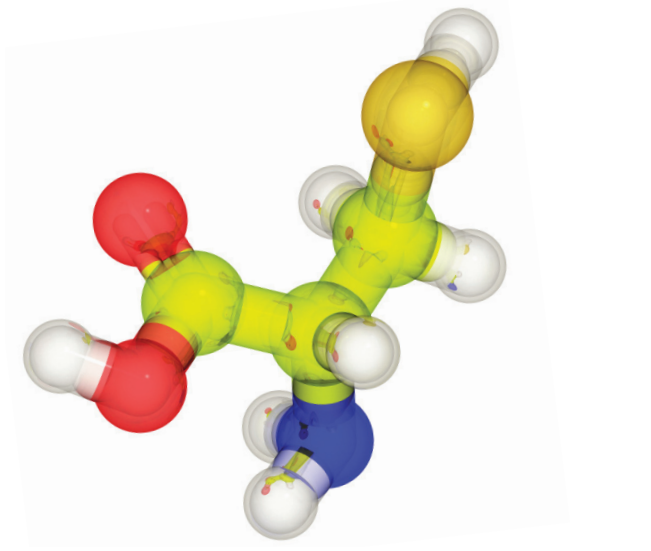


Applied Food Protein Chemistry

Edited by Zeynep Ustunol



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Edited by

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Zeynep Ustunol is a Professor of Food Science and Human Nutrition and Director of Graduate Programs at Michigan State University. She received her BS and MS degrees from Utah State University in Food Science and her PhD degree in Food Science with a minor in Biochemistry at University of Kentucky. After two years of a postdoctoral fellowship in the Department of Animal Science at University of Kentucky, she joined the faculty in the Department of Food Science and Human Nutrition at Michigan State University. She teaches Food Chemistry, Advanced Food Chemistry, Principles of Food Science, and Dairy Processing courses at Michigan State University. She also has an active research program in dairy chemistry/processing focused on enhancing the value of dairy and dairy-based products. For many years Dr. Ustunol has served as the Faculty Coordinator for *R.J. Brunner Protein Symposium*, which was held yearly at Michigan State University in honor of Dr. Brunner. This symposium has been the source of inspiration for this book.

Dr. Ustunol is the recipient of the Fulbright Senior Research Scholar Award to New Zealand, and recipient of the American Dairy Science Association Milk Industry Foundation Outstanding Teaching Award. Dr. Ustunol is an active member of the American Dairy Science Association (ADSA), Institute of Food Technologists (IFT), American Chemical Society (ACS), and American Association for Advancement of Science (AAAS). During 2004–2008, she served as the Senior Editor and Editor of the *Journal of Dairy Science.*

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Preface

The intent of this book is to provide an updated applied reference book for those who work with or do research on food proteins. This book is also intended to provide an updated text on applied food protein chemistry for upper-level students or graduate students in Food Science programs.

The information in the book is grouped into three sections: (1) overview of food proteins, (2) plant proteins, and (3) animal proteins. The first section on the overview of food proteins covers amino acid, peptide, and protein chemistry, reviews physical and chemical properties of food proteins, their chemical, physical, and enzymatic modification. Functional properties, nutritional aspects of proteins as well as biologically active and antioxidant peptides are also covered in separate chapters. The focus of the remaining two sections is to cover in depth use of both plant proteins (soy, canola, wheat, rice, sorghum, millet) and animal proteins (muscle, dairy, egg, seafood). Each chapter discusses global production, distribution, utilization, physicochemical properties, their functional properties, and food applications. The authors for each of the chapters have been carefully selected from those actively working in the topic area and have a reputation of being an expert in the field. All chapters are peer-reviewed. The book is designed to augment the related books currently in the market.

I am indebted to the contributing authors of this book and the scientific review panel for their hard work, contributions, and high level of professionalisms. I hope that readers of this book will find it useful, and will direct their comments, and any unavoidable errors that they detect to my attention.

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I
Protein Properties

1
Introduction to Food Proteins

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The word protein was coined by Jons J. Berzelius in 1838, and is derived from the Greek word *proteios* which refers to being "of the first rank." Over the past 100 years proteins were studied extensively and food proteins have been of much interest not only because of their importance nutritionally and for their functionality in foods, but also for their detrimental effects. Food proteins include proteins from milk, meats (including fish and poultry), eggs, cereals, legumes, and oilseeds. Although these have been the traditional sources of protein in the human diet, potentially any proteins from a biological source could serve as a food protein. However, a food protein must be nontoxic, nutritionally adequate, digestible, have functionality desirable in foods, be readily available, and agriculturally sustainable.

The primary role of proteins in the diet is to provide the building materials for synthesis of muscle and other tissues. Proteins play a critical role in many biological processes. For example, proteins such as myoglobin and ferritin are involved in the transport of important biological molecules; oxygen and iron, respectively. Proteins are also major components of muscle and skin, and are essential for providing mechanical support in the body. Antibodies are highly specific proteins, important in immune defenses. Nerve cell responses to specific stimuli are mediated by protein receptors. Growth and differentiation of cells are also controlled by growth factors that are often proteins. Enzymes are proteins with catalytic activity which stimulate many chemical reactions in biological systems. Recently, there has been much interest in proteins due to the satiety they provide, as well as the bioactive peptides derived from them because of their potential as neutraceuticals. Proteins and bioactive peptides have the potential to improve health and reduce risk of various diseases.

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The nutritional value of a protein is determined by its amino acid composition. A protein containing all of the essential amino acids in life and growth sustaining proportions is considered a complete protein and will have a high biological value. Many animal proteins generally have high biological value, whereas plant proteins generally are not as high in biological value due to their deficiency in some of the essential amino acids. However, incomplete proteins can be supplemented with the missing essential amino acids. This has been an important practice in improving world's food sources. The body's daily protein requirements vary by person, with typical demand being greatest during growth, pregnancy, and lactation. Protein malnutrition can be reversed by proper diet. However, when protein intake is inadequate for too long the recovery may not be complete and the damage is irreversible, possibly leading to mental retardation.

Food proteins are responsible for texture, color, and flavor. Today they are extracted, modified, and incorporated into processed foods to impart specific functional properties. For example, proteins can function as buffering agents, emulsifiers, and fat mimetics in foods. Certain proteins can also form gels and foams. Because proteins contain both hydrophilic and hydrophobic characteristics they can orient themselves at the oil–water interface and can stabilize emulsions, which is important for the stability of foods such as salad dressings, sauces and mayonnaise. Foams are colloidal dispersion of gas in liquid. The protein orients itself at the air–water interface to trap air, similarly as emulsions. Foams are important in foods such as dessert toppings and ice creams. Egg and milk proteins are good foaming agents. Proteins can also form a well-ordered protein matrix or a gel which then traps water, fat, and other food components. Food products like yogurt, tofu, and gelatin dessert rely on the gelation properties of proteins.

Enzymes in food can be desirable or undesirable. Enzymes may serve as processing aids in food processing. For example, lactose-free (or lactose-reduced) milk and lactose-free dairy products are produced from milk where lactose has been hydrolyzed through a controlled enzymatic process. Enzymes also have undesirable aspects including their involvement in deteriorative reactions in foods. For example, polyphenoloxidase catalyzes browning reactions in fruits like apples in the presence of oxygen, and lipoxygenase is involved in lipid oxidation of polyunsaturated oils.

Food proteins can also have adverse effects in the diet. Food proteins can be powerful allergens for some people. Peanut, various tree nuts (such as walnuts, pecans, almonds, and cashews), soybean, wheat, milk, egg, crustacean, and fish proteins have been demonstrated to induce immunoglobulin E (IgE)-mediated food allergies. These eight foods account for approximately 90% of the food allergy reactions in the United States and are sometimes referred to as the "big eight." There are also some proteins that have antinutritional properties. Trypsin inhibitors (which reduce digestibility of protein) and avidin (which binds biotin, a B vitamin) are common examples. There are also proteins, or amino acids that may react to form toxins. For example, acrylamide in fried potatoes is formed from the reaction of amino acid asparagine with a reducing sugar.

It is important to note that food processing can alter the nutritional value and functional properties of proteins, along with enzyme activity.

This book will review the properties of food proteins, and provide in-depth information on important plant and animal proteins consumed around the world.

2
Overview of Food Proteins

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2.1 Overview of food proteins

This book, *Applied Food Protein Chemistry,* is divided into three main sections. The first section reviews amino acid, peptide, and protein chemistry. It covers the properties of proteins important in foods. The second section provides an in-depth review of the chemistry, properties, and application of food proteins derived from plant sources. The third and last section is on the chemistry, properties and application of food proteins from animal sources.

2.1.1 Section I. Protein properties

Food proteins are essential source of amino acids in the diet which are necessary for normal growth and maintenance of the body. Chapter 3 provides a review of amino acid, peptide, and protein chemistry. Many of our food proteins are denatured and/or altered at the time of their consumption. Thus Chapter 4 is dedicated to protein denaturation, chemical modification of proteins, as well as processing-induced changes. The effects of these changes on specific food proteins are further discussed in the specific chapter dedicated to that protein. Protein denaturation may or may not be desirable, and the nutritional value of a particular protein may be dependent on the extent of denaturation; this is addressed in several of the chapters.

Proteins are essential in the human diet, therefore it is critical to accurately define protein quality and the amount of protein required to meet nutritional needs of different populations. Since the optimum balance between the amino acid supply (provided by dietary proteins) and the dietary needs is important for the health and well-being of

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human populations, dietary applications for proteins are determined by the nutritional value of the protein and the human requirements for protein and amino acids. Chapter 8 provides a critical review of the most relevant method for evaluation of food protein quality, the relevance of the pig as a model for assessment of protein quality, and the most recent development in amino acid requirement estimates for humans.

Food proteins also encode a series of bioactive peptides within their structure. Bioactive peptides, once ingested and absorbed, can play a key biological role as regulators of the immune system, blood pressure, or as signalling molecules. Bioactive peptides derived from food proteins are reviewed in detail in Chapter 6.

In addition to their importance nutritionally, food proteins are used as ingredients in foods because of their functional properties and are important in providing structure to the food matrix. Chapter 4 reviews functional properties of food proteins. However, as the authors indicate, this chapter is not a comprehensive review of food protein functionality, rather it highlights some of the novel aspects and recent advances in the methodologies used to study protein functionality. Understanding protein structure provides insight into different aspects of protein functionality; food texture may be manipulated by manipulating the structure and aggregation of proteins. This may be accomplished through processing, by altering environmental conditions, or the interaction of protein with other components in foods. It is important to emphasize that food matrices are complex. The presence of other food components and phases in food provide for additional complexity to studying these systems. Food proteins' ability to form colloidal structures is also discussed in Chapter 4 as these are important in the processing and nutritional properties of the food matrix they form.

Proteins or peptides derived from food proteins also offer potential as natural antioxidants. They can serve as food preservatives, against lipid oxidation. Lipid oxidation is the primary way lipids and lipid-containing foods deteriorate. Lipid oxidation can lead to loss of nutrients and color, and produce off-flavors and off-aromas. Lipid oxidation can also impact texture and functionality because of its effect on protein structure. Chapter 7 provides an overview of lipid oxidation mechanisms in foods and offers an insight into natural antioxidant options derived from proteins and peptides, among the variety of existing natural antioxidants, to manage lipid oxidation in food and beverage systems. Allergenicity of proteins of course is a concern when using proteins as ingredients/additives. Hydrolyzed pea protein currently looks most promising in food applications. A section in this chapter is dedicated to pea proteins.

2.1.2 Section II. Plant proteins

Proteins from soybeans, canola, corn, wheat, rice, sorghum, and millet are the most significant sources of plant proteins around the world. The nutrient composition of plants and thus proteins are influenced by cultivar, breeding, growth conditions, processing, and refinement.

Soy proteins is one of the most widely consumed oilseed protein in the world. They have been of interest due to their low cost and data accumulating on their health benefits. Soy protein products have experienced significant increase in sales in the United States.

Their application in foods range from functional ingredients to biopolymers, biofilms, and nanocomposite. Particularly popular are energy bars, soy beverages, flavored soy nuts texturized meat substitutes. Changes in their physicochemical properties can further extend their applications in food formulations by improving on their functional, sensory, rheological, and nutritional properties. Chapter 9 reviews chemical composition and principal physicochemical modifications of soybean proteins, and their application in foods.

Canola is a variety of rapeseed that was developed in Canada. It is characterized by its low erucic acid and glucosinolate content in the oil and the meal. Canola is a major oilseed crop. Canola proteins are found in the meal that results as a by-product of oil extraction. The nutritional profile and functional properties of canola proteins make them good candidates as ingredients for use in the food and beverage industries. However, a number of antinutritional components such as glucosinolates, phenolics, phytates, tannins, sinapine, and high fiber in the canola meal have limited their food use. These components also contribute to the inferior physicochemical properties of canola proteins, their digestibility, color, and taste. Over the years research has focused on removing or reducing the amount of these undesirable components, which has provided canola proteins with desirable functional properties with potential use in foods. Chapter 10 discusses the characteristics of canola and low erucic acid rapeseed proteins as well as their functional and health-related properties.

Among cereals grains, wheat, rice, and maize make up approximately 87% of the world production. Wheat is one of the oldest cereal grains to be domesticated. Today it is grown in almost all continents. Among cereal grains wheat has the highest protein content. Wheat proteins are storage proteins whose primary application are bakery products. When the grain is milled and mixed with water, the water-insoluble storage proteins form an elastic dough, due to the unique rheological properties of the gluten proteins. This dough has the ability to retain gas bubbles. Wheat flour is well suited for making the many wheat-based food products that are an important part of the diet for millions around the world. These products included bread in many different forms, noodle and pasta products, pastries, and other baked goods. Gluten was one of the first proteins to be purified and identified as contributing to wheat's unique dough-forming properties. Today, great complexity of wheat grain proteins is well known. Chapter 11 provides an in-depth review of wheat proteins. Growing interest and expanding markets for "gluten-free" products are also covered in this chapter.

After wheat, maize (or corn) contains the highest amount of protein among cereal grains. Maize is one of the top three cereal crops in terms of world consumption and economic importance. It is the main source of calories and protein for majority of the population in about 20 of the developing countries in Central and South America, Asia, and Africa. It is also used as a weaning food for babies in these countries. Much research has been conducted on improving the nutritional value of maize. There are several excellent books dedicated to maize and maize proteins, therefore, maize is not covered in this book.

Rice is a cereal grain also consumed as a staple food in parts of the world particularly Asia and parts of Central and South America, and to a lesser extent Africa. Rice is a tropical cereal, typically grown in paddies. Rice has one of the lowest protein contents

among cereals. Proteins are present in rice hull, bran, and endosperm but the highest protein content is found in the bran. The best sources of rice proteins are the bran and broken rice because these by-products of rice processing are undervalued and with limited applications. Contrary to other cereal proteins which tend to be deficient in lysine, rice has a good amount of lysine. Recently rice proteins have been of interest due to their hypoallergenicity. The functional properties of protein hydrolysates from rice bran and rice endosperm and their various food applications are described in Chapter 12.

Chapter 13 is dedicated to sorghum and pearl millet which rank fifth and sixth worldwide in production among the cereal grains. They serve as the major source of energy and protein for a large segment of the population in parts of Africa and Asia in the semi-arid tropics. Sorghum is generally a tropical or a subtropical crop, and is considered drought resistant. Millets are a group of variable plants that adapt to low moisture and hot climates. Pearl millet is the most popular among millets. Both sorghum and millets are ground into flour for production of various foods. White sorghum recently has been of interest as a source of gluten-free flour and meal.

2.1.3 Section III. Animal proteins

Animal proteins, meat, poultry, fish, milk, and eggs are nutritionally more complete compared to proteins from plant sources. However, traditionally they have not been consumed to the same extent particularly in the developing countries due to their higher cost.

Meat has been consumed as a food and protein source all throughout history. Chapter 14 is dedicated to meat proteins and it covers key factors affecting raw meat production, structure and functionality of skeletal muscle, muscle protein utilization, protein quality control, and value-added processing of meat.

Surimi is stabilized myofibrillar proteins extracted from mechanically deboned fish meat that is washed with water and blended with cryoprotectants. Surimi is the common seafood protein that is commercially used as raw material; surimi seafood is the finished product. Surimi, forms thermo-irreversible gels upon heating, thus, makes it useful as a food ingredient. Surimi serves as an intermediate product in a variety of products such as kamaboko products of Japan, or surimi seafood products including crabstick. Chapter 15 covers world production, distribution, and utilization of surimi. It reviews the chemistry and rheology of proteins involved in surimi gelation, as well as the biochemical and immunochemical assays used for proteins, differentiation of fish species, and allergen identification.

Milk is a biological fluid produced by mammals; its intent in nature is to nourish the newborn. Although, cow's milk is most widely used around the world for the production of dairy foods, milk from other species are also utilized. Milk proteins contribute approximately 25% of the daily protein intake of the US diet and are an important source of protein in the diet around the world. Milk proteins include caseins, whey proteins, milk fat globule membrane proteins, and enzymes that naturally occur in milk. Chapter 16 provides an in-depth review of cow's milk proteins, their structure, and their functional properties.

Eggs from avian species have also been recognized as a good source of proteins for humans. Eggs have high nutritional value and unique functional properties. They are commonly used as ingredients in the food industry. Chapter 17 provides a detailed review of egg proteins, their chemistry, and functional properties. Better understanding of egg protein chemistry and functional properties is important for the application of eggs in food products, improving existing products, as well as in developing novel food products.

2.2 Projected needs for the future

In an in-depth review of food proteins, their chemical properties, and importance in our food and diet—some key issues facing the future became apparent. These include but are not limited to population growth, food availability, and energy. The world population is estimated to reach 9.5 billion by 2075. Currently, majority of the population growth is in the developing world. The challenge ahead is to provide food security and adequate nutrition to the growing world population. Traditional plant breeding methods and advances in agricultural biotechnology has provided for production of higher yields with less use of agrochemicals. Various other tools including biotechnology for modifications in plant and animal foods that can benefit society are available. Clearly, as with any new scientific advancement, the risk versus benefit to society as a whole needs to be evaluated. Public knowledge and consumer attitudes also need to be considered.

In assessing need for the future however, a more urgent issue at hand is global food waste. Approximately one-third of the food produced for human use is lost or wasted globally (Gustaffson et al., 2011). This waste occurs at various stages; at the initial stages of food production as well as in the household at the consumption stage. For example, cereal grains are harvested at certain times during the year. Losses during storage due to insects, molds, and rodent infestation can be as high as 50% in hot climates. With animal products again losses can be significant due to lack of proper processing, storage, and refrigeration. In the low-income countries losses are during the early stages of food production and less food is wasted in the household at the consumer level. In the medium- and high-income countries the waste tends to be at the consumption stage. When compared on the per capita basis, overall, more food is wasted in the industrial world than in developing countries (Gustaffson et al., 2011). So efforts and resources dedicated to food production are in vain. Research on preventing global food waste is urgent. Proper preservation and storage is a key area for improvement and essential for the future of food security.

Reference

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3
Amino Acids, Peptides, and Proteins

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3.1 Introduction

Proteins are polymers of amino acids that are covalently joined by a substituted amide linkage named a peptide bond. There are 20 different amino acids that make up food proteins. The 20 amino acids differ in their side groups, which vary in size, structure, and charge. Due to the ionizable amine and carboxyl groups amino acids can act as acids and bases. Several amino acids also have ionizable side groups, thus, the properties of proteins containing these amino acids are pH dependent. There are four levels of protein structure. These are defined as primary, secondary, tertiary, and quaternary structures. The primary structure of a protein is the amino acid sequence of the polypeptide. The primary structure of a food protein is very important in that it determines the subsequent conformation of the protein; how that protein folds into its unique three-dimensional structure, and ultimately the function of that protein. A variety of intra- and intermolecular forces provide for the stability of food proteins. Food proteins occur in a wide variety of sizes and amino acid composition, and they can be small or very large. This chapter will provide an overview of amino acid, peptide, and protein chemistry as it relates to food proteins.

3.2 Amino acids

Amino acids are the basic structural unit and building block of proteins. There are 20 amino acids that are coded genetically that most commonly make up food proteins. Selenocysteine is a rare amino acid, it is derived from serine. It contains selenium instead

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of the sulfur of cysteine. Selenocysteine is a constituent of just a few known proteins (Nelson and Cox, 2013). An amino acid consists of a hydrogen (H), an amine (NH_2) group, a carboxyl (COOH) group, and a side group (R) which are covalently linked to a central α-carbon. The structures of amino acids differ only in the different structure of their side group (R). Proline is the only exception in that it is an *imino* acid, because of its cyclic α-amino structure. The side chain of proline is covalently linked to the central α-carbon and to the nitrogen of the amine group forming a cyclic structure. Contrary to the other amino acids, the amine group in proline is a secondary rather than a primary amine. The structure and the properties of the side group (R) determines to a great extent the size, net charge, solubility, chemical reactivity and hydrogen bonding ability of the amino acids, and the resulting protein (Nelson and Cox, 2013). This in turn determines the physicochemical properties and functionality of the protein. The Joint Commission of Biochemical Nomenclature (JCBN) of International Union of Pure and Applied chemistry (IUPAC)-International Union of Biochemistry (IUB) has established the standard symbols and nomenclature for amino acids and peptides.

Amino acids are asymmetric and can exist as mirror images of each other, both as D and L enantiomers. The only exception to this is glycine, since it contains single hydrogen as its R group. Amino acid residues in proteins including food proteins are exclusively L-amino acids, D-amino acid residues being extremely rare. This nomenclature is derived from D- or L-glyceraldehyde rather than levorotation, the direction of rotation of linearly polarized light. The D or L prefix is omitted when it is obvious that the amino acid is derived from a food protein source thus it is assumed to be an L-amino acid (Damodaran, 2008; Li-Chan, 2012).

Based on their side group R, amino acids at physiological pH can be grouped into five main classes. Positively charged or basic amino acids include Arg, His, Lys, negatively charged or acidic are Asp, Glu. Polar uncharged amino acids include Asn, Gln, Ser, Thr, Cys, aliphatic and nonpolar are Ala, Ile, Leu, Val, Gly, Pro, Met; aromatic and nonpolar include Phe, Tyr, Trp. It should be noted that a few of the amino acids particularly Gly, His, and Cys do not fit perfectly into a particular group, thus their assignments into a particular group should not be considered absolute (Nelson and Cox, 2013). Physicochemical properties of the 20 amino acids common to food proteins, their one and three letter abbreviations are summarized in Table 3.1. Molecular weights of amino acids range from 75.1 to 204.2 daltons. The molecular weight of an amino acid residue is calculated by subtracting the molecular weight of water, 18 daltons. Hydrophobicity (or hydrophilicity) of the side groups of amino acids that make up the protein influences protein folding and thus the physical properties of that protein. One of the more direct methods to estimate hydrophobicity of a protein is to determine the free energy changes for dissolution of the amino acid side chain in water and in an organic solvent. A high positive number indicates an amino acid with a hydrophobic side group which prefers to be in the organic phase rather than water phase. In a protein this amino acid will orient itself in the interior of the molecule to avoid water and be in an organic phase with similar polarity. Whereas a negative value indicates a hydrophilic side group. In a protein these amino acids will orient toward the surface of the molecule and try to interact with water. Clearly, within this

Table 3.1 Some physicochemical properties of the 20 amino acids found in food proteins

Amino Acid	Abbreviation	Molecular weight (Daltons)	pKa side group R	pI	Estimated hydrophobic effect (Kcal/mol)	Hydropathy
Alanine	Ala, A	89.1	–	6.00	1.0	1.8
Arginine	Arg, R	174.2	12.0	10.76	1.1	−4.5
Asparagine	Asn, N	133.1	–	5.41	−0.1	−3.5
Aspartic acid	Asp, D	132.1	3.9–4.0	2.77	−0.1	−3.5
Cysteine	Cys, C	121.1	9.0–9.5	5.02	0.0	2.5
Glutamic acid	Glu, E	146.1	4.3–4.5	3.24	0.5	−3.5
Glutamine	Gln, Q	147.1	–	5.65	0.5	−3.5
Glycine	Gly, G	75.1	–	5.97	0.0	−0.4
Histidine	His, H	155.2	6.0–7.0	7.59	1.3	−3.2
Isoleucine	Ile, I	131.2	–	6.02	2.7	4.5
Leucine	Leu, L	131.3	–	5.98	2.9	3.8
Lysine	Lys, K	146.2	10.4–11.1	9.82	1.9	−3.9
Methionine	Met, M	149.2	–	5.74	2.3	1.9
Phenylalanine	Phe, F	165.2	–	5.48	2.3	2.8
Proline	Pro, P	115.1	–	6.30	1.9	−1.6
Serine	Ser, S	105.1	–	5.68	0.2	−0.8
Threonine	Thr, T	119.1	–	5.64	1.1	−0.7
Tryptophan	Trp, W	204.2	–	5.89	2.9	−0.9
Tyrosine	Tyr, Y	181.2	9.7	5.66	1.6	−1.3
Valine	Val, V	117.1	–	5.96	2.2	4.2

Source: From Kyte and Doolittle (1982); Damodaran (2008); Li-Chan (2012).

range amino acids vary in their degree of hydrophobicity depending on the constituents of the side groups (Damodaran, 2008; Li-Chan, 2012).

Amino acids display ionization behavior and can serve as hydrogen donors or acceptors, therefore they can behave as acids and bases. The pH of its environment will determine ionization and thus whether or not an amino acid will bear charge. The *isoelectric point (pI)* of an amino acid is the pH at which the amino acid has no net charge. At pH values above their pI (pH > pI) amino acids will be negatively charged. At pH values below their pI (pH < pI) amino acids will be positively charged. The pI for amino acids with no acidic or basic side chains range from 5.0 to 6.3. The dissociation of the α-carboxyl group provides pK_a values of 1.8–2.7. The pK_a for protonation of the α-amino group is in the range of 8.2–10.6. If both groups are ionized (e.g., at neutral pH), then the molecule is a dipolar ion or a *zwitterion* and has no net charge. Furthermore, seven of the amino acids (Arg, Asp, Cys, Glu, His, Lys, Tyr) have ionizable side group (Table 3.1). Aspartic acid and glutamic acid are acidic amino acids containing ionizable carboxyl side chains with pK_a of 3.9–4.0 and 4.3–4.5, respectively. Arginine, lysine, and histidine contain ionizable side groups with pK_a values of 12.0, 10.4–11.1, and 6.0–7.0, respectively. Tyrosine and cysteine also have ionizable side chains with pK_a of 9.7 and 9.0–9.5 for the phenolic hydroxyl and the thiol groups, respectively. The total charge of an amino acid will be the sum of the charge due

to all ionizable groups of that amino acid, therefore charge due to amine, carboxyl, and ionizable side group at the given pH.

3.2.1 Derived amino acids and conjugated proteins

There are also amino acids that are derivatives of primary amino acids. *Derived amino acids* have covalently or noncovalently bound moieties or maybe cross-linked. The proteins that contain derived amino acids are called *conjugated proteins.* Covalently bound groups may include phosphate or carbohydrate moieties, as in the case of phosphoproteins and glycoproteins, respectively. Noncovalent groups may include lipids or nucleic acids as with lipoproteins, and nucleoproteins. Cysteine is a common cross-linked amino acid found in foods, others include desmosine, isodesmosine, and di- and trityrosine. Several simple derivatives of amino acids are found in several proteins. *Derived proteins* are not naturally occurring proteins instead they are obtained by further chemical or enzymatic modification, in the cell or derived due to food processing (Damodaran, 2008; Li-Chan, 2012).

3.3 Peptides, oligopeptides, polypeptides, proteins

Amino acids are linked covalently through α-carboxyl group of one amino acid and the α-amino group of another amino acid through an amide or peptide bond to form peptides, oligopeptides, polypeptides, and proteins. Generally, peptides with 10–20 residues are called oligopeptides; those with more are called polypeptides. Polypeptides of more than 50 residues are referred to as proteins (Nelson and Cox, 2013). The peptide bond formation is a condensation reaction which results in the removal of one molecule of water (Figure 3.1). The free α-amino group of the first amino acid is the N-terminal, and the free α-carboxyl of the last amino acid is the C-terminal end of a peptide or protein. Although the peptide bond is usually represented as a single covalent bond, it has partial double bond characteristics due to the resonance structure provided by delocalization of electrons. This resonance structure of the peptide bond has important consequences. First, it impedes protonation of the peptide N-H group. Second, the rotation of the CO-NH is

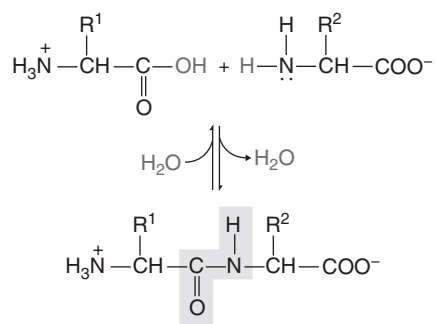

Figure 3.1 Formation of a peptide bond

restricted to a maximum of 6° (ω-angle), thus each six atom segment unit including the peptide bond (Cα – CO-NH-Cα) lie in a single plane. So the polypeptide can be represented as a series of Cα – CO-NH-Cα planes connected by Cα, with only N-Cα and Cα-C φ and ψ dihedral angles, respectively, having rotational freedom. However, the rotations of N-Cα and Cα-C bonds are also restricted due to stearic hindrances from side chain groups. Due to these restrictions, the flexibility of a peptide and protein is also restricted. Finally, with the exception of when proline is involved in the peptide bond, the peptide bond is normally in a trans configuration. Furthermore, the delocalization of the electrons imparts a partial negative and partial positive charge to the C=O and NH group, respectively, providing the potential for hydrogen bonding for these groups (Nelson and Cox, 2013; Voet et al., 2008).

