. Phytochemical Signals and Plant–Microbe Interactions. Recent Advances in Phytochemistry, Vol. 32. New York: Plenum Press, 1998, pp 95–117.
183. KS Boo, IB Chung, KS Han, JA Pickett, LJ Wadhams. J Chem Ecol 24:631–643, 1998.
184. P Barbosa, DK Letourneau, eds. Novel Aspects of Insect–Plant Interactions. New York: Wiley, 1988.
185. NE Gomez, L White, T Hartmann. J Chem Ecol 25:1007–1027, 1999.
186. X Belles, F Camps, J Coll, MD Piulachs. J Chem Ecol 11:1439–1445, 1985.
187. T Amano, R Nishida, Y Kuwahara. Biosci Biotechnol Biochem 61:1518–1522, 1997.
188. R Nishida, H Fukami. J Chem Ecol 16:151–164, 1990.
189. T Eisner, JS Johnessee, J Carrel. Science 184:996–999, 1974.
190. DN Young, BM Howard, W Fenical. J Phycology 16:182–185, 1980.
191. ML Bittner, M Silva, VJ Paul, W Fenical. Phytochemistry 24:987–989, 1985.
192. T Irie, M Suzuki, E Kurosawa, T Masamune. Tetrahedron 26:3271–3277, 1970.
193. P Crews, BL Myers, S Naylor, EL Clason, RS Jacobs, GB Staal. Phytochemistry 23:1449–1451, 1984.
194. P Crews, E Kho. J Org Chem 40:2568–2570, 1975.
195. L Castedo, ML Garcia, E Quiñoá, R Riguera. J Nat Prod 47:724–726, 1984.
196. WH Gerwick, W Fenical. J Org Chem 46:22–27, 1981.
197. FJ McEnroe, KJ Robertson, W Fenical. In: DJ Faulkner, WH Fenical, eds. Marine Natural Products Chemistry. New York: Plenum Press, 1977, pp 179–189.
198. DR Hirschfeld, W Fenical, GHY Lin, RM Wing, P Radlick, JJ Sims. J Am Chem Soc 95: 4049–4050, 1973.
199. HH Sun, FJ McEnroe, W Fenical. J Org Chem 48:1903–1906, 1983.
200. RW Binkley, DJ Koholic. J Org Chem 44:2044–2048, 1979.
201. AB Alvarado, WH Gerwick. J Nat Prod 48:132–134, 1985.
202. E Fattorusso, S Magno, L Mayol, C Santacroce, D Sica, V Amico, G Oriente, M Piattelli, C Tringali. J Chem Soc Chem Commun 575–576, 1976.
203. ME Hay, JE Duffy, CA Pfister, W Fenical. Ecology 68:1567–1580, 1987.
204. P Crews, TE Klein, ER Hogue, BL Myers. J Org Chem 47:811–815, 1982.
205. HH Sun, OJ McConnell, W Fenical, K Hirotsu, J Clardy. Tetrahedron 37:1237–1242, 1981.
206. S De Rosa, S De Stefano, S Macura, E Trivellone, N Zavodnik. Tetrahedron 40:4991–4995, 1984.
207. D Schlenk, WH Gerwick. Phytochemistry 26:1081–1084, 1987.
208. M Ochi, M Watanabe, I Miura, M Taniguchi, T Tokoroyama. Chem Lett 1229–1232, 1980.
209. J Tanaka, T Higa. Chem Lett 231–232, 1984.
210. AJ Blackman, RJ Wells. Tetrahedron Lett 2729–2730, 1976.
211. V Amico, G Oriente, M Piattelli, C Tringali, E Fattorusso, S Magno, L Mayol. Tetrahedron Lett 3593–3596, 1978.
212. AJ Blackman, RJ Wells. Tetrahedron Lett 3063–3064, 1978.
213. VJ Paul, W Fenical. Tetrahedron 40:2913–2918, 1984.
214. VJ Paul, W Fenical. Phytochemistry 24:2239–2243, 1985.
215. VJ Paul, W Fenical. Science 221:747–749, 1983.

216. VJ Paul, W Fenical. Tetrahedron 40:3053–3062, 1984.
217. HH Sun, W Fenical. Tetrahedron Lett 685–688, 1979.
218. VJ Paul, W Fenical. Mar Ecol Prog Ser 34:157–169, 1986.
219. VJ Paul, W Fenical. In: PS Scheuer, ed. Bioorganic Marine Chemistry. Berlin: Springer-Verlag, 1987, pp 1–29.
220. VJ Paul, MM Littler, DS Littler, W Fenical. J Chem Ecol 13:1171–1185, 1987.
221. VJ Paul, JM Cronan Jr., JH Cardellina II. J Chem Ecol 19:1847–1860, 1993.
222. VJ Paul, KL Van Alstyne. Proc. Sixth Int Coral Reef Symp 3:133–138, 1988.
223. VJ Paul. In: VJ Paul, ed. Ecological Roles of Marine Natural Products. Ithaca, NY: Cornell University Press, 1992, pp 24–50.
224. VJ Paul, KL Van Alstyne. J Exp Marine Biol Ecol 160:191–203, 1992.
225. JJ Sims, MS Donnell, JV Leary, GH Lacy. Antimicrob Agents Chemother 7:320–321, 1975.
226. T Nakatsu, BN Ravi, DJ Faulkner. J Org Chem 46:2435–2438, 1981.
227. AG González, V Darias, E Estévez. Planta Med 44:44–46, 1982.
228. N Enoki, K Tsuzuki, S Õmura, R Ishida, T Matsumoto. Chem Lett 1627–1630, 1983.
229. E Quiñoa, L Castedo, R Riguera. Comp Biochem Physiol 92B:99–101, 1989.
230. L Minale, R Riccio. Tetrahedron Lett 2711–2714, 1976.
231. A Kelecom, VL Teixeira. Sci Total Environ 58:109–115, 1986.
232. C Ireland, DJ Faulkner, J Finer, J Clardy. J Am Chem Soc 98:4664–4665, 1976.
233. G Cimino, S De Rosa, S De Stefano, G Sodano. Tetrahedron Lett 22:1271–1272, 1981.
234. G Cimino, S De Rosa, S De Stefano, G Sodano, G Villani. Science 219:1237–1238, 1983.
235. C Avila, VJ Paul. Marine Ecol Prog Ser 150:171–180, 1997.
236. C Avila. Oceanogr Marine Biol Annu Rev 33:487–559, 1995.
237. G Cimino, G Sodano. In: TMG van Kempen, JC Brakman, eds. Sponges in Time and Space. Biology, Chemistry, Paleontology. Rotterdam: AA Balkema, 1994, pp 469–472.
238. C Ireland, MO Stallard, DJ Faulkner, J Finer, J Clardy. J Org Chem 41:2461–2465, 1976.
239. SC Pennings, VJ Paul. Ecology 73:1606–1619, 1992.
240. JA Elix. In: TH Nash, ed. Lichen Biology. Cambridge: Cambridge University Press, 1996, pp 14–180.
241. JD Lawrey. Bryologist 89:111–122, 1986.
242. D Fahselt. In: TH Nash, ed. Lichen Biology. Cambridge: Cambridge University Press, 1996, pp 181–198.
243. CR Gardner, DMJ Mueller. Am J Bot 68:87–95, 1981.
244. CF Culberson. Chemical and Botanical Guide to Lichen Products. Chapel Hill, NC: University of North Carolina Press, 1969.
245. JD Lawrey. Lichenologist 9:137–142, 1977.
246. WR Goldner, FM Hoffman, RJ Medve. Can J Bot 64:1586–1590, 1986.
247. JD Lawrey. Lichenologist 23:205–214, 1991.
248. NK Honda, W Vilegas. Quimica Nova 21:110–125, 1998.
249. S Huneck, I Yoshimura. Identification of Lichen Substances. New York: Springer-Verlag, 1996, pp 494.
250. S Huneck, G Follmann. Z Naturforsch 20:611–612, 1965.
251. RE Corbett, H Young. Delaware J Chem Soc 1564–1567, 1966.
252. S Huneck, G Follmann. Z Naturforsch 22:666–670, 1967.
253. C Wetmore. Michigan State Univ Biol Ser 1:369–452, 1960.
254. I Yosioka, T Nakanishi, I Kitagawa. Chem Pharmacol Bull 14:804–807, 1966.
255. M Reitz. Pharmazeut Ind 57:II/27–II/28, 1995.
256. Y Asakawa. In: W Herz, GW Kirby, RE Moore, W Steglich, CH Tamm, eds. Progress in the Chemistry of Organic Natural Products. New York: Springer-Verlag, 1995.
257. A Evidente, L Sparapano, O Fierro, G Bruno, F Giordano, A Motta. Phytochemistry 45: 705–713, 1997.

258. JT Romeo, KR Downum, R Verpoort, eds. Phytochemical Signals and Plant–Microbe Interactions. Recent Advances in Phytochemistry, Vol. 32. New York: Plenum Press, 1998.
259. T Akino, T Turushima, Yamaoka R. Nippon Nogeikagaku Kaishi 69:1581–1586, 1995.
260. F Osborn, K Jaffe. J Chem Ecol 24:1173–1186, 1998.
261. JW Gassett, DP Wiesler, AG Baker, DA Osborn, KV Miller, RL Marchinton, M Novotny. J Chem Ecol 23:569–578, 1997.
262. S Abarzua, S Jakubowski. Marine Ecol Prog Ser 123:301–312, 1995.
263. H Hirota, Y Tomono, N Fusetani. Tetrahedron 52:2359–2368, 1996.
264. H Komaki, A Nemoto, Y Tanaka, H Takagi, K Yazawa, Y Mikami, H Shigemori, J Kobayashi, A Ando, Y Nagata. J Antibiot 52:13–19, 1999.
265. H Shibata, A Irie, Y Morita. Biosci Biotechnol Biochem 62:2450–2452, 1998.
266. RE Estell, EL Frederickson, DM Anderson, KM Havstad, MD Remmenga. J Chem Ecol 24: 1–12, 1998.
267. L Lajide, P Escoubas, J Mizutzni. Phytochemistry 40:1105–1112, 1995.
268. L Lajide, P Escoubas, J Mizutani. Phytochemistry 40:1101–1104, 1995.
269. ML Cornelius, JK Grace, JR Yates. J Econ Entomol 90:320–325, 1997.
270. K Osawa, H Yasuda, T Maruyama, H Morita, K Takeya, H Itokawa, K Okuda. Chem Pharm Bull 42:922–925, 1994.
271. T Kato, B Frei, M Heinrich, O Sticher. Planta Med 62:66–67, 1996.
272. NH Fischer, T Lu, CL Cantrell, AJ Castaneda, L Quijano, SG Franzblaus. Phytochemistry 49:559–564, 1998.
273. S Bharel, A Gulati, MZ Abdin, PS Srivastava, SK Jain. Fitoterapia 67:387–402, 1996.
274. Y Takaya, KI Kurumada, Y Takeuji, HS Kim, Y Shibata, N Ikemoto, Y Wataya, Y Oshima. Tetrahedron Lett 39:1361–1364, 1998.

17

Lipases: Structure, Function, and Properties

ALEJANDRO G. MARANGONI

University of Guelph, Guelph, Ontario, Canada

1 AN OVERVIEW OF LIPASE STRUCTURE

1.1 Introduction

Lipases are a class of water-soluble enzymes that act on water-insoluble substrates and are stable in both polar and nonpolar environments [1,2]. The first two published crystallographic structures of lipases were those of *Rhizomucor miehei* lipase [3] and human pancreatic lipase [4]. The elucidation of the structure of lipases, in the early part of this decade, has allowed us to better understand their function. Lipases can act both in bulk solution and at interfaces; however, their activity is greatly enhanced at interfaces. This phenomenon is referred to as ''interfacial activation.'' Crystallographic studies of lipases have shown that interfacial activation results partly from a conformational change in the enzyme upon interfacial binding [5,6]. This conformational change involves a loop (lid) movement: A helical fragment of about 20 amino acids, called the ''lid'' or ''flap,'' seals or closes the active site, preventing substrate molecules from entering. Upon interfacial binding, however, this lid is displaced by a conformational change and the active site becomes exposed to solvent and substrate [5,6]. The structure and activity of a cutinase from *Fusarium solani*, which lacks a lid, lend support for this mechanism of lipase interfacial activation [7,8]. The crystal structure of this cutinase [7] shows that the protein folding pattern is very similar to that of *R. miehei* lipase [9]. Cutinases are α/β proteins and their active site is composed of the catalytic triad Asp-His-Ser [7]. However, this enzyme lacks a ''lid'' covering the active site; hence, the active site is exposed to solvent and has a performed oxyanion hole [8]. Moreover, this cutinase is not activated at oil–water interfaces.

1.2 Active-Site Triad

The observed inhibition of lipases by serine proteinase inhibitors indicated that a Ser residue might be directly involved in catalysis [3]. Moreover, based on the inhibition of enzyme activity by chemical modification, lipases have been classified as Ser hydrolases [2]. These enzymes have a common GX_1SX_2G consensus sequence which contains the essential Ser residue, where X represents any amino acid [2]. In addition to the catalytic serine, histidine and an acidic amino acid (Asp/Glu) are also required for catalysis [3,4,10]. The discovery of the catalytic triad in lipases revived interest in this configuration of catalytic amino acids and has provided new information on the function of the triad. More recently, however, Noble et al. [11] reported that the acidic residue (Asp^{203}) of the catalytic triad in *Pseudomonas glumae* lipase was not essential for lipase activity.

1.3 Structural Features: The α/β Hydrolase Fold and the Lid

All of the lipases whose structures have been elucidated are α/β-type proteins, with a trypsinlike catalytic triad (Asp/Glu-His-Ser) [2]. In addition, lipases have been included in the α/β hydrolase group of enzymes [12]. The structure and function of this class of enzymes became clearer as a result of the crystal structure determination of human pancreatic lipase (hPL) [4], *R. miehei* lipase (RML) [3,9], *Geotricum candidum* lipase (GCL) [10,13], *F. solani* cutinase (FSC) [7,8], *Candida rugosa* lipase [14,15], and *Pseudomonas glumae* lipase (PGL) [11]. These studies have shown that lipases contain a central mixed β-pleated sheet center surrounded by a number of loops and α-helices.

The active-site catalytic triad of lipases is covered by amphipathic loops. As mentioned earlier, this loop structure is known as the flap. X-ray diffraction studies have shown that this flap, which prevents solvent and substrate access to the active site in the native structure, can rearrange upon association with a substrate or substrate interface [5,6,14,15]. The movement of this flap exposes the active-site serine and a number of hydrophobic residues to solvent and substrates. In addition, this conformational change increases the hydrophobicity of the enzyme surface in the vicinity of the active site, enhancing lipase interfacial binding in this region [2]. An elegant study by Dugi et al. [16] has shown that the structure of the lid is a key factor influencing the ability of human lipoprotein lipase to interact with lipid substrates.

A more detailed discussion of the relationship between the structure of lipases and their function will be presented next using *C. rugosa* lipase (CRL) as an example. We chose CRL for this purpose because we have used this enzyme extensively in our research work.

2 THE STRUCTURE OF *CANDIDA RUGOSA* LIPASE

2.1 General Aspects

Five isoforms of CRL have been characterized. The isolated genomic sequences encode 5 mature proteins consisting of 534 amino acids with a molecular mass of about 60 kDa [17–19]. The protein sequences predicted from the nucleotide sequences have approximately 80% homology to each other but differ in their isoelectric point and extent of glycosylation [19]. It has also been determined that the sequence Phe/Tyr-Gly-Glu-Ser-Ala-Gly surrounding the active-site Ser-209 [2] is similar to that of the acetylcholinesterase

(AchE), indicating a close relation of CRL enzyme with the AChE family (25% homology). Moreover, CRL has a high degree of homology (about 40%) with *G. candidum*. In addition, CRL shares a substantial homology with several esterases [19].

2.2 Genetic Aspects of *Candida rugosa* Lipase

Two genes coding for *C. rugosa* lipases (*Lip* 1 and *Lip* 2) have been isolated and their nucleotide sequences determined [17]. Interestingly, in CRL, the nucleotide sequence CTG was shown to code for serine rather than leucine [20]. Based on the *Lip* 1 clone sequence, other three lipase-encoding genomic sequences (*Lip* 3, *Lip* 4, and *Lip* 5) have been identified by the same group of researchers [18]. *Lip* 3, *Lip* 4, and *Lip* 5 encode for proteins of 534 amino acids, highly homologous in sequence to each other and to *Lip* 1 and *Lip* 2. These lipases all had a similar molecular mass of about 58 kDa. The five amino acid sequences share an overall homology of 84%. The predicted isoelectric points for these enzymes were 4.5 for Lip 1, 4.9 for Lip 2, 5.1 for Lip 3, 5.7 for Lip 4, and 5.5 for Lip 5 [18]. Lotti et al. [19] also showed that these lipases are not strongly related to other lipases. However, significant homology was observed to two lipase isoforms of *G. candidum* (40–44% identity over 544 amino acids aligned) and the enzymes from the AChE family [19]. A comparison of the primary structure CRL with that of *G. candidum* lipase and *Torpedo califorinica* AChE demonstrated a 30% homology in the central regions and 70% homology around the active-site Ser. From an evolutionary point of view, this observation suggests that GCLs, CRLs, and AChE may have a common ancestral gene [21].

2.3 Secondary Structure of *Candida rugosa* Lipase

Candida rugosa lipase, a single-domain protein, is an ellipsoidal molecule with approximate dimensions of $66 \times 55 \times 45$ Å^3 [14]. In addition, the secondary structure indicates that CRL has 14 sheets (3 small and 11 large β-sheets), 16 α-helices, and 3 loop structures (Fig. 1). The three small β-pleated sheets are located at the amino terminus and are perpendicular to the large central parallel and antiparallel β-sheet segments [14].

The catalytic triad is formed by Ser-209, His-449, and Glu-34. Ser-209 is embedded in the characteristic supersecondary structural motif, strand-turn-helix (Fig. 1), found in all other lipases, cutinases [7,8], and acetylcholinesterases [22,23].

2.4 The Flap of *Candida rugosa* Lipase

The flap of *Candida rugosa* lipase was first proposed to include residues 62–92 [14] and was located between two hinge points at Glu-66 and Pro-92 by the same workers [15]. In addition, the loop consists of a deformed helical turn (residues 69–72) and an α-helical segment (residues 75–84) [15]. Moreover, it is no surprise that this long loop, located on the protein surface, is an amphipathic structure. The side exposed to the solvent includes most of hydrophilic residues and forms a hydrophilic surface. In contrast, the inside face consists of hydrophobic residues and interacts with hydrophobic amino acid residues surrounding the active site [14]. Upon conformational change from a closed to an open form, the flap moves almost 90° to open a substrate binding tunnel (Fig. 2). In addition, there are three proline residues within the flap. Proline 92, which rotates from a cis to a trans conformation, plays a key role in flap movement during the interfacial activation [15].

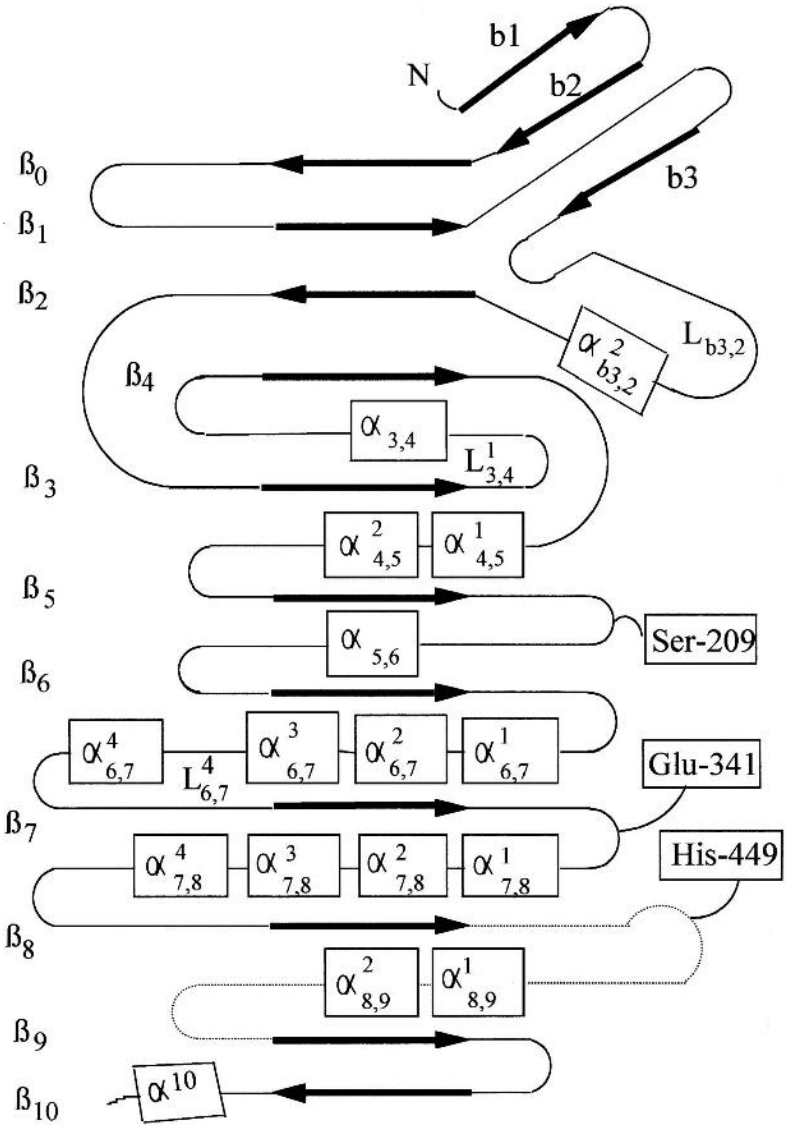


Figure 1 Secondary structure of *Candida rugosa* lipase.

2.5 Two Conformational States: Open and Closed Forms

Two conformational states of *C. rugosa* lipase, corresponding to the open and closed forms, have been identified by x-ray crystallography [14,15]. The open structure of *C. rugosa* lipase (CRLo), determined at 2.06 Å resolution, reveals a conformation with a solvent-accessible active site [14]. Moreover, the open structure represents the active conformation adopted by the enzyme near or at the lipid–water interface. In the open conformation, the central part of the flap helix is extended toward the solvent [14]. The ''closed'' structure of *C. rugosa* lipase (CRLc) was determined and refined to 2.1 Å resolution [15]. This closed structure represents an inactive conformation where the active site is covered

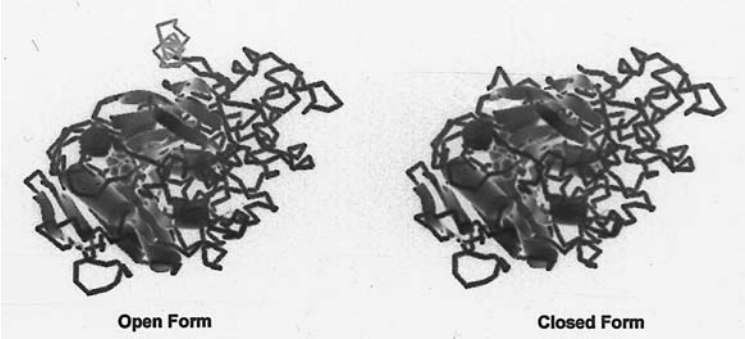


Figure 2 Crystal structure of *Candida rugosa* lipase in its open and closed forms.

by the flap (Fig. 3). Unlike CRLo, the active-site Ser-209, which together with His-449 and Glu-341 forms the catalytic triad, is completely occluded from the solvent by the flap [15].

2.6 Oxyanion Hole

In 1970, Henderson [23] pointed out that the presence of the so-called oxyanion hole for chymotrypsin is another essential feature of the active site of serine proteases. Lipases catalyze the hydrolysis of triglycerides and are activated by interfaces formed by their natural substrates. In addition, the amino acids that make up the active site are believed to create a ''hole'' either on the surface or as a buried structure, into which the substrate must fit.

In the CRL structure, the oxyanion hole is formed by the backbone amino (NH) groups of Ala-210 and Gly-124, a part of the $L_{3,4}$loop (within the third and fourth β-sheet [14]). Moreover, the active-site Ser-209 lies at the bottom of the oxyanion hole with its

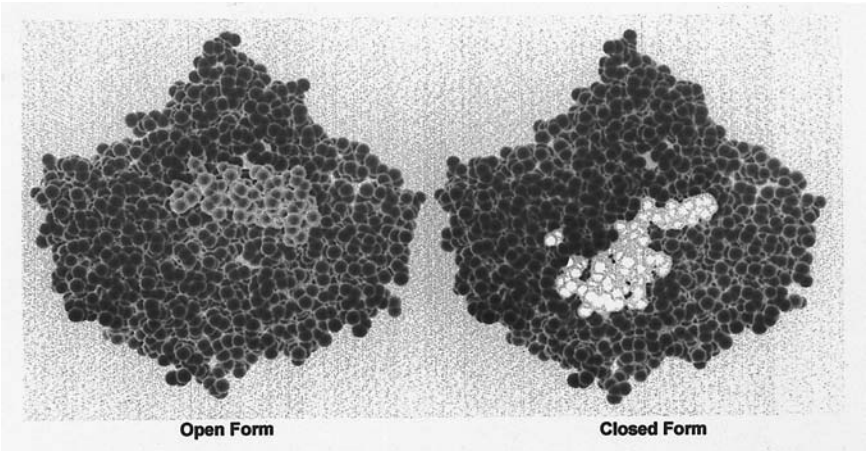


Figure 3 Crystal structure of *Candida rugosa* lipase in its open and closed forms. The oxyanion hole and active site are exposed in the open form, whereas in the closed form, the oxyanion hole and active sites are covered by the flap.

O_r atom (from the OH group of serine) exposed to the solvent. More hydrophobic residues are exposed to solvent in the vicinity of the active-site Ser-209 residue than in any other solvent-exposed region of the molecule [14].

Based on the CRLo and CRLc structures [14,15], the conformational change, the flap movement, does not include the formation of the oxyanion hole, but rather the oxyanion hole is already performed in the closed form of CRL [15]. This observation is in agreement with research on AChE [24], RML [5], and hPL [4]. More recently, Norin et al. [25] showed the existence of a long tunnel extending from the active-site Ser-209 toward the center of the oxyanion hole. This structure has been observed in all lipases studied to date and is involved in the binding of fatty acid chains [25].

3 CONFORMATIONAL CHANGES AND INTERFACIAL ACTIVATION OF *CANDIDA RUGOSA* LIPASE

Comparison of the CRLo and CRLc structures indicated that interfacial activation of the lipase required the movement and refolding of the flap, including a cis to trans isomerization of a proline residue, and movement of a single surface loop to expose a large hydrophobic surface, which would likely interact with the lipid interface [15]. The movement of the flap in CRL from a closed to an open form increased the molecular surface by about 1530 $Å^2$, which is about 10% of the total surface area of CRLc [15]. Furthermore, the proportion of hydrophobic residues at the surface increased dramatically in the CRLo conformation relative to the CRLc conformation. The total hydrophobic surface area in CRLc is about 890 $Å^2$, about 6% of the total surface area of CRLc [15]. This hydrophobic surface area increased to 1530 $Å^2$ in CRLo. Increases in hydrophobic surface area were responsible for 85% of the total increase in surface area [15].

In addition, a comparison of the two CRL structures shows that the transition between open and closed forms requires only the movement of the flap. However, the flap movement cannot be described as a single polypeptide rotation between two hinge points, because a significant change in CRL secondary structure also occurs during opening [15].

4 CONFORMATIONAL CHANGES IN OTHER LIPASES

The crystal structures of lipases confirmed that conformational changes are associated with interfacial activity [2]. Brzozowski et al. [5] demonstrated that a helical flap structure is displayed during the conformational change associated with lipid binding and catalytic activity in *R. miehei* lipase. The hydrophobic side of the flap becomes exposed, thus expanding the amount of nonpolar surface around the active site. This conformation change allows access of solvent and substrate to the active-site serine.

The activity-site serine is located at the base of a helix (the ''elbow'') whose dipole may help stabilize the transition state during catalysis. Brzozowski et al. [5] studied RML interfacial activation using the inhibitor diethyl *p*-nitrophenyl phosphate. Using x-ray diffraction spectroscopy, they reported that, in comparison with the native structure, the enzyme-inhibitor complex structure showed that the Ser-82 side chain assumes a favorable conformation for oxyanion interaction only after the lid had moved away from the active site. In RML, the total newly exposed area, taking into account these residues alone, amounts to 732 $Å^2$ or 7% of the total surface [5]. This was accompanied by a significant loss of the predominantly polar surface. All these changes occur around the area of the

active site, and a similar phenomenon has been observed during the interfacial activation of GCL [13] and CRL [15].

The crystal structure of hPL has been refined to 2.3 Å [4]. The 449 amino acid residue sequence of the mature protein was deduced from the cloned cDNA sequence. The N-terminal domain contains the active site and the Ca^{2+}-binding site, whereas the C-terminus contains the colipase-binding site [4,26]. The crystal structure suggests that an actual conformational change must occur before hPL can bind substrate. It was noted that the flap residues, in particular Trp-252, must move out of the active-site region, and additionally, conformational adjustments in other parts of the molecule must take place, in order to create enough room for a triglyceride substrate molecule [4,27].

Thus, exposure of the catalytic residues to substrate, or a substrate interface, is accompanied by a marked increase in the nonpolarity of the surrounding surface. Interfacial activation is thus explained by the stabilization of this nonpolar surface by the lipid environment which would, in effect, create a catalytically competent enzyme which is able to attack triglyceride molecules within the lipid interface [2,5,6].

5 THE MECHANISM FOR INTERFACIAL ACTIVATION

A number of hypotheses have been put forward in order to explain interfacial activation, which is characterized by an often dramatic increase in the activity of lipolytic enzymes observed when the critical micellar concentration of insoluble substrate molecules is exceeded [28]. These hypotheses include increased effective substrate concentration and structural organization at the interface [29] and conformational changes of the enzyme upon binding [5].

The ''enzyme theory'' postulates that, concomitant with binding to the interface, the lipase undergoes a conformational change [5]. Evidence for the enzyme theory has been previously mentioned in Section 2. The ''substrate theory'' attributes interfacial activation to changes in the concentration and organization of substrate molecules at the interface. The properties of an interface such as charge, lipid composition, and lipid-packing density have been termed the ''quality'' of the interface [30]. The quality of the interface has been considered a key determinant in the regulation of the lipase catalytic activity [29,31]. Interfacial binding is a complex process. Difficulties with the development of a mechanistic model for interfacial activation arise because lipase interfacial binding and activation are influenced by many factors, including lipase–lipid interactions as well as structural changes in the enzyme upon binding.

6 KINETIC MODEL FOR LIPOLYSIS OF INSOLUBLE LIPIDS

One of the most characteristic features of lipases is their interfacial activation. In 1958, Sarda and Desnuelle [28] demonstrated that esterases displayed Michaelis–Menten patterns for the dependence of reaction velocity on substrate concentration. In contrast, lipases displayed very low activity at the same substrate concentration; however, lipolytic activity increased greatly when the substrate solubility limit was exceeded.

Verger et al. [32] and Verger and de Haas [33] proposed a mechanism for lipase catalysis which included a reversible enzyme adsorption or penetration into the interface, followed by a Michaelis–Menten catalytic cycle:

$$E \underset{k_d}{\overset{k_p}{\rightleftharpoons}} \begin{matrix} E^* \\ \updownarrow \\ E_i^* \end{matrix} + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} E^*S \overset{k_{cat}}{\rightsquigarrow} P$$

This proposed mechanism is described by the following differential equations and mass balance:

$$\frac{d(E^*S)}{\mathrm{dt}} = k_1(E^*)(S) - (k_{cat} + k_{-1})(E^*S)$$

$$\frac{d(E_I^*)}{dt} = k_I E^* - k_{-I}(E_I^*)$$

$$\frac{d(E^*)}{dt} = k_p(E) + k_{-I}(E_I^*) - (k_d + k_I + k_I S)(E^*) + (k_{cat} + k_{-I})(E^*S)$$

$$\frac{d(P)}{dt} = v_m = v_b \frac{V}{I} = k_{cat}(E^*S)$$

$$E_T = E + E^* \frac{I}{V} + E^*S \frac{I}{V} + E^* \frac{I}{V}$$

where E_T is the total enzyme concentration (mol/m^3), E is the concentration of bulk free enzyme (mol/m^3), I/V is the interfacial area per volume ratio (m^2/m^3), E^* is the concentration of interfacial enzyme (mol/m^2), E_I^* is the concentration of inactive interfacial enzyme (mol/m^2), E^*S is the concentration of the interfacial enzyme–substrate complex (mol/m^2), V_m is the interfacial-phase reaction velocity (mol/m^2/sec), and V_b is the bulk-phase reaction velocity (mol/m^3/sec).

This model involves two sequential equilibria, where E is the water-soluble enzyme and E^* is the interfacially penetrated enzyme with more efficient interfacial catalytic properties. The penetration of a water-soluble enzyme into an interface, and the concomitant conformational change, is described by the first equilibrium ($E \rightleftarrows E^*$). After the first step, the penetrated enzyme molecule binds to a single-substrate molecule and forms the E^*S complex. The water-soluble products are released after the catalytic cycle is complete. The model assumes that the physicochemical properties of the interface do not change with time because the products of reaction are water soluble and diffuse rapidly into the water phase. This, however, may not be true because partial glycerides and free fatty acids accumulate at interfaces. The reversible inactivation of E^* is represented by the step $E^* \rightleftarrows E_I^*$ [33]. This inactivation competes with the formation of E^*S and is enhanced at low surface pressures. Also, detergent, which sometimes is added to help emulsify the substrate, is a competitive inhibitor of the reaction [34].

Solutions for these equations have been reported for the case of no interfacial inactivation [32], the case of reversible inhibition at the interface [33,34], and for the case of irreversible inhibition of the enzyme at the interface [35].

Even though these attempts at modeling interfacial kinetics are commendable, there are some serious problems with the assumptions of the model. In our opinion, the most troubling assumptions are that the properties of the interface do not change in time and that the reaction products leave the interface after the catalytic cycle is complete. Free fatty acids are known to inhibit lipase activity [36]. Long-chain fatty acids, particularly, tend to accumulate at oil–water interfaces because their solubility in aqueous media

is low [36]. This accumulation of fatty acids will also lead to a drop in pH, affecting enzyme activity. Monoglycerides and diglycerides are also surface-active compounds which will accumulate at interfaces, changing the nature of the interface. The time-dependent alteration of lipase activity (some of which may be inactivation) should therefore always be taken into account in modeling exercises, because the interfacial environment is in constant flux. As well, one should consider that the lipase will be acting on different substrates at the same time: Triglycerides, diglycerides, and monoglycerides are all substrates for lipase reactions. The relative amount of all these species will be changing in time during the course of hydrolysis, both increasing and decreasing in time. The affinity of the enzyme for each of these substrates may be different. All of these treatments consider lipase reactions as single-substrate reactions, which they are not; however, water is in such excess that the reaction can be safely assumed to be a single-substrate reaction.

This short discussion on interfacial kinetics should highlight the difficulties associated with modeling the kinetics of lipase reactions. The reader will quickly appreciate the complexity of the processes. Immobilized lipases would behave in a completely different fashion, because they already are bound to a surface. In that case, substrate and product partitioning and mass transfer effects control much of the observed kinetics. Under those circumstances, the use of the Michaelis–Menten model for homogeneous enzymatic catalysis becomes strictly a phenomenological exercise.

7 REACTIONS CATALYZED BY LIPASES AND MOLECULAR MECHANISM OF CATALYSIS

Because the reactions they catalyze are fully reversible, lipases can be used to carry out hydrolytic as well as interesterification reactions, depending on the water content of the system. Using glycerides as an example,

$$\text{TAG} + \text{H}_2\text{O} \rightleftharpoons \text{DAG} + \text{FFA}$$
$$\text{DAG} + \text{H}_2\text{O} \rightleftharpoons \text{MAG} + \text{FFA}$$
$$\text{MAG} + \text{H}_2\text{O} \rightleftharpoons \text{Glycerol} + \text{FFA}$$

where TAG represents triacylglycerols, DAG represents diacylglycerols, MAG represents monoacylglycerols, and FFA represents free fatty acids. In excess water, hydrolysis is enhanced, whereas in water-limiting conditions, esterification is enhanced. Because lipases are merely catalysts, they do not affect the position of equilibrium, but rather the velocity of the reaction.

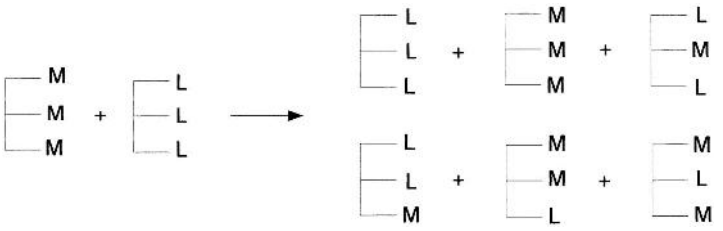


Figure 4 Transesterification reaction between a triglyceride containing medium-chain fatty acids and a triglyceride containing long-chain fatty acids, catalyzed by a *sn*-1,3-specific lipase.

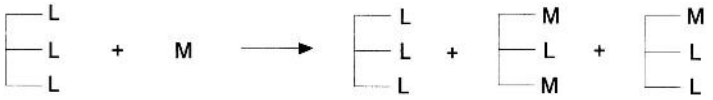


Figure 5 Acidolysis reaction between a medium-chain fatty acid and a triglyceride containing long-chain fatty acids, catalyzed by a *sn*-1,3-specific lipase.

Among interesterification reactions, of commercial interest are transesterification, acidolysis, and alcoholysis. Transesterification is the exchange of acyl groups between two esters, namely two triacylglycerols, or a triacylglycerol and a fatty acid or a methyl/ethyl ester of a fatty acid (Fig. 4). Acidolysis is the transfer of an acyl group between an acid and an ester (Fig. 5). Alcoholysis is the reaction between an alcohol and an ester (Fig. 6).

Interesterification is a multisubstrate reaction, with the main substrates being glycerides, fatty acids, and water. This reaction can be considered a special case of chemical group transfer, involving sequential hydrolysis and esterification reactions [37]. Lipase-catalyzed interesterification follows a Ping Pong Bi reaction for multisubstrate reactions [37,38]. The actual mechanism of acylation and deacylation in the active site of lipases is shown in Figure 7. During acylation, a covalent acyl–enzyme complex is formed by nucleophilic attack of the active-site serine on the carbonyl carbon of the substrate. The active-site serine is made a stronger nucleophile by the presence of histidine and aspartic acid residues. The histidine imidazole ring becomes protonated and positively charged, stabilized by the negative charge of the active-site aspartic acid or glutamic acid residues. A tetrahedral intermediate is subsequently formed, stabilized by two hydrogen bonds formed with oxyanion-stabilizing residues [39]. A break in the carbon–oxygen bond of the ester causes the release of the alcohol. During the reaction, the acylglycerol is associated with the catalytic triad through covalent bonds. Histidine hydrogen bonds with both

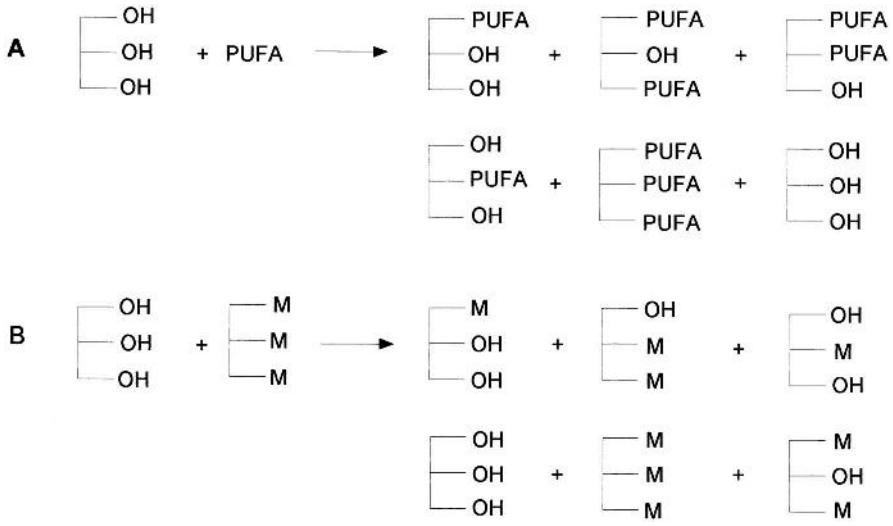


Figure 6 Alcoholysis reaction between glycerol and a polyunsaturated fatty acid (A) and glycerol and a triglyceride containing medium-chain fatty acids (B), catalyzed by a *sn*-1,3-specific lipase.

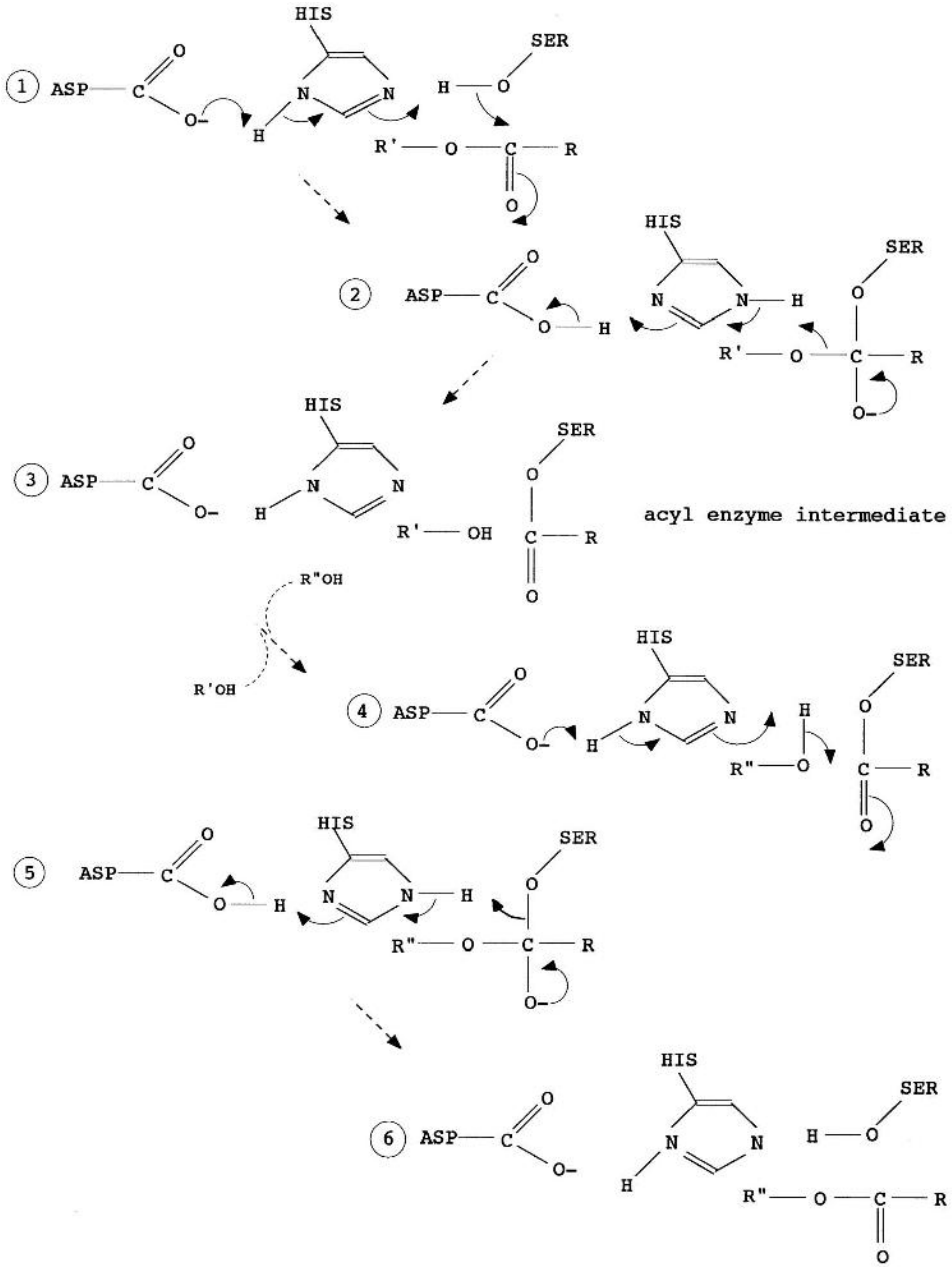


Figure 7 Reactions involved in the catalytic cycle of lipases.

serine and the oxygen of the leaving alcohol. Nucleophilic attack by water or an alcohol causes the addition of a hydroxyl group to the carbonyl carbon, producing a tetrahedral intermediate, which will rearrange, releasing the altered acylglycerol and regenerating the active-site serine [40,41].

The first stage of interesterification involves hydrolysis of triacylglycerols with consumption of water to produce diacylglycerols, monoacylglycerols, and free fatty acids. Accumulation of hydrolysis products will continue during interesterification until an equilibrium is established [37].

8 SPECIFICITY

The main advantage of lipases which differentiates enzymatic interesterification from chemical interesterification is their specificity. The fatty acid specificity of lipases has been exploited to produce structured lipids for medical foods and to enrich lipids with specific fatty acids to improve the nutritional properties of fats and oils. There are three main types of lipase specificity: positional, substrate, and stereospecificity. Positional and fatty acid specificity are usually determined by partial hydrolysis of synthetic triacylglycerols and separation by thin-layer chromatography with subsequent extraction and analysis of the products. Other methods include conversion of the fatty acids produced during hydrolysis to methyl esters for gas chromatographic analysis [42].

8.1 Nonspecific Lipases

Certain lipases show no positional or fatty acid specificity during interesterification. Interesterification with these lipases after extended reaction times gives complete randomization of all fatty acids in all positions and gives the same products as chemical interesterification. Examples of nonspecific lipases include lipases derived from *Candida cylindraceae*, *Corynebacterium acnes*, and *Staphylococcus aureus* [43,44].

8.2 Positional Specificity

Positional specificity (i.e., specificity toward ester bonds in positions *sn*-1,3 of the triacylglycerol) results from an inability of lipases to act on position *sn*-2 on the triacylglycerol, due to steric hindrance. Steric hindrance prevents the fatty acid in position *sn*-2 from entering the active site [43,45]. An interesterfication reaction using a 1,3-specific lipase will initially produce a mixture of triacylglycerols, 1,2- and 2,3-diacylglycerols, and free fatty acids [45]. After prolonged reaction periods, acyl migration can occur, with the formation of 1,3-diacylglycerols, which allows some randomization of the fatty acids existing at the middle position of the triacylglycerols. When compared to chemical interesterification, 1,3-specific lipase-catalyzed interesterification of oils with a high degree of unsaturation in the *sn*-2 position of the triacylglycerols will decrease the saturated to unsaturated fatty acid level [46]. Lipases that are 1,3-specific include those from *Aspergillus niger*, *Mucor miehei*, *R. arrhizus*, and *Rhizopus delemar* [43]. The specificity of individual lipases can change, due to microenvironmental effects on the reactivity of functional groups or substrate molecules [47,48]. For example, lipase from *Pseudomonas fragi* is known to be 1,3-specific, but has also produced random interesterification, possibly due to a microemulsion environment. As of now, lipases that are specific toward fatty acids in the *sn*-2 position have been difficult to identify. Under aqueous conditions, one such lipase from *Candida*

parapsilosis hydrolyzes the *sn*-2 position more rapidly than either of the *sn*-1 and *sn*-3 positions and is also specific toward long-chain polyunsaturated fatty acids [49].

The differences in the nutrition of chemically interesterified fats and oils compared to enzymatically interesterified samples can be linked to the positional specificity exhibited by some lipases. In fish oils and some vegetables oils that contain high degrees of essential polyunsaturated fatty acids, these fatty acids are usually found in greater quantities in the *sn*-2 position. In the intestines, 2-monoacylglycerols are more easily absorbed than *sn*-1 or *sn*-3-monoacylglycerols. Using a 1,3-specific lipase, the fatty acid composition of positions 1 and 3 can be changed to meet the targeted structural requirements while retaining the nutritionally beneficial essential fatty acids in position 2. Using random chemical interesterification, the retention and improvement in beneficial fatty acid content cannot be accomplished due to the complete randomization of the fatty acids in the triacylglycerols [50].

8.3 Stereospecificity

In triacylglycerols, the *sn*-1 and *sn*-3 positions are sterically distinct. Very few lipases differentiate between the two primary esters at the *sn*-1 and *sn*-3 positions, but when they do, the lipases possess stereospecificity. In reactions where the lipase is stereospecific, positions 1 and 3 are hydrolyzed at different rates. Stereospecificity is determined by the source of the lipase and the acyl groups and can also depend on the lipid density at the interface, where an increase in substrate concentration can decrease specificity due to steric hindrance. Differences in chain length can also affect the specificity of the lipase [39]. Lipase from *Pseudomonas* species and porcine pancreatic lipase have shown stereoselectivity when certain acyl groups are hydrolyzed [51].

8.4 Fatty Acid Specificity

Many lipases are specific toward particular fatty acid substrates. Most lipases from microbial sources show little fatty acid specificity, with the exception of lipase from *G. candidum*, which is specific toward long-chain fatty acids containing *cis*-9 double bonds [43]. Lipases can also demonstrate fatty-acid-chain-length specificity, with some being specific toward long-chain fatty acids and others being specific toward medium-chain and short-chain fatty acids. For example, porcine pancreatic lipase is specific toward shorter-chain fatty acids, whereas lipase from *Penicillium cyclopium* is specific toward long-chain fatty acids. Also, lipases from *A. niger* and *Aspergillus delemar* are specific toward both medium-chain and short-chain fatty acids [52,53]. Other lipases have been found to be specific toward fatty acids of varying lengths [54]. Marangoni [55] found that in the hydrolysis of butter oil, lipase from *C. rugosa* showed specificity toward butyric acid compared to *Pseudomonas fluorescens* lipase. With interesterification reactions in organic media, lipases can also be specific toward certain alcohol species. A large group of lipases from sources such as *C. cylindracea*, *M. miehei*, and *R. arrhizus* have been found to be strongly specific against fatty acids containing the first double bond from the carboxyl end at an even-numbered carbon, such as *cis*-4, *cis*-6, and *cis*-8, resulting in slower esterification of these fatty acids compared to other unsaturated and saturated fatty acids. Fatty acid specificity by certain lipases can be used in the production of short-chain fatty acids for use as dairy flavors and in the concentration of EPA and DHA in fish oils by lipases with lower activity toward these fatty acids.

9 REACTION SYSTEMS

9.1 Enzymatic Interesterification in Microaqueous Organic Solvent Systems

Because the main substrates of lipases are long-chain triacylglycerols, which are insoluble in water, many experiments have been conducted in the presence of organic solvents. Organic solvents allow the fat or oil to be solubilized and convert two-phase systems into a one-phase system [56]. Stability can be improved by covalent attachment of poly(ethylene glycol) (PEG) to free amino groups of the lipase, giving lipases amphiphilic properties and allowing their dissolution in organic solvents [57]. It has been reported that the thermal stability of lipases can be improved in microaqueous organic solvent systems because the lack of water prevents unfolding of the lipase at high temperatures [58]. Elliott and Parkin [58] found that porcine pancreatic lipase had optimum activity at 50°C in an emulsion, whereas the optimum increased to 70°C in a microaqueous organic solvent system using hexane. Lipase activity in organic solvents depends on the nature and concentration of the substrate and source of enzyme [56]. The specific organic solvent used can dramatically affect the activity of the lipase [59]. Lipases are more active in *n*-hexane and iso-octane than other solvents such as toluene, ethyl acetate, and acetonitrile [60,61]. The polarity of solvents can be described by *P*, the partition coefficient of a solvent between water and octanol. This is an indication of the hydrophobicity of the solvent. No lipase activity is observed in solvents with a value for log *P* less than 2 [62,63]. The hydrophobicity of the solvent can also affect the degree of acyl migration during interesterification using a 1,3-specific lipase. Hexane tends to promote acyl migration due to the low solubility of free fatty acids and partial glycerides in hexane, which forces them into the microaqueous region around the lipase, providing optimum conditions for acyl migration. In contrast, the use of diethyl ether, in which free fatty acids and partial glycerides are more soluble, removes the products from the microaqueous environment and reduces the risk of acyl migration [64]. Because the choice of organic solvents based on minimization of acyl migration may conflict with maximization of interesterification, acyl migration is usually minimized simply by reducing reaction times. Lipases can be made more active and soluble in organic solvent systems by attachment of an amphiphilic group such as PEG. PEG reacts with the N-terminal or lysine amino groups, rendering the lipase more soluble in organic solvents [65]. The activity of lipases in organic solvent depends on the solubility of the solvent in water. Lipases are only active in water-immiscible solvents, because water miscible organic solvents extract the water of hydration layer from the vicinity of the enzyme, thereby inactivating them [61]. Because the success of an interesterfication reaction depends on the concentration of water in the system, the hydration state of the lipase plays a key role, as a minimum amount of water is needed to maintain the enzyme in its active form. The use of hydrophobic solvents limits the flexibility of the enzyme, preventing it from assuming its most active conformation. Therefore, if organic solvents are used, the enzyme must be in its active conformation before addition of the organic solvent. This can be accomplished by exposing the enzyme to an inhibitor or substrate and then drying it in its active conformation [39,66]. The advantage of using organic solvents in lipase-catalyzed interesterification reactions is that the water content can be carefully controlled. A water content higher than 1% can produce high degrees of hydrolysis, whereas water levels lower than 0.01% can prevent full hydration of the lipase and reduce the initial rate of hydrolysis [67]. Therefore, water levels between these two extremes are necessary to maximize the effectiveness of enzymatic interesterification in or-

ganic solvents. In microaqueous organic solvent systems, the effect of pH on lipase activity is complex because water levels are so low. It has been proven that enzymes in organic solvent systems have a memory of the pH of the last aqueous environment in which they were. Elliott and Parkin [58] found that porcine pancreatic lipase has an optimum activity in hexane after being exposed to pHs between 6.5 and 7.0. At pH 8.5, the decrease in activity was attributed to a change in the ionization state of the histidine in the active site.

A common form of organic solvent system used in lipase-catalyzed interesterification is that of reverse micelles. Reverse micelles, or microemulsions, are defined as nanometer-sized water droplets dispersed in organic media with surfactants stabilizing the interface [68,69]. A common surfactant used is an anionic double-tailed surfactant called sodium-bis-(2-ethylhexyl)sulfosuccinate (AOT). Reverse micelles are used in interesterification reactions because they increase the interfacial area and improve the interaction between the lipase and substrate [68]. Also, the use of microemulsions makes it possible to use polar and nonpolar reagents in the same reaction mixture [70]. Reverse micelles can be formed by gently agitating a mixture of AOT, lipid substrate, organic solvent, and lipase dissolved in buffer until the solution becomes clear. The lipase is trapped in an aqueous medium in the core of the micelle, avoiding direct contact with the organic medium [53]. Lecithin has been used to promote the formation of reverse micelles and to protect the lipase from nonpolar solvents [71,72]. At ionic strengths higher than 1 *M*, activity is decreased due to decreased solubility and activity of the lipase. The water content required for microemulsion systems is dependent on the desired reaction, although some level of water is necessary to hydrate the enzyme. For example, Holmberg et al. [73] found that 0.5% water was the optimum for production of monoacylglycerols from palm oil in a microemulsion. The composition of the substrate can also affect the rate of interesterification in reverse micelles. Substrates with more amphiphilic properties are better because they can partition to the interface. More polar substrates tend to stay in the water phase and interact less with the interface [74]. The disadvantages of reverse-micelle systems are that lipase activity is decreased rapidly, and the system can alter lipase specificity [71,74,75]. Reverse micelles can also be used with immobilized lipases, where the reverse micelle is formed around the support and immobilized lipase. This method has been used with hexane to produce cocoa butter equivalents [71]. Although they have been used in experimental form to produce triacylglycerols from diacylglycerols and oleic acid [76], as well as triacylglycerols suitable for use as cocoa butter substitutes [72], reverse micelles are not used in industrial enzymatic interesterification applications.

9.2 Immobilization

Immobilization of lipases has become increasingly popular for both hydrolysis and synthesis reactions. The advantages of immobilized enzyme systems compared to free enzyme systems include reusability, rapid termination of reactions, lowered cost, controlled product formation, and ease of separation of the enzyme from the reactants and products. Also, immobilization of different lipases can affect their selectivity and chemical and physical properties. Immobilization also provides the possibility of achieving both purification of the lipase from an impure extract and immobilization simultaneously, with minimal inactivation of the lipase [77]. Methods for immobilization of enzymes include chemical forms, such as covalent bonding, and physical forms such as adsorption and entrapment in a gel matrix or microcapsules [78,79].

The easiest and most common type of immobilization used in interesterification

reactions is adsorption, which involves contacting an aqueous solution of the lipase with an organic or inorganic surface-active adsorbent. The objective of immobilization is to maximize the level of enzyme loading per unit volume of support. The process of adsorption can be accomplished through ion exchange or through hydrophobic or hydrophilic interactions and van der Waals interactions [80]. After a short period of mixing the free enzyme and support, the immobilized enzyme is washed to remove any free enzyme that is left, after which the product is dried [77]. The same adsorption process can be accomplished by precipitating an aqueous lipase solution onto the support using acetone, ethanol, or methanol, then drying as previously described [43,80]. Although desorption can occur, most immobilized lipase preparations are stable in aqueous solutions for several weeks. The preparations are stable because as the lipase adsorbs to the support, it unfolds slightly, allowing several points of interaction between the lipase and support. In order for desorption to occur, simultaneous loss of interactions at all contact sites must occur, which is unlikely [81].

The degree of immobilization depends on several conditions, including pH, temperature, solvent type, ionic strength, and protein and adsorbent concentrations. The choice of carrier is dependent on its mechanical strength, loading capacity, cost, chemical durability, functionality, and hydrophobic or hydrophilic character [82]. In general, lipases retain the highest degree of activity when immobilized on hydrophobic supports, where desorption of lipase from the support after immobilization is negligible, and improved activity has been attributed to increased concentrations of hydrophobic substrate at the interface [38,78]. The disadvantages of using hydrophilic supports include high losses of activity due to changes in conformation of the lipase, steric hindrance, and prevention of access of hydrophobic substrates [78]. Common hydrophobic supports include polyethylene, polypropylene, styrene, and acrylic polymers, whereas hydrophilic supports include Duolite, Celite, silica gel, activated carbon, clay, and Sepharose [78]. The effectiveness of the immobilization process is influenced by the internal structure of the support. If a support with narrow pores is used, most of the enzyme will be immobilized on the surface of the support, which prevents the occurrence of internal mass transfer limitations. If a support containing larger pore sizes is used, such as Spherosil DEA, with an average diameter of 1480 Å, some lipase will be immobilized inside the pores, which can prevent access of the substrate to the some of the lipase. This is due to preferential filling of pores and crevices by the lipase during immobilization [83,84]. The activity of lipases tends to decrease upon immobilization, with activity being reduced by 20% to 100% [77,80]. The activity of an immobilized enzyme relative to the free form can be compared by an effectiveness value, which is defined as the activity of immobilized enzyme divided by the activity of an equal amount of free enzyme determined under the same operating conditions. The effectiveness value can be used as a guide to the degree of inactivation of the enzyme caused by immobilization. For values close to 1.0, very little enzyme activity has been lost upon immobilization, whereas values much lower than 1 indicate high degrees of enzyme inactivation [79].

The performance of an immobilized lipase can also be affected by handling and reaction conditions. Freeze-drying of the immobilized enzyme before interesterification substantially reduced the moisture content and has been reported to dramatically improve activity. Molecular sieves can also be added to reaction systems to reduce the amount of water that accumulates during the reaction, which would, in turn, reduce the degree of hydrolysis [85]. The main disadvantage associated with adsorption as an immobilization method is that changes in pH, ionic strength, or temperature can cause desorption of lipase

than has been adsorbed by ion exchange. Lipases adsorbed through hydrophobic or hydrophilic interactions can be desorbed by changes in temperature or substrate concentration [77].

9.2.1 Factors Affecting Immobilized Lipase Activity

Immobilization can have an impact on the activity of lipases through steric, mass transfer, and electrostatic effects. During immobilization, the enzyme conformation can be affected and parts of the enzyme can be made inaccessible to the substrate due to steric hindrance.

Mass Transfer Effects

The kinetics of lipase-catalyzed interesterification can be affected by mass transfer limitations. The substrate must diffuse through the fluid boundary layer at the surface of the support, into the pore structure of the support, and react with the lipase. Once products have been released by the lipase, they must diffuse back out of the pore structure and away from the surface of the support. Mass transfer limitations fall into two categories: internal and external mass transfer. Internal mass transfer is the transport of substrate and product within the porous matrix of the support and is affected by the size, depth, and smoothness of the pores. Internal mass transfer is diffusion limited only. When the rate of diffusion inward is slower than the rate of conversion of substrate to product, the reaction is diffusion limited, as there is not enough substrate available for the amount of enzyme present [86]. A diffusion coefficient for internal mass transfer in immobilized enzyme systems compared to free enzyme systems is defined as

$$D_e = \frac{D\Psi}{\tau}$$

where D_e is the effective diffusion coefficient inside the support particles, D is the diffusion coefficient in free solution, Ψ is the porosity of the particles, and τ is the tortuosity factor, defined as the distance of the path length traveled by molecules between two points in a particle.

The effective diffusion coefficient varies inversely with the molecular weight of the substrate [79]. Internal diffusional limitations can be recognized if the activity increases when the support particles are crushed, as crushing would decrease the length of the pathway that the substrate would have to travel to reach the enzyme. The Thiele modulus, Φ, can be used to evaluate the extent of internal mass transfer limitations:

$$\phi = L\lambda = L\sqrt{\frac{V_{\max}}{K_m D_e}}$$

where L is the half-thickness of the particles. Internal mass transfer limitations can also be identified by measuring the initial velocity of the reaction at increasing enzyme concentrations. If the rate of the reaction remains constant at increasing enzyme concentrations (amount of enzyme per gram of support), the reaction is mass transfer limited. If the rate of reaction increases linearly with increasing enzyme concentration, the reaction is kinetically limited. Internal diffusion limitations can be reduced by decreasing the support particle size, increasing pore size and smoothness, using low-molecular-weight substrates, and using high substrate concentrations [79]. The difficulty with using smaller-sized support particles in fixed-bed reactors where internal mass transfer limitations are high is that it tends to increase the back-pressure of the system [83].

External mass transfer limitations are the resistance to transport between the bulk solution and a poorly mixed fluid layer surrounding each support particle. External mass transfer can occur in packed bed and membrane reactors and is affected by both convection and diffusion [83]. If the reaction is faster than the rate of diffusion of the substrate to the surface or product from the surface, this can affect the availability of substrate for lipase catalysis. If inadequate substrate quantities reach the enzyme, the rate of reaction will be lower than that of the free enzyme. An increasing external mass transfer coefficient can be identified during kinetic analysis by an increasing slope of a Lineweaver–Burk plot [87]. In stirred-reaction systems, external mass transfer limitations have been eliminated when there is no increase in the reaction rate with increasing rates of stirring. External mass transfer limitations can be reduced in packed-bed reactors by increasing the flow rate, reducing the viscosity of the substrate, and increasing the substrate concentration [79]. Changing the height-to-diameter ratio of a fixed-bed reactor can also reduce external mass transfer limitation as it increases the linear velocity of the substrates.

The Nernst Layer and Diffusion Layer

Immobilized lipases are surrounded by two different layers that can create differences in substrate concentration between them and the bulk phase. The Nernst layer is a thin layer directly next to the surface of the support. In the case of hydrophobic supports and hydrophobic substrates such as triacylglycerols, the concentration of substrates in the Nernst layer is more concentrated than in the bulk solution because the hydrophobic substrate tends to partition toward the hydrophobic support material. Another layer surrounding the support particles is a diffusion or boundary layer. A concentration gradient is established between the diffusion layer and the bulk phase, as the substrate is converted to product by the lipase. The product concentration in the diffusion layer is higher than in the bulk phase, as it must diffuse from the surface of the support into the bulk phase. Consequently, due to the higher product concentration in the diffusion layer, the substrate concentration is lower than in the bulk phase, producing a concentration gradient with more substrate diffusing toward the support and immobilized lipase. Differences in substrate concentration between the Nernst layer and/or the boundary layer and the bulk phase can affect the determination of K_m because the substrate concentration will be measured in the bulk layer, which may not be the concentration of substrate closer to the lipase. With a lower substrate concentration at the support compared to the bulk phase, the apparent K_m will appear higher and the activity will appear lower than its actual values. The opposite will occur with a higher substrate concentration at the interface [88].

A third factor that can affect the activity of immobilized lipase is electrostatic effects. If the support and substrate possess the same charge, then they will experience repulsion, whereas if they have opposite charges, they will be attracted. This factor can have an effect on the apparent K_m [88]. Also, electrostatic effects can have an impact on other components in the reaction. For example, if the support were anionic, the local concentration of hydrogen ions would be higher in the vicinity of the immobilized lipase, which would cause a decrease in the pH around the enzyme.

Combining the electrostatic effects and the effect of the Nernst layer, the value of the apparent K_m can be modified as follows [88]:

$$K'_m = K_m + \frac{X}{D} V_{\max}$$

where K'_m is the apparent K_m of the lipase, X is the thickness of Nernst layer, and D is the diffusion coefficient.

If the thickness of the Nernst layer decreases, then the ratio X/D would decrease and K'_m to approach K_m.

9.2.2 Stability of Immobilized Enzymes

The stability of immobilized enzymes depends on the method of immobilization and the susceptibility of the enzyme to inactivation. Inactivation can be caused by contaminants and changes in temperature, pH, and ionic strength. High shear, microbial contamination, fouling, and breakage of support particles have also been found to inactivate immobilized enzymes. Depending on the strength of the immobilization method, the enzyme can also be desorbed from the support. The stability of immobilized enzymes is evaluated by determining the half-life of the enzyme under the reaction conditions. In diffusion-limited systems, there is a linear decay in enzyme activity in time, as enzymes on the surface of the support are inactivated and the substrate diffuses further into the pores to reach enzyme molecules that have not been inactivated. In systems free of diffusional limitations, enzyme inactivation follows a first-order decay. The half-life of lipases in interesterification systems have been reported to range from 7 min to 7 months, with the large variability attributed to the source of lipase and different reaction conditions [38]. As previously stated, the half-life of the immobilized enzyme can be used to determine the productivity of the system. In order to avoid losses in productivity as the activity of the immobilized lipase decreases, the temperature can be increased to increase the reaction rate or, in fixed-bed reactor systems, the flow rate can be decreased [79]. Although these measures can improve the conversion rate, they can also increase the rate of enzyme inactivation in the case of temperature increases or decrease the throughput in the case of reduced flow rate.

9.2.3 Immobilized Enzyme Kinetics

The previous discussion on the kinetics of lipase action was developed for soluble lipases acting on an insoluble substrate, but assuming that diffusional and mass transfer effects are not rate limiting, the same theories can be applied to immobilized lipases. When using immobilized lipases, the level of substrate compared to the level of enzyme must be considered. In general, there is a low average concentration of substrate in direct contact with the immobilized lipase due to high conversion rates, producing first-order, mixed first- and zero-order, or zero-order kinetics [79]. The kinetics of immobilized lipases are also affected by the type of reactor used, as reactors differ in the amount of immobilized lipase used and in the method of substrate delivery, product removal, and degree of mixing.

9.3 Enzymatic Interesterification Reactors

Reactors designed for immobilized enzyme reactions differ from one another based on several criteria. Reactors can be batch or flow-through systems and can differ in the degree of mixing involved during the reaction. For all reactor systems, the productivity of the system is defined as the volumetric activity times the operational stability of the immobilized enzyme, with units of kilograms of product per liter of reactor volume per year. The volumetric activity is determined as the mass of product obtained per liter of reactor per hour, and the operational stability is the half-life of the immobilized enzyme [79]. The

most common reactor systems used include fixed-bed, batch, continuous-stirred tank, and membrane reactors.

9.3.1 Fixed-Bed Reactor

A fixed-bed reactor is a form of continuous-flow reactor, where the immobilized enzyme is packed in a column or as a flat bed and the substrate and product streams are pumped in and out of the reactor at the same rate. The main advantages of fixed-bed reactors are their easy application to large-scale production, high efficiency, low cost, and ease of operation. A fixed-bed reactor also provides more surface area per unit volume compared to a membrane reactor system [78]. A model fixed-bed reactor for interesterification would consist of two columns in series, one for the reaction and a precolumn for fat-conditioning steps such as incorporation of water. Reservoirs attached to the columns would contain the feed streams and products streams. A pump would be required to keep the flow rate through the system constant, and the system would have to be water-jacketed to keep the reaction temperature constant. Because water is required in minimal amounts for hydration of the enzyme during the reaction, the oil is first passed through a precolumn containing water-saturated silica or molecular sieves which would allow the oil to become saturated with sufficient water to allow progression of the interesterification reaction, without increasing the rate of hydrolysis. Interesterification in a fixed-bed reactor can lead to increases in product formation through increased residence time in the reactor. Complete conversion to products will never be achieved, and with an increase in product levels, a loss in productivity will occur [89]. Using a fixed-bed reactor with a silica precolumn for water saturation of the oil phase, Posorske et al. [89] produced a cocoa butter substitute from palm stearine and coconut oil. These authors found that decreasing the flow rates to increase the total product concentration caused a decrease in the productivity. Decreasing flow rates to increase product levels from 20% to 29% leads to a significant decrease in productivity. Fixed-bed reactors are more efficient than batch reactors but are prone to fouling and compression. Dissolution of the oil in an organic solvent to reduce viscosity for flow through the packed bed may be required [89]. Also, the substrate has to be treated to remove any particulates, inhibitors, and poisons that can build up over time and inactivate the lipase [90]. Macrae [43] found that after treating palm oil midfraction and stearic acid to remove particulates, inhibitors, and poisons, acidolysis reached completion after 400 hr and there was no appreciable loss in lipase activity even after 600 hr of operation. Wisdom et al. [91] performed a pilot-scale reaction using a 2.9-L fixed-bed reactor to esterify shea oleine with stearic acid. It was found that with high-quality substrates, only a small loss of activity was exhibited after 3 days with the production of 50 kg of product. However, when a lower-grade shea oil was used, there was rapid inactivation of the lipase.

The kinetics of a packed-bed reactor are assumed to be the same as for a soluble lipase, where

$$-\frac{d\mathrm{S}}{dt} = \frac{V_{\max}\,[\mathrm{S}]}{K'_m + [\mathrm{S}]}$$

This can be rearranged and integrated to

$$[\mathrm{S}_0]p = K'_m \ln(1 - p) + \frac{kEV_v}{Q}$$

where $[S_0]$ is the initial substrate concentration, p is the fraction of S reacted at any given time, Q is the flow rate, V_v is the void volume of the pore space in the bed, and E is the total number of moles of enzyme per unit volume of bed [79,92]. The residence time, τ, is based on the porosity of the packed bed and is defined as [93]

$$\tau = V_{tot} \frac{P}{Q}$$

where V_{tot} is the volume of the reactor, P is the porosity of the bed, and Q is the flow rate of the substrate.

The porosity of the bed in a fixed-bed reactor can produce internal transfer limitations. Ison et al. [83] studied the effects of pore size on lipase activity in a fixed-bed reactor using Spherosil with a mean pore size of 1480 Å, and Duolite with a mean pore size of 190 Å. The larger pore size of the Spherosil was found to produce a decrease in lipase activity. This loss in activity was due to the higher degree of enzyme loading during immobilization, making some of the lipase inaccessible to substrate. With the smaller pore size of Duolite, the lipase was immobilized only on the surface of the support, eliminating internal mass transfer limitations.

9.3.2 Stirred-Batch Reactor

A stirred-batch reactor is a common system used in laboratory experiments with lipase-catalyzed interesterification due to its simplicity and low cost. No addition and removal of reactants and products is performed except at the initial and final stages of the reaction The equation to characterize the kinetics of a stirred-batch reactor is

$$[S_0]p = -\frac{p}{1-p} K'_m + \frac{kEt}{V}$$

where $[S_0]$ is the initial substrate concentration, p is the fraction of substrate reacted at any given time, t is the reaction time, kE is the maximum activity of the enzyme in moles of substrate converted per unit time, and V is the volume of the reactor.

Kurashige [94] found that a batch reactor was useful in reducing the diacylglycerol content in palm oil by converting existing diacylglycerols and free fatty acids into triacylglycerols. Using lipase coadsorbed with lecithin on Celite under vacuum to keep the water content below 150 ppm, the author was able to increase the triacylglycerol content from 85% to 95% in 6 hr. The rate of conversion in a stirred-batch reactor decreases over time because there is a high initial level of substrate, which is reduced over time, with conversion to product. In order to maintain the same rate of conversion throughout the reaction, it would be necessary to add more immobilized enzyme to the reaction mixture [79]. A stirred-batch reactor has the advantage that it is relatively easy to build and free enzymes can be used, but it has the disadvantage that, unless immobilized, the enzyme cannot be reused. Also, a larger system or longer reaction times are required to achieve equivalent degrees of conversion compared to other systems, and side reactions can be significant [56]. Macrae [43] used a batch reactor to produce cocoa butter equivalents from the interesterification of palm oil midfraction and stearic acid. Although product yields were high, by-products such as diacylglycerols and free fatty acids were formed. Therefore, it was necessary to isolate the desired triacylglycerols products using fat fractionation techniques.

9.3.3 Continuous-Stirred Tank Reactor

A continuous-stirred tank reactor combines components of both fixed-bed and batch reactors. It is an agitated tank in which reactants and products are added and removed at the same rate while providing continuous stirring to eliminate mass transfer limitations encountered in a fixed bed. Stirring also prevents the formation of temperature and concentration gradients between either substrates or products. A continuous-stirred tank reactor can be in the form of a tank with stirring from the top or bottom or a column with stirring accomplished by propellers attached to the sides of the column [63]. The kinetics for a continuous-stirred tank reactor, developed by Lilly and Sharp [95], first consider the substrate balance in the system as

$$Q[S_i] - Q[S_0] = \frac{dS}{dt} V_L$$

where Q is the flow rate, $[S_i]$ is the initial substrate concentration entering the reactor, $[S_0]$ is the substrate concentration leaving the reactor, and V_L is the liquid volume in the tank.

Rearrangement and integration gives

$$[S_0]p = -\frac{p}{1-p} K'_m + \frac{kE}{Q}$$

where $[S_0]$ is the initial substrate concentration, Q is the flow rate, kE is the maximum enzyme activity, and p is the fraction of the substrate reacted at any given time.

The main disadvantages of continuous-stirred tank reactors are the higher power costs associated with continuous stirring, the possibility of breaking up support particles with agitation, and the requirement for a screen or filter at the outlet to prevent losses of the immobilized lipase [78,79].

9.3.4 Membrane Reactors

Immobilization of enzymes onto semipermeable membranes is an attractive alternative for lipase-catalyzed interesterification reactions. Membrane reactors involve two-phase systems, where the interface of two phases is at a membrane. The advantages of membrane systems are reduced pressure drops, reduced fluid channeling, high effective diffusivity, high chemical stability, and a high membrane surface area-to-volume ratio [96]. Membranes are commonly produced in the form of a bundle of hollow fibers and can be hydrophilic of hydrophobic in nature. Materials used in membrane systems are polypropylene, polyethylene, nylon, acrylic resin, and poly(vinyl chloride). In a membrane such as microporous polypropylene, the pores have dimensions of 0.075×0.15 μm and the fibers have an internal diameter of 400 μm, providing 18 m^2 of surface area per gram of membrane [81]. With a hydrophilic membrane such as cellulose, the oil phase circulates through the inner fiber side while the aqueous components circulate on the shell side [56]. Immobilization of lipase can be accomplished by submerging the fibers in ethanol, rinsing in buffer, and then submerging them in lipase solution [81]. Another method involves dispersing the enzyme in the oil phase, and using ultrafiltration to deposit the lipase on the inner fiber side. One of the substrates can diffuse through the membrane toward the interface where the enzyme is immobilized. Van der Padt et al. [56] used hollow fibers made from cellulose to perform glycerolysis of decanoic acid. Using a hydrophilic membrane bioreactor, the lipase activity was similar to the activity in emulsion systems. The hydrophilic

membrane was found to be more effective for glycerolysis because the lipase was immobilized on the oil-phase side, with the membrane preventing it from diffusing into the glycerol phase and being lost. Hoq et al. [97] used a hydrophobic polypropylene membrane to esterify oleic acid and glycerol. The lipase was adsorbed on the glycerol side, resulting in the loss of some enzyme in this phase. Therefore, using a hydrophobic membrane would require the addition of more lipase to prevent losses in activity [57,78]. Membrane reactors have been used in glycerolysis and acidolysis reactions and have an advantage over more conventional stirred tank reactors in that the reaction and separation of substrates and product can be accomplished all in one system. Having the substrates and products separated during the reaction is especially useful during esterification reaction where water is produced. Hoq et al. [97,98] found that during esterification of oleic acid and glycerol, the excess water produced could be removed by passing the oleic acid stream through molecular sieves, thereby preventing losses in productivity from hydrolysis.

9.3.5 Fluidized-Bed Reactor

Fluidized-bed reactors are reactors in which the immobilized enzyme and support are kept suspended by the upward flow of substrate or gas at high flow rates [79]. The advantages of fluidized-bed reactors are that channeling problems are eliminated and there is less change in pressure at high flow rates and less coalescence of emulsion droplets. Also, particulates do not have to be removed from the oil and there are no concentration gradients [78]. The main disadvantage of fluidized-bed reactors is that small concentrations of enzyme can be used because a large void volume is required to keep the enzyme and support suspended. Mojovic et al. [99] used a gas-lift reactor to produce a cocoa butter equivalent by interesterifying palm oil midfraction with stearic acid, using hexane as the solvent. These authors immobilized lipase encapsulated in lecithin reverse micelles in hexane, the reaction in the gas-lift reactor was more efficient than in a stirred batch reactor. Equilibrium was reached 25% earlier and productivity was 2.8 times higher in the gas-lift reactor.

9.4 Factors Affecting Lipase Activity During Interesterification

In considering all the factors involved in enzymatic interesterification, all components of the system must be examined, namely pH, water content, temperature, substrate composition, product composition, and lipase content.

9.4.1 pH

Lipases are only catalytically active at certain pHs, depending on their origin and the ionization state of residues in their active sites. Although lipases contain basic, neutral, and acidic residues, the residues in the catalytic site are only active in one particular ionization state. The pH optima for most lipases lie between 7 and 9, although lipases can be active over a wide range of acid and alkaline pHs, from about pH 4 to pH 10 [38,100]. For example, the optimum pH for lipase from *Pseudomonas* species is around 8.5, whereas fungal lipases from *A. niger* and *R. delemar* are acidic lipases [101]. The effect of immobilization on the pH optimum of lipases is dependent on the partitioning of protons between the bulk phase and the microenvironment around the support and the restriction of proton diffusion by the support. If the lipase is immobilized on a polyanion matrix, the concentration of protons in the immediate vicinity of the support will be higher than in the bulk phase, thereby reducing the pH around the enzyme, compared to the pH of the bulk phase. Because there is a difference in the perceived pH of the solution as

measured by the pH of the bulk phase, the lipase would exhibit a shift in pH optimum toward a more basic pH. For instance, for a free lipase that has a pH optimum of 8.0, when immobilized on a polyanionic matrix, with the bulk solution at pH 8.0, the pH in the immediate vicinity of the lipase might only be 7.0. Therefore, although the reaction pH is 8.0, the lipase is operating at pH 7.0, which is below its optimum. The pH of the bulk solution would have to be increased to pH 9.0 to get the pH around the lipase to its optimum of 8.0. This phenomenon is only seen in solutions with ionized support and low-ionic-strength systems [102]. If protons are produced in the course of interesterification, the hydrogen ion concentration in the Nernst layer can be higher than in the bulk phase, thereby decreasing the pH in the vicinity of the lipase. Running an interesterification reaction with lipases at a pH well removed from the optimum can lead to rapid inactivation of the enzyme.

9.4.2 Temperature

In general, increasing the temperature increases the rate of interesterification, but very high temperatures can reduce the reaction rates due to irreversible denaturation of the enzyme. Animal and plant lipases are usually less thermostable than extracellular microbial lipases [100]. In a solvent-free system, the temperature must be high enough to keep the substrate in the liquid state [83,103]. Temperatures do not need to be as high in systems containing organic solvents because they easily solubilize hydrophobic substrates. However, for food industry applications, where organic solvents are avoided, the reaction temperatures are usually higher. Sometimes, the temperature has to be increased as high as 60°C to liquefy the substrate. Such high temperatures can seriously reduce the half-life of the lipase, although immobilization has been found to improve the stability of lipases under high-temperature conditions. Immobilization fixes the enzyme in one conformation which reduces the susceptibility of the enzyme to denaturation by heat. The optimum temperature for most immobilized lipases falls within the range of 30–62°C, whereas it tends to be slightly lower for free lipases [38]. Immobilized lipases are more stable to thermal deactivation because immobilization restricts movement and can reduce the degree of unfolding and denaturation. Hansen and Eigtved [104] found that even at a temperature of 60°C, immobilized lipase from *M. miehei* has a half-life of 1600 hr.

9.4.3 Water Content and Water Activity

The activity of lipases at different water contents or water activity is dependent on the source of the enzyme. Lipases from molds seem to be more tolerant to low water activity than bacterial lipases. The optimum water content for interesterification by different lipases ranges from 0.04% to 11% (w/v), although most reactions require water contents of less than 1% for effective interesterification [38,105,106]. The water content in a reaction system is the determining factor as to whether the reaction equilibrium will be toward hydrolysis or ester synthesis. Ester synthesis depends on low water activity. Too low a water activity will prevent all reactions from occurring because lipases need a certain amount of water to remain hydrated, which is essential for enzymatic activity [107,108]. As stated previously, lipases tend to retain the greatest degree of original activity when immobilized on hydrophobic supports. When the immobilized lipase is contacted with an oil and water emulsion, the oil phase tends to associate with and permeate the hydrophobic support, so that there is not an aqueous shell surrounding the enzyme and support. It can be assumed that there is an ordered hydrophobic network of lipid molecules surrounding the support. Any water that reaches the enzyme for participation in hydrolysis and interes-

terification reactions must diffuse there from the bulk emulsion phase. Therefore, in order to avoid diffusional limitations, the oil phase must be well saturated with water [38]. Too much water can inhibit interesterification, probably due to decreased access of hydrophobic substrates to the immobilized enzyme. Abraham et al. [109] found that in a solvent-free system, interesterification dominated hydrolysis up to a water-to-lipase ratio of 0.9, after which hydrolysis became the predominant reaction. During interesterification, the reaction equilibrium can be forced away from ester synthesis due to accumulation of water, 1 mol of which is produced for every mole of ester synthesized during the reaction. The equilibrium can be pushed back toward ester synthesis by continuous removal of water produced during the reaction. Water activity can be kept constant by having a reaction vessel with a saturated salt solution in contact with the reaction mixture via the gas phase in order to continuously remove the water produced in the course of interesterification. Another method of water activity control that has proven useful with interesterification reactions is the use of silicone tubing containing the salt solution, immersed in the reaction vessel. Water vapor can be transferred out of the reaction system across the tubing wall and into the salt solution [108]. A very simple method of water removal involves adding molecular sieves near the end of the reaction, or running the reaction under a vacuum so that the water produced is continuously removed while still allowing the lipase to retain its water of hydration [61,94,110]. Kurashige [94] ran an effective interesterification reaction with less than 150 ppm water maintained by running the reaction under vacuum.

9.4.4 Enzyme Purity and Presence of Other Proteins

During immobilization, adsorption of protein to surface-active supports is not limited solely to lipases. Other protein sources in the lipase solution can be adsorbed and this can have an effect on the loading and activity of the immobilized enzyme. Using a pure lipase solution for immobilization has been found to reduce activity of the lipase, whereas the presence of other proteins on the support can increase the activity of the immobilized lipase [91]. Nonprotein sources of contamination during immobilization are usually not a problem, because the lipase is preferentially adsorbed to the support.

9.4.5 Substrate Composition and Steric Hindrance

The composition of the substrate can have an effect on the rate of hydrolysis and interesterification by lipase. The presence of a hydroxyl group in the *sn*-2 position has a negative inductive effect, so triacylglycerols are hydrolyzed at a faster rate than diacylglycerols that are hydrolyzed at a faster rate than monoacylglycerols [52]. Although the nucleophilicity of substrate is important to the rate of reaction, steric hindrance can have a much greater negative effect. If the composition of the substrate is such that it impedes access of the substrate to the active site, any improvements in the nucleophilicity will not improve the activity [111].

The conformation of the substrate can also have an effect on the rate of reaction. The hydrophobic tunnel in the lipase accepts aliphatic chains and aromatic rings more easily than branched structures [52,61]. For example, using carboxylic acids of differing chain lengths, Miller et al. [61] found that increasing the acyl group chain length up to seven carbons increased the esterification rate for lipase from *M. miehei*.

Oxidation of substrates, especially polyunsaturated fatty acids, is possible and can cause inhibition and a decrease in activity of lipases especially in reactions containing organic solvents. Inhibition is seen at hydroperoxide levels greater than 5 mEq/kg oil and

is attributed to the breakdown of hydroperoxides to free radicals [112]. Therefore, before running interesterification reactions, especially in flow-through systems like fixed-bed reactors which are more susceptible to poisoning and inactivation, oils containing high levels of polyunsaturated fatty acids must be refined [89].

9.4.6 Surface-Active Agents

The presence of surface-active agents used during the immobilization process can improve lipase activity during interesterification. The addition of lecithin or sugar esters as surface-active agents during the immobilization process can increase activity 10-fold when the preparation is used under microaqueous conditions [113]. In contrast, using surface-active agents to form an emulsion can dramatically decrease the rate of interesterification because they prevent contact between the lipase and substrate [114]. Adsorption at the interface can be inhibited by the presence of other nonsubstrate molecules such as proteins. The presence of proteins other than lipase at the interface reduces the ability of the lipase to bind to the interface. Addition of protein in the presence of lipase can cause desorption of lipase from the interface.

Phospholipids, such as phosphatidylcholine, phosphatidylethanolamine, and phosphatidylinositol, can be found as minor components in oil, in quantities of 0.1–3.2%. The presence of phospholipids can have a negative effect on lipase activity. The initial rate of reaction can be decreased due to initial competition between phosphatidylcholine and the triacylglycerols for the active site of the lipase. Phosphatidylethanolamine seems to have the most inhibitory effect on lipase action, possibly due to the presence of the amine group. Due to their effects, the phospholipid content of oils needs to be less than 500 ppm in order to prolong the half-life of immobilized lipases during interesterification [115].

9.4.7 Product Accumulation

During interesterification of two triacylglycerols, the production of monoacylglycerols and diacylglycerols can lead to an increase in the rate of reaction, whereas the presence of high levels of free fatty acids can inhibit the initial hydrolysis of triacylglycerols [37]. In lipase-catalyzed interesterification, where hydrolysis is extensive or in acidolysis reactions, the level of free fatty acids can have an impact on the rate of the reaction. During acidolysis of butter oil with undecanoic acid, Elliott and Parkin [58] reported that concentrations of undecanoic acid greater than 250 m*M* decreased the activity of porcine pancreatic lipase. Inhibition of lipase activity by free fatty acids agreed with the Michaelis–Menten model for uncompetitive inhibition by a substrate [58].

The loss of activity by lipase in the presence of high concentrations of free fatty acids has been attributed to several factors. High levels of free fatty acids would produce high levels of free or ionized carboxylic acid groups which would acidify the microaqueous phase surrounding the lipase or cause desorption of water from the interface. Also, with short- and medium-chain fatty acids, there could be partitioning of fatty acids away from the interface into the surrounding water shell due to their increased solubility in water. This would limit access by the substrate to the interface [116]. Kuo and Parkin [116] found that there was less inhibition when longer-chain fatty acids such as C13:0 and C17:0 were used during acidolysis as compared to C5:0 and C9:0. The decrease in lipase activity was attributed to both increased solubility of the short-chain fatty acids in, and acidification of, the aqueous phase.

10 CONCLUSIONS

The elucidation of the structure of free lipases has greatly increased our understanding of their catalytic activity in free solution. Our understanding of the mechanism of interfacial catalysis, however, remains limited. In particular, we lack a basic understanding of the catalytic mechanism of immobilized lipases at high substrate concentrations, where the substrate for the reaction is also the solvent of the reaction. On the other hand, we now possess a good understanding of the factors which affect the performance of immobilized lipases in bioreactors. The development of a more profound mechanistic understanding of lipase catalysis at interfaces and on solid surfaces will most probably depend on the needs of particular industries for such knowledge.

ACKNOWLEDGMENTS

The author would like to thank Ms. Sue Hall for her help with the preparation of the manuscript and Mr. Yih-Cherng Liou and Ms. Wendy Willis for their advice. The financial support of the Natural Sciences and Engineering Research Council of Canada is gratefully acknowledged.

REFERENCES

1. HL Brockman. In: Lipases. New York: Elsevier Science, 1984, pp 4–36.
2. ZS Derewenda, AM Sharp. Trends Biochem Sci 18:20–25, 1993.
3. L Brady, AM Brzozowski, ZS Derewenda, E Dodson, G Dodson, S Tolley, JP Turkenburg, L Christiansen, B Huge-Jensen, L Norskov, L Thim, U Menge. Nature 343:767–770, 1990.
4. FK Winkler, A D'Arcy, W Hunziker. Nature 343:771–774, 1990.
5. AM Brzozowski, U Derewenda, ZS Derewenda, GG Dodson, DM Lawson, JP Turkenburg, F Bjorkling, B Huge-Jensen, SA Patkar, L Thim. Nature 351:491–494, 1991.
6. U Derewenda, AM Brzozowski, DM Lawson, ZS Derewenda. Biochemistry 31:1532–1541, 1992.
7. C Martinez, PD Geus, M Lauwereys, G Matthyssens, C Cambillau. Nature 356:615–618, 1992.
8. C Martinez, A Nicolas, H van Tilbeurgh, M-P Egloff, C Cudrey, R Verger, C Cambillau. Biochemistry 33:83–89, 1994.
9. ZS Derewenda, U Derewenda, GG Dodson. J Mol Biol 227:818–839, 1992.
10. JD Schrag, Y Li, S Wu, M Cygler. Nature 351:761–764, 1991.
11. MEM Noble, A Cleasby, LN Johnson, MR Egmond, LGJ Frenken. FEBS Lett 331:123–128, 1993.
12. DL Ollis, E Cheah, M Cygler, B Dijkstra, F Frolow, SM Franken, M Harel, SJ Remington, I Silman, J Schrag, JL Sussman, KHG Vershueren, A Goldman. Protein Eng 5:197–211, 1992.
13. JD Schrag, M Cygler. J Mol Biol 230:575–591, 1993.
14. P Grochulski, Y Li, JD Schrag, F Bouthillier, P Smith, D Harrison, B Rubin, M Cygler. J Biol Chem 268:12,843–12,847, 1993.
15. P Grochulski, Y Li, JD Schrag, M Cygler. Protein Sci 3:82–91, 1994.
16. KA Dugi, HL Dichek, GD Talley, HB Brewer, S Santamarina-Fojo. J Biol Chem 267: 25,086–25,091, 1992.

17. S Longhi, F Fussetti, R Grandori, M Lotti, M Vanoni, L Alberghina. Biochem Biophys Acta 1131:227–232, 1992.
18. S Longhi, M Lotti, F Fusetti, E Pizzi, A Tramontano, L Alberghina. Biochim Biophys Acta 1165:129–133, 1992.
19. M Lotti, R Grandori, F Fussetti, S Longhi, S Brocca, A Tramontano, L Alberghina. Gene 124:45–55, 1993.
20. Y Kawaguchi, H Honda, J Taniguchi-Morimura, S Iwasaki. Nature 341:164–166, 1989.
21. WA Hide, L Chan, W-H Li. J Lipid Res 33:167–178, 1992.
22. JL Sussman, M Harel, F Frolow, C Onfner, A Goldman, L Toker, I Silman. Protein Sci 253: 872–879, 1991.
23. R Henderson. J Mol Biol 54:341–354, 1970.
24. M Cygler, JD Schrag, JL Susuman, IS Harel, MK Gentry, BP Doctor. Protein Sci 2:366–382, 1993.
25. M Norin, F Haeffner, A Achour, T Norin, K Hult. Protein Sci 3:1493–1503, 1994.
26. H van Tilbeurgh, L Sarda, R Verger, C Cambillau. Nature 359:159–162, 1992.
27. H van Tilbeurgh, M-P Egloff, C Martinez, N Rugani, R Verger, C Cambillau. Nature 362: 814–820, 1993.
28. L Sarda, P Desnuelle. Biochim Biophys Acta 30:513–521, 1958.
29. JM Muderhwa, HL Brockman. J Biol Chem 267:24184–24192, 1992.
30. R Verger. Methods Enzymol 64:340–392, 1980.
31. JM Smaby, JM Muderhwa, HL Brockman. Biochemistry 33:1915–1922, 1994.
32. R Verger, MCE Mieras, GH De Haas. J Biol Chem 248:4023–4034, 1973.
33. R Verger, GH de Hass. Annu Rev Biophys Bioeng 5:77–117, 1976.
34. S Ransac, C Riviere, JM Soulie, C Gancet, R Verger, GH de Haas. Biochim Biophys Acta 1043:57–66, 1990.
35. BH Havsteen, R Varon Castellanos, M Molina, MMJ Garcia, E Valero, MM Garcia. J Theoret Biol 157:523–533, 1992.
36. RW Lencki, N Smink, H Snelting, J Arul. J Am Oil Chem Soc 75:1195–1200, 1998.
37. HR Reyes, CG Hill Jr. Biotechnol Bioeng 43:171–182, 1994.
38. FX Malcata, HR Reyes, HS Garcia, CG Hill Jr, CH Amundson. Enzyme Microb Technol 14:426–446, 1992.
39. K-E Jaeger, S Ransac, BW Dijkstra, C Colson, M van Heuvel, O Misset. FEMS Microbiol Rev 15:29–63, 1994.
40. DS Wong. Food Enzymes: Structure and Mechanism. New York: Chapman & Hall, 1994, pp 170–211.
41. AG Marangoni, D Rousseau. Trends Food Sci Technol 6:329–335, 1995.
42. PE Sonnet, JA Gazzillo. J Am Oil Chem Soc 68:11–15, 1991.
43. AR Macrae. J Am Oil Chem Soc 60:291–294, 1983.
44. FD Gunstone. In: FD Gunstone, JL Harwood, FD Padley, eds. Chemical Properties, The Lipid Handbook. 2nd ed. London: Chapman & Hall, 1994, pp 594–595.
45. AR Macrae, P How. US Patent 4,719,178, 1988.
46. S Sil Roy, DK Bhattacharyya. J Am Oil Chem Soc 70:1293–1294, 1993.
47. S Parida, JS Dordick. J Am Chem Soc 113:2253–2259, 1991.
48. F Pabai, S Kermasha, A Morin. Appl Microbiol Biotechnol 43:42–51, 1995.
49. A Riaublanc, R Ratomahenina, P Galzy, M Nicolas, J Am Oil Chem Soc 70:497–500, 1993.
50. S Ray, DK Bhattacharyya. J Am Oil Chem Soc 72:327–330, 1995.
51. H Uzawa, T Noguchi, Y Nishida, H Ohrui, H Meguro. Biochim Biophys Acta 1168:253–260, 1993.
52. P Desnuelle. In: PD Boyer, ed. The Lipases, The Enzymes. Volume VII. 3rd ed. New York: Academic Press, 1972, pp 575–616.
53. H Stamatis, A Xenakis, M Provelegiou, FN Kolisis. Biotechnol Bioeng 42:103–110, 1993.
54. P Villeneuve, TA Foglia. INFORM 8:640–650, 1997.

55. AG Marangoni. J Food Sci 59:1096–1099, 1994.
56. A van der Padt, MJ Edema, JJW Sewalt, K van't Riet. J Am Oil Chem Soc 67:347–352, 1990.
57. M Murakami, Y Kawasaki, M Kawanari, H Okai. J Am Oil Chem Soc 70:571–574, 1993.
58. JM Elliott, KL Parkin. J Am Oil Chem Soc 68:171–175, 1991.
59. E Santaniello, P Ferraboschi, P Grisenti. Enzyme Microb Technol 15:367–382, 1993.
60. CC Akoh, BH Jennings, DA Lillard. J Am Oil Chem Soc 72:1317–1321, 1995.
61. C Miller, H Austin, L Posorske, J Gonziez. J Am Oil Chem Soc 65:927–931, 1988.
62. RH Valivety, GA Johnston, CJ Suckling, PJ Halling. Biotechnol Bioeng 38:1137–1143, 1991.
63. G Carta, JL Gainer, AH Benton. Biotechnol Bioeng 37:1004–1009, 1991.
64. SH Goh, SK Yeong, CW Wang. J Am Oil Chem Soc 70:567–570, 1993.
65. K Takahashi, Y Saito, Y Inada. J Am Oil Chem Soc 65:911–916, 1988.
66. F Monot, E Paccard, F Borzeix, M Badin, J-P Vandecasteele. Appl Microbiol Biotechnol 39:483–486, 1993.
67. HL Goderis, G Ampe, MP Feyton, BL Fouwé, WM Guffens, SM Van Cauwenbergh, PP Tobback. Biotechnol Bioeng 30:258–266, 1987.
68. P Quinlan, S Moore. INFORM 4:580–585, 1993.
69. H Stamatis, A Xenakis, U Menge, FN Kolisis. Biotechnol Bioeng 42:931–937, 1993.
70. PDI Fletcher, RB Freedman, BH Robinson, GD Rees, R Schomacker. Biochim Biophys Acta 912:278–282, 1987.
71. L Mojovíc, S Šiler-Marinkovic, G Kukíc, G Vunjak-Novakovíc. Enzyme Microb Technol 15:438–443, 1993.
72. AG Marangoni, RD McCurdy, ED Brown. J Am Oil Chem Soc 70:737–744, 1993.
73. K Holmberg, B Larsson, M-B Stark. J Am Oil Chem Soc 66:1796–1800, 1989.
74. DG Hayes, E Gulari. Biotechnol Bioeng 35:793–801, 1990.
75. M Safari, S Kermasha. J Am Oil Chem Soc 71:969–973, 1994.
76. F Ergan, M Trani, G Andre. J Am Oil Chem Soc 68:412–417, 1991.
77. OR Zaborsky. In: Immobilized Enzymes. Cleveland, OH: CRC Press, 1973, pp 75, 119–123.
78. FX Malcata, HR Reyes, HS Garcia, CG Hill Jr, CH Amundson. J Am Oil Chem Soc 67: 890–910, 1990.
79. PSJ Cheetham. In: A Wiseman, ed. Principles of biotechnology. 2nd ed. New York: Surrey University Press Chapman & Hall, 1988, pp 164–201.
80. A Mustranta, P Forssell, K Poutanen. Enzyme Microb Technol 15:133–139, 1993.
81. FX Malcata, HS Garcia, CG Hill Jr, CH Amunson. Biotechnol Bioeng 39:647–657, 1992.
82. I Karube, Y Yugeta, S Suzuki. Biotechnol Bioeng 19:1493–1501, 1977.
83. AP Ison, AR Macrae, CG Smith, J Bosley. Biotechnol Bioeng 43:122–130, 1994.
84. RA Wisdom, P Dunnill, MD Lilly, A Macrae. Enzyme Microbiol Technol 6:443–446, 1984.
85. HM Ghazali, S Hamidah, YB Che Man. J Am Oil Chem Soc 72:633–639, 1995.
86. G Abraham. In: TH Applewhite, ed. Proceedings of World Conference on Biotechnology for the Fats and Oils Industry. Champaign, IL: American Oil Chemist's Society, 1988, pp 219–225.
87. BK Hamilton, CR Gardner, CK Colton. In: AC Olson, CL Cooney, eds. Immobilized Enzymes in Food and Microbial Processes. New York: Plenum Press, 1974, pp 205–219.
88. T Richardson, DB Hyslop. In: OR Fennema, ed. Enzymes, Food Chemistry. 2nd ed. New York: Marcel Dekker, 1985, pp 391–394.
89. LH Posorske, GK LeFebvre, CA Miller, TT Hansen, BL Glenvig. J Am Oil Chem Soc 65: 922–926, 1988.
90. AR Macrae. In: J Tramper, HC van der Plas, P Linko, eds. Proceedings of an International Symposium held at Noorwijkerhout, The Netherlands, (14–17 April 1985). Amsterdam: Elsevier Science, 1985, pp 195–208.

91. RA Wisdom, P Dunnill, MD Lilly. Biotechnol Bioeng 29:1081–1085, 1987.
92. WE Hornby, MD Lilley, EM Crook. Biochem J 107:669–674, 1968.
93. P Forssell, P Parovuori, P Linko, K Poutanen. J Am Oil Chem Soc 70:1105–1109, 1993.
94. J Kurashige. In: TH Applewhite, ed. Proceedings of World Conference on Biotechnology for the Fats and Oils Industry. Champaign, IL: American Oil Chemist's Society, 1988, pp 219–225.
95. MD Lilly, AK Sharp. Chem Eng 215:CE12–CE18, 1968.
96. J Kloosterman IV, PD van Wassenaar, WJ Bel. In: TH Applewhite, ed. Proceedings of World Conference on Biotechnology for the Fats and Oils Industry. Champaign, IL: American Oil Chemist's Society, 1988, pp 219–225.
97. MM Hoq, H Tagami, T Yamane, S Shimizu. Agric Biol Chem 49:335–342, 1985.
98. MM Hoq, T Yamane, S Shimizu. J Am Oil Chem Soc 61:776–781, 1984.
99. L Mojovic, S Šiler-Marinkovíc, G Kukíc, B Bugarski, G Vunjak-Novakovic. Enzyme Microb Technol 16:159–162, 1994.
100. T Yamane. J Am Oil Chem Soc 64:1657–1662, 1987.
101. M Iwai, Y Tsujisaka. Agric Biol Chem 38:1241–1247, 1974.
102. MD Trevan. Immobilized Enzymes. New York: Wiley, 1980, pp 16–26.
103. P Forssell, K Poutanen. In: J Tramper, MH Vermue, HH Beeftink, U von Stockar, eds. Biocatalysis in Non-Conventional Media. Amsterdam: Elsevier Science, 1992, pp 491–495.
104. TT Hansen, P Eigtved. In: AR Baldwin, ed. Proceeedings of the World Conference on Emerging Technologies in the Fats and Oils Industry, Cannes, France, Nov. 3–8, 1985. Champlain, IL: American Oil Chemist's Society, 1986, pp 365–369.
105. S Bornaz, J Fanni, M Parmentier. J Am Oil Chem Soc 71:1373–1380, 1994.
106. Z-Y Li, OP Ward. J Am Oil Chem Soc 70:745–748, 1993.
107. D Briand, E Dubreucq, P Galzy. Biotechnol Lett 16:813–818, 1994.
108. I Svensson, E Wehtje, P Adlercreutz, B Mattiasson. Biotechnol Bioeng 44:549–556, 1994.
109. G Abraham, MA Murray, VT John. Biotechnol Lett 10:555–558, 1988.
110. PE Sonnet, GP McNeill, W Jun. J Am Oil Chem Soc 71:1421–1423, 1994.
111. HS Bevinakatti, AA Banerji. Biotechnol Lett 10:397–398, 1988.
112. SB Pedersen, G Holmer. J Am Oil Chem Soc 72:239–243, 1995.
113. J Kurashige, N Matsuzaki, H Takahashi. J Am Oil Chem Soc 70:849–852, 1993.
114. D Briand, E Dubreucq, P Galzy. J Am Oil Chem Soc 72:1367–1373, 1995.
115. Y Wang, MH Gordon. J Am Oil Chem Soc 68:588–590, 1991.
116. S-J Kuo, KL Parkin. J Am Oil Chem Soc 70:393–399, 1993.

18

Industrial Uses of Lipase

CHING T. HOU

National Center for Agricultural Utilization Research, Agricultural Research Service, U.S. Department of Agriculture, Peoria, Illinois

1 INTRODUCTION TO LIPASES

Lipases (triacylglycerol hydrolases E.C. 3.1.1.3) are enzymes that catalyze the hydrolysis of triglycerides to glycerol and fatty acids. Lipase was first identified in the pancreas by Eberle in 1834 and Bernard in 1856. Together with amylase and protease, it became one of the three major known digestive enzymes. However, because of its difficulty in handling water-insoluble substrate and heterogeneous reaction system, lipase was rarely in the main stream of research. Plants, animals, and microorganisms produce lipases. Lipases are found in several different animal tissues described as pancreatic, gastric, and pregastric lipases. Earlier investigations were concerned mainly with enzymes that participated in lipid metabolism in animals. The most thoroughly studied was lipase from pancreas. Recently, increasingly more attention is being paid to lipases produced from bacteria and fungi. Microbial lipases are relatively stable and are capable of catalyzing a variety of reactions; they are potentially of importance for diverse industrial applications.

Although lipase has been perceived by scientists as being one of the most important classes of industrial enzymes, its annual sales account at present for about 4% of the worldwide enzyme market. The major obstacle to practical use of lipase in industries remains the cost of the enzymes. The significance of lipases rests on their potential rather than their current level of use. In recent years, interest in the use of enzymes as hydrolytic or synthetic chiral catalysts have risen rapidly. In particular, the search for selective enzyme inhibitors and receptors, agonists, or antagonists is one of the keys for target-oriented research in the pharmaceutical industry. Extracellular microbial lipases are particularly suited for this application. Because of their availability, more than one dozen commercial lipases have been studied extensively by researchers in both industry and academia. Indus-

try continues to look for economical sources of lipases with high activity. New lipases from microbial sources have been reported sporadically [1–3]. Hou and Johnston [4] systematically screened lipase activity from over 1000 microbial cultures, including bacteria, yeast, and fungi. They found many new lipase activities previously not reported. These new lipase activities were divided based on their pH dependence and thermostability [5]. The regioselectivity of some good lipases from yeast and bacteria were characterized [6,7]. Johann and Rozor also screened 6000 microorganisms for stereoselective and regioselective enzymes [8].

In recent years, information on the mechanistic properties of lipases has become available [9]. The notion that lipases have a catalytic triad consisting of Ser-Asp-His was confirmed by the structures of the human pancreatic lipase (HPL) [10] and *Rhizomucor miehei* lipase (RML), whereas for the *Geotrichum candidum* lipase (GCL), the catalytic triad was found to be Ser-Glu-His. In all three cases, the side chains of the active-site amino acids form a configuration which is stereochemically very similar to that of serine proteases. In contrast to the proteases, the lipases share the common feature that the active site is buried in the protein. In the case of HPL and RML, the active site is covered by a short amphipathic helix or ''lid,'' whereas the active site of GCL seems to be covered by two nearly parallel amphipathic helices. The lid moves away upon interaction with the substrate. It has been proposed that this conformational change results in activation of these enzymes at an oil–water interface [11]. However, this interfacial activation phenomenon does not occur in all lipases. For example, lipases from *Pseudomonas aeruginosa* and *Candida antarctica* B [12] do not have a ''lid'' and, therefore, lack interfacial activation. These enzymes constitute a bridge between lipases and esterases. Accordingly, lipases can be defined as esterases that are able to catalyze the hydrolysis of long-chain triacylglycerides. Lipase specificities and their potential application in lipid bioconversion were reviewed [13].

2 SPECIFICITY

Lipases can be divided generally into the following four groups according to their specificity in hydrolysis reaction.

2.1 Substrate-Specific Lipases

Lipases are defined by their ability to preferentially hydrolyze particular glycerol esters such as triglycerols, diglycerols, and monoglycerols and phospholipids. For example, during digestion, the hydrolysis of triacylglycerol (TAG) is incomplete. The resulting diacylglycerol (DAG) is transformed into monoacylglycerol (MAG), but the hydrolysis of the latter is very slow. Accordingly, TAG is the favored substrates for most animal, plant, and microbial lipases. However, a few lipases such as *Penicillium camembertii* are reported to hydrolyze partial glycerides faster than TAG [14,15]. Recently, a lipase from *Penicillium* sp. that discriminates against DAG has been reported [16].

2.2 Regioselective Lipases

Regioselectivity is defined as the ability of lipases to distinguish between the two external positions (primary ester bonds) and the internal position (secondary ester bond) of the TAG backbone. During lipolysis of TAG substrates, 1,3-regioselective lipases preferentially hydrolyze the *sn*-1 and *sn*-3 positions over the *sn*-2 position [17]. Examples of this type

are lipases from pig pancreas, *Aspergillus niger* [18], *Rhizopus arrhzus* [17], and *Mucor miehei* [17]. True *sn*-2-regioselective lipase is very rare. The only lipase reported in this category is from *Candida antarctica* A [19].

Many lipases display little, if any, regioselectivity and hydrolyze all ester bonds in TAG. Examples of these random specificity lipases are numerous and include the lipases from *Penicillium expansum* [20], *Aspergillus* sp. [21], and *Pseudomonas cepacia* [22].

2.3 Fatty-Acid-Specific Lipases

Lipases can be specific for particular fatty acids or, more generally, for a class of fatty acids. Such lipases will hydrolyze glyceride esters of these acids regardless of their position on the glycerol backbone. Examples of this type are lipases from *Penicillium roquefortii* [23] and premature infant gastric lipase [24,25], which are specific for short-chain fatty acids, and lipase from *G. candidum* [26], which is specific for *cis*-9-unsaturated fatty acids.

2.4 Stereospecific Lipases

This type of specificity is defined as the ability of lipases to distinguish between *sn*-1 and *sn*-3 position of TAG. Reports for this type of specificity are relatively recent. Examples of this type are human lingual [27], *C. antarctica* B, and dog gastric lipases [19] for *sn*-3 and *Humicola lanuginosa* and *Pseudomonas fluorescens* lipases [19] for *sn*-1-specific. Recently, Chandler et al. [28] described lipase-catalyzed synthesis of chiral triglycerides. Under certain reaction conditions, the acidolysis of tripalmitin with oleic acid using immobilized lipase from *R. miehei* resulted in a higher level of monosubstituted oleoyldipalmitoyl triglycerides. Chiral high-performance liquid chromatography (HPLC) analysis of the reaction products indicated that the enzyme was more active at the *sn*-1 position of the triglyceride. Lipases are also able to differentiate between enantioisomers of chiral molecules. This ability has recently become very important in producing pure chiral isomer as intermediates for drug synthesis.

3 INDUSTRIAL USES OF LIPASES

The use of lipases in oils and fats bioconversion has many advantages over classical chemical catalysts. Lipases operate under milder reaction conditions over a range of temperatures and pressures that minimize the formation of side products. The usefulness of microbial lipases in commerce and research stems from their physiological and physical properties [29]. In particular, large amounts of purified lipases are usually available, microbial lipases are generally more stable than animal or plant lipases, and microbial lipases have unique characteristics compared with plant and animal lipases. In either an aqueous or nonaqueous system, lipases catalyze many reactions: hydrolysis, ester synthesis, transesterification, and enantioresolution of esters. Yamane gave an engineering overview of lipase in the lipid industry [30] and a review on the application of lipase was also published [31]. Recently, a lipase-catalyzed reaction was demonstrated in supercritical fluid with immobilized *C. antarctica* lipase [32].

3.1 Hydrolysis of Esters

The Colgate–Emery Process operated at high temperature (250°C) and high pressure (50 atm) is used for industrial production of fatty acids from tallow. There have been many

attempts to replace this chemical process with a cleaner and milder bioprocess using lipases. For example, Miyoshi Oil Company in 1981 reported for the first time the lipase-catalyzed hydrolysis of oils for the production of soaps. It was reported that the process was not only cost-effective but the products produced were of high quality. However, as long as the cost of lipases is not reduced dramatically, there is no sign of using lipases in the large-scale hydrolysis of tallow. Small quantities of high-quality fatty acids are currently produced by lipases in batch processes. The hydrolysis of oils and fats has been carried out using lipase in an aqueous phase [33,34], or as an emulsion [33,35,36], or two phase [37], or reverse micelle [34,38]. Several articles have been published on the application of immobilized lipase for hydrolysis and esterification. Review articles on different aspects of immobilized lipase application were published by Balco et al. [39] and Malcata et al. [40]. Kimura et al. [41] have used lipase entrapped in a photo cross-linkable resin for the hydrolysis of olive oil. Covalent binding of lipase with polyacrylamide gel, nylon supports [42], and activated silica [43] have also been reported. Immobilization by adsorption of lipase on hydrophobic [44–46] and hydrophillic [44] matrices have also been reported. Continuous hydrolysis of oil in a fluidized-bed reactor [47] and a loop reactor fitted with an oil–water separator [48] and in a countercurrent reactor were also studied. Lipase immobilized on polypropylene beads was used for studying hydrolysis of animal fats on an industrial scale [49].

Lipases are also used in the concentration of γ-linolenic acid from borage oil [50]. With *Candida rugosa* lipase, Shimada et al. [51] reported that production of γ-linolenic acid from borage oil at greater than 49% yield in a reaction mixture containing 90% water. γ-Linolenic acid was also produced from borage oil on a large scale [52]. Furthermore, a two-step enzymatic procedure for the isolation of erucic acid from rapeseed oil based on chain-length discrimination of this lipase has been developed [53].

Flavors for use in foods for human and animal consumption have been changed and/or enhanced by the lipase-catalyzed partial hydrolysis of triglycerides [54,55]. Commercial processes include the enzymatic modification of milkfat [56,57], as well as the development of a number of enzyme preparations for use in the manufacture of Italian [58,59], American [60,61] and cheddar cheeses [62]. Examples of patented lipolyzed flavors and processes for their manufacture include lipolyzed milk compositions [63] and lipolyzed milkfat products, such as butter flavors [64,65], cultured cream flavors [66], blue cheese flavors [67], and cheeselike flavors [68,69].

In leather manufacture, lipase is used in the processing of hides and skins to remove residual fats. It is now a common practice to utilize a mixture of lipases and proteases for this purpose.

In waste treatment, lipases are utilized in activated sludge and other aerobic waste processes, where thin layers of fats must be continuously removed from the surface of aerated tanks to permit oxygen transport.

3.2 Synthesis of Esters

For lipase-catalyzed synthesis and interesterification reactions, a given water activity is needed to obtain good lipase activity and minimize competitive hydrolysis reactions.

Monoacylglycerol is one of the most used Food and Drug Administration (FDA)-approved emulsifiers in food, cosmetics, and drug industries. Total world consumption is estimated at 60,000 tons annually. Currently, MAG is produced from tallow and glycerol

in a continuous process using an alkali catalyst at a high temperature (220°C). The yield is 40–50%. Because of high temperature, the product MAG tends to have decoloration and burnt odor. Reports [70] on the production of MAG by using lipases include (1) synthesize MAG from fatty acid and glycerol using high-specificity lipase from *Penicillium camembertii*, (2) blockage of two of the three hydroxy groups of glycerol with acetol first and then reacting with fatty acid in the presence of lipase to produce MAG, and (3) mixing tallow, glycerol, and *P. fluorecens* lipase at low temperature. Process (3) with 70–90% yields is the best one for industrial production of MAG so far [71,72]. Commercial lipases were also screened for their production of monoacylglycerols and diacylglycerols [73]. The ester synthesis activity of the commercially available lipases can be summarized as follows: Bacterial lipases such as *Pseudomonas* sp. lipases show highest activity, fungal lipases such as *Mucor* sp. show lower activity, and yeast lipases such as *Candida* lipases show negligible activity [70]. The reason for this is still unknown.

Lipases have been shown to have a catalytic function in esterifying various alcohols with fatty acids [74]. Lipase-catalyzed synthesis of sugar esters is also known. A regioselective synthesis of 6′-*O*-acyl sucrose monoesters has been developed through the lipase-catalyzed esterification of sucrose acetals with fatty acids in both organic solvents and under a solvent-free condition [75]. The products were obtained in overall yields of 20–27%. Although the cost of lipase-catalyzed esterification typically remains too high for the manufacturing of many bulk products, the synthesis of several speciality esters has found its way into the market. Unichem International produced isopropyl myristate, isopropyl palmitate, and 2-ethylhexyl palmitate [76] for use as personal-care products.

3.3 Transesterification (Interesterification)

Geraniol ester is one of the most important natural fragrances. Traditional methods, such as extraction from plant materials and direct biosynthesis by fermentation, are used for flavor and fragrance production. However, these methods exhibit a high cost of processing and a low yield of the desired flavor component. Synthesis of geranyl acetate by lipase-catalyzed transesterfication (*Mucor miehei* lipase) in hexane could reach 85% yield after 3 days of reaction. Enzymatic synthesis of geranyl acetate in hexane was also studied with *C. antactica* lipase [77] and with *Pseudomonas* sp. lipase immobilized on glass beads, which achieved a yield of up to 97% [78].

Macrocyclic lactones (C14 to C16) are high-grade, expensive aromatic substances of musky fragrance. Synthetic chemical methods used so far have been based on polycondensation of ω-polyhydroxycarboxylic acids to respective polyesters and then on catalytic-thermal depolymerization and cyclization at high temperature and under vacuum. These methods are technically arduous because of the necessity of using high temperatures and high vacuum. Lipases have been used to produce macrocyclic lactones. Examples are as follows: (1) Lipase from *Pseudomonas* sp. and porcine pancreas catalyzed the lactonization of methyl esters of ω-hydroxy acids with C12–C16, producing high yields of monolactones and dilactones [79]; (2) porcine pancreatic lipase lactonized various γ-hydroxy acid esters with high yield and high enantioselectivity [80]; (3) lipases from *Candida cylindracea, Pseudomonas* sp., and porcine-pancreas-esterified dicarboxylic acids and diols with various carbon chain lengths to macrocyclic lactones; and (4) lipase from *Mucor javanicus* catalyzed the lactonization of 15-hydroxypentadecanoic and 16-hydroxyhexadecanoic acids to macrocyclic lactones.

Cocoa butter is the most expensive triglyceride and consists of saturated fatty acid such as stearic at its 1,3-positions and an unsaturated fatty acid at its 2-position (SUS). The most important features of cocoa butter, vital for its use and responsible for its unique sensory characteristic, are its crystal structure and a very sharp melting profile between 25°C and 35°C. Thus, it is a brittle solid at room temperature, but melts completely just below body temperature, leaving no ''greasy'' sensation in the mouth. The potential of 1,3-specific lipases for the manufacture of cocoa butter substitutes was clearly recognized about two decades ago when Unilever [81] and Fuji Oil [82] filed their patent applications. In both cases, the process relied on lipase-catalyzed transesterification or acidolysis of inexpensive oil such as palm midfraction, which contains a significant amount of 1,3-dipalmitoyl-2-oleoyl-glycerol with tristearin or stearic acid, respectively. For example, palm oil and stearic acid or tristearylglycerol were interesterified with a 1,3-specific lipase from *A. niger* or *Rhizopus japonicus* as a catalyst to produce the high-value cocoa butter [83]. A fat very similar to cocoa butter in its chemical composition was manufactured by lipase-catalyzed modification of olive oil and palm oil with saturated fatty acids [84]. The purpose of the lipase-catalyzed reaction is to introduce more stearate into the triglycerides by interesterification producing 1-palmitoyl-2-oleoyl-3-stearoyl-glycerol (POS) or 1,3-distearoyl-2-oleoyl-glycerol (SOS). The cocoa butter equivalent can be used in the chocolate and confectionery industry.

The following is a typical process [70]. *Rhizopus chinensis* was grown on porous polyurethane particles. The mycelium-covered particles were collected, washed with acetone, and dried to form immobilized cells (lipase). These immobilized cells were used to produce SOS at 40% yields from olive oil and methyl stearate by interesterification. When this process was operated at less than 100 ppm water content, the half-life of the biocatalyst (lipase) was 1.7 months. Synthesis of cocoa butter equivalent was also conducted in supercritical fluid [85].

3.4 Synthesis of Structured Lipids

1,3-Regioselective lipases have been used widely on an industrial scale to obtain new fats with nutritionally improved properties. Human milkfat equivalent was synthesized by interesterifying tripalmitin with polyunsaturated fatty acid (PUFA). The result was a TAG rich in palmitic acid in position *sn*-2 and with PUFA on the 1,3-positions. Human milkfat substitute was also produced solely from vegetable sources for infant formula. The product with a saturated fatty acid (palmitic) at the *sn*-2 position and oleic acid at both 1 and 3 positions were produced from palm oil and oleic acid through interesterification using lipases [86–88]. TAG with a saturated fatty acid in the *sn*-2 position was shown to improve digestibility and enhance the absorption of other nutrients.

Similarly, TAG with PUFA at the *sn*-2 position and medium-chain fatty acids at the 1,3-positions can be produced by interesterification using lipases and was shown to be more rapidly absorbed than natural TAG. Short-chain preference lipases may be used in the production of low-calorie-structured TAG. The short-chain specificity of *C. antarctica* may prove useful in interesterification reactions to increase the ration of medium-chain TAG in different oils [89]. *R. miehei* lipase was used in the synthesis of position-specific low-calorie-structured lipids by interesterification of tristearin and tricaprin. The resulting structured lipids have their specific fatty acids at the *sn*-1,3 position.

The production of structured lipids containing medium-chain caprylic acid by immobilized *Rhizopus delemar* lipase was reported by Shimada et al. [90]. Using commercial

lipases from various microbial organisms, Kwon et al. [91] synthesized medium-chain glycerides, such as monocaprin, dicaprin, and tricaprin in iso-octane from glycerol and capric acid. Recently, structured lipids have been synthesized using immobilized lipase [92]. The resulting lipid was enriched with eicosapentaenoic acid (EPA) at specific positions.

Lipases were also applied to the interesterification of palm oil and soy oil in an effort to reduce the solid content of palm oil [93].

3.5 Improved PUFA Content in Fish Oil

The worldwide production of fish oil is around 1.6 million tons annually. Most of the fish oil is hydrogenated for use in margarine and shortening. However, fish oil has the highest n-3 polyunsaturated fatty acid (*n*-3 PUFA) content (20–30%) among all kinds of oils. Recently, n-3 PUFA, such as EPA and docosahexaenoic acid (DHA), were proved to have many physiological activities (anti-blood-coagulation, enhanced memory, etc.) [94,95]. Physical methods, such as solvent fractionation and winterization, can increase *n*-3 PUFA content up to 40% in fish oil. With this limitation, many companies developed bioprocesses to concentrate *n*-3 PUFA in fish oil; for example, a two-step process was developed to concentrate *n*-3 PUFA [96]. Through selective hydrolysis by lipase, fish oil was converted to partial glycerides and free fatty acids. Partial glycerides were esterified with free PUFA by lipase to produce PUFA-enriched fish oil. Another example is that PUFA-enriched fish oil was produced by urea adduct and acidolysis or interesterification using lipases [97]. PUFA was also concentrated from fish oil by transesterification of fish oil with ethanol using *Pseudomonas* lipase [98]. Haraldsson et al. tested 17 lipases and found that *Pseudomonas* lipase had the highest activity toward the saturated and monounsaturated fatty acids in the fish oil, much lower activity toward EPA and DHA, and, at the same time, good tolerance toward the anhydrous alcoholic conditions. They obtained a mixture comprised of approximately 50% EPA + DHA.

McNeill et al. [99] used lipases from *C. rugosa* and *G. candidum* to hydrolyze fish oil and obtained an increase in the content of total ω-3 acids from about 30% in the feed oil to 45% in the partial glycerides. The lipase from *C. rugosa* was effective in selectively enriching either DHA or EPA, resulting in a change of either the DHA/EPA ratio or the EPA/DHA ratio from approximately 1:1 to 5:1. Homogeneous triglycerides containing solely EPA or DHA were synthesized by using *C. antarctica* lipase [100]. The ability of *R. miehei* and *C. cylindracea* lipases to discriminate against the n-3 family of PUFA has been used for the selective harvesting of PUFA from fish oil [101,102].

3.6 Enantioresolution of Esters

One important property of lipases is their ability to differentiate between enantiomers of chiral molecules. Microbial lipases are increasingly used in kinetic resolution of chiral compounds that serve as synthons in the synthesis of chiral pharmaceuticals and agrochemicals. Hydroxy acids and their derivatives are major target molecules because they constitute the framework of many chiral natural products and biologically active agents. Lipases have also been widely used for the resolution of racemic alcohols and carboxylic acids through asymmetric hydrolysis of the corresponding esters. There has been an explosion in the number of patents and publications over the past few years. The following are some examples of industrial and potential industrial uses of lipases in the production of enantioselective isomers.

Lipase from *C. cylindracea* catalyzes the acidolysis between racemic 2-methylalkanoates and fatty acids in heptane with a preference for the (*S*)-configured esters [103]. The enzymatic enantioselective resolution of 2-substituted propionic acids has been the subject of intense investigation. Much of this effort has centered on the production of (*R*)-2-chloro-propionic acid due to its high value as an intermediate in the synthesis of a number of commercially important herbicides. Chemie Linz Co. (Austria) under a license from the Massachusetts Institute of Technology [104] carried out the production on a 100-kg scale. Typically, greater than 99% enantiomeric excess (e.e.) is obtained at 75% of the theoretical yield, and the resolution is complete in several hours. A substantial body of literature also exists on the production of (*S*)-2-arylpropionic acids, which are valuable as anti-inflammatory agents [105–107].

Lipases are used in the synthesis of chiral synthon as intermediates for the synthesis of paclitaxel (taxol). Taxol, a complex polycyclic diterpene, exhibits a unique mode of action on microtubule proteins that are responsible for the formation of the spindle during cell division. Taxol has been used to treat various cancers, especially ovarian cancer. Currently, taxol is produced from extracts of the bark of a Pacific yew tree by a cumbersome purification process. Alternative methods produce the chiral intermediate, 3(*R*)-*cis*-acetyloxy-4-phenyl-2-azetidinone, using lipases from *P. cepacia* and another *Pseudomonas* sp. Both lipases achieve over 95% yield and 99.4% optical purity [108].

Lipases are used in the synthesis of a lactol, [3*aS*-(3*a*α,4α,7α,7*a*α)]-hexahydro-4,7-epoxy-isobenzo-furan-1-(3*H*)-one, which is a key chiral intermediate for the total synthesis of a new cardiovascular agent useful in the treatment of thrombolic disease [108]. *P. fluorescens* and *P. cepacia* lipase-catalyzed reactions achieved a greater than 85% yield and 97% optical purity of the chiral intermediate for the synthesis of a thromboxane A2 antagonist.

Lipases are also used in the production of an intermediate for the synthesis of an antihypertensive drug. Captopril is designated chemically as 1-[(2*S*)-3-mercapto-2-methylpropionyl]-L-proline. Its *S* configuration compound is 100 times more active than its corresponding *R* enantiomer. Captopril prevents the conversion of angiotension I to angiotensin II by inhibition of an angitensin-converting enzyme. Immobilized lipases from both *P. cepacia* and a *Pseudomonas* sp. catalyzed the production in methanol of the *S* isomer at greater than 32% yields and 96% optical purity [108].

Immobilized *P. cepacia* lipase was used in organic solvent for the selective acylation of a key alcohol intermediate, which is used for the synthesis of Camptosar, a drug used in the treatment of ovarian cancer [109]. A yield of greater than 46% conversion and 0.79 e.e. was achieved.

Lipases are also used in the production of stereospecific isomer for the production of β-blockers. A β-blocker is a common name for a group of antihypertensive and cardiovascular drugs which contain an aryloxypropanolamine structure with an asymmetric carbon. There are over 24 drugs containing this type of moiety, with over 3 billion dollars sales annually. Traditionally, racemic arylpropanolamine was used in the synthesis. Recently, due to social and economic demand, only the physiologically active pure (*S*) enantiomer is used for the synthesis of the β-blocker. Both lipases and esterases are used in this respect [110]. Almost every pharmaceutical company has its own lipase process in producing synthons for the synthesis of their patented drugs. With the rapid progress in molecular modeling of protein three-dimensional structure and molecular biology, within a few years it is possible to have ''tailor-made'' lipases with improved properties in activity, stability, and designed specificity.

REFERENCES

1. Y Kokusho, H Machida, S Iwasaki. Agric Biol Chem 46:1159–1164, 1982.
2. H Machida, T Higashi, Y Kokusho. Nippon Nogei Kagaku Kaishi 58:799–804, 1984.
3. Y Wang, BB Saha. J Am Oil Chem Soc 70:1135–1138, 1993.
4. CT Hou, TM Johnston. J Am Oil Chem Soc 69:1088–1097, 1992.
5. CT Hou. J Ind Microbiol 13:242–248, 1994.
6. CT Hou. J Am Oil Chem Soc 74:1391–1394, 1997.
7. AC Lanser, LK Manthey, CT Hou. Characterization of new bacterial lipases. 90th AOCS Annual Meeting, Orlando, FL, 1999, Abst Bio-P6.
8. P Johann, P Razor. In: Proceedings of ''Chiral-92 Symposium.'' Manchester, UK: Spring Innovation Ltd., 1992, pp 47–50.
9. L Alberghina, RD Schmid, R Verger, eds. Lipases: Structure, Mechanism and Genetic Engineering. New York: VCH, 1991.
10. FK Winkler, A d'Aray, W Hunziker. Nature 343:771–774, 1990.
11. KE Jaeger, S Ranasac, BW Dijkstra, C Colson, M Vanheuvel, O. Misset. FEMS Microbiol Rev 15:29–63, 1994.
12. M Martinelle, M Holmquist, K Hult. Biochim Biophys Acta 1258:272–276, 1995.
13. TA Foglia, P Villeneuve. INFORM 8:640–650, 1997.
14. S Yamaguchi, T Mase. Appl Microbiol Biotechnol 34:720–725, 1991.
15. H Tornquist, P Blefrage. J Biol Chem 251:813–819, 1976.
16. K Gulomova, E Ziomek, JD Schrag, K Davranov, M Cygler. Lipids 31:375–384, 1996.
17. M Semeriva, G Benzonna, P Desnuelle. Bull Soc Chim Biol 49:71–81, 1967.
18. S Okumura, M. Iwai, Y Tsujisaka. Agric Biol Chem 40:655–660, 1976.
19. E Rogalska, C Cudrey, F Ferrato, R Verger. Chirality 5:24–30, 1993.
20. W Stocklein, H Sztajer, U Menge, RD Schmidt. Biochim Biophys Acta 1168:81–189, 1993.
21. X Fu, X Zhu, K Gao, J Duan. J Am Oil Chem Soc 72:527–531, 1995.
22. PE Sonnet, TA Foglia, SH Fearheller. J Am Oil Chem Soc 70:381–391, 1993.
23. T Mase, Y Matsumiya, A Matsuura. Biosci Biotechnol Biochem 59:329–330, 1995.
24. RG Jensen, FA Djong, LG Lambert Davis, M Hamosh. Lipids 29:433–435, 1994.
25. RH Barton, CJ O'Connor, KW Turner. J Dairy Sci 79:27–32, 1996.
26. RG Jensen, FA Dejong, RM Clark. Lipids 18:239–252, 1983.
27. RG Jensen, FA Dejong, RM Clark, L Palmgren, TH Liao, M Hamosh. Lipids 17:570–572, 1982.
28. IC Chandler, PT Quinlan, GP McNeill. J Am Oil Chem Soc 75:1513–1518, 1998.
29. AR Marcrae, RC Hammond. Biotechnol Genet Eng Rev 3:193–217, 1985.
30. T Yamane. J Am Oil Chem Soc 64:1657–1662, 1987.
31. NN Gandhi. J Am Oil Chem Soc 74:621–634, 1997.
32. MA Jackson, JW King. J Am Oil Chem Soc 74:103–106, 1997.
33. Y Kosugi, H Suzuki, T Funada. Biotechnol Bioeng 31:349–356, 1988.
34. D Jan, JS Ree. Biotechnol Bioeng 28:1250, 1986.
35. WM Linfield, DJ O'Brian, S Serota. J Am Oil Chem Soc 61:1067–1071, 1984.
36. YJ Wang, FF Wang, JY Sheu, JF Shaw. Biotechnol Bioeng 31:628–633, 1988.
37. KH Kim, DY Kwon, JS Rhee. Lipids 19:975–977, 1984.
38. F Yang, AJ Russell. Biotechnol Bioeng 47:60–70, 1995.
39. VM Balco, AL Paiva, FX Malcata. Enzyme Microbiol Technol 18:392–416, 1996.
40. FX Malcata, HR Reyes, HS Garcia, CG Hill Jr, CH Amundson. J Am Oil Chem Soc 67: 890–910, 1990.
41. Y Kimura, A Tanaka, K Sonomoto, T Nihara, S. Fukui. Eur J Appl Microbiol Biotechnol 17:107–112, 1983.
42. G Carta, JL Gainer, ME Gibson. Enzyme Microbiol Technol 14:904–910, 1992.
43. MB tark, K Holmberg. Biotechnol Bioeng 34:942–950, 1989.

44. E Lie, A Fersson, G Molin. Appl Microbiol Biotechnol 35:19–20, 1991.
45. JF Shaw, RC Chang, FF Wang, YJ Wang. Biotechnol Bioeng 35:132–137, 1990.
46. Y Kosugi, H. Tanaka, N. Tomizuka. Biotechnol Bioeng 36:617–622, 1990.
47. Y Kosugi, K Takahashi, C Lopez. J Am Oil Chem Soc 72:1281–1285, 1995.
48. Y Kpsugo, N Tomozuka. J Am Oil Chem Soc 72:1329–1332, 1995.
49. MD Virto, S Montero, A Balco, LC Landeta, I Agud, R Solozabal, JM Lascaray, M deRenobales, MJ Llama, JL Serra. Enzyme Microbiol Technol 16:61–65, 1994.
50. TA Foglia, PE Sonnet. J Am Oil Chem Soc 72:417–420, 1995.
51. Y Shimada, N Fukushima, H Fujita, Y Honda, A Sugihara, Y Tominaga. J Am Oil Chem Soc 75:1581–1586, 1998.
52. Y Shimada, N Sakai, A Sugihara, H Fujita, Y Honda, Y Tominaga. J Am Oil Chem Soc 75:1539–1544, 1998.
53. PE Sonnet, TA Foglia, SH Fearheller. J Am Oil Chem Soc 70:387–391, 1993.
54. JH Nelson. J Am Oil Chem Soc 49:559–562, 1972.
55. KM Shahani, RG Arnold, A Kilara, BK Dwivedi. Biotechnol Bioeng 18:891, 1976.
56. HE Otting (to M & R Dietetic Labs Inc.). US Patent 1,966,460, 1934.
57. HE Otting (to M & R Dietetic Labs Inc.). US Patent 2,169,278, 1939.
58. MG Farnham (to Dairyland Food Laboratories Inc.). US Patent 2,531,329, 1950.
59. MG Farnham (to Dairyland Food Laboratories Inc.). US Patent 2,794,734, 1957.
60. Kraftco Corp. British Patent 1,240,345, 1971.
61. MJ Roberts (to Kraftco Corp.). US Patent 3,650,768, 1971.
62. MV Arbige, PR Freund, SC Silver, JT Zelko. Food Technol 40:91, 1986.
63. CA Kempf, JS Niedlinger, AP Stewart, PF Sharp (to Golden State Co. Ltd.). US Patent 2,638,418, 1953.
64. Morton International Inc. British Patent 1,251,272, 1971.
65. AC Buhler, E Engel, JH Nelson, CH Amundson (to Morton-Norwich Products). US Patent 3,653,921, 1972.
66. DJ Pangier (to Miles Laboratory Inc.). US Patent 3,469,993, 1969.
67. SC Knight. US Patent 3,100,153, 1963.
68. WS Clus (to Carnation Co.). US Patent 3,190,753, 1965.
69. JD Johnson, DL Southworth (to Kraftco Corp.). US Patent 3,780,182, 1973.
70. T Yamane. Yukagaku 40:193–201, 1991.
71. GP McNeill, S Shimizu, T Yamane. J Am Oil Chem Soc 68:1–5, 1991.
72. GP McNeill, T Yamane. J Am Oil Chem Soc 68:6–10, 1991.
73. B Yang, WJ Harper, KL Parkin, J Chen Int Diary J 4:1–13, 1994.
74. S Basheer, M Nakajima, U Cogan. J Am Oil Chem Soc 73:1475–1479, 1996.
75. DB Sarney, MJ Barnard, DA McManus, EN Vulfson. J Am Oil Chem Soc 73:1481–1487, 1996.
76. A Macrae, EI Rochl, HM Brand. Drug Cosmetic Ind 147:36–39, 1990.
77. PA Claon, CC Akoh. J Am Oil Chem Soc 71:575–578, 1994.
78. LN Yee, CC Akoh. J Am Oil Chem Soc 73:1379–1384, 1996.
79. A Makita, T Nihira, Y Yamada. Tetrahedron Lett 28:805–808, 1987.
80. A Gutman, K Zuobi, A Boltansky. Tetrahedron Lett 28:3861–3964, 1987.
81. MH Coleman, AR Macrae (to Unilever Co.). UK Patent 1,577,933, 1980.
82. T Matsuo, N Sawamura, Y Hashimoto, W Hashida (to Fuji Oil Co.). European Patent 0 035 883, 1981.
83. AR Macrae, P How. UK Patent Appl. GB2119397A1, November 16, 1983.
84. Y Ota, M Dantsuji. J Jpn Oil Chem Soc 37:38–44, 1988.
85. KJ Liu, HM Cheng, RC Chang, JF Shaw. J Am Oil Chem Soc 74:1477–1482, 1997.
86. P Quinlan, R Moore. INFORM 4:580–585, 1993.
87. DM King, FB Padley. European Patent 0 209 327, 1990.
88. U Schmid, UT Bornscheuer, MM Soumanou, GP McNeill, RD Schmid. J Am Oil Chem Soc 75:1527–1531, 1998.

89. R Jandacek, JA Whiteside, BN Holcombe, RA Volpenheim, JD Taubee. Am J Clin Nutr 45: 940–945, 1987.
90. Y Shimada, A Sugihara, H Nakano, T Yokota, T Nagao, S Komemushi, Y Tominaga. J Am Oil Chem Soc 73:1415–1420, 1996.
91. DY Kwon, HN Song, SH Yoon. J Am Oil Chem Soc 73:1521–1525, 1996.
92. CC Akoh. INFORM 6:1055–1061, 1995.
93. J Graille, M Pina, D Montet, JM Muderwha. Elaeis 4:1–10, 1992.
94. JE Kinsella, B Lokesh, RA Stone. Am J Clin Nutr 52:1–28, 1990.
95. V Young. Lipid Technol 2:7–10, 1990.
96. Y Tanaka, S Imamura, Y Kosugi. In: Food Industry Bioreactor Systems Technical Research Group, ed. Food Chemistry News, P391, 1990.
97. GG Haraldsson, PA Hoskuldsson, ST Sigurdsson, F Thorsteinsson, S Gudbjarnason. Tetrahedron Lett 30:1671–1674, 1989.
98. GG Haraldsson, B Kristinsson, R Sigurdardottir, GG Gudmundsson, H Breivik, J Am Oil Chem Soc 74:1419–1424, 1997.
99. GP McNeill, RG Ackman, SR Moore. J Am Oil Chem Soc 73:1403–1407, 1996.
100. GG Haraldsson, BO Gudmundsson, O Almarsson. Tetrahedron 51:941–952, 1995.
101. MJ Hills, I Keiwitt, KD Mukherjee. J Am Oil Chem Soc 67:561–567, 1990.
102. G Haraldsson, B Kristinsson. J Am Oil Chem Soc 75:1551–1556, 1998.
103. KH Engel. J Am Oil Chem Soc 69:146–150, 1992.
104. G Kirchner, MP Scollar, AM Klibanov. J Am Chem Soc 107:7072–7076, 1985.
105. MJ Barton, JP Hamman, KC Fichter, GJ Carlton. Enzyme Microbiol Technol 12:577–583, 1990.
106. K Yamamoto, Y Ueno, K Otsubo, K Kawakami, K Komatsu. Appl Environ Microbiol 56: 3125–3129, 1990.
107. CJ Sih, QM Gu, G Fulling, SH Wu, DR Reddy. Dev Ind Microbiol 29:221–229, 1988.
108. RN Patel, A Banerjee, LJ Szarka. J Am Oil Chem Soc 73:1363–1375, 1996.
109. JC Sih. J Am Oil Chem Soc 73:1377–1378, 1996.
110. M Kloosterman, VHM Elferink, J van Lersel, JH Roskan, EM Meijer, LA Hulshof, RA Sheldon. Trends Biotech 6:251–256, 1988.

19

Plant Lipases as Biocatalysts

KUMAR D. MUKHERJEE

Institute for Biochemistry and Technology of Lipids, H. P. Kaufmann-Institute, Federal Center for Cereal, Potato and Lipid Research, Münster, Germany

1 INTRODUCTION

Triacylglycerol acylhydrolases (E.C. 3.1.1.3) that hydrolyze the ester bonds of storage triacylglycerols of seeds, such as oilseeds and cereal grains, constitute a major group of plant lipases. Further lipases occurring in diverse plant tissues include nonspecific lipid acylhydrolases exhibiting combined action of phospholipases A1 (E.C. 3.1.1.32), A2 (E.C. 3.1.1.4), B (E.C. 3.1.1.5), glycolipase, sulfolipase and monoacylglycerol lipase, and phospholipases C (E.C. 3.1.4.3) and D (E.C. 3.1.4.4). Several reviews have extensively covered the occurrence, properties, and physiological role of various lipases in plants [1–6]. Sources of lipases from plant tissues, techniques for their isolation and purification, as well as their properties are well documented [7,8]. This chapter will focus on the application of lipases from plants as catalysts for biotransformation of lipids.

2 CHARACTERISTICS AND APPLICATIONS OF PLANT LIPASES AS BIOCATALYSTS

As early as in the beginning if this century, lipase preparations obtained from plants have been used as catalysts for hydrolysis and esterification of lipids [9,10]. Several publications have appeared in later literature [11–15] on the isolation of lipases from plant tissues for their possible applications in biotransformations. Although the purification of plant lipases to homogeniety is time-consuming, preparations with good lipolytic activity are relatively easy to isolate from plant tissues [12] and pronounced substrate specificities of such biocatalysts [6] can be utilized in lipid biotechnology [16].

2.1 Triacylglycerol Lipases

Germination of oil-rich seeds and cereal grains is accompanied by hydrolysis of the storage triacylglycerols to fatty acids and glycerol by the action of the endogenous triacylglycerol lipase, and the products of lipolysis are metabolized further in glyoxysomes and other organelles of the seedling. Especially, the seedlings of germinating oilseeds are rich sources of triacylglycerol lipases that have been isolated, partially purified, and used as a catalyst for biotransformation of lipids [7,8].

2.1.1 Triacylglycerol Lipases from Oilseeds

Biocatalysts with good lipolytic activity are easily obtained from germinating cruciferous oilseeds, such as rape (*Brassica napus*) and mustard (*Sinapis alba*), simply by homogenization of the seedlings in a suitable buffer followed by centrifugation; the supernatant contains the bulk of the lipase activity [12]. Such lipase preparations or their ''acetone powder'' preparations (i.e., acetone-insoluble residue obtained by extraction of the seedling homogenates with chilled acetone) catalyze the hydrolysis of oils (e.g., sunflower oil) with preferential cleavage of fatty acids esterified at the *sn*-1,3 positions of the triacylglycerols [12].

Crude homogenate in Tris-HCl buffer (pH 8.0) [17] or acetone powder [18] isolated from germinating seedlings of low-erucic rape catalyzes the hydrolysis of the exogenous triacylglycerols of low-erucic rapeseed oil at a similar rate. Using the crude homogenate of rape seedlings as a biocatalyst, good rates of hydrolysis of the exogenous triacylglycerols are found at day 4 of germination, pH 8.0, and at a temperature of 30°C [17].

The endogenous oil (i.e., storage triacylglycerols contained in the seeds of low-erucic rape) has been efficiently hydrolyzed by homogenization of the germinating seedlings in Tris-HCl buffer (pH 8.0) followed by incubation at ambient temperature [17]. The fatty acids formed undergo very little β-oxidation and they can be recovered from the homogenate by solvent extraction or centrifugation [17]. These findings show an alternative mild biotechnological approach for the production of fatty acids from seed oils via lipolysis of the seed storage triacylglycerols in situ, as opposed to conventional fat splitting at high temperatures and pressures.

Lipase contained in the crude homogenates of germinating rape seedlings catalyzes the hydrolysis of the triacylglycerols of various oils in the following order: low-erucic and high-erucic rapeseed oil > linseed oil > *Hydnocarpus wightiana* oil > borage oil > hake (*Merluccius hubbsi*) liver oil > castor oil > coriander oil; however, very little hydrolysis of the wax esters contained in jojoba oil and orange roughy (*Hoplostethus atlanticus*) oil takes place [19].

Partially purified lipase from rape seedlings has been immobilized on Celite [20,21] and some unique substrate specificities of such preparations in hydrolysis and esterification have been reported [22,23]. Thus, in esterification with *n*-butanol, the rapeseed lipase discriminates against fatty acids having a *cis*-6 double bond [e.g., petroselinic (*cis*-6-octadecenoic), γ-linolenic (all-*cis*-6,9,12-octadecatrienoic), and stearidonic (all-*cis*-6,9,12,15-octadecatetraenoic) acid] or a *cis*-4 double bond (e.g., all-*cis*-4,7,10,13,16,19-docosahexaenoic acid) [22]. In analogy, tripetroselinin and tri-γ-linolenin are hydrolyzed by rapeseed lipase at much lower rates than triolein [22].

Some of the above fatty acid specificities are also exhibited by the crude homogenate from rape seedlings [19]. Thus, in the hydrolysis of borage oil, coriander oil, *Hydnocarpus wightiana* oil, and hake liver oil, the lipase from rape seedlings discriminates against

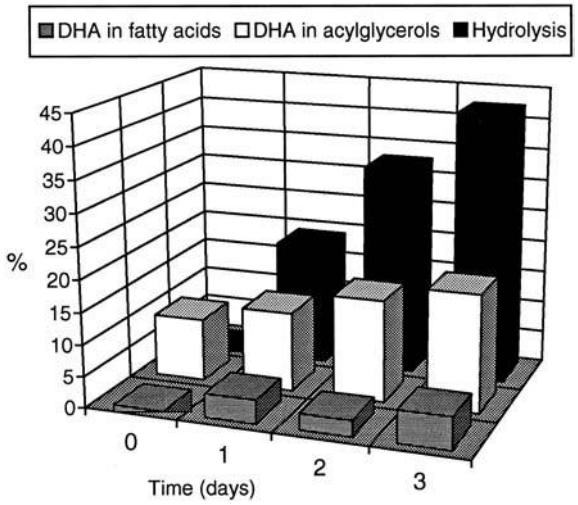


Figure 1 Content of docosahexaenoic acid (DHA) in fatty acids and acylglycerols during hydrolysis of hake liver oil, catalyzed by lipase in rape seedling homogenate. (Adapted with permission from Ref. 19, copyright [1995] American Chemical Society.)

the characteristic acyl constituents of these oils [i.e., γ-linolenoyl, petroselinoyl, gorlioyl (13-[cyclopent-2-en-1-yl]tridec-6-enoic) and docosahexaenoyl moieties, respectively, which are acyl moieties having Δ^4 or Δ^6 *cis*-double bonds]; therefore, these acyl moieties are enriched in the unhydrolyzed triacylglycerols as well as diacylglycerols and monoacylglycerols [19]. Figures 1 and 2 show, for example, that during the hydrolysis of hake liver oil and coriander oil, catalyzed by rape seedling homogenate, some enrichment

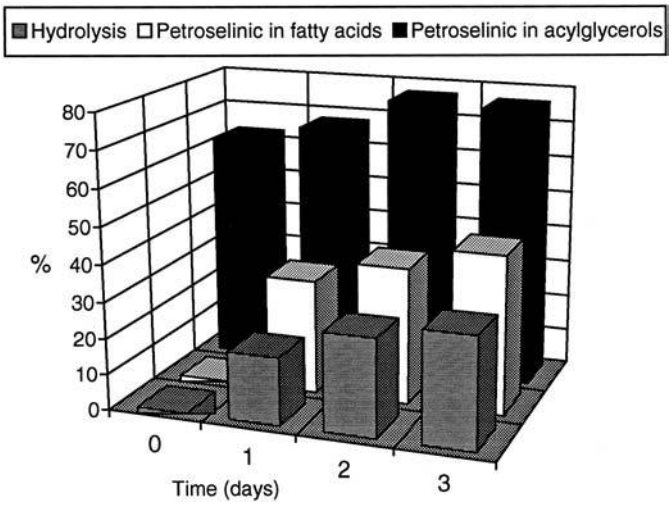


Figure 2 Content of petroselinic acid in fatty acids and acylglycerols during hydrolysis of coriander oil, catalyzed by lipase in rape seedling homogenate. (Adapted with permission from Ref. 19, copyright [1995] American Chemical Society.)

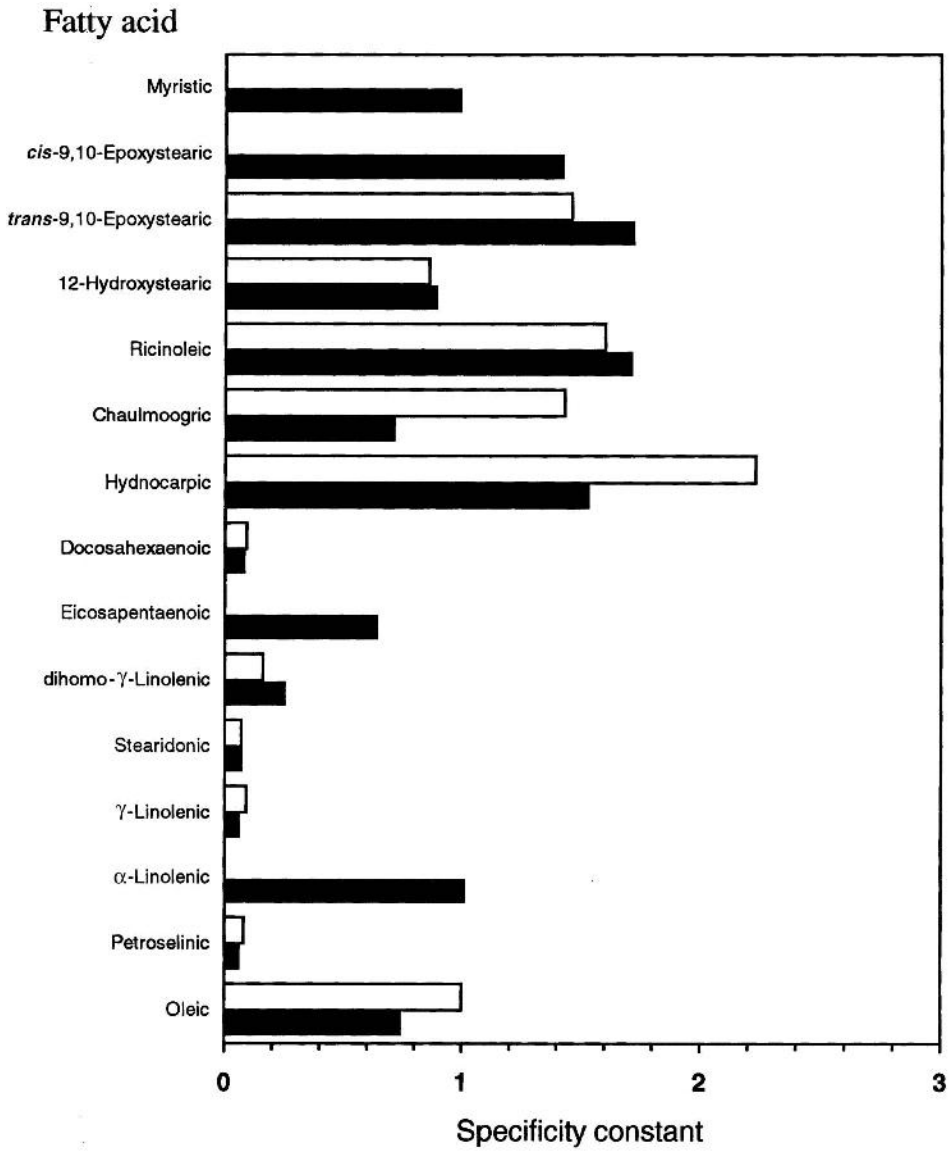


Figure 3 Substrate selectivity (specificity constant) of individual fatty acids in the esterification with *n*-butanol using rapeseed lipase (white bar) and papaya lipase (black bar) as biocatalysts [specificity constant of the reference substrates oleic acid (rapeseed) and myristic acid (papaya) = 1.00]. (Adapted with permission from Ref. 24 copyright [1996] Springer Verlag and from Ref. 25 copyright [1996] American Chemical Society.)

of docosahexaenoic acid and petroselinic acid, respectively, in the acylglycerols takes place.

Acetone powders from germinating rape seedlings catalyze the esterification of fatty acids (e.g., oleic acid with long-chain alcohols, such as oleyl alcohol, and with short-chain alcohols, *n*-butanol and interesterification of methyl oleate with erucic acid) [18]. Figure 3 shows the specificity constants of individual fatty acids in the esterification with *n*-butanol, catalyzed by lipase in acetone powder from rape seedlings. In esterification reactions with *n*-butanol, fatty acids having Δ^4, Δ^6, and Δ^8 *cis* double bonds [e.g., petroselinic, γ-linolenic, stearidonic, dihomo-γ-linolenic (all-*cis*-8,11,14-eicosatrienoic), docosahexaenoic, and gorlic acids) are strongly discriminated against, whereas most of the common long-chain fatty acids, such as oleic acid, are very well accepted as substrates; less common fatty acids, such as ricinoleic (12-hydroxy-*cis*-9-octadecenoic), 12-hydroxystearic, *trans*-9,10-epoxystearic, chaulmoogric (13-[cyclopent-2-en-1-yl]tridecanoic), and hydnocarpic (11-[cyclopent-2-en-1-yl]undecanoic) acids, are distinctly preferred as substrates over oleic acid (Fig. 3) [24]. This contrasts with the observed specificity of rapeseed lipase in the hydrolysis of castor oil [19]. The above findings indicate the potentials of germinating rapeseed as an inexpensive and easily accessible biocatalyst for complete hydrolysis of oils for the preparation of fatty acids and for partial selective hydrolysis of oils or selective esterification of fatty acids for the enrichment of definite fatty acids from mixtures via kinetic resolution as shown in Figure 4.

Several examples are known on the enrichment of definite fatty acids via kinetic resolution utilizing the substrate selectivity of the rape lipase. γ-Linolenic acid has been enriched from the fatty acids of evening primrose oil via selective esterification with *n*-butanol in hexane using the immobilized rapeseed lipase as a biocatalyst [26,27]. The proportion of γ-linolenic acid in unesterified fatty acids increases in the course of esterification with concomitant decrease of this fatty acid esterified in the butyl esters (Fig. 4). Simultaneously, the level of linoleic acid in the unesterified fatty acids decreases sharply

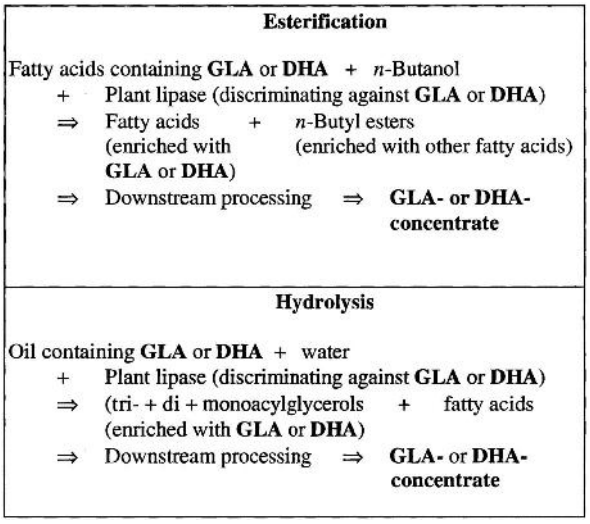


Figure 4 Enrichment of γ-linolenic acid (GLA) or docosahexaenoic acid (DHA) from natural oils via kinetic resolution by esterification or hydrolysis catalyzed by plant lipases.

and a corresponding increase of this fatty acid esterified in butyl esters occurs. After about 86% esterification, the level of γ-linolenic acid in the unesterified fatty acids is increased from about 10% in the starting material to almost 65% [26,27].

In a similar manner, acetone powder of germinating rape seedlings has been used as a biocatalyst for the enrichment of γ-linolenic acid from fatty acids of borage oil via selective esterification with *n*-butanol [18]. The γ-linolenic acid content of borage oil fatty acids is thus raised from about 20% in the starting material to about 65% in the unreacted fatty acids (Fig. 5).

Selective hydrolysis of the triacylglycerols of evening primrose oil utilizing the substrate selectivity of the rape lipase has also been employed for the enrichment of γ-linolenic acid [19,26]. The triacylglycerols of evening primrose oil, emulsified with gum arabic, have been subjected to selective hydrolysis using solubilized rapeseed lipase [26]. During the progress of hydrolysis, γ-linolenic acid is enriched in the acyl moieties of the unhydrolyzed triacylglycerols and diacylglycerols as well as monoacylglycerols. After 83% hydrolysis of the oil, the γ-linolenic acid content of the acylglycerols increases from about 10% in the starting material to 28%; the highest enrichment of γ-linolenic acid occurs in the monoacylglycerol fraction [26].

Immobilized rapeseed lipase has been used as a biocatalyst for the enrichment of docosahexaenoic acid via selective esterification of fatty acids of cod liver oil with *n*-butanol in hexane [27]. The proportion of docosahexaenoic acid in unesterified fatty acids increases in the course of esterification with a concomitant decrease in the level of this fatty acid in the butyl esters. After about 85% esterification, the level of docosahexaenoic acid in unesterified fatty acids is raised from about 9% in the starting material to about 17% [27].

In a similar manner, acetone powder of germinating rape seedlings has been used as a biocatalyst for the enrichment of docosahexaenoic acid from fatty acids of hake liver oil via selective esterification with *n*-butanol [18]. The level of docosahexaenoic acid of

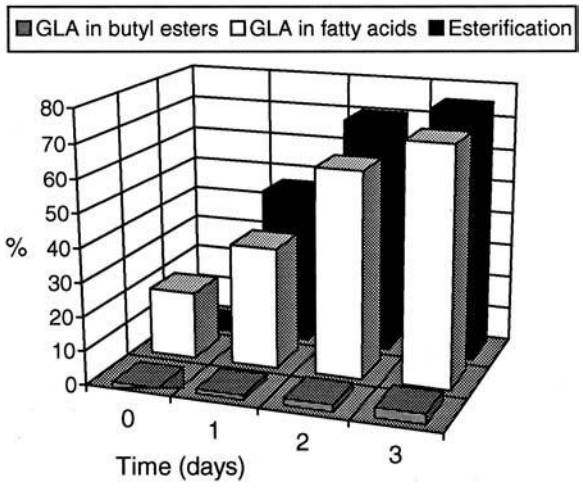


Figure 5 Content of γ-linolenic acid (GLA) in fatty acids and butyl esters during esterification of fatty acids of borage oil with *n*-butanol, catalyzed by acetone powder from rape seedlings. (Adapted with permission from Ref. 18, copyright [1996] AOCS Press.)

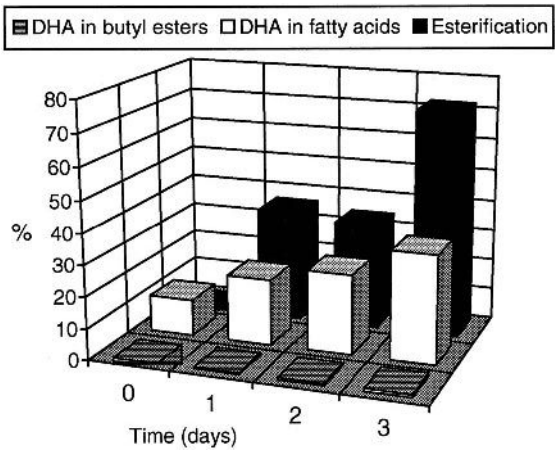


Figure 6 Content of docosahexaenoic acid (DHA) in fatty acids and butyl esters during esterification of fatty acids of hake liver oil with *n*-butanol, catalyzed by acetone powder from rape seedlings. (Adapted with permission from Ref. 18, copyright [1996] AOCS Press.)

hake liver oil fatty acids is thus raised from about 9% in the starting material to about 25% in the unreacted fatty acids (Fig. 6).

Acetone powder of germinating rape seedlings has also been used as a biocatalyst for the enrichment of petroselinic acid from fatty acids of coriander oil via selective esterification with *n*-butanol [18]. The level of petroselinic acid in coriander oil fatty acids is thus raised from about 80% in the starting material to about >95% in the unreacted fatty acids (Fig. 7).

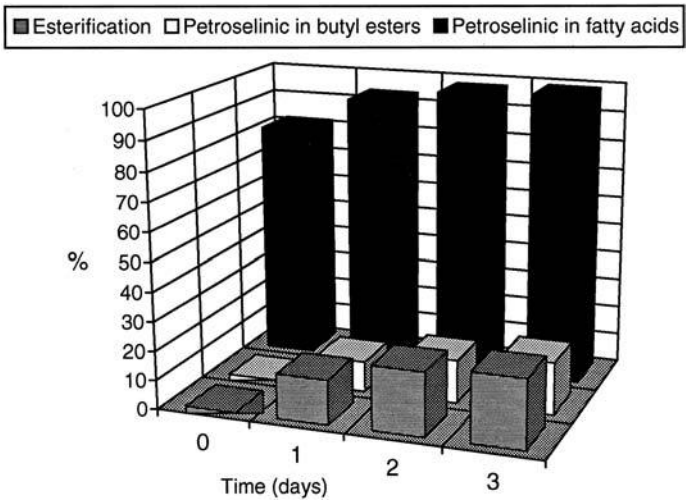


Figure 7 Content of petroselinic acid in fatty acids and butyl esters during esterification of fatty acids of coriander oil with *n*-butanol, catalyzed by acetone powder from rape seedlings. (Adapted with permission from Ref. 18, copyright [1996] AOCS Press.)

The ability of the rapeseed lipase to esterify fatty acids exclusively to the primary alcohols, but neither to secondary nor tertiary alcohols [22] can possibly be utilized for the synthesis of a wide variety of ''designer'' esters. Moreover, the regiopreference of the rape lipase for the *sn*-1,3 positions of the glycerol backbone [13] can be utilized for the preparation of structured triacylglycerols.

A lipase, partially purified from rape seedlings and immobilized on Celite, has been used for the transesterification of lauric acid with tricaprin [15]. Incorporation of lauric acid into tricaprin is strongly dependent on water activity, and the highest activity of the rape lipase preparation is found to be almost as high as the maximal activity of a lipase from *Candida rugosa*, based on the amount of protein in the lipase preparations [15].

The enantioselectivity of a partially purified rape lipase, immobilized on Celite [28], has been evaluated in the esterification of *rac*-alkylglycerols and *rac*-acylglycerols with oleic acid in a reaction medium consisting of supercritical carbon dioxide [29]. Esterification of 1-*O*-octadecyl-*rac*-glycerol, 1-monopalmitoyl-*rac*-glycerol, and 1,2-dipalmitoyl-*rac*-glycerol with oleic acid proceeds with enantiopreference for the *sn*-3-position [29]. These findings show that rape lipase can be suitable for the separation of enantiomers by kinetic resolution.

The oil-rich seeds of *Vernonia anthelmintica*, a rich source of vernolic (*cis*-12,13-epoxy-*cis*-9-octadecenoic) acid, contain an active lipase in the dormant ungerminated seeds. Lipolysis in situ of the oil in the seeds of *Vernonia anthelmintica* using the endogenous lipase of the seed has been carried out to yield 1,3-divernoloylglycerol; however, the yield is rather low [30]. A lipase isolated from *Vernonia galamensis*, another rich source of vernolic acid [31], has been used for hydrolysis of the seed oil of *V. galamensis* to prepare 1,3-divernoloylglycerol in high yield; subsequent chemical hydrolysis of 1,3-divernoloylglycerol affords vernolic acid in high purity [32].

A lipase has been isolated as an acetone powder from the ungerminated seeds of *V. galamensis* for use in biotechnology [33]. Although the acetone powder from *V. galamensis* does not have a fatty acid specificity in hydrolysis of soybean or coconut oils, in the transesterification of fatty acids with triacylglycerols, the short- and medium-chain triacylglycerols are the preferred substrates [33]. The acetone powder from *V. galamensis* lipase preferentially cleaves the fatty acids from the *sn*-1,3 positions of triacylglycerols, and this biocatalyst has been successfully used for the hydrolysis of soybean oil dissolved in 2,2,4-trimethylpentane [33]. A partially purified lipase from *V. galamensis* catalyzes the hydrolysis of trivernolin, the predominant constituent of the seed oil of *V. galamensis*, much faster than triolein or other triacylglycerols [34]. Similarly, in the transesterification of tricaprylin with fatty acids, catalyzed by purified *V. galamensis* lipase, a strong preference for vernolic acid is observed [34]. It appears that the lipase isolated by Ncube et al. [34] has somewhat different properties from the acetone powder preparation used in the hydrolysis of *V. galamensis* oil [32].

Transesterification of triacylglycerols has been carried out using a lipase, isolated from the cotton plant, as a biocatalyst [35].

The seed oil of black cumin (*Nigella sativa*) has been hydrolyzed in situ by subjecting the ground seeds to the action of the native lipase in seed in the presence of moisture and heat [36]. It appears that even the dormant seed of black cumin has an active lipase, because the triacylglycerols contained in used frying oil are extensively ($>$90%) hydrolyzed by treatment with the ground seeds of black cumin, defatted by hydraulic pressing [37,38].

Ground and defatted seeds of black cumin also catalyze the esterification of oleic acid and fatty acids of sunflower seed oil and coconut oil to glycerol to yield up to 70–80% triacylglycerols [38,39]. Using the same biocatalyst, oleic acid is esterified with methanol to an extent of about 50% [38]. Acetone powder of black cumin seeds, prepared according to Ref. 40, catalyzes the glycerolysis (i.e., interesterification with glycerol) of a used frying oil (sunflower seed oil) yielding up to 66% monoacylglycerols [41].

2.1.2 Triacylglycerol Lipases from Castor Bean

Castor bean contains active triacylglycerol lipase even in the dormant state, in contrast to most oilseeds that develop lipase activity during seed germination. Early literature shows that lipase preparations from the castor bean have been used to hydrolyze fats for the preparation of fatty acids [9] and esterify fatty acids with glycerol for the synthesis of acylglycerols [10].

Ricinoleic acid—the predominant constituent fatty acid of castor oil and a valuable starting material for a variety of products [42]—cannot be produced by conventional steam splitting at high temperatures and pressures [43] due to dehydration of ricinoleic acid yielding conjugated fatty acids and intermolecular esterification yielding estolides [44]. Therefore, saponification of castor oil followed by acidification at moderate temperatures are carried out so far for the production of ricinoleic acid.

Ricinoleic acid can also be produced enzymatically by hydrolysis of castor oil using lipase preparations isolated from the castor bean as a biocatalyst [45–47]. Lipolysis in situ of castor oil present in the bean, without prior isolation of the oil or the lipase from the castor bean, has been carried out by treatment of the ground castor beans with dilute acetic acid [48]. After only a 1 hr reaction at 30°C and pH 4.8, as much as 84% of the endogenous oil in the castor bean is hydrolyzed [48]. Using a homogenate of castor bean as a biocatalyst, about 90% lipolysis of castor oil, extracted from the beans, has been achieved after a 5-hr reaction without any formation of estolides [49].

Recently, pressed castor bean, pretreated with phosphate–citrate buffer solutions, has been used as a biocatalyst for the esterification of fatty acids to glycerol for the preparation of triacylglycerols [50]. The formation of triacylglycerols is highest (about 46%) when a stoichiometric ratio of glycerol to fatty acids is used at a reaction temperature of 40°C.

2.1.3 Triacylglycerol Lipases from Cereals

The occurrence of native lipase in dormant oats [51], especially on the surface of oat caryopses [52], is well documented.

Olive oil and tallow have been hydrolyzed in a two-phase system using a crude lipase preparation from oat seeds [14]. Oleoyl moieties are selectively cleaved during lipolysis of tallow, which leads to enrichment of oleic acid in the fatty acids and concentration of the saturated acyl moieties in the triacylglycerols, diacylglycerols, and monoacylglycerols [14]. These findings agree with the substrate selectivity of the oat lipase [53,54].

In the hydrolysis of pure monoacid triacylglycerols, oat lipase rapidly cleaves oleoyl, elaidoyl, linoleoyl, and linolenoyl moieties, whereas palmitoyl, stearoyl, and petroselinoyl moieties are discriminated against [55]. Oils containing polyunsaturated acyl moieties have been hydrolyzed using ground caryopses of oat, defatted with diethyl ether, as a lipase preparation [56]. Almost complete lipolysis of soybean oil occurs with oat lipase at 35°C in about 19 hr in the presence of 2,2,4-trimethylpentane as reaction medium [56].

Extensive lipolysis of castor oil also occurs with ground and delipidated oat caryopses as biocatalyst [57].

Whole caryopses of oat, moistened with water, have also been used as a source of lipase for biotransformation of fats [58]. The overall rate of lipolysis using the whole oat caryopses is low [58] as compared to lipolysis catalyzed by the ground and defatted oat caryopses [14,57]. The addition of nonpolar solvents, such as hexane, to the reaction medium and gentle agitation increase the rates of lipolysis [58]. With the moistened whole oat caryopses as the biocatalyst, the fatty acids are cleaved from all the three positions of the glycerol backbone, and no accumulation of either monoacylglycerols or diacylglycerols occurs [58]. Further reactions catalyzed by moistened oat caryopses are transesterification of oleic acid with triacylglycerols of soybean oil, esterification of oleic acid with glycerol, and transesterification of triacylglycerols of soybean oil with primary alcohols; however, the reaction rates are rather low [58].

Moist oat caryopses produced by a impact-type dehuller are quite efficient as a biocatalyst for the hydrolysis of oils, such as beef tallow, lard, soybean oil, and crambe oil; however, castor oil and corn oil are hydrolyzed at lower rates [59]. Lipolysis of milkfat by moist oat caryopses results in preferential cleavage of the C_6–C_{10} fatty acids [59]. Also, rice bran oil has been hydrolyzed by lipase from oat seeds [60].

The ability of moist oat caryopses to hydrolyze triacylglycerols of soybean oil depends to a great extent on variety and growth conditions of the oat cultivars [60]. Caryopses of oat, harvested 15 days postanthesis, have a greater lipolytic activity than those harvested 30 days postanthesis [61].

In the hydrolysis of trihexanoylglycerol, oat seed lipase cleaves the fatty acids most quickly from the *sn*-3 position, moderately from the *sn*-1 position and hardly from the *sn*-2 position [62]. Such positional specificity of oat lipase [62] and the ability of rice bran lipase to cleave preferentially the fatty acids esterified at the *sn*-1,3 positions of triacylglycerols [63] can possibly be utilized for the enrichment of such fatty acids via selective hydrolysis.

Wheat-germ lipase is known to hydrolyze simple esters, triacylglycerols [64–66], and water-soluble long-chain fatty acid esters of sorbitan [67].

2.1.4 Triacylglycerol Lipases from Miscellaneous Plants

Triacylglycerol lipases from a variety of other plants, such as oil palm (*Elaeis guineensis*) mesocarp [68,69], rubber (*Hevea brasiliensis*) seed [70] and pepper (*Piper nigrum*) powder [71] have been examined; however, possible applications of these lipases in biotransformation of lipids are not known so far.

Latex from papaya (*Carica papaya*)—a well-known commercially available enzyme preparation containing the proteolytic enzymes papain and chymopapain, that has been employed for a long time in food and beverage industries—also has a good activity in the hydrolysis of tributyroylglycerol and long-chain triacylglycerols [72]. The lipase in papaya latex catalyzes the hydrolysis of triacylglycerols stereoselectively at the *sn*-3 position and it has a preference for short-chain acyl moieties [73]. Moreover, in the interesterification of a chiral triacylglycerol, 1-butyroyl-2-stearoyl-3-palmitoylglycerol with trimyristin, catalyzed by papaya latex lipase, a preferential acyl exchange at the *sn*-3 position of triacylglycerols has been observed [74]. In the interesterification of tricaprylin with various homogeneous triacylglycerols, catalyzed by papaya latex lipase, short-chain acyl moieties are preferentially exchanged over the long-chain ones [74].

Papaya latex lipase has been evaluated as biocatalyst in the esterification of various fatty acids with *n*-butanol in the presence of myristic acid as the reference standard [25].

Similar to rapeseed lipase [18,19,22,24], papaya latex lipase strongly discriminates against fatty acids having a *cis*-4 unsaturation (e.g., docosahexaenoic acid), *cis*-6 unsaturation (e.g., petroselinic, γ-linolenic, and stearidonic acids), as well as *cis*-8 unsaturation (e.g., dihomo-γ-linolenic acid) (Fig. 3) [25]. Fatty acids having *cis*-5 unsaturation (e.g., all-*cis*-5,8,11,14,17-eicosapentaenoic acid) and those having a *cis*-9 unsaturation (e.g., oleic and α-linolenic acids) are very well accepted as substrates (Fig. 3) [25]. Fatty acids having hydroxy groups (e.g., ricinoleic and 12-hydroxystearic acid), epoxy groups, (e.g., *trans*-9,10-epoxystearic acid), and cyclopentenyl groups (e.g., hydnocarpic and chaulmoogric acids) are also well accepted as substrates (Fig. 3) [25]. The observed substrate specificities are similar to those reported for lipase preparations from microorganisms, animals, and plants.

In addition to the above proteolytic enzyme preparation from papaya latex, other commercially available protease preparations from plants have been screened for their biocatalytic activity in the esterification of various fatty acids with *n*-butanol [75]. Of all the plant protease preparations tested, only that from pineapple (bromelain) contains active lipase. Similar to lipases from microorganisms, animals, and plants, such as papaya (*Carica papaya*) latex, lipase in the protease preparations of bromelain strongly discriminates against fatty acids having a *cis*-4, *cis*-6, and *cis*-8 unsaturation, whereas fatty acids having hydroxy, epoxy, and cyclopentenyl groups are well accepted as substrates [75].

The strong regioselectivity of papaya latex lipase for the acyl moieties at the primary positions of glycerol, especially at the *sn*-3 position [73,74], has been utilized for the enzymatic synthesis of structured triacylglycerols resembling human milkfat for use in infant nutrition [76].

The triacylglycerols of human milk contain palmitoyl moieties predominantly at the *sn*-2 position of the glycerol backbone and C_{18}-saturated and unsaturated fatty acids esterified at the *sn*-1,3 positions [77]. Structured triacylglycerols resembling human milkfat are produced commercially for use in infant food formulations by transesterification of tripalmitin, with oleic acid or polyunsaturated fatty acids using *sn*-1,3–specific microbial lipases as biocatalysts [78].

Lipases from edible plants may find a wider acceptance as biocatalysts for the preparation of the above type of designer lipid for use in infant food formulations and nutraceuticals rather than those from transgenic microorganisms. Therefore, the lipase in inexpensive crude papaya latex, which is commercially available in bulk scale, has been used as a biocatalyst for the preparation of structured triacylglycerols resembling human milkfat. Such structured triacylglycerols have been prepared by transesterification of tripalmitin with fatty acids of low-erucic rapeseed oil using crude papaya latex as a biocatalyst [76]. Transesterification of tripalmitin with fatty acids of low-erucic canola-type rapeseed oil, catalyzed by papaya latex lipase, leads to the incorporation of 18:1 and 18:2 acids predominantly into the *sn*-1(3-) positions of triacylglycerols, thus yielding structured triacylglycerols resembling human milkfat (Fig. 8) [76].

Selectivity of papaya latex lipase for the *sn*-1(3-) positions of triacylglycerols and short-chain fatty acids has been utilized for the synthesis of low-calorie structured triacylglycerols of the SALATRIM (short and long acyltriglyceride molecule) type [79]. Thus, interesterification of fully hydrogenated soybean oil with tributyrin, catalyzed by papaya latex lipase, yields saturated structured triacylglycerols containing butyryl moieties predominantly at the *sn*-1(3-) positions and stearoyl and palmitoyl moieties mainly at the *sn*-2 position [80].

In transesterification of tricaprylin with various acyl donors, catalyzed by papaya latex lipase, unesterified lauric acid reacts much faster than its alkyl esters, such as methyl or ethyl laurate [81].

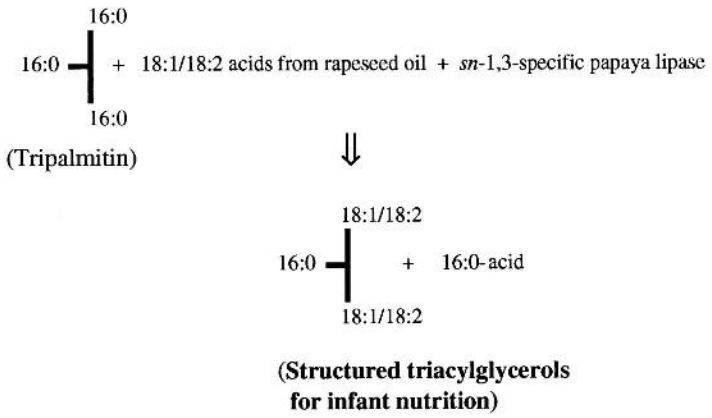


Figure 8 Structured triacylglycerols resembling human milkfats prepared by transesterification of tripalmitin with fatty acids of canola-type rapeseed oil, catalyzed by papaya latex lipase.

2.2 Nonspecific Acylhydrolases

Patatin constitutes a family of major soluble storage glycoproteins of potato tubers which is also known to have a strong lipid acylhydrolase activity [82–86]. Potato lipid acylhydrolase catalyzes the hydrolysis of a wide variety of lipids, such as phospholipids, glycolipids, sulfolipids, and monoacylglycerols [83,85,86], whereas it has a very low activity with diacylglycerols and triacylglycerols [86].

Potato lipid acylhydrolase also catalyzes the esterification of an alkanol with a fatty acid and transesterification of an alkanol with a phospholipid, yielding, in each case, an alkyl ester of fatty acids [82,87].

Recently, potato acylhydrolase has been isolated as a freeze-dried protein extract from potato tubers and applied as a biocatalyst for the esterification of fatty acids with glycerol to prepare monoacylglycerols [88]. In hydrolysis of acylglycerols, the potato acylhydrolase is highly specific for monoacylglycerols, and this specificity increases with increasing purity of the enzyme, accomplished via ammonium sulfate precipitation and hydrophobic interaction chromatography on octyl-Sepharose [88]. Esterification of oleic acid with glycerol at 50°C under vacuum using freeze-dried potato protein extract as a biocatalyst leads to >90% conversion into monoacylglycerols with very little formation of diacylglycerols [88]. Similarly, esterification of oleic acid with diols, such as ethane diol, 1,2-propane diol, or 1,3-propane diol, catalyzed by freeze-dried potato protein extract, leads to 30–45% conversion of oleic acid into diol monoesters with very little formation of diesters [88].

2.3 Phospholipases

Phospholipases A1, A2, and C of plant origin can be used for the biotransformation of phospholipids, such as regiospecific hydrolysis and transesterification of diacylglycerophospholipids, and for their conversion to the corresponding diacylglycerol molecular species [16,89]. So far, the only phospholipase from plants used often for biotransformation of phospholipids is phospholipase D isolated from cabbage leaves.

2.3.1 Phospholipase D from Cabbage

Phospholipase D from cabbage has been successfully employed for transphophatidylation reactions in which the polar head groups of phospholipids are interchanged as shown in Figure 9 [90–99]. For example, transphosphatidylation of phosphatidylcholines with ethanolamine yields phosphatidylethanolamines and choline. Transphosphatidylation is often accompanied by the undesirable hydrolysis of the phospholipids that yields the phosphatidic acids and choline, ethanolamine, glycerol, serine, and so forth. The conditions of transphosphatidylation should therefore be chosen such that the hydrolytic cleavage of the terminal phosphate diester bond of glycerophospholipids is kept at a minimum.

Phospholipase D from cabbage leaves catalyzes the transphosphatidylation of phosphatidylcholines with glycerol, yielding phosphatidylglycerols which are useful as artificial lung surfactants in infants suffering from respiratory distress syndrome [90]. Phosphatidylcholines, dissolved in diisopropyl ether, have been reacted with a solution of glycerol containing phospholipase D and calcium chloride in a membrane reactor to give good yields of phosphatidylglycerols with only a minor extent of formation of phosphatidic acids [90]. An almost quantitative yield of phosphatidylglycerols is obtained when the transphosphatidylation process is carried out in a stirred reactor using an emulsion of phosphatidylcholines (diethyl ether solution) with glycerol (aqueous buffer solution) [92].

Phosphatidylglycerols are also prepared in almost quantitative yields by transphosphatidylation, carried out in batch and continuous operations using cabbage phospholipase D, immobilized on octyl-Sepharose CL-4B as the biocatalyst [93]. A stirred reactor is used in a biphasic system in which the aqueous phase consists of immobilized cabbage phospholipase D in acetate buffer (pH 5.6) containing calcium chloride and glycerol, whereas the substrate (phosphatidylcholines) and products (mainly phosphatidylglycerols) are dissolved in diethyl ether [93].

Phosphatidylethanolamines are prepared by transphosphatidylation of phosphatidylcholines with ethanolamine, using phospholipase D from cabbage as a biocatalyst [94]. Transphosphatidylation is performed in a stirred reactor using ethyl acetate as the solvent for the substrate (phosphatidylcholines) and the product (phosphatidylethanolamines), and the aqueous phase contains the phospholipase D in a buffer, to which calcium chloride and ethanolamine are added. Phospholipase D from cabbage, as compared to the microbial phospholipase D preparations, gives almost quantitative conversion of phosphatidylcholines to phosphatidylethanolamines, with essentially no formation of phosphatidic acids [94].

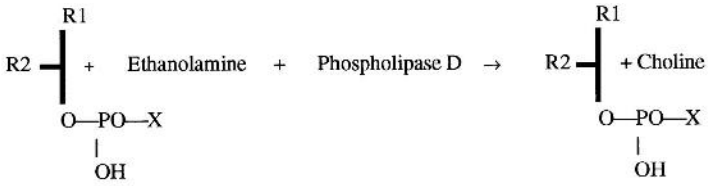


Figure 9 Transphosphatidylation, catalyzed by phospholipase D from cabbage.

Phosphatidylserines that are useful for the preparation of liposomes as drug delivery systems are obtained via transphosphatidylation of phosphatidylcholines with serine using phospholipase D from cabbage [95] in a manner similar to that described earlier [94]. Phospholipase D from cabbage, as opposed to the corresponding enzyme preparations from microbial sources, is highly stereoselective in the transphosphatidylation reactions. Thus, transphosphatidylation of phosphatidylcholines with L-serine gives phosphatidylserine in a yield of about 40%, whereas with D-serine, no transphosphatidylation occurs and the substrate (phosphatidylcholines) is completely hydrolyzed to phosphatidic acids [95].

Commercially available phospholipids, such as soybean lecithin and egg lecithin, have been subjected to transphosphatidylation with choline chloride using various preparations of phospholipase D in order to increase the phosphatidylcholine content of such products [96]. In the above transphosphatidylation reaction, the phospholipase D from cabbage is a rather poor biocatalyst when compared to several microbial phospholipase D preparations [96].

Radiolabeled ethanolamine plasmalogens have been subjected to transphosphatidylation, catalyzed by phospholipase D from cabbage, to prepare the dimethylethanolamine and choline analogs of these substances [91].

Phospholipase D from cabbage efficiently catalyzes the hydrolysis of phosphatidylcholines, dissolved in diethyl ether, but not in *n*-hexane, to yield phosphatidic acids [100]. This reaction has been utilized to increase the phosphatidic acid content of commercial phosphatides and thus improve their emulsifying properties and the ability to mask the taste of bitter food ingredients and pharmaceuticals [99].

Phospholipase D from cabbage also catalyzes the transphosphatidylation of phosphatidylcholines, dissolved in either diethyl ether or *n*-hexane, with 1-butanol to yield phosphatidyl-1-butanol; however, extensive hydrolysis of phosphatidylcholines to phosphatidic acids also occurs [100].

Phospholipid analogs, such as 1,2-dioleoyl-*sn*-glycero-3-phospholipids with N-heterocyclic head groups have been prepared by cabbage phospholipase D-catalyzed transphosphatidylation of 1,2-dioleoyl-*sn*-glycero-3-phosphocholines with primary or secondary alcohols bearing N-heterocyclic groups, such as *N*-(2-hydroxyethyl)-piperidine, 4-hydroxy-*N*-methyl-piperidine, and so on [101]. Similarly, phospholipid analogs, such as alkylphosphate esters, have been prepared by the exchange of choline in hexadecylphosphocholine or octadecylphosphocholine by N-heterocyclic alcohols via transphosphatidylation, catalyzed by phospholipase D from cabbage [101]. Hydrolysis of alkyl phosphorylcholines, catalyzed by phospholipase D from cabbage, yields alkyl phosphatidic acids [102].

3 SUMMARY AND PERSPECTIVES

Lipases used so far for biotransformation of lipids are mostly of microbial origin. Some interesting properties of triacylglycerol lipases from plants and the relatively simple techniques of their isolation as partially purified enzymes from inexpensive starting materials, such as germinating or dormant seeds, might stimulate their future use as catalysts for biotransformation of lipids in a variety of areas of commercial interest. For example, triacylglycerol lipases in acetone powder from germinating rape seedlings or dormant vernonia seeds and in crude papaya latex have great potential as biocatalysts for the enrichment of definite fatty acids, especially polyunsaturated fatty acids, from fats and oils via selective hydrolysis or esterification. The ability of the plant lipases to cleave fatty acids

from the *sn*-1,3 positions of triacylglycerols can possibly be utilized for the preparation of erucic acid (e.g., a valuable starting material for oleochemicals and technical products), which can be enriched by selective hydrolysis of seed oils of many cruciferae that contain large proportions of erucic acid almost exclusively esterified at the primary positions of triacylglycerols. It should also be possible to synthesize a wide variety of structured triacylglycerol as designer lipids for nutrition and dietetics by esterification and transesterification using *sn*-1,3–regiospecific triacylglycerol lipases from plants. Lipid acylhydrolase from potato has great potential as biocatalyst for the preparation of monoacylglycerols that are widely used as emulsifiers.

Storage triacylglycerols of oilseeds can be hydrolyzed in situ simply by germinating the seeds and subsequently homogenizing the seedlings and incubating the homogenate, in order to subject the oil to lipolysis by the action of the endogenous lipase. It is conceivable that such a biotechnical processing strategy should provide fatty acids and glycerol or acylglycerols directly from the oilseeds without prior extraction of the oil. Similarly, storage triacylglycerols in seeds containing dormant lipase can be hydrolyzed in situ by homogenizing the seeds and incubating the homogenate, in order to subject the oil to lipolysis by the action of the endogenous lipase.

REFERENCES

1. H Brockerhoff, RG Jensen. Lipolytic Enzymes. New York: Academic Press, 1974.
2. T Galliard. In: T Galliard, EI Mercer, eds. Recent Advances in the Chemistry and Biochemistry of Plant Lipids. New York: Academic Press, 1975, pp 319–337.
3. T Galliard. In: PK Stumpf, E Conn, eds. The Biochemistry of Plants, Vol. 4. New York: Academic Press, 1980, pp 85–116.
4. AHC Huang. In: B Borgström, HL Brockman, eds. Lipases. Amsterdam: Elsevier, 1984, pp 419–442.
5. AHC Huang, In: PK Stumpf, E. Conn, eds. The Biochemistry of Plants, Vol. 9. New York: Academic Press, 1987, pp 91–119.
6. AHC Huang, Y-H Lin, S-M Wang. J Am Oil Chem Soc 65:897–899, 1988.
7. KD Mukherjee. Prog Lipid Res 33:165–174, 1994.
8. KD Mukherjee, MJ Hills, In: P Woolley, SB Petersen, eds. Lipases. Cambridge: Cambridge University Press, 1994, pp 49–75.
9. W Connstein, E Hoyer, H Wartenberg. Ber Dtsch Chem Ges 35:3988–4006, 1902.
10. YW Jalander, Biochem Z 36:435–476, 1911.
11. RL Ory, AJ St Angelo, AM Altschul. J Lipid Res 3:99–105, 1962.
12. FR Hassanien, KD Mukherjee. J Am Oil Chem Soc 63:893–897, 1986.
13. MJ Hills, KD Mukherjee. Appl Biochem Biotechnol 26:1–10, 1990.
14. G Piazza, A Bilyk, D Schwartz, M Haas. Biotechnol Lett 11:487–492, 1989.
15. I Ncube, P Adlercreutz, J Read, B Mattiasson. Biotechnol Appl Biochem 17:327–336, 1993.
16. KD Mukherjee. Biocatalysis 3:277–293, 1990.
17. I Jachmanían, M Perifanova-Nemska, M-A Grompone, KD Mukherjee. J Agric Food Chem 43:2992–2996, 1995.
18. I Jachmanían, KD Mukherjee. J Am Oil Chem Soc 73:1527–1532, 1996.
19. I Jachmanían, KD Mukherjee. J Agric Food Chem 43:2997–3000, 1995.
20. MJ Hills, I Kiewitt, KD Mukherjee. Appl Biochem Biotechnol 27:123–129, 1991.
21. MJ Hills, JN O'Sullivan. Biochem Soc Trans 17:480, 1989.
22. MJ Hills, I Kiewitt, KD Mukherjee. Biochim Biophys Acta 1042:237–240, 1990.
23. MJ Hills, I Kiewitt, KD Mukherjee. Biochem Soc Trans 17:478, 1989.
24. I Jachmanían, E Schulte, KD Mukherjee. Appl Microbiol Biotechnol 44:563–567, 1996.

25. KD Mukherjee, I Kiewitt. J Agric Food Chem 44:1948–1952, 1996.
26. MJ Hills, I Kiewitt, KD Mukherjee. Biotechnol Lett 11:629–632, 1989.
27. MJ Hills, I Kiewitt, KD Mukherjee. J Am Oil Chem Soc 67:561–564, 1990.
28. C Fuchs, G Hansen. Z Naturforsch 49c:293–301, 1994.
29. M Bariszlovich, C Fuchs, G Glowacz, JTh Mörsel. In: Oils-Fats-Lipids 1995, Proceedings 21st World Congress of the International Society for Fat Research (ISF), The Hague, Vol 2. Bridgwater, England: PJ Barnes & Assoc. 1996, pp 61–68.
30. CF Krewsen, JS Ard, RW Riemenschneider. J Am Oil Chem Soc 39:334–340, 1962.
31. KD Carlson, WJ Schneider, SP Chang, LH Princen. In: EH Pryde, LH Princen, KD. Mukherjee eds. New Sources of Fats and Oils. Champaign, IL: American Oil Chemists' Society, 1981, pp 297–318.
32. FO Ayorinde, CP Nwaonicha, VN Parchment, KA Bryant, M Hassan, MT Clayton. J Am Oil Chem Soc 70:129–132, 1993.
33. I Ncube, JS Read. Ind Crops Prod 3:285–292, 1995.
34. I Ncube, T Gitlesen, P Adlercreutz, JS Read, B Mattiasson. Biochim Biophys Acta 1257: 149–156, 1995.
35. ZKh Kadyrova, SA Abdurakhimov, KK Khalinyazov. Chem Nat Compd 19:411–413, 1983.
36. L Dandik, HA Aksoy. J Am Oil Chem Soc 69:1239–1241, 1992.
37. L Dandik, G Arioglu, HA Aksoy. Appl Biochem Biotechnol 42:119–126, 1993.
38. L Dandik, HA Aksoy. Enzyme Microb Technol 19:277–281, 1996.
39. S Mert, L Dandik, HA Aksoy. Appl Biochem Biotechnol 50:333–342, 1995.
40. OA Afolabi, MA Ologunde, WA Anderson, JS Read, MD Dacosta, FA Epps, FO Ayorinde. J Chem Technol Biotechnol 51:41–46, 1991.
41. N El, L Dandik, HA Aksoy. J Am Oil Chem Soc 75:1207–1211, 1998.
42. R Vignolo, F Naughton. Int News Fats, Oils Relat Mater 2:692–699, 1991.
43. NOV Sonntag. In: RW Johnson, E Fritz, eds. Fatty Acids in Industry. New York: Marcel Dekker, 1989, pp 23–72.
44. G Lakshminarayana, R Subbarao, YSR Sastry, V Kale, TC Rao, A Gangadhar. J Am Oil Chem Soc 61:1204–1206, 1984.
45. RL Ory. Lipids 4:177–185, 1969.
46. HE Longenecker, DE Haley. J Am Chem Soc 57:2019–2021, 1935.
47. RL Ory, AJ St Angelo, AM Altschul. J Lipid Res 1:208–213, 1960.
48. KSVA Rao, MM Paulose, G Lakshminarayana. Biotechnol Lett 12:377–380, 1990.
49. KSVA Rao, MM Paulose. Res Ind 37:36–37, 1992.
50. M Tüter. J Am Oil Chem Soc 75:417–420, 1998.
51. EG Hammond. In: PJ Barnes ed. Lipids in Cereal Technology. New York: Academic Press, 1983, pp 331–352.
52. HF Martin, FG Peers. Biochem J 55:523–529, 1953.
53. DL Berner, EG Hammond. Lipids 5:572–573, 1970.
54. GJ Piazza, A Bilyk. J Am Oil Chem Soc 66:489, 1989.
55. G Piazza, A Bilyk, DP Brower, MJ Haas. J Am Oil Chem Soc 69:978–981, 1992.
56. GJ Piazza. Biotechnol Lett 13:173–178, 1991.
57. GJ Piazza, HM Farrell Jr. Biotechnol Lett 13:179–184, 1991.
58. I Lee, EG Hammond. J Am Oil Chem Soc 67:761–765, 1990.
59. S Parmar, EG Hammond. J Am Oil Chem Soc 71:881–886, 1994.
60. J Kaur, V Ramamurthy, RM Kothari. Biotechnol Lett 15:257–262, 1993.
61. S Parmar, EG Hammond, KJ Frey. Cereal Chem 71:537–539, 1994.
62. Y Ota, T Minesaki, A Oshima. Biosci Biotech Biochem 61:166–167, 1997.
63. M Funatsu, Y Aizono, K Hayashi, M Inmasu, M Yamaguchi. Agric Biol Chem 35:734–742, 1971.
64. TP Singer, BJH Hofstee. Arch Biochem 18:229–244, 1948.
65. TP Singer, BJH Hofstee. Arch Biochem 18:245–259, 1948.

20

Phospholipases in Enzyme Engineering of Phospholipids for Food, Cosmetics, and Medical Applications

YUGO IWASAKI and TSUNEO YAMANE

Nagoya University, Nagoya, Japan

1 INTRODUCTION

Phospholipids (PLs) are naturally occurring lipids that contain phosphorus and are ubiquitous in all organisms. One of the most important characteristics of PLs is their amphiphilicity, which arises from the hydrophobic acyl or alkyl groups and hydrophilic polar head groups. Their amphiphilic nature makes it possible to form several aggregates with water, such as micelles, reverse micelles, and bilayer vesicles. Physiologically, PLs are major components of biomembranes, which maintain the cell shape, support membrane proteins, and provide the substrate for phospholipases (PLases) involved in transmembrane signaling.

Physical properties, biocompatibility, and nutritional functions of PLs make them useful in industrial fields such as food, cosmetics, and pharmaceuticals. In many cases, PLs seem to be employed for the benefits coming from the physical properties and biocompatibility rather than their nutritional benefits, even in food industries. They are used as emulsifiers, components of cosmetics, medical formulations, and for liposome preparations.

Phospholipases are enzymes which hydrolyze PLs. Based on their mode of action, PLases are classified into several classes: A_1, A_2, B, C, and D (PLA_1, PLA_2, PLB, PLC, and PLD, respectively). Figure 1 shows the mode of action of each PLase on phosphatidylcholine (PC). PLA_1 and PLA_2 hydrolyze the acyl ester bond at *sn*-1 and *sn*-2 positions,

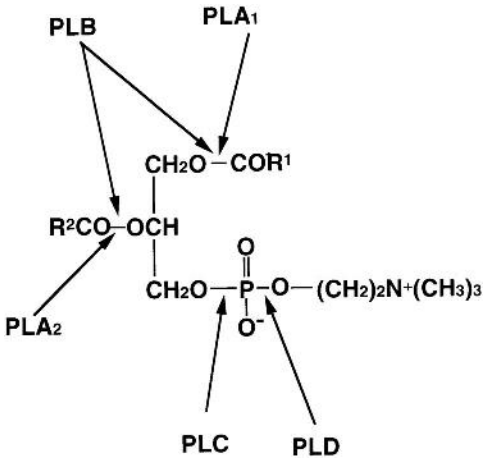


Figure 1 Modes of action of phospholipases on phosphatidylcholine.

respectively, and PLB hydrolyzes both. PLC and PLD acts on the phosphodiester bond, but PLC cleaves the linkage between the glycerol and the phosphate, and PLD attacks the bond between the phosphate and the hydroxyl group of the polar head.

Enzymatic reactions catalyzed by PLases can be used for PL processing or syntheses, as complements to chemical reactions. Among the five types of PLases (i.e., A_1, A_2, B, C, and D types), PLA_2 and PLD are mostly studied with respect to their applications. The purpose intended for their uses in synthetic approaches are (1) enrichment or purification of particular species from nonhomogeneous mixture of various molecular species which are different in the acyl groups and the polar head groups (available from natural sources such as egg yolk and soybean lecithins), (2) improving properties (e.g., solubility in water) of natural PLs, (3) obtaining particular species for which an appropriate natural source is not available [e.g., phosphatidylserine (PS) whose possible natural source is animal brains], and (4) creating novel molecular species with value-added characteristics (e.g., PLs with bioactive compounds at the polar head groups).

This chapter summarizes the uses of PLases (and lipases) in processing or synthesis of PLs as commodity chemicals used in food industry, as fine chemicals used in cosmetics and pharmaceutical formulations, as well as laboratory reagents.

2 MODIFICATION OF ACYL GROUPS

2.1 Use of PLA_2 and Lipase

Acyl group modification is often intended to modify specifically either the *sn*-1 or *sn*-2 position. Many sources for PLA_1 are known and are well studied from their biochemical and physiological points of view [1,2]. However, with respect to their usages in PL engineering, some of these enzymes are not well studied partly because they are not commercially available. Lipases (triacylglycerol acylhydrolase) are known to act on PLs as well as acylglycerols. Many kinds of lipases are commercially available, some of which possess regiospecificity toward *sn*-1,3 positions. Hence, for the modification at the *sn*-1 position of PLs, such 1,3-specific lipases (pancreatic and fungal enzymes) are mostly employed instead of PLA_1.

There are two types of PLA_2: secreted PLA_2 and cytosolic PLA_2. The former with a molecular mass of about 14 kDa is one of the best studied PLases and is found in digestive juice of animals or in animal venom. The latter, with a molecular mass of about 85 kDa, is involved in transmembrane signaling in eukaryotic cells [3]. The secreted PLA_2, especially the one from porcine pancreas, is commercially available and can be used for the modification at the *sn*-2 position of PLs.

2.2 Production of Lysolecithin

Lecithins are inferior to other food emulsifiers such as monoacylglycerol, sugar ester, and polyglycerol ester due to their strong hydrophobicity. In contrast, lysolecithin or lysophospholipid (usually the *sn*-1-acyl-2-lyso form) has superior emulsifying properties because they are more soluble in water, thus forming stable oil-in-water (O/W) type emulsions. Three possible strategies for the production of lysolecithins are shown in Figure 2. For these purposes, chemical methods are obviously complicated because of the difficulty in deacylation or acylation of the substrate specifically at the desired position.

The first strategy, hydrolysis of PL with PLA_2, is already applied industrially [4] (Fig. 2a). Soybean lecithin and porcine pancreatic PLA_2 are used as starting material and catalyst, respectively. Soybean lecithin dispersed in aqueous buffer containing Ca^{2+} ion as a cofactor is hydrolyzed by the enzyme. The hydrolysate is dehydrated under reduced pressure, followed by acetone removal of the fats, which are mainly liberated free fatty acids (FA). Then, the product is dried under reduced pressure to give the final product as a powder.

The second strategy includes deacylation at the *sn*-1 position by *sn*-1,3–specific lipase to obtain *sn*-1-lyso-2-acyl-PL. The resultant *sn*-1-lyso form is further treated with

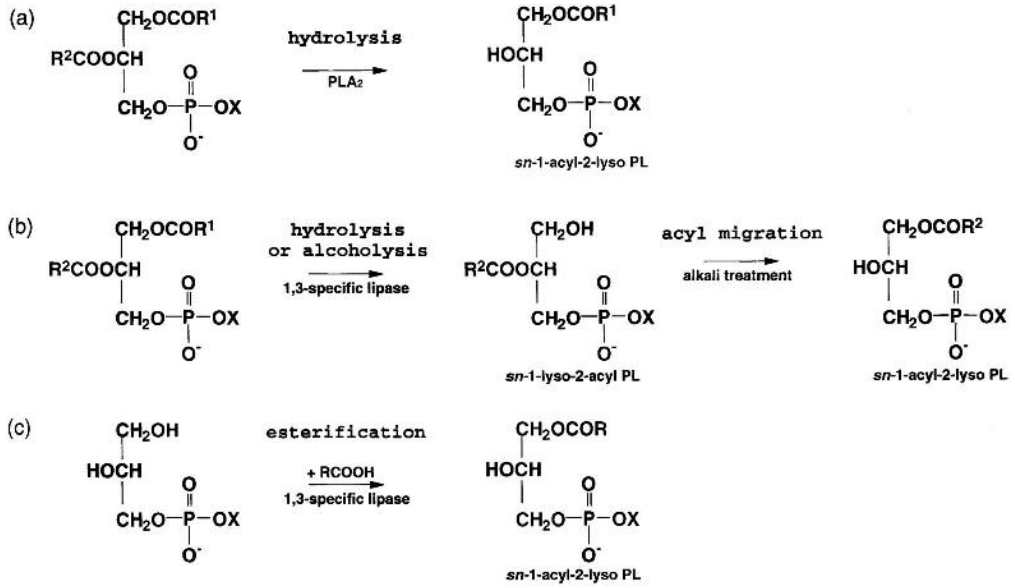


Figure 2 Three strategies for the synthesis of lysophospholipids: (a) PLA_2-mediated hydrolysis; (b) hydrolysis with 1,3-specific lipase, followed by acyl migration promoted by alkali; (c) esterification (condensation) by 1,3-specific lipase.

alkali to promote acyl migration from the *sn*-2 to *sn*-1 position, resulting in the *sn*-2-lyso form (Fig. 2b). An example is lysofungin (*sn*-1-linoleoyl-2-lyso-phosphatidylinositol), an antifungal agent [5]. Lysofungin was synthesized from soybean phosphatidylinositol by hydrolysis with *Rhizopus arrhizus* lipase, and subsequent acyl migration in N-Tris(hydroxymethyl)methyl-3-aminopropanesulfonic acid (TAPS) buffer at pH 8.5. In another example, Sarney et al. transesterified PC with ethanol by *Rhizomucor miehei* lipase (Lipozyme™) and acyl migration of the product was promoted by the exposure to ammonium vapors [6]. The advantages claimed for their strategy are the following: (1) the reaction mixture (PL solution in ethanol) is homogeneous, facilitating overall process control and allowing continuous operation, (2) easy product recovery after the reaction, because, unlike PLA_2, the lipase does not require Ca^{2+} ion as a cofactor and because the reaction can be performed in a predominantly organic medium (ethanol); (3) both *sn*-1-lyso and *sn*-2-lyso forms can be obtained. Sarney et al. applied this strategy for continuous production using a column reactor packed with immobilized lipase [6]. The reactor was capable of producing 0.5 g of product per gram of the catalyst per day, without inactivation of the catalyst over 1180 hr.

The third method is esterification of glycerophosphorylcholine (GPC) or glycerol phosphate with FA by *sn*-1, 3–specific lipase (Fig. 2c). An example that appeared in literature is lysophosphatidic acid (lysoPA) synthesis completed by Han and Rhee [7]. LysoPA, a potent bioactive compound, was synthesized from glycerol phosphate and FAs with *R. miehei* lipase with a yield of 32%. Because this is a condensation reaction, water is generated during the reaction, and it is assumed that an efficient removal of water (by vacuum or addition of molecular sieves) might improve the yield significantly.

2.3 Preparation of PLs with Particular Chemical Structure

Natural PLs are heterogeneous mixtures of various molecular species with different FAs and polar head groups, but it is often necessary to use a pure PL with a defined chemical structure. In the liposome technology, for example, such pure PLs should be used to control the properties of the vesicles.

Phosphatidylcholine with identical FA residues at both *sn*-1 and *sn*-2 positions (symmetric PC) can be synthesized chemically (Fig. 3a). Natural PC is deacylated chemically to obtain GPC, followed by acylation with an appropriate FA derivative such as FA-chloride and FA-anhydride [8]. Preparation of PC by the above method often handles GPC as an adduct with cadmium chloride to facilitate its recrystalization and to improve its dispersity in solvent [9]; however, the use of such a toxic heavy metal should be avoided. A Japanese company developed and industrialized an alternative production process, where GPC absorbed on an inorganic support (e.g., Celite) is used instead of a cadmium adduct [10].

Phosphatidylcholine species with different FA residues at the *sn*-1 and *sn*-2 positions (asymmetric PC) are prepared by hydrolysis of the corresponding symmetric PC by PLA_2 and further chemical acylation at the *sn*-2 position [11] (Fig. 3a).

2.4 Introduction of Particular Fatty Acids into PLs by Enzymes

The chemical and chemoenzymatic methods described in the previous subsection are very useful. Yet, there might be several drawbacks in the chemical methods: (1) They require an activated acyl donor such as FA anhydride or chloride, or otherwise condensation agents such as carbodiimides and (2) depending on the PL species, certain functional

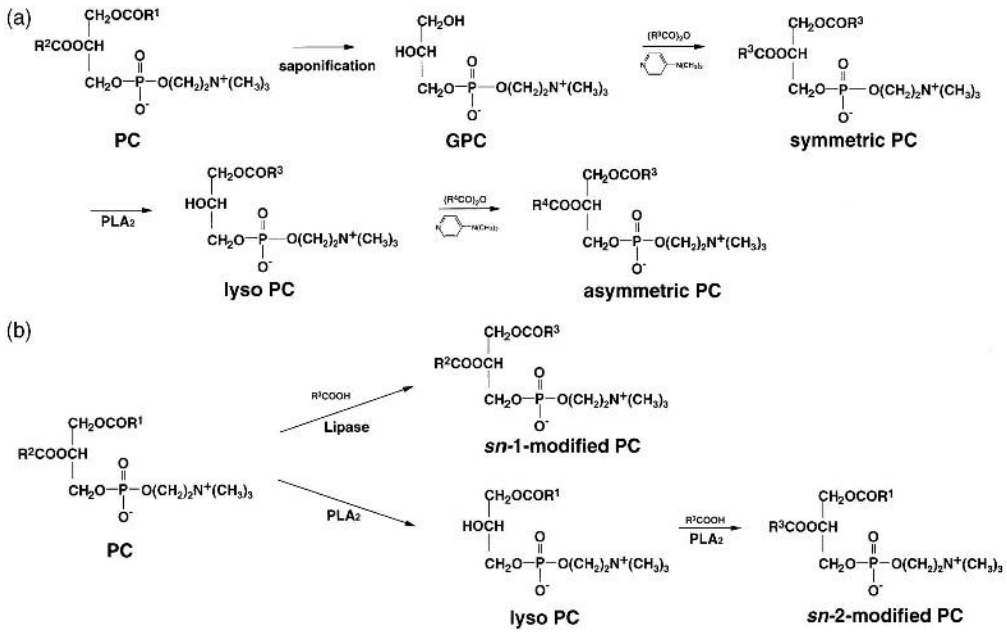


Figure 3 Preparation of PLs containing particular FAs at a specific position: (a) chemical methods; (b) enzymatic methods.

groups [e.g., amino groups of phosphatidylethanolamine (PE) or PS, hydroxyl groups of phosphatidylinositol (PI) or phosphatidylglycerol (PG)] should be protected prior to deacylation/reacylation and deprotected thereafter [12,13]. To simplify the synthesis, several attempts were made to enzymatically introduce particular fatty acids into PLs using lipase and PLA_2.

1,3-Specific lipase-catalyzed transesterification is employed to introduce particular fatty acids exclusively into the *sn*-1 position of PLs (Fig. 3b) [14,15]. A major problem in the lipase-catalyzed reaction is the formation of deacylated PLs (e.g., lysoPLs) via hydrolysis of the substrate. Svenson et al. [15] performed the transesterification of PC and heptadecanoic acid with *R. arrhizus* lipase; by optimizing the water activity in the reaction system, almost 100% incorporation in the *sn*-1 position with 60% of PC recovery was achieved.

Introduction of FA into the *sn*-2 position is done with PLA_2. Because PLA_2 scarcely catalyzes transesterification, the enzyme is used in the condensation reaction of a particular FA and lysoPL (i.e., the reverse reaction of hydrolysis) (Fig. 3b). This reverse reaction was first reported by Pernas et al. [16] with a very low yield (6%). Afterward, several researchers improved the yield. So far, the highest yield of the condensation achieved, which was reported by Egger et al. [17] and Hosokawa et al. [18], was approximately 60%.

Polyunsaturated fatty acids (PUFAs), such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), are known to be bioactive compounds. Because PUFAs are very unstable, enzymatic conversion of PUFAs under mild conditions is worthwhile. A good example was reported by Hosokawa et al. [18]. PLs containing PUFA at the *sn*-1 position was prepared by Lipozyme-catalyzed acidolysis of PC with a mixture of EPA and DHA. Use of a combination of water and propylene glycol as a water mimic gave

80% of the theoretical maximum incorporation with the PC recovery of 80%. In addition, PC containing PUFA at the *sn*-2 position was prepared by PLA_2-mediated condensation of lysoPC and PUFA in glycerol as a reaction medium. The addition of a small amount of formamide to the reaction mixture gave best results with 60% yield.

3 MODIFICATION OF HEAD GROUPS

3.1 Transphosphatidylation by PLD

In addition to hydrolysis, PLD catalyzes a reaction in which polar head groups of PLs are replaced with other hydroxyl compounds [19,20]. This reaction (so-called transphosphatidylation) can be used to prepare a PL with a particular polar head group (Fig. 4a). Various PLs can be synthesized from naturally abundant PLs such as PC or lecithin and corresponding hydroxyl compounds.

Important parameters for evaluating a reaction are selectivity and yield defined as follows:

$$\text{Selectivity } (\%) = \frac{[\text{PX}]}{[\text{PA}] + [\text{PX}]} \times 100$$

$$\text{Yield } (\%) = \frac{[\text{PX}]}{[\text{PC}]_0} \times 100$$

where PX is the desired product, PA is phosphatidic acid (in this case, biproduct formed by hydrolysis of the substrate), and the square brackets denotes the concentration of each component.

The reaction is typically carried out in a biphasic system consisting of a water-immiscible organic solvent (e.g., diethyl ether and ethyl acetate) containing lipids and an

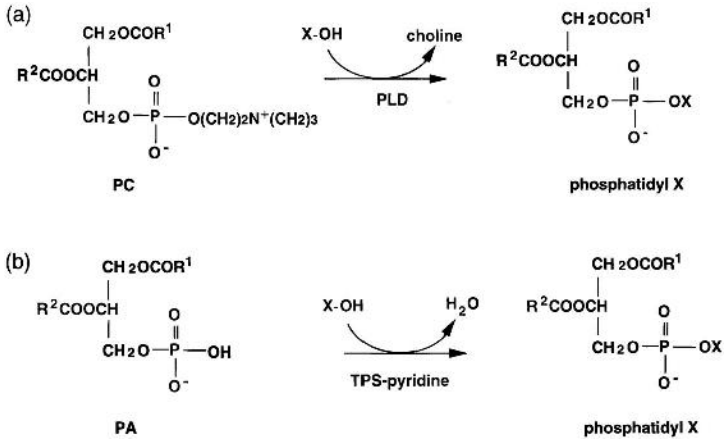


Figure 4 Introduction of particular hydroxyl compounds into the polar heads: (a) Transphosphatidylation catalyzed by PLD. PC is converted into phosphatidyl X in the presence of hydroxyl compounds (X–OH). When X is H, the reaction is hydrolysis. (b) Chemical method. Phosphatidic acid and hydroxyl compound (X–OH) is condensed using 2,4,6-triisopropylbenzenesulfonyl chloride (TPS) and pyridine as a condensation agent.

aqueous solution of enzyme and hydroxyl compounds (i.e., acceptor compounds such as ethanolamine, glycerol, or serine). Although PLD is intrinsically a hydrolytic enzyme, transphosphatidylation even occurs in a water-rich system under optimized conditions. Thus, there is no need to control water content in the reaction system or dehydrate the enzyme prior to the reaction, unlike the cases of lipase-catalyzed transesterification reactions. An advantage of the biphasic system is that the desired product is soluble in the organic phase and can be separated easily from the aqueous phase by a simple phase separation.

It is also possible to introduce particular hydroxyl compounds into the polar head group by a chemical method, where the hydroxyl compound and phosphatidic acid (PA) is condensed in the presence of 2,4,6-triisopropylbenzenesufonyl chloride as a condensing agent [21] (Fig. 4b). However, the most convenient method of preparation of PA from lecithin as a starting material is, in fact, hydrolysis by PLD. Thus, the enzymatic transphosphatidylation is preferable over the chemical reaction, unless other special purposes are intended (e.g., synthesis of D-PLs, which is the stereoisomer of naturally occurring L-PLs, using an appropriate chiral building block [22]). To achieve high selectivity, a quite high concentration of the acceptor is necessary. This might be a drawback of the enzymatic method compared to the chemical one, especially when the acceptor compound is expensive. However, the use of the biphasic system might overcome this problem, because the separated aqueous phase (containing the enzyme and the residual acceptor compound) can be reused several times in other batches of organic phase containing fresh lipid substrate [23].

3.2 Syntheses of Natural Pure PLs

Juneja et al. synthesized several natural PLs such as PG [24,25], PE [26], or PS [27,28] from lecithin or PC using PLD-catalyzed transphosphatidylation. Under the optimized conditions, the syntheses were very successful with yields of almost 100%. Transphosphatidylation with L- and D-serine gave phosphatidyl-L- and D-serine, respectively. Interestingly, bacterial PLD showed a twofold higher reaction rate with D-serine than with L-serine, whereas cabbage PLD reacted only with L-serine. In addition, the reaction of egg lecithin containing 75% PC and 25% PE with choline gave PC with almost 100% purity [29]. Generally, volumetric productivity as high as possible is favorable. When a higher concentration of PC was used, the efficiency of the reaction became worse due to product inhibition by the released choline. Removal of the choline by coexisting choline oxidase and catalase enabled the reaction with a high concentration of the starting substrate [27].

As shown above, most of natural pure PLs can be synthesized enzymatically. In addition, starting from PC with defined FA residues described in Sections 2.3 and 2.4, most of the natural PLs with a completely defined structure can be obtained. An exception is the synthesis of PI from PC and inositol with PLD, which is not yet successful.

3.3 Acceptor Compounds for Transphosphatidylation

The PLD-mediated transphosphatidylation can be extended to syntheses of PLs with unnatural polar heads. Until now, a large variety of artificial lipids were synthesized. There are several factors determining whether a hydroxyl compound can be an acceptor of the phosphatidyl group:

1. Type of the hydroxyl group. The reaction occurs with a preference of primary hydroxyl groups over secondary ones. Thus, aliphatic primary alcohols are good

acceptors. Some secondary alcohols can be also transphosphatidylated, but the selectivity for such compounds seems lower than those for primary alcohols [30]. In the cases of compounds with both primary and secondary hydroxyl groups (e.g., polyols), the primary hydroxyl groups are selectively transphosphatidylated. For example, the transphosphatidylation of PC with L-ascorbic acid gave predominantly 6-phosphatidyl-L-ascorbic acid (PAsA), in which the sixth primary hydroxyl group of the ascorbic acid moiety is linked to the phosphatidyl group (Fig. 5, compound **1**) [31]. Tertiary hydroxyl groups can not be acceptors.

2. Molecular size. Kokusho et al. synthesized various phosphatidylsaccharides-containing sugars such as glucose and maltose with good yields [32]. However, oligosaccharides higher than trimers, such as maltotriose and dextrin, could not react at all.
3. Substituent effects. This was pointed out by Takami et al., who synthesized various PLs with a series of *p*-substituted phenols [33]. There was a correlation between the efficiency and the Hammets's sigma constants (σ_p) of the substituents. Phenols with more electron-donating substituents (lower σ_p) became more effective acceptors.
4. Solubility. The concentration of the acceptor should be high to ensure high selectivity. In fact, it is not always necessary for the hydroxyl compounds of interest to be water soluble if they are soluble in the solvent phase. For example, 2-naphthol, which has ~0.1% solubility in water, could be reacted with 41%

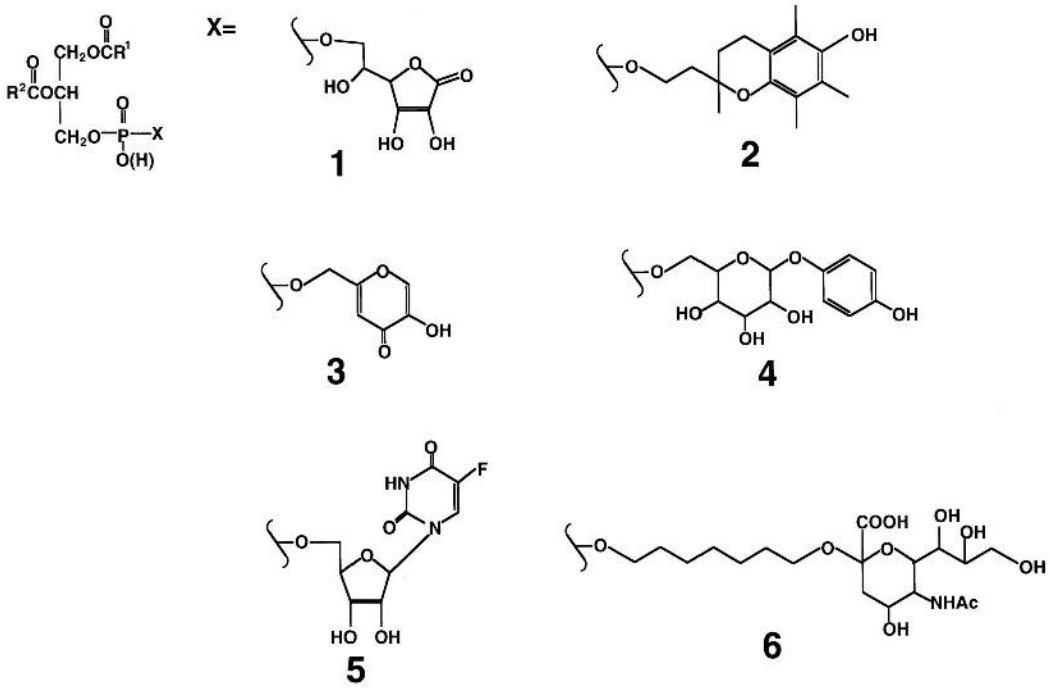


Figure 5 Structure of several functional phospholipids: Phosphatidyl derivatives of ascorbic acid (**1**), 2,5,7,8-tetramethyl-6-hydroxy-2-(hydroxyethyl)chroman (**2**), arbutin (**3**), kojic acid (**4**), 5-fluorouridine (**5**), and *N*-acetylneuraminic acid (connected with octanediol linker) (**6**) are shown.

yield [33]. However, it might be difficult to carry out the reaction with hydroxyl compounds with low solubility in both the water and the organic phases.

3.4 Synthesis of PLs Containing Bioactive Compounds

The ability of PLD to transphosphatidylate a large number of compounds is applied for syntheses of novel PLs containing functional polar head groups. Because the phosphatidyl moiety itself is biocompatible, it is considered as a nontoxic carrier of bioactive compounds. The purpose for the syntheses of such functional PLs is to add physical and biological properties of PLs to the acceptor compounds. Examples intended for the use in food, cosmetics, and medical applications are shown below.

Nagao et al. synthesized phosphatidylascorbic acid (PAsA) (Fig. 5, compound **1**) [31]. It was revealed that PAsA suppressed oxidation of PC in multilamelar liposomes [34]. The result implicated that the ascorbic acid moiety of PAsA was localized on the water–lipid surface of the liposomes, enhancing the effective concentration of the ascorbic acid moiety for scavenging aqueous peroxy radicals. Similarly, Koga et al. introduced 2,5,7,8-tetramethyl-6-hydroxy-2-(hydroxyethyl)chroman into the polar head, resulting in phosphatidylchromanol (PCh) (Fig. 5, compound **2**) [35]. The chroman ring, which is a portion of α-tocopherol (i.e., vitamin E), is involved in the antioxidant activity of α-tocopherol. PCh suppressed autoxidation of lard more effectively than the original compound. The explanation for this phenomenon is that PCh formed reverse micelles in the oil and trapped the residual water which dissolved a trace of metal ions such as iron which initiated the oxidation [36].

Both arbutin and kojic acid prevent overproduction of melanin in epidermal cells. Arbutin is a competitive inhibitor of tyrosinase, whereas kojic acid inhibits the enzyme as a chelator and as an antioxidant. Because these compounds are water soluble, their instability was a problem for their use in cosmetic products. Takami et al. converted these compounds into PLs in order to improve their physical characteristics (Fig. 5, compounds **3** and **4**) [37]. The resultant phosphatidylarbutin and phosphatidylkojic acid retained the inhibitory activity toward tyrosinase to similar extent of the original compound in vitro.

The phosphatidyl moiety has a high affinity for cell membranes, so that phosphatidyl derivatives can easily penetrate into cells. Shuto et al. synthesized various PL derivatives of nucleoside analogs, anticancer agents such as 5-fluorouridine (Fig. 5, compound **5**) [38]. Some of them showed superior antitumor activities than the parent compounds [39].

A sialic-acid-containing PL was synthesized from *N*-acetylneuraminic acid (NeuAc) and PC by a combination of chemical and enzymatic methods (Fig. 5, compound **6**) [40]. Derivatives of NeuAc are expected to be potent antiviral agents. The inhibitory effect of the resultant liposomes for rotavirus infection was 10^3–10^4 fold higher than that of NeuAc. A speculation for its enhanced antiviral activity is that the synthesized lipid forms bilayers with multivalent NeuAc displayed on the surface, which then interacts with the virus in a multivalent manner.

In addition, phosphatidyl derivatives of a peptide-based inhibitor (against fibronectin adhesion to integrin) [41] and dihydroxyacetone (tanning agent for cosmetics) [42] are also interesting examples.

3.5 Use of Actinomycete PLDs for Transphosphatidylation

In early days, PLD from white cabbage leaves had primarily been used for the transphosphatidylation partly because there had been few other sources of the enzyme. Afterward, attempts were made for isolation of PLD-producing microorganisms by screening from

Table 1 Properties of Several Actinomycetes PLDs

Strain	T activity[a]	Optimum pH	Chelator	Gene cloning	Ref.
S. chromofuscus	Weak	8.0	Sensitive	Yes	23,26,46,47
Streptomyces sp.	Weak	7.5	Sensitive	No	23,26
S. lydicus	Strong	6.0	Tolerant	No	26,48
S. prunicolor	Strong	7.0	NR[b]	No	26
Streptomyces sp. AA 586	Strong	5.5	Tolerant	Yes	26,49
S. antibioticus	Strong	5.5	Tolerant	Yes	26,50,51
Streptomyces sp. PMF	Strong	4–6	Tolerant	No	52
Streptomyces sp. PM43	Strong	6–7	Tolerant	No	52
Stv. hachijoense IFO12782[c]	Strong	(5.0)[d]	Tolerant	No	23
	Weak	(8.0)[d]	Sensitive	No	23
Stv. cinnamoneum IFO12852[c]	Strong	6.0	Tolerant	Yes	23,53
	Weak	(8.0)[d]	Sensitive	No	23
Stv. mediocidicus IFO13202[c]	Strong	(5.0)[d]	Tolerant	No	23
	Weak	(8.0)[d]	Sensitive	No	23
Actinomadura sp. No 362	Strong	5.5	Tolerant	No	54

[a] Transphosphatidylation activity.
[b] Not reported.
[c] Considered to produce two kinds of PLDs.
[d] Speculated from the experiments using the culture supernatants.

other natural sources [43–45]. Interestingly, most of the isolated PLD-producing microbial strains were actinomycetes such as genus *Streptomyces*, *Streptoverticilium*, and *Actinomudra* (Table 1). For example, Kato et al. screened about 3000 strains of microorganisms isolated from soil for PLD production and found 200 positive strains, all of which were actinomycetes [45]. Some of these actinomycete PLDs showed higher selectivity than the cabbage PLD, whereas the others showed lower selectivity (Fig. 6) [26]. These actinomycete PLDs might be categorized into at least two classes with respect to the properties, here tentatively named classes I and II. The common characteristics of the enzymes of class I are (1) strong transphosphatidylation activity (or high selectivity), (2) acidic opti-

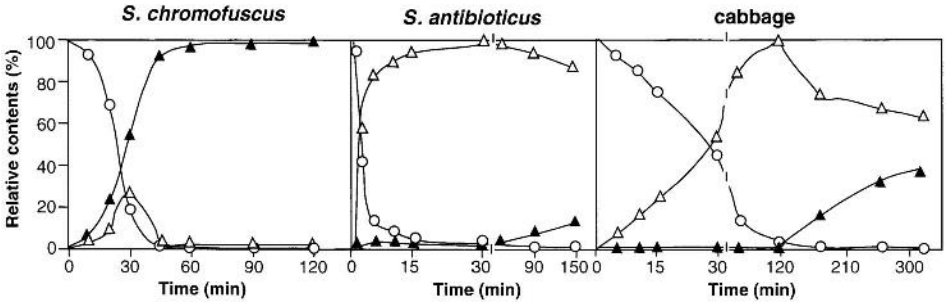


Figure 6 Comparison of PE synthesis by PLDs from *S. chromofuscus*, *S. antibioticus*, and cabbage. PC was reacted with PLD in the presence of ethanolamine. Contents of PC (○, substrate) PE (△, the transesterified product) and PA (▲, by-product) were monitored intermittently. (From Ref. 26.)

mum pH, and (3) tolerance against chelating agent such as EDTA (no absolute requirement for Ca^{2+}). The contrary of the above-mentioned characteristics are given to class II enzymes. Nakashima et al. suggested that there are several strains that produce both classes of PLDs [23].

This classification is explained from the molecular structure of the enzymes. Several class I enzymes, such as those of *S. antibioticus* [51], *Streptomyces* sp. AA586 [49], and *Stv. cimamoneum* [53], are very closely related to each other with respect to the primary sequences (Fig. 7). These class I PLDs are members of the so-called ''PLD superfamily'' in which many other eukaryotic PLDs are included [55]. In contrast, *S. chromofuscus* PLD, the only example which has been cloned among class II enzymes, is less homologous to the class I enzymes. *S. chromofuscus* PLD is rather similar to a nonspecific phosphatase/phosphodiesterase of *B. subtilus* [56]. These class II enzymes are not suitable as catalysts for transphosphatidylation [23,26]; rather, they are useful tools for quantitative analysis of PLs due to their strong hydrolytic activity and broad substrate specificity (PLD of *S. chromofuscus* can hydrolyze even PI, which is scarcely hydrolyzed by class I enzymes). A diagnostic protocol for analyses of PLs was developed using *S. chromofuscus* PLD [57].

4 ENZYMATIC CONVERSION BY OTHER PHOSPHOLIPID-RELATED ENZYMES

4.1 Transesterification by PI–PLC

There are three classes of PLCs, which are PC-preferring, PI-specific and sphingomyelin (SM)-specific classes. Of these, PI-specific PLC (PI–PLC) is reported to catalyze a kind of transesterification, as explained below [58]. This enzyme is a hydrolytic enzyme which cleaves specifically phosphatidylinositols to diacylglycerol and *myo*-inositol phosphates. The hydrolysis occurs in two steps. In the first step, the 2-hydroxyl group of the inositol ring attacks the phosphorus atom, resulting in the formation of *myo*-inositol 1:2-cyclic phosphate. The second step is the hydrolysis of the formed cyclic ester, generating *myo*-inositol-1-phosphate (Fig. 8) [59]. In the presence of an alcohol, transesterification from the cyclic ester to the alcohol takes place, resulting in the formation of the corresponding phosphodiester (Fig. 8). Generally, chemical syntheses dealing with derivatives of inositol–phosphate are very complicated, because such methods include many protection and deprotection steps for the hydroxyl groups of the inositol ring and optical resolution steps to obtain the chiral compound [60]. Thus, this enzymatic reaction might be valuable as an alternative method for syntheses of inositol–phosphate derivatives. Some of these compounds could be inhibitors for eukaryotic PI–PLC and might be used as reagents for cell biology and drugs for medical purposes.

4.2 Sphingolipid-Related Enzymes

Phosphosphingolipid (PSL) is another class of naturally occurring PLs. PSLs are usually categorized as a subclass of sphingolipids that includes glycosphingolipids as well, because of a common structure, the sphingosine moiety. Because these sphingolipids are not as abundant in natural sources as glycerophospholipids, their bulk usage (e.g., such as food emulsifiers) seems difficult. In the last years, however, much attention has been paid to these lipids due to their specific biological functions involved in cellular recognition events and transmembrane signaling.

Figure 7 Comparison of primary structure of several actinomycete PLDs. Amino acid residues identical among three enzymes are indicated with asterisks.

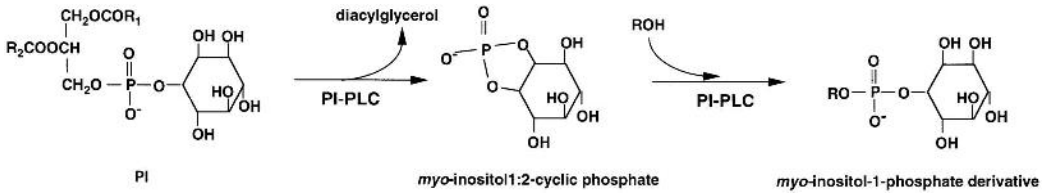


Figure 8 Transesterification catalyzed by phosphatidylinositol-specific phospholipase C. R represents alkyl group (transesterification) or H (hydrolysis).

Sphingosinephosphorylcholine (SPC) is an important compound from which various derivatives can be synthesized. SPC is usually prepared by acid methanolysis of SM [61]. However, this chemical method causes epimerization of the configuration at C3 of the sphingosine moiety, resulting in the formation of the L-*threo*-(2*S*,3*R*) form, which differs from the naturally occurring D-erythro-(2*S*,3*S*) form. Ito et al. [62] discovered a novel type of enzyme, sphingosine-ceramide-*N*-acylase (SCDase), which cleaves the *N*-acyl linkage of sphingolipids. Hydrolysis of sphingolipids (SM or other glycosphingolipids) with SCDase gave the corresponding lysosphingolipids without epimerization (Fig. 9) [63]. In addition, SCDase catalyzes condensation between fatty acid and lysosphingolipids, enabling preparation of sphingolipids with various *N*-acyl groups by a simple enzymatic reaction [64].

5 CONCLUDING REMARKS AND FUTURE PERSPECTIVES

Phospholipases as well as lipases have become powerful tools for enzymatic conversion of phospholipids. Many of the reactions described here are, in principle, very simple and well established.

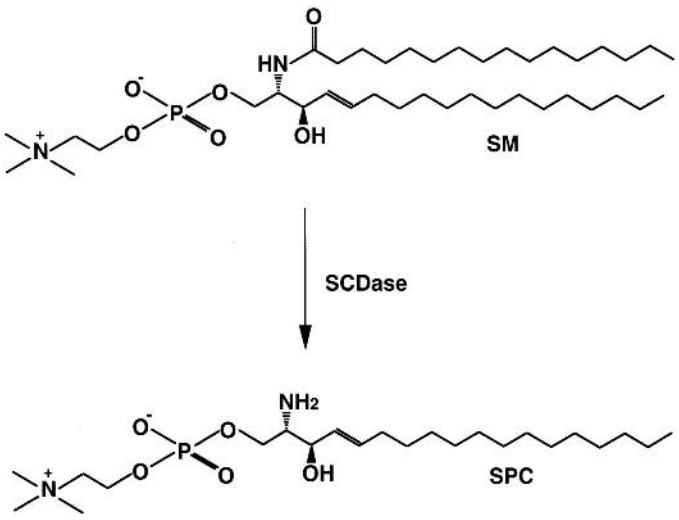


Figure 9 Enzymatic preparation of SPC.

Reducing production costs is very important for practical implementation of those methods. Production costs are influenced by many factors such as choice of the substrates, catalysts, volumetric productivity, downstream purification of products, and formation of by-products. Among them, the enzymes' costs are usually most predominant. Immobilized enzyme would facilitate continuous operation or repeated use of the catalysts, thereby lowering the costs. Effective production of enzymes themselves by recombinant DNA techniques is also promising. In addition, obtaining novel enzymes with enhanced stability by screening or by mutagenesis techniques is worthwhile.

Versatility of these reactions is another point to be considered. For example, in the case of PLD-catalyzed transphosphatidylation, phosphatidylinositols cannot be synthesized, possibly due to steric hindrance. Relaxing the enzyme's specificity by mutagenesis techniques would make the reaction more versatile.

More importantly, what to synthesize as well as how to synthesize should be considered, because these reactions might surely become beneficial when they are applied to syntheses of useful compounds for certain purposes. Collaborative works among people who study nutritional chemistry, pharmacology, and synthetic chemistry might help in designing novel useful target compounds.

REFERENCES

1. Y Nakagawa, M Setaka, S Nojima. Methods Enzymol 197:309–315, 1991.
2. AG Diagne, J Fauvel, H Chap. Methods Enzymol 197:316–325, 1991.
3. JH Exton. Biochim Biophys Acta 1212:26–42, 1994.
4. H Nanbu, N Aoi, N Kadota, Y Toda, N Yamazaki. Japanese Patent S63-279753, 1988.
5. F VanMiddlesworth, M Lopez, M Zweerink, AM Edison, K Wilson. J Org Chem 57:4753–4754, 1992.
6. DB Sarney, G Fregapane, EN Vulfson. J Am Oil Chem Soc 71:93–96, 1994.
7. JJ Han, JS Rhee. Biotechnol Lett 17:531–536, 1995.
8. CM Gupta, R Radhakrishnan, HG Khorana. Proc Natl Acad Sci USA 74:4315–4319, 1977.
9. JS Chadha. Chem Phys Lipids 4:104–108, 1970.
10. Y Nanba, T Sakakibara. US Patent 4,690,784, 1987.
11. JT Mason, AV Broccoli, CH Hung. Anal Biochem 113:96–101, 1981.
12. R Aneja, JS Chadha, EC Robels, RV Daal. Biochim Biophys Acta 187:439–441, 1969.
13. S Murakami, S Tokuyama, K Ozawa, R Kiyoda. Japanese Patent S63-157993, 1988.
14. I Svensson, P Adlercreutz, B Mattiasson. Appl Microbiol Biotechnol 33:255–258, 1990.
15. I Svensson, P Adlercreutz, B Mattiasson. J Am Oil Chem Soc 69:986–991, 1992.
16. P Pernas, JL Olivier, MD Legoy, G Bereziat. Biochem Biophys Res Commun 168:644–650, 1990.
17. D Egger, E Wehtje. P Adlercreutz. Biochim Biophys Acta 1343:76–84, 1997.
18. M Hosokawa, K Takahashi, Y Kikuchi, M Hatano. J Am Oil Chem Soc 72:1287–1291, (1995).
19. SF Yang, S Freer, AA Benson. J Biol Chem 242:477–484, 1967.
20. RMC Dawson. Biochem J 102:205–210, 1967.
21. R Aneja, JS Chadha. Biochim Biophys Acta 248:455–457, 1971.
22. R Kumar, MF Gardner, DD Richman, KY Hostetler. J Biol Chem 267:20,288–20,292, 1992.
23. J Nakajima, T Nakashima, Y Shima, H Fukuda, T Yamane. Biotechnol Bioeng 44:1193–1198, 1994.
24. LR Juneja, N Hibi, N Inagaki, T Yamane, S Shimizu. Enzyme Microbiol Technol 9:350–354, 1987.
25. LR Juneja, N Hibi, T Yamane, S Shimizu. Appl Microbiol Biotechnol 27:146–151, 1987.
26. LR Juneja, T Kazuoka, T Yamane, S Shimizu. Biochim Biophys Acta 960:334–341, 1988.

27. LR Juneja, T Kazuoka, N Goto, T Yamane, S Shimizu. Biochim Biophys Acta 1003:277–283, 1989.
28. LR Juneja, E Taniguchi, S Shimizu, T Yamane. J Ferment Bioeng 73:357–361, 1992.
29. LR Juneja, T Yamane, S Shimizu. J Am Oil Chem Soc 66:714–717, 1989.
30. P D'Arrigo, L de Ferra, V Piergianni, A Ricci, D Scarcelli, S Servi. J Chem Soc Chem Commun:1709–1710, 1994.
31. A Nagao, N Ishida, J Terao. Lipids 26:390–394, 1991.
32. Y Kokusho, A Tsunoda, S Kato, H Machida, S Iwasaki. Biosci Biotechnol Biochem 57:1302–1305, 1993.
33. M Takami, N Hidaka, Y Suzuki. Biosci Biotechnol Biochem 58:2140–2144, 1994.
34. A Nagao, J Terao. Biochem Biophys Res Commun 172:385–389, 1990.
35. T Koga, A Nagao, J Terao, K Sawada, K Mukai. Lipids 29:83–89, 1994.
36. T Koga, J Terao. J Agric Food Chem 42:1291–1294, 1994.
37. M Takami, N Hidaka, S Miki, Y Suzuki. Biosci Biotechnol Biochem 58:1716–1717, 1994.
38. S Shuto, H Itoh, S Ueda, S Imamura, K Fukawa, A Matsuda, T Ueda. Tetrahedron Lett 28: 199–202, 1987.
39. S Shuto, H Itoh, S Ueda, S Imamura, K Fukawa, M Tsujino, A Matsuda, T Ueda. Chem Pharm Bull 36:209–217, 1988.
40. M Koketsu, T Nitoda, H Sugino, LR Juneja, M Kim, T Yamamoto, N Abe, T Kajimoto, CH Wong. J Med Chem 40:3332–3335, 1997.
41. P Wang, M Schuster, YF Wang, CH Wong. J Am Chem Soc 115:10,487–10,491, 1993.
42. M Takami, Y Suzuki. Biosci Biotechnol Biochem 58:2136–2139, 1994.
43. T Yamaguchi, Y Okawa, K Sakaguchi, N Muto. Agric Biol Chem 37:1667–1672, 1973.
44. K Shimbo, H Yano, Y Miyamoto. Agric Biol Chem 53:3083–3085, 1989.
45. S Kato, Y Kokusho, H Machida, S Iwasaki. Agric Biol Chem 48:2181–2188, 1984.
46. S Imamura, Y Horiuchi. J Biochem 85:79–95, 1979.
47. I Yoshioka, J Mizoguchi, M Takahara, S Imamura, T Beppu, S Horinouchi. Japanese Patent H3-187382, 1991.
48. K Shimbo, H Yano, Y Miyamoto. Biosci Biotechnol Biochem 54:1189–1193, 1990.
49. N Tuchiya, S Miura, K Suzuki, K Yshioka. In: Industry Research Association of Technology for Conversion of Enzymatic Function in Food Industries, ed. Protein Engineering for Food. Tokyo: Shokuhin Kagaku Shimbun Shya, 1992, pp 160–182 (in Japanese).
50. K Shimbo, Y Iwasaki, T Yamane, K Ina. Biosci Biotechnol Biochem 57:1946–1948, 1993.
51. Y Iwasaki, H Nakano, T Yamane. Appl Microbiol Biotechnol 42:290–299, 1994.
52. G Carrea, P D'Arrigo, V Piergianni, S Roncaglio, F Secundo, S Servi. Biochim Biophys Acta 1255:273–279, 1995.
53. C Ogino, Y Negi, T Matsumiya, K Nakaoka, K Kondo, S Kuroda, S Tokuyama, U Kikkawa, T Yamane, H Fukuda. J Biochem 125:263–269, 1999.
54. Y Kokusho, S Kato, H Machida. Agric Biol Chem 51:2515–2524, 1987.
55. SM Hammond, YM Altshuller, TC Sung, SA Rudge, K Rose, J Engebrecht, AJ Morris, MA Frohman. J Biol Chem 270:29,640–29,643, 1995.
56. S Eder, L Shi, K Jensen, K Yamane, FM Hulett. Microbiology 142:2041–2047, 1996.
57. T Yamane. J Jpn Oil Chem Soc (YUKAGAKU) 47:1005–1013, 1999 (in Japanese).
58. KS Bruzik, Z Guan, S Riddle, MD Tsai. J Am Chem Soc 118:7679–7688, 1996.
59. DW Heinz, M Ryan, TL Bullock, OH Griffith. EMBO J 14:3855–3863, 1995.
60. T Desai, J Gigg, R Gigg, E Martin-Zamora. In: JHP Tyman, ed. Synthesis in Lipid Chemistry. Cambridge: The Royal Society of Chemistry, 1996, pp 67–91.
61. RC Gaver, CC Sweeley. J Am Oil Chem Soc 42:294–298, 1965.
62. M Ito, T Kurita, K Kita. J Biol Chem 270:24,370–24,374, 1995.
63. N Sueyoshi, H Izu, M Ito. J Lipid Res 38:1923–1927, 1997.
64. S Mitsutake, K Kita, N Okino, M Ito. Anal Biochem 247:52–57, 1997.

21

Enzymatic Synthesis of Structured Lipids

CASIMIR C. AKOH, SUBRAMANI SELLAPPAN, LYDIA B. FOMUSO, and VIVIENNE V. YANKAH

The University of Georgia, Athens, Georgia

1 INTRODUCTION

Natural fats and oils extracted from plants and animals serve as a primary source of lipid in human diet. Lipids are an important nutrient for energy, essential fatty acids (linoleic, linolenic, and eicosanoic fatty acids), and fat-soluble vitamins. They also play a very important role in growth and development, disease prevention, and maintenance of good health. Primarily, the composition and combination of fatty acids in a particular fat or oil determines its physicochemical property and nutritional value. The nutritional quality of lipids is determined by 1) content of essential fatty acids, 2) ratio of unsaturated (monounsaturated and polyunsaturated) to saturated fatty acids, 3) presence or absence of medium-chain fatty acids, and 4) presence or absence of cholesterol. However, there is no single source of lipid that can meet all of these requirements. The conventional lipids derived from plants are normally rich in unsaturated long-chain fatty acids and animal lipids are high in saturated fatty acids. Fish oils, on the other hand, are known for their high content of docosahexaenoic (DHA) and eicosapentaenoic (EPA) acids. Further, the physical properties, such as melting behavior, physical state (solid, semisolid, and liquid) at ambient temperature, and stability, determine the functional and commercial value of lipids such as cocoa butter.

Recent advances in understanding the role of each type of fatty acid and its caloric contribution, positional distribution at *sn*-1, *sn*-2, and/or *sn*-3 positions in glycerol moiety, digestion, absorption, and metabolism further defines the characteristics of good lipids for human nutrition. A delicate balance between the amount of lipid in the diet and its nutritional quality is very important in maintaining optimal health. Any lipid that contains

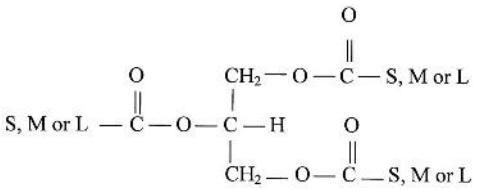


Figure 1 General structure of structured lipids; S, M, and L: short-, medium-, and long-chain fatty acids, respectively.

medium-chain and long-chain unsaturated fatty acids with less caloric value (<9 kcal/g) may be desirable for certain applications and functionality. In addition, the positional distribution of fatty acids in the glycerol backbone also plays a role in improving the nutritional quality of a lipid. The presence of long-chain unsaturated fatty acids at the *sn*-2 position is highly desirable because the action of pancreatic lipase during digestion leaves it intact and unhydrolyzed. Conservation of long-chain unsaturated fatty acids at the *sn*-2 position is physiologically important because these monoacylglycerols (MAGs) can be easily absorbed and efficiently used for the synthesis of structural components of tissues and cells rather than being metabolized or rearranged in the *sn*-1 and/or *sn*-3 positions. Nevertheless, the natural fats and oils have a randomized distribution of fatty acids among *sn* positions and the presence of long-chain unsaturated fatty acids at *sn*-2 is not always commonly observed in all kinds of lipids.

To meet these requirements, redistributing or incorporating the desirable fatty acids at specific positions led to the concept of structured lipids (SLs) (see Fig. 1) [1]. By definition, structured lipids are triacylglycerols (TAGs) which have been modified to incorporate a new fatty acid or restructured to change the positions of fatty acids or to produce novel TAGs [2].

Structured lipids with unique characteristic requirements, such as specific melting behavior, functionality, and metabolism, can be designed by introducing desirable fatty acids to enhance their use in foods, nutrition, and therapeutics. These modified fats are often considered as ''nutraceuticals'' (i.e., food or part of food that provides medical or health benefits for potential treatment and/or prevention of disease) [3]. Sometimes, they are also referred to as ''functional foods'' (provide specific health benefits) or ''medical foods'' (developed and used to treat or manage particular disease or nutritional deficiency under medical supervision). They are also known and referred to as ''physiologic foods,'' ''pharma foods,'' ''nutritional foods,'' ''designer foods,'' or ''engineered foods.''

2 ENZYMATIC SYNTHESIS OF STRUCTURED LIPIDS

The recent trend in green chemistry and natural processes using already available natural ingredients is highly preferred because of environmental and health concerns. The enzymatic synthesis of structured lipids is a relatively new concept in lipid modification. Mostly, lipases are used for this purpose. Although lipases have better ability to carry out hydrolytic reactions, the manipulation of reaction system to low water content enables their use for synthetic purposes. Nevertheless, the type of lipase plays a critical role in the synthesis of desired SLs. Any process that involves enzymes is considered to be natural.

2.1 Lipase

Lipases (E.C. 3.1.1.3) are a class of enzymes that normally catalyze the hydrolysis of lipids. They are widely distributed in animals, plants, and microorganisms. Lipases from these sources vary in their specificity and stability even though they have a common reaction pattern and substrate, lipids. Almost all lipases possess the ability to synthesize structured lipids; however, the vast majority of the studies were carried out using microbial lipases over their plant and animal counterparts. There are many advantages of using microbial lipases over their plant and animal counterparts, including 1) large-scale availability, 2) choice of specificity or nonspecificity, and 3) higher activity and stability. The sources of lipases that are primarily studied for their ability to synthesize structured lipids are listed in Table 1.

2.2 Selectivity and Specificity of Lipases

Specificity of a lipase is an important consideration in the synthesis of structured lipids. It allows the incorporation of desired fatty acids at specific positions of the glycerol moiety or to remove selectively some of the fatty acids from specific positions to concentrate a particular type of fatty acid. For example, the content of DHA and EPA in fish oil could be increased up to 80% and 90%, respectively, using *Pseudomonas* lipase [30]. The majority of lipases are either nonspecific or *sn*-1,3–specific. The *sn*-1,3–specific lipase allows the retention of the fatty acids at the *sn*-2 position while modifying at *sn*-1,3 positions. The selectivity of lipase depends on the reaction type and conditions. Some lipases, like oat seed lipase, are very selective in hydrolyzing fatty acids at the *sn*-3 position more quickly than *sn*-1 (moderately) and *sn*-2 (hardly at all) [31]. *Candida parapsilosis* lipase shows rare selectivity toward the *sn*-2 position. In addition to hydrolysis, the lipase of *C. parapsilosis* catalyzes an alcoholysis reaction in the strict sense of the term (i.e., the transfer of fatty acyl groups from acylglycerols to various alcohols without direct involvement of water). In the presence of alcohol in microaqueous medium, alcoholysis occurred preferentially to hydrolysis. The enzyme thus displays transferase activity in which the acyl acceptor may be either water or alcohol [32]. Lipases from papaya latex have been demonstrated to synthesize structured lipids [29] and exhibit *sn*-3 selectivity. Under certain conditions, this enzyme may also be selective for short-chain fatty acids [33]. The commercial protease preparations from pineapple and *Rhizopus* sp. were found to contain active lipases having a *cis*-4 unsaturation; for example, all-*cis*-4,7,10,13,16,19-docosahexaenoic acid; *cis*-6 unsaturation, such as petroselinic (*cis*-6-octadecenoic), gamma-linolenic (all-*cis*-6,9,12-octadecatrienoic), and stearidonic (all-*cis*-6,9,12,15-octadecatetraenoic) acid; and *cis*-8 unsaturation [dihomo-γ-linolenic (all-*cis*-8,11,14-eicosatrienoic)] acid [34]. The specificity of mutant prolipase from *Rhizopus delemar* showed pH dependency on fatty acid selectivity [35]. The dependence of lipase selectivity on water activity was also observed. An increase in water activity was found to increase the specificity of *Penicillium roquefortii* lipase toward *sn*-1,2 positions in dilaurin synthesis [36]. The alteration of lipase specificity through physicochemical modifications with defined natural polymers, such as carbohydrates and proteins, has been observed. The polarity of polymer seems to influence the liberated fatty acid ratio by stabilization of the modified lipase–oil/water interface complex and the different energy levels in the transition state during lipolysis [37]. The use of organic solvents also changes the substrate specificity that is influenced by the nature of the solvent [38]. This diversity in lipase selectivity allows the preparation of specific and various kinds of structured lipids.

Table 1 Sources of Lipases for Synthesis of Various Structured Lipids

Source of lipase	Substrate	Structured lipid	Ref.
Rhizomucor miehei	Tricaprylin + peanut oil	Structured lipid with essential fatty acid in *sn*-2 and medium-chain fatty acids in *sn*-1 and *sn*-3 position	4
Rhizomucor miehei	Trilinolein + medium-chain fatty acids	High essential fatty acid structured lipid	5
Rhizomucor miehei	Tricaprylin, tricaprin, trilaurin + EPA ethyl esters	TAGs containing EPA and medium-chain fatty acids	6
Rhizomucor miehei	EPA and DHA + vegetable oil	TAGs containing EPA and DHA	7
Rhizomucor miehei	Cod-liver oil	Enrichment of DHA and EPA	8
Rhizomucor miehei	EPA and DHA + corn oil	TAGs incorporated with EPA and DHA fatty acids	9
Rhizomucor miehei	EPA and DHA + trilinolein	TAGs incorporated with EPA and DHA	10
Rhizomucor miehei	EPA and DHA + ground nut oil	TAGs with EPA and DHA	11
Rhizomucor miehei	Capric and caprylic acid methyl esters + coconut oil	Enriched TAGs with capric and caprylic acids	12
Rhizomucor miehei	EPA and DHA + PUFA	Acylglycerols with EPA and DHA	13
Rhizomucor miehei	*Cis*-4 or *cis*-6 fatty acids, DHA + evening primrose, cod-liver oil	TAGs with DHA and gamma-linolenic acid	14
Rhizomucor miehei	FFA, methyl fatty acid ester + sand eel oil	Interesterified sand eel oil with DHA	15
Candida antarctica	Trilinolein + tricaprin	TAGs with essential fatty acid and medium-chain fatty acid	5
Candida antarctica (SP 435)	EPA and DHA + vegetable oil	TAGs with EPA and DHA	7
Candida antarctica	EPA + evening primrose oil	TAGs with EPA	16
Candida antarctica	EPA and DHA ethyl esters + trilinolein	TAGs incorporated with EPA and DHA	10
Candida antarctica DHA ethyl esters	Glycerol + EPA, and EPA	TAGs with DHA	17
Candida cylindraceae	EPA and DHA + sardine oil	EPA- and DHA-enriched sardine oil	18
Candida cylindraceae	EPA and DHA + glycerol	EPA and DHA containing TAGs	19
Candida cylindraceae	*B. orientalis* seed oil + *cis*-5 20:3 and 20:4	Enriched *B. orientalis* seed oil	20
Candida cylindraceae	DHA + acylglycerol	Enriched DHA in partial acylglycerol	21
Candida cylindraceae	Tuna oil	Enrichment of DHA in tuna oil	22
Candida cylindraceae	Cod liver, sardine oil	Enrichment of DHA and EPA	23
Choromobacterium viscosum	Peanut oil + tricaprylin	TAGs with essential fatty acids at *sn*-2 position	4

Table 1 Continued

Source of lipase	Substrate	Structured lipid	Ref.
Choromobacterium viscosum	Tuna oil	Enriched tuna oil with DHA	24
Choromobacterium viscosum	EPA and DHA fatty acids	TAGs with DHA and EPA	19
Choromobacterium viscosum	Seal and whale oil	MAGs with EPA and DHA	25
Choromobacterium viscosum	Chilean fish oil	Enrichment of EPA and DHA	26
Choromobacterium viscosum	Tuna oil	DHA-enriched tuna oil	24
Choromobacterium viscosum	Sardine oil	Enriched with EPA and DHA	18
Pseudomonas sp.	Sardine oil	Enriched with EPA and DHA	18
Pseudomonas sp. (PS-30)	Glycerol + PUFA	EPA and DHA high acyl-glycerols	13
Pseudomonas sp.	Seal and whale oil	MAGs with EPA and DHA	25
Rhizopus arrhizus	Glycerol + capric acid and ester	TAGs with medium-chain fatty acids	27
Rhizopus delemar	Seal and whale oil	MAGs containing EPA and DHA	25
Carica papaya (papaya latex)	Tripalmitin + rapeseed oil fatty acids	TAGs resembling human milk	28
Carica papaya (papaya latex)	Hydrogenated soy oil + tributyrin	Short-chain triacylglycerols	29

2.3 Mechanism of Lipase Action in Low-Water Media

An aqueous environment is important for the formation of active three-dimensional structure and expression of lipase activity. The nature of lipolytic reaction by lipases is more complex than other hydrolases because the substrate is often water insoluble. The immiscibility of lipids in water leads to a heterogeneous reaction media by forming a liquid–liquid interface for oils and solid–liquid interface for solid fats.

Generally, the lipase activity in a two-phase or emulsion system is influenced by the nature of the interface and interfacial area. This is also true for aggregated substrates in water [39]. This is due to the interfacial nature of lipase and the presence of the hydrophobic domain. Adsorption of lipase at interface activates the enzyme by opening the lid. All types of interface like solid–liquid, liquid–liquid, or liquid–gas can influence lipase adsorption and activity that depend on the interfacial hydrophobicity. The influence of adsorption on the activity of lipase has been demonstrated [40]. The amount of adsorbed enzyme is directly proportional to the interfacial area. That means the increased interfacial area would increase lipase activity. A sequence of events occurs before complete catalysis is achieved. Initially, the enzyme is activated by adsorption, followed by substrate binding and then catalysis. As the reaction proceeds, the accumulation of products on the interface causes the surface pressure to decrease, which corresponds to high surface energy. These effects, in turn, strongly exert denaturing effect on the enzyme molecule. These phenomena predominantly occur in high-water reaction systems as in two phase or emulsions,

where the enzyme is in completely solubilized state and has absolute flexibility in its three-dimensional structure. However, the reaction environment in structured lipid synthesis is much different and primarily contributed by the hydrophobic environment, in the presence of bulk organic solvents (mainly hexane) with very little water.

Water, specifically water activity, a_w, plays a very important role in determining the direction of reaction toward either hydrolysis or synthesis. The reduction of water content in the synthesis of triolein from glycerol and oleic acid by castor seed lipase was demonstrated by Jalander in 1911 [41]. In 1938, Kaufmann [42] established that the free energy for TAG hydrolysis is on the order of 0 kcal/mol. Thus, a thermodynamic basis was established for the observation of ester synthesis by the reverse reaction of lipase. However, interest in further application was not renewed until the 1960s. The reduction in water content by introduction of organic solvents to replace water was initiated by Dastoli and co-workers in 1966 and 1967 [43–45]. Further, the dispersed lipase was found to be active in organic solvents [46,47]. The esterification potential of lipase over a broad substrate spectrum was investigated by many authors [48–53]. These remarkable properties of enzymes suspended in organic solvents made possible the synthesis of many types of structured lipids of interest. Iwai et al. [48] demonstrated that a_w plays a crucial role in the synthetic capability of lipase. Control of water activity (usually $a_w < 1$) is one of the crucial factors in the synthesis of structured lipids. Reductions of water content to such low levels have a positive effect on stability (increased) and a negative effect on activity (decreased). Water enhances enzyme flexibility by shielding interactions between polar residues [54]. The control of water content directly influences the rigidity of lipases and thus the marked increase in stability and reduction in activity. During catalysis, enzymes undergo conformational changes upon binding with the substrate at the active site and back to the original conformation after catalysis in a continuous fashion. Any change in the surrounding solvent influences the overall flexibility. Suspended enzymes in nonpolar solvents have lower activity due to reduced flexibility. However, the introduction of denaturants like guanidine chloride, dimethyl sulfoxide, and formide increased the activity by enhancing the interaction between protein polar groups and the solvent due to increased flexibility [55,56]. The decrease in lipase activity in organic solvents is not due to inactivation but to lack of interfacial activation. Although the enzyme is surrounded by molecular layer of water, it could not be effectively adsorbed on the interface because of molecular rigidity. The interfacial activation is not observed in this system because the enzyme is insoluble and suspended as particles. The insolubility also changes the nature of interface, from the liquid–liquid interface (as in emulsion) to the solid–liquid (in organic solvents) interface. Further, the presence of insolubilized enzyme brings in other complications such as conformational or steric effect, partitioning, microenvironment, diffusion, and mass transfer effects. All these factors contribute to the lower synthetic activity of lipase in organic solvents than in other systems such as microemulsions.

3 TYPES OF LIPID MODIFICATION REACTIONS

3.1 Hydrolysis

Hydrolysis reactions involve an attack on the ester bond of fats in the presence of water to produce glycerol, fatty acids (FA), and partial acylglycerols, depending on the positional specificity of the enzyme. The hydrolysis of water-soluble carboxylic acid esters by lipases is rather slow. Preferred substrates are hydrophobic FA esters and TAG with mainly long-

chain FAs [57]. These compounds have low solubility in water and, thus, water-soluble lipases are forced to catalyze the hydrolysis of ester bonds at the interface between the aqueous phase in which the enzyme is dissolved and an insoluble substrate phase. Partial acylglycerols and soaps of free fatty acids (FFAs) formed during lipase reactions are surface active and tend to accumulate near the interface. This may prevent access of the lipase to further substrate, making it difficult to maintain a constant reaction rate over a long period of time, as is required for an extensive hydrolysis of the substrate [58]. Different technologies have been applied to improve the rate of lipase-mediated hydrolysis reactions. These include the use of two lipases [59] to affect complete hydrolysis. The use of organic solvents sometimes increases the rate of hydrolysis [60]. The most common reason for using organic media for enzymatic reactions is that the substrate to be converted is poorly soluble in water. The addition of a moderate amount of organic solvent increases the solubility of hydrophobic substrates, thereby making the reaction feasible. Both water-miscible and water-immiscible solvents can be used [61]. Lipase-catalyzed hydrolysis is carried out in a biphasic medium, and various types of membrane reactors have been used for this process [62].

In the synthesis of SLs, hydrolysis reactions are usually employed singly [63] or in combination with other transesterification reactions to give a higher yield of the desired product [64]. In two-step enzymatic reactions, selective hydrolysis is used to concentrate a particular fatty acid as the 2-MAG. This is then reacted with fatty acids using a position-specific enzyme to obtain incorporation at positions 1 and 3 [65]. This technique has been shown to produce not only high yields but also high-purity SLs. The regioselective fat-splitting ability of pancreatic lipase is useful for selective hydrolysis. A method of selective hydrolysis has also been used in the production of DHA-rich oil as an alternative to traditional winterization. Winterization gave low yields and the value of DHA enrichment was about 35% [66]. Selective hydrolysis was used to concentrate DHA in the undigested acylglycerols by hydrolyzing tuna (or bonito) oil with a lipase that shows low activity on DHA [21–23]. *Candida* lipase was effective in the production of oil containing a high concentration of DHA at 57% [21]. Nutritionally speaking, the acylglycerol form of PUFA is metabolized better than their methyl or ethyl esters. Intestinal absorption of alkyl esters of n-3 fatty acids was reportedly impaired in laboratory animals [67–69]. It has also been shown that methyl and ethyl esters of unsaturated fatty acids hydrolyze at a slower rate than their corresponding acylglycerols [70]. Food flavors have also been enhanced by partial hydrolysis of triacylglycerols. Well-known examples include the manufacture of various cheeses (Italian, American, cheddar) and lipolyzed milk fat products such as butter flavors and cultured cream flavors [71].

3.2 Direct Esterification

Direct esterification is the opposite of hydrolysis. The shift in equilibrium between the forward reaction (hydrolysis) and the reverse reaction (direct esterification) is affected by a number of variables such as the water content of the reaction mixture. For practical purposes, not only the reaction rate but also the yield is important. The equilibrium yield of the ester formed can be increased by choosing a solvent that favors solubilization of the ester groups of the substrates [72]. The a_w and water levels in direct esterification reactions control the equilibrium position of reactants and are very essential to the product yield. A study of the effect of water activity on reaction rates and equilibrium positions in enzymatic esterification showed that the true reaction medium is comprised of a mixture

of solvent, substrates, and water. High substrate concentrations can increase the polarity of the reaction medium and increase the solubility of water. On the other hand, the amount of water also influences the polarity of the medium, which, in turn, increases the solvation of the substrates. These two effects, put together, influence the equilibrium position of the esterification reaction [73]. Solvent-free lipase-catalyzed esterifications [74,75] as well as the use of supercritical fluid as a reaction medium [76,77] have been successfully conducted. The major advantage of supercritical fluid is that it can be vented to the atmosphere, allowing products to be recovered without a trace of solvent. This aspect is very important in the food industry [78]. An application of lipase-catalyzed esterification reaction is the synthesis of sugar esters, which can be used as emulsifiers or fat substitutes, depending on the degree of acyl group incorporation into the sugar molecule. Esters of glucose, fructose, sorbitol, sucrose, and alkyl glycosides were synthesized with lipases [79–81]. Flavor esters mimicking flavor profiles found in the dairy products have been produced enzymatically [82].

A common concern with direct esterification reactions is the accumulation of water in the system. This may inhibit the activity of the lipase or enhance hydrolysis of the formed ester [83]. Formed water is continuously removed by carrying out the reaction at high temperature (60–75°C) in open test tubes or under reduced pressure (0.1–0.05 mmHg) or in the presence of molecular sieves [74,84,85].

3.3 Transesterification

Transesterification involves the exchange of acyl groups or radicals between an ester and an acid (acidolysis), an ester and an alcohol (alcoholysis), two esters (namely two triacylglycerols or TAGs), and an ester (interesterification or ester–ester interchange), or an ester and an amine (aminolysis). With transesterification reactions, the problem of water encountered in direct esterification reactions is eliminated. Generally, hydrolysis precedes esterification in transesterification reactions.

3.3.1 Acidolysis

Acidolysis is an effective means of incorporating novel fatty acids into TAG in the synthesis of structured lipids. Based on the metabolic effects of individual fatty acids, regiospecific enzymes are used with acidolysis for restructuring TAG to obtain a specific arrangement to serve the desired purpose, including nutritional, medical/therapeutic, low caloric value, and functionality. Acidolysis has been used to incorporate free acid forms of EPA and DHA into vegetable and fish oils to improve their nutritional properties. Shimada et al. [86] incorporated caprylic acid in tuna oil by acidolysis reaction. Their results showed that fatty acids in the tuna oil were exchanged for caprylic acid and that hydrolysis rarely occurred. In addition, the enzyme used in catalyzing the reaction, *Rhizopus* lipase, exhibited stricter specificity in acidolysis than in hydrolysis. Performing an acidolysis reaction between cod-liver oil and free EPA and DHA, Yamane et al. [87] used immobilized lipase from *Rhizomucor miehei* to increase the EPA content in the oil from 8.6% to 25%, and the DHA content from 12.7% to 40%. Ethyl esters of EPA have been used to enrich fish oil by interesterification, yielding contents of 40 wt% EPA and 23 wt% DHA [88]. During acidolysis in a fixed-bed reactor, Yamane et al. [8] increased the polyunsaturated fatty acid content of cod-liver oil by reducing the temperature in the product reservoir to between −10°C and −20°C. This led to crystallization and removal of more saturated fatty acids present in the fish oil. Acidolysis has also been used to incorporate oleic acid into

milk fat [89]. This process led to an increase in the level of unsaturated fatty acids in butter without loss in the characteristic flavor of butter. Acidolysis of milk fat with oleic acid was also found to decrease the crystallization temperature and lower the melting range of the milk lipids [90].

3.3.2 Alcoholysis

An alcoholysis reaction between an alcohol and an ester produces methyl esters. During alcoholysis, hydrolysis of triacylglycerols to produce diacylglycerols (DAG) and MAG can occur, although the presence of small amounts of alcohol can inhibit hydrolysis [90]. Alcoholysis reactions have been performed using nonspecific lipases from *Candida cylindracea* to produce menthyl acetate and butyrate from menthol, triacetin, and tributyrin [91]. Recently, formation of methyl esters from TAGs has received considerable attention due to their potential use as petrodiesel substitutes [92,93]. Problems, such as the slowing of reaction rate, were encountered with methanolysis of vegetable oils, due to the formation of a two-phase reaction system, preventing the occurrence of second-order kinetics. Increasing the molar ratio of methanol to vegetable oil to 27, produced a one-phase system of higher polarity, with product yield increasing to 99.4% in 7 min [94]. Acetic acid inhibits lipase activity in direct esterification reactions [51]. To minimize this inhibitory effect, Chulalaksananukul et al. [95,96] proposed an alcoholysis reaction to synthesize geranyl acetate from geraniol and propyl acetate using Lipozyme IM 60, a *Rhizomucor miehei* lipase. Castro et al. [97] considered alcoholysis a better method for synthesis of citronellyl acetate as compared to other methods such as transesterification and esterification. Substrate partitioning between the immobilization support and the organic medium seemed to greatly influence the catalytic performance of the lipase preparation. Alcoholysis in aqueous medium with lipase from *Candida parapsilosis* was made possible by methanolic inhibition of hydrolysis of the esters [98]. The main application of alcoholysis is for glycerolysis reactions. Glycerolysis is the exchange of acyl groups between glycerol and triacylglycerol to produce MAGs, DAGs, and TAGs. This reaction method has been discussed as an alternative to glycerolysis by chemical interesterification for synthesis of MAG. Monoacylglycerols have a wide range of applications in the food industry. Besides functioning as surfactants in emulsions, foams, aerosols, and suspensions, they are also utilized to modify starch or protein-containing products via complex formation with amylose or proteins, or to modify physical characteristics of fats by controlling fat crystal polymorphism [99]. MAGs produced from coconut oil by solid-phase glycerolysis catalyzed by lipase PS-30 from *Pseudomonas* sp. and purified by hexane fractionation could be used to control *Listeria monocytogenes* in certain dairy products or in other foods that contain reduced fat [100]. Maximum yields of MAG produced via lipase-catalyzed synthesis range from 74% to 96% conversion of starting materials and an estimated 15 kg of MAG per gram of enzyme. Weiss [101] reported a 96% yield using *Penicillium cyclopium* and *Rhizopus* sp., whereas Holmberg and Osterberg [102] achieved an 80% yield of MAGs using an oil-rich microemulsion system. Continual improvements in these lipase-catalyzed systems, especially bioreactors utilizing immobilized enzyme, may increase MAG yields and purity to the point of economic feasibility [99].

Nonspecific lipases are usually used in glycerolysis reactions, giving a wide range of reaction products. Lipases derived from *Pseudomonas fluorescens* and *Chromobacterium viscosum* have been shown to have high glycerolysis activity [103]. To obtain high yields in lipase-catalyzed monoacylglycerol synthesis, newly formed MAGs are removed from the reaction mixture by temperature-induced crystallization. This pushes equilibrium of

the reaction toward increased MAG production. Glycerolysis of lipids containing saturated fatty acids leads to an increased concentration of saturated monoacylglycerols in the reaction product mixture, because they crystallize at lower temperatures than unsaturated monoacylglycerols [104]. Vegetable oils have low critical temperatures (T_c) at 5–10°C as compared to animal fats with T_c between 30°C and 46°C, which is mainly attributed to the presence of long-chain unsaturated fatty acids [90]. By reducing the reaction temperature to a value below T_c, yields of MAGs can be increased from 30% to 90% [103]. Yang et al. [105] found the best supporting conditions for the formation of 50–55% MAG at 35°C, 2.5–4.8% water in glycerol, and a molar ratio of acyl groups to glycerol of 0.66–0.85. At temperatures greater than 35°C, up to 50% reduction in yield of MAG was reported. The temperature control of reaction equilibria was not attributable to changes in availability of water. However, the ability of the chosen lipases to mediate glycerolysis reactions with butter oil and other oils appeared to be principally controlled by water availability in the reaction mixture [106]. Increasing the water content between 0.5% and 5.7% increases the production of MAGs, and water content above this level does not increase the yield any further [103]. Depending on the physical characteristics of substrates used for glycerolysis, the temperature control can be limiting. Glycerolysis of melted tallow with immobilized *Rhizomucor miehei* lipase for synthesis of MAG at 42°C resulted in MAG crystallization, which improved the yield up to 50%, but further yield increases were prevented by solidification of the reaction mixture. The main problem with lipase-catalyzed glycerolysis is the long reaction time. About 4–5 days is required to produce high yields of MAGs [103]. Enzymatic glycerolysis of soybean oil has been studied using nine different lipases [107].

3.3.3 Interesterification

In general, lipase-catalyzed transesterification produces fat with a slightly lower solid fat content than is obtained by means of chemical interesterification. This may be attributed to contamination by MAGs, DAGs, and FFAs, which are produced in the early stages of transesterification. Kalo et al. [108] compared lipase-catalyzed transesterification to chemical interesterification of butter. They found that the solid fat content of butter increased from 41.2% to 42.2% at 20°C when lipase-catalyzed interesterification was used, whereas chemical interesterification produced butter with a solid fat content of 57.8% at 20°C.

The main disadvantage of using cocoa butter in chocolate and confections is its high cost. A cocoa butter equivalent can be made from inexpensive fats and oils by interesterification. By transesterifying fully hydrogenated cottonseed and olive oils, Chang et al. [109] produced a cocoa butter substitute with similar POS levels and slightly higher SOS (where P = palmitic, O = oleic, S = stearic acids) levels than those found in cocoa butter. Interesterification reactions are preferably carried out in hydrocarbon solvents, which facilitate the reaction by lowering the viscosity of the reaction medium; however, for nutritional safety, the reaction product has to be bleached, deodorized, and the solvent completely removed [110]. Therefore, interesterification in the absence of solvents has been preferred by an increasing number of researchers [108,110,111] although solvent-free systems require higher processing temperatures. Balcao et al. [112] produced butterfat with improved nutritional properties using *sn*-1,3-specific lipase in solvent-free hydrolysis and ester interchange reactions with controlled water activity. Their lipase-modified butterfat possessed a wider melting temperature range than regular butter and had less long-chain fatty acid residues (10.9% lauric, 10.7% myristic, and 13.6% palmitic acids), with an increase in

the proportion of (hypocholesterolemic) monoene TAG, than those of the original butter fat.

3.4 Factors Affecting the Success of Synthesis

3.4.1 Water

The amount of water present in a reaction mixture influences biocatalysis in several ways. The presence of water affects the activity of the enzyme, as well as creating an immiscible system of an organic and aqueous phase. In interesterification reactions, water is needed to initiate hydrolysis before synthesis of TAG can occur. However, depending on the enzyme used, increasing the water content of the system above the levels required to maintain the enzyme structure in the activated state will result in decreases in the yield of synthesis. In direct esterification reactions, water is produced and must be removed to enable the reaction to proceed toward synthesis of esters. Removal of water from the reaction mixture can be achieved by use of molecular sieves or by microporous membranes. Molecular sieves are added after the reaction has proceeded for a period, probably to a level where the initiating hydrolysis reactions have already taken place. In glycerolysis reactions, with one of the substrates being glycerol, water formation is expected in the reaction system and this has to be removed to prevent hydrolysis. The presence of water also causes acyl migration to occur, leading to formation of a high level of MAGs as opposed to ester formation.

Water activity can be kept constant by having a saturated salt solution in contact with the gas phase of the reaction mixture, whereby continuous removal of the water produced is made possible through the course of the reaction [90]. Another method for controlling water activity in interesterification reactions is the immersion of silicone tubing containing the salt solution in the reaction vessel. Water vapor can be transferred out of the reaction system across the tubing wall and into the salt solution [73]. A convenient and simple method to maintain a constant water activity is the addition of a suitable salt hydrate pair to the reaction medium [113,114]. A salt hydrate pair acts as a buffer by releasing or removing water molecules as required, keeping the a_w during the reaction at a characteristic a_w value at a given temperature, as long as some of both salt hydrate pairs remains in the reaction medium [113].