Peptides, oligopeptides, and proteins only have one free α-amino and α-carboxyl group at either end of the molecule, which ionize similar to a free amino acid, and contribute to the acid–base properties of a protein, thus the total charge of the molecule; whereas, the groups involved in the peptide bond formation do not, since they do not ionize. However, the ionizable side groups are free to contribute to the charge of these molecules. It is important to note that the pK_a values of the ionizable side groups in oligopeptides, polypeptides, and proteins are slightly different than the corresponding free amino acids due to the influence of the neighboring residues and other environmental factors. In proteins, the typical pK_a value of terminal carboxyl and amino groups are 3.75 and 7.8, respectively, while pK_a values of side chain carboxyl, and imidazole, sulfhydryl, phenolic, amino, and guanidine groups are 4.6, 7.0, 8.8, 9.6, 10.2, and >12, respectively. The acid–base behavior and charge of a peptide and a protein can be determined by adding the contribution of each ionizable group. Similarly to amino acids, peptides, oligopeptides, and proteins have their characteristic pI. The degree of charge on a protein, in turn, determines many of the protein's properties such as solubility, water holding capacity (Damodaran 2008; Li-Chan, 2012).

3.3.1 Protein structure

There are four levels of protein structure defined as primary, secondary, tertiary, and quaternary structure.

Primary structure of a protein is its specific amino acid sequence which in turn determines the secondary, tertiary, and quaternary structures of a protein. The primary structure is the linear sequence of the amino acids that make-up the peptide or protein. The primary structure is determined by its genetic code and post-translational covalent modifications. The primary structure of a protein ultimately determines the physicochemical, and thus the functional properties of that food protein. The biological function of a protein is determined by its secondary, tertiary, and quaternary structures.

Secondary structure of a protein is the local three-dimensional arrangement of a protein which is determined by the amino acid sequence of the polypeptide. Secondary structure may result from aperiodic or periodic structures. *Random coil* is an aperiodic structures and regions in the protein where consecutive amino acids residues possess different sets

of dihedral angles. Whereas, *helical* and *extended* structures are periodic structures; which result from consecutive amino acid residues in a segment having the same recurring set of φ and ψ angles. Stability to these structures are provided by the decrease in local free energy by the rotation of the φ and ψ angles, local noncovalent interactions between amino acid side chains, as well as hydrogen bonding between C=O and N-H groups (Nelson and Cox, 2013; Voet et al., 2013).

Helical structure: In food proteins, although α-, 3_{10}- and β-helix, are found, the right-handed α-helix is the most common and is also the most stable. The 3_{10}- and β-helix are not so common. In the α-*helix* each helical rotation has 3.6 amino acid residues. The helical structure is formed by the φ and ψ angles of the consecutive amino acid residues being rotated in a similar manner. The stability to this structure is provided by the hydrogen bonding of the N-H and C=O groups of the fourth preceding residues. In the α-helix, the amino acids are positioned perpendicular to the axis of the helix and the hydrogen bonds are oriented parallel to the helix axis. Thus, the groups associated with hydrogen bonding N, H, and O atoms are positioned almost in a straight line. The *amphiphilic* nature of the α-helical structure is due to the helix surface being made up of hydrophobic (or nonpolar) residues as well as hydrophilic (or polar) residues. The nonpolar residues may also position themselves in the hydrophobic interior of the protein molecule. The α-helix is sometimes also termed the 3.6_{13}-helix, due to the 13 backbone atoms in the hydrogen-bonded loop in the structure of this molecule (Damodaran, 2008).

The cyclic imino acid proline cannot form α-helices, thus, is known to be an "α-helix breaker' due to its ring structure. Because of its ring structure the N-Cα bond is unable to twist and the φ angle remains fixed at 70°. It also cannot form hydrogen bond due to the lack of hydrogen on the nitrogen atom. Proteins containing high levels of proline residues will take random aperiodic structures. If high levels of proline residues are in a repeating periodic arrangement in a protein then the protein will form the helical structures polyproline I and polyproline II, where the peptide bond is in cis and trans configurations, respectively. It should be noted that of the 20 amino acids commonly found in foods, only proline is likely to adopt the cis isomer (Damodaran, 2008; Nelson and Cox, 2013).

β-*sheet structure:* The β-sheet is an extended structure where C=O and N-H groups are positioned perpendicular to the direction of the polypeptide chain, which then allows for hydrogen bonding between the two segments or β-strands. The side chains are positioned perpendicularly above or below the plane of the sheet. The resulting sheet-like structure is called a β-pleated sheet or simply β-sheet. There are two types of β-pleated sheet structures, parallel β-sheet or antiparallel β-sheet depending on the direction of the polypeptide strands. In parallel β-sheet as the name indicates the N → C strands run parallel, whereas in the antiparallel β-sheet N → C strands run in the opposite direction. In the antiparallel β-sheet structure hydrogen bonds form a straight line which provides additional stability to the structure making antiparallel β-sheet more stable than its parallel β-sheet counterpart. In the parallel β-sheet structure hydrogen bonds are formed at an angle, thus the stability of the hydrogen bonds, and therefore the stability of the structure are reduced. Also, generally, β-sheet is more stable than the α-helix structure; therefore, proteins with large segments of β-sheet structures are likely to be more heat stable or have higher denaturation temperatures (Nelson and Cox, 2013; Voet et al., 2013).

Supersecondary structure or *motifs* are grouping of secondary structural elements in dissimilar globular proteins. These structures may be important in terms of protein structure and function. The most common supersecondary structure is the βαβ motif where the α-helix connects two parallel strands of β-sheets. Others supersecondary structures include the β-*hairpin*, β-*bend* or β-*turn*, which is the tight reverse turn of a polypeptide chain in β-sheet that consist of antiparallel strands stabilized by hydrogen bonds. The αα motif consists of two consecutive antiparallel helices that are packed together with their axes inclined. The β-*barrel* results when extended β-sheets are rolled-up into a barrel shape (Voet et al., 2008, 2013).

Tertiary structure of a protein is the three-dimensional structure of the entire polypeptide. It is the arrangement of the linear polypeptide which contains segments of secondary structure which has further folded to produce a more compact structure. The amino acid sequence of a polypeptide determines to a great extent the formation of the tertiary structure of that protein. The folding of the protein into a tertiary structure defines the size and shape of that protein. The tertiary structure is stabilized through hydrogen bonding between the CO and NH groups. Additional rearrangement of the molecule that enhance hydrophobic, electrostatic, van der Waals interactions, and hydrogen bonding among the different groups reduce net free energy and also contributes to the stability of this structure. Folding of the protein into tertiary structure may bring distant amino acid residues closer together and provide for hydrophobic residues to be further situated at the interior of the molecule thus away from water if not restricted by stearic constraints. Hydrophilic and charged residues are positioned on the surface of the molecule that allow them to interact with water at the protein–water interface. The segments of the protein that are far apart in the polypeptide sequence and are in different secondary structure may also interact with additional folding of the protein. The interfacial area at the protein–water interface is reduced as the protein polypeptide folds to a tertiary structure (Voet et al., 2008; Nelson and Cox, 2013).

It is the distribution of the hydrophilic and hydrophobic residues present in the polypeptide that determines to a great extent as to whether a protein will fold to an elongated, rod-like or a globular shape. Globular proteins tend to be abundant in hydrophobic residues; elongated or rod-shaped proteins tend to be more abundant in uniformly distributed hydrophilic residues (Nelson and Cox, 2013).

A *domain* is the structure formed in large polypeptides typically with more than 200 residues. A domain is the region of the polypeptide that folds independently into a globular tertiary structure; thus its stability is independent of others. A domain may provide for the distinctive tertiary structure of a protein. The number of domains in a protein is determined by the size of the protein; larger proteins will have multiple domains (Voet et al., 2008).

Quaternary structure of a protein is the three-dimensional arrangement of more than one polypeptide; which are also referred to as subunits or oligomers. Not all proteins have quaternary structures. Large proteins (i.e., larger than 100 kDa) are likely to have more than one polypeptide, therefore more likely to have quaternary structures. The three-dimensional arrangement of the subunits in a quaternary structure may be homogeneous (composed of identical) or heterogeneous (composed of unidentical) polypeptide

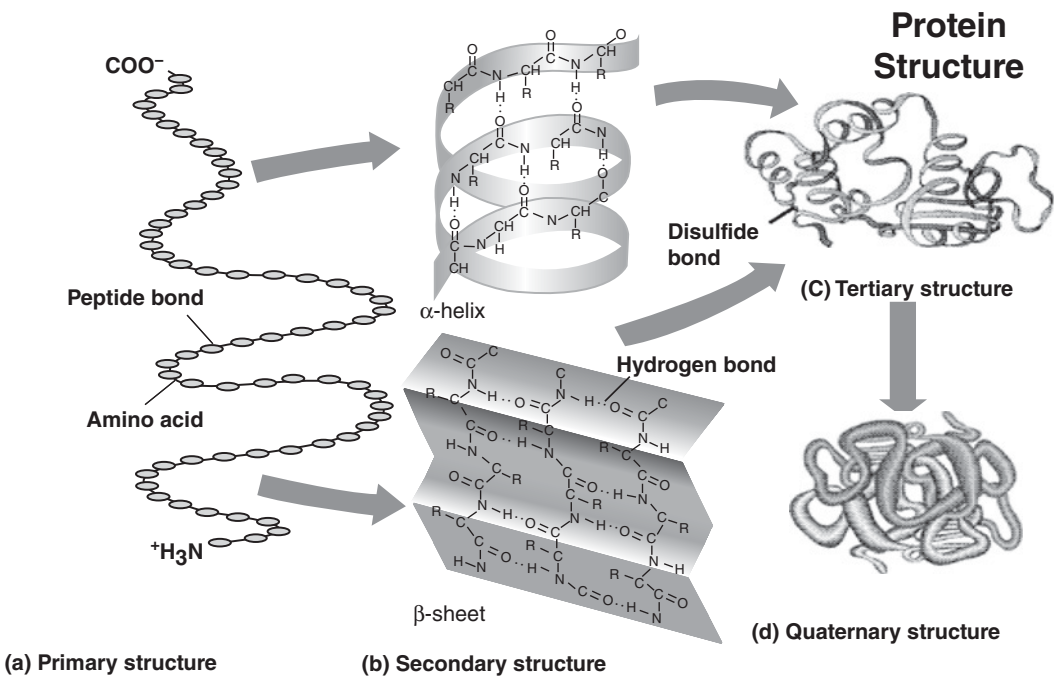

Figure 3.2 Four levels of protein structure. Estelle Levetin and Karen McMahon, Botany Visual Resource Library © 1998 The McGraw-Hill Companies, Inc. All rights reserved

monomers. There are many food proteins that have quaternary structures, for example, cereal and soy proteins, have different polypeptide subunits. Bovine milk β-lactoglobulin is interesting in that it is a monomer of 18 kDa at pH >8. Its structure forms a dimer at pH 5–8 and an octamer at pH 3–5 (Damodaran, 2008).

The formation of quaternary structure in a protein is determined by the thermodynamics of the positioning of the hydrophobic and hydrophilic residues. The quaternary structure is further stabilized by noncovalent interactions such as hydrogen bonding, hydrophobic, and electrostatic interactions. Figure 3.2 summarizes the four levels of protein structure.

Based on their higher levels of structures food proteins may be classified as *globular* or *fibrous* proteins. Globular proteins are those that the polypeptide chain folds to form a globular shape. Fibrous proteins are those where the polypeptide chains are arranged in long strands or sheets.

3.3.2 Forces involved in stability of proteins

The folding of a polypeptide chain into its three-dimensional structure is complex. The biological native confirmation of a protein is encoded in its amino acid sequence. It is

facilitated by a variety of intra- and intermolecular interactions. The intramolecular interactions result from forces intrinsic to the protein molecule (i.e., stearic, van der Waals), whereas intermolecular interactions result from the surrounding solvent (i.e., hydrogen bonding, electrostatic, and hydrophobic interactions). In its native conformation a protein molecule folds to lower its overall free energy as much as possible. Thus, during protein folding all the thermodynamically favorable interactions are optimized and the thermodynamically unfavorable interactions are minimized. However, it should be noted that under physiological conditions native proteins are only marginally stable.

3.3.2.1 Hydrophobic interactions Hydrophobic interactions are the main force that drives protein folding; therefore, they are important in determining the native structure of a protein. Hydrophobic interactions are due to thermodynamically unfavorable interaction of nonpolar groups with water, thus, minimizing their association with water. *Hydropathies* describe hydrophobic and hydrophilic tendencies of each amino acid residue; greater the hydropathy of an amino acid residue the more likely it will orient or bury itself to the interior of the protein molecule. Table 3.1 provides the hydropathies of the 20 common amino acids. Hydrophobic interactions are the main reason proteins fold into their tertiary structures, and hydrophobic free energy provides stability to this structure.

3.3.2.2 Electrostatic interactions *Van der Waals interactions* are the attractive or repulsive forces resulting from induced dipole by the polarization of the electron cloud between neutral atoms in protein molecules. Although the van der Waals interactions are relatively weak, in the interior of a closely packed protein molecule they are an important stabilizing influence. The strength of these forces decreases rapidly, with increase in distance. They become negligible beyond 6 Å.

Hydrogen bonds are ionic interactions in that it involves the interaction of a hydrogen atom that is covalently attached to an electronegative atom such as O, N, or S with a second electronegative atom. Hydrogen bonds most commonly are formed between N-H and C=O groups in α-helix and β-sheet structures of a protein as discussed previously. Although hydrogen bonds are essential to protein structure, their contribution to protein stability is minor. This is because water can also form hydrogen bond with the N-H and C=O groups in proteins, and this is as energetically favorable.

Ionic interactions involve attractive forces between ionizable groups of a protein that has a negative and a positive charge. As to whether a protein will bear a charge is influenced by the pH of the environment as well as the pK_a of the side chains. An *ion pair* or a *salt bridge* is the association of two ionic protein groups of opposite charge. Although electrostatic interactions between oppositely charged ion pairs are strong, and certainly have an influence on protein folding patterns, they contribute little to the stability of a protein since these charged groups can also interact with water (Damodaran, 2008; Li-Chan, 2012).

3.3.2.3 Covalent bonds or chemical cross-links The only covalent cross-linking of side groups found in proteins are *disulfide bonds (S–S)*. They are produced when the

Table 3.2 Energy of forces involved in the stability of proteins structure

Molecular forces	Energy (kJ/mol)
Covalent bonds	330–380
Electrostatic interactions	42–84
Hydrogen bonds	8–40
Hydrophobic interaction	4–12
Van der Waals	1–9

Source: From Li-Chan (2012).

sulfhydryl (thiol) groups of two cysteine molecules are in an oxidizing environment. Disulfide bonds may be inter- or intramolecular, and they help stabilize the folded protein structure.

It is important to note that the folding of a polypeptide and protein stability are also determined by stearic hindrances from side chain atoms. Because of the stearic hindrances of the side groups the polypeptide may only assume a limited number of configurations.

In summary, the formation and the stability of the three-dimensional structure of a protein is the net result of a variety of noncovalent and covalent interactions. Among noncovalent forces that have been discussed above, hydrophobic and van der Waals interactions are more significant in providing stability to native protein structure in an aqueous environment. Because of their potential interaction with water, hydrogen bonds and ionic interactions are less significant. There is no competing interaction with water for nonpolar groups. In the presence of nonpolar groups water molecules can form hydrogen bonds among themselves forcing nonpolar groups together to minimize interaction with water. The hydrophobic interactions are created by folding of the protein molecule so that nonpolar groups are oriented in the interior of the molecule. However, some nonpolar groups may be located on the surface of the molecule to be involved in intermolecular interactions. Although energy of van der Waals interactions appear to be weak, because numerous atom pairs are involved, the sum of their contribution to protein stability is significant. Among covalent interaction disulfide bonds are the most significant in protein stability (Damodaran, 2008; Li-Chan, 2012). Table 3.2 summarizes the energy of the forces involved in the stability of protein structure.

3.4 Conclusion

Understanding the chemistry of components that make up food proteins, peptides, and oligopeptides and understanding the forces that contribute to their stability will provide us the tools to better utilize food proteins in food products.

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4

Physical, Chemical, and Processing-Induced Changes in Proteins

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4.1 Introduction

The native structure of a protein is a creation of its environment. It is the most stable thermodynamically, and it is in the lowest free energy state. The native structure of a protein is due to various attractive and repulsive forces, resulting from different intramolecular forces and the interactions of the different groups with the surrounding solvent water. Denaturation of a protein is the change in its secondary, tertiary, and quaternary structure without altering its primary structure; it is due to the change in the original environment of the native protein. Denaturation alters the properties of a protein; enzymes lose their activity. Protein denaturation can result from physical conditions such as temperature, pressure shear or can be due to chemical denaturant such as changes in ionic strength or pH (Damodaran, 2008). Most food proteins are consumed in their denatured state. Protein denaturation may be desirable or undesirable depending on the circumstances. Denaturation increases the digestibility of a protein, often makes it more palatable, and inactivates deteriorative enzymes. However, depending on the extent of denaturation and modification, its nutritional value may also be lost.

Functional properties of denatured proteins are different from that of native proteins; thus denaturation is used to alter functional properties of food proteins (Culbertson,

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2012). Properties and functionality of food proteins can be altered through chemical and enzymatic modifications.

Processing of foods can also result in changes in properties of food proteins and their functionality. Among processing methods, heating is the most common and traditional method of processing foods. However, more recently there has been great interest in nonthermal processes such as high pressure and pulsed electric field (PEF) processing.

This chapter will review denaturation, chemical modification of proteins as well as processing-induced changes. These changes and their effect on specific food proteins are further discussed in the specific chapter dedicated to that protein and chapter dedicated to protein functionality.

4.2 Protein denaturation

The native structure of a protein is the result of the repulsive and attractive forces, the interactions among the amino acid side groups of proteins, as well as the environment the protein is in. *Protein denaturation* refers to changes in the secondary and tertiary structure of the protein. The primary structure (the amino acid sequence) of a protein remains unchanged. Denaturation uncoils the protein from a well-defined native state (under physiological conditions) into an unfolded random shape under nonphysiological conditions. This change may or may not be reversible. During denaturation the interactions responsible for the secondary and tertiary structure are disrupted. Therefore, any factor that contributes to the changes in the environment surrounding the protein and disruption of these interactions that stabilize the secondary and tertiary structure will denature the protein. These factors include physical factors such as temperature, high pressure, shear; or chemical factors such as pH, polarity of solvents, or additives that impact the ionic environment of the protein (Damodaran, 2008).

A denatured protein will undergo physical and chemical changes; and it will lose its biological activity. A denatured protein may lose solubility; actually monitoring changes in solubility is a crude (and an old method) that is used to monitor protein denaturation. Denatured proteins will aggregate, however, protein aggregation occur in a concentration dependent manner (Minton, 2005). A critical concentration of denatured protein may be needed to initiate aggregation (Mahler et al., 2009; Neudecker et al., 2012). In case of enzymes, denaturation may result in loss of enzyme activity, or changes in reaction rate of the enzyme, affinity for its substrate, its optimum pH or temperature, or optimum reaction specificity (Damodaran, 2008). In food processing denaturation, thus, loss of enzyme activity is sometimes monitored to determine adequate processing of a food product. For examples milk is assayed for alkaline phosphatase (ALP) activity to determine adequate pasteurization (Rocco, 2004). Denatured proteins because they are unfolded are also more susceptible to the action of proteolytic enzymes. Although, these changes in proteins may be viewed negatively, with food proteins these may be considered desirable. Denaturation often improves digestibility of proteins, inactivates antinutritional factors, inactivates deteriorative enzymes, and provides methods to monitor processing of foods. Furthermore, denaturation may also improve functional properties of food proteins that are used as ingredients in processed foods.

4.2.1 Physical denaturants

4.2.1.1 Temperature-induced denaturation Both high and low temperatures can cause proteins to denature. Depending on the protein and the severity of the conditions these changes that cause the protein to denature may or may not be reversible. *Heat* is one of the most common ways food proteins are denatured. Most food processing applications require heat. Effect of heat on food proteins have been extensively studied (Bull and Breese, 1973; Caldarelli and Los Rios, 2001; Damodaran, 2008). Denaturation temperature (T_d) or melting temperature (T_m) of a protein is the critical temperature unique to the protein, where the protein transitions from its native to denatured state. Measure of melt transition temperature is widely used as a measure of protein stability (Cooper, 1999). At $T < T_m$ the native state is more stable, thermal denaturation occurs at $T > T_m$ (Oshima and Kinoshita, 2013).

Temperature primarily affects the stability of noncovalent interactions. When temperatures are increased hydrogen bonding and electrostatic interaction that are exothermic in nature, are weakened. As these bonds are weakened and broken, the protein becomes more flexible providing more of the side groups to be exposed to the surrounding solvent. If the heating ceases at this point the protein may be able to fold back and revert back to its native structure. However, hydrogen bonds that stabilize the secondary structure may be disrupted if the heating continues. As these bonds are disrupted the water surrounding the protein will form new hydrogen bonds with the newly exposed groups. Nearby hydrogen bonds may further weaken and increase the nearby dielectric constant. As hydrogen bonds are disrupted the protein unfolds hydrophobic groups also become exposed. Hydrophobic interactions, which are endothermic in nature, are enhanced upon heating, contrary to hydrogen bonding and electrostatic interactions. However, at 70–80°C the strength of hydrophobic interactions reach a maximum. The hydrogen bonds stabilizing the protein will remain intact during heating if the nonpolar environment surrounding the protein is maintained. Conformational entropy of the protein chain increases upon heating favoring the unfolded state. The net stability of a particular protein is therefore the total sum of all these interactions at a giving temperature. Denaturation of a protein is a balance of forces between the various interactions that tend to favor the folded state and the conformational entropy of the chain that favor the unfolded state. Experimentally, stability of a protein is determined by measuring the difference between free energy of the folded and unfolded state under equilibrium conditions. Most proteins experience irreversible denaturation on exposure to high temperatures (Damodaran, 2008).

It is well known that thermal stability of proteins is determined by the amino acid composition of the proteins. Hydrophobic amino acid residues, especially Val, Ile, Leu, and Phe provide for higher stability than proteins with more hydrophilic amino acids. Other amino acid that may positively influence protein's thermostability include Asp, Cys, Glu, Lys, Leu, Arg, Trp, and Tyr, and that may negatively influence thermostability include Ala, Asp, Gly, Gln, Ser, Thr, Val, and Tyr. Furthermore, studies on proteins from thermophilic microorganism indicate that proteins that can withstand high temperatures contain lower level of Asn and Gln residues because these two amino acids are liable to deamination at high temperatures. The Cys, Met and Trp content, are also typically low

since they can oxidize readily at high temperatures. Thermostable proteins tend to have high levels of Ile and Pro (Ponnuswamy et al., 1982; Polyakov et al., 1997). The Ile provides for tighter packing of the interior core of the protein reducing void spaces. Tight packing of a protein reduces its mobility at high temperatures and minimizes the increase in configurational entropy thus unfolding of the protein. High Pro content provides rigidity to the protein structure. Also, there appears to be high number of ion-pairs particularly in the crevices and higher amount of buried water molecules involved in hydrogen bonding bridge between segments (Weber, 1992).

Thermal denaturation of proteins is facilitated in the presence of water (Fujita and Noda, 1981; Damodaran, 2008). When hydrated, proteins have higher mobility and flexibility; therefore upon heating they are more susceptible to unfolding and denaturation. Proteins under dry conditions have restricted mobility. Thus, they are more resistant to unfolding and thus denaturation. In foods there are typically other components and ingredients that may be associated with water and/or protein in addition to water. Ingredients like sugars and salts also impact thermal stability of proteins in aqueous solutions. Sugars such as sucrose, lactose, glucose, and glycerol stabilize proteins against thermal denaturation. Thermal stability increases with increase in sugar concentration. Disaccharides are more effective than monosaccharides at providing stability to the protein (Oshima and Kinoshita, 2013). This is attributed primarily to increases in "solvent crowding" caused by sugar addition. Similarly to sugars, salt at 0.5 M concentration also is reported to increase denaturation temperature of proteins β-lactoglobulin, soy proteins, serum albumin, and oat globulin (Damodaran, 2008).

Some proteins also denature at *cold* temperatures. Cold-induced denaturation is much less studied. The effect of cold temperatures on proteins is often reversible, contrary to high temperatures, although irreversible denaturation is also reported (Jaenicke, 1990; Weber, 1992; Franks, 1995). Cold denaturation or cryoinactivation is often seen with oligomeric (tetrameric or higher) proteins, where the protein dissociates into its monomers, and the native oligomeric structure of the protein is lost (Kunugi and Tanaka, 2002). These changes are reversible, in that re-association back to oligomeric structure can be restored when the protein is brought to ambient temperature. However, in certain cases (i.e. virus proteins) the protein will aggregate after the initial cold-induced dissociation. Then, if reassociation occurs it is into inactive aggregates. Cold denaturation has been also observed for a variety of monomeric or dimeric globular proteins, which does not involve dissociation as an initial step. Their denaturation is caused by the unfolding and loss of original structure of the protein. This was initially reported with chymotrypsionogen by Brandts and Brandts et al. (1964, 1970).

Cold temperatures weaken hydrophobic interactions, therefore food proteins stabilized primarily by these interactions are destabilized when refrigerated or frozen. During refrigerated storage of milk, β-casein as well as calcium, magnesium, and phosphorus will dissociate from the casein micelles due to weakening of hydrophobic interactions. Thus, the relative amount of β-casein in the micellar phase is decreased significantly. The amount of micellar α_{s2}-casein also tends to decrease. Due to the dissociation of these caseins serum casein concentration (in the water phase) increases. As a direct consequence, rennetting properties of casein micelles (and of milk) are altered. Cold storage prolongs rennet

coagulation time as well as the aggregation or the secondary phase of milk coagulation (Ustunol, 1983; Ustunol and Brown, 1985). Changes in the composition and the distribution of micellar and serum caseins induced by cold storage of milk, is reversible when milk is re-equilibrated to ambient temperature. Glycinin which is one of the storage proteins of soybean will also denature as evidenced by the formation of aggregates and precipitates when stored at 2°C. This cold-induced denaturation of glycinin is also reversible. The protein again becomes soluble when equilibrated to ambient temperature (Damodaran, 2008). There are also a number of oligomeric enzymes important in foods (i.e. lactate dehydrogenase) where the subunits dissociate thus loosing enzyme activity upon cold storage. However, when warmed to and held at an ambient temperature for a few hours they reassociate and completely gain their activity (Kunugi and Tanaka, 2002).

4.2.1.2 High-pressure-induced denaturation Bridgman (1914) was the first to report the role of high pressure on protein unfolding. Since then there have been numerous studies, however, the physical basis for high-pressure-induced denaturation has not been well explained. High-pressure-induced denaturation of proteins result from the pressure-dependent changes in the structure of the bulk water and water associated with the protein as well as from the loss of internal cavities in the folded state of the protein, or from a combination of these three factors (Ando, 2008; Grigera and McCarthy, 2010). Roche et al. (2012) demonstrated that pressure unfolds proteins primarily due to the cavities that are found in the folded state of the protein and that are absent in the unfolded state. It has been thought that high pressure can push water molecules to the inside of the cavities, causing the protein to swell, and denature (Bianco et al., 2012). These cavities can also provide a significant contribution to the volume differences between folded and unfolded states that direct the pressure-induced unfolding of proteins. So, greater is the contribution of void spaces to partial specific volume of the protein the more unstable the protein will be when pressurized. Proteins such as fibrous proteins that are lacking cavities in their structure tend to be more stable to hydrostatic pressure than globular proteins (Damodaran, 2008). Presence of internal cavities might also determine the pressure dissociation of protein oligomers and aggregates (Foguel, 2003). The changes in the solvent density due to high pressure and hydration of the exposed groups were proposed in the past as a major contributing factor to the volume change of unfolding. Although Roche et al. (2012) did not find a correlation; they indicated pressure unfolding was not significantly driven by the structural changes of bulk water. Dissanayake et al. (2012) reported if free thiol groups are present, disulfide bonds that originally stabilize the native protein conformation, are likely to engage in inter and intramolecular disulfide bond exchange under high pressure. The mechanism of pressure-induced denaturation is still under debate.