3.4.2 Substrate and Product Effects

The composition of the substrate affects the yield of synthesized TAG in interesterification reactions. This is particularly related to the properties of the lipase used for synthesis. The chain length of the substrates seems to affect the activity of enzyme. In an acidolysis reaction of stearic acid and oleic acid as compared to stearic acid with caprylic acid, *Rhizomucor miehei* lipase seemed to have a preference for longer-chain fatty acids as compared to medium chains [115]. The hydrophobic tunnel in lipase accepts aliphatic chains and aromatic rings more easily than branched structures [116,117]. Using carboxylic acids of differing chain lengths, Miller et al. [117] found that increasing the acyl group chain lengths up to seven carbons increased the esterification rate of lipase from *R. miehei*. The activity of esterase 30,000 from *R. miehei* in organic solvent increases when the acid carbon chain length increases [118]. Inhibition of the enzyme by short-chain acids occurs as a result of lowering the local pH around the enzyme, causing an alteration in the protonation state of the enzyme, and the molar concentrations of these

acid functions decrease when the acid carbon chain length increases [119]. However, another report [120] showed that the activity of lipase from *R. miehei*, immobilized on celite, decreased when the acid or alcohol carbon chain length was increased. It seems that the activity of enzymes toward the substrates depends on the enzymes' pretreatment (immobilization, chemical modification). It can also depend on the type of culture medium of the organisms from which the enzymes were extracted [119]. With free-enzyme systems, the buildup of FFAs will reduce lipase activity until the enzyme eventually loses its catalytic ability [121]. Inhibition of lipase activity by FFA can be related to the Michaelis–Menten model for uncompetitive inhibition by a substrate:

$$v = \frac{V_{\max}(S_0)}{(S_0)[1 + (I)/K_i] + K_m}$$

where (S_0) is the initial free fatty acid concentration, I is inhhibitor concentration, K_i is the inhibition constant, and K_m is the Michaelis constant. Immobilization of lipase onto a solid support can minimize product inhibition, but the process may cause a large loss in activity [122]. Another method of counteracting the effect of product inhibition is to remove the inhibiting species as they are being produced. This approach has proven effective in increasing the productivity of product-inhibited enzymatic reactors [123]. The nucleophilicity of the substrate is important to the rate of the reaction. The presence of a hydroxyl group in the *sn*-2 position has a negative inductive effect, so TAGs are hydrolyzed faster than DAGs, which are hydrolyzed faster than MAGs [116]. High concentrations of substrate can cause substrate inhibition effect and lower the amount of synthesized products. The hydrolysis activity of a chlorophyllase on refined, bleached, and deodorized (RBD) canola oil decreased with increase in the oil content [124]. This is an example of steric hindrance to lipase activity and impedes the substrates from reaching the active site of the enzyme. Oxidation of substrates, particularly polyunsaturated fatty acids (PUFA), can cause inhibition and inactivity of lipases. Inhibition is seen at hydroperoxide levels greater than 5 mEq/kg oil and is attributed to the breakdown of hydroperoxides to free radicals [125]. The mechanism to account for the resistance of lipases toward long-chain n-3 PUFA in marine oils has been illustrated by Bottino et al. [126]. The presence of carbon–carbon cis double bonds in the fatty acids result in bending of the chains. The terminal methyl group of the fatty acid thus lies close to the ester bond, thereby causing a steric hindrance effect on lipases. The presence of five and six double bonds in EPA and DHA, respectively, enhances this steric hindrance effect, increasing rigidification and making the substrates less accessible to the enzyme. Saturated or monounsaturated fatty acids do not present as many barriers and are more easily hydrolyzed by lipases. Branched and secondary alcohols are poor substrates for lipases [127].

3.4.3 Temperature

Temperature aids in solubilizing reactants for biocatalysis. However, high temperatures cause denaturation and do not favor enzyme activity. The optimum temperature for most immobilized lipases falls within the range of 30°C to 62°C, whereas it tends to be slightly lower (30–40°C) for free lipases [128]. The distribution of activities of lipases relative to various triacylglycerols changes with temperature; as temperature is increased, the rates of release of long-chain fatty acids increase faster than those of the corresponding short-chain acids [129]. The temperature for synthesis can be regulated to effect the crystalliza-

tion of product, which can be removed to direct the equilibruim toward more synthesis. This technique has been applied in glycerolysis reactions [130].

High temperature reduces the viscosity of the reaction mixture, which is related to mass transfer and the overall reaction rate. Temperature also has an effect on acyl migration.

3.4.4 Enzyme

Lipases have been used extensively as stereoselective catalysts in the conversion of fats and oils to produce desirable high-value products. The type of enzyme selected will affect the expected SL synthesis. As proteins, lipases are affected by the conditions in the reaction mixture as water (a_w), pH, temperature, solvent system, and so forth. Because substrates used for synthesis are often more hydrophobic than hydrophilic, recent developments are directed toward performing synthetic reactions in hydrophobic solvents to aid solubility of substrates and reduce reaction temperatures. Lipases active in organic media typically require a certain amount of water to confer conformational flexibility necessary for catalysis. Both water and the solvent system play a critical role in determining lipase activity. Enzyme immobilization increases the thermostability of enzymes and allows enzyme separation and reuse to reduce the cost of their production, thus extending their practical use.

The specificity of lipases have classically been divided into five major types: 1) lipid class, 2) positional, 3) fatty acid, 4) stereochemical, and 5) combinations thereof [131]. Thus, absolute specificity is considered debatable because this characteristic is subject to the conditions of the reaction mixture.

3.4.5 Solvent System

The effect of solvent systems on biocatalysis is an important aspect of enzymatic SL synthesis. Information on the choice of solvent systems indicates the potential advantages of conducting biocatalytic reactions in aqueous–organic solvent systems or in pure organic solvents. These include 1) the relative solubility of many compounds in organic solvents, 2) the ability to reverse hydrolytic reactions into synthetic reactions in nonaqueous media, 3) the possibility of diminishing undesirable side reactions in organic media, as well as substrate and product inhibition, and 4) the relative ease of product and biocatalyst recovery from systems containing an organic phase [132]. Use of organic solvents has its limitations because many organic solvents are known to inactivate or to denature biocatalysts. The polarity or hydrophobicity of solvents can have a profound effect on the three-dimensional structure of an enzyme and the retention of enzyme-associated water necessary for catalysis. It has been reported that the polarity (or strictly referred to as the hydrophobicity) of the solvent system is better expressed as the partition coefficient ($\log P$) and has been correlated to the rate of synthesis [132]. For mixtures of two solvents, the general semiempirical formula

$$\log P_{\text{mixture}} = X_1 \log P_1 + X_2 \log P_2$$

can be used as a qualitative measure of polarity, where X_1 and X_2 are the mole fractions of components 1 and 2, respectively [133]. In the case of not too apolar substrates, higher activities are feasible using organic solvents having a log P value between 2 and 4. In this case, the gain in activity has to be weighed against the possible loss in biocatalytic stability. The conformation of biocatalysts can be stabilized by substitution of H_2O with D_2O, which may be advantageous in nearly anhydrous reaction media. Matrices that

bind water very tightly will help protect the biocatalyst against the water-distorting activity of the surrounding organic solvent; hence high activities for prolonged periods can be expected in solvents having a log $P < 4$ [134]. Recently, more attention was drawn to the use of highly hydrophobic solvents, such as hexane, heptane, octane, and cyclohexane, in synthetic reactions. Due to the need for a monophasic system and to avoid mass transfer limitations in hydrolysis reactions, hydrophilic solvents, such as acetone, dimethylsulfoxide (DMSO), dimethylformamide (DMF), tetrahydrofuran (THF), dioxane, acetonitrile, and many alcohols, are used in enzymatic and organic synthesis reactions [135].

3.4.6 Acyl Migration

During lipase-catalyzed interesterification, some acyl migrations occur as a result of formation of partial acylglycerols, especially DAG, a necessary and unavoidable intermediate in the formation of TAG. Acyl migration in a typical DAG is initiated by the nucleophilic attack of a lone pair of electrons of the free hydroxyl oxygen on the ester carbonyl carbon, formation of an unstable cyclic intermediate, resulting in a five-member ring intermediate [136]. When the ring opens, two products are formed, the original conformation of DAG and another DAG with a migrated fatty acid position. Acyl migration can occur from the *sn*-2 position to the *sn*-3 or *sn*-1 position, or from positions *sn*-1 and *sn*-3 to the *sn*-2 position. This migration occurs until an equilibrium is achieved. In all reaction mixtures, acyl migration is expected because the 2-DAG is continuously produced [137–139] and 1,2(2,3)-DAGs are not thermodynamically stable compounds. They tend to change into 1,3-DAG at a ratio of about 2:3 when equilibrium is reached [140]. Different chain lengths [141] as well as different double bonds in FAs [142] have different migration rates, with more unsaturated fatty acids migrating faster.

The presence of a strong acyl donor in the reaction mixture can cause acyl migration. Strong acids have been reported as a source of acyl migration of partial acylglycerols. In acidic environments, the attraction of a proton to the negative charge on the carbonyl oxygen will accentuate the electrophilicity of the carbon atoms. However, because fatty acids are weak acids, their protonating effects will probably be minimal [142]. Methyl esters are alternatives to FFA as acyl donors and produce more DAG during lipase-catalyzed interesterification [143]. Nonpolar solvents cause higher rates of acyl migration [144]. Polar solvents, such as acetone and chloroform or the presence of small amounts of water in a solvent-free system, reduce the rate of acyl migration [142]. In solvent-free systems, water did not induce acyl migration. Reported effects of acyl migration rates are probably due to the differences in DAG quantities produced at different water levels [86,145].

The lack of ''absolute specificity'' of enzymes is, to an extent, a result of acyl migration. Reports on absolute specificity of enzymes have been controversial [137,145], although it is generally accepted that most lipases are specific in reaction systems. Besides, the specificity of individual lipases can change as a result of microenvironmental effects on the reactivity of functional groups or substrate molecules [146]. *R. miehei* lipase, an enzyme known to be 1,3-specific, has been used successfully in the synthesis of triolein from glycerol and oleic acid. This was possible because the ester enzymatically formed with the primary alcohol isomerizes, through acyl migration, to an ester on the secondary hydroxyl, and the freed primary hydroxyl group may then undergo further enzymatic conversion [136]. Some enzyme immobilization supports and salt hydrate additives used to control water activity in enzymatic reaction systems have been reported to influence acyl

migration [147]. It is therefore important to optimize immobilization processes and reactor design to reduce acyl migration [142].

Temperature and reaction time are two thermodynamic factors that influence the equilibrium of the reaction and, thus, acyl migration. If the acyl migration rate in a reaction could be related to the Arrhenius equation, the temperature would directly relate to the acyl migration rate as follows:

$$k = A\exp\left(\frac{-E}{RT}\right)$$

where k is the rate, A is a constant, E is the activation energy, R is the gas constant, and T is the temperature. The reaction time also relates to temperature, and less time is needed to reach equilibrium when high temperature is used. This was the case when acyl migration of 1,2-dipalmitoyl-glycerol at different temperatures and time was studied [140].

4 STRUCTURED LIPIDS TARGETED FOR NUTRITIVE AND THERAPEUTIC PURPOSES

With an increasing understanding of the different properties of fatty acids, more research was focused on the possible use of fats as nutraceuticals. The nature and type of fatty acid in a TAG determines its functional and physical properties as well as its metabolic fate and health benefits. A variety of fatty acids can be used in the synthesis of SLs taking advantage of the functions and properties of each to obtain maximum benefits from any given SL [2]. These fatty acids include short-chain fatty acids (SCFA), medium-chain fatty acids (MCFA), long-chain unsaturated and polyunsaturated fatty acids, and saturated long-chain fatty acids (LCFA). SLs are suited for use as nutraceuticals because their structure can be manipulated to suit specific patient requirements.

Lipase specificity is the main advantage of lipase-catalyzed interesterification over chemical interesterification. This is of nutritional significance because 2-MAGs are preferentially absorbed in the small intestine over *sn*-1 or *sn*-3 fatty acids. They are preferentially absorbed because they readily form mixed micelles with bile acids and cannot form insoluble soaps with divalent cations, as seen especially with *sn*-1,3-liberated saturated fatty acids [148]. Lipase-catalyzed interesterification using a 1,3-specific lipase can be used to alter the fatty acid composition in positions 1 and 3 to meet targeted structural requirements while retaining the nutritionally beneficial essential fatty acids at the *sn*-2 position [149]. Medium-chain triacylglycerols (MCTs) have been used for years to meet the nutritional needs of patients with symptoms of fat malabsorption, such as patients with Crohn's disease, cystic fibrosis, colitis, and premature infants. MCTs provide a rapid energy source because they are absorbed through the portal system, are not carnitine dependent for entry into the cell mitochondria, do not require chylomicron formation, and are easily oxidized and utilized as fuel and energy. They have a higher plasma clearance and improved nitrogen-sparing action, provide better than twice the caloric density of proteins and carbohydrates, and have little tendency to deposit as body fat. They therefore provide necessary calories in an easy-to-digest form that is suitable for enteral and parenteral feeding [3]. Total parenteral nutrition (TPN) is the provision of all nutrient requirements by the intravenous route without the use of the gastrointestinal tract (GIT). However, lack of essential fatty acids, thermogenicity, and limited enteral and parenteral tolerance has made MCTs

less ideal substrates for intravenous use. Essential fatty acids are the precursors of the various prostaglandins that are of great physiological importance. MCTs may lead to hyperketonemia and elevated levels of lactic acid in some patients and have been associated with central nervous system (CNS) toxicity. Conventional fats and oils are composed of acylglycerols of LCFA and are designated as long-chain triacylglycerols (LCT) such as soy, corn, safflower, olive, and sunflower oils. Although LCT emulsions are too slow in clearing from the blood and oxidize too slowly to supply fuel and energy, MCTs may be too rapid in clearing. An SL with sufficient essential fatty acids will serve to slow down the clearance of the MCT backbone to a more acceptable level for enteral and parenteral feeding [1]. An SL having about 25% linoleic acid (Captex 810B) appears to be suitable for such use [1]. Structured lipids comprised of long- and medium-chain fatty acids are not equivalent to physical mixtures of TAGs of the same fatty acids, and the modified absorption rates seem to play a role in the oxidation of SLs for energy. An SL made from safflower oil and MCFA was compared with a physical mix in injured rats; the animals receiving the SL were found to have greater gain in body weight, greater positive nitrogen balance, and higher serum albumin concentration [150]. An SL emulsion containing MCFA and LCFA esterified randomly to glycerol compared with LCT in postoperative patients was well tolerated, rapidly cleared from the plasma compartment, and was rapidly oxidized without any significant hyperlipidemia or ketosis. SLs were associated with a higher whole-body fat oxidation than LCT [151].

Jandacek et al. [148] demonstrated that an SL containing octanoic acid at the 1 and 3 position and an LCFA in the 2-position is more rapidly hydrolyzed and efficiently absorbed compared to typical LCTs. They proposed that SL may be synthesized to provide the most desirable features of LCFA and MCFA for use as nutrients in cases of pancreatic insufficiency. Both n-3 and n-6 PUFAs are precursors of hormonelike compounds known as eicosanoids, which are involved in many biological processes in the human body [152]. Researchers have suggested that the current Western diet does not contain the proper balance of n-6 to n-3 required for proper biological function [152,153]. This imbalance is believed to cause a variety of disease symptoms ranging from cardiovascular disease, hypertension, inflammatory and autoimmune disorders, and certain disrupted neurological functions [154]. The inhibitory effects of PUFAs on many of these conditions seem to be related to their mediation of synthesis of eicosanoid precursors [155]. Dietary n-3 fatty acids appear to be rapidly incorporated into cell membranes, favor production of eicosanoid species with less inflammatory potential, and result in improved immune function and reduced endogenous production of cytokines such as tumor necrosis factor (TNF) and interleukins (ILs). Studies of fish-oil-structured lipid-based diet on prostaglandin release from mononuclear cells in cancer patients after surgery demonstrated trends toward improved renal and liver functions as well as a reduced number of gastrointestinal and infectious complications [156]. These studies show evidence of an associated reduction of eicosanoid production from peripheral blood mononuclear cells, which was presumed to be the principal mechanism for the effects observed [156]. Teo et al. [157] demonstrated that an MCT/fish-oil structured lipid can improve whole-body and tissue nitrogen balance and, in addition, reduce the hypermetabolic responses following burn injury. The new structured lipid retains the beneficial protein sparing qualities seen in other studies as well as the lowered energy expenditure associated with fish-oil feeding [150].

Palmitic acid appears to be useful as an energy source for infants. Human milk contains 20–30% palmitic acid, 70% of which is present in the *sn*-2 position of the TAG [158]. This is important in terms of the metabolism of palmitic acid, which is best absorbed

as a 2-MAG and only minimally absorbed in free-acid form [158]. An SL produced by reacting tripalmitin with unsaturated fatty acids using a 1,3-specific lipase closely mimics the fatty acid distribution of human milk and is currently being marketed under the trade name Betapol. A study of the in vivo fate of fat emulsions demonstrates that SLs could also potentially be used as core material in fat-emulsion-based drug delivery systems [159].

5 STRUCTURED LIPIDS TARGETED FOR FUNCTIONAL APPLICATIONS

Structured lipids are texturally important in the manufacture of plastic fats such as margarine, modified butters, and shortenings. The physical nature of any fat or oil is determined by the chain length and unsaturation of the fatty acids and their distribution among the three positions of the glycerol molecule [160]. Fats with a higher percentage of saturated fatty acids tend to be solid at room temperature and those with a higher percentage of unsaturated fatty acids tend to be liquid. Interesterification alters the original order of distribution of fatty acids in the glycerol moiety, producing fats with different melting and crystallization characteristics than the parent fat [160]. This allows for fats to be tailor-made to suit particular foods. Some studies have shown that the rate of autoxidation and melting properties of TAGs can be affected by the position of unsaturated fatty acids on the glycerol molecule [161–163]. In margarine production, the melting point, spreadability, shelf life, and nutritional properties of the natural fats and oils can be modified and custom-made [164]. Also, 1,3-positional specificity of lipase has been exploited in a number of applications to obtain added-value specialty fats such as cocoa butter substitutes [165,166] and vegetable spreads [165]. In the manufacture of margarine, the objective is to produce a fat mixture with a steep solid fat content that will give a stiff product when in the refrigerator, but spreads easily upon removal, and melts quickly in the mouth [167]. When SCFA or MCFA and LCFA are interesterified, they can produce TAGs with good spreadability and temperature stability [167]. Due to the growing concern about the health implications of trans fatty acids in margarine, research interest has been focused in the production of zero-trans fatty acid margarine or shortenings. A palm stearin–sunflower (40:60 and 50:50, w/w) blend subjected to transesterification using *Pseudomonas* lipase was shown to be suitable for table margarine formulation [164]. Seriburi and Akoh [163] were able to produce a soft-type margarine blend by interesterifying lard and high oleic sunflower oil (60:40, w/w) using SP 435 lipase from *Candida antarctica*. Palm stearin and palm kernel olein were interesterified using 1,3-specific *R. miehei* lipase at a 40:60 ratio to produce an experimental table margarine [168]. The experimental blend exhibited larger linear viscoelastic regions and higher elasticity than its commercial counterpart.

The most expensive ingredient used in the manufacture of chocolate is cocoa butter. Its unique physical characteristics coupled with limited supply has an enormous amount of research for cocoa butter alternatives. There is no other naturally occurring fat with the same physical properties as cocoa butter. It is brittle at room temperature and is completely and fast melting at body temperature. Most cocoa butter alternatives are made by blending and/or modifying fats [169]. Fats can be modified to form three types of cocoa butter alternatives: 1) cocoa butter equivalents which are similar in their physical and chemical properties to cocoa butter and are mixable with it in every amount without altering the properties of cocoa butter, 2) cocoa butter replacers which are not lauric fats with a distribution of fatty acids similar to cocoa butter, but a completely different structure of triacylglycerols only in small ratios compatible to cocoa butter, and 3) cocoa butter

substitutes: lauric plant fats, chemically different from cocoa butter, with some physical similarities and suitable to substitute for cocoa butter to 100% [169].

Salatrim preparations have been made to closely emulate the melting properties of cocoa butter by manipulating the ratio of SCFA to LCFA of the TAGs [170]. Caprenin is a commercially available SL that consists of MCFA and behenic acid. It is intended to replace cocoa butter in soft candy and confectionary coatings. A 1,3-specific lipase can be used to interesterify palm oil mid-fraction and ethyl stearate to produce cocoa butter equivalents with a TAG structure similar to cocoa butter [171]. Palm oil mid-fraction can also be interesterified with stearic acid using *Rhizopus arrhizus* lipase to produce a similar cocoa butter substitute [172]. The most commonly used starting substrate for lipase-catalyzed synthesis of cocoa butter substitutes is palm oil mid-fraction, with the primary goal being the introduction of stearate into its component TAGs. Other vegetable fats can be enzymatically modified to form cocoa butter substitutes such as sal (*Shorea robusta*), kokum (*Garcinia indica*), and mango (*Mangifera indica*) from India [173] and Piqui (*Caryocar brasiliennse Camb*) oil from Brazil [174].

6 STRUCTURED LIPIDS AS LOW-CALORIE FATS

Structured lipids may be produced to serve as low-calorie fats. This is usually done by replacing a poorly absorbed long-chain saturated fatty acid with a SCFA or MCFA. SCFAs are useful in the synthesis of low-calorie SLs because they provide fewer calories per unit weight than LCFAs [170]. This technique of replacing long-chain saturated fatty acids and MCFAs or SCFAs has been utilized in industry by Procter and Gamble to chemically interesterify coconut, palm kernel, and rapeseed oils to produce Caprenin, an SL containing C8:0, C10:0, and C22:0. Because of the presence of behenic acid, Caprenin® is only partially absorbed by the body and, therefore, supplies 5 kcal/g versus the 9 kcal/g supplied by conventional fats. Caprenin fed as an SL diet to male subjects for 6 days did not alter plasma cholesterol concentration but decreased high-density lipoprotein(HDL)-cholesterol by 14% [175]. The physical properties of Caprenin are similar to those of cocoa butter; as a result, it is suitable for use in soft candy and confectionery coatings. The principle of combining SCFAs and LCFAs has also been used by Nabisco Foods Group in the chemical synthesis of Salatrim, now marketed as Benefat by Cultor Food Science. Benefat is produced by base-catalyzed interesterification of highly hydrogenated vegetable oils with TAGs of acetic and/or propionic and/or butyric acids [170]. The resulting TAGs can include an infinite number of low-calorie fat products with various applications in foods, such as cocoa butter substitutes, baked products, salad dressings, and filled dairy products. The caloric availability of Salatrim molecules has been determined to be approximately 5 kcal/g. Fomuso and Akoh [176] produced SLs with potential for use as reduced-calorie structured lipids using a 1,3-specific lipase from *R. miehei*. Butyric and caproic acids were interesterified with triolein to form SLs containing C4:0, C6:0, and C18:1. The average caloric value of C4:0 and C6:0 being 6.8 kcal/g reduces the overall caloric value of the SLs [176,177].

7 METHODS FOR LARGE-SCALE PRODUCTION OF STRUCTURED LIPIDS

The most important difference between enzyme reactors and chemical reactors is that enzyme reactors are operated at lower temperatures and pressures. An enzyme reactor can

be defined as the container in which a reaction catalyzed by free or immobilized enzymes or cells takes place, with associated sampling and monitoring devices [178]. Many different types of reactor have been used with enzymes either in their free or immobilized form. Limiting factors in the use of enzymes in industry are the high cost of enzymes and the limited number of highly functional products requiring enzymatic catalysis. Many pharmaceutical chiral intermediates and drugs are produced with enzymes. Enzyme immobilization can be defined as the attachment of enzymes to artificial matrices whereby movement is completely or severely restricted [178]. Immobilization enables enzyme recovery, thus converting labor-intensive processes into more capital-intensive operations and permitting continuous automated production with better quality control [179]. For lipases to be used on a large scale in the food industry, the process has to be both technically and economically feasible. Enzyme immobilization, although a costly process, extends the enzymes' operational lifetime and allows for enzyme reuse and prevention of product contamination. Several reactor types have been investigated for use in industry. Four commonly used reactors are stirred tank, continuous-stirred tank, plug flow and packed-bed, and membrane reactors.

7.1 Plug Flow and Packed-Bed Reactors

Packed-bed reactors are most commonly used in industrial-scale applications because of their ease of construction, relatively low cost, and ease of operation. The substrate solution can be fed into the reactor either from the top or the bottom of the column or bed. A pump is required to pump substrate and product in and out at a constant flow rate. The substrate moves along the column such that there is negligible mixing in the direction of flow, but it is well mixed in the radial direction [180]. The molecules move as a fluid plug without mixing with the previous or subsequent fluid elements in the reactor [79]. In plug flow reactors, efficiency is high near the input end and lower near the exit end. The ratio between substrate and enzyme is much lower in a continuous-packed-bed reactor than is the case with conventional batch reactors. This results in shorter reaction times, reduced acyl migration, and, consequently, purer structured lipids. Other researchers were able to transesterify rapeseed oil and lauric acid in a continuous reactor with maximum yields after 20 min [181,182]. They calculated residence time, using the equation

$$\tau = V_{\text{tot}} \frac{\varepsilon}{v}$$

where τ is the residence time, V_{tot} is the volume, ε is the porosity of the enzyme bed, and v is the flow rate of the substrate. Mu et al. [181] showed that incorporation of acyl donors was most affected by residence time than any of the other parameters studied. The residence time and reaction flow rate are inversely proportional. Therefore, flow rates in the reactor can be reduced to increase residence time, or increased to reduce residence time. The activity of a biocatalyst reactor decreases with time due to enzyme inactivation and release and due to microbial or other contamination. Therefore, in industrial practice, a number of reactors are usually used either in series or in parallel to eliminate product quality fluctuations [183]. The acidolysis reaction for the production of structured lipids containing γ-linolenic acid (GLA) and caprylic acid scarcely changed even though the substrate mixture was continuously fed for 60 days [184]. Enzyme stability differs depending on the purity of the substrate used and also the conditions of the column, such as temperature. Wisdom et al. [185] found that in the esterification of shea olein with

stearic acid, only a small loss of activity was exhibited with high-quality substrates, but with poor quality substrates, there was rapid inactivation of the lipase. The temperature of the column has to be maintained at levels high enough to allow the substrate to be liquid but not enough to deactivate the enzyme. Increased reactor temperature and MCFA content were observed to improve incorporation of MCFAs to form SLs [181]. A study by Xu et al. [186] on the production of lipase-catalyzed specific-structured lipids in a continuous-packed-bed reactor showed that high incorporation was favored by high substrate ratios between reactants, and long residence times. Productivity of the reactor can be determined by the degree of conversion of the substrate molecules into product molecules in the column reactor, which could be expressed as kilogram of product formed per liter of reactor volume per year [111].

7.2 Continuous-Stirred-Tank Reactors

A continuous-stirred-tank (CSTR) is a reactor in which substrate is added at the same rate as the product is removed. With ideal mixing, the composition of the liquid phase is constant throughout the reactor vessel. Agitation in a CSTR is usually provided by an impeller. Increased shear due to high stirring speeds may destroy the enzyme and, therefore, make enzyme recovery inefficient. This is significant in industrial scale-up, where enzyme cost can be a limiting factor. Substrate concentrations are constant in a CSTR and this results in fairly constant reaction rates. Fresh catalyst can be added during the course of the reaction, whereas with other reactors, the system has to be shut down to replenish the enzyme supply. For the simple case of an enzyme acting on a single reactant where the reaction rate is described by the Michaelis–Menten equation, the performance of a CSTR can be compared with a packed-bed reactor by the following equations [187]:

Packed-bed reactor:

$$PC_0 - K\ln(1 - P) = k\frac{E}{q}$$

CSTR:

$$PC_0 - K\frac{P}{1 - P} = k\frac{E}{q}$$

where C_0 is the reactant feed concentration, q is the feed rate, P is the proportion of the reactant converted to product in the reactor, E is the total enzyme in the reactor, k is a rate constant, and K is the apparent Michaelis constant. When C_0 is much greater than K, the two equations become identical, but when C_0 is much less than K, the performances are very different [187]. When C_0 is very low, more enzyme is required in the CSTR to maintain the same performance as in the packed-bed reactor.

7.3 Stirred-Tank Batch Reactors

Batch reactors are traditionally used in bench-scale lipase-catalyzed reactions where demonstrating the technical feasibility of a process is the primary goal. They are versatile and simple to operate. In stirred-batch reactors, the composition of the reactants varies during the reaction but remains constant throughout the reactor system [178]. As there is no flow in or out of the system, the substrate-to-product ratio reduces over time, with a correspond-

ing decrease in reaction rate. Parameters, such as pH, temperature, and substrate-to-product ratio, also remain constant during the course of a reaction. Better mixing can be achieved by increasing the stirrer speed, decreasing the viscosity of the substrate, or by baffling the reactor more efficiently [178]. Lee and Akoh [188] reported that increasing the mixing speed in a stirred-tank batch reactor from 200 rpm to 640 rpm improved yields of desired structured lipid. The high ratio between substrate and enzyme in batch reactors requires long reaction times, which encourages the formation of DAGs and, therefore, decreases the purity of SL synthesized using 1,3-specific lipases. A stirred-batch reactor also has the disadvantage of destroying biocatalysts due to high shear forces brought about by the stirrer.

7.4 Membrane Bioreactors

These are reactors that consist of two fluids separated by a permeable membrane such that the reaction and separation of substrates and/or products can take place in one unit [189]. Permeable solutes can be separated from the reaction mixture by the action of a driving force (e.g., chemical potential, pressure, electric field) present across the membrane [190]. The membrane may be used to separate phases when more than one phase is in use, and it can also provide interfacial contact area and act with the enzyme as an interfacial catalyst. In lipase-catalyzed reactions, the lipase is usually immobilized on the side of the membrane that faces the hydrophobic side of the membrane. Products from the reaction should be able to move across the membrane barrier for collection, either by diffusion (induced by a concentration gradient) or by convection (induced by a pressure gradient) [190]. Usually, to optimize the reactor, an appropriate mathematical model is necessary to assess the influence of parameters such as enzyme concentration, enzymatic activity and diffusion coefficients of reactants [191]. For lipase applications in bioreactors, hydrophilic membranes are preferred over hydrophobic ones because less enzyme is required [191]. Membrane reactors can be classified into three types: 1) direct-contact membranes: substrate and membrane are in direct contact; 2) diffusion membranes: enzyme–substrate contact occurs after a simple passive diffusion step; 3) multiphase membrane reactors: interfacial contact occurs between enzyme and substrate in the membrane matrix [190]. Membrane modules commonly found in the marketplace are flat sheet (spiral wound, tubular, and hollow fibers) [192]. Most applications of membrane reactor technology in lipase-catalyzed reactions are hydrolysis reactions, but a significant amount of work has also been done in the synthesis of acylglycerols. *Candida rugosa* lipase has been used to catalyze the esterification of decanoic acid with glycerol in a hydrophilic membrane reactor to produce monocaprin, dicaprin, and tricaprin [193]. Continuous synthesis of oleic acid acylglycerols using *Pseudomonas fluorescens* in a microporous hydrophobic membrane bioreactor has also been successfully accomplished with a molar ratio yield of 3:4:1 (MAG:MAG:TAG) [194].

8 DOWNSTREAM PROCESSING OF SL

All of the processes used for the purification and isolation of the products of SLs synthesis can be referred to as downstream processing. This is an important and sometimes costly process. On a laboratory scale, SLs can be purified using thin-layer chromatography (TLC) techniques, where TAG can be separated from FFA, MAG, and DAG and other artifacts of the reaction. In addition to TLC methods, high-performance liquid chromatography

(HPLC) can also be used to isolate individual structured lipid species that is useful for small-scale analysis but impractical for large-scale use. On a larger scale, short-path distillation, column chromatography, or deacidification by alkaline extraction can be used to separate out FFAs from SLs [2].

Short-path distillation is advantageous over other methods, in that separation of heat-sensitive materials can be accomplished at low temperatures. This is done by combining the concept of distillation at low pressures with a wiped film distribution of the feed for improved heat transfer, short residence time, and low product holdup to achieve the best fractionation. In our laboratory, free fatty acids were separated from an interesterified blend of melon seed oil and high-oleic sunflower oil using short-path distillation. The conditions used were as follows: feed heat 20°C, evaporator heat 205°C, vacuum 0.04–1 mm Hg, condenser temperature −2.5°C, feed rate 400 mL/hr, and a wiper speed of 400 rpm [195]. Distilling the blend three times under identical conditions produced an oil with a residual free fatty acid content of 0.08–2.4% [195]. Lee and Akoh [6] used column chromatography to isolate TAGs resulting from the interesterification of eicosapentaenoic acid ethyl ester (EPAEE) and MCT. Silicic acid and silica gel were used as the stationary phase and the column was eluted first with 95:5 (v/v) hexane:diethyl ether and then with 90:10 (v/v) hexane:diethyl ether. The second fraction contained the SL and unreacted MCT. Alkaline deacidification is achieved by reacting FFA with alkali to form a soapstock. Removal of the soapstock is crucial in this process due to the possibility of high losses of neutral lipids.

Other processes used in the purification of oils are also applicable in the purification of SL from transesterification reactions. Deodorization is needed to remove undesirable odors and flavors such as those that arise from residual FFAs (particularly caprylic or caproic acids). Deodorization should also remove peroxide decomposition products, resulting in oils with a peroxide value of near zero and FFA content of less than 0.03% [196]. Briefly, deodorization involves steam distillation performed at high temperatures and under high vacuum. Addition of antioxidants is necessary after the production of SLs, especially when highly unsaturated fatty acids such as EPAs or DHAs are part of the TAG backbone. Synthetic antioxidants, such as butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA), and tertiary butylhydroxyquinone (TBHQ), may be added or natural antioxidants such as tocopherols and tocotrienols may be added at appropriate levels. Often, blends of antioxidants perform well.

9 ADVANTAGES OF ENZYMATIC SYNTHESIS

Products of enzymatic processes can be easily purified and leave less wastes, which, in turn, reduces environmental load [197]. This may be termed a ''green process'' with consumer appeal. Transesterification using an *sn*-1,3–specific lipase conserves the fatty acid originally present at the *sn*-2 position. This is very significant from a nutritional quality point of view, as the pancreatic lipase can only hydrolyze *sn*-1,3 fatty acids [198]. Fatty acids at the *sn*-2 position are more efficiently absorbed than those at the *sn*-1 position and *sn*-3 position. The auto-oxidation and melting properties of TAGs can be affected by the distribution of fatty acids at the *sn*-2 position. The use of enzymes at low temperatures reduces the processing cost by conserving energy.

Advantages of the enzymatic synthesis of structured lipids cannot be overstated and these include 1) position-specific incorporation of desired fatty acids, 2) regioselective synthesis (specific to bond to be cleaved), 3) enantioselective synthesis (optical selectiv-

ity), 4) chemoselective synthesis (specific to functional group), 5) custom synthesis of structured lipids for specific applications like food or therapeutic use, 6) synthesis of novel products, 7) mild reaction conditions, 8) few or no unwanted side reactions or products, 9) easy and accurate control of overall process, 10) ease of product recovery, 11) added value to fats and oils from cheap sources, and 12) improved functionality and properties of fats [2].

10 LIMITATIONS

In spite of the above advantages, the commercialization of the enzymatic process is still in its infancy. There are many factors that currently hinder the commercial development of these engineered lipids. They include enzyme cost, lack of *sn*-2–specific lipase, scale-up problems, regulatory requirements, and competition with plant breeding and genetic modification of oil crops.

10.1 Cost

The cost of enzymes for the commercial production of SL is considered high. Despite a few successful commercializations of enzymatic process, such as the synthesis of cocoa butter equivalent and Betapol, the cost for the manufacture of other kinds of fats still remains high. The commercial demand, value, and nutritional quality of a particular product determines whether an enzymatic process can be used. Currently, the enzymatic synthesis of SLs for medical or therapeutic applications is feasible. If the enzyme cost is reduced, then there is a great opportunity to adopt the process for large-scale structured-lipid synthesis.

10.2 Genetically Engineered Plant Seed Oils

Genetic modification of fatty acid profiles in oilseeds has been attempted on a commercial scale by Calgene, Inc. [199]. However, the absolute success of this method is still challenged by a lack of knowledge in crop management, disease resistance, and biochemical regulation of oilseed metabolic process [200]. If such a process becomes generally acceptable, then the final product could be much less expensive than that of the enzymatic process. However, genetically modified products do create concern in some European countries. There needs to be a dialogue aimed at resolving the acceptance and regulatory issues posed by genetically modified organisms (GMO) and foods.

10.3 Regulatory and Safety Aspects

Structured lipids are considered safe. The safety study of modified fats such as Salatrim showed no significant clinical effects when fed at 30 g/day [201]. Caprenin, a randomized TAG, was tested for toxicity by feeding more than 15% (w/w) in the diet (or more than 83% of total dietary fat). No adverse effects from the ingestion of caprenin were detected [202]. These studies show the reliability and safety of these new kinds of lipid. However, there are no regulatory guidelines on these products from any regulatory agencies so far, including the United States Food and Drug Administration. There are no current regulations on the use of enzymatically produced structured lipids. Hopefully, it will not be long before we have some directives.

REFERENCES

1. VK Babayan. Lipids 22:417–420, 1987.
2. CC Akoh. In: CC Akoh, DB Min, eds. Food Lipids: Chemistry, Nutrition, and Biotechnology. New York: Marcel Dekker, 1998, pp 699–727.
3. JP Kennedy. Food Technol 45:76–83, 1991.
4. MM Soumanou, UT Bornscheuer, U Menge, RD Schmid. J Am Oil Chem Soc 74:42–433, 1997.
5. KT Lee, CC Akoh. J Am Oil Chem Soc 74:579–584, 1997.
6. KT Lee, CC Akoh. J Am Oil Chem Soc 73:611–615, 1996.
7. K Huang, CC Akoh. J Am Oil Chem Soc 71:1277–1280, 1994.
8. T Yamane, T Suzuki, T Hosino. J Am Oil Chem Soc 70:1285–1287, 1993.
9. ZY Li, OP Ward. Biotechnol Lett 15:185–188, 1993.
10. CC Akoh, BH Jennings, DA Lillard. J Am Oil Chem Soc 72:1317–1321, 1995.
11. R Sridhar, G Lakshminarayan. J Am Oil Chem Soc 69:1041–1042, 1992.
12. S Ghosh, DK Bhattacharyya. J Am Oil Chem Soc 74:593–595, 1997.
13. ZY Li, OP Ward. J Am Oil Chem Soc 70:745–748, 1993.
14. MJ Hills, I Kiewitt, KD Mukherjee. J Am Oil Chem Soc 67:561–564, 1990.
15. P Langholz, P Andersen, T Forskov, W Schmidtsdorff. J Am Oil Chem Soc 66:120–1123, 1989.
16. CC Akoh, BH Jennings, DA Lillard. J Am Oil Chem Soc 73:1059–1062, 1996.
17. Y Kosugi, N Azuma. J Am Oil Chem Soc 71:1397–1403, 1994.
18. S Adachi, K Okumura, V Ota, M Mankura. J Ferment Bioeng 75:259–264, 1993.
19. K Osada, K Takahashi, M Hatano. J Am Oil Chem Soc 67:921–922, 1990.
20. MSF Lie Ken Jie, MSK Syed Rahmatullah. J Am Oil Chem Soc 72:245–249, 1995.
21. Y Shimada, K Maruyama, M Nakamura, M Nakayama, A Sughihara, Y Tominaga. J Am Oil Chem Soc 71:951–954, 1994.
22. Y Tanaka, J Hirano, T Funada. J Am Oil Chem Soc 69:1210–1214, 1992.
23. T Hoshino, T Yamane, S Shimizu. Agric Biol Chem 54:1459–1467, 1990.
24. Y Tanaka, J Hirani, T Funada. J Am Oil Chem Soc 71:331–334, 1994.
25. B Myrnes, H Barstad, RL Olsen, EO Elvevoll. J Am Oil Chem Soc 72:1339–1344, 1995.
26. GP McNeill, RG Ackman, SR Moore. J Am Oil Chem Soc 73:1403–1407, 1996.
27. A Millqvist Fureby, P Adlercreutz, B Mattiasson. J Am Oil Chem Soc 73:1489–1495, 1996.
28. KD Mukherjee, I Kiewitt. Biotechnol Lett 20:613–616, 1998.
29. TA Foglia, P Villeneuve. J Am Oil Chem Soc 74:1447–1450, 1997.
30. GG Haraldsson, B Kristinsson, R Sigurdardottir, H Breivik. J Am Chem Soc 74:1419–1424, 1997.
31. Y Ota, T Minesaki, A Oshima. Biosci Biotechnol Biochem 61:166–167, 1997.
32. D Briand, E Dubreucq, P Galzy. Eur J Biochem 228:169–175, 1995.
33. P Villeneuve, M Pina, A Skarbek, J Graille, TA Foglia. Biotechnol Tech 11:91–94, 1997.
34. KD Mukherjee, I Kiewitt. J Agric Food Chem 46:2427–2432, 1998.
35. RR Klein, G King, RA Moreau, GP McNeill, P Villeneuve, MJ Haas. J Am Oil Chem Soc 74:1401–1407, 1997.
36. AM Fureby, L Tian, P Adlercreutz, B Mattiasson. Enzyme Microb Technol 20:198–206, 1997.
37. O Chmiel, H Traitler, WP Hammes, W Bauer. Food Biotechnol 8:35–56, 1994.
38. S Tawaki, AM Klibanov. J Am Chem Soc 114:1882–1884, 1992.
39. L Sarda, P Desnuelle. Biochim Biophys Acta 30:513–521, 1958.
40. G Benzonana, P Desnuelle. Biochim Biophys Acta 105:121–136, 1965.
41. YW Jalander. Biochem J 36:435–437, 1911.
42. HP Kaufmann. Fett Seifen 44:105–108, 1937.
43. FR Dastoli, NA Musto, S Price. Arch Biochem Biophys 115:44–47, 1966.

44. FR Dastoli, S Price. Arch Biochem Biophys 118:163–165, 1967.
45. FR Dastoli, S Price. Arch Biochem Biophys 122:289–291, 1967.
46. A Zaks, AM Klibanov. Science 224:1249–1251, 1984.
47. A Zaks, AM Klibanov. Proc Natl Acad Sci USA 82:3192–3196, 1985.
48. M Iwai, Y Tsujisaka, J Fukumoto. J Gen Appl Microbiol 10:13–17, 1964.
49. Y Tsujisaka, S Okumura, M Iwai. Biochim Biophys Acta 489:415–422, 1977.
50. S Okumura, M Iwai, Y Tsujisaka. Biochim Biophys Acta 575:156–165, 1979.
51. M Iwai, S Okumura, Y Tsujisaka. Agric Biol Chem Tokyo 44:2731–2732, 1980.
52. S Okumura, M Iwai, Y Tsujisaka. Agric Biol Chem Tokyo 45:185–189, 1981.
53. S Okumura, MI Wai, Y Tominaga. Agric Biol Chem Tokyo 48:2805–2808, 1984.
54. S Bone, R Pethig. J Mol Biol 157:571–575, 1982.
55. G Garza-Ramos, A Fernández-Velasco, L Ramirez, L Shoshani, A Darszon. Eur J Biochem 205:509–517, 1992.
56. O Almarsson, AM Klibanov. Biotechnol Bioeng 49:87–92, 1996.
57. J Van Camp, A Huyghebaert, P Goeman. In: AB Christophe, ed. Structured Modified Food Fats: Synthesis, Biochemistry and Use. Champaign, IL: AOCS Press, 1998, pp 20–45.
58. AR Macrae, RC Hammond. Biotechnol Genet Eng Rev 3:193–217, 1985.
59. YH Park, GM Pastore, M de Almelda. J Am Oil Chem Soc 65:252–254, 1988.
60. CC Akoh. In: RE McDonald, DB Min, eds. Food Lipids and Health. New York: Marcel Dekker, 1996, pp 117–138.
61. P Adlercreutz. In: AMP Koskinen, AM Klibanov, eds. Enzymatic Reactions in Organic Media. New York: Blackie Academic and Professional, 1996, pp 9–42.
62. KD Mukherjee. Biocatalysis 3:277–293, 1990.
63. UN Wanasundara, F Shahidi. J Am Oil Chem Soc 75:945–951, 1998.
64. YH Ju, FC Huang, CH Fang. J Am Oil Chem Soc 75:961–965, 1998.
65. MM Soumanou, UT Bornscheuer, RD Schmid. J Am Oil Chem Soc 75:703–710, 1998.
66. K Takahashi. Yugakaku 40:931–941, 1991.
67. TA Hamazaki, A Hirai, T Terano, J Sajiki, S Kondo, T Fuhita, Y Tamura, A Kumagai. Prostaglandins 23:557–567, 1982.
68. S El-Boustani, SC Colette, L Monnier, B Descomps, CA Paulet, F Mendey. Lipids 22:711–714, 1987.
69. LD Lawson, BG Hughes. Biochem Biophys Res Commun 152:328–335, 1988.
70. LY Yang, A Kuksis, JJ Myher. Biochem Cell Biol 67:192–204, 1989.
71. FX Malcata, HR Reyes, HS Garcia, CG Hill Jr, CH Amundson. J Am Oil Chem Soc 12: 890–910, 1990.
72. PJ Halling. Biotechnol Bioeng 35:691–701, 1990.
73. I Svensson, E Wehtje, P Adlercreutz, B Mattiasson. Biotechnol Bioeng 44:549–556, 1994.
74. E Ergan, M Trani, G Andre. Biotechnol Lett 11:194–211, 1988.
75. CC Akoh. Biotechnol Lett 15:949–954, 1993.
76. DA Hammond, M Karel, AM Klibanov. Appl Biochem Biotechnol 12:393–399, 1985.
77. A Marty, W Chulalaksananukul, RM Willemot, JS Condoret. Biotechnol Bioeng 39:273–280, 1992.
78. JS Dordick. Enzyme Microb Technol 11:194–211, 1989.
79. H Seino, T Uchibori, T Nichitani, S Inamasu. J Am Oil Chem Soc 61:1761–1765, 1984.
80. A Lazar, A Weiss, RD Schmid. In: AR Baldwin, ed. Proceedings of the World Conference on Emerging Technologies in the Fats and Oil Industry. Champaign, IL: American Oil Chemists Society, 1986, pp 346–354.
81. LN Mutua, CC Akoh. J Am Oil Chem Soc 70:43–46, 1993.
82. J Kim, DH Altreuter, DS Clark, JS Dordick. J Am Oil Chem Soc 75:1109–1113, 1998.
83. AM Klibanov. Trends Biochem Sci 14:141–149, 1989.
84. PA Claon, CC Akoh. Biotechnol Lett 15:1211–1216, 1993.
85. CC Akoh. J Am Oil Chem Soc 71:319–323, 1994.

86. Y Shimada, A Sugihara, K Maruyama, T Nagao, S Nakayama, H Nakano, Y Tominaga. J Ferment Bioeng 81:299–303, 1996.
87. T Yamane, T Suzuki, Y Sahashi, L Vikersveen, T Hoshino. J Am Oil Chem Soc 69:1104–1107, 1992.
88. GG Haraldsson, PA Hoskulsson, ST Sigurdsson, F Thorsteinsson, S Gudbjarnason. Tetrahedron Lett 30:1671–1674, 1995.
89. T Oba, B Witholt. J Dairy Sci 77:1790–1797, 1994.
90. WM Willis, AG Marangoni. Enzymatic Interesterification. In: CC Akoh, DB Min, eds. Food Lipids: Chemistry, Nutrition, and Biotechnology. New York: Marcel Dekker, 1998, pp 665–698.
91. CJ Gray, JS Narang, SA Barker. Enzyme Microb Technol 12:800–807, 1990.
92. MA Mazed, JD Summers, DG Batchelder. Trans ASAE 28:1375–1377, 1985.
93. RO Dunn, MO Bagby. J Am Oil Chem Soc 72:895–904, 1995.
94. DG Boocock, SK Konar, V Mao, C Lee, S Buligan. J Am Oil Chem Soc 75:1167–1172, 1998.
95. W Chulalaksananukul, JS Condoret, D Combes. Enzyme Microb Technol 14:293–298, 1992.
96. W Chulalaksananukul, JS Condoret, D Combes. Enzyme Microb Technol 5:691–698, 1993.
97. HF Castro, PC de Oliveira, EB Pereira. Biotechnol Lett 19:229–232, 1997.
98. D Briand, E Dubreucq, P Galzy. Biotechnol Lett 16:813–818, 1994.
99. E Boyle. Food Technol 51:52–59, 1997.
100. LL Wang, BK Yang, KL Park, EA Johnson. J Agric Food Chem 41:1000–1005, 1993.
101. A Weiss. Fat Sci Technol 92:392–396, 1990.
102. K Holmberg, E Osterberg. J Am Oil Chem Soc 65:1544–1548, 1988.
103. GP McNeill, S Shimizu, T Yamane. J Am Oil Chem Soc 68:1–5, 1991.
104. GP McNeill, D Borowitz, RG Berger. J Am Oil Chem Soc 69:1098–1103, 1992.
105. B Yang, WJ Harper, KL Parkin. J Agric Food Chem 41:1905–1909, 1993.
106. B Yang, WJ Harper, KL Parkin. J Agric Food Chem 41:1899–1904, 1993.
107. H Noureddini, SE. Harmeier. J Am Oil Chem Soc 75:1359–1365, 1998.
108. P Kalo, P Paarviainen, K Vaara, S Ali-Yrrk, M Antila. Milchwiss 41:82–85, 1986.
109. MK Chang, G Abraham, VT John. J Am Oil Chem Soc 67:832–834, 1990.
110. P Kalo, H Houtori, M Antila. Milchwiss 45:281–285, 1990.
111. LH Posorske, GK LeFebvre, CA Miller, TT Hansen, BL Glenvig. J Am Oil Chem Soc 65: 922–926, 1988.
112. VM Balcao, A Kemppinen, FX Malcata, PJ Kalo. J Am Oil Chem Soc 75:1347–1358, 1998.
113. PJ Halling. Biotechnol Tech 6:271–276, 1992.
114. JE Kim, JJ Han, JH Yoon, JS Rhee. Biotechnol Bioeng 57:121–125, 1998.
115. VV Yankah, CC Akoh. J Am Oil Chem Soc 77:495–500, 2000.
116. P Desnuelle. In: PD Boyer, ed. The Enzymes, Vol III. 3rd ed. New York: Academic Press, 1972, pp 575–616.
117. C Miller, H Austin, L Posorske, J Gonziez. J Am Oil Chem Soc 65:927–931, 1988.
118. G Langrand, N Rondot, C Triantaphylides, J Baratti. Biotechnol Lett 12:581–586, 1990.
119. M Karra-Chaabouni, S Pulvin, D Touraud, D Thomas. J Am Oil Chem Soc 75:1201–1206, 1998.
120. A Manjon, JL Iborra, A Arocas. J Am Oil Chem Soc 13:339–344, 1998.
121. JM Elliot, KL Parkin. J Am Oil Chem Soc 68:171–175, 1991.
122. Y Kosugi, H Suzuki. Biotechnol Bioeng 40:369–374, 1992.
123. RW Lencki, N Smink, H Snelting, J Arul. J Am Oil Chem Soc 75:1195–1200, 1998.
124. A Khamessan, S Kermasha. J Food Biochem 20:311–328, 1996.
125. SB Pederson, G Holmer. J Am Oil Chem Soc 72:239–243, 1995.
126. NR Bottino, GA Vandenburg, R Reiser. Lipids 2:489–493, 1967.
127. S Parida, JS Dordick, J Org Chem 58:3238–3244, 1993.
128. FX Malcata, HR Reyes, HS Garcia, CG Hill Jr, CH Amundson. Enzyme Microb Technol 14:426–446, 1992.

129. M. Suguira, M Isobe. Chem Pharm Bull 23:681–682, 1975.
130. GP McNeil, D Borowitz, RG Berger. J Am Oil Chem Soc 69:1098–1103, 1992.
131. RG Jensen, DR Galluzza, VJ Bush. Biocatalysis 3:307–316, 1990.
132. C Laane, S Boeren, K Voos, C Veeger. Biotechnol Bioeng 30:81–87, 1987.
133. MH Hilhorst, R Spruyt, C Laane, C Veeger. Eur J Biochem 144:459–466, 1984.
134. C Laane, S Boeren, K Voos, C Veeger. Biotechnol Bioeng 30:81–87, 1987.
135. SC Mohapatra, JT Hsu. Biotechnol Bioeng 55:399–407, 1997.
136. R Lortie, M Trani, F Ergan. Biotechnol Bioeng 41:1021–1026, 1993.
137. GG Haraldsson, O Almarsson. Acta Chem Scand 45:723–730, 1991.
138. GG Haraldsson, BO Gudmundsson, O Almarsson. Tetrahedron 51:941–952, 1995.
139. HM Ghazali, S Hamidah, YB Che Man. J Am Oil Chem Soc 72:633–639, 1995.
140. DR Kodali, A Tercyak, DA Fahey, DM Small. Chem Phys Lipids 52:163–170, 1990.
141. G Boswinkel, JTP Derkson, K van't Riet, FP Cuperus. J Am Oil Chem Soc 73:707–711, 1996.
142. X Xu, ARH Skands, C-E Hoy, H Mu, S Balchen, J Adler-Nissen. J Am Oil Chem Soc 75: 1179–1186, 1998.
143. S Bloomer, P Adlercreutz, B Mattiasson. J Am Oil Chem Soc 69:966–973, 1992.
144. SH Goh, SK Yeong, CW Wang. J Am Oil Chem Soc 70:567–570, 1993.
145. S Bloomer, P Adlercreutz, B Mattiasson. Biocatalysis 5:145–162, 1991.
146. F Pabai, S Kermasha, A Morin. Appl Microbiol Biotechnol 43:42–51, 1995.
147. BJ Sjursnes, L Kvittingen, T Anthonsen. J Am Oil Chem Soc 72:533–537, 1995.
148. RJ Jandacek, JA Whiteside, BN Holcombe, RA Volpenhein, JD Taulbee. Am J Clin Nutr 45:940–945, 1987.
149. WM Willis, RW Lencki, AG Marangoni. Crit Rev Food Sci Nutr 38:639–674, 1998.
150. KT Mok, A Maiz, K Yamakazi, J Sobrado, VK Babayan, L Moldawen, BR Bistrian, GL Blackburn. Metabolism 33:910–915, 1984.
151. R Sandstrom, AH Hyltander, U Kokrner, K Lundholm. J Parent Enter Nutr 19:381–386, 1995.
152. AP Simopoulos. Am J Clin Nutr 54:438–463, 1991.
153. N Salem Jr, GR Ward. World Rev Nutr Diet 71:128–147, 1993.
154. IS Newton. J Food Lipids 3:233–249, 1996.
155. LM Braden, KK Caroll. Lipids 21:285–288, 1986.
156. WS Swails, AS Kenler, DF Driscoll, SJ Demichele, TJ Babineau, T Utsunamiya, SC Chavali, RA Forse, BR Bistrian. J Parent Enter Nutr 21:266–274, 1997.
157. TC Teo, SJ Demichele, KM Selleck, VK Babayan, GL Blackburn, BR Bistrian. Ann Surg 210:100–107, 1988.
158. SM Innis, R Dyer, P Quinlan, D Diersen-Schade. J Nutr 125:73–81, 1995.
159. H Hedeman, H Bronsted, A Mullertz, S Frokjaer. Pharm Res 13:725–728, 1996.
160. B Sreenivasan. J Am Oil Chem Soc 55:796–805, 1978.
161. KG Raghuveer, EG Hammond. J Am Oil Chem Soc 44:239–243, 1967.
162. S Wada, C Koizumi. J Am Oil Chem Soc 60:1105–1109, 1983.
163. V Seriburi, CC Akoh. J Am Oil Chem Soc 75:1339–1345, 1998.
164. OM Lai, HM Ghazali, CC Let. Food Chem 64:83–88, 1998.
165. JBM Rattray. J Am Oil Chem Soc 61:1701–1712, 1984.
166. CN Chong, YM Hoh, CW Wang. J Am Oil Chem Soc 69:137–145, 1992.
167. D Rousseau, AG Marangoni. In: CC Akoh, DB Min, eds. Food Lipids: Chemistry, Nutrition, and Biotechnology. New York: Marcel Dekker, 1998, pp 251–281.
168. OM Lai, HM Ghazali, DM Hashim. J Food Lipids 6:25–46, 1999.
169. M Lipp, E Anklam. Food Chem 62:73–97, 1998.
170. RE Smith, JW Finley, GA Leveille. J Agric Food Chem 42:432–434, 1994.
171. S Bloomer, P Adlercreutz, B Mattiasson. J Am Oil Chem Soc 67:519–524, 1990.
172. YH Ju, CK Chiu, FC Huang. J Chin Inst Chem Eng 27:65–70, 1996.

173. R Sridhar, G Lakshminarayana, TNB Kaimal. J Am Oil Chem Soc 68:726–730, 1991.
174. NL Facioli, LAG Gonzales. Quimica Nova 21:16–19, 1998.
175. LL Swift, JO Hill, JC Peters, HL Greene. Am J Clin Nutr 56:881–866, 1992.
176. LB Fomuso, CC Akoh. J Am Oil Chem Soc 74:269–272, 1997.
177. LB Fomuso, CC Akoh. J Am Oil Chem Soc 75:405–410, 1998.
178. PSJ Cheetham. In: A Wiseman, ed. Principles of Biotechnology. New York: Surrey University Press/Chapman & Hall, 1988, pp 164–201.
179. JF Roland. In: WH Pitcher, ed. Immobilized Enzymes for Food Processing. Boca Raton, FL: CRC Press, 1975, pp 55–77.
180. HW Blanch, DS Clark. In: Biochemical Engineering. New York: Marcel Dekker, 1996, pp 276–342.
181. H Mu, X Xu, CE Hoy. J Am Oil Chem Soc 75:1187–1193, 1998.
182. P Forsell, P Parovuori, P Linko, K Poutanen. J Am Oil Chem Soc 70:1105–1109, 1993.
183. YY Linko, P Linko. In: J Tramper, HC Van Der Plas, P Linko, eds. Biocatalysts in Organic Syntheses. Amsterdam: Elsevier Science, 1985, pp 159–178.
184. Y Shimada, M Suenaga, A Sugihara, S Nakai, Y Tominaga. J Am Oil Chem Soc 76:189–193, 1999.
185. RA Wisdom, P Dunhill, MD Lilly. Biotechnol Bioeng 29:1081–1085, 1987.
186. X Xu, S Balchen, CE Hoy, J Adler-Nissen. J Am Oil Chem Soc 75:1573–1579, 1998.
187. MD Lilly, AK Sharp. Chem Eng 215:CE12–CE18, 1968.
188. KT Lee, CC Akoh. J Am Oil Chem Soc 75:1533–1537, 1998.
189. J Kloosterman IV, PD van Wassenar, WJ Bel. In: TH Applewhite, ed. Proceedings: World Conference on Biotechnology for the Fats and Oils Industry. Champaign, IL: American Oil Chemists Society, 1988, pp 219–225.
190. DMF Prazeres, JMS Cabral. Enzyme Microb Technol 16:738–750, 1994.
191. ST Bouwer, FP Cuperus, JTP Derksen. Enzyme Microb Technol 21:291–296, 1997.
192. AL Santos, M Mateus, JMS Cabral. In: CA Costa, JS Cabral, eds. Chromatographic and Membrane Processes in Biotechnology. Dordrecht: Kluwer Academic, 1996, pp 177–205.
193. A Van der Padt, MJ Edema, JJW Sewalt, K van't Riet. J Am Oil Chem Soc 67:347–352, 1990.
194. MM Hoq, H Tagami, T Yamane, S Shimizu. Agric Biol Chem 49:335–342, 1985.
195. CO Moussata, CC Akoh. J Am Oil Chem Soc 75:1155–1159, 1998.
196. LA Johnson. In: CC Akoh, DB Min, eds. Food Lipids: Chemistry, Nutrition, and Biotechnology. New York: Marcel Dekker, 1998, pp 181–228.
197. FD Gunstone. Biochem Soc Trans 17:1141–1142, 1989.
198. S Ray, DK Bhattacharyya. J Am Oil Chem Soc 72:327–330, 1995.
199. BF Haumann. INFORM 8:804–811, 1997.
200. M Miquel, J Browse. INFORM 6:108–111, 1995.
201. JW Finley, GA Leveille, RM Dixon, CG Walchak, JC Sourby, RE Smith, KD Francis, MS Otterburn. J Agric Food Chem 42:581–596, 1994.
202. DR Webb, FE Wood, TA Bertram, NE Fortier. Food Chem Toxicol 31:935–946, 1993.

22

Enzymatic Production of Betapol and Other Specialty Fats

CASIMIR C. AKOH

The University of Georgia, Athens, Georgia

XUEBING XU

Technical University of Denmark, Lyngby, Denmark

1 INTRODUCTION

Specialty fats, including Betapol, generally refer to any fats that have special functional or nutritional property intended for edible or pharmaceutical use. They can be produced by either modification or restructuring of existing fats and oils. This chapter focuses only on specialty fats that are produced by enzymatic methods. Structured lipids, polyunsaturated fatty-acids-enriched oils, modified milkfats, and modified oils from marine organisms have already been included in other chapters of the volume. Therefore, this chapter covers the following, based on nutritional or functional differences: Betapol and other specialty fats for infant formula, cocoa butter equivalents and cocoa-butter-like fats, and margarine and other plastic fats.

2 BETAPOL AND OTHER SPECIALTY FATS FOR INFANT FORMULA

Betapol is a brand name for a newly developed human milkfat substitute from Loders Croklaan. The product is based on vegetable oils which more closely mimic the specific structure of the human milkfat (HMF) as well as closely matching HMF fatty acid composition. Betapol is produced by modern enzyme technology which involves position-targeted reactions catalyzed by *sn*-1,3–specific lipases such as *Rhizomucor miehei* lipase (Novo Nordisk A/S, Denmark) under anhydrous conditions. This product resembles breast milk more closely in terms of its nutritional value and high content of palmitic acid at

the *sn*-2 position than other milkfat substitutes. In addition, Betapol's use in infant formula can lead to improved mineral and fat absorption, as well as softer stools, which are associated with breast feeding, and less risk of constipation (McNeill and Craig, personal communication, 1999).

The diet of infants during the first months of life is critical to their growth and development. Mother's milk is still the best food for infants. Breast milk provides vital nutrients and protective antibodies in a balanced combination which is easily digested, absorbed, and well tolerated by the infant. For these reasons, breast milk is often referred to as the ''gold standard'' to which infant-formula manufacturers aspire (McNeill and Craig, personal communication, 1999). To better understand the advantages of Betapol over other current milkfat substitutes for infant formula, a description of breast milkfat is necessary. About 50–60% of the dietary energy that an infant receives is provided by HMF [1,2]. Breast milk fatty acid composition varies widely and is influenced by the mother's diet. However, the most abundant saturated fatty acid in all human milks evaluated is palmitic acid (C16:0), which represents 20–25% of the total milk fatty acids and more than 10% of the infant's total energy intake (Table 1). Oleic acid represents about 40% of total milk fatty acids. Palmitic acid in HMF is located predominantly at the *sn*-2 position, whereas unsaturated fatty acids occupy mainly the *sn*-1 and *sn*-3 positions of the triacylglycerol (TAG). Betapol is a TAG mixture rich in palmitic acid (30–45 wt%).

The unique fatty acid structure in HMF plays a specific and valuable role in baby growth. After ingestion, the milk fat is emulsified and lipases hydrolyze the TAGs to release free fatty acids from the *sn*-1 and *sn*-3 positions. The free fatty acids and the

Table 1 Fatty Acid Composition (%) and Positional Distribution of HMFs

	HMF (Ref. 2)	HMF (Ref. 3)	HMF (Ref. 1)		HMF (Ref. 4)	
Fatty acids	TAG	TAG	TAG	*sn*-2	TAG	*sn*-2
8:0	0.36	0.29				
10:0	2.56	1.69	2.9	1.6		
12:0	6.86	7.85	7.3	6.9	4.9	5.3
14:0	8.01	9.04	9.4	15.4	6.6	11.3
16:0	22.70	22.24	27.0	57.1	21.8	44.5
16:1n-7	2.29	2.22	3.6	1.6		
18:0	5.79	6.40	7.1	4.9	8.0	1.2
18:1n-9	30.54	35.11	34.2	8.1	33.9	9.2
18:2n-6	14.10	10.29	7.9	3.7	13.2	7.1
18:3n-3	0.75	0.47			1.2	—
20:0	0.30	0.21				
20:1n-9	1.35					
20:2n-6	1.45					
20:3n-3	0.79					
20:4n-6	0.95	0.48				
20:5n-3	0.07	0.07				
22:0	0.19					
22:1n-9	0.30					
22:4n-6	0.39					
22:6n-3		0.26				

Note: TAG = fatty acid composition of intact triacylglycerols.

monoacylglycerols (MAGs) produced are absorbed in the small intestine. However, not all fatty acids are easily absorbed. Longer-chain, saturated fatty acids (C12:0 to C18:0) are generally less well absorbed than medium-chain (C6:0 to C10:0) and unsaturated fatty acids. The reduced absorbability with increasing chain length of saturated fatty acids is probably due to the formation of insoluble calcium soaps of these fatty acids and may result in loss of dietary energy [5–7]. Recent studies in piglets as well as infants have shown that the fatty acid at *sn*-2 is preserved at this position throughout the digestive processes and must be absorbed as an *sn*-2 MAG.

An approximate match of the fatty acid composition of HMF can be achieved by blending vegetable oils in the correct proportions to give a fatty acid profile as close as possible to HMF. Unfortunately, there remains a significant difference between vegetable oils and breast milk in terms of the specific location of fatty acids. The palmitic acid in vegetable oils is located predominantly at the *sn*-1 and *sn*-3 positions. Betapol is commercially available. For the production of this fat, enzymes are used to adjust the position of fatty acids in the TAG molecule. As a result, up to 60% or more of the C16:0 is found at the *sn*-2 position of the Betapol TAGs. A description of the fatty acid composition and distribution is given in Table 2. The TAG composition is listed in Table 3.

Numerous studies have been conducted on the nutritional applications of Betapol [8–20]. The effects of Betapol-containing formulas on fat absorption have been studied in both premature and full-term infants. Infants fed the Betapol-containing formulas showed significant improvement in C16:0 absorption both in preterm (+22%) and in full-term infants (+18%) [4,14,19]. A study in adult rats showed that C16:0 was more rapidly absorbed from Betapol than from the control diet [11]. Overall, a more efficient fat absorption was reported.

As indicated earlier, free long-chain saturated fatty acids may react with mineral ions to form insoluble soaps. The impact of soap formation on calcium absorption can be

Table 2 Fatty Acid Composition (%) and Positional Distribution of Betapol

	Betapol (Refs. 8–10)		Betapol (Refs. 11 and 12)		Betapol (Ref. 13)		Betapol (Ref. 14)		Betapol (Ref. 15)	
Fatty acids	TAG	*sn*-2	TAG	*sn*-2	TAG	*sn*-2	TAG	*sn*-2	TAG	*sn*-2
8:0	0.6	—					1.7			
10:0	0.4	—					1.3			
12:0	3.3	4.7			1.0	1.4	10.1			
14:0	1.8	1.7	1.0	1.9	2.7	4.3	4.2		1.3	1.9
16:0	29.6	69.9	30.4	72.7	27.4	53.5	23.9	73.9	31.1	67.8
16:1n-7			0.1	—			0.4		0.2	—
18:0	3.1	2.6	3.2	6.9	8.1	6.9	3.1		1.9	2.7
18:1n-9	40.6	13.7	51.5	14.7	42.1	20.9	36.0		51.7	22.6
18:2n-6	16.4	6.5	13.3	3.6	11.2	7.2	16.3		12.6	3.6
18:3n-3	3.2	0.8	0.1	—			2.1		0.1	1.4
20:0	0.4						0.2			
20:1n-9	0.1									
22:0	0.1									

Note: TAG = fatty acid composition of intact triacylglycerols.

Table 3 Triacylglycerol Profile (mol%) of Betapol

Types[a]	Betapol
SSS	0.3
SOS	0.9
OSS	6.6
SLS	<0.2
SSL	3.1
SOO	5.8
OSO	54.1
OSL	22.8
SOL	1.8
OOO	0.8
>3DB[b]	4.2

[a] Only *sn*-2 position is specified (β form).
[b] Triacylglycerols that have more than three double bonds.
Note: S = saturated fatty acids; O = oleic acid; L = linoleic acid; DB = double bonds.
Source: Ref. 16.

significant. One study of newborn full-term infants on high C16:0 formulas in which the C16:0 position on TAG was not specifically defined showed that calcium absorption was only 6% of intake compared to 51% for breast-fed infants. As a consequence, stool hardness was increased due to the formation of soaps from free long-chain saturated fatty acids [17]. This is a major contributing factor to constipation and, in extreme cases, this can lead to bowel obstruction. Betapol was shown to have similar absorption to that of HMF and to improve the metabolic processes in infants.

Enzymes are now widely used to produce food-grade specialty fats and have proven to be effective and safe catalysts in fat processing. The enzymatic reactions are carried out under mild conditions and are generally more consumer and environmental friendly than the harsh chemical reactions. Regiospecificity is one of the most important advantages of the lipase application for the production of Betapol; otherwise, it is impossible to produce it by chemical methods.

Acidolysis is the main reaction employed for the production of Betapol. The model reaction is between tripalmitin from vegetable oil and oleic acid (acyl donor), as depicted in Figure 1. An immobilized *Rhizomucor miehei* lipase is utilized in a packed-bed reactor and the reaction performed continuously up to 70°C under practically anhydrous condition. The contents of the three TAGs in the reaction, after reaction equilibrium has been reached, depend very much on the ratios between oleic acid and tripalmitin. Higher ratios would certainly move the reaction equilibrium to the product (OPO) formation. Here, O represents oleic acid and P represents palmitic acid; the order of letters indicates the position of each fatty acid in the TAG molecule. However, the equilibrating time will also increase with an increase in substrate molar ratio, as illustrated in Figure 2. Therefore, it is very important to choose a suitable substrate molar ratio to increase reaction efficiency (incorporation level of oleic acid per unit time) and productivity (product quantity per unit time)

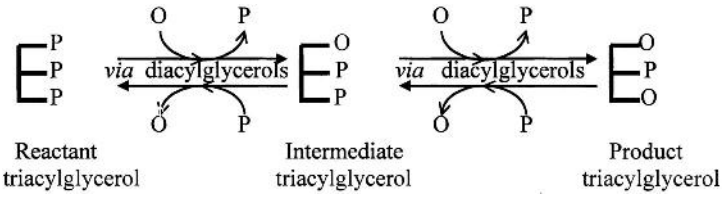


Figure 1 Reaction principle for the production of Betapol by enzymatic interesterification with *sn*-1,3–specific lipases. P = palmitic acid; O = oleic acid. (Adapted from Ref. 21.)

in a specific reaction system. The choice of substrate molar ratio is also related to the downstream processing cost and associated difficulties of separating free fatty acids or acyl donors by evaporation and/or distillation. A high substrate molar ratio may reduce the reaction time to obtain a suitable product, but the purification of the product may also be more difficult. A 3:1 substrate molar ratio (O:PPP) will, after equilibrium, result in 48% PPO/OPP, 36% OPO, and 16% PPP [22,23]. After purification and fractionation, PPP can be removed. The fractionation step can be either solvent or solvent-free. After blending with other oils, a TAG composition can be produced that is similar in structure and composition to human breast milk (McNeill and Craig, personal communication, 1999). The resulting product, therefore, will contain 57.2% PPO/OPP and 42.8% OPO, which is equivalent to an oleic acid incorporation level of 71.4% at *sn*-1,3 positions and 47.6% based on the intact TAGs.

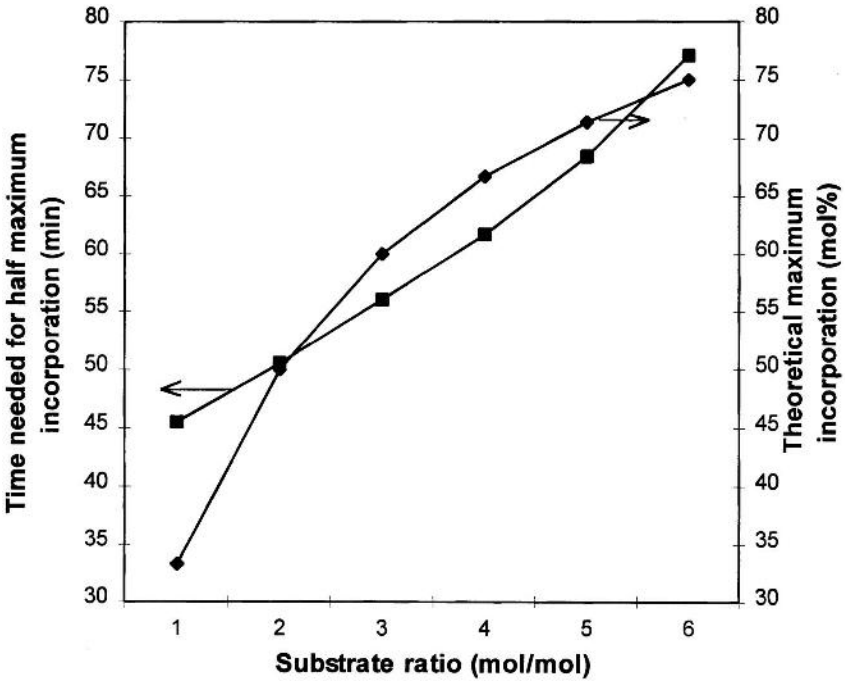


Figure 2 Effect of substrate molar ratio on the maximal incorporation of acyl donors and the time needed to reach the half-maximum incorporation. (Adapted from Ref. 22.)

The production of Betapol in the industrial plant is conducted in a two-stage reaction process [24]. A packed-enzyme-bed bioreactor is used. The substrate passes the first bed and the first-stage product is purified by distillation to separate the free fatty acids. The purified product is further mixed with oleic acid and passed to the second-stage packed-bed reactor. The second-stage product is purified by distillation. The purified product is further fractionated to separate unreacted PPP and formed diacylglycerols (DAGs). A final refining, including bleaching and deodorization, is necessary to make it suitable for edible purposes. A full process outline in depicted in Figure 3.

The materials for Betapol production are not pure PPP and oleic acids as described earlier, which, in effect, will lead to increased cost of production and, certainly, the price of the product. No report showed the exact source of the vegetable oil or oleic acid used for the production of Betapol. According to market price and availability, palm stearin is probably the best source of PPP. Palm stearin normally contains 50–60% PPP according to fractionation processes [25]. The rest mainly contains POP and PPO/OPP. If special fractionation procedures are applied, PPP content can be increased up to 70–80%. The sources for oleic acid can be from the high-oleic-acid sunflower-oil fatty acids, which are comparatively inexpensive. Mixed fatty acids from olive oil and teaseed oil that contain 70–80% oleic acid can also be used. The specification for commercial Betapol (Betapol 45) is as follows: free fatty acid, 0.08%; typical fatty acid composition (%); 12.0 C12:0, 4.7 C14:0, 22.9 C16:0, 3.3 C18:0, 39.6 C18:1, 12.9 C18:2, 1.6 C18:3, and others 3.0; total C16:0 esterified at the *sn*-2 position is 45% (from the catalog published by Loders

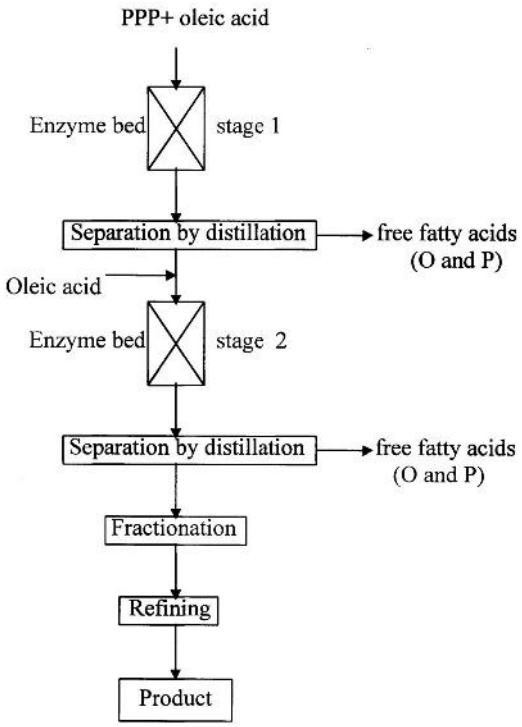


Figure 3 Process scheme for Betapol production by lipase-catalyzed interesterification in industrial scale. O = oleic acid; P = palmitic acid. (Redrawn from Ref. 24.)

Croklaan). From this datasheet, it can be deduced that the material for the production only contains about 50% PPP or the incorporation level is not very high. However, a recent personal communication from McNeill and Craig (personal communication, 1999) indicates that the fatty acid composition range for Betapol is as follows (wt%): C12:0, up to 0.5; C14:0, up to 2.2; C16:0, 29.0–42.0; C18:0, up to 3.9; C18:1, 41.5–58.0; C18:2, 10.6; C18:3, up to 1.6; others, up to 1.3; total C16:0 at the *sn*-2 position ranges between 60.0% and 80.0%.

A tree fruit fat called *urushi wax* was reported to contain 60% PPP and could be developed as a source of substrate for Betapol production [26]. The tree is mainly seen in China and some other Asian countries. The fat is currently used as a material for soap or fatty acid production.

Regiospecificity of lipases play a key role in the production of Betapol. Lipozyme IM, a commercially available immobilized lipase, is widely used by many researchers for lipid modifications and for Betapol production. This *Rhizomucor miehei* lipase is immobilized on an ion-exchange resin by physical adsorption [27,28]. It is *sn*-1,3–specific on the TAGs under suitable conditions. However, the reaction process is not simple as described in Figure 1. By-reactions do occur during the reaction due to the formation of DAGs, which are inevitable intermediates in the reaction process [29]. The reaction is depicted in Figure 4. Many factors affect acyl migration and by-product formation during reaction. An increase in temperature, reaction time, water content, and water activity will enhance acyl migration and by-product formation. However, these factors also affect the main reaction and desired product formation. Therefore, a compromise may be necessary to

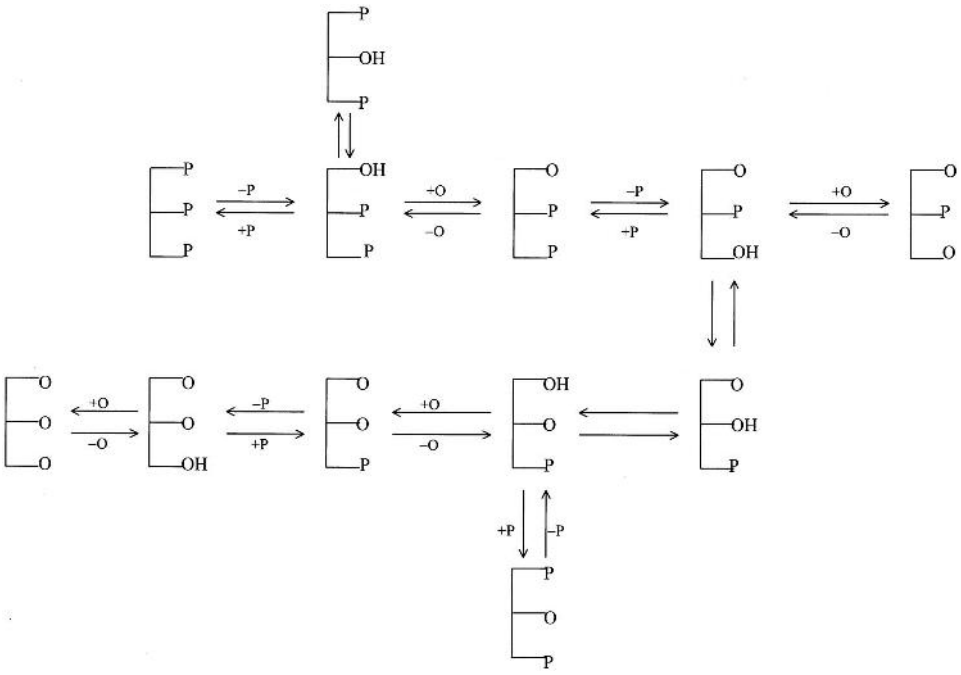


Figure 4 Scheme for *sn*-1,3–specific lipase-catalyzed interesterification between triacylglycerols and free fatty acids including main reactions and by-reactions. P = palmitic acid; O = oleic acid. (Adapted from Ref. 30.)

achieve optimal product yield [31]. Reactor types also affect the degree of acyl migration and the formation of by-products. It was demonstrated that packed-enzyme-bed reactors have advantages over stirred-tank reactors in reducing acyl migration [22,32]. This is probably due to the shorter reaction time, no breaking of enzyme particles, and system control in packed-bed reactors. Breaking of enzyme particles in stirred-tank reactors would affect the productivity, final product quality, and downstream processing. Purification by evaporation or distillation was reported to affect acyl migration as well [23]. High temperature and long residence time during distillation will greatly impact the degree of acyl migration. An efficient process is necessary for the reduction of acyl migration and the improvement of product quality as well.

A ''two-step'' process was introduced for the production of Betapol, in which alcoholysis was the first step and esterification the second step [33]. A schematic representation is given in Figure 5. The first step was a reaction between PPP and ethanol catalyzed by a lipase to produce MAG in the solvent system. The reacted mixture in the solvent was crystallized and fractionated at low temperature. The MAG was then separated from the system. This MAG was further reacted with oleic acid in the solvent to produce OPO. A 90% purity of the product was reported. This process is suitable for the production of high-purity product, however, scale-up trials need to be conducted to check the performance. MAGs are very unstable even at room temperature. The *sn*-2 MAG could rearrange into *sn*-1 or *sn*-3 MAGs during the separation and reaction, especially in large-scale operations. Therefore, POO and OOP could be formed after reaction.

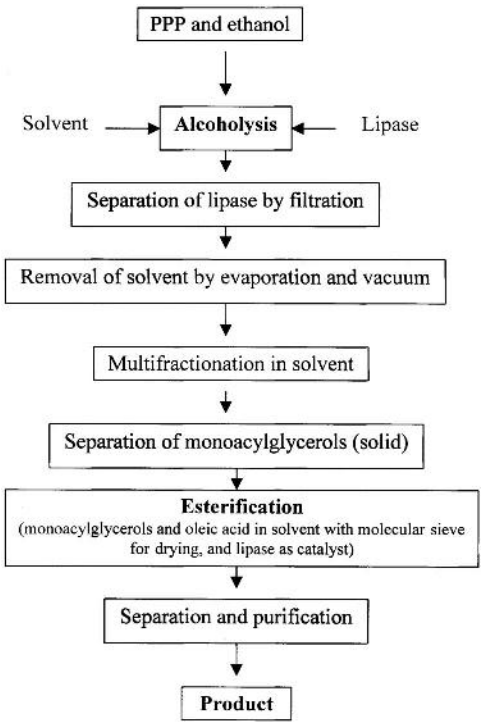


Figure 5 A two-step process (alcoholysis and esterification) for the production of Betapol by lipase-catalyzed reactions. (Drawn according to data from Ref. 33.)

Table 4 Comparison Between Human Milkfat and Modified Butter Oil in Terms of Their Fatty Acid Composition and Distribution

	Human milkfat		Modified butter oil	
Fatty acids	TAG	*sn*-2	TAG	*sn*-2
C6:0			0.48	—
C8:0			0.43	—
C10:0			1.77	—
C12:0	2.54	5.00	2.10	0.75
C14:0	5.07	8.99	6.57	11.74
C14:1	0.26	1.19	0.98	0.45
C15:0	0.36	1.84	0.57	0.90
C16:0	27.73	50.65	22.50	34.96
C16:1n-7	3.31	4.00	2.14	4.10
C17:0	0.21	2.08	0.30	0.74
C18:0	8.73	4.64	6.03	7.78
C18:1n-9	38.82	14.32	43.52	31.48
C18:2n-6	8.68	4.21	10.76	5.54
C18:3n-3	0.85	0.55	0.70	0.45
C20:1n-9	1.12	1.18	0.37	0.09
C18:4n-3	—	—	0.03	0.10
C20:4n-6	0.40	0.64	0.09	0.18
C22:1n-9	0.16	0.72	0.08	0.08
C20:4n-3	0.27	—	—	—
C20:5n-3	0.18	—	0.31	—
C22:4n-6	0.37	—	0.03	0.04
C22:5n-6	0.24	—	0.03	—
C22:5n-3	0.29	—	0.08	—
C22:6n-3	0.41	—	0.13	0.08

Note: TAG = fatty acid composition of intact triacylglycerols.
Source: Ref. 34.

Other types of fat for infant formula were produced to enhance their EPA and DHA contents that exist in small amounts in HMF [34]. EPA and DHA are helpful for infant brain growth, but do not exist in butter oil. Therefore, the modification of butter oil by Lipozyme IM-catalyzed acidolysis was performed to produce HMF substitute. The product was compared with HMF (Table 4) and a similar fatty acid composition was obtained. Papaya (*Carica papaya*) latex was tested for the production of Betapol-type fats [35]. A similar activity to Lipozyme IM was observed.

3 COCOA BUTTER EQUIVALENTS AND COCOA-BUTTER-LIKE FATS

Cocoa butter is an important major constituent of the chocolate formulations. Cocoa butter is composed predominantly (more than 70%) of symmetrical TAGs with oleic acid in the *sn*-2 position, mainly POP, POSt, and StOSt, where P is palmitic acid, O is oleic acid, and St is stearic acid. A typical fatty acid composition of cocoa butter is (mol%): 24.4 C16:0, 33.6 C18:0, 37.0 C18:1, 3.4 C18:2, and others 1.6. The fatty acid distribution

at the *sn*-2 position is (mol%) 0.4 C16:0, 1.6 C18:0, 87.0 C18:1, 10.0 C18:2, and others, 1.0. A typical TAG composition is (mol%) 16 POP, 35 POSt, 26 StOSt, 4 POO, 6 StOO, 2 PLP, 4 PLSt, 2 StLSt, 1 PLO, and others, 4 [36].

Cocoa butter has a melting range of 32–35°C. Due to its unique TAG composition and the extremely low levels of DAGs, cocoa butter has special physical properties for chocolate making. Cocoa butter has a sharp melting range and has ability to recrystallize during processing to a stable crystal mode. The typical solid fat content (SFC) measured by pulse nuclear magnetic resonance (NMR) is listed as follows at different temperatures: 82.1 (20°C), 78.7 (25°C), 58.3 (30°C), and 2.4 (37°C).

Cocoa butter is obtained mainly from Africa, South and Central America, south Asia, and some other tropical countries. The price of cocoa butter very much depends on the price of cocoa bean and varies year by year. The market demand for confections has increased steadily over the years. The uncertainty in cocoa butter supply and the volatility in cocoa butter prices have forced confection producers to seek alternatives to replace cocoa butter. This search started a long time ago and many cocoa-butter-like fats were used by industry as confectionery fats such as lauric cocoa butter substitutes (CBS), non-lauric cocoa butter substitutes, and cocoa butter equivalents (CBE) [37]. CBE are closest to cocoa butter in this classification and are totally compatible with cocoa butter. The European Union (EU) requirements for CBE are as follows: 1) SOS $\geq$ 65% (S = saturated fatty acids; O = oleic acid), 2) unsaturated fatty acids at the *sn*-2 position $\geq$ 85%, 3) total unsaturated fatty acids $\leq$ 45%, 4) unsaturated fatty acids with two or more double bonds $\leq$ 5%, 5) content of lauric acid $\leq$ 1%, and 6) content of trans fatty acids $\leq$ 2%.

The development of enzyme technology makes it possible to produce CBE from other vegetable oils that better mimic the composition and properties of cocoa butter. The POP component in cocoa butter can be found in palm oil [25] and Chinese vegetable tallow [26,38,39]. StOSt can be found in sal fats, mango fat, illipe fat, kokum fat, and shea oil [40]. However, formulations from all these sources have no or little POSt components that abundantly exists in cocoa butter. By using lipase-catalyzed reactions, all the three components can be produced with oils in which the *sn*-2 position is mainly occupied by oleic acid. The main reaction is depicted in Figure 6.

Production of CBE is one of the most promising applications of lipase-catalyzed reactions due to the structural characteristics of cocoa butter and regiospecificity of lipases.

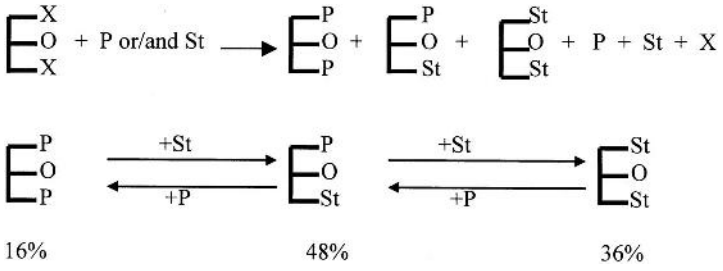


Figure 6 Scheme of reactions for the production of cocoa butter equivalents by lipase-catalyzed interesterification and the balanced contents of the three main triacylglycerol species (POP, POSt, and StOSt) after equilibrium is reached at the specified substrate molar ratio. Quantities of each TAG at equilibrium after reaction with a substrate molar ratio of 3:1 between acyl donors (free fatty acids or their ethyl esters) and substrate oils. P = palmitic; O = oleic; St = stearic acids; X = any fatty acids. (Adapted from X. Xu, internal project report, 1998.)

From the initial investigations of the possibilities of reverse reactions to hydrolysis by lipases in microaqueous media, a promising application seemed to be the production of CBE [41,42]. In early 1980s, many reports appeared [43–49] and some patents were issued [50–55]. To date, commercial or large-scale production of CBE by enzyme-catalyzed interesterification has been reported [24,56–58]. More investigations were conducted recently [59–70].

The structural modification of oils and fats by lipase-catalyzed interesterification was based on the properties of lipases, the source oils, and low prices. Governed by the *sn*-1,3 specificity of the lipases, products such as POP, StOSt, or suitable mixtures of POP, POSt, and StOSt can be creatively produced using available source oils. The starting oils can be the following: olive oil [43], high-oleic-acid sunflower oil, high-oleic-acid canola oil [71], and teaseed oil [42,62], which mainly contain OOO; palm oil mid-fraction [56,59–61,65–69] and Chinese vegetable tallow [63,70], which mainly contain POP; and shea butter, kokum fat, dhupa fat, and sal fat [40], which mainly contain StOSt. Possible reaction routes are illustrated in Figure 7.

A similar process for the production of Betapol was used for the production of cocoa butter equivalents by Unilever's Loders Croklaan, as described in Figure 3 [24]. The process is a two-stage reaction. Palm oil midfraction is the oil source and stearic acid is the acyl donor for the production. Due to the high melting points for both the palm oil mid-fraction and acyl donor, a system that involves solvent as a medium or high temperature was needed for the reaction [52,55]. Otherwise, the viscosity would be very high and the mass transfer at the packed-bed bioreactor would be limited. However, no detailed information was reported as to the actual system used by Loders Croklaan in its industrial plant.

Fuji Oil Company used a different process for the production of cocoa butter equivalents by lipase-catalyzed interesterification [56–58]. The schematic presentation of the process is given in Figure 8. As seen from the process, Fuji Oil used a stirred-tank reactor for the reaction system and hexane as the medium. Stearic acid ethyl ester was used as an acyl donor to reduce the melting point and boiling point. This will increase the substrate solubility in hexane, reduce the viscosity of the system, and reduce the distillation temperature during downstream processing.

Normally after the reaction, free fatty acids or their alkyl esters are removed by evaporation and/or distillation under vacuum. This process represents the cost of the whole

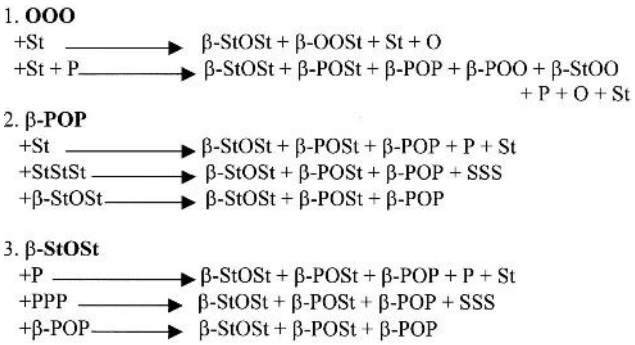


Figure 7 Schematic routes for the production of cocoa butter equivalents by lipase-catalyzed reactions from different source oils. O = oleic acid; P = palmitic; St = stearic acids; S = saturated fatty acids. The β-indicated *sn*-2 position is specified. (Adapted from Ref. 70.)

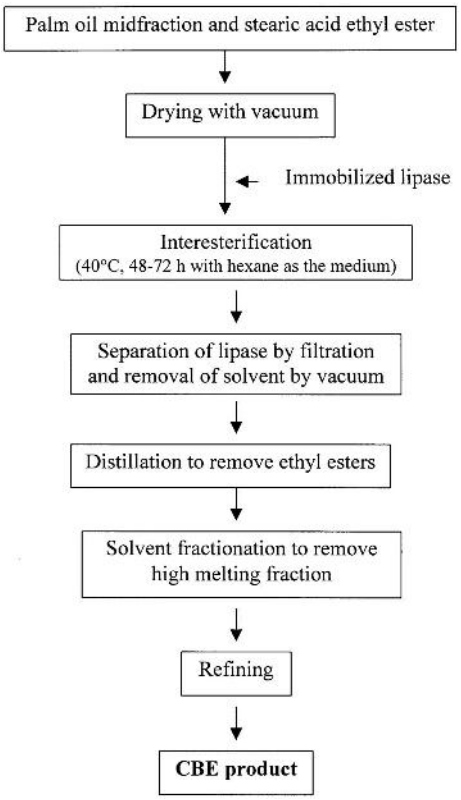


Figure 8 Fuji Oil process for the production of cocoa butter equivalents by lipase-catalyzed interesterification. (Redrawn from Ref. 58.)

process and it may also lead to deterioration of the product quality. High temperatures probably increase acyl migration [23]. Xu et al. studied the process using solvent fractionation to remove the free fatty acids after the reaction [70]. The process is depicted in Figure 9. This process took advantage of the solvent reaction medium which, after reaction and filtration of enzyme, was directly used for solvent fractionation. This process can also remove the fully saturated triacylglycerols (PPP, PPSt, PStSt, StStSt) when formed and part of the DAGs. The rest of the free fatty acids can be removed by alkali neutralization using a solvent system.

The production of cocoa butter equivalents also employed the regiospecificity of lipases. By-reactions and formation of by-products occur as described in Figure 4, as PStP and StStSt may be formed. The formation of DAGs will affect the quality of the product. DAGs will affect the formation of stable crystals and the conditioning process during chocolate making [56]. The content of DAGs should be minimized to improve product quality. The effect of reaction parameters on the formation of DAGs has been studied [30]. Water content or water activity is a crucial parameter in the study of DAGs formation [30,31]. Other parameters, such as temperature, enzyme load, and substrate molar ratio, also had impact on the content of DAGs in the products. It was reported that the choice of solvent and types of acyl donor also influenced the content of DAGs [61].

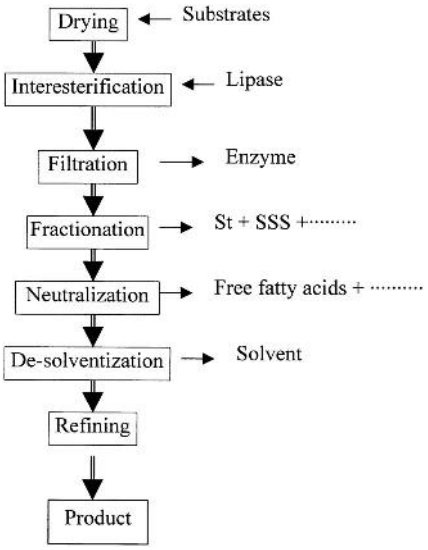


Figure 9 Pilot process for the production of cocoa butter equivalents from Chinese vegetable tallow by lipase-catalyzed interesterification. Fractionation was used for the separation of free fatty acids. St = stearic acid; S = saturated fatty acids. (Adapted from Ref. 70.)

In addition to the use of packed-bed reactors and stirred-tank reactors, with or without solvents as reactor media in studies and industrial productions, other systems have also been studied. Mojovic et al. [65] studied the gas-lift reactor for the production of cocoa butter equivalents between palm oil mid-fraction and stearic acid in hexane. They found that a 2.8-fold increase in the productivity of interesterification by Celite-immobilized and lecithin-protected lipase was achieved in the gas-lift reactor when compared with shake-flask experiments. It was concluded that this was partially attributed to the reactor design.

Liu et al. [66] and Nakaya et al. [69] studied the production of cocoa butter equivalents using supercritical carbon dioxide as the media. Liu et al. found that the best lipase in this system was Lipozyme IM, which was most effective and specific in synthesizing CBE product by interesterification. They found that the yields of cocoa butter equivalents were affected by pressure, substrate oil composition, solubility, and cosolvent. Their best reaction conditions were as follows: reaction pressure at 1500 psi, reaction medium with 5.0% water,and reaction temperature of 50°C. Nakaya et al. studied the reaction between triolein and stearic acid by lipase-catalyzed interesterification using liquid CO_2 as the medium. They found three regions which could be classified according to the pressure. In the nonsolvent region (below 5 MPa), the reaction was limited and very slow. In the near-critical region, from 5 to 10 MPa, the reaction rate was maximal at 5.9 MPa because of the stabilization of the enzyme–substrate complex. In the supercritical region, above 10 MPa, the reaction rate increased with an increase in pressure, reflecting the increase in solubility of substrate in supercritical carbon dioxide.

Ju et al. [68] studied the interesterification of palm oil mid-fraction and stearic acid under controlled water activity in hexane. They found that a combination of water activity (0.432) and enzyme loading (10.33 U/mg) yielded optimal activity.

Many different *sn*-1,3–specific lipases have been studied for the production of cocoa butter equivalents. However, the two most promising lipases were *Rhizomucor miehei* [27,28,40,59,61,64,66,69] and *Rhizopus arrhizus* [56–58,60,65,67,68] lipases, which attracted a large number of studies. Among animal lipases, pancreatic lipase attracted most studies [62,63] and the pilot trial was also reported [70].

4 MARGARINE AND OTHER PLASTIC FATS

Margarines are fatty foods prepared by blending fats and oils with other ingredients, such as water and/or milk products, suitable edible proteins, salt, flavoring and coloring materials, and vitamins A and D. By U.S. and EEC regulations, margarine must contain at least 80% fat. The margarine industry also produces reduced-calorie margarines containing 40% fat and spreads containing 50–60% fat.

Many types of margarine are produced by industry for different purposes such as stick and tub margarines [72]. For nutritional improvement and operational convenience, fluid margarines are also produced by industry for different uses. A solid fat profile for general-purpose margarine at different temperatures (°C in parenthesis) is as follows: 38 (10), 30 (15), 23 (20), 17 (25), 13 (30), 10 (35), 7 (40), and 4 (45).

Margarine plastic fats are normally produced by blending different TAGs (liquid oils and hydrogenated fats) or by randomizing a mixture of liquid oil and a solid fat by chemical transesterification. The plastic range or solid fat content profile is mainly determined by the ratio between the solid fat and the liquid oil and relates to types of solid fats or liquid oils and processing procedures [72]. Applications of lipase technology for the production of margarine fats provide a few advantages when compared with chemical methods. The process is more natural and ''green.'' The products are better accepted by consumers. The conditions are mild and would be better for nutritional margarine production in terms of nutrient stability and product quality. Furthermore, the formation of partial acylglycerols due to lipase hydrolysis poses no harm to the processing of margarine; on the contrary, they act as emulsifiers, which are important ingredients for margarine production. This is different from confectionery production in which stable β crystals are formed. In margarine production, intermediate β′ crystals are needed, in which partial acylglycerols assist in the formation of needed crystals.

Two oils and fats (liquid oil and solid fat) are normally used for the production of margarine fats by lipase-catalyzed transesterification. Certainly, more than two oils and fats or only one oil and one fat can be used, if necessary, to produce a suitable solid fat profile and some other physical properties. Free fatty acids or their ethyl esters are not recommended in this case because the purification process for their removal is costly.

Two types of lipase were used for the production of margarine fats: *sn*-1,3–specific and nonspecific lipases. The reactions catalyzed by these two types of lipases produce similar TAG components, but the proportion of each TAG is different because nonspecific lipases will result in a fully randomized product. The two reactions are depicted in Figure 10. Due to acyl migration, reactions with *sn*-1,3–specific lipases may be partially randomized or fully randomized in extreme conditions [73].

The processes must be, in cases where quality is maintained, as simple as possible, to reduce the production cost because margarines are daily-used products with relatively low market price. The lipase-catalyzed reactions can be conducted in stirred-tank reactors and packed-bed reactors, normally without solvents. After reaction, the mixture of lipids

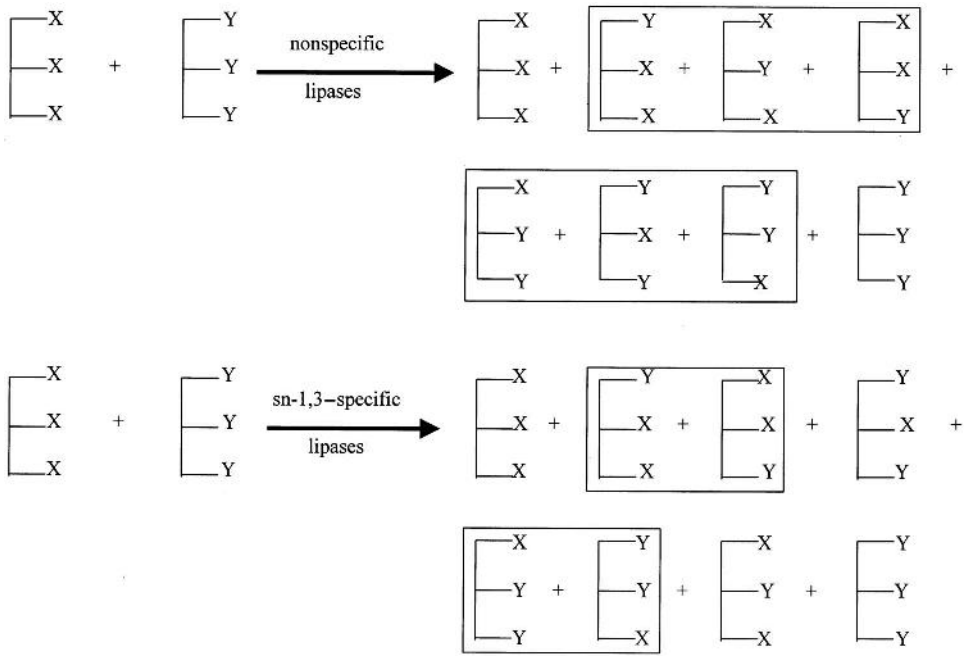


Figure 10 Reaction schemes for the lipase-catalyzed transesterification between two triacylglycerols with nonspecific and *sn*-1,3–specific lipases. Triacylglycerols in each rectangle have the same content if nonspecific lipases are nonspecific both on positions and fatty acids, and *sn*-1,3–specific lipases have the same selectivity on *sn*-1,3 positions and are nonspecific on fatty acids. X and Y are different fatty acids. (Adapted from Xu, internal project report, 1998.)

are directly moved to the refining stage to remove free fatty acids, color, and smell by bleaching and physical refining, or neutralization, bleaching, and deodorization.

Limited studies have been conducted by a few groups worldwide on margarine fat production by lipase-catalyzed transesterification. Lai et al. [74–79] investigated the production of margarine fats by lipases using palm stearin (PS) together with sunflower oil (SO) or palm kernel olein (PKO), and they characterized the products therefrom. They tested a few lipases for the reaction and found that *Pseudomonas* lipase had the fastest rate of reaction (50.0 per hour) followed by *Rhizomucor miehei* lipase (27.1 per hour). *Pseudomonas* lipase-catalyzed mixtures lead to the largest drop in slip melting point and solid fat content in all the mixtures studied. They found five mixtures (i.e., the *Pseudomonas* lipase-catalyzed PS/PKO mixtures at 40/60 and 50/50, *Rhizomucor miehei* lipase-catalyzed PS/PKO mixtures at 40/60, and *Pseudomonas* lipase-catalyzed PS/SO mixtures at 40/60 and 50/50 ratios) to be suitable for the formulation of table margarine. In polymorphic studies, *Pseudomonas* lipase seemed more efficient, as it produced blends with exclusively β′ forms, whereas a mixture of β′ and β forms was obtained for *Rhizomucor miehei* lipase-catalyzed fat blends. They also studied the physical, chemical, rheological, and sensory performance of the produced margarines from *Rhizomucor miehei* lipase-catalyzed PS/PKO (40/60) blend. The results indicated that the produced margarine fats by lipase-catalyzed transesterification showed some promise for industrial adaptation.

Seriburi and Akoh [80,81] studied the production of plastic fats by *Candida antarctica* lipase-catalyzed interesterification of lard/high-oleic-sunflower oil, and triolein/tristearin mixtures. They found that a 60/40 ratio of lard/high-oleic-sunflower oil had the widest plastic range (3–26°C). The product had 60.1% oleic acid at the *sn*-2 position compared to 44.9% for the physical blend. The solid fat profile of the 60/40 interesterified mixture resembled soft-type margarine fat. When using triolein/tristearin as the starting materials, a 1:2 molar ratio gave a maximal yield (47%) of OStSt/StStO (O-oleic; St-stearic acids).

Mohamed et al. [82–84] studied the blends from refined cottonseed oil (RCO) and fully hydrogenated soybean oil (FHSO) for the production of plastic fats via lipase-catalyzed reactions in solvent-free systems. Two types of lipase were used, namely *Rhizomucor miehei* and *Candida antarctica* lipases, in which the former was *sn*-1,3–specific and the latter was nonspecific. Changes in acylglycerol structure were investigated. The interesterification decreased the levels of trisaturated and triunsaturated TAGs and increased the amounts of monosaturated and disaturated TAGs in the blends. The TAG products obtained by the two lipases were similar in composition but different in the proportions: Levels of high-melting acylglycerols were higher in the products obtained with the nonspecific lipase. They also found that the content of *sn*-1,3 DAGs formed exceeded that of *sn*-1,2 DAGs and low levels of MAGs were formed during the reaction. The presence of DAGs in the interesterified fat reduced the amount of crystallized solids. They found that a mixture of 86.5% RCO and 13.5% FHSO had properties similar to conventional soft margarine fats. The crystallization behavior and rheological properties of the products were also studied and an increase in the relative stability of the β′ form crystal was observed after interesterification.

Some other studies were performed using palm oil or palm stearin as the solid fat source together with other liquid oils [85,86]. Similar products or performance characteristics of the products were obtained.

REFERENCES

1. RG Jensen, GL Jensen. J Pediatr Gastroenterol Nutr 15:232–245, 1992.
2. CH Winter, EB Hoving, FAJ Muskiet. J Chromatogr 616:9–24, 1993.
3. VP Carnielli, G Verlato, F Pederzini, IHT Luijendijk, A Boerlage, D Pedrotti, PJJ Sauer. Am J Clin Nutr 67:97–103, 1998.
4. EL Lien, FG Boyle, R Yuhas, RM Tomarelli, P Quinlan. J Pediatr Gastroenterol Nutr 25: 167–174, 1997.
5. EL Lien. J Pediatr 125:S62–S67, 1994.
6. SM Innis. J Pediatr 120:S56–S61, 1992.
7. DM Small. Annu Rev Nutr 11:413–434, 1991.
8. SM Innis, R Dyer, P Quinlan, D Diersen-Schade. J Nutr 125:73–81, 1995.
9. SM Innis, P Quinlan, D Diersen-Schade. Am J Clin Nutr 57:382–390, 1993.
10. SM Innis, R Dyer, P Quinlan, D Diersen-Schade. Lipids 31:497–505, 1996.
11. NJ de Fouw, GAA Kivits, PT Quinlan, WGL van Nielen. Lipids 29:765–770, 1994.
12. A Zampelas, CM Williams, LM Morgan, J Wright, PT Quinlan. Br J Nutr 71:401–410, 1994.
13. PL Zock, J Gerritsen, MB Katan. Eur J Clin Invest 26:141–150, 1996.
14. A Lucas, P Quinlan, S. Abrams, S Ryan, S Meah, PJ Lucas. Arch Dis Child 77:F178–F184, 1997.
15. LKM Summers, BA Fielding, V Ilic, P Quinlan, KN Frayn. Br J Nutr 79:141–147, 1998.
16. DA Pufal, PT Quinlan, AM Salter. Biochim Biophys Acta 1258:41–48, 1995.

17. PT Quinlan, S Lockton, J Irwin, AL Lucas. J Pediatr Gastroenterol Nutr 20:81–90, 1995.
18. SM Innis, R Dyer, CM Nelson. Lipids 29:541–545, 1994.
19. VP Carnielli, IHT Luijendijk, JB van Goudoever, EJ Sulkers, AA Boerlage, HJ Degenhart, PJJ Sauer. Am J Clin Nutr 61:1037–1042, 1995.
20. VP Carnielli, IHT Luijendijk, JB van Goudoever, EJ Sulkers, AA Boerlage, HJ Degenhart, PJJ Sauer. J Pediatr Gastroenterol Nutr 23:553–560, 1996.
21. H Mu, X Xu, C-E Hoy, J Adler-Nissen. Fett/Lipid 101:158–164, 1999.
22. X Xu, S Balchen, C-E Hoy, J Adler-Nissen. J Am Oil Chem Soc 75:1573–1579, 1998.
23. X Xu, S Balchen, C-E Hoy, J Adler-Nissen. J Am Oil Chem Soc 75:301–308, 1998.
24. P Quinlan, S Moore. INFORM 4:580–585, 1993.
25. FD Gunstone. Palm Oil. New York: Chichester, 1987, pp 68–83.
26. X Xu. J Malaysian Oil Sci Technol 3:46–50, 1994.
27. LH Posorske, GK LeFebvre, CA Miller, TT Hansen, BL Glenvig. J Am Oil Chem Soc 65: 922–926, 1988.
28. TT Hansen, P Eigtved. In: Proceedings of Emerging Technologies in the Oil and Fat Processing. Champaign, IL: AOCS Press, 1987, pp 365–369.
29. X Xu, ARH Skands, C-E Høy, H Mu, S Balchen, J Adler-Nissen. J Am Oil Chem Soc 75: 1179–1186, 1998.
30. X Xu, H Mu, A Skands, C-E Høy, J Adler-Nissen. J Am Oil Chem Soc 76:175–181, 1999.
31. X Xu, A Skands, J Adler-Nissen, C-E Høy. Fett/Lipid 100:463–471, 1998.
32. H Mu, X Xu, C-E Høy. J Am Oil Chem Soc 75:1187–1193, 1998.
33. U Schmid, UT Bornscheuer, MM Soumanou, GP McNeill, RD Schmid. J Am Oil Chem Soc 75:1527–1531, 1998.
34. TC Christensen, G Holmer. Milchwissenschaft 48:543–547, 1993.
35. KD Mukherjee, I Kiewitt. Biotechnol Lett 20:613–616, 1998.
36. R Liendo, FC Padilla, A Quintana. Food Res Int 30:727–731, 1997.
37. M Lipp, E Anklam. Food Chem 62:73–97, 99–108, 1998.
38. Y Xin, K Aitzetmuller. Fat Sci Technol 94:123–130, 1992.
39. BSJ Jeffrey, FB Padley. J Am Oil Chem Soc 68:123–127, 1991.
40. R Sridhar, G Lakshminarayana, TNB Kaimal. J Am Oil Chem Soc 68:726–730, 1991.
41. RW Stevenson, FE Luddy, HL Rothbart. J Am Oil Chem Soc 56:676–680, 1979.
42. T Tanaka, E Ono, M Ishihara, S Yamanaka, K Takinami. Agric Biol Chem 45:2387–2389, 1981.
43. K Yokozeki, S Yamanada, K Takinami, Y Hirose, A Tanaka, K Sonomoto, S Fukui. J Appl Microb Biotechnol 14:1–5, 1982.
44. AR Macrae. J Am Oil Chem Soc 60:243A–246A, 1983.
45. AR Macrae. In: C Ratledge, P Dawson, J Rattray, ed. Biotechnology for the Oils and Fats Industry. Champaign, IL: AOCS Press, 1984, pp 189–198.
46. LH Pososke. J Am Oil Chem Soc 61:1758–1760, 1984.
47. T Nielsen. Fette Seif Anstrich 87:15–19, 1985.
48. AR Macrae. In: J Tramper, HC van der Plas, and P Linko, eds. Biocatalysts in Organic Syntheses. Amsterdam: Elsevier Science, 1985, pp 195–208.
49. AR Macrae, RC Hammond. Biotechnol Genet Eng Rev 3:193–217, 1985.
50. MH Coleman, AR Macrae. U.S. Patent Specification 1,577,933, 1980.
51. T Matsuo, N Sawamura, Y Hashimoto, W Hashida. UK Patent Appl 2 035 359 A, 1980.
52. AR Macrae, P How. European Patent Specification 0093602B1, 1983.
53. T Tanaka, E Ono. US patent 4,275,011, 1981.
54. T Matsuo, N Sawamura, Y Hashimoto, W Hashida. European Patent Specification 0035883A2, 1981.
55. PJ Halling, AR. Macrae. European Patent Specification 0064855A1, 1982.
56. Y Hashimoto. In: A Tanaka, T Tosa, T Kobayashi, eds. Industrial Application of Immobilised Biocatalysts. New York: Marcel Dekker, 1993, pp 337–352.

57. N Sawamura. Ann NY Acad Sci 542: Enzyme Eng 9:266–269, 1988.
58. YJ Owusu-Ansah. In: BS Kamel, Y Kakuda, eds. Technological Advances in Improved and Alternative Sources of Lipids. London: Blackie Academic & Professional, 1994, pp 360–389.
59. CN Chong, YM Hoh, CW Wang. J Am Oil Chem Soc 69:137–140, 1992.
60. L Mojovic, S Siler-Marinkovic, G Kukic, G Vunjak-Novakovic. Enzyme Microb Technol 15: 438–443, 1993.
61. S Bloomer, P Adlercreutz, B Mattiasson. J Am Oil Chem Soc 67:519–524, 1990.
62. X Xu, X Hu, G Zhang. In: Proceedings of International Symposium on New Approaches in the Production of Foodstuffs and Intermediate Products from Cereal Grains and Oil Seeds. Beijing: Chinese Cereal and Oil Association, 1994, pp 808–811.
63. G Zhang, X Hu, X Xu. In: Proceedings of International Symposium on New Approaches in the Production of Foodstuffs and Intermediate Products from Cereal Grains and Oil Seeds. Beijing: Chinese Cereal and Oil Association, 1994, pp 382–389.
64. S Rasmussen, S Bækgaard. In: VKS Shukla, G Hølmer, eds. Proceedings of 15th Scandinavian Symposium on Lipids. Rebild Bakker: Intl Food Sci Center AS, 1989, pp 264–267.
65. L Mojovic, S Siler-Marinkovic, G Kuke, B Bugarski, G Vunjak-Novakovic. Enzyme Microb Technol 16:159–162, 1994.
66. KJ Liu, HM Cheng, RC Chang, JF Shaw. J Am Oil Chem Soc 74:1477–1482, 1997.
67. FC Huang, YH Ju. J of the Chin I. Ch E 28:73–78, 1997.
68. YH Ju, CK Chiu, FC Huang. J of the Chin I. Ch E 27:65–70, 1996.
69. H Nakaya, O Miyawaki, K Nakamura. Biotechnol Tech 12:881–884, 1998.
70. X Xu, S Balchen, X Hu, G Zhang, J Adler-Nissen. In: Proceedings of International Symposium on the Approaches to Functional Cereals and Oils. Beijing: Chinese Cereal and Oil Association, 1997, pp 104–112.
71. T Gitlesen, I Svensson, P Adlercreutz, B Mattiason, J Nilsson. Ind Crops Products 4:167–171, 1995.
72. G Hoffmann. The Chemistry and Technology of Edible Oils and Fats and Their High Fat Products. London: Academic Press, 1989, pp 292–303.
73. HM Ghazali, S Hamidah, YB Che Man. J Am Oil Chem Soc 72:633–639, 1995.
74. OM Lai, HM Ghazali, CL Chong. Food Chem 64:83–88, 1999.
75. OM Lai, HM Ghazali, F Cho, CL Chong. Food Chem 64:221–226, 1999.
76. OM Lai, HM Ghazali, CL Chong. Food Chem 63:155–159, 1998.
77. OM Lai, HM Ghazali, CL Chong. J Am Oil Chem Soc 75:881–886, 1998.
78. OM Lai, HM Ghazali, CL Chong. J Am Oil Chem Soc 75:953–959, 1998.
79. OM Lai, HM Ghazali, DZ Hashim. J Food Lipids 6:25–46, 1998.
80. V Seriburi, CC Akoh. J Am Oil Chem Soc 75:711–716, 1998.
81. V Seriburi, CC Akoh. J Am Oil Chem Soc 75:1339–1345, 1998.
82. HMA Mohamed, S Bloomer, K Hammadi. Fat Sci Technol 95:428–431, 1993.
83. HMA Mohamed, K Larsson. Fat Sci Technol 96:56–59, 1994.
84. HMA Mohamed, MH Iskandar, B Sivik, K Larsson. Fat Sci Technol 97:336–340, 1995.
85. S Ghosh, DK Bhattacharyya. J Am Oil Chem Soc 74:589–592, 1998.
86. J Kurashige, N Matsuzaki, H Takahishi. J Am Oil Chem Soc 70:849–852, 1993.

23

Enzyme-Mediated Modification of Milkfat

VICTOR M. BALCÃO

Universidade Fernando Pessoa, Porto, Portugal

F. XAVIER MALCATA

Universidade Católica Portuguesa, Porto, Portugal

1 GENERAL OVERVIEW

Several commercial processes have used existing enzymes that can be produced in large amounts, at low prices, and are designed to provide unique selectivity and activity [1]. It is particularly true that milkfat can be improved by changing or redesigning its physical, chemical, and/or nutritional properties. Although bulk chemical processes are still used to a large extent, lipase-mediated modifications require only mild reaction conditions (e.g., room temperature and absence of organic solvents). Lipase also displays high selectivity with reduced generation of by-products, and mimics natural pathways that feature controlled hydrolysis, transesterification, acidolysis, and alcoholysis reactions.

Public demand for convenience, safety, nutritional balance, and sensory satisfaction is a basic consideration for modification of existing or creation of new food products [2]. Consumers prefer healthier, more palatable fat spreads that can be used for cooking and have good textural properties. Modified butters, butter-based spreads, and defatted milk products are good examples. The related worldwide increase of surplus milkfat [3,4] has promoted global R&D efforts for developing innovative uses that give added value to milkfat.

Fats are not only an essential component of human diet but also provide a high level of palatability, flavor, satiety, and mouthfeel to food [5]. Fats may be categorized into three chemical groups based on the degree of saturation of their constituent fatty acids: saturated, monounsaturated, and polyunsaturated fats. Saturated fats usually originate from

animals (good examples are butterfat, tallow, and lard) but can also be found in vegetable products such as coconut oil (92% saturated), palm kernel oil (84%), and palm oil (50%). They are normally solid at room temperature and play a key role in providing structure to food. Monounsaturated fats, such as high-oleic-acid safflower oil (80% monounsaturated), olive oil (72%), and canola oil (60%), are found primarily in plants. They are generally liquid at room temperature and are used for frying and seasoning of food. Polyunsaturated fats are found mostly in plants such as safflower (77% polyunsaturated), sunflower (70%), soybean (60%), and corn (57%). Fish oils from anchovy, codfish liver, mackerel, herring, and sardine also contain significant amounts of polyunsaturated fatty acids (1–18%) [6]. They are liquid at room temperature and appear to possess nutraceutical activities [6]. In general, the unsaturated fats are more beneficial than the saturated ones; that is, unsaturated fats in food may be associated with decreases in human serum cholesterol levels and in the incidence of coronary heart disease, and tumor development [5]. Examples of the physical properties altered by fatty foods include the structure of chocolate and margarine, the body of mayonnaise and salad dressings, the aeration within icings and cakes, the barrier properties of cereals, and the preservation of olive-oil-submerged feta cheese.

Although milkfat as a food component possesses an unparalleled and universal appeal in culinary science, its high content of saturated fat is being increasingly regarded as undesirable by the consumers. Accordingly, the industry seeks to reformulate milkfat. Reformulated and so-called healthier fats have thus been obtained using several technological approaches: 1) *biotechnological modifications*; for example, microbial strains modified by genetic engineering producing modified fats, and enzymatic techniques using lipases (discussed in detail herein); 2) *fat substitutes*; for example, direct replacement of conventional fats by providing similar functionality [7,8], such as Bindex™ (Sanofi, France), Caprenin™ (Procter & Gamble, U.K.), Finesse™ (Reach Associates, U.S.), Jojoba Oil™ (Nestlé, Switzerland and Lever Bros., U.K.), Lita™ (Opta Foods, U.S.), Litesse™ (Pfizer, U.S.), Lycadex™ (Roquette, France), Maltrin™ (Grain Processing, U.S.), Oatrim™ (Rhône Poulenc, France and Quaker Oats, U.S.), Olestra™ (Procter & Gamble, U.K.), Paselli SA2™ (Avebe, The Netherlands), Salatrim™ (Nabisco, U.S.), Simplesse™ (NutraSweet, U.S.), Slendid™ (Copenhagen Pectin, Denmark), Stellar™ (Staley Man. Corp., U.S.), Tapiocaline™ (Tipiak, France), and Trailblazer™ (Kraft General Foods, U.S.); and 3) *nonconventional plant crops*; for example, purslane, which is rich in ω-3 fatty acids, *Lesquerella fendleri*, whose seeds contain approximately 25% oil (of which 55% are lesquerolic acid and 45% oleic, linoleic, and linolenic acids), and *Limnanthes alba*, which produces meadowfoam oil containing >95% C20:1 and longer-chain monounsaturated fatty acids [5,9].

Enzymes are biological catalysts that selectively lower the activation energies of chemical reactions [10] without affecting their chemical equilibria; therefore, very high specificities and accelerations can be achieved over nonenzymatic rates [11]. Thus, enzymatic modification of fats is promising. The mechanism of more favorable activation energies and the reason why enzymes are highly efficient and selective catalysts lies on their linear polymeric amino acid sequence. The extremely long backbone of an enzyme can bend, twist, and fold back upon itself to allow the few amino acids that constitute the catalytic site to be precisely arranged, giving the three-dimensional architecture that can interact with reactive groups on the substrate with unusually high efficiency [10,12].

Lipases hydrolyze fats and oils, acting preferentially at the oil/water interfaces. Their versatility and suitability for specific catalyses have attracted increased interest throughout the industrial world, and account at present for a market that is valued at more than $US

20 million [13–15]. Although the market for other hydrolytic enzymes is currently worth 10 times more [15], lipase-based technologies are newly developed processes with high potential. Lipases have been called ''solutions in search of problems.'' Thus, these versatile enzymes may be useful for the modification of milkfat also. Improvements in extraction and purification of lipases, and in lipase production by genetic engineering and microbial cloning techniques are expected to enhance their industrial utilization and feasibility. In fact, current and proposed industrial applications of lipases exceed those of proteases or carbohydrases [15]; hence, the technical significance of lipases likely rests on their potential rather than on their actual use.

2 CHARACTERIZATION OF MILKFAT

Lipids consist of a broad group of compounds that are generally soluble in organic solvents but are only sparingly soluble in water, owing to their hydrophobic hydrocarbon chain(s). The distinction between a fat and an oil is made on the basis of whether the respective material is solid or liquid at room temperature. The predominant long-chain fatty acids in natural lipids possess an even number of carbon atoms resulting from the mechanism of biosynthesis [16]. Many long-chain fatty acids, either saturated or unsaturated, can be esterified on the hydroxyl groups of a glycerol molecule to form acylglycerides in fats and oil. Although the glycerol molecule is symmetrical, the central carbon atom acquires chirality when one of the primary hydroxyl groups (on carbons 1 or 3) is esterified or if these two primary hydroxyl groups are esterified with different acids [17]. The esterification position of carbon 1, 2, and 3 of glycerol is designated as *sn*-1, *sn*-2, and *sn*-3, respectively.

Milk is thought to be a near-perfect food because of such balanced constituents as its content for protein, fat, sugar, minerals, and vitamins. In addition to being an important part of the diet, milkfat imparts superior flavor and mouthfeel to dairy products. The various components of milkfat have many desirable and critical functions, such as organoleptic (buttery aroma, creamy mouthfeel, sensory satisfaction, and satiety effects), physical (buttery structure and texture), and nutritional (energy source, essential fatty acids, and fat-soluble vitamins). However, the health value of milkfat has been questioned [18] because it can lead to a hypercholesterolemic condition [19] which is implicated in coronary heart diseases [20]. The hypercholesterolemic effect of milkfat is associated mainly with lauric, myristic and palmitic fatty acid residues [19,21]. On the contrary, recent work [18,22] has provided evidence that stearic and oleic acids are effective in lowering plasma cholesterol levels when either one replaces palmitic acid in the human diet. Apparently, short-chain fatty acids containing less than 12 carbon atoms do not raise the blood cholesterol level [23]. Although polyunsaturated fatty acids decrease the levels of both the high-density lipoprotein (HDL) and low-density lipoprotein (LDL) cholesterol, monounsaturated fatty acids lower the level of LDL cholesterol only [21,23]. Therefore, selective biomodification of the fatty acyl component of the triacylglycerol molecules of milkfat may be suitable to produce a healthier type of milkfat.

Natural milkfat is highly complex [24] and contains a combined mixture of more than 100,000 different triacylglycerols with a wide range of molecular weights [470–890 g/mol] and with 24–54 acyl carbon atoms [25]. The fatty acyl components of milkfat are as follows: (1) they contain approximately 50 mol% long-chain saturated fatty acid and 15 mol% short-chain to medium-chain saturated fatty acid mixtures [24] and (2) their distribution on the glycerol moiety is nonrandom (e.g., butyric acid is acylated almost

exclusively at the *sn*-3 position and caproic acid at the *sn*-1 and *sn*-3 positions). As many as 400 different fatty acyls have been found in milkfat [26], 25% of which are short-chain saturates and 45% are long-chain saturates [27].

In nutritional terms, milkfat contains a high percentage of such hypercholesterolemic factors as saturated fatty acyls in the *sn*-2 position [28]. However, the combination of this myriad of acyls in triacylglycerols is responsible for the specific flavor and physical behavior that occurs in butterfat [29]. Accordingly, medium-chain and long-chain saturated fatty acyls and their stereochemical distribution among the glycerol backbones [29] can produce a variety of relatively high melting points [30]. At room temperature, milkfat is a mixture of oil (containing triacylglycerols with 26–40 acyl carbon atoms) and semihard to hard fat (containing triacylglycerols with 42–54 acyl carbon atoms) [31], with a melting range spanning from −30°C to 37°C [24]. The C16 and longer-chain saturated fatty acyls are not absorbed as well as their unsaturated counterparts, particularly when they are esterified to the *sn*-1,3 positions. The saturated fatty acyls in the *sn*-1,3 positions are hydrolyzed as free fatty acids by pancreatic lipase in the digestive tract and tend to form insoluble calcium soaps that are poorly absorbed [32]. These fatty acyls, when esterified in the *sn*-2 position, are efficiently absorbed as their *sn*-2 monoglyceride, partly because soap formation is prevented. Indeed, this may be a major reason why human milkfat, which contains a large proportion of the major saturated fatty acyls in the *sn*-2 position, is so well absorbed by infants when compared with plant fats. In plant fats, which possess similar fatty acid composition as human milkfats, such saturated fatty acids are acylated mainly in the *sn*-1,3 positions [32].

3 CHARACTERIZATION OF LIPASE

Lipase, specifically a glycerol ester hydrolase (E.C. 3.1.1.3), is an ester-hydrolyzing enzyme for water-insoluble substrates. It also catalyzes ester synthesis, provided that microaqueous conditions are maintained. These two reversible reactions can then be combined in a sequential fashion to generate interesterifications, which are described comprehensively elsewhere [33]. Because lipase-catalyzed reactions resemble natural metabolism, applied processes associated with lipases may be more environment-friendly than those associated with bulk chemical syntheses, which employ extreme processing conditions or produce hazardous by-products. In addition, the low reactional activation energy brought about by the enzyme makes feasible mild reaction conditions of temperature and pH. The mild conditions, in turn, lead to low energy consumption and low thermal damage of reactants and products.

Lipases catalyze reactions with a broad spectrum of esterified compounds, and protein engineering on lipases may further improve their selectivity [34]. Industrial efforts to modify the protein have attempted to increase the hydrolytic efficiency, peracid generation (perhydrolysis), and protease stability, all of which are especially useful to the detergent industry. However, protein engineering for improving specificity is still a minor area because industrial applications of fine chemistry still lag commercially well behind industrial manufacture of detergents. Because of a growing worldwide scientific interest, it is expected that better industrial processes based on the use of engineered lipases will soon become a reality.

4 ENGINEERING OF MILKFAT

A dairy flock can be conceptually seen as a processing unit that takes food as feedstock and produces milkfat as the finished product. Thus, three types of milkfat modifications

are possible: those taking place upstream by changing the feeding regime, those taking place in the process itself by genetic engineering of the cow's mammary gland, and those taking place downstream by physical fractionation and biochemical modification. Feeding efforts aimed at obtaining a desirable milkfat may affect the protein content and other qualities of milk [35]. On the other hand, genetic engineering of a dairy cow is a long-term project with unpredictable outcome [36]. Therefore, the most likely modification of milkfat may occur downstream in the processing stage. For example, an area of current interest concerns the use of butterfat in place of cocoa butter in confections [37]. In the United States and several other countries, milkfat is a legally recognized ingredient of milk and dark chocolates and is less expensive than cocoa butter. However, milkfat cannot be added to chocolate confections in excessive amounts because it produces undesirable softening, as well as changes in tempering, gloss, and contraction properties [37].

Milkfat with physically modified properties can be brought about by biochemical fractionation techniques. Also, milkfat can be modified by hydrogenation, interesterification, hydrolysis, and alcoholysis. Biochemical modification of the fatty acid profile of milkfat can be effectively and efficiently catalyzed by lipases. With excess amounts of water, these enzymes catalyze the hydrolysis of ester bonds releasing free fatty acids. In a microaqueous environment, ester hydrolysis and synthesis can occur sequentially as an interesterification process. An extensive literature search [2,3,30,31,33,37–82] indicates that interesterifications account for approximately 36%, hydrolysis 34%, transesterification 12%, acidolysis 10% and alcoholysis 8% of all references concerning lipase-catalyzed modification of milkfat.

4.1 Modification by Hydrolysis

Because triacylglycerols of milkfat have an unusually large proportion of short-chain fatty acyls, lipase-catalyzed release of these moieties as free fatty acids can impart sensations of richness, creaminess, buttery flavor, and cheeselike aromas. The enzymatic hydrolysis of milkfat in a controlled fashion is a traditional part of the dairy industry, producing butterlike flavor or cheeselike flavor and additive products [13,83]. Thus, lipolyzed milkfat is used extensively in oils, fats, cereals, snacks, and baked goods. Another interesting example is either the oil used to pop corn or to season popped corn. Lipolyzed milkfat can give a satisfying variety of food flavors. At low concentrations, a mouthfeel sensation of richness is imparted without the free fatty acidic flavor. At high concentrations, the modified milkfat resembles either cream, butter, or cheese.

In general, the manufacturing steps of lipolyzed milkfat include: (1) production of condensed milk or butteroil as substrate, (2) preparation of the lipase system in water, (3) combination of the milkfat substrate and lipase system, (4) homogenization of the mixture, producing a stable emulsion and increasing the surface area, thereby promoting the lipolyzing reaction, (5) incubation at a controlled temperature until a specified degree of hydrolysis has been achieved, (6) pasteurization to inactivate completely any residual lipase, and (7) a finishing process of spray-drying, or alternative formulation and packaging. The formulated products incorporating lipase-modified milkfat include the following [64,84]: (1) chocolate products (e.g., milk chocolate, compound coatings, chocolate flavor syrups, and chocolate beverages; (2) butter flavors (e.g., margarine, butter creams, and butter sauces); (3) milk and cream flavors (e.g., coffee whiteners, imitation sour cream, and imitation milks; and (4) cheese flavor additives (e.g., Italian cheese flavor). Lipolyzed milkfat emulsions are effective carriers for such flavor adjuncts as synthetic fatty acids, diacetyl, butter esters, and lactones. Addition to bakery/cereal products (cake and cookie

mixes, chemically leavened bakery formulations, sweet doughs, cheesecake mixes, pancake mixes, and cereal flakes), to candy/confectionery products (milk chocolate, creams, toffee, and caramel fudges), to dairy products (cheese dips and coffee whiteners), and to other miscellaneous products (margarines, popcorn, popcorn oils, salad dressings, sauces, snack foods, soups, cooking oil, and cooking fats) gives each a distinctive buttery flavor [85]. Lipase-modified milk cream and cultured cream can be incorporated also as dairy flavor enhancers in coffee whiteners, candies, cheese cakes, dips, sauces, sweet doughs, soups, and baked goods [85]. Lipolyzed milkfat emulsions, with fat content ranging between 25% and 95% [64] (average of 50% [84]), are the usual form of lipase-treated milkfat. Whole lipolyzed milk powder, analogous in gross composition to unmodified whole milk powder, and spray-dried lipolyzed emulsions containing either a carrier of plain whey solids or added milk solids, have been advertised also [84]. In addition, butterfats modified by lipases originating from different sources [83] have been reported to improve the flavor of bread after storage for 24 hr if 35–40% of the shortening is replaced by enzyme-modified butterfat [83].

The potential of milkfat toward the production of flavors has prompted numerous applications of lipase-catalyzed hydrolyses in food industry and, consequently, led to several patents pertaining to enzyme-modified milkfat products and additives in baked products and other foods [13]. In order to modify milkfat emulsions for a posteriori incorporation in baked goods, a number of lipases originating from milk (lipoprotein lipase), pancreas (pancreatic lipase), molds (*Aspergillus niger*, *Geotrichum candidum*, and *Penicillium roquefortii*), bacteria (*Achromobacter lipolyticum* and *Pseudomonas fluorescens*), and gastrointestinal tract (kid and lamb pregastric esterases) have been studied [13]. Milkfat products modified by the action of these enzymes possess better and more distinctive flavors than those derived from other sources of lipase. However, modified milkfats using *A. lipolyticum*, *P. roquefortii*, and *G. candidum* lipases produce soapy and sometimes musty flavors when incorporated in bread recipes [13]. Kid and lamb esterases impart a rancid flavor [13]. On the contrary, a lipase preparation from *P. roquefortii* used to lipolyze milkfat has produced a cheeselike product when previously emulsified with fermented, condensed skim milk [86]. Also, a lipase from *Rhizopus delemar* has been used industrially [86] for enhancing flavors of such dairy products as milk, butter, and cheese.

To modify milkfat in milk, Otting [87,88] has used steapsin, and Kempf et al. [89] have used a milk lipase for lipolyzed milk products that had part of the volatile fatty acids removed by steam distillation. Farnham et al. [90,91] have described the use of pregastric esterase in the preparation of modified whole milk powder. The pregastric esterase reacts specifically to release short-chain fatty acids from milkfat [65] for flavor development and possible interesterification purposes. Garcia et al. [45] have used lipase from *A. niger* to modify butteroil emulsions, and from *Candida cylindracea* to react with plain butterfat [51]. The lipase from *C. cylindracea* was immobilized onto a spiral-wound membrane reactor. Malcata [12] and Malcata and Hill [2,68] have assessed the technical feasibility of an immobilized lipase from *A. niger* for controlling the hydrolysis of melted butterfat in a hollow-fiber reactor. Claus [92] has employed a lipase extracted from papaya to manufacture low-moisture modified fats. Furthermore, Pangier [93] has described a sequential treatment using lactic cultures and lipolytic enzymes to produce modified milkfat products.

Milkfat subjected to lipolyses by various microbial lipases [13,49,64,82,84] yields different types and amounts of fatty acid; for example, *A. lipolyticum* produces a lipase that releases linoleic acid selectively, and *G. candidum* yields a lipase that releases more

linoleic and oleic acid than those by *A. lipolyticum* lipase. Furthermore, an *A. niger* lipase facilitates the release of stearic acid preferentially. Because the free fatty acid profiles vary significantly depending on the source of the lipase, tailor-made variation of flavors can be imparted to the final product.

When milkfat in cream is pretreated with lipases, subjected to thermal processing, and then inoculated with *Lactobacillus bulgaricus*, a further acidity of chiefly lactic acid develops, so alternative products, generally termed lipolyzed cultured-cream products, can be produced. These products generate an enhanced dairy flavor in candies, cheesecakes, sauces, dips, salad dressings, sweet doughs, soups, and baked goods.

It is noted that a lipolysis treatment may preceed other important flavor pathways. For instance, a lipase from *P. roquefortii* acts on milkfat to release free fatty acids which can serve as precursors for such additional flavor compounds as methyl ketones and secondary alcohols [85]. Both of the latter compounds are directly implicated in the typical flavor of blue cheese [94,95].

4.2 Modification by Intramolecular Interesterification

Interesterification, also known as ester interchange or randomization, involves the exchange and redistribution of acyl groups among its triacylglycerol substrates. Interesterification has been developed initially using a high-temperature chemical process [4] and a chemical catalyst (sodium methoxide). Recently, lipases have been used to produce modified milkfat that exhibits different triacylglycerol stereochemical composition [4,31,41, 44,82] but have the same total fatty acid residue composition as the starting material. Such enzymatic interesterifications are employed for the manufacture of margarines, shortenings, and confectionery fats. Unlike the lipase-mediated process, chemical catalysts promote random interesterification that requires bleaching and deodorization of the final product. The latter treatments could be harmful to nutrition and destroy the fine buttery flavor which is savored by consumers [44].