As evidence from changes in spectral properties most proteins undergo pressure-induced denaturation when exposed to 1–12 kbar pressure with a mid-point of pressure-induced transition occurring at 4–8 kbar (Damodaran, 2008). Several studies have reported that both mechanistically and conformationally, pressure- and cold-induced processes are alike, while heat-induced unfolding is different. Structural differences have been reported between cold and heat denatured form of β-lactoglobulin (Hedoux et al.,

2011). Hedoux et al. (2011) compared both pressure- and heat-induced transformations. They observed that both pressure and heat denatured states were obtained through an intermediate state characterized by intact secondary structure and enhanced water penetration in the tertiary structure. But they proposed that the mechanism of pressure-induced denaturation was related to the disruption of hydrogen bond network of water onto a set of clusters characterized by strengthened O-H interactions. This induced a hardening of protein dynamics. However, heating was the opposite; there was a softening of the hydrogen bond network of water inducing a softer protein dynamics.

Pressure-induced denaturation is reversible. Enzymes in dilute solutions regain their activity after equilibration to the atmospheric pressure. However, the effect of high pressure on oligomeric proteins is not as well studied. Subunits of oligomeric proteins and enzymes will dissociate at 0.001–2 kbar and further exposure to high pressure will denature the dissociated subunits (Damodaran, 2008).

High-pressure processing has gained interest over the last decade as a nonthermal process for processing and preservation of foods. High-pressure processing is effective in inactivating microorganism and certain enzymes, and leaving molecules like vitamins and flavor compounds intact (San Martin et al., 2002). Effects of high-pressure processing on food proteins will be further discussed in Section 4.5.2.

4.2.1.3 Shear-induced denaturation Denaturation of food proteins can result from exposure to mechanical shear forces generated by processes such as shaking, whipping, mixing, sonication, vortexing, flow through conduits, centrifugation, and texturization. Shear rates less than $10^3 s^{-1}$ have been found to significantly alter the three dimensional structure of proteins (Bekard et al., 2011). Further exposure to higher shear can cause proteins to aggregate (Bekard et al., 2011). Although there is some literature on shear denaturation of proteins, in most cases the experimental evidence is indirect, or results are complicated by the methods used to study denaturation (Jaspe and Hagen, 2006). Thus, the literature on shear-induced denaturation of proteins can be somewhat confusing to interpret. Furthermore, in case of food processing operations high temperatures and pressure may be involved, thus further confounding the results. Effects of shear stress on protein structure is not only of interest to food scientist but also to the pharmaceutical industry as protein-based biotherapeutics and bioreagents such as enzymes may encounter significant agitation during bioprocessing steps, as well as during shipping and handling (Weiss et al., 2009).

In general, the extent of structural change depends on the protein. Flexible globular proteins are more susceptible to shear-induced denaturation. Upon exposure to high shear denaturation results due to the incorporation of air bubbles and adsorption of protein molecules at the air-liquid interface. The protein undergoes conformational change at the interface due to energy of the air-liquid interface being greater than that of the bulk phase. Typically, the nonpolar residues of the unfolded protein position toward the air and the more polar residues are positioned toward the aqueous phase (Damodaran, 2008). The strain history has been reported to be an important parameter in the shear-effect. Even exposures that may appear to be low-shear can lead to shear-induced structural changes.

Given the great diversity of food proteins, the diversity of their sizes, their conformations, and nature of intramolecular interactions, their susceptibility to shear may vary.

There are several food processing operations that use high temperatures, pressure, and shear; one such example is texturization. *Texturization* involves use of mechanical shear to unfold globular structure of native proteins, may involve breaking of intramolecular bonds and rearrangement of disulfide bonds. The shear action typically is accompanied by use of heat and/or pressure (Bhattarcharyam and Padmanabhan, 1999). Texturization of proteins in most cases is accomplished using an extruder. In the extruder proteins are subjected to the action of the internal rotating screws which press the protein against the fixed heated barrel wall. The molten protein mass is pushed through the die which aligns the protein mass in the direction of the rotational flow (Onwulata et al., 2010). Texturization imparts unique functional properties to a protein, which then can result in new food protein-based ingredients. Texturization of soy proteins has been explored extensively. More recently, texturization of milk proteins has also been studied. For a review of extrusion texturized dairy proteins see the review by Onwulata et al. (2011).

4.2.2 Chemical denaturants

4.2.2.1 pH-induced denaturation The charge of a food protein is dependent on the pH of the food system. Under physiological or neutral pH conditions most proteins are above their isoelectric point and have a net negative charge, although a few may be positively charged. The isoelectric point of a protein is the pH where the net charge on the protein is zero. At its isoelectric point, repulsion of charges is minimized and protein will aggregate, and precipitate. However, even if the protein does remains in solution, the isoelectric point is usually the pH of minimum solubility. At pH values below the isoelectric point, the protein will have a net positive charge. Both above and below the isolectric point the presence of surface charges will provide for intermolecular repulsion thus enhanced solubility of the protein (Culbertson, 2012). In case of extreme pH conditions, the high net negative or positive charge on the proteins and the strong intramolecular electrostatic repulsions resulting can result in the swelling and unfolding of the protein (Damodaran, 2008; Culbertson, 2012). The extent of the unfolding in extreme pH conditions is reported to be greater at alkaline pH, than acid pH conditions; may be in part due to ionization of partially buried carboxyl, phenolic and sulfhydryl groups that try to interact with the aqueous environment thus result in the unfolding of the proteins. Most pH-induced denaturation of proteins is reversible (Damodaran, 2008).

4.2.2.2 Organic-solvent-induced denaturation Organic solvents that are less polar than water such as ethanol, will lower the dielectric constant of the protein's environment, thus effecting protein stability (Mirejovsky and Arnett, 1983; Jamadagni et al., 2011). Nonpolar solvents weaken hydrophobic interactions, because of the interactions of the nonpolar side groups of the protein with the nonpolar solvent. However, in certain situations an organic solvent may enhance formation of the peptide hydrogen bonds, particularly in low permittivity environment, and provide for stability (Silverstain et al., 1998; Franzese et al.,

2011). Electrostatic interactions are also impacted by organic solvents. Decrease in permittivity due to organic solvent enhances electrostatic interactions if oppositely charged groups are present, or will enhance repulsion if like charges are present (Finney and Soper, 1994). The net result therefore will be a sum of all the effects of the organic solvent on the polar and nonpolar interactions of the protein. In addition to polarity, the concentration of the organic solvent is also important. At high concentrations all organic solvents are protein denaturants, even though they may provide stability at low concentrations. Reversibility of denaturation depends to a great extent on the polarity of the organic solvent and its concentration (Mirejovsky and Arnett, 1983; Damodaran, 2008).

Solvent precipitation is often used in the extraction, purification, and concentration of enzymes.

4.2.2.3 Salt and other additive-induced denaturation Protein's solubility is enhanced in diluted salt solutions of low ionic strength (below 0.2 M), and is independent of the salt type. In diluted salt solutions, the salt will associate with oppositely charged proteins decreasing the electrostatic free energy of the protein and increasing the activity of the solvent. This provides for additional water binding to the protein, more than the protein's charge groups do alone. Thus protein hydration is increased. This is referred to as *"salting in."* At low salt concentration protein–solvent interactions are stronger than protein–protein interactions. However, when salt concentration is high (>1 M), the salt will interact with the surrounding water, and decrease the amount of water available to interact with charged groups of proteins; thus, dehydrating the protein. In this case protein–protein interactions are stronger than protein–solvent interactions. If the protein is sufficiently dehydrated it will lose solubility and precipitate. This phenomenon is referred to as *"salting out"* (Damodaran, 2008). Different proteins will precipitate at different salt concentrations.

The Hofmeister series is a classification of ions, based on their ability to salt-in or salt-out proteins. It originated from their ability to precipitate a mixture of egg proteins. A partial listing is provided below, although many more salts have been studied. Relative order for anions and cations are given as:

Kosmotropic ↔ Chaotropic

cations NH_4^+, K^+, Na^+, Li^+, Mg^{2+}, Ca^{2+}

Anions SO_4^{2-}, HPO_4^{2-}, acetate$^-$, citrate$^-$, Cl^-, NO_3^-, ClO_3^-, I^-, ClO_4^-, SCN^-

Today, the Hofmeister series is used in terms of the ability of the ions to stabilize the protein structure. However, the series is not universal. It should be noted that the relative order of anion may reverse depending on the surface properties such as charge, change in pH, hydrophobicity, and hydrophilicity. The relative order of cations associated may also be reversed with different anions due to ion pair effect (Medda et al., 2012; Lo Nostro and Ninham, 2012). However, while certain salts such as sulfate, phosphate, and fluoride salts of sodium stabilize protein structure, others such as bromide, iodide, perchlorate, and thiocyanate destabilize the protein structure and are denaturants. The terms *kosmotrope* (order maker) and *chaotrope* (disorder maker) refer to solutes that stabilize or destabilize proteins, respectively. However, these terminologies may be somewhat

misleading; because these properties may vary depending on the circumstances and methods used. Also, the ionic kosmotropes and chaotropes should be differentiated from nonionic kosmotropes and chaotropes which have no net charge, thus can impact proteins differently.

Salts affect the structural stability of proteins altering the hydration properties of proteins, and bulk structure of water. In general, salts that stabilize protein structure enhance the hydrogen-bonded structure of water (Lo Nostro and Ninham, 2012). Those that destabilize protein structure break-down bulk water structure. Therefore kosmotropic and chaotropic salts actually affect protein structure by stabilizing or disrupting water structure, respectively.

4.3 Chemical modification of proteins

Chemical modification allows altering and improving functional properties of food proteins (Kumagai, 2012), and may denature proteins. The modification of the protein is accomplished by chemical modification of the side groups of the amino acids that make up the primary structure of the protein. These side groups include the ε-amino group of lysine, thiol group of cysteine, carboxyl group of glutamic and aspartic acids, hydroxyl groups of tyrosine, serine, and threonine, the imidazole group of histidine, thioether group of methionine, and the indole group of tryptophan. It is important to note, however, that such derivatization may alter the nutritional value of the amino acid and thus the protein. So, not all chemical modification of proteins are suitable for food applications.

During chemical modification, the changes in the properties of a protein depend on the reaction's conditions and also to a great extent on the degree of the modification on the protein.

4.3.1 Acylation

Acylation is the chemical modification that covalently links an acyl group to the amino group of proteins. The ε-amino group of lysine is most readily acylated (Shukla, 1982). Acylation increases the solubility of the native protein, although it may impair other functional properties of the protein such as gelation. Acylated proteins are poorly absorbed and are less digestible due to their resistance to digestive enzymes (Damodaran, 2008). Most common acylations, *acetylation* and *succinylation*, are accomplished using acetic anhydride and succinic anhydride, respectively. The reaction is carried out at a pH of about 8 (Kumagai, 2012). Upon acetylation of a protein the positive charge on lysine is eliminated and there is an increase in the electronegativity of the molecule. Succinylation results in the replacement of positive charge of lysine with a negative charge (Damodaran, 2008). In both cases the electronegativity of the protein is increased. Acylation and the resulting functional properties of soybean, myofibrillar, oat, faba bean, jack bean, pea, rapeseed, peanut, flax, wheat, corn, rice bran, whey, egg, milk, and yeast proteins have been reported. See the review by Kumagai (2012) for more details.

The covalent linking of long-chain fatty acids to the ε-amino group of lysine has been reported to enhance the lipophilicity of a protein. This is achieved through the reaction of the fatty acylchloride or N-hydroxy-succinimide ester of a fatty acid with a protein. These

lipophilic protein molecules can then facilitate the formation of novel micellar structures and protein aggregates (Damodaran, 2008).

Since all acylation reactions involve covalent cross-linking, they are irreversible.

4.3.2 Alkylation

Alkylation is the chemical reaction of iodoacetate ($I\text{-}CH_2\text{-}COOH$) or iodoacetamide ($I\text{-}CH_2\text{-}CONH_2$) with free sulfhydryl or ε-amino group of a protein. The reaction is carried out under alkaline conditions of pH 8–9. Reaction with iodoacetate eliminates the positive charge on lysine, and covalently attaches to the free sulfhydryl thus preventing further reactions of the free sulfhydryl group. It introduces a negative charge both to lysyl and cysteine residues; thus increasing the electronegativity of the protein. (Damodaran, 2008).

Reductive alkylation can occur with aldehydes and ketones, in the presence of a reductant such as sodium borohydride ($NaBH_4$) or sodium cyanoborohydride ($NaCNBH_3$), at a pH 7–10 (Damodaran, 2008; Kumagai, 2012). In this reaction, the amino group of a protein and the carbonyl group of the aldehyde (or ketone) condense to produce an imine or Schiff base. In the presence of a reductant as mentioned above the alkylated protein is formed. Aldehydes are more reactive than ketones.

Depending on the extent, protein alkylation improves the functional properties of proteins such as emulsifying properties, water binding capacity. It can also make a protein resistant to proteolysis. Alkylation most often will prevent or delay Maillard reactions. Similar to acylation reactions, alkylation reactions are also irreversible.

4.3.3 Glycosylation

Chemical glycosylation of a food protein can be accomplished by reductive alkylation if a reducing sugar is selected as the reactant. The reaction occurs under alkaline conditions. Pentoses typically are more reactive than hexoses, and monosaccharides react faster than disaccharides and oligosaccharides. Glycosylation of a protein makes a protein more hydrophilic, thus more soluble, and provides for improved emulsifying and foaming abilities. Glycosylation of ovalbumin, β-lactoglobulin, casein, pea legumin, buckwheat protein, rice endosperm protein, and fish myofibrillar protein have been reported. For a more comprehensive review see Kumagai (2012).

4.3.4 Phosphorylation

Phosphorylation is the chemical reaction between hydroxyl groups of serine, threonine, or amino group of lysine with phosphorous oxychloride, $POCl_3$. While the O-P bond is stable under acidic conditions, the N-P bond is acid labile but stable in alkaline pH (Vojdani and Whitaker, 1996). Therefore, it is thought that chemical phosphorylation does not impair the digestibility of lysine. Phosphorylation results in an increase in the negative charge of a protein, and an increase in its solubility. Phosphorylated proteins are more sensitive to gelation in the presence of calcium ions (Woo and Richardson, 1983; Matheis and Whitaker, 1984).

4.3.5 Sulfitolysis

Sulfitolysis is the formation of the *S*-sulfonate derivative of a protein. The chemical reaction involves cleaving off the disulfide bond and addition of SO_3^-. In this reaction one S-SO_3^- and a free sulfhydryl group are formed. The reaction is typically carried out using sulfite and Cu^{+2}, and is reversible (Damodaran, 2008).

4.4 Enzymatic modification of proteins

Although numerous enzymatic modifications occur in biological systems, only a few enzymatic modifications of proteins are practical and used for modifying food proteins as discussed in the following sections.

4.4.1 Hydrolysis by proteases

Hydrolysis of a protein involves breaking of the peptide bond with the addition of a water molecule. Hydrolysis reduces the size of the protein molecule. Proteases such as chymosin and papain have been used to coagulate milk and tenderize meat in the food industry, respectively. Extensive hydrolysis by proteases can impact the functional properties of proteins. Nonspecific proteases, such as papain, can hydrolyze insoluble proteins to smaller peptides that are more soluble. In general, hydrolysis of proteins changes their water binding, foaming, and emulsifying properties (Kumagai, 2012). More recently, controlled hydrolysis by proteases has been studied in the production of bioactive peptides. Bioactive peptides are discussed in detail in Chapter 6.

4.4.2 Cross-linking by transglutaminase

Transglutaminase (TGase) (E.C. 2.3.2.13) provides for covalent cross-linking of proteins. The reaction involves the acyl transfer between the ε-amino group of lysyl residues (acyl acceptor) and the amide group of glutamine residue (acyl donor). In the absence of an amine substrate the reaction results in the deamination of the glutamine residue with the water molecule serving as the acyl acceptor (Motiki and Seguro, 1998; Jaras and Rohm, 2011; Mariniello et al., 2013). Until the end of 1980 TGase was only obtained from animal tissue; most commonly from guinea pig liver. The enzyme was too expensive for application in food because of high production costs. However, microbial TGase from *Streptomyces mobaraense* obtained through a fermentation process has reduced the cost of TGase, making it readily available for use in foods. This organism was identified by screening thousands of TGase producing strains. Research is currently ongoing to further reduce production costs by genetically engineering the enzyme. The microbial TGase is calcium independent, has low substrate specificity, and is the only one currently available for the food industry. Microbial TGase is a polypeptide of 331 amino acids with a pI of 8.9. It is active in the pH range of 4–9 with an optimum range of pH 6–7, and optimally at 50°C. It is inactivated at 70°C but is stable under high hydrostatic pressure (HHP) and retains

some activity at near-freezing conditions (Motiki and Seguro, 1998; Jaras and Rohm, 2011; Mariniello et al., 2013). TGase rarely has natural inhibitors in foods.

TGase may aid in the gelation of proteins, it also provides for gel stability at high temperatures. TGase has been used in restructured meat products in the binding of meat pieces at temperatures below 10°C, and therefore sometimes is referred to as "meat glue." These restructured meat products are stable under high temperatures of cooking. TGase is also used in the development of desirable texture for fish product such as surimi and kamaboko gels that are used for imitation products like fish sticks or crab sticks (Li-Chan, 2012). In the dairy industry the most significant application is in fermented milks, in improving textural properties. TGase is used to improve firmness and reduce syneresis in yogurt. In plant-based foods TGase is applied mainly on wheat proteins. TGase cross-linked flour has higher water-holding capacity and improved thermal properties. The dough produced has higher tensile strength. The volume and crumb characteristics of the baked bread are also improved. TGase has also been used in the production of tofu (Motiki and Seguro, 1998; Jaras and Rohm, 2011; Mariniello et al., 2013).

More recently TGase drew interest in macromolecular formation for the production of conjugates for food and pharmaceutical applications (Zhu and Tramper, 2008; Mariniello et al., 2013). These macromolecules include protein/polyethylene glycol (PEG) conjugates, noncovalent TGase cross-linked protein polysaccharide complexes, glycosylated proteins, casein/konjac glucomannan. The formation of these macromolecules may combine the individual traits of proteins and polysaccharides to produce food ingredients with broader range of functional properties. An enzymatic approach to the formation of these molecules provides an alternative to chemical coupling of these molecules. Enzymes allow for selectivity and eliminate by-products formation during chemical reactions, as well as other concerns of chemical processes.

4.4.3 Plastein reaction

Plasteins were discovered nearly 100 years ago, and are formed when concentrated solutions of enzymatically hydrolyzed proteins are incubated with proteases to re-form proteins. The plastein products are water insoluble and gel forming (Douchet et al., 2003). Typically, proteases such as papain, trypsin, or chymotrypsin are used. Plastein formation can be considered a three-step process. The first step in the process involves enzymatic hydrolysis of low concentration of protein solution of 3–5% at the optimum pH of the specific protease used. This step can also be accomplished by partial acid hydrolysis. Regardless, the hydrolyzed protein is lower in molecular weight than the original protein and is soluble in water. The next step involves the concentration of the hydrolyzed protein, thus removal of the moisture, which can be accomplished by a variety of methods such as evaporation, freeze drying, ultrafiltration. Once the hydrolyzed protein is concentrated to approximately 30–50%, the final step involves incubation of the concentrated protein with the same enzyme or a different protease for the plastein reaction to occur. A pH of 4–7 is reported to be optimum for plastein reactions, however rate of the reaction is also influence by the size of the proteins in the concentrated hydrolysate (Fujimaki et al.,

1971). The reaction can also take place in one step where a concentrated protein solution of 30–35% is incubated with papain in the presence of L-cysteine (Damodaran, 2008).

Over the years, the mechanism of plastein formation has been somewhat controversial. Some have reported on the re-synthesis of the peptide bond through condensation or transpeptidation reactions (Yamashita et al., 1975), others have reported that the formation of the plastein products is through a physical process resulting in aggregation of the protein (Andrews and Alichanidis, 1990). Regardless, plastein products form thixotropic gels, or thixotropic viscous solutions depending on concentration, are stable over a wide pH range (Fujimaki et al., 1971; Andrews and Alichanidis, 1990) and temperature conditions (Sukan and Andrews, 1982a, 1982b), and display altered functional properties compared to the original protein. Plastein products have been of interest to the food industry in developing new functional protein ingredients as well as for improving nutritional value of certain proteins through plastein reactions (Douchet et al., 2003). Plastein reactions in soy proteins are further discussed in Chapter 9.

4.5 Processing-induced changes in food proteins

The main purpose of processing foods is to prevent undesirable changes in the wholesomeness, nutritive value, and sensory properties of a food. The intent is to control the metabolism of microorganisms, prevent other undesirable physical and chemical changes in foods, and also prevent contamination. The effect of traditional methods such as thermal processing on food proteins is well known. However, the effect of newer emerging technologies on food proteins and enzymes is not as extensively studied, thus the literature is limited.

4.5.1 Heat processing

4.5.1.1 Moderate and high heat Many food processing operations such as pasteurization, drying, evaporation, blanching, sterilization, canning, extrusion, cooking, baking, roasting, and frying involve the application of heat. Heat treatment is one of the most common ways food proteins denature during processing. Moderate temperatures of 60–90°C will denature food proteins. Partial denaturation often improves digestibility of proteins and may enhance the biological availability of the essential amino acids. Moderate heat treatments also inactivate antinutritional factors found in plant proteins such as legumes and oilseed proteins. Antinutritional factors such as trypsin, chymotrypsin, lectins impair efficient digestion of proteins, reducing their biological availability, and may cause intestinal malabsorption of other nutrients. Protease inhibitors found in egg and milk proteins also lose their activity when exposed to moderate heat treatment. Moderate heat treatments will also inactivate enzymes such as proteases, lipases, polyphenol oxidase (PPO), lipoxygenase (LOX), amylase, and other enzymes involved in deteriorative reactions of foods. Blanching is a heat process to inactivate such deteriorative enzymes. Protein toxins, such as botulinum toxin, from *Clostridium botulinum* and enterotoxin from *Staphylococcus aureus* are inactivated at temperatures of 100°C or above (Damodaran, 2008; Li-Chan, 2012).

More severe heat treatment such as at temperatures above 200°C as in the case of broiling, baking, grilling, frying, drying, sterilization can lead to the decomposition and pyrolysis of some amino acids. Under these conditions not only the essential amino acids may be lost, but potential carcinogens or mutagens may be produced. High temperature processing may also lead to the formation of *isopeptides* which are amide cross-linkages formed by transamidation reactions involving ε-amino groups of lysyl residues, and β- and γ-carboxamide groups of asparaginyl and glutaminyl residues, respectively. This produces ε-N-(β-aspartyl)lysyl and ε-N-(γ-glutamyl)lysyl cross-links. It should be noted that these bonds are different from the peptide bond formed between the carboxyl and the amine groups associated with the C_α atom of the protein (Li-Chan, 2012).

4.5.1.2 Heat under alkaline conditions Texturized vegetable proteins are produced using thermal processing under alkaline pH conditions. Nixtamalization is a process where maize is soaked and cooked with lime under alkaline conditions in the production of corn for making tortillas. Maize that undergoes nixtamalization is more easily ground, and its flavor and aroma is believed to be improved. Heat processing under alkaline pH leads to partial *racemization* of L-amino acid residues to D-amino acids (Damodaran, 2008, Li-Chan, 2012). Acid hydrolysis of proteins and high temperature roasting may also lead to racemization. Residues such as Asp, Ser, Cys, Glu, Phe, Asn, and Thr undergo racemization more readily than other amino acid residues (Liardon and Friedman, 1987).

Additionally extreme heat processing conditions can lead to the formation of dehydroalanine (DHA) due to β-elimination reaction of cysteine and phosphoserine residues, thus these amino acid residues will not undergo racemization under alkaline conditions. Formation of lysinolalanine (LAL), lanthionine (LAN), or histidinoalanine (HAL) will result from the reaction of DHA with the ε-amino group of lysine, thiol group of cysteine, and imidazoyl group of histidine, respectively. These may then produce cross-link in proteins. LAL formation is of particular concern in foods such as wheat where lysine may be the limiting amino acid (Damodaran, 2008; Li-Chan, 2012).

Other than racemization and β-elimination reactions discussed above, heating of proteins under alkaline pH conditions destroys Arg, Ser, Thr, and Lys.

4.5.1.3 Maillard reaction and acrylamide formation Proteins may also interact with other components present in food during heat processing such as roasting, baking, frying, and drying. The *Maillard reaction*, also referred to as *nonenzymatic browning*, is the reaction between the amino groups in protein or amino acid and a carbonyl compound typically a reducing sugar such as glucose upon heating (Jaeger et al., 2010). The initial step involves the formation of a Schiff base followed by Amadori rearrangement and Strecker degradation. The advanced stages of the Maillard reaction involve complex series of reactions, but the end result is the formation of brown pigments called melanoidin pigments. The Maillard reactions are influenced by temperature, pH, water activity, and metal ions. Alkaline conditions promote Maillard reactions. Maillard reactions cause significant loss of lysine. Furthermore, Met, Tyr, Hist, and Trp may undergo oxidation (Damodaran, 2008). Maillard reactions are important in the formation of color and flavor in processes such as roasting, baking, and frying. However, Maillard reactions and the resulting

compounds formed are not so desirable during pasteurization, sterilization, and drying processes (Jaeger et al., 2010). The safety of some compounds formed during Maillard reactions has recently been a concern because of suspicions that they are carcinogens (Moon and Shibamoto, 2011).

Acrylamide is formed from the reaction of asparagine with a reducing sugar via the Maillard reaction during heat processing at temperatures above 120°C. A number of potential mechanisms were published in the early to mid-2000s. The mechanism involves the formation of Schiff base followed by decarboxylation and elimination of ammonia, or substituted imine, under heat processing conditions resulting in acrylamide formation (Figure 4.1) (Zyzak et al., 2003). This was established by isotope substitution and mass spectrometric analysis which confirmed the presence of key reaction intermediates. Further confirmation of the mechanism came from selective removal of asparagine with asparaginase resulting in reduced levels of acrylamide (Zyzak et al., 2003). Over the past few years several nonasparagine routes have been proposed. One involved the reaction of acrylic acid with ammonia released during thermal degradation of amino acids and proteins to produce acrylamide (Yaylayan and Stadler, 2005). Acrylic acid may be formed from aspartic acid through Maillard reaction, or from acrolein a lipid degradation product. Acrelein may provide a carbonyl group and also react with asparagine through Maillard reaction (Yasuhara et al., 2003). Claus et al. (2006, 2008) also suggested that specific amino acid of wheat gluten may release acrylamide under certain conditions, but at low yields. However, studies with potato and cereal-based foods have demonstrated the importance of asparagine in the acrylamide formation by effectively eliminating acrylamide by the use of asparaginase.

Acrylamide in foods was first detected in fried potatoes. When other foods were analyzed, acrylamide was found in many carbohydrate-rich foods. Foods derived from potatoes and cereals tend to contain the highest amount of acrylamide, due to the natural presence of the reactants. Control of sugar content, frying, and baking temperatures are considered most effective in reducing acrylamide content in potato products. Acid treatment that reduces the pH of the food also is effective in reducing acrylamide. In cereal products asparagine is the main determinant of acrylamide formation rather than reducing sugars. Concentrations of asparagine in grains vary significantly, therefore asparagine levels in these crops are difficult to control. Therefore the strategy to reduce acrylamide formation in cereal products is to control baking conditions and ingredients. It is important to note that products made with fermented dough tend to have lower acrylamide levels when compared to similar nonfermented products. Yeast assimilates asparagine and sugars, thus lowering the reactants formed. Treatment with asparaginase is reported to be effective both with potatoes and with cereal products (Lineback et al., 2012; Zhang et al., 2012).

Halford et al. (2012) reported on the efforts to reduce acrylamide formations by development of crop varieties with lower free asparagine and/or reducing sugar contents, and the best agronomic practices to keep their levels low. Some of the strategies include the genetic modification of crops or other genetic techniques; such as identifying of the quantitative trait loci. However, genetic modification of crops still remains somewhat controversial around the world.

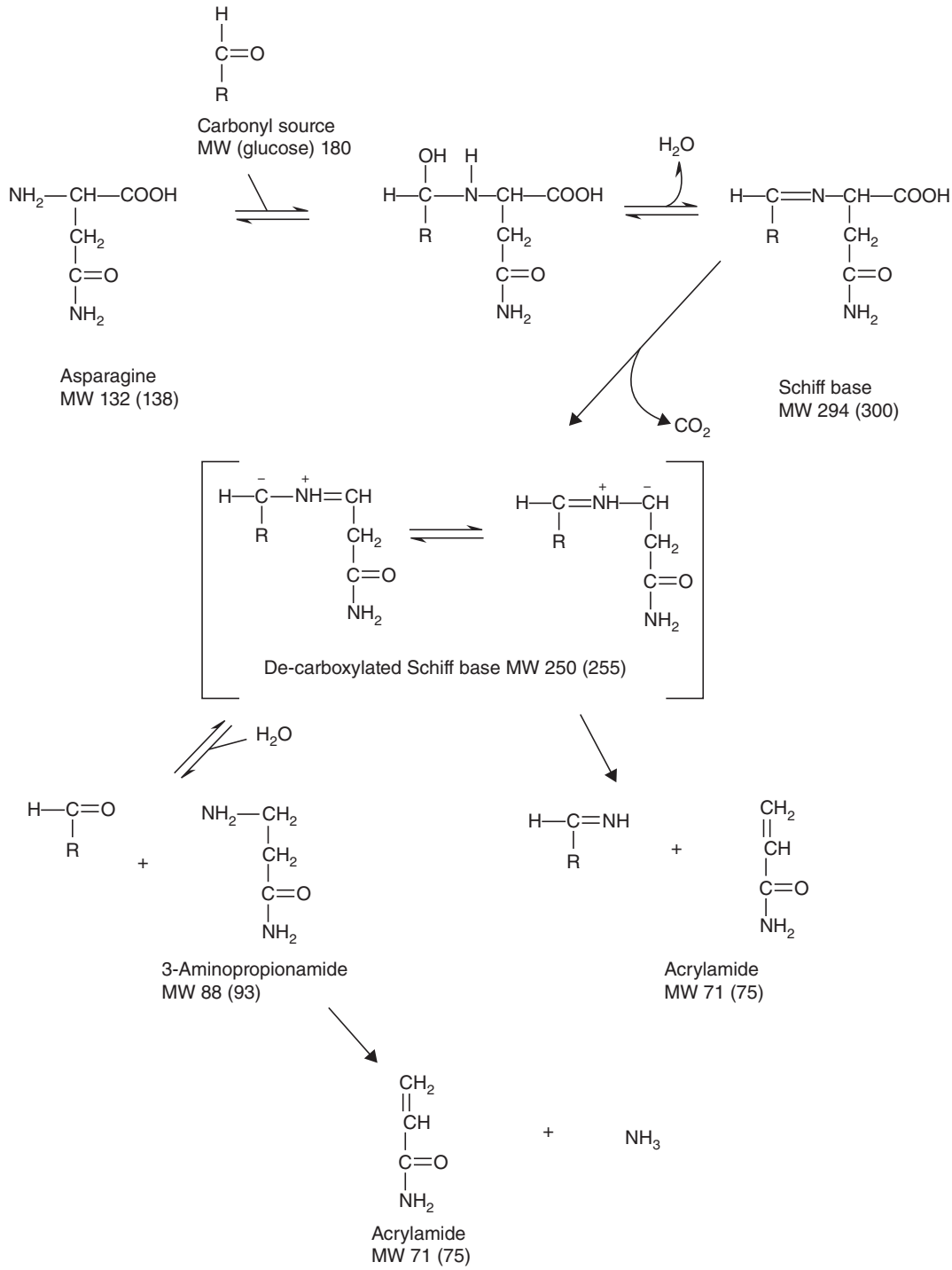

Figure 4.1 Mechanism of acrylamide formation in foods. Reprinted with permission from Zyzak et al. (2003). *J. Agric. Food Chem.* 51:4782–4787. Copyright American Chemical Society

The main health concern regarding acrylamide in foods is its potential to cause cancer in humans (JECFA, 2011). U.S. National Toxicology Program classifies acrylamide as being "reasonably anticipated to be a human carcinogen" (NTP, 2011). The Environmental Protection Agency refers to it as "likely to be a carcinogen to humans" (EPA, 2011). In both cases the classification is based on sufficient evidence from animal studies. Acrylamide is also known to be a potent neurotoxin. The announcement of presence of acrylamide in certain foods has created a need to monitor as well as collect data on the extent of acrylamide occurrence in foods. U.S. Food and Drug Administration (FDA) and the European Commission (EC) maintain large data bases. The European Food Safety Authority (EFSA) has its member states monitor acrylamide contents of foods, and reports the findings on an annual basis. This reporting should provide information on dietary exposure and be useful in risk assessment. Also, it should provide important information whether the efforts in place are effective at reducing acrylamide content in foods (Lineback et al., 2012).

4.5.2 High-pressure processing

HHP processing or pascalization is a method of food preservation where the food product is processed under high pressures ranging 100–900 MPa. Commercial systems typically use 400–700 MPa. During HPP processing, the food product is sealed in its package and placed into a steel pressure vessel most often containing water, and pressure is applied isostatically for the desired treatment time, then pressure is released. HPP has been shown to inactivate enzymes as well as impact protein structure. HHP processed foods have been commercially successful due to demand for minimally processed foods, particularly fruits and vegetables. Currently, HPP is the most effective commercial technology to process avocado paste (San Martin et al., 2002; Jacobo-Valazquez and Hernandez-Brenes, 2011). The mechanism of high-pressure-induced denaturation was discussed in Section 4.2.1.2. The next two sections will discuss changes in food proteins due to high-pressure processing.

4.5.2.1 Protein gelation Coagulation of proteins under pressure was first documents as early as 1914 (San Martin et al., 2002). High pressures can induce gelation under ambient temperatures. However, these gels are typically weaker than heat-induced gels. Gelation of egg white, 16% soy protein solution, or 3% actomyosin solution by application of 1–7 kbar hydrostatic pressure for 30 minutes at 25°C was reported. These gels were not as firm as thermally induced gels (Damodaran, 2008). High-pressure-induced whey proteins gels were also low in firmness compared with their heat-induced counterparts (Patel et al., 2005). Exposure of beef muscle to 1–3 kbar resulted in partial fragmentation of myofibrils. This was suggested as a potential for tenderizing meat (Damodaran, 2008). However, Dissanayake et al. (2012) showed that with concentrated solutions of whey proteins (80% w/w), proteins were resistant to physicochemical denaturation under high pressures (600 MPa for 15 minutes). The native confirmation of secondary and tertiary structures was confirmed. Surimi gels obtained with HPP from Pacific whiting were less opaque than heat set gels (Chung et al., 1994), Ashie and Lanier (1999) also reported that surimi gels

formed under high pressure were less opaque, more glossy, and weaker than heat-induced gels. Turkey pastes did not form gels under high pressure but high-pressure-induced gelation was aided with TGase (Ashie and Lanier, 1999), but again these gels were weaker than heat-induced gels.

4.5.2.2 Enzymes The effect of HPP on enzymes depends on type of enzymes, source, substrate, and processing conditions such as pressure and time (San Martin et al., 2002). Some enzymes like PPO are reported to show increased enzyme activity upon high-pressure treatment; particularly in mushrooms, potatoes, and apples at pressures 200–600 MPa (Gomes and Ledward 1996; Weemaes et al., 1996). Palou et al. (1999) demonstrated that PPO may be inactivated to residual levels when puree is blanched prior to HPP, however the residual activity was sufficient to initiate browning after 6 days. Estiaghi and Knorr (1993) reported on complete inactivation of PPO in potato cubes at 400 MPa within 15 minutes, when the cubes were acidified with 0.5% citric acid solution prior to high-pressure treatment. Complete inactivation of PPO was obtained at 800 MPa (Gomes and Ledward, 1996; Weemaes et al., 1996). Weemaes et al. (1996) also demonstrated that inactivation of PPO by HPP followed first-order kinetics. In apples, grapes, avocado, and pear PPO reduction in enzyme activity was seen at 600, 700, 800, and 900 MPa, at 25°C, respectively. Whereas plum PPO started losing activity at 900 MPa and 50°C. Increase in enzyme activity due to HPP in the range of 400–600 MPa was reported for α- and β-amylase. However, at high pressures 700–800 Mpa the activity was significantly decreased (Gomes et al., 1998).

Van der Ven et al. (2005) reported on inactivation of trypsin inhibitor and LOX. For complete inactivation of LOX either very high pressures (800 MPa) or a combined temperature/pressure (60°C/600 MPa) was needed. For 90% inhibition of the trypsin inhibitor, a treatment time of <1 minute, temperatures between 77–90°C, and pressures of 750 and 525 MPa were needed. Mazri et al. (2012) reported on inactivation of lactoperoxidase. There was no substantial inactivation of lactoperoxidase at pressures of 450–700 MPa at 20°C in skim milk, whey, or in buffer. More recently, Teixeira et al. (2013) reported on the changes in enzyme activity and protein profiles of HPP treated sea bass. They reported on decrease in activity of the deteriorative enzymes when fillets were treated at 400 MPa, suggesting that HPP can be a potential tool to prevent or delay the softening seas bass muscle and loss of prime quality.

4.5.3 Pulsed electric field processing

PEF is a nonthermal food preservation technology that has generated much interest over the years. It can be an alternative or may complement traditionally used processes for processing of fluid foods. The advantage of nonthermal processes such as PEF is that it has minimum impact on the sensory, nutritional, and quality attributes of the processed food. The food does not undergo the changes typically associated with thermal processing. PEF has been of interest particularly in processing foods and food components that are particularly sensitive to heat damage. PEF processing of fluid foods involves applying an electric field, ranging from 20 to 80 kV/cm, in short pulses of about 1–5 μs. This is done

as the food is confined or flowing through a pair of electrodes (Martin-Belloso and Elez-Martinez, 2005; Marco-Moles et al., 2011).

Much research has been conducted on the inactivation of microorganism by PEF. It has also been reported that intensive PEF treatment can result in inactivation of many enzymes. Some of the enzymes studied include: pectin methyl esterase (PME), polygalacturonase (PG), PPO, peroxidase (POD), LOX, ALP, protease, lipase, α-amylase, glucose oxidase, lysozyme, pepsin, lactate dehydrogenase, and papain (Martin-Belloso and Elez-Martinez, 2005). These studies are on the effects of PEF on enzymes suspended in aqueous solutions, and in liquid foods such as fruit juices and milk. Most enzymes are almost completely inactivated, while others show resistance to PEF processing, or show increased activity. The mechanism of PEF-induced protein unfolding is not fully understood. Strength of the electric field, treatment time, number of pulses used and pulse width, field polarity, frequency, and treatment temperature are reported to be factors that influence enzyme inactivation. It is suggested that intense electric fields may disrupt interactions among charged groups (Martin-Belloso and Elez-Martinez, 2005). Aguilo-Aguayo et al. (2010) reported on inactivation of PPO with high-intensity electric field. The treatments were more effective in bipolar than in monopolar mode; and longer durations were more effective. These authors were able to minimize PPO activity down to 2.5% by selecting bipolar treatments, frequencies greater than 229 Hz, and pulse width 3.23–4.23 μs for a total of 2000 μs of treatment time.

4.5.4 Texturization

Texturization is the conversion of a protein to a fibrous physical structure with altered textural properties. Vegetable proteins (i.e., soy protein) are typically preferred for texturization. Proteins can be texturized by two methods, spun fiber or extrusion (Damodaran, 2008). In the *spun-fiber method* soy protein isolates are concentrated, pH adjusted to >12, and aged. The increase in viscosity is due to protein denaturation and cross-linking due to alkaline conditions. This viscous mass is passed through a spinneret and into a phosphoric acid and salt bath at a pH 2.5. At this point the coagulated protein becomes fibrous. The fibrous protein is compressed and stretched using steel rolls, and is washed to remove excess acidity and salt. Other additives like fat, flavor, color, binders may be added. The mixture is then heated to 80–90°C to induce gelation of the binder (Damodaran, 2008). In the case of *extrusion* the soy protein is steam conditioned and concentrated to approximately 25% moisture. The protein mass is then extruded in an extruder which contains rotating screws in the barrel which is heated to 150–180°C. The proteins that denature under these conditions form fibers as they move through the extruder. In both cases the denaturation of the proteins is due to heat, shear, pressure, and alkaline conditions (Damodaran, 2008). The mechanism of denaturation of proteins is discussed in previous.

4.6 Oxidizing agents

Amino acids and amino acid residues may undergo oxidation upon treatment of food with hydrogen peroxide, benzoyl peroxide, and sodium hypochlorite. These chemicals are

used as bleaching and antimicrobial agents in foods. Irradiation of foods also may cause oxidation of proteins through formation of hydrogen peroxide. Met, Cys, Trp, and His are the most susceptible to oxidation and Tyr to a lesser extent. Oxidized amino acids may or may not be biologically available (Damodaran, 2008; Li-Chan, 2012). In the case of wheat proteins, oxidation may play a role in cross-linking to provide for the dough and bread characteristics. This is further discussed in Chapter 11 which deals with wheat proteins.

4.7 Conclusion

Denaturation and modifications of proteins can be intentional improving on their functional properties, inactivating antinutritional factors, or deteriorating enzymes. The nutritional values of proteins can be unintentionally lost during processing. Thermal processing is commonly used when processing foods can result in significant changes in proteins. Understanding the conditions under which these changes occur helps to establish processes that safely minimize undesirable changes while maintaining optimal nutritional, sensory, and functional properties. A number of relatively new processing technologies are at various stages of development and approval for food use. Their effects on food proteins and enzymes must be further studied.

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5
Functional Properties of Food Proteins

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5.1 Introduction

The primary structure of proteins is the key of their unique character. The interactions between the amino acids within a chain and between chains are at the basis of the structural as well as functional features of proteins as they lead to formation of helices, β-sheets and turns, and the tridimensional structure of the proteins. From the nutritional standpoint, in addition to being the source of essential amino acids, food proteins encode a series of bioactive peptides within their structure. Once absorbed by the intestinal cells, bioactive peptides play a major role in the biological functions of gastrointestinal tract, for example, as regulators of the immune system and blood pressure, or as signaling molecules. The research on bioactive peptides derived from food proteins has grown in the past few years, as part of a renewed interest for a deeper understanding of the connection between food and health.

Besides their nutritional properties, proteins are employed as ingredients in foods because of their processing functionality. They often serve as structural building blocks of the food matrix. Depending on their concentration, processing history, and environmental conditions, proteins have the ability to self-assemble in supramolecular structures which, in turn, impart texture to food. For example, milk proteins comprise the building blocks that impart texture in cheese and yogurt, and gluten is fundamental to the formation of the network that holds structure in bread dough. It is possible to obtain a variety of final food textures by manipulating the structure and aggregation of proteins through processing, changes in environmental conditions and interactions with other molecules in the matrix. As food texture is critical to sensory perception (van der Sman and van der Goot, 2009)

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modifying protein structures and their behavior to cause textural changes becomes imperative in today's efforts to develop food matrices not only of high nutritional value, but also appealing to consumers.

The way the proteins assemble under various conditions makes a profound difference to the final texture of the product. As an example, a higher extent of heat-induced denaturation of the whey proteins in milk causes significant differences to the texture of yogurt-like type of gels. During heating, whey proteins denature and form aggregates that interact with casein micelles, and when present in sufficient amount, form bridges between the micelles, resulting in greater yogurt firmness and serum retention (Lucey et al., 1999).

Many aspects of protein functionality can be derived by an in-depth study of their structure, as the principles for the structure–function relationships are well established. However, proteins in foods normally undergo a number of processing steps and the challenges lay in the complexity of the matrix. A material science approach is often used to study the functional properties of the proteins. For instance, the molecules can impart viscosity to the food matrix, which can be studied by means of rheological techniques. During heating proteins can denature and unfold, or can maintain a globular structure, and form polymeric structures resembling those of synthetic biopolymer particle gels. The gels may also be thermally reversible (e.g., gelatin). Protein molecules can also interact or be thermodynamically incompatible and phase separate with other biopolymers in the food matrix imparting novel structures, such as large aggregates (coacervates) or water in water emulsions. In addition, some proteins have very unique structural features, such as particular distributions of charges, or hydrophilic and hydrophobic moieties, making them particularly prone to interacting with various phases, as for example in the case of amphiphilic proteins able to adsorb at oil–water or air–water interfaces, and impart stability to foams and emulsions.This is an important characteristic of food proteins, as food matrices are very complex systems often composed of more than one phase and, therefore, the use of components aiding in their stabilization is critical.

Most of the research on food protein functionality has been carried out on relatively pure protein systems. It is indeed necessary to be able to control the processing history of the protein as well as its interactions with the environment and other components to truly understand the functional behavior and to derive some principles that will apply to more complex matrices. Studies in model systems have provided a significant scientific base on the structure–function relationship of food proteins and many reviews have been published (e.g., Dickinson, 2011; Keerati-u-rai and Corredig, 2010). However, it is important to consider that foods are the result of the interactions of different ingredients, and often protein sources are mixtures of various proteins (e.g., whey proteins, egg white proteins, soy proteins, etc.). Hence, it is expected that multiple reactions will occur simultaneously, a distribution of protein aggregates may be present, and multiple components will contribute to the overall processing functionality. This is a challenge for the food product developer, who needs to clearly possess the necessary skills to evaluate the processing history of the ingredient as well as the properties of the various components, to be able to predict the overall functionality. In addition, in the majority of the cases research studies on protein structure–function relationships are performed with protein concentrations that are much lower than those needed in manufacturing processes, under very controlled

conditions, and with less sophisticated processes than those typical of food manufacturing (Dickinson, 2011).

Recently, food protein functionality has been defined as the ability of a protein to form the colloidal structures serving as building blocks of the food matrix (Foegeding and Davis, 2011). This definition may be able to relate more closely to the structural features of the protein with the processing and environmental factors, and therefore better bridge the gap between the fundamental principles and ingredient transformation. Studies utilizing ingredients at concentration and compositions used in manufacturing processes and preparation techniques that simulate the industrial ones are necessary, considering that in the majority of the cases the purified components of food proteins will show different behavior compared to the protein ingredients. Any factor which modifies the structure of proteins will affect their ability to form colloidal or supramolecular aggregates and hence will affect their processing functionality. It is therefore important to fully understand the principles that lead to such changes, since modulating the environmental conditions, processing treatments, and protein assembly and behavior may result in the development of new food ingredients or novel food structures. This chapter is focused on the ability of proteins to form colloidal structures, as these structures will have tremendous consequences in the processing and nutritional properties of the food matrix. Furthermore, the factors that influence the functionality as well as an overview of the methods and techniques used to characterize functional properties of food proteins are presented. Changes in protein functionality due to chemical and enzymatic modifications as well as processing are mentioned only briefly and mainly as examples, as they are the subject of other chapters and are not a main focus here. Also, the experimental approaches employed to follow changes during processing have been extensively reviewed in the past (e.g., Li-Chan, 1998; Pelton and McLean, 2000), and they will not be discussed in this chapter.

Once the structure of the protein has been elucidated, the ability of the protein to form colloidal building blocks and then larger structures needs to be evaluated, most often using a multitechnique approach, which relates to answering various aspects of the specific role played by the protein in the formation of the food matrix. For example, rheological measurements may be carried out to characterize gels, as well as follow structure formation or identify a specific gelation point. This technique, however, has to be coupled with other experimental methods that would elucidate initial stages of aggregation, interactions between the biopolymers, or the final microstructure of the gels. The need for a multitechnique approach increases with the complexity of the matrix under study. When studying emulsions, gel electrophoresis may be employed to identify which proteins are adsorbed at the interface, but physical techniques such as light scattering and interfacial dilatational and shear rheometry may be used to better understand the emulsifying behavior of the proteins. Some tests to determine protein functionality are quite specific and practical, as for example, test for flour functionality. These tests have been developed for specific food applications and will not be discussed in this chapter. It is important to point out at this point that, in general, the methods employed to study protein structure often focus on laboratory model systems prepared with purified protein solutions.

This work outlines the principles dictating functionality of food proteins, with multiple examples on structure–function relations. The intent of this work is not to be a comprehensive review of the functionality of food proteins, but to highlight some novel aspects as well as recent advances in the methodologies employed to study protein functionality.

5.2 Interfacial properties

Because of their hydrophilic and hydrophobic moieties, proteins can adsorb spontaneously at interfaces, and are often employed to stabilize multiple phase foods such as foams and emulsions. The interfacial adsorption of the protein results in conformational changes which lead to a new free energy minimum and a reduction of surface tension (Dickinson, 2011). The ability of the protein to adopt a different structure at the interface depends on its molecular flexibility. Figure 5.1 illustrates some examples of protein adsorption at interfaces.

Flexible proteins, such as β-casein, adsorb rapidly at the interface and adapt their structure, so that the hydrophobic moieties are sheltered from the water phase, and the flexible, hydrophilic parts of the structure instead protrude into the water phase. In studies of model systems consisting of isolated proteins it has been shown that once adsorbed at the interface, proteins can assume a variety of configurations with trains, loops, and tails (Dickinson, 1998). For example, β-casein adsorbs at the interface in a configuration of train with small loops and a long tail of N-terminus dangling away from the surface, while α_{s1}-casein assumes a loop-like conformation. Because of their charge, the proteins form a polyelectrolyte layer with a thickness of about 10 nm, resulting in electrostatic and steric stabilization (Dickinson, 1998). On the other hand, less flexible globular proteins

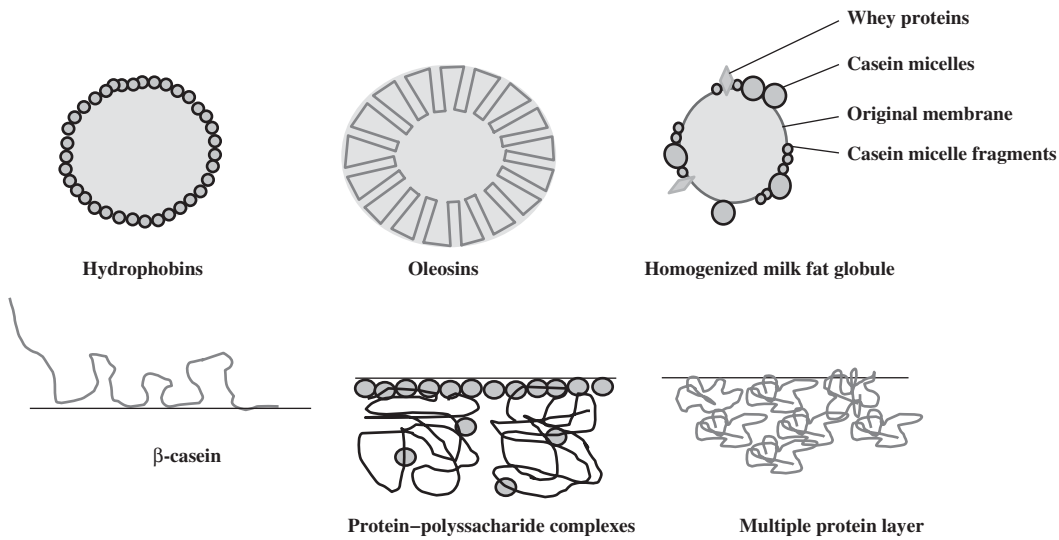

Figure 5.1 Examples of protein structures at the interface, not to scale. Adapted from Kallio et al. (2007); Dickinson (2011); Gallier and Singh (2012)

like lysozyme or α-lactalbumin, do not undergo extensive surface denaturation, because of the presence of internal crosslinks, and may still show enzymatic activity once adsorbed at the interface as in the case of lysozyme, (Haynes and Norde, 1994). However, Desfougeres et al. (2011) claimed that lysozyme still shows structural modifications at the interface, with an increase in the percentage of β-sheets. In this case, the monolayer of these proteins would show a thickness of a few nanometers, and the stabilization would be mostly because of electrostatic repulsive forces.

The pattern of distribution of hydrophobic and hydrophilic groups of the protein is more important that the total surface hydrophobicity on influencing surface activity of proteins (Damodaran et al., 2008). A unique example of the significance of distribution of hydrophobic and hydrophilic groups on the interfacial characteristics of a protein is the structure of hydrophobins, fungal proteins with unique interfacial properties. These proteins have a highly amphiphilic structure that stems from a patch of hydrophobic side chains (Kallio et al., 2007). Hydrophobins diffuse fast at the interface, retain their globular shape, and self-assemble into films, forming a very stable interface with high surface viscosity (Green et al., 2013). Another interesting example of stabilizing proteins in nature is present in seed oil bodies. The size of oil bodies is inversely proportional to the concentration of these proteins, called oleosins. They are composed of three domains, namely, an N-terminal amphipathic domain, a central hydrophobic domain that extends into the oily core of the oil bodies, and a C-terminal amphipathic domain which allows oleosins to stabilize the oil body by connecting to the phospholipid outer monolayer and the triacylglycerol inside core (Huang, 1992). It has been shown recently that their digestion in the gastric stage with pepsin is critical to the destabilization and digestion of almond oil (Gallier and Singh, 2012).

Limited hydrolysis of the proteins or other mild structural changes like glycosylation or phosphorylation may cause substantial changes to the adsorption behavior of the proteins. For example, limited (only to about 3–5%) hydrolysis of sunflower protein isolate, showed improved foaming characteristics compared to unhydrolysed protein (Ruiz-Henestrosa et al., 2007), especially at pH values close to the isoelectric point of the isolate.

Unlike small molecular weight surfactants, protein adsorption at the interface is slow, but once at the interface they may not be easily removed (Dickinson, 1998). There is an energy barrier to desorption, that will depend on the structures formed at the interface. When desorption occurs, a recovery of the structure may not occur. For example, the changes in the secondary and tertiary structures of lysozyme and α-lactalbumin upon adsorption are partially reversible between the solution and adsorbed state, whereas β-lactoglobulin does not show any thermal transitions once adsorbed, indicating surface denaturation, and these conformational changes are irreversible once desorbed (Corredig and Dalgleish, 1995). The structural changes occurring at the interface are also significant from a biological standpoint, as it has been demonstrated that the susceptibility of the proteins to proteolysis is affected. For example, *in vitro* digestion experiments of whey-protein stabilized emulsions showed that the resistance to pepsin substantially decreases when β-lactoglobulin is adsorbed at the interface; in contrast, α-lactalbumin is only partly hydrolyzed and a large peptide is recovered at the interface (Malaki Nik et al., 2010).

During homogenization, when more than one protein is present in solution, the composition of the interface may change over time. Protein exchanges may occur, for example, with changes in storage conditions or with processing (Mackie et al., 2000; Dalgleish et al., 2002). Competitive displacement of caseins and whey proteins has been shown in the presence of small molecular weight surfactant (e.g., polysorbates), after homogenization, as their presence at the interface may be thermodynamically favorable (Mackie et al., 1999, 2000). Small molecular weight emulsifiers easily migrate to the interface and displace proteins considering their high mobility and lack of conformational constrains. Small surfactant orient at the interface in defect areas of protein network, and as they rearrange their adsorption increases, causing compression of the protein to the degree of failure of protein network and orogenic displacement of the protein from the interface (Mackie et al., 1999). Not all proteins readily exchange or are displaced at the interface. The type of protein, the extent of unfolding, the strength of intermolecular interactions, and the structures formed at the interface will determine the composition of the interfacial layer. For example, β-lactoglobulin and α-lactalbumin form films at the interface, and they are more resistant to displacement by polysorbates than β-casein (Mackie et al., 1999).

The concentration of proteins on a thin interfacial film is quite high, about 20–25% (Damodaran, 2004). Surface loads between 2 and 8 mg/m^2 of surface have been reported for milk proteins adsorbed at the interface, and the differences in concentration depend on the aggregation state of the proteins (Hunt and Dalgleish 1994; Damodaran et al., 2008). Depending on protein concentration in the soluble phase, proteins can be present as single or multiple layers (Dickinson, 1998). The close proximity of the proteins once adsorbed may induce further interactions between neighboring molecules, causing changes in the viscoelastic properties of the interface as a function of time. During homogenization, because of the high frequency of collisions, large particles such as casein micelles or fragments of protein particles will adsorb at the interface first, but the composition at the interface at equilibrium will depend on the various molecular species present in the mixture. Protein aggregates also may adsorb at the interface, and in this case, their mechanism of stabilization is closer to a pickering-type stabilization (Dickinson, 2012). In these systems, solid particles surround the interface in a densely packed layer that provides a steric barrier which prevents flocculation and coalescence of emulsion droplets (Dickinson, 2010). Examples of protein aggregates that may be adsorbed at the interface are casein micelles or soy protein particles.