After random interesterification of milkfat by *Chromobacterium viscosum* lipase [64], the resulting product exhibits better spreadability [64] but has a waxlike mouthfeel. Lipase-catalyzed randomization of acyl groups in milkfat has also been suggested [37] to yield a cocoa butter substitute for the manufacture of chocolate. When compared to a human diet rich in native milkfat, randomized milkfat appears to have a beneficial nutritional effect on health by reducing the plasma cholesterol level by 12% [96]. However, other studies [64] did not confirm such cholesterol reductions following long-term ingestion of the modified milkfat. Similar studies on butterfat include modifications by: (1) an *sn*-1,3–specific lipase from *Mucor javanicus* that is immobilized by adsorption onto hydrophobic hollow fibers [31], (2) a nonspecific lipase from *C. cylindracea* that is immobilized on Celite using hexane as solvent [39,70,71], (3) a nonspecific lipase from *P. fluorescens* that is immobilized on Celite in the presence or absence of solvents [40,41,44], (4) a lipase from *Pseudomonas fragi* that is suspended in phosphate buffer and acts on a microemulsion system [66], and (5) lipases from *Rhizopus niveus, R. delemar, M. javanicus*, and *Mucor miehei* that are immobilized in other ways [67].

4.3 Modification by Acidolysis

Acidolysis encompasses the exchange reaction between an acyl moiety of acylglycerol and a free carboxylic acid [33]. Because the physical properties of milkfat depend directly on the type of fatty acyl esterified to glycerol, modifying milkfat by introducing specific

fatty acids in the acyl positions may lead to especially designed fats and, thus, may increase the added value of the modified milkfat [97].

Studies on acidolysis indicate that acyl to fatty acid interchanges occur with interesterification of milkfat in the absence of any solvent and in the presence of either a nonspecific lipase from *P. fluorescens* immobilized on Celite [44,56,98] or an *sn*-1,3–specific lipase from *M. miehei* immobilized on a macroporous anion-exchange resin [74]. Acidolysis of milkfat has also been reported to occur with oleic acid in the presence of a lipase from *Rhizopus oryzae* immobilized on controlled pore glass particles [30] and a lipase from *M. javanicus* immobilized by physical adsorption onto a bundle of hydrophobic hollow fibers [73,80,81]. Acidolysis with caprylic acid has been demonstrated with a specific lipase from *Pseudomonas cepacia* immobilized onto microporous polypropylene powder [63], and it occurs with free undecanoic acid also [3]. During the initial stages of acidolysis, a portion of the triacylglycerols appears to be hydrolyzed, thus consuming water and releasing free fatty acids [99]. After this transient hydrolysis, it is suggested that interesterification then proceeds smoothly because the available pool of water molecules is lowered and maintained in proper balance.

A major portion of energy used by infants fed on human milk or infant formulas is contributed by lipids [100]. Thus, the modified fats and oils in infant formulas should have not only the correct fatty acid composition but also the same positional acyl distribution as in human milkfat. Recently, a modified milkfat having these properties has been described [32,60]. The milkfat is interesterified with concentrates of unsaturated fatty acids by using an immobilized *sn*-1,3–specific lipase from *Rhizomucor miehei*. Thus, a milkfat analog can be made for inclusion in infant formulas. The total content of short-chain and medium-chain fatty acids is reduced from 23.1% to 9.5%, whereas the linoleic acid content increases from 2.0% to 11.3% and long-chain polyunsaturated fatty acids can be introduced easily. Diets enriched in monounsaturated fatty acids are advantageous in infant feeding because they are absorbed better than those with saturated fatty acids of the same chain length. The milkfat enriched with monounsaturated fatty acids causes less interference with calcium absorption and is less prone to peroxidation than polyunsaturated fatty acids [60]. Although acidolysis may be used to lower the saturated fatty acid content, the milkfat modified by different reaction conditions and by lipases with different selectivities can have glycerides with altered short-chain fatty acid profiles, so that the desirable milky flavor is no longer present. Hence, special care must be exercised in efforts aimed at increasing the oleic acid content of butterfat [30,72,73,80,81].

4.4 Modification by Alcoholysis

Alcoholysis is defined as a displacement of an acyl moiety between an acylglycerol and an alcohol [33]. Even though alcoholysis of milkfat is not often described, some examples do exist. For example, lipase-mediated alcoholysis reactions can modify milkfat in the presence of primary alcohols (ethanol, 1-butanol, 1-octanol, 1-undecanol, 1-dodecanol, and pentadecanol), secondary alcohols (*sec*-butanol and 2-octanol) and tertiary alcohols (*t*-butanol and linalool) [3]. Butanol, methanol, ethanol, 1-propanol, 2-propanol, 1-dodecanol, cyclohexyl methanol, butane-1,4-diol, and 2-fluoroethanol are other alcohols that may be used to alter milkfat via catalysis by a lipase from *M. miehei* immobilized on an ion-exchange resin [77].

Monoacylglycerols (MAG) and diacylglycerols (DAG) are widely used as emulsifiers of food [79], accounting for approximately 75% of the world production of emulsifiers

[101]. MAG and DAG are used also in the pharmaceutical and cosmetic industries [102]. They are produced by a controlled hydrolysis of triacylglycerols or controlled esterification of glycerol and fatty acids. Alternatively, they are produced by glycerolysis, the acyl exchanges then taking place between free (excess) glycerol and triacylglycerols [79]. Yang et al. [79] have screened several commercial lipase preparations (viz. porcine pancreatic lipase, *C. cylindracea*, *Rhizopus arrhizus*, *Pseudomonas* spp., *Rhizopus javanicus*, *R. delemar*, *G. candidum*, and *M. javanicus*) for alcoholysis of milkfat in the presence and absence of solvent.

4.5 Modification by Transesterification

In transesterification, two acyl moieties are exchanged between two acylglycerols [33]. This sort of reaction occurs when two fats or oils are blended and then mixed with lipase. Therefore, changes in milkfat structure by an interchange of fatty acyl moieties can lead to a modified fat or oil in a controlled manner, as demonstrated by a transesterification of the solid milkfat fraction with rapeseed oil using *C. cylindracea* lipase [78]. A major disadvantage of transesterification is a downstream requirement to separate the reaction mixture of modified milkfat and the oil by-product, which are similar compounds, unlike what happens with modified milkfat and free fatty acids or alcohols; such a disadvantage limits a widespread use of this technique.

5 FROM BENCH SCALE TO INDUSTRIAL SCALE

Enzymes are convenient for industrial processing. They are specific, controlling the variety of products generated and increasing yield by reducing other by-products. Also, enzymes react under mild processing conditions, which ensure low cost of energy, capital equipment, by-product removal and effluent treatment costs [103]. Thus, lipases have numerous industrial uses for the production of esters and specialty fats, acceleration of cheese ripening, fermentation of vegetables, curing of meat products, processing of fish, refinement of rice flavor, modification of soybean milk, pretreatment of smoked carps, improvement of flavor of alcoholic beverages, improvement of whipping quality of egg whites, formulation of cosmetic products, manufacture of pharmaceutical products, treatment of leathers, preparation of aliphatic acids (from dark and highly acid oils and fats), chewing gum and toothpaste products, sewage treatment, flavor enhancement, and improvement of detergents [86,103]. However, industrial lipases as alternatives to bulk chemicals or other physical processes have been initiated recently for the modification of edible oils and fats, including milkfat. Lipases should, in particular, be stable against proteolytic action, thermal processing, and oxidative damage and detergent effects [104]. Although improvement of lipases has been possible by genetic engineering, site-directed mutagenesis, and random mutagenesis [34,104–106], more extensive R&D efforts are required to give economical and beneficial products.

A strong impetus exists for use of lipases in food and pharmaceutical areas; hence, studies are still needed for understanding the stereopreference of lipases when acting on milkfat triacylglycerols or other compounds of pharmaceutical interest. The investigations may lead eventually to specific changes at the active site and the binding site of lipases [104] using protein engineering techniques. In addition, the economical and efficient use of lipases requires development of suitable means to make enzymes easily accessible to the reactant system, and to keep them in a mechanically distinct phase for recovery and

subsequent reuse [107]. These goals can be reached by either 1) attaching the enzyme to a support that is immiscible with the reaction or medium, 2) changing the surrounding medium to permit precipitation of the enzyme, or 3) confining the enzyme to a localized space using a solid, permeable barrier [33,107]. Hence, engineering of both the medium and the protein is expected to play an important role in the future.

6 TOWARD ENVIRONMENT-FRIENDLY PROCESSES

The environment would be much cleaner if industries used ''green technologies.'' Within limits, the enzyme industry can contribute to the transnational goal of a cleaner environment. First, enzyme technology can offer entrepreneurs and consumers the opportunity to replace bulky, traditional chemicals with mild, nontoxic processes. Second, enzyme-mediated processes have a marginal impact on the environment [108] and resemble the natural course of metabolism. Consequently, the reaction mechanisms and processes associated with enzymes may be viewed as more environment-friendly than those associated with bulk chemical syntheses. Third, enzymes are, unlike inorganic catalysts, highly specific (e.g., stereospecific- and substrate-specific). Thus, they allow the easy manufacture of high-value-added products without producing harmful by-products. Fourth, their catalytic efficiency resulting from a low activation energy and a concomitant requirement of mild reaction temperatures reduce the requirements of nonrenewable energy. With less thermal damage, the food products become safer and less degraded.

There are several ways to use lipases for reducing a high biochemical oxygen demand caused by the waste discharged into water ponds and streams. One way seeks to reduce the fat load of effluent streams from dairy industries by producing emulsions and, subsequently, generating free fatty acids via lipase-catalyzed hydrolyses [2,12,68]. Alternatively, lipases can be combined with a microbial cocktail (available commercially as Combizyme™, produced by Biocatalysts, U.K.) for the treatment of fat-rich effluents from ice cream plants [15].

7 MARKETING CONCERNS

For the past two decades, world consumption of butter has steadily declined [4] because of several factors that range from nutritional profile to functional limitations [29]. For example, important barriers to increased butter sales can be ranked according to price, health image (e.g., negative consumer attitudes toward cholesterol and saturated fats), poor spreadability, nonexistent product innovation, and legislation and regulatory restrictions (e.g., legislated restrictions on fat content in commercial milk) [4]. The increasing demand for milk with higher protein and lower fat contents is another reason for the increasing surplus of milkfat. The problem is that although the improved feeding of cows gives milk with higher protein contents, it also leads to higher fat contents. Also, the extensive defatting of milk generates even larger surpluses of milkfat. The manufacture of butter continues to serve as a safety valve for the dairy industry owing to its capacity for using milkfat. To counteract growing stocks of surplus milkfat, however, a common practice in more developed countries has been to dump products below cost [109].

The decline of milkfat consumption in Europe and North America, where major consumers of butter live, is the result of three factors [24]: 1) the cost of butter in virtually all countries is higher than that of margarine; 2) the spreadability of refrigerated butter is poor compared with that of margarine; and 3) the bad publicity of butter consumption

relates to its high cholesterol and saturated fat contents, which have been linked to a high incidence of coronary heart diseases. Although the flavor and mouthfeel of milkfat are superior to those of any other fat, such a rheological property as its poor spreadability at refrigerated temperatures is not attractive for most consumers. Furthermore, the margarine and spread industry can easily tailor a low-priced, modified product that has good spreadability, despite the many advances in the ability to alter the texture and rheological properties of butter via lipase-mediated pathways. However, consumer demand for better spreadability is limited by their lack of willingness to pay more for such convenience. Hence, it is doubtful that a single market will be found for milkfat that will replace the decreasing sale of butter; thus, a large number of relatively small market products is anticipated.

8 FUTURE PROSPECTS

Neither compulsive cuts in milk production nor aggressive advertisements of dairy products are likely to reduce the ever-growing surplus of milkfat worldwide. Any workable solution should rely on a compromise between innovative biotechnology to create new tailor-made products and publicication to sell the modified products. Although modified glycerides are based on chemical catalyses in the fat and oil industry, alteration with lipases is technically feasible and environmentally preferable. In fact, economic benefits for the production of enzyme-modified milkfat are justified by its improved and more natural qualities, decreased pollution, and selective catalyses. Interesterification mediated by lipases is a powerful tool for the modification and optimization of both structure and properties of milkfat. With the advent of fatty-acid-specific lipases (e.g., *G. candidum* lipase which hydrolyzes such fatty acyls with *cis*-Δ9 double bonds as oleic, linoleic, and linolenic acids [110–114]), exciting new developments are possible. For example, the incorporation of unsaturated fatty acids may produce a more spreadable milkfat [115,116]. The incorporation of ω-3 fatty acids into milkfat, which has been a long-time goal of the dairy industry, may be feasible also through enzymatic interesterification with fish oils.

With the introduction of less expensive and more stable lipases coupled with more cost-efficient bioreactors, the near future will likely see many fat and oil manufacturers switch from bulk, aggressive chemical treatments to lipase-mediated (green) biotechnological processes. Although the catalytic efficiency of lipases is superior to that of chemical catalysts and the manufacturing costs of lipases are dropping, enzymatic processes will always be more expensive than conventional chemical ones; thus, extensive industrial applications will be feasible provided that the new lipase-mediated products have a high added value arising from an improved or unique nutritional/functional property. According to this rationale, lipase-mediated randomization of milkfat does not seem economically feasible at present. However, transesterification of milkfat with polyunsaturated fatty acid concentrates catalyzed by *sn*-1,3-specific lipases does have a potential. Lipase-catalyzed interesterification is currently being used by food technologists and nutritionists to study the relationship between structure and function of triacylglycerols, which may lead eventually to development of new products. Nevertheless, several problems remain before governmental and consumer acceptance. Even though these problems may be solved in the coming years, their impact on the dairy industry will be surely felt. With knowledge concerning the relationship between lipase structure and its catalytic and stability properties, new edible fats, which meet the nutritional and governmental demands of the growing world markets, will be designed and manufactured from surplus milkfat that is modified with specific lipases in a more and more regular fashion.

9 CONCLUDING REMARKS

The decrease in consumption of high-fat milk and dairy products, caused by their association with coronary heart diseases, has raised a major concern to the dairy industry. As surpluses of milkfat have steadily increased in the past decade, alternative uses for milkfat are being sought. Research has been undertaken using lipases as vectors for modification of milkfat because these and similar enzymes are readily available on a commercial scale for industrial applications. This chapter has discussed the state of the art pertaining to lipase-mediated modification of milkfat. Industrial applications of lipases that yield structured and modified milkfat products with improved physical behavior, digestibility, reduced caloric value, and flavor enrichment were thus mentioned. Industrial feasibility, marketability, environmental concerns, and future prospects were accordingly discussed.

REFERENCES

1. A Tanaka, T Tosa, T Kobayashi. Industrial Application of Immobilized Biocatalysts. New York: Marcel Dekker, 1993.
2. FX Malcata, CG Hill. In: R Paterson, ed. Third International Conference on Effective Membrane Processes—New Perspectives. London: Mechanical Engineering Publications, 1993, pp 107–122.
3. SJ Kuo, KL Parkin. J Am Oil Chem Soc 70:393–399, 1993.
4. D Hettinga. In: YH Hui, ed. Bailey's Industrial Oil and Fat Products, Vol. 3: Edible Oil and Fat Products: Products and Application Technology. New York: Wiley, 1996.
5. J McGrady. Food Technol 148, November 1994.
6. BF Haumann. INFORM 8:428–447, 1997.
7. DE Blenford. Int Food Ingredients 41–53, May 1993.
8. JD Dziezak. Food Technol 66–74, July 1989.
9. Anon. INFORM 2:685–699, 1991.
10. IH Segel. Enzyme Kinetics—Behavior and Analysis of Rapid Equilibrium and Steady-state Enzyme Systems. New York: Wiley, 1993.
11. TE Creighton. Protein—Structures and Molecular Properties. New York: Freeman, 1984.
12. FX Malcata. Hydrolysis of Butterfat by Immobilized Lipase Using Three-phase Membrane Reactors. PhD dissertation, University of Wisconsin, Madison, 1991.
13. A Kilara. Process Biochem 20:33–45, 1985.
14. MV Arbige, WH Pitcher. Trends Biotechnol 7:330–335, 1989.
15. EN Vulfson. In: P. Woolley, SB Petersen, eds. Lipases, Their Structure, Biochemistry and Applications. Cambridge: Cambridge University Press, 1994, pp 271–288.
16. AL Lehninger. Principles of Biochemistry. New York: Worth Publishers, 1993.
17. WW Nawar. In: OR Fennema, ed. Food Chemistry. New York: Marcel Dekker, 1985, p 139.
18. CL Davide. Philippine Agriculturist 77:1–16, 1994.
19. DM Ney. J Dairy Sci 74:4002–4012, 1991.
20. E Renner, M Gurr. Dairy Ind Int 56:34–35, 1991.
21. B Vessby. INFORM 5:182–185, 1994.
22. JA O'Donnell. J Dairy Sci 76:1797–1801, 1993.
23. H Glaeser, M Keane. Dairy Ind Int 57:39–42, 1992.
24. A Boudreau, J Arul. J Dairy Sci 76:1772–1781, 1993.
25. K Larsson. Lipids—Molecular Organization, Physical Functions and Technical Applications. Dundee: Oily Press, 1994, pp 1–41.
26. RG Jensen, AM Ferris, CJ Lammi-Keefe. J Dairy Sci 74:3228–3243, 1991.
27. E Deffense. J Am Oil Chem Soc 70:1193–1201, 1993.
28. S Kermasha, S Kubow, M Safari, A Reid. J Am Oil Chem Soc 70:169–173, 1993.

29. D Rousseau, K Forestière, AR Hill, AG Marangoni. J Am Oil Chem Soc 73:963–972, 1996.
30. T Oba, B Witholt. J Dairy Sci 77:1790–1797, 1994.
31. VM Balcão, A Kemppinen, FX Malcata, PJ Kalo. J Am Oil Chem Soc 75:1347–1358, 1998.
32. P O'Carroll. World of Ingredients 32–36, May–June 1995.
33. VM Balcão, AL Paiva, FX Malcata. Enzyme Microb Technol 18:392–416, 1996.
34. A Svendsen. INFORM 5:619–623, 1994.
35. J McNair. Dairy Foods 91:76, 1990.
36. J O'Donnell. Dairy Foods 91:93, 1990.
37. CE Bystrom, RW Hartel. Lebensm Wiss Technol 27:142–150, 1994.
38. P Kalo, M Perttila, A Kemppinen, M Antila. Meijeritieteellinen Aikakauskirja 46:36–47, 1988.
39. A Kemppinen, P Kalo, M Antila. Meijeritieteellinen Aikakauskirja 46:48–55, 1988.
40. P Kalo, H Huotari, M Antila. Fat Sci Technol 91:276–280, 1989.
41. P Kalo, H Huotari, M Antila. Meijeritieteellinen Aikakauskirja 47:29–38, 1989.
42. P Kalo, H Huotari, A Kemppinen, M Antila. Characterization of interesterified butterfat by gas chromatography. Proceedings of Congrès International ''Chevreul'' pour l'Étude des Corps Gras—Premier Congrès Eurolipid, Angers, France, 1989, pp 256–263.
43. P Kalo, V Harjunpaa, A Kemppinen. Lipase-catalysed interesterification of butterfat under microaqueous reaction conditions. Proceedings of the XXIII International Dairy Congress ''Dairying in a Changing World,'' Montreal, Canada, 1990, p 351.
44. P Kalo, H Huotari, M Antila, Milchwiss 45:281–285, 1990.
45. HS Garcia, CH Amundson, CG Hill. J Food Sci 56:1233–1237, 1991.
46. FX Malcata, CG Hill, CH Amundson. Biotechnol Bioeng 38:853–868, 1991.
47. GP McNeill, S Shimizu, T Yamane. J Am Oil Chem Soc 68:1–5, 1991.
48. JP Chen, SD McGill. Food Biotechnol 6:1–18, 1992.
49. JP Chen, B Yang. J Food Sci 57:781–782, 1992.
50. K Dewettinck, W De Greyt, A Huyghebaert. Med Fac Landbouww Univ Gent 57(4b):1905–1907, 1992.
51. HS Garcia, FX Malcata, CG Hill, CH Amundson. Enzyme Microb Technol 14:535–545, 1992.
52. FX Malcata, HS Garcia, CG Hill, CH Amundson. Biotechnol Bioeng 39:647–657, 1992.
53. FX Malcata, CG Hill, CH Amundson. Biotechnol Bioeng 39:984–1001, 1992.
54. FX Malcata, CG Hill, CH Amundson. Biotechnol Bioeng 39:1002–1012, 1992.
55. FX Malcata, CG Hill, CH Amundson. Biotechnol Bioeng 39:1097–1111, 1992.
56. P Kalo, A Kemppinen. J Am Oil Chem Soc 70:1209–1217, 1993.
57. P Kalo, J Rinne, H Huotari, M Antila. Fat Sci Technol 95:58–62, 1993.
58. A Kemppinen, P Kalo. J Am Oil Chem Soc 70:1203–1207, 1993.
59. FX Malcata, CG Hill, CH Amundson. Biocatalysis 7:177–219, 1993.
60. TC Christensen, G Hølmer. Milchwiss 48:543–548, 1993.
61. TA Foglia, K Petruso, SH Feairheller. J Am Oil Chem Soc 70:281–285, 1993.
62. FX Malcata, CG Hill, CH Amundson. Biocatalysis 8:201–228, 1993.
63. HR Reyes, CG Hill. Biotechnol Bioeng 43:171–182, 1994.
64. W de Greyt, A Huyghebaert. Lipid Technol 10–12, January 1995.
65. HS Garcia, A Qureshi, L Lessard, S Ghannouchi, CG Hill. Lebensm Wiss Technol 28:253–258, 1995.
66. F Pabai, S Kermasha, A Morin. Appl Microbiol Biotechnol 43:42–51, 1995.
67. M Safari, S Kermasha. J Am Oil Chem Soc 71:969–973, 1994.
68. FX Malcata, CG Hill. Ann NY Acad Sci 750:401–407, 1995.
69. P Kalo, A Kemppinen, M Antila. Meijeritieteellinen Aikakauskirja 45:19–32, 1987.
70. P Kalo, P Parviainen, K Vaara, S Ali-Yrrkö, M Antila. Milchwiss 41:82–85, 1986.
71. P Kalo, P Elo, M Antila. Milchwiss 43:416–422, 1988.
72. VM Balcão, FX Malcata. J Mol Catal B Enz 3:161–169, 1997.
73. VM Balcão, A Kemppinen, FX Malcata, P Kalo. Enzyme Microb Technol 23:118–128, 1998.
74. S Bornaz, J Fanni, M Parmentier. J Am Oil Chem Soc 71:1373–1380, 1994.

75. MT Patel, R Nagarajan, A Kilara. J Food Sci 61:33–38, 73, 1996.
76. JP Chen, H Pai. J Food Sci 56:234–238, 1991.
77. DE Stevenson, RA Stanley, RH Furneaux. Enzyme Microb Technol 16:478–484, 1994.
78. P Kalo, K Vaara, M Antila. Fett Seifen Anstrichm 88:362–365, 1986.
79. B Yang, WJ Harper, KL Parkin, J Chen. Int Dairy J 4:1–13, 1994.
80. VM Balcão, FX Malcata. Enzyme Microb Technol 22:511–519, 1998.
81. VM Balcão, FX Malcata. Biotechnol Bioeng 60:114–123, 1998.
82. VM Balcão, FX Malcata. Biotechnol Adv 16:309–341, 1998.
83. RG Arnold, KM Shahani, BK Dwivedi, J Dairy Sci 58:1127, 1975.
84. JH Nelson. J Am Oil Chem Soc 49:559–562, 1972.
85. JD Dziezak. Food Technol 114–120, April 1986.
86. EW Seitz. J Am Oil Chem Soc 51:12–16, 1974.
87. HE Otting (to M. & R. Dietetic Labs). US Patent 1,966,460, July 17, 1934.
88. HE Otting (to M. & R. Dietetic Labs). US Patent 2,169,278, August 15, 1939.
89. CA Kempf, JS Niedlinger, AP Stewart, PF Sharp (to Golden State). US Patent 2,638,418, May 12, 1953.
90. MG Farnham (to Dairyland Food Labs). US Patent 2,794,743, June 4, 1957.
91. MG Farnham, JH Nelson, AR Kemp. In: CD Pratt, ed. Twenty Years of Confectionering and Chocolate Progress. Westport CT: AVI Publishing, 1970, Chap 15.
92. WS Claus (to Carnation). US Patent 3,190,753, June 22, 1965.
93. DJ Pangier (to Miles Labs). US Patent 3,469,993, September 30, 1969.
94. R Jolly, FV Kosikowski. J Dairy Sci 56:624, 1973.
95. D Stead. J Dairy Res 53:481–505, 1986.
96. A Christoph, G Verdonk, J Decatelle, A Huyghebaert. Arch Int Physiol Biochim 90:B100–B101, 1982.
97. LH Posorske, GK LeFebvre, CA Miller, TT Hansen. J Am Oil Chem Soc 65:922–926, 1988.
98. P Kalo. Kemia-Kemi 16:899–902, 1989.
99. HL Goderis, G Ampe, MP Feyten, BL Fouwé, WM Guffens, SM van Cauwenbergh, PP Tobback. Biotechnol Bioeng 30:258–266, 1987.
100. AR Kavanagh. World of Ingredients 41–42, March–April 1996.
101. NJ Krog. In: K Larsson, SE Friberg, eds. Food Emulsions. New York: Marcel Dekker, 1990, p 127.
102. UT Bornscheuer. Enzyme Microb Technol 17:578–586, 1995.
103. LH Posorske. J Am Oil Chem Soc 61:1758–1760, 1984.
104. MR Egmond, J de Vlieg, HM Verhey, GH de Haas. In: FX Malcata, ed. Engineering of/with Lipases. Dordrecht: Kluwer Academic, 1996, pp 193–202.
105. L Alberghina, M Lotti. In: FX Malcata, ed. Engineering of/with Lipases. Dordrecht: Kluwer Academic, 1996, pp 219–228.
106. JS Okkels, A Svendsen, SA Patkar, K Borch. In: FX Malcata, ed. Engineering of/with Lipases. Dordrecht: Kluwer Academic, 1996, pp 203–217.
107. FX Malcata. Trends Chem Eng 1:359–379, 1993.
108. EA Falch. Biotechnol Adv 9:643–658, 1991.
109. Anon. US Department of Commerce, Bureau of the Census, (1993) Statistical Abstracts of the United States. Washington, DC: US Government Printing Office, 1993.
110. RG Jensen. Lipids 9:149–157, 1974.
111. RG Jensen, M Hamosh. In: FX Malcata, ed. Engineering of/with Lipases. Dordrecht: Kluwer Academic, 1996, pp 17–29.
112. RG Krishnamurthy. INFORM 3:1130–1135, 1992.
113. J Kurashige, N Matsuzaki, K Makabe. J Dispers Sci Technol 10:531–559, 1989.
114. A Sugihara, Y Shimada, M Nakamura, T Nagao, Y Tominaga. Protein Eng 7:585–588, 1994.
115. AG Marangoni, D Rousseau. Trends Food Sci Technol 6:329–335, 1995.
116. P Quinlan, S Moore. INFORM 4:582–585, 1993.

24

Enzymatic Enrichment of Polyunsaturated Fatty Acids

YUJI SHIMADA, AKIO SUGIHARA, and YOSHIO TOMINAGA

Osaka Municipal Technical Research Institute, Osaka, Japan

1 INTRODUCTION

Polyunsaturated fatty acids (PUFAs) have various physiological functions and are used as pharmaceutical substances, ingredients of cosmetics, health foods, and food materials. PUFA-rich oil and a highly absorbable structured lipid with medium-chain fatty acid at 1,3 positions and functional fatty acid at the 2 position can be produced by taking advantage of lipase specificity, such as fatty acid and positional specificity. These high-value-added lipids show greater physiological effects by a small intake. In addition, they are easily emulsified because the amount of addition can be reduced. On the other hand, highly purified PUFAs themselves are expected as pharmaceutical substances and ingredients of cosmetics and can be efficiently purified by enzymatic method. In this chapter, we describe the production of PUFA-rich oils by selective hydrolysis and the purification of free PUFAs by selective esterification.

2 FUNCTION AND APPLICATION OF TARGET PUFA

Eicosapentaenoic acid (EPA, 20:5n-3) can be purified industrially by a combination of rectification and urea adduct fractionation and has been used in the treatment of arteriosclerosis obliterans and hyperlipemia since 1991 in Japan [1]. Thus, we dropped EPA from our target PUFAs, and selected docosahexaenoic acid (DHA, 22:6n-3), γ-linolenic acid (GLA, 18:3n-6), and arachidonic acid (AA, 20:4n-6).

Docosahexaeonic acid competes with AA [2,3] and plays a role in the prevention of a number of human diseases, including cardiovascular disease [4–6], inflammation [7], and cancer [8,9]. It has been also reported that DHA shows important functions in the

brain [10] and retina [11] and that it accelerates the growth of preterm infants [12,13]. For this reason, tuna oil containing DHA has been used as a food material, an ingredient of baby milk, and a health food [14], and medical application of DHA has received increasing attention.

Gamma-linolenic acid is biosynthesized from linoleic acid by Δ6-desaturase, which is the rate-limiting enzyme in the essential fatty acid cascade [15,16]. GLA is also an intermediate precursor of local hormones (prostaglandins, thromboxanes, and leukotrienes) [17]. These hormones participate in the development and regulation of immunological and inflammatory response together with eicosanoids derived from n-3 series of PUFAs [18]. Thus, an abnormal fatty acid profile is implicated in the impairment of the immune function and pathogenesis of inflammatory, autoimmune, and neoplastic diseases [19]. For example, patients with atopic eczema were shown to have higher levels of linoleic acid and lower levels of GLA, suggesting impaired Δ6-desaturase activity [20,21]. In a number of clinical trials, dietary supplementation with GLA increased epidermal levels of eicosanoid precursors [22] and produced significant improvement in itching and antihistamine use [23,24]. Furthermore, GLA-containing oil was effective for curing rheumatoid arthritis [25,26] and multiple sclerosis [27]. Based on these studies, GLA-containing borage oil has been used as a health food and an ingredient in infant formula [28], and the highly purified GLA has been desired as a medicine.

Arachidonic acid is a rare fatty acid that has potential pharmaceutical value and is a precursor of local hormones, such as prostaglandins, thromboxanes, and leukotrienes, involved in the AA cascade [17,29]. An AA-containing oil can be produced by a microorganism [30], and industrial production of the oil containing 25–40% AA has been achieved. The fatty acid is also important for the growth of preterm infants as is DHA [12,13] and the oil was recently used in a preterm infant formula. In addition, arachidonylethanolamide (anandamide) [31] and 2-arachidonylglycerol [32] can be bound to the receptor of cannabinoid, which is a psychotropic agent, and attention has been given to their medical applications.

3 FUNDAMENTAL FEATURE OF LIPASE

3.1 Lipase Reactions Applicable to Oil Processing

Lipase hydrolyzes triglycerides to partial glycerides or glycerol and fatty acids. The reaction is reversible, and esterification and transesterification can also be catalyzed [33]. Figure 1 shows lipase reactions applicable for oil processing. In general, hydrolysis occurs preferentially in a system containing a large amount of water, and esterification proceeds effectively in a system containing only a small amount of water [34]. Transesterification is efficiently catalyzed in a mixture without water using an immobilized enzyme. When lipase is used as a catalyst, it should be kept in mind that the enzyme acts on liquid-state substrates strongly but on solid-state ones very weakly.

3.2 Substrate Specificity of Lipase

Substrate specificity of lipase can be classified into fatty acid specificity, alcohol specificity, positional specificity, triglyceride specificity, and glyceride specificity. These specificities, except for alcohol specificity, are important for the construction of a suitable system for oil processing. Lipase has also attracted attention as a catalyst for optical resolution, and the reactions can be mainly achieved by the application of alcohol specificity.

1. Hydrolysis

$ROCOR^1 + H_2O \rightarrow ROH + R^1COOH$

2. Esterification

$ROH + R^1COOH \rightarrow ROCOR^1 + H_2O$

3. Transesterification

3-1 Acidolysis

$ROCOR^1 + R^2COOH \rightarrow ROCOR^2 + R^1COOH$

3-2 Alcoholysis

$R^1OCOR + R^2OH \rightarrow R^2OCOR + R^1OH$

3-3 Interesterification

$R^1OCOR^2 + R^3OCOR^4 \rightarrow R^1OCOR^4 + R^3OCOR^2$

Figure 1 Lipase reactions applicable to oil processing.

The classification based on the reactivity of lipase is very useful for selecting an enzyme suitable for the desired oil processing. Microbial lipases can be classified into five groups, as shown in Table 1, from the homologies of their primary structures [35–52]. We noted that the lipases in the same group possessed similar reactivity as well as similar structure and production mechanism [53,54]. The enzymes in groups II, IV, and V are particularly effective for oil processing.

3.2.1 Fatty Acid Specificity

When lipase is applied to the processing of PUFA-containing oil, the fatty acid specificity is one of the most important factors. The specificity has been investigated mainly by measuring the activity on simple triglycerides or methyl (ethyl) esters [55] or by measuring the release rates of the constituent fatty acids from a natural oil [56]. The evaluation by

Table 1 Classification of Lipases Based on Their Primary Structure

Group	Origin	Ref.
I	*Staphylococcus aureus*	35
	Staphylococcus hyicus	36
II	*Pseudomonas cepacia*	37
	Pseudomonas glumae	38
	Pseudomonas aeruginosa	39
	Pseudomonas sp. KWI-56	40
	Pseudomonas sp. 109	41
III	*Pseudomonas fluorescens*	42
	Serratia marcescens	43
IV	*Candida rugosa*	44
	Geotrichum candidum	45, 46
V	*Rhizomucor miehei*	47
	Rhizopus delemar	48
	Rhizopus niveus	49
	Fusarium heterosporum	50
	Humicola langinosa	51
	Penicillium camembertii	52

the former method is not precise because of the different physical states of substrates: liquid and solid. By the latter method, the specificity of 1,3-positional–specific lipase cannot be evaluated because the fatty acids on triglyceride molecules in the natural oils are not randomly distributed. Therefore, several natural oils and simple triglycerides were mixed to give almost the same content of each fatty acid, and the fatty acids were then distributed randomly on the triglyceride molecules by the conventional interesterification using Na-methylate as a catalyst. Fatty acid specificity of lipase in hydrolysis can be precisely evaluated by investigating the release rates of the constituent fatty acids from the interesterified oil [57–59]. We selected one enzyme from groups II, IV, and V in Table 1, and their fatty acid specificity was investigated using the interesterified oil (Fig. 2). As a result, all lipases acted on PUFAs weakly. In particular, lipases from *Candida rugosa* and *Rhizopus delemar* acted on GLA, AA, EPA, and DHA very weakly.

We also recognized that fatty acid specificity changed a little under different reaction conditions. An example is shown in Table 2. *Rhizopus* lipase acted on DHA moderately in hydrolysis, and the activity decreased in esterification [60]. Furthermore, the immobilized enzyme scarcely acted on DHA in acidolysis [61]. Thus, when lipase is applied to oil processing, we should correctly evaluate the fatty acid specificity in the reaction system.

3.2.2 Positional Specificity

Lipases can be classified into two groups; one acts on ester bonds at 1,3 positions of triglyceride, and the other acts on all the ester bonds of triglyceride [33,62]. Lipases in groups II and IV in Table 1 are positionally nonspecific enzymes, and lipases in group V and *Serratia* lipase in group III are 1,3-positional–specific enzymes. The lipase showing 2-positional specificity has not been found, although isozymes III and IV from *Geotrichum candidum* in group IV preferentially acted on the 2-position of triglyceride [63–65]. When 1,3-specific lipase was used as a catalyst in the hydrolysis of triglyceride, part of the fatty acids esterified at the 2-position migrate spontaneously to the 1 or 3 position during the reaction for more than 20 min, and they are liberated from glycerides. Thus, positional specificity must be determined by analyzing the glyceride components generated in a short reaction time [62,66].

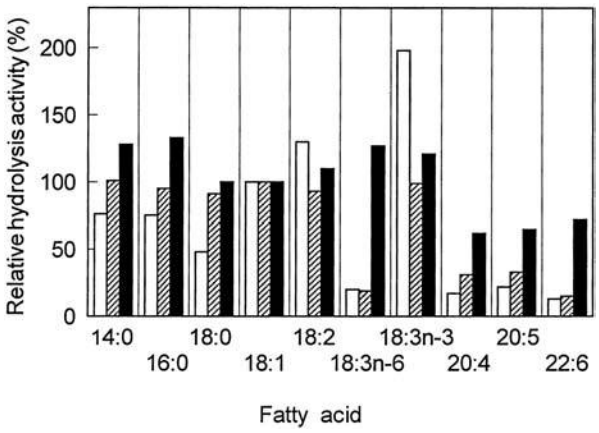


Figure 2 Fatty acid specificities of lipases from *C. rugosa* (□), *R. delemar* (▨), and *P. aeruginosa* (■). The activity was expressed relative to that on oleic acid.

Table 2 Fatty Acid Specificity of *R. delemar* Lipase in Hydrolysis, Esterification, and Acidolysis

	Activity (%)		
Fatty acid	Hydrolysis[a]	Acidolysis[b]	Esterification[c]
14:0	101	105	94
16:0	95	97	93
16:1	105	117	108
18:0	91	88	75
18:1	100	100	100
18:2	93	89	91
18:3n-6	19	nd[d]	1
18:3n-3	99	106	95
20:4n-6	31	21	11
20:5n-3	33	26	13
22:6n-3	15	nd	5

[a] Randomly interesterified oil was hydrolyzed in a mixture containing 50% water.
[b] Randomly interesterified oil was acidolyzed with caprylic acid using immobilized lipase.
[c] Fatty acids derived from randomly interesterified oil were esterified with lauryl alcohol in a mixture containing 20% water.
[d] nd = Not determined.

Cocoa butter substitute is a structured lipid with stearic or behenic acid at the 1,3-positions and oleic acid at the 2 position, and Betapol is a structured lipid with oleic acid at the 1,3-positions and palmitic acid at the 2 position and is used in infant formula [67]. These lipids have been produced industrially by changing fatty acids at the 1,3 positions of natural oils for desired fatty acids. The immobilized 1,3-positional–specific lipase is used for the production. In addition, structured lipid with medium-chain fatty acid at the 1,3 positions and functional fatty acid at the 2 position is expected as a highly absorbable oil [68,69], and this type of structured lipid can be efficiently produced by acidolysis of natural oil with medium-chain fatty acid using an immobilized 1,3-specific lipase as a catalyst [61,70–75].

3.2.3 Triglyceride Specificity

A lipase recognizes the structure of triglyceride. Tanaka et al. [76] investigated the activity of *C. rugosa* lipase on tridocosahexaenoin (TG-DDD), triglyceride with 2 mol of DHA and 1 mol of oleic acid (TG-DDO), triglyceride with 1 mol of DHA and 2 mol of oleic acids (TG-DOO), and triolein (TG-OOO). As a result, *Candida* lipase acted strongly in the order TG-OOO, TG-DOO, TG-DDO, and TG-DDD. They called this specificity ''triglyceride specificity.''

3.2.4 Glyceride Specificity

In general, lipase shows different activity on triglyceride (TG), diglyceride (DG), and monoglyceride (MG). We found that *C. rugosa* lipase acted strongly in the order MG, DG, and TG, and we named the specificity ''glyceride specificity'' [77]. *Penicillium camembertii* lipase is an enzyme in group V and acts on only MG and DG [78]. The lipase is an example showing strong glyceride specificity.

4 PRODUCTION OF PUFA-RICH OIL BY SELECTIVE HYDROLYSIS

The oil containing higher PUFA concentration is predicted to have the following characteristics: 1) A greater physiological effect can be expected by a small amount of intake; 2) the PUFA-rich oil can be easily emulsified in various kinds of food, because the amount of addition is reduced; 3) higher PUFA concentration is effective as a starting material of PUFA purification. Traditional winterization is available for the enrichment of PUFA, but it is not effective in attaining the higher PUFA concentration (e.g., winterization of tuna oil raised the DHA content to only 35%) and the yield is low. Because lipase acts on PUFA weakly (Fig. 2), it can be used to enrich PUFA in undigested glycerides by selective hydrolysis of a natural PUFA-containing oil [58,60,77,79–82].

4.1 Production of DHA-Rich Oil from Tuna Oil

Candida rugosa and *Geotrichum candidum* lipases (group IV in Table 1) act on DHA very weakly, and their hydrolysis activities are strong. Thus, these lipases are most effective for the production of DHA-rich oil by selective hydrolysis of tuna (or bonito) oil. In particular, *Candida* lipase is useful because of the industrially available enzyme (Meito Sangyo Co., Aichi, Japan). The optimum reaction conditions were as follows: A mixture of equal amounts of the oil and water was stirred at 35°C along with 200 U/g *Candida* lipase [60]. Here, one unit of lipase activity was defined as the amount which liberated 1 μmol of fatty acid from olive oil per minute. Figure 3 shows a typical time course of the selective

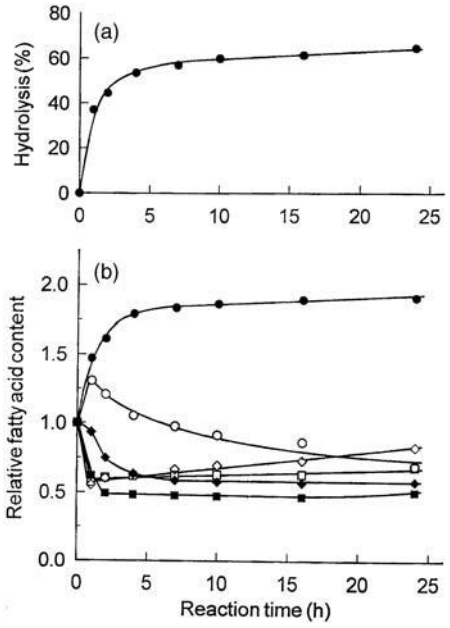


Figure 3 Time course of hydrolysis of tuna oil with *C. rugosa* lipase: (a) hydrolysis extent; (b) main fatty acid contents in undigested glyceride fraction. The fatty acid contents in glycerides were expressed relative to those in the original tuna oil. Symbols: ■, palmitic acid (the original content, 18.2%); ◇, palmitoleic acid (4.6%); ◆, stearic acid (4.4%); □, oleic acid (18.5%); ○, EPA (6.9%); ●, DHA (24.4%).

hydrolysis of tuna oil under these conditions. The hydrolysis extent increased rapidly up to 4 hr and then increased gradually. The DHA content in glycerides increased with increasing the hydrolysis extent. The contents of palmitic, palmitoleic, and oleic acids decreased rapidly in the early stage of the reaction. The stearic acid content decreased after a 1-hr lag, and the EPA content decreased gradually after an increase during the first hour. After a 24-hr reaction, the DHA content in glycerides reached 48%, and the contents of triglycerides, diglycerides, and monoglycerides were 88%, 10%, and 2%, respectively. This time course showed that the activities of *Candida* lipase on the constituent fatty acids were in the order of palmitoleic and oleic acids > palmitic acid > stearic acid > EPA > DHA. The order agreed completely with the fatty acid specificity evaluated using randomly interesterified oil as a substrate (Fig. 2).

When hydrolysis was conducted in a mixture containing 50% water, the DHA content in glycerides mainly depended on the hydrolysis extent. Figure 4 shows the correlation between hydrolysis extent and the DHA content in glycerides. The hydrolysis extent could be increased by using a larger amount of lipase or by extending the reaction time, and the DHA content in glycerides increased with increasing the hydrolysis extent. The DHA content was raised from 23% to 45% at 60% hydrolysis, and 22% of total DHA was released into the free fatty acid (FFA) fraction. By increasing the hydrolysis extent to 70%, the DHA content was raised to 50%, although 35% of DHA was released into the FFA fraction. The reaction system described here can be applied to the industrial production of 50% DHA-containing oil, and the product has been used as a health food in Japan since 1994.

4.2 Production of GLA-Rich Oil from Borage Oil

Because *Candida* lipase acts on GLA very weakly as well as on DHA (Fig. 2), borage oil (GLA content, 22%) was selectively hydrolyzed at 35°C for different times. Figure 5 shows the correlation between the hydrolysis extent and the GLA content in undigested glycerides. Hydrolysis of tuna oil gradually increased the DHA content even though the hydrolysis extent was more than 60% (Fig. 4). However, the hydrolysis rate of GLA ester was the same as those of the other fatty acid esters when the hydrolysis extent exceeded 65%. Therefore, *Candida* lipase did not show the fatty acid selectivity at more than 65%

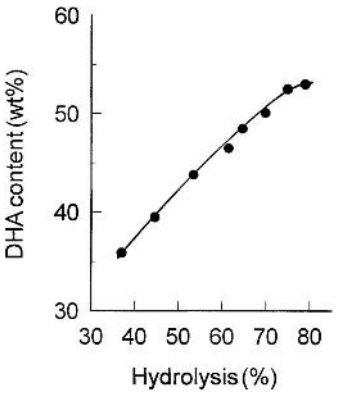


Figure 4 Correlation between hydrolysis extent of tuna oil and DHA content in undigested glycerides. Tuna oil was hydrolyzed at 35°C for 24 hr in a mixture containing 50% water with 20–2000 U/g of *C. rugosa* lipase.

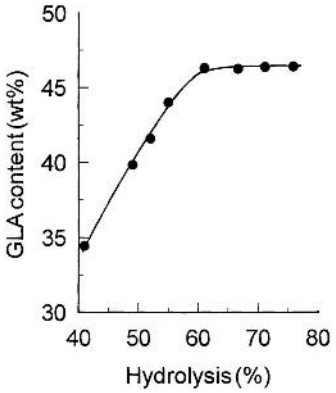


Figure 5 Correlation between hydrolysis extent of borage oil and GLA content in undigested glycerides. Borage oil was hydrolyzed at 35°C for 0.25–24 hr in a mixture containing 50% water with 100 U/g of *C. rugosa* lipase.

hydrolysis, and the GLA content in glycerides was not raised above 46% [81,82]. The 65% hydrolysis was achieved most efficiently under the following conditions: Borage oil was stirred at 35°C for 16–24 hr in a mixture of 50% water and 20 U/g of *Candida* lipase [82]. The lipase amount in hydrolysis of borage oil was one-tenth of that in hydrolysis of tuna oil, showing that borage oil was a better substrate of *Candida* lipase than tuna oil.

The GLA content in glycerides was not raised above 46% in hydrolysis of borage oil in a mixture of 50% water. However, the hydrolysis in 90% water raised the GLA content to 49% at 75% hydrolysis. This result suggested that FFAs accumulating in the reaction mixture affected the apparent fatty acid specificity of the lipase and interfered with the increase of the GLA content [82]. To investigate the kinetics of the selective hydrolysis in a mixture without FFAs, triglycerides containing 22%, 35%, and 46% GLA were hydrolyzed with *Candida* lipase. The result showed that the hydrolysis rate decreased with increasing GLA content of glycerides but that the release rate of GLA did not change. Thus, it was found that the apparent fatty acid specificity of the lipase in the selective hydrolysis was also affected by the triglyceride structure (triglyceride specificity) [82].

To produce an oil containing more than 46% GLA, the first reaction was conducted at 35°C for 16 hr in a mixture of 50% water using 20 U/g of *Candida* lipase and the undigested glycerides were recovered. The selective hydrolyses were then repeated under the same conditions (Table 3). As a result, the GLA content was raised to 54% at 20% hydrolysis. In addition, when the second reaction was done in 90% water using 20 U/g lipase or in 50% water using 200 U/g lipase, the GLA content was raised to 59% at 46% or 60% hydrolysis. These results showed that repeated hydrolysis was effective in producing an oil containing a higher concentration of GLA. The repeated selective hydrolysis of tuna oil with *Candida* lipase was also effective, and 59% DHA oil was produced.

4.3 Production of AA-Rich Oil from *Mortierella* Single-Cell Oil

We next attempted to produce AA-rich oil using *Candida* lipase. AA-containing oil is produced by *Mortierella alpina* [30], and the oils of 25% and 40% AA have been commercialized (SUN-TGA25 and SUN-TGA40; Suntory Ltd., Osaka, Japan) [83,84]. When

Table 3 Production of GLA-Rich Oil by Repeated Hydrolysis with *C. rugosa* Lipase

Reaction	Reaction mixture		Hydrolysis (%)	GLA content (%)	GLA recovery[a] (%)
	Water (%)	Lipase (U/g)			
None (Borage oil)	—	—	—	22.2	100
First	50	20	62.3	45.3	76.5
Second	50	20	19.8	54.1	73.4
	90	20	46.1	59.0	53.7
	50	200	60.4	59.3	39.6

[a] Recovery of the initial GLA content of the original borage oil. The recovery was calculated by assuming that glycerides were completely recovered by the extraction with *n*-hexane.

TGA25 oil was selectively hydrolyzed at 35°C for 16 hr in a mixture containing 50% water and 90 U/g of the lipase, the resulting glycerides contained 50% AA at 52% hydrolysis [58]. Because repeated hydrolysis was effective in increasing the AA content, the glycerides recovered from the reaction mixture were allowed to react again under the same conditions (Table 4). The extents of the second and third hydrolyses were 18 and 17%, respectively. The AA content in glycerides was increased by the repetition of hydrolysis and raised to 60% by three-times hydrolysis. In addition, when TGA40 oil was hydrolyzed at 35°C for 20 hr in a mixture of 50% water with 15 U/g of lipase, the AA content was raised to 57% at 36% hydrolysis [85]. In the selective hydrolysis, the recovery of AA in glycerides reaches 91%, if it is assumed that all glycerides can be recovered. This result shows that the starting material containing a higher concentration of PUFA is effective for the production of PUFA-rich oil by enzymatic selective hydrolysis.

4.4 A Method Available for Industrial Purification of PUFA-Rich Oil

When PUFA-rich oil is separated from the reaction mixture, FFAs have to be removed. Solvent extraction is not suitable as an industrial-scale separation method because of time, cost, and risk of explosion. On the other hand, film distillation may be preferable for the following reasons. 1) The reaction mixture consists of FFA and glycerides (triglycerides were more than 80%), and their average molecular weights are ~280 and 900, respectively. 2) In general, distillation can recover a desired compound in a high yield.

Table 4 Main Fatty Acid Content in Glycerides Obtained by Repeated Selective Hydrolysis

Reaction	Hydrolysis (%)	Fatty acid content (wt%)						AA recovery[b] (%)
		16:0	18:0	18:1	18:2	18:3[a]	20:4	
None (TGA-25)	—	13.4	6.3	14.8	21.3	3.2	24.9	100
First	52.4	5.7	3.6	6.7	9.6	4.7	49.5	52.4
Second	18.1	3.8	3.2	4.0	5.5	4.9	54.3	87.2
Third	17.2	2.4	2.3	2.5	3.3	5.2	59.7	75.0

[a] γ-Linolenic acid.

[b] Recovery of the initial AA content of the original TGA-25 oil. The recovery was calculated by assuming that all glycerides were recovered by the extraction with *n*-hexane.

Table 5 Large-Scale Purification of GLA-Rich Oil from Reaction Mixture Obtained by Selective Hydrolysis of Borage Oil

Procedure	Weight (kg)	Acid value	Amount of glycerides[a] (kg)	GLA content[b] (%)	GLA recovery[b] (%)
Original borage oil	7.00	nd[c]	7.00	22.2	100
Hydrolysis	6.29	122	2.45	45.7	71.8
Film distillation					
Distillate 1-1	3.26	197	0.05	nt[c]	—
Distillate 1-2	0.67	151	0.16	nt	—
Residue 1-2	2.28	4	2.23	46.4	64.8

[a] Calculated by assuming that the acid value of free fatty acids originating from borage oil is 200.
[b] The content and the recovery of GLA in the glyceride fraction.
[c] nd = Not determined; nt = Not tested.

Table 5 is an example of the purification of GLA-rich oil from reaction mixture obtained by selective hydrolysis of borage oil [82]. Borage oil (7 kg) was hydrolyzed at 35°C for 24 hr in a mixture containing an equal amount of water and 20 U/g *Candida* lipase. The reaction was conducted with agitating at 200 rpm using a 30-L reactor, and the GLA content in glycerides was raised to 46% at 61% hydrolysis. Film distillation of the reaction mixture was performed at 170°C and 0.05 mmHg. Most of FFAs were recovered in the distillate 1-1, but the acid value of the residue was still high. Hence, the second cycle of distillation was carried out after increasing the temperature to 190°C, and 2.3 kg of the residue (acid value 4) was obtained. The distillation recovered 91% of glycerides in the reaction mixture, showing that the film distillation was very effective for the large-scale purification of PUFA-rich oil obtained by selective hydrolysis.

4.5 Reaction Mechanism of Selective Hydrolysis

The hydrolysis extent exceeded 50% in selective hydrolyses of several PUFA-containing oils, but the main components in the undigested glyceride fraction were triglycerides (>80%). Thus, we investigated what reactions occurred with the hydrolysis and found that partial glycerides were esterified with PUFA but glycerol was not and that interesterification between partial glycerides also occurred. In addition, the hydrolysis activity of *Candida* lipase on PUFA glycerides was in order of monoglycerides > diglycerides > triglycerides, and the main component after hydrolysis of PUFA partial glycerides was triglyceride. On the basis of these results, the selective hydrolysis of PUFA-containing oil was concluded to proceed as follows [77]. The lipase hydrolyzed the ester bonds of fatty acids other than PUFAs in the early stage of the reaction. The PUFA partial glycerides generated were converted to PUFA triglycerides by interesterification between partial glycerides, and by esterification of partial glycerides with a small amount of PUFA existing in the reaction mixture. The lipase action on PUFA glycerides is in the order monoglycerides > diglycerides > triglycerides (glyceride specificity) and that on triglycerides is in the order zero > one > two > three molecules of PUFA in the triglycerides (triglyceride specificity). PUFA triglyceride was the poorest substrate in the reaction mixture and the lipase acted on it only very weakly. The simultaneous reactions and the specificities of the lipase resulted in the accumulation of PUFA-rich triglycerides in the reaction mixture.

5 PURIFICATION OF PUFA BY SELECTIVE ESTERIFICATION

Several methods of purifying PUFA have been proposed, such as high-performance liquid chromatography [86] and silver ion-exchange column chromatography [87]. However, these methods have not been adopted for the industrial purification because of high cost. In addition to these methods, enzymatic methods have drawn attention, and selective hydrolysis and esterification have been reported [58–60,77,79–82,85,88–94]. As described earlier, the content of PUFA could not be raised to more than 60% by selective hydrolysis. However, selective esterification was effective for increasing the PUFA content to near 90%, but it required a large amount of organic solvents [88,89,91]. Thus, we attempted to develop a new enzymatic method, which gave more than 90% purity and could be applied industrially. We describe herewith the two-step enzymatic method for purifying DHA from tuna oil [90,92], GLA from borage oil [59,94], and n-6 series of PUFAs from *Mortierella* single-cell oil [85,93].

5.1 Two-Step Enzymatic Method

A desired PUFA can be enriched in the FFA fraction by hydrolyzing PUFA-containing oil with a lipase acting on the fatty acid very strongly. However, this type of lipase has not been found yet. Thus, we attempted the purification of PUFA by a two-step enzymatic method [59,85,90,92–94]. The first step is the preparation of FFAs by hydrolyzing PUFA-containing oil with a lipase acting on PUFA as strongly as the other fatty acids, and the second step is the enrichment of PUFA in the FFA fraction by esterifying the resulting FFAs with alcohol and a lipase acting on PUFA very weakly.

5.1.1 First Step: Hydrolysis of PUFA-Containing Oil

Polyunsaturated-fatty-acid-containing oil can be hydrolyzed by heating with a large amount of ethanol under the alkaline condition. However, the procedure requires a larger-scale reactor and contains the risk of the isomerization of PUFA. The wastewater also contains ethanol and shows a high chemical oxigen demand (COD) value. Furthermore, to recover the FFAs, the pH of the reaction mixture has to be returned to the acidic condition. On the other hand, when the hydrolysis is conducted using a lipase, FFAs can be easily recovered from the reaction mixture by film distillation. Thus, we attempted the hydrolysis with a lipase that acts on PUFA comparatively well and gives the higher hydrolysis extent. Screening of industrial enzymes showed that *Pseudomonas* lipases (group II in Table 1) were suitable for the hydrolysis of PUFA-containing oil.

5.1.2 Second Step: Selective Esterification

Polyunsaturated fatty acids can be enriched in the unesterified FFA fraction by the esterification of FFAs originating from PUFA-containing oil, with an alcohol and a lipase acting on PUFA only very weakly. Because the esterification proceeds efficiently in a mixture containing a smaller amount of water, dehydrated substrates have been used and water generated by the reaction has been removed by reducing pressure in the reactor or by adding molecular sieves into the reaction mixture [95,96]. It has been also reported that the reaction in organic solvent is effective [88,89,97]. However, a vacuum pump is necessary to remove water, and the use of organic solvent requires a larger-scale reactor and contains the risk of explosion. Thus, we attempted to develop a system in which the esterification proceeded efficiently, even though a mixture contained a large amount of water.

Table 6 Screening for a Fatty Alcohol Suitable for Enrichment of DHA by Selective Esterification of Free Fatty Acids Originating from Tuna Oil[a]

Alcohol	Esterification (%)	DHA content (%)	Recovery of DHA (%)
None	—	23.2	100
Methanol	14.1	23.2	83.7
Ethanol	19.1	23.8	80.9
Propanol	26.5	27.9	86.2
Butanol	36.7	32.7	87.0
Pentanol	56.9	49.2	89.1
Hexanol	66.1	62.4	88.9
Octanol	70.0	67.6	85.2
Decanol	72.4	70.3	81.5
Lauryl alcohol	72.7	70.9	81.3
Oleyl alcohol	66.4	60.7	85.7

[a] A mixture of 4 g FFAs/alcohol (1:2, mol/mol), 1 g water, and 1000 U *R. delemar* lipase was incubated at 30°C for 16 hr with stirring at 500 rpm.

In general, fatty alcohols are good substrates of lipases, but their esters are poor ones. The esterification of fatty acids with fatty alcohol can, therefore, proceed efficiently in spite of the presence of a large amount of water, because the esterification products (fatty alcohol esters) are weakly hydrolyzed by lipases. Table 6 shows the result of the screening for a fatty alcohol suitable for selective esterification of fatty acids originating from tuna oil [90]. Long-chain fatty alcohols enhanced esterification, and the DHA content in the FFA fraction depended on the esterification extent. Lauryl alcohol was the most effective substrate and the use of the alcohol raised the DHA content to 71% at the esterification extent of 73% [90,93]. On the basis of the results, we chose lauryl alcohol as a substrate for the selective esterification.

5.2 Purification of DHA from Tuna Oil by the Two-Step Enzymatic Method

5.2.1 First Step: Hydrolysis of Tuna Oil

Industrially available lipases were screened for hydrolysis of tuna oil. As shown in Figure 2, *Pseudomonas* lipases in group II acts on PUFA somewhat. Actually, *Pseudomonas* lipases acted on DHA as strongly as the other fatty acids, and the hydrolysis extents were also high (Table 7). In particular, *Pseudomonas* lipase (Lipase-AK; Amono Pharmaceutical Co., Aichi, Japan) hydrolyzed the DHA ester more strongly than the EPA ester, and DHA was recovered in the FFA fraction in the highest yield. Thus, Lipase-AK was chosen for hydrolysis of tuna oil.

Several factors affecting the hydrolysis were investigated, and the reaction conditions were set as follows: A reaction mixture of oil/water (1:1, wt/wt) and 500 U/g lipase was incubated at 40°C with stirring [92]. When tuna oil was hydrolyzed for 48 hr under the conditions, the hydrolysis extent reached 79%. After the reaction, FFAs were extracted from the reaction mixture by the extraction with *n*-hexane and were named Tuna-FFA (the DHA recovery, 83%).

Table 7 Screening for a Lipase Suitable for Hydrolysis of Tuna Oil[a]

Lipase	Hydrolysis (%)	Fatty acid content (%)[b]		Recovery of DHA (%)
		EPA	DHA	
Rhizopus delemar	38.5	5.1	15.3	25.7
Candida rugosa	64.4	6.8	13.1	36.8
Pseudomonas aeruginosa	64.8	6.6	23.9	67.6
Pseudomonas glumae	65.4	6.5	23.5	67.1
Pseudomonas sp. KWI-56	58.8	6.7	23.1	59.3
Pseudomonas sp.[c]	68.4	4.3	24.2	71.9
Pseudomonas sp.[d]	78.9	5.2	19.8	68.2

[a] A mixture of 2.5 g tuna oil, 2.5 g water, and 2500 U of lipase was incubated for 16 hr with stirring at 500 rpm.
[b] Content in the FFA fraction. The contents of EPA and DHA in the original tuna oil were 6.5% and 22.9%, respectively.
[c] Lipase-AK (Amono Enzyme Inc., Aichi, Japan).
[d] LIPOSAM (Showa Denko K.K., Tokyo, Japan).

5.2.2 Second Step: Selective Esterification

Several lipases were tested for the selection of an enzyme suitable for selective esterification of Tuna-FFA with lauryl alcohol (Table 8). It is expected that lipases in groups IV and V, which act on PUFA very weakly, are suitable for the selective esterification. Actually, *Pseudomonas* lipase acted on DHA moderately, and the DHA content was not raised over 51%. When *C. rugosa* lipase was used, DHA was scarcely esterified with lauryl alcohol, and 98% DHA was recovered in the unesterified fatty acid fraction. However, the DHA content in the fatty acid fraction was only 42% because of the low esterification extent. Meanwhile, *R. delemar* and *Fusarium heterosporum* lipases achieved high esterification extents, and the activities on DHA were also low. Therefore, DHA was successively purified as an FFA; DHA was purified from 23% to 65% at 68% esterification by *Rhizopus* lipase. From these results, *Rhizopus* lipase (Ta-Lipase, Tanabe Seiyaku Co., Osaka, Japan) was selected as the most effective enzyme.

Several factors affecting the selective esterification were investigated and the reaction conditions were determined as follows: A reaction mixture of 8 g Tuna-FFA/lauryl alcohol

Table 8 Screening for a Lipase Suitable for Selective Esterification of Tuna-FFA[a]

Lipase	Esterification (%)	DHA content[b] (%)	Recovery of DHA[c] (%)
Rhizopus delemar	67.8	65.4	90.8
Fusarium heterosporum	63.9	58.5	91.0
Candida rugosa	45.6	41.9	98.2
Pseudomonas aeruginosa	72.0	50.6	65.9

[a] A reaction mixture of 2.7 g lauryl alcohol/Tuna-FFA (2:1, mol/mol), 0.3 g water, and 600 U lipase was stirred at 30°C for 16 hr.
[b] DHA content in the unesterified fatty acid fraction. The DHA content in Tuna-FFA was 23.2%.
[c] DHA amount recovered in unesterified fatty acid fraction.

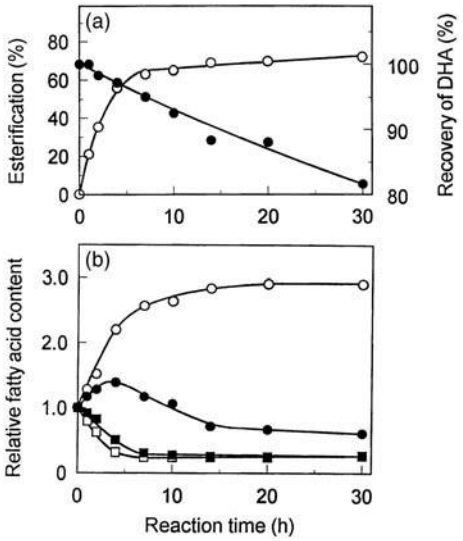


Figure 6 Time course of selective esterification of Tuna-FFA with *R. delemar* lipase. (a) The esterification extent (○) and DHA recovery (●). (b) The content of unesterified fatty acids. The contents were expressed as relative values to their initial ones. Symbols: ○, DHA (initial content, 23.2%); ●, EPA (5.8%); □, oleic acid (23.0%); ■, palmitic acid (17.9%).

(1:2, mol/mol), 2 g water, and 200 U/g *Rhizopus* lipase was incubated at 30°C with stirring [90]. Figure 6 shows a typical time course under these conditions. The esterification extent did not increase much after 7 hr and the contents of oleic and palmitic acids rapidly decreased. The EPA content increased in the early stage of the reaction and then gradually decreased. The DHA content increased efficiently with increasing the esterification extent and reached 73% after 20 hr with 84% of recovery. To further increase the DHA content, unesterified FFA were recovered from the reaction mixture and then esterified again with lauryl alcohol under the same conditions. As a result, the DHA content was raised to 91% with a recovery of 60% of the initial content in tuna oil (Table 9).

An oil with 50 wt% DHA has been produced industrially by selective hydrolysis of tuna oil with *C. rugosa* lipase, and the FFAs generated have been treated as a waste material. Because the DHA content in the FFA fraction was 13–15%, we attempted to

Table 9 Purification of DHA by Two-Step Enzymatic Method

	Fatty acid content (wt%)[a]						Recovery of DHA[b]
Procedure	16:0	16:1	18:0	18:1	20:5	22:6	(%)
Original tuna oil	18.6	4.6	4.5	20.7	6.5	22.9	100
Hydrolysis	19.4	4.8	4.5	21.1	4.3	24.2	83.0
Esterification (1st)	5.2	1.2	1.1	5.7	4.0	71.6	68.5
Esterification (2nd)	0.8	nd[c]	nd	0.9	1.7	90.6	60.3

[a] The contents of main fatty acids in the FFA fraction.
[b] The recovery was calculated by assuming that FFAs were completely recovered.
[c] nd = Not determined.

purify DHA from the FFAs by repeated selective esterification with lauryl alcohol. As a result, the DHA content was raised from 13% to 81% with 70% recovery [92].

5.3 Purification of GLA from Borage Oil by the Two-Step Enzymatic Method

5.3.1 Small-Scale Purification

The two-step enzyme method was applied to the purification of GLA. In general, *Pseudomonas* lipases in group II act on GLA as strongly as they do on the other long-chain fatty acids (e.g., palmitic, stearic, oleic, linoleic, and α-linolenic acids). Because *Pseudomonas* sp. lipase (Lipase-PS; Amano Pharmaceutical Co.) was found to be most suitable for hydrolysis of borage oil among the lipases tested, several factors affecting the hydrolysis were investigated using the lipase as a catalyst. On the basis of the results, the hydrolysis conditions were set as follows: A reaction mixture of borage oil/water (2:1, w/w) and 250 U/g Lipase-PS was incubated at 35°C with stirring [59,94]. When borage oil was hydrolyzed for 24 hr under these conditions, hydrolysis extent reached 92%, and 93% of GLA was recovered as an FFA.

The FFAs obtained by the hydrolysis (named Borage-FFA) were selectively esterified with lauryl alcohol using *Rhizopus* lipase under the same conditions as those of selective esterification of Tuna-FFA. The GLA content was raised from 22% to 70% by a single esterification. To further increase the GLA content, unesterified fatty acids were extracted with *n*-hexane and were esterified again under the same conditions. As a result, the GLA content was raised to 94% with a recovery of 68% of the initial content [59]. Table 10 shows the results of the purification of GLA from 50 g borage oil by the two-step enzymatic method.

5.3.2 Large-Scale Purification

Small-scale purification of GLA was achieved by the two-step enzymatic method. We next attempted large-scale purification of GLA on the assumption of the industrial process. Because the oil containing a higher concentration of GLA is generally effective as a starting material for the purification of GLA, we chose 45% GLA oil (GLA45; Nippon Supplement Inc., Osaka, Japan), which was produced by selective hydrolysis of borage oil with

Table 10 Purification of GLA by Two-Step Enzymatic Method

Procedure	Degree of reaction (%)	Amount of FFA[a] (g)	Fatty acid composition (%)								Recovery of GLA[b] (%)
			16:0	18:0	18:1	18:2	18:3	20:1	22:1	24:1	
Original borage oil	—	47.3[c]	9.7	3.9	17.5	38.3	22.2	4.3	2.6	1.6	100
Hydrolysis	91.5	43.3	9.8	4.1	17.5	37.8	22.5	4.3	2.5	1.5	92.7
Esterification (1st)	74.4	11.1	4.7	2.0	6.3	14.3	70.2	1.3	0.8	nd[d]	74.1
Esterification (2nd)	31.7	7.6	0.8	0.4	1.0	2.8	93.7	0.2	nd	nd	67.5

[a] The amount of FFA was calculated from its acid value, 200.
[b] The recovery was calculated by assuming that FFAs were completely recovered.
[c] The FFA amount in 50 g borage oil.
[d] nd = Not determined.

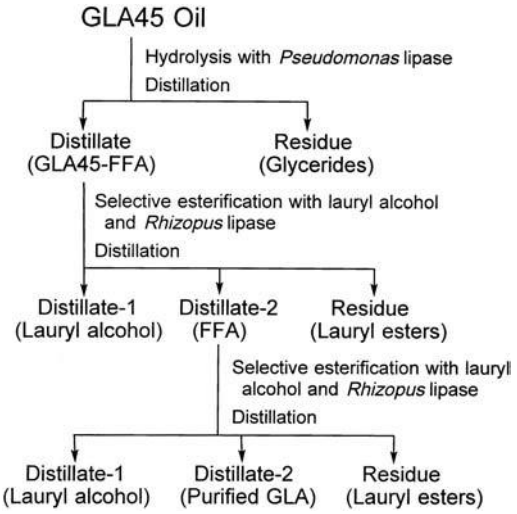


Figure 7 Strategy of large-scale purification of GLA by the two-step enzymatic method.

C. rugosa lipase (see Sec. 4.4). Figure 7 shows the strategy of the purification procedure. GLA45 oil is hydrolyzed with *Pseudomonas* lipase, and FFAs are recovered by distillation. The reaction mixture of selective esterification of the resulting FFA with lauryl alcohol contains lauryl alcohol (molecular weight, 186), FFAs (~280), and lauryl esters (>460). These compounds can be separated by distillation because of their different molecular weights.

Several preliminary experiments were performed according to the above strategy. First, the separation of FFAs from reaction mixture was attempted. Film distillation was very effective in recovering FFAs from the reaction mixture of hydrolysis of GLA45 with Lipase-PS. However, because FFAs and lauryl esters in the reaction mixture after selective esterification were not separated by film distillation, we tried simple distillation to separate them. As a result, FFA-rich fraction was obtained, but 15–20% of lauryl esters were contained in the fraction and they were completely removed by urea adduct fractionation. Next, we examined the effect of the ester contaminants on the repeated esterification (Fig. 8). The lauryl-ester-free FFAs were prepared by eliminating lauryl ester with *n*-hexane and esterified at 30°C for 16 hr with lauryl alcohol and different amounts of *Rhizopus* lipase. When FFAs were esterified with 70 U/g of the lipase, the GLA content in the FFA fraction was raised from 91.0% to 98.7% at the esterification extent of 15.8% (Fig. 8b). The GLA recovery in the FFA fraction was 91.3%. Even though the extent of esterification was increased by using a larger amount of the lipase, the content of GLA was not increased and its recovery was decreased. On the other hand, when FFAs contaminated with 15.5% lauryl esters was esterified under the same conditions, the GLA content was raised to 97.1% at the esterification extent of 15.4% and the GLA recovery was 90.3% (Fig. 8a). These results show that low level of lauryl esters had little effect on the increase of the GLA content in the FFA fraction [94].

On the basis of these preliminary experiments, we attempted the purification of GLA from 10 kg of GLA45 oil [92]; the material balance is shown in Table 11. Because the saponification value of GLA45 oil was 183 and the GLA content was 45.1%, the amounts

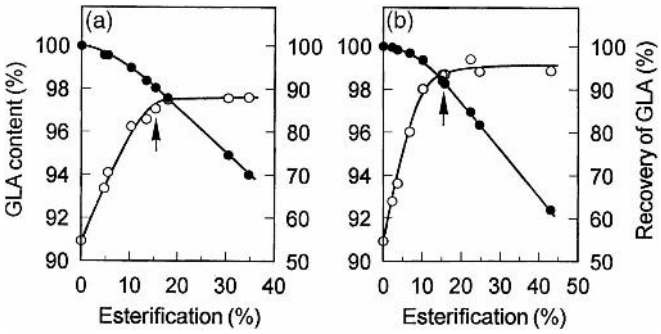


Figure 8 Effect of lauryl ester contaminants on selective esterification of FFAs with lauryl alcohol. The FFAs contaminated with 15.5% lauryl esters (a) and the lauryl-ester-free FFAs (b) were esterified at 30°C for 16 hr with two molar equivalents of lauryl alcohol in 6 g of reaction mixture containing 20% water and 5–400 U/g of *R. delemar* lipase. Arrows indicate the reactions with 70 U/g of lipase. Symbols: ○, the content of GLA in the FFA fraction; ●, the recovery of GLA in the FFA fraction. The recovery was calculated by assuming that FFAs were completely recovered.

Table 11 Large-Scale Purification of GLA from GLA45 Oil

			GLA in FFA fraction		
Step	Weight (kg)	Amount of FFA[a] (kg)	Content (%)	Amount (kg)	Recovery (%)
GLA45 oil	10.00	9.15[b]	45.1[c]	4.13[c]	100
Hydrolysis[d]	8.69	7.95	46.3	3.68	89.1
Distillation[e]	7.55	7.51	46.3	3.48	84.2
Esterification[f]	16.54	3.41	89.5	3.05	73.9
Distillation[g]	3.87	3.15	89.4	2.82	68.2
Esterification[h]	7.61	2.53	97.3	2.46	59.6
Distillation[g]	2.67	2.28	98.1	2.24	54.2
Urea fractionation	2.09	2.07	98.6	2.04	49.4

[a] The amount of FFA was calculated from its acid value, 200.
[b] The amount of fatty acid in GLA45 oil.
[c] The content and amount of GLA in GLA45 oil.
[d] Reaction was conducted at 35°C for 24 hr in a mixture of GLA45 oil/water (2:1, w/w) and 250 U/g of Lipase-PS.
[e] The first cycle of film distillation was performed at 150°C and 0.05 mmHg using molecular distillation apparatus. The residue was distillated again at 160°C and 0.05 mmHg. Distillates obtained by the first and the second distillations were combined.
[f] Reaction was conducted at 30°C for 16 hr in a mixture of FFAs/lauryl alcohol (1:2, mol/mol) and 50 U/g of *R. delemar* lipase.
[g] Simple distillation was done. The distillate at 185°C and 0.2 mmHg was recovered after removing the distillate at 105°C and 0.2 mmHg.
[h] Reaction was conducted at 30°C for 16 hr in a mixture of FFAs/lauryl alcohol (1:2, mol/mol) and 70 U/g of *R. delemar* lipase.

of FFAs and GLA in the oil were estimated to be 9.15 and 4.13 kg, respectively. The oil was hydrolyzed at 35°C for 24 hr in a mixture containing 5 kg water and 250 U/g of Lipase-PS. After the hydrolysis, 8.69 kg of the dehydrated oil layer was recovered and the acid value was 183. Thus, the FFA amount in the oil layer was calculated to be 7.95 kg, showing that the hydrolysis extent was 91.5%. Because the GLA content in the FFA fraction was 46.3%, 89.1% of GLA in the oil was recovered as a FFA by the hydrolysis. The FFA fraction separated by film distillation showed an acid value of 199, and the recovery of GLA was 94.5%. The resulting FFAs were esterified at 30°C for 16 hr in a mixture containing 20% water with two molar equivalents of lauryl alcohol and 50 U/g of *Rhizopus* lipase. The GLA content in the FFA fraction was raised to 89.5% at the esterification extent of 52.0%. Simple distillation of the dehydrated reaction mixture separated 3.87 kg of FFA fraction, in which 18.5% of lauryl esters were present, because the acid value of the FFA was 163. The recovery of GLA by esterification and distillation were 87.6% and 92.5%, respectively. The FFAs containing lauryl esters were allowed to react again with two molar equivalents of lauryl alcohol and 70 U/g of *Rhizopus* lipase. The GLA content in the FFA fraction was raised to 97.3% at the esterification extent of 15.2%. The FFAs recovered by simple distillation was contaminated with 13.5% of lauryl esters and 1.1% of lauryl alcohol, and the esters were completely removed by urea adduct fractionation, although 0.8% of lauryl alcohol remained. The FFA with 98.6% GLA were prepared with a recovery of 49.4% of initial content of GLA45 oil by a series of the purification procedures. The result shows that GLA can be efficiently purified by the combination of enzymatic method, distillation, and urea adduct fractionation. In addition, the procedure should be applicable to the purification of the other PUFAs.

5.4 Purification of n-6 PUFA by the Two-Step Enzymatic Method

Because we confirmed that the two-step enzymatic method is very effective for the purification of PUFA, this method was applied to the laboratory-scale purification of AA from TGA25 oil. Screening for a lipase suitable for the first step, hydrolysis of TGA25 oil, showed that *Pseudomonas* lipase (Lipase-PS) was the best among the commercial lipases tested. When a mixture of TGA25/water (1:1, w/w) and 800 U/g of Lipase-PS was incubated at 40°C for 40 hr with stirring, the hydrolysis extent reached 89%, and 90% of AA was recovered in the FFA fraction. The FFAs originating from TGA25 oil was named TGA25-FFA. Selective esterification of TGA25-FFA was performed under the same conditions as those of esterification of Tuna-FFA and Borage-FFA using *Rhizopus* lipase as a catalyst. However, the lipase acted on AA moderately, and the fatty acid was not efficiently enriched. Screening for a lipase acting on AA very weakly selected *C. rugosa* lipase (Lipase-OF) as a suitable enzyme. After investigating several factors affecting selective esterification, the reaction conditions were determined as follows: A reaction mixture of TGA25-FFA/lauryl alcohol (1:1, mol/mol), 30% water, and 200 U/g of *Candida* lipase was incubated at 30°C with stirring [93].

Enrichment of AA was attempted by using 100 g TGA25 oil as a starting material (Table 12). TGA25-FFA was prepared from the hydrolysate of the oil with Lipase-PS. The selective esterification of the resulting FFAs raised the AA content in the FFA fraction from 25% to 51% with a recovery of 92%. Because *Candida* lipase acted on GLA, dihomo-GLA, behenic acid, and lignoceric acid as weakly as on AA, these fatty acids were also enriched in the unesterified fatty acid fraction. The lipase activity on stearic acid was somewhat weak, and the content was not decreased. Unesterified FFA mixture extracted

Table 12 Purification of n-6 PUFA Rich in AA by Two-Step Enzymatic Method

Step	Degree of reaction (%)	Fatty acid composition (%)[a]									Recovery of arachidonic acid[b] (%)
		16:0	18:0	18:1	18:2	18:3	20:3	20:4	22:0	24:0	
TGA25 oil[c]	—	13.9	6.2	13.7	24.2	2.1	3.2	24.6	2.0	4.1	100
Hydrolysis[d]	90.1	13.7	6.1	13.4	23.6	2.4	3.1	24.9	1.9	3.8	91.2
Esterification[e]	55.3	5.2	6.7	3.5	6.5	4.0	6.1	51.0	3.8	7.5	83.5
Urea adduct	—	4.5	2.9	3.5	6.4	5.1	7.9	63.0	nd[f]	nd	78.0
Esterification[e]	23.0	1.8	2.8	1.4	2.5	5.4	8.9	74.9	nd	nd	71.4

[a] Fatty acid composition in the FFA fraction.
[b] The recovery was calculated by assuming that FFAs were completely recovered.
[c] Fatty acid composition of TGA25 oil.
[d] A mixture of TGA25 oil/water (1:1, w/w) and 800 U/g of Lipase-PS was stirred at 40°C for 40 hr.
[e] A mixture of TGA25-FFA/lauryl alcohol (1:1, mol/mol), 30% water and 200 U/g *C. rugosa* lipase was stirred at 30°C for 16 hr.
[f] nd = Not determined.

with *n*-hexane was in the solid state at 30°C because of the presence of long-chain saturated fatty acids. Thus, the conventional urea adduct formation was conducted to eliminate these saturated fatty acids. As a result, behenic and lignoceric acids were completely eliminated, and a part of palmitic and stearic acids were also removed with an AA recovery of 93%. To further elevate the AA, the selective esterification was performed again. The esterification extent was 23% and the AA content was raised to 75% with a recovery of 71% of the initial content. The total content of the n-6 series of PUFA was raised from 30% to 89%.

In the purification of a PUFA, it is effective to use the oil containing a higher concentration of PUFA as a starting material. Therefore, we attempted the purification of a n-6 PUFA mixture rich in AA using 40% AA-containing single-cell oil (TGA40 oil) as a starting material. After hydrolysis of TGA40 oil, FFAs recovered were applied to the urea adduct fractionation. The resulting liquid-state FFAs were selectively esterified twice. As a result, the AA content was raised to 81% with a recovery of 80% of the initial content, and the total content of n-6 PUFA was 96% [85].

6 CONCLUSION

We have described the enrichment of PUFA by the selective hydrolysis and esterification. Selective hydrolysis in an organic solvent-free system is very useful for the industrial production of PUFA-rich oil as a food, because the main components of undigested glycerides are triglycerides and film distillation is available for the separation of the product. The purification of PUFA by the two-step enzymatic method has the following advantages: 1) Enzyme reaction under mild conditions suppresses denaturation of PUFA (e.g., isomerization, oxidation, epoxydation, polymerization, and so on). 2) Ethanol is unnecessary for hydrolysis of oil with a lipase. The hydrolysis with chemical catalyst requires a large amount of ethanol, and the treatment of the wastewater containing ethanol is a serious problem in the industrial process. 3) Because selective esterification with lauryl alcohol does not require any organic solvent, the reaction scale can be made small and the risk of explosion can be avoided. 4) The esterification proceeds effectively even in the reaction mixture containing 20% water, so it is not necessary to remove the water generated by esterification as well as the water in the substrates. 5) The conventional distillation and urea adduct fractionation is available for the separation of free PUFA from the enzyme reaction mixture. In view of these advantages, we believe that the two-step enzymatic method may be suitable for purifying PUFA industrially.

REFERENCES

1. K Hara. Yushi 46(1):91–99, 1993 (in Japanese).
2. MA Belury, KE Patrick, M Locniskar, SM Fischer. Lipids 24:423–429, 1989.
3. ML Garg, ABR Thomson, MT Clandinin. J Lipid Res 31:271–277, 1990.
4. ML Garg, EB Bosschietor, CDL Coulander. N Engl J Med 312:1205–1209, 1985.
5. T Kanayasu, I Morita, J Nakao-Hayashi, N Asuwa, N Fujisawa, T Ishii, M Ito, S Murota. Lipids 26:271–276, 1991.
6. BE Phillipson, DW Rothrock, WE Connor, WS Harris, DR Illingworth. N Engl J Med 312: 1210–1216, 1985.
7. T Lee, RL Hoover, JD Williams, RI Sperling, J Ravalese, BW Spur, DR Robinson, EJ Corey, RA Lewis, KF Austen. N Engl J Med 312:1217–1224, 1985.

8. MG Bravo, RJ Antueno, J Toledo, ME Tomas, OF Mercuri, C Quintans. Lipids 26:866–870, 1991.
9. W Stillwell, W Ehringer, LJ Jenski. Lipids 28:103–108, 1993.
10. R Uauy, I DeAndraca. J Nutr 125:2278–2280, 1995.
11. M Nuringer, WE Conner, CV Petten, L Barstad. J Clin Invest 73:272–276, 1984.
12. SE Carlson, SH Werkman, JM Peeples, RJ Cooke, EA Tolley. Proc Natl Acad Sci USA 90: 1073–1077, 1993.
13. CI Lanting, V Fidler, M Huisman, BCL Touwen, ER Boersma. Lancet 344:1319–1322, 1994.
14. K Maruyama, M Nishikawa. Food Chem 1995(4):31–37, 1995 (in Japanese).
15. RR Brenner, ME De Tomas, RO Peluffo. Biochim Biophys Acta 106:640–642, 1965.
16. YL Marcel, K Christiansen, RT Holman. Biochim Biophys Acta 164:24–34, 1968.
17. P Borgeat, M Nadeau, H Salari, P Poubelle, BF De Laclos. In: R Paoletti, D Kritchevsky eds., Advance in Lipid Research, Vol. 21. New York: Academic Press, 1985, pp 47–77.
18. JS Goodwin, J Ceuppens. J Clin Immunol 3:295–308, 1983.
19. D Wu, SN Meydani. In: YS Huang, DE Mills, eds. γ-Linolenic Acid: Metabolism and Its Roles in Nutrition and Medicine. Champaign, IL: AOCS Press, 1996, pp 106–117.
20. MS Manku, DF Horrobins, NL Morse, S Wright, JL Burton. Br J Dermatol 110:643–648, 1984.
21. R Lindskov, G Holmer. Allergy 47:517–521, 1992.
22. CC Miller, VA Ziboh. Biochem Biophys Res Commun 154:967–974, 1988.
23. A Fiocchi, M Sala, P Signoroni, G Banderai, C Agostoni, E Riva. J Intern Med Res 22:2–32, 1994.
24. A Bordoni, PL Biagi, M Masi, G Ricci, C Fanelli, A Patrizi, E Ceccolini. Drugs Exp Clin Res 14:291–297, 1988.
25. RB Zurier, P Deluca, D Rothman. In: YS Huang, DE Mills, eds. γ-Linolenic Acid: Metabolism and Its Roles in Nutrition and Medicine. Champaign, IL: AOCS Press, 1996, pp 129–136.
26. J Jantti, E Seppala, H Vapaatalo, H Isomaki. Clin Rheumatol 8:238–244, 1989.
27. AJ Barber, Pharmaceut J 240:723–725, 1988.
28. GL Crozier, MC Secretin. In: YS Huang, DE Mills, eds. γ-Linolenic Acid: Metabolism and Its Roles in Nutrition and Medicine. Champaign, IL: AOCS Press, 1996, pp 246–251.
29. GD Minno, AM Cerbone, A Postiglione. In: R Paoletti, D Kritchevsky, eds. Advance in Lipid Research, Vol. 22. New York: Academic Press, 1987, pp 63–82.
30. Y Shinmen, S Shimizu, K Akimoto, H Kawashima, H Yamada. Appl Microbiol Biotechnol 31:11–16, 1989.
31. WA Devane, L Hanus, A Breuer, RG Pertwee, LA Stevenson, G Griffin, D Gibson, A Mandelbaum, A Etinger, R Mechoulam. Science 258:1946–1949, 1992.
32. N Stella, P Schweltzer, D Plomelli. Nature 388:773–778, 1997.
33. T Yaname. J Am Oil Chem Soc 64:1657–1660, 1987.
34. M Iwai, Y Tsujisaka, J Fukumoto. J Gen Appl Microbiol 10:13–22, 1964.
35. CY Lee, JJ Iandolo. J Bacteriol 166:385–391, 1986.
36. F Götz, F Popp, E Korn, H Schleifer. Nucleic Acids Res 13:5895–5906, 1985.
37. S Jøgensen, KW Skov, B Diderichsen. J Bacteriol 173:559–567, 1991.
38. LGL Frenken, MR Egmond, AM Batenburg, JW Bos, C Visser, CT Verrips. Appl Environ Microbiol 58:3787–3791, 1992.
39. M Chihara-Shiomi, K Yoshikawa, N Oshima-Hirayama, K Yamamoto, Y Sogabe, T Nakatani, T Nishioka, J Oda. Arch Biochem Biophys 296:505–513, 1992.
40. T Iizumi, K Nakamura, Y Shimada, A Sugihara, Y Tominaga, T Fukase. Agric Biol Chem 55:2349–2357, 1991.
41. F Ihara, Y Kageyama, M Hirata, T Nihira, Y Yamada. J Biol Chem 266:18,135–18,140, 1991.
42. GH Chung, YP Lee, GH Jeohn, OJ Yoo, JS Rhee. Agric Biol Chem 55:2359–2365, 1991.
43. H Akatsuka, E Kawai, K Omori, S Komatsubara, T Shibatani, T Tosa. J Bacteriol 176:1949–1956, 1994.

44. Y Kawaguchi, H Honda, J Taniguchi-Morimura, S Iwasaki. Nature 341:164–166, 1989.
45. Y Shimada, A Sugihara, Y Tominaga, T Iizumi, S Tsunasawa. J Biochem 106:383–388, 1989.
46. Y Shimada, A Sugihara, T Iizumi, Y Tominaga. J Biochem 107:703–707, 1990.
47. E Boel, B Huge-Jensen, M Christensen, L Thim, NP Fiil. Lipids 23:701–706, 1988.
48. MJ Haas, J Allen, R Berka. Gene 109:107–113, 1991.
49. W Kugimiya, Y Otani, M Kohno, Y Hashimoto. Agric Biol Chem 56:716–719, 1992.
50. T Nagao, Y Shimada, A Sugihara, T Tominaga. J Biochem 116:536–540, 1994.
51. E Boel, B Huge-Jensen. European Patent 0,305,216,A1, 1989.
52. S Yamaguchi, T Mase, K Takeuchi. Gene 103:61–67, 1989.
53. Y Shimada, A Sugihara, Y Tominaga. In: Y Murooka, T Imanaka, eds. Recombinant Microbes for Industrial and Agricultural Applications. New York: Marcel Dekker, 1994, pp 359–371.
54. Y Shimada, A Sugihara, Y Tominaga. In: AB Christophe, ed. Structural Modified Food Fats: Synthesis, Biochemistry, and Use. Champaign, IL: AOCS Press, 1998, pp 73–88.
55. Y Tsujisaka, M Iwai, Y Tominaga. In: G Terui, ed. Fermentation Technology Today. Osaka: Society of Fermentation Technology, 1972, pp 315–320.
56. M Matori, Y Kushida, S Adachi, Y Ota. Yukagaku 41:347–348, 1992 (in Japanese).
57. Y Shimada, A Sugihara, T Nagao, S Kitai, M Nakamura, S Gemba, Y Tominaga. Kagaku to Kogyo 69:95–99, 1995 (in Japanese).
58. Y Shimada, A Sugihara, K Maruyama, T Nagao, S Nakayama, H Nakano, Y Tominaga. J Am Oil Chem Soc 72:1323–1327, 1995.
59. Y Shimada, A Sugihara, M Shibahiraki, H Fujita, H Nakano, T Nagao, T Terai, Y Tominaga. J Am Oil Chem Soc 74:1465–1470, 1997.
60. Y Shimada, K Maruyama, S Okazaki, M Nakamura, A Sugihara, Y Tominaga. J Am Oil Chem Soc 71:951–954, 1994.
61. Y Shimada, A Sugihara, H Nakano, T Nagao, M Suenaga, S Nakai, Y Tominaga. J Ferment Bioeng 83:321–327, 1997.
62. S Okumura, M Iwai, Y Tsujisaka. Agric Biol Chem 40:655–660, 1976.
63. A Sugihara, Y Shimada, Y Tominaga. Appl Microbiol Biotechnol 35:738–740, 1991.
64. A Sugihara, S Hata, Y Shimada, K Goto, S Tsunasawa, Y Tominaga. Appl Microbiol Biotechnol 40:279–283, 1993.
65. A Sugihara, Y Shimada, M Nakamura, T Nagao, Y Tominaga. Protein Eng 7:585–588, 1994.
66. Y Shimada, C Koga, A Sugihara, T Nagao, N Takada, S Tsunasawa, Y Tominaga. J Ferment Bioeng 75:349–352, 1993.
67. BF Haumann. INFORM 8:1004–1011, 1997.
68. MS Christensen, CE Hoy, CC Becker, TG Redgrave. Am J Clin Nutr 61:56–61, 1995.
69. I Ikeda, Y Tomari, M Sugano, S Watanabe, J Nagata. Lipids 26:369–373, 1991.
70. Y Shimada, A Sugihara, K Maruyama, T Nagao, S Nakayama, H Nakano, Y Tominaga. J Ferment Bioeng 81:299–303, 1996.
71. Y Shimada, A Sugihara, H Nakano, T Yokota, T Nagao, S Komemushi, Y Tominaga. J Am Oil Chem Soc 73:1415–1420, 1996.
72. Y Shimada, M Suenaga, A Sugihara, S Nakai, Y Tominaga. J Am Oil Chem Soc 76:189–193, 1999.
73. CC Akoh. INFORM 6:1055–1061, 1996.
74. CC Akoh, LN Yee. J Am Oil Chem Soc 74:1409–1413, 1997.
75. MM Soumanou, UT Bornscheuer, RD Schmid. J Am Oil Chem Soc 75:703–710, 1998.
76. Y Tanaka, T Funada, J Hirano, R Hashizume. J Am Oil Chem Soc 70:1031–1034, 1993.
77. Y Shimada, K Maruyama, M Nakamura, S Nakayama, A Sugihara, Y Tominaga. J Am Oil Chem Soc 72:1577–1581, 1995.
78. S Yamaguchi, T Mase. Appl Microbiol Biotechnol 34:720–725, 1991.
79. T Hoshino, T Yamane, S Shimizu. Agric Biol Chem 54:1459–1467, 1990.
80. Y Tanaka, J Hirano, T Funada. J Am Oil Chem Soc 69:1210–1214, 1992.

81. MSK Syed Rahmatullah, VKS Shukla, KD Mukherjee. J Am Oil Chem Soc 71:569–573, 1994.
82. Y Shimada, N Fukushima, H Fujita, Y Honda, A Sugihara, Y Tominaga. J Am Oil Chem Soc 75:1581–1586, 1998.
83. H Kawashima, E Sakuradani, N Kamada, K Akimoto, K Konishi, J Ogawa, S Shimizu. J Am Oil Chem Soc 75:1495–1500, 1998.
84. K Higashiyama, T Yaguchi, K Akimoto, S Fujikawa, S Shimizu. J Am Oil Chem Soc 75: 1501–1506, 1998.
85. Y Shimada, T Nagao, A Kawashima, A Sugihara, S Komemushi, Y Tominaga. Kagaku to Kogyo 73:125–130, 1999 (in Japanese).
86. Y Yamamura, Y Shimomura. J Am Oil Chem Soc 74:1435–1440, 1997.
87. M Yamaguchi, I Tanaka, Y Ohtsu. Yukagaku 40:959–964, 1991 (in Japanese).
88. MJ Hills, I Kiewitt, KD Mukherjee. J Am Oil Chem Soc 67:561–564, 1990.
89. TA Foglia, PE Sonnet. J Am Oil Chem Soc 72:417–420, 1997.
90. Y Shimada, A Sugihara, H Nakano, T Kuramoto, T Nagao, M Gemba, Y Tominaga. J Am Oil Chem Soc 74:97–101, 1997.
91. FC Huang, YH Ju, CW Huang. J Am Oil Chem Soc 74:977–981, 1997.
92. Y Shimada, K Maruyama, A Sugihara, S Moriyama, Y Tominaga. J Am Oil Chem Soc 74: 1441–1446, 1997.
93. Y Shimada, A Sugihara, Y Minamigawa, K Higashiyama, K Akimoto, S Fujikawa, S Komemushi, Y Tominaga. J Am Oil Chem Soc 75:1213–1217, 1998.
94. Y Shimada, N Sakai, A Sugihara, H Fujita, Y Honda, Y Tominaga. J Am Oil Chem Soc 75: 1539–1543, 1998.
95. Y Tanaka, T Funada, J Hirano. Yukagaku 41:563–567, 1992 (in Japanese).
96. S Yamaguchi, T Mase. J Ferment Bioeng 72:162–167, 1991.
97. K Liu, J Shaw. J Am Oil Chem Soc 72:1271–1274, 1995.

25

Production of Tailor-Made Polyunsaturated Phospholipids Through Bioconversions

KORETARO TAKAHASHI and MASASHI HOSOKAWA

Graduate School of Fisheries Sciences, Hokkaido University, Hakodate, Hokkaido, Japan

1 INTRODUCTION

Long-chain polyunsaturated fatty acids (PUFAs), such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), have received a great deal of attention concerning their health benefits. Uptake studies of PUFA indicate that PUFA is a more beneficial component of phospholipids than of triglycerides or ethyl esters [1–3]. When PUFA resided at the secondary position (*sn*-2 position) of a phospholipid molecule, PUFA is especially effective. Accordingly, tailor-made phospholipids may provide alternative nutritional, medical, and industrial uses. Apparently, the polar head, different species, and binding position of fatty acid moieties play important roles in the expression of biological functions. For example, phospholipids containing DHA at the *sn*-2 position are known to enhance survivals of tumor-bearing mouse [4] and to promote cell differentiation of erythroleukemia cancer cells [5]. Also, phospholipids containing EPA are known to decrease the weight of adipose tissue among the major organs [6]. Many other health benefits have also been reported on n-3 PUFA-bound phospholipids [7–11].

Tailor-made phospholipid applications other than n-3 PUFA bound phospholipids have been reviewed by D'Arrigo and Servi [12]. Unlike chemical synthesis, enzymatic bioconversions attempt to avoid by-products which are often harmful to animals and difficult to separate. Soybean phospholipids, corn phospholipids, and egg-yolk phospholipids are economical raw materials for the glycerophospholipid backbone, which incorporates PUFA at the *sn*-2 position. In this chapter, we describe the biocatalytic approach for pre-

paring tailor-made n-3 PUFAs bound to glycerophospholipid from convenient raw materials.

2 SYNTHESIS OF n-3 PUFA-BOUND PHOSPHOLIPID BY BIOCATALYTIC REACTIONS

In 1986, Yoshimoto et al. [13] succeeded in synthesizing EPA-bound phosphatidylcholine by using a polyethylene glycol–modified *Candida cylindracea* lipase in a transesterification reaction. The biocatalysis occurred in a water-saturated benzene solvent. Recovery of n-3 PUFA-bound phospholipid was limited (3%), and the conversion from dipalmitoylphosphatidylcholine to EPA-bound phosphatidylcholine was 8%. The uneconomical modification process of lipase was also discouraging. Na et al. [1] used a microemulsion system to synthesize n-3 PUFA-bound phosphatidylcholine as mediated by a phospholipase-catalyzed esterification. However, the yield of product was low. Totani and Hara [14] proposed a one-step reaction to produce n-3 PUFA-bound phospholipids. They used a high n-3 PUFA-containing triglyceride (sardine oil) as acyl donor and soybean phospholipid as substrate. The total percentage of n-3 PUFA incorporated into soybean phospholipid was 18.4%. Overall, a concomitant side reaction of hydrolysis, which seriously impaired the recovery or yield, was unavoidable.

3 WATER ACTIVITY OF LIPASE AND PHOSPHOLIPASE FOR PREPARING n-3 PUFA-BOUND PHOSPHOLIPIDS

Several studies [15–20] have demonstrated that water activity is crucial for bioconversions in organic solvents. Valivety et al. [15] showed that different enzymes require different levels of water activity to give optimum bioconversions. Adlercreutz [16,17] and Valivety et al. [18] demonstrated that supporting materials on which enzymes were adsorbed and different solvent polarity also affected the optimum water activity. For optimum reactivity, less water is required in hydrophobic solvents, such as *n*-hexane and isooctane, than in hydrophilic solvent. In hydrophobic mixtures, most of the water apparently came from a hydrated immobilized enzyme [19]. Therefore, the localized water at the active site or water activity is considered to be crucial. It is suggested that the water activity determined the degree of enzyme hydration and, consequently, its catalytic activity [20]. Water activities beyond the optimum point would lead to hydrolytic reactions, which decreased the yield of n-3 PUFA-bound product.

4 PHOSPHOLIPASE A_2 AND LIPASE TO TAILOR-MADE n-3 PUFA-BOUND PHOSPHATIDYLCHOLINE

Two kinds of enzyme could be used to incorporate n-3 PUFA into α-lysophosphatidylcholine that was prepared from soybean phosphatidylcholine. As illustrated in Figure 1 (unpublished data), optimal yield of the phospholipase A_2-mediated system was higher than the lipase-mediated system when lysophospholipid was a substrate. (When transesterification was carried out with diacylphospholipids, however, lipase was more efficient than phospholipase A_2 [21]). The extent of acyl incorporation was better and n-3 PUFA was incorporated exclusively into the *sn*-2 position of lysophosphatidylcholine when using the phospholipase A_2-mediated system. Therefore, it is noteworthy that phospholipase A_2

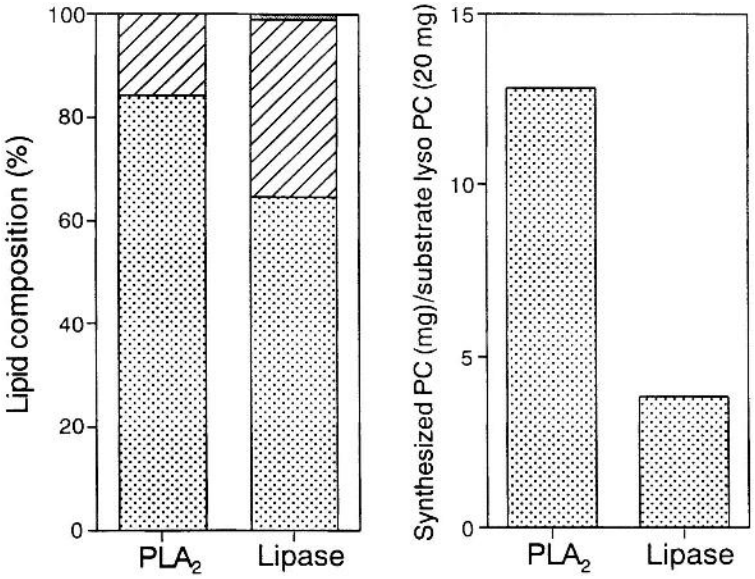


Figure 1 Comparison of phospholipase A_2 with lipase for the synthesis of phosphatidylcholine by the esterification of lysophosphatidylcholine. Phospholipase A_2 (PLA_2) mixture contained 54.5 mg PLA_2, 100 mg lysophosphatidylcholine (lyso PC; produced from soybean lecithin hydrolysate), 163 mg EPA, 5000 mg glycerol, 0.45 mL formamide, and 2.7 μmol $CaCl_2$. The reaction included 40°C temperature, 1000 rpm agitation rate, and 48 hr incubation. Lipase mixture contains 2 mL toluene, 20 mg lysophosphatidylcholine, 180 mg EPA, and 30 mg dehydrated Novozyme 435 (Novo Nordisk Bioindustrials, Inc.). The lipase-mediated reaction was conducted at 40°C with 75 strokes/min agitation and 72 hr incubation. Symbols: ⬚, PC; ◪, lyso PC.

produces n-3 PUFA-bound phosphatidylcholine with n-3 PUFA residing at the *sn*-2 position exclusively.

Phospholipase A_2 can be used either for esterification of lysophosphatidylcholine or for transesterification of phosphatidylcholine. The phospholipase A_2-mediated reaction shows an inverse relationship between the incorporation of n-3 PUFA and the yield of the desired phosphatidylcholine, which is illustrated in Figure 2 [22].

Egger et al. [20] characterized the relationship between the synthetic and the hydrolytic reactions at different water activities of phospholipase A_2-mediated systems. Figure 3 shows that the final phospholipid composition of the reaction mixtures after both synthesis and hydrolysis have occurred at the same level when the equilibrium point of water activity and phospholipase A_2 is reached.

Egger et al. [20] proposed a water-activity gradient technique in order to avoid a concomitant side reaction of hydrolysis, which impaired the yield. Starting with a high water activity, one can obtain a correspondingly high reaction rate. The water activity can then be decreased to achieve higher product yields. This technique with a three-step water-activity gradient is described in Figure 4, giving a 60% yield of n-3 PUFA-bound phosphatidylcholine. Another technique to avoid the undesirable hydrolysis is to use water mimics for enzyme activation. Although water mimics activate lipases and phospholipases, they will not participate in hydrolysis like a water substrate. For this reason, hydrolysis is suppressed considerably. Polar solvents with high dielectric points appear to be necessary

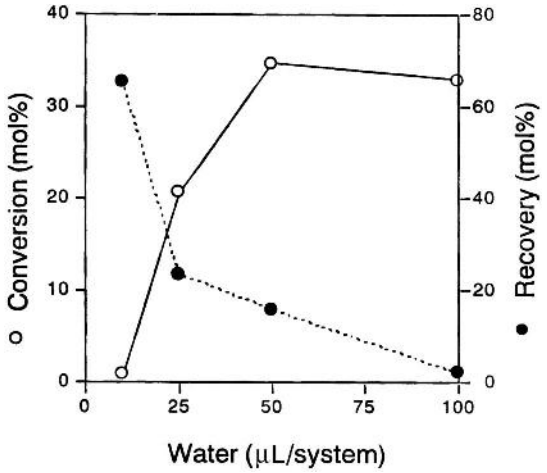


Figure 2 Effect of water content on phospholipase A_2-mediated transesterification between soybean phosphatidylcholine and EPA. Reaction was with phospholipase A_2 10 mg, soybean phosphatidylcholine 14 μmol, EPA 60 μmol, glycerol 550 mg, and varying levels of water for 48 h at 25°C. Recovery (%) = (mg recovered PC/mg applied PC before acidolysis) × 100. Conversion (%): incorporation rate of EPA into PC was defined as the EPA increase in PC fatty acid composition. (From Ref. 22.)

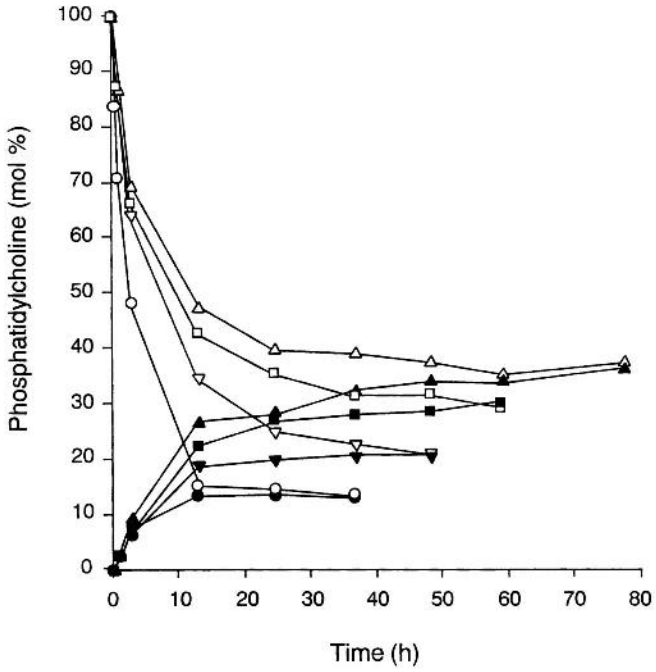


Figure 3 Time course of the synthesis (filled symbols) and the hydrolysis (open symbols) reactions of phospholipid at different water activities. The phospholipid concentration was 10 m*M* and oleic acid 800 m*M* in both the synthesis and hydrolysis reactions. The reactions were conducted at 25°C. Water activities: 0.22 (△, ▲); 0.33 (□, ■); 0.43 (▽, ▼); 0.53 (○, ●). (From Ref. 20.)

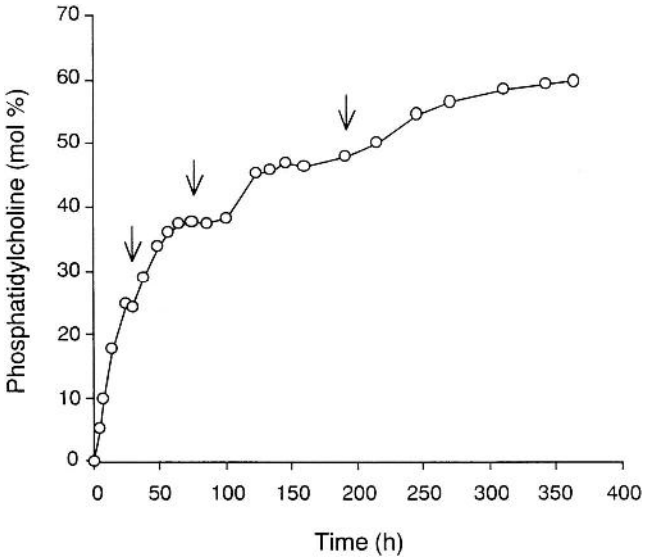


Figure 4 Time course for the synthesis of phospholipid by stepwise adjustments of water activity. Lysophosphatidylcholine concentration was 10 m*M* and oleic acid was 1.8 *M*. The reaction mixture with toluene in a volume of 3 mL contains 150 mg phospholipase A_2. The reactions were conducted at 25°C. The water activity was 0.43 initially and decreased to 0.33, 0.22, and 0.11 stepwise by placing the reaction vial into salt containers with corresponding water activities. The arrows indicate stepwise times (hours) when the reaction vial was transferred to a salt container with lower water activity. (From Ref. 20.)

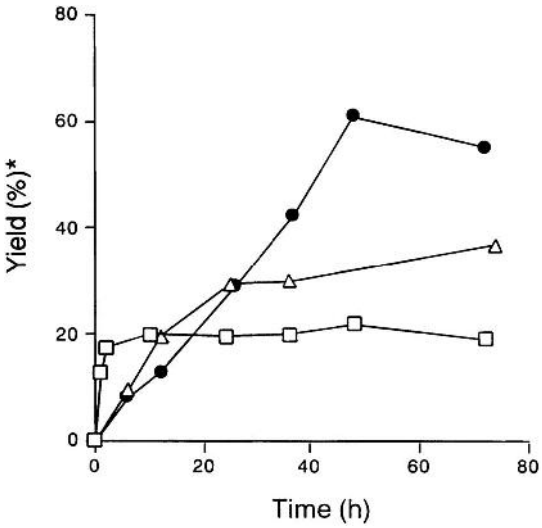


Figure 5 Boosting effect of formamide on phospholipase A_2-mediated esterifications. Reaction mixture: 23 mg phospholipase A_2, 110 mg lysophosphatidylcholine, 180 mg EPA, 5500 mg glycerol (moisture 0.15% in weights), and 3 μmol $CaCl_2$ at 25°C and 800 rpm agitation rate. Symbols: (●) formamide, 0.5 mL; (△) 0.2 *M* Tris-HCl buffer (pH 8.0), 0.2 mL; (□) 0.2 *M* Tris-HCl, 0.5 mL. *Yield (%) = (mg synthesized PC/mg substrate lyso PC) × 100. (From Refs. 23 and 24.)

in order to mimic water. Figure 5 shows that the formamide substitution for water results in a high yield and enhanced incorporation of EPA into lysophosphatidylcholine [23,24]. Degree of incorporation into the *sn*-2 position approximates the theoretical maximum level, giving a product yield of 60% by weight (about 40 mol%). In order to avoid oxidation of n-3 PUFA, the reaction temperature is kept at a constant 25°C. However, L-Hallberg and Härröd [19] suggested that reaction temperatures up to 60°C did not seriously affect the oxidation when the reaction was within 80 hr. Therefore, by combining a nitrogen gas treatment to purge undesirable oxygen from an antioxidant added substrate prior to the reaction, it is possible to obtain high yields at high temperatures, such as 60°C, and under vacuum. Heat denaturation of phospholipase A_2 is slight at this temperature because the enzyme is thermostable.

5 BIOCONVERSION AT THE *sn*-1 POSITION OF AN *sn*-2 PUFA-BOUND PHOSPHATIDYLCHOLINE

The inverse relationship between the recovery of phospholipid and PUFA incorporation rate at the *sn*-1 position is also observed in the transesterifications at the *sn*-1 position (Fig. 6). As the water activity increased, the conversion at the *sn*-1 position increased, especially at the early stage of the reaction. However, the recovery of the desired product decreased because of the concomitant hydrolysis [24]. Conversion of n-3 PUFA into phosphatidylcholine was also enhanced when nonaqueous solvents were used as water mimics (Fig. 7 [24]). The nonaqueous solvent used for this study was propylene glycol. A partial but not full substitution of propylene glycol for water gave the best transesterification.

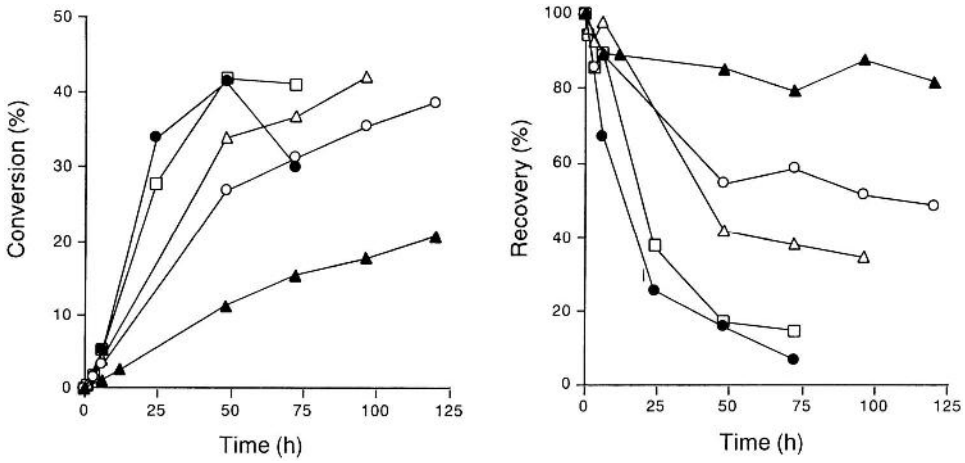


Figure 6 Effect of water activity on lipase-mediated transesterification for the incorporation of EPA into soybean phosphatidylcholine. Reaction mixture: *n*-hexane solution (0.5 mL) containing 10 mg soybean phosphatidylcholine, 60 mg EPA, and 40 mg Lipozyme IM-20 at 40°C temperature and with 75 strokes/min agitation. Water activities of Lipozyme IM-20: (▲) P_2O_5 dehydration; (○) 0.064; (△) 0.13; (●) 0.75; (□) no adjustment of water activity. Definitions of recovery and conversion are the same as in Figure 2. (From Ref. 24.)

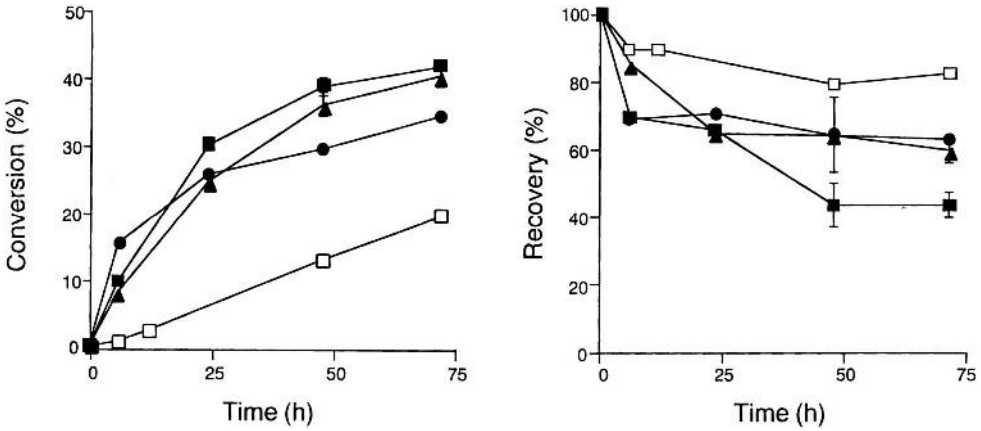


Figure 7 Effect of propylene glycol and water on lipase-mediated transesterification for the incorporation of EPA into soybean phosphatidylcholine. Reaction mixture: *n*-hexane solution (0.5 mL) containing 10 mg soybean phosphatidylcholine, 60 mg EPA, and 23 mg Lipozyme IM-60 at 40°C and with 75 strokes/min agitation. Symbols: (▲) propylene glycol + water (1:1, v/v), 1 μL; (●) propylene glycol, 1 μL; (■) water, 1 μL; (□) no water or water mimics. Definitions of recovery and conversion are the same as in Figure 2. (From Ref. 24.)

This observation was unlike the esterification mediated by phospholipases A_2 (Fig. 5), which was free of water and contained a formamide solvent. It was suggested that glycerol might act as a disperser of such substrates for lysophosphatidylcholine esterification (Fig. 5). Glycerol contains a small amount of water, approximately 0.15% by weight. Therefore, only a partial substitution of water mimic for water is required for the optimal effect. Excess water may act exclusively as a nucleophilic substrate for the hydrolysis rather than the esterification of desired fatty acids. It was suggested that water mimics facilitate the activation of enzyme without being a nucleophilic substrate like water and elude hydrolysis. Accordingly, lipase and phospholipase activate a net synthesis of EPA and DHA into glycerolipids in a nonaqueous medium containing appropriate amounts of water and water mimic.

6 APPLICATION OF A PARTIAL HYDROLYTIC REACTION FOR PRODUCING TAILOR-MADE PHOSPHOLIPIDS CONTAINING DHA

As has been depicted earlier, it is crucial for optimal synthesis of tailor-made phospholipids that the water activity of the reaction mixture be adjusted with a proper addition of water mimic. Without a water mimic, the water activity of such lipase as Lipozyme (immobilized lipase produced by Novo Nordisk Bioindustrials, Inc.) is adjusted to a critical intermediate level. Under the suitable level of water activity to initiate a partial hydrolytic reaction of phospholipids containing small amounts of DHA, the DHA-enriched phospholipids can be obtained [25]. Figure 8 shows that initial water activity of approximately 0.44 is desirable for the partial hydrolytic reaction with this hydrophobic solvent system.

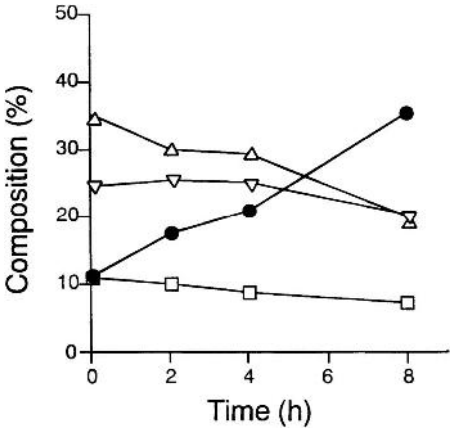


Figure 8 Time course of the concentration of DHA on the acyl moiety of phosphatidylcholine that has small amounts of acylated DHA by partial hydrolysis with a specific lipase. Reaction mixture: *n*-hexane solution (2 mL) containing 20 mg of egg-yolk phosphatidylcholine from hens fed with fish oil and 53 mg of Lipozyme IM at 40°C and with 75 strokes/min agitation. Symbols: (△) palmitic acid; (▽) oleic acid; (□) stearic acid; (●) DHA. (From Ref. 25.)

7 MODIFICATION OF POLAR HEAD OF PHOSPHOLIPID CONTAINING n-3 PUFA

Beneficial functionalities of phosphatidylserine for brain are of current interest. Accordingly, phosphatidylserine-containing n-3 PUFA, especially DHA, at the *sn*-2 position may

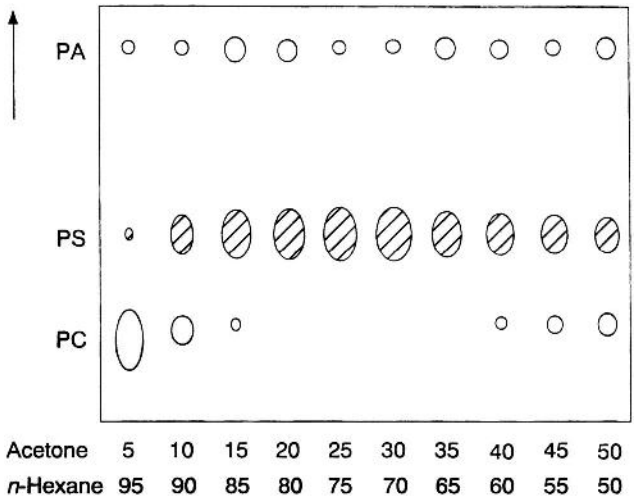


Figure 9 Effect of *n*-hexane/acetone solvent mixtures on phospholipase D-mediated transphosphatidylation, converting the choline head group into serine as analyzed by thin-layer chromatography. Reaction mixture: various ratios of *n*-hexane/acetone solvent (2.5 mL) containing 20 mg of squid skin phosphatidylcholine, 1 mL of 3.4 *M* serine, and 32.4 units of phospholipase D (Asahi Chemical Industry Co., Ltd.) at 40°C and with 75 strokes/min agitation for 1 hr. Abbreviations: PA, phosphatidic acid; PS, phosphatidylserine; PC, phosphatidylcholine.

be useful for nutritional and pharmaceutical purposes. This highly tailor-made phospholipid can be obtained via a transphosphatidylation reaction using squid skin phospholipid, which contains >30% total fatty acids as DHA. Kaneda et al. (unpublished data) found that using *n*-hexane together with 20–30% acetone as a solvent mixture favored a transphosphatidylation that was mediated by *Streptomyces* phospholipase D (Asahi Chemical Industry Co., Ltd.). Figure 9 confirms that the phosphatidylcholine compound was transphosphatidylated completely to phosphatidylserine and phosphatidic acid compounds within 1 hr.

8 PRODUCTION OF DI-DHA-BOUND PHOSPHOLIPIDS

It is feasible to produce a wide variety of glycerophospholipid molecular species through bioconversions, as has been depicted. However, a di-DHA phospholipid compound was difficult to produce. Recently, Hirano et al. [26] had shown that a di-DHA phosphatidylcholine could be biosynthesized from an *sn*-2 DHA-bound lysophosphatidylcholine using lysophosphatidylcholine/transacylase enzyme from bonito muscle cytosol for the bioconversion. A notable feature of this enzymatic system is that its synthetic activity exceeds hydrolytic activity even when the substrate concentration is low (e.g., when the substrate and critical micelle concentrations are similar.) However, a large-scale synthesis by this method seems impractical due to a limited source of the enzyme.

Because tailor-made n-3 PUFA glycerophospholipids are now available, studies can be conducted to determine their therapeutic potential and their role in cellular function.

ACKNOWLEDGMENTS

Permissions granted from AOCS Press [Fig. 2, p. 1289, J Am Oil Chem Soc 72(11), 1995], Elsevier Science (Figs. 6 and 10, pp. 81 and 83, Biochim Biophys Acta 1343, 1997), Chapman & Hall [Fig. 3, p. 585, Biotechnol Tech 12(8), 1998], Harwood Academic Publishers [phrases, pp. 55 and 58, Biocatal Biotransform 12(1), 1995], and Japan Oil Chemists' Society [Fig. 3, p. 870, J Jpn Oil Chem Soc 46(8), 1997]. Original sources are gratefully acknowledged.

REFERENCES

1. A Na, C Eriksson, S-G Eriksson, E Österberg, K Holmberg. J Am Oil Chem Soc 67:766–770, 1990.
2. C Galli, CR Sirtori, C Mosconi, L Medini, G Gianfranceschi, V Vaccarino, C Scolastico. Lipids 27:1005–1012, 1992.
3. BC O'Brien, VG Andrews. Lipids 28:7–12, 1993.
4. LJ Jenski, M Zerouga, W Stillwell. Proc Soc Exp Biol Med 210:227–233, 1995.
5. M Suzuki, K Asahi, K Isono, A Sakurai, N Takahashi. Develop Growth Differ 34:301–307, 1992.
6. K Yazawa, K Watanabe, C Ishikawa, K Kondo, S Kimura. In: DJ Kyle, C Ratledge, eds. Industrial Application of Single Cell Oils. Champaign, IL: American Oil Chemists Society, 1992, pp 29–51.
7. JS Parks, TY Thuren, JD Schmitt. J Lipid Res 33:879–887, 1992.
8. O Mori, T Suzuki, K Takama. Effect of phospholipid type ω-3 PUFA feeding on reducing levels of plasma cholesterol and triglyceride. Abstracts Book 1 on XV International Congress of Nutrition, Adelaide, 1993, p 308.

9. M Ono, M Hosokawa, K Takahashi, Y Inoue. Hemorheol Related Res 1:7–11, 1998 (in Japanese).
10. K Tochisawa, M Hosokawa, H Kurihara, H Kohno, S Odashima, K Takahashi. J Jpn Oil Chem Soc 46:382–390, 1997.
11. K Matsumoto, I Morita, H Hibino, S Murota. Prostaglandins Leukot Essent Fatty Acids 49: 861–866, 1993.
12. P D'Arrigo, S Servi. Tibtech 15:90–96, 1997.
13. T Yoshimoto, M Nakata, S Yamaguchi, T Funada, Y Saito, Y Inada. Biotechnol Lett 8:771–776, 1986.
14. Y Totani, S Hara. J Am Oil Chem Soc 68:848–851, 1991.
15. RH Valivety, PJ Halling, AR Macrae. In: J Tramper, ed. Effect of Water Activity on Rate of Lipase-Catalyzed Esterification. Amsterdam: Elsevier, 1992, pp 549–555.
16. P Adlercreutz. Eur J Biochem 199:609–614, 1991.
17. P Adlercreutz. Biochim Biophys Acta 1163:144–148, 1993.
18. RH Valivety, PJ Halling, AR Macrae. Biochim Biophys Acta 1118:218–222, 1992.
19. M L-Hallberg, M Härröd. Biocatal Biotransform 12:55–66, 1995.
20. D Egger, E Wehtje, P Adlercreutz. Biochem Biophys Acta 1343:76–84, 1997.
21. A-M Aura, P Forssell, A Mustranta, K Poutanen. J Am Oil Chem Soc 72:1375–1379, 1995.
22. M Hosokawa, M Ito, K Takahashi. Biotechnol Techniques 12:583–586, 1998.
23. M Hosokawa, K Takahashi, Y Kikuchi, M Hatano. J Am Oil Chem Soc 72:1287–1291, 1995.
24. M Hosokawa. Enzymatic Synthesis of Phospholipids Containing Polyunsaturated Fatty Acids and Their Physiological Functions. PhD dissertation, Hokkaido University, Hakodate, Japan, 1996 (in Japanese).
25. M Ono, M Hosokawa, Y Inoue, K Takahashi. J Jpn Oil Chem Soc 46:867–872, 1997.
26. K Hirano, E Okada, T Tanaka, K Satouchi. Proceeding of the Japanese Conference on the Biochemistry of Lipids, Kamakura, 1999, pp 139–142 (in Japanese).

26

Enzymatic Preparation of Chiral Pharmaceutical Intermediates by Lipases

RAMESH N. PATEL

Bristol–Myers Squibb Pharmaceutical Research Institute, New Brunswick, New Jersey

1 INTRODUCTION

Currently, much attention has been focused on the interaction of small molecules with biological macromolecules. The search for selective enzyme inhibitors and receptor agonists or antagonists is one of the keys for target-oriented research in the pharmaceutical industry. Increasing understanding of the mechanism of drug interaction on a molecular level has led to the increasing awareness of the importance of chirality as the key to the efficacy of many drug products. It is now known that, in many cases, only one stereoisomer of a drug substance is required for efficacy and the other stereoisomer is either inactive or exhibits considerably reduced activity. Pharmaceutical companies are aware that, where appropriate, new drugs for the clinic should be homochiral to avoid the possibility of unnecessary side effects due to an undesirable stereoisomer. In many cases where the switch from racemate drug substance to enantiomerically pure compound is feasible, there is the opportunity to double the use of an industrial process. The physical characteristic of enantiomers versus racemates may confer processing or formulation advantages.

Chiral drug intermediates can be prepared by different routes. One is to obtain them from naturally occurring chiral synthons produced mainly by fermentation processes. The chiral pool primarily refers to inexpensive, readily available, optically active natural products.

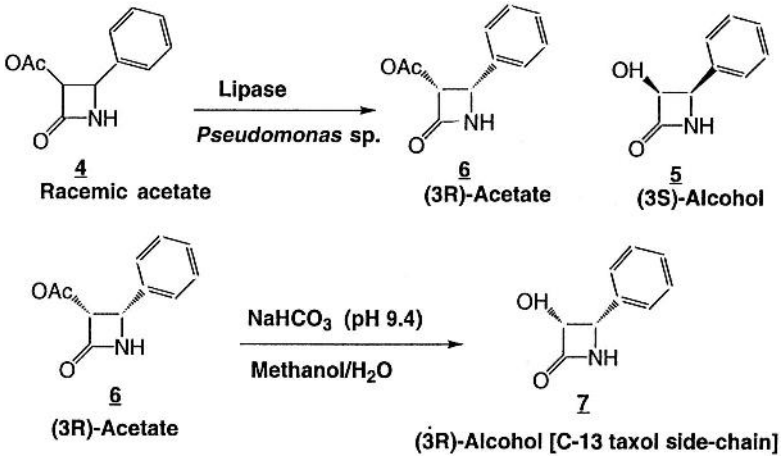
The second is to carry out the resolution of racemic compounds. This can be achieved by preferential crystallization of stereoisomers or diastereoisomers and by kinetic

resolution of racemic compounds by chemical or biocatalytic methods. Finally, chiral synthons can also be prepared by asymmetric synthesis by either chemical or biocatalytic processes using microbial cells or enzymes derived therefrom. The advantages of microbial or enzyme-catalyzed reactions over chemical reactions are that they are stereoselective and can be carried out at ambient temperature and atmospheric pressure. This minimizes problems of isomerization, racemization, epimerization, and rearrangement that generally occur during chemical processes. Biocatalytic processes are generally carried out in aqueous solution. This will avoid the use of environmentally harmful chemicals used in the chemical processes and solvent waste disposal. Furthermore, microbial cells or enzymes derived therefrom can be immobilized and reused many cycles.

Recently, a number of review articles [1–15] have been published on the use of enzymes in organic synthesis. This chapter provides some specific examples of the use of lipases in stereoselective catalysis and preparation of some chiral drug intermediates.

2 ANTICANCER (PACLITAXEL SEMISYNTHESIS)

Among the antimitotic agents, paclitaxel (taxol) **1** (Scheme 1) [16,17], a complex, polycyclic diterpene, exhibits a unique mode of action on microtubule proteins responsible for the formation of the spindle during cell division. In contrast to other ''spindle formation inhibitors,'' such as vinblastine or colchicine, both of which prevent the assembly of tubulin, paclitaxel is the only compound known to inhibit the depolymerization process of microtubulin [18]. Because of its biological activity and unusual chemical structure, paclitaxel represents the prototype of a new series of chemotherapeutic agents. Various types of cancers have been treated with paclitaxel and the results in treatment of ovarian cancer are very promising. In collaboration with the National Cancer Institute, Bristol–Myers Squibb developed paclitaxel for treatment of refractory ovarian cancer. Paclitaxel was originally isolated from the bark of the yew, *Taxus brevifolia* [16], and has also been found in other *Taxus* species in relatively low yield. Paclitaxel was initially obtained from *T. brevifolia* bark in about 0.07% yield. It required cumbersome purification of paclitaxel from the other related taxanes. It is estimated that about 20,000 lbs. of yew bark (the



Scheme 1

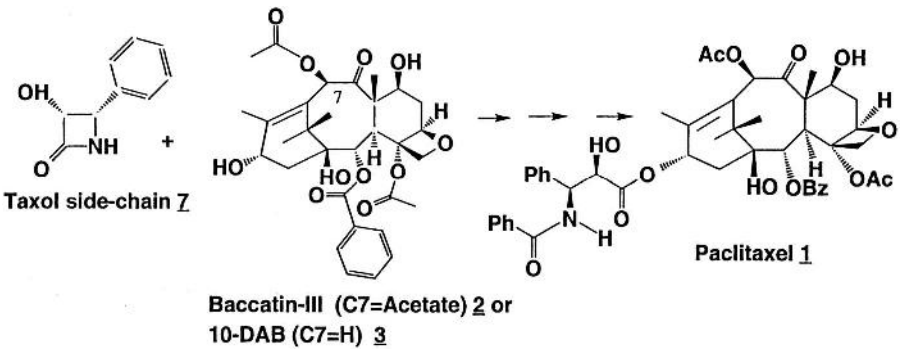
equivalent of about 3000 trees) are needed to produce 1 kg of purified paclitaxel [19]. This created a concern among the environmentalists about the mass destruction of trees to produce the required amount of taxol.

Alternative methods for production of paclitaxel by cell-suspension cultures and by semisynthetic processes are being evaluated by various groups [20–22]. The development of a semisynthetic process for the production of paclitaxel from baccatin III **2** or 10-deacetylbaccatin III (10-DAB) **3** and C-13 paclitaxel side chain was a very promising approach. Paclitaxel and related taxanes, baccatin III and 10-DAB, can be derived from renewable resources such as extract of needles, shoot, and young *Taxus* cultivars.

An enzymatic process was developed for the preparation of chiral C-13 paclitaxel side-chain synthon. The stereoselective enzymatic hydrolysis of racemic *cis*-3-(acetyloxy)-4-phenyl-2-azetidinone **4** (Scheme 2) was carried out [23]. Among commercially available enzymes evaluated, lipases from *Pseudomonas cepacia* (PS-30), *Geotrichum candidum* (GC-20), *Candida cylindracea* (AY-30), and porcine pancreatic lipase catalyzed the hydrolysis of the undesired enantiomer in racemic acetate **4** to the corresponding *S*(−)-alcohol **5**. The desired enantiomer (3*R*-*cis*) acetate **6** remained unreacted (Scheme 2). The reaction yield of 40–96% and the enantiomeric excess (ee) 94–99.6% were obtained for chiral acetate **6** depending up on lipase used in the reaction mixture. Amano lipase PS-30 from *Pseudomonas cepacia* gave a 98% reaction yield and 99.8% ee of the desired (3*R*-*cis*) acetate **6** (Table 1).

For an in-house source of enzyme, a lipase fermentation and recovery process was developed using *Pseudomonas* sp. SC 13865. The highest lipase activity achieved in a fed-batch (soybean oil feed) fermentation process using *Pseudomonas* sp. SC 13865 was 1500 units/mL. Most of the glucose and soybean oil was consumed during the first 36 hr, which corresponded to the period of rapid cell growth and lipase production (Fig. 1). Crude BMS lipase (1.7 kg) containing 140,000 U/g of lipase activity was recovered from the fermentation broth by ethanol precipitation. BMS lipase was immobilized on Accurel polypropylene with 98% adsorption efficiency. The immobilized BMS lipase was evaluated in the resolution of racemic acetate **4**. The substrate was used at 10 g/L and the enzyme was used at 3 g/L concentration. A reaction yield of 96 mole% and ee of 99.5% were obtained for (3*R*-*cis*) acetate **6** after 40 hr of reaction time.

For an alternative enzyme source, commercially available lipase PS-30 (Amano International Enzyme Company) was also immobilized on Accurel polypropylene in a similar manner. The kinetics of hydrolysis of racemic acetate **4** were investigated indepen-



Scheme 2

Table 1 Enzymatic Resolution of *Cis*-3-acetyloxy-4-phenyl-2-azetidinone **4** by Lipases

Enzyme	Reaction time (hr)	Yield of **6** (%)	Enantiomeric excess of **6**
BMS lipase	42	95	99.4
Pseudomonas lipase (Lipase PS-30)	48	96	99.6
Geotrichum candidum (Lipase GC-20)	48	84	99
Rhizopus niveus (Lipase N)	48	64	98.5
Aspergillus niger (Lipase APF)	48	40	99.5
Candida cylindracea (Lipase AY 30)	48	90	94
Porcine pancreatic lipase	48	90	99.2
Pseudomonas sp. (Lipase AK)	48	74	98.3

dently using immobilized BMS lipase and lipase PS-30. The reaction was conducted at 10 g/L substrate and 3 g/L enzyme concentrations. The hydrolysis of the undesired enantiomer of racemic acetate **4** was observed, enriching **4** with the desired (3*R-cis*) acetate **6** as the reaction progressed. The initial rate (0–24 hr) of hydrolysis was faster than the later rate of hydrolysis (24–40 hr). Using each enzyme, a reaction yield of >96% and ee >99.5% were obtained for the desired (3*R-cis*) acetate **6** (Table 2). The immobilized BMS lipase and lipase PS-30 were reused for 10 cycles without any loss of activity, productivity,

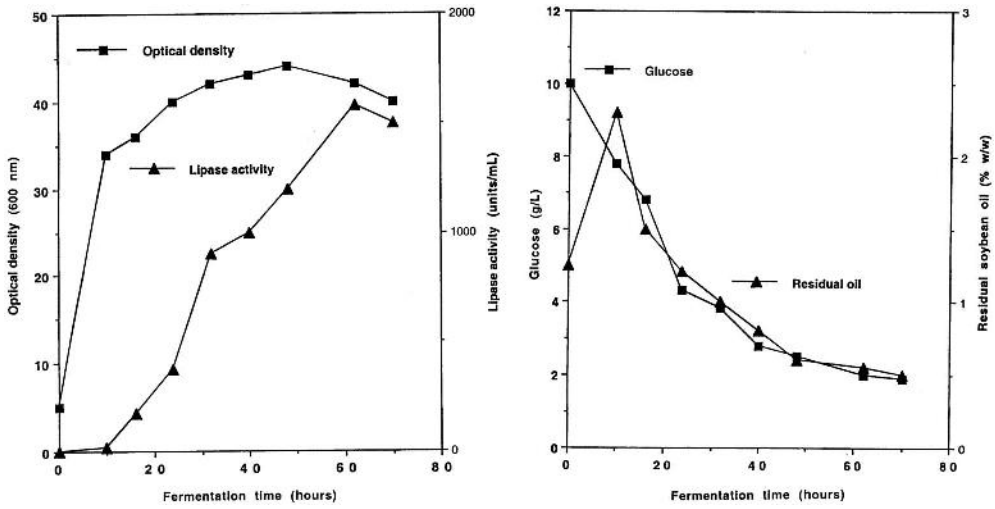


Figure 1 Production of BMS lipase: fermentation of *Pseudomonas* sp. SC 13856 for the production of lipase.