Although the ability of proteins to act as emulsifiers and foaming agents stems from their surface active properties, this attribute is not enough to explain the stability of emulsions and foams. Their ability to stabilize colloidal structures is imparted by their charge, as well as their ability to form a thick and well-hydrated film to form complexes at the interface. The viscoelasticity of the film formed by the proteins is a main contributor in the stability of foams and emulsions (Dickinson, 1999; van Aken et al., 2003). In addition, the presence of a charged polymer at the interface will ensure steric repulsion between the colloidal particles (i.e., emulsion droplets). Interfaces containing multiple protein structures as well as complexes of proteins and polysaccharides, either in the form of coacervates or glycoconjugates are often present in food products, because of their effectiveness at stabilizing interfaces. In protein–polysaccharide mixed interfacial layers,

the primary layer that adsorbs directly at the interface is composed of the protein, which represents the surface active component whereas the secondary layer consists of a polysaccharide that can interact with attractive electrostatic interactions with the primary layer (Dickinson, 2011).

In food systems pure protein interfaces are not common, and the interfacial behavior of proteins in complex mixtures may differ from that of the pure protein components. In addition, the composition at the interface may depend on environmental conditions, such as salt, pH, temperature. During homogenization, the newly formed interfacial area is covered with multiple surface active molecules, but the composition of the protein layer will depend on the kinetics of adsorption and the collision rates. In milk, for example, the native milk fat globules are surrounded by a membrane composed of phospholipids and proteins, while globular proteins as well as aggregated colloidal particles, casein micelles, are present in the dispersed phase. During homogenization of milk, the fat globules are disrupted, and the newly formed interface increases to about 10 times the original area. As a result, intact and fragments of casein micelles, as well as monomer proteins are adsorbed at the surface to stabilize the homogenized fat globules (Walstra, 1975). When small molecular weight emulsifiers, such as polysorbates or mono and diglycerides are present in homogenized milk systems, as in the case of ice cream mixes, further rearrangements at the interface will occur, including some displacement of the proteins (Goff and Jordan, 1989). If homogenized milk is thermally treated, whey proteins will also be present at the interface, as they interact with caseins by disulfide bridging (Sharma and Dalgleish, 1993).

5.2.1 Factors affecting interfacial properties

Several environmental and processing factors affect the interfacial behavior of proteins. Essentially, anything that causes changes in the protein structure may influence interfacial behavior. Even subtle modifications to the protein structure, little differences in the amino acid sequence of genetic variants or mild hydrolysis can affect the functional properties of the proteins. Therefore, knowledge of the processing history of protein ingredients is critical to be able to predict their processing functionality. For example, although dry heating of lysozyme does not alter its secondary structure, it modifies its interfacial behavior (Desfougeres et al., 2011). Dry-heated lysozyme adsorbs fast at the interface and forms a highly viscoelastic film in contrast to the native protein that forms a monolayer film with properties similar to those of other globular proteins.

Any processing condition that influences protein structure (e.g., induces aggregation and/or reduces solubility), will affect the interfacial properties of the proteins. The aggregation state of the proteins will not only affect the surface load (as more protein will be required to fully cover the interface), but also the ability to lower the interfacial tension and ultimately stabilize the colloidal structure. It has been recently shown, for example, that although the composition of the mixed proteins is similar at the interface, heated and unheated soy protein isolates show very different solubility and interfacial properties (Keerati-u-rai et al., 2011).

The processing order also has a significant impact in forming aggregates at the interface. The structure of the proteins adsorbed is different if heating is carried out before or after homogenization. This has been recently demonstrated for soy protein isolate emulsions as well as homogenized milk (Keerati-u-rai and Corredig, 2009; Ion-Titapiccolo et al., 2013). The process of heating soy protein isolate stabilized oil-in-water emulsion mixtures before homogenization results in all protein components being adsorbed at the interface in aggregated form, whereas, when heating is carried out after emulsification some β-conglycinin and the acidic glycinin polypeptides are recovered in the adsorbed phase (Keerati-u-rai and Corredig, 2009). In the case of homogenized milk, although the composition seems similar, the thickness of the interface and the effective displacement by small molecular weight emulsifier depends on the order of the processing steps (Ion-Titapiccolo et al., 2013).

Ionic strength and pH are among the most important environmental factors that affect food protein functionality, hence interfacial properties. Most proteins exhibit poor emulsifying properties at pH close to their isoelectric point, due to poor solubility and charge neutralization. For example, the adsorption at the air–water interface of soy protein components β-conglycinin and glycinin decreases drastically at pH 5 (close to the isoelectric point of β-conglycinin) and low ionic strength (0.05 M), but their foaming properties improve at pH 7 and low ionic strength (Pizones Ruiz-Henestrosa et al., 2007). Decrease in the overall charge of the protein often causes decrease in hydration and increases the extent of aggregation of the proteins, both in solution as well as at the interface. When adsorbed at the interface, a decrease in the charge of the polyelectrolyte layer around oil droplets induced by a change in pH or an increased ionic strength will lead to decreased charge repulsion, causing flocculation and coalescence.

The presence of sugar molecules (sucrose, lactose, etc.) impairs foaming properties, reducing protein ability to adsorb and form a stable film. However, increased sugar concentration increases the viscosity of the bulk phase, causing improved foam stability. The effect of sucrose on foam stability depends on the protein type (Davis and Foegeding, 2007; Yang and Foegeding, 2010). Egg white proteins are more resistant to drainage, and show higher yield stress and increased dilatational elasticity of the interface in the presence of sucrose, while whey proteins show the opposite effect (Davis and Foegeding, 2007).

5.2.2 Experimental approaches to measuring interfacial properties of proteins

Food proteins are often employed as ingredients because of their emulsifying properties, and numerous analytical approaches are available to describe the adsorption and stabilization behavior of proteins at air–water and oil–water interfaces. As previously indicated, protein adsorption decreases the interfacial tension, but the thickness of the interface, the structures formed, the charge, and the viscoelastic properties of the film are key to the stabilization of the colloidal structure formed. Hence, various aspects of protein adsorption behavior need to be studied, to enable the formation of predictive models on the ability of the protein to adsorb and stabilize interfaces.

At the molecular level, it is important to define the changes occurring to the protein structure upon adsorption. This is at times challenging as many of the spectroscopic techniques employed to determine structural modifications require very clean model systems and very dilute solutions. On the other hand, when looking at emulsions and foams, samples are often too turbid even at low oil volume fractions and protein concentrations. A variety of techniques can be employed to study the process of adsorption at air–water and solid interfaces. Ellipsometry provides information on the amount of protein adsorbed and the layer thickness (Malmsten, 1995). Interfacial measurements on flat interfaces can clearly demonstrate adsorption kinetics and quantify changes in interfacial tension. Using drop tensiometry or other interfacial rheology techniques it is possible to quantify the viscoelastic properties of protein layers. A variety of spectroscopic techniques, such as x-ray and neutron reflectivity, circular dichroism (CD) spectroscopy, infrared, or fluorescence spectroscopy can better define details of structural modifications and changes occurring at the interface (Lopez-Rubio and Gilbert, 2009; Richter and Kuzmenko, 2013). In complex systems more than one technique needs to be employed to follow the interfacial behavior of the protein (de Jongh et al., 2004). In the case of oil–water interfaces, the scattering of the emulsion droplets brings challenges to the use of spectroscopic techniques. Fourier transform infrared spectroscopy and front face fluorescence can overcome such challenges, and identify changes to the secondary and tertiary structure of the proteins once adsorbed (Fang and Dalgleish, 1997; Fang and Dalgleish, 1998; Miriani et al., 2011). Thermal transitions can also be measured with micro-differential scanning calorimetry, and can be used to probe structural changes in emulsions (Corredig and Dalgleish, 1995; Keerati-u-rai and Corredig, 2009). More recently, atomic force microscopy and force probe measurements have been used to unravel the protein at the interface and identify secondary structures of proteins adsorbed on oil droplets (Touhami et al., 2011). These approaches are important as they allow for *in situ* studies of the emulsion droplet interface, without dilution and independently from the extent of scattering of the light.

As previously mentioned, a very important aspect related to protein adsorption is the ability of the molecules to form viscoelastic films capable to resist stresses and deformation, limiting droplets coalescence (Dickinson, 1999). Surface shear and dilational rheological properties are frequently employed to measure the viscoelasticity of the interface, as they are often related to the stability of the emulsions and foams and provide information on the structure and interactions of proteins at the interface (Dickinson et al., 1990). Drop tensiometry allows for measurement of the changes in the interfacial tension on an air bubble or a liquid droplet formed at the tip of a syringe. The image of the shape of the drop is recorded with a camera and analyzed to determine the values of interfacial tension as a function of time or concentration of surfactant, while the viscoelasticity of the interfacial film is measured following dilational surface deformation (Gülseren and Corredig, 2012; Martin et al., 2002). Although with this technique it is possible to derive information on the interfacial properties of the proteins at the interface and on adsorption kinetics and displacement properties, it is not possible to predict the behavior of emulsion droplets stabilized by the proteins as it cannot count for the collision of particles during the emulsification process. Hence, when studying emulsifying properties of proteins it is important to observe the behavior of the emulsion droplets formed after homogenization,

as in this case, the rate of collisions and the kinetics of adsorption are fundamental to determine the composition of the interface.

Furthermore, measurements of particle size distributions and surface load (the amount of protein adsorbed at the interface), and determination of the thickness of the adsorbed layers are necessary when characterizing the emulsifying properties of proteins. The amount of protein adsorbed at the interface, as well as the distribution of protein, if a mixture of protein is used, are also two aspects of great importance when characterizing emulsifying properties of food ingredients. Although exchanges at the interface and competitive displacement can be observed using drop tensiometry, as already mentioned above, composition studies are usually conducted by separating the colloidal particles from the continuous phase, and analyzing the two phases separately to determine the protein concentration and distribution in the two phases. Once the amount of protein adsorbed at the interface is estimated, by combining the information with the size of the droplets, it is possible to calculate a surface load as the amount of protein per interfacial area (Hunt and Dalgleish, 1994).

With light scattering, the measurement of the oil droplet size distribution is obtained by observing the intensity of the scattered light at different angles. In emulsion systems, light scattering is often employed to characterize the emulsions obtained with protein isolates by determining the particle size distribution, which allows for characterization and comparison of different protein samples, and prediction of their ability to stabilize emulsions under different conditions. Figure 5.2 illustrates the typical changes occurring to the average diameter and the particle size distribution as a function of protein concentration.

Upon homogenization, small oil droplets form, and proteins adsorb at the interface. If the amount of protein is not sufficient to cover the newly created surface, recoalescence or bridging of protein between the droplets will occur. With sufficient amount of protein, the average droplet size of the emulsion will reach a plateau, determined by the type of emulsification device used and the pressure applied. At this point, the distribution of the oil droplets is usually monomodal. This analytical approach can be used to determine the ability of proteins to form emulsion, and compare protein types or processing history.

In addition, the techniques available for characterization of emulsions cannot be well adapted to measure the foaming properties of food proteins. Besides the whipping methods which are empirical and less reproducible, image analysis for foam volume measurements combined with conductometry measurements of the liquid entrapped in the foam have been used in an automated bubbling apparatus to quantify foaming properties of various food proteins like egg albumen, β-casein, bovine serum albumin, ovotransferrin, and pea albumin (Baniel et al., 1997; Sarker et al., 1998; Lu et al., 2000).

Imaging techniques, light scattering, or ultrasonic devices are often employed to observe the changes occurring to the physical properties of the emulsions over time, and determine the stability of the emulsion droplets and foams to coalescence, creaming, and drainage. A number of reviews are available on the mechanisms involved in destabilization of foams and emulsions and the principles behind the techniques usually employed to follow the changes in physical properties (e.g., McClements, 2007). Ideally, measurements of these properties should be conducted without dilution, as properties are dependent on volume fraction. Techniques like diffusing wave spectroscopy (DWS)

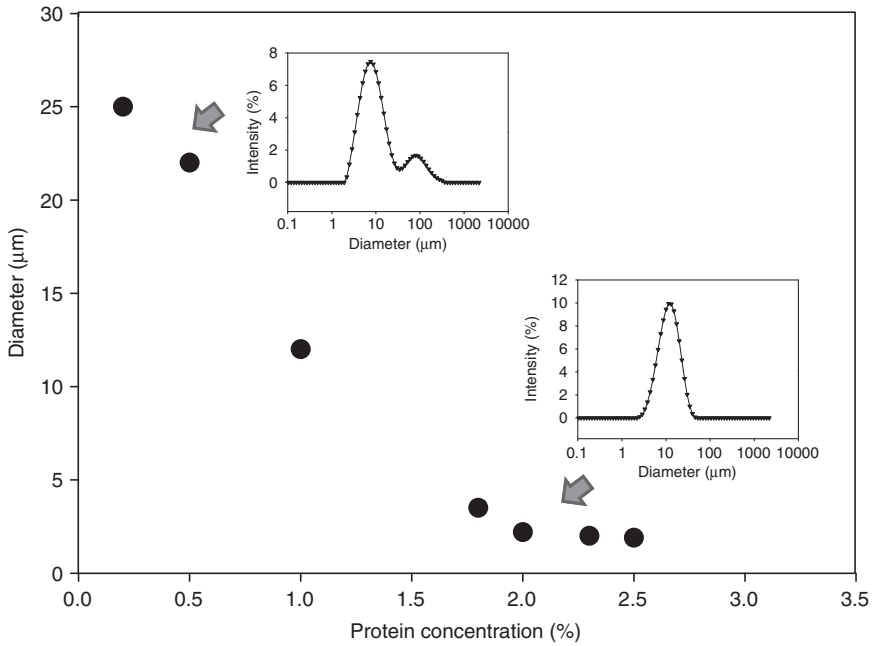

Figure 5.2 Example of the effect of protein concentration on average diameter of emulsion droplets. At low concentrations, protein is not sufficient to cover the newly formed surface, and coalescence or flocculation occurs. Once enough protein is present, a monomodal distribution of oil droplets can be measured using light scattering

and acoustic spectroscopy have been successfully used to characterize emulsion *in situ* under non-diluted conditions and in a non-invasive way (Corredig and Alexander, 2008; Gülseren and Corredig, 2011).

The charge distribution of the colloidal particles is often estimated using zeta potential measurements. The velocity of motion of charged droplets in an applied electric field depends on the size and charge of the emulsion droplets and can provide information on these characteristics of the droplets. For this purpose droplet movement is monitored with dynamic light scattering technique (McClements, 2005). Another technique that allows the measurement of electrophoretic mobility and zeta potential of the droplets is electroacoustics that combines ultrasonic and electric fields by measuring either the acoustic signal that results from particles that oscillate under an electric signal applied to the emulsion (electrosonic amplitude approach) or the electric signal of particles that oscillates under the acoustic signal applied to the emulsion (colloid vibration potential approach). The advantage of electroacoustics over dynamic light scattering is that in the former, the emulsion is studied in concentrated state, without any dilution, which in turn is a requirement for the DLS measurements. Electroacoustics has been successfully used to monitor changes in the stability of concentrated soy oil-in-water emulsions stabilized with sodium caseinate in the presence or not of non-adsorbing polymers (Gülseren and Corredig, 2011).

5.3 Proteins as structure formers leading to aggregation and network formation

The ability of food protein to undergo conformational changes, denature, and aggregate is of great relevance to the formation of food structures. Protein aggregates can form in solution, and the shape and size of the aggregates can be controlled by processing, environmental conditions or presence of other components interacting with the proteins. A large number of studies are available in this area, since the aggregation of proteins leads to the formation of the building blocks of texture and structure of food products. When adsorbed at the interface, proteins impart specific characteristics and functionality to the particles. The physical and chemical properties, and stability to environmental conditions or processing are determined by the protein present on the surface. For example, a sodium caseinate stabilized emulsion droplet will be destabilized close to the isoelectric point of the protein. In addition, particle interactions with polysaccharides will depend on the protein adsorbed at the interface (Liu et al., 2007).

Protein structures are versatile, and for a given protein, depending on the extent of the processing treatments, more than one type of structure could be present at the same time in a food matrix. For example, during processing of a globular protein with oil-in-water emulsion system, the same protein might exist in various forms, such as surface denatured, monomeric and aggregated in solution. Such complexity of the system makes it challenging to predict the behavior of the food matrix and it is further complicated by the presence of more than one protein, as well as other components (polysaccharides, small solutes). Hence, depending on the process and the environmental conditions, different molecules will play a different role in the formation of structure. In milk, for example, both caseins and whey proteins are present. However, depending on the heating applied, acidified milk will have a different structure, because of a different extent of participation of the whey proteins in the network. In unheated milk where whey proteins are not denatured, only caseins participate in the gel matrix, whereas when heat-induced complexes are present, the whey proteins act as a bridge between casein micelles strengthening the gel (Lucey et al., 1997).

In addition, multiple competing reactions may occur in protein matrices, causing countless pathways to the formation of aggregates and networks. Mixed gelation, for example, may occur by subjecting the same protein to acidification and enzymatic treatment at the same time (as in the case of some cheese matrices) or by causing multiple proteins to gel in sequence, controlling the order of the activation process.

5.3.1 Factors affecting protein aggregation

Depending on the concentration, ionic strength, pH, and the presence of other biopolymers, proteins may form supramolecular aggregates, may increase the viscosity, precipitate, or form networks. The changes in structure can be caused by enzymatic reactions, pH, and ionic strength changes, as well as heating or high-pressure treatments. The denaturation and assembly of the proteins need to be controlled, and the mechanisms of these

processes need to be well understood, considering that gelation and aggregation might be desirable or undesirable processes for the final product.

The driving forces leading to protein aggregation have been recently reviewed (Mezzenga and Fischer, 2013). The linkages formed can be covalent in nature, as in the case of disulfide bridges between cysteine-containing proteins after heating, or by using crosslinking enzymes such as transglutaminase. Other interactions can be physical in nature, mostly driven by hydrogen bonding or hydrophobic forces. For example, whey proteins undergo structural changes at temperatures >70°C and can form soluble aggregates and gel networks depending on the ionic strength, pH, and concentration. At neutral pH, the aggregation is driven by hydrophobic interactions as well as disulfide bridging. On the other hand, although heating is a prerequisite for soy protein gelation as the proteins need to dissociate and unfold, the network is strengthened with cooling, because of the formation of hydrogen bonds (Renkema and van Vliet, 2002). Gelatin, a derivate of collagen, also forms physical, thermoreversible gels. The protein is soluble at temperatures >40°C, and when the solution is cooled, a coil to helix transition occurs at above a critical concentration of protein and a three-dimensional network forms driven by hydrogen bonds between chains (Haug and Draget, 2011). In the recent years, crosslinking of proteins with agents like enzyme transglutaminase has been explored as means to improve their functionality. Open structure proteins like caseins are good candidates for transglutaminase crosslinking since they allow easy access of the enzyme in the reaction sites while globular proteins in their native form are poor substrates for this purpose (Buchert et al., 2010). Transglutaminase crosslinking transforms caseins in nanogel particles that are stable when treated with 6 M urea or when calcium is removed with chelators (de Kruif et al., 2012). Gluten is another very important protein in food with unique functional properties that plays a major role in dough formation. Stretching and kneading of dough leads to the development of high molecular weight glutenin aggregates through covalent disulfide bridging while gliadins, the other protein component of gluten, interact with each other and glutenin fraction through hydrogen bonds and hydrophobic interactions. The result is a rubbery viscoelastic protein network that forms the basis of wheat flour products (Delcour et al., 2012).

Structural changes leading to denaturation are often a prerequisite for protein aggregation. The amino acid sequence in the protein is organized in such a way that the conformation allows for a minimum free energy. The conformation of globular proteins is a great example of a secondary structure well packed in a globular form, hiding all the hydrophobic amino acids within the structure to minimize their exposure to water, and allowing for a balance of hydrogen bonds and attractive and repulsive van der Waals interactions. Covalent bonds between cysteine residues may also contribute in structure stabilization, making the protein more resistant to denaturation. Heating treatments modify protein structure, and expose previously buried moieties, altering the balance between attractive and repulsive interactions. For example, in the case of β-lactoglobulin, heating exposes hydrophobic residues initially present in the core of the protein structure. These changes in conformation lead to a new equilibrium whereby protein–protein interactions occur to minimize entropically unfavorable interactions, such as the contact of the hydrophobic residues with the water. Once unfolded, protein–protein interactions lead to the formation

of aggregates, clusters, and three-dimensional networks. Aggregation is initiated by the formation of clusters, and proceeds until a tridimensional network is formed, under favorable environmental conditions. The balance between attractive and repulsive forces, as well as the concentration of protein present in the original solution will affect the structure and size of the aggregates formed. Hence, the type of clusters formed and the final gel structure will depend strongly on the pH, ionic strength, and protein concentration. For example, in the case of β-lactoglobulin gels, heating at different pH values will result in fibrillar, spherical, or worm – like aggregates (Jung et al., 2008; Pouzot et al., 2004; Alting et al., 2004).

Protein-containing cysteine groups contribute covalent bonds in the gelation process, strengthening the aggregates and the gel network. Disulfide bridging is thermally activated and promoted by protein unfolding and the subsequence exposure of the cysteine residues which were protected by the folded tertiary structure prior to thermal unfolding. Protein unfolding is also important to increase access to enzymatic crosslinking, as in the case of transglutaminase-induced gels. As mentioned above, milk proteins with flexible structure, like caseins, are more susceptible to transglutaminase crosslinking, compared to the whey proteins, which need to undergo some degree of denaturation to allow for easier access of the enzyme to the protein structure (Sharma et al., 2001).

In addition to heat, changes in solvent quality, high pressure, or interfacial adsorption can also cause a modification of the protein from the folded tertiary structure to a more exposed structure prone to protein–protein interactions. Following protein structural changes cold gelation can occur by modifications of environmental conditions of the soluble phase. In some cases, heating forms supramolecular colloidal structures stable in a certain environment, but prone to destabilization with changes in pH or salt (Alting et al., 2004; Maltais et al., 2008). Thus, supramolecular structures of protein are first formed and aggregation is then induced by the addition of salt or change in pH (Remondetto et al., 2002). This is the case of cold gelation of whey proteins, which, under specific conditions of temperature, pH, and ionic strength, can gel by addition of cations at cold temperatures. Divalent cations are effective at concentrations lower than the monovalent cations since they can screen more effectively the charges and can form salt bridges (Bryant and McClements, 1998).

Acidification of a protein suspension can also induce aggregation, precipitation, or gelation. In this case, pH causes an overall change in the charge density of the proteins causing a shift in the balance between attractive and repulsive forces. This type of gelation is of great importance in the creation of food texture, and is often used to destabilize colloidal protein particles, such as soy milk particles, casein micelles, or heat-induced whey protein aggregates (Alting et al., 2004; Morand et al., 2011).

Some of the mechanisms of destabilization of proteins present in foods during processing are pH adjustment or acidification, enzymatic treatments, and addition of salts. As an illustration, soy milk gels can be obtained by the addition of ions such as Ca^{2+} and Mg^{2+}, or by acidification with glucono delta-lactone. As mentioned before, heating is an important step for ensuring gelation of soy milk protein particles. Gelation of soy milk occurs at a pH of about 5.8, and the stiffness of the gel matrix depends on the volume fraction of the soy protein particles. Recently, it has been shown that in this multi-protein system,

glycinin proteins destabilize first, followed by the β-conglycinin subunits (Ringgenberg et al., 2013). At refrigeration temperatures, the gels are stiffer and gelation seems to occur at an earlier pH than gels prepared at 30°C, suggesting that hydrogen bridging plays an important role in the network formation (Ringgenberg et al., 2013).

Similar to protein particles, protein-covered emulsion droplets can also be destabilized using heat or by changes in ionic strength and pH. As for the proteins, cold gelation can be induced in emulsions, where the droplet surface is stabilized by proteins (whey, soy proteins) (Rosa et al., 2006; Li et al., 2011). The amount of salt added, the heating temperature, the volume fraction of the oil droplets, and the particle size are all important factors in determining the final properties of the gel. In binary systems consisting of emulsion droplets behaving as filler particles, and dispersed proteins forming a gel network, the filler particles play a major role in imparting the final textural and sensory properties to the gel (Mosca et al., 2012). The colloidal properties of the oil droplets and their interfacial composition affect the rheological properties of the final gel, depending on their affinity to participate in the gel network (Sala et al., 2007).

In addition to protein gels, an area of great interest in foods is the formation of fibrils from hydrolyzed proteins. A number of proteins have been shown to form fibrils by acid catalyzed hydrolysis. Peptides interact via β-sheets associations (driven by hydrogen bonding and enthalpy gain associated with the association of the β-sheets) and stack perpendicular to the fibril axis forming fibril structures with potential functions, as they have shown to enhance viscosity and form gels at low protein concentrations (Loveday et al., 2009). Differences in the structure of the fibrils occur depending on the concentration, pH, ionic strength, and degree of hydrolysis, and have been reported for β-lactoglobulin, α-lactalbumin, lysozyme, ovalbumin, and soy proteins (Lara et al., 2011; Akkermans et al., 2007; Loveday et al., 2009).

5.3.2 Protein structures caused by interactions with polysaccharides

Proteins behave as biopolymers and may interact with other biopolymers in the solution. For example, gelatin interacts with polysaccharides forming cold setting structures with properties that depend on the processing conditions and the properties of the polysaccharide present (Lorén and Hermansson, 2000). The ability of the protein to interact with polysaccharides should be considered as an important functional property of the protein, which is affected by the structure and size of the protein and its aggregates. The structuring properties of the proteins in the presence of other biopolymers will also depend on the response of the system to changes in environmental conditions, pH, ionic strength, and concentration of the solutes (Corredig et al., 2011).

The molecular and supramolecular structure of the protein play an important role in the interactions with polysaccharides, and different structures form depending on polymer ratios and concentrations. The interactions can be segregative or associative in nature. Phase separation occurs because of the thermodynamic incompatibility of proteins and polysaccharide molecules, when the interactions between the protein and the solvent

are favored compared to polysaccharide–protein interactions (Grinberg and Tolstoguzov, 1997). These phase-separated systems can be considered as water in water emulsions where the proteins form micro or macroscopic phase-separated regions, with polysaccharide droplets surrounded by protein solutions, droplets of protein surrounded by polysaccharide or bicontinuous structures, depending on the relative concentration of the biopolymers.

Phase separation in systems where the biopolymers cause density gradients will result in bulk phase separation, showing two discrete macroscopic phases; however, different structures may form when phase separation also is combined with gelation, and reaction kinetics are well controlled. The incompatibility between the protein and the polysaccharide can be depicted in a phase diagram, whereby the binodal curve shows the concentration of protein and polysaccharide present in the upper and lower phase after separation. Figure 5.3 depicts a typical phase separation diagram, and some of the microstructures that can form with mixing. These diagrams are specific to the protein and polysaccharides present in the mixture, as well as the ionic conditions or the environmental conditions. Mixtures below the binodal curve will not phase separate while mixtures above the binodal curve will show macroscopic phase separation. However, even at low concentrations, the microscopic phase separation occurs causing important rheological and textural changes to the food matrix. This is especially the case in gelling systems with controlled kinetics (Lorén and Hermansson, 2000; Acero-Lopez et al., 2009).

The interactions can result in complex coacervates of proteins with polysaccharides, which can also be used as encapsulating agents (Aberkane et al., 2012; Zimet and Livney, 2009). The forces involved in the formation of the complexes can be entropic or

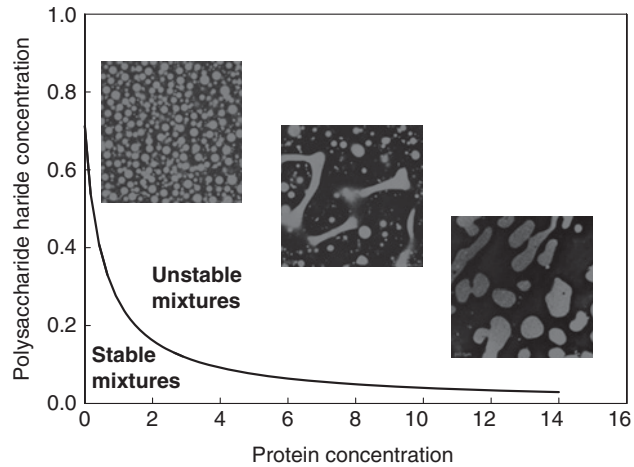

Figure 5.3 Example of phase separation diagram with the bimodal curve separating conditions where the mixtures will show macroscopic destabilization. Confocal images are also provided as an example of microstructures that could form during mixing. At low concentrations of protein, dispersed phase is composed of protein, while at high concentrations the dispersed phase is composed of polysaccharide. A bicontinuous system forms in the intermediate concentrations

electrostatic in nature (Turgeon et al., 2007). The extent of interactions is dependent on environmental conditions such as pH and ionic strength, as well as on the structural features of the molecules (stiffness, charge density, intrinsic viscosity).

5.3.3 Experimental approaches in the study of structure formation

In the study of structure formation, the structure has to be characterized at various length scales from the micrometer to the nanometer scale. Various means are available to characterize the final matrix, mostly adapted from material science approaches (Foegeding et al., 2011). The structure formed in the food matrix is the foundation to the textural and sensorial properties of the food, and although many techniques are available to characterize the food matrix, they may not be able to give any information on the pathways that led to the final structure and texture of the product, or to identify differences in the building blocks that have formed the structure. Microscopic observations are a valuable tool to evaluate the structural features of the clusters and the type of gel matrices formed. As an example, electron microscopy has been used successfully to identify the structure of particulate and fine stranded gels (Langton and Hermansson, 1992). Electron microscopy techniques can clearly describe the structural features in the gel network or aggregate structures; however, they require considerable sample preparation which increases the chances of introducing artifacts in the sample. Confocal microscopy is also often employed to characterize the microstructure of protein-based gels. In this case, less sample preparation is necessary, but fluorescent labels are employed to identify the signal coming from the protein strands, or the distribution of the various components in a phase-separated mixture.

An important method to quantify the extent of the linkages or aggregation in protein systems is rheology. The resistance to the flow (viscosity) or changes in the elastic and viscous modulus during small deformation experiments can identify differences in the rearrangement of the gels or the extent of bridges between the proteins. Although very detailed information can be derived on the viscoelastic properties of the protein network once aggregation has occurred, or on the precise point of gelation, rheology does not provide information on the beginning stages of aggregation, the porosity of the gel, structural heterogeneities, and molecular rearrangements.

The prerequisite to aggregation is the change in the structure of the protein from native to a conformation that exposes reactive sites with dimerization, polymerization, or the creation of a number of new linkages, eventually resulting in a three-dimensional network. Hence, to look at the initial stages of gelation, aggregates need to be analyzed using preparative methods such as chromatography or electrophoresis, to obtain information on the size and composition of the aggregates. The formation of the gel and the clusters characterization are difficult to study, especially in systems that are concentration dependent, and the environmental conditions play a major role in the assembly of the proteins.

In the study of structure formation, scattering techniques like small angle X-ray scattering (SAXS) and small angle neutron scattering (SANS) have been used to look at the building blocks of the protein gel formed (Lopez-Rubio and Gilbert, 2009; Alting et al.,

2002, 2004). Light scattering is an established technique for investigating protein–protein interactions. For example, it has been used to determine the molecular details of the aggregation of whey proteins at neutral pH and low ionic strength. Below a critical concentration, the proteins aggregate in isolated clusters and the solution does not gel; in this case light scattering can be used to measure the size and dimensions of the clusters (Pouzot et al., 2004). Traditional light scattering techniques can be successfully applied to study protein aggregation, but only in very low scattering regimes using highly diluted systems. This becomes a shortcoming during the study of phase separating or aggregating systems, which are dependent on concentration. Cross correlation light scattering techniques such as backscattering or transmission DWS, need to be used in the case of opaque systems. *In situ* systems are particularly interesting because concentration and volume fraction are the limiting factors (e.g., in gelation and phase separation). Techniques such as DWS have shown great promise in the study of structure formation in food matrices (Corredig and Alexander, 2008).

Molecular diffusion can also be employed to characterize the protein gel matrices and especially the microrheological properties of the network, using multiple particle tracking, fluorescence recovery after photobleaching or pulsed field NMR diffusometry (Lorén et al., 2009; Tzoumaki et al., 2011; Salami et al., 2013). These techniques show great potential to advance our understanding of protein network formation and the ability of the proteins to form structural heterogeneities and aqueous phase voids.

5.4 Binding properties of food proteins

A functional property of increasing interest in foods is the ability of proteins to bind to hydrophobic molecules. A number of studies have recently become available on the use of proteins and protein aggregates as carriers of molecules of interest such as flavors, vitamins, fatty acids, and polyphenols (Livney, 2010). The ability of a protein to assemble either spontaneously or to form aggregates during processing represents a great area of development for the use of proteins as nano- or microscopic-size delivery systems. In addition, the structures are tunable with processing, and the encapsulated structures have been shown to protect labile compounds (such as flavors, vitamins, drugs, polyphenols), and deliver bioactive molecules in particular areas of the gastrointestinal tract. The interest in the use of food proteins as matrices for the delivery of bioactive molecules through diet also derives from the fact that most proteins play a major role not only in structural scaffolding, but also in nutrition, as within their primary structure they encode sequences of amino acids with bioactive properties.

Proteins associate with hydrophobic and hydrophilic molecules via non covalent interactions, and the association can protect flavor molecules from oxidation (Zimet and Livney, 2009). Proteins may interact with hydroxyl and carboxyl groups of ligand molecules through hydrogen bridging and electrostatic interactions. Denatured proteins may have exposed hydrophobic regions and more binding sites for interactions. Beta-lactoglobulin has been used often as a model molecule to study binding of protein with physiologically relevant molecules. Often the binding between proteins and biological

molecules, such as folic acid, resveratrol, polyphenols, retinol are quantified using fluorescence techniques, although care has to be taken in using very diluted systems (Liang and Subirade, 2010; Augustin et al., 2011). It has been shown that folic acid binds to the hydrophobic pocket of β-lactoglobulin, in a groove between the α-helix and the β-barrel, and that the photostability of folic acid to ultraviolet (UV) radiation is improved when in complex with β-lactoglobulin (Liang and Subirade, 2010). Retinol binds in the calyx cavity of β-lactoglobulin (Cho et al., 1994), while in the case of resveratrol, a less hydrophobic molecule, the association seems to occur mostly on the surface of β-lactoglobulin (Liang et al., 2008). This protein has also been shown to interact with tea catechins, and the binding causes an increase in β-sheet and α-helix (Kanakis et al., 2011). The binding of β-lactoglobulin with hydrophobic molecules seems to stabilize the structure and cause a shift in the heat denaturation temperature of the protein (Considine et al., 2007).

Proteins are also known to form complexes with polyphenols, which have been studied for many years, as they have profound consequences on cloud stability in juices, the destabilization or clarification of wine, and sensory astringence sensations. Recently this functionality has become of interest as the complexes formed may help stabilize polyphenols in nutraceutical beverages, help overcome the limitations related to the low solubility and help deliver these molecules to the gastrointestinal tract.

Catechins readily interact with proteins rich in proline that have an open and flexible structure (Papadopoulou and Frazier, 2004; Maiti et al., 2006). In addition, binding is clearly affected by protein characteristics including protein structure and amino acid composition, as well as polyphenol structure (e.g., glycosylated or not) and molecular size (Poncet-Legrand et al., 2006; Richard et al., 2006; Frazier et al., 2010).

The presence of polyphenols causes an unpleasant astringency sensation in the mouth (Charlton et al., 2002; Lesschaeve and Noble, 2005). Caseins resemble the salivary proline-rich proteins in that they have a high number of proline residues and relatively open structure, and have been used to study polyphenol–protein interactions (Jöbstl et al., 2004; Schwarz and Hofmann, 2008; Yan et al., 2009). The binding of tea catechins to β-caseins decreases the sensation of astringency caused by tea polyphenols (Lesschaeve and Noble, 2005; Schwarz and Hofmann, 2008). Besides monomeric caseins (especially β-casein), known avid binders of polyphenols, milk proteins in general, including whey proteins, have been shown to bind to tea polyphenols, resveratrol, and curcumin (a hydrophobic molecule) (Shpigelman et al., 2010; Sahu et al., 2008; Haratifar and Corredig, 2014; Rahimi-Yazdi and Corredig, 2012).

Only recently studies have been focused on the effect of protein binding on bioefficacy of bioactives (Liang et al., 2008; Remondetto et al., 2004). As the complex formation between bioactives and proteins becomes increasingly applied in the food industry as a means to stabilize labile molecules, more research will be needed to evaluate if the binding will affect the processing functionality of the proteins, such as their ability to stabilize interfaces or form gels upon heating or enzymatic treatment. As previously mentioned, the complex formation may cause a shift in the denaturation temperature (Considine et al., 2007; O'Connell et al., 1998) or cause long term gelation of whey proteins (Yuno-Ohta and Corredig, 2007, 2011). In addition, it has been recently shown that the binding of tea

catechins to casein micelles inhibits the rennet-induced gelation of casein micelles (Haratifar and Corredig, 2014).

Aggregated protein structures at the nano and microscale have shown great potential for encapsulation of bioactives. Recently there has been significant interest in the study of protein nanoparticles, as their small size and low scattering allow for their use in transparent beverages (Zimet and Livney, 2009). In addition, their high surface area causes high efficiency of encapsulation and absorption. Various proteins have been used as encapsulating agents for bioactive molecules including gelatin, egg albumin, whey proteins, and caseins (Digenis et al., 1994; Torrado and Torrado, 1996; Beaulieu et al., 2002). Cold-set gelation of whey proteins has been successfully employed to obtain whey protein particles for encapsulation of retinol (Beaulieu et al., 2002). Methods to prepare protein micro and nanoparticles include emulsification and solvent extraction, acid or enzyme treatments, desolvation, salting out or protein–polysaccharide coacervation (Chen and Subirade, 2005; Ko and Gunasekaran, 2006; Aberkane et al., 2012; Zimet and Livney, 2009). The size of the particles depends on the balance between the attractive and repulsive forces that determines the level of molecular aggregation. β-lactoglobulin nanoparticles can be obtained by desolvation at alkaline pH, where the charge distribution of the protein ensures charge repulsion between the molecules (Ko and Gunasekaran, 2006; Gülseren et al., 2012a). Desolvated nanoparticles prepared with whey protein isolates, after preparation can be stabilized in acidic solutions and used as carriers in acidic beverages (Gülseren et al., 2012a, 2012b).

Another technique that has recently gained attention for production of thin protein fibrils is electrospinning. During the spinning process high voltage is applied to polymeric solutions, conditions that make the polymers align to form fibrils. Most proteins cannot be spun under food grade conditions, as the polymers need to be highly soluble, at high enough concentrations that the protein chains can entangle, and under the appropriate conditions of conductivity, viscosity, and surface tension for the proteins to form fibrils. Zein and gelatin have been shown to have great potential to be used for electrospinning. Zein, the storage protein from maize, is unstructured and readily soluble in ethanol. These characteristics make it suitable for electrospinning since the solvent is evaporated during the process and the fibers can be collected (Kanjanapongkul et al., 2010; Zhang et al., 2009; Zhang et al., 2005). Gelatin can also be successfully spun, as reported (Zhang et al., 2009). Recently nanofibers were obtained from zein–soy protein isolate blends using ethanol as a solvent and formic or acetic acid. The type of acid is known to affect the microstructure of the fibers (Phiriyawirut et al., 2008). These fibers show the potential to deliver bioactive components (Wang et al., 2013).

5.5 Conclusions and outlook

Proteins are widely employed as ingredients in foods for their nutritional value as well as for their technological function. They indeed play a major role in the formation of the building blocks of the structure of food matrices. Proteins in food undergo changes during processing operations such as heating, acidification, or emulsification that result in functional colloidal building blocks ultimately leading to the final food structure (Foegeding

and Davis, 2011). The structural differences between proteins allow for a wide range of supramolecular structures to form. The growth of the building blocks needs to be controlled by changing environmental and processing conditions; this will ultimately result in a multitude of structural solutions. In addition, proteins can be found adsorbed at the interface, an important aspect in foods, whereby more than one phase—air bubbles, oil droplets, or liquid–liquid emulsions—is present. In summary, the ability of proteins to form a variety of colloidal structures, interact with other biopolymers, stabilize interfaces, and form gel networks, will bring endless opportunities to their use, and only an evidence-based approach will lead to the development of more sophisticated structures in food products aimed at better digestion and bioefficacy of nutrients.

In the past decades we have experienced significant progress in understanding the structure–function relationship of pure proteins and simple model systems. These studies have led to an increased capacity to predict processing behavior and functionality of protein-based ingredients. Nonetheless, the proteins used in food products are usually isolates or concentrates, and their processing history and how it impacts their functional behavior needs to be fully understood. In addition, studies conducted so far have mostly been carried out in model, diluted systems, and only recently novel experimental approaches have allowed studying more complex systems. It is now important to bridge the gap between the fundamental principles and ingredients transformation. Studies utilizing ingredients at concentration and compositions used in manufacturing processes and preparation techniques that simulate the industrial ones are then necessary. Furthermore, it is very important to develop better methods to evaluate novel functionality aspects, such as binding of bioactive molecules. Using proteins as carriers or protein structures as matrices for delivery of additional health benefits will become increasingly important in food development; however, the overall processing behavior of the proteins and the newly formed structures as well as their implication on bioefficacy need to be better understood.

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6

Biologically Active Peptides from Foods

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6.1 Introduction

The human body is continuously facing physiological imbalances and other aberrations, such as exposure to extrinsic toxic substances, which eventually cause or develop diseases (Udenigwe and Aluko, 2011). Proteins function as macronutrients in foods and as a source of energy and amino acids, which are necessary for normal growth and maintenance of the body. Recently, protein hydrolysates, especially bioactive peptides, have been found to exhibit a variety of biological activities in addition to their established nutritional value. A protein is made up of polypeptide subunits while peptides are short copolymers of amino acids linked by peptide bonds. Generally, bioactive peptides contain 2–20 amino acid units, but exceptions also exist like lunasin, a 43 amino acids peptide from soybean, which is well known as an anticarcinogen having a molecular weight (MW) of 5400 Da (Pihlanto-Leppälä, 2000; Jeong et al., 2002).

6.2 Production of bioactive peptides

Bioactive peptides may be liberated in the small intestine due to the action of several proteases such as pepsin, trypsin, and/or chymotrypsin. It has been suggested that protein hydrolysis is necessary for peptides to render bioactivity, because bioactive peptides are generally inactive within their precursor proteins. The processing approaches that are commonly used for production of bioactive peptides include digestive proteolysis *in vivo*, chemical or enzymatic hydrolysis *in vitro*, bacterial fermentation, gene expression, and chemical synthesis (Shahidi and Zhong, 2008).

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During *in vivo* proteolysis, most of the peptides produced exhibit their physiological activities after being absorbed from the gastrointestinal (GI) tract and transported to different sites like the peripheral blood and cellular receptor sites, while some of the peptides can function without being absorbed in the GI or transported through GI. The drawback of digestive proteolysis is that it may not afford the same peptides each time, because different proteolysis patterns lead to different peptide compositions. This disadvantage can lead to altered bioactivity or loss of efficacy. As such, source proteins hydrolyzed *in vitro* may offer better and more predictable performance as well as convenience of operation.

Bioactive peptides are generally produced by *in vitro* enzymatic hydrolysis based on physiological properties of products. In order to isolate protein-rich fractions, operations such as cleaning, milling, sieving, extrusion, and even autolysis under controlled pH and temperature of the seeds, grains or fruits are needed. The optimization of the extraction procedure is usually performed on the basis of the angiotensin-converting enzyme I (ACEI) activity results. After a protein source is selected, it may be subjected to a single or multiple specific or nonspecific proteases in order to release the peptides of interest.

In enzymatic proteolysis, production of bioactive peptides may be affected by the type of enzyme, duration of hydrolysis process, degree of hydrolysis, and pretreatment of the protein before hydrolysis, among others. Different types of proteinases (endo- and exoproteinases) such as trypsin, chymotrypsin, thermolysin, pepsin, carboxypeptidases as well as commercial proteases, such as Alcalase and Flavourzyme (Novozyme, Bagsvaerd, Denmark) are the main enzymes used for peptide production.

After protein hydrolysis, a problem that might be encountered is the low purity and low yield of peptides of interest. Generally, further processing of the primary enzymatic food protein hydrolysates aids to select and purify the peptides of interest in order to obtain better quality peptides. Appropriate post-hydrolysis selection method is selected based on the physicochemical and structural properties of the constituent peptides to enhance their bioactivities.

According to their targeted pharmacological uses, the properties of peptides considered include size, hydrophobicity, and net charge. To accumulate peptides of defined MW range, membrane ultrafiltration and size-exclusion chromatography may be employed, especially for low-molecular-weight fractions resisting further *in vivo* proteolytic digestion. Moreover, based on their hydrophobic properties, peptides can be separated by reverse-phase high performance liquid chromatography (HPLC) on a hydrophobic column matrix, aiding in the study of peptide structures with functionalities (Pownall et al., 2010). In addition, chromatography with selective ion-exchange columns can acquire peptide fractions with particular net charges; this processing mean is valuable and beneficial when the disease targets are inactivated by molecules with strong net positive or negative charges (Li and Aluko, 2005; Pownall et al., 2011). Adsorption chromatography is used to develop specific amino acids of interest in peptides by decreasing the content of aromatic amino acids to remove the bitterness (Clemente, 2000). For example, a peptide with less aromatic amino acids can be obtained after protein hydrolysis by passing the hydrolysates through a column packed with activated carbon or simply by mixing with activated carbon (Udenigwe and Aluko, 2010; Humiski and Aluko, 2007). Activated carbon is broadly

used to reduce the bitterness of the resultant peptides; bitterness is a main drawback for widespread application of protein hydrolysates. For instance, hydrophobic character is important for ACE inhibitory peptides, which is commonly displayed by the N-terminal of the peptide (Rho et al., 2009; Wijesekara and Kim, 2010). This is because hydrophilic peptides are incompatible with the active sites of ACE leading to a weak or no ACE inhibitory activity. Therefore, removal of hydrophobic amino acids or peptides could affect the inhibition ability of ACE, because hydrophobic peptides possess better affinity for the active sub-sites of ACE (Maruyama et al., 1987; Li et al., 2004; Matsui et al., 2006).

A novel membrane technology, known as electrodialysis-ultrafiltration (EDUF), has displayed high efficiency in selectively separating and accumulating low-molecular-weight bioactive peptides with a net charge. EDUF can separate cationic, anionic, and neutral peptides from each other under defined molecular sizes (Firdaous et al., 2009)

6.3 Bioactive peptides in health and disease

Bioactive peptides are known to render different biological activities, hence affecting human health. Benefits of bioactive peptides, including cardiovascular, GI, immune, and nervous systems are summarized in Table 6.1.

6.3.1 Antihypertensive peptides

Hypertension is a leading preventable risk factor affecting cardiovascular health. Blood pressure is regulated by several biochemical systems including the renin-angiotensin system (RAS), the major system involved in blood pressure responses, the renin-chymase system (RCS), the kinin-nitric oxide system (KNOS), and the neutral endopeptidase system (NEPS) (Beldent et al., 1993; Schror, 1992; Husain, 1993; Weber, 2001).

To date, antihypertensive peptides are the most extensively researched bioactive peptides from exogenous sources like food. Antihypertensive peptides have been effective in preventing/treating hypertension mainly by inhibiting the ACE and/or renin. (Suetsuna et al., 2004) ACE can convert angiotensin I (AT-I) to angiotensin II (AT-II), while AT-II is a potent vasoconstrictor; ACE can also inactivate bradykinin, a vasodilator. Renin behaves in the first stage of angiotensinogen conversion to AT-I. The RAS is a main regulator related to blood pressure control in the body. Angiotensinogen is the starter of RAS, the only known precursor of AT-I, and it is also known as the only substrate for renin (Maruyama et al., 1987). Renin is obtained from the inactive precursor pro-renin by

Table 6.1 Physiological functionalities of bioactive peptides

Human system	Functions of bioactive peptides		
Cardiovascular	Antihypertensive	Antioxidant	Hypocholesterolemic
Gastrointestinal	Mineral binding	Antimicrobial	Opioid
Immune	Immunomodulatory	Antimicrobial	Opioid
Nerves	Opioid		

kallikrein (Ondetti and Cushman, 1982) and is usually derived from granular cells on the kidney, submaxillary gland, and amniotic fluid (Fitzgerald et al., 2004).

The RCS is similar to RAS, containing all the components of RAS. Compared to RAS, RCS uses chymase to convert AT-I into AT-II instead of ACE (Vermeirssen et al., 2004). Chymase-dependent AT-II formation mostly appears in the left ventricle of the human heart, while ACE-dependent AT-II formation governs the other chambers of the heart (Urata et al., 1996). Therefore, blood pressure control systems contain a series of various reactions and interactions with several metabolic pathways.

6.3.1.1 Characterization of angiotensin-converting enzyme inhibitory peptides, renin inhibitory peptides, and their mechanisms These ACE-inhibitory peptides may share common structural properties such as the hydrophobic amino acid residues and positive charge terminal of peptides. ACE prefers binding to substrates or competitive inhibitors containing tripeptides with hydrophobic amino acids in the C-terminal (Vermeirssen et al., 2004). Detailed review of the molecular mechanism of ACE inhibitory peptides has recently been provided by Pan et al. (2012).

ACE degrades BK and generates AT-II, leading to dual effects such as the prevention of vasodilation and the activation of vasoconstriction. As such, inhibitors of this enzyme can be used as antihypertensive agents. The mechanism of ACE inhibitory peptides derived from food proteins has been identified as competitive inhibition (Maruyama et al., 1987). This inhibition is characterized by competition of the peptides with ACE substrate for the catalytic sites of enzymes. Moreover, some peptides also exhibit noncompetitive (for example, leucine-tryptophan (LW) and isoleucine-tyrosine (IY) and uncompetitive (e.g., IW and FY) modes of inhibition (Sato et al., 2002), where the peptides bind to other sites on the enzyme resulting in changes in enzyme conformation and decreased activity. A detailed action mechanism of the ACE inhibitory peptides is illustrated by FitzGerald et al. (2004).

Food-derived peptides have also been shown to inhibit the activity of renin thus providing a better blood pressure (BP)-lowering properties than by only inhibiting ACE activity, since it prevents the synthesis of AT-I, which can be converted to AT-II in some tissues via an ACE-independent alternative chymase-catalyzed pathway (FitzGerald and Meisel, 2000).

The presence of an N-terminal small hydrophobic amino acid (e.g., isoleucine (I), leucine (L), alanine (A), and valine (V), and a C-terminal bulky amino acid (e.g., tryptophan (W), phenylalanine (F), and tyrosine (Y)) is necessary to inhibit human renin (Udenigwe and Aluko, 2011). Even though no correlation existed between ACE- and renin-inhibitory activities of the dipeptides, the features of dipeptide structural requirements were similar to each other.

6.3.2 Food-derived sources of antihypertensive peptides

6.3.2.1 Dairy protein sources of antihypertensive peptides Bioactive peptides with ACE-inhibitory activity are produced during enzymatic hydrolysis of milk proteins by various proteases such as pepsin, pancreatin, Alcalase, and Flavourzyme, during microbial

Table 6.2 Examples of antihypertensive peptides from milk proteins produced by using different enzymes

Enzyme hydrolysis treatment	Peptide sequence	Reference
Trypsin	MAIPPKK	Contreras et al., 2009
Trypsin	AVPYPQR	Martinez-Maqueda et al., 2012
Pepsin	AYFYPEL	Martinez-Maqueda et al., 2012
Pepsin	YQKFPQY	Martinez-Maqueda et al., 2012
Pepsin	PYVRYL	Martinez-Maqueda et al., 2012
Pepsin	RYLGY	Miguel et al., 2007
Pepsin	WQ	Martinez-Maqueda et al., 2012
Gastric and pancreatic enzymes	YGLF	Martinez-Maqueda et al., 2012
Pepsin, chymotrypsin, and trypsin	IAK	Martinez-Maqueda et al., 2012
Proteinase K	VYP	Martinez-Maqueda et al., 2012
Thermolysin	LQKW	Martinez-Maqueda et al., 2012
Thermolysin	LLF	Martinez-Maqueda et al., 2012
AS1.398 neutral protease	DERF; RYPSYG	Martinez-Maqueda et al., 2012
Prozyme 6 and mixture of lactic acid	GVW or GTW	Martinez-Maqueda et al., 2012

fermentation of milk, or in some cases by combined use of enzymes and microorganisms. The type of enzymes used and species of bacteria involved during peptide production greatly influence the cleavage pattern of peptides from the protein, which determines the inhibitory activity of the resultant peptides against ACE. For example, milk fermented with *Lactobacillus helveticus* bacteria had an increased level of proline-containing short-chain peptides, such as isoleucine-proline-proline (IPP) and valine-proline-proline (VPP) showing greater antihypertensive activity than those fermented with other lactic acid bacteria species (Mizushima et al., 2004; Martinez-Maqueda et al., 2012). ACE-inhibitory peptides have also been isolated from other dairy products such as yogurt and cheese. Examples of antihypertensive peptides from milk proteins obtained by different hydrolysis are summarized in Table 6.2.

6.3.2.2 Animal protein sources of antihypertensive peptides Meat and marine animal sources of antihypertensive peptides have mostly been studied in the past; especially from by-products or processing discards of meat and seafood. This utilization of by-products has become a trend that may provide substantial environmental and cost benefits (Wijesekara and Kim, 2010). Collagen and gelatin, as popular products, have been recently extracted from marine sources by an optimized method (Gomez-Guillen et al., 2011). Collagen and gelatin are generally produced from porcine skin and bovine hide in addition to bones, tendons, and cartilages. Moreover, interest in evaluating by-products from non-mammalian species, such as poultry or fish, has been increasing in recent years (Gomez-Guillen et al., 2011; Harnedy and FitzGerald, 2012). A more detailed review about fish gelatin is beyond the scope of this contribution and can be found in the recent literature (Li-Chan et al., 2011).

Pepsin or orientase is commonly used to produce antihypertensive peptides from animal sources (Ko et al., 2006; Katayama et al., 2008; Nakade et al., 2008; Lee et al., 2010). For examples, sea cucumbers have been subjected to consecutive treatments with

different enzymes like bromelain and Alcalase (Zhao et al., 2007, 2009), while Protamex and Flavourzyme are used as treatments in freshwater clams (Tsai et al., 2006). In addition, a thermolysin hydrolysate of dried bonito, a traditional Japanese food, had special relevance among antihypertensive food-derived products (Nakade et al., 2008). Based on *in vivo* studies in spontaneously hypertensive rat (SHR) and clinical studies in borderline and mildly hypertensive subjects, a US patent has been issued and the technology has been implemented in production of a commercial product named Vasotensin® in the United States, and PeptACE™ and Levenorm™ in Canada, that ensures healthy blood pressure levels by supplying bioactive peptides from bonito fish (Thorkelsson et al., 2009).

Among animal protein sources, egg is a leading source of bioactive peptides, especially in antihypertensive peptides (Miguel and Aleixandre, 2006), since eggs are consumed in a large amount daily. Two egg-derived antihypertensive peptides were obtained from a hydrolysate of ovalbumin, which is the major egg white protein. Both of these peptides exhibited antihypertensive activity in SHRs with an associated vaso-relaxing mechanism (Fang et al., 2008). Ovokinin (FRADHPFL), resulting from the pepsin hydrolysate, had an important vasorelaxing activity in canine mesenteric arteries by bradykinin B1 receptors (Fujita et al., 1995). In comparison to ovokinin, the second egg-derived antihypertensive peptide derived by chymotrypsin ovalbumin hydrolysis, characterized as ovokinin 2–7 (RADHPF), obtained higher activity in SHR. Bioactive peptide with ACE inhibitory activity from various animal protein sources are listed in Table 6.3.

6.3.2.3 Plant protein sources of antihypertensive peptides In comparison with milk and other animal proteins, plant proteins have been less studied as a source of antihypertensive peptides. However, interest in studying plant proteins has been growing rapidly in recent years. Soybean proteins have been the most studied source of bioactive peptides among all plants. As the major protein resource in many countries, soybean is consumed as tofu, soy milk, soy flour, soy protein isolate, tempeh, and miso.

Two hypotheses about the mechanism of antihypertensive activity of soybeans have been proposed. In comparison to animal-derived bioactive peptides, the inherently associated isoflavones in soybean-derived products may affect uniquely the activity found. Isoflavones are thought to exert a favorable effect in reducing cardiovascular risk factors as well as vascular function (Messina, 2002). Another hypothesis, on the basis of *in vitro* results indicates that the contribution of isoflavones to BP-lowering effect in soybean ACE inhibitory peptides may be negligible (Wu and Muir, 2008). Similarly, it has also been reported that the reduction of hypertension of a fermented product from soy milk was mainly contributed by peptides with MW of 800–900 Da. As such, further research is needed to determine the mechanism of antihypertensive activity of soy peptides.

Other widely consumed plant foods such as wheat, maize, rice, pea, corn, mung bean, and apricot almond showed antihypertensive effects, when derived peptide products were administered to SHRs (Martinez-Maqueda et al., 2012). Following the isolation and extraction of proteins, proteolysis with Alcalase, thermolysin, α-chymotrypsin, subtilisin, Neutrase, papain, or other proteases was carried out, leading to a variety of products. Several examples of bioactive peptides with ACE inhibitory activity obtained from various plant sources are provided in Table 6.3.