Table 2 Kinetics of Hydrolysis of *Cis*-3-acetyloxy-4-phenyl-2-azetidinone **4** by Immobilized Lipase PS-30 and BMS Lipase

Reaction time (hr)	(3*R*)-Acetate (g/L)	(3*S*)-Acetate (g/L)	(3*S*)-Alcohol (g/L)	(3*R*)-Alcohol (g/L)	Conversion (%)	EE of (3*R*)-acetate (%)
Lipase PS-30						
0.5	5	4.5	0.4	0	8	54
16	4.95	2.5	2.5	0	50	75
24	4.92	1.2	3.8	0.013	72	86
32	4.89	0.4	4.3	0.07	92	96
40	4.82	0	4.95	0.14	96.4	99.6
BMS lipase						
0.5	5	4.2	0.8	0	14	57
16	4.98	2.48	2.49	0.02	50	75
24	4.96	1.5	3.6	0.04	66	86
32	4.9	0.26	4.7	0.1	95	97.4
40	4.8	0	4.92	0.16	96	99.5

Note: The reaction mixture in 1 L of 25 m*M* potassium phosphate buffer (pH 7.0) contained 10 g of substrate **4** and 3 g of immobilized lipase. Reactions were carried out at 30°C, 200 rpm.

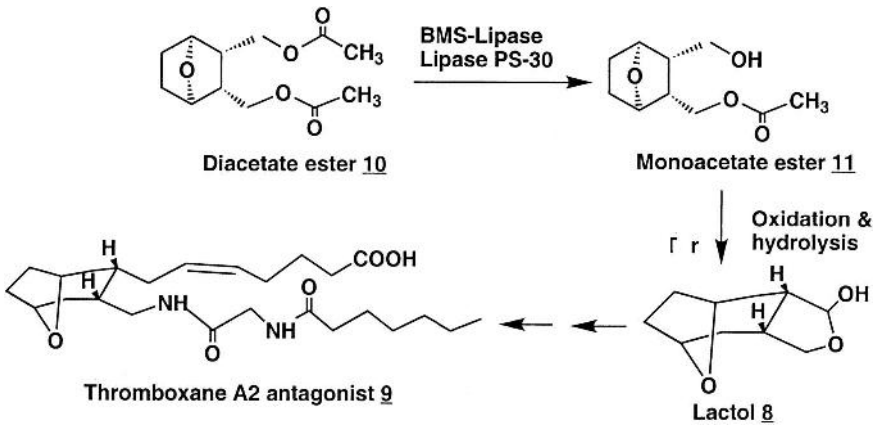
or the ee of the product. An average reaction yield of 94% and ee of 99.3% were obtained for chiral acetate **6**. The rate of hydrolysis of **4** remained constant over 10 cycles (0.12 g/L/hr). Similar results were obtained in reusability studies using BMS lipase, except the reaction was completed in 36 hr.

Preparative-scale hydrolyses of racemic acetate **4** were carried out in a 75-L and 150-L volume using immobilized BMS lipase and lipase PS-30, respectively. After 38 hr reaction time, 94 M% reaction yield and 99.4% ee of **6** were obtained in each batch. From the reaction mixtures, 331 and 675 g of chiral acetate **6** were isolated (overall yield of 88 M% and 90 M%, respectively) from the two batches. The isolated acetate **2** ($[\alpha]_D = -15.6°$) from both batches gave an ee of 99.5% by chiral high-pressure liquid chromatography (HPLC) and 99.9 area % purity by HPLC.

The C-13 paclitaxel side chain (**7**) produced by the resolution process could be coupled to baccatin III or 10-DAB after protection and deprotection of each compound to prepare paclitaxel by a semisynthetic process (Scheme 2). The preparation of taxol side-chain precursors by the lipase-catalyzed enantioselective esterification of methyl *trans*-β-phenylglycidate has been demonstrated [24]. The lipase-catalyzed enantioselective hydrolysis and transesterification of racemic esters and alcohols, respectively, have also described by Brieva et al. [25].

3 THROMBOXANE A_2 ANTAGONIST

Thromboxane A_2 (TxA2) is an exceptionally potent proaggregatory and vasoconstrictor substance produced by the metabolism of arachidonic acid in blood platelets and other tissues. Together with the potent antiaggregatory and vasodilator, it is thought to play a role in the maintenance of vascular homeostasis and to contribute to the pathogenesis of a variety of vascular disorders. Approaches toward limiting the effect of TxA2 have focused on either inhibiting its synthesis or blocking its action at its receptor sites by means



Scheme 3

of an antagonist [26,27]. The lactol [3*aS*-(3*a*α,4α,7α,7*a*α)]-hexahydro-4,7-epoxyisobenzo-furan-1-(3*H*)-ol **8** (Scheme 3) is a key chiral intermediate for the total synthesis of [1*S*-[1α,2α(*Z*),3α,4α[[-7-[3-[[1-oxoheptyl)-amine] acetyl]]methyl]]]-7-oxabicyclo-[2.2.1] hept-2-yl]-5-heptanoic acid **9**, a new cardiovascular agent useful in the treatment of thrombolic disease [28,29].

The stereoselective asymmetric hydrolysis of (exo, exo)-7-oxabicyclo [2.2.1] heptane-2,3-dimethanol, diacetate ester **10** to the corresponding chiral monoacetate ester **11** (Scheme 3) has been demonstrated with lipases [30]. Lipase PS-30 from *Pseudomonas cepacia* was most effective in asymmetric hydrolysis to obtain the desired enantiomer of monoacetate ester (Table 3). A reaction yield of 90 M% and ee of >98% were obtained when the reaction was conducted in a biphasic system with 10% toluene at 5 g/L substrate concentration. Lipase PS-30 was immobilized on Accurel polypropylene (PP) and the

Table 3 Enzymatic Asymmetric Hydrolysis of Diacetate Ester **10** to Chiral Monoacetate Ester **11**

Enzyme	Diacetate ester **10** (mg/mL)	Monoacetate ester **11** (mg/mL)	Conversion (mol%)	Monoacetate ester ee (%)
Pseudomonas fluorescens (Biocatalysts Ltd.)	0.45	3.5	85	97
Pseudomonas lipase (Lipase PS-30, Amano)	0.33	3.75	90	98
Geotrichum candidum (Lipase GC-20, Amano)	1.6	1.8	44	60
Chromobacterium viscosum Lipase (Sigma)	1.8	2.1	51	78
Candida cylindracea (Lipase AY 30)	1.2	2.5	61	81
Pseudomonas lipase (Enzymatics)	0.52	3.42	82	75

Table 4 Kinetics of Hydrolysis of Diacetate Ester **10** to Monoacetate Ester **11** by Lipase PS-30: 80 L Preparative Batch

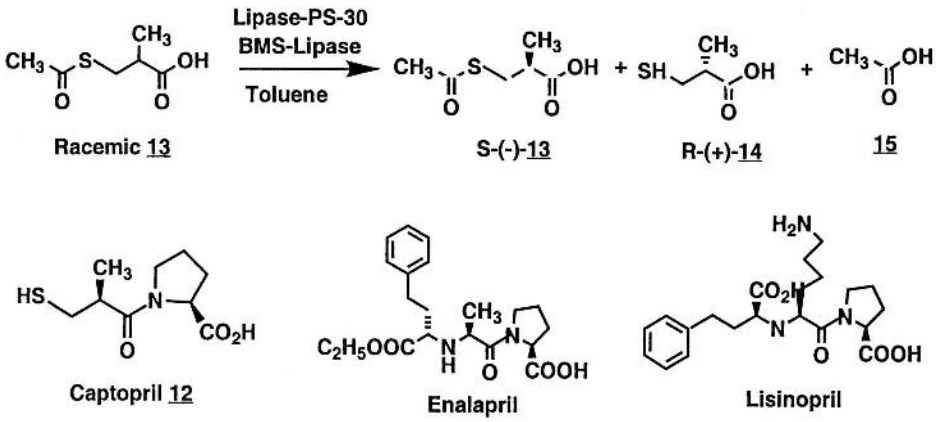
Reaction time (hr)	Diacetate ester **10** (g/L)	Monoacetate ester **11** (g/L)	Conversion (mol%)	Monoacetate ester ee (%)
4	2.2	1.8	43	
8	1.4	2.6	62	
16	0.6	3.3	79	
20	0.5	3.5	85	
24	0.4	3.7	89	
28	0.3	3.9	93	99.5

Note: The reaction mixture (80 L) contained 72 L of 50 m*M* potassium phosphate buffer (pH 7.0), and 8 L of toluene containing 400 g of substrate **10** and 200 g of immobilized lipase PS-30. The reaction was carried out at 5°C, 230 rpm. The pH was maintained at 7.0 with 25% NaOH.

immobilized enzyme was reused (five cycles) without loss of enzyme activity, productivity, or ee. The reaction process was scaled-up to 80 L (400 g of substrate) and monoacetate ester **11** was isolated in 80 M% yield with 99.5% ee, as determined by chiral HPLC and nuclear magnetic resonance (NMR) analysis. The isolated product gave a gas chromatography (GC) homogeneity index (HI) of 99.5%. Kinetics of asymmetric hydrolysis of diacetate ester is as shown in Table 4. The chiral monoacetate ester **11** was oxidized to its corresponding aldehyde and subsequently hydrolyzed to give chiral lactol **8** (Scheme 3). The chiral lactol **8** obtained by this enzymatic process was used in chemoenzymatic synthesis of thromboxane A_2 antagonist.

4 ANGIOTENSIN-CONVERTING ENZYME INHIBITORS

Captopril is designated chemically as 1-[(2*S*)-3-mercapto-2-methyl propionyl]-L-proline **12** (Scheme 4). It is used as an antihypertensive agent through suppression of the renin–



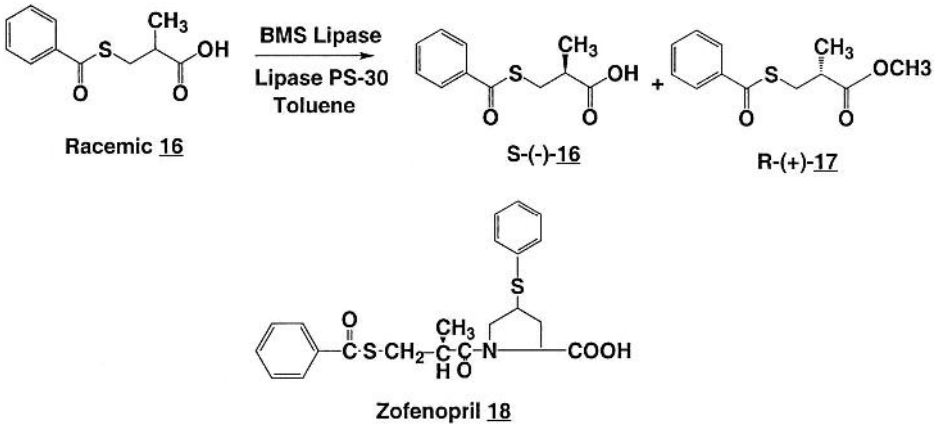
Scheme 4

angiotensin–aldosterone system [31–33]. Captopril and other compounds such as enalapril and lisinopril (Scheme 4) prevent the conversion of angiotensin I to angiotensin II by inhibition of the angiotensin-converting enzyme (ACE).

The potency of captopril **12** as an inhibitor of ACE depends critically on the configuration of the mercaptoalkanoyl moiety; the compound with the (*S*) configuration is about 100 times more active than its corresponding (*R*) enantiomer [31]. The required 3-mercapto-(2*S*)-methylpropionic acid moiety has been prepared from the microbially derived chiral 3-hydroxy-(2*R*)-methylpropionic acid, which is obtained by the hydroxylation of isobutyric acid [34,35].

The use of extracellular lipases of microbial origin to catalyze the stereoselective hydrolysis of 3-acylthio-2-methylpropanoic acid ester in an aqueous system has been demonstrated to produce optically active 3-acylthio-2-methyl propanoic acid [36–38]. The synthesis of chiral side chain of captopril by the lipase-catalyzed enantioselective hydrolysis of the thioester bond of racemic 3-acetylthio-2-methyl propanoic acid **13** to yield *S*-(−)-**13** has been demonstrated [39]. Among various lipases evaluated, lipase from *Rhizopus oryzae* ATCC 24563 (heat-dried cells) and lipase PS-30 in an organic solvent system (1,1,2-trichloro-1,2,2-trifluoroethane or toluene) catalyzed hydrolysis of the thioester bond in the undesired enantiomer of racemic **13** to yield desired *S*-(−)**13**, *R*-(+)-3-mercapto-2-methylpropanoic acid **14**, and acetic acid **15** (Scheme 4). A reaction yield of >24% (theoretical maximum 50%) and ee of >95% were obtained for *S*-(−)-**12** using each lipase in an independent experiment.

In an alternate approach to prepare the chiral side chain of captopril **12** and zofenopril **18** (Scheme 5), the lipase-catalyzed stereoselective esterification of racemic 3-benzoylthio-2-methylpropanoic acid **16** in an organic solvent system was demonstrated (Table 5) to yield *R*-(+) methyl ester **17** and unreacted acid enriched in the desired *S*-(−)-enantiomer **16** [40]. Using lipase PS-30 with toluene as solvent and methanol as nucleophile, the desired *S*-(−)-**16** was obtained in 38% reaction yield (theoretical maximum 50%) and 97% ee (Table 6). The substrate was used at 22 g/L concentration. The amount of water and concentration of methanol supplied in the reaction mixture were very critical. Water was used at 0.1% concentration in the reaction mixture. Higher than 1% water led to the aggregation of enzyme in the organic solvent, with a decrease in the rate of reaction due



Scheme 5

Table 5 Evaluation of Lipases for the Stereoselective Esterification of Racemic 3-Benzylthio-2-methyl Propanoic Acid **16**

Lipase source	Reaction time (hr)	Conversion to **17** (%)	Yield of **16** (%)	ee of *S*-(−)-**16** (%)
Candida cylindraceae (Sigma)	1.3	64	36	38.7
Pseudomonas lipase (Lipase PS-30)	27	62	38	97
Rhizopus sp. (Amano N)	26	58	42	56
Mucor sp. (Amano MAP)	0.5	78	22	58.4
Aspergillus niger (Lipase APF)	99	86	14	67
Pseudomonas sp. (Biocatalysts)	27	68	32	96
Pseudomonas sp. (Enzymatics)	27	58	42	92
Pseudomonas sp. (Amano AK)	27	56	44	86.5
Pseudomonas sp. (Amano CES)	27	59	41	87
Penicillum sp. (Amano P)	99	61	39	42

Note: The reaction mixture in 5 mL of toluene contained 0.025 *M* of racemic **16** (5.6 mg), 0.1 *M* methanol, 0.1% water, and 1 g of crude lipase. The reaction was carried out at 40°C, 200 rpm.

to mass transfer limitation. The rate of esterification and ee of desired product *S*-(−)-**16** decreased as the methanol to substrate ratio was increased from 1:1 to 8:1 (Table 7). Higher methanol concentrations probably inhibited the esterification reaction by stripping the essential water from the enzyme. Crude lipase PS-30 was immobilized on three different resins, XAD-7, XAD-2, and Accurel PP, in adsorption efficiencies of about 68%, 71%, and 98.5%, respectively. These immobilized lipases were evaluated for the ability to stereoselectively esterify racemic **16**. Enzyme immobilized on Accurel PP catalyzed efficient esterification, giving 36–45% reaction yield and 97.7% ee of *S*-(−)-**16**. The immobilized enzyme under identical conditions gave similar optical purity and yield of product in 23 additional reaction cycles without any loss of activity and productivity. *S*-(−)-**16** is a key chiral intermediate for the synthesis of captopril **12** [41] or zofenopril **18** [42]; both are antihypertensive drugs (Scheme 5).

S-(−)-α-[(Acetylthio)methyl]benzenepropanoic acid **19** is a key chiral intermediate for the neutral endopeptidase inhibitor **20** (Scheme 6) [43,44]. The lipase-catalyzed stereoselective hydrolysis of thioester bond of racemic α-[(acetylthio)methyl] benzenepropanoic acid **19** (Table 8) has been demonstrated in organic solvent to yield *R*-(+)-α [(mercapto)methyl] benzenepropanoic acid **21** and *S*-(−)-**19** [39]. Using lipase PS-30, the *S*-(−)-**19** was obtained in 40% reaction yield (theoretical maximum 50%) and 98% ee (Scheme 6).

Table 6 Screening of Alcohols for the Stereoselective Esterification of Racemic **16**

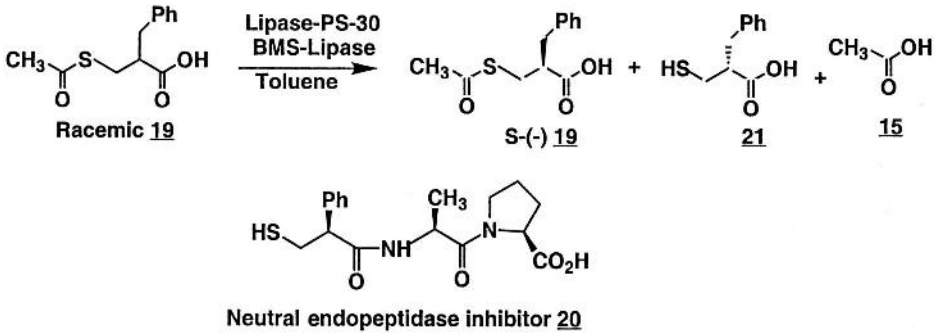
Alcohol	Reaction time (hr)	Conversion to **17** (%)	Yield of **16** (%)	ee of *S*-(−)-**16** (%)
Methanol	112	50.3	49.7	91.7
	136	55.1	44.9	95.4
	162	63	37	97.3
2-Amino-1-ethanol	16	68	32	50
	40	93	7	50
Trifluoroethanol	162	29	71	62
	328	38	62	63
Isopropylidene glycerol	112	33	67	67
	280	58	42	87
Benzyl alcohol	112	39	61	72
	280	67	33	96
1-Octanol	112	34	66	68

Note: The reaction mixture in 25 mL of toluene contained 0.1 *M* of racemic **16** (22.4 mg), 0.4 *M* alcohol, 0.1% water, and 1 g of crude lipase PS-30. The reaction was carried out at 28°C, 280 rpm.

Table 7 Effect of Methanol Concentration on the Esterification of Racemic **16**

Methanol concentration (*M*)	Reaction time (hr)	Conversion to **17** (%)	Yield of **16** (%)	ee of *S*-(−)-**16** (%)
0.5	5.5	55	45	93.6
1	8	61	39	96
1.5	21	60	40	96.5
2	30	62	38	95.5
3	64	13	87	56
4	64	6	94	56

Note: The reaction mixture in 10 mL of toluene contained 0.5 *M* racemic **16** and methanol as indicated, 0.1% water, and 1.0 g of immobilized lipase PS-30 on Accurel PP. The reactions were carried out at 40°C, 280 rpm.



Scheme 6

Table 8 Evaluation of Lipases for the Stereoselective Deacylation of Racemic α-[(Acetylthio)methyl]benzene Propanoic Acid **19**

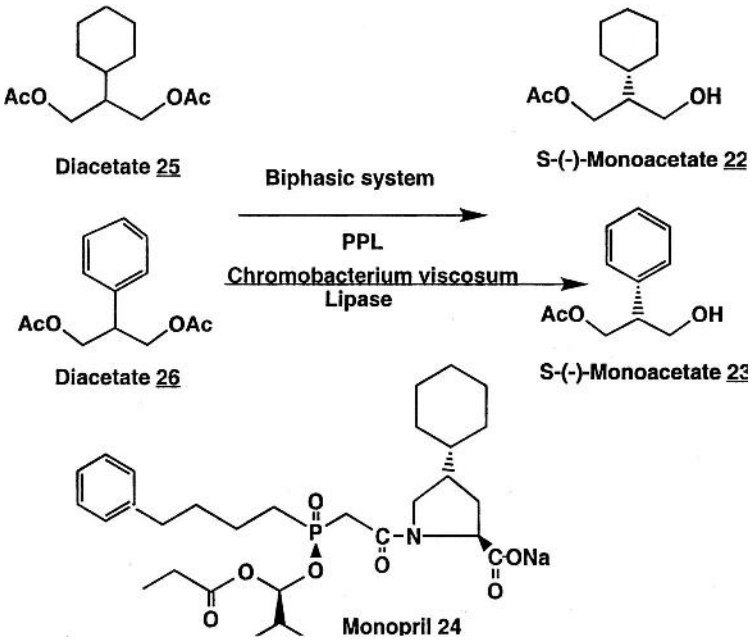
Lipase source	Reaction time (hr)	Yield of **19** (%)	ee of *S*-(−)-**19** (%)
Candida cylindraceae (Amano)	25	36	55
Pseudomonas cepacia (Lipase PS-30, Amano)	25	40	98
Mucor sp. (Enzymatics)	4	48	52
Aspergillus niger (Lipase APF, Amano)	48	39	68
Pseudomonas sp. (Lipase B1, Biocatalysts)	40	19	86
Geotrichum candidum (Amano GC)	160	50	58
Pseudomonas sp. (Enzymatics)	27	42	92
Pseudomonas sp. (Amano AK)	25	64	74
Pseudomonas sp. (Amano CE)	25	26	55

Note: The reaction mixture in 10 mL of toluene contained 250 mg of racemic **19**, 0.1% water, and 1 g of crude lipase. The reaction was carried out at 28°C, 200 rpm.

S-(−)-2-Cyclohexyl 1,3-propanediol monoacetate **22** and *S*-(−)-2-phenyl-1,3-propanediol monoacetate **23** (could be chemically converted to a cyclohexyl derivative) are key chiral intermediates for the chemoenzymatic synthesis of monopril **24** (Scheme 7), a new hypertensive drug which acts as an ACE inhibitor. The asymmetric hydrolysis of 2-cyclohexyl-1,3-propanediol diacetate **25** and 2-phenyl-1,3-propanediol diacetate **26** to the corresponding *S*-(−) monoacetate **22** and *S*-(−) monoacetate **23** by porcine pancreatic lipase (PPL) and *Chromobacterium viscosum* lipase has been demonstrated by Patel et al. [45]. In a biphasic system using 10% toluene, the reaction yield of >65% and ee of 99% were obtained for *S*-(−)-**22** using each enzyme. *S*-(−)-**23** was obtained in a 90% reaction yield and 99.8% ee using *C. viscosum* lipase under similar conditions.

A number of research groups have been interested in the synthesis of the aminodiol **27**. This compound is believed to mimic the transition state for the renin-catalyzed hydrolysis of the peptide angiotensinogen, and therefore has been useful as a potential antihypertensive drug. The enzymatic resolution of the 3-acetoxy-β-lactam **28** (Scheme 8) by lipase PS-30 has been demonstrated by Spero et al. [46]. A new approach to the *N*-α-butoxycarbonyl-protected amino diol **29** via opening of 3,4-*cis*-disubstituted β-lactam has been demonstrated (Scheme 8).

The stereoselective hydrolysis of dimethyl esters of symmetrical dicarboxylic acids including meso-diacids such as *cis*-1,2-cycloalkane dicarboxylic acids and diacids with prochiral centers has been demonstrated by Mohr et al. [47]. The products of these stereoselective hydrolyses, chiral monoacetate of dicarboxylic acids, were obtained with an ee

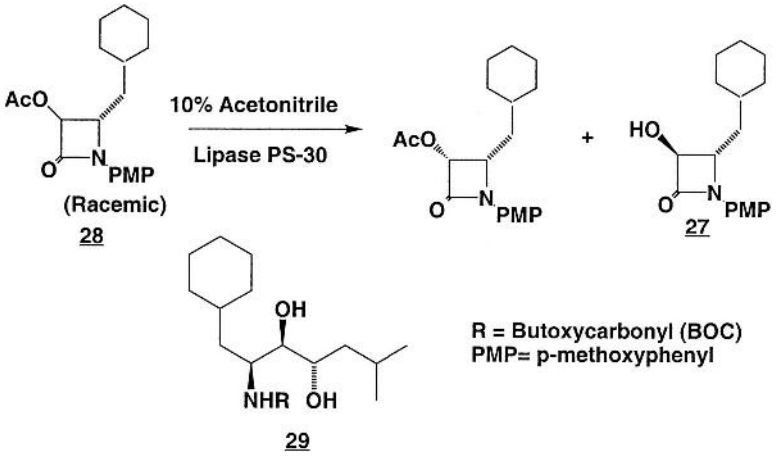


Scheme 7

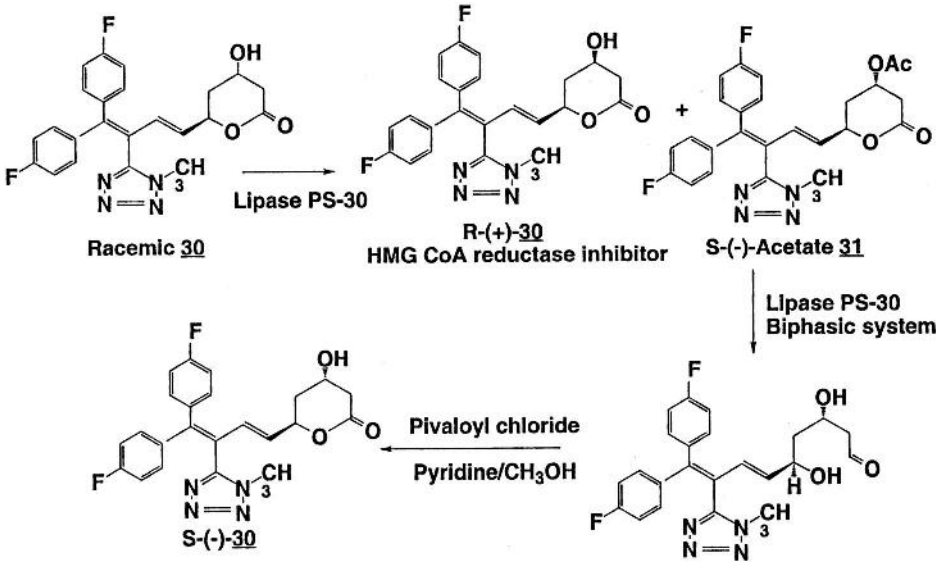
from 10% to 90% depending on the substrate. Enantioselective hydrolysis of *cis*-1,2-diacetoxy cycloalkane and 2-substituted 1,3-propanediol diacetate has been demonstrated using PPL by Laumen and Schneider [48] and Tombo et al. [49], respectively.

5 ANTICHOLESTEROL DRUGS

Chiral β-hydroxy esters are versatile synthons in organic synthesis, specifically in the preparation of natural products [50–52]. The asymmetric reduction of carbonyl compounds using baker's yeast has been demonstrated and reviewed [53,54]. The compound



Scheme 8



Scheme 9

[4-[4a,6β(*E*)]]-6-[4,4-*bis*[4-fluorophenyl]-3-(1-methyl-1*H*-tetrazol-5-yl)-1,3-butadienyl]-tetrahydro-4-hydroxy-2H-pyren-2-one, *R*-(+)-**30** (Scheme 9), a new anticholesterol drug, acts by inhibition of 3-hydroxy-3-methyl glutaryl coenzyme A (HMG CoA) reductase [55].

Using a resolution process, chiral alcohol *R*-(+)-**30** was prepared by the lipase-catalyzed stereoselective acetylation of **30** in organic solvent [56]. Various lipases were evaluated, among which lipase PS-30 (Amano International Enzyme Co.) and BMS lipase (Table 9) efficiently catalyzed the acetylation of the undesired enantiomer of racemic **30**

Table 9 Evaluation of Lipases for the Stereoselective Acetylation of Racemic Compound **30**

Lipase source	Reaction time (hr)	Conversion to **31** (%)	Yield of **30** (%)	ee of *R*-(+)-**30** (%)
Candida sp. AY-30 (Amano)	48	40	60	78
Pseudomonas lipase (Lipase PS-30)	24	52	48	98.7
Rhizopus sp. (Amano N)	48	4	96	ND[a]
Geotrichum candidum (Amano)	48	40	60	48
Porcine pancreatic	48	8	92	ND
Pseudomonas sp. (Biocatalysts)	24	53	47	98.5

Note: The reaction mixture in 10 mL of toluene contained 40 mg of racemic **30**, 800 μL of isopropenyl acetate, 0.1% water, and 1 g of crude lipase. The reaction was carried out at 28°C, 200 rpm.

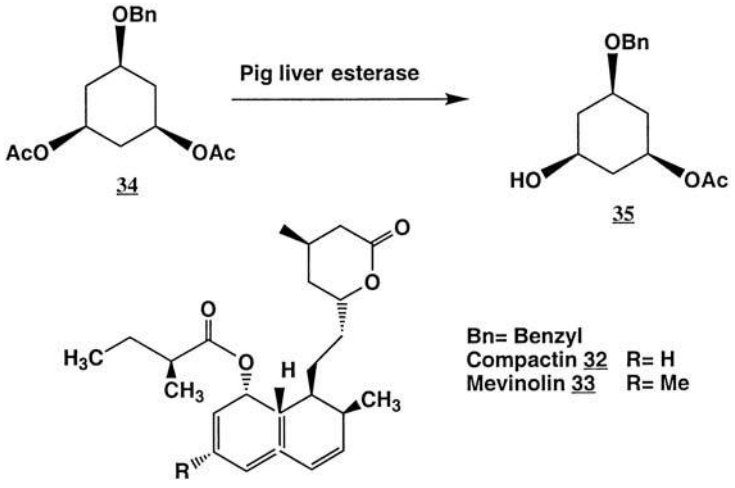
[a] ND = not determined.

to yield *S*-(−)-acetylated product **31** and unreacted desired *R*-(+)-**30** (Scheme 9). A reaction yield of 49 M% (theoretical maximum 50 M%) and ee of 98.5% were obtained for *R*-(+)-**30** when the reaction was conducted in toluene as the solvent in the presence of isopropenyl acetate as the acyl donor. The substrate was used at 4 g/L concentration. In methyl ethyl ketone at 50 g/L substrate concentration, a reaction yield of 46 M% and optical purity of 96.4% were obtained for *R*-(+)-**30**.

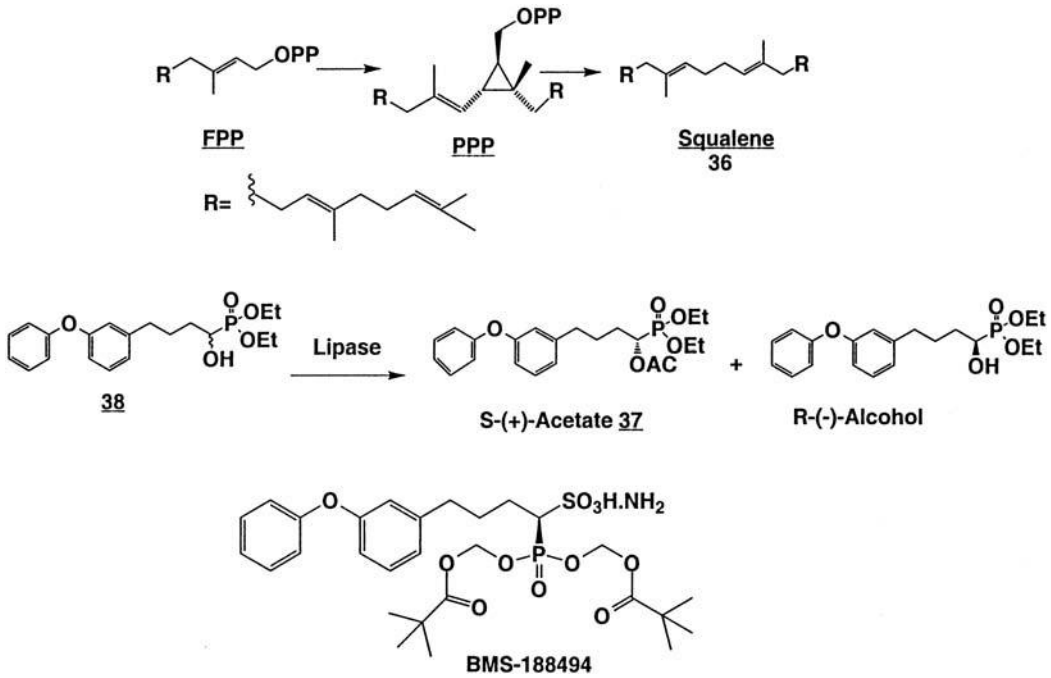
Lipase PS-30 was immobilized on Accurel PP and the immobilized enzyme was reused five times without any loss of activity or productivity in the resolution process to prepare *R*-(+)-**30**. The enzymatic process was scaled-up to a 640-L preparative batch using immobilized lipase PS-30 at 4 g/L racemic substrate **30** in toluene as the solvent. From the reaction mixture, *R*-(+)-**30** was isolated in 35 M% overall yield (theoretical maximum is 50%) with 98.5% ee and 99.5% chemical purity. The undesired *S*-(−)-acetate **31** produced by this process was enzymatically hydrolyzed by lipase PS-30 in a biphasic system to prepare the corresponding *S*-(−)-alcohol **30**. Thus, both enantiomers of alcohol **30** were produced by the enzymatic process.

Compactin **32** and mevinolin **33** (Scheme 10) are potent competitive inhibitors of 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMG CoA reductase), the rate-limiting enzyme in cholesterol biosynthesis. A synthetic route to the chiral lactone moiety of **32** and **33** has been prepared from meso-diacetate **34**. The chiral compound **35** has been prepared by the asymmetric hydrolysis of meso-diacetate **34** by pig liver esterase [57]. A reaction yield of 62% and ee of 90% were obtained for chiral compound **35** (Scheme 10).

Squalene synthase is the first pathway-specific enzyme in the biosynthesis of cholesterol and catalyzes the head-to-head condensation of two molecules of farnesyl pyrophosphate (FPP) to form squalene **36** (Scheme 11). It has been implicated the transformation of FPP into presqualene pyrophosphate (PPP) [58]. FPP analogs are a major class of inhibitors of squalene synthase [59,60]. However, this class of compounds lack specificity and are potential inhibitors of other FPP-consuming transferases such as geranyl geranyl pyrophosphate synthase. To increase enzyme specificity, analogs of PPP and other mechanism-based enzyme inhibitors have been synthesized [61,62].



Scheme 10



Scheme 11

BMS-188494 is a potent squalene synthase inhibitor effective as an anticholesterol drug [63,64]. (*S*)[1-(acetoxyl)-4-(3-phenyl)butyl]phosphonic acid, diethyl ester **37**, is a key chiral intermediate required for the total chemical synthesis of BMS-188494. The stereoselective acetylation of racemic [1-(hydroxy)-4-(3-phenyl)butyl]phophonic acid, diethyl ester **38** (Scheme 11) was carried out using *Geotrichum candidum* lipase in tolune as the solvent and isopropenyl acetate as the acyl donor [65]. A reaction yield of 38% (theoretical maximum yield is 50%) and an ee of 95% were obtained for chiral **37** (Table 10).

Table 10 Evaluation of Lipases for the Stereoselective Acetylation of Racemic **38**

Substrate added (mg/mL)	Reaction time (hr)	Substrate remained (mg/mL)	Product **37** (mg/mL)	Yield of **37** (%)	ee of *S*-(+)-**37** (%)
1	24	0.84	0.16	16	95
	100	0.62	0.34	34	95
2.5	42	1.45	0.55	27	94.6
	100	1.7	0.77	31	94.5
5	42	4	1	20	94
	100	3.7	1.3	26	94

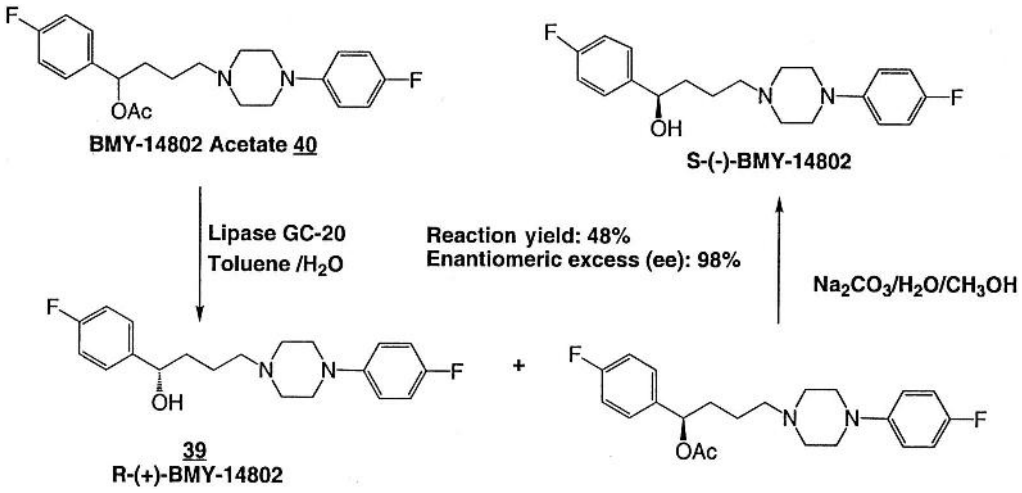
Note: The reaction mixture in 10 mL of toluene contained racemic **38** as indicated, 200 µL of isopropenyl acetate, 0.1% water, and 50 mg of crude lipase. The reaction was carried out at 37°C, 200 rpm.

6 ANTIPSYCHOTIC AGENTS

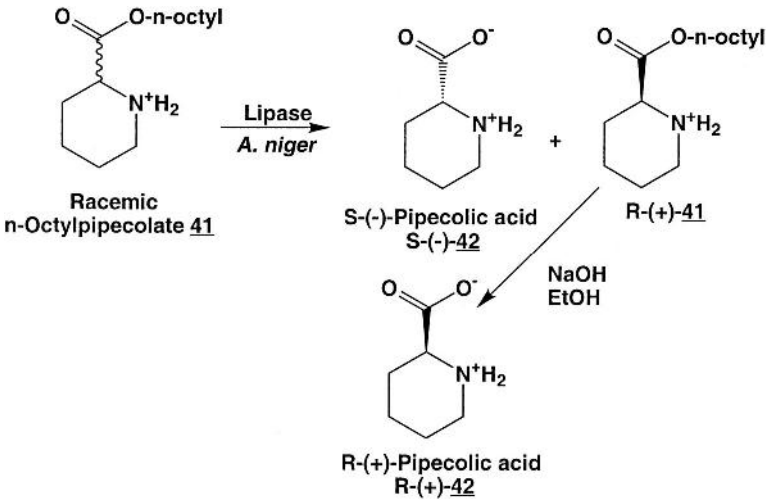
During the past few years, much effort has been directed toward the understanding of the Sigma receptor system in the brain and endocrine tissue. This effort has been motivated by the hope that the Sigma site may be a target for a new class of antipsychotic drugs [66–68]. Characterization of the Sigma system helped to clarify the biochemical properties of the distinct haloperidol-sensitive Sigma binding site, the pharmacological effects of Sigma drugs in several assay systems, and the transmitter properties of a putative endogenous ligand for the Sigma site [69–72].

The *R*-(+) compound **39** (BMY-14802) is a Sigma ligand and has a high affinity for Sigma binding sites and antipsychotic efficacy. *R*-(+)-BMY 14802 **39** (Scheme 12) has been prepared by lipase-catalyzed resolution of racemic BMY-14802 acetate ester **40** [73]. Lipase from *Geotrichum candidum* (GC-20 from Amano Enzyme Co.) catalyzed the hydrolysis of acetate **40** to *R*-(+)-BMY 14802 (Scheme 12) in a biphasic solvent system in 48% reaction yield and 98% ee. The rate and enantioselectivity of the hydrolytic reaction was dependent on the organic solvent used. The enantioselectivity (*E* values) ranged from 1 in the absence of solvent to >100 in dichloromethane and toluene. *S*-(−)-BMY 14802 was also prepared by the chemical hydrolysis of undesired BMY-14802 acetate obtained during the enzymatic resolution process.

Pipecolic acid (2-piperidinecarboxylic acid) is a precursor of various bioactive compounds such as thioridazine (antipsychotic agent), pipradol (anticonvulsant agent), a potassium opioid analgesic, and immunosuppressants FK 506 [74–76]. Ng-Youn-Chem et al. [77] has developed an enzymatic process for the kinetic resolution of racemic *n*-octyl pipecolate **41** using partially purified lipase from *Aspergillus niger* (Scheme 13). The reaction yield of 20% (theoretical maximum is 50%) and the ee of 97% were obtained for *S*-(−)-pipecolic acid **42**. The unreacted *R*-(+)-*n*-octyl pipecolic **41** was obtained in 26% reaction yield (theoretical maximum is 50%) and 96% ee and was hydrolyzed with sodium hydroxide in ethanol to yield *R*-(+)-pipecolic acid **42** in 96% ee (Scheme 13).



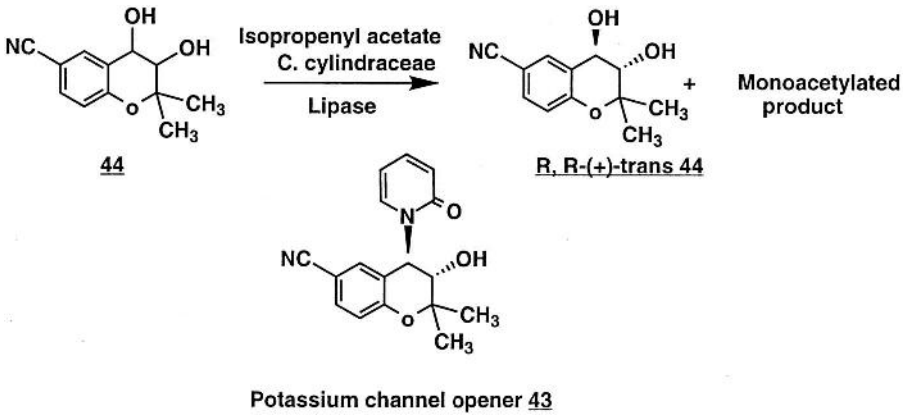
Scheme 12



Scheme 13

7 POTASSIUM CHANNEL OPENERS

The study of potassium (K) channel biochemistry, physiology, and medicinal chemistry has flourished, and numerous articles and reviews have been published in recent years [78,79]. It has long been known that K channels play a major role in neuronal excitability and it is now clear that K channels play a critical role in the basic electrical and mechanical function of a wide variety of tissues, including smooth muscle, cardiac muscle, and glands [80]. A new class of highly specific pharmacological compounds has been developed which either open or block K channels [81,82]. The K channel openers are powerful smooth muscle relaxants with in vivo hypotensive and bronchodilator activity. Recently, the synthesis and antihypertensive activity of a series of novel K channel openers [83–85] based on monosubstituted *trans*-4-amino-3,4-dihydro-2,2-dimethyl-2*H*-1-benzopyran-3-ol **43** (Scheme 14) have been demonstrated. Chiral diol **44** is a potential intermediate



Scheme 14

Table 11 Kinetics of Acetylation of Racemic Diol **44** by *Candia cylindraceae* Lipase

Reaction time (hr)	Diol remained (g/L)	Monoacetylated product (g/L)	ee of (+)-*trans* diol **44** (%)
0	10	0	50
12	9.1	1.2	54
24	7.9	1.94	61
36	6.8	3.4	72
48	6.1	3.95	82
60	5.4	4.8	91
80	4.35	5.9	96

Note: The reaction mixture (1 L) contained 920 mL of toluene, 80 mL of isopropenyl acetate, 1 mL of water, 10 g of racemic diol **44**, and 10 g of crude lipase. The reaction was carried out at 30°C, 150 rpm.

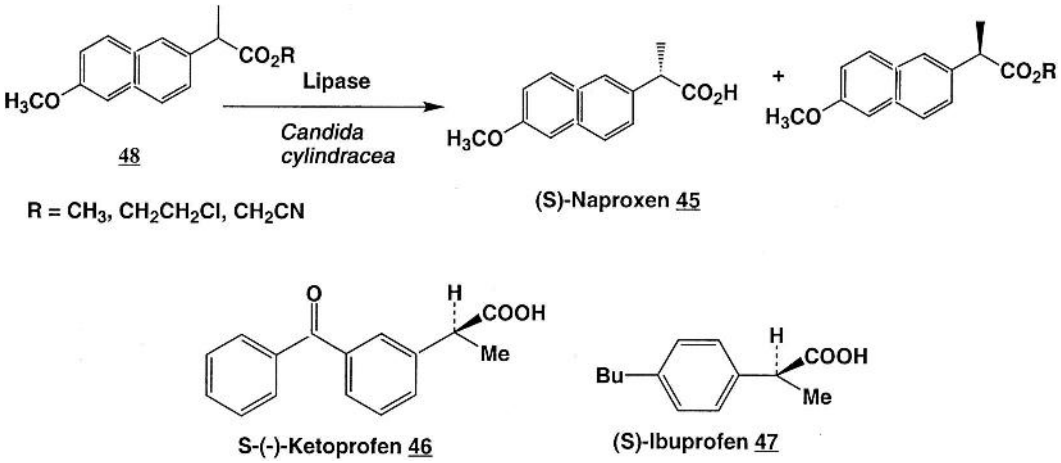
for the synthesis of K channel activators important as an antihypertensive and bronchodilator agent.

In an enzymatic resolution approach, chiral diol **44** was prepared by the stereoselective acetylation of racemic diol with lipases from *Candida cylindracea* and *Pseudomonas cepacia* (Table 11). Both enzymes catalyzed the acetylation of the undesired enantiomer of racemic diol to yield monoacetylated product and unreacted desired (+)-*trans* diol **44** (Scheme 14). A reaction yield of >42% (theoretical maximum is 50%) and an ee of >96% were obtained using each lipase [86]. Kinetics of acetylation reaction using *Candida cylindracea* lipase are as shown in Table 11.

8 ANTI-INFLAMMATORY DRUGS

Arylpropionic acids are an important class of nonsteroidal anti-inflammatory drugs. The two most commonly prescribed drugs are Motrin (*p*-isobutylhydratropic acid) and Naproxen [(+)-*S*-2-(6-methoxy-2-naphthyl) propionic acid]. They are widely used in the treatment of the symptoms of arthritis. Naproxen first developed by Syntex [87,88]. Biologically active desired *S*-naproxen has been prepared by enantioselective hydrolysis of the methyl ester of naproxen by esterase derived from *Bacillus subtilis* Thai 1-8 [89]. The esterase was subsequently cloned in *Escherichia coli* with over an 800-fold increase in activity of the enzyme. The resolution of racemic naproxen amide and ketoprofen amides has been demonstrated by amidases from *Rhodococus erythropolis* MP50 and *Rhodococcus* sp. C311 [90–93]. *S*-Naproxen **45** and *S*-ketoprofen **46** (Scheme 15) were obtained in 40% yield (theoretical maximum 50%) and 97% ee. Recently, the enantioselective esterification of naproxen has been demonstrated using lipase from *Candida cylindracea* in iso-octane as solvent and trimethylsilyl as alcohol. The undesired isomer of naproxen was esterified leaving the desired *S* isomer unreacted [94].

Ibuprofen **47** is another well-known analgesic, anti-inflammatory drug and it is believed that it will be marketed as a single isomer drug. The kinetic enzymatic resolution of racemic ibuprofen has been reported [95]. The reaction for the resolution has been scaled-up to make gram quantities of *S*-ibuprofen. This was accomplished by two enantioselective reactions each catalyzed by Novozyme 435. In the first reaction, 300 g of racemic



Scheme 15

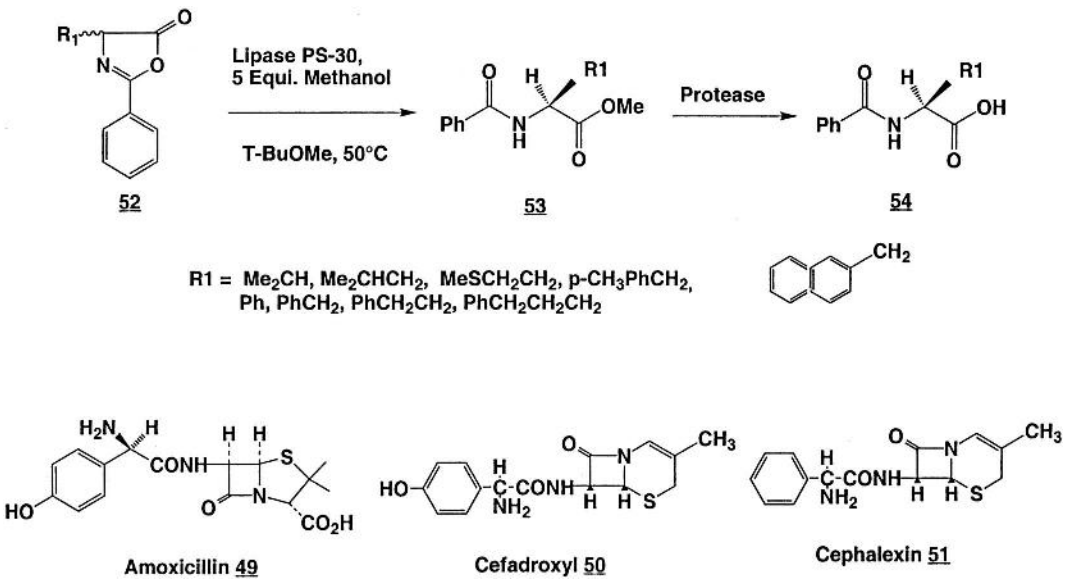
ibuprofen were esterified with 1-dodecanol to yield the *R* ester and *S*-ibuprofen to produce 89 g of *S*-ibuprofen in 85% enantiomeric excess. In the second reaction, 75 g of the 85% ee material were used to prepare 39 g of *S*-ibuprofen with a 97.5% ee.

Another approach for the enzymatic preparation of *S*-ibuprofen has been demonstrated by de Zoete et al. [96]. The enantioselective ammonolysis of ibuprofen 2-chloroethyl ester by *Candida antarctica* lipase (lipase SP435) gave the remaining ester *S*-(+)-enantiomer in 44% yield and 96% ee. The enantioselective enzymatic esterification of racemic ibuprofen has also been demonstrated using lipase from *Candida cylindracea* [97]. The reaction was carried out in a water-in-oil microemulsion [*bis*-(2-ethylhexyl) sulfosuccinate (AOT)/iso-octane]. The lipase showed high preference for the *S*-(+) enantiomer of ibuprofen which was esterified and the *R*-(−) enantiomer remained unreacted. The reaction yield of 35% was obtained using *n*-propanol in the reaction mixture as a nucleophile.

Gu et al. [98] has demonstrated the preparation of Naproxen via enzymatic enantioselective hydrolysis of racemic chloroethyl-2-(6-methoxy-2-naphthyl) propionate **48** by lipase from *Candida cylindracea* (Scheme 15). A reaction yield of 40% and ee of >96% were obtained for desired *S*-(−) product.

9 ANTI-INFECTIVE DRUGS

During the past several years, synthesis of α-amino acids has been pursued intensely [99–101] because of their importance as building blocks of compounds of medicinal interest particularly anti-infective drugs. The asymmetric synthesis of β-hydroxy-α-amino acids by various methods has been demonstrated [102–104] because of their utility as starting materials for the total synthesis of monobactum antiobiotics. D-Hydroxy phenylglycine (Scheme 16) is used in the production of amoxicillin **49** and cefadroxyl **50** [105,106], and D-phenylglycine is required for the semisynthetic antibiotic amphicillin and cephalexin **51**. The use of D-*p*-hydroxy phenylglycine will significantly increase because new drugs, such as aspoxicillin, cefbuperazine, and cepyramide are expected to be marketed.



Scheme 16

Recently, Crich et al. [107] have developed a general enzymatic asymmetric synthesis of α-amino acids. This method describes the use of the lipase PS-30 from *Pseudomonas cepacia* to catalyze the enantioselective methanolysis of a variety of 4-substituted 2-phenyloxazolin-5-one derivatives **52** in a nonpolar organic solvent to yield optically active *N*-benzoyl-L-α-amino acid methyl esters **53** in 70–98% ee (Scheme 16). The ee was further improved by the protease-catalyzed kinetic resolution to yield enantiomerically pure *N*-benzoyl-L-α-amino acids **54**.

In contrast to the method described in Scheme 16, D-amino acids are also commercially produced by a chemoenzymatic route using D-hydantoinase. In this process, chemically synthesized DL-5-substituted hydantoin is hydrolyzed to *N*-carbamoyl-D-amino acid and L-hydantoin by microbially derived D-hydantoinase. The latter compound undergoes rapid and spontaneous racemization under the reaction conditions; therefore, theoretically, a 100% yield can be obtained. The *N*-carbamoyl-D-amino acid is further chemically converted to the corresponding D-amino acid [108–111]. Recently, microbial *N*-carbamoylase has been demonstrated which catalyzed the conversion of *N*-carbamoyl-D-amino acid to the corresponding D-amino acid. Some organisms contained both D-hydantoinase and *N*-carbamoylase activity [112–115].

L-Hydantoinase activity has also been described from a microbial source that catalyzes the conversion of DL-5-substituted hydantoin to *N*-carbamoyl-L-amino acid. This process has been used in the production of L-amino acids [116,117].

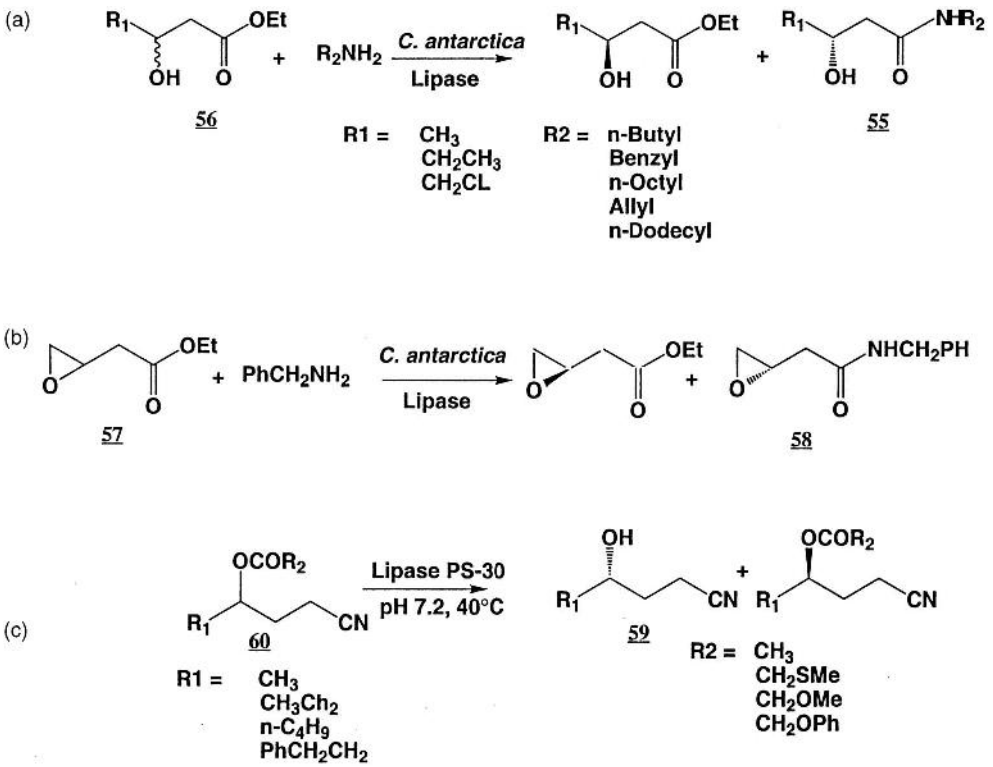
D-Amino acids and L-amino acids have also been prepared by D-specific or L-specific acylases derived from microbial sources. In this process, DL-*N*-acetyl amino acid is resolved by hydrolytic reaction to yield the D- or L-amino acid, depending on D- or L-selective acylase used in the reaction [118–120].

L-α-Amino acids have been prepared by the resolution of racemic α-amino acid amide by the L-specific aminopeptidase from *Pseudomonas putida* ATCC 12633 [121].

Enzyme from *Pseudomonas putida* ATCC 12633 cannot be used to resolve α-alkyl-substituted amino acid amides. Amino amidase from *Mycobacterium neoaurum* ATCC 25795 has been used in the preparation of the L-acid and D-amide of α-alkyl-substituted amino acids by an enzymatic resolution process using racemic α-alkylamino acid amide as a substrate [109,121]. Amidase from *Ochrobactrum anthropi* catalyzed the resolution of α,α-disubstituted amino acids, *N*-hydroxyamino acids, and α-hydroxy acid amides. The resolution process could lead to the production of chiral amino acids or amides in 50% yield. Recently, amino acid racemases have been used to achieve 100% yield of chiral amino acids [121].

The synthesis of amides is critical for the development of many therapeutic agents, as the amide bond is present in a large range of compounds [122]. Garcia et al. [123] have demonstrated the synthesis of optically active 3-hydroxyamides **55** (Scheme 17). Lipase from *Candida antarctica* efficiently catalyzed enantioselective aminolysis of various racemic 3-hydroxyesters **56** with aliphatic amines. A reaction yield of >54% and ee of >90% were obtained depending on the substrate used in the reaction mixture (Scheme 17a). *Candida antarctica* lipase also catalyzed the aminolysis of racemic ethyl-3,4-epoxybutyrate **57** to yield epoxyamide **58** in >92% ee and 27% reaction yield (Scheme 17b).

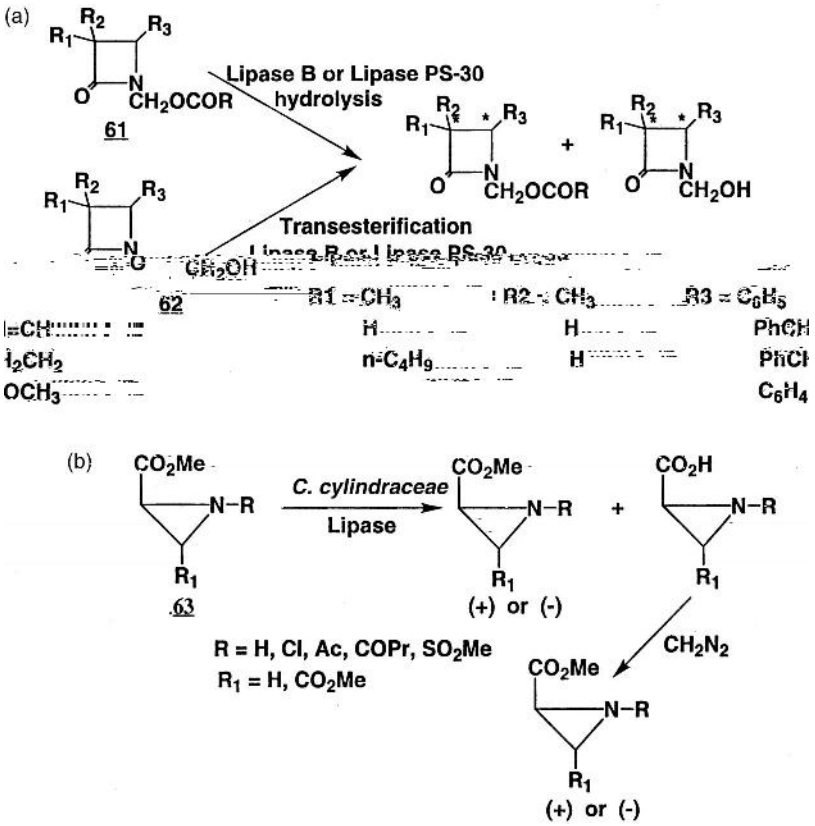
Optically active 4-hydroxyalkanenitriles **59** are useful building blocks for asymmetric synthesis, as the cyano group is a functional precursor group of the amino and carbonyl



Scheme 17

group. Lipase PS-30 catalyzed enantioselective hydrolysis of the ester of 4-hydroxy alkanenitriles **60** (Scheme 17c) has been demonstrated by Takagi and Itoh [124]. Enantiomeric excesses of 80–98% were obtained depending on the compound used in the reaction mixture.

After the discovery of the antibiotic thienamycin, compounds which contain the carbapenem and penem ring system have attracted much attention. The importance of stereochemistry of the hydroxyethyl group is demonstrated by the fact that this group must be in the (*R*) configuration for antimicrobial activity. Previously, syntheses of carbanepem and penem compounds have often utilized the optically active β-lactam intermediates [125–127]. For the synthesis of β-lactam antibiotics, the presence of asymmetric carbon at the 3- and 4-positions is critical to prepare optically active β-lactams [128]. Nagai et al. [129] have developed an enzymatic synthesis of optically active β-lactams by lipase-catalyzed kinetic resolution. They used the enantioselective hydrolysis of *N*-acyloxymethyl β-lactams **61** in an organic solvent (isopropylether saturated with water) and the transesterification of *N*-hydroxymethyl β-lactam **62** in an organic solvent (methylene chloride) in the presence of vinyl acetate as the acyl donor (Scheme 18a). Reaction yields of 35–50% (theoretical maximum is 50%) and ee's of 86–98% were



Scheme 18

obtained, depending on the specific substrate used in the reaction mixture. Lipase B from *Pseudomonas fragi* and lipase PS-30 from *Pseudomonas* sp. were used in the reaction mixture.

The use of enantioselective ester hydrolysis in the synthesis of optically active *N*-unsubstituted and *N*-substituted aziridine carboxylate by *Candida cylindracea* lipase has been demonstrated by Bucciareli et al. [130]. Racemic methyl aziridine-2-carboxylate and 2,3-dicarboxylate **63** were used as substrates both for enzymatic hydrolysis and for the synthesis of *N*-chloro, *N*-acyl and *N*-sulfonyl derivatives (Scheme 18b). Reaction yields of 35–45% (theoretical maximum is 50%) and the ee's of 80–96% were obtained depending on the substrate used in the reaction mixture. Aziridinecarboxylates are chiral intermedates for the synthesis of β-lactams and amino acids [131].

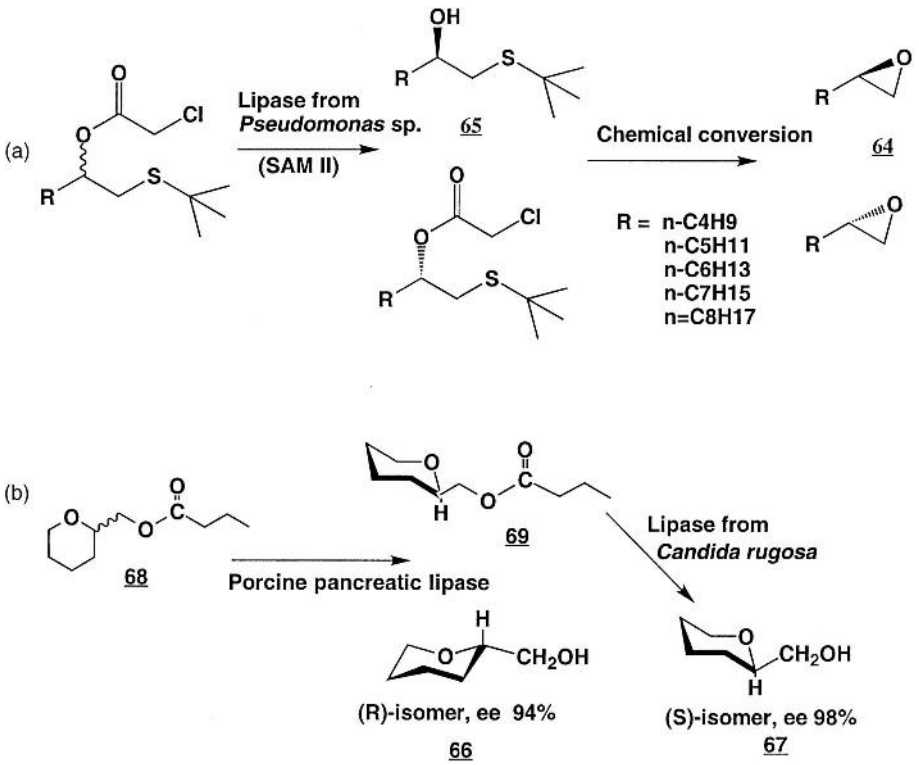
(*S*)- and (*R*)-3-hydroxyglutaric acid monoesters are chiral precusors for the synthesis of L-carnitine, carbapenem, and compactin, an anticholesterol drug. Monterio et al. [132] demonstrated the enantioselective hemihydrolysis of diethyl-3-hydroxyglutaric acid with esterase 30,000 to prepare (*S*)-3-hydroxyglutaric acid monoester in 90% yield and >98% ee. They also demonstrated the quantitative synthesis of (*R*)-3-hydroxyglutaric acid monoester by catalyzing the hydrolysis of ethyl *t*-butyl 3-hydroxyglutaric acid by esterase 30,000. Pig-liver-esterase-catalyzed enantioselective hydrolysis of dimethyl 3-hydroxy, 3-methoxyethoxymethoxy, and 3-benzyloxy glutarates has been demonstrated by Lam and Jones [133]. The enzymatic preparation of optically active 2,4-dimethylglutaric acid monomethyl esters from the corresponding diester has been demonstrated by Chen et al. [134] using lipase from *Glicocledium roseum*. Asymmetric hydrolysis of diethyl 3-hydroxy-3-methylglutarate to its corresponding monoester in high ee by pig liver esterase has been demonstrated [135,136]. Chiral monoesters are synthons for the chemoenzymatic preparation of β-lactam.

Chiral 6-substituted 5,6-dihydro-2H-pyran-2-ones (α,β-unsaturated δ-lactones) are key structural intermediates for a variety of natural products which exhibit antifungal activity [137]. Hasse and Schneider [138] prepared both enantiomeric series of a variety of optically pure 6-alkylated δ-lactones via an enzyme-mediated route. The key step in the process was the ring opening of enantiomerically pure alkyl oxiranes **64**, accessible via the corresponding β-hydroxythioethers **65** which were obtained enantiomerically pure by the lipase-catalyzed kinetic resolutions (Scheme 19a).

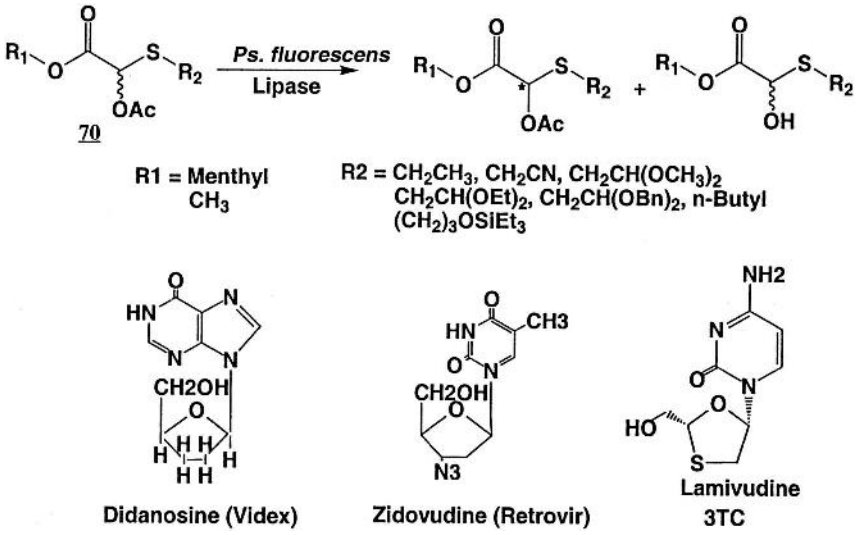
Tetrahydropyran-2-methanol and tetrahydrofurfuryl alcohol are key chiral intermediates for the synthesis of polyether antibiotics. Such antibiotics are known as ionophores, which are able to transport metal ions across biological membranes, and some of their medicinal application are in diuretics and potent analgesics [139,140]. Chiral (*R*)- and (*S*)-tetrahydropyran-2-methanol **66** and **67** has been prepared from the hydrolysis of its butyrate ester **68** using porcine pancreatic lipase [140]. The unreacted (*S*) enantiomer of ester **69** was hydrolyzed by lipase from *Candida rugosa*. Both enantiomers were prepared in >95% enantiomeric excess (Scheme 19b).

10 ANTIVIRAL AGENTS

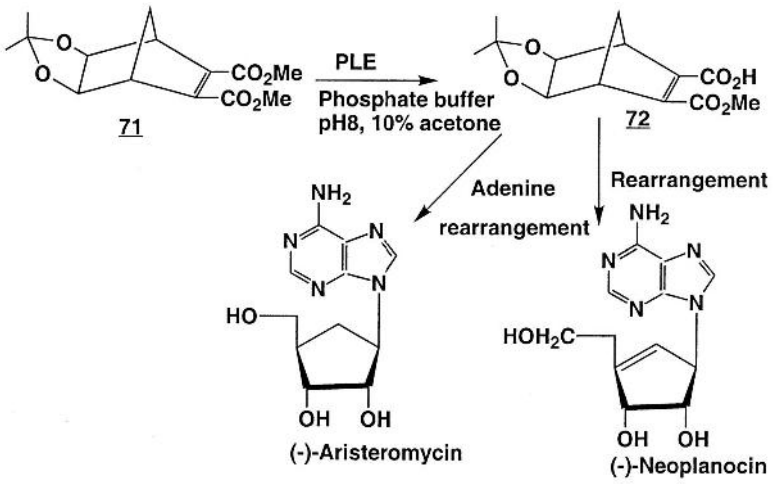
Purine nucleoside analogs have been used as antiviral agents [141]. lamivudine, zidovudine, and didanosine (Scheme 20) are effective antiviral agents. Lamivudine, a highly promising drug candidate for HIV 2 and HIV 3 infection provides a challenge to the synthetic chemist due to the presence of two acetal chiral centers, both sharing the same oxygen



Scheme 19



Scheme 20



Scheme 21

atom. The use of cytidine deaminase from *E. coli* [142] has been demonstrated to deaminate 2′-deoxy-3′-thiacytidine enantioselectively to prepare optically pure (2′*R*-*cis*)-2′-deoxy-3′-thiacytidine (3TC, lamivudine).

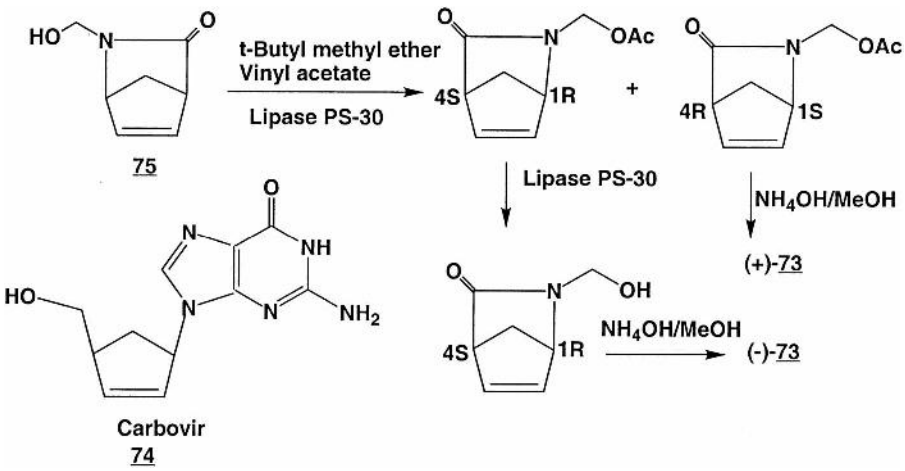
A novel enzymatic resolution process has been developed for the preparation of chiral intermediate for lamivudine synthesis. An enzymatic resolution of α-acetoxysulfides **70** (Scheme 20) by *Pseudomonas fluorescens* lipase has been demonstrated to give chiral intermediate in >45% yield (theoretical maximum is 50%) and 95% ee [143].

Biologically important carbocyclic nucleosides such as (−)-aristeromycin and (−)-neoplanocin have been prepared by the lipase-catalyzed asymmetric hydrolysis with pig liver esterase [144]. Asymmetric hydrolysis of the meso-epoxy diesters dialkyl 5,6-epoxybicyclo[2.2.1]hept-2-ene-2,3-dicarboxylate **71** with pig liver esterase quantitatively produced the corresponding optically active monoester **72** in >92% ee (Scheme 21).

Racemic 2-azabicyclo[2.2.1]hept-5-ene-3-one has great potential as a synthetic intermediate. The bicyclic lactam is a synthon for carbocyclic sugar amines, carbonucleosides, and carbocyclic dinucleotide analogs [145]. Chiral 2-azabicyclo[2.2.1]hept-5-ene-3-one **73** (Scheme 22) is a key intermediate for the synthesis of (−)-carbovir **74**, an antiviral agent effective against HIV. Nakano et al. [146] have prepared chiral **73** by enantioselective transesterification of hydroxmethyl-2-azabicyclo[2.2.1]hept-5-ene-3-one **75** (Scheme 22). A reaction yield of 40% and ee of 92% were obtained.

11 PROSTAGLANDINS SYNTHESIS

Optically active epoxides are useful chiral synthons in the pharmaceutical synthesis of prostaglandins. Microbial epoxidation of olefinic compounds was first demonstrated by van der Linden [147]. Subsequently, May et al. [148] demonstrated the epoxidation of alkenes in addition to hydroxylation of alkanes by ω-hydroxylase system. Oxidation of alk-1-enes in the range C6–C12, α,ω-dienes from C6–C12, alkyl benzene, and allyl ethers were demonstrated using the ω-hydroxylase enzyme system from *Pseudomonas oleovorans*. *R*-Epoxy compounds in greater than 75% ee were produced by epoxidation reactions

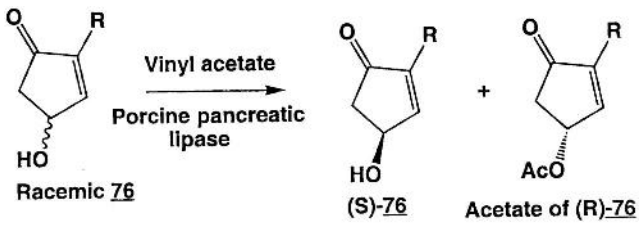


Scheme 22

using the ω-hydroxylase system [149,150]. The epoxidation system from *Nocardia corallina* is very versatile and has broad substrate specificity and reacts with unfunctionalized aliphatic as well as aromatic olefins to produce *R* epoxides [151,152].

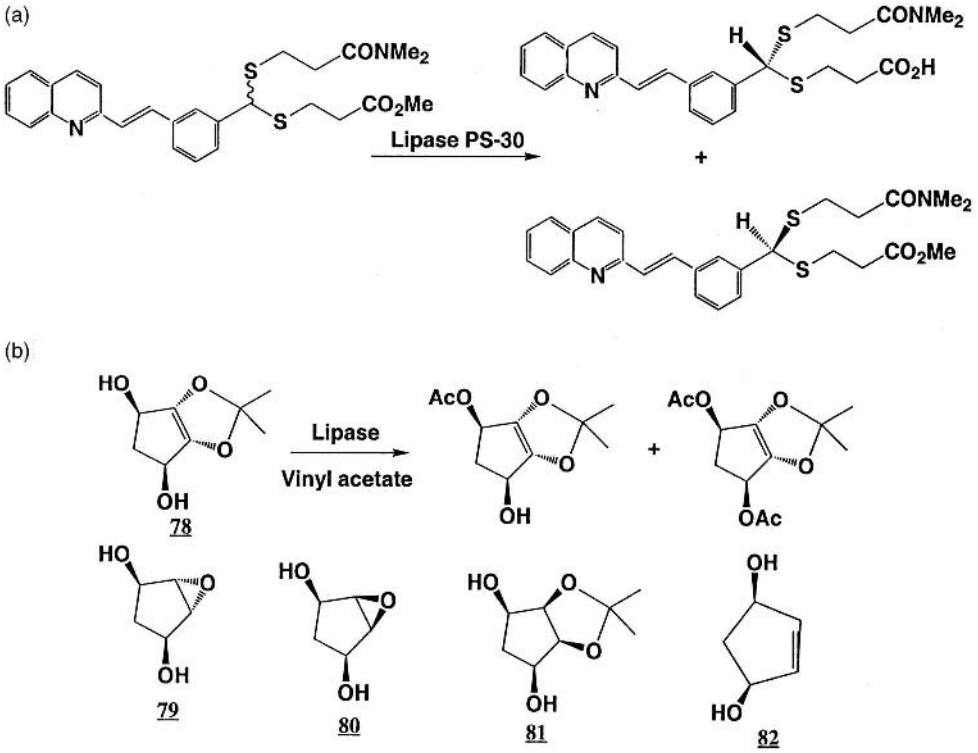
Chiral compounds **76** and **77** (Scheme 23) are key intermediates for the chemoenzymatic synthesis of some prostaglandin analogs used for the treatment of peptic ulcer disease. Babiak et al. [153] evaluated several lipases, including that from *Pseudomonas* sp., *Candida cylindracea*, *Aspergillus niger*, and porcine pancreas, to catalyze the resolution of racemic compounds **76** and **77**. It was demonstrated that all of the enzymes were selective in acylating the *R*-hydroxyl isomer of the starting hydroxy enone compound. Porcine pancreatic lipase, either free or immobilized on Amberlite XAD-8, gave a 45% reaction yield (theoretical maximum is 50%) and 98% ee of products (Scheme 23).

An efficient chemoenzymatic synthesis of both enantiomers of an LTD4 antagonist have been prepared by lipase-catalyzed asymmetric hydrolysis of prochiral and racemic dithioacetal esters having up to five bonds between the prochiral/chiral center and the ester carbonyl group. An ee of 99% and reaction yield of 45% were obtained using lipase PS-30 (Scheme 24a). LTD4 antagonists have potential for therapeutic treatment of asthma [154].



R = $(CH_2)_6CO_2Me$
$(CH_2)_2$-C=C$(CH_2)_2CO_2Me$ 77
CH_2-C=C-$(CH_2)_3CO_2Me$

Scheme 23



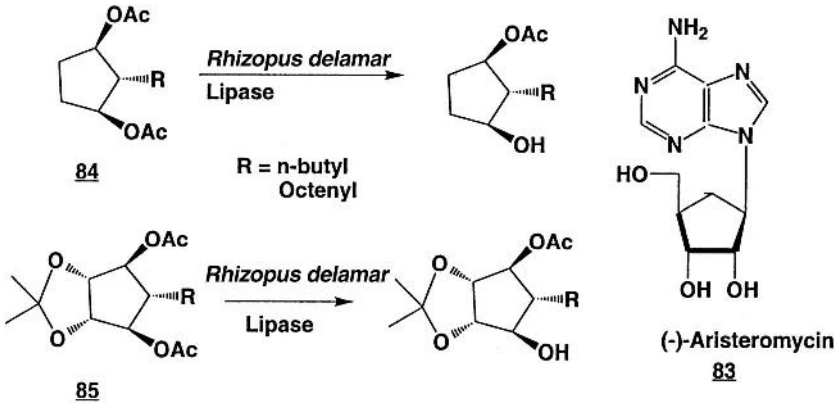
Scheme 24

Lipase-catalyzed transesterification of meso-cyclopentane diols **78–82** have been demonstrated by Theil et al. [155] with vinyl acetate as the acyl donor and tetrahydrofuran/triethylamine as solvent (Scheme 24b). The chiral monoacetate was obtained in 65–85% reaction yields and 95% ee, depending on the substrate used in the transesterification reaction. The chiral monoacetate serves as the starting material for the prostaglandin synthesis.

Tanaka et al. [156] have demonstrated the enzymatic synthesis of the sugar moiety of carbocyclic nucleosides required for the total synthesis of (−)-aristeromycin **83** (Scheme 25). Using lipase from *Rhizopus delamar*, the enantioselective hydrolysis of meso-1,3-*bis*-(acetoxymethyl)-2-*trans*-alkylcyclopentane **84** and **85** were carried out to prepare the corresponding chiral monoacetate in >98% ee (Scheme 25). The chiral monoacetate was also used in the synthesis of optically active 11-deoxyprostagladins [157].

12 CALCIUM CHANNEL ANTAGONISTS

Dilthiazem is a calcium channel antagonist useful as a coronary vasodilator and has been widely used in controlling hypertension. A chiral intermediate, (2*R*,3*S*)-3-(4-methoxyphenyl) glycidic acid methyl ester [(−)-MPGM] **86**, is required for the synthesis of dilthiazem **87** (Scheme 26). Matsumae et al. [158] screened over 700 microorganisms and identified lipase from *Serratia marcescens*, which catalyzed the enantioselective hydrolysis of racemic MPGM in a biphasic system using toluene as the organic phase (Scheme 26). A reaction yield of 48% and ee of 98% were obtained for (−)-MPGM.

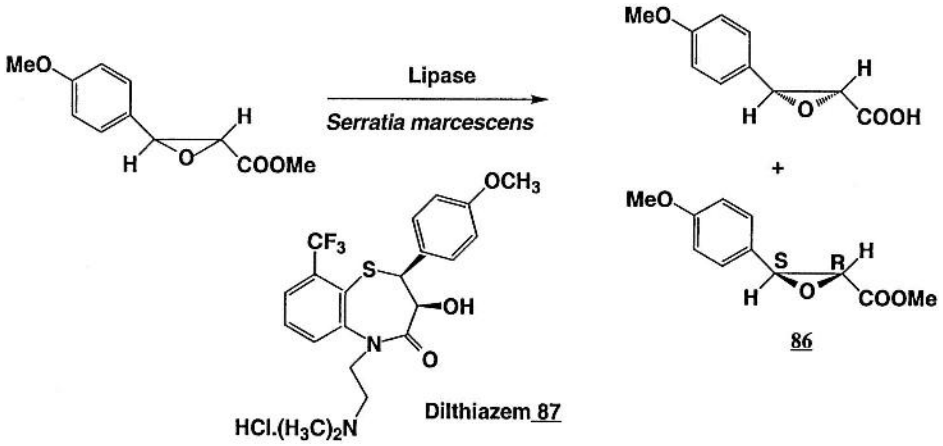


Scheme 25

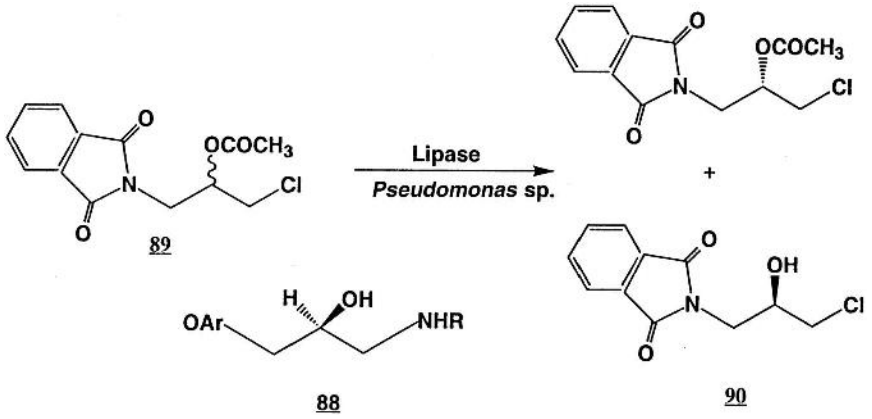
13 ANTIARRHYTHMIC AGENTS

Larsen and Lish [159] reported the biological activity of a series of phenethanolamine-bearing alkyl sulfonamido groups on the benzene ring. Within this series, some compounds possessed adrenergic and antiadrenergic actions. D-(+)-Sotalol is a β-blocker [160] that, unlike other β-blockers, has antiarrhythmic properties and has no other peripheral actions [161]. The β-adrenergic blocking drugs, such as propanolol and sotalol, have been separated chemically into the dextro and levo rotatory optical isomers, and it has been demonstrated that the activity of the levo isomer is 50 times that of the corresponding dextro isomer [162].

The chiral compound **88** (Scheme 27) belongs to a group of a 1,2,3-trisubstituted propane derivatives, which are a valuable source of chiral building blocks in the synthesis of β-adrenergic blocking agents, derivatives of 1-alkylamino-3-aryloxy-2-propanol (X = ArO, Y = HNR). (*S*)-Propranolol has a 1-naphthyloxy for the ArO group and an isopropylamino for the HNR group, whereas in (−)-timolol, the ArO group is 4-mortholino-1,2,5-thiadiazol-3-yloxy and the HNR group is *tert*- butylamino. The racemic compound **89** has



Scheme 26



Scheme 27

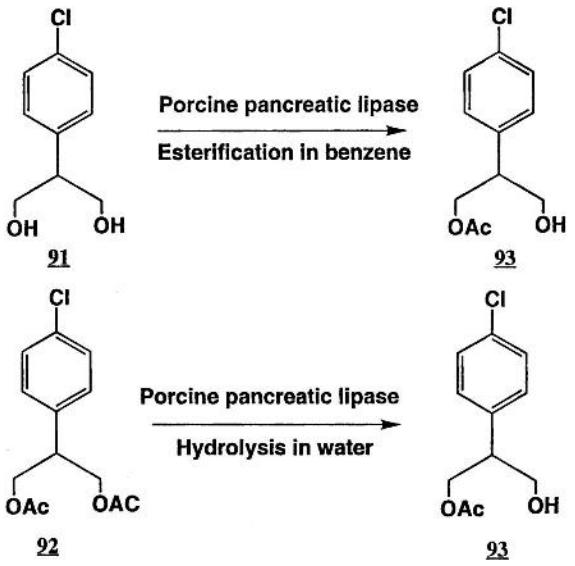
been prepared by Gelo and Sunjic [163] to evaluate the lipase-catalyzed enantiomeric hydrolysis. Lipase from *Pseudomonas* sp. catalyzed the enantioselective hydrolysis of racemic **89** in the presence of 10% acetone and Triton X-100 to yield chiral alcohol **90** in 40% yield and 99% ee (Scheme 27).

γ-Aminobutyric acid (GABA) is an inhibitory neurotransmitter related to the control of neuronal activity in the central nervous system, which regulates several physiological mechanisms. GABA has been shown to act through at least two different receptor sites with different binding properties ($GABA_A$ and $GABA_B$ receptors). Baclofen, or 4-amino-3-(4-chlorophenyl) butanoic acid, is an analog of GABA and is a selective agonist for the GABA receptor and has been used as an antispastic agent. The *R* enantiomer of baclofen is more active. Chenevert and Desjardins [164] have recently reported the chemoenzymatic preparation of (*R*)-baclofen using porcine pancreatic lipase. The enzymatic esterification of baclofen precursor 2-(4-chlorophenyl)-1,3-propanediol **91** was carried out in an organic solvent in the presence of acetic anhydride or hydrolysis of diacetate **92** in aqueous system to prepare desired enantiomer of monoacetate **93** (Scheme 28).

14 IMMUNOSUPPRESSIVE AGENT (15*S*-DEOXYSPERGAULIN)

An antitumor antibiotic spergualin was discovered in the culture filtrate of a bacterial strain BMG162-aF2, which is related to *Bacillus laterosporus*, and its structure was determined to be (−)-(15*S*)-1-amino-10-guanidino-11,15-dihydroxy-4,9,12-triazanonadecane-10,13-dione [165,166]. The total synthesis was accomplished by the acid-catalyzed condensation of 11-amino-1,1-dihydroxy-3,8-diazaundecane-2-one with (*S*)-7-guanidino-3-hydroxy heptanamide, followed by the separation of the 11-epimeric mixture [167]. Antibacterial or antitumor activity of the enantiomeric mixture of spergualin was about half of that of the natural spergualin, indicating the importance of the configuration at C-11 for antitumor activity [168].

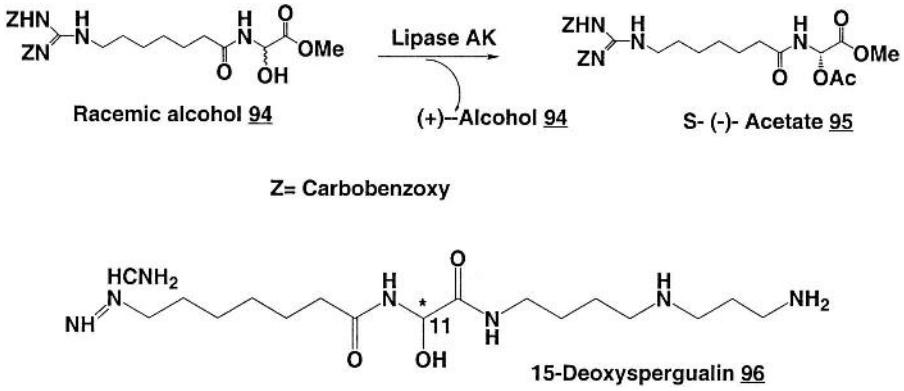
Umeda et al. [169] have demonstrated the optical resolution of the key intermediate of 15-deoxyspergualin, racemic *N*-(7-guanidino-heptanoyl)-α-alkoxyglycine, by use of an exopeptidase (serine carboxypeptidase) and racemic *N*-(7-guanidino-heptanoyl)-α-alkoxyglycyl-L-amino acid as the substrate. Carboxypeptidase from *Penicillum janthinellum*



Scheme 28

catalyzed the hydrolysis of peptide bond of racemic *N*-(7-guanidinoheptanoyl)-α-methoxyglycyl-L-phenylalanine to yield (−)-*N*-(7-guanidinoheptanoyl)-α-methoxyglycine. They deduced that the absolute configuration of the carbon at 11 (C-11) of the bioactive (−)-enantiomer, and so that of the natural spergualin, is *S*. The (−)-enantiomer of 15-deoxyspergualin was active against mouse leukemic L1210, whereas the (+)-enantiomer was almost inactive.

We have demonstrated an alternate and more direct route, the lipase-catalyzed stereoselective acetylation of racemic 7-[*N*,*N′-bis*(benzyloxycarbonyl)-N-(guanidinoheptanoyl)]-α-hydroxy-glycine **94** to the corresponding *S*-(−)-acetate **95** and unreacted alcohol (+)-**94**. *S*-(−)-acetate **95** is a key intermediate for the total chemical synthesis of (−)-15-deoxyspergualin **96** (Scheme 29), a related immunosuppressive agent and antitumor antibiotic [170].



Scheme 29

Commercially available lipases [lipase PS-30, lipase AY-30, lipase PG, lipase AK, lipase OF, lipase R, lipase MAP-10, lipase GC-20, porcine pancreatic lipase (PPL), and esterase 3000] were screened for the stereoselective acetylation of racemic **94** in an organic solvent (toluene) in the presence of isopropenyl acetate (IPAC) as the acyl donor. Lipase AY-30, lipase PG, lipase R, PPL, and lipase GC-20 were inactive in the enzymatic resolution process. Lipase AK from *Pseudomonas* sp. efficiently catalyzed the enantioselective acetylation of the desired enantiomer of racemic **94**. A reaction yield of 46% and an ee of 98% were obtained for *S*-(−)-acetate **95**. A reaction yield of 41% and an ee of 98.8% were obtained for the unreacted (+)-alcohol **94** [170].

REFERENCES

1. CJ Sih, CS Chen. Angew Chem Int Ed Engl 23:570–578, 1984.
2. JB Jones. Tetrahedron 42:3351–3403, 1986.
3. DHG Crout, S Davies, RJ Heath, CO Miles, DR Rathbone, BEP Swoboda. Biocatalysis 9: 1–30, 1994.
4. HG Davies, RH Green, DR Kelly, SM Roberts. Biotechnology 10:129–152, 1990.
5. R Csuz, BI Glanzer. Chem Rev 96:556–566, 1991.
6. J Crosby. Tetrahedron 47:4789–4846, 1991.
7. J Kamphuis, WHJ Boesten, QB Broxterman, HFM Hermes, JAM van Balken, EM Meijer, HE Schoemaker. Adv Biochem Eng Biotechnol 42:133–186, 1990.
8. CJ Sih, Q-M Gu, Q-M, X Holdgrun, K Harris. Chirality 4:91–97, 1992.
9. SC Stinson. Chem Eng News 46, September 28, 1992.
10. E Santaneillo, P Ferraboschi, P Grisenti, A Manzocchi. Chem Rev 92:1071–1140, 1992.
11. AL Margolin. Enzyme Microb Technol 15:266–280, 1993.
12. DC Cole. Tetrahedron 50:9517–9582, 1994.
13. C-H Wong, GM Whitesides. Enzymes in Synthetic Organic Chemistry. Tetrahedron Organic Chemistry Series Vol. 12. Tarrytown, NY: Pergamon/Elsevier Science, 1994.
14. K Mori. Synlett 1097–1109, November 1995.
15. AM Klibanov. Science 219:722–727, 1983.
16. MC Wani, HL Taylor, ME Wall, P Coggon, AT McPhail. J Am Chem Soc 93:2325–2327, 1971.
17. DGI Kingston. Pharmac Ther 52:1–34, 1991.
18. PB Schiff, J Fant, SB Horowitz. Nature 277:665–667, 1979.
19. KC Nicolaou, W-M Dai, RK Guy. Angew Chem Int Ed Engl 33:15–44, 1994.
20. AA Christen, DM Gibson, J Bland. US Patent 5,019,504, 1991.
21. J-N Denis, AE Greene, AA Serre, M-J Luche. J Org Chem 51:46–50, 1986.
22. RA Holton, RR Juo, HB Kim, AD Williams, S Harusawa, RE Lowenthal, S Yogai. J Am Chem Soc 110:6558–6560, 1988.
23. RN Patel, A Banerjee, RY Ko, JM Howell, W-S Li, FT Comezoglu, RA Partyka, LJ Szarka. Biotechnol Appl Biochem 20:23–33, 1994.
24. D-M Gou, Y-C Liu, C-S Chen. J Org Chem 58:1287–1289, 1993.
25. R Brieva, JZ Crich, CJ Sih. J Org Chem 58:1068–1075, 1993.
26. M Nakane. US Patent 4,663,336, 1987.
27. AW Ford-Hutchinson. Clin Exper Allergy 21:272–276, 1991.
28. J Das, MF Haslanger, JZ Gougoutas, MF Malley. Synthesis 12:1100–1103, 1987.
29. N Hamaka, T Seko, T Miyazaki, A Kawasaki. Adv Prostaglandin Thromboxane Leukotri Res 21:359–362, 1990.
30. RN Patel, M Liu, A Banerjee, LJ Szarka. Appl Microbiol Biotechnol 37:180–183, 1992.
31. MA Ondetti, DW Cushman. J Med Chem 24:355–361, 1981.
32. MA Ondetti, B Rubin, DW Cushman. Science 196:441–444, 1977.

33. DW Cushman, MA Ondetti. Biochem Pharmacol 29:1871–1875, 1980.
34. CT Goodhue, JR Schaeffer. Biotechnol Bioeng 13:203–214, 1971.
35. J Hasegawa, M Ogura, H Kanema, N Noda, H Kawaharada, KJ Watanabe. J Ferment Technol 60:501–508, 1982.
36. CJ Sih. European Patent 87264125, 1987; Derwent International Publication #W087105328, 1987.
37. QM Gu, CS Chen, CJ Sih. Tetrahedron Lett 27:1763–1766, 1986.
38. A Sakimas, K Yuri, N Ryozo, O Hisao. European Patent 0172614, 1986.
39. RN Patel, JM Howell, CG McNamee, KF Fortney, LJ Szarka. Biotechnol Appl Biochem 16:34–47, 1992.
40. RN Patel, JM Howell, A Banerjee, KF Fortney, LJ Szarka. Appl Microbiol Biotechnol. 36: 29–34, 1991.
41. JL Moniot US Patent Appl CN 88-100862, 1988.
42. MA Ondetti, A Miguel, J Krapcho. US Patent 4,316,906, 1982.
43. NG Delaney, EN Gordon, JM DeForrest, DW Cushman. European Patent EP361365, 1988.
44. B Shadid, HC van der Plas, WHJ Boesten, J Kamphuis, EM Meijer, HE Schoemaker. Tetrahedron 46:913–920, 1990.
45. RN Patel, RS Robison, LJ Szarka. Appl Microbiol Biotechnol 34:10–14, 1990.
46. DM Spero, S Kapadia, V Farina. Tetrahedron 36:4543–4546, 1995.
47. P Mohr, N Waespe-Sarcevic, C Tamm. Helv Chim Acta 66:2501–2511, 1983.
48. K Laumen, M Schneider. Tetrahedron 26:2073–2076, 1985.
49. GMR Tombo, HP Schar, XF Busquets, O Ghisalba. Tetrahedron 27:5707–5710, 1986.
50. K Mori, K Tanida. Tetrahedron 40:3471–3479, 1984.
51. M Hirama, M Uei. J Am Chem Soc 104:4251–4253, 1982.
52. AS Gopalan, CJ Sih. Tetrahedron 25:5235–5238, 1984.
53. OP Ward, CS Young. Enzyme Microb Technol 12:482–493, 1990.
54. CJ Sih, BN Zhan, AS Gropalan, CS Chen, G Girdaukais, F vanMiddlesworth. Ann NY Acad Sci 434:186–193, 1984.
55. SY Sit, RA Parker, I Motoe, HW Balsubramanian, CD Cott, PJ Brown, WE Harte, MD Thompson, J Wright. J Med Chem 33:2982–2999, 1990.
56. RN Patel, CG McNamee, LJ Szarka. Appl Microbiol Biotechnol 38:56–60, 1992.
57. H Suemune, M Takahashi, S Maeda, Z-F Xie, K Sakai. Tetrahedron Asymm 1:425–428, 1990.
58. ARPM Valentijn, R de Hann, E de Kant, GA van der Marel, LH Cohen, JH van Boom. Recl Trav Chim Pays-Bas 114:332–336, 1995.
59. SA Biller, MJ Sofia, B DeLange, C Foster, EM Gordon, T Harrity, LC Rich, CP Ciosek. J Am Chem Soc 113:8522–8525, 1991.
60. SA Biller, C Foster, EM Gordon, T Harrity, LC Rich, J Maretta, CP Ciosek. J Med Chem Soc 34:1912–1918, 1991.
61. A Steiger, HJ Pyun, RM Coates. J Org Chem 57:3444–3449, 1992.
62. AC Oehlschlanger, SM Singh, S Sharma. J Org Chem 56:3856–3861, 1991.
63. SA Biller, D Majnin. US Patent 5,428,028, 1995.
64. M Lawrence, SA Biller, OM Fryszman. US Patent 5,447,922, 1995.
65. RN Patel, A Banerjee, LJ Szarka. Tetrahedron Asymm 8:1055–1059, 1997.
66. CD Ferris, DJ Hirsch, BP Brooks, SH Snyder. J Neurochem 57:729–737, 1991.
67. JL Junien, BE Leonard. Clin Neuropharmacol 12:353–374, 1989.
68. LM Walker, WD Bower, FO Walker, RR Matsumoto, BD Costa, KC Rice. Pharm Rev 42: 355–402, 1990.
69. GF Steinfels, SW Tam, L Cook. Neuropsychopharmacology 2:201–207, 1989.
70. T Massamiri, SP Duckles. J Pharmacol Exp Ther 253:124–129, 1990.
71. JA Martinez, L Bueno. Eur J Pharmacol 202:379–383, 1991.
72. DP Taylor, MS Eison, SL Moon, FD Yocca. Adv Neuropsychol Psychopharm 1:307–315, 1991.

73. RL Hanson, A Banerjee, FT Comezoglu, KD Mirfakhrae, RN Patel, LJ Szarka. Tetrahedron Asymm 5:1925–1934, 1994.
74. KS Patrick, JL Singletary. Chirality 3:208–212, 1991.
75. PS Portoghese, TL Pazdernik, WL Kuhn, G Hite, A Shafike. J Med Chem 11:12–20, 1968.
76. V Vechetti, A Giordani, G Giardina, R Colle, D Clarke. J Med Chem 34:397–402, 1991.
77. MC Ng-Youn-Chen, AN Serreqi, Q Huang, RJ Kazlaukas. J Org Chem 59:2075–2081, 1994.
78. G Edwards, AH Weston. TIPS 11:417–422, 1990.
79. DW Robertson, MI Steinberg. Ann Med Chem 24:91–100, 1989.
80. DW Robertson, MI Steinberg. J Med Chem 33:1529–1533, 1990.
81. TC Hamilton, AH Weston. Gen Pharmacol 20:1–9, 1989.
82. VA Ashwood, RE Buckingham, F Cassidy, JM Evans, TC Hamilton, DJ Nash, G Stempo, KJ Willcocks. J Med Chem 29:2194–2201, 1986.
83. EN Jacobsen, W Zhang, AR Muci, JR Ecker, L Deng. J Am Chem Soc 113:7063–7064, 1991.
84. R Bergmann, V Eiermann, RJ Gericke. J Med Chem 33:2759–2761, 1990.
85. K Atwal, GJ Grover, KS Kim. US Patent 5,140,031, 1991.
86. RN Patel, A Banerjee, CG McNamee, LJ Szarka. Tetrahedron Asymm 6:123–130, 1995.
87. JH Fried, IT Harrison. British Patent 1211134, 1967.
88. IT Harrison, B Lewis, P Nelson, W Rooks, A Roszwski, A Tomolonis, JH Fried. J Med Chem 13:203–208, 1970.
89. C Giordano, M Villa, S Panossian. In: AN Collins, GN Sheldrake, J Crosby, eds. Chirality in Industry. New York: Wiley, 1992, pp 303–312.
90. QM Gu, DR Reddy, CJ Sih. Tetrahedron Lett 27:5203–5206, 1986.
91. E Battistel, D Bianchi, P Cesti, C Pina. Biotechnol Bioeng 38:659–664, 1991.
92. N Layh, A Stolz, J Bohme, F Effenberger, H-J Knackmuss. J Biotechnol 33:175–182, 1994.
93. K Yamamoto, Y Uneo, K Otsubo, K Kawakami, K Komatsu. Appl Environ Microbiol 56: 3125–3129, 1990.
94. S-W Tsai, H-J Wei. Enzyme Microb Technol 16:328–333, 1994.
95. M Trani, A Ducret, P Pepin, R Lortie. Biotechnol Lett 17:1095–1098, 1995.
96. MC de Zoete, J Kock-van Dalen, F van Rantwijk, RA Sheldon. Biocatalysis 10:307–316, 1994.
97. G Hedstrom, M Backlund, JP Slotte. Biotechnol Bioeng 42:618–624, 1993.
98. QM Gu, C-S Chen, CJ Sih. Tetrahedron 27:1763–1766, 1986.
99. RM Williams. In: JE Baldwin, PD Magnus, eds. Synthesis of Optically Active α-Amino Acids, Vol. 7, Oxford: Pergamon, 1989, pp 130–150.
100. MJ O'Donnell, WD Bennett, S Wu. J Am Chem Soc 111:2353–2355, 1989.
101. U Schmidt, M Respondek, A Lieberknecht, J Werner, P Fischer. Synthesis 28:256–261, 1989.
102. G Bold, RO Duthaler, M Riediker. Angew Chem Int Ed Engl 28:497–498, 1989.
103. Y Ito, M Sawamura, E Shirakawa, K Hayashizaki, T Hayashi. Tetrahedron 44:5253–5262, 1988.
104. EM Gordon, MA Ondetti, J Pluscec, CM Cimarusti, DP Bonner, RB Sykes. J Am Chem Soc 104:6053–6060, 1982.
105. K Aida, I Chibata, K Nakayama, K Takinami, H Yamada, eds. Biotechnology of Amino Acid Production. Progress Industrial Microbiology Vol. 24. Amsterdam: Elsevier, 1986.
106. C Syldatk, D Cotoras, G Dombach, C Grob, H Kallwab, F Wagner. Biotechnol Lett 9:25–30, 1987.
107. JZ Crich, R Brieva, P Marquart, R-L Gu, S Flemming, CJ Sih. J Org Chem 58:3252–3258, 1993.
108. A Moller, C Syldatk, M Schulze, F Wagner. Enzyme Microb Technol 10:618–625, 1988.
109. J Kamphuis, HFM Hermes, JAM van Balken, HE Schoemaker, WHJ Boesten, ME Meijer. In: G Lubec, GA Rosenthal eds. Amino Acids, Chemistry, Biology and Medicine. Vienna: ESCOM Science, 1990, pp 119–125.

110. A Morin, W Hummel, M-R Kula. Appl Microbiol Biotechnol 25:91–96, 1986.
111. H Yamada, S Takahashi, Y Kii, H Kumagai. J Ferment Technol 56:484–491, 1978.
112. R Oliveiri, E Fascetti, L Angelini, L Degen. Enzyme Microb Technol 1:201–204, 1979.
113. S Runser, N Chinski, E Ohleyer. Appl Microbiol Biotechnol 33:382–388, 1990.
114. K Yokozeki, S Nakamori, S Yamanaka, C Eguchi, K Mitsugi, F Yoshinaga. Agric Biol Chem 51:715–719, 1987.
115. G-J Kim, H-S Kim. Enzyme Microb Technol 17:63–67, 1995.
116. Y Nishida, K Nakamichi, K Nabe, T Tosa. Enzyme Microb Technol 9:721–725, 1987.
117. R Tsugawa, S Okumura, T Ito, N Katsuya. Agr Biol Chem 30:27–34, 1966.
118. M Sugie, H Suzuki. Agric Biol Chem 44:1089–1095, 1989.
119. I Chibata, T Tosa, T Sato, T Mori. Methods Enzymol 44:746–759, 1976.
120. K Sakai, K Oshima, M Moriguchi. Appl Environ Microbiol 57:2540–2543, 1991.
121. J Kamphuis, EM Meijer, WHJ Boesten, QB Broxterman, B Kaptein, HFM Hermes, HE Schoemaker. In: D Rozzell, F Wagner, eds. Production of Amino Acids and Derivatives. Munich: Hanser Publishers, 1992, pp 117–200.
122. BC Challis, JA Challis. Comprehensive Organic Chemistry, Vol. 2. New York: Pergamon, 1979, pp 957–985.
123. M Garcia, C del Campo, EF Llama, JM Sanchez-Montero, JV Sinisterra. Tetrahedron 49: 8433–8440, 1993.
124. Y Takagi, T Itoh. Bull Chem Soc Japan 66:2949–2955, 1993.
125. K Fujimoto, Y Iwano, K Hirai, S Sugawara. Chem Pharm Bull 34:999–1014, 1986.
126. T Shibata, K Iino, T Tanaka, T Hashimoto, Y Kameyama, Y Sugimura. Tetrahedron 26: 4739–4742, 1985.
127. M Alpegiani, A Bedeschi, F Giudichi, E Perrone, G Franceschi. J Am Chem Soc 107:6398–6399, 1985.
128. R Labila, C Morin. J Antibiot 37:1103–1129, 1984.
129. H Nagai, T Shizawa, K Achiwa, Y Terao. Chem Pharm Bull 41:1933–1938, 1993.
130. M Bucciareli, A Forni, I Moretti, F Prati, G Torre. J Chem Soc Perkin Trans 1:3041–3045, 1993.
131. D Tanner, C Birgersson, HK Dhaliwal. Tetrahedron 31:1903–1906, 1990.
132. J Monteiro, J Braun, F LeGoffic. Synthetic Commun 20:315–319, 1990.
133. L K-P Lam, JB Jones. Can J Chem 66:1422–1424, 1987.
134. C-S Chen, Y Fujimoto, CJ Sih. J Am Chem Soc 103:3580–3582, 1981.
135. F-C Haung, LFH Lee, RSD Mittal, PR Ravikumar, JA Chan, CJ Sih, E Caspi, CR Eck. J Am Chem Soc 97:4144–4145, 1975.
136. H-J Gais, KL Lukas. Angew Chem Int Ed Engl 23:142–143, 1984.
137. MT Davies-Coleman, DEA Rivett. Fortschr Chem Org Naturst 55:1–6, 1989.
138. B Hasse, MP Schneider. Tetrahedron Asymm 4:1017–1026, 1993.
139. H Merz, K Stockhaus. J Med Chem 22:1475–1481, 1979.
140. EG Quartey, JA Hustad, K Faber, T Anthonsen. Enzyme Microb Technol 19:361–365, 1996.
141. M Mansuri, JC Martin. Ann Report Med Chem 22:125–140, 1987.
142. M Mahmoudian, BS Baines, CS Drake, RS Hale, P Jones, JE Piercey, DS Montgomery, IJ Purvis, R Storer, MJ Dawson, GC Lawrence. Enzyme Microb Technol 15:749–755, 1993.
143. J Milton, S Brand, MF Jones, CM Rayner. Tetrahedron 36:6961–6964, 1995.
144. S Niwayama, S Kobayashi, M Ohno. J Am Chem Soc 116:3290–3295, 1994.
145. R Vince, M Hua. J Med Chem 33:17–24, 1990.
146. H Nakano, Y Okauyama, K Iwasa, H Hongo. Tetrahedron Asymm 5:1155–1156, 1994.
147. AC van der Linden. Biochim Biophys Acta 77:157–162, 1963.
148. SW May, RB Schwartz, BJ Abbott, OR Zaborsky. Biochim Biophys Acta 403:245–255, 1975.
149. BJ Abbott, CT Hou. Appl Microbiol 26:86–91, 1973.
150. M-J deSmet, J Kingma, H Wynberg, B Witholt. Enzyme Microb Technol 5:352–356, 1983.

151. M Takagi, N Uemura, K Furuhashi. Ann NY Acad Sci 613:697–702, 1990.
152. K Furuhashi. In: AN Collins, GN Sheldrake, J Crosby, eds. Chirality in Industry. New York: Wiley, 1992, pp 167–188.
153. KA Babaik, JS Ng, JH Dygos, CL Weyker, Y-F Wang, C-H Wong. J Org Chem 55:3377–3381, 1990.
154. DL Hughes, JJ Bergan, JS Amato, M Bhupathy, JL Leazer, JM McNamara, DR Sidler, PJ Reider, EJJ Grabowski. J Org Chem 55:6252–6259, 1990.
155. F Theil, H Schick, G Winre, G Reck. Tetrahedron 47:7569–7582, 1991.
156. M Tanaka, M Yoshioka, K Sakai. Tetrahedron Asymm 4:981–996, 1993.
157. AD Borthwick, K Biggadike. Tetrahedron 48:571–623, 1992.
158. H Matsumae, M Furui, T Shibatani. J Ferment Bioeng 75:93–98, 1993.
159. AA Larsen, PM Lish. Nature 203:1283–1284, 1964.
160. RH Uloth, JR Kirk, WA Gould, AA Larsen. J Med Chem 9:88–96, 1966.
161. PM Lish, JH Weikel, KW Dungan. J Pharmacol Exp Ther 149:161–173, 1965.
162. P Somani, T Bachand. Eur J Pharmacol 7:239–247, 1969.
163. M Gelo, V Sunjic. Synthesis 9:855–857, September 1993.
164. R Chenevert, M Desjardins. Can J Chem 72:2312–3217, 1994.
165. T Takeuchi, H Iinuma, S Kunimoto, T Masuda, M Ishizuka, M Hamada, H Naganawa, S Kondo, H Umezawa, H. J Antibiot 34:1619–1621, 1981.
166. H Umezawa, S Kondo, H Iinuma, Y Kunimoto, H Iwasawa, D Ikeda, T Takeuchi. J Antibiot 34:1622–1624, 1981.
167. S Kondo, H Iwasawa, D Ikeda, Y Umeda, H Ikeda, H Inuma, H Umezawa. J Antibiot 34: 1625–1627, 1981.
168. H Iwasawa, S Kondo, D Ikeda, T Takeuchi, H Umezawa. J Antibiot 35:1665–1669, 1982.
169. Y Umeda, M Moriguchi, K Ikai, H Kuroda, T Nakamura, A Fujii, T Takeuchi, H Umezawa. J Antibiot 40:1316–1324, 1987.
170. RN Patel, A Banerjee, LJ Szarka. Tetrahedron Asymm 8:1767–1771, 1997.

27

Production of C_{20} Polyunsaturated Fatty Acids by an Arachidonic Acid–Producing Fungus *Mortierella alpina* 1S-4 and Related Strains

JUN OGAWA, EIJI SAKURADANI, and SAKAYU SHIMIZU

Graduate School of Agriculture, Kyoto University, Kyoto, Japan

1 INTRODUCTION

The C_{20} polyunsaturated fatty acids (PUFAs), such as Mead acid (20:3n-9), dihomo-γ-linolenic acid (DGLA, 20:3n-6), arachidonic acid (AA, 20:4n-6), and 5,8,11,14,17-*cis*-eicosapentaenoic acid (EPA, 20:5n-3) (see Fig. 1 for structures and biosynthetic pathways), are of interest, as they are precursors for prostaglandins, thromboxanes, leukotrienes, prostacyclins, and so on, all of which have hormonelike activities. Possibly due to the physiological activities of these eicosanoids, PUFAs exhibit several unique biological activities, such as lowering of plasma cholesterol level, prevention of thrombosis, and so on. The inclusion of AA and 4,7,10,13,16,19-*cis*-docosahexaenoic acid (DHA, 22:6n-3) as supplements of infant formula has been recommended for the growth of preterm babies [1]. Also, EPA and DHA as dietary supplements has been recommended for the prevention of heart diseases [2], and γ-linolenic acid (18:3n-6) has been recommended for the relief of eczema [3]. Accordingly, PUFAs and/or lipids containing them are highly important substances in the pharmaceutical, medical, and nutritional fields. Because food sources rich in these PUFAs are limited to a few seed oils which contain 18:3n-6 and fish oil which contains EPA and DHA, recent investigations have been focused on microorganisms as alternative sources of these PUFAs.

Microorganisms are thought to be promising lipid sources because of their extremely high growth rates in simple media and the simplicity of their manipulation. Furthermore,

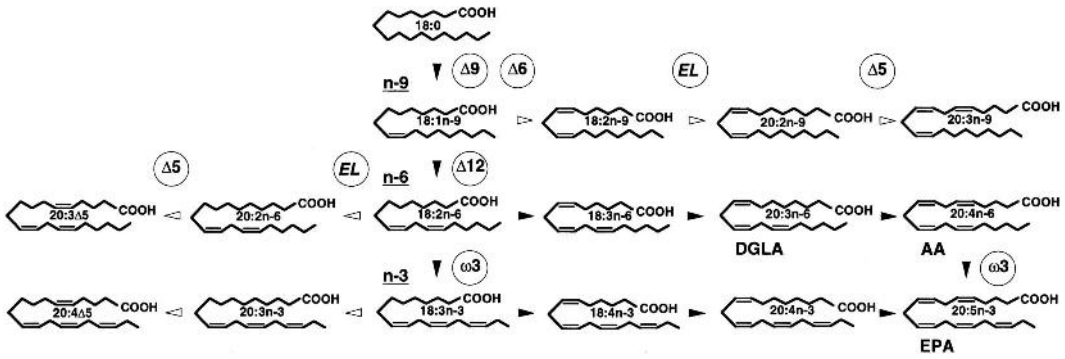


Figure 1 Proposed biosynthetic pathways for PUFA biosynthesis in *M. alpina* 1S-4. *M*:*N*n-*X* PUFA, polyunsaturated fatty acids containing *M* carbon atoms and *N* double bonds, in which the final double bond is located *X* carbon atoms (inclusive) from the terminal carbon atom of the fatty acid chain. n-9, n-6, and n-3 represent the families of PUFA which are defined by the position of the double bond closest to the methyl end of the fatty acid molecules and also represent the biosynthetic routes for these families in which the desaturation occurs toward the carboxyl end of the molecule and chain elongation at the carboxyl end, leaving the methyl end unsaturated. Δ*N*, desaturation at the *N*th carbon atom from the carboxyl terminus, or the enzyme catalyzing the Δ*N* desaturation (Δ*N* desaturase). EL, elongation. Open arrowheads show by-paths through which n-9 fatty acids and nonmethylene-interrupted PUFAs are formed in mutants Mut48 and Mut49 from the parental 1S-4 strain, respectively.

microbial oils are usually characterized by the presence of only a few PUFAs, making purification of individual fatty acids an easier task than it is from other sources. A variety of PUFAs have been detected in microorganisms, including bacteria, fungi, algae, mosses and protozoa. Erwin and Bloch [4] suggested that lower classes of organisms, including microorganisms, can be classified into several groups based on their ability to produce PUFAs. Shaw [5] pointed out that some fungi belonging to the Mucorales accumulate relatively large amounts of 18:3n-6 in their mycelia. Based on these early observations, several groups have started to screen for microorganisms capable of accumulating lipids containing PUFAs in order to obtain more suitable sources for large-scale preparation of important nutritional components. We found that several filamentous fungi belonging to the genus *Mortierella*, especially *M. alpina* 1S-4, produce large amounts of triacylglycerol rich in AA. Various kinds of mutants defective in desaturation ability, which were derived from *M. alpina* 1S-4, selectively produce all the C_{20} PUFAs of n-6, n-3, and n-9 families. They are new and promising sources of C_{20} PUFAs [6–9].

2 PRODUCTION OF PUFAS BY *M. ALPINA* 1S-4 AND RELATED FUNGI

2.1 Arachidonic Acid

Since 1986, we have been assaying the C_{20} PUFA productivity of a wide variety of microorganisms. Most C_{20} PUFA producers were found to be filamentous fungi belonging to the orders Mucorales and Entomophthorales. They primarily produce C_{20} PUFAs of the n-6 family (i.e., AA and DGLA) together with C_{18} PUFA of the same family (i.e., 18:3n-6). Most of the PUFA-producing isolates from natural sources were found to belong to

the genus *Mortierella*. Interestingly, all of the *Mortierella* strains found as C_{20} PUFA producers belong to the subgenus *Mortierella*. Neither the stock cultures nor isolates belonging to the subgenus *Micromucor* showed any detectable accumulation of C_{20} PUFAs, although they were good producers of 18:3n-6 [10]. As a result of this screening, we obtained a soil isolate which was taxonomically identified as *Mortierella alpina* 1S-4, as a potent producer of AA [11,12]. The fungus effectively utilized not only glucose but also glycerol, maltose, palmilate, stearate, oleate, *n*-hexadecane, and *n*-octadecane as carbon sources for AA production.

Using a 50-kL fermentor, a mycelial mass of 60 kg/kL of medium (dry wt) containing about 60% lipid (triacylglycerol) was produced by *M. alpina* 1S-4. The AA content reached 40–50% of the total fatty acids in the lipid. This fermentation involved intermittent feeding of glucose and 7–10 days of cultivation at 28°C (Fig. 2) [13,14]. The AA content of the lipid further increased nearly 70% when the harvested mycelia was allowed to stand at room temperature for a further 6 days. The triacylglycerol containing AA was obtained from the mycelia of *M. alpina* 1S-4 with a good recovery (80–90%) through the steps involving (1) separation of the mycelia by filtration, (2) drying, (3) crushing by ball mill, (4) extraction of lipids with *n*-hexane, (5) removal of insoluble materials by centrifugation, (6) decolorization and deodorization with active charcoal, and (7) concentration. If necessary, the AA in the purified oil could be isolated as the ethyl ester, with a good recovery, after successive transesterification, liquid–liquid partition chromatography, and high-performance liquid chromatography.

2.2 Dihomo-γ-linolenic Acid

Most AA-producing fungi accumulate small amounts of DGLA when they are cultivated under the conditions optimized for AA production [15]. Because AA is derived from DGLA through the n-6 route, suggesting that all of the AA-producing fungi potentially should produce large amounts of DGLA if one can block its Δ5 desaturation to AA.

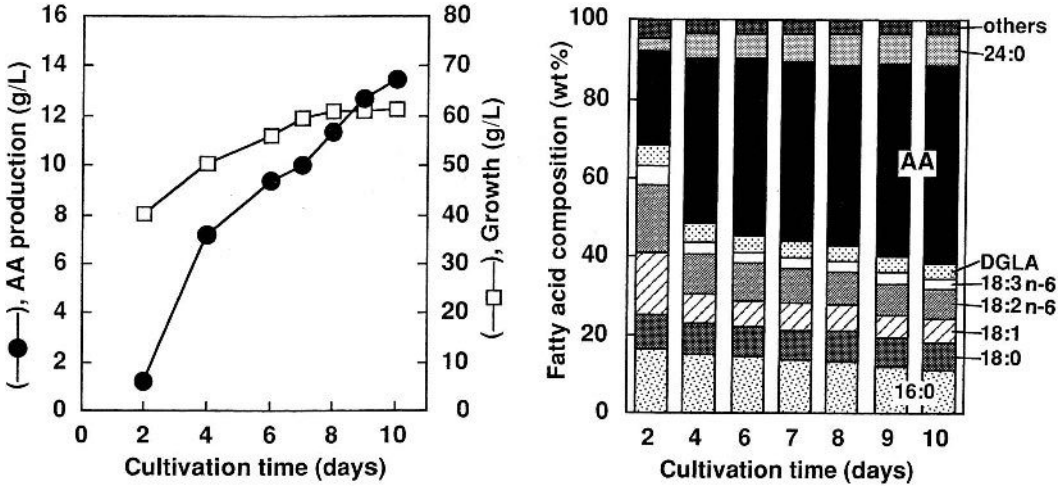


Figure 2 Time course of AA production by wild strain, *M. alpina* 1S-4. The left panel represents AA production and cell growth. The right panel represents the fatty acid composition of the triacylglycerol produced.

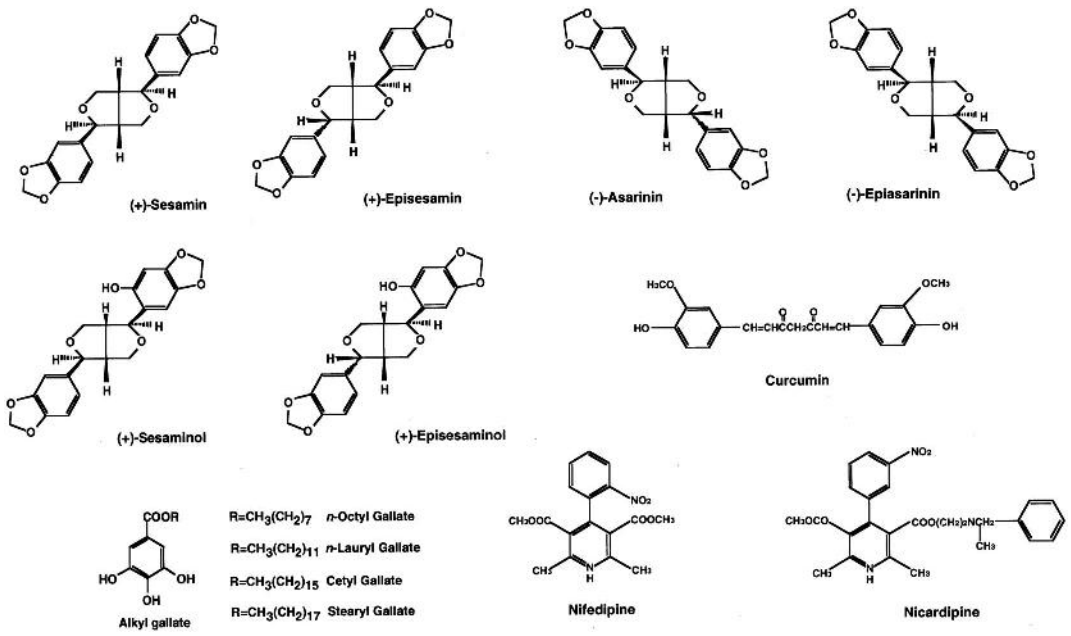


Figure 3 Desaturase inhibitors of PUFA biosynthesis.

The first approach to blocking the Δ5 desaturation began with screening for Δ5-desaturase inhibitors. Among various substances tested, sesame oil and peanut oil were found to cause a marked decrease in AA content [16,17] when added to the culture medium. On addition of 3% sesame oil to a glucose–yeast extract medium, the production of DGLA with *M. alpina* 1S-4 reached 1.7 g/L, whereas the production of AA was only 0.7 g/L. The effective factors responsible for this phenomenon were isolated from sesame seeds extracts or sesame oil and identified to be lignan compounds [i.e., (+)-sesamin, (+)-episesamin, (+)-sesaminol, and (+)-episesaminol (Fig. 3)] [18,19]. The results obtained from experiments using either cell-free extracts of *M. alpina* 1S-4 or a rat liver microsomal fraction clearly demonstrated that these lignan compounds specifically inhibited Δ5 desaturase at low concentrations. At these concentrations, none of the Δ6, Δ9, and Δ12 desaturases were inhibited by these lignan compounds [18]. (−)-Asarinin and (−)-epiasarinin, the stereoisomers of (+)-episesamin and (+)-sesamin, respectively, which were isolated from a Chinese crude medicine ''Saishin'' (*Asiasari radix*), also showed specific noncompetitive inhibition of Δ5 desaturase [20]. Another desaturase inhibitor, curcumin, was found in turmeric. The compound inhibited both Δ5 and Δ6 desaturase in vitro, but it was not as effective as sesamin in stimulation of DGLA production [21,22]. An antioxidant, alkyl gallate, and Ca^{2+} channel blockers nicardipine and nifedipine were also shown to inhibit both desaturases [23,24].

In a study on optimization of the culture conditions for the production of DGLA by *M. alpina* 1S-4, a medium containing glucose, yeast extract, and the nonoil fraction of sesame oil was found to be suitable. Under the optimal conditions in a 50-L fermentor, the fungus produced DGLA at 2.2 g/L (107 mg/g dry mycelia) (Table 1). This value accounted for 23.1% of the total mycelial fatty acids [19].

Table 1 Comparison of DGLA Productivities and Mycelial Fatty Acid Profiles of Some *Mortierella* Strains Under Different Culture Conditions

Strain and conditions	Productivity			Mycelial fatty acid composition (wt%)			
	Mycelial mass (g/L)	DGLA content (mg/g dry mycelia)	DGLA yield (g/L)	18:2n-6	18:3n-6	DGLA	AA
M. alpina 1S-4							
+ sesame oil	20.3	107	2.2	6.6	4.1	23.1	11.2
Mutant Mut44							
− inhibitors[a]	26.0	123	3.2	8.9	6.5	23.4	3.7
+ inhibitors[a]	28.4	74	2.1	10.0	6.8	16.1	1.3
Mutant S14							
− inhibitors[a]	17.1	140	2.4	4.4	5.8	43.3	1.5

[a] Inhibitors, a mixture of sesamin and episesamin (50:50 by mol).

2.3 Eicosapentaenoic Acid

We found that lowering the cultivation temperature caused the additional accumulation of EPA by all the AA producers tested [15,25]. Most of these AA producers grow well at low temperature (6–16°C) and produce enough mycelia in simple growth media. The results of experiments with cell-free extracts of *M. alpina* 1S-4 demonstrated that the enzyme(s) that catalyzes the conversion of AA to EPA (Δ17 desaturation) is inducibly activated on cold adaptation [15]. As shown in Table 2, *M. alpina* 1S-4 produced EPA at levels of more than 0.3 g/L at 12°C.

Several AA-producing *Mortierella* strains accumulate detectable amounts of EPA in their mycelia when grown in media containing α-linolenic acid (18:3n-3) [26]. This observation suggests that the conversion of 18:3n-3 to EPA through the n-3 route occurs in *Mortierella* fungi as well as in animals. This conversion is independent of the growth temperature, because EPA production takes place even at 28°C. Linseed oil, in which 18:3n-3 amounts to about 60% of the total fatty acids, was found to be the most suitable for EPA production. Under the optimal culture conditions, *M. alpina* 1S-4 effectively converted the 18:3n-3 in the added linseed oil into EPA, and the EPA production reached 0.99 g/L (41 mg/g dry mycelia). This value is 3.3-fold higher than that obtained under low-temperature growth conditions (Table 2). EPA production was further stimulated when AA-producing fungi were grown in a medium containing linseed oil at low temperature. This phenomenon was suggested to be mainly due to the low temperature-dependent production of EPA from AA formed through the n-6 route and the conversion of the 18:3n-3 in the added linseed oil to EPA through the n-3 route took place at the same time [27]. Stimulation of the n-3 route itself at low temperature was also suggested to contribute to the increased EPA production. The amount of EPA accumulated reached 1.88 g/L (66.6 mg/g dry mycelia) on cultivation of *M. alpina* 1S-4 with 3% linseed oil at 12°C (Table 2).

2.4 Rare PUFAs

Mortierella and several related filamentous fungi convert *n*-alkanes of 15 or 17 carbon atoms to unusual C_{19} PUFAs efficiently (Fig. 4) [28–30]. In the same way, *M. alpina* 1S-

Table 2 Comparison of EPA Productivities and Mycelial Fatty Acid Profiles of Some *Mortierella* Strains Under Different Culture Conditions

Strain and conditions	Productivity			Mycelial fatty acid composition (wt%)					
	Mycelial mass (g/L)	EPA content (mg/g dry mycelia)	EPA yield (g/L)	18:3n-6	18:3n-3	DGLA	AA	EPA	Others
M. alpina 1S-4									
12°C	11.1	27.0	0.30	4.5	—	2.9	63.8	10.9	17.9
28°C + linseed oil	24.1	41.0	0.99	1.4	29.3	1.5	13.8	9.2	44.8
12°C + linseed oil	28.2	66.6	1.88	1.6	28.2	3.0	15.7	12.0	39.5
M. alpina 2O-17									
12°C	17.0	29.0	0.49	3.5	—	2.3	60.0	13.5	20.7
28°C + linseed oil	32.4	41.5	1.35	0.9	38.5	1.4	12.3	7.1	39.8
M. hygrophila									
12°C	15.2	20.4	0.31	3.5	—	1.6	7.2	6.7	81.0
Mutant Mut48									
20°C + linseed oil	15.1	64.0	0.97	0.3	20.3	0.8	7.8	19.5	51.3

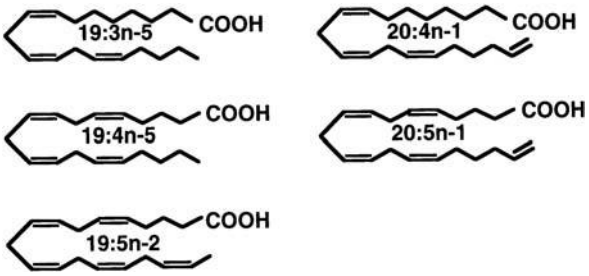


Figure 4 Rare PUFAs produced by *M. alpina* 1S-4 and related strains.

4 accumulates a novel 5,8,11,14,19-eicosapentaenoic acid (20:5n-1) together with additional n-1 fatty acids, such as 15-hexadecenoic acid, 17-octadecenoic acid, and 8,11,14,19-eicosatetraenoic acid (20:4n-1) on growth with 1-hexadecene or 1-octadecene (Fig. 4) [31]. Based on the hypothesis that a terminal double bond has no effect on the PUFA biosynthesis, it is suggested that the 20:5n-1 is formed through the n-6 route. The ω methyl group, which is opposite the double-bond end, may be first oxidized to a carboxyl group, and the resultant n-1 fatty acid may be introduced to the n-6 route.

3 *M. ALPINA* MUTANTS DEFECTIVE IN FATTY ACID DESATURATION

Because the studies so far have concerned with the wild strain, *M. alpina* 1S-4, it seemed to reach the limits of our abilities to produce new PUFAs or to increase the PUFA production. Thus, we have focused on the isolation of PUFA biosynthesis mutants of this fungus. Upon mutagenizing the parental spores, we succeeded in the isolation of eight mutants which could be divided into six groups (Fig. 5) [32,33]. Characteristics of some mutants are presented in the following subsections (Table 3).

3.1 Δ5-Desaturase-Defective Mutants

Several mutants of this type which are characterized by a high DGLA level and a reduced AA level were obtained and two (Mut44 and S14) of them were studied in detail [34,35].

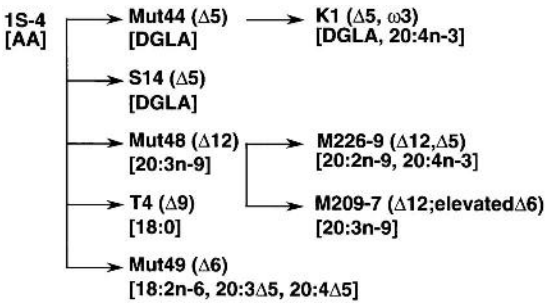


Figure 5 Mutants derived from *M. alpina* 1S-4. Desaturases missing are shown in parentheses; major fatty acids produced de novo or from suitable precursor fatty acids added are shown in square brackets. See Figure 1 for chemical structure of PUFAs, biosynthetic routes, reactions, and enzymes.

Table 3 Comparison of Fatty Acid Compositions of the Fatty Acid–Desaturation-Defective Mutants of *M. alpina* 1S-4[a]

	Growth temperature (°C)								
	28						12		
Fatty acid (wt%)	1S-4	Mut48	Mut49	Mut44	S14	T4	Mut49	Mut44	K1
16:0	11.1	9.9	12.7	10.8	16.9	8.2	5.8	10.4	10.7
18:0	5.3	6.1	10.7	7.8	7.5	38.0	10.2	9.8	6.5
18:1n-9	13.7	49.3	15.3	11.1	10.7	5.2	8.7	13.7	21.9
18:2n-6	7.4	—[b]	31.8	5.6	2.9	3.9	25.5	4.3	5.5
18:2n-9	—	12.1	—	—	—	—	—	—	—
18:3n-6/20:0[c]	4.6	0.7	1.7	9.6	6.7	11.9	2.3	10.0	11.6
18:3n-3/20:1n-9[d]	1.1	2.7	1.0	1.1	0.6	—	2.7	1.0	1.2
18:4n-3	—	—	—	—	—	—	—	1.3	—
20:2n-6	0.6	—	2.1	1.0	0.5	—	6.1	0.6	0.8
20:2n-9	—	2.1	—	—	—	—	—	—	—
DGLA/22:0[c]	6.5	2.2	2.9	32.8	48.8	1.1	1.7	30.8	32.7
20:3n-9	—	9.3	—	—	—	—	—	—	—
20:3Δ5	—	—	7.8	—	—	—	6.4	—	—
AA/20:3n-3[d]	44.4	—	6.8	11.7	0.9	31.7	22.7	7.1	8.1
20:4n-3	—	—	—	—	—	—	—	6.4	—
20:4Δ5	—	—	—	—	—	—	3.2	—	—
24:0	5.2	5.6	7.8	8.5	4.2	—	3.3	1.5	1.0
EPA	—	—	—	—	—	—	1.5	3.1	—

[a] All strains were grown in medium of 2% (w/v) glucose and 1% (w/v) yeast extract (pH 6.0) at either 28°C for 7 days (28°C) or at 12°C for 6 days after preincubation for 1 day at 28°C (12°C), except that K1 was grown at 12°C for 10 days after a 1-day cultivation at 28°C (12°C).

[b] Undetectable.

[c] Both were found in all strains except for Mut48, in which GLA and DGLA were not detected. 20:0, arachidic acid; 22:0, behenic acid.

[d] 18:3n-3 and 20:3n-3 were found as an additional fatty acid on growth at 12°C except for Mut48.

The mycelial fatty acid compositions of the two mutants are compared in Table 3. S14 has a higher DGLA level than Mut44, its AA level being about one-tenth that in Mut44. On cultivation for 6 days at 28°C in a 10-L fermentor, Mut44 produced 3.2 g DGLA per liter of culture broth (123 mg/g dry mycelia), which accounted for 23.4% of the total mycelial fatty acids [34]. The mycelial AA amounted to only 19 mg/g dry mycelia (0.5 g/L culture broth), which accounted for 3.7% of the total mycelial fatty acids (Table 1). The level of AA in Mut44 could be further decreased by growing the fungus in the presence of Δ5-desaturase inhibitors (i.e., sesamin and episesamin). On cultivation in a medium supplemented with 0.075 g/L of the inhibitors, the production of DGLA was 2.1 g/L culture broth (16.1% of total fatty acids) and the AA production was 0.2 g/L (1.3%) [36]. The DGLA production of S14 was 2.4 g/L culture broth (43.3% of total fatty acids) when grown at 28°C for 7 days in a 5-L jar fermentor [35]. The production of DGLA by using mutants is superior to the above-mentioned method of using inhibitors because it does not require inhibitors, and the content of DGLA in the mycelial triacylglycerol was higher (Table 1). The same mutant can be used for the production of 8,11,14,17-*cis*-eicosatetrae-

noic acid (20:4n-3). The production of 20:4n-3 was 1.7 g/L (66 mg/g dry mycelia; 11.6% of total fatty acids), on cultivation in a medium containing linseed oil [37].

3.2 Δ12-Desaturase-Defective Mutant

Mutant Mut48 is characterized by a high oleic acid (18:1n-9) level and the absence of n-6 PUFAs in its mycelia. In contrast to the wild strain, this mutant produces three n-9 PUFAs [i.e., 6,9-*cis*-octadecadienoic acid (18:2n-9), 8,11-*cis*-eicosadienoic acid (20:2n-9), and 20:3n-9] [38]. On cultivation at 20°C for 10 days in a 5-L fermentor, the production of 20:3n-9 reached about 0.8 g/L (56 mg/g dry mycelia), accounting for 15% of the total mycelial fatty acids. About 70 mol% of 20:3n-9 was present in the triacylglycerol and the remainder was in the phospholipid fraction, especially in phosphatidylcholine. The formation of 20:3n-9 would occur through the same sequential reactions suggested by Fulco and Mead [39] (i.e., 18:1n-9 was desaturated at the Δ6-position into 18:2n-9 followed by elongation and Δ5 desaturation to 20:3n-9) (Fig. 1).