Table 6.3 Bioactive peptide with angiotensin-converting enzyme (ACE) inhibitory activity produced from various food protein sources

Food protein source	Peptide sequence	Reference
Egg white	YRGGLEPINF	Contreras et al., 2009
Egg ovotransferrin	KVREGTTY	Lee et al., 2006
Egg ovalbumin	Ovokinin (FRADHPPL) Ovokinin (2–7) (KVREGTTY)	Miguel and Aleixandre, 2006
Fish muscle	LKP, IKP, LRP (derived from sardine, bonito, tuna, squid)	Nagai et al., 2006
Meat muscle	IKW, LKP	Vercruysse et al., 2005
Silkworm fibroin	GVGY	Igarashi et al., 2006
Milk α-LA, β-LG	Lactokinins (e.g., WLAHK, LRP, LKP)	Hartmann and Meisel, 2007
Milk α-, β-, k-CN	Casokinins (e.g., FFVAP, FALPQY, VPP)	Lee et al., 2006
Porcine troponin	KRQKYDI	Katayama et al., 2008
Porcine myosin	MNPPK	Nakashima et al., 2002
	VKKVLGNP	Muguruma et al., 2009
	KRVIQY	Katayama et al., 2007
Wheat gliadin	IAP	Motoi and Kodama, 2003
Broccoli plant protein	YPK	Lee et al., 2006
Soy	NWGPLV	Kodera and Nio, 2006
Rice protein	TGVY	Li et al., 2007
Corn oligopeptides	AY	Lin et al., 2011
Sake	HY, VY, RF, VW, YW	Saito et al., 1994
Rapeseed protein	IY, RIY, VW, VWIS	Marczak et al., 2003
Wakame (*Undaria pinnatifida*)	YH, KY, FY, IY	Suetsuna et al., 2004
Spirulina platensis	IQP	Lu et al., 2011
Pleurotus cornucopiae	RLPSEFDLSAFLRA RLSGQTIEVTSEYLFRH	Lu et al., 2010

6.3.3 Antioxidant peptides

Bioactive peptides often exhibit antioxidant activity due to their radical scavenging activities and efficacy in metal ion chelation. Humans and biological systems act against oxidants by the removal of reactive oxygen species (ROS) through enzymatic and nonenzymatic antioxidants (Johansen et al., 2005). However, the endogenous defense system may fail to protect the body against reactive radicals by itself under certain circumstances, thus resulting in oxidative stress, which is more a promoter than an initiator of chronic diseases (Rice-Evans and Diplock, 1992; Kaneto et al., 1999).

6.3.3.1 Sources of antioxidant peptides Plants and herbs contain substantial amounts of peptidic antioxidants that can prevent the oxidation process, thus protecting food against rancidity development. Moreover, according to the Food and Agriculture Organization (FAO), leaf proteins are regarded as rich sources of high-quality protein for human consumption due to their abundance in nature, nutritive value, and for being cholesterol-free. Besides, plant-derived bioactive peptides are acceptable for vegetarians.

Table 6.4 Antioxidant peptides from various food protein sources

Food protein source	Peptide sequence	Reference
Rice endosperm protein	FRDEHKK, KHDRGDEF	Zhang et al., 2010
Algae protein waste	VECYGPNRPQF	Sheih et al., 2009
Peptide from frog skin	LEELEEELEGCE	Qian et al., 2008
α_{s1}-casein	YFYPEL	Pihlanto, 2006
β-casein	VKEAMAPK, AVPYPQR, KVLPVPEK	Pihlanto, 2006
β-lactoglobulin	WYSLAMAASDI, MHIRL, YVEEL	Pihlanto, 2006
Sardine muschle	MY	Hernandez-Ledesma et al., 2009
Gelatin (Alaska Pollock skin)	GEHGPHGPHGPHGPHG GPHGPHGPHGPHG	Kim et al., 2001
Hoki (*Johnius belangerii*) skin	HGPLGPL	Mendis et al., 2005
Hoki (*J. belangerii*) frame	GSTVPERTHPACPDFN	Kim et al., 2007

Food protein sources of antioxidant peptides, without consideration of any specific order, include pea, soy, canola, corn, potato, peanut, sunflower, *Brassica carinata*, quinoa, flaxseed, whey, rice bran, sunflower, alfalfa leaf, corn gluten meal, buckwheat, curry leaves, milk casein, fish sea cucumber, porcine collagen, peanut kernels, frog skin, yam, egg yolk, milk kefir, soymilk kefir, medicinal mushroom, mackerel, cotton, casein, algae waste, capelin, fish skin gelatin, Alaska Pollock skin, tuna backbone, and other seafood by-products (Aluko and Monu, 2003; Amarowicz and Shahidi, 1997; Je et al., 2007; Cumby et al., 2008; Shahidi and Zhong, 2008; Udenigwe and Aluko, 2010; Harnedy and FitzGerald, 2012). Table 6.4 provides a list of food sources which could afford antioxidant peptides.

6.3.3.2 Mechanism of action of antioxidant peptides and factors influencing their activities The antioxidant properties of bioactive peptides include scavenging or quenching of ROS and/or free radicals, chelation of transition metal ions, ferric reducing power and inhibition of ROS-induced oxidation of biological molecules such as lipids, proteins, and DNA. The ability to participate in single electron transfer (ET) reaction or hydrogen atom transfer (HAT) causes the radical-quenching activities of food antioxidants (Huang et al., 2005). Antioxidative property may be contributed by the peptidic amino acid residues which can transfer electrons or hydrogen atom to the free radicals under physiological pH conditions.

The specific contribution of individual amino acid residues to the antioxidant activity of a peptide depends largely on the nature of the ROS, and the reaction medium. However, how these "antioxidant" amino acid residues contribute to the overall antioxidant activity of a peptide mixture typical of food protein hydrolysates is still unknown. It is worthwhile to consider all these possible amino acid contributions in order to strategically process proteins to yield peptide mixtures containing amino acid residues of interest.

6.3.3.3 Structural characterization of antioxidant peptides and factors influencing their antioxidant activities Several factors that may affect the antioxidant activity of bioactive peptides include specificity of proteases used for hydrolysis, the degree of

hydrolysis; as well as the structural properties of the resulting peptides, such as molecular size, hydrophobicity, amino acid sequences, and amino acid composition (Pihlanto, 2006). Like protein fractions with other activities, bioactive peptides exhibit greater antioxidant activity than intact proteins or amino acid mixtures.

Studies have shown that histidine residues of peptides can chelate metal ions, quench active oxygen, and scavenge ·OH (hydroxyl radical) due to their imidazole group (Chen et al., 1996, 1998), which is involved in both HAT and ET reactions (Chan and Decker, 1994) Similarly, a histidine-containing dipeptide like carnosine (β-Ala-His), which is derived from muscle cells, also displays potent antioxidant activity (Chan et al., 1994). The tripeptide unit PHH (proline-histidine-histidine) was found to be responsible for the antioxidant activity of soy protein hydrolysates based on the comparison of antioxidant capacity of 28 peptides structurally related to LLPHH (leucine-leucine-proline-histidine-histidine) (Chen et al., 1996). It was also proven that the removal of histidine residue from C-terminal can reduce its antioxidative activity.

The hydrophobicity of peptides has also been shown to greatly affect the antioxidative properties, since it can increase the accessibility of the antioxidant peptides to hydrophobic cellular targets like the polyunsaturated chain of fatty acids of biological membranes (Chen et al., 1998). However, a detailed coverage of this topic is beyond the scope of this chapter. It is also known that the peptide fractions with high 1,1-diphenyl-2-picrylhydrazyl (DPPH) scavenging capacity possess high levels of histidine and hydrophobic amino acids. The radical scavenging activity of peptides is also possibly via hydrogen donor activity of tyrosine through its aromatic hydroxyl group, as observed in most phenolic antioxidants.

Moreover, the electron-dense aromatic rings of phenylalanine, tyrosine, and tryptophan residues of peptides can contribute to the chelation of prooxidant metal ions. Phenylalanine can also scavenge hydroxyl radicals to form more stable para-, meta-, or ortho-substituted hydroxylated derivatives (Sun et al., 1993). Besides, -SH (sulfhydryl) group in cysteine has an independent antioxidant property since it can interact with radicals directly (Qian et al., 2008).

Peptide linkage and/or specific structural features of peptides may influence their antioxidant capacity. It has been shown that specific amino acids can render higher antioxidative activities when present within dipeptides (Nagasawa et al., 2001), whereas other studies have shown that peptide bonds or its structural conformation can reduce the antioxidant activity of the constituent amino acids. As such, peptide conformation behaves as a double-edged sword.

It has been stated that the configuration of peptides can also affect the antioxidant activity. Studies have found that substitution of L-histidine by D-histidine of peptide leads to the reduction of the activity (Chen et al., 1996). In addition to the presence of proper amino acids, their positioning in peptide sequence plays an important role in the antioxidant activity of peptides (Rajapakse et al., 2005). It is concluded that the proper positioning of the imidazole group has a major influence on the antioxidant activity.

Antioxidant activity of bioactive peptides can also be affected by other factors, such as the degree of hydrolysis, type of protease (Pena-Ramos and Xiong, 2002; Gibbs et al., 2004), peptide concentration, and operational conditions. Protein concentration had an impact on the antioxidant activity of peanut protein hydrolysates (Chen et al., 2007). The antioxidant activity of corn gluten meal hydrolysates was related to their concentration

and MW. Peptides of MW 500–1500 Da display stronger antioxidant activity than those with a MW above 1500 Da or below 500 Da (Li et al., 2008).

6.3.4 Hypocholesterolemic peptides

Bioactive peptides with hypocholesterolemic and hypolipidemic activity reduce the risk of cardiovascular disease (CVD). Enzymatic hydrolysis of food proteins is the general method to produce hypocholesterolemic and hypolipidemic peptides.

Soybean is perhaps the most widely explored food source with hypocholesterolemic activity due, in part, to soy 7S globulin (Fang et al., 2008). The α + α′ subunit of soy 7S globulin is believed to be responsible for upregulating the expression of low-density lipoprotein (LDL) to further enhance LDL uptake and degradation, which was demonstrated on cultured hepatocytes (Lovati et al., 1998). Moreover, the specific functional region of the bioactive peptide has been located to be in the α′ subunit and sequenced; it is a 24-amino acid peptide, LRVPAGTTFYVVNPDNDENLRMIA (leucine-arginine-valine-proline-asparagine-glycine-threonine-threonine-phenylalanine-tyrosine-valine-valine-asparagine-proline-aspartic acid-asparagine-aspartic acid-glutamic acid-asparagine-leucine-arginine-methionine-isoleucine-alanine), that corresponds to position 127–150 of the α′ subunit. This peptide modulates cholesterol homeostasis by increasing LDL receptor-mediated LDL uptake in Hep G2 cells (Lovati et al., 2000).

However, not only large peptides like the one from α′ subunit are able to render cholesterol-lowering effect, small peptides derived during proteolysis of soy protein possess strong cardioprotective effects. For instance, an octapeptide (FVVNATSN; phenylalanine-valine-valine-asparagine-alanine-threonine-serine-asparagine) from enzymatic digestion of soybean proteins was identified as the most active stimulator of LDL receptor transcription in Hep T9A4 human hepatic cells (Table 6.5) (Lovati et al., 2000).

Other than altering gene expression, soy protein hydrolysates and constituent peptides with bile acid-binding capacity can also display hypocholesterolemic activity, generally in two ways. This is achieved by either reducing micellar solubility of bile acids and neutral sterols in the small intestine epithelial cells or suppressing reabsorption of bile acid in the ileum. Although the capacity of bile acid-binding and neutral sterols may partially be attributed to fractions of high-molecular-weight insoluble bioactive peptides, these bioactive peptides might also have drawbacks such as difficulties to pass the intestinal epithelium into blood circulation to exhibit cholesterol-lowering effects due to their large molecular size (Nagaoka et al., 2001).

Table 6.5 Hypocholesterolemic peptide from various food protein sources

Food protein source	Peptide sequence	Reference
Soy glycinin	LPYPR	Wang and de Mejia, 2005
Soy glycinin	IAVPGEVA	Pak et al., 2005
Milk β-LG	IIAEK	Nagaoka et al., 2001
Soy	Lactostatins (IIAGK, GLDI, QK, ALPMH)	Pihlanto, 2011
	FVVNATSN	Cho et al., 2008

Lunasin, a 43 amino acid peptide from soybean, inhibits acetylation of histone H3 Lys14 residue to display hypocholesterolemic activity. In addition, lunasin is able to increase cellular production of LDL receptors to discard plasma LDL cholesterol (Shahidi and Zhong, 2008). Table 6.5 shows hypocholesterolemic peptides derived from food sources.

6.3.4.1 Application of hypocholesterolemic peptides Increased research on cholesterol-lowering effect of bioactive peptides has led to the development and commercialization of Lunasin XP and LunaSoy. Lunasin inhibits acetylation of histone H3 Lys14 residue to function as a hypocholesterolemic agent. In addition, lunasin is able to increase cellular production of LDL receptors to discard plasma LDL cholesterol. Further studies of structural influence on peptide properties are needed in order to better understand the essential structural features of hypolipidemic and hypocholesterolemic peptides (Udenigwe and Aluko, 2011).

6.3.5 Anticancer peptides

Bioactive peptides also exhibit anticancer activity as shown in several studies (Messina, 2002). Carcinogenesis is a multistage process derived from a combination of multiple heritable and environmental factors. It has been shown that populations consuming large amounts of plant products, such as soybean, face a lower risk of cancer incidence and experience longer life expectancies (Hernandez-Ledesma et al., 2009). As noted already, lunacin is the predominant peptide from soy protein and is capable of suppressing chemical and viral oncogene-induced cancers by inhibiting core histone acetylation and deacetylation in mammalian cells. The mechanism of action of lunasin is via inhibition of HAT to modify histone acetylation and deacetylation dynamics leading to inhibition of acetylation of H3 and H4 repression of cell cycle progression, modification of chromatin, apoptosis in cancer cells, and suppression of carcinogenesis (Hernandez-Ledesma et al., 2009). Research has shown that Bowman–Birk protease inhibitor (BBI), a widely studied bioactive substance from soybean, can prevent lunasin from degradation when orally administrated (Udenigwe and Aluko, 2011).

6.3.5.1 Other sources of anticancer peptides Other than soybean, peptides with anticancer activity are also discovered in a variety of other sources, such as mistletoe, *Amaranthus hypochondriacus*, and tuna. A lunasin-containing glutelin fraction derived from *A. hypochondriacus* can induce apoptosis in cervical cancer (HeLa) cells upon digestion with trypsin. Two large peptides, LPHVLTPEAGAT and PTAEGGVTMVT, derived from tuna dark muscle by-product upon hydrolysis with papain and protease XXII displayed dose-dependent antiproliferative activities against cultured breast cancer (MCF-7) cells. Leguminous plants, such as Yunnan bean and pinto bean, as well as plant sources like buckwheat and ginseng, have been shown to serve as good sources of bioactive peptides with antiproliferative activities (Shahidi and Zhong, 2008; Leung and Ng, 2007). A peptide isolated from buckwheat seeds with a MW of 4 kDa exhibited antiproliferative activity against hepatoma, leukemia, and breast cancer cells (Leung and Ng, 2007).

In addition to plant sources, antiproliferative peptides have been found in dairy milk proteins, eggs, marine animals, and their by-products. Cheese slurries have been found to contain antiproliferative hydrophobic peptide fractions, and these purified peptide fractions exhibited high cytotoxic activity against tumor cell lines SNU-C2A, SNU-1, and P388D1 (Kim et al., 1995). It has been shown that egg protein hydrolysates can exert an antiproliferative effect on mouse lymphoma cells (Yi et al., 2003). Moreover, anticancer peptides derived from marine animals and microorganisms can be present either as dipeptides or tripeptides composed entirely of tryptophan.

6.3.6 Antimicrobial peptides

Bioactive peptides with antimicrobial activity are the focus of a number of studies because they have been discovered in a wide array of natural sources from microorganisms to plants and animals. Their application in food preservation and for therapeutic purposes in health care has also been of interest. A database about the antimicrobial peptides is also available at http://aps.unmc.edu/AP/main.php (Wang et al., 2009). The antimicrobial peptides are applied to inhibit microbe-caused food deterioration as these microbes *in vivo* enhance the innate immunity and resistance against microorganisms and diseases of organisms by inhibiting the invasion of those pathogens (Shahidi and Zhong, 2008). Moreover, these antimicrobial peptides from natural sources demonstrate effective multidrug-resistant activities.

6.3.6.1 Structural requirements of antimicrobial peptides Most antimicrobial peptides have less than 50 amino acids (6–100 amino acids), and half of these amino acids are hydrophobic. Studies also show that basic amino acids such as lysine and arginine are abundant; positive charge and amphipathic three-dimensional structural fold by amino acids are significant in exhibiting antimicrobial activity (Peters et al., 2010).

Antimicrobial peptides are categorized into four classes of α-helix, β-sheet, extended, and loop peptides according to their secondary structures (Guani-Guerra et al., 2010). Cathelicidins and defensins are the most extensively studied antimicrobial peptides in humans (Guani-Guerra et al., 2010) while bacteriocins have been of interest in the food industry, especially in meat curing.

6.3.6.2 Application of antimicrobial peptides and application challenges Due to selectivity of antimicrobial peptides, some of them might not be effective in replacing conventional applications. These peptides prefer interacting with microbial cells than mammalian cells, which leads to the belief that these peptides are harmless to mammalian cells. The selectivity of antimicrobial peptides is due to factors such as polarization, membrane composition, and structural characteristics (Bradshaw, 2003; Yeaman and Yount, 2003; Harris et al., 2009). In addition, antimicrobial peptides can behave as synergistic therapeutic agents by inserting into the microbial cells to improve the strength of antimicrobials present, because antimicrobial peptides exhibit permeability toward target microbial cell membranes (Tang et al., 2002; Yeaman and Yount, 2003). Recently, antimicrobial peptides have also been shown to demonstrate immunomodulatory activities, endotoxin

neutralization, wound healing, and anti-neoplastic properties (Brown and Hancock, 2006; Mader and Hoskin, 2006; Mookherjee and Hancock, 2007; Diamond et al., 2009). Therefore, antimicrobial peptides have become new predominant therapeutic agents which might be employed not only as antimicrobials, but also as modifiers of inflammation, or in cancer treatment (Peters et al., 2010)

Although antimicrobial peptides can exert various beneficial effects, several challenges remain that may hinder their commercialization and use in food processing. High production costs and susceptibility to proteolytic degradation are the two leading impediments; some studies suggest that these hindrances might be overcome by extensive chemical modification (Bradshaw, 2003).

6.3.7 Immunomodulatory peptides

6.3.7.1 Mechanism and structural requirement of immunomodulatory peptides

Generally, immunomodulatory peptides exert their activities on the immune system in three ways: regulation of cytokine expression, production of antibody, and ROS-induced immune functions (Hartmann and Meisel, 2007). Immunosuppression and immunostimulation are involved in disease control. Immune cell functions can be improved by immunomodulatory peptides; immune cell functions are evaluated by lymphocyte proliferation, natural killer (NK) cell activity, antibody synthesis, and cytokine regulation (FitzGerald and Murray, 2006).

The immunomodulatory property is due to the structural characteristics of peptide molecules. Immunomodulatory peptides produced upon the digestion of rice and soybean with trypsin affect non-specific immune defense systems by activating superoxide anions (Kitts and Weiler, 2003; Mine, 2007). The peptides derived from eggs can exhibit immunostimulating activities in cancer immunotherapy to enhance immune function (Kitts and Weiler, 2003). Wheat protein hydrolysates have been reported to enhance the immune system of human volunteer subjects after 6 days of consumption (3 g/d) (Horiguchi et al., 2005). In addition, a recent study shows that a pea protein hydrolysate administrated orally to mice can decrease NO production by activating macrophages and reduce secretion of proinflammatory cytokines, tumor necrosis factor (TNF)-α and interleukin (IL)-6 (Ndiaye et al., 2012). Dairy products have been demonstrated to perform an important role in the immune system of human beings, especially newborn infants. Immunomodulatory peptides might decrease allergic reactions in humans and increase mucosal immunity in the GI tract.

6.3.7.2 Applications and impediments in the use of immunomodulatory peptides

Immunomodulatory peptides may be used as blueprints for the development of innovative therapeutic agents for which action mechanism and supportive data from clinical experiments are necessary (Shahidi and Zhong, 2008).

Immunomodulatory peptides might decrease allergic reactions in atopic applications in human subjects and increase mucosal immunity in the GI tract. Immunomodulatory peptides from different food protein sources are displayed in Table 6.6.

Table 6.6 Immunomodulatory peptides from various food protein sources

Food protein sources	Peptide sequence	Reference
Bovine milk α_{s1}-casein	–	Gill et al., 2000
Bovine milk isracidin	N-terminal sequence 1–23	Gill et al., 2000
Bovine milk β-casomorphin	YPFPGPIPDSL	Gill et al., 2000
Para-κ-casein	FFSDL (17e21)	Gill et al., 2000
α-Lactalbumin	Hydrolysed α-lactalbumin	Gill et al., 2000
Rice albumin	Oryzatensin (GYPMYPLR)	Takahashi et al., 1994

6.3.8 Mineral-binding peptides

Peptides derived from food proteins may also exhibit mineral-binding activity leading to improvement of antioxidant activity and mineral absorption. For example, caseinophosphopeptides (CPPs), in β-casein, can prevent lipid oxidation and food quality deterioration as well as oxidative stress-mediated cellular damage via chelation of prooxidant metal ions like those of iron and copper. CPPs may also function as carriers for different minerals, especially calcium. Specific CPPs can bind to calcium at the negatively charged side chains and form a soluble organophosphate salt complex (Meisel, 1998). This leads to enhancement of calcium absorption across enterocytes in the distal intestine (Rutherfurd-Markwick and Moughan, 2005). CPPs are also used for preventing dental caries due to their role in recalcification of the dental enamel. Published data on the effect of CPP on mineral solubility and absorption are inconsistent, partly due to the diversity of the experimental approaches employed such as the amount of Ca in the diet that may determine the effect of CPP on Ca absorption (Vegarud et al., 2000).

More importantly, mineral-binding peptides are effective in enhancing *in vivo* absorption of metals, including copper, calcium, iron, zinc, and other trace elements and therefore improve their bioavailability (Vegarud et al., 2000). Binding of copper to certain amino acids such as histidine, methionine, and cysteine in small peptides mediates absorption of copper through these amino acid transporters (Gaetke and Chow, 2003).

6.3.9 Opioid peptides

Opioid peptides from food proteins were first reported by Zioudrou et al. (1979). Based on the structural similarity of opioid peptides to endogenous ligands (endorphins and enkephalins), opioid peptides can interact with opioid receptors of the d-, m-, or k-type. Endogenous and exogenous ligands share common structural motif, which are an N-terminal tyrosine residue (except α-casein opioids) and the presence of another aromatic residue in the third or fourth position from the N terminus (phenylalanine or tyrosine) (Hartmann and Meisel, 2007). Agonistic activity is comparable to that of endogenous ligands, whereas antagonistic peptides exert inhibitory effects similar to naloxone, which is a potent opiate receptor antagonist usually used as a drug (e.g., in the case of heroin overdose) (Nagai et al., 2006).

Table 6.7 Opioid peptides found in different food protein sources

Food protein sources	Peptide sequence	Reference
Milk α-LA, β-LG	α-Lactorphins, β-lactorphins	Silva and Malcata, 2005
κ-Casein	Casoxin A, B, C (25–34: YIPIQYVLSR, 35–42: YPSYGLNN; 58–61: TPTY)	Pihlanto, 2006
Serum albumin	Serorphin (399–404: YGFNA)	Pihlanto, 2006
Bovine hemoglobin	YPLSTQEF	Pihlanto, 2006
Gluten	GYYP, -T and YGGW, -L	Pihlanto, 2006
Spinach	Rubiscolin-5, -6 (YPLDL, -F)	Pihlanto, 2006
Soybean	Soymorphin-5 (YPFVV)	Pihlanto, 2006
Wheat gluten	Gluten-exorphins A4, A5 (GYYPT), B4, B5, and C (YPISL)	Takahashi et al., 2000

Casomorphin, an opioid peptide derived from casein hydrolysate, may be involved in regulating functions of the gut and enhance net water and electrolyte absorption, subsequently decelerating intestinal transit of the chime and thus acting as an antidiarrheal agent (Meisel, 2005). Unlike endomorphins found in human organisms, exorphins such as casomorphins from casein are also found in other milk proteins (β-lactoglobulin, α-lactalbumin, lactoferrin), in cereal proteins such as wheat (gluten, gliadin), barley (hordein, avenin, secalin, zein), rice (albumin), in vegetables such as soybeans (α-protein), spinach (rubisco protein), and in meat/poultry (albumin, hemoglobin, γ-globulin), and egg (ovalbumin). Exorphins primarily affect the intestinal lumen and mucosa by regulating GI motility as well as gastric and pancreatic secretions. Examples of opioid peptides found in different food protein sources are listed in Table 6.7.

Multifunctional peptides have been reported to possess more than one significant physiologically relevant bioactive property. For instance, the β-lactoglobulin-derived β-lactorphin (YLLF) inhibits ACE activity and also possesses opioid-like activity (Mullally et al., 1997). The multiple bioactivities displayed by these peptides can increase their impact toward the amelioration of more than one disease target or multiple symptoms of a disease, such as CVD, since many human diseases are interrelated in terms of their etiology and progression. Therefore, the conditions for generating and processing bioactive food protein hydrolysates can be carefully designed to yield multifunctional peptides with diverse applications in maintaining optimum health.

6.3.10 Anti-obesity peptides

In recent years, obesity has already become a common health problem which can lead to increased risk for type 2 diabetes, CVD, stroke, and sleep-breathing disorder. Some bioactive peptides derived from marine organisms show a significant ability to reduce obesity. For example, shrimp head protein hydrolysates with low-molecular-weight peptides (1–1.5 kDa) have been discovered to serve as an effective agent for stimulation of cholecystokinin release in STC-1 cells (Cudennec et al., 2008) Therefore, anti-obesity peptides are

suggested as a promising functional food against obesity via regulation of cholecystokinin release.

6.4 Application and development of bioactive peptides

6.4.1 Bioactive peptides absorption and *in vivo* activity

Bioactive peptides possess a variety of beneficial effects; however, research on their bioactivity *in vitro* may not reflect their effect *in vivo*. Several properties of bioactive peptides, such as absorption, bioavailability, and susceptibility, usually influence how bioactivity could translate into pharmacological effects *in vivo*. Since bioactive peptides can be employed as nutraceuticals and pharmaceuticals, bioactive products are already commercialized and are available in the marketplace in some countries such as Poland and Japan, among others (Sarmadi and Ismail, 2010).

Food-derived bioactive peptides are required to maintain their effective original peptide sequence, while they are passing through enterocytes to serum without degradation by enzymes to act on the target cellular sites. Peptides and proteins can escape digestion and be absorbed in an intact form through the interstitial space into the intestinal lymphatic system. However, the ability of such compounds to enter the intestinal lymphatic system is affected by their permeability via the capillary of the portal circulation and lipid solubility (Deak and Csáky, 1984). It has been proposed that drugs transported through GI lymphatic system can escape the hepatic metabolism (Wasan, 2002). Therefore, the ability to maintain stability against enzymes, such as serum protease, and to be absorbed in serum to function on targets are necessary for bioactive peptides to exhibit their effects.

In addition, several studies indicate that physiological properties of bioactive peptides, such as charge, molecular size, lipophilicity, and solubility, determine their bioavailability. For instance, molecular size is a well-studied factor. Research shows that smaller peptides can cross the enterocytes via intestinal-expressed peptide transporters. Nevertheless, these small-size peptides are still able to render biological effects at the tissue level (Gardner, 1988, 1998). On the other hand, by means of passive transport, larger molecular size peptides might pass through hydrophobic regions of membrane epithelia or tight junctions. Moreover, research findings indicate that peptides with 2–6 amino acids are absorbed more readily in comparison to protein and free amino acids (Grimble, 1994). Small (di- and tripeptides) and large (10–51 amino acids) peptides are demonstrated to cross the intestinal barrier intact and exhibit their biological functions at the tissue level (Roberts et al., 1999). However, as the MW of peptides increases, their chance to pass the intestinal barrier decreases. It has been recorded that the presence of proline and hydroxyproline results in peptide resistance to digestive enzymes, especially tripeptides with proline-proline at the C-terminal that are resistant to proline-specific peptidases (Fitzgerald and Meisel, 2000). Moreover, it was observed that the amount of peptide in human plasma increased in a dose-dependent manner. Thus, it was proposed that saturation of peptide transporters may affect the amount of peptides entering peripheral blood (Matsui et al., 2002).