Like the wild strain, Mut48 converts exogenous 18:3n-3 to EPA [40]. On cultivation at 20°C for 10 days in a 5-L fermentor containing a medium supplemented with linseed oil, the production of EPA was approximately 1 g/L culture broth (64 mg/g dry mycelia), accounting for about 20% of the total mycelial fatty acids. The mycelial AA content was 26 mg/g dry mycelia (0.4 g/L), accounting for 7.8% of the total mycelial fatty acids. As shown in Table 2, the EPA contents were lower than AA contents in the oils produced by all strains except for Mut48, in which the content of EPA was 2.5-fold that of AA. This is probably due to the inability of Mut48 to supply 18:2n-6 from glucose.

Recently, enhanced production of 20:3n-9 by a mutant M209-7, derived from Mut48, was reported [41]. The mutant showed enhanced Δ6 desaturation activity, which was 1.4 times higher than that of the parent Mut48. The production of 20:3n-9 by M209-7 was 1.3 times greater than that by Mut48.

3.3 Δ6-Desaturase-Defective Mutants

Mutant Mut49 was considered to be defective in Δ6-desaturase because of the high 18:2n-6 level and low levels of 18:3n-6, DGLA, and AA in its mycelia (Table 3). In addition to the accumulation of 18:2n-6, two nonmethylene-interrupted PUFAs [i.e., 5,11,14-*cis*-eicosatrienoic acid (20:3Δ5) and 5,11,14,17-*cis*-eicosatetraenoic acid (20:4Δ5)] were found [42]. The amount of 20:3Δ5 was greatest (27 mg/g dry mycelia) on growth at 20°C, and it accounted for about 7% of the total mycelial fatty acids. 20:4Δ5 was detected only when the mutant was grown at a temperature lower than 24°C or in a culture medium supplemented with either 18:3n-3 or 20:3n-3. 20:3Δ5 is considered to be converted from linoleic acid (18:2n-6) by two subsequent reactions (i.e., elongation and Δ5 desaturation) (Fig. 1).

3.4 Δ9-Desaturase-Defective Mutant

Mutant T4 is considered to be defective in Δ9 desaturation [33]. Its mycelial fatty acids included 38% stearic acid (18:0), the level being only 5% for the wild strain. Upon growth at 24–28°C, its mycelial lipids included a markedly high level (up to 50 mol% of total mycelial lipids) of free fatty acids, of which about 90 mol% was 18:0. However, the levels of free fatty acids were markedly decreased with a concomitant increase in the

triacylglycerol level when the mutant was grown at 20°C or lower, or when it was grown in a culture medium supplemented with 18:2n-6 or linseed oil, which includes about 60% 18:3n-3. Because the fatty acid composition of phosphatidylethanolamine and phosphatidylcholine at the *sn*-2 position included more than 75% of PUFAs, the accumulation of free fatty acids were assumed to be due to the lack of PUFAs.

3.5 ω3-Desaturase-Defective Mutant

Mutant K1, which was derived from strain Mut44, was considered to be defective in the conversion of n-6 to n-3 fatty acids (ω3 desaturation) [43]. The mutant cannot produce 20:4n-3 or any other n-3 fatty acids, of which about 10% was found in its parental strain upon cultivation at 12°C. The growth rate of the mutant was comparable to that of the parental strain when grown at 28°C, but it became much slower when the mutant was grown at 12°C. At 12°C, the lag phase for Mut44 was about 2 days, but 5 days for the mutant.

3.6 Double Mutant Defective in Δ12 and Δ5 Desaturation

A mutant strain, M226-9, was derived from the Δ12-desaturase-defective mutant Mut48. In M226-9, Δ5- and Δ12-desaturases were completely defective and this mutant produced large amounts of 20:2n-9 (1.68 g/L; 101 mg/g dry mycelia). No detectable 20:3n-9 occurred in the resultant fungal oil. The major fatty acids other than 20:2n-9 (17.6%) were palmitic acid (6.1%), 18:0 (7.9%), 18:1n-9 (44.0%), and 18:2n-9 (17.6%). The addition of olive oil, in which 18:1n-9 is abundantly present, to the growth medium enhanced the production of 20:2n-9 [44]. The same mutant is useful for the production of 20:4n-3 from 18:3n-3 (linseed oil) [45].

4 CONCLUDING REMARKS

An AA-producing fungus *M. alpina* 1S-4 and related fungi are good producers of several useful single-cell oils, especially those containing PUFAs such as AA, DGLA, and EPA. Furthermore, mutants of *M. alpina* 1S-4 have expanded abilities for producing new oils and PUFAs or increased productivities for existing ones. They are useful not only as producers of some useful fatty acids and PUFAs such as AA, DGLA, 20:3n-9 and so on, but they also provide useful information on PUFA biosynthesis. The enzymes (i.e., desaturases and elongase) that are involved in the PUFA biosynthesis in this fungus seem to have a wide substrate specificity. For example, Δ5-desaturase can act on odd-numbered fatty acids (19:3n-5), PUFAs with an ω-terminal double bond (20:4n-1), n-3 PUFA (20:4n-3), n-6 PUFA (DGLA), n-9 PUFA (20:2n-9), and PUFAs with no C-8 double bond (20:2n-6 and 20:3n-3). However, the first double bond needs to be inserted at the C-9 position. The genes encoding PUFA biosynthesis of *M. alpina* [46–56] together with those of its mutants may also be used as tools for making either transgenic plants or animals that have improved fatty acid compositions.

REFERENCES

1. SE Carlson, SH Werkman, JH Peeples, RJ Cooke, EA Tolley. Proc Natl Acad Sci USA 90: 1073–1077, 1993.
2. PM Herold, JK Kinsella. Am J Clin Nutr 43:566–598, 1986.

3. L Oxdale. Chem Br 21:813, 1990.
4. J Erwin, K Bloch. Science 143:1006–1012, 1964.
5. R Shaw. Biochim Biophys Acta 98:230–237, 1965.
6. M Certik, E Sakauradani, S Shimizu. Trends Biotechnol 16:500–505, 1998.
7. M Certik, S Shimizu. J Biosci Bioeng 87:1–14, 1999.
8. M Certik, S Shimizu. Agro-Food-Industry Hi-Tech 10:26–32, 1999.
9. S Shimizu, J Ogawa. In: MC Flickinger, SW Drew eds. Encyclopedia of Bioprocess Technology: Fermentation, Biocatalysis, and Bioseparation. New York: Wiley, 1999, pp 1839–1851.
10. N Amano, Y Shinmen, K Akimoto, H Kawashima, T Amachi, S Shimizu, H Yamada. Mycotaxon 44:257–265, 1992.
11. H Yamada, S Shimizu, Y Shinmen. Agric Biol Chem 51:785–790, 1987.
12. Y Shinmen, S Shimizu, K Akimoto, H Kawashima, H Yamada. Appl Microbiol Biotechnol 31:11–16, 1989.
13. K Higashiyama, T Yaguchi, K Akimoto, S Fujikawa, S Shimizu. J Am Oil Chem Soc 75: 1501–1505, 1998.
14. K Higashiyama, T Yaguchi, K Akimoto, S Fujikawa, S Shimizu. J Am Oil Chem Soc 75: 1815–1819, 1998.
15. S Shimizu, Y Shinmen, H Kawashima, K Akimoto, H Yamada. Biochem Biophys Res Commun 150:335–341, 1988.
16. S Shimizu, K Akimoto, H Kawashima, Y Shinmen, H Yamada. J Am Oil Chem Soc 66:237–244, 1989.
17. S Shimizu, K Akimoto, H Kawashima, Y Shinmen, S Jareonkitmongkol, H Yamada. Agric Biol Chem 53:1437–1438, 1989.
18. S Shimizu, K Akimoto, Y Shinmen, H Kawashima, M Sugano, H Yamada. Lipids 26:512–516, 1991.
19. S Shimizu, K Akimoto, M Sugano, H Yamada. In: A Sinclair, R Gibson, eds. Essential Fatty Acids and Eicosanoids. Champaign, IL: AOCS Press, 1993, pp 37–41.
20. S Shimizu, H Kawashima, K Akimoto, Y Shinmen, H Sugano, H Yamada. Phytochemistry 31:757–760, 1992.
21. S Shimizu, S Jareonkitmongkol, H Kawashima, K Akimoto, H Yamada. Lipids 27:509–512, 1992.
22. H Kawashima, K Akimoto, S Jareonkitmongkol, N Shirasaka, S Shimizu. Biosci Biotechnol Biochem 60:108–110, 1996.
23. H Kawashima, K Akimoto, S Jareonkitmongkol, N Shirasaka, S Shimizu. Biosci Biotechnol Biochem 60:1672–1676, 1996.
24. H Kawashima, K Akimoto, N Shirasaka, S Shimizu. Biochim Biophys Acta 1299:34–38, 1996.
25. S Shimizu, H Kawashima, Y Shinmen, K Akimoto, H Yamada. J Am Oil Chem Soc 6:1455–1459, 1988.
26. S Shimizu, H Kawashima, K Akimoto, Y Shinmen, H Yamada. J Am Oil Chem Soc 66:342–347, 1989.
27. S Shimizu, H Kawashima, K Akimoto, Y Shinmen, H Yamada. Appl Microbiol Biotechnol 32:1–4, 1989.
28. S Shimizu, H Kawashima, K Akimoto, Y Shinmen, H Yamada. J Am Oil Chem Soc 68:254–258, 1991.
29. N Shirasaka, T Yokochi, S Shimizu. Biosci Biotechnol Biochem 59:1963–1965, 1995.
30. S Shimizu, H Kawashima, K Akimoto, Y Shinmen, H Yamada. In: Proceedings of Session Lectures and Scientific Presentation on ISF–JOCS World Congress 1988, Vol. II. Tokyo: Japan Oil Chemists Society, 1989, pp 1102–1106.
31. S Shimizu, S Jareonkitmongkol, H Kawashima, K Akimoto, H Yamada. Arch Microbiol 156: 163–166, 1991.
32. S Jareonkitmongkol, S Shimizu, H Yamada. J Gen Microbiol 138:997–1002, 1992.

33. S Jareonkitmongkol, E Sakuradani, H Kawashima, S Shimizu, Nippon Nogeikagakukaishi 67:531, 1993. (in Japanese).
34. S Jareonkitmongkol, H Kawashima, N Shirasaka, S Shimizu, H Yamada. Appl Environ Microbiol 58:2196–2200, 1992.
35. S Jareonkitmongkol, E Sakuradani, S Shimizu. Appl Environ Microbiol 59:4300–4304, 1993.
36. S Jareonkitmongkol, H Kawashima, H Yamada. J Ferment Bioeng 76:406–407, 1993.
37. H Kawashima, N Kamada, E Sakuradani, S Jareonkitmongkol, K Akimoto, S Shimizu. J Am Oil Chem Soc 74:1431–1434, 1997.
38. S Jareonkitmongkol, H Kawashima, S Shimizu, H Yamada. J Am Oil Chem Soc 69:939–944, 1992.
39. AJ Fulco, JF Mead. J Biol Chem 234:1411–1416, 1959.
40. S Jareonkitmongkol, S Shimizu, H Yamada. J Am Oil Chem Soc 70:119–123, 1993.
41. H Kawashima, M Nishihara, Y Hirano, N Kamada, K Akimoto, K Konishi, S Shimizu. Appl Environ Microbiol 63:1820–1825, 1997.
42. S Jareonkitmongkol, S Shimizu, H Yamada. Biochim Biophys Acta 1167:137–141, 1993.
43. S Jareonkitmongkol, E Sakuradani, S Shimizu. Arch Microbiol 161:316–319, 1994.
44. N Kamada, H Kawashima, E Sakuradani, K Akimoto, J Ogawa, S Shimizu. J Am Oil Chem Soc 76:1269–1274, 1999.
45. H Kawashima, E Sakuradani, N Kamada, K Akimoto, K Konishi, J Ogawa, S Shimizu. J Am Oil Chem Soc 75:1495–1500, 1998.
46. E Sakuradani, M Kobayashi, S Shimizu. Eur J Biochem 260:208–216, 1999.
47. P Wongwathanarat, LV Michaelson, AT Carter, CM Lazarus, G Griffiths, AK Stobart, DB Archer, DA MacKenzie. Microbiology 145:2939–2946, 1999.
48. E Sakuradani, M Kobayashi, T Ashikari, S Shimizu. Eur J Biochem 260:812–820, 1999.
49. E Sakuradani, M Kobayashi, S Shimizu. Gene 238:445–453, 1999.
50. YS Huang, S Chaudhary, JM Thurmond, EG Bobik Jr, L Yang, GM Chan, SJ Kirchner, P Mukerji, DS Knutzon. Lipids 34:649–659, 1999.
51. DS Kuntzon, JM Thurmond, YS Huang, S Chaudhary, EM Bobik Jr, GM Chan, SJ Kirchner, P Mukerji. J Biol Chem 273:29,360–29,366, 1998.
52. LV Michaelson, CM Lazarus, G Griffith, JA Napier, AK Stobart. J Biol Chem 273:19,055–19,059, 1998.
53. N Kouzaki, H Kawashima, MCM Chung, S Shimizu. Biochim Biophys Acta 1256:319–326, 1995.
54. M Kobayashi, E Sakuradani, S Shimizu. J Biochem 125:1094–1103, 1999.
55. E Sakuradani, M Kobayashi, S Shimizu. Appl Environ Microbiol 65:3873–3879, 1999.
56. M Certik, E Sakuradani, M Kobayashi, S Shimizu. J Biosci Bioeng 88:667–671, 1999.

28

Microbial Transformations of Steroids

HERBERT L. HOLLAND

Brock University, St. Catharines, Ontario, Canada

1 INTRODUCTION

The microbial transformation of steroid is long established as a method for the preparation of hydroxysteroids, particularly the corticosteroids, and other specific target molecules; it is also a valuable tool for the investigation of steroid metabolism. The use of microbial systems to modify a steroidal substrate has been extensively investigated and discussed in several classic books [1–3], reviews [4–8], and review series [9] and continues to be the focus of attention at many levels ranging from primary research to product development. This chapter will focus on recent developments in the microbial transformations of steroids. It is not intended as a comprehensive compilation of steroid biotransformations as this material is covered in the ongoing review series by Mahato and co-workers [9], but it will deal in detail with the more recent advances in both fundamental and applied aspects of steroid transformations by microorganisms.

2 HYDROXYLATION REACTIONS

Hydroxylation, the direct conversion of a C—H bond into a C—OH unit, is one of the more valuable reactions of microbial transformation and was first developed as a synthetic tool in the 1950s in the context of the preparation of corticosteroids. Although efficient microbial methods have now been developed for the introduction of key hydroxyl groups at C-11α and C-11β of progesterone (**1**) (Fig. 1), and a large number of microbial biocatalysts capable of the introduction of hydroxyl groups at other sites of the steroid nucleus has been identified [10], researchers in this area continue to investigate new substrate groups and identify new classes of products from microbial biotransformations.

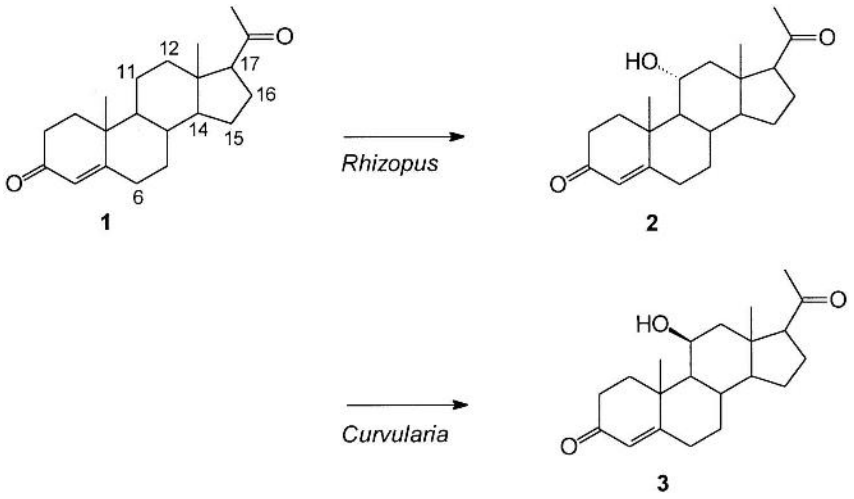


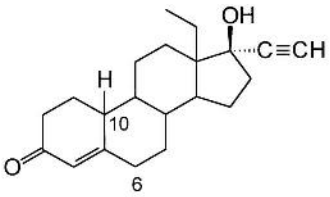
Figure 1 Conversions of progesterone (**1**) into 11α-hydroxyprogesterone (**2**) and 11β-hydroxyprogesterone (**3**).

2.1 Substrates

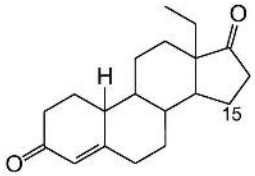
The transformation of bile acids, although not a novel process, has been relatively underinvestigated in comparison with the transformation of other substrate groups, but it has received increased attention recently in the development of processes that may lead to useful pharmaceutical products. The scope of these reactions, many of which are unique to this substrate group, has been recently reviewed [7].

Microbial transformations of 13-ethyl steroids related to the contraceptives Gestoden and Desogestrel have been examined recently. Kinetic resolution of racemic **4** (**4–9**) was observed during its conversion to the natural configuration 6β, 10β-diol by *Cunninghamella echinulata* [11], and extensive screening led to the identification of two biocatalysts (*Fusarium nivale* and *Mortierella pusilla*) for the conversion of **5** to its commercially important 15α-hydroxy derivative, obtained in up to 76% yield with *F. nivale* [12]. In a search for new pharmacologically active materials, the hypolipemic agent *E*-guggulsterone (**6**) was hydroxylated at C-11α and C-15β by *Cephalosporium aphidicola*, and at C-7β and C-15β by *Aspergillus niger*, concurrent with other reactions occurring at the 17(20) double bond [13].

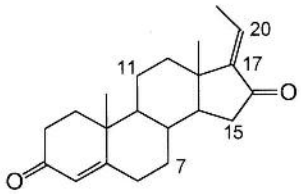
The hydroxylations of a series of 3- and 17-acetylamino-substituted 5α- and 5β-androstanes and 4-androstenes (**7**) and 3- and 20-acetylamino-substituted 5α-pregnanes and 4-pregnenes (**8**) by microorganisms known for their active role in the hydroxylation of conventional steroids (*Aspergillus ochraceus* at C-11α, *Bacillus megaterium* at C-15β, *Curvularia lunata* at C-11β/C-14α, and *Rhizopus arrhizus* at C-6β/C-11α) resulted for the most part in hydroxylation at the sites specified above, although some new redox activities were observed [14]. Biotransformation by these microorganisms of 2-oxatestosterone (**9**) also resulted primarily in hydroxylations only at the above-listed sites listed [15].



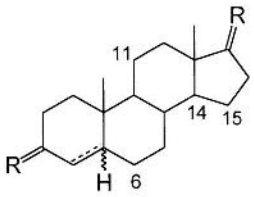
4



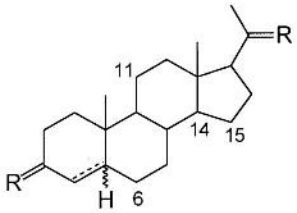
5

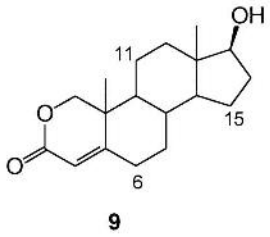


6



7, R = H,OH or O; or H,$NHCOCH_3$





9

8, R = H,OH or O; or H,$NHCOCH_3$

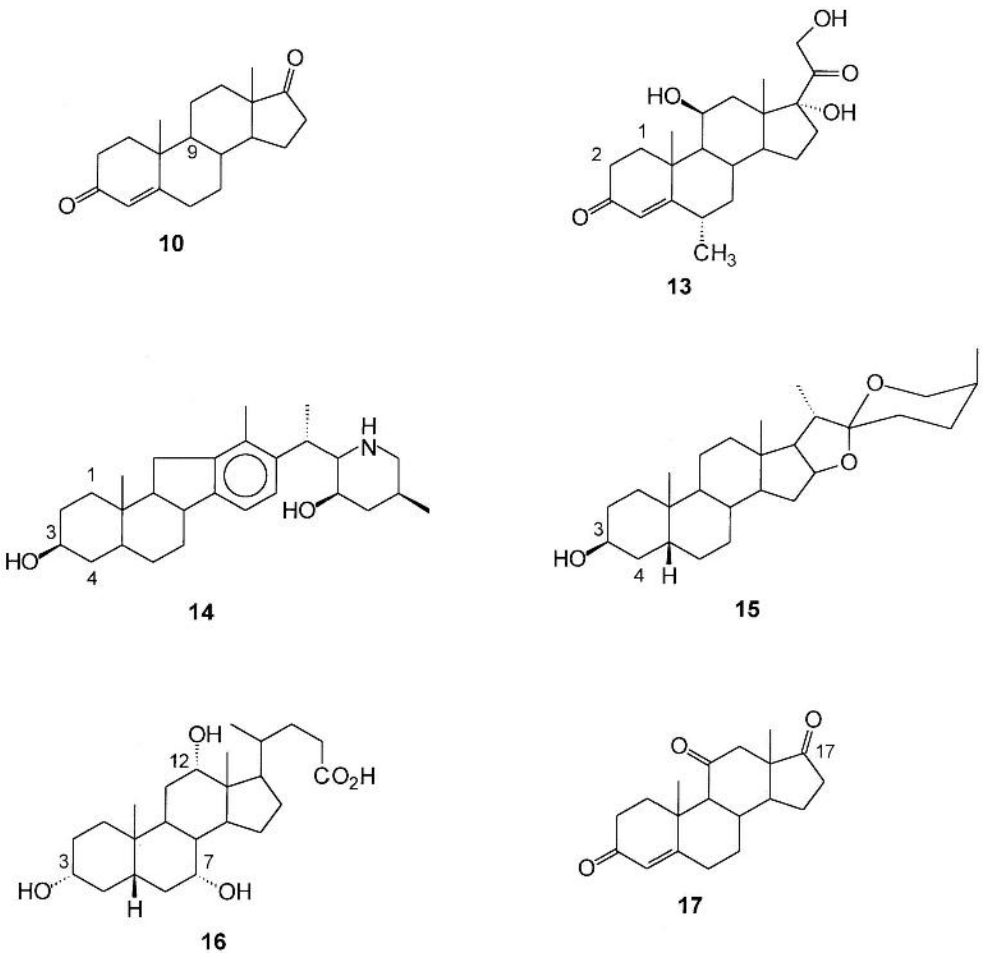
Structures 4–9

2.2 Products

Microbial methods for the conversion of steroids into pharmaceutically important derivatives hydroxylated at C-9α, C-14α, or C-25 have received particular attention recently. A *Rhodococcus* species with high activity for the conversion of 4-androstene-3, 17-dione (**10**) (**10–17**) to its 9α-hydroxy derivative has been identified [16], and the use of various fungal strains for the 14α-hydroxylation of a number of steroidal substrates related to progesterone (**1**) has been systematically explored [17]. Hydroxylation at C-25 of cholesterol and related compounds has been targeted for the development of analogues of vitamin D and is carried out by (*inter alia*) various *Streptomyces* species [18].

3 DEHYDROGENATION REACTIONS

The classical microbial dehydrogenation of steroids, of value for the conversion of corticosteroids into the related prednisones, is the introduction of a double bond at the C-1(2) position of a Δ^4-3-ketosteroid, represented by the conversion of **11** to **12** in Figure 2. This reaction is carried out with high efficiency by a number of microbial species, notably



Structures 10, 13–17

Arthrobacter simplex [1], but it is nevertheless still subject to attempts at development and improvement. The addition of β-cyclodextrin, for example, is reported to lead to an increase in the rate of C-1(2) dehydrogenation of 6α-methylhydrocortisone (**13**) by *Arthrobacter globiformis*, an effect attributed to the reduction of substrate inhibition [19].

Dehydrogenations at other positions adjacent to carbonyl groups of the substrate are also common and have been reported recently for the biotransformation of the cardiovascu-

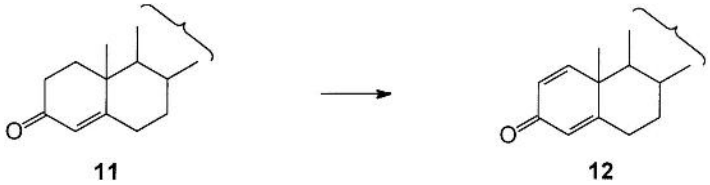


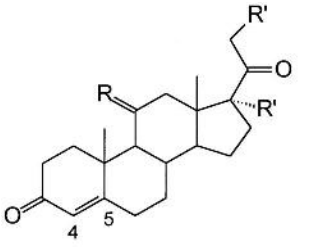
Figure 2 Dehydrogenation of a Δ^4-3-ketosteroid.

lar-active steroidal alkaloid veratramine (**14**), converted to the corresponding 3-oxo-4-ene and 3-oxo-1,4-diene derivatives by *Nocardia* species ATCC 21145 [20], and for conversion of sarsasapogenin (**15**) to the corresponding 3-oxo-4-ene in 62% yield by *Mycobacterium* species NRRL B-3805 [21].

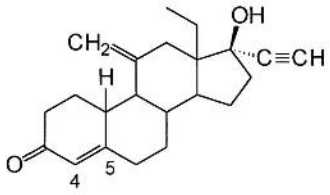
4 OTHER REACTIONS

Other microbial transformations of steroids include the redox interconversion of secondary alcohol and carbonyl groups (CHOH ↔ C═O), the reduction of carbon–carbon double bonds (HC═CH → CH_2—CH_2), and oxidative processes such as the epoxidation of olefins and degradation of the sterol chain [1]. The alcohol–carbonyl interconversion is commonly encountered during bile acid biotransformation and has been systematically explored as a means for the regioselective oxidation of C-3, C-7, or C-12 secondary hydroxyl groups of substrates such as cholic acid (**16**) [22]. The oxidation of the C-3 alcohol group is a necessary prerequisite for the subsequent dehydrogenations of veratramine (**14**) [20] and sarsasapogenin (**15**) [21] discussed earlier. The regiospecific reduction of a carbonyl group is observed in the specific reduction of adrenosterone (**17**) at C-17 to the corresponding C-17β alcohol by the microalga *Scenedesmus quadricauda* in 62% yield [23].

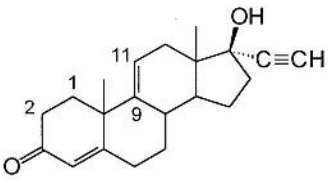
Reduction of C═C bonds conjugated with keto groups is observed for positions C-1(2) and C-4(5) of unsaturated 3-ketosteroids. A series of corticosteroids and related compounds, **18**, (**18–21**) is reduced to the corresponding saturated 5α-steroids by *Penicillium decumbens* ATCC 10436 in yields up to 68% [24], and 3-ketodesogestrel (**19**) is reduced specifically at the C-4(5) position by several biocatalysts: *Clostridium paraputrificum*, giving products with the C-5β configuration, and *Mycobacterium smegmatis*, producing C-5α derivatives [25] (**18–21**). Other alkene positions conjugated with ketones are also susceptible to reduction, exemplified by reduction of the C-17(20) double bond



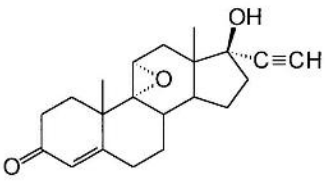
18 R = H,OH or O, R' = H or OH



19

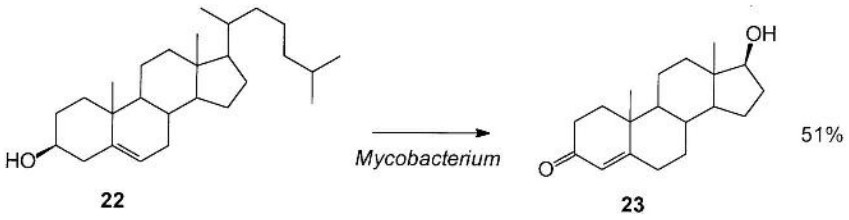


20



21

Structures 18–21



22 23

Figure 3 Production of testosterone from cholesterol.

of *E*-guggulsterone (**6**) concomitant with its hydroxylation at C-7β and C-15β by *Aspergillus niger* [13].

Microbial oxidation of alkenes to the corresponding epoxides is often accompanied by hydrolysis of the latter to geminal diol products, but the transformation of 9(11)-dehydro-17α-methyltestosterone (**20**) by *Nocardia restrictus* ATCC 14887 resulted in the isolation of the corresponding 9(11)α-epoxide **21**, together with its analog having a C-1(2) double bond, as the only products [26].

Oxidative degradation of the sterol side chain has a long and successful history of application for the production of useful steroids with the androstane skeleton, but in the past, the process has focused largely on the use of plant sterols as substrates [1–3]. More recently, however, attention has been given to the use of animal products, such as cholesterol as substrates for this process, exemplified by the conversion shown in Figure 3 of cholesterol (**22**) into testosterone (**23**) in 51% yield in a single-step transformation by a *Mycobacterium* species, a process which also involves oxidation of the C-3 alcohol and migration of the C-5(6) bond to the C-4(5) position [27].

5 METHODOLOGY

In spite of the long history of successful microbial transformations of steroids, efforts continue to be made to identify new biocatalysts and to find methods for improvement in the use of biocatalysts that have a proven ability to perform useful reactions. The following subsections will discuss the development of new biocatalysts and focus on two methods, cell immobilization and the use of low-water environments, for the improvement of existing biocatalysts.

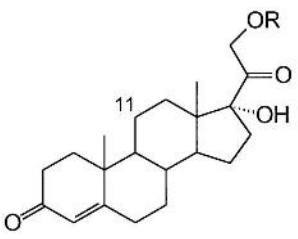
5.1 New Biocatalysts

The importance of hydroxylation at C-11 of the steroid nucleus in the production of the corticosteroids is emphasised by continuing work on the development of biocatalysts for this process. The significance of the physiological state of the mycelium of *Rhizopus nigricans* for the production of 11α-hydroxyprogesterone (see Fig. 1) has been systematically examined [28], and the screening for an improved strain of *Curvularia lunata* for the C-11β hydroxylation reaction has also been recently reported [29]. The biotransformation of Δ^4-3-ketosteroids by *Fusarium culmorum* leads to products of hydroxylation at C-6β, C-12β, or C-15α [30], whereas the hydroxylation of progesterone **1** at both C-6α and C-6β is carried out by the moderate thermophile *Bacillus thermoglucosidasius* [31]. A strain of *Metarhizium anisopliae* for hydroxylation of 13-ethyl steroids at C-11α was recently reported and conditions for its use have been optimized [32].

The use of the fungus *Cephalosporium aphidicola* for hydroxylation of a variety of steroid substrates has been developed [33–37]. The predominant position of hydroxylation by *C. aphidicola* was found to be substrate dependent; the presence of a C-5(6) double bond resulted in nonstereospecific hydroxylation at the allylic C-7 position, but in the absence of this functionality in the substrate, hydroxylations of a variety of substrate types occurred mainly at C-6β, C-7α, or C-11α.

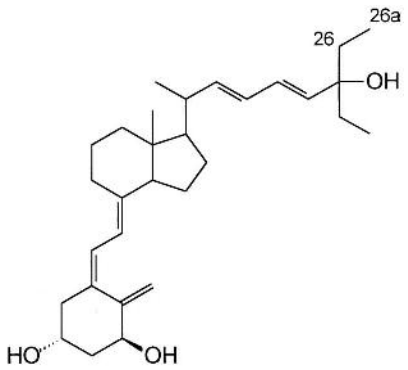
The C-11β hydroxylating enzyme system of *Cochliobolus lunatus* has been examined in detail with respect to the nature and location of enzyme within the cell and the role of plant defense compounds as inducers of enzyme activity in the intact fungus [38,39]. The ability of this fungus to oxidize the C-11β hydroxyl group to a keto function has also been examined at the enzymatic level [40]. The commercially significant hydroxylation of cortexolone (**24**) at C-11β by *Curvularia lunata* to yield hydrocortisone has been re-examined by the use of protoplast preparations of a high-producing strain of *C. lunata* (IM 2901/366), and elevated levels of activity were observed [41] (**24, 25, 30**).

The limitations of the direct use of mammalian steroid-metabolizing enzymes for metabolic studies is illustrated by the use of cultured human cells for the transformation of the synthetic vitamin D analog EB1089 (**25**), which gave levels of C-26 and C-26a hydroxylated products insufficient for conventional chemical isolation [42]. However, the use of enzymes from mammalian sources for the preparative transformation of steroids has gained impetus in recent years through the use of recombinant gene technology to express such enzymes in microorganisms. The expression of human cytochrome P4503A4 in yeast produced a strain capable of the hydroxylation of progesterone (**1**) at C-6β and C-16α [43], and *Escherichia coli* expressing bovine cytochrome P450c17 has been reported to carry out the hydroxylations of progesterone (**1**) and of pregnenolone (**26**) (Fig. 4) at C-17α [44]. The hydroxylation of testosterone (**23**) at C-6β by human cytochrome P450 expressed in *E. coli* has also been noted [45]. The construction of a strain of *Saccharomyces cerevisiae* expressing enzymes of the steroidogenic pathway from various sources, but lacking a key biosynthetic enzyme for the formation of ergosterol, has resulted in a microorganism capable of producing the mammalian steroids pregenolone (**26**) and progesterone (**1**) directly from galactose [46]. This process makes use of the yeast's native biosynthetic machinery to construct ergosta-5,7-diene-3β-ol (**27**), which then diverted



24 R = H

30 R = $COCH_3$



25

Structure 24, 25, and 30

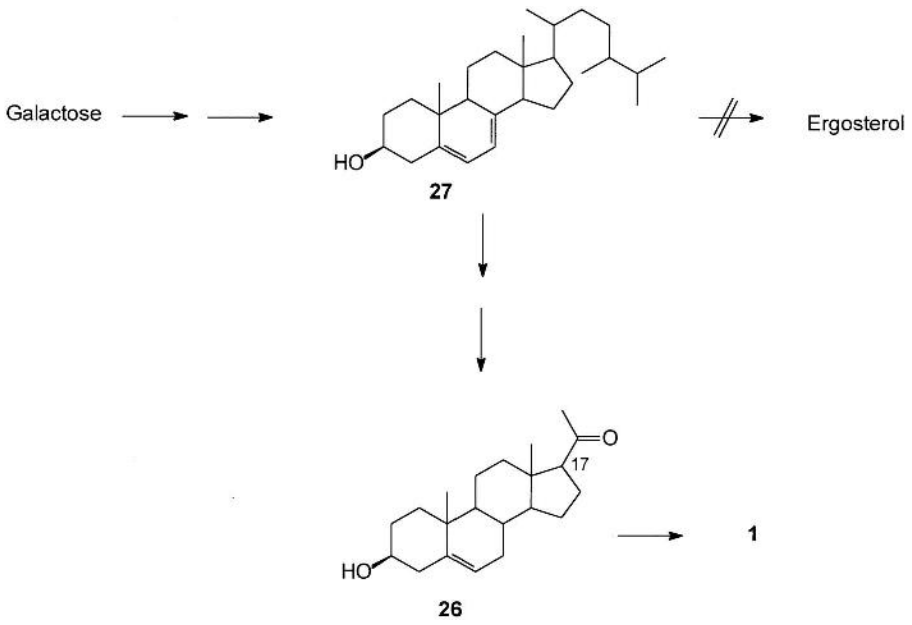


Figure 4 Production of progesterone from galactose by engineered yeast.

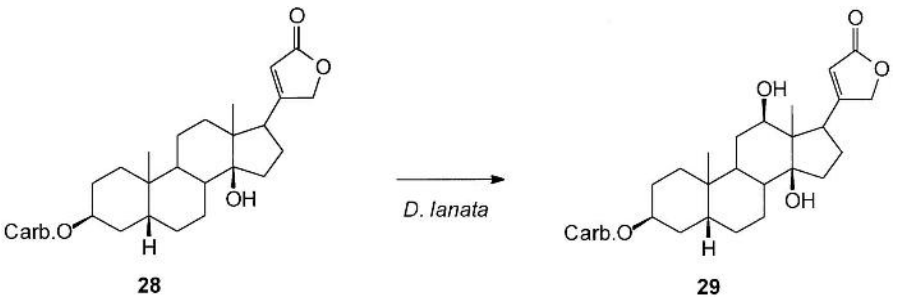
from its normal biosynthetic pathway by the action of inserted Δ^7-reductase and bovine side-chain cleavage enzymes to give end products with the pregnane skeleton (Fig. 4).

5.2 New Techniques

5.2.1 Immobilization

The possible advantages arising from the use of immobilized biocatalysts and low-water environments have prompted investigation of these techniques for the microbial transformation of steroids. The former method allows for the simple recovery of a reusable biocatalyst, whereas the latter has the potential to overcome limitations of substrate solubility inherent with the use of water-insoluble substrates in an aqueous environment. The use of immobilized cells has been briefly reviewed recently [47] and described for the 14α-hydroxylation of progesterone (**1**) by alginate bead preparations of *Mortierella isabellina* ATCC 42613 [48]. The latter process was found to proceed in acceptable yields (up to 70%) only in the presence of 5–10% methanol, a phenomenon attributed to effect of the solvent in permeabilizing the alginate bead to the substrate. Immobilized cells of *Penicillium raistrickii* were obtained by the growth of spore preparations immobilized in microcapsules, alginate beads, or photo-cross-linked polymers, and they were used for the 15 α-hydroxylation of 13-ethylgon-4-en-3,17-dione (**5**) with an efficiency for the first two preparations equal to that observed for non-immobilized cells [49].

Spores of *Rhizopus nigricans* immobilized in polyacrylamide, agar, or chitosan matrices result in preparations that can be reused for the 11α-hydroxylation of progesterone (**1**) [50], and spores of *Aspergillus niger* NCIM 589 immobilized on high-density polyethylene gave rise to a biocatalyst for the same reaction that showed a higher level of conversion than alginate-entrapped or free mycelia of the latter fungus [51]. The immobilization



28 29

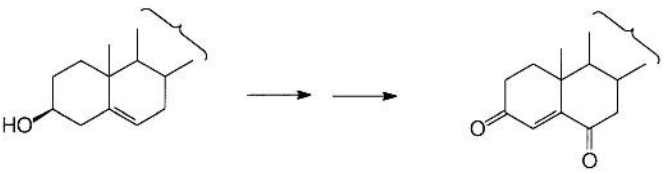
Figure 5 Conversion of digitoxin to digoxin by cells of *Digitalis lanata*.

of a plant cell suspension of *Digitalis lanata* in polyurethane foam or calcium alginate resulted in preparations that could be used for the conversion of digitoxin (**28**) to digoxin (**29**) (Fig. 5), but no significant increases in activity over the use of free cells was observed [52].

5.2.2 Low-Water Environments

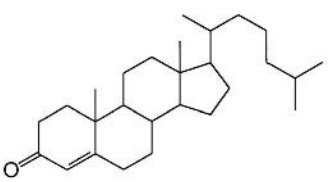
Although the use of organic solvents can facilitate substrate and product solubility, it can also result in altered biocatalytic function. Immobilized spores of *Rhizopus nigricans* show normal biocatalytic activity [50], but new catalytic functions were observed for immobilized spores of *Aspergillus ochraceus* when used under low-water conditions [53]. This organism, which converts progesterone (**1**) by hydroxylation at C-11α under conventional conditions, gave products arising (*inter alia*) from C-17α hydroxylation when alginate-immobilized spores were used under conditions of low water activity. The proportion of stereoisomeric C-11α- and C-11β-hydroxylated products produced by biotransformation of cortexolone-21-acetate (**30**) by *Absidia orchidis* was also found to be dependent on the presence of organic solvents: The ratio of 11β to 11α hydroxylation of 0.9 observed under conventional aqueous conditions changed to 2–2.5 when the reaction was carried out using an alginate-immobilized cell preparation in the presence of 10% propane-1,2-diol as a cosolvent [54].

The oxidation of cholesterol and related Δ^5-3β-hydroxysteroids in aqueous–organic two-phase systems has received attention. A cyclohexane-tolerant strain of *Pseudomonas putida* converts cholesterol and related substrates to Δ^4-3,6-diones **31** in an aqueous-toluene mixture as outlined in Figure 6 [55], and *Arthrobacter simplex* is reported to carry out the conversion of cholesterol (**22**) to cholest-4-en-3-one (**32**) in 95% yield in an aqueous carbon tetrachloride mixture [56]. A celite-immobilized preparation of *Mycobacterium*

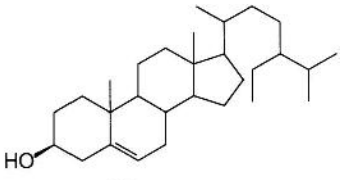


31

Figure 6 Oxidation of Δ^5-3β-hydroxysteroids by *Pseudomonas putida*.



32



33

Structures 32, 33

sp. NRRL B-3805 can convert β-sitosterol (**33**) to 4-androstene-3,17-dione (**9**) (**32–33**) in 80–90% yield using bis(2-ethylhexyl)phthalate as the conversion medium, but the system could not take advantage of high solubility levels due to diffusional limitations [57].

6 CONCLUSIONS

In spite of a long history of development, the microbial transformation of steroids remains an active research area. Although the search for new natural biocatalysts and new transformations is not as productive as it once was, the application of genetic methods for the construction of biocatalysts with specific activities promises to be a fruitful area for further progress, and the development of new methods for conducting established microbial transformations can also present significant opportunities for the improvement of existing processes.

REFERENCES

1. W Charney, HL Herzog. Microbial Transformations of Steroids. New York: Academic Press, 1967.
2. H Iizuka, A Naito. Microbial Transformation of Steroids and Alkaloids. State College, PA: University Park Press, 1967.
3. H Iizuka, A Naito. Microbial Conversion of Steroids and Alkaloids. Berlin: Springer-Verlag, 1981.
4. LL Smith. In: KH Overton, ed. Terpenes and Steroids, Vol. 4. London: Royal Society of Chemistry, 1974, pp 394–530.
5. HL Holland. Acc Chem Res 17:398–402, 1984.
6. HL Holland. Chem Soc Rev 11:371–395, 1982.
7. O Bortolini, A Medici, S Poli. Steroids 62:564–577, 1997.
8. HL Holland. Steroids 64:178–186, 1999.
9. SB Mahato, S Garai. Steroids 62:332–345, 1997.
10. HL Holland. Organic Synthesis with Oxidative Enzymes. New York: VCH, 1992.
11. S Hu, X Tian, G Han. Steroids 63:88–92, 1998.
12. A Jekkel, E Ilkoy, G Horváth, I Pallagi, J Süto, G Ambrus. J Mol Catal B:Enzym 5:385–387, 1998.
13. A Rahman, MI Choudhary, F Shaheen, M Ashraf, S Jahan. J Nat Products 61:428–431, 1998.
14. HL Holland, G Lakshmaiah, PL Ruddock. Steroids 63:484–495, 1998.
15. HL Holland, G Lakshmaiah. J Mol Catal B:Enzym 6:83–88, 1999.
16. S Mutafov, B Angelova, T Avramova, L Boyadjieva, I Dimova. Process Biochem 32:585–589, 1997.
17. S Hu, G Genain, R Azerad. Steroids 60:337–352, 1995.

18. K Suzuki, M Kato, K Sasahara. Japanese Patent 07,123,997, 16 May 1995; Chem Abs 123: 81766n, 1995.
19. VV Fokina, AV Karpov, IA Sidorov, VA Andrjushina, AY Arinbasarova. Appl Microbiol Biotechnol 47:645–649, 1997.
20. KA El Sayed. J Nat Products 61:149–151, 1998.
21. S-S Lee, J-L Yan, KC Wang. J Nat Products 61:275–278, 1998.
22. G Fantin, S Ferrarini, A Medici, P Pedrini, S Poli. Tetrahedron 54:1937–1942, 1998.
23. MD Greca, A Fiorentino, G Pinto, A Pollio, L Previtera. Biotechnol Lett 18:639–642, 1996.
24. HL Holland, DH Nguyen, NM Pearson. Steroids 60:646–649, 1995.
25. H Groh, R Schön, M Ritzau, H Kasch, K Undisz, G Hobe. Steroids 62:437–443, 1997.
26. HK Kang, SS Lee. Arch Pharm Res 20:525–528, 1997.
27. W-H Liu, C-K Lo. J Ind Microbiol Biotechnol 19:264–272, 1997.
28. P Znidarsic, R Komel, A Pavko. J Biotechnol. 60:207–216, 1998.
29. G Wang, M Wang, L Du. Weishengwuxue Zazhi 18:23–26, 1998; Chem Abs 129:22,9702, 1998.
30. T Kolek, A Swindor. J Steroid Biochem Mol Biol 67:63–69, 1998.
31. O Sideso, RAD Williams, SG Welch, KE Smith. J Steroid Biochem Mol Biol 67:163–169, 1998.
32. J Shi, L Ye, G Shen, L Chen. Shanghai Yike Daxue Xuebao 24:265–269, 1997; Chem Abs 129:104,177, 1998.
33. JR Hanson, PB Hitchcock, AC Hunter. Phytochemistry 49:1287–1292, 1998.
34. JR Hanson, AC Hunter. Phytochemistry 49:2349–2353, 1998.
35. CM Benasson, JR Hanson, AC Hunter. Phytochemistry 49:2355–2358, 1998.
36. JR Hanson, AC Hunter. Phytochemistry 49:2359–2362, 1998.
37. CS Benasson, Y Chevolot, JR Hanson, J Quinton. Phytochemistry 50:25–30, 1999.
38. M Vitas, D Rozman, R Komel, SL Kelly. J Biotechnol 42:145–150, 1995.
39. M Vitas, KE Smith, J Plavec, J Kesselmeier, T Pajic, A Ferlan, D Zigon, SL Kelly, R Komel. Chemosphere 38:853–863, 1999.
40. M Vitas, T Pajic, SL Kelly, R Komel. J Steroid Biochem Mol Biol 63:345–350, 1997.
41. K Paraszkiewicz, J Dlugonski. J Biotechnol 65:217–224, 1998.
42. V Narayanaswamy, FJ Dilworth, HLJ Makin, NJ Schroeder, DJH Trafford, A-M Kissmeyer, MJ Calverley, E Binderup, G Jones. Biochem Pharmacol 53:783–793, 1997.
43. Z Mehmood, DE Kelly, SL Kelly. Biotechnol Lett 17:83–88, 1995.
44. MS Shet, CW Fisher, RW Estabrook. Arch Biochem Biophys 339:218–225, 1997.
45. H Iwata, K Fujita, H Kushida, A Suzuki, Y Konno, K Nakamura, A Fujino, T Kamataki. Biochem Pharmacol 55:1315–1325, 1998.
46. C Duport, R Spagnoli, E Degryse, D Pompon. Nature Biotechnol 16:186–189, 1998.
47. SB Chincholkar. Microb Biotechnol 7–11, 1994; Chem Abs 128:19,1593, 1998.
48. HL Holland, S Poddar, B Tripet. J Ind Microbiol 10:195–197, 1992.
49. D Schlosser, S Irrgang, H-P Schmauder. Acta Biotechnol 15:161–172, 1995.
50. AS Vidyarthi, SJ Nagar. Biol Mem 20:15–19, 1994.
51. AG Kulkarni, SS Lele, PR Kulkarni. J Ferment Bioeng 86:510–512, 1998.
52. Y-S Park, H-K Kim, H-J Hong, D-I Kim. Kongop Hwahak 6:222–229, 1995; Chem Abs 123: 11,0231, 1995.
53. TK Dutta, TB Samanta. Bioorg Med Chem Lett 7:629–632, 1997.
54. J Wang, C Chen, B Li, J Zhang, Y Yu. Enzyme Microb Technol 22:368–373, 1998.
55. R Aono, N Doukyu. Biosci Biotech Biochem 60:1146–1151, 1996.
56. W-H Liu, W-C Horng, M-S Tsai. Enzyme Microb Technol 18:184–189, 1996.
57. P Fernandes, JMS Cabral, HM Pinheiro. Prog Biotechnol 11:431–436, 1996.

29

Enzymatic Approaches to the Production of Biodiesel Fuels

MICHAEL J. HAAS, GEORGE J. PIAZZA, and THOMAS A. FOGLIA

Agricultural Research Service, U.S. Department of Agriculture, Wyndmoor, Pennsylvania

1 INTRODUCTION

The idea of using vegetable oil as a substitute for diesel fuel was demonstrated by the inventor of the diesel engine, Rudolph Diesel, around 1900. Since that time, the concept of using fats and oils as renewable fuels has been periodically reintroduced, most recently during the fuel shortages of the 1970s. Since then, research in this area has continued, with various vegetable- and animal-fat-derived biofuels having been widely tested as alternative diesel fuels [1]. To overcome problems (high viscosity and fuel injector fouling) associated with the use of intact triglycerides as diesel fuels, the oil and/or fat is converted to simple alkyl esters (primarily methyl and ethyl esters). Today, ''biodiesel'' is the term applied to simple alkyl fatty acid esters used as alternatives to petroleum-based diesel fuels.

The relatively high cost of refined oils and fats makes biodiesel produced from these materials more expensive than petroleum-derived diesel fuel. To reduce the cost of biodiesel, there has been considerable investigation of the use of lower-quality/lower-value lipids, such as spent fryer grease and by-products of edible oil refining, as feedstocks for biodiesel production.

Biodiesel is presently in commercial production in several countries worldwide. In Europe, rapeseed and sunflower oils are the major feedstocks, along with waste grease. In Malaysia, palm oil esters are of interest. In the United States, soybean oil esters feature prominently, as do the esters of tallow and spent restaurant oils. For most technical applications, methyl esters are produced because methanol is readily available and relatively inexpensive. In some cases, however, it may be preferable to prepare ethyl esters because ethanol is less toxic than methanol. Moreover, because ethanol can be produced from

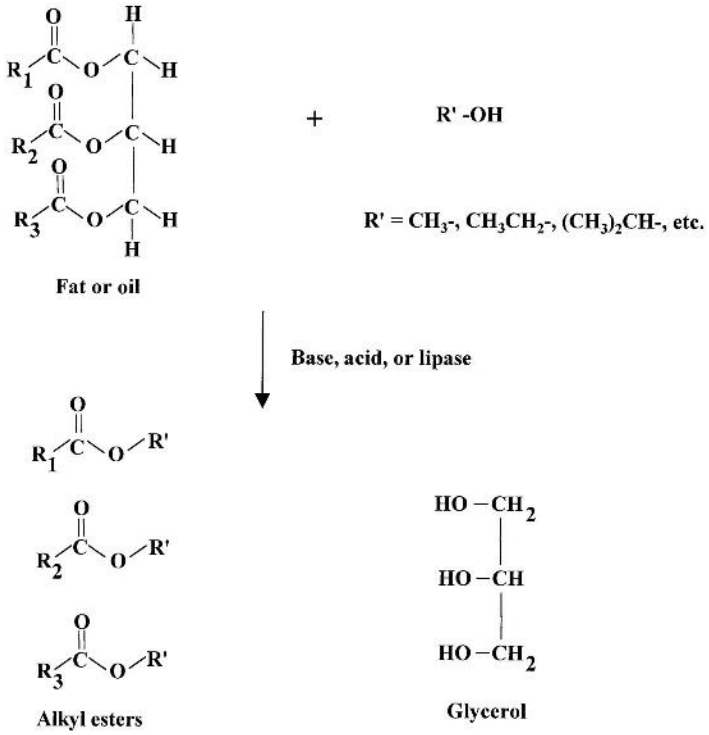
grain or biomass, ethyl ester biodiesel is a fuel that can be totally derived from renewable resources.

Presently, the driving forces behind the use of biodiesel fuels in the United States are mainly environmental and energy concerns. Vegetable oil and animal fat biodiesel fuels, being alkyl esters, have the following advantages over diesel fuel: as a neat fuel or in blends with diesel fuel, they produce less smoke and particulates, have higher cetane numbers, produce lower carbon monoxide and hydrocarbon emissions, and are biodegradable and nontoxic. Biodiesel is less volatile than petroleum diesel, which is reflected by its higher flash point. The latter, however, does result in its safer handling and storage. Another significant advantage of biodiesel is that it is derived from renewable sources that are produced domestically. Conversely, biodiesel fuels present technical challenges of their own, such as higher pour, cloud, and cold filter plugging points and low-temperature flow tests, elevated NO_x emissions (although under some conditions, and/or with slight modifications to engine timing, a reduction in the emissions of oxides of nitrogen can be achieved), possible reduced oxidative stability in storage, and incomplete combustion. The advantages and disadvantages of fat- and oil-derived alkyl ester diesel fuels with respect to fuel properties, engine performance, and emissions have been reviewed recently [2].

Currently, biodiesel is most commonly made by the alkali-catalyzed transesterification of an oil or fat with an alcohol, usually methanol, a process that shifts the glyceride fatty acids from glycerol to methanol, producing fatty acid methyl esters (FAME) and glycerol (Scheme 1). After separation of the glycerol and FAME products, the latter are water-washed to remove traces of glycerol, alcohol, and catalyst, before being blended with conventional diesel or used neat as fuel. The most commonly used blend is 20 vol% in petroleum diesel, which is referred to as a B-20 blend. This is the preferred blend level because the biodiesel component provides a desirable final 2.2 wt% of oxygenate in the fuel. This level of oxygenate imparts the desired reduction in emissions when using biodiesel, while minimizing the effects of power reduction that results from the lower-energy content of biodiesel compared to petroleum diesel.

Several procedures are available that use various catalysts for the alcoholysis of fats and oils [3]. The most commonly used catalysts are alkali hydroxides, but in some instances, alcoholates are used. When the feedstock has a high free fatty acid (FFA) content, as is common with rendered fats and spent restaurant oils, some operators merely add excess alkali, causing loss of the free acids as their insoluble soaps. This decreases the final yield of ester and consumes alkali. As an alternative, one can conduct an acid-catalyzed reaction, which simultaneously achieves transesterification of the glyceride and esterification of the free fatty acids. However, compared to alkali-catalyzed transesterification, this requires higher reaction temperatures and longer reaction times. More commonly, for FFA-containing feedstocks, a two-step process is used. The first step is acid-catalyzed esterification of the FFA, followed by neutralization of the acid and alkali-catalyzed transesterification of glycerides. A disadvantage of these chemical procedures is that the catalysts are removed with the side stream containing the coproduct glycerol and cannot be reused. Moreover, spent-catalyst removal increases the cost of glycerol purification.

Full conversion of FFA and glycerides in the feedstock to alkyl esters is a necessary feature for any method used to produce biodiesel, because even low residual amounts of these materials reduce the handling and performance characteristics of the fuel. Thus, strict standards are being developed for the maximum allowable levels of these materials



Scheme 1 Transesterification reaction of triacylglycerols to simple alkyl esters catalyzed by inorganic catalysts or by lipases.

in biodiesel. Current specifications set by the American Society for Testing and Materials for neat biodiesel call for a maximum residual level of free and bound glycerol of 0.240 wt%, and for free fatty acids, an acid number of 0.8 mg potassium hydroxide per gram [4]. Failure to attain these values during esterification necessitates costly cleanup steps that further increase the cost of the fuel.

2 LIPASE-CATALYZED FATTY ACID ESTER SYNTHESIS

Biochemical catalysis, mediated by enzymes, offers an alternative route for the esterification of FFA and the transesterification of glycerides. The classes of enzymes most associated with the hydrolysis of fatty acid esters in biological systems are the lipases. Ester hydrolysis is a reversible reaction, but it does not run in the direction of ester synthesis in biological systems, due to their high natural concentration of water, a coreactant in the hydrolysis reaction. The concept that lipases might be useful catalysts for ester synthesis was considered unlikely through the mid-1980s, due to the commonly held belief that enzymes were inactive in organic solvents. These data were gathered via experiments that employed mixtures of water and polar organic solvents, systems that do largely inactivate enzymes. However, the demonstration of marked enzymatic activity in virtually anhydrous hydrophobic organic solvents [5] opened many minds, which led to a flurry of research defining the boundaries and uses of this phenomenon, including the clear demonstration

of the abilities of lipases to synthesize esters. Since the mid-1980s, lipases have been exploited as catalysts for a number of transformations of fats and oils. Their main applications have been in modifying the fatty acid composition of triglycerides by interesterification [6–9] or acidolysis (acyl exchange) [10], the hydrolysis of triacylglycerols [11], and the direct synthesis of esters [12]. The use of lipases for the transesterification of triglycerides, producing simple fatty acid esters, also has been reported. For example, the lipase-catalyzed alcoholysis of various vegetable oils in aqueous oil systems has been described [13], although large amounts of catalyst, alcohol, and water were needed. The advantages of lipase catalysis over nonenzymatic methods for the production of simple alkyl esters include room-temperature reaction conditions, catalyst reuse, the ability to esterify both glyceride-linked and unesterified fatty acids in one step, and production of a glycerol side stream with minimal water content and little or no inorganic material. Bottlenecks to their use include the high cost of biocatalysts compared to inorganic catalysts without effective schemes for their multiple usage and stabilization, inactivation of the lipase by contaminants in the triglyceride feedstocks, and/or substrate inhibition or inactivation especially with polar short-chain alcohols.

2.1 Enzymatic Conversion of Triglycerides to Alkyl Esters

Mittelbach, using sunflower oil as the feedstock [14], conducted early work on the application of enzymes for biodiesel synthesis. The ability of various lipases to perform alcoholysis was tested in petroleum ether. Only three lipases were found to catalyze methanolysis: a free lipase from *Pseudomonas fluorescens* (Rohm GmbH) and immobilized lipase preparation of a selected strain of a *Candida* sp. (SP382, Novozyme) and *Rhizomucor miehei* (Lipozyme™, Novozyme). The *Pseudomonas* enzyme gave the best ester yields, with maximum conversion (99%) obtained with ethanol (Table 1). When the reaction was repeated without petroleum ether, only 3% product was produced with methanol as alcohol, whereas with absolute ethanol, 96% ethanol and 1-butanol conversions ranged between 70% and 82%. Reaction rates with a series of homologous primary alcohols (C1–C5), with or without the addition of 0.17 wt% water, showed that reaction rates increased

Table 1 Conversion of Sunflower Oil to Alkyl Esters[a]

Lipase	Alcohol	Alkyl ester[b]
Pseudomonas lipase	Methanol	79
	Ethanol	99
	1-Propanol	81
Candida lipase	Methanol	53
	Ethanol	79
	1-Propanol	29
Mucor lipase	Methanol	25
	Ethanol	82
	1-Propanol	80

[a] Reactions run in petroleum ether solvent for 5 hr at 45°C [14].

[b] Conversion rates expressed as mole product/mole triglyceride fatty acid.

with increasing chain length of the alcohol. In the case of methanol, the highest conversion was obtained without the addition of water, but for the other alcohols, the addition of water increased the esterification rate by twofold to fivefold.

In a subsequent study by Shaw and Wang [15], a *Pseudomonas* lipase immobilized on Celite was used to catalyze the alcoholysis of long-chain fatty-acid-containing triglycerides with ethanol, isopropanol, and amyl alcohol. Initial rates of alcoholysis of saturated fatty acid triglycerides were faster in isopropanol than in ethanol, but the opposite was true for unsaturated fatty-acid-containing triglycerides. Final ester concentrations were greater than 90% of theoretical maximum with tripalmitin and triolein in isopropanolysis reactions, but lower in ethanolysis reactions. Olive oil also was an acceptable substrate in both esterification reactions. The immobilized lipase retained some activity even after 10 days of use, with better activity being retained during isopropanolysis (83%) than in ethanolysis (25%).

Linko et al. [16] screened 25 commercial lipases to assess their activity in catalyzing the esterification of oleic acid with 1-butanol. Lipases from *C. rugosa, Chromobacterium viscosum, R. miehei*, and *P. fluorescens* had the highest activity, producing ester yields >85% in 20 hr. The initial water content of the system, amount of lipase used, and mole ratio of reactants were important factors in determining ester yields, and it was possible to obtain 100% esterification with the *R. miehei* lipase. The lipase from *C. rugosa* was used to study the transesterification of rapeseed oil without additional organic solvent. The highest conversions to esters were obtained with 1% water added, a molar ratio of alcohol to rapeseed oil of 2.8, and reaction temperature of 37°C. Increasing reaction temperatures to 55°C gave faster conversions but did not affect overall ester yields. Mechanical mixing was investigated as a means of keeping the lipase suspended in larger reactions. It was found that when mixing was rapid enough to keep the lipase suspended, product yields increased. With such an approach, when *C. rugosa* lipase was immobilized on Amberlite XAD-7 resin, a >90% conversion of rapeseed oil to esters was achieved in 8 hr for 2-kg batches using 3.4 wt% of lipase at 37°C.

Recently, Selmi and Thomas [17] reported the ethanolysis of sunflower oil with Lipozyme in a medium totally composed of sunflower oil and ethanol. Conditions studied for the conversion of the oil to esters included substrate molar ratio, reaction temperature and time, and enzyme load. Ethyl ester yields did not exceed 85%, even under the optimized reaction conditions. The addition of water (10 wt%), in addition to that associated with the immobilized enzyme, decreased ester yields significantly. The affect of added water in this instance is to be contrasted to the result obtained for reactions in organic solvent. These authors reported that ester yields could be improved with the addition of silica to the medium. The positive affect of silica on yield was attributed to the adsorption of the polar glycerol coproduct onto the silica, which inhibited glycerol deactivation of the enzyme. Enzyme reuse was also investigated, but ester yields decreased significantly with enzyme recycle (four cycles), even in the presence of added silica.

One drawback to the use of esters prepared from refined vegetable oils is their relatively high cost compared to other triglyceride substrates. A lower cost substrate is tallow. However, because tallow contains lower levels of unsaturated fatty esters and higher proportions of saturated fatty acids, biodiesel prepared from tallow has poor low-temperature properties compared to vegetable-oil-derived biodiesel. One way to partially overcome this difficulty is to prepare branched-chain esters from tallow [18]. Here, it was found that branching in the alcohol moiety of alkyl esters improved their low-temperature properties. For example, the pour point of isopropyl tallowate was 9°C lower than that of the

n-propyl ester, and that of the 2-butyl ester was 6°C lower than the *n*-butyl ester. A similar lowering in pour point for branched alkyl esters of soy oil compared to their normal-chain counterparts also has been reported [19]. A study was conducted using different lipases, vegetable oils, and tallow, under different reaction conditions in order to determine the best conditions for preparing biodiesel [20]. Using hexane as solvent, several commercially available lipases were screened for their abilities to transesterify the triacylglycerols (TAG) of olive, soybean, rapeseed, and tallow with short-chain alcohols. The enzymes studied included (1) immobilized Lipozyme IM60, which is a 1,3-regioselective lipase, (2) SP435, an immobilized form of the nonspecific *C. antarctica* lipase (Novozyme), and (3) PS-30, a nonimmobilized nonspecific lipase from *P. cepacia* (Amano Enzyme Co.). The immobilized lipase from *R. miehei* (Lipozyme) was the most effective in converting the oils and tallow to their corresponding methyl ester derivatives (Table 2). Using 10 wt% of Lipozyme (relative to the weight of tallow) and a reaction time of 8 hr resulted in a 95% conversion to esters. The efficiencies of esterification with methanol and ethanol

Table 2 Lipase-Catalyzed Transesterification of Triglycerides to Alkyl Esters with Primary Alcohols[a]

Substrate	Alcohol	Lipase	Temp. (°C)	% Composition of product[b,c]			
				MG	DG	TG	Ester
Tallow	Methanol	*R. miehei*[d]	45	0.5 e	8.2 e	13.6 f	77.8 b
Tallow[e]	Methanol	*R. miehei*	45	0.1 e	3.5 f-i	1.5 g	94.8 a
Soybean	Methanol	*R. miehei*	45	1.4 e	12.5 d	10.7 f	75.4 b,c
Rape	Methanol	*R. miehei*	45	1.9 d,e	7.8 e,f	13.0 f	77.3 b
Tallow	Methanol	*C. antarctica*[d]	45	5.1 c	12.8 d	53.5 d	25.7 d
Tallow	Methanol	*P. cepacia*	45	0.0 e	6.9 e,f,g	79.2 b	13.9 e,f
Soybean	Methanol	*P. cepacia*	45	2.4 d,e	17.8 c	65.3 c	14.5 e,f
Olive	Methanol	*P. cepacia*	45	1.3 e	24.2 a,b	50.1 d	24.4 d
Tallow	Ethanol	*R. miehei*	45	0.1 e	0.9 h,i	0.7 g	98.3 a
Tallow	Ethanol[f]	*R. miehei*	45	14.4 b	22.4 b	1.6 g	68.0 f,g
Tallow	Ethanol	*R. miehei*	35	0.0 e	4.6 e-h	1.4 g	93.9 a
Tallow	Ethanol	*R. miehei*	55	0.4 e	3.3 g,h,i	1.8 g	94.5 a
Soybean	Ethanol	*R. miehei*	45	0.6 e	1.2 h,i	0.8 g	97.4 a
Rape	Ethanol	*R. miehei*	45	0.8 e	0.3 h,i	0.3 g	98.2 a
Tallow	Propanol	*R. miehei*	45	0.2 e	1.5 h,i	0.1 g	98.3 a
Tallow	Propanol[f]	*R. miehei*	45	0.7 e	0.5 h,i	0.3 g	98.6 a
Tallow	Butanol	*R. miehei*	45	0.1 e	0.1 i	0.2 g	99.6 a
Tallow	Butanol[f]	*R. miehei*	45	0.6 e	0.5 h,i	0.8 g	98.1 a
Tallow	Isobutanol	*R. miehei*	45	0.1 e	0.8 h,i	0.8 g	98.5 a
Tallow	Isobutanol[f]	*R. miehei*	45	0.2 e	0.2 i	0.2 g	99.4 a
Tallow	Isobutanol	*P. cepacia*	45	6.8 c	27.1 a	37.3 e	28.8 d

[a] Reaction conditions for transesterification were as follows: 0.34 *M* triglyceride in hexane (8 mL), 200 rpm, 5 hr reaction time [20].

[b] Determined by gas chromatography.

[c] Means ($n = 3$) in the same column with no letter in common are significantly different ($p < 0.05$) by Bonferroni LSD.

[d] *R. miehei* lipase used was Lipozyme IM60; *C. antarctica* lipase used was SP435.

[e] Reaction time was 8 hr.

[f] Water, 6.0 mol% based on triglyceride, was added to reaction.

were sensitive to the water content of the reaction mixtures, with water greatly reducing the amount of ester formed. The use of 95% ethanol instead of absolute ethanol reduced esterification from 98% to 68% in the course of a 5-hr reaction (Table 2). *n*-Propyl, *n*-butanol, and isobutyl esters were prepared at high conversion efficiencies (94–100%) during a 5-hr reaction using 12.5 wt% of Lipozyme and an alcohol-to-tallow molar ratio of 3 : 1. Minor amounts of water did not appear to affect ester production in these instances. The conversions were nearly constant over temperature ranges between 35°C and 55°C, as exemplified by ethanolysis of tallow (Table 2).

Screening reactions for transesterification of tallow with secondary alcohols (Table 3) showed a different trend, in which the lipases from *C. antarctica* (SP435) and *P. cepacia* (PS30) gave higher ester conversions than Lipozyme [20]. This is in agreement with the findings of Shaw and Wang [15], who found immobilized *P. cepacia* to be effective in the ethanolysis/isopropanolysis of tripalmitin and triolein. For the production of esters from secondary alcohols, the amount of enzyme used was 25 wt% based on triglyceride. Reactions run without the addition of water were sluggish for both the SP435 and PS30 lipases. In both cases, conversions of 60–84% were obtained overnight (16 hr). The addition of small amounts of water improved the yields. The opposite effect was observed in the case of methanolysis, which was extremely sensitive to the presence of water. For branched-chain alcohols, better conversions were obtained when the reactions were run neat, as seen with isopropanol and 2-butanol (Table 3). Reduced yields when using the normal alcohols, methanol and ethanol, in solvent-free reactions were attributed to unfavorable viscosity conditions, which affected mixing of substrates with the lipase, or to enzyme deactivation by the alcohol.

Tallow transesterification was scaled up (120 g of triglyceride, solvent-free reactions, low molar ratio of alcohol : tallow), resulting in yields of greater than 95% for the primary alcohols and greater than 90% for secondary alcohols, with minimum production

Table 3 Lipase-Catalyzed Transesterification of Tallow with Alcohols[a]

Alcohol	Solvent	Lipase	Time (hr)	% Composition of products[b,c]			
				MG	DG	TG	Ester
Methanol	Hexane	*R. miehei*[d]	5	0.5 c,d	8.2 b	13.6 d	73.8 d
Methanol	Hexane	*R. miehei*	8	0.1 d	3.5 c	1.5 e	94.8 a
Methanol	None[e]	*R. miehei*	8	5.2 a	10.0 a	67.6 a	19.4 g
Ethanol	Hexane	*R. miehei*	5	0.2 d	1.2 d	0.6 e	98.0 a
Ethanol	None[e]	*R. miehei*	5	1.8 b,c	3.7 c	29.0 c	65.5 e
Isopropanol	Hexane	*C. antarctica*[d]	16	0.0 d	1.1 d	47.2 b	51.7 f
Isopropanol	None[e]	*C. antarctica*	16	1.2 b	4.0 b	0.9 e	94.3 b
Isobutanol	Hexane	*R. miehei*	5	0.1 d	0.8 d	0.6 e	98.5 a
Isobutanol	None[e]	*R. miehei*	5	0.8 c,d	0.9 d	1.0 e	97.4 a
2-Butanol	Hexane	*C. antarctica*	16	0.0 d	1.1 d	15.4 d	83.8 c
2-Butanol	None[e]	*C. antarctica*	16	1.3 b,c,d	1.3 d	1.0 e	96.4 a

[a] Transesterification reactions were run as follows: 0.34 *M* tallow in hexane (8 mL), 45°C, 200 rpm [20].
[b] Determined by gas chromatography.
[c] Means ($n = 3$) in the same column with no letter in common are significantly different ($p < 0.05$) by Bonferroni LSD.
[d] *R. miehei* lipase employed as Lipozyme IM60; *C. antartica* lipase employed as SP435.
[e] Reaction conditions the same, except no solvent used.

Table 4 Continual Conversion of Vegetable Oil to Corresponding Methyl Esters by Immobilized *C. antarctica* Lipase[a]

Cycle	Composition (%)				Hydrolysis (%)
	ME[b]	MG[b]	DG[b]	TG[b]	
1	97.4	1.6	1.0	nd[b]	1.0
50	96.2	1.7	2.1	nd	0.2

[a] Data taken from the literature [22].
[b] Abbreviations: ME = methyl esters; MG = monoglycerides; DG = diglycerides; TG = triglycerides; nd = not detected.

of partial glycerides. By modification of these conditions, similar conversions also could be obtained for both the methanolysis and isopropanolysis of soybean and rapeseed oils [21].

In another study [22], mixtures of soybean and rapeseed oils were treated with various immobilized lipase preparations in the presence of methanol. It was found that the lipase from *C. antarctica* was the most effective in promoting the formation of methyl fatty acid esters. It was not necessary to pretreat the lipase with water, and the addition of extra water reduced the conversion of oil to methyl ester. When more than 1.5 mol methanol was added per mole of oil, conversion to ester was decreased. Once lipase activity had been diminished by the addition of extra methanol, it was not possible to restore lipase activity by incubation in reduced methanol. In order to achieve high levels of conversion to methyl ester, it was necessary to add three equivalents of methanol. Because this level of methanol resulted in lipase deactivation, it was necessary to add the methanol in three separate additions. The best procedure was to add methanol at 0, 10, and 14 hr, and then let the reaction proceed for 24 hr. Under these conditions, greater than 97% conversion of oil to methyl ester was achieved. The results of analysis of products after the first cycle and 50 cycles of reused lipase are shown in Table 4. It shows that the conversion of oil to methyl ester was barely diminished even after 50 cycles with one lipase preparation. It also was found that merely allowing the reaction mixture to stand could separate the methyl ester and glycerol layers.

2.2 Enzymatic Conversion of Greases to Biodiesel

Recent research on low-temperature properties and diesel engine performance of selected monoalkyl esters derived from tallow and spent restaurant grease strongly suggested that ethyl esters of grease (i.e., ethyl greasate) might be an excellent source of biodiesel [23]. Ethyl esters of grease have low-temperature properties, including cloud point, pour point, could filter plugging point, and low-temperature flow test, closely resembling those of methyl soyate, the predominant form of biodiesel currently marketed in the United States. Results of diesel engine performance and emission tests were obtained for 20% blends of ethyl greasate and isopropyl tallowate in No. 2 diesel fuel in a matched dual-cylinder diesel engine. Data from the 5-hr test runs indicated adequate performance, reduced fuel consumption, and no apparent difference in carbon buildup characteristics, CO, CO_2, O_2, and NO_x emissions compared to No. 2 diesel [23]. The biodiesel used in these tests was synthesized enzymatically. Low-value lipids, such as waste deep-fat-fryer grease, usually

have relatively high levels of FFA (8% or greater). Lipases are of particular interest as catalysts for the production of fatty esters from such feedstocks, because they accept both free and glyceride-linked fatty acids as substrates for ester synthesis. In contrast, biodiesel production from such mixed feedstocks using inorganic catalysts requires multistep processing [24].

To exploit these attractive features of lipase catalysis, a study was conducted using a lipase from *P. cepacia* and recycled restaurant grease with 95% ethanol [25]. The effects of temperature, reaction time, lipase level, and ethanol concentration on the degree of esterification were tested in a systematic method. The data were analyzed by response surface methodology, a statistical method for optimizing chemical and lipase-catalyzed reactions. The response surface analysis (Fig. 1) showed that time and temperature had significant effects on the yield of ethyl esters, and lipase level had a modest effect. The concentration of ethanol did not have any significant effect on the yield of ethyl esters within the range of mole ratios of ethanol to grease of 3:1 to 6:1, and water activity had modest effects.

The regression equation derived from this experiment predicted that the yield of ethyl esters would not exceed 85%. At near-optimal levels of temperature, lipase, and ethanol, it was found that the rate of ethyl ester synthesis deteriorated after a 1-hr reaction time. Accordingly, a second portion of the lipase from *P. cepacia* was added after 1 hr. This second addition of lipase did not significantly improve the conversion of grease to ethyl esters. However, it was found that if an immobilized preparation from *C. antarctica*

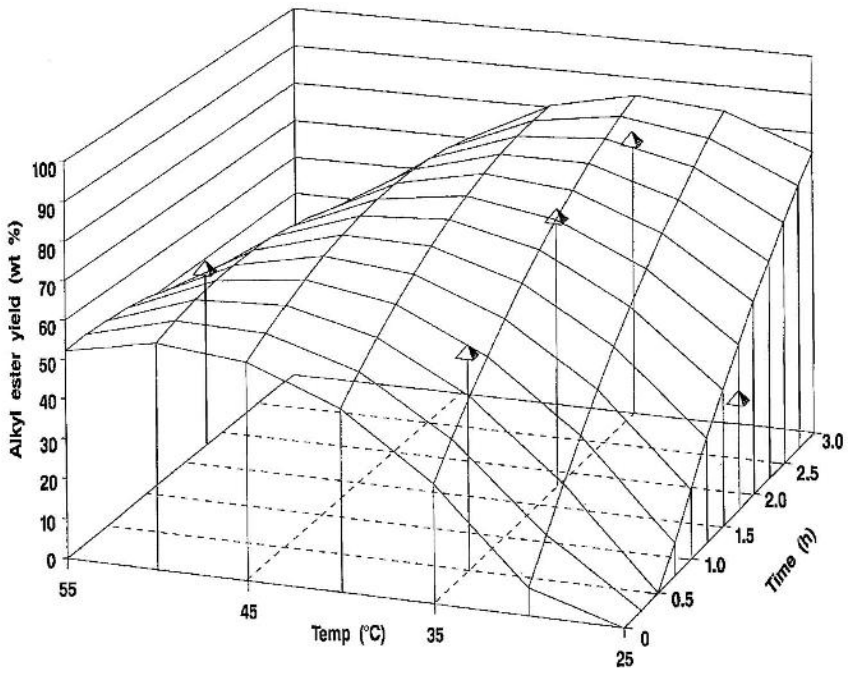


Figure 1 Contour plot of time and temperature versus percent alkyl esters. The spikes represent the observed yields where molar excess of ethanol is 46.7% and lipase level is 6.75 wt%. (From Ref. 25.)

lipase (SP435) was added, instead of *P. cepacia* lipase, after the first hour of incubation, the conversion to ethyl esters was increased to greater than 96%.

Similarly, response surface methodology in combination with principal-component analysis methods was applied for optimizing the enzymatic transesterification of rapeseed oil methyl esters [26]. The reaction of interest was the alcoholysis of rapeseed oil methyl esters (biodiesel) and trimethylolpropane. This study also showed that the water activity and alcohol (methanol) content of the reaction were the principal factors affecting product yields.

2.3 Enzymatic Conversion of Soapstocks to Biodiesel

One step in the refining process for edible vegetable oils involves the addition of water and alkali, causing precipitation of a semisolid material known as ''soapstock.'' This fraction contains residual triglycerides, monoglycerides and diglycerides, FFA, phosphoglycerides, pigments, and other minor components [27]. Although soapstock is used to prepare several products, it is nevertheless considered to be an underused by-product, and there is considerable interest in finding ways to convert soapstocks to industrial intermediates.

The acidulation of soapstock generates a fraction termed ''acid oil.'' This fraction contains protonated FFA (40–80 wt%), neutral glycerides (20–50 wt%), and other materials. A process using immobilized *Mucor miehei* lipase (Novozyme IM20) to catalyze simultaneous alcoholysis of the neutral glycerides and esterification of FFA has been developed [28]. The reactions contained 10 wt% catalyst, based on the weight of acid oil, and were conducted at 60°C. No solvent other than the substrate was employed. Butyl ester production after a 4-hr incubation amounted to approximately 80% of theory. The work did not specifically target the production of biodiesel and, in fact, did not investigate esterification with alcohols shorter than four carbons in length. It did, however, establish the feasibility of using enzymatic catalysis to esterify acid oils.

A partial semienzymatic process recently was devised that directly converted soapstocks to fatty esters suitable for biodiesel production [29]. In this two-step, solvent-free process, dried soapstock was first treated with alcohol and potassium hydroxide to promote transesterification of the fatty glyceride and phosphoglyceride fatty esters. Time-course studies with five alcohols (methyl, ethyl, *n*-butyl and isobutyl, and isoamyl alcohol) revealed that all measured glyceride and phospholipid fractions were rapidly transesterified, except for the diglyceride fraction. However, because the diglyceride fraction was only a very small proportion of the total fatty material, actual yields of fatty esters were only slightly less than theoretical. Transesterification predominated, although in some situations as much as 20% of the glyceride fatty acids were converted to free acids because of ester hydrolysis. It was postulated that the phosphoglycerides in the feedstock might have caused retention of water, despite lyophilization prior to the transesterification reaction, and that this water then fostered ester hydrolysis instead of transesterification.

In the second step, enzymatic catalysis was employed to esterify the free fatty acids in the mixture. These are recalcitrant to esterification by an alcoholic base. An immobilized lipase from *Candida antarctica* (SP435) gave the best yield of fatty esters. This enzyme-catalyzed reaction was pH dependent, with the best activity demonstrated at pH 6, although the reaction proceeded almost as well at pH 7. However, due to inefficient enzymatic

esterification, the combined process of base-catalyzed alcoholysis and lipase-catalyzed esterification resulted only in an 81% yield of simple fatty acid esters.

3 ANALYTICAL METHODS

It is important to determine the degree of conversion of fatty acids to esters in any biodiesel synthesis. It also is advisable to establish the degree of purity and the type of contaminants present in a biodiesel prior to its use. Accordingly, methods have been developed to monitor the composition of the products of the transesterification reaction and assess the quality of the biodiesel produced by either chemical or enzymatic procedures.

One laboratory measured glyceride transesterification mixtures prepared as biodiesel by thin-layer chromatography with flame-ionization detection (TLC/FID) [30]. This method was used subsequently to study the variables affecting the yields of fatty esters from transesterified vegetable oils [31]. This analytical technique, although quantitative, was time-consuming, difficult to conduct, and labor intensive. The transesterification reaction mixtures subsequently were analyzed by capillary gas chromatography (GC), which detected and quantitated esters, triglycerides, diglycerides, and monoglycerides in one run [32]. Because it is important to determine the amount of residual glycerol in biodiesel, the capillary GC approach was subsequently augmented to include the determination of glycerol [33].

Analysis of biodiesel by GC [32,33] required that the hydroxyl groups of the glycerides and glycerol be derivatized by silylation with *N*-methyl-*N*-trimethylsilyltrifluoroacetamide. Recently, a high-performance liquid chromatographic (HPLC) method has been developed for analyzing reaction mixtures obtained from transesterified fats and oils [34]. Advantages of the method are that derivatization of the sample is not required, analysis time is less than 30 min and all neutral lipid classes, including alkyl esters, FFA, triglycerides, 1,2- and 1,3-diglycerides, and 1(2)-monoglycerides, are readily quantitated.

A rapid analytical method that is particularly suitable for continuous analysis is near-infrared spectroscopy. Although infrared spectra of triacylglycerols and their corresponding methyl esters are similar, there are weak but distinguishing signals at 4425–4430 cm^{-1} and 6005 cm^{-1} that are representative for TAG and FAME. The potential utility of the signal at 4425–4430 cm^{-1} had been recognized [35]. With the recent availability of fiber-optic probes for infrared spectroscopy, it is now possible to monitor the degree of alcoholysis directly in a reaction medium, provided that the starting materials are reasonably pure [36]. This provides a useful method for real-time monitoring of the progress of ester synthesis.

4 CONCLUSION

From the foregoing, it is demonstrated that lipase-catalyzed transesterification is a viable method for the production of alkyl esters from vegetable oils, tallow, greases, and soapstocks. Work is still ongoing to maximize conversions of these feedstocks with specific alcohols, particularly secondary alcohols, to improve conversions for solvent-free methanolysis and ethanolysis reactions, and to scale-up reactions to provide sufficient quantities of materials for determination of their cold-temperature properties and characterization of their fuel properties. Because of the high cost of these biocatalysts, the potential of this technology rests with the development of processes for their stabilization and reuse. This

is needed despite their advantages even when using lower-cost alternative feedstocks such as recovered restaurant oils and greases.

REFERENCES

1. G Knothe, RO Dunn, MO Bagby. In: BC Saha, J Woodward, eds. Fuels and Chemicals form Biomass, American Chemical Society Symposium Series No. 666. Washington DC: American Chemical Society, 1997, pp 178–208.
2. MS Graboski, RL McCormick. Prog Energy Combust Sci 24:125–164, 1998.
3. G Vicente, A Coteron, M Martinez, J Aracil. Ind Crops Prod 8:29–35, 1998.
4. Specifications for Biodiesel (B100), National Biodiesel Board, Jefferson City, MO, January 1999.
5. AM Klibanov, CHEMTECH 16:354–359, 1986.
6. AR Macrae. J Am Oil Chem Soc 60:243A–246A, 1983.
7. MK Chang, G Abraham, VT John. J Am Oil Chem Soc 67:832–834, 1990.
8. TA Foglia, K Petruso, SH Feairheller. J Am Oil Chem Soc 70:281–285, 1993.
9. P Forssell, R Kervinen, M Lappi, P Linko, T Suortti, K Poutanen. J Am Oil Chem Soc 69: 126–129, 1992.
10. F. Ergan, M Trani. Biotechnol Lett 13:19–24, 1991.
11. S-W Tsai, C-S Chang. J Chem Tech Biotechnol 57:147–154, 1993.
12. KD Mukherjee, I Kiewitt. J Agric Food Chem 36:1333–1336, 1988.
13. G Lazar. Fette Seifen Anstrichm 87:394–400, 1985.
14. M Mittelbach. J Am Oil Chem Soc 67:168–170, 1990.
15. J-F Shaw, D-L Wang. Enzyme Microbiol Technol 13:544–546, 1991.
16. Y-Y Linko, M Lämsä, A Huhtala, O Rantanen. J Am Oil Chem Soc 72:1293–1299, 1995.
17. B Selmi, D Thomas. J Am Oil Chem Soc 75:691–695, 1998.
18. TA Foglia, LA Nelson, RO Dunn, WN Marmer. J Am Oil Chem Soc 74:951–955, 1997.
19. I Lee, LA Johnson, EG Hammond. J Am Oil Chem Soc 72:1155–1160, 1995.
20. LA Nelson, TA Foglia, WN Marmer. J Am Oil Chem Soc 73:1191–1195, 1996.
21. TA Foglia, LA Nelson, WN Marmer. US Patent 5,713,965, 1998.
22. Y Shimada, Y Watanabe T Samukawa, A Sugihara, H Noda, H Fukuda, Y Tominaga. J Am Oil Chem Soc 76:789–793, 1999.
23. W-H Wu, TA Foglia, WN Marmer, RO Dunn, CE Goering, TE Briggs. J Am Oil Chem Soc 75:1173–1178, 1998.
24. M Mittelbach, H Enzelsberger. J Am Oil Chem Soc 76:545–550, 1999.
25. W-H Wu, TA Foglia, WN Marmer, JG Phillips. J Am Oil Chem Soc 76:517–521, 1999.
26. E Uosukainen, M Lamsa, Y-Y Linko, P Linko, M Leisola. Enzyme Microbiol Technol 25: 236–243, 1999.
27. HE Snyder, TW Kwon. Soybean Utilization. New York: Van Nostrand Reinhold, 1987, pp 110–112.
28. S Ghoshray, DK Bhattacharya. J Am Oil Chem Soc 72:1541–1544, 1995.
29. MJ Haas, KM Scott. J Am Oil Chem Soc 73:1393–1401, 1996.
30. B Freedman, EH Pryde, WF Kwolek. J Am Oil Chem Soc 61:1215–1220, 1984.
31. B Freedman, H Pryde, TL Mounts. J Am Oil Chem Soc 61:1638–1643, 1984.
32. B Freedman, WF Kwolek, EH Pryde. J Am Oil Chem Soc 63:1370–1375, 1986.
33. C Plank, E Lorbeer. J Chromatogr A 697:461–468, 1995.
34. TA Foglia, KC Jones. J Liq Chromatogr Rel Technol 20:1829–1838, 1997.
35. RT Holman, PR Edmondson. Anal Chem 28:1533–1538, 1956.
36. G Knothe. J Am Oil Chem Soc 76:795–800, 1999.

30

Lipase-Catalyzed Synthesis of Value-Added Fatty Acid Esters

JEI-FU SHAW

Institute of Botany, Academia Sinica, Taipei, Taiwan

1 INTRODUCTION

Lipase can catalyze hydrolysis of esters, synthesis of esters, and interesterification reactions that include alcoholysis, acidolysis, and ester exchange. The diverse functions and the enzyme specificity (stereospecificity and regiospecificity) make lipase one of the most important biocatalysts in biotechnological applications. Several recent reviews on the biochemistry, molecular biology, and biotechnological applications have appeared in the literature [1–5]. This chapter describes a few examples of the synthesis of value-added fatty acid esters by lipase-catalyzed reactions that we have discovered in our laboratories in the past few years. The process has been patented [11].

2 PRODUCTION OF FATTY ACID ESTERS BY LIPASE-CATALYZED ALCOHOLYSIS

Esters of fatty acid are useful intermediate raw materials for the production of a number of fatty acid derivatives, including superamides, fatty alcohols, and certain other fatty acid esters [6]. They are also useful as additives in food industry and as fragrances in the cosmetics industry [7]. In industry, fatty acid esters are usually obtained by heating vegetable oils in methanol at 100–200°C. Because of the high energy cost of the conventional chemical process and the anticipated lower prices of enzymes, application of lipase in the oleochemical industry has become more attractive.

Alcoholysis of tripalmitin by *Pseudomonas* lipase was higher in isopropanol than in ethanol. After 6 hr of alcoholytic reaction, the conversion of triplamitin to isopropyl ester was 65%, which was about three times the observed 20% conversion of tripalmitin

to the corresponding ethyl ester. Extensive alcoholysis of tripalmitin resulted in 99% and 100% conversion in isopropanol and ethanol, respectively. A different relationship between the isopropanolysis rate and the ethanolysis rate of triolein was observed. The initial rate of alcoholysis of triolein in ethanol was higher than that in isoproanol. However, as time progressed, the two reaction curves crossed over and eventually reached equilibrium after prolonged incubation. At the end of the reaction, about 91% and 97% of triolein was converted to esters in ethanol and isopropanol, respectively.

To investigate the feasibility of using lipase-catalyzed alcoholysis for the production of fatty acid esters from vegetable oil, olive oil was used as a substrate for the reaction in isopropanol. The equilibrium concentration for the isopropyl esters of oleic, palmitic, linoleic, and stearic acids from alcoholysis, as catalyzed by 0.9 g Celite-immobilized lipase, were 74, 12.4, 9.4, and 3.1 m*M*, respectively. This final reaction–product distribution correlates well with the composition of acyl groups in olive oil. Different alcohols also affected the yields of these esters. The concentrations of oleic acid esters obtained after 24 hr of alcoholysis by ethanol, isopropanol, and amyl alcohol were 63, 74, and 73 m*M*, respectively. This result is consistent with that observed in the alcoholysis of triolein in which isopropanolysis gave a higher yield of ester than the ethanolysis.

The immobilized lipase was stable for 4 and 30 days in the alcoholytic reaction of triolein in ethanol and isopropanol, respectively. Although the initial rate of triolein alcholysis was higher in ethanol (160 μmol/hr) than in isopropanol (110 μmol/hr), lipase appeared to lose activity much faster in ethanol. Therefore, the Celite-immobilized lipase could be used more economically in isopropanol than in ethanol for long-term alcoholytic reaction.

Fatty acid esters can also be synthesized by direct esterification of fatty acid with alcohol [8,9]. However, the formation of water during the reaction greatly affected enzyme activity and decreased the yield of esters because it favors the ester hydrolysis. The use of alcohols as both the substrate and solvent for the alcoholysis of triglycerides in our system is advantageous because it not only maintains an excessive amount of reactant but also simplifies the subsequent separation process [10]. The direct alcoholysis of vegetable oils or animal fat for the production of fatty acid esters is obviously very cost-effective. The process has been patented [11].

3 PRODUCTION OF CARBOHYDRATE ESTERS BY LIPASE-CATALYZED HYDROLYSIS OR ALCOHOLYSIS

Esters of partially acylated saccharides have a number of interesting and potentially useful properties, including surface activity, antitumor activity, and plant growth-inhibiting activity. This potential has not been fully explored because the production of oligoesters (e.g., di-, tri-, and tetra-) of sugars is a difficult problem in organic chemistry due to the abundance of hydroxyl groups in sugar molecules and the similar reactivity of most of them. At the same time, all hydroxyl groups in saccharides can be readily acylated; for example, treatment of glucose with acetic anhydride results in efficient preparative production of glucose penta-acetate. It occurred to us that glucose penta-acetate (or other peracylated sugars) can serve as a pool for the preparation of glucose esters of various degrees of acylation, provided that the acyl groups can be selectively cleaved off, leading to the accumulation of some species. This approach has been experimentally confirmed using lipase (E.C. 3.1.1.3) as a catalyst; glucose diacetates, triacetates, and tetra-acetates have been produced on a gram scale [9]. 1-Hydroxy-glucose tetra-acetate was prepared with

74% yield by *Aspergillus niger* lipase-catalyzed hydrolysis of β-D-glucose tetra-acetate at room temperature.

The acyl derivatives of 1-hyrdoxy aldose can be prepared efficiently, with a yield over 98%, by the alcoholysis of peracylated aldose in the presence of a Celite-immobilized *A. niger* lipase [12,13]. The order of reaction rates in isopropanol is as follows: β-Gal-5Ac > β-Glu-5Ac > α-Gal-5Ac > α-Man-5-Ac. The K_m values of glucose penta-acetate in isopropanol and butanol were 0.047 *M* and 0.055 *M*, respectively. The optimal reaction temperature was 35°C. The operational half-lives of the Celite-immobilized lipase in isopropanol were 11, 6.6, and 4.8, days at 28°C, 35°C, and 40°C, respectively.

The lipase-catalyzed hydrolysis or alcoholysis was possibly a kinetic process carrying out the conversion of glucose penta-acetate → glucose tera-acetate → glucose triacetate → glucose diacetate → glucose monoacetate → glucose with rate constants of k_1, k_2, k_3, k_4, and k_5 for the consecutive reaction steps. By choosing conditions which have a high k_1/k_2 ratio, we can selectively prepare glucose tetra-acetate in nearly 100% yield.

It appears that the same lipase-catalyzed process may be applicable for the production of other acyl derivatives of 1-hydroxy aldose starting with their peracylated precursors. The enzymatic approach offers many advantages over the chemical process disclosed by a Japanese patent [14]. The Japanese patent disclosed that refluxing glucose penta-acetate in CF_3CO_2H and $(CF_3CO)_2O$ for 16.5 hr followed by methanolysis gave 75% of 2, 3, 4, 6-glucose tetra-acetate. In the enzymatic process described in this article, we can obtain 100% yield of 2, 3, 4, 6-glucose tetra-acetate in room temperature by simple lipase-catalyzed alcoholysis. This process has four advantages: (1) high yield and no need for product purification, (2) conserve energy, (3) simple operation, and (4) conserve reagents such as CF_3CO_2H and $(CF_3CO)_2O$.

Following our discoveries that lipase can catalyze regioselective hydrolysis or alcoholysis of peracylated glucose penta-acetate [9,12,13], several reports showed that the approach can also be applied to other carbohydrate esters [14–19]. The *Candida rugosa* lipase is the best enzyme for selective deacylation of the primary position (C6) of peracylated methyl pyranosides, whereas the procine pancreatic lipase is the best for selective hydrolysis of the 1-*O*-acetyl group from peracylated pyranoses [14]. Lipase AP-6 is the best enzyme for the preparation of 2,3,6,2′,3′,4′,6′-hepta-*O*-acetyl maltose/cellobiose octa-acetate [17]. In conclusion, different enzymes have different substrate specificities and regioselectivities in the production of various partially acylated carbohydrate esters. The reaction conditions could greatly affect the catalytic rates and enzyme specificity.

Enzymatic regioselective acylation of carbohydrates using lipases or proteases is an alternative for the synthesis of carbohydrate esters in organic solvents [20]. Supercritical carbon dioxide ($SCCO_2$) offers an attractive alternative to organic media for the enzymatic synthesis of carbohydrate esters for food and pharmaceutical industries because the $SCCO_2$ has advantages such as nontoxicity and easy separation of esters from unreacted fatty acids. It has been demonstrated that *C. rugosa* lipase can effectively catalyze the acylation of glucose with lauric acid in $SCCO_2$ [21]. The acylation depends on the water activity, temperature, and sugar/acyl donor ratio.

4 LIPASE-CATALYZED SYNTHESIS OF PROPYLENE GLYCOL FATTY ACID ESTERS AND PUFA-BASED EMULSIFIERS

Propylene glycol (1,2-propanediol) monoesters are good water-in-oil emulsifiers with low hydrophilic–lipophilic balance values [22]. These compounds have been approved by the

U.S Food and Drug Administration for use in foods and are most often used in cakes, cake mixes, whipped toppings, and bread [23]. They can be used in combination with monoglycerides to obtain excellent cake batter behavior, resulting in increased cake volume and uniform structure. They are also good for whipped toppings due to their aerating and foam-stabilizing properties. Synthesis of propylene glycol monoesters by chemical methods (e.g., esterification of propylene glycol with fatty acids in the presence of acid or alkaline catalysts usually results in a complex mixture) [24]. Enzyme-catalyzed conversion is more efficient and selective. We have discovered that a *Pseudomonas* lipase (Amano PS) can catalyze the acylation of propylene glycol with fatty acids, fatty acid ethyl esters, anhydrides, and triglycerides as acyl donors in anhydrous organic solvents for the facile synthesis of propylene glycol monoesters [25].

Fatty acids are better acyl donors than fatty acid esters for the lipase-catalyzed synthesis of propylene glycol monoesters. The carbon number of fatty acid and unsaturation also affect the yield. In general, organic solvents with high log*P* values (log*P* is defined as the logarithm of the partition coefficient in a standard octanol–water two-phase system). are better solvents for the synthesis. The Amano PS lipase performs better under lyophilized conditions. Fatty acid anhydrides are the best acyl donors for the acylation of propylene glycol. The best substrates in toluene and hexane are lauric anhydride and stearic anhydride, respectively. This suggests that lipase specificity can be changed by solvent engineering. Because eicosapentaenoic acid (EPA, $C_{20:5}$) and docosahexaenoic acid (DHA, $C_{22:6}$) are known to reduce both thrombotic tendency and hyperglyceridemia [26], propylene glycol esters of EPA and DHA are potentially health-beneficial PUFA-based emulsifiers which have both surfactant and biological activities. We found that immobilized *Mucor miehei* lipase (Lipozyme IM20) is the best enzyme for the synthesis of both propylene glycol monoesters of EPA and DHA among nine lipases tested [27]. The yields are affected by reaction conditions such as temperature, pH memory, fatty acid/propylene glycol ratio, and water content.

The polyunsaturated fatty acid (PUFA)-based emulsifiers are useful for nutraceutical biomedical and drug delivery applications [28]. The same idea is applicable to other enzyme-catalyzed syntheses of PUFA-based emulsifiers, including lipase-catalyzed synthesis of γ-linolenic acid (GLA) and DHA esters of carbohydrates [29], and phospholipase-catalyzed synthesis of PUFA phospholipids [30,31].

5 LIPASE-CATALYZED SYNTHESIS OF KOJIC ACID ESTERS

Kojic acid [5-hydroxy-2(hydroxymethyl)1,4-pyrone] is an inhibitor of plant polyphenol oxidase [32] and animal tyrosinase [33], which is useful in industries such as for the prevention of food browning and skin melanin formation. It is also an antioxidant [34]. However, kojic acid is water soluble and unstable for cosmetic use. Esterification of kojic acid is suitable to improve the lipophilicity because the ester moiety serves as nontoxic carriers with a high affinity for cell membranes and a great hydrophobicity to prevent degradation. We discovered that *Pseudomonas cepacia* (Amano PS) lipase and *Penicillium camembertii* lipase (Amano G) are effective biocatalysts for the synthesis of kojic acid monolaurate and kojic acid mono-oleate, respectively, in anhydrous acetonitrile [35].

6 CONCLUDING REMARKS

Lipases can be used for the synthesis of a wide variety of value-added fatty acid esters. Lipases from different organisms are highly versatile with respect to their diverse substrate

and reaction specificities. Although lipases share similar catalytic triad Ser-Asp/Glu-His (similar to serine proteases), their overall sequence homologies are usually quite low and, therefore, their substrate binding sites are quite different. No single lipase can be applied to the synthesis of all fatty acid esters. For the synthesis of a particular fatty acid ester, lipases from various resources should be tested to find the best lipase for application.

In a number of cases, organisms produce lipase isoforms that exhibit quite different substrate specificity [36]. Crude enzyme preparations are used in most applications, and lipase obtained from different supplies have been shown variations in catalytic efficiency and stereospecificity [37]. We have discovered that multiple enzyme forms with different substrate specificity and thermostability in a commercial *C. rugosa* lipase preparation [38]. Recently, we demonstrated that five *C. rugosa* lipase genes are differentially expressed in the presence of different inducers [39]. Traditionally, the culture conditions are optimized for the maximum production of enzyme activity units. Our results indicate that different culture conditions might result in heterogeneous compositions of isozymes, which display different catalytic activities and specificities. By engineering the culture conditions, selected enzymes can be enriched for particular biotechnological applications.

Many factors affect the lipase-catalyzed synthesis of fatty acid esters, including enzyme structure, immobilization methods [40], reaction media (organic solvents and supercritical fluid), temperature, pH (or pH memory in organic solvents), water activity, and log*P* in organic media, acyl donors and receptors. To obtain the best yield of fatty acid esters, all the factors should be optimized. Screening and directed evolution are current popular technologies for obtaining best enzyme for particular applications. Protein engineering and computer modeling based on three-dimensional structure are expected to lay the foundation for the rational-designed, tailor-made lipases used in various biotechnological applications.

REFERENCES

1. ARM Yahya, WA Anderson, M Moo-young. Enzyme Microbiol Technol 23:438–450, 1998.
2. RD Schmid, R. Verger. Angew Chem Int Ed Engl 37:1608–1633, 1998.
3. B Rubin, EA Dennis, eds. Methods in Enzymology: Lipase, Part A: Biotechnology (Vol. 284). San Diego, CA: Academic Press, 1997; Methods in Enzymology: Lipase, Part B: Enzyme Characterization and Utilization (Vol. 286). San Diego, CA: Academic Press, 1997.
4. FX Malcata, ed. Engineering of/with Lipases. Dordrecht: Kluwer, 1996.
5. NN Gandhi. J Am Oil Chem Soc 74:621–634, 1997.
6. NOV Sonntag. J Am Oil Chem Soc 61:229–232, 1984.
7. G Langrand, C Triantaphylides, J Baratti. Biotech Lett 10:549–554, 1988.
8. K Holmberg, E Osterber. J Am Oil Chem Soc 65:1544–1548, 1988.
9. JF Shaw, AM Klibanov. Biotechnol Bioeng 29:648–651, 1987.
10. JF Shaw, DL Wang, YJ Wang. Enzyme Microbiol Technol 13:544–546, 1991.
11. JF Shaw. Chinese Patent CP3990, June 11, 1990.
12. JF Shaw, ET Liaw. In: C Laane, J Tramper, MD Lilly, eds. Biocatalysis in Organic Media. Amsterdam: Elsevier, 1987, pp 233–239.
13. JF Shaw, ET Liaw, J. Chin Biochem Soc 18:73–80, 1989.
14. WJ Hennen, HM Sweers, YF Wang, CH Wong. J Org Chem 53:4939–4945, 1988.
15. EW Holla. Angew Chem Int Ed Engl 28:220–221, 1989.
16. S Riva, J Chopineau, APG Kieboom, AM Klibanov. J Am Chem Soc 110:584–589, 1988.
17. GT Ong, KY Chang, SH Wu, KT Wang. Carbohydr Res 265:311–318, 1994.
18. KY Chang, SH Wu, KT Wang. Carbohydr Res 222:121–129, 1991.

19. GT Ong, KY Chang, SH Wu, KT Wang. Carbohydr Res 241:327–333, 1993.
20. DR Patil, DG Rethwish, JS Dordick. Macromolecules 24:3462–3463, 1991.
21. C Tsitsimpikou, H Stamatis V Sereti, H Daflos, FN Kolisis. J Chem Technol Biotechnol 71: 309–314, 1998.
22. RL Stutz. AJ Del Vecchio, RJ Tenney. Food Prod Dev 7:52–58, 1973.
23. PE Macrae. J Am Oil Chem Soc 60:291–294, 1983.
24. PE Sonnet, GG Moore. Lipids 23:955–960, 1988.
25. JF Shaw, S Lo. J Am Oil Chem Soc 71:715–719, 1994.
26. JB Hansen, JO Olsen, L Wilsgard, V. Lyngmo, B Sevensson. Eur J Clin Nutr 47:497–501, 1993.
27. KJ Liu, JF Shaw. J Am Oil Chem Soc 72:1271–1274, 1995.
28. I Gill, R Valiverty. TIBTECH 15:470–478, 1997.
29. OP Ward, J Fang, Z Li. Enzyme Microbiol Technol 20:52–56, 1997.
30. M Härröd, I Elfman. J Am Oil Chem Soc 72:641–646, 1995.
31. M Hosokawa, K Takahasi, Y Kikuchi, M Hatano. J Am Oil Chem Soc 72:1287–1291, 1995.
32. R Saruno, F Kato, T Ikeno. Agric Biol Chem 43:1337–1339, 1979.
33. JS Chem, C Wei, RS Rolle, WS Otwell, MO Balaban, MR Marshall. J Agric Food Chem 39: 1396–1401, 1991.
34. JS Chen, C Wei, MR Marshall. J Agric Food Chem 39:1897–1901, 1991.
35. KJ Liu, JF Shaw. J Am Oil Chem Soc 75:1507–1511, 1998.
36. RC Chang, SJ Chou, JF Shaw. Biotechnol Appl Biochem 19:93–97, 1994.
37. MJ Barton, JP Hamman, KC Fichter, CJ Calton. Enzyme Microbiol Technol 12:577–583, 1990.
38. JF Shaw, CH Chang, YJ Wang. Biotech Lett 11:779–784, 1989.
39. GC Lee, SJ Tang, KH Sun, JF Shaw. Appl Environ Microbiol 65:3888–3895, 1999.
40. JF Shaw, RC Chang, FF Wang, YJ Wang. Biotechnol Bioeng 35:132–137, 1990.

31

Microbiological Conversions of Fatty Acids to Value-Added Products

TSUNG MIN KUO, TSUNEO KANESHIRO*, and CHING T. HOU

National Center for Agricultural Utilization Research, Agricultural Research Service, U.S. Department of Agriculture, Peoria, Illinois

1 INTRODUCTION

Vegetable oils are inexpensive, renewable feedstocks for industrial-scale fermentations to give value-added products with many food and nonfood uses. Vegetable oils generally have many saturated and unsaturated fatty acids in common [1], but castor oil is exceptional because of its unique, ready-made hyroxy fatty acid component that requires no chemical modification. Owing to its versatile usage in industry, ricinoleic [12-hydroxy-9(*Z*)-octadecenoic] acid, the major component of castor oil, has been studied extensively for production by genetically engineered plants [2,3]. Development of strategies for producing commercially useful levels of ricinoleate in transgenic soybeans is discussed by McKeon and Lin in Chapter 7. Alternatively, microbiological conversions of vegetable oils and their component fatty acids can provide new products with enhanced functionality and reactivity. These bioconversions are stereospecific and generate less undesirable byproducts and residues and, so, have become a major focus of our research effort [4]. This chapter reviews the production and potential uses of monohydroxy, dihydroxy, and trihydroxy fatty acids, fatty amides, wax esters, sphingolipids, furans, and lactones, as well as other ancillary products resulting from microbiological conversions of vegetable oil and fatty acids.

Names are necessary to report factually on available data; however, the USDA neither guarantees nor warrants the standard of the product, and the use of the name by USDA implies no approval of the product to the exclusion of others that may also be suitable.

* Retired.

2 α- AND β-HYDROXY FATTY ACIDS

A mixture of the 2(*R*)-hydroxy fatty acids (C_{14}–C_{18} in chain length) possesses antimicrobial activity against *Vibrio tyrogenuses* [5]. Short-chain, branched 2-hydroxyalkanoic acids are part of the ring structure of several peptide antibiotics such as sporidesmolides, amidomycin, and valinomycin [6–8]. 2(*R*)-Hydroxyhexadecanoic acid is a metabolite of unknown function in yeast [9]. Also, 2-hydroxy saturated C_{20}–C_{25} fatty acids are constituents found in the phospholipids of marine sponges [10], and 2-hydroxy saturated and monounsaturated C_{22}–C_{24} are fatty acid constituents of phospholipids in a Caribbean sea urchin [11]. α-Hydroxy fatty acids are major components of fatty acids found in *Arthrobacter simplex*, where the cell-free extracts contain enzymic activity to convert palmitic acid to α-hydroxypalmitic acid [12]. The sphingolipids of a wide variety of organisms are rich in α-hydroxy fatty acids, and in *Sphingobacterium paucimobilis*, α-hydroxymyristic acid is the major fatty acid component [13]. The α-hydroxylase of *S. paucimobilis* responsible for converting myristate to 2-hydroxymyristate is a member of the P450 superfamily [14], but the enzyme uses hydrogen peroxide instead of NADH and molecular oxygen [15]. Moreover, the enzyme exhibits high substrate specificity for α-hydroxylation reactions [16].

3-Hydroxydodecanoic acid is a constituent of the peptide antibiotics serratamolide [17] and isariin [18], whereas 3-hydroxy C_{10}–C_{12} fatty acids are components of the peptide antibiotic esperin [19]. 3(*R*)-Hydroxypalmitic acid is an extracellular metabolite of the yeast *Saccharomycopsis malanga* NRRL Y-6954 [20]. In *Mucor* sp. A-73, formation of 3-hydroxyalkanoic acid may serve as a physiological mechanism to prevent intracellular accumulation of undesirable metabolites [21]. Physiotrophic β-hydroxy-β-methylbutyric acid, which can increase muscle strength and lean mass gains in animals and humans undergoing resistance-exercise training, is produced from α-ketoisocaproate metabolism [22]. It can be synthesized from the bioconversion of β-methylbutyric acid by *Galactomyces reessii* CBS 179.60 [22]. A direct β-hydroxylation of oleic acid at the C-3 position occurs in *Alcaligenes* sp. 5-18, producing 3-hydroxyoleic acid and 3-hydroxyhexadecenoic acid [23]. The yeast *Dipodascopsis uninucleata* UOFS-Y128 can transform exogenous arachidonic acid to a stable 3-hydroxy-5,8,11,14-eicosatetraenoic acid (3-HETE) [24], a compound possessing signal transduction activity in human neutrophils [25]. Formation of 3-HETE is inhibited by aspirin and, apparently, differs from cyclooxygenase and lipoxygenase enzyme systems that initiate the formation of other arachidonic acid metabolites. Chiral-phase high-performance liquid chromatography analysis of 3-HETE and other metabolites reveals that they are 3(*R*)-hydroxy fatty acids. The regiospecificity of 3-HETE hydroxylation rules out the biosynthesis via a normal β-oxidation pathway but, perhaps, suggests a direct monooxygenase reaction at C-3 or a 2-enoyl-CoA hydratase with opposite steric specificity [26]. The *Dipodascopsis* yeast also converts exogenous linoleic acid and 11(*Z*),14(*Z*),17(*Z*)-eicosatrienoic acid to the 3(*R*)-hydroxylated metabolites of shorter chain length, but hydroxylates neither oleic acid, linolelaidic acid, γ-linolenic acid, nor eicosanoic acid [26].

2.1 Polyhydroxyalkanoates

In *Aeromonas caviae* cultures [27], a (*R*)-specific enol-CoA hydratase gene is expressed, and the gene products from alkanoic acids are polyhydroxyalkanoates (PHAs). Wallen and Rohwedder [28] have shown in an earlier study that the structure of PHA found in activated sludge is composed primarily of β-hydroxyvaleric and β-hydroxybutyric acids

of C_4–C_7 chain lengths. Subsequent studies by others show that the majority of PHAs contain 3(*R*)-hydroxyalkanoic acid monomers ranging from 3 to 14 carbons in length. The PHAs are produced in a wide variety of bacteria as an energy source and regarded as an attractive renewable product that is biodegradable and biocompatible as polymers. These metabolites may be useful in thermoplastics, fibers, and chiral-building fine chemicals. Various reviews written by Poirier et al. [29], Steinbüchel and Füchtenbusch [30], and Lee and Choi [31] present a large volume of literature on the biosynthesis, potential uses, and bioprocessing development of PHAs in microbial fermentations and transgenic plants. Interestingly, *Pseudomonas aeruginosa* 44T1 cultivated on vegetable oils, instead of sugars and short-chain alkanoic acids, forms novel PHAs [32]. In addition to the usual C_6–C_{14} 3-hydroxy saturated fatty acids, PHAs formed from euphorbia oil also contain $\Delta^{8,9}$-epoxy-3-hydroxy-5*c*-tetradecenoate and probably $\Delta^{6,7}$-epoxy-3-hydroxydodecanoate and $\Delta^{4,5}$-epoxy-3-hydroxydecanoate, whereas those formed from castor oil contain 3,8-dihydroxy-5*c*-tetradecenoate and 3,6-dihydroxydodecanoate [32]. PHAs can also be synthesized by *Alcaligenes* sp. AK201 in a mineral medium containing both even and odd carbon numbers of α,ω-alkanedioic acids or hydroxylated fatty acids as carbon substrates [33].

3 ω-HYDROXY FATTY ACIDS

The cell-free enzyme system of *Pseudomonas oleovorans* catalyzes ω-oxidation of saturated, even-numbered fatty acids ranging from $C_{8:0}$ to $C_{18:0}$. Medium-chain fatty acids (octanoate, decanoate, laurate, and myristate) are the most active substrates, whereas long-chain fatty acids (palmitate and stearate) are oxidized at lower rates, but hexanoate short-chain fatty acid is not oxidized [34]. The same enzyme system can also catalyze epoxidation reaction of alkenes, such that 1,7-octadiene is converted to both 7,8-epoxy-1-octene and 1,2-7,8-diepoxyoctane [35]. The ω-hydroxylation system of *P. oleovorans*, as catalyzed by the action of alkane 1-hydroxylase (simply as ω-hydroxylase), requires Fe^{2+} ions, NADH, molecular oxygen, and two other protein components, rubredoxin and an NADH–rubredoxin reductase [36]. The ω-hydroxylase is purified [37] and shown to be a nonheme iron protein requiring phospholipid [38] and ferrous irons [39] for full activity. The enzymatic complex transfers electrons in the sequence of NADH → reductase → rubredoxin → ω-hydroxylase → O_2 [40]. The enzyme can also be purified to homogeneity by various approaches and shown to contain a di-iron cluster for catalytic activity [41]. The ω-hydroxylase belongs to a large family of such functionally diverse enzymes as desaturase, hydroxylase, acetylenase, and epoxidase that may share a generalized mechanism for modification of fatty acids [42].

Oxygen-dependent hydroxylations also occur on carbon atoms adjacent to the terminus of long-chain fatty acids. ω- and (ω-1)-Hydroxy fatty acids are components of yeast sorphorose glycolipids belonging to a group of surface-active compounds [43]. In the production of extracellular hydroxy fatty acid sophorosides, strains of *Torulopsis* spp. hydroxylate long-chain $C_{16:0}$ and $C_{18:0}$ or $C_{18:1}$ to the corresponding 15-hydroxy C_{16} and 17-hydroxy C_{18} fatty acids [43,44]. The ω-1 hydroxylation is stereospecific in the introduction of an oxygen atom from molecular oxygen [44,45]. The involvement of cytochrome P-450 in the hydroxylation reaction is also well characterized in *Candida apicola* [46,47]. Under aerobic conditions, strains of *Bacillus pumilus* hydroxylate on the ω-1, ω-2, and ω-3 carbon atoms of oleic acid to produce 15-, 16-, and 17-hydroxy-9-octadecenoic acids, having 17-hydroxyoctadecenoic acid as the most abundant product [48]. A strain of *Bacil-*

lus sp. U88 converts 12-hydroxyoctadecanoic acid to 12,15-, 12,16-, and 12,17-dihydroxyoctadecanoic acids [49].

A soluble cell-free system from *Bacillus megaterium* ATCC 14581 hydroxylates *n*-saturated and monounsaturated fatty acids on the ω-1, ω-2, and ω-3 carbon atoms, primarily in the ω-2-position in the presence of NADPH and O_2 [50,51]. It does not hydroxylate the terminal methyl (ω) group of either fatty acids, alcohols, or amides, and hydoxylations of methylene carbon beyond ω-3 position are insignificant [51]. Formation of ω-hydroxylate isomers involves a single species of ω-2 hydroxylase that requires a ferredoxin-type component and is dependent on a P-450-type cytochrome [52,53]. The ω-2 hydroxylase is a unique, self-sufficient biocatalyst (designated as cytochrome P-450_{BM-3}), having both functions of an active cytochrome-*c* reductase and a monooxygenase resided in a single polypeptide with a molecular mass of 119 kDa [54]. The soluble recombinant P-450_{BM-3}, which is purified from the *E. coli* strain DH5α transformed with the P-450_{BM-3} gene [55], shows fatty acid chain-length specificity [56]. The soluble P-450_{BM-3} converts lauric and myristic acids to their corresponding ω-2 hydroxy fatty acids, but fails to react with capric acid. When palmitic acid is the substrate, the resulting products are dependent on substrate concentrations [56], and the fatty acid is converted to a mixture of ω-1, ω-2, and ω-3 hydroxy analogs at concentrations greater than 250 μ*M*. When the concentration of palmitic acid is less than 250 μ*M*, a mixture of 14-ketohexadecanoic acid, 15-ketohexadecanoic acid, 13-hydroxy-14-ketohexadecanoic acid, 14-hydroxy-15-ketohexadecanoic acid, and 13, 14-dihydroxyhexadecanoic acid are produced, indicating that the soluble P-450_{BM-3} can also function as a dehydrogenase [56]. Cytochrome P-450_{BM-3} also converts polyunsaturated arachidonic acid to 18(*R*)-hydroxyeicosatetraenoic acid and 14(*S*), 15(*R*)-epoxyeicosatrienoic acid [57].

4 DICARBOXYLIC ACIDS

Long-chain dicarboxylic acids (DCs) are useful raw materials for the preparation of fragrances, polyamides, adhesives, lubricants, macrolide antibiotics, and polyesters [33,58,59]. The DCs can also be used by microorganisms to produce PHAs, which can, in turn, be used to manufacture biodegradable plastics [33]. Tridecane-1,13-dicarboxylic acid (DC-15) is useful as a pharmaceutical stock chemical for synthesis of muscone, a cyclopentadecanone drug used in treating heart disease and inflammation of joints [60]. Aliphatic dicarboxylic acids are formed by a reaction mechanism first described as diterminal oxidation by Kester and Foster [61] in the production of dicarboxylic acids from the C_{10}–C_{14} alkanes by a *Corynebacterium* sp. strain 7E1C. A mutant strain of *Candida cloacae* 310 unable to assimilate DC as a sole carbon source produces large amounts of DCs, predominantly with the same number of carbon atoms as those of *n*-alkanes (C_9–C_{18}) used in the culture [62]. Among the reaction products, *n*-tetradecane ω,ω′-dicarboxylic acid (DC-16) from *n*-hexadecane (*n*-C_{16}) and DC-15 from *n*-C_{15} are most abundant. Conversion of *n*-C_{15} to DC-15 is stimulated by organic solvent- and detergent-treated *Cryptococcus neoformans* and *P. aeruginosa* but is also inhibited by elevated levels of DC-15 [63]. To avoid product inhibition, use of a continuous process with immobilized *Cryptococcus* cells can lead to a fivefold increase in yield as compared with the batch type of DC-15 production [60].

Besides long-chain alkanes, diterminal oxidation also occurs on long-chain saturated and unsaturated fatty acids. The ω-hydroxy fatty acids formed in the cell-free enzyme system of *P. oleovorans* can easily be oxidized further to the corresponding dicarboxylic

acids by an NAD-dependent ω-hydroxy fatty acid dehydrogenase [34]. A mutant strain S_{76} of *Candida tropicalis* produces long-chain dioic acid, not only from alkane but also from its alcohol, monoic acid, α,ω-diol, and ω-hydroxy acid of corresponding chain length by the reaction mechanisms of diterminal oxidation and/or ω-oxidation of the terminal methyl group [64]. The formation of α,ω-dioic acids from *n*-alkanes is carried out by a metabolic pathway that involves α,ω-diols as plausible intermediates [64]. The same microorganism also converts oleic acid and its alcohol and ester derivatives to form 9(*Z*)-1,18-octadecenedioic acid by ω-oxidation of the terminal methyl group, which can be metabolized further to form shorter, even-numbered carbon atoms of saturated or unsaturated dioic acids [65,66].

Conversion of *n*-alkanes to α,ω-dioic acids and ω- and (ω-1)-hydroxylation of fatty acids is well known for strains of *Candida maltosa*. Isoforms of cytochromes P-450 encoded by P450alk genes [67] are inducible by various long-chain *n*-alkanes and fatty acids and exhibit chain-length preferences in the terminal hydroxylation reactions [67–69]. An active alkane monooxygenase system can be reconstituted from purified recombinant proteins of P-450 52A3 and the corresponding NADPH-dependent reductase [70]. The enzyme system converts hexadecane to produce 1-hexadecanol, hexadecanal, hexadecanoic acid, 1,16-hexadecanediol, 16-hydroxyhexadecanoic acid, 1,16-hexadecanedioic acid, thus demonstrating the complete catalytic activity for sequentially converting *n*-alkanes to α,ω-dioic acids [71].

A genetically engineered *C. tropicalis* in which the β-oxidation pathway has been sequentially disrupted to fully redirect alkane and fatty acid substrates to the ω-oxidation pathway greatly improves the production of long-chain dioic acids [59]. Amplification of genes encoding the rate-limiting ω-hydroxylase of the β-oxidation-blocked strain further enhances the productivity [59]. The addition of pristane to the culture to act as an inert carrier for the sparingly water-soluble fatty substrates improves the production of dioic acids from long-chain fatty acids in a bioreactor by *C. cloacae* FERM-P736, a selected mutant strain with an impaired β-oxidation pathway [72].

A mutant yeast strain M 25 is derived from *C. tropicalis* DSM 3152 mutagenized with *N*-methyl-*N*-nitro-*N*′-nitrosoguanidine and selected with oleic acid as the sole carbon source [73]. Strain M 25 converts oleic acid to produce 3-hydroxy-9(*Z*)-1,18-octadecenedioic acid [73]. The mutant strain also transforms linoleic acid to (*Z*),(*Z*)-octadeca-6,9-dienedioic acid, (*Z*),(*Z*)-3-hydroxyoctadeca-9,12-dienedioic acid, and (*Z*),(*Z*)-3-hydroxytetradeca-5,8-dienedioic acid [74], and ricinoleic acid to *R*-(*Z*)-7-hydroxy-9-octadecenedioic acid and (*Z*)-3,12-dihydroxy-9-octadecenedioic acid. In addition, sunflower oil and rapeseed oil are utilized to contain *R*-(*Z*)-3-hydroxy-9-octadecenedioic acid in the fermentation culture [75]. During bioconversion, therefore, the configuration of the double bond is not changed and the hydroxylation is site-specific and regiospecific.

5 10-HYDROXYSTEARIC ACID AND 10-KETOSTEARIC ACID

Such hydroxy saturated fatty acids as 14-hydroxyeicosanoic acid and 10-hydroxystearic acid are used as commercial grease thickeners [76]. Also, keto fatty acids or derivatives of keto fatty acids are ingredients of multipurpose greases [77]. It is notable that specific microorganisms can be used to hydrate such fatty acids as oleic acid to form 10-hydroxystearic acid (10-HSA) and/or 10-ketostearic acid (10-KSA).

Wallen et al. [78] first described a *Pseudomonas* sp. (NRRL B-2994) possessing an intracellular enzyme that converts oleic acid to 10-HSA under anaerobic conditions with

a 14% yield. The anaerobic hydroxylation indicates that the hydroxyl oxygen is derived from water rather than molecular oxygen and that the enzyme is a hydratase rather than an oxygenase [79]. The finding that NRRL B-2994 fed with deuterium oxide produces 10-HSA from oleic acid with the deuterium shown on carbon atom 9, and in the *S*-configuration, it supports a hydration reaction [80]. Consistent with this result, a cell-free preparation of NRRL B-2994 catalyzes the interconversion of oleic acid and 10(*R*)-HSA and does not act upon 9(*R*)-HSA [81]. Similar enzyme preparations catalyze the hydration of *Z* (*cis*)- and *E* (*trans*)-9,10-epoxystearic acids, respectively [82], with stereospecific incorporation of the oxygen of water at carbon atom 10 [83]. Another strain of *Pseudomonas* sp. (NRRL B-3266) also shows that such a hydration reaction is specific for all unsaturated fatty acids having a *Z* configuration in the 9,10-position and that aerobiosis leads to the formation of 10-keto fatty acids [79]. Moreover, anaerobic cultures of NRRL B-3266 convert ricinoleic acid to 10,12-dihydroxystearic acid, and linoleic acid and linolenic acid to 10-hydroxy-12(*Z*)-octadecenoic acid and 10-hydroxy-12(*Z*),15(*Z*)-octadecadienoic acid, respectively, with no migration of the unreacted double bonds at C_{12} and C_{15} of the fatty acids [84]. Under anaerobic conditions, strains of *Nocardia cholesterolicum* (NRRL 5767 and NRRL 5768) and a *Nocardia* sp. (NRRL 5636) convert linoleic acid and linolenic acid to 10-hydroxy-12(*Z*)-octadecenoic acid and 10-hydroxy-12(*Z*),15(*Z*)-octadecadienoic acid, respectively [85]. A *Flavobacterium* sp., strain DS5, also converts linoleic acid to 10-hydroxy-12(*Z*)-octadecenoic acid with a 55% yield [86]. The strain DS5 hydratase is a C-10 positional and cis-unsaturation-specific enzyme [87].

Other bioconversions of oleic acid to 10-HSA have been found with *Corynebacterium* sp. S-401 [88], *Rhodococcus rhodochrous* (*Nocardia aurantia*) [89], *N. cholesterolicum* [90], *Saccharomyces cerevisiae* NRRL Y-2034, *Candida intermedia* UI 5159 [91], *Micrococcus luteus* [92], *Sphingobacterium thalpophilum* NRRL B-14797 [93], and *Staphylococcus* sp. NRRL B-14813 [94]. Two species of anaerobic ruminal bacteria, *Selenomonas ruminantium* and *Enterococcus faecalis*, can convert oleic acid to form 10-HSA [95]. Resting cells of *Nocardia paraffinae* CBS 255.58 also convert oleic acid to 10-HSA anaerobically [96].

Such hydrations of oleic acid by microbial cultures may lead to produce enantiomers [97]. *Nocardia aurantia* ATCC 12674, *N. restrictus* ATCC 14887, *Mycobacterium fortuitum* UI-53387, and baker's yeast produce a mixture of enantiomeric 10(*R*)-HSA and 10(*S*)-HSA, whereas *Pseudomonas* sp. NRRL B-3266 and NRRL B-2994 produce optically pure 10(*R*)-HSA [97]. *Corynebacterium* sp. S-401 [88] and *S. thalpophilum* NRRL B-14797 [93] also convert oleic acid to 10(*R*)-HSA. In addition to HSA, 10-KSA is a major product of oleic acid bioconversions under the usual, aerobic conditions of one *Mycobacterium* and two *Nocardia* species [91], *Aspergillus terreus* strains UI 58 and ATCC 11156 [91], *Staphylococcus warneri* sp. [98], *Flavobacterium* sp. strain DS5 [99] and strain 12-4A [100], and *S. thalpophilum* strain O22 [101]. Among these oleic acid bioconverters, *S. cerevisiae* NRRL Y-2034, *C. intermedia* UI 5159 [91], and *S. thalpophilum* NRRL B-14797 [93] produce 10-HSA exclusively, whereas *Flavobacterium* sp. 12-4A [100] and a newly examined *Bacillus sphaericus*-like organism [Kuo and Nakamura, unpublished results] produce 10-KSA solely.

Microbial hydration of oleic acid may also lead to produce different amounts of 10-HSA and 10-KSA, often depending on the conditions employed during the conversion. For example, under anaerobic conditions, *N. cholesterolicum* strain NRRL 5767 converts oleic acid to 98% hydroxy acid and 2% keto acid, whereas under aerobic conditions, the products become 88% hydroxy acid and 12% keto acid [90]. Likewise, the growing cells

of *Flavobacterium* sp. strain DS5 (NRRL B-14859) convert oleic acid to 90% keto acid and 10% hydroxy acid, whereas the rest-cell suspension of strain DS5 produces 75% keto acid and 25% hydroxy acid. Moreover, the crude extract obtained from ultrasonically disrupted suspension cells of strain DS5 converts oleic acid to 97% hydroxy acid and 3% keto acid [99]. These results suggest that oleic acid is converted first to 10-HSA and, subsequently, to 10-KSA (Fig. 1). Therefore, cell-free extracts of aerobic NRRL B-14797 cultures, which convert oleic acid to 10-HSA exclusively, may be devoid of secondary alcohol dehydrogenase activity [93]. *Flavobacterium* sp. 12-4A and the *B. sphaericus*-like organism, which produce only 10-KSA from oleic acid conversions, are likely to possess high activities of secondary alcohol dehydrogenase for converting 10-HSA to 10-KSA.

Other bioconversion reactions may also occur at the middle of C—C chains of fatty acids. For example, the cytochrome P-450-dependent enzyme system from *B. megaterium* [102] acts much like the ω-hydroxylation system of *P. oleovorans* to epoxidize palmitoleic acid, forming a 9,10-epoxypalmitate. *Alcaligenes* sp. 5-18 is a bacterium tolerant of high concentrations of oleic acid [23]. It transforms oleic acid to produce dienoic acids of C_{14}–C_{18} in addition to 3-hydroxyoleic acid and 3-hydroxyhexadecenoic acid. These compounds are presumably intermediates in the β-oxidation pathway of oleic acid. Also, 9,12-hexadecadienoic acid (16:2ω4) can be produced from palmitoleic acid by *Trichoderma* sp. AM076, presumably by a Δ^{12} desaturating enzyme [103]. Strains of *Eubacterium lentum* carry out extensive cis–trans isomerization reactions on the unsaturated long-chain fatty acids and the reaction is inhibited by sulfhydryl reagents and metal chelators [104]. During the conversion of oleic acid by *Pseudomonas* sp. strain 42A2 [105,106] and strain PR3 (NRRL B-18602) [107], cis–trans isomerization of the substrate also occurs to produce 10-hydroxy-8(*E*)-octadecenoic acid in the fermentation culture. A few monohydroxy unsaturated fatty acids found in the stromata of *Epichlöe typhina* [108] and those isolated from leaves of wild rice [109] are known to be antifungal substances. Isoricinoleic acid [9-hydroxy-12(*Z*)-octadecenoic acid] is the major fatty acid of *Wrightia* oil and its derivatives possess antifungal activity [110]. Sulfonation of isoricinoleic acid produces a useful anionic surfactant [111].

Ricinoleic acid, the major fatty acid component of castor oil, is a versatile industrial raw material used in coatings, lubricants, hydralic fluid, plastics, pharmaceuticals, food additives, and detergents [112–114]. It is reported [4] that the U.S. military stockpiles

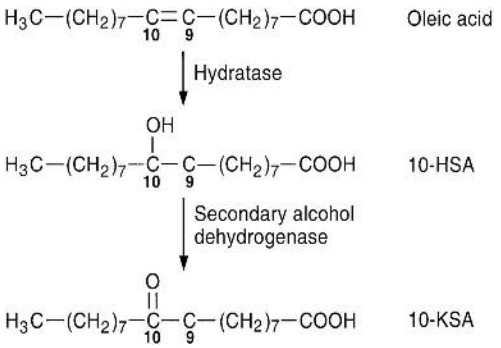


Figure 1 The postulated conversion pathway of oleic acid to 10-hydroxystearic acid and 10-ketostearic acid. (From Refs. 90 and 99.)

castor oil as an imported product necessary in strategic defense materials. Several strains of undesignated soil bacteria are also reported to convert oleic acid to ricinoleic acid [115]. To date, however, a direct microbial hydroxylation in the mid-carbon atoms of a long-chain unsaturated fatty acid, producing ricinoleic-acid-like compounds, has yet to be confirmed. Recently, the gene encoding oleoyl-12-hydroxylase for producing ricinoleic acid in castor beans has been cloned and expressed in transgenic plants, although the yields of hydroxy products are relatively low at present [2,3]. It is conceivable that the gene can be cloned into a bacterium for the commercial production of ricinoleic acid.

6 MULTIHYDROXY FATTY ACIDS

As mentioned earlier, a cytochrome P-450 system from *B. megaterium* catalyzes both an epoxidation and a hydroxylation of monounsaturated fatty acids, epoxidizing such unsaturated fatty acids as palmitoleic acid to give 9,10-epoxypalmitate [102]. The epoxy compound can be reduced further by enzymatic hydration to produce a 9,10-dihydroxypalmitate [116]. A strain of *Pseudomonas* sp. also converts 9,10-epoxystearic acid to 9,10-dihydroxystearic acid [83], a product that can accumulate to some 60% of the total lipid fraction of *Claviceps sulcata* sclerotia [117]. Oleic acid may also be hydroxylated to 7-hydroxy-16-oxo-9(*Z*)-octadecenoic acid and 7-hydroxy-17-oxo-9(*Z*)-octadecenoic acid by a *Bacillus* strain NRRL BD-447 [118,119]. A yeast closely related to *Rhodotorula* (*Torulopsis*) *fujisanensis* (NRRL YB-2501) produces still other extracellular lipids that are analyzed to be comprising 8,9,13-trihydroxy-docosanoic acid and 8,9-dihydroxy-13-oxodocosanoic acid [120]. Moveover, bioconversions mediated by *Pseudomonas* sp. and *Clavibacter* sp. ALA2 in order to produce multihydroxy compounds are characterized extensively [121–124].

6.1 Fatty Acid Conversion by *Pseudomonas* sp.

Pseudomonas sp. strain 42A2 isolated from an oil-contaminated water sample produces a dihydroxy unsaturated fatty acid using olive oil as the sole carbon source [121]. The compound is 7,10-dihydroxy-8(*E*)-octadecenoic acid with a cultural conversion yield of 20% [125]. Another *Pseudomonas* strain 44T1 isolated from the same source produces two glycolipids in addition to the dihydroxy compound [125]. Strain 42A2 produces the dihydroxy acid during the logarithmic phase and ceases production at the beginning of the stational phase; thus, it is a growth-linked process [126]. The fermentation culture also forms 10-hydroperoxy-8(*E*)-octadecenoic acid and 10-hydroxy-8(*E*)-octadecenoic acid [105]. The bioconversions reach maximum yields at 24 hr for both monohydroxy and dihydroxy compounds and at 18 hr for the hydroperoxide compound [105]. It is thus postulated that the oxidation of oleic acid substrate at C-10 and cis–trans isomerization leading to a hydroperoxide intermediate is the initial stage to 7,10-dihydroxy-8(*E*)-octadecenoic acid formation [105]. The lipoxygenase-like enzyme responsible for this initial bioconversion is located in a periplastic fraction [105].

Pseudomonas aeruginosa PR3 (NRRL B-18602) is effective in the conversion of unsaturated fatty acids to multihydroxy analogs [127]. Strain PR3 was isolated from a water sample on a pig farm at Morton, Illinois [122]. DNA thermal melting and reassociation measurements were used to identify the strain as *P. aeruginosa* [128]. Glycerol and dextrose as carbon sources support its maximal growth in the screening medium (SM) at pH 7, but cultures grown in glycerol fail to convert oleic acid to 7,10-dihydroxy-8(*E*)-

octadecenoic acid (DOD) [122]. The optimal production of DOD (a yield of 63%) is 48 hr after addition of oleic acid at 30°C and 200 rpm aeration rate [122]. Further incubation periods lead to reduction in DOD yields, indicating that strain PR3 metabolizes DOD. Also, the bioconversion is sensitive to composition of the culture medium. Recently, the stabilization and the yield of DOD production have been improved from 60% [122] to 89% [124]. Replacing 10 g/L $(NH_4)_2HPO_4$ in SM with 2 g/L $(NH_4)_2HPO_4$ and 1 g/L NH_4NO_3, and excluding $MgSO_4$ from the medium, the DOD production is increased from 7% to 89%. On the other hand, when $MnSO_4$ is excluded from the optimal medium, the yield decreases to about 7% [124]. Therefore, Mn^{2+} is required for the conversion of oleic acid to DOD. Moreover, strain PR3 does not accumulate a hydroperoxide intermediate in the fermentation culture, as does strain 42A2 [105]. Our results are consistent with the DOD formation that involves an intermediate 10(*S*)-hydroxy-8(*E*)-octadecenoic acid (HOD) [107] (Fig. 2).

Pseudomonas aeruginosa PR3 converts ricinoleic acid to a novel compound, 7,10,12-trihydroxy-8(*E*)-octadecenoic acid (TOD) [124]. The yield of TOD production was always higher in cultures grown on a rich Wallen fermentation (WF) medium rather than a minimal SM [129] (Table 1). Extending the conversion time from 48 to 72 hr prior to lipid extraction leads to a 65% reduction in yield, indicating that TOD is metabolized further by strain PR3 and that the reaction time is important for achieving optimal yield of TOD. The optimum culture density, substrate reaction time, pH, temperature, and substrate concentration for TOD production are found to be 20–24 hr cultural growth, 48 hr substrate reaction, pH 7.0, 25°C incubation, and 1% substrate (v/v), respectively. Under the optimal conditions, the TOD yield is greater than 45%. Also, the bioconversion pathway is consistent with an intermediate 10,12-dihydroxy-8(*E*)-octadecenoic acid (DHOD) [130]. Thus, the mechanism of TOD formation from ricinoleic acid is the same as that of DOD formation from oleic acid [107], involving one hydration, one hydroxylation, and a double-bond rearrangement of the substrate molecule (Fig. 2). Strain PR3 also converts linoleic acid to isomeric compounds identified as 9,10,13(9,12,13)-trihydroxy-11*E*(10*E*)-octadecenoic acid [131].

6.2 Linoleic Acid Conversion by *Clavibacter* sp.

Clavibacter sp. ALA2, a gram-positive, nonmotile, and rod-shaped bacterium isolated from a dry soil sample at McCalla of Alabama, converts linoleic acid to a novel compound, 12,13,17-trihydroxy-9(*Z*)-octadecenoic acid (THOA) [123]. Production of THOA reaches a maximum level with a 25% yield after 85 hr and maintains this level as the reaction extends to 160 hr [132]. This indicates that strain ALA2 does not metabolize THOA further. Additionally, strain ALA2 converts linoleic acid to produce two minor hydroxy unsaturated fatty compounds, 12-(5-ethyl-2-tetrahydrofuranyl)-12-hydroxy-9(*Z*)-dodecenoic acid and 12-(5-ethyl-2-tetrahydrofuranyl)-7,12-dihydroxy-9(*Z*)-dodecenoic acid [133].

6.3 Potential Uses of Multihydroxy Fatty Acids

The dihydroxy unsaturated fatty acid, 7,10-dihydroxy-8(*E*)-octadecenoic acid produced from oleic acid conversions by *Pseudomonas* sp. 42A2 [125] and by *P. aeruginosa* PR3 [123], can lower surface tension [121,134]. Along with other chemically synthesized allylic monohydroxy and dihydroxy fatty acids, these compounds may be suitable for usage in microemulsion or additive of various commercial products [135]. Besides its surface-

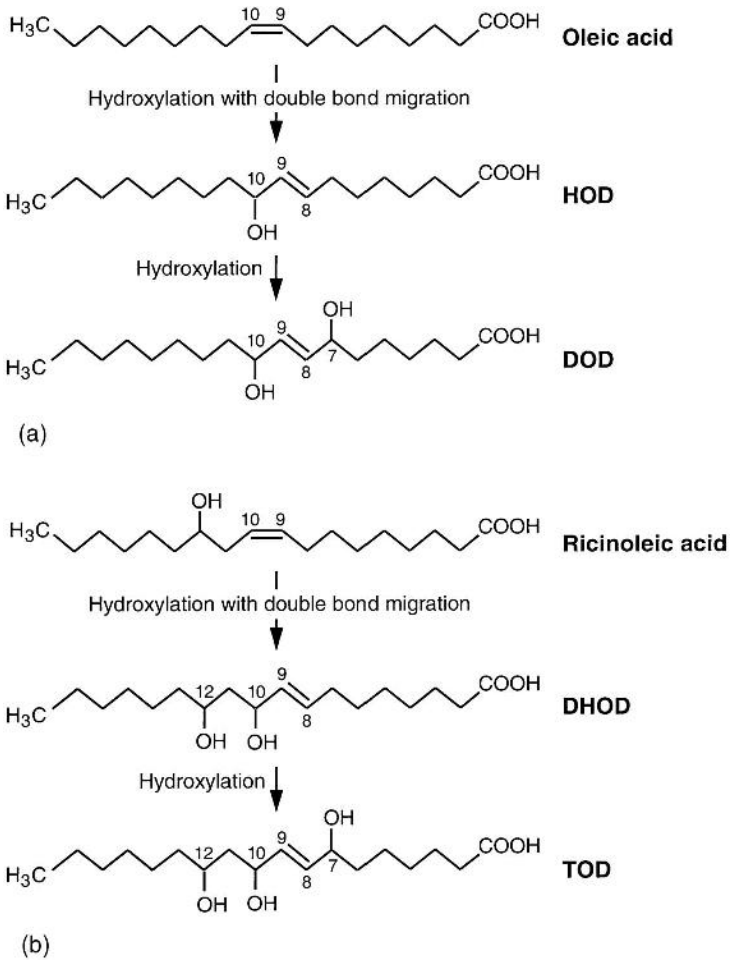


Figure 2 The postulated conversion pathway of (a) oleic acid and (b) ricinoleic acid by *P. aeruginosa* strain PR3 (NRRL B-18602) [107,130]. In the oleic acid conversion by *Pseudomonas* sp. strain 42A2, a hydroperoxy monounsaturated intermediate may also be present [105]. HOD, 10(*S*)-hydroxy-8(*E*)-octadecenoic acid, an intermediate for the formation of DOD; DOD, 7,10-dihydroxy-8(*E*)-octadecenoic acid; DHOD, 10,12-dihydroxy-8(*E*)-octadecenoic acid; TOD, 7,10,12-trihydroxy-8(*E*)-octadecenoic acid.

active property, the dihydroxy unsaturated fatty acid also possesses some antimicrobial activity against *Bacillus subtilis* and a common pathogen, *Candida albicans* [127,136].

Additionally, many oxygenated metabolites of unsaturated fatty acids exhibit a variety of important roles in biological systems. Enzymatic conversion of lipid hydroperoxides to multihydroxy fatty acids is well documented in higher plants [137]. Many of these oxygenated fatty acids are involved in plant defense against pathogen invasion [138]. Yeast produces 8,9,13-trihydroxy docosaenoate as an extracellular lipid component [139]. Beer contains 9,10,13-trihydroxy-10(*E*)- and 9,12,13-trihydroxy-11(*E*)-octadecenoic acids [140] that may be produced from linoleic acid conversions during the barley malting process [141]. Rice plants infected with blast disease also contain trihydroxy unsaturated

Table 1 Production of DOD and TOD by *Pseudomonas aeruginosa* PR3 in Different Culture Media

	DOD		TOD	
Medium	mg	% Yield	mg	% Yield
WF	188	63	44	22
SM6	260	87	27	14
SM7	79	26	0	0

Abbreviations: DOD, 7,10-dihydroxy-8(*E*)-octadecenoic acid; TOD, 7,10,12-trihydroxy-8(*E*)-octadecenoic acid; WF, Wallen fermentation medium; SM, screening medium. WF contains the following (g/L): 4 glucose, 5 yeast extract, 4 K_2HPO_4, 0.5 $MgSO_4 \cdot 7H_2O$, and 0.015 $FeSO_4 \cdot 7H_2O$, pH 7.3. SM6 contains the following (g/L): 4 glucose, 1 yeast extract, 1 $(NH_4)_2HPO_4$, 4 K_2HPO_4, 0.1 $MgSO_4 \cdot 7H_2O$, 0.02 $FeSO_4 \cdot 7H_2O$, and 0.02 $MnSO_4 \cdot H_2O$, pH 7.2. SM7 contains the following (g/L): 2 glucose, 1 yeast extract, 1 NH_4NO_3, 6 K_2HPO_4, 0.1 $MgSO_47H_2O$, 0.02 $FeSO_4 \cdot 7H_2O$, and 0.02 $MnSO_4 \cdot H_2O$, pH 7.2. Reactions were done at 28°C, 200 rpm for 2 days using 300 mg oleic acid and 200 mg ricinoleic acid in 30 mL medium for the production of DOD and TOD, respectively.

Note: Values were estimated according to GC responses and the amount of internal standard, $C_{16:0}$, added to each sample prior to lipid extraction. Each figure is the average of at least two separate experiments, each with duplicate runs.

Source: Ref. 129.

fatty acids, 9(*S*),12(*S*),13(*S*)-trihydroxy-10(*E*)-octadecenoic acid and 9(*S*),12(*S*),13(*S*)-trihydroxy-10(*E*),15(*Z*)-octadecadienoic acid, which inhibit the elongation of conidial germ tubes of the rice blast fungus [142]. 9,12,13-Trihydroxy-10(*E*)-octadecenoic acid isolated from taro (*Colocasia antiquorum*) that is infected with black rot fungus (*Ceratocystis fimbriata*) possesses antifungal activity [143]. 12,13,17-Trihydroxy-9(*Z*)-octadecenoic acid (THOA) [123], the trihydroxyfatty acid discussed previously, also exhibits antimicrobial activity [136] (Table 2). TOD, as discussed earlier, has a structure similar to that of known plant-derived defense substances and THOA. TOD is also an antifungal agent most active against the rice blast fungus, which causes disease affecting rice production worldwide (Table 2).

6.4 Relationship with Phenazine 1-Carboxylic Acid Accumulation

Phenazine 1-carboxylic acid (PCA) is an antibiotic (tubermycin B) against a variety of microorganisms and is a fungicide which inhibits the growth of several wheat pathgens [144]. *Pseudomonas fluorescens* strain 2-79 (NRRL B-15132), originally isolated from a wheat field in spontaneous ''take-all'' decline, produces PCA [145,146]. The phenomenum of ''take-all'' decline is a natural resistance response of crop plants to ''take-all'' disease after the same crop has been grown in a field for 4–6 years. Physiological and nutritional requirements of *P. fluorescens* strain 2-79 for regulating accumulation of PCA and considerations for mass production of the microorganism as a biocontrol agent have been reviewed recently [147]. Although it is unknown whether vegetable oils or fatty acids can affect PCA accumulation with strain 2-79, it is suggested that the bioconversion of fatty acids is related to PCA accumulation in the *Pseudomonas* sp. fermentation broth.

Table 2 Test Results of THOA and TOD for Growth Inhibition of Pathogenic Fungi That Cause Plant Diseases

	Percent growth inhibition	
Plant disease (fungus)	THOA at 200 ppm	TOD at 5 ppm
Cucumber botrytis (*Botrytis cinerea*)	63	ND
Peach blossom blight (unknown fungal sp.)	ND[a]	20
Potato late blight (*Phytophthora infestans*)	56	1
Rice blast (*Pyricularia grisea*)	0	29
Rice sheath blight (*Rhizoctonia solani*)	0	21
Wheat foot rot (*Pseudocercosporella herpotrichoides*)	0	0
Wheat glume blotch (*Septoria nodorum*)	0	0
Wheat leaf rust (*Puccinia recondita*)	86	ND
Wheat powdery mildew (*Erisyphe graminis*)	77	ND

Note: The results are adapted from Ref. 136 for 12,13,17-trihydroxy-9(*Z*)-octadecenoic acid (THOA) and from Ref. 129 for 7,10,12-trihydroxy-8(*E*)-octadecenoic acid (TOD).
[a] ND: not determined.

During the conversion of oleic acid to DOD by *P. aeruginosa* strain PR3, a yellowish compound is also formed and identified as PCA [128]. Strain PR3 produces more PCA as conversion of oleic acid to DOD decreases [128]. On the other hand, several fatty acids (e.g., 12-hydroxystearic acid, ricinoleic acid, and oleic acid) can profoundly enhance the production of PCA by *P. aeruginosa* strain 2HS [144]. Strain 2HS, isolated from sheep manure obtained from a farm in Macomb, Illinois, U.S.A., also converts oleic acid to DOD [144]. The mechanism for the formation of PCA by *Pseudomonas* sp., however, is not understood.

7 FATTY AMIDES

Such long-chain fatty amides as erucamide [13(*Z*)-docosenamide] can be produced for industrial applications [148] from plant-produced erucic acid (e.g., crambe seed or industrial rapeseed oil) treated with ammonia. These compounds are used for nonsticking plastic films, lubricants, and protective coatings [148,149]. In addition, some unique medical properties include an angiogenic (stimulation of blood-vessel formation) activity in omental and adipose tissues [150] and the sleep-inducing factor of *cis*-9,10-octadecenoamide and *cis*-13,14-docosenoamide [151].

Microbial transformations by *Bacillus* spp. indicate tightly coupled cellular syntheses of fatty amides, thus leading to less efficient conversion rates of 5–20%. *B. megaterium* (NRRL B-3437) [152] and *B. cereus* (NRRL B-14812) [94] produce 9(*Z*)-octadecenamide and other fatty amides from oleate substrate. *B. cereus* strain 50 produces 12-hydroxyoctadecanamide in a 21.5% yield from 12-hydroxyoctadecanoic acid [153].

8 WAX ESTERS

Jajoba (*Simmondsia californica*) seed oil has become an industrial standard for liquid wax production and a proven substitute for sperm whale oil [154]. The wax esters from jojoba consisting of monounsaturated fatty acids and their alcohols contain an esterified mixture

with an even number of C atoms from C_{34} to C_{48} [155]. The wax ester product is used in such diverse formulations as lubricants, cosmetics, solid wax coatings, and biofuel additives. As an alternative, designed wax esters can be produced by microbial fermentation using specific C_8–C_{18} alcohols and medium to long-chain C_{12}–C_{18} fatty acids [155].

A proper choice of microbial strain and fermentation conditions seems critical for efficient transformation of fatty acids and extracellular production of wax esters. For example, a *de novo* synthesis by *Euglena gracilis* (ATCC 12716) yields less wax esters with predominantly saturated C_{14} fatty acid and saturated C_{14} alcohols [156]. *Acinetobacter* sp. H01-N (ATCC 14987) is demonstrated to produce a C_{32}–C_{40} mixture of saturated and unsaturated waxes from C_{16} to C_{20} *n*-alkanes as substrates [157]. Analogous to triacylglycerols stored in yeasts, an *Acinetobacter* (NRRL B-14920) culture either stores or accumulates large amounts of oleyl oleate (C_{36}) liquid ester when exogenous oleic acid and oleyl alcohol are used as substrates [155].

In addition, *Acinetobacter calcoaceticus* RAG-1 (ATCC 31012) has been utilized in the biodegradation of oil pollutants and found to elicit an extracellular emulsifier patented as emulsan [158]. It is noteworthy that *Corynebacterium, Moraxella*, and other *Acinetobacter* strains [159–161] grown in broths produce de novo lesser amounts of wax ester with C_{24} to C_{42} chain lengths. Under low-temperature growth conditions, however, *Moraxella atlantae* and *M. osloensis* [159] produce long-chained ($>C_{40}$) wax esters. *Micrococcus cryophilus* [162] and numerous *Acinetobacter* spp. [163–165] have been utilized also for biochemical and genetic studies related to wax ester synthesis.

Unlike seed oils, liquid wax esters produced by *Acinetobacter* (strain NRRL B-14920) cultures may be easily varied according to fatty alcohol [155] added together with a fatty acid substrate. Apparently, oleate is readily metabolized to C_{16} and C_{14} fatty acids, whereas the more stable oleyl alcohol component may be designed to control the C length and saturation of wax esters. With oleyl alcohol and triacylglycerols from soybean oil, our best 100-mL *Acinetobacter* culture yields approximately 260 mg wax esters, starting with approximately 800 mg of combined fatty alcohol and acid substrates. However, fermentation and optimal conditions on larger batches are yet to be assessed.

8.1 Biosynthesis and Gene Analysis of Wax Ester Formation

Biosynthesis of wax esters has been extensively documented with *A. calcoaceticus* and other *Acinetobacter* spp. With *A. calcoaceticus* 69V grown on hexadecane [163], lipopolysaccharide-rich vesicles are formed that contain an enriched source of phosphatidylethanolamine and are susceptible to exogenous phospholipase. Psychrophilic *Acinetobacter* spp. [165] grown on a complex broth medium produce extracellular lipase as well as cell-bound and extracellular esterases. The lipase formation is affected by nutrient media and the addition of taurocholate emulsifier. Another psychrophile, *M. cryophilus* [162] is suggested for eliciting an acyl-CoA : alcohol transacylase enzyme for wax ester formation. Accordingly, a long-chain fatty acid substrate may be both reduced by a $NADP^+$-linked alcohol dehydrogenase [161] to the corresponding alcohol component and converted to an acyl-CoA component for biosynthesis of the wax ester. A third esterification enzyme system, an acyl-CoA : alcohol transferase (transacylase), is required to complete synthesis of the wax esters. Furthermore, the multiplicity of cellular alcohol dehydrogenases, aldehyde dehydrogenases, and β-oxidized fatty acids [155] prevents a uniform cellular biosynthesis by which a singular wax ester with the same number of C atoms (e.g., 100% oleyl oleate) is produced (Fig. 3).

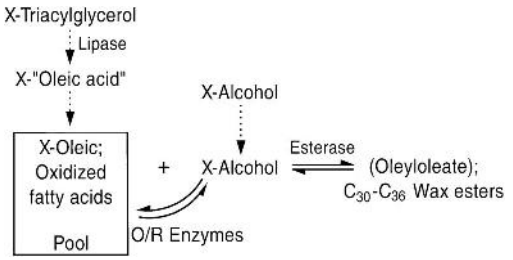


Figure 3 Biosynthetic pathways of wax ester formation by *Acinetobacter* spp. (From Ref. 155.)

It is noteworthy that when oleic acid and hexadecanol are emulsified with gum arabic polysaccharide, both animal and *P. fluorescens* lipases favor wax ester over free-acid formation by a 9:1 equilibrium ratio [166]. It is suggested that wax esters are synthesized with lipases from either free fatty acids or through degradation of triacylglycerols. Perhaps, the lipase system can be considered to have a secondary, extracellular transesterification activity when fatty substrates and lipase are emulsified at a favorable pH.

Acinetobacter genes encoding for esterase and alcohol dehydrogenase [158] have been analyzed in relation to emulsan biosynthesis. Notably, a RAG-1 strain extracellular esterase gene is expressed in an *Escherichia coli* host as demonstrated by growth on a triacetin substrate. Genetic analysis [164] of *A. calcoaceticus* BD413 that produces both extracellular lipase and cell-bound esterase activities indicates at least two esterases from the incomplete esterase deletions observed with deleted *estA* gene products. However, both esterases appear to be expressed under the same regulatory mechanism and produced at the end of exponential growth in batch cultures.

9 SPHINGOLIPIDS AND SPHINGOIDS

Sphingolipid bases (18 C atoms) share a common molecular terminus of —CHOH—CHN-H_2—CH_2OH. Generally, various yeasts possess phytosphinganine (phytosphingosine) and sphinganine (dihydrosphingosine) bases as a constituent part of intracellular lipids. However, an exception is *Pichia* (*Hansenula*) *ciferrii* NRRL Y-1031, isolated and characterized by Maister et al. [167], which is observed to secrete fully acetylated tetraacetylphytosphinganine and triacetylsphinganine (yields of 300–500 mg/L culture) in 600-gal fermentors. A more recent study [168] of small-batch NRRL Y-1031 cultures (100–500 mL range), maintained as part of a lyophilized stock collection, gives significantly less amounts of sphingolipid even when determined as total *N*-acetylsphingolipid bases (10–30 mg/L culture). In NRRL Y-1031 cultures treated with a related sphingoid inhibitor fumonisin B_1, however, the extractable sphingolipids from a particulate–cellular fraction increase 15–30-fold.

Fumonisin B_1 (—CHOH—CH_2—CHOH—$CHNH_2$—CH_3) and AAL toxin (—CHO-H—CHOH—CH_2—CHOH—CH_2NH_2) are fungus-produced toxins [169,170] that have sphingoid (sphingolipidlike) bases [171]. In addition to secondary alcohol and CH_3 groups, these fungal toxins have tricarballylic substituents (propane-1,2,3-tricarboxylic acid) esterified to the sphingoid bases [171]. Sphingoid compounds, which are demonstrated to be metabolic inhibitors, are toxic and carcinogenic for animals [172,173]. As a mycotoxin, fumonisin induces a dramatic equine leucoencephalomalacia [171]. Sphingoids are toxic

to tomato [169], jimsonweed [174], and duckweed [170] plant cultures. Parboiled-rice cultures of *Fusarium moniliforme* and *F. proliferatum* have been shown to produce up to 15,000 ppm and 6100 ppm of fumonisin B_1, respectively [170].

Another category of sphingolipid bases (—CHOH—$CHNH_2$—CH_2OH) biosynthesized by *Sphingobacterium* spp. [175] and *Sphingomonas paucimobilis* (syn. *Pseudomonas paucimobilis* and *Flavobactrium devorans* [16,176] contains both sphinganine (*n*-alkane) and *iso* hydrocarbon groups [CH_3—$CH(CH_3)$—CH_2—] at the ω ends. Because cultural production appears coupled rigidly to cellular growth, however, the sphingolipid is limited in yield and is bound to acetone-dried cellular constituents [93,175]. Thus, *Sphingomonas* (*Flavobacterium* ATCC 10829 and NRRL B-54) yields a ceramide glucuronic acid—more specifically, an *N*(2-hydroxymyristyl) dihydrosphingosine 1-glucuronic acid cellular constituent [176].

9.1 Biosynthesis of Sphingolipid Bases

Earlier investigations with *P. ciferrii* extracts as discussed in Ref. 168, indicate that a condensation reaction of palmityl CoA and serine is crucial for the synthesis of sphinganine and phytosphinganine bases. In addition, other intermediate metabolites have been found in *P. ciferrii* extracts. Cellular cultures of *P. ciferrii*, however, may be induced to accumulate sphingolipids by a fumonisin B_1 inhibitor [168]. More recent studies on *Sphingobacterium* [16,175,176] suggest a fresh alternative for explaining sphingolipid metabolism and their functions in cells.

Cellular particles of *Sphingobacterium* and *Sphingomonas* genera [175,176] are found to possess both 1,3-dihydroxy-2-amino-15-methylhexadecane (*iso*-C17 sphinganine) and sphinganine as major base products. The sphingolipids found in sphingobacterial cells are extractable as ceramides [176]. Their study [16] has shown further that various α-hydroxy long-chain fatty acids of ceramides are synthesized by a cytochrome P-450 enzymatic reaction. However, inhibitory effect of fumonisin B_1 has not been explored with these sphingobacteria cultures.

9.2 Mechanism of Fumonisin B_1 Inhibition

Sphingolipid metabolism in plants [169,174] and animals [172,173,177] is inhibited by fumonisin B_1. From animal feedstuff [172,177,178], to various cellular tissues [173], and to microsomal proteins [179,180], fumonisin B_1 appears to inhibit a ceramide synthetase activity uniformly. It is noteworthy that Riley et al. [178] and Wang et al. [177] have analyzed changes of the sphinganine : sphingenine ratio for animals affected by fumonisin toxicity. Results indicate an abnormal accumulation of sphinganine base that is caused by the inhibition of a ceramide synthetase activity [173,178]. Further, it is demonstrable that a synthetic sphingoid base containing an *N*-palmitoyl-aminopentol structure [180] is a more potent inhibitor than fumonisin B_1. A fortuitous effect related to fumonisin inhibitions reveals the presence of a desaturase enzyme leading to synthesis of sphingenine from a sphinganine-containing ceramide substrate. Also, fumonisin B_1 appears to inhibit effectively a serine/threonine phosphatase reaction [181].

10 FURANS AND LACTONES

The microbial conversion of lipids and fatty acids leading to various potential flavor and fragrance products and γ- and δ-lactones as aroma compounds have been discussed

[182,183]. Additionally, the fungus *Cephalosporium recifei* NRRL 5161, when grown on glucose solution, produces a 12-membered lactone, 11-hydroxy-8(*E*)-dodecenoic acid, and δ-lactone of 3,5-dihydroxydecanoic acid [184,185]. Two strains of *Penicillium roquefortii* originally isolated from French blue cheese convert fatty acids from soybean and copra oils to γ-lactones of 4-dodecanolide, *cis*-6-dodecen-4-olide, and 4-hexanolide that possess a peach odor [186]. Conversion of oleic acid to optically active γ-dodecalactone can be achieved in two steps: (1) producing (*R*)10-HSA by a gram-positive, rod-shaped soil bacterium and (2) converting the hydroxy acid to the γ-lactone by baker's yeast [187]. A batch-fed culture technique with supplemental feeding of castor hydrolysate that contains approximately 83.4% ricinoleic acid greatly enhances the production of γ-decalactone by *Sporobolomyces odorus* strain AHU3246 [188]. *Sporidiobolus ruinenii* is better suited than *Sporidiobolus salmonicolor* to give an efficient industrial process for γ-decalactone production from ricinoleic acid. *S. ruinenii* is more tolerant to the toxicity of γ-decalactone and can produce both lactone and its precursor, 4-hydroxydecanoic acid [189].

Both *Corynebacterium* sp. FUI-2 and *Bacillus lentus* 5d2 (NRRL B-14864) convert 12-HSA to a major compound, 5-*n*-hexyl-tetrahydrofuran-2-acetic acid (5-HTFA), and other minor intermediate compounds, 6-hydroxydodecanoic acid, 4-hydroxydecanoic acid, 4-ketodecanoic acid, and γ-decalactone with a 43% yield for 5-HTFA and 5% for γ-decalactone [190]. 5-HTFA shows juvenile hormone activity on 3-day-old dried fruit beetle (*Carprophilus hemipterus*) pupae when tested in pure form but not in a 10% concentration [191]. Both microorganisms also convert various saturated monohydroxyfatty acids to γ-lactones and tetrahydrofurans but are inactive with monounsaturated and monohydroxyfatty acids [191]. Enzyme inhibitors, 2-bromooctanoic acid, sodium azide, and oligomycin inhibit the production of 5-HTFA from 12-HSA by strain NRRL B-14864, indicating that the bioconversion is mediated most likely through a fatty acid β-oxidation pathway [191].

Table 3 Selective Enrichment of Compost Bacterial Isolates Capable of Fatty Acid Conversions

Compost source/ Selective fatty acid	Bacterial isolates	Reaction products
Fresh compost		
Oleic acid	*Sphigobacterium thalpophilum*, *Acinetobacter* spp.	10-Ketostearic acid (10-KSA); 10-hydroxystearic acid (10-HSA)
10-KSA	*Enterobacter cloacae*	6-Ketotetradecanoate; 4-ketododecanoate
Ricinoleic acid	*E. cloacae; Escherichia* sp., *Pseudomonas aeruginosa*	12-C and 14-C homologs of ricinoleic acid
	P. aeruginosa	7,10,12-Trihydroxyoctadecenoate
Linoleic acid	*Enterobacter agglomerans*	Hexadecadienoate
Linoleic acid with hexane	*Pseudomonas putida*, *P. viridilivida*: *Serratia plymuthica*	14-C and 16-C homologs of linoleic acid
Commercial compost		
Oleic acid	*S. thalpophilum*, *Staphylococcus* sp., *Flavobacterium gleum*	10-HSA; 10-KSA
	Bacillus cereus	Octadecenamide
	Acinetobacter spp., coryneform	Oleyl wax esters

Source: Refs. 94 and 196.

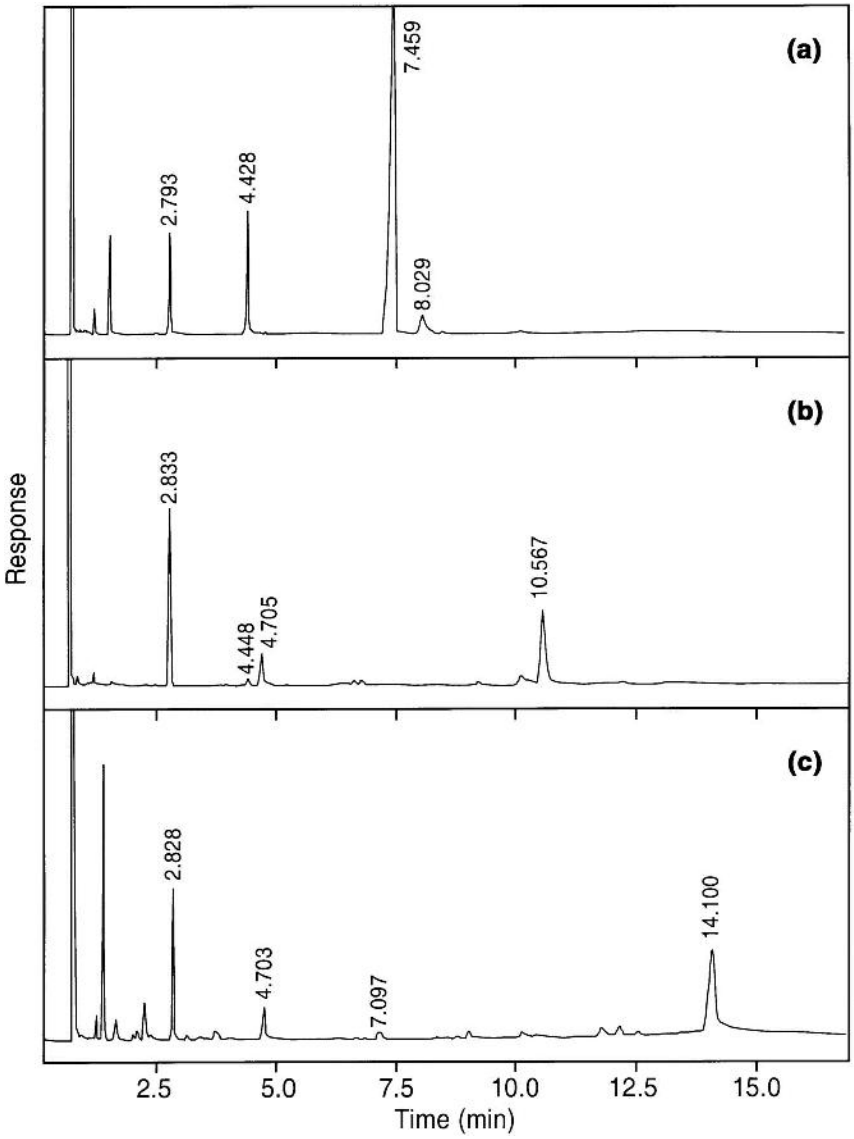


Figure 4 Gas chromatograms of methyl esters recovered after conversion of fatty acids by reactive microbes isolated from composted material: (a) Oleic acid to 10-hydroxystearic acid and 10-ketostearic acid by *S. thapophilium* O22 (NRRL B-23210) [101], (b) Oleic acid to 7,10-dihydroxy-8(*E*)-octadecenoic acid (DOD) by *P. aeruginosa* Rn30 (NRRL B-23260) [Kuo, unpublished results], and (c) ricinoleic acid to 7,10,12-trihydroxy-8(*E*)-octadecenoic acid (TOD) by *P. aeruginosa* Rn30 [196]. GC peaks are palmitic acid internal standard (RT = 2.79–2.83 min), oleic acid (4.43–4.45 min), ricinoleic acid (7.10 min), 10-ketostearic acid (7.46 min), 10-hydroxystearic acid (8.03 min), DOD (10.57 min), and TOD (14.10 min); the 4.70-min GC peak is presumably phenazine 1-carboxylic acid.

11 ENRICHMENT CULTURE SELECTION

Extensive screenings of microorganisms in soil and water samples have led to the identification of *Pseudomonas* sp. strains that can convert oleic acid to a novel dihydroxyoctadecenoic acid [121,122,144] and a *Clavibacter* sp. that converts linoleic acid to a 12,13,17-trihydroxyoctadecenoic acid [123]. A survey of microorganisms from our ARS Culture Collection (NRRL) have yielded several strains that convert oleic acid or linoleic acid to mono-oxygenated and dioxygenated fatty acids [48,79,85,119]. Bioremediation studies have demonstrated that microbial enrichments in soils follow a predictable isolation of pattern: *Arthrobacter* strain KCC201 from degrading crude petroleum [192]. *P. aeruginosa* from gasoline waste [193], *Alcaligenes eutrophus* and *Burkholderia* sp. from decomposing 2,4-dichlorophenoxyacetate [194], and *Sphingomonas* sp. RA2 from decomposing pentachlorophenol [195]. Analogously, an enrichment culture procedure that promotes microbial proliferation in a selective medium can yield reactive microbes that can degrade or convert such selective agents as specific unsaturated fatty acids (UFAs). Microorganisms from composted manure can be employed to carry out effective conversion reactions of UFA into value-added products [94,196]. Such enrichment cultures involved in fatty acid biotransformations have been described in a review [197]. When different fatty acids are used as selective agents, a specific type of bacteria appears to transform UFAs, involving decarboxylation, hydroxylation, dehydrogenation, cis–trans isomerization, and hydroperoxidation reactions (Table 3). Figure 4 shows GC analyses of a few conversion products of UFAs when incubated with compost bacteria. Notably, using oleic acid as a selective fatty acid appears to direct compost cultures toward an enrichment of *S. thalpophilum* strains [93,198]. These strains display a wide spectrum of hydration and dehydrogenation activity upon conversion of oleic acid to produce predominantly 10-KSA or 10-HSA (Table 4).

12 CONCLUDING REMARKS

Microbial conversions of vegetable oil and component fatty acids have yielded diverse products that are useful potentially as value-added food and nonfood items. A number of potential new products biosynthesized by microbial enzymic actions are mentioned and discussed herein: α-, β-, and ω-hydroxy fatty acids, polyhydroxyalkanoates (PHAs), dicar-

Table 4 Conversion of Oleic Acid to 10-Ketostearic Acid and 10-Hydroxystearic Acid by *Sphingobacterium thalpophilum* Strains Isolated from Compost Cultures

Group	Isolate/NRRL No.	10-KSA (% Total product)	10-HSA (% Total product)	Total product (mg)	Yield (%)
I	O6/B-23206	94	6	126	49
	O18/B-23208	93	7	124	48
II	O21/B-23209	93	7	190	73
	O22/B-23210	94	6	197	76
	O27/B-23211	94	6	185	71
III	O42/B-23212	87	13	80	31
IV	142b/B-14797	0	100	180	69

Source: Refs. 93 and 198.

boxylic acids (DCs), monohydroxy-saturated and unsaturated fatty acids, dihydroxy-saturated and unsaturated fatty acids, trihydroxy-unsaturated fatty acids, fatty amides, wax esters, sphingolipids, furans, and lactones. Perhaps with the exception of PHAs and DCs, other lipid products often are produced in low yield or conversion rate. To enhance their potential as industrial products, therefore, it is necessary to improve the productivity and efficiency by developing better microbial strains and more efficient fermentation processes. For example, reactive strains may become more efficient bioconverters after an improved modification of either their metabolite requirements or bioreactor designs and cell/enzyme immobilization technology.

ACKNOWLEDGMENTS

We thank Ms. Karen Ray for the technical assistance with the manuscript, Mr. Raymond F. Sylvester for preparing the figures, and Dr. Cletus P. Kurtzman for critically reading the manuscript.

REFERENCES

1. CE Stauffer. Cereal Foods World 41:782–786, 1996.
2. FJ van de Loo, P Broun, S Turner, C Somerville. Proc Natl Acad Sci USA 92:6743–6747, 1995.
3. P Broun, C Somerville. Plant Physiol 113:933–942, 1997.
4. MO Bagby, KD Carlson. In: RC Cambie, ed. Fats for the Future. Chichester: Ellis Horwood, 1989, pp 301–317.
5. CP Kurtzman, RF Vesonder, MJ Smiley. Appl Microbiol 26:650–652, 1973.
6. DW Russell. J Chem Soc 1962:753–761, 1962.
7. MM Shemyakin, YA Ovchinnikov, VT Ivanov, AA Kiryushkin. Tetrahedron Lett 28:1927–1932, 1963.
8. MM Shemyakin, EI Vinogradova, MY Feigina, NA Aldanova. Tetrahedron Lett 6:351–356, 1963.
9. RF Vesonder, FH Stodola, WK Rohwedder, DB Scott. Can J Chem 48:1985–1986, 1970.
10. NM Carballeira, F Shalabi, V Negrón. Lipids 24:229–232, 1989.
11. NM Carballeira, F Shalabi, M Reyes. J Nat Products 57:614–619, 1994.
12. I Yano, Y Furukawa, M Kusunose. Biochim Biophys Acta 239:513–516, 1971.
13. I Matsunaga, E Kusunose, I Yano, K Ichihara. Biochem Biophys Res Commun 201:1554–1560, 1994.
14. I Matsunaga, N Yokotani, O Gotoh, E Kusunose, M Yamada, K Ichihara. J Biol Chem 272: 23,592–23,596, 1997.
15. I Matsunaga, M Yamada, E Kusunose, T Miki, K Ichihara. J Biochem 124:105–110, 1998.
16. I Matsunaga, T Sumimoto, A Ueda, E Kusunose, K Ichihara. Lipids 35:365–371, 2000.
17. HH Wasserman, JJ Keggi, JE McKeon. J Am Chem Soc 84:2978–2982, 1962.
18. LC Vining, WA Taber. Can J Chem 40:1579–1584, 1962.
19. DW Thomas. Tetrahedron 25:1985–1990, 1969.
20. RF Vesonder, LJ Wickerham, WK Rohwedder. Can J Chem 46:2628–2629, 1968.
21. S Tahara, Y Suzuki, J Mizutani. Agric Biol Chem 41:1643–1650, 1977.
22. IY Lee, SL Nissen, JPN Rosazza. Appl Environ Microbiol 63:4191–4195, 1997.
23. N Esaki, S Ito, W Blank, K Soda. J Ferment Bioeng 77:148–151, 1994.
24. MS van Dyk, JLF Kock, DJ Coetzee, OPH Augustyn, S Nigam. FEBS Lett 283:195–198, 1991.

25. JLF Kock, DJ van Vuuren, A Botha, MS van Dyk, DJ Coetzee, PJ Botes, N Shaw, J Friend, C Ratledge, AD Roberts, S Nigam. System Appl Microbiol 20:39–49, 1997.
26. P Venter, JLF Kock, GS Kumar, A Botha, DJ Coetzee, PJ Botes, RK Bhatt, JR Falck, T Schewe, S Nigam. Lipids 32:1277–1283, 1997.
27. T Fukui, N Shiomi, Y Doi. J Bacteriol 180:667–673, 1998.
28. LL Wallen, WK Rohwedder. Environ Sci Technol 8:576–579, 1974.
29. Y Poirier, C Nawrath, C Somerville. Bio/Technology 13:142–150, 1995.
30. A Steinbüchel, B Füchtenbusch. Trends Biotechnol 16:419–427, 1998.
31. SY Lee, JI Choi. In: AL Demain, JE Davies, eds. Manual of Industrial Microbiology and Biotechnology. 2nd ed. Washington, DC: American Society for Microbiology, 1999, pp 616–627.
32. G Eggink, P de Waard, GNM Huijberts. Can J Microbiol 41:14–21, 1995.
33. M Akiyama, Y Doi. Biotechnol Lett 15:163–168, 1993.
34. M Kusunose, E Kusunose, MJ Coon. J Biol Chem 239:1374–1380, 1964.
35. SW May, BJ Abbott. J Biol Chem 248:1725–1730, 1973.
36. JA Peterson, D Basu, MJ Coon. J Biol Chem 241:5162–5164, 1966.
37. EJ McKenna, MJ Coon. J Biol Chem 245:3882–3889, 1970.
38. RT Ruettinger, ST Olson, RF Boyer, MJ Coon. Biochem Biophys Res Commun 57:1011–1017, 1974.
39. RT Ruettinger, GR Griffith, MJ Coon. Arch Biochem Biophys 183:528–537, 1977.
40. GR Griffith, RT Ruettinger, EJ McKenna, MJ Coon. Methods Enzymol 53D:356–360, 1978.
41. J Shanklin, C Achim, H Schmidt, BG Fox, E Münck. Proc Natl Acad Sci USA 94:2981–2986, 1997.
42. J Shanklin, EB Cahoon. Annu Rev Plant Physiol Plant Mol Biol 49:611–641, 1998.
43. AP Tulloch, JFT Spencer, PAJ Gorin. Can J Chem 40:1326–1338, 1962.
44. E Heinz, AP Tulloch, JFT Spencer. J Biol Chem 244:882–888, 1969.
45. E Heinz, AP Tulloch, JFT Spencer. Biochim Biophys Acta 202:49–55, 1970.
46. RK Hommel, S Stegner, K Huse, HP Kleber. Appl Microbiol Biotechnol 40:724–728, 1994.
47. K Lottermoser, WH Schunck, O Asperger. Yeast 12:565–575, 1996.
48. AC Lanser, RD Plattner, MO Bagby. J Am Oil Chem Soc 69:363–366, 1992.
49. JK Huang, KC Keudell, SJ Seong, WE Klopfenstein, L Wen, MO Bagby, RA Norton, RF Vesonder. Biotechnol Lett 18:193–198, 1996.
50. Y Miura, AJ Fulco. J Biol Chem 249:1880–1888, 1974.
51. Y Miura, AJ Fulco. Biochim Biophys Acta 388:305–317, 1975.
52. RS Hare, AJ Fulco. Biochem Biophys Res Commun 65:665–672, 1975.
53. PP Ho, AJ Fulco. Biochim Biophys Acta 431:249–256, 1976.
54. LO Narhi, AJ Fulco. J Biol Chem 261:7160–7169, 1986.
55. LP Wen, AJ Fulco. J Biol Chem 262:6676–6682, 1987.
56. SS Boddupalli, BC Pramanik, CA Slaughter, RW Estabrook, JA Peterson. Arch Biochem Biophys 292:20–28, 1992.
57. JH Capdevila, S Wei, C Helvig, JR Falck, Y Belosludtsev, G Truan, SE Graham-Lorence, JA Peterson. J Biol Chem 271:22,663–22,671, 1996.
58. I Shiio, R Uchio. Agric Biol Chem 35:2033–2042, 1971.
59. S Picataggio, T Rohrer, K Deanda, D Lanning, R Reynolds, J Mielenz, LD Eirich. Bio/Technology 10:894–898, 1992.
60. EC Chan, J Kuo. Enzyme Microb Technol 20:585–589, 1997.
61. AS Kester, JW Foster. J Bacteriol 85:859–869, 1963.
62. R Uchio, I Shiio. Agric Biol Chem 36:426–433, 1972.
63. EC Chan, J Kuo, HP Lin, DG Mou. Appl Microbiol Biotechnol 34:772–777, 1991.
64. ZH Yi, HJ Rehm. Eur J Appl Microbiol Biotechnol 15:175–179, 1982.
65. ZH Yi, HJ Rehm. Appl Microbiol Biotechnol 28:520–526, 1988.

66. ZH Yi, HJ Rehm. Appl Microbiol Biotechnol 30:327–331, 1989.
67. M Ohkuma, T Zimmer, T Iida, WH Schunck, A Ohta, M Takagi. J Biol Chem 273:3948–3953, 1998.
68. U Scheller, T Zimmer, E Kärgel, WH Schunck. Arch Biochem Biophys 328:245–254, 1996.
69. T Zimmer, M Ohkuma, A Ohta, M Takagi, WH Schunck. Biochem Biophys Res Commun 224:784–789, 1996.
70. E Kärgel, R Menzel, H Honeck, F Vogel, A Böhmer, WH Schunck. Yeast 12:333–348, 1996.
71. U Scheller, T Zimmer, D Becher, F Schauer, WH Schunck. J Biol Chem 273:32,528–32,534, 1998.
72. KD Green, MK Turner, JM Woodley. Enzyme Microb Technol 27:205–211, 2000.
73. D Fabritius, HJ Schäfer, A Steinbüchel. Appl Microbiol Biotechnol 45:342–348, 1996.
74. D Fabritius, HJ Schäfer, A Steinbüchel. Appl Microbiol Biotechnol 48:83–87, 1997.
75. D Fabritius, HJ Schäfer, A Steinbüchel. Appl Microbiol Biotechnol 50:573–578, 1998.
76. JW Hagemann, JA Rothfus. J Am Oil Chem Soc 68:139–143, 1991.
77. HE Kenney, ET Donahue, G Maerker. US Patents 3,832,368 and 3,836,468, 1974.
78. LL Wallen, RG Benedict, RW Jackson. Arch Biochem Biophys 99:249–253, 1962.
79. EN Davis, LL Wallen, JC Goodwin, WK Rohwedder, RA Rhodes. Lipids 4:356–362, 1969.
80. GJ Schroepfer Jr. J Biol Chem 241:5441–5447, 1966.
81. WG Niehaus Jr, A Kisic, A Torkelson, DJ Bednarczyk, GJ Schroepfer, Jr. J Biol Chem 245: 3790–3797, 1970.
82. WG Niehaus, Jr., GJ Schroepfer Jr. J Am Chem Soc 89:4227–4228, 1967.
83. WG Niehaus, Jr., A Kisic, A Torkelson, DJ Bednarczyk, GJ Schroepfer, Jr. J Biol Chem 245:3802–3809, 1970.
84. LL Wallen, EN Davis, YV Wu, WK Rohwedder. Lipids 6:745–750, 1971.
85. S Koritala, MO Bagby. J Am Oil Chem Soc 69:575–578, 1992.
86. CT Hou. J Am Oil Chem Soc 71:975–978, 1994.
87. CT Hou. J Am Oil Chem Soc 72:1265–1270, 1995.
88. CW Seo, Y Yamada, N Takada, H Okada. Agric Biol Chem 45:2025–2030, 1981.
89. JH Litchfield, GE Pierce. US Patent 4,582,804, April 1986.
90. S Koritala, L Hosie, CT Hou, CW Hesseltine, MO Bagby. Appl Microbiol Biotechnol 32: 299–304, 1989.
91. SH El-Sharkawy, W Yang, L Dostal, JPN Rosazza. Appl Environ Microbiol 58:2116–2122, 1992.
92. N Esaki, S Ito, W Blank, K Soda. Biosci Biotechnol Biochem 58:319–321, 1994.
93. T Kaneshiro, JK Huang, D Weisleder, MO Bagby. J Ind Microbiol 13:351–355, 1994.
94. T Kaneshiro, LK Nakamura, MO Bagby. Curr Microbiol 31:62–67, 1995.
95. JA Hudson, CAM MacKenzie, KN Joblin. Appl Microbiol Biotechnol 44:1–6, 1995.
96. A Latrasse, S Paitier, B Lachot, P Bonnarme, G Féron, A Durand, JL le Quéré. Biotechnol Lett 19:715–718, 1997.
97. W Yang, L Dostal, JPN Rosazza. Appl Environ Microbiol 59:281–284, 1993.
98. AC Lanser, LK Nakamura. Curr Microbiol 32:260–263, 1996.
99. CT Hou. Appl Environ Microbiol 60:3760–3763, 1994.
100. H Kawashima. Biotechnol Lett 17:493–496, 1995.
101. TM Kuo, AC Lanser, T Kaneshiro, CT Hou. J Am Oil Chem Soc 76:709–712, 1999.
102. RT Ruettinger, AJ Fulco. J Biol Chem 256:5728–5734, 1981.
103. N Shirasaka, T Umehara, T Murakami, H Yoshizumi, S Shimizu. J Am Oil Chem Soc 75: 717–720, 1998.
104. A Verhulst, G Parmentier, G Janssen, S Asselberghs, H Eyssen. Appl Environ Microbiol 51:532–538, 1986.
105. A Guerrero, I Casals, M Busquets, Y Leon, A Manresa. Biochim Biophys Acta 1347:75–81, 1997.

106. J Bastida, C de Andrés, J Culleré, M Busquets, A Manresa. Biotechnol Lett 21:1031–1035, 1999.
107. H Kim, HW Gardner, CT Hou. J Am Oil Chem Soc 77:95–99, 2000.
108. H Koshino, S Togiya, T Yoshihara, S Sakamura. Tetrahedron Lett 28:73–76, 1987.
109. Y Suzuki, O Kurita, Y Kono, H Hyakutake, A Sakurai. Biosci Biotechnol Biochem 59:2049–2051, 1995.
110. SM Ahmed, F Ahmad, SM Osman. J Am Oil Chem Soc 62:1578–1580, 1985.
111. I Ahmad, J Singh. J Am Oil Chem Soc 67:205–208, 1990.
112. KT Achaya. J Am Oil Chem Soc 48:758–763, 1971.
113. FC Naughton. J Am Oil Chem Soc 51:65–71, 1974.
114. FT Orthoefer. In: YH Hui, ed. Bailey's Industrial Oil and Fat Products. Vol. 1: Edible Oil and Fat Products: General Applications. 5th ed. New York: Wiley, 1996, pp 19–43.
115. K Soda. In: TH Applewhite, ed. World Conference on Biotechnology for the Fats and Oils Industry. Champaign, IL: American Oil Chemists Society, 1988, pp 178–179.
116. JF Buchanan, AJ Fulco. Biochem Biophys Res Commun 85:1254–1260, 1978.
117. LJ Morris. Lipids 3:260–261, 1968.
118. AC Lanser. J Am Oil Chem Soc 75:1809–1813, 1998.
119. AC Lanser, LK Manthey. J Am Oil Chem Soc 76:1023–1026, 1999.
120. RF Vesonder, FH Stodola. Can J Chem 47:1247–1249, 1969.
121. E Mercadé, M Robert, MJ Espuny, MP Bosch, MA Manresa, JL Parra, J Guinea. J Am Oil Chem Soc 65:1915–1916, 1988.
122. CT Hou, MO Bagby. J Ind Microbiol 7:123–130, 1991.
123. CT Hou. J Am Oil Chem Soc 73:1359–1362, 1996.
124. TM Kuo, LK Manthey, CT Hou. J Am Oil Chem Soc 75:875–879, 1998.
125. JL Parra, J Pastor, F Comelles, MA Manresa, MP Bosch. Tenside Surfact Deterg 27:302–306, 1990.
126. C de Andrés, E Mercadé, J Guinea, A Manresa. World J Microbiol Biotechnol 10:106–109, 1994.
127. TM Kuo, CT Hou. Recent Res Dev Oil Chem 3:1–10, 1999.
128. CT Hou, LK Nakamura, D Weisleder, RE Peterson, MO Bagby. World J Microbiol Biotechnol 9:570–573, 1993.
129. TM Kuo, H Kim, CT Hou. Curr Microbiol 43:198–203, 2001.
130. H Kim, TM Kuo, CT Hou. J Ind Microbiol Biotechnol 24:167–172, 2000.
131. H Kim, HW Gardner, CT Hou. J Ind Microbiol Biotechnol 25:109–115, 2000.
132. CT Hou, W Brown, DP Labeda, TP Abbott, D Weisleder. J Ind Microbiol Biotechnol 19: 34–38, 1997.
133. CT Hou, H Gardner, W Brown. J Am Oil Chem Soc 75:1483–1487, 1998.
134. G Knothe, MO Bagby, RE Peterson, CT Hou. J Am Oil Chem Soc 69:367–371, 1992.
135. G Knothe, RO Dunn, MO Bagby. J Am Oil Chem Soc 72:43–47, 1995.
136. CT Hou. In: BC Saha, DC Demirjian, eds. Applied Biocatalysis in Specialty Chemicals and Pharmaceuticals. Washington, DC: American Chemical Society, 2001, pp 92–102.
137. BA Vick, DC Zimmerman. In: PK Stumpf, ed. The Biochemistry of Plants: A Comprehensive Treatise, Vol. 9. Orlando, FL: Academic Press, 1987, pp 53–90.
138. E Blee. INFORM 6:852–861, 1995.
139. FH Stodola, RF Vesonder, LJ Wickerham. Biochemistry 4:1390–1394, 1965.
140. C Baur, W Grosch. Z Lebensm Unters-Forsch 165:82–84, 1997.
141. A Graveland. J Am Oil Chem Soc 47:352–361, 1970.
142. T Kato, Y Yamaguchi, N Abe, T Uyehara, T Namai, M Kodama, Y Shiobara. Tetrahedron Lett 26:2357–2360, 1985.
143. H Masui, T Kondo, M Kojima. Phytochemistry 28:2613–2615, 1989.
144. KC Keudell, JK Huang, L Wen, WE Klopfenstein, MO Bagby, AC Lanser, RD Plattner, RE Peterson, D Weisleder. Microbios 102:27–38, 2000.

145. DM Weller, RJ Cook. Phytopathology 73:463–469, 1983.
146. LS Thomashow, DM Weller. J Bacteriol 170:3499–3508, 1988.
147. PJ Slinger, JE VanCauwenberge, MA Shea-Wilber, RJ Bothast. In: GJ Boland, LD Kuykendall, eds. Plant–Microbe Interactions and Biological Control. New York: Marcel Dekker, 1998, pp 329–353.
148. KD Carlson, DL Van Dyne. In: KD Carlson, DL Van Dyne, eds. Industrial Uses for High Erucic Acid Oil from Crambe and Rapeseed. Columbia, MO: University of Missouri, 1992.
149. KI Ekpenyong, JR Walls. Ind Eng Chem Res 29:121–125, 1990.
150. K Wakamatsu, T Masaki, F Itoh, K Kondo, K Sudo. Biochem Biophys Res Commun 168: 423–429, 1990.
151. M Rouhi. Chem Eng News 73:7, 1995.
152. T Kaneshiro, RF Vesonder, RE Peterson, D Weisleder, MO Bagby. J Am Oil Chem Soc 71: 491–494, 1994.
153. JK Huang, KC Keudell, WE Klopfenstein, L Wen, MO Bagby, RF Vesonder, RA Norton, D Weisleder. J Am Oil Chem Soc 74:601–603, 1997.
154. TK Miwa, JA Rothfus, E Dimitroff. J Am Oil Chem Soc 56:765–770, 1979.
155. T Kaneshiro, LK Nakamura, JJ Nicholson, MO Bagby. Curr Microbiol 32:336–342, 1996.
156. S Koritala. J Am Oil Chem Soc 66:133–134, 1989.
157. S Dewitt, JL Ervin, D Howes-Orchison, D Dalietos, SL Neidleman, J Geigert. J Am Oil Chem Soc 59:69–74, 1982.
158. DL Gutnick, R Allon, C Levy, R Petter, W Minas. In: KT Towner, E. Bergogne-Bérézin, CA, Fewson, eds. The Biology of Acinetobacter. New York: Plenum Press, 1991, pp 411–441.
159. K Bryn, E Jantzen. J Gen Microbiol 102:33–43, 1977.
160. C Ratledge. In: C Ratledge, SG Wilkinson, eds. Microbial Lipids, Volume 2. London: Academic Press, 1989, pp 567–668.
161. RA Pieringer. In: C Ratledge, SG Wilkinson, eds. Microbial Lipids, Vol. 2. London: Academic Press, 1989, pp 51–114.
162. GM Lloyd, NJ Russell. J Gen Microbiol 129:2641–2647, 1983.
163. P Borneleit, T Hermsdorf, R Claus, P Walther, HP Kleber. J Gen Microbiol 134:1983–1992, 1988.
164. RG Kok, VM Christoffels, B Vosman, KJ Hellingwerf. J Gen Microbiol 139:2329–2342, 1993.
165. C Breuil, DJ Kushner. Can J Microbiol 21:423–433, 1975.
166. T Tsujita, M Sumiyoshi, H Okuda. Lipids 34:1159–1166, 1999.
167. HG Maister, SP Rogovin, FH Stodola, LJ Wickerham. Appl Microbiol 10:401–406, 1962.
168. T Kaneshiro, RF Vesonder, RE Peterson. Curr Microbiol 24:319–324, 1992.
169. CJ Mirocha, DG Gilchrist, WT Shier, HK Abbas, Y Wen, RF Vesonder. Mycopathologia 117:47–56, 1992.
170. RF Vesonder, W Wu, D Weisleder, SH Gordon, T Krick, W Xie, HK Abbas, CE McAlpin. J Nat Toxins 9:103–112, 2000.
171. SC Bezuidenhout, WCA Gelderblom, CP Gorst-Allman, RM Horak, WFO Marasas, G Spiteller, R Vleggaar. J Chem Soc, Chem Commun 1988:743–745, 1988.
172. RT Riley, E Wang, JJ Schroeder, ER Smith, RD Plattner, H Abbas, HS Yoo, AH Merrill Jr. Nat Toxins 4:3–15, 1996.
173. E Wang, WP Norred, CW Bacon, RT Riley, AH Merrill Jr. J Biol Chem 266:14,486–14,490, 1991.
174. HK Abbas, T Tanaka, SO Duke, JK Porter, EM Wray, L Hodges, AE Sessions, E Wang, AH Merrill Jr, RT Riley. Plant Physiol 106:1085–1093, 1994.
175. Yano, I Tomiyasu, E Yabuuchi. FEMS Microbiol Lett 15:303–307, 1982.
176. A Yamamoto, I Yano, M Masui, E Yabuuchi. J Biochem 83:1213–1216, 1978.
177. E Wang, PF Ross, TM Wilson, RT Riley, AH Merrill Jr. J Nutr 122:1706–1716, 1992.
178. RT Riley, E Wang, AH Merrill Jr. J AOAC Int 77:533–540, 1994.

179. C Michel, G van Echten-Deckert, J Rother, K Sandhoff, E Wang, AH Merrill Jr. J Biol Chem 272:22,432–22,437, 1997.
180. HU Humpf, EM Schmelz, FI Meredith, H Vesper, TR Vales, E Wang, DS Menaldino, DC Liotta, AH Merrill Jr. J Biol Chem 273:19,060–19,064, 1998.
181. H Fukuda, H Shima, RF Vesonder, H Tokuda, H Nishino, S Katoh, S Tamura, T Sugimura, M Nagao. Biochem Biophys Res Commun 220:160–165, 1996.
182. C Ratledge, FM Dickinson. Colloq Inst Nat Recher Agron 75:153–166, 1995.
183. A Endrizzi, Y Pagot, A le Clainche, JM Nicaud, JM Belin. Crit Rev Biotechnol 16:301–329, 1996.
184. RF Vesonder, FH Stodola, LJ Wickerham, JJ Ellis, WK Rohwedder. Can J Chem 49:2029–2032, 1971.
185. RF Vesonder, FH Stodola, WK Rohwedder. Can J Biochem 50:363–365, 1972.
186. P Chalier, J Crouzet. Biotechnol Lett 14:275–280, 1992.
187. S Gocho, N Tabogami, M Inagaki, C Kawabata, T Komai. Biosci Biotech Biochem 59:1571–1572, 1995.
188. SL Lee, SJ Lin, CC Chou. J Ferment Bioeng 80:195–199, 1995.
189. G Feron, L Dufosse, E Pierard, P Bonnarme, JL le Quéré, HE Spinnler. Appl Environ Microbiol 62:2826–2831, 1996.
190. JK Huang, KC Keudell, J Zhao, WE Klopfenstein, L Wen, MO Bagby, AC Lanser, RD Plattner, RE Peterson, TP Abbott, D Weisleder. J Am Oil Chem Soc 72:323–326, 1995.
191. JK Huang, PF Dowd, KC Keudell, WE Klopfenstein, L Wen, MO Bagby, AC Lanser, RA Norton. J Am Oil Chem Soc 73:1465–1469, 1996.
192. SS Radwan, NA Sorkhoh, IM El-Nemr, AF El-Desouky. J Appl Microbiol 83:353–358, 1997.
193. JM Foght, DWS Westlake, WM Johnson, HF Ridgway. Microbiology 142:2333–2340, 1996.
194. J Dunbar, S White, L Forney. Appl Environ Microbiol 63:1326–1331, 1997.
195. GM Colores, PM Radehaus, SK Schmidt. Appl Biochem Biotechnol 54:271–275, 1995.
196. T Kaneshiro, TM Kuo, LK Nakamura. Curr Microbiol 38:250–255, 1999.
197. TM Kuo, T Kaneshiro. In: BC Saha, DC Demirjian, eds. Applied Biocatalysis in Specialty Chemicals and Pharmaceuticals. Washington, DC: American Chemical Society, 2001, pp 55–64.
198. TM Kuo, AC Lanser, LK Nakamura, CT Hou. Curr Microbiol 40:105–109, 2000.

32

Biosynthesis and Applications of Glycolipid and Lipopeptide Biosurfactants

DAVID C. HERMAN

University of Houston, Houston, Texas

RAINA M. MAIER

University of Arizona, Tucson, Arizona

1 INTRODUCTION

A wide variety of microorganisms produce surface-active compounds referred to as biosurfactants and there are many putative roles for biosurfactants [1–4]. For example, biosurfactant production can stimulate microbial assimilation of low-water-solubility compounds (e.g., hydrocarbons [5–7]. Antibiotic effects of biosurfactants have been observed toward viruses [8], bacteria [9,10], and other microorganisms, including the zoosporic plant pathogens [11]. Biosurfactants can complex heavy metals [12] and protect against metal toxicity during biodegradation of organics [13]. Studies indicate a role for biosurfactants in the adhesion and deadhesion of bacteria to surfaces [3]. Finally, biosurfactants are one of the virulence factors associated with pathogenic microorganisms [14]. In this chapter, we review the chemical nature of biosurfactants, their biosynthetic pathways, and their possible applications to the field of biotechnology.

2 PROPERTIES OF BIOSURFACTANTS

All surfactants are amphipathic, meaning that they combine both a hydrophilic and a hydrophobic moiety. The amphipathic nature of surfactants causes them to accumulate and orient in a regular manner at the interface between two phases, such as a liquid–air

or a liquid–liquid interface. This results in a reduction in the interfacial tension between the two phases, facilitating a greater interaction between the phase components. For example, surfactants can promote the dispersion of low-water-solubility organic compounds into the aqueous phase (also called solubilization). They can also be used for emulsification, de-emulsification, foaming, and wetting. Exploitation of these properties has created a multibillion dollar market for surfactants in the food, cosmetic, medical, petrochemical, and environmental industries. Currently, this market is primarily for synthetic surfactants, but interest in biosurfactants has recently increased. The increased interest is partly because of the specificity of biosurfactants for particular applications and partly because biosurfactants are natural products and may, therefore, be more environmentally compatible than their synthetic surfactant counterparts [4,15–17].

Biosurfactants are considered environmentally compatible because often they are less toxic and more easily biodegraded than their synthetic counterparts. For example, rhamnolipid biosurfactants are known to be readily degraded by soil microorganisms [13,18] and are not toxic to soil microorganisms [19]. Using several different microbial toxicity assays, Poremba et al. [20] compared four synthetic surfactants and six biosurfactants in terms of biodegradability and toxicity. Their results indicate that the biosurfactants were more biodegradable and generally less toxic than the synthetic surfactants tested. Lang and Wagner [21] describe a field study concerning removal of oil contamination that compares synthetic surfactants and biosurfactants applied to coastal sediments artificially contaminated with oil. They found that biosurfactants removed more of the oil and were more successful in preventing oil penetration into the sediments than the synthetic surfactants. Biosurfactants were also less toxic to brine crayfish (*Corophium volutator*), a marine organism, and to *Artemia* larva than the synthetic surfactants.

2.1 Surface Activity and Critical Micelle Concentration

Surfactant activity can be compared on the basis of their ability to lower surface (air–water interface) or interfacial (liquid–liquid interface) tension. Distilled water has a surface tension of 72 mN/m, and the addition of an effective surfactant will lower this value to approximately 30 mN/m. As the surfactant concentration in a solution increases, surface tension will decrease until the critical micelle concentration (CMC) is reached. Once the CMC is reached, further addition of surfactant does not change the surface tension (Fig. 1). The CMC represents the point where individual surfactant molecules spontaneously

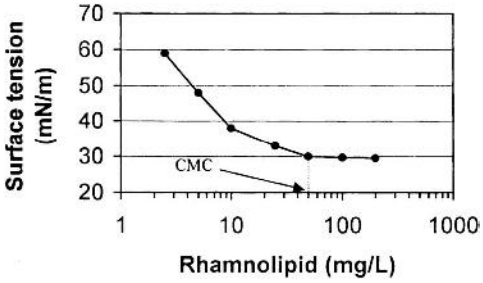


Figure 1 Typical plot of surface tension against the log concentration of rhamnolipid biosurfactant. (From Ref. 24.)

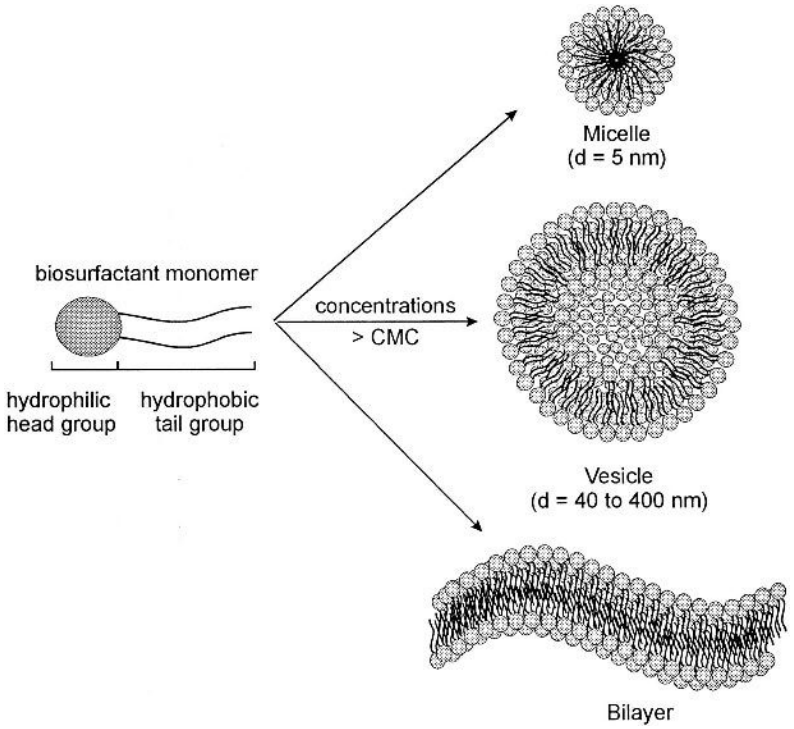


Figure 2 Rhamnolipid biosurfactant structures formed above the critical micelle concentration (CMC). (From Ref. 23.)

aggregate into complex structures, including micelles, bilayers, and vesicles (Fig. 2). The type of aggregate formed is dependent on surfactant structure, ionic strength, and pH [22,23].

A comparison of the minimum surface and interfacial tension values as well as the CMC for several biosurfactants is provided in Table 1. The minimum surface and interfacial tension values shown in Table 1 are similar to those reported for synthetic surfactants, but few studies have directly compared the activity of synthetic surfactants and biosurfactants under the same set of conditions [42]. In one such study, Kretschmer et al. [43] compared a glycolipid biosurfactant from *Rhodococcus erythropolis* with several synthetic surfactants. They found that the biosurfactant was more stable against hydrolysis at extreme pH values and that the surface and interfacial activity of the biosurfactant was stable over a wide range of pH and ionic strengths. In fact, the stability of biosurfactants over a wide range of conditions makes them highly relevant to some environmental and industrial applications. For example, the activity of a lipopeptide biosurfactant produced by *Bacillus licheniformis* is not affected by high pH, salinity, or temperature, making it suitable for use in oil recovery where extreme conditions are prevalent [38,44].

2.2 Classification of Biosurfactants

Surfactants of microbial origin contain a lipophilic moiety composed of the hydrocarbon chain from a fatty acid, and a hydrophilic moiety which can be from various sources,

Table 1 Physical Properties of Selected Biosurfactants

Biosurfactant	Microorganism	Surface tension (mN/m)	Interfacial tension (mN/m)	CMC (mg/L)	Ref.
Rhamnolipid (mix)	*Pseudomonas* sp. DSM 2874	26	<1[a]	25	25
Rhamnolipid (mix)	*P. aeruginosa* YP380	30	n.d.[b]	50	26
Rhamnolipid (mix)	*P. aeruginosa* 44TI	27	n.d.	n.d.	27
Monorhamnolipid (R1 only)	*P. aeruginosa* ATCC 9027	28	2	50	28
Dirhamnolipid (R2 only)	Unknown[c]	36	5	65	28
Dirhamnolipid methyl ester	Synthesized (36)	31	<0.1	21	29
Trehalolipid	*Rhodococcus erythropolis* DSM43315	36–43[d]	18–17[a]	0.7–1.7	30
Trehalolipid	*Rhodococcus* sp. 51T7	30	n.d.	n.d.	31
Trehalolipid	*Rhodococcus* sp. H13-A	n.d.	0.25[a]	1.5	32
Trehalolipid	*Tsukamurella* sp. nov	25	n.d.	n.d.	33
Glycolipid (mannose monoester)	*Arthrobacter* sp. DSM2567	40	19[a]	5	34
Glycolipid (glucose monoester)	*Arthrobacter* sp. DSM2567	40	9[a]	10	34
Glycolipid (maltose monoester)	*Arthrobacter* sp. DSM2567	33	1[a]	1	34
Glycolipid (maltose diester)	*Arthrobacter* sp. DSM2567	46	13[a]	10	34
Glycolipid (cellobiose monoester)	*Arthrobacter* sp. DSM2567	35	1[a]	3	34
Glycolipid (maltotiose triester)	*Arthrobacter* sp. DSM2567	44	19[a]	20	34
Sophorolipid	*Torulopsis bombicola* ATCC 22214	27	1–2[e]	82	35
Sophorolipid	*T. apicola* IMET 43747	22.7	<1[f]	n.d.	36
Lipopeptide (surfactin)	*Bacillus subtilis* ATCC 21332	27	1[a]	25	37
Lipopeptide (lichenysin A)	*B. licheniformis* BAS50	28	n.d.	12	38
Lipopeptide	*B. licheniformis* JF-2	27	<1[a]	20	39
Lipopeptide	*Arthrobacter* sp.	24	n.d.	13.5	40
Lipopeptide	*B. licheniformis* JF-2	n.d.	<1[f]	10	41

[a] Against hexadecane.
[b] n.d., Not determined.
[c] Purified dirhamnolipid was obtained from Kyowa Hakko Kogyo Co., Ltd. (Tokyo, Japan).
[d] Depending on the medium.
[e] Against hexadecane and vegetable oils.
[f] Against decane.

including a sugar molecule (monosaccharide or disaccharide) or a peptide sequence. Biosurfactants are broadly classified into several different groups based on the nature of the hydrophilic moiety. These include glycolipids, lipoproteins, phospholipids, neutral lipids, and fatty acids. Herein, we focus on two well-characterized groups of low-molecular-weight biosurfactants: glycolipids and lipopeptides. In the following sections, we will first discuss the chemical diversity within these biosurfactant groups and then describe their biosynthetic pathways as well as the physiological factors related to their production. Where possible, the genetic basis of synthesis and regulation is provided.

3 TYPES OF BIOSURFACTANTS

3.1 Glycolipids

The most commonly described biosurfactants are the glycolipids, which combine a sugar molecule (hydrophilic moiety) with a long-chain aliphatic side chain (lipophilic moiety). The three best studied glycolipids, namely rhamnolipids, trehalolipids, and sophorolipids, are discussed next. The type of glycolipid formed is a function of both the bacterial isolate and of the growth medium, the carbon source in particular. Therefore, the possibility of creating ''designer'' glycolipids for commercial application may be possible through a judicious choice of strain and growth media.

3.1.1 Rhamnolipids

Rhamnolipids are the principal anionic glycolipids produced by *Pseudomonas aeruginosa*. Rhamnolipids are secreted from the cell after synthesis and are considered extracellular biosurfactants. *P. aeruginosa* strains are ubiquitous in soil and freshwater environments, but are considered opportunistic pathogens, especially in hospital settings. The predominant forms of rhamnolipid (Fig. 3) contain one (monorhamnolipid) or two (dirhamnolipid) 1,2-linked rhamnose sugars combined with two 3-hydroxydecanoic acid moieties. These forms are also referred to as rhamnolipid 1 (R1) (L-rhamnosyl-3-hydroxydecanoyl-3-hydroxydecanoate) and rhamnolipid 2 (R2) (L-rhamnosyl-L-rhamnosyl-3-hydroxydecanoyl-3-hydroxydecanoate). Syldatk et al. [25,45] noted the production of two, more hydrophilic, forms of rhamnolipid during growth of *Pseudomonas* sp. DSM2874 on *n*-alkanes. In addition to R1 and R2, DSM2874 produced a monorhamnolipid and dirhamnolipid that contained an aliphatic side chain composed of only a single 3-hydroxydecanoic acid group. These are referred to as rhamnolipids 3 (R3) and 4 (R4).

Most *P. aeruginosa* produce rhamnolipid mixtures [46,47]. Usually, the mixtures contain R1 and R2 forms that vary in the length and saturation of the fatty acid moiety. Rendell et al. [48] examined rhamnolipid production by a clinical *P. aeruginosa* isolate and reported the presence of R1 and R2 forms with C_{10}—C_8, C_8—C_{10}, C_{10}—C_{10}, C_{12}—C_{10}, C_{10}—C_{12}, and C_{10}—$C_{12:1}$ fatty acids. Deziel et at. [49] compared rhamnolipids produced by a *P. aeruginosa* strain grown on a water-soluble substrate, mannitol, or on an aromatic compound, naphthalene, which has limited water solubility. The rhamnolipid mixture produced on mannitol contained 21 different rhamnolipids, dominated by a C_{10}—C_{10}—R2. In contrast, the rhamnolipid complex produced on naphthalene contained 16 rhamnolipids and was dominated by a C_{10}—R3. These results are supported by other studies showing that the rhamnolipid mixture produced is influenced by the carbon source [27]. It should also be mentioned that some of the rhamnolipid forms detected may be degradation products of R1 and R2 [2].

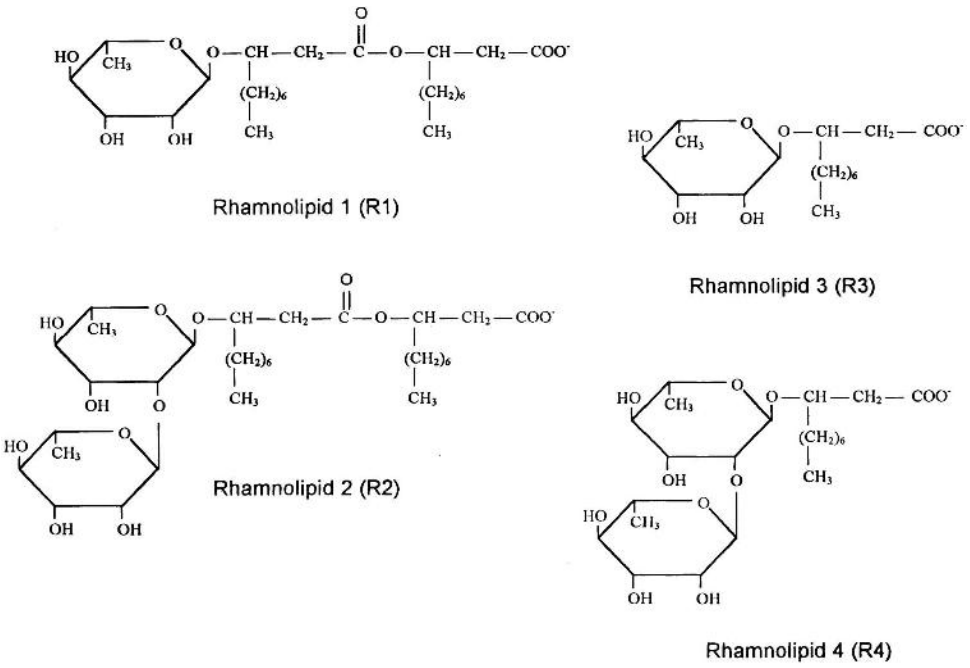


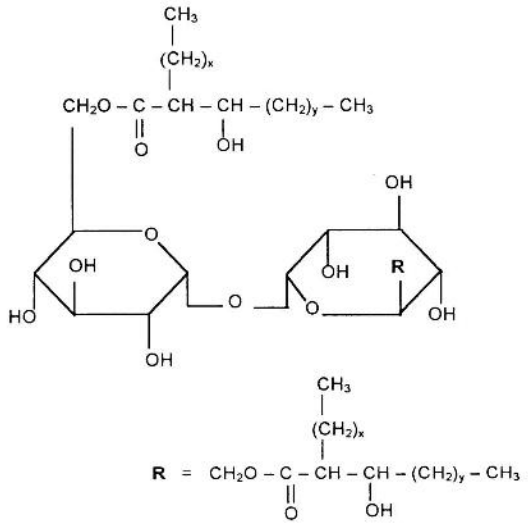
Figure 3 Structure of rhamnolipids formed by *Pseudomonas aeruginosa*. (From Ref. 16.)

There is currently no explanation of how the diversity in rhamnolipids produced contributes to the activity or role of rhamnolipids in the growth and survival of *P. aeruginosa*. However, the form and composition of the rhamnolipids produced does affect their chemical properties. For example, Hirayama and Kato [50] reported a *P. aeruginosa* strain which produces a novel nonionic form of R2 (a rhamnolipid-methyl ester). A comparison of anionic R2 with nonionic R2 revealed that the nonionic form was more effective in lowering interfacial tension and promoting microbial utilization of hydrocarbons compared to the acidic form of R2 [29]. Other properties affected by rhamnolipid structure include the ability to solubilize hydrocarbons [28], the ability to complex metals [51], effectiveness as a biological control agent for zoosporic plant pathogens [11], and sorption to soil [51].

3.1.2 Trehalolipids

Trehalolipids can be nonionic or anionic in nature and are composed of a trehalose disaccharide linked by ester bonds to long-chain fatty acids [52]. One type of acid is mycolic acid, which is an α-branched β-hydroxy fatty acid [53]. Mycolic acids are often found as part of glycolipid cell-wall components of *Mycobacterium*, *Nocardia*, and *Cornyebacter*. They are referred to as ''cord factors'' because these bacteria have a characteristic cordlike form of growth [54]. Rapp et al. [53] examined the trehalolipids formed by *Rhodococcus erythropolis* grown on *n*-alkanes, and they found that the lipid moiety consisted predominantly of saturated long-chain α-branched β-hydroxy fatty acids ranging from $C_{32}H_{64}O_3$ to $C_{38}H_{76}O_3$, with $C_{34}H_{68}O_3$, and $C_{35}H_{76}O_3$ as the dominant structures (Fig. 4a). The lipid moiety is linked to the trehalose via ester bonding, resulting in the formation of a nonionic compound.

(a) Trehalose dicorynomycolate (x + y = 27 to 31)



(b) Trehalose-tetraester

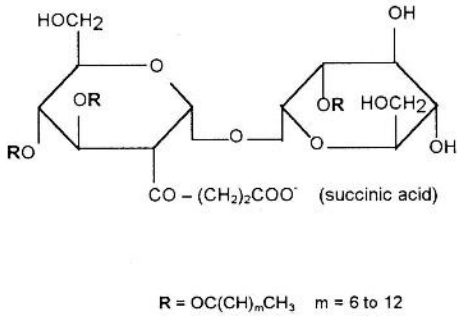


Figure 4 Structure of trehalolipids formed by *Rhodococcus erythropolis*: (a) nonionic trehalose dicorynomycolate (from Ref. 53); (b) anionic trehalose tetraester. (From Refs. 42 and 55.)

Rhodococcus erythropolis has also been reported to produce an anionic trehalolipid. Ristau and Wagner [55] and Uchida et al. [56] described the formation of trehalose tetraesters which are composed of trehalose linked to three long-chain fatty acids and also to succinic acid, which imparts an anionic character to the molecule (Fig. 4b). Singer et al. [32] found that hexadecane-grown *Rhodococcus* species H13-A produced an anionic biosurfactant composed of trehalose acylated with normal C_{10} to C_{22} saturated and unsaturated fatty acids, C_{35} to C_{40} mycolic acids, 10-methyl-hexadecanoic and 10-methyl-octadecanoic acids, and hexanedioic and decanedioic acids, the latter accounting for the anionic character of the glycolipid. The composition of the lipid moiety of a trehalolipid can vary

depending on the carbon source utilized for growing the cells. Espuny et al. [31] grew *Rhodococcus* sp. 51T7 on different hydrocarbons and found that the trehalose tetraester formed contained different alkyl substituents. For example, growth on *n*-decane produced a trehalose linked to four decanoyl groups, whereas growth on tridecane produced a trehalose linked to a succinoyl, octanoyl, nonanoyl, and decanoyl group.

3.1.3 Sophorolipids

Sophorolipids are formed from the disaccharide sophorose, which is linked to a long-chain hydroxy fatty acid. Sophorolipids are produced by yeast such as *Torulopsis petrophilum* [57], *T. bombicola* [35,58], later reclassified as a species of *Candida*, and *C. apicola* [59,60]. In acidic sophorolipids, the sophorose sugar moiety is attached to the lipid end of the fatty acid leaving the carboxyl functional group free (Fig. 5a). Neutral lactone forms also occur which have internal esters derived from the interaction of the carboxyl group with a sugar hydroxyl group (Fig. 5b). Mixtures of sophorolipids are often formed which differ in terms of fatty acid saturation, lactonization, and acetylation of the sugar moiety. The nature of the fatty acid in the sophorolipid also varies depending on the type of hydrophobic organic substrate utilized by the yeast for growth [52]. This is because alkanes and other lipophilic substrates can be directly incorporated into the sophorolipids of *Torulopsis* strains during growth.

3.1.4 Other Glycolipids

Other microbially produced glycolipids exist, although they have not been well studied. For example, one *Arthrobacter* sp. that produces trehalolipid can also produce other glycolipids. These glycolipids contain a trehaloselike α-branched β-hydroxy fatty acid, but the sugar moiety reflects the carbon source provided. For example, when resting cells of *Arthrobacter* sp. strain DSM2567 were incubated with different sugars, including mannose, maltose, glucose, and cellobiose, the glycolipids produced contained the sugar moiety corresponding to the sugar supplied as the carbon source [34]. A comparison of the biosurfactant properties of these different glycolipids found that the more hydrophilic glycolipids, cellobiose and maltose monoesters, were capable of the strongest reductions in surface and interfacial tension (Table 1).

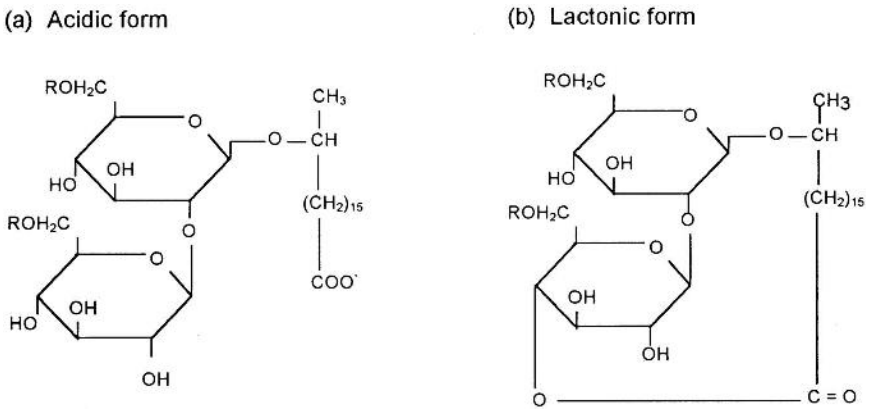


Figure 5 Structure of sophorolipids formed by *Candida bombicola*; R = acetyl. (From Ref. 58.)

Vollbrecht et al. [33] reported the production of glycolipids by the bacterium *Tsukamurella* sp. during growth on sunflower oil. These glycolipids contained tetrahalose, trisaccharides, and tetrasaccharides moieties linked to a unique sequence of fatty acids which varied in chain length from C_4 to C_{18}. The disaccharide, trisaccharide, and tetrasaccharide lipids were formed in sequence at different growth stages, suggesting that the disaccharide and trisaccharide lipids may be precursors to the tetrasaccharide form. These glycolipids were found to have strong surfactant and antimicrobial properties.

3.2 Lipopeptides

Lipopeptides combine a cyclic peptide chain (hydrophilic moiety) with a long-chain fatty acid (lipophilic moiety). Examples of lipopeptides include surfactin (Fig. 6a), which is produced by *Bacillus subtilis* ATCC 21332 [37], lichenysin (Fig. 6b) produced by *Bacillus licheniformis* [38,44], and a lipopeptide produced by *Arthrobacter* sp. [40]. Initial interest in lipopeptides produced by *Bacillus* focused on antibiotic properties. However, lipopeptides are among the most powerful biosurfactants known in terms of their ability to reduce surface tension at low CMC values [4]. The lipopeptides formed by different species of

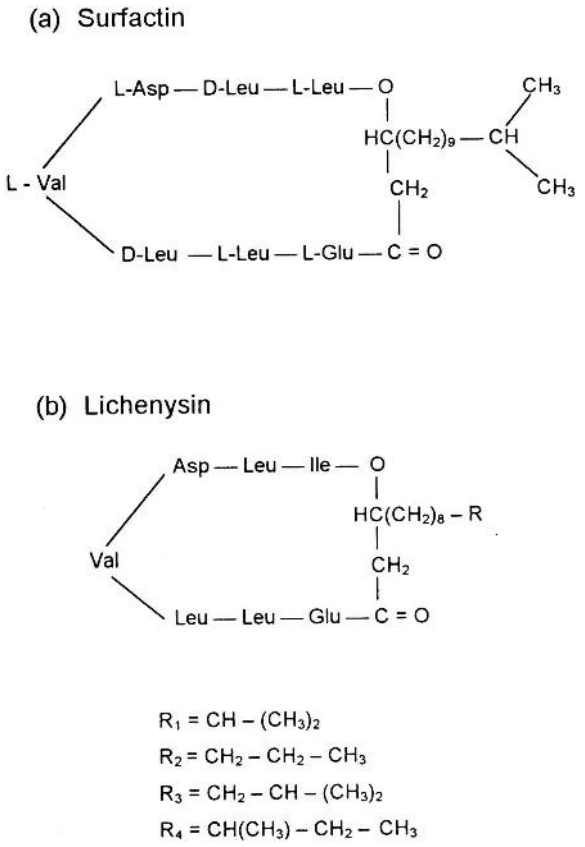


Figure 6 Structure of lipopeptides: (a) surfactin formed by *Bacillus subtilis* ATCC 21332 (from Ref. 4) and (b) lichenysin formed by *Bacillus licheniformis*. (From Ref. 1.)

bacteria vary in terms of the types of amino acids present in the peptide ring, as well as in the chain length and structure of the fatty acid component. In one example, the lipopeptide from *B. licheniformis* BAS50 (referred to as lichenysin A) was composed of a 7-amino-acid cyclic peptide moiety linked to a mixture of 14 different linear and branched β-hydroxy fatty acids ranging in size from C_{12} to C_{17} [38]. In comparison to the lipopeptide surfactin, lichenysin A contains a dominant fatty acid moiety that is longer in chain length and peptide moiety composed of amino acids that are less polar. As a result, the CMC of lichenysin A is approximately one-half (12 mg/L) of the CMC of surfactin (25 mg/L).

Several *B. licheniformis* strains were able to grow and produce biosurfactants under conditions of high salinity, pH, and temperature and are also able to produce biosurfactants when grown under anaerobic conditions [38,44,61]. These strains have been investigated for their use in enhancing oil recovery from underground reservoirs [39].

4 BIOSYNTHESIS OF BIOSURFACTANTS

The properties of biosurfactants result from their unique structures. Biosynthesis of biosurfactants generally involves separate pathways to form the hydrophilic and hydrophobic moieties which are then subsequently combined [52]. These two moieties may be formed through de novo synthesis or they may be derived from carbon substrates available to the cells from their environment (e.g., a preformed sugar or lipid). Knowledge of these pathways is critical to understanding how the carbon source affects biosurfactant structure. Ultimately, this knowledge can be used to manipulate biosynthesis pathways to direct biosurfactant synthesis.

4.1 Rhamnolipid

Rhamnolipid and surfactin are currently the best characterized biosurfactants in terms of synthesis and genetic regulation. Burger et al. [62] proposed a pathway for rhamnolipid biosynthesis in which thymidine-diphospho-rhamnose (TDP-rhamnose) is the donor substrate for two sequential enzymatic reactions (Fig. 7). The first reaction involves an enzyme, rhamnosyltransferase (Rt1), which links rhamnose to the aliphatic side chain (3-hydroxydecanoyl-3-hydroxydecanoate) to form R1. The second reaction involves a second rhamnosyltransferase (Rt2), which adds a second rhamnose forming R2. An assay to monitor the activity of rhamnosyltransferase was devised by Burger et al. [62] and later modified, as described by Ochsner et al. [2]. The assay involves combining [^{14}C]-TDP rhamnose with either the aliphatic side-chain substrate (3-hydroxydecanoyl-3-hydroxydecanoate) or with R1. After a short incubation period, the product of rhamnosyltransferase activity, either R1 or R2, is extracted, identified, and quantified using thin-layer chromatography and liquid scintillation counting. TDP-Rhamnose is formed from TDP-glucose by a three-step transformation (Fig. 8) involving enzymes belonging to the *rfb* gene cluster [63]. These enzymes are dTDP-D-glucose 4,6-dehydratase (Rfb B protein), dTDP-6-deoxy-D-*xylo*-4-hexulose 3,5-epimerase (Rfb C protein), and NADPH:dTDP-6-deoxy-L-*lyxo*-4-hexulose 4-reductase (Rfb D protein) [64]. The sugar rhamnose is not unique to rhamnolipids because it is also a component of the lipopolysaccharide (LPS) found in the *O*-antigen polysaccharide of the outer membrane of *P. aeruginosa* [65]. TDP-Rhamnose is the form in which rhamnose is donated during assembly of the *O*-antigen.

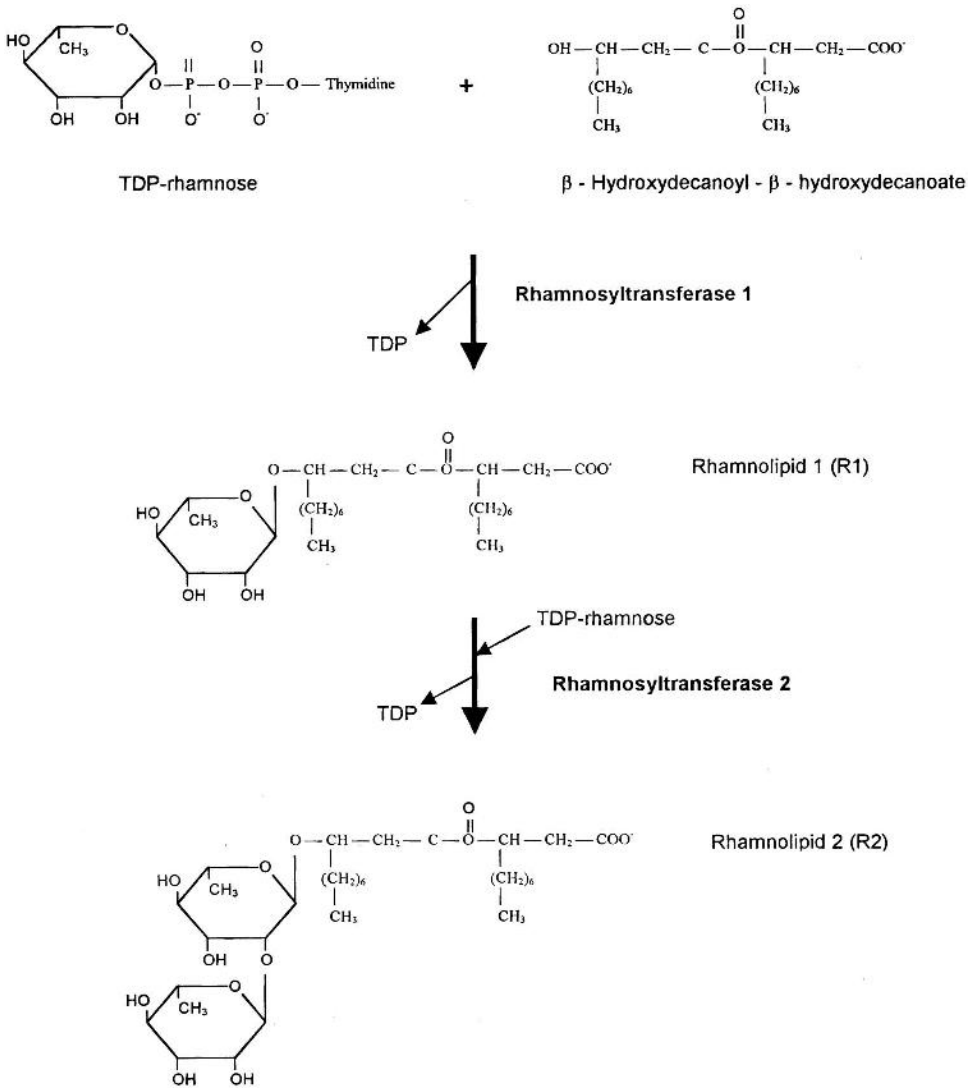


Figure 7 Pathway of rhamnolipid biosynthesis by *Pseudomonas aeruginosa*. (From Refs. 2 and 16.)

The aliphatic side chain, 3-hydroxydecanoyl-3-hydroxydecanoate, is formed by the esterification of two molecules of 3-hydroxydecanoic acid [2]. Studies show that the pathway for the formation of the side chain is linked to de novo fatty acid synthesis. Rehm et al. [66] described a gene involved in the synthesis of polyhydroxyalkanoic acids (PHA), which is a storage product deposited as granules in many pseudomonads. The de novo synthesis of fatty acids leading to the production of PHA involves the synthesis of 3-hydroxy fatty acids, which are joined to form medium-chain-length polymers, including poly-3-hydroxydecanoate. The polymerization of 3-hydroxy acids involves the use of PHA synthases. This pathway is similar to the synthesis of the fatty acid moiety of rhamnolipid,

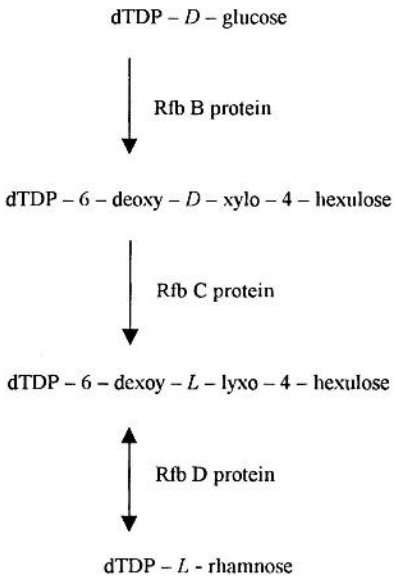


Figure 8 Pathway for the biosynthesis of dTDP-L-rhamnose from dTDP-glucose. (From Ref. 64.)

which can involve the polymerization of two molecules of 3-hydroxydecanoic acid to form 3-hydroxydecanoyl-3-hydroxydecanoate. Compos-Garcia et al. [67] reported a *P. aeruginosa* mutant strain which did not produce rhamnolipid, but had similar growth rate and total lipid production as the wild type. They described a *rhlG* gene which produced a reductase protein required for the production of the fatty acid moiety of rhamnolipid. The RhlG protein is an NADPH-dependent acyl carrier protein (ACP) reductase specifically required for the production of 3-hydroxy fatty acid moiety of rhamnolipids and is similar to the enzymes involved in PHA synthesis. The authors suggest that the substrate for this enzyme is an ACP-linked 3-hydroxy fatty acid, which is an intermediate common to both PHA synthesis and rhamnolipid synthesis. The RhlG enzyme converts this substrate into a precursor of the 3-hydroxydecanoyl-3-hydroxydecanoate side chain of rhamnolipid.

Several of the genes involved in rhamnolipid production have been identified and a mechanism has been proposed for regulation of a rhamnolipid operon for Rt1 [68–71]. As shown in Figure 9, four genes involved in rhamnolipid production have been designated *rhlA*, *rhlB*, *rhlR*, and *rhlI*. The genes *rhlA* and *rhlB* encode subunits of the Rt1 enzyme that catalyzes the transfer of rhamnose from TDP-rhamnose to 3-hydroxydecanoyl-3-hydroxydecanoate [68]. The products of *rhlR* and *rhlI* control the expression of *rhlAB* rhamnosyltransferase genes [70]. The RhlR protein is a transcriptional activator and its activity is controlled by an autoinducer which is the product of the *rhlI* gene. The RhlR protein is activated by binding to the autoinducer and the activated protein upregulates expression of the *rhlAB* genes. The autoinducer is a homoserine lactone, which is a class of small diffusible molecules that are released from the cell and mediate cell-to-cell communication. Therefore, the autoinducer is most effective at the high cell densities characteristically found during late log and stationary phases of growth. As a result, rhamnolipids are characteristically produced during this time frame.

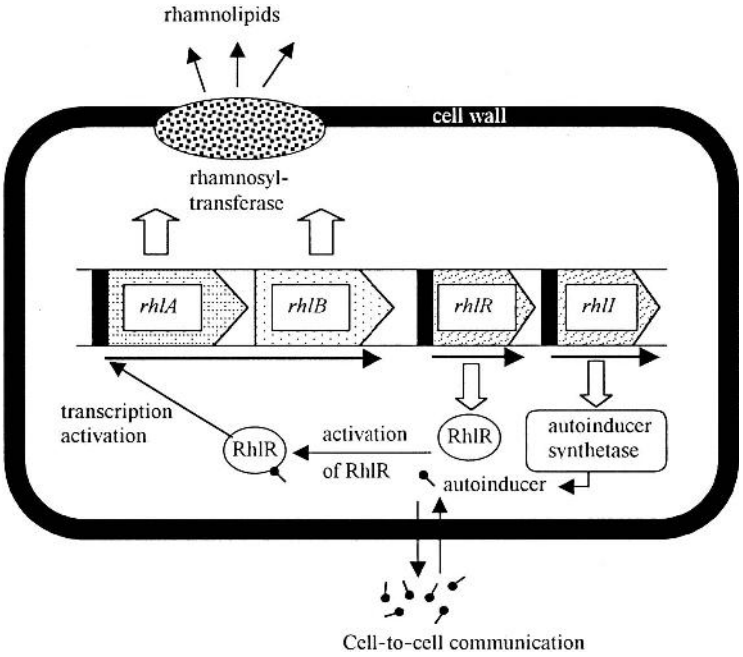


Figure 9 Model for the regulation of rhamnolipid production by *Pseudomonas aeruginosa*. (From Ref. 2.)

Rahim et al. [72] recently described the gene (*rhlC*) that encodes the Rt2 enzyme. This enzyme is homologous to rhamnosyltransferases involved in lipopolysaccharide (LPS) biosynthesis. In addition, Compos-Garcia et al. [67] have identified the gene (*rhlG*) encoding the enzyme responsible for draining the fatty acid precursors of rhamnolipid from the general biosynthetic pathway. The RhlG enzyme shows significant sequence homology with numerous NADPH-dependent ketoacyl reductases. Synthesis of the fatty acid moiety of rhamnolipid separates from the *P. aeruginosa* general fatty acid biosynthetic pathway at the level of the ketoacyl reduction [67]. Therefore, the RhlG protein is required for rhamnolipid production, but is not necessary for fatty acid synthesis. Although *rhlR* and *rhlI* regulate expression of the *rhlA* and *rhlB* genes, it is still unclear how the *rhlC* and *rhlG* genes are regulated.

A comparison of the structure of the RhlR and RhlI proteins indicates that they are similar to the LuxR–LuxI type of autoinducer-dependent bacterial regulator system [69]. The LuxR–LuxI system mediates the cell-density-dependent control of luminescence (*lux*) gene expression in the bacteria *Vibrio fischeri*. *P. aeruginosa* uses the autoinducer system to mediate cell–cell communication, thereby regulating the production of exoproducts, including rhamnolipid and other virulence factors, such as proteases and toxins, which are expressed when cell density reaches a high level [71].

4.2 Surfactin

Production of the lipopeptide surfactin is controlled by the expression of the *srf* operon, which encodes for the subunits of the enzyme surfactin synthetase [73]. Surfactin synthe-

tase catalyzes the incorporation of seven amino acids into the peptide moiety of surfactin. The enzyme is a large multifunctional complex which forms the polypeptide by a process referred to as the thiotemplate mechanism for nonribosomal peptide formation [34,75]. This mechanism is similar to the mechanism used by *Bacillus* to produce peptide antibiotics. The mechanism involves the activation of amino acids by ATP to form an aminoacyl-adenylate, which is stabilized by thioester bonds at a specific location on the multisite enzyme. A transport enzyme then moves along the multisite enzyme and assembles the amino acids into a peptide chain. The lipopeptide is then formed by linking the peptide to a β-hydroxy fatty acid using an acyltransferase enzyme.

The regulation of expression of the *srf* operon involves the pheromone ComX and a two component regulatory system composed of ComP and ComA (Fig. 10). The transcription regulation of the *srf* operon is similar to the regulation of the rhamnolipid *rhlA-BRI* genes in that their expression is induced at a high cell density and that the control mechanism involves a diffusible substrate that mediates cell–cell communication. ComX is a peptide pheromone that accumulates to high concentrations when *B. subtilis* has been grown to a late-exponential or stationary growth phase. ComX activates the signal transduction system composed of ComP and ComA. The sensor protein ComP, which is embedded in the cytoplasmic membrane, recognizes ComX and donates a phosphate to the response regulator ComA. In the phosphorylated state, ComA is a transcriptional activator and stimulates the expression of the *srf* operon.

Included in the *srf* operon is a competence regulatory gene *comS* [77]. The *comS* gene product is involved in a complicated process of preparing the cell to be ''competent'' for the assimilation of DNA. Competence is a physiological state in which a cell is able to bind and assimilate DNA and possibly other substances which have been released from lysed cells into the surrounding medium. Therefore, competence and biosurfactant production are both linked to high cell density. Cosby et al. [76] speculated that it is advantageous for the cell to become competent at the same time it produces a lytic agent (surfactin),

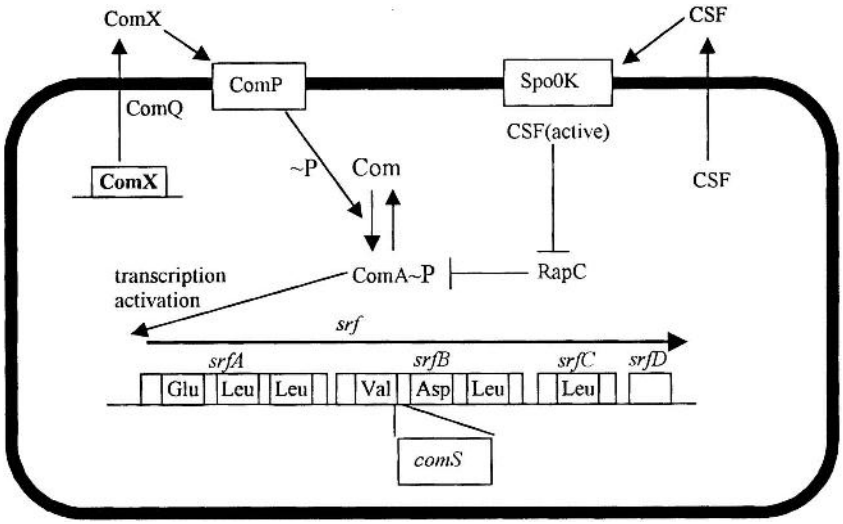


Figure 10 Model for the regulation of surfactin production by *Bacillus subtilis*. (From Ref. 76.)

because the lytic agent can disperse hydrophobic aggregates, thereby aiding their assimilation by competent cells.

At about the same time that *B. subtilis* develops competence, it also begins to sporulate. Sporulation is a developmental state which allows the bacterium to enter a dormancy period in which it can survive adverse conditions such as nutrient deprivation. The regulation of sporulation is highly complicated, but it is tied to competence and surfactin production because some of the regulatory genes required for sporulation are also required for the development of competence and surfactin production. The pheromone CSF (competence and sporulation stimulatory factor) participates in the regulation of sporulation [78]. CSF is believed to be imported into the cell via the oligopeptide permease encoded by the *spoOK* gene. CSF is thought to regulate *srf* transcription by inhibiting the activity of the enzyme RapC. RapC acts to remove the phosphate from the ComA-P signal system, thereby inactivating ComA. The inactivation of ComA is prevented by the involvement of CSF in the inhibition of RapC activity.

5 FACTORS INFLUENCING BIOSURFACTANT PRODUCTION

The role biosurfactants play in the life cycle of microbes living in soil or aquatic habitats is unknown. However, the complex genetic regulatory network just described for rhamnolipid and surfactin suggests that these compounds are of adaptive value to microbes living in the environment. Two factors that are known to impact biosurfactant production are the growth substrate and the stage of growth of the microbe. These factors are discussed next.

5.1 Substrate

Glycolipid biosurfactants are known to play a role in promoting microbial assimilation of substrates with low water solubility, such as hydrocarbons. Therefore, the carbon substrate available influences both the structure of the surfactant produced, as previously discussed, as well as the amount of surfactant produced. For example, a higher level of trehalolipid production by *Rhodococcus* was observed when the bacterium was grown on *n*-alkanes (C_{10} to C_{18}) instead of water-soluble substrates such as glucose or glycerol [31,53,79]. Similarly, Hommel et al. [36] found greater sophorolipid production from *C. apicola* when grown on *n*-alkanes. Finally, the highest levels of rhamnolipid production reported are from hydrophobic substrates like soybean oil (112 g/L [16]) and corn oil (46 g/L [80]) although a high level of production was also reported for the more water-soluble substrate, ethanol (32 g/L [81]). These yields are in comparison to a yield of approximately 2 g/L when grown on glucose [82].

5.2 Growth Stage and Limiting Nutrients

Some surfactants are produced during exponential growth (e.g., surfactin) and others are produced during late-exponential and stationary growth phases (e.g., rhamnolipid). Two factors are important for surfactant production at later growth stages. The first is high cell density, which helps induce the biosynthetic pathway, and the second is growth-limiting conditions that cells experience at this growth stage [25,33,79]. For example, Ochsner et al. [2] demonstrated that rhamnolipid production was stimulated in late-exponential or stationary-growth-phase cells when nitrate in the media had been completely depleted.

Similarly, Hommel et al. [36] showed that most of the sophorolipid production in *C. apicola* occurs in late exponential and in early stationary phases of growth and that increasing the C:N ratio increases lipid formation.

The nutritional balance optimal for glycolipid production has been explored. Promoting the slow growth of cells by limiting essential nutrients, such as Mg, Ca, K, Na, and Fe ions, while maintaining high levels of carbon substrate can increase the production of biosurfactant [83]. Ochsner et al. [2] found that nitrogen- and iron-limiting conditions were modulators of rhamnolipid production and reported that a C-to-N ratio from 15:1 to 23:1 and a C-to-Fe ratio of 60,000:1 were optimal for rhamnolipid production. Mulligan and Gibbs [84] reported a direct correlation between rhamnolipid production and the activity of the enzyme glutamine synthetase. The activity of glutamine synthetase, which is involved in the assimilation of ammonia, will increase when ammonia becomes limiting. In *P. aeruginosa*, glutamine synthetase activity was greatest during rhamnolipid production.

When *Rhodococcus erythropolis* was grown under optimal, nonlimiting conditions, the production of trehalolipid was found to parallel the increase in cell biomass and then ceased when the cells had reached the stationary phase [42]. Altering the media to create a nitrogen-limiting environment and suddenly dropping the incubation temperature at the point where nitrogen was depleted resulted in continued production of the glycolipid into the stationary growth phase. The overproduction of biosurfactant from stationary (or resting)-phase cells has led to the investigation of a two-stage production system. Cells are first grown to the stationary phase under optimal growth conditions. The cells are then washed and transferred into a buffer solution limited in nutrients but containing high levels of hydrocarbon substrate. Resting cells have been shown to efficiently convert the hydrocarbon into trehalolipid exoproduct [42,79,85].

The bacterial production of lipopeptide biosurfactants differs in several important features from production of glycolipid biosurfactants. Lipopeptides are produced during the exponential-growth phase by cells growing on water-soluble substrates such as glucose [38,44]. The production of lipopeptides is not associated with the utilization of hydrocarbons as a growth substrate. For example, the addition of hexadecane to the medium was found to eliminate surfactin production by *Bacillus subtilis* [37]. However, surfactin production is strongly affected by iron concentrations [37,86]. Wei and Chu [86] increased surfactin production by adding iron sulfate to the media of glucose-grown *B. subtilis* ATCC 21332. The bacterium was first grown on media containing limiting levels of iron, and then the medium was spiked with iron, which increased the iron concentration from an initial level of 0.004 m*M* to 1.7 m*M* iron sulfate. The addition of iron resulted in an increase in both biomass and surfactin production. However, iron addition also resulted in a decrease in the pH of the growth medium, which led to the precipitation of the surfactin. Therefore, the addition of iron to slowly growing cells as a technique to enhance surfactin production was effective only if the culture pH was maintained above 5.0 by adding NaOH. The mechanism by which iron is a modulator of surfactin production is not known. Cooper et al. [37] suggested that surfactin could chelate iron, thereby sequestering it away from the cells. This could explain why the addition of iron stimulated growth.

Recent work has also suggested that nitrogen limitation may stimulate surfactin production. In a study by Davis et al. [87], it was found that surfactin yield was highest under nitrate-limited/oxygen-depleted conditions. This system was compared to ammonium-limited/oxygen-depleted, carbon-limited/oxygen-depleted, carbon-limited/aerobic, and nitrogen-limited/aerobic systems.

6 INDUSTRIAL APPLICATIONS FOR BIOSURFACTANTS

Research demonstrates that biosurfactants (e.g., rhamnolipid and sophorolipid) can be commercially produced at levels exceeding 100 g/L [16,88]. At this level, the cost of producing biosurfactants becomes competitive with the cost of producing synthetic surfactants. As the production cost becomes competitive and as the commercial availability of biosurfactants increases, one can expect the commercial use of biosurfactants to grow. Important to note is that only two biosurfactants have been studied extensively. However, the analytical and molecular tools now available make the tasks of identifying new biosurfactants and understanding how and why biosurfactants are produced more feasible and rapid. The following subsections explore various uses of glycolipid and lipopeptide biosurfactants that have been proposed in the scientific literature.

6.1 Bioremediation

Bioremediation is the use of microorganisms and/or plants to aid in the cleanup of sites contaminated with organics, metals, or, in some cases, a mixture of these two types of contaminants. According to a recent National Research Council report [89], the estimated cleanup cost for the United States exceeds $1 trillion. As a result, interest in cleanup alternatives such as bioremediation is increasing. Surfactants, both synthetic and biological, have been explored for their bioremediation potential and show promise for application to sites impacted by both organic and metal contaminants. Biosurfactants have several potential advantages over synthetic surfactants for bioremediation applications. These advantages include low toxicity, better specificity for some applications, and the potential for in situ production of biosurfactants.

6.1.1 Biodegradation of Organics

There is an extensive body of literature documenting the effects of biosurfactants on biodegradation of hydrocarbons, both aliphatic and aromatic. Interestingly, the majority of research has involved glycolipids and little data exist for lipopeptides. Research indicates that glycolipids, in particular rhamnolipids, can enhance biodegradation of a variety of alkanes including hexadecane, octadecane, and *n*-paraffins, as well as phenanthrene in pure culture [6,7,10,28,29,58,90–93]. Further, glycolipids have been shown to enhance degradation in soil systems containing hexadecane [94–96], tetradecane [94], pristane [94], creosote [18], a hydrocarbon mixture [97], crude oil [98], and polychlorinated biphenyls [99]. Although the success of biosurfactants is clearly documented, there are also reports involving both pure culture and soil systems, which suggest that the addition of biosurfactant can inhibit biodegradation. Whether biodegradation is enhanced or inhibited in pure culture seems to be dependent on the degrading genus, wherein the biosurfactant-producing genus is more likely to be stimulated than other genera [90]. However, even biosurfactant-producing strains vary in their response to biosurfactant addition [6,96]. For soils, it is unclear why biosurfactants cause inhibition although recent findings suggest that biosurfactants can serve as a preferred carbon source [13,100,101].

How do biosurfactants enhance biodegradation? Both the structure and the physical state of an organic contaminant affect its bioavailability and, therefore, biodegradation. In general, bioavailability and biodegradation rates increase with increasing aqueous solubility of the contaminant. Biosurfactants can aid in increasing contaminant bioavailability in two ways. First, they can increase contaminant bioavailability either by increasing the

apparent solubility of the contaminant or by increasing the rate of desorption of the contaminant from soil surfaces [7,102]. Second, they can induce alterations in cell surface properties to allow better contact between the cell and the contaminant [29,96,103]. Recent research has further examined the interaction of biosurfactants with degrading cells. Evidence suggests that rhamnolipids cause a loss of lipopolysaccharide (LPS), an important hydrophilic component of the gram-negative cell surface [103]. Loss of LPS results in an increase in the relative hydrophobicity of the cell. Because LPS loss can occur at very low sub-CMC concentrations of surfactant, the second mechanism may be more important for hydrocarbon bioremediation. This is because, although high levels of rhamnolipid are required to substantially increase hydrocarbon solubility, only low levels of rhamnolipid are required to alter the cell surface. This is important for several reasons. First, because rhamnolipids are biodegradable, they can serve as a preferred carbon source in a mixed population. In this case, the addition of high amounts of rhamnolipid would suppress degradation of hydrocarbons until a substantial portion of the rhamnolipid was degraded. Second, lower levels of rhamnolipid are less apt to cause mobilization of hydrocarbons which could result in undesirable spreading of a contaminant plume [95]. Finally, in economic terms, the less material required, the better.

It is estimated that 37% of sites contaminated with organics are also contaminated with metals [104]. The presence of toxic metals, such as lead, cadmium, or arsenic, can cause inhibition of biodegradation of organic compounds [105–107]. Rhamnolipids have been demonstrated to enhance hydrocarbon degradation in systems cocontaminated with organics and toxic metals. This has been shown both in pure culture with cadmium and naphthalene [108] and in two soils amended with cadmium and phenanthrene [13]. In the soil tests, it was found that rhamnolipid biodegradation occurred over 8–12 days. Therefore, rhamnolipid was added in several pulses to each soil such that phenanthrene biodegradation in the cadmium-containing microcosms reached levels similar to the microcosms that contained no cadmium.

6.1.2 Biosurfactants as a Flushing Agent: Organics

Biosurfactants have been studied for their potential as soil washing or flushing additives for organics. In some instances, where biodegradation processes are too slow or infeasible, it may be necessary to remove organics from soil using ex situ soil washing or in situ soil flushing, also known as pump and treat. This pertains to two types of organic compounds: nonaqueous-phase liquids (NAPL) (e.g., petroleum or chlorinated solvents) and solid-phase organics [e.g., polyaromatic hydrocarbons (PAH)]. In both cases, the low aqueous solubility of the organic constrains removal by water alone. Additives that can enhance organic solubility have been researched, including the use of surfactants. Although various synthetic surfactants have been studied in this regard, biosurfactants are of interest because they are biodegradable and nontoxic and, hence, are considered environmentally compatible.

For NAPL, the addition of a surfactant to a flushing solution can enhance flushing efficiency by either mobilization of NAPL which results from a decrease in interfacial tension between the NAPL and the surfactant solution, or by an increase in solubilization of the NAPL. Thus, to be effective, a surfactant must have a good solubilization capacity and/or be able to reduce interfacial tension. Rhamnolipid has been shown to have a solubilization capacity (expressed as a molar solubilization ratio, MSR) for the model NAPL hexadecane that is 20 times greater than the MSR for hexadecane–alkyl benzyl sulfonate

[109]. In studies examining the use of rhamnolipid for removal of residual hexadecane from soil, it was shown that rhamnolipid (20% removal) was more effective than either sodium dodecyl sulfate (SDS) (negligible removal) or Tween-80 (6% removal) [110]. This experiment compared the surfactants on an equal mass basis (500 mg/L) and for an equal number of flushings (100 pore volumes). It was further shown that NAPL removal could be optimized by altering pH and ionic strength to achieve a maximum reduction in interfacial tension. Thus, in the same system, under conditions of pH 6 and 320 m*M* sodium, rhamnolipid removed 60% of residual hexadecane in only 2 pore volumes [111]. A *Rhodococcus* biosurfactant has also been examined for its efficacy in removal of oil from sand [98]. Crude biosurfactant extracts from 10 different *Rhodococcus* strains were tested with 4 different oils. Each strains performed slightly differently in terms of oil removal, with overall removal ranging between 9.8% and 98.0% (in comparison to the removal by water alone, which ranged between 1.9% and 31.3%).

Similar promising results are reported for rhamnolipid solubilization of solid-phase materials. Specifically, the rhamnolipid–octadecane MSR [102] was 10-fold higher than the MSR for Triton X-114–octadecane and 5-fold higher than the MSR for Corexit 0600–octadecane [112]. The MSR for rhamnolipid–phenanthrene ranged from 1.7 times higher to 2.8 times lower than for 13 different synthetic surfactants that have been tested with phenanthrene [28,113,114]. In a comparison of removal of a hydrocarbon mixture from soil, it was found that rhamnolipid performed more effectively than either Triton X-100 or Tween-60 for all hydrocarbon components. The hydrocarbon mixture contained undecane, pentadecane, hexadecane, octadecane, pristane, naphthalene, phenanthrene, and pyrene [115]. Rhamnolipid-enhanced removal of phenanthrene [116,117], pyrene [118], and polychlorinated biphenyls and a variety of PAH [116] from soil has also been demonstrated.

6.1.3 Rhamnolipid as a Flushing Agent: Metals

Sites may be contaminated with metals alone or may be cocontaminated with metals and organics. In either case, in some instances, it may be necessary to remove the toxic metals from the site. There are a variety of agents that have been shown to complex metals. Although the strength of the metal complexation is important, it is also requisite that these materials be environmentally compatible. The strength of the complexation is quantified by determining stability constants for the metal–organic ligand in question. Table 2 compares the stability constants for several different organic ligands that have been proposed for metal remediation. Although synthetic materials such as NTA, EDTA, and DTPA are extremely effective at metal complexation, as indicated by their high stability constants, their use in the field is questionable because of potential toxicity effects. NTA is a Class II carcinogen [120] and DTPA is a potential carcinogen. Both EDTA and DTPA are toxic as measured by invertebrate toxicity tests [121,122]. These effects are suggested to be due to formation of chelates with biologically important trace metals such as manganese, iron, and zinc. Thus, these materials are such effective metal chelates that they remove required as well as toxic metals from the soil. A further concern is biodegradability. EDTA is a decontamination agent that was used in nuclear facilities and routinely buried with radioactive wastes. EDTA's limited biodegradability in the environment is demonstrated by its presence in groundwater near these nuclear facilities [123]. These examples illustrate the importance of choosing environmentally compatible alternatives.

Rhamnolipid is one such alternative [124]. Rhamnolipid is readily degraded as determined by the OECD 301D Ready Biodegradability test [125] and by Maslin and Maier

Table 2 Stability Constants for Various Organic Ligands with Cadmium (Cd) and Lead (Pb)

Organic ligand	Naturally occurring or synthetic	Stability constants[a]	
		Cadmium	Lead
DTPA	Synthetic	19.00	18.66
EDTA	Synthetic	16.36	17.88
NTA	Synthetic	9.78	11.34
Rhamnolipid	Naturally occurring	6.89	8.58
Oxalic acid	Naturally occurring	2.75	4.00
Citric acid	Naturally occurring	2.73	4.08
SDS	Synthetic	1.95	N.D.[b]
Acetic acid	Naturally occurring	1.56	2.15

[a] Stability constants are expressed in log values. All stability constants are from Ref. 119, except for rhamnolipid [51] and SDS (unpublished results).
[b] N.D. = not determined.
Source: Ref. 17.

[13]. Further, invertebrate toxicity tests performed in accordance with OECD 202 [126] on rhamnolipid biosurfactant produced by Jeneil Biosurfactant Co. (Saukville, WI) show that rhamnolipid has very low toxicity. As shown in Table 1, rhamnolipid has significantly better metal complexation abilities than other naturally occurring, environmentally compatible materials. Research suggests that rhamnolipids have high affinity for a variety of metals of concern, including cadmium, copper, lanthanum, lead, and zinc [51,127,128]. Their use to remove metals from soil was demonstrated in bench-scale column experiments for both cadmium [12] and lanthanum (unpublished results). The lipopeptide surfactin was also used to demonstrate removal of zinc and copper from a contaminated soil with a 12.6% oil and grease content [129].

6.2 Use in Industrial Processes

A number of industrial applications have been investigated for biosurfactants. Helle et al. [130] report the use of sophorolipid and rhamnolipid to enhance the heterogeneous enzymatic hydrolysis of cellulose and steam-exploded wood. In this study, sophorolipid was able to increase the hydrolysis of steam-exploded wood by 67% and also decrease the amount of cellulase adsorbed onto the cellulose.

Another application proposed for biosurfactants is as an additive for microbially enhanced oil recovery (MEOR) and cleaning oily sludges from storage tanks. In a thorough review of this topic, Banat [131] described several MEOR field trials that have been performed. In general, the results of these field trials are positive, with increases in oil production ranging from 11% to 250% following injection of microorganisms and nutrients. Similarly, injection of a combination of kerosene and biosurfactant resulted in a 500% increase in oil production. However, as Banat pointed out, it is difficult to provide controls for field studies that isolate each parameter tested. Therefore, it is difficult to conclusively say that the addition of biosurfactants or biosurfactant-producing microbes were the cause of the increased oil recoveries observed. However, as the price of petroleum continues to

increase and petroleum reserves shrink, enhanced oil recovery will receive more attention. Clearly, the use of MEOR is an attractive option that will be worth pursuing in the future.

6.3 Use in Medicine

Biosurfactants are known to have antibiotic properties. In some cases, this property has potential for medical research. For example, Vollenbroich et al. [132] showed that surfactin has antimycoplasma properties useful for protecting cell cultures from mycoplasma infection. Kracht et al. [8] reported the antiviral activity of surfactin. In that case, surfactin effectiveness was dependent on its structure. Surfactin with fatty acid chains of 14 or 15 carbons inactivated an enveloped virus, whereas surfactin with a fatty acid chain of 13 showed little antiviral activity. Finally, Kim et al. [133] described an anti-inflammatory effect of surfactin. This effect occured through the selective inhibition of cytosolic phospholipase A_2 (PLA_2). The PLA_2's are a family of enzymes that catalyze the hydrolysis of the fatty acid ester bonds of membrane phospholipids. The result of the hydrolysis is the formation of inflammatory mediators such as prostaglandins and leukotrienes.

6.4 Production of Fine Chemicals

Biosurfactants and chemically modified biosurfactants are being investigated as alternatives to high-value synthetic chemicals whose use may have toxic environmental impacts. For example, Ishigami and Suzuki [134] reported the synthesis of a pyrenacylester of rhamnolipid (R-PE) for use in monitoring the polarity and fluidity of solid surfaces and the attendant impact of coatings on these surface properties. The R-PE was synthesized to facilitate the use of pyrene, which is one of the most effective fluorescent probes in monitoring the micropolarity and microfluidity of surfaces. However, pyrene alone is difficult to use in aqueous systems because of its extremely low aqueous solubility and its propensity to bind to hydrophobic surfaces.

Rhamnolipids are also a source of stereospecific L-rhamnose, a compound used commercially in the production of high-quality flavor compounds and as a starting material for synthesis of some organic compounds [80]. Rhamnose can be obtained from a variety of sources, including quercitrin from oak bark, naringin from citrus peels, or rutin, which is found in a variety of plants. Rhamnose can also be obtained from rhamnose-containing polysaccharides produced by either plants or microorganisms. However, processing rhamnose from these materials is difficult. Plant sources of rhamnose are bulky and generate unwanted waste products. Rhamnose in polysaccharides must be separated from other sugar components. An alternative to these rhamnose sources is the use of rhamnolipid produced by *Pseudomonas aeruginosa*. Rhamnolipids are excreted by *Pseudomonas* in the late log and stationary phase, allowing easy separation from cells. Once isolated, rhamnolipid can be hydrolyzed to produce a mixture of L-rhamnose and the fatty acid 3-hydroxydecanoic acid [80].

The cosmetic and health care industries use large amounts of surfactants for a wide variety of products, including insect repellents, antacids, acne pads, antidandruff products, contact lens solutions, hair color and care products, deodorants, nail care products, lipstick, eyeshadow, mascara, toothpaste, denture cleaners, antiperspirants, lubricated condoms, baby products, foot care products, antiseptics, shaving and depilatory products, and moisturizers [135]. Biosurfactants in general are considered to have some advantages over synthetic surfactants, the predominant type of surfactant used in this industry. These ad-

vantages are low irritancy or anti-irritating effects and compatibility with skin [135]. Sophorolipids and rhamnolipids, in particular, are being used as cosmetic additives [4,136]. There are currently patents for use of rhamnolipids to make liposomes [137] and emulsions [138], both important in the cosmetic industry. Similarly, there are patents for the use of sophorolipid as a humectant in cosmetics [139,140].

6.5 Biological Control

There are several reports on the efficacy of biosurfactants in biological control. Rhamnolipids have been shown to have activity against zoosporic plant pathogens, specifically *Pythium aphanidermatum*, *Phytophthora capsici*, and *Plasmopara lactucae-radicis*. In a study reported by Stanghellini and Miller [11], monohamnolipid and dirhamnolipid were found to cause loss of motility and lysis of zoospores in less than 1 min. Effective concentrations ranged between 5 and 30 mg/L, depending on the rhamnolipid tested. The same group also demonstrated the efficacy of rhamnolipid in a near-commercial hydroponic recirculating cultural system. In this system, the recirculating tubs were inoculated with a *Pseudomonas* strain isolated from the greenhouse system and received inputs of olive oil as a substrate for growth. Therefore, rhamnolipids were produced in the system. Although positive results were observed, it was noted that results were not consistent in performance and/or sustained control.

Research indicates that surfactin and a similar lipopeptide, iturin A, produced by *Bacillus subtilis* RB14, play a role in the suppression of damping-off disease of tomato seedlings caused by *Rhizoctonia solani* [141]. This group compared the effect of RB14 with a RB14 mutant, RΔ1, deficient in production of surfactin and iturin A, on prevention of damping-off. Results showed that the wild-type suppressed *R. solani* and prevented damping-off, whereas the mutant did not. This result was further confirmed using a transformant of RΔ1 in which the production of iturin A was restored and the production of surfactin was partially restored. This isolate was able to prevent damping-off. Further, this group provides evidence that iturin A is stable in soil for 1–2 weeks and surfactin is stable for even longer periods of time.

7 SUMMARY

Demand for new specialty chemicals in the agriculture, cosmetic, food, pharmaceutical, and environmental industries is steadily increasing. Because these chemicals must be both effective and environmentally compatible, it is natural to turn to the microbial world to try to meet this demand. Microbes offer a largely unexplored variety of chemicals, such as biosurfactants, that have exciting potential for industrial application. However, to realize the application of any new chemical product is difficult. First and foremost is to obtain demonstrations of product efficacy. Then, the new microbial product must be produced at an economical scale and yield, as well as meet regulatory testing requirements. This chapter documents the applications for biosurfactants as additives for environmental remediation, as fine chemicals, as antimicrobial agents, and as biological control agents. Biosurfactants, including rhamnolipid and sophorolipid, can now be produced at high yield in large-scale fermentors and are becoming economically competitive with synthetic surfactant alternatives. Therefore, it seems likely that in the future, biosurfactants will be competitive for certain industrial and medical applications currently employing synthetic surfactants.

ACKNOWLEDGMENTS

Research originating in my (RMM) laboratory and discussed in this chapter was supported by grants from EPA, NIEHS, and DOE.

REFERENCES

1. A Fiechter. Trends Biotechnol 10:208–217, 1992.
2. UA Ochsner, T Hembach, A Fiechter. Adv Biochem Bioeng 53:89–118, 1995.
3. TR Neu. Microbiol Rev 60:151–166, 1996.
4. JD Desai, IM Banat. Microbiol Mol Biol Rev 61:47–64, 1997.
5. AK Koch, O Kappeli, A Fiechter, J Reiser. J Bacteriol 173:4214–4219, 1991.
6. Y Zhang, RM Miller. Appl Environ Microbiol 60:2101–2106, 1994.
7. G Shreve, S Inguva, S Gunnam. Mol Mar Biol Biotechnol 4:331–337, 1995.
8. M Kracht, H Rokos, M Ozel, M Kowall, G Pauli, J Vater. J Antibiot 52:613–619, 1999.
9. S Lang, F Wagner. In: N Kosaric, ed. Biosurfactants: Production, Properties, Applications. New York: Marcel Dekker, 1993, pp 251–268.
10. S Arino, R Marchal, J-P Vandecasteele. J Appl Microbiol 84:769–776, 1998.
11. ME Stanghellini, RM Miller. Plant Dis 81:4–12, 1997.
12. JL Torrens, DC Herman, RM Miller-Maier. Environ Sci Technol 32:776–781, 1998.
13. P Maslin, RM Maier. Biorem J 4:295–308, 2000.
14. CD McClure, NL Schiller. Curr Microbiol 33:109–117, 1996.
15. R Muller-Hürtig, F Wagner R Blaszczyk, N Kosaric. In: N Kosaric, ed Biosurfactants: Production, Properties, Applications. New York: Marcel Dekker, 1993, pp 447–469.
16. S Lang, D Wullbrandt. Appl Microbiol Biotechnol 51:22–32, 1999.
17. RM Maier, G Soberón-Chávez. Appl Microbiol Biotechnol 54:625–633, 2000.
18. MA Providenti, CA Flemming, H Lee, JT Trevors. FEMS Microbiol Ecol 17:15–26, 1995.
19. L Deschenes, P Lafrance, J-P Villeneuve, R Samson. Appl Microbiol Biotechnol 46:638–646, 1996.
20. K Poremba, W Gunkel, S Lang, F Wagner. Environ Toxciol Water Qual 6:157–163, 1991.
21. S Lang, F Wagner. In: N Kosaric, ed. Biosurfactants: Production, Properties, Applications. New York: Marcel Dekker, 1993, pp 391–417.
22. Y Ishigami, Y Gama, H Nagahora, M Yamaguchi, H Nakhara, T Kamata. Chem Lett 5:763–766, 1987.
23. JT Champion, JC Gilkey, H Lamparski, J Retterer, RM Miller. J Colloid Interf Sci 170:569–574, 1995.
24. RM Miller, Y Zhang. In: D Sheehan, ed. Methods in Biotechnology, Vol. 2: Bioremediation Protocols. Totowa, NJ: Humana Press, 1997, pp 59–67.
25. C Syldatk, S Lang, U Matulovic, F Wagner. Z Naturforsch 40c:61–67, 1985.
26. J-H Cho, Y-L Jeong, O-J Park, B-D Yoon, J-W Yang. J Microbiol Biotechnol 8:645–649, 1998.
27. M Robert, ME Mercade, MP Bosch, JL Parra, MJ Espuny, MS Manresa, J Guinea. Biotechnol Lett 11:871–874, 1989.
28. Y Zhang, WJ Maier, RM Miller. Environ Sci Technol 31:2211–2217, 1997.
29. Y Zhang, RM Miller. Appl Environ Microbiol 61:2247–2251, 1995.
30. A Kretschmer, F Wagner. Biochim Biophys Acta 753:306–313, 1983.
31. MJ Espuny, S Egido, I Rodon, A Manresa, ME Mercade. Biotechnol Lett 18:521–526, 1996.
32. MEV Singer, WR Finnerty, A Tunelid. Can J Microbiol 36:746–750, 1990.
33. E Vollbrecht, R Heckman, V Wray, M Nimtz, S Lang. Appl Micrbiol Biotechnol 50:530–537, 1998.
34. Z-Y Li, S Lang, F Wagner, L Witte, V Wray. Appl Environ Microbiol 48:610–617, 1984.
35. DG Cooper, DA Paddock. Appl Environ Microbiol 47:173–176, 1984.

36. RK Hommel, O Stuwer, W Stuber, D Haferburg, H-P Kleber. Appl Microbiol Biotechnol 26:199–205, 1987.
37. DG Cooper, CR MacDonald, JB Duff, N Kosaric. Appl Environ Microbiol 42:408–412, 1981.
38. MM Yakimov, KN Timmis, V Wray, HL Fredrickson. Appl Environ Microbiol 61:1706–1713, 1995.
39. MJ McInerney, M Javaheri, DP Nagle Jr. J Ind Microbiol 5:95–102, 1990.
40. M Morikawa, H Daido, T Takao, S Murata, Y Shimonishi, T Imanaka. J Bacteriol 175:6459–6466, 1993.
41. S-C Lin, MA Minton, MM Sharma, G Georgiou. Appl Environ Microbiol 60:31–38, 1994.
42. S Lang, JC Philip. Antonie van Leeuwenhoek 74:59–70, 1998.
43. A Kretschmer, H Bock, R Wagner. Appl Eviron Microbiol 44:864–870, 1982.
44. GE Jenneman, MJ McInerney, RM Kanpp, JB Clark, JM Feero, DE Revus, EE Menzie. Dev Ind Microbiol 24:485–492, 1983.
45. C Syldatk, S Lang, F Wagner, V Wray, L Witte. Z Naturforsch 40c:51–60, 1985.
46. S Arino, R Marchal, J-P Vandecasteele. Appl Microbiol Biotechnol 45:162–168, 1996.
47. CG DeKoster, B Vos, C Versluis, W Heerma, J Haverkamp. Biol Mass Spectrom 23:179–185, 1994.
48. NB Rendell, GW Taylor, M Somerville, H Todd, R Wilson, PJ Cole. Biochim Biophys Acta 1045:189–193, 1990.
49. E Deziel, F Lepine, D Dennie, D Boismenu, OA Mamer, R Villemur. Biochim Biophys Acta 1485:145–152, 1999.
50. T Hirayama, I Kato. FEBS Lett 139:81–85, 1982.
51. F Ochoa-Loza. PhD dissertation, University of Arizona, Tucson, 1998.
52. RK Hommel, C Ratledge. In: N. Kosaric, ed. Biosurfactants: Production, Properties, Applications. New York: Marcel Dekker, 1993, pp 3–63.
53. P Rapp, H Bock, V Wray, F Wagner. J Gen Microbiol 115:491–503, 1979.
54. MT Madigan, JM Martinko, J Parker. Brock Biology of Microorganisms. 8th ed. Englewood Cliffs, NJ: Prentice-Hall, 1997, pp 734–736.
55. E Ristau, F Wagner. Biotechnol Lett 5:95–100, 1983.
56. Y Uchida, R Tsuciya, M Chino, J Hirano, T Tabuchi. Agric Biol Chem 53:757–763, 1989.
57. DG Cooper, DA Paddock. Appl Environ Microbiol 46:1426–1429, 1983.
58. S Ito, S Inoue. Appl Environ Microbiol 43:1278–1283, 1982.
59. L Weber, J Stach, G Haufe, R Hommel, H-P Kleber. Carbohydr Res 206:13–19, 1990.
60. RK Hommel, L Weber, A Weiss, U Himmelreich, O Rilke, HP Kleber. J Biotechnol 33: 147–155, 1994.
61. M Javaheri, GE Jenneman, MJ McInerney, RM Knapp. Appl Environ Microbiol 50:698–700, 1985.
62. MM Burger, L Glaser, RM Burton. J Biol Chem 238:2595–2602, 1963.
63. XM Jiang, B Neal, F Santiago, SJ Lee, LK Romana, PR Reeves. Mol Microbiol 5:695–713, 1991.
64. K Marumo, L Lindqvist, N Verma, A Weintraub, PR Reeves, AA Lindberg. Eur J Biochem 204:539–545, 1992.
65. HL Rocchetta, LL Burrows, JS Lam. Microbiol Mol Biol Rev 63:523, 1999.
66. BH Rehm, N Kruger, A Steinbuchel. J Biol Chem 273:24,044–24,051, 1998.
67. J Compos-Garcia, AD Caro, R Najera, RM Miller-Maier, RA Al-Tahhan, G Soberon-Chavez. J Bacteriol 180:4442–4451, 1998.
68. UA Ochsner, A Fiechter, J Reiser. J Biol Chem 269:19,787–19,795, 1994.
69. UA Ochsner, AK Koch, A Fiechter, J Reiser. J Bacteriol 176:2044–2054, 1994.
70. UA Ochsner, J Reiser. Proc Natl Acad Sci USA 92:6424–6428, 1995.
71. JP Pearson, EC Pesci, BH Iglewski. J Bacteriol 179:5756–5767, 1997.

72. R Rahim, C Olvera JS Lam, G Soberón-Chávez. Proceedings Pseudomonas. E99: Biotechnology and Pathogenesis, Maui, Hawaii, 1999.
73. F Peypoux, JM Bonmatin, J Wallach. Appl Microbiol Biotechnol 51:553–563, 1999.
74. H Kleinkauf, H von Dohren. Eur J Biochem 192:1–5, 1990.
75. S Doekel, MA Marahiel. Chem Biol 7:373–384, 2000.
76. WM Cosby, D Vollenbroich, OH Lee, P Zuber. J Bacteriol 180:1438–1445, 1998.
77. LW Hamoen, H Eshuis, J Jongbloed, G Venema Sinderen, D Van. Mol Microbiol 15:55–63, 1995.
78. JM Solomon, BA Lazazzera, AD Grossman. Genes Dev 10:2014–2024, 1996.
79. Kim, J-S, M Powalla, S Lang, F Wagner, H Lunsdorf, V Wray. J Biotechnol 13:257–266, 1990.
80. RJ Linhardt, R Bakhit, L Daniels, F Mayerl, W Pickenhagen. Biotechnol Bioeng 33:365–368, 1989.
81. M Matsufuji, K Nakata, A Yoshimoto. Biotechnol Lett 19:1213–1215, 1997.
82. LH Guerra-Santos, O Käppeli, A Fiechter. Appl Environ Microbiol 48:301–305, 1984.
83. LH Guerra-Santos, O Käppeli, A Fiechter. Appl Microbiol Biotechnol 24:443–448, 1986.
84. CN Mulligan, BF Gibbs. Appl Environ Microbiol 55:3016–3019, 1989.
85. S Lang, F Wanger. In: N. Kosaric, ed. Biosurfactants: Production, Properties, Applications. New York: Marcel Dekker, 1993, pp 205–227.
86. Y-Y Wei, I-M Chu. Enzyme Microb Technol 22:724–728, 1998.
87. DA Davis, HC Lynch, J. Varley. Enzyme Microb Technol 25:322–329, 1999.
88. U Rau, C Manzke, F Wagner. Biotechnol Lett 18:149–154, 1996.
89. National Research Council. In Situ Bioremediation: When Does It Work? Washington DC: National Academy Press, 1993.
90. K Hisatsuka, T Nakahara, N Sano, K Yamada. Agric Biol Chem 35:686–692, 1971.
91. S Itoh, T Suzuki. Agric Biol Chem 36:2233–2235, 1972.
92. Y Zhang, RM Miller. Appl Environ Microbiol 58:3276–3282, 1992.
93. SA Churchill, RA Griffin, LP Jones, PF Churchill. J Environ Qual 24:19–28, 1995.
94. DK Jain, H Lee, JT Trevors. J Ind Microbiol 10:87–93, 1992.
95. DC Herman, RJ Lenhard, RM Miller. Environ Sci Technol 31:1290–1294, 1997.
96. DC Herman, Y Zhang, RM Miller. Appl Environ Microbiol 63:3622–3627, 1997.
97. A Oberbremer, R Müller-Hurtig, F Wagner. Appl Microbiol Biotechnol 32:485–489, 1990.
98. IB Ivshina, MS Kuyukina, JC Phip, N Christofi. World J Microbiol Biotechnol 14:711–717, 1999.
99. PM Golyshin, HL Fredrickson, L Giuliano, R Rothmel, KN Timmis, MM Yakimov. Microbiologica 22:257–267, 1999.
100. TPA Bregnard, P Hohener, J Zeyer. Environ Toxicol Chem 17:1222–1229, 1998.
101. C Vipulanandan, XP Ren. J Environ Eng 126:629–634, 2000.
102. RM Miller. In: H Skipper, R. Turco, eds. Bioremediation—Science and Applications. Madison, WI: Soil Science Society of America, 1995, pp 33–54.
103. RA Al-Tahhan, TR Sandrin, AA Bodour, RM Maier. Appl Environ Microbiol 66:3262–3268, 2000.
104. W Kovalick. Abstracts 4th World Congress of Chemical Engineering, Karlsruhe, Germany, 1991, Abstract 3.3–1.
105. MN Hughes, RK Poole. In: RK Poole, GM Gadd, eds. Metal Mimicry and Metal Limitations in Studies of Metal–Microbe Interactions. Oxford: IRL Press, 1989, pp 1–17.
106. T Duxbury. FEMS Microbiol Lett 11:217–220, 1981.
107. E Baath. Water Air Soil Poll 47:335–379, 1989.
108. TR Sandrin, AM Chech, RM Maier. Appl Environ Microbiol. In press.
109. S Thangamani, GS Shreve. Environ Sci Technol 28:1993–2000, 1994.
110. G Bai, ML Brusseau, RM Miller. J Contam Hydrol 25:157–170, 1997.

111. G Bai, ML Brusseau, RM Miller. J Contam Hydrol 30:265–279, 1998.
112. LT Thai, WJ Maier. Proceedings of the 47th Annual Purdue University Industrial Waste Conference, Chelsea, MI, 1992.
113. DA Edwards, RG Luthy, Z Liu. Environ Sci Technol 25:127–133, 1991.
114. A Tiehm. Appl Environ Microbiol 60:258–263, 1994.
115. K Scheibenbogen, RG Zytner H Lee JT Trevors. J Chem Technol Biotechnol 59:53–59, 1994.
116. MI Van Dyke, P Couture, M Brauer, H Lee, JT Trevors. Can J Microbiol 39:1071–1078, 1993.
117. WH Noordman, W. Ji, ML Brusseau, DB Janssen. Environ Sci Technol 32:1806–1812, 1998.
118. P Lafrance, M Lapointe. Ground Water Monitoring and Remediation 18:139–147, 1998.
119. AE Martell, RM Smith. Critical Stability Constants, Vols. 1 and 3. New York: Plenum Press, 1976.
120. RW Peters. J Haz Materials 66:155–210, 1999.
121. U Borgmann, WP Norwood. Can J Fisheries Aquat Sci 52:875–881, 1995.
122. RA van Dam, MJ Barry, JT Ahokas, DA Holdway. Aquat Toxicol 46:191–210, 1999.
123. MH Baik, KF Lee. Ann Nucl Energy 21:81–96, 1994.
124. RM Miller. Environ Health Perspect 103:59–62, 1995.
125. OECD's Guidelines for the Testing of Chemicals Section 3. www.oecd.org/ehs/test/degrad.htm.
126. OECD's Guidelines for the Testing of Chemicals Section 2. www.oecd.org/ehs/test/Biotic.htm.
127. H Tan, JT Champion, JF Artiola, ML Brusseau, RM Miller. Environ Sci Technol 28:2402–2406, 1994.
128. DC Herman, JF Artiola, RM Miller. Environ Sci Technol 29:2280–2285, 1995.
129. CN Mulligan, RN Yong, BF Gibbs, S James, HPJ Bennett. Environ Sci Technol 33:3812–3820, 1999.
130. SS Helle, SJB Duff, DG Cooper. Biotechnol Bioeng 42:611–617, 1993.
131. IM Banat. Bioresource Technol 51:1–12, 1994.
132. D Vollenbroich, G Pauli, M Ozel, J Vater. Appl Environ Microbiol 63:44–49, 1997.
133. K Kim, SU Jung, KK Lee, J-K Jung, JK Park, KK Kim, C-H Lee. Biochem Pharm 55:975–985, 1998.
134. Y Ishigami, S Suzuki. Prog Org Coat 31:51–61, 1997.
135. V Kleckner, N Kosaric. In: N Kosaric, ed. Biosurfactants, Production, Properties, Applications. Surfactant Science Series Vol. 48, New York: Marcel Dekker, 1993, pp 373–389.
136. Personal communication, Ichiro Matsuura, Iwata Co., Japan.
137. Y Ishigami, Y Gama, H Nagahara, T Motomiya, M Yamaguchi. Japanese Patent Kokai 63–182,029, 1988.
138. Y Ishigami, Y Gama, Y Uji, K Masui, Y Shibayama. Japanese Patent Kokai 63–77,535, 1988.
139. S Inoue, Y Kimwa, M Kinta (to Kao Soap Co, Japan) German Patent 2905252, 1979.
140. S Inoue, Y Kimwa, M Kinta (to Kao Soap Co., Japan) German Patent 2905295, 1979.
141. O Asaka, M Shoda. Appl Environ Microbiol 62:4081–4085, 1996.

33

Application of Lipolytic Enzymes in Detergents

MAARTEN R. EGMOND

University of Utrecht, Utrecht, and Unilever Research, Vlaardingen, The Netherlands

1 INTRODUCTION

The use of enzymes as an aid in fabric stain removal dates back to the early sixties. In those days, chances for sustained enzyme action during fabric washing were rather limited, because washing was performed at relatively elevated temperatures (i.e., around 60°C and higher). Proteolytic enzymes were added mainly with the aim to prevent redeposition of denatured proteins to the fabric. Since then, washing habits have changed. For instance, the washing temperatures were lowered to around 40°C in Europe and the United States due to economic and environmental reasons. This improved the possibilities for the application of other enzymes [1] such as lipases. Although washing systems already contain surfactants to dissolve greasy stains, the need for additional detergency was found to be stronger when the temperature was lowered. Lipases, or lipolytic enzymes in general, can bring about this additional action under proper conditions (e.g., see Ref. 2 for a recent overview). Greasy stains (such as food stains, cosmetics stains like lipstick, and body stains like sebum) usually consist of proteins, lipids, starch, and pigments, requiring the combined action of several enzymes and many other detergent components for optimal stain removal. As a consequence, the application of lipases for greasy stain removal needs to take into account that other enzyme activities are present—apart from the need to optimize lipolytic action in the presence of surfactants. For optimal lipase action in concert with other enzyme activities, tailor-made lipases need to be developed. Protein engineering of lipolytic enzymes, either using random mutagenesis or site-directed methods, provides the facilities to accomplish this objective. Particularly for site-directed methods, the recent advances in our understanding of structural and functional properties of many lipases

provide a strong asset in the endeavor to yield optimal lipolytic action in greasy stain removal from fabrics.

2 APPLICATION ISSUES

2.1 Lipolase

The first lipase to appear on the market for application as an aid in stain removal from fabrics was Lipolase™ [3]. Novo Nordisk obtained this breakthrough in 1987 after having transferred the lipase gene from the original organism *Humicola lanuginosa* (or *Thermomyces lanuginosus*) to an *Aspergillus* host species. The enzyme (molecular mass 24.5 kDa excluding glycosylation) was found to be stable under application conditions in the presence of proteolytic enzyme activity [4]. However, the contribution of Lipolase activity in single-wash tests was found to be very modest indeed [3]. Only after repeated wash and dry cycles, the benefit of lipolytic action was observed. As it turned out, lipolytic action of greasy stains is strongly inhibited by the surfactants present in the washing liquor. Also, the presence of sequestrants added to lower free-calcium concentrations in the washing systems resulted in decreased activity of Lipolase. Despite these drawbacks, however, no other lipolytic enzyme has replaced Lipolase since its introduction.

Figure 1 shows the structure of *Humicola lanuginosa* lipase (HLL) that was resolved recently (pdb code 1 tib). The structural model indicates that the active-site residues (Ser146, Asp201, and His258) are not accessible to solvent, requiring a helical element of the molecule to move away (the so-called ''flap'' or ''lid''). The open structure exposes a large hydrophobic surface ideally suited for substrate binding. This essential conformational change was identified indeed [5] and is often referred to as interfacial activation, the onset of catalytic activity of lipases brought about by the presence of a lipid–water interface (e.g., a micellar interface or an oil–water emulsion) [6]. The rather complex kinetic behavior of lipases is generally accepted to be related with the presence of a flap

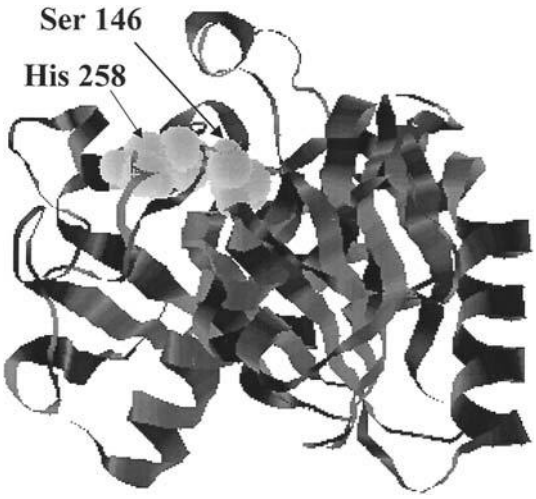


Figure 1 Structure of *Humicola lanuginosa* lipase (HLL) with active-site residues Ser146 and His258 indicated and the helical lid covering these active-site residues.

and explains, at least in part, why lipase activity is highly dependent on the presence of surfactants.

2.2 *Pseudomonas* Lipases

Even before the introduction of Lipolase into the detergent market, screening studies of a series of microbial organisms yielded a high-performing lipase from *Pseudomonas glumae* (now known as *Burkholderia glumae*, formerly also known as *Pseudomonas gladioli* or *Chromobacterium viscosum*) [7]. In multicycle-wash tests, *Pseudomonas glumae* lipase (PGL; molecular mass 33 kDa) showed superior wash performance relative to other lipases, among which is the lipase from *Humicola lanuginosa* [8]. The data obtained are reproduced in Figure 2.

Surfactants present in the wash liquor also inhibit PGL, like Lipolase, leading to poor lipase performance when tested in single washes. However, in repeated wash tests, PGL is apparently more robust, leading to a higher wash performance. Where Lipolase was found to be sensitive toward nonionic surfactants, PGL is strongly inhibited by anionic surfactants such as linear alkyl sulfonates (LAS). The adverse effect of anionic surfactants becomes even more pronounced in the presence of proteolytic activity of the subtilisin from *Bacillus lentus*, Savinase™. Ever since the sixties, proteases have been added to the detergent liquor with the aim of removing proteinaceous stains from fabrics. PGL activity is irreversibly lost due to the proteolytic cleavage of a single peptide bond, in later studies identified to be the Ser153–His154 linkage [9]. In this work, mutations were made around the cleavage site with the aim of improving the proteolytic stability of PGL. A threefold to fivefold higher stability was obtained upon the mutations His154Pro, Ser153Arg/

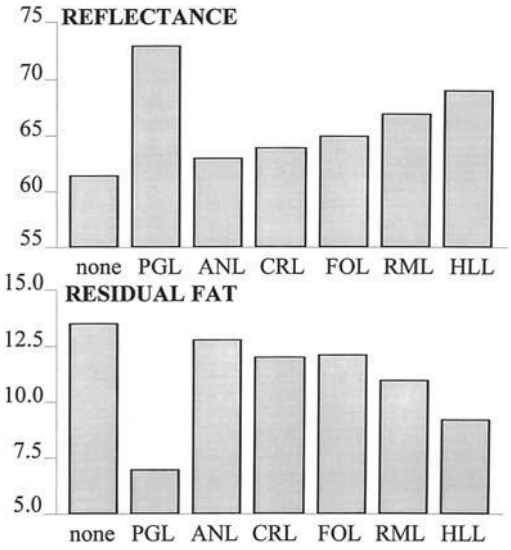


Figure 2 Performance of several lipases in fatty stain removal. The top panel gives an indication of cleanliness (reflectance in arbitrary units) and the bottom panel shows the percentage residual fat after four wash cycles. PGL, *Pseudomonas glumae* lipase; ANL, *Aspergillus niger* lipase; CRL, *Candida rugosa* lipase; FOL, *Fusarium oxysporum* lipase; RML, *Rhizomucor miehei* lipase; HLL, *Humicola lanuginosa* lipase.

His154Arg, or Ser153Arg/His154Pro. Although proteolytic cleavage was not completely abolished by these mutations, the breakdown of PGL was considerably slowed down. A clue to the high proteolytic sensitivity of the residues near positions 150–155 was provided when the crystal structure of PGL was resolved (pdb code 1tah [10] or 1qge [11]). The residues mentioned are located in a flexible region that links helix 5 (the ''flap'') with the main body of the lipase, as shown in Figure 3.

The flap opening was considered to be essential for lipolytic activity to occur, as the active site (containing the triad Ser87, Asp263, and His285) is deeply buried in the closed form of PGL. This hypothesis was later confirmed when the crystal structure of the open form of yet another, but highly homologous, *Pseudomonas* lipase (*Burkholderia cepacia* lipase, pdb code 3lip) was discovered [12,13]. The structure of the latter enzyme is also shown in Figure 3, demonstrating the complete rearrangement of the top right half of the structure when opening up to allow access to the active-site residues. Although the stabilized PGL variants were shown to provide lipolytic action in washing liquor containing proteolytic activity [8], these lipases were apparently not considered of interest for application in those early days. A relatively poor production level was one of the stumble blocks limiting applicability, even when the original host system was used. Recently, a new variant *Pseudomonas alcaligenes* lipase has appeared on the market called Lipomax. This *Pseudomonas* lipase was manufactured originally by Gist-Brocades on a large scale (see Ref. 14 and references cited therein), but so far failed application on a large scale in detergent systems.

In more general terms, the complexity of lipolytic action displayed by the so-called ''true'' lipases containing a buried active site (such as HLL, PGL, and PCL) prompted a search for simpler esterases, possibly with an accessible active site not requiring major conformational changes. This search yielded the lipolytic esterase cutinase as a likely candidate for application in the presence of detergents.

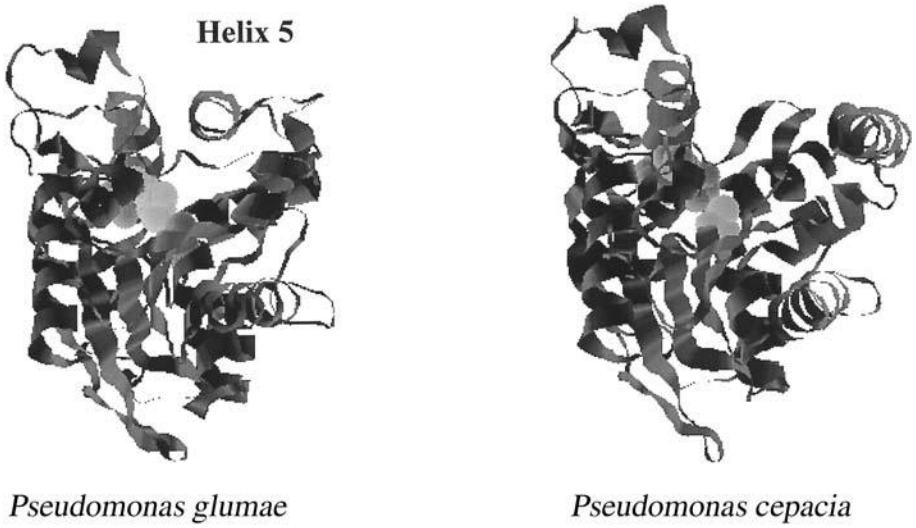


Figure 3 PGL (left-hand model) and PCL (right-hand model) representing the closed and open forms of *Pseudomonas* lipases, respectively.

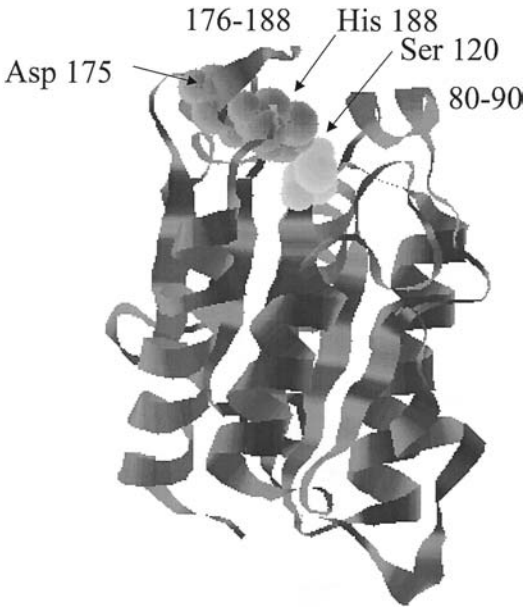


Figure 4 Structure of cutinase with active-site residues Ser120, Asp175, and His188. The hydrophobic regions close to the active site are indicated (80–90, 176–188).

2.3 Cutinase

The wild-type cutinase (molecular mass 20.6 kDa) is secreted by a plant pathogenic fungus *Fusarium solani pisi*. The enzyme was named after its ability to degrade the water-insoluble biopolyester cutin [15], but cutinase also displayed high catalytic activity on a broad range of triacylglycerols. In 1992, the structure of cutinase was resolved (code 1 agy [16]) and subsequently refined to 1.0 Å resolution (code 1 cex [17]). A three-dimensional model of cutinase is shown in Figure 4.

The active-site residues are readily accessible to solvent without a requirement for major conformational changes. A closer look at amino acid residues around the active site shows that many of these are hydrophobic in nature (i.e., Leu81, Leu86, Leu176, Val177, Leu182, Ile183, Val184) and also readily solvent-accessible. For a water-soluble enzyme like cutinase acting on poorly water-soluble lipid substrates, hydrophobic amino acids should be available to interact with the substrate, but too many of these hydrophobic residues would severely limit water solubility. Interestingly, several highly exposed and positively charged residues surrounding the active site (including Arg17, Arg78, Arg88, Arg96, Arg138, Arg156, Arg158, Arg166, Lys168, Arg196) may further improve the water solubility of cutinase and help to remove negatively charged reaction products from the active site.

2.4 Interaction of Cutinase with Surfactants

Although it was originally anticipated that cutinase would only modestly interact with surfactants, the combined surface hydrophobicity near the active site and accumulation

of positively charged residues actually provided the ideal basis for strong interaction with anionic surfactants. Below the critical micellar concentration (CMC) of anionic surfactants, such as sodium dodecyl sulfate (SDS), cutinase forms mixed micelles with a total mass of 86 kDa [18]. Unfortunately, cutinase is unstable and reversibly unfolds under these conditions [18,19]. This work shows that strong interactions between lipases and surfactants (e.g., charged) are not exclusively correlated with the presence of a flap in the lipase molecules. A comparable behavior has previously been found for phospholipases A_2 [20]. For cutinase, the interaction with SDS diminishes with increasing pH, or when the overall charge is made more negative by site-directed mutagenesis [9]. Recently, the effect of several surfactants was tested on cutinase adsorbing to a nonhydrolyzable lipid layer [21]. These studies revealed strong interference by SDS, but less so by bile salts such as taurodeoxycholate. Interestingly, zwitterionic surfactants, such as *n*-alkylphosphorycholine derivatives, did not prevent cutinase adsorption nor showed any adverse effects on cutinase integrity, whereas negatively charged *n*-alkylphosphoglycols only gave rise to modest effects on adsorption and stability.

2.5 Removal of Fatty Material After Lipolysis

Obviously, the application of lipases to improve detergency of washing systems only pertains to hydrolysis of fatty esters, not to their removal from fabrics. It is generally believed that the hydrolysis products are sufficiently water soluble to be washed away by the detergent product. Recent studies, however, have shown that calcium soap formation in detergent systems retaining high free-calcium levels severely limits detergency in single washes [22]. In this work, lipolysis was optimized by sequentially dosing lipases and surfactants, thus avoiding strong inhibition of lipolytic action. Due to its requirement for calcium, Lipolase did not perform properly at low calcium levels or gave rise to calcium soap formation limiting detergency at high calcium levels. In contrast, cutinase not requiring calcium for lipolytic action hydrolyzed 65% of the material present during a half-hour wash, leading to complete removal of the reaction products in the next step at pH 10 or above.

3 CONCLUDING REMARKS

The state of the art of lipase application in detergent systems demonstrates that the limited lipolytic action during single washes only marginally adds to general detergency—if at all—unless precautions are taken. Multiple soil and wash application tests have benefited from the continued lipolytic action after the wash processes. One way to boost lipolysis during the wash, for instance, is a sequential dosing process, but free-calcium levels should be kept low to prevent calcium soap formation. It is clear that full benefits from lipolytic action in detergency can only be obtained when the detergent system is adapted to the lipases being applied. This can be accomplished by using compatible surfactants or other compounds aiding in fatty soil removal or by modifying the washing process itself.

ACKNOWLEDGMENTS

The author wishes to acknowledge the very fruitful collaboration and discussion with Dr. H.T.W.M. van der Hijden, Dr. J.A.C. Flipsen, Dr. M.L.M. Mannesse, and Dr. C.T. Verrips.

REFERENCES

1. C Dambmann, P Holm, V Jensen, MH Nielsen. Dev Ind Microbiol 12:11–23, 1971.
2. AM Wolff, MS Showell. In: JH van Ee, O Misset, EJ Baas, eds. Enzymes in Detergency. New York: Marcel Dekker, 1997, pp 93–106.
3. E Boel, IB Huge-Jensen (to Novo). European Patent Applic EP-305216, 1989.
4. D Aaslyng, E Gormsen, H Malmos. J Chem Tech Biotechnol 50:321–330, 1991.
5. AM Brzozowski, U Derewenda, ZS Derewenda, GG Dodson, DM Lawson, JP Turkenburg, F Bjorkling, B Huge-Jensen, SA Patkar, L Thim. Nature 351:491–494, 1991.
6. OG Berg, Y Cajal, GL Butterfoss, RL Grey, MA Alsina, BZ Yu, MK Jain. Biochemistry 37: 6615–6627, 1998.
7. D Thom, T Swarthoff, J Maat (to Unilever). European Patent Applic EP-A-206390, 1986.
8. MR Egmond, WP Antheunisse, CJ van Bemmel, P Ravestein, LGJ Frenken, MEM Noble, LN Johnson. In: FX Malcata, ed. Engineering of/with Lipases. 1995, pp 103–113.
9. MR Egmond, J de Vlieg, HM Verheij, GH deHaas. In: FX Malcata, ed. Engineering of/with Lipases. Dordrecht: Kluwer Academic Publishers; 1995, pp 193–202.
10. MEM Noble, A Cleasby, LN Johnson, MR Egmond, LGJ Frenken. Protein Eng 7:559–562, 1994.
11. DM Lang, B Hofmann, L Haalck, HJ Hecht, F Spener, RD Schmid, D Schomburg. J Mol Biol 259:704–717, 1996.
12. JD Schrag, YG Li, M Cygler, DM Lang, T Burgdorf, HJ Hecht, R Schmid, D Schomburg, TJ Rydel, JD Oliver, LC Strickland, CM Dunaway, SB Larson, J Day, JA McPherson. Structure 5: 187–202, 1997.
13. KK Kim, HK Song, DH Shin, KY Hwang, SW Suh. Structure 5:173–185, 1997.
14. O Misset. In: JH vanEe, O Misset, EJ Baas, eds. Enzymes in Detergency. New York: Marcel Dekker, 1997, pp 107–131.
15. W Koller, PE Kolattukudy. Biochemistry 21:3083–3090, 1982.
16. C Martinez, P deGeus, M Lauwereys, G Matthyssens, C Cambillau. Nature 356:615–618, 1992.
17. S Longhi, M Czjzek, V Lamzin, A Nicolas, C Cambillau. J Mol Biol 268:779–799, 1997.
18. MR Egmond. In: FX Malcata, ed. Engineering of/with Lipases. 1995, pp 183–191.
19. DJ Pocalyko, M Tallman. Enzyme Microb Technol 22:647–651, 1998.
20. GH deHaas, GJM van Scharrenburg, AJ Slotboom. Biochemistry 26:3402–3408, 1987.
21. JAC Flipsen. The Action of Cutinase at Oil–Water Interfaces. PhD dissertation, University of Utrecht, The Netherlands, 1998.
22. JAC Flipsen, ACM Appel, HTWM van der Hijden, CT Verrips. Enzyme Microb Technol 23: 274–280, 1998.

34

Supercritical Fluid Technology for Lipid Extraction, Fractionation, and Reactions

JERRY W. KING

National Center for Agricultural Utilization Research, Agricultural Research Service, U.S. Department of Agriculture, Peoria, Illinois

1 INTRODUCTION

Critical fluids are substances held above their critical temperature (T_c) and pressure (P_c) or liquids sustained in their liquid state by the application of pressure, which can be used for the extraction of natural products or as an alternative reaction medium. By far the most utilized critical fluid has been supercritical carbon dioxide (SC-CO_2) or its liquified analogue (LCO_2), due to its benign effect on the environment, low toxicity, nonflammability, and compatibility with processed foodstuffs. Several well-known applications of the technology exist, including the decaffeination of coffee [1], extraction of hop essence for flavoring [2], production of spice and aroma concentrates [3], and isolation of natural antioxidants [4]. More recently, critical fluids have been applied for the production of fine particles, or as a versatile reaction medium, and for the modification of novel materials such as polymers and cements.

This chapter is concerned with applying critical fluids for the extraction, fractionation, and reaction of lipid moieties, particularly those operations which have implications for lipid biotechnology. An excellent example of combining these two technologies is the use of critical fluids with enzymatic catalysis to produce unique lipid materials. This will

Names are necessary to report factually on available data; however the USDA neither guarantees nor warrants the standard of the product, and the use of the name by USDA implies no approval of the products to the exclusion of others that may also be suitable.

be illustrated with examples from the author's research [5] and further discussed in Chapter 35. Both critical fluid technology and lipid biotechnology share in common the goal to modify natural products, thereby producing either a superior product or a process that is both consumer and environmentally acceptable. Today, it is estimated that there are over 44 production plants employing critical fluid technology throughout the world, and the technology is currently receiving additional interest with the development of the nutraceutical and functional food markets [6].

Supercritical fluid extraction (SFE) became an industrial reality in the early 1970s, but as we shall see later, it is a technique which has limitations with respect to the resolution that it can achieve between molecular species. Modern trends in supercritical fluid processing emphasize fractionation schemes that can separate lipid species having similar physical properties or the enrichment of target lipid compounds from complex oleochemical mixtures. Reaction chemistry in supercritical agents such as SC-CO_2 is not limited to enzymatically catalyzed transformations, but includes hydrogenation and hydrolysis of lipids utilizing mixtures of binary fluids [6] and subcritical water [7], respectively. Such processes support the hypothesis of ''green'' processing and products which are recognized by the general public as highly desirable.

In the next section, some of the fundamental principles which govern the application of critical fluids in SFE and supercritical fluid fractionation (SFF) will be discussed. The use of SC-CO_2 will be emphasized because of its use in industry, low cost, and the favorable critical properties. Then, the mechanics of SFE, generic equipment requirements, and examples of this technique will be presented. This will be followed by fractionation methodology utilizing columnar fractionation approaches and preparative chromatography. Finally, the use of the critical fluids as a reaction medium will be presented with examples of how this can be coupled with SFE and SFF.

It should be noted that critical fluids have also found widespread application for the analysis of lipids both in extraction (SFE) [8] and chromatographic mode (SFC) [9]. To cover this subject in detail would require a separate chapter; hence, we will only note this application as it supports research and development in the above-mentioned areas. Indeed, analytical-scale SFE and SFC can be used to model extraction and reaction processes in critical fluids and can be used in a combinatorial fashion [10] to improve extraction efficiency and select catalysts for use in supercritical media. However, their most important application in analytical chemistry has been as an alternative extraction technique to replace organic-solvent-intensive methodology, thereby reducing the use of hazardous solvents in the laboratory environment [11].

2 FUNDAMENTALS OF CRITICAL FLUID TECHNOLOGY AS APPLIED TO LIPIDS

The conditions defining a supercritical fluid (SF) are frequently described by a region of the phase diagram of pressure versus temperature for the common critical fluid, CO_2. This is shown in Figure 1, in which the SF region in the upper right-hand sector is defined by carbon dioxide's T_c (31°C) and P_c (1070 psi or 73 atm). It should be emphasized that this is an arbitrary definition, because others define the SF state as simply being above the critical temperature of a substance. This broader definition of a SF fluid acknowledges the fact that critical fluids such as carbon dioxide under modest compression can also interact with more volatile lipid species as well as higher-molecular-weight compounds (e.g., triglycerides).

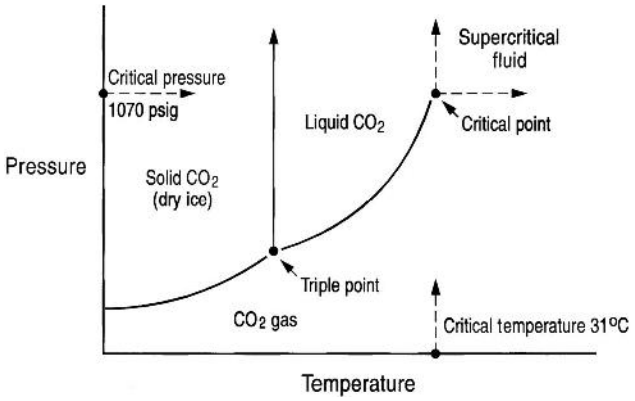


Figure 1. Phase diagram for carbon dioxide.

Movement across the arbitrary boundaries defined in Figure 1 can be quite easily affected by changing the temperature and pressure of the fluid. Thus, LCO_2 has also been used to extract natural products [12] but suffers from reduced extraction selectivity because its density can only be varied slightly as a function of temperature and pressure. On the other hand, SC-CO_2's density can be varied from that associated with a dilute gas (10^{-3} g/cm^3) to densities in excess of unity simply by altering the applied mechanical pressure. This ''tunable'' solvent power is one of the major attractive features in using supercritical fluids as processing agents.

Other factors besides CO_2's low cost, environmental compatibility, and low toxicity make it the most popular SF. The extraction fluxes obtained using SC-CO_2 are both a function of the solubility and diffusivity of the dissolved solutes (i.e., lipids) in CO_2; therefore, the mass transfer properties of SFs such as diffusivity and viscosity also play an important role in processes using SFs. For example, SF solvents exhibit self-diffusivities of the order of 10^{-3} cm^2/sec, whereas liquids have diffusion coefficients of approximately 10^{-6} cm^2/sec. This ''gaslike'' nature of SFs gives them superior penetration properties into substrates, such as oilseeds, relative to that obtained by using liquid solvents. Likewise, the viscosity of SF solvents are of the order of 10^{-4} g/cm · sec, two decades lower than those exhibited by liquids [13].

The mechanically adjustable solvent power of an SF can be nicely correlated with the aid of the solubility parameter concept, where the solubility parameter, δ, as defined by Giddings et al. [14] is given by

$$\delta = 1.25 P_c^{1/2} \frac{\rho_{r,f}}{\rho_{r,l}} \tag{1}$$

where $\rho_{r,f}$ is the reduced density of the fluid at actual experimental conditions, and $\rho_{r,l}$ is the reduced density of the fluid at infinite compression [i.e., the liquified gas (values range between 2.6 and 3.1)]. These reduced parameters are simply the fluid's actual density over the critical density of the fluid ($\sim$ 0.44 g/cm^3 for CO_2). A plot of the δ of several common gases as a function of pressure is illustrated in Figure 2. Here, it can be seen that as one compresses CO_2, its δ increases substantially as a function of pressure relative to the δ of nitrogen and helium at temperatures between 50°C and 70°C. Note that between 8000 and 10,000 psi, the δ of SC-CO_2 reaches values which match those exhibited by most

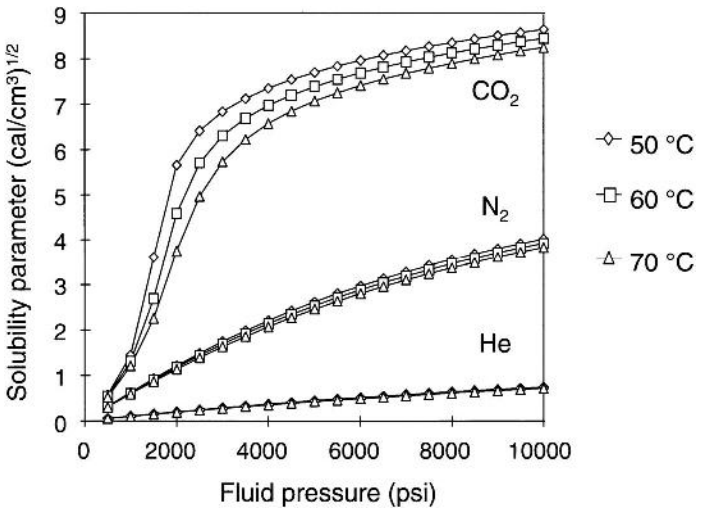


Figure 2. Solubility parameters of various supercritical fluids versus applied pressure.

lipid species [15]. It is under these conditions that the solubility of most lipid species is maximized in SC-CO_2 (i.e., $\delta_{r,f} = \delta_{\text{lipid solute}}$). This does not imply, however, that smaller but finite solubility levels are not attained in SC-CO_2 at lower levels of fluid compression.

The asymptotical values for CO_2's solubility parameter in Figure 2 correspond to reduced densities for CO_2 between 2.0 and 2.2. Table 1 lists solubility parameters associated with lipid-type species found in vegetable oil mixtures as well as the $\rho_{r,f}$ associated with extraction conditions to maximize their solubility in SC-CO_2. Note that the reduced densities for each solute (lipid) class is in the range of the asymptotic values for the solubility parameter for SC-CO_2. It is for this reason that SFE of oils at these high levels of compression yield extraction products (oils) that are equivalent to those obtained with liquid hydrocarbon solvents (i.e., hexane). Table 1 also illustrates a limitation of SFE.

Table 1 Solubility Parameters for Lipid Groups Found in Seed Oils and the Reduced Densities Required to Optimize Extraction of These Lipid Groups

Lipid type	Solubility parameter ($cal^{1/2}/cm^{3/2}$)	Reduced density
Hydrocarbons	8.34	2.08
Carotenoids	8.72	2.17
Tocopherols	8.86	2.21
Triglycerides	8.91	2.22
Ubiquinones	9.08	2.26
Fatty acids	9.10	2.27
Diglycerides	9.45	2.35
Sterols	9.52	2.37
Monoglycerides	10.2	2.54

Note that the reduced density interval between the lipid classes is relative small (only 0.44), suggesting that fractionation of individual lipid species can be difficult at best. This is in agreement with the observation that a difference in δ values of at least 2.5 is required for complete separation of one species from another.

The theoretical considerations presented above translate into significant differences in lipid solubility in SC-CO_2 as a function of pressure and temperature. As shown in Figure 3, extraction of triglycerides from soybean oil [16] reaches levels of only 3 wt% at 40°C over a pressure range from 100 to 1000 bar. However, as the extraction temperature is increased, the reinforcing effect of solute (lipid) vapor pressure along with the solubility of the triglycerides in SC-CO_2 can yield a substantial increase in the yield of lipid species using extraction temperatures of 70–80°C. This increase in a solute's solubility in SC-CO_2 as temperature is increased as a function of pressure is an example of the well-known crossover phenomenon which occurs for many solutes in SFE [17].

The above-stated conditions are close to the recorded solubility maximum observed for lipid-type seed oil in SC-CO_2. Figure 4 shows the trend in solubility for sunflower oil in SC-CO_2 as a function of temperature and pressure. Again, an increasing yield of oil can be attained by conducting the extraction at higher temperatures. It is interesting to note that when the δ for SC-CO_2 is 8.7 $cal^{1/2}/cm^{3/2}$, it matches the solubility maxima recorded by Stahl et al. [18] for sunflower oil in SC-CO_2 (Fig. 4). This is very close to the theoretically predicted δ and reduced density conditions for solubilizing triglycerides as a solute class as given in Table 1.

It should not be inferred from the above discussion that a partial fractionation of mixtures of lipids is not feasible nor undesirable by selective SFE. This is usually accomplished by selecting discrete intervals of extraction pressure or density for a finite amount of time and collecting the resultant fraction. As shown in Figure 5, an enrichment of components in a triglyceride mixture is possible by changing the reduced pressure, P_r ($P_r = P_{exp}/P_c$), or reduced density and, ultimately, the solubility parameter of the fluid, over the range of associated values presented. Thus, a mixture enriched in the C_{34} triglyceride is initially achieved using a reduced fluid density of 1.87, whereas as the extraction is continued at higher values of reduced density, the higher-molecular-weight triglycerides are preferentially extracted. The resultant compositions of the extracts noted in Figure 5

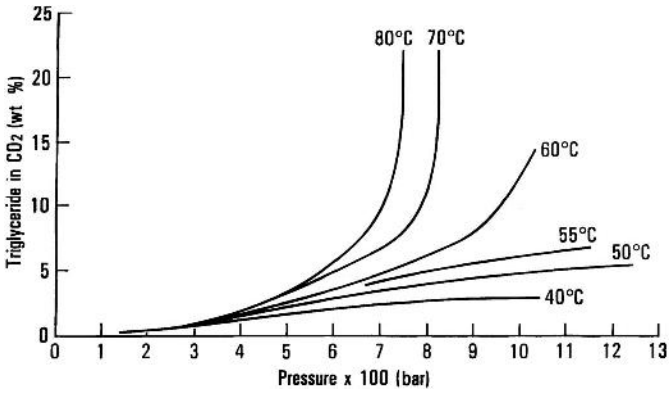


Figure 3. Solubility of soybean oil triglycerides in SC-CO_2 as a function of temperature and pressure.

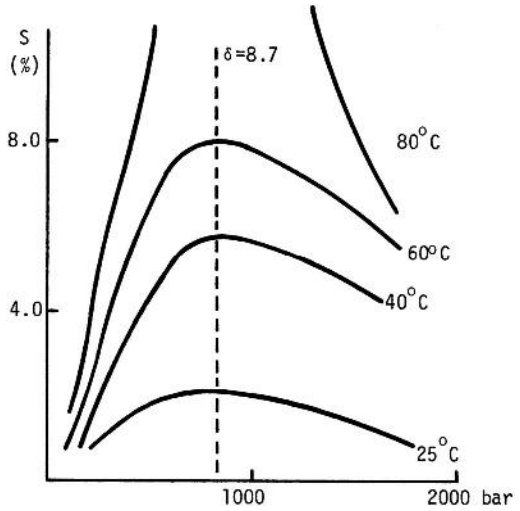


Figure 4. Solubility of sunflower oil (wt%) versus extraction pressure.

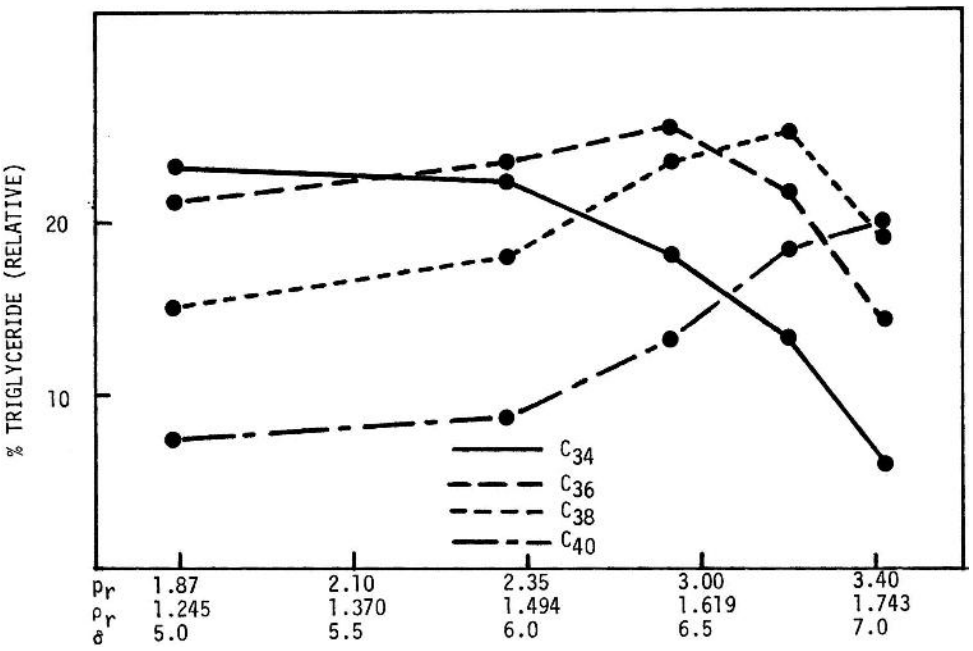


Figure 5. Percent triglyceride extracted versus extraction fluid reduced pressure, density, and solubility parameter.

are really a function of the chosen extraction density and the time over which the extraction is conducted.

Perhaps the most important result that can be ascribed to the mass transfer characteristics of supercritical fluids are the kinetics of extraction. This is illustrated in Figure 6 for the SFE of a high-value lipid product, evening primrose oil (EPO), from its crushed seed. These results are taken from the author's modeling study [19] of the extraction kinetics of EPO, but the results are generally applicable to the extraction of most lipid species, including pure compounds. The actual experimental data are designated by points representing different extraction pressures (1 MPa = 145 psi), and the lines are theoretical fits to the various extraction curves at 40°C based on the models of Brunner [20] and Hong et al. [21]. It can be seen in Figure 6 that the rate of EPO extraction (i.e., mass of EPO extracted versus time) is a function of the extraction pressure, suggesting that the initial predominating factor governing extraction rate is the oil's solubility in the SC-CO_2. Indeed, as shown in Figure 3, higher extraction pressures correlate with higher levels of triglyceride solubility in SC-CO_2; curves such as those in Figure 6 can be used to determine lipid solubilities in supercritical fluids [21]. Figure 6 also reveals that later in the SFE rate curve, as the oil becomes depleted from the seed matrix, there is the onset of a mass transfer region where further extraction of the residual oil is difficult. This can be due to several factors, including, particularly, the shape and particle size of the comminuted seeds; the problem has been elegantly described by Reverchon et al. [22]. This retardation of the oil-extraction kinetics has been termed the ''diffusion phase'' by Stahl et al. [23], or the nonequilibrium region of the extraction curve by the author.

As noted previously, SC-CO_2 reigns supreme as the principle processing agent in critical fluid technology. However, like any other extraction solvent, SC-CO_2 cannot be utilized for all tasks and is a relatively poor solvent for polar compounds. For certain applications, the addition of a minimal amount of a polar cosolvent (usually an organic solvent with a critical temperature, T_c, higher than CO_2) suffices to improve the extraction

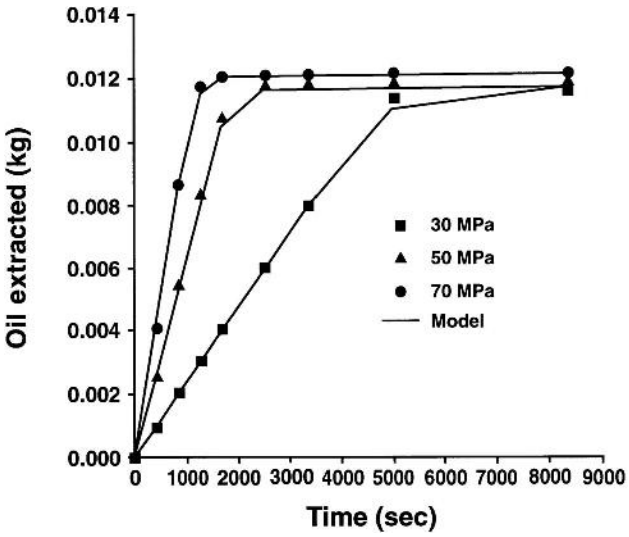


Figure 6. Extraction rate curves for evening primrose oil at 50°C as a function of extraction pressure; experimental data versus predicted extraction curve.

of compounds from natural products. The number of GRAS (generally regarded as safe) cosolvents suitable for this purpose is rather limited (e.g., water, ethanol, acetic acid). Cosolvents can be used in conjunction with SC-CO_2 to achieve fractionation of lipids, either in a single-step or multistep extraction process to produce the desired end result, a most notable example being the deoiling of seed moieties followed by SC-CO_2/ethanol solubilization of polar phospholipids, which have residual solubility in neat SC-CO_2 [15].

Propane has also been evaluated for critical processing of lipids, as it has a lower P_c (P_c = 48 atm, T_c = 97°C) than CO_2 and the solubility of lipids in near-critical propane is higher than in CO_2. Propane has been used for the extraction and fractionation of fish oil in the Selexol process [24], and a CO_2/propane mixture has been used for the deoiling of lecithin and fractionation of glycerides [25]. However, despite these advantages and applications, CO_2 seems to be more acceptable to the food industry than propane because it is nonflammable and a ''green'' solvent.

Other alternatives, which embrace the ''green'' processing concept, are binary fluid mixtures and specific fluorocarbon fluids. The use of liquefied gases (e.g., LCO_2) and pressurized liquids (i.e., liquids under an appropriate external pressure, so as to not vaporize at extraction temperatures above their boiling point) can produce superior results in specific cases, compared to conventional liquid solvent extraction [26]. For the processing of thermally sensitive lipids, the selection of extraction temperature is critical to avoid degradation of the solutes. It is for this reason that liquefied gases, such as LCO_2 are used at near-ambient or subambient temperatures in natural-product extractions [27–29]. In addition, pumping a liquefied gas does not require as much expenditure of energy as pumping and compressing a gas for specific processing conditions [30]. Recently, water has been used under subcritical conditions for the extraction and reaction of lipid moieties; however, these higher temperature conditions can alter the composition of some lipid material [31] if due caution is not exercised.

3 CRITICAL FLUID EXTRACTION OF LIPIDS AND OILS

The critical fluid extraction of lipids and oils has been reviewed by the author [32] and is the subject of an extensive monograph edited by King and List [33]. The reader is referred to these sources and the recent tome by Mukhopadhyay [13] for a historical appreciation of the SFE of lipid-bearing substrates. With respect to biotechnology applications, it is often the information presented in the proceeding section and the use of SFE as the fundamental basis for conducting fractionations and reactions in supercritical fluids that is of critical importance. For this reason, it is worth commenting on the equipment and mechanical aspects of SFE.

Evaluation of the potential and efficacy of SFE is often done on a bench scale, laboratory apparatus employing a tubular extraction vessel, and a continuous flow of the extraction fluid. In Figure 7, a schematic of a simple, bench-top extraction system that has proven very versatile in our laboratories is presented. The fluid source (e.g., CO_2), A, can be either in the gaseous or liquefied state, the liquid state being more appropriate when using larger extraction systems. A compressor or liquid pump, C, delivers the fluid through a tandem switching valve, SV-1 and SV-2, into the extraction cell held at a controlled temperature. The fluid passes through the cell containing the material to be extracted and is passed on through a similar switching valve arrangement to either a micrometering valve, MV, or back-pressure regulator, where the pressure of the fluid is reduced. These back-pressure regulators or metering valves are usually heated to compensate for

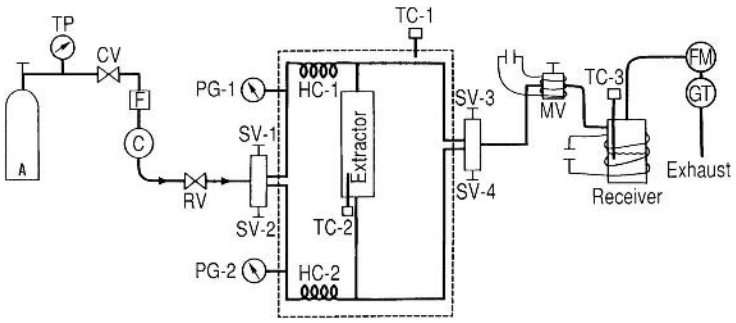


Figure 7. Laboratory bench-top supercritical fluid extraction system.

the attendant Joule–Thomson cooling effect that occurs when depressurizing the fluid. The fluid is then directed to a collection (receiver) vessel, which can exist in several formats, and may be packed with internals or kept at a very low temperature (−15°C) to eliminate entertainment of the extracted components in the rapidly expanding critical fluid. Gas flow under ambient conditions is assessed with the aid of a flow meter, FM, and fluid totalizing module, GT. This type of unit can be reformatted for different types of experiment involving critical fluids and has proven to be extremely flexible for a modest cost [34].

Although the above-described system is entirely adequate for laboratory SFE experiments, it does not meet the requirements of an actual production SFE operation. In these cases, the fluid is reused by circulating it over and over again through the substrate in the extraction vessel. Thus, the solvent (fluid) supply remains constant except for small losses, an attractive feature of process SFE. To illustrate this principle in greater detail, a schematic of the semicontinuous pilot plant at our research center is illustrated in Figure 8. Here, continuous SFE is assured by the use of several 4-L extraction vessels utilized in tandem. The extraction solvent can be directed in a sequential fashion to one or more of the extraction vessels; thus, vessel A can be extracted while another vessel, B, is being loaded with the material to be processed. The third pictured vessel, C, will occupy an intermediate processing state undergoing either pressurization or depressurization. The receiver vessel, R, must be of sufficient size (2 L) to allow separation of the extracted solutes by depressurizing the fluid. A sorbent-filled column is inserted on the low-pressure side of the fluids recycle line to remove unwanted odoriferous volatile compounds. Note that a provision is made for making up fluid lost during the removal of the extract from the receiver vessel. Utilizing the above scheme and equipment has permitted the exhaustive deoiling of oil seeds such as soya in as little as 10 min, using mass flow rates of SC-CO_2 of 0.5 kg/min at 80°C and 10,000 psi [35]. Actual industrial-scale SFE installations use lock hoppers or screw conveyors under reduced pressure to continuously feed solid substrates into multiple extraction vessels.

The SF processing of specialty oils is perhaps of most interest when applying SFE in the field of biotechnology. This is because these oils are marketed as nutraceuticals and sold on the basis of their unique fatty acid composition and/or presence of lipid-soluble minor components in their composition, as these ingredients have implied health benefits. Such specialty oils also find applications in medicine and skin care products and they feature antioxidant activities that are reported to be inhibiting against cancers. Re-

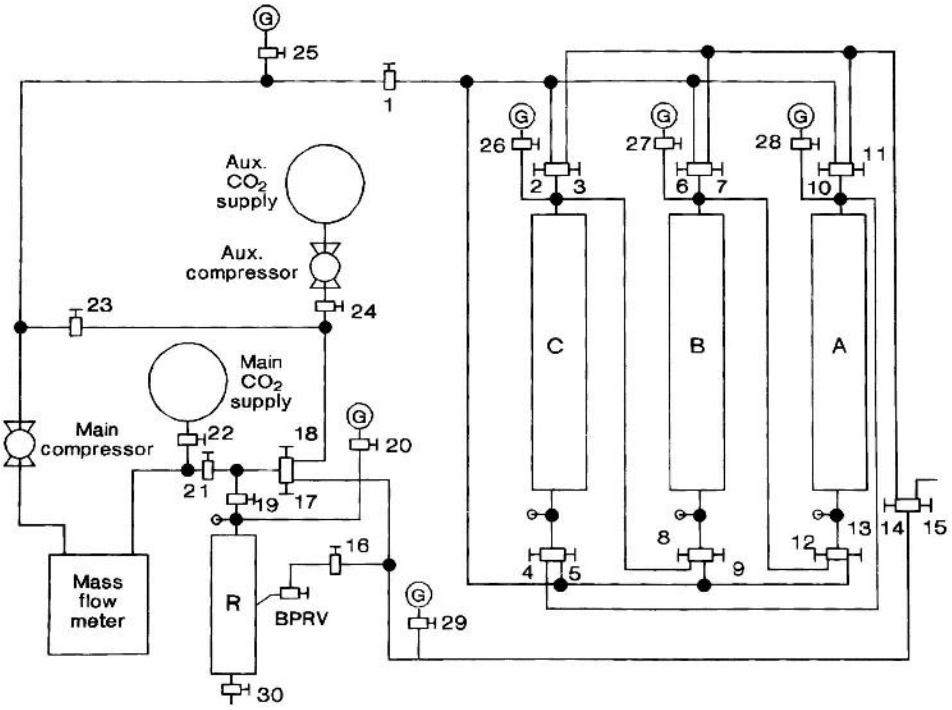


Figure 8. Batch semicontinuous supercritical fluid pilot plant with provision for extraction fluid recirculation.

cently, the presence of minor lipid components in some speciality oils has also been shown to reduce serum cholesterol levels in human subjects [36].

Examples of these therapeutic oils are those derived from oat and barley oils, because they are rich in tocopherols and tocotrienols. Oat oil has been extracted using SC-CO_2 by Fors and Eriksson [37], whereas barley oil can be extracted from whole or pearling flour because the majority of the extractable lipids are located in the outer layers of the seed kernel. Another source of natural antioxidants is rice bran oil which has been extracted using SC-CO_2 to yield an extract rich in tocopherols, oryzanol, and sterols (campesterol, stigmasterol, β-sitosterol) [38,39]. Recently, Xu and Godber [40] have extracted rice bran with both SC-CO_2 and organic solvents and have shown that the γ-oryzanol content of the SC-CO_2-derived extract is four times greater than the extract obtained with organic solvents. In a similar fashion, neat SC-CO_2 was shown to yield a ferulate–phytosterol ester containing extract from corn fiber by Moreau et al. [41] or a sterol content in a SC-CO_2 extract of saw palmetto, which was approximately four times that of a corresponding ethanol extract. Such SC-CO_2-derived oil extracts offer a potential alternative to the cholesterol-reducing properties of chemically altered tall oil extracts that have been recently commercialized and sold in margarine formulations [42].

As previously noted, EPO can readily be extracted with SC-CO_2. The interest in this oil is due to its high γ-linolenic acid content. Extracts obtained between 200 and 690 atm and 40–60°C have been characterized by Favati et al. [43]. Similarly, other oils that are rich in γ-linolenic acid (borage, blackcurrent, and flax) have been extracted using

critical fluids. In our laboratory, oils have been obtained using SC-CO_2 extraction from wheat germ, avocado, and sorghum bran that have implied therapeutic value. It also has been reported that critical fluids can also be used to extract oils which are devoid of cholesterol [44] from fungi or marine-derived plant material, such as spirulina. Extraction of natural lipid-type pigments, such as carotenoids and xanthophylls from similar type substrates, has been reported as well as from freeze-dried substrates of alfalfa leaf protein concentrate [45]. An excellent review of the SFE of natural pigments is provided by Mukhopadhyay [13].

4 FRACTIONATION OF LIPIDS USING SUPERCRITICAL FLUIDS

Prior to the mid-1980s, critical fluid processing was largely accomplished using SFE. As noted previously, selective fractionation of extracts was achieved by either altering the extraction fluid density as a function of processing time or, in some cases, by selectively decreasing the pressure after the extraction stage to achieve the desired extract. The latter fractionation technique makes use of successive receiver vessels in which the extracting fluid density is progressively lowered by manipulating the temperature and pressure. Examples of this technique include the dewaxing of essential oils by Della Porta et al. [46] and the isolation of tocopherols from olive oil by Ibanez and co-workers [47]. Useful separations have been attained using the above techniques, but largely between compounds which differ significantly in their physicochemical properties (e.g., molecular weight, vapor pressure, or polarity).

Fractionation processes utilizing critical fluid media have been improved by combining principles utilized in supercritical fluid extraction with other separation techniques. These improved methods often make use of fractionating columns or chromatography to yield improvements in the resolution of the desired molecular species. The fractionating column or tower approach is somewhat analogous to operating a distillation column, but there are differences when using critical fluid media. For an understanding of the fundamentals involved in using this technique, one should consult the primer by Clifford [48].

Figure 9 illustrates the components and principles involved in column-based fractionation using the simple column in our research laboratory. Although this is not the most sophisticated SFF column approach, it will serve as an example of how the process works. In this case, SC-CO_2 is brought to the desired extraction pressure where upon fluid is directed to flow upward inside the fractionating column. The fractionating column contains a packing to facilitate contact between the SC-CO_2 and the components being separated. The components to be separated are injected with a pump into the flowing SC-CO_2, prior to its entry into the column (components 5 and 10 in Fig. 9). The fluid–solute mixture then enters the first heated zone of the fractionating column and the separation process is initiated. The SC-CO_2–solute mixture then ascends the column encountering zones of increasing temperature, which amplify the separation of solutes based on their relative solubilities in SC-CO_2 and respective vapor pressures. In effect, the column is operating as a density gradient in SC-CO_2 because the fluid is kept isobaric.

The fractionating column described in Figure 9 can be operated in either the batch or semicontinuous mode with cocurrent flow of the solute and supercritical fluid streams. Using this approach, we have demonstrated the enrichment of lipid monoglycerides (MAG) from a mixture of glycerides [49]. The effect is nicely demonstrated by capillary SFC profiles shown in Figure 10. Here, the feed material is a mixture of glycerides in which the MAG content is 45 wt% of the mixture. As the fractionation of the mixed

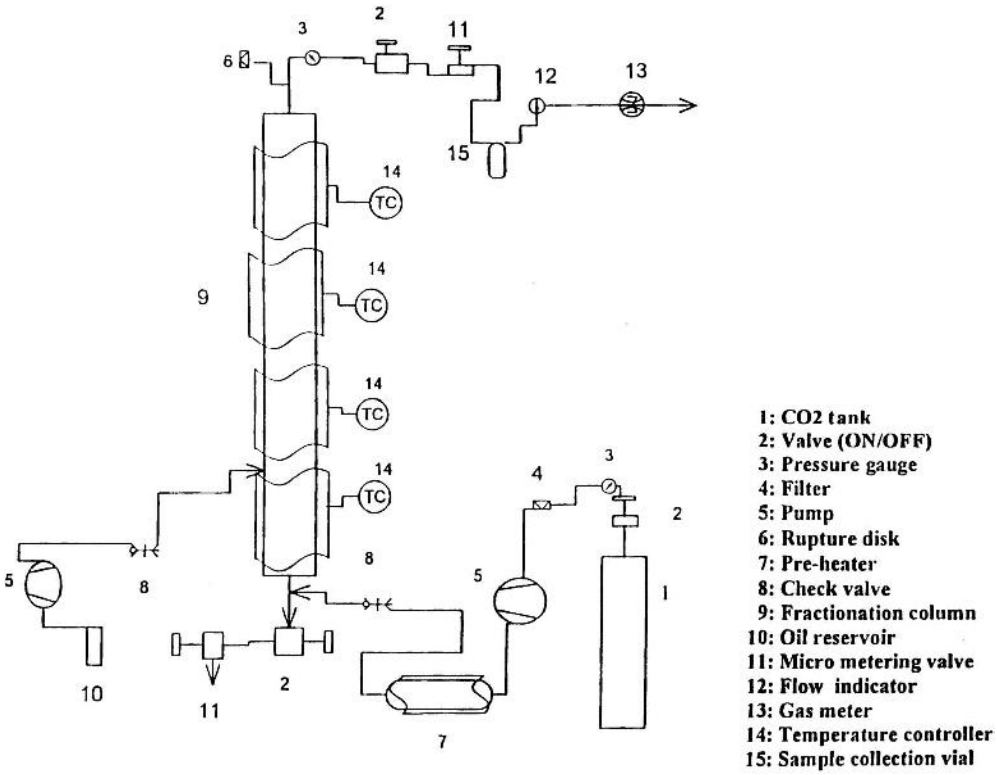


Figure 9. Schematic of a cocurrent critical-fluid-packed fractionation column system.

glyceride feed proceeds, the extract that is removed from the top of the column is considerably enriched in MAG content. Using this approach, an extract containing 95 wt% of MAG can be achieved [50].

A similar fractionation approach [51] has been recently used to produce enriched nutraceutical extracts from deodorizer distillate (DD). DD is a complex by-product from the refining of vegetable oils and contains many components, as illustrated by the capillary SFC chromatogram in Figure 11. Many of these components are desired in a more pure or enriched fraction. Therefore, in a recent study, we used a fractionating column to remove undesired free fatty acids from DD while enriching the free sterols and steryl ester content of the raffinate (top product).

The results of this study are presented in Table 2 for both rice bran and soybean oil DD. In this case, the starting material (the feed into the column) contained between 32 and 38 wt% free fatty acids and between 13 and 18 wt% sterols, whereas the raffinate collected after the fractionation had a free fatty acid content of between 5 and 8 wt% and sterol–steryl ester enrichments of between 27 and 34 wt%. These results could undoubtably be improved upon by employing an even longer fractionating column or by preferentially operating a column in the countercurrent mode, which other investigators have employed in Germany and Italy. For example, tocopherols have been enriched from soybean oil DD by Brunner and colleagues [52] and the same research group separated the ethyl esters of fish oil basestock using the countercurrent column approach [53].

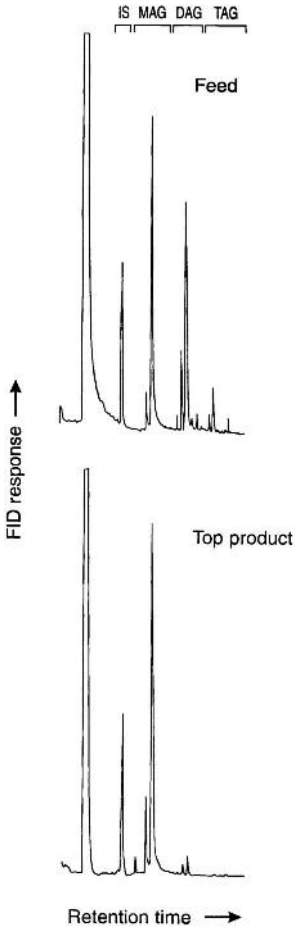


Figure 10. Capillary supercritical fluid chromatographic profile of feed and top (extracted) product glyceride profiles using critical-fluid-packed column fractionation. MAG = monoacylglycerides, DAG = diacylglycerides, TAG = triacylgylycerides, IS = internal standard.

Another SFF option is to employ chromatography in the preparative or production mode, in its own right or coupled with a preliminary SFE enrichment stage. Our research group at NCAUR has utilized the latter approach several times to achieve extracts with target compounds enriched at levels that were not possible by using SFE alone. There are a number of different SF chromatographic-based methods that could be used for this purpose, but the process described in Figure 12 has been designed with scale-up and economics in mind. As shown in Figure 12, flaked soybeans are initially extracted at a relatively low pressure to enrich the target components of interest, tocopherols. This fraction is then moved sequentially on to a sorbent-filled column for further fractionation to yield a tocopherol-enriched extract. Enrichment factors relative to the tocopherol content in the original soybean flakes are tabulated in Table 3. Note that these are only modest for application of the single SFE-based separation; however, significant enrichment of the desired components is attained by applying the chromatographic fractionation step [54].

Figure 11. Capillary SFC profile of soybean oil DD.

Using this approach, we have been able to enrich other lipid moieties in addition to tocopherols, such as phospholipids or steryl esters from vegetable oils, seeds, and by-products of the milling or vegetable oil refining processes. Figure 13 illustrates the case for the separation, enrichment, and fractionation of phospholipids (PLs) from vegetable oils or seeds [55]. Here, soybean flakes are initially extracted with SC-CO_2 to remove the oil, followed by extraction of the PLs from the deoiled flakes with a SC-CO_2–ethanol mixture. This second extraction produces an extract which is enriched in PLs, because PLs are not appreciably soluble in neat SC-CO_2 but can be solubilized in SC-CO_2 ethanol mixtures.

Table 2 Raffinate Compositions (wt%) from Fractionation Tower Separation of Vegetable Oil Deodorizer Distillates (DD) Using SC-CO_2

Component	Rice bran DD	Soybean DD
Free fatty acids	5	8
Sterols	20	31
Steryl Esters	7	3
Triglycerides	38	30

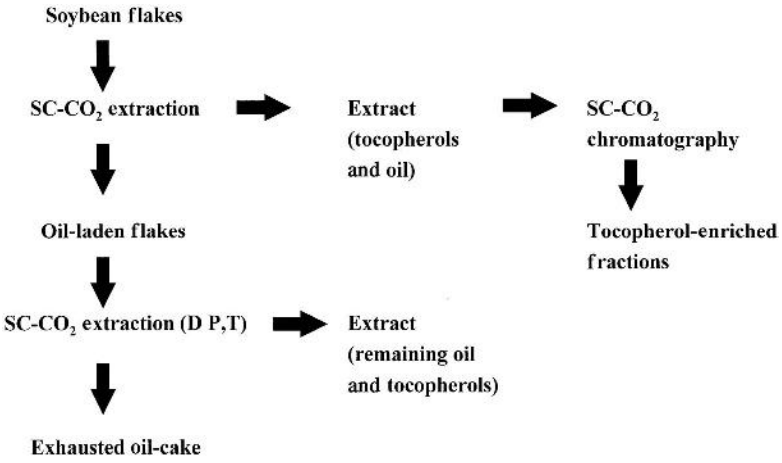


Figure 12. Tocopherol enrichment/fractionation scheme using supercritical fluid techniques.

As shown in Table 4, the second SFE using SC-CO_2–ethanol produces an extract containing a total 43.7% by weight of PLs. This is a considerable enrichment relative to the concentration of the PLs in the starting oil or seed matrix. Further PL enrichment is facilitated as noted earlier, by transferring this extract enriched in PLs to an alumina preparative SFC column, where SC-CO_2 modified with 5–30 vol%, 9:1 ethanol:water eluent is used to elute and fractionate the PLs. In the case of the SFC enrichment step, eluent fractions can be collected as a function of time and their PL content quantitated. As indicated by the data given in Table 4, collection of discrete fractions during the SFC process can produce purities in excess of 75% for the individual PLs, phosphatidylcholine and phosphatidylethanolamine. It should be noted in the described process that in the SFC steps, only GRAS (generally regarded as safe) solvents are being used for the enrichment process.

Recently, a similar SFE/SFC process has been used to isolate sterols and phytosterol esters from corn bran and fiber oil [56]. For example, by using both SFE and SFC, it has been possible to isolate a fraction containing up to 53% by weight of ferulate phytosterol esters (FPE) from corn fiber oil. Moreau et al. have isolated similar moieties in a total oil extract using SC-CO_2, which is called ''Amaizing Oil'' [57]. Intended for use as a cholesterol-lowering agent, this extract unfortunately contains mostly triglycerides and

Table 3 Enrichment Factors of Tocopherols from Soybeans by SFE and SFE/SFC

Tocopherol	SFE	SFE/SFC
Alpha	4.33	12.1
Beta	1.83	2.4
Gamma	3.94	15.0
Delta	3.75	30.8

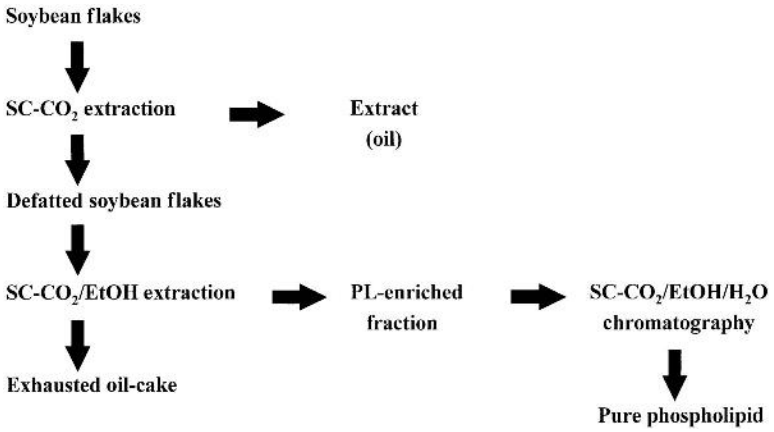


Figure 13. Phospholipid enrichment/fractionation scheme using supercritical fluid techniques.

about 6 wt% FPEs. Whereas this extract may have some utility in the nutraceutical marketplace, it is limited for formulating or chemical testing purposes due to the limited enrichment of FPEs. The tandem SFE/SFC process provides an even greater enrichment of the active principle and could be improved on further by using successive chromatographic purification.

Recently, the principle of simulated moving-bed (SMB) chromatography has been applied to the purification of lipids such as fish oil esters. A special facility that deserves some mention is the relatively new KD-Pharma/Industries Quimicas Asociadas plant for producing concentrates of ω-3 fatty acid esters [eicosapentaenoic (EPA) and docosahexaenoic acids (DHA)] from fish oil in Tarragona, Spain. This production facility employs a combination of SFE/SFC to produce fish oil ester mixtures of 95% purity. The process uses a proprietary silica-based packing material in the SFC stage to separate the ω-3 fatty acids from the ω-6 moieties, as described in detail by Lembke [58] for the pilot-plant-scale operation. Increasing the EPA content of this natural product improves its nutraceutical functionality and is an excellent example of what can be achieved using supercritical-fluid-based fractionation methods.

Table 4 Relative Amount of Phospholipids from Soybeans in SFE Isolates and in SFC Collected Fractions

Phospholipid	SFE[a]	SFC
Phosphatidylethanolamine	16.1	74.9
Phosphatidylinositol	9.2	20.8
Phosphatidic acid	2.8	55.8
Phosphatidylcholine	15.6	76.8

[a] All data in percent of that component relative to other eluting constitutents (oil and unidentified peaks).

5 LIPID REACTION CHEMISTRY IN SUPERCRITICAL FLUIDS

The ability to conduct reactions in critical fluid media has been under extensive study over the past 9 years [59]. Three types of reaction (enzymatic, hydrogenation, and conversions) in subcritical water are of particular interest with respect to their application for lipid materials. Critical fluids offer some unique advantages when conducting reactions, including improvements in mass transfer of reactants and products and potential control of the final product distribution by altering the fluid density in which the reaction takes place. Additional options include the possibility of performing conversions at low temperatures, *in situ* regeneration of catalysts, and combining the reaction step sequentially with SFE or SFF.

It should be noted that the Gibbs free energy of reaction is also sensitive to pressure, as defined by Eq. (2):

$$\left(\frac{\partial RT \ln K_x}{\partial P}\right)_T = \nabla V \tag{2}$$

where K_x is the mole fraction equilibrium constant and ∇V is the excess partial molar volumes of the products over the reactants in the equilibrium mixture. Therefore, regardless of any benefits that come from conducting a reaction in critical fluid media, the application of pressure will have an influence on the reaction.

Experimental parameters which can influence the conversion of lipid-based substrates in critical fluid media are listed in Table 5. The composition of fluid phase is important because it ultimately influences the solute (reactant) solubility and introduction of reagents (H_2) into the critical fluid system. Therefore, phase equilibria and solute solubility relationships are important, not only with respect to assuring that adequate solute (reactant) solubility occurs in the critical fluid media but also that an adequate throughput of converted product is feasible to make the synthetic process kinetically feasible and economical. Other important relationships are the optimization of reaction conditions via proper selection and activation of the catalyst (if required) and the moisture content of the substrate (in the case of certain types of enzymatic catalysis). The flow rate in tubular reactor systems is also critical, not only with respect to the critical fluid but also for the introduction of reactants and their solubilization in the critical fluid media. In addition, the flow rate is linked to product throughput and must be optimized to allow proper kinetic conversion of the reactants.

One promising area for applying supercritical fluid reactions (SFR) for the conversion of lipids is the use of enzymes for initiating reactions such as esterifications, transesterifications, oxidation, alcoholysis, and hydrolysis. Immobilized enzymes on porous supports for conducting conversions in a tubular flow reactor are particularly amenable for

Table 5 Experimental Parameters That Influence Lipid Reaction Chemistry in Critical Fluids

Fluid type and composition	Optimization of reaction conditions
Pressure	Catalyst type and activity
Temperature	Moisture content of substrate
Phase equilibria	Effect of flow rate
Solute (lipid) solubility	Solute throughput

conversions using critical fluid media. The coupling of enzyme catalysis with SC-CO_2 is particularly attractive because both are ''natural'' agents that avoid the use of chemical solvents or catalyst residues in the final product. The above transformations can be conducted in the presence of a lipase, but particular attention must be paid to the temperature, pressure, and presence of water in the supercritical fluid system. Of particular note is Novozym SP 435, a lipase derived from *C. antarctica*, as a catalyst supported on polyacrylate resin, an enzyme that has proven to be a particularly effective catalyst in the presence of SC-CO_2 [59–62].

Jackson and King [60] have demonstrated the compatibility of an enzymatically catalyzed transesterification in SC-CO_2 on triacylglycerol (TAG)-based oils extracted directly from seeds (soybean) using an enzyme flow reactor system. Such transesterifications and simple esterifications can be conducted using Novozym SP 435 at pressures from 2500 to 5000 psi and temperatures from 40°C to 70°C. The utilization of higher temperatures and pressures has been reported [61] and is desirable in terms of increasing the solubility of the substrate (TAGs), but this can also reduce the service lifetime of the enzyme. An example of the conversion possible in a SC-CO_2/Novozym SP-435-based transesterification system is shown in Figure 14, where analytical capillary SFC shows a 97% conversion of soybean trigylcerides to the corresponding fatty acid methyl esters (FAMES) at 2500 psi and 60°C. This formation of fatty acid methyl esters is so complete, reproducible, and quantitative that it has served as a basis for analytical SFE/SFR methods developed in our laboratory for nutritional fat analysis [63].

Lipolysis has been used to successfully methylate other lipid moieties such as sterols and phospholipids [64]. Such results pave the way for esterification of these compounds to other synthetic compounds having different fatty alcohol chain lengths. Recently, steryl esters have been synthesized by our research group using SC-CO_2 and various lipases,

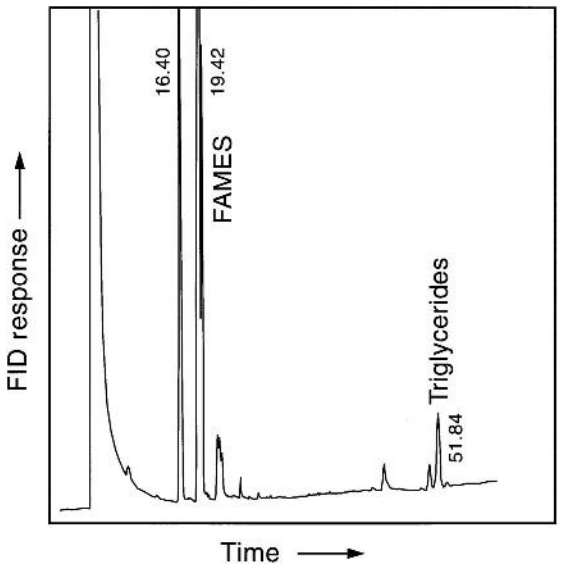


Figure 14. Capillary SFC profile of end-product mixture from enzymatic conversion of TAGs to FAMES.

including partially dehydrated soapstock feeds containing fatty acids which have also been esterified using the above conditions [65].

The presence of moisture in natural product substrates can have an effect on enzymatic-based synthesis. A previous study [66] showed that there is a minimal amount of water that must be associated with the enzyme in the presence of the critical fluid to assure retention of activity. However, excessive water can denature the enzyme, leading to loss of activity and conversion of reactants. This is illustrated by the results in Table 6, in which the effect of added water on the methanolysis of corn oil to form FAMES is described. Note that in terms of volume percent of water in SC-CO_2, this is quite a small quantity (0.05 vol%) and must be rigorously controlled to prevent loss of activity and conversion. Fortuitously, the solubility of water in SC-CO_2 is quite small [67] and this aids in maintaining the activity of the enzyme for long periods of time when extracting and reacting lipid moieties from natural products. Another convenient way of maintaining the hydration level critical for maintenance of the enzyme's activity in the presence of a critical fluid is to add the requisite amount of water via a syringe pump directly into the critical fluid.

The rate addition of reactants to a flow reaction system operating under critical fluid conditions can be quite critical in assuring the maximum yield of end products. For example, Jackson and King [60] have shown that the addition of methanol for conducting a transesterification of a vegetable oil must be optimized or the relative activity of the enzyme will not be realized. This is required to assure that there is an adequate stoichiometry of the reactants as well as time for these moieties to react during their passage over the supported enzyme catalyst. This is also true in the case of hydrogenation of triglyceride-based vegetable oils in SC-CO_2 or SC-C_3H_8 in the presence of a supported catalyst, as will be discussed shortly.

Another type of reaction that can be catalyzed by an enzyme in the presence of a supercritical fluid is the interesterification of vegetable oils to produce a ''randomized'' product having quite different physical and chemical properties than the starting materials. Jackson et al. [62] interesterified a variety of starting materials by dissolving them in SC-CO_2 and transporting them over immobilized beds of Novozym SP-435 lipase at 27.5 MPa (3988 psi) and 65°C. The end effect of this interesterification is quite striking because liquid vegetable oil feedstocks can be randomized to products of a semisolid nature having potential as margarine base stocks.

Table 6 Effect of Added Water on the Methanolysis of Corn Oil in SC-CO_2

Volume% water in carbon dioxide	Relative activity
0	100
0.05	99
0.10	81
0.20	56
0.30	18

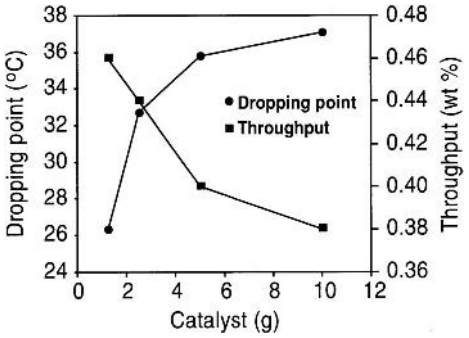


Figure 15. Effect of catalyst (enzyme) concentration on fat dropping point and end product throughput for randomization of palm olein in SC-CO_2 via enzymatic catalysis.

For palm olein, the resultant physical end products of the interesterified product is highly dependent on the amount of catalyst (enzyme) that is used. In this study [62], palm olein was randomized using varying amounts of Novozym SP-435 under the above conditions with an expanded CO_2 flow rate of 12.5 L/min. As would be expected, increasing the amount of catalyst resulted in a fat with a higher dropping point (a measure of solid–liquid content of the resultant fat) as indicated by the trend shown in Figure 15. The dropping point of the initial palm olein was 21.7°C, indicating that even the smallest amount of enzyme had a sizable effect on the dropping point of the triglyceride mixture. Also indicated in Figure 15 is that the production rate (throughput) of randomized palm olein through the SF reactor decreased with an increase in the quantity of enzyme used. Thus, it would appear that the dropping point and throughput are inversely related and that at 2.5 g of enzyme, the maximum throughput and dropping point can be realized for this particular interesterification.

Figure 16 shows the solid fat content of a high-stearate soybean oil and palm olein both before and after randomization. After randomization, both of these oils show an increase in solid-fat content, as measured by wide-line nuclear magnetic resonance, at

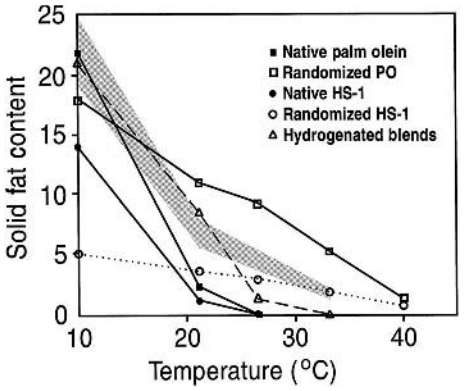


Figure 16. Solid-fat content of palm olein and HS-1 soybean oil before and after randomization in SC-CO_2 at 27.5 MPa and 65°C.

higher temperatures and a decrease at lower temperatures. The randomized HS-1 and palm olein (PO) begin to have a higher fat content than their native state at approximately 15°C and 20°C, respectively. Typical solid-fat content for soft-tub margarine oils obtained by blending hydrogenated and liquid soybean oils shows a thermal behavior indicated by the shaded area in Figure 16. At 26°C, both enzymatically randomized oils have higher solid-fat contents than the hydrogenated blend. It is apparent that the randomized palm olein product has a SCI that exceeds that typically found for commercial products and that the randomized HS-1 product approaches the SCI values found for soft commercial margarines.

Another way of producing semisolid oleochemical formulations for food use is through hydrogenation of native oils and fats. Harrod et al. [68] and Tacke and co-workers [69,70], have hydrogenated a variety of oleochemicals and shown that hydrogenation of fats/oils is feasible under supercritical conditions using SC-CO_2 and SC-C_3H_8. Recently, we have studied the hydrogenation of FAMES using binary fluid mixtures of SC-CO_2/H_2 and SC-C_3H_8/H_2 in a flow reactor under SF conditions at quite high temperatures (150–250°C) [71]. Using conventional inorganic catalysts, FAMES were exhaustively hydrogenated to yield the corresponding fatty alcohols using a critical fluid phase which can contain up to 25 mol% H_2. High conversion rates (>98%) were achieved quite rapidly due to the enhanced contact made between the fixed–bed catalyst and the methyl esters dissolved in the fluid mixtures. Greater product throughput was achieved by using the SC-C_3H_8/H_2 mixture due to the higher solubility of FAMES in SC-C_3H_8 versus SC-CO_2; however, the SC-CO_2/H_2-based process gives less by-product *n*-alkanes relative to the SC-C_3H_8/H_2 system.

The above hydrogenation reaction can be combined advantageously with an initial transesterification step to synthesize FAMES directly from vegetable oils extracted in SC-CO_2. In this case, soybean oil triglycerides were methylated using the conditions established by Jackson and King [60] and then transported from the supported enzyme reactor to flow through the hydrogenation reactor. Hydrogenation of the FAMES was then accomplished by continuously facilitating the total reduction of the FAMES to the saturated alcohols (hexadecanol and octadecanol), splitting off methanol. Using this procedure, the methanol by-product can be fed back into the first stage of the synthesis process (transesterification) to produce FAMES.

In the studies mentioned so far, SC-CO_2 has been the predominant critical fluid media used due to its low environmental impact. Another medium that meets this criteria is subcritical water, which can be defined as hot compressed water held between 1 and 218 atm and between its normal boiling point and critical temperature of 374°C. Under these conditions, water does not boil away and can act as a solvent whose solubility characteristics are determined predominantly by the extraction or reaction temperature of the water [72]. Research conducted in our laboratory has utilized subcritical water for the hydrolysis of vegetable oils to synthesize fatty acid mixtures [73]. The results of this subcritical water hydrolysis are given in Table 7 and demonstrate how complete this conversion can be under a variety of conditions. Note that residence times under 10 min can produce over a 90% conversion of the vegetable oil feedstock (in this case soybean oil) to the component fatty acids. Although this approach uses higher water-to-oil feed ratios than those currently used in industrial hydrolysis processes, it requires no catalyst.

In conclusion, the future would appear bright for reaction chemistry in critical fluid media. As noted earlier, there are a variety of media that can support synthesis above and below their respective critical points (SC-CO_2, SC-C_3H_8, and subcritical H_2O), with the

Table 7 Conversion of Soybean Oil to Free Fatty Acids Using Subcritical Water in an Open-Tubular Flow Reactor

Residence time (min)	12.6	9.9	7.5
Temperature (°C)	335	335	335
Pressure (atm)	125	125	134
Water : oil ratio	2.5 : 1	5 : 1	2.5 : 1
% Free fatty acid	98	100	90.4

resultant synthetic processes being potentially capable of being coupled with SFE and SFF in pre-reaction and postreaction enrichment schemes to yield a variety of lipid-processing possibilities.

6 CONCLUDING REMARKS

In the preceding sections, it was demonstrated how critical fluids, applied as an overall technological approach, can be extremely useful in isolating, fractionating, and converting lipids into useful industrial products. The high capital costs of implementing critical fluid technology makes it imperative that plants and processing facilities be adaptable to other roles besides just the extraction mode. The examples given in this chapter suggest that such options are feasible and can be coupled to considerable advantage.

The conditions for solubilizing lipids under supercritical conditions do not always favor the use of biocatalysis, primarily because high lipid solubility in SC-CO_2 is commensurate with the use of high pressures and temperatures, which may denature bioactive proteins. Recent developments in the field of high-pressure food processing [74] suggest that enzyme and protein functionality may be more resilient than previously thought and hold the key to the development of better thermophilic and hyperbaric enzymes. Compressed, subcritical water's use may also be extended in the reaction area in the near future.

Supercritical fluid technology can also serve the needs of lipid biotechnology through its use in analytical chemistry. As has been amply demonstrated in this chapter, analytical SFC is an excellent and rapid form of chromatography for separating various lipid classes without the need for extensive sample preparation. Additional examples of its use in lipids separation and analysis are contained in the review by King and Snyder [75]. Analytical SFE has had a major impact as one of several competing technologies to reduce the time, cost, labor, and extensive use of organic solvents in sample preparation methods [76]. Major application areas for analytical SFE have been in fat and lipid analysis [77], toxicant residue determination [78], and as a benign method to study lipid degradative processes [79].

In the author's view, analytical supercritical fluid technology can serve as a testing ground for assessing the feasibility of conducting extractions, fractionations, and reactions in critical fluid media. The use of analytical supercritical fluid instrumentation, when automated and miniaturized, can save considerable effort, particularly when working with expensive and scarce lipid biochemicals. Such equipment allows a combinatorial technological approach to evaluating the myriad of possibilities presented by lipid biotechnology while blending a third technology with the two previously mentioned.

REFERENCES

1. MA McHugh, VJ Krukonis. Supercritical Fluid Extraction: Principles and Practice. 2nd ed. Boston: Butterworth-Heinemann, 1994, pp 417–426.
2. FR Sharpe, D Crabb. J Inst Brew 86:60–64, 1980.
3. DA Moyler. In: MB King, TR Bott, eds. Extraction of Natural Products Using Near-Critical Solvents. Glasgow: Blackie Academic, 1993, pp 140–183.
4. N Nguyen, DA Evans, G Frakman. In: SSH Rizvi, ed. Supercritical Fluid Processing of Biomaterials. London: Blackie Academic, 1994, pp 103–113.
5. JW King. In: E Kiran, PG Debenedetti, CJ Peters. Supercritical Fluids: Fundamentals and Applications. Dordrecht: Kluwer Academic, 2000, pp 451–488.
6. M Yalpani, ed. New Technologies for Healthy Foods and Nutraceuticals. Shrewsbury, MA: AT Press, 1997.
7. PT Anastas, TC Williamson, eds. Green Chemistry. Washington, DC: American Chemical Society, 1996. pp. 251.
8. J King. INFORM 4:1089–1098, 1993.
9. P Mantinea, P Laakso, H Kallio. J Am Oil Chem Soc 72:1001–1008, 1995.
10. JW King, FJ Eller, SL Taylor, AL Neese. Utilization of analytical critical fluid instrumentation in non-analytical applications. Proceedings of the 5th International Symposium on Supercritical Fluids, Atlanta, GA, 2000 (CD disk).
11. JW King, ML Hopper. J Assoc Off Anal Chem Int 75:375–378, 1992.
12. C Grimmett. Chem Ind 1981(10):359–362, 1981.
13. M Mukhopadhyay. Natural Extracts Using Supercritical Carbon Dioxide. Boca Raton, FL: CRC Press, 2000, pp 265–302.
14. JC Giddings, MN Myers, L McLaren, RA Keller. Science 162:67–73, 1968.
15. J King. In: PJ Wan, PJ Wakelyn, eds. Technology and Solvents for Extracting Oilseeds and Nonpetroleum Oils. Champaign, IL: AOCS Press, 1997, 283–310.
16. JP Friedrich. US Patent 4,446,923, August 21, 1984.
17. RT Kurnik, SJ Holla, RC Reid. J Chem Eng Data 26:47–51, 1981.
18. E Stahl, K-W Quirin, D Gerard. Dense Gases for Extraction and Refining. Berlin: Springer-Verlag, 1988, pp 85–94.
19. JW King, M Cygnarowicz-Provost, F. Favati. Ital J Food Sci 9:193–204, 1997.
20. G Brunner. Ber Bunsenges Phys Chem 88:887–891, 1984.
21. IK Hong, SW Rho, KS Lee, WH Lee, KP Yoo. Korean J Chem Eng 7:40–46, 1990.
22. E Reverchon, G Donsi, LS Osseo. Ind Eng Chem Res 32:2721–2726, 1993.
23. E Stahl, K-W Quirin, D Gerard. Dense Gases for Extraction and Refining. Berlin: Springer-Verlag, 1988, p 6.
24. AF Williams, WL Lom. Liquefied Petroleum Gases. New York: Wiley, 1974, p 367.
25. S Peter. In: J King, GR List, eds. Supercritical Fluid Technology in Oil and Lipid Chemistry. Champaign, IL: AOCS Press, 1996, pp 65–100.
26. AA Clifford, A Basile, MM Jimenez-Carmona, SHR AI-Saidi. Extraction of natural products with superheated water. Proceedings of the GVC-Fachausschss ''High Pressure Chemical Engineering,'' Karlsruhe, Germany, 1999.
27. MB King, TR Bott. Extraction of Natural Products Using Near-Critical Solvents. Glasgow: Blackie Academic, 1993, pp. 435.
28. E Stahl, E Schutz, HK Mangold. J Agric Food Chem 28:1153–1157, 1980.
29. WG Schultz, JM Randall. Food Technol 24(11):94–98, 1970.
30. G Brunner. Gas Extraction. Darmstadt: Steinkopf Verlag, 1994, pp 170–174.
31. RL Holliday, J King, GR List. Ind Eng Chem Res 36:932–935, 1997.
32. J King. In: PJ Wan, PJ Wakelyn, eds. Technology and Solvents for Extracting Oilseeds and Nonpetroleum Oils. Champaign, IL: AOCS Press, 1997, pp 283–310.
33. JW King, GR List. Supercritical Fluid Technology in Oil and Lipid Chemistry. Champaign, IL: AOCS Press, 1996, pp. 435.

34. JW King. Trends Anal Chem 14:474–481, 1995.
35. GR List, JP Friedrich, JW King. Oil Mill Gaz 95(6):28–34, 1989.
36. BD Oomah, G Mazza. Trends Food Sci Tech 10:193–198, 1999.
37. SM Fors, CE Eriksson. Lebensm Wiss Technol 23:390–395, 1990.
38. Z Shen, MV Palmer, SST Ting, RJ Fairclough. J Agric Food Chem 44:3033–3039, 1996.
39. NT Dunford, JW King. Supercritical carbon dioxide fractionation of crude rice bran oil using a packed column with characterization of the resultant fractions. Proceedings of the 5th International Symposium on Supercritical Fluids, Atlanta, GA, 2000 (CD disk).
40. Z Xu, JS Godber. J Am Oil Chem Soc 77:547–551, 2000.
41. RA Moreau, MJ Powell, KB Hicks. J Agric Food Chem 44:2149–2154, 1996.
42. BA Haumann. INFORM 9:6–13, 1998.
43. F Favati, JW King, M Mazzanti. J Am Oil Chem Soc 68:422–427, 1991.
44. QH Hu. J Agric Food Chem 47:2705–2706, 1999.
45. F Favati, JW King, JP Friedrich, K Eskins. J Food Sci 53:1532–1536, 1988.
46. G Della Porta, R Taddeo, E D'Urso, E Reverchon. Lebensm Wiss Technol 31:454–460, 1998.
47. E Ibanez, J Palacios, FJ Senorans, G Santa-Maria, J Tabera, G Reglero. J Am Oil Chem Soc 77:187–190, 2000.
48. T Clifford. Fundamentals of Supercritical Fluids. Oxford: Oxford University Press, 1999, pp 130–154.
49. JW King, E Sahle-Demessie, F Temelli, JA Teel. J Supercrit Fluids 10:127–136, 1997.
50. E Sahle-Demessie. Ind Eng Chem Res 36:4906–4913, 1997.
51. NT Dunford, JW King. Enrichment of phytosterols in vegetable oils utilizing supercritical carbon dioxide and a fractionating tower. Abstracts of the 2000 IFT Annual Meeting, Dallas, TX, 2000, p 58, Abstract #37-10.
52. G Brunner, T Malchow, K Sturken, T Gottschau. J Supercrit Fluids 4:72–80, 1991.
53. V Riha, G Brunner. J Supercrit Fluids 17:55–64, 2000.
54. JW King, F Favati, SL Taylor. Separ Sci Tech 31:1843–1857, 1996.
55. SL Taylor, JW King, L Montanari, P Fantozzi, MA Blanco. Ital J Food Sci 12:65–76, 2000.
56. SL Taylor, JW King. J Am Oil Chem Soc 77:687–688, 2000.
57. D Stanley. Agric Res 45(12):12–13, 1997.
58. P Lembke. In: K Anton, C Berger, eds. Supercritical Fluid Chromatography in Packed Columns. New York: Marcel Dekker, 1998, pp 429–443.
59. JW King, MA Jackson, GR List, E Sahle-Demessie, RL Holliday, F Temelli. In: SS Koseoglu, KC Rhee, and RF Wilson, eds. Proceedings of the World Conference on Oilseed and Edible Oils Processing, Champaign, IL: AOCS Press, 1998, pp 314–320.
60. MA Jackson, JW King. J Am Oil Chem Soc 73:353–356, 1996.
61. MA Jackson, JW King. J Am Oil Chem Soc 74:103–106, 1997.
62. MA Jackson, JW King, GR List, WE Neff. J Am Oil Chem Soc 74:635–639, 1997.
63. A Overmeyer, S Schrader-Leppelt, V Kasche, G Brunner. Biotechnol Lett 21:65–69, 1999.
64. JM Snyder, J King, MA Jackson. J Chromatogr 750:2101–2107, 1996.
65. JW King, SL Taylor, JM Snyder, RL Holliday. J Am Oil Chem Soc 75:1291–1295, 1998.
66. TW Randolph, HW Blanch, DS Clark. In: JS Dordick, ed. Biocatalysis for Industry. New York: Plenum Press, 1991, pp 219–237.
67. KA Evelein, RG Moore, RA Heidemann. Ind Eng Chem Process Des Dev 15:423–428, 1976.
68. M Harrod, MB Macher, J Hogberg, P Moller. Hydrogenation at supercritical conditions. Proceedings of the 4th Italian Conference on Supercritical Fluids. Capri, Italy, 1997, pp 319–326.
69. T Tacke, S Wieland, P Panster. Selective and complete hardening of edible oils and free fatty acids in supercritical fluids. 4th International Meeting on Supercritical Fluids, Sendai, Japan, 1997, pp 511–514.
70. T Tacke, S Wieland, P Panster. In: PR von Roar, C Repp, eds. High Pressure Chemical Engineering. Amsterdam: Elsevier Science, 1996, pp 17–21.

71. JW King, MBO Andersson. Synthetic modification of oleochemicals in critical fluids. Proceedings of the 6th Meeting on Supercritical Fluids, Nottingham, 1999, pp 195–203.
72. DJ Miller, SB Hawthorne. Anal Chem 70:1618–1621, 1998.
73. JW King, RL Holliday, GR List. Green Chem 1:261–264, 2000.
74. NS Issacs, ed. High Pressure Food Science, Bioscience and Chemistry. Cambridge: The Royal Society of Chemistry, 1998, p 511.
75. JW King, JM Snyder. In: R McDonald, M Mossoba, eds. New Techniques and Applications in Lipid Analysis. Champaign, IL: AOCS Press, 1997, pp 139–162.
76. JW King. J Assoc Off Anal Chem Int 81:9–17, 1998.
77. FJ Eller, JW King. Sem Food Anal 1:145–162, 1996.
78. SJ Lehotay. J Chromatogr A 785:289–312, 1997.
79. JM Snyder, JW King. J Am Oil Chem Soc 71:261–265, 1994.

35

Enzyme Reactions in Supercritical Fluids

OSATO MIYAWAKI and KOZO NAKAMURA

The University of Tokyo, Tokyo, Japan

1 INTRODUCTION

Enzyme reactions can be conducted in a nonaqueous system [1]. Enzyme reactions in nonaqueous systems, using organic solvents, have the following advantages: (1) increase the solubility of hydrophobic substrates; (2) shift the reaction equilibrium from hydrolysis to synthesis of products; (3) inhibit the microbial contamination.

Supercritical fluids are materials existing above their respective critical temperature and the critical pressure and can serve as alternative reaction media, as opposed to organic solvents for conduction of enzyme reactions [2–4]. As supercritical fluid is intermediate between a gas and liquid, it can have unique physical properties, such as exhibiting high diffusivities and low viscosity relative to a gas, with densities approaching those of a liquid, and surface tensions which are normally very low. In addition, these physicochemical properties of the supercritical fluid are changeable over a wide range simply by changing pressure.

In this chapter, the current status of basic investigations on enzyme reactions in supercritical fluids and their applications are overviewed.

2 ENZYME REACTIONS IN SUPERCRITICAL FLUIDS

2.1 Supercritical Fluid as an Enzyme Reaction Medium

Table 1 shows the list of critical temperature, T_c, and critical pressure, P_c, of supercritical fluids, which are considered to be applicable as media for enzyme reactions [3]. These supercritical fluids have T_c and P_c in the ranges so that enzyme proteins are not physically affected by them (i.e., denaturation).

Table 1 Supercritical Fluids Available for Enzyme Reaction Media and Their Physical Properties

Material	T_c (°C)	P_c (MPa)	ρ_c (g/mL)
Ethylene	9.2	5.04	0.218
Fluoroform	25.9	4.84	0.526
Carbon dioxide	31.0	7.38	0.468
Ethane	32.3	4.88	0.210
Sulfur hexafluoride	45.6	3.76	0.734

Source: Ref. 3.

Enzyme reactions in supercritical fluids were first investigated by Hammond et al. [5], Randolph et al. [6], and Nakamura et al. [7]. Table 2 lists typical investigations carried out so far in this field. Among supercritical fluids, supercritical carbon dioxide (SC-CO_2) is the most dominant because it is environmentally benign, nontoxic, nonflammable, and a natural solvent having a low price that can be widely used in the food and pharmaceutical field. Among the enzymes, lipases are most frequently used because they catalyze various useful reactions, as shown in Table 3, with relatively low substrate specificity.

2.2 Enzymes in Supercritical Fluids

When enzymes are used in a supercritical fluid, the enzyme proteins are not solubilized nor dispersed in the reaction medium. This often causes a low catalytic efficiency to be realized. To overcome this problem, immobilized enzymes are frequently used on solid support materials. Recently, Okahata et al. [34,35] applied lipid-coated enzymes in SC-CO_2. Triglyceride synthesis using such lipid-coated lipases [34], as well as transgalactosylation rates using lipid-coated β-D-galactosidase [35] in SC-CO_2, were found much higher as compared with the case in which native enzymes were used.

Stability of enzymes in supercritical fluid is very important in practice. Taniguichi et al. [36] studied the stabilities of α-amylase, glucoamylase, β-galactosidase, glucose oxidase, glucose isomerase, lipase, thermolysin, alcohol dehydrogenase, and catalase in SC-CO_2 at 35°C, 20 MPa for 1 hr. They showed that enzymes are compatible with SC-CO_2 containing ethanol less than 6% and water less than 0.1%. Lipase, however, lost its activity by two-thirds when 50% water coexisted. Kao et al. [37] measured the stability of lipase in SC-CO_2 and in supercritical SF_6 at 60°C and 8.2 MPa for 2 days. The enzyme was stable in both solvents when dry, but it became very unstable, especially in SC-CO_2 when the water content was higher than 1%.

Lozano et al. [38] reported that the stability of the immobilized α-chymotrypsin in SC-CO_2 was dependent on the fluid density. The half-life of the enzyme changed in the following order: liquid > supercritical > gas. The stability was also strongly affected by the water content. Overmeyer et al. [39] showed a surprising high stability of lipase from *Candida antarctica* B (Novozyme 435), with a maximal activity at 90°C in SC-CO_2. These results suggest that enzymes are fairly stable in dry supercritical fluid in most cases. Therefore, it is most important to find the optimal condition for each enzyme in a supercritical fluid at the desired water content level.

Table 2 Investigations on Enzyme Reactions in Supercritical Fluids

Supercritical fluid	Reaction (substrate)	Enzyme	Ref.
CO_2	Trilaurin + palmitic acid	Lipase	8
CO_2	Triolein + stearic acid	Lipase	7
CO_2	Cod-liver oil + ethanol	Lipase	9–11
CO_2	Canola oil + milkfat	Lipase	12
CO_2	Triolein + ethylbehenate	Lipase	13,14
CO_2	1-Nonanol + ethylacetate	Lipase	15
CO_2	*n*-Valeric acid + citroneroll	Lipase	16
CO_2	Oleic acid + ethanol	Lipase	17,18,19
CO_2	Myristic acid + ethanol	Lipase	20
CO_2	Butanol + lauric acid	Lipase	21
CO_2	Isoamyl alcohol + ammonium acetate	Lipase	22
CO_2	*N*-(Benzoyloxycarbonyl)-L-aspartic acid + L-phenylalanine methyl ester	Thermolysin	23
CO_2	*p*-Cresol	Polyphenol oxidase	5
CO_2	*p*-Nitorphenylphosphate	Alkaline phospha-tase	6
CO_2	Cholesterol	Cholesterol oxidase	24–26
CO_2	Hexanol + hexanoic acid	Cutinase	27
CO_2	Avicel	Cellulase	28
CO_2, ethylene	1-Phenylethanol	Cutinase	29
Fluoroform	Methyl methacrylate + 2-ethyl-1-hexanol	Lipase	30
Fluoroform	*N*-Acetyl-L-phenylalanine ethyl ester + methanol, etc.	Subtilisin	31,32
SF_6, ethane, ethylene, CO_2	Methyl methacrylate + 2-ethylhexanol	Lipase	33

Table 3 Reactions Catalyzed by Lipase

A. Hydrolysis

$R_1COOR_2 + H_2O \rightarrow R_1COOH + R_2OH$

B. Ester synthesis

$R_1COOH + R_2OH \rightarrow R_1COOR_2 + H_2O$

C. Transesterification

C1. Acidolysis

$R_1COOR_2 + R_3COOH \rightarrow R_3COOR_2 + R_1COOH$

C2. Alcoholysis

$R_1COOR_2 + R_3OH \rightarrow R_1COOR_3 + ROH$

C3. Ester–ester transesterification

$R_1COOR_2 + R_3COOR_4 \rightarrow R_1COOR_4 + R_3COOR_2$

2.3 Comparison of Supercritical Fluid with Organic Solvent as Enzyme Reaction Media

As a reaction medium, SC-CO_2 has been compared with hexane, which is an organic solvent frequently used for enzyme reactions. Chi et al. [40] carried out hydrolysis of triolein and its transesterification with stearic acid catalyzed by lipase in SC-CO_2 and hexane. In both reactions of hydrolysis and transesterification, the reaction rates were higher in SC-CO_2 than in hexane, as shown in Figure 1. Similar results were reported for the ester synthesis from oleic acid and primary alcohols [12,17,41].

The reaction mechanism of lipase is reported to be described by a Ping-Pong Bi-Bi model [19,20] and the kinetic parameters were measured in SC-CO_2 and hexane. When the immobilized lipase from *Mucor miehei* was used, the maximum velocity was higher for SC-CO_2 than hexane in the esterification of myristic acid with ethanol [20] but not much different in the esterification of oleic acid with ethanol [19]. Steytler et al. [21]

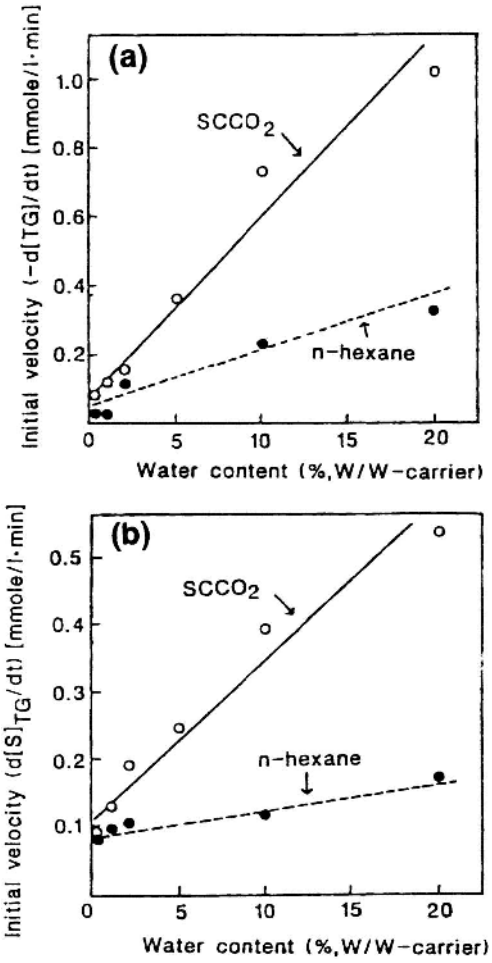


Figure 1. Comparison of SC-CO_2 (29.4 MPa) and hexane at 323 K as lipase reaction media for (a) hydrolysis and (b) transesterification between triolein and stearic acid. (From Ref. 40.)

showed that the synthesis rate of butyl laurate from butanol and lauric acid by lipase (*Candida*) was higher in SC-CO_2 than in toluene and hexane by saturating the supercritical fluid with water.

On the contrary, the synthesis rate of isoamyl acetate by lipase-catalyzed acylation of the corresponding alcohol was higher in hexane than SC-CO_2 [22]. In the synthesis of geranyl acetate [42] and 2-ethylhexylmethacrylate [43] by lipase-catalyzed transesterification, the reaction rate was much higher in hexane than SC-CO_2. Thus, the comparison of SC-CO_2 as an enzyme reaction medium with organic solvents is not straightforward. Marty et al. [18] recommended to use SC-CO_2 in the water-producing enzyme reaction for its hydrophilicity compared with hexane. As SC-CO_2 exhibits a great variability in density and, consequently its solvent power as a function of temperature and pressure, SC-CO_2 may have advantages over organic solvents when it is used in an integrated process of reaction and separation [44].

2.4 Comparison Among Supercritical Fluids

As shown in Table 1, SC-CO_2 is not a sole supercritical fluid available as a medium for enzyme reactions. Kamat et al. [33] carried out lipase-catalyzed transesterification of methyl metacrylate in various supercritical fluids and showed that sulfur hexafluoride gave the highest and SC-CO_2 gave the lowest reaction rate. SC-CO_2 reversibly forms covalent complexes with free-amine groups of the enzyme. Barreiros et al. used subtilisin [45], lipase [46], and cutinase [29] in compressed, near-critical, and supercritical fluids and showed an adverse effect of CO_2 in the enzyme reactions. These results suggest that SC-CO_2 is not the best supercritical fluid, depending on the enzyme system employed with respect to the reaction rate.

3 FACTORS AFFECTING ENZYME REACTIONS IN SUPERCRITICAL FLUIDS

3.1 Effect of Pressure

Nakaya et al. [47] reported the effect of pressure on the lipase-catalyzed transesterification rate between triolein and stearic acid in SC-CO_2. The reaction rate has a sharp peak at the pressure near the critical point, as shown in Figure 2. A similar phenomenon has been reported by Ikushima et al. [16] in a lipase-catalyzed esterification of *n*-valeric acid and citroneroll in SC-CO_2. They explained the sharp increase in the activity at the near-critical point by the increase in the electron-accepting power of SC-CO_2 expressed by the Kamlet–Taft α-value [48]. They also observed a sharp increase at the near-critical point in stereoselectivity in the esterification between oleic acid and ($\pm$)-citroneroll [49]. This phenomenon was explained by the sharp increase in the interaction between carbon dioxide and the enzyme causing a sharp change in the secondary structure of the protein, which was measured by Fourier transform infrared spectroscopy and microgravimetry [50,51].

Randolph et al. [24–26] investigated cholesterol oxidation in SC-CO_2 catalyzed by cholesterol oxidase from *Gloeacysticum chrysocreas* also in the near-critical region. They showed that the reaction rate increased with an increase in pressure in the near-critical region or with the addition of small amount of cosolvents such as *tert*-butanol and isobutanol. From electron spin resonance (ESR) spectroscopic measurements, this increase in the reactivity in the near-critical region or with the cosolvent addition was explained by the aggregation of cholesterol molecules, not by the change in the protein structure.

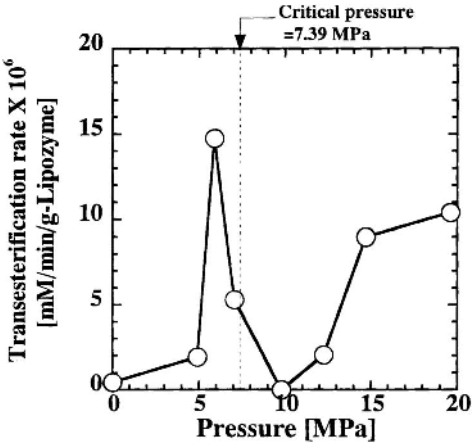


Figure 2. Effect of pressure on lipase-catalyzed transesterification rate between triolein and stearic acid in pressurized CO_2 at 323 K. (From Ref. 47.)

In the supercritical region in SC-CO_2 using pressures >10 MPa, the effect of pressure on the enzyme reactivity seems complicated for the homogeneous system operated under the solubility limit of substrates. Steytler et al. [21] reported an increase of enzyme reactivity with pressure (150–500 MPa) in the lipase-catalyzed synthesis of butyl laurate from butanol and lauric acid. They explained this by the change in the partition of the

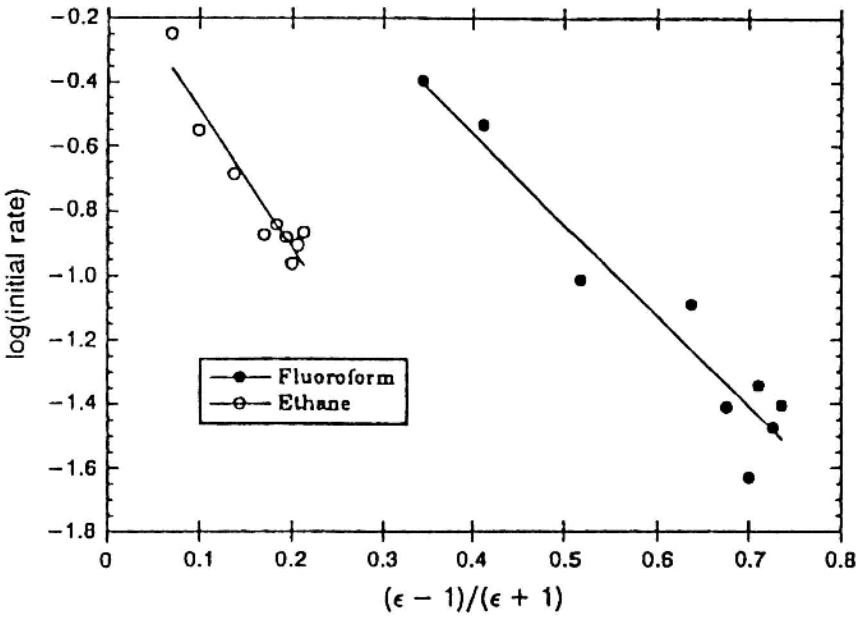


Figure 3. Relationship between solvent dielectric constant and activity of lipase in fluoroform (●) and ethane (○). (Adapted from Ref. 30.)

substrate between the solid enzyme phase and the SC-CO_2 phase. On the contrary, Erickson et al. [8] reported a decrease of the enzyme reactivity with pressure in the transesterification of trilaurin and palmitic acid catalyzed by lipase. They explained that an increase in pressure led to the dilution effect of the substrates on the molar fraction basis. Vermue et al. [15] studied the transesterification of nonanol and ethyl acetate catalyzed by lipase (*Mucor miehei*) in SC-CO_2. They also estimated the Hildebrand solubility parameter as an index of the solubilization power of SC-CO_2, which increased with pressure. This increase, however, hardly influenced the transesterification rate by the enzyme. Fontes et al. [52] carried out subtilisin-catalyzed transesterification of *N*-acetyl-L-phenylalanine ethyl ester (APEE) with 1-propanol in compressed propane, near-critical SC-CO_2, and *tert*-amyl alcohol at various pressures, keeping the molar fractions of substrates constant. In all of the cases, increasing pressure lowered the catalytic activity of the enzyme and showed positive activation volumes in these reactions.

For heterogeneous systems over the solubility limit of substrates in far supercritical

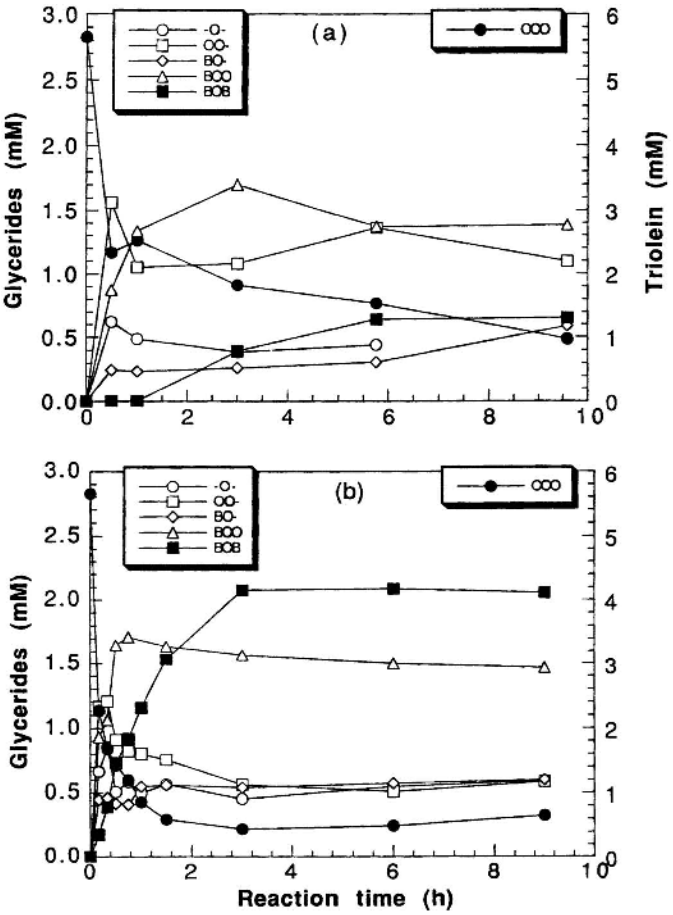


Figure 4. Changes in glycerides in the lipase-catalyzed transesterification between triolein and (a) behenic acid or (b) ethylbehenate in SC-CO_2 at 50°C, 15 MPa. (From Ref. 14.)

CO_2, an increase in pressure caused an increase in the reactivity simply because of an increase in the solubility of substrates, as shown by the lipase-catalyzed transesterification between triolein and stearic acid in Figure 2 [47].

For supercritical fluids other than SC-CO_2, the effect of pressure on enzyme reactivity seems more straightforward. Kamat et al. [30] observed a large increase in enzyme reactivity with pressure (5.9–28 MPa) for lipase-catalyzed transesterification of methylmethacrylate with 2-ethyl-1-hexanol in supercritical fluoroform. In this pressure range, the dielectric constant changed from 1 to 8, and a good correlation with the change in the reactivity could be expressed through the Kirkwood expression (Fig. 3) as follows:

$$\text{Log(rate)} = \frac{\varepsilon - 1}{\varepsilon + 1} \tag{1}$$

where ε is the dielectric constant. The same approach was also applied to the enantioselectivity of subtilisin (*Carlsberg*) and *Aspergillus* protease [31] and the substrate selectivity of subtilisin [32] in supercritical fluoroform. These results strongly suggest the possibility of the control of enzyme reactivity in supercritical fluids simply by changing pressure.

3.2 Effect of Substrate Solubility

Solubility of substrates in supercritical fluids is very important and this substantially affects enzyme reactions in some cases. Jackson and King [53] carried out lipase-catalyzed alcoholysis of soybean oil with glycerol, 1,2-propanediol, ethylene glycol, and methanol in SC-CO_2. The reactivity of the alcohols paralleled with their solubility in SC-CO_2.

Yoon et al. [13,14] carried out the lipase-catalyzed transesterification between triolein and behenic acid or ethylbehenate in SC-CO_2 to produce 1,3-dibehenoyl-2-oleoyl glycerol (BOB), used as an antiblooming agent for chocolate. In this case, the production rate of the final product, BOB, was much higher when ethylbehenate was used compared to behenic acid, as shown in Figure 4. These two materials were found to exhibit differential solubility in SC-CO_2 of three order of magnitude, as shown in Figure 5. This seemed to partly explain the difference in the reactivity shown in Figure 4 [14,54]. The solubility

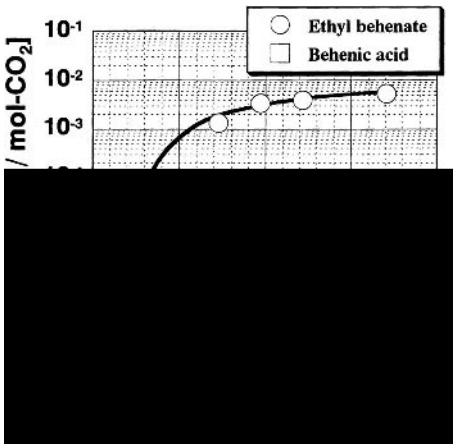


Figure 5. Solubility of behenic acid and ethylbehenate in SC-CO_2 at 313 K. Fitting curves were based on the regular solution model coupled with the Flory–Huggins theory. (From Ref. 54.)

of these materials was described well by the regular solution model modified with Flory–Huggins theory [55,56], expressed by the solid lines in Figure 5 [54]. For the transesterification of triglycerides in SC-CO_2, an ester form of fatty acid, instead of a free fatty acid, is recommended for use as a substrate, due to its higher solubility in the supercritical fluid [54].

3.3 Effect of Water

The importance of water in enzyme reactions is not restricted only in supercritical fluids. Water affects enzyme stability, the reaction equilibrium, and the reaction kinetics in any reaction media. The effect of water on enzyme stability in a supercritical fluid was already described in Sec 2.2. In the hydrolytic enzyme reactions, water is one of the substrates and it affects both the reaction equilibrium and kinetics. Figure 6 shows the effect of the water content on the transesterification between triolein and ethylbehenate catalyzed by lipase in SC-CO_2 [13]. Both the hydrolysis and the transesterification activities increased with an increase in the water content, but the transesterification rate reached maximum

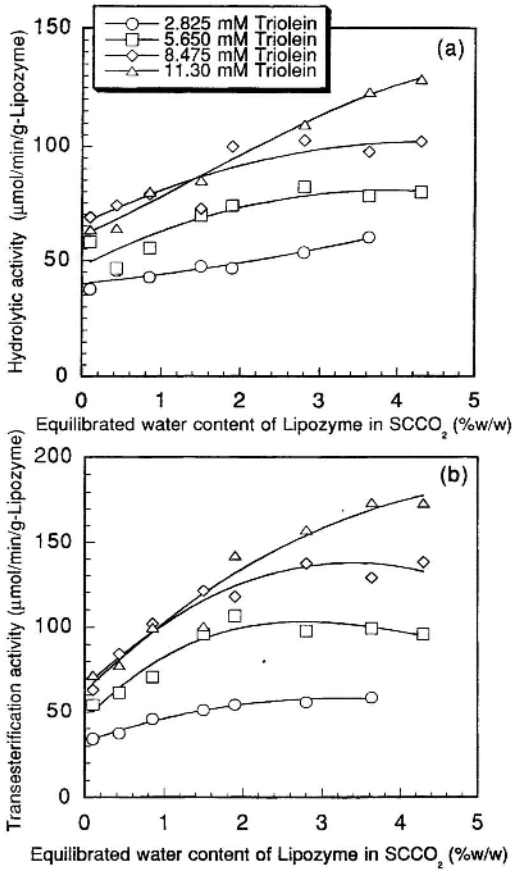


Figure 6. Effect of water content on (a) hydrolytic activity and (b) transesterification activity of lipase (Lipozyme) for reaction between triolein and ethybehenate in SC-CO_2 at 50°C, 30 MPa. (From Ref. 13.)

because of the competition with the hydrolysis reaction, which monotonously increased with an increase in the water content. In some cases, however, too much water around the enzyme physically hinders the interaction between hydrophobic substrates and the enzyme. Hence, it is necessary to find the optimal water content for each system.

4 APPLICATIONS OF ENZYME REACTIONS IN SUPERCRITICAL FLUIDS

4.1 Analytical Field

In the analytical field, the application of supercritical fluid chromatography is already established as a routine method. In this method, the pressure gradient technique is available, as well as temperature gradient, to control the solvent power of a mobile phase. For the total-fat analysis in various meat and oilseed samples, Snyder et al. [57,58] used SC-CO_2 for extraction of fat followed by lipase-catalyzed methylation of the extracted fat prior to gas chromatography. Good recovery compared to Soxhlet extraction with hexane and good conversions to methyl ester were reported for fats from soybean, sunflower, bacon, beef, and sausage. The same group [59] also applied the same technique of coupling supercritical fluid extraction with supercritical fluid reaction to analyze the total fatty acid contents of soybean- and corn-oil–based soapstocks. The results were comparable with those obtained by the AOCS official method but could be done in about one-half the time. In addition, the amount of solvent used in each analysis was reduced from 575 mL using an official AOCS method to only 1.8 mL using this method.

4.2 Food and Pharmaceutical Area

Although SC-CO_2 may not be the best supercritical fluid for conducting enzyme reactions, as was described in Sec. 2.4, SC-CO_2 is most frequently used in the food and pharmaceutical area because of its inherent safety and naturalness. As an enzyme, lipases are most dominantly used because of their solvent tolerance and multifunctionality (Table 3). Table 4 summarizes the potential applications of enzyme reactions in SC-CO_2 in the food and pharmaceutical area.

For example, oleyl oleate was synthesized by lipase-catalyzed reaction in SC-CO_2, which was shown to be more effective than the solvent-free system [41]. Oleyl oleate finds uses in cosmetics, pharmaceutical and food additives, and high-pressure lubricants. Fatty acid esters of carbohydrate are useful as nonionic surfactants applied in food and pharmaceutical industries. Lipase-catalyzed acylation of glucose with lauric acid can be carried out in SC-CO_2, providing conversions up to 60% [60]. Geranyl acetate, a flavor compound naturally found in lemon oil, can be synthesized from geraniol and propyl acetate [42]. Thioesters, serving as artificial fruits flavors, can also be synthesized from oleic acid and buthanethiol [61].

For the preferential extraction of n-3 polyunsaturated fatty acids, such as eicosapentanoic acid and docosahexanoic acid, lipase-catalyzed alcoholysis by ethanol has been applied to cod-liver oil [9–11]. At a relatively low pressure (9 MPa), the synthesized ethyl ester was preferentially extracted. Monoglycerides, which are emulsifiers in the food and pharmaceutical industries, have been produced by lipase-catalyzed glycerolysis of soybean oil, providing yields higher than 80% [53]. 1,3-Dibehenoyl-2-oleoyl glycerol, an anti-blooming agent for chocolate, was produced by the lipase-catalyzed transesterification between triolein and ethylbehenate [13,14].

Table 4 Application of Enzyme Reaction in SC-CO_2 in Food and Pharmaceutical Areas

Reaction category	Final product	Starting material	Enzyme	Ref.
Ester synthesis	Oleyl oleoate	Oleic acid/oleyl alcohol	Lipase (Lipozyme IM)	41
Ester synthesis	Carbohydrate fatty acid ester	Lauric acid/glucose	Lipase (*Candida rugosa*)	60
Ester synthesis	Geranyl acetate	Geraniol/propyl acetate	Lipase (Lipozyme)	42
Ester synthesis	Thioester	Oleic acid/butanethiol	Lipase (Lipozyme)	61
Alcoholysis	Ethylester of fatty acid	Cod-liver oil/ethanol	Lipase (Novozyme 435)	9–11
Glycerolysis	Monoglyceride	Soybean oil/glycerol	Lipase (Novozyme 435)	53
Transesterification	BOB[a]	Triolein/ethylbehanate	Lipase (Lipozyme)	13, 14
Trandesterification	Cocoa butter equivalent	Palm oil/tristearin	Lipase (Lipozyme IM-20)	62
Transesterification	Interestrified fat	Milkfat/canola oil	Lipase (*Candida cylindracea*)	12
Transesterification	Randomized fat	Soybean oil, palm oil, etc.	Lipase (Novozyme 435)	63
Peptide synthesis	Aspartame precursor	*Z*-L-Asp/L-PheOMe[b]	Thermolysin	23
Oxidation	4-Cholesten-3-one	Cholesterol/O_2	Cholesterol oxidase	24–26

[a] 1,3-Dibehenoyl-2-oleoyl glycerol.
[b] *N*-(Benzyloxycarbonyl)-L-aspartic acid β-benzyl ester/L-phenylalanine methyl ester.

Physical properties of fat and oil, such as melting point, are very important in their practical use in food and medicine. These properties have been modified by lipase-catalyzed transesterification in SC-CO_2. A cocoa butter equivalent was produced by transesterification between palm oil and tristearin [62]. The melting point of the transesterified product was 34.3°C. Interesterification between canola oil and milkfat has been carried out to produce a high-value confectionary fat [12]. The starting mixture, consisted mainly of the triglycerides of C_{52}–C_{56} and C_{32}–C_{38} for the respective canola oil and milkfat, were changed to a C_{42}–C_{52} and C_{54} distribution. Lipase-catalyzed randomization of fats and oils has also been attempted for soybean oil, palm oil, cocoa butter, tallow, and so forth [63]. Substantial changes in the melting point of these starting substances can be observed after the randomization.

As for enzymes other than lipase, thermolysin was used in SC-CO_2 for the synthesis of the precursor of aspartame, an artificial sweetener [23]. A favorable conversion above 30% was reported with the case using liquid CO_2. Cholesterol oxidase was applied to produce 4-cholesten-3-one, a precursor of interest in the pharmaceutical production of androst-1,4-diene-3,17-diene, from cholesterol and oxygen [24–26].

4.3 Stereospecific Separation

Stereospecificity is the one of the most important feature of enzyme reactions. This is applicable for the kinetic resolution of stereospecific compounds and serves as a basis for separation using supercritical fluids. This is important especially in pharmaceutical industries. Lipase-catalyzed kinetic resolution of racemic 3-hydroxy ester was carried out in SC-CO_2 and a good enantiometric excess up to 99% was reported [64]. 3-Hydroxy esters represent a class of chiral building blocks for the synthesis of various compounds. Stereospecific hydrolysis of 3-(4-methoxyphenyl) glycidic ester, for the production of a material with activity as a calcium antagonist, was performed to a 87% stereospecific excess at 53% conversion level [65]. Stereospecificity of porcine pancreatic lipase was investigated and the effect of water content was reported on the enzyme specificity [66]. Enantioselective hydrolysis of bicyclo [3.2.0] heptanoyl ester for precursors to a number of physiologically active compounds was carried out by lipase catalysis in SC-CO_2 [67]. Lipase-catalyzed kinetic resolution of ibuprofen, an anti-inflammatory drug, was performed at temperatures from 40°C to 160°C in SC-CO_2 [39].

5 CONCLUDING REMARKS

Supercritical fluids have been expected to be the most unique solvent for enzyme reactions because their solvent properties are easily modified simply by changing a pressure as well as temperature. This have been elegantly proved for lipase-catalyzed reaction in supercritical fluoroform and ethane, as shown in Figure 3 [30].

In practice, however, SC-CO_2 will be a most promising medium, but enzyme-catalyzed reactions in SC-CO_2 are much more complicated than those in fluoroform and ethane. Among supercritical fluids, SC-CO_2 seems very special for its hydrophilicity. SC-CO_2 directly interacts with enzyme proteins [16,33] that may cause a change in pH in the local environment and even in the structure of enzyme protein [50]. Enzyme reactions in SC-CO_2 show a singularity around the near-critical point in many cases [16,26,47]. These seem to make a general discussion and categorization on enzyme reactions in SC-CO_2 difficult. Therefore, enzyme reactions in SC-CO_2 should be discussed on an individual

enzyme basis considering four major state parameters: temperature, pressure, water content, and cosolvent.

Practical application of these processes is determined by a balance between the added value of the product and the cost of the process. Rapidly increasing interests for the safety from the environmental impact increase the application of SC-CO_2 enzyme processes and derived products so that a breakthrough will be expected in the near future for the processes listed in Table 4. Before that, however, a more complete understanding of the solvent properties of SC-CO_2 [15,48] as an enzyme reaction medium, followed by the optimization of the process parameters, will be necessary, coupled with possible integration with downstream processing for product separation and purification [44].

REFERENCES

1. A Zaks, AM Klibanov. J Biol Chem 263:3194–3201, 1988.
2. K Nakamura. TIBTECH 8:288–292, 1990.
3. SV Kamat, EJ Beckman, AJ Russel. Crit Rev Biotechnol 15:41–71, 1995.
4. AJ Mesiano, EJ Beckman, AJ Russel. Chem Rev 99:623–633, 1999.
5. DA Hammond, M Karel, AM Klibanov. Appl Biochem Biotechnol 11:393–400, 1985.
6. TW Randolph, HW Blanch, JM Prausnitz. CR Wilke. Biotechnol Lett 7:325–328, 1985.
7. K Nakamura, YM Chi, Y Yamada, T Yano. Chem Eng Commun 45:207–212, 1986.
8. JC Erickson, P Schyns, CL Cooney. AIChE J 36:299–301, 1990.
9. H Gunnlaugsdottir, B Sivik. J Am Oil Chem Soc 72:399–405, 1995.
10. H Gunnlaugsdottir, B Sivik. J Am Oil Chem Soc 74:1483–1490, 1997.
11. H Gunnlaugsdottir, AA Karlsson, B Sivik. J Am Oil Chem Soc 74:1491–1494, 1997.
12. ZR Yu, SSH Rizvi, JA Zollweg. Biotechnol Prog 8:508–513, 1992.
13. SH Yoon, O Miyawaki, KH Park, K Nakamura. J Ferment Bioeng 82:334–340, 1996.
14. SH Yoon, H Nakaya, O Ito, O Miyawaki, KH Park, K Nakamura. Biosci Biotech Biochem 62:170–172, 1998.
15. MH Vermue, J Tramper, JPJ de Jong, WHM Oostrom. Enzyme Microb Technol 14:649–655, 1992.
16. Y Ikushima, N Saito, M Arai. J Chem Eng Japan 29:551–553, 1996.
17. A Marty, W Chulalaksananukul, JS Condoret, RM Willemot, G Durand. Biotechnol Lett 12: 11–16, 1990.
18. A Marty, V Dossat, JT Condoret. Biotechnol Bioeng 56:232–237, 1997.
19. A Marty, W Chulalaksananukul, JS Condoret, RM Willemot, JS Condoret. Biotechnol Bioeng 39:273–280, 1992.
20. T Dumont, D Barth, C Corbier, G Branlant, M Purrut. Biotechnol Bioeng 39:329–333, 1992.
21. DC Steytler, PS Moulson, J Reynolds. Enzyme Microb Technol 13:221–226, 1991.
22. H Vija, A Telling, V Tougu. Bioorg Med Chem Lett 7:259–262, 1997.
23. M Kamihira, M Taniguchi, T Kobayashi. Agric Biol Chem 51:3427–3428, 1987.
24. TW Randolf, HW Blanch, JM Prausnitz. AIChE J 34:1354–1360, 1988.
25. TW Randolf, DS Clark, HW Blanch, JM Prausnitz. Science 238:387–390, 1988.
26. TW Randolf, DS Clark, HW Blanch, JM Prausnitz. Proc Natl Acad Sci USA 85:2979–2983, 1988.
27. V Sereti, H Stamatis, FN Kolosis. Biotechnol Tech 11:661–665, 1997.
28. Y Zheng, GT Tsao. Biotechnol Lett 18:451–454, 1996.
29. N Fontes, MC Almeida, C Peres, S Garcia, J Grave, MR Aires-Barros, CM Soares, JMS Cabral, CD Maycock, S Barreiros. Ind Eng Chem Res 37:3189–3194, 1998.
30. SV Kamat, B Iwaskewycz, EJ Beckman, AJ Russell. Proc Natl Acad Sci USA 90:2940–2944, 1993.
31. SV Kamat, EJ Beckman, AJ Russell. J Am Chem Soc 115:8845–8846, 1993.

32. AK Chaudhary, SV Kamat, EJ Beckman, D Nurok, RM Kleyle, P Hajdu, AJ Russell. J Am Chem Soc 118:12,891–12,901, 1996.
33. S Kamat, J Berrera, EJ Beckman, AJ Russell. Biotechnol Bioeng 40:158–166, 1992.
34. T Mori, A Kobayashi, Y Okahata. Chem Lett 921–922, 1998.
35. T Mori, Y Okahata. Chem Commun 2215–2219, 1998.
36. M Taniguchi, M Kamihira, T Kobayashi. Agric Biol Chem 51:593–594, 1987.
37. FJ Kao, SA Ekhorutomwen, SP Sawan, Biotechnol Tech 11:849–852, 1997.
38. P Lozono, A Avellaneda, R Pascual, JL Iborra. Biotechnol Lett 18:1345–1350, 1996.
39. A Overmeyer, S Schrader-Lippelt, V Kasche, G Brunner. Biotechnol Lett 21:65–69, 1999.
40. YM Chi, K Nakamura, T Yano. Agric Biol Chem 52:1541–1550, 1988.
41. M Habulin, V Krmelj, Z Knez. J Agric Food Chem 44:338–342, 1996.
42. W Chulalaksananukul, JS Condoret, D Combes. Enzyme Microb Technol 15:691–698, 1993.
43. S Kamat, G Critchley, EJ Beckman, AJ Russel. Biotechnol Bioeng 46:610–620, 1995.
44. A Marty, D Combes, AS Condoret. Biotechnol Bioeng 43:497–504, 1994.
45. IB Carvalho, TC Sampaio, S Barreiros. Biotechnol Bioeng 49:399–404, 1996.
46. MC Almeida, R Ruivo, C Maria, L Freire, TC Sampaio, S Barreiros. Enzyme Microb Technol 22:494–499, 1998.
47. H Nakaya, O Miyawaki, K Nakamura. Biotech Tech 12:881–884, 1998.
48. Y Ikushima, N Saito, M Arai, K Arai. Bull Chem Soc Japan 64:2224–2229, 1991.
49. Y Ikushima, N Saito, T Yokoyama, K Hatakeda, S Ito, M Arai, HW Blanch. Chem Lett 109–112, 1993.
50. Y Ikushima, N Saito, M Arai, HW Blanch. J Phys Chem 99:8941–8944, 1995.
51. Y Ikushima. Adv Colloid Interf Sci 71–72:259–280, 1997.
52. N Fontes, E Nogueiro, AM Elvas, TC de Sampaio, S Barreiros. Biochim Biophys Acta 1383: 165–174, 1998.
53. MA Jackson, JW King. J Am Oil Chem Soc 74:103–106, 1997.
54. H Nakaya, O Miyawaki, K Nakamura. Kagaku Kogaku Ronbunshu 25:237–239, 1999.
55. Y Iwai, Y Koga, T Fukuda, Y Arai. J Chem Eng Japan 25:757–760, 1992.
56. Y Iwai, Y Koga, H Maruyama, Y Arai. J Chem Eng Data 38:506–508, 1993.
57. JM Snyder, JW King, MA Jackson. J Am Oil Chem Soc 74:585–588, 1997.
58. HB Ffrykman, JM Snyder, JW King. J Am Oil Chem Soc 75:517–520, 1998.
59. JW King, SL Taylor, JM Snyder, RL Holliday. J Am Oil Chem Soc 75:1291–1295, 1998.
60. C Tsitsimpikou, H Stamatis, V Sereti, H Daflos, FN Kolisis. J Chem Technol Biotechnol 71: 309–314, 1998.
61. M Caussette, A Marty, D Combes. J Chem Technol Biotechnol 68:257–262, 1997.
62. KJ Liu, HM Cheng, RC Chang, JF Shaw. J Am Oil Chem Soc 74:1477–1482, 1997.
63. MA Jackson, JW King, GR List, WE Neff. J Am Oil Chem Soc 74:635–639, 1997.
64. A Capewell, V Wendel, U Bornscheur, HH Meyer, T Scheper. Enzyme Microb Technol 19: 181–186, 1996.
65. M Rantakyla, M Alkio, O Aaltonen. Biotechnol Lett 18:1089–1094, 1996.
66. G Glowacz, M Bariszlovich, M Linke, P Richter, C Fuchs, JT Mosel. Chem Phys Lipids 79: 101–106, 1996.
67. O Parve, I Vallikivi, L Lahe, A Metsala, U Lille, V Tougu, H Vija, T Pehk. Bioorg Med Chem Lett 7:811–816, 1997.