In conclusion, it is necessary to enhance and strengthen the stability and absorption of bioactive peptides during delivery by different methodologies, including microencapsulation. In comparison to large molecular size peptides, small peptides, like dipeptides and tripeptides, have more advantages *in vivo* due to their resistance to peptidolysis and ability to be absorbed into blood circulation. However, some specific peptides only exert their functions in the GI tract, for example, hypocholesterolemic peptides. Therefore, it is not necessary to consider their ability to be absorbed in to blood circulation.

6.4.2 Safety concerns of bioactive peptides

Nutraceutical products are usually considered as double-edge swords; however, food-derived bioactive peptides have been considered as being safe until now. Several studies indicate lack of toxicity of bioactive peptides in cell cultures. This is not only because these bioactive peptides are similar to those from digestion of food proteins by human beings, but also food-grade enzymes and processes are usually applied in the industrial production.

Recent research shows that casein hydrolysates possessing antihypertensive peptides demonstrate no side effects on the clinical parameters of rats, such as blood biochemical, hematology, organ weight ratios, histopathological, or death rate. Moreover, lactotripeptides (IPP, which is isoleucine-proline-proline) has been shown to be tolerated by prehypertensive and hypertensive human subjects, and no vital adverse effect has been found in serum chemistry or in urine parameters (Boelsma and Kloek, 2008).

To sum up, food-derived bioactive peptides are relatively safe for human consumption; nevertheless, it is still necessary to ensure the safety and quality of bioactive peptides, as well as avoiding the use of any processing techniques or procedures that could trigger adverse effects of bioactive peptides. For further interest, Schaafsma (2009) has published a comprehensive review about the safety of protein hydrolysates and their relation to human nutrition.

6.5 Conclusion

Biologically active peptides from foods have remarkable activities both *in vitro* and *in vivo*. Among all these, antihypertensive peptides have been studied most, while anticancer peptides have not received much attention in the existing literature. Peptides with antioxidant, cholesterol-lowering, immunomodulatory, antimicrobial, mineral-binding, opioid, and multifunctional properties have been reported in the past few decades. Several aspects reviewed in this contribution have also attracted much interest, such as bioavailability, molecular mechanism action, safety issues, and evaluation of bioactive peptides *in vivo*.

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7

Protein and Peptide-Based Antioxidants

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7.1 Introduction

Oxidation is an unavoidable outcome of aerobic respiration in living organisms. It leads to the generation of several types of radical oxygenated species (ROS), all of which are capable of reacting and impacting several vital biological building blocks such as DNA, protein, and most certainly lipids (Ames et al., 1993). Of the many antioxidant defense mechanisms that involve several *in vivo* mechanisms and strategies, one is the ingestion of antioxidants, perhaps the most commonly known being Vitamin E from grains and oils, in addition to carotenoids from vegetables, flavanoids found in fruits and juices, and polyphenolic compounds such as those found in green tea. Studying and quantifying the antioxidant activity of these natural products in a model test (*in vitro*) is very straightforward and simple; however, assessing the health effect of these antioxidants *in vivo* is not straightforward at all for many reasons, such as genetic background, environmental conditions, life style and diet, bioavailability, metabolic structural consequences, and lack of quantification possibility of "what's healthy." These overlapping factors make it harder to quantify and subsequently guaranty a health effect of ingested antioxidants.

There is another category of commonly used antioxidants, the ones that serve as food preservatives, against lipid oxidation and its effect on shelf-life deterioration of foods and beverages. Whereas the chemistry of oxidation in foods is often very complex and hard to elucidate, the effect of antioxidants can be guaranteed in quantitatively measured freshness and extended shelf life. Along with spoilage, oxidation constitutes a leading cause in shortening of shelf life, especially in lipid-containing foods. Lipid oxidation generates

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off flavors and off aromas; it leads to loss of color through the oxidative degradation of pigments, as well as loss of nutrients and vitamins; and it could change the texture and functionality by impacting protein structure – all of which happen through co-oxidation of lipids (Schaich, 2013).

Most of the food and beverage products found in a grocery store suffer from oxidative deterioration, to various extents. Fats and oils from plant and animal and marine sources are known to suffer the most from rancidity. Development of warmed-over flavor (mainly beef, poultry, pork, and sea food) is also a result of lipid oxidation; nevertheless, many other types of foods are subject to oxidative quality damage, such as dairy products and their corresponding non-dairy alternatives, canned foods and soups, breakfast cereal, snacks, baked goods, dressings, condiments and sauces, confectionary, beverages, and many others.

A current trend in food manufacture is the replacement of stable, hydrogenated fats, with the unstable-but-more-healthy polyunsaturated fats. As the food industry continues to make this substitution, it will face enormous food preservation challenges that will require more powerful strategies for controlling oxidation in foods. In addition to improvements in the technologies of packaging and food processing, the industry will need to develop novel, targeted, naturally derived antioxidant systems to achieve the goal of stable, wholesome foods for consumers. The best strategy to manage oxidation through antioxidants involves blends and combinations that operate on different components of the oxidation mechanism and act synergistically to provide extraordinary increases in stability. The food industry needs to reach a more advanced state to meet the challenge of providing extraordinary stability in today's complex foods, which requires better understanding of the available antioxidant options—their chemical structures, mechanisms of action, sources, applications where they work best, and those where they do not (Nahas, 2012).

The purpose of this chapter is to provide an overview of lipid oxidation mechanisms, in foods. Consequently, the ultimate purpose is to offer an insight into natural antioxidant options that can be derived from proteins and peptides, among the variety of existing natural antioxidants, to manage lipid oxidation in today's foods and beverages.

7.2 Background

Oxidation can be a main issue in foods and a major factor in shortening shelf life by generating off flavors and aromas in lipid-containing foods. It can even change the texture and functionality by impacting protein structure (i.e., decrease in water-holding capacity and undesirable texture); therefore, oxidation affects a wide array of foods, such as meats, oils, fried foods, dressings, dairy products, baked goods and extruded snacks. Using antioxidants constitute a major oxidation defense strategy.

It is well established that oxidation affects food quality. The link between oxidized fats on blood lipid profile, fatty liver development, carnitine homeostasis, thyroid function, thyroid hormone, glucose tolerance, insulin sensitivity, inflammation, and both exogenous and endogenous defense mechanisms have been reported (Eder, 2010); however, the effect of oxidized fats and their derived secondary oxidation products (e.g., aldehydes

and ketones) on our health has not been as extensively studied. Oxidized foods may have various proposed health effects and interferences with multiple body functions.

Lipid oxidation is a free radical reaction of three stages—initiation, propagation, and termination. In the initiation stage, free radicals are formed through mechanisms mediated by heat, light, or transition metal ions such as iron (i.e., Fenton's Reaction) (Berdahl et al., 2010):

$$H_2O_2 + Fe^{2+} \rightarrow HO^{\bullet} + HO^{-} + Fe^{3+}$$

Hydroxyl radicals can abstract hydrogen directly from a fatty acid lipid (LH), forming a lipid radical ($L^{\bullet}$). The lipid radical will react instantly with molecular oxygen to form a hydroperoxyl radical ($LOO^{\bullet}$):

$$LH + HO^{\bullet} \rightarrow L^{\bullet} + O_2 \rightarrow LOO^{\bullet}$$

In turn, the hydroperoxyl radical ($LOO^{\bullet}$) can abstract a hydrogen from another lipid, causing the formation of a new and additional hydroperoxyl radical, hence, the propagation stage:

$$LOO^{-} + LH \rightarrow L^{\bullet} + O_2 \rightarrow LOO^{\bullet}$$

In the termination stage, two radical species will react to form a non radical species, such as:

$$L^{\bullet} + L^{\bullet} \rightarrow \text{Non-radical species.}$$

Hydroperoxide species formed in the propagation stage (also known as primary oxidation markers) will eventually decompose to form secondary oxidation products such as aldehydes and ketones, which are directly responsible for the off aroma and off flavor found and associated with oxidized foods.

Antioxidants are classified on the basis of the step and mechanism of the lipid oxidation cycle they interfere with or prevent. The most commonly known type consists of the primary antioxidants (also known as chain-breaking or radical scavenger antioxidants), most of which are phenolic compounds capable of donating a hydrogen to the free radical and becoming a radical themselves but low enough in energy or stable enough so that it does not propagate further radical formation. The second main type would be preventative antioxidants such as metal chelators, which can sequester transition metal ions and prevent them from initiating oxidation altogether, hence are "preventative." Oxygen scavengers constitute a different category, with the mechanism of action being reducing oxygen and removing it from the system being stabilized. Ascorbic acid is among the few known antioxidants known to function in this manner. A completely different category consists of antioxidants that have a functionality based on deactivating high-energy species such as singlet oxygen. Carotenoids like β-carotene are known to function in this manner. Finally, certain antioxidants are referred to as regenerators because of their ability to reduce primary antioxidants once the latter donate a proton to a radical, and regenerate them in this manner, for additional use and allowing lower levels

of primary antioxidants to perform better when combined with regenerators; therefore, it is clear that antioxidants can be classified according to their mode of action, whether it is synthetic or natural (plant phenolics, ascorbic acid, tocopherols, and proteins). For the non-technical consumers though, the classification falls more along the line of synthetic/artificial versus natural. Synthetic antioxidants generally work very well in preserving foods, are certainly cost effective, and can be readily prepared in large amounts and maintain high purity. The introduction of new ones requires extensive testing and regulatory effort. More importantly, these are derived from fossil fuels because of which they have a negative consumer image. Natural antioxidants such as those found in herbs and spice, on the other hand, have been used in culinary applications since ancient times. Consequently, they have a favorable consumer image; however, they might not be as pure as their synthetic counterparts and certainly not as cost effective. Nevertheless, their market growth is exceeding that of synthetic antioxidants.

7.3 Classes of natural antioxidants

The most commonly found natural antioxidants can be divided into the following categories: (i) herb and spice extracts, (ii) tocopherols, (iii) ascorbic acid, (iv) proteins and peptides. The next sections will briefly review natural antioxidants and focus on protein- and peptide-based antioxidants.

7.3.1 Herb and spice extracts

The *Labiatae* family provides some of the most potent and recognized natural antioxidant extracts for food preservation, with rosemary being the most famous. Rosemary has been used in culinary applications since ancient times, and there are many reports on its antioxidant activity dating back to the 1950s (Rac and Ostric, 1955; Chipault et al., 1956). Today, it is the dominant herb extract on the market, both in Europe and the United States, with exact figures-related usage volume hard to assess with accuracy. The antioxidant activity of rosemary is mostly attributed to its predominantly oil-soluble phenolic diterpenes, such as carnosic acid and carnosol, and water-soluble phenolics, such as rosmarinic acid, and to a much lesser extent epirosmaniol, rosmanol, isorosmanol, methylepirosmanol, and others (Nahas, 2012). Carnosic acid is a super stoichiometric radical scavengers because of its ability to regenerate sequential phenolic intermediate antioxidant structures, as illustrated by Masuda et al. (2001) (Figure 7.1). Because of those numerous and abundant active antioxidant compounds in rosemary, commercially available rosemary extracts are found today in a multitude of food applications (Table 7.1).

7.3.2 Tocopherols

Tocopherols are natural antioxidants, derived from grains, and consist of a family of four congeners α-tocopherol (vitamin E), β-tocopherol, δ-tocopherol, and γ-tocopherol. They are allowed as food additives and are relatively stable and effective lipid-soluble antioxidants. Tocopherols are normally available on a large scale but with some supply chain

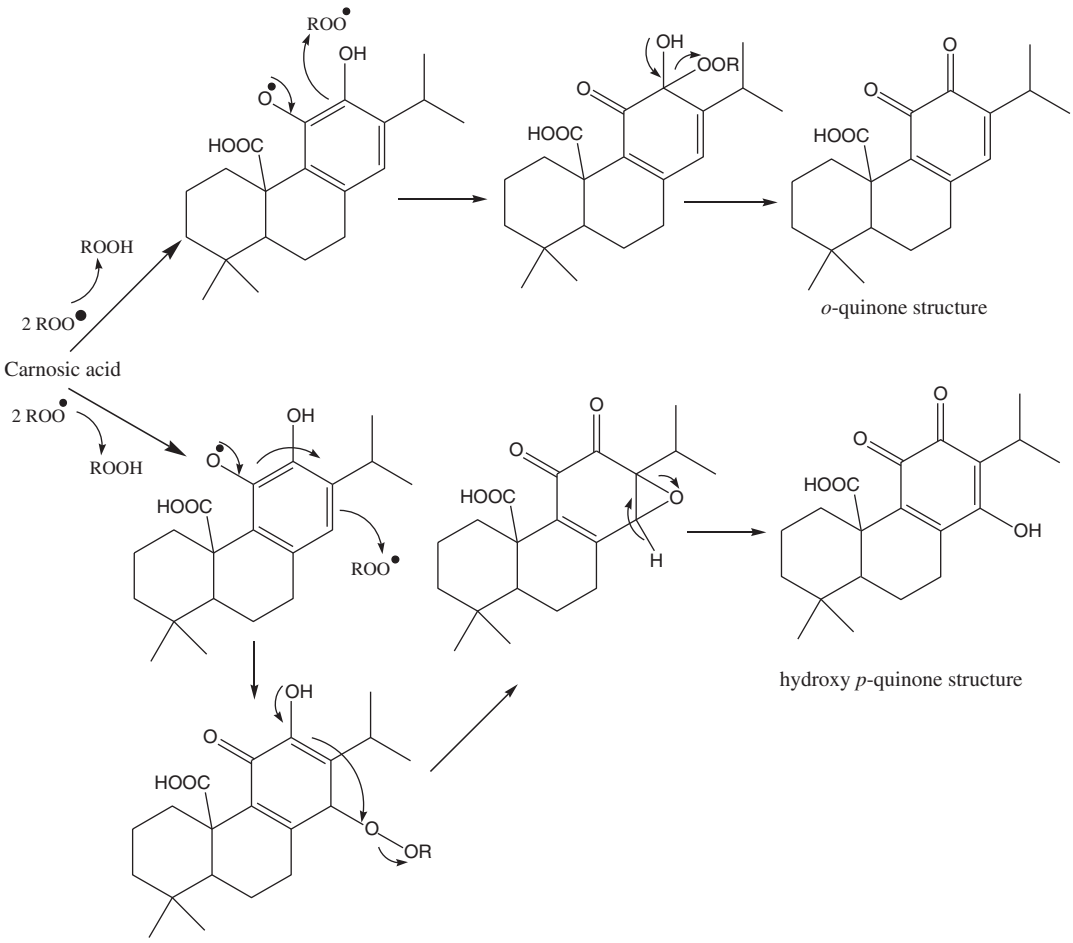

Figure 7.1 Proposed antioxidant mechanism of carnosic acid. Adapted from Masuda et al. (2001)

inconsistency, depending on environmental factors and grains market. They are commonly used in fats, oils, meats, snacks, cereals, baked good, and beverages. Radical scavenging is their main mode of antioxidant action, as with most of phenolic compounds. It is also reported that tocopherols are good singlet oxygen quenchers through a charge transfer mechanism (Kim and Min, 2008). One of their disadvantages (besides supply chain) is their potential prooxidant effect, as it is documented in reports (Frankel, 1996).

7.3.3 Ascorbic acid

Ascorbic acid is a water-soluble antioxidant, also known as Vitamin C (the L-ascorbic acid). Ascorbic acid has many possible modes of actions, but of the proposed ones is its ability to scavenge oxygen, by consuming oxygen and producing water as ascorbic acid is

Table 7.1 Examples of studies on rosemary extracts

Food system	Method	Effect	Reference
Cooked ground chicken (high-oxygen atmosphere)	TBA Sensory Hexanal	Significant improvement on oxidative stability, color stability and sensory	(Keokamnerd et al., 2008)
Chicken breakfast sausage	TBA	Effect similar to synthetic BHA-BHT mixture	(Lee et al., 1997)
Irradiated beef myoglobin	Colorimetric	Significantly preserved color, better than citric acid	(Stetzer et al., 2009)
Fried beef patties	Formation of heterocyclic amines	Reduced formation of heterocyclic amines by 49–77%	(Tsen et al., 2006)
Cooked ground beef	TBA Sensory Colorimetric	TBA reduced by 92% Significantly reduced hexanal Slight color improvement	(Ahn et al., 2007)
Vitamin E supplemented beef, MAP packaged	TBA	Significantly better than synthetic BHA/BHT mixture	(Formanek et al., 2001)
Frozen vacuum-packaged beef and pork	TBA Colorimetric Sensory	Slight improvement	(Rojas and Brewer, 2008)
Fish (fillets) supplemented with antioxidants during feeding	TBA	Notable improvement over control	(Sant'ana and Manchini-Filho, 2000)
Frying flaxseed oil	Retained fatty acids	Significant improvement	(Irwandi et al., 2004)
Deep-fat frying palm oil	Response Surface Methodology	Rosemary was the most important factor among sage extract and citric acid for sensory acceptability	(Irwandi and Yaakob, 1999)
Palm Olein	PV and TBA Fatty Acids Polymers Viscosity Color	Rosemary>BHA>Sage>BHT>control	(Che Man and Tan, 1999)
Fish oil	PV, Anisidine GC	Effective	(Kendrick and MacFarlane, 2003)
Soybean oil	OSI	Significant improvement	(Reynhout, 1991)
Food oil systems	Differential Scanning Calorimetry	Protective effect	(Irwandi et al., 2000)
Fragrances and aromas	Color	Effective	(Christopher and Pisano, 2003)

converted to dehydroascorbic acid (Cort, 1974). Ascorbic acid has proven useful in stabilizing beverages and various aqueous systems, as well as oils and lipid-containing foods; however, prooxidant effects have been observed in some systems, such as mayonnaise and energy bars, and even in milk and dressings. This could be attributed to its metal reducing power (Jacobsen et al., 2008).

7.3.4 Proteins and peptides

Because of the constant increase in demand for natural antioxidants, more sources are being investigated to generate effective, label clean, economical and (most importantly) safe antioxidants. Although the examples of natural antioxidants listed thus far have been successful in several food applications, they do not completely fulfill the need of the food and beverage industry. There is certainly room for additional natural antioxidants that can replace existing natural antioxidants that are either too expensive or do not deliver the required oxidative shelf-life extension. Proteins and peptides might be able to play that role and fill the gap as additional effective natural antioxidants by complementing the preexisting commercial natural antioxidants through synergistic effects. Proteins function as antioxidants through commonly occurring mechanisms such as free radical and reactive oxygen species scavenging and metal chelation, and non-commonly occurring mechanisms, such as quenching of volatile aldehydes, two-electron reduction of lipid hydroperoxides, and enzymatic functionality (Elias and Decker, 2010).

7.3.4.1 Antioxidative animal proteins *Milk proteins* are one of the most extensively studied animal proteins for their antioxidant activity. The activity was attributed to different proteins, such as caseins, whey protein, and lactoferrin, which work by different functionalities such as metal chelation and radical scavenging, hence constituting a rich source of protein-based metal chelators and also radical-scavenging individual amino acids. More studies are needed, however, to conclusively elucidate the real modes of action of milk antioxidant proteins (Pihlanto, 2006). The milk protein hydrolysates also seem to be good antioxidants, especially those from casein-derived proteins. Casein-phosphopeptides constitute a good example of the latter category, derived from enzymatic hydrolysis of casein, and are believed to have metal-chelating abilities because of the presence of phosphate groups originating from serine amino acids in the sequence. These polar functional groups create a medium that is favorable for chelating metals, such as calcium, zinc, copper, manganese and iron (Kitts, 2005). Metal chelation, however, does not explain all the activity of casein-phosphopeptides; casein hydrolysates were shown to be more effective than the enriched casein-phosphopeptides at the same phosphorus level. This excluded the theory of attributing the antioxidant activity to the metal-chelating activity of the characteristic phosphoseryl residues in the casein-phosphopeptides (Díaz et al., 2003). Regardless, casein, casein hydrolysates, and the enriched phosphorylated peptides from casein all inhibited lipid oxidation in the emulsion model used in the study. In a subsequent study, casein hydrolysates and casein-phosphopeptides were tested in a phosphatidylcholine liposome system against oxidation promoted by ferric/ascorbate system. Inhibition of

Thiobarbituric Acid Reactive Species (TBARS) in cooked ground beef was also investigated (Díaz and Decker, 2004). The hydrolyzed milk proteins were shown to be effective in these systems, depending on the dosage, which is promising for their application as potential real foods natural antioxidants. Laparra et al. (2008) went a step further and assessed the antioxidant protective effect of casein-phosphopeptides on oxidative stress. The testing included mitochondrial enzyme activity, intracellular glutathione (GSH) levels and GSH reductase activity, and cell cycle analysis with RNA-induced changes. The data reported prompted the authors to postulate a possible synergistic effect between the milk peptides and other antioxidants found in fruit juices, but more studies are needed for conclusive *in vivo* effects. Whey protein also has its share of reported antioxidant activity, such as in a salmon-oil-in-water emulsion model (Tong et al., 2000). The antioxidants activity was attributed to the metal-chelating potential of high molecular weight fractions from pasteurized milk. The research was also extended to show that whey protein can stabilize algal oil emulsions as well (Djoredjevic et al., 2004).

Hen's egg white protein obtained from yolk-free egg white has been shown to have a protective antioxidant effect on a high polyunsaturated fat. The protein was mixed with safflower or sardine oil, at a 9:1 oil–protein ratio that was stored in its powdered form at 37°C and tested for oxidation markers periodically. Egg protein exhibited antioxidant protective effect by the progression of peroxide values and TBARS (Taguchi et al., 1988).

Fish proteins are another available rich source of animal protein that can be potentially used as a beneficial antioxidant. The fish protein hydrolysate is of particular interest, especially those from lower value fish. Fish protein hydrolysates are prepared by solubilizing the fish muscle proteins under alkaline conditions and leaving behind connective tissues, bones, and lipids. Protein isolates can then be purified by selective precipitation and hydrolyzed enzymatically. Theodore et al. (2008) assessed the antioxidant activity of hydrolyzed catfish protein by measuring the metal-chelating potential, radical-scavenging activity, ferric-reducing power, and oxygen radical absorbance capacity. The results showed that antioxidant activity somehow depended on the degree of achieved hydrolysis and also on the molecular size of fractions of the protein hydrolysate. Similarly, silver carp protein can be isolated and hydrolyzed to yield antioxidant beneficial products, with the activity proportional to the degree of hydrolysis, hydrolysis time, and molecular weight of hydrolysate fractions. This can make silver carp more marketable and turn it from low-market value to a value-added fish-derived product (Dong et al., 2008).

7.3.4.2 Antioxidative vegetable proteins *Soybean proteins* have been investigated for their antioxidant activity. Cinch proteins isolated from soybean are perhaps among the most commonly available vegetable proteins, and there are certainly numerous studies that have documented their antioxidant activity. Pihlanto (2006) attributed the antioxidative properties to peptides containing histidine in particular, with the mode of action being attributed to metal-chelating activity, oxygen quenching and hydroxyradical scavenging. Another area of active protein antioxidant research focuses on hydrolyzing the protein enzymatically and consequently obtaining smaller peptide chains, with increased antioxidant activity. Peñta-Ramos and Xiong (2002) showed that hydrolyzing soybean protein

with purified enzymes (pepsin and chymotrypsin) and purified enzymes (Alcalase®, Protamex™, and Flavourzyme™) resulted in active peptides that decreased the TBARS in meat. In another study, the commercial microbial proteases from *Bacillus subtilis* and *Bacillus licheniformis* were used and found to hydrolyze soybean protein into active hydrolysate fractions. Those fractions not only showed *in vitro* antioxidant activity such as the oxygen radical absorbance capacity (ORAC), 1,1,-diphenyl-2picrylhydrazyl (DPPH) radical-scavenging activity and metal chelation potential, but also real antioxidant activity in meat (Zhang et al., 2010). The explanation of the mode of action of the antioxidant activity of soybean protein and hydrolyzed peptides remained somehow inconclusive and ranging between radical and metal chelation. While Lv et al. (2009) isolated and identified various metal-chelating peptides chains ranging from 1–30 kDa, Lei et al. (2010) focused on attributing the antioxidant activity of soybean proteins and peptides to free radical–scavenging amino acids, such as Ala, Gly, Leu, Ile, Asx (Asp+Asn), Glx (Glu+Gln) and Trp, but not Val, Pro, Met, Phe, Lys, and His. Recently, Phoon et al. (2014) hydrolyzed a globulin protein from soy that is β-conglycinin, using both trypsinization and acid hydrolysis. The effect of several factors was investigated, but only select conditions showed an improvement in the antioxidant activity, especially pH 7 under low ionic strength. This was attributed to an increase in interfacial barrier property because of substantial increment of intermolecular β-sheets. Interestingly, various hydrolyzed fractions were prooxidant at pH3, which has implications on the processing method and specific end application.

Potatoes are an important source of protein, and there is also documented antioxidant activity with potato protein and potato protein hydrolysate. Storage proteins of sweet potato (*Ipomoe batatas* L.) were obtained and tested along with their corresponding hydrolyzed peptides for antioxidant activity. The *in vitro* testing included scavenging activity of the following: DPPH, hydroxyl radical by spectrophotometry, superoxide radical by spectrophotometry and prevention of Cu^{2+}-induced low-density lipoprotein peroxidation by Tis and protecting calf thymus DNA from hydroxyl radical–induced damages, and others. Although the antioxidant activity depended on the dose, the effect levels was dependent on and varied with the type of *in vitro* testing (Hou et al., 2005). Wang and Xiong (2008) took an approach focusing on protecting meat products by oxidation, using hydrolyzed potato protein as a functional ingredient. The potato protein isolate was hydrolyzed by Alcalase®, and tested against oxidation of myofibril protein from pork. Measurement of lipid oxidation and corresponding antioxidant activity of the potato protein hydrolysate showed a positive effect by TBARS, protein carbonyls (protein oxidation markers), ATPase assay (markers for monitoring structural changes in myosin), and UV spectra (marker for oxidation related structural changes in protein).

Chickpea is a widely used legume, with its seed rich in protein; hence, its protein has naturally been assessed for antioxidant activity, along with the corresponding hydrolysate. Water-soluble proteins from chickpeas have been shown to be thermally stable and are known to carry both high radical-scavenging potential (compared with Trolox as standard) and Fe^{2+} metal-chelating capacity (Arcan and Yemenicioğlu, 2007). Megías et al. (2007) took a different approach, however, where they went one step further and hydrolyzed the chickpea protein and also isolated peptide fractions and tested them individually. The activity that they examined consisted of copper chelation, measured by the

inhabitation of the oxidation and disappearance of β-carotene in a phosphate-buffered emulsion model. The antioxidant activity was somehow hard to pinpoint on one factor as it varied on the basis of the amino acid profile and sequence as well as the size of peptides. Interestingly, the study also suggested that these metal-chelating peptides could play a functional role outside the world of lipid oxidation and antioxidants in the area of food mineral fortification for increasing the bioavailability of the bound metals/minerals. Along the same lines of nutritional functional benefit, the study suggested that enzymatic hydrolysis of peptides (including those derived from chickpea protein isolate) are more active than the parent protein and retain a better structural integrity and no toxicity compared with their chemically hydrolyzed counterparts. Moreover, they also could also have additional bioactive properties and a potential as metal chelators. Those include angiotensin-converting enzyme (ACE) activity and calmodulin (CaM)-dependent enzymes. The former is an established hypertension marker (Aluko, 2008).

Yellow pea (Pisum sativum L.) is a rich source of hypoallergenic protein that has been gaining momentum in the last few years because of the allergenicity advantage it has over soybean and milk-abundant proteins. There has been a number of commercially available protein isolates from pea, used as functional ingredients, especially as emulsifiers and other functional ingredients or substituents to other ingredients. Pea protein hydrolysates are being assessed for their antioxidant potential. A relatively recent study (Pownall et al., 2010) found that multiple peptide fractions of pea protein hydrolysates outperformed the peptide glutathione in inhibiting linoleic acid oxidation, with the responsible mode of action being metal chelation.

Canola or rapeseed proteins have also been investigated as part of the search for additional hypoallergenic protein as a source of antioxidants as well as the search for a functional, bioactive use from a (somewhat) waste product or by-product. The canola meal is what typically results from deoiled canola seeds and is a by-product usually used as animal feed. Proteins from canola have higher molecular weights than the undesirable co-products such as glucosinolates, phytates, and other small molecules. Yoshie-Stark et al. (2008) precipitated the protein by pH control precipitation, and by ultrafiltration, analyzed the protein content and composition, and studied a variety of functional properties, including the radical-scavenging activity of the protein hydrolysate. Although the latter property was not found having the same magnitude as the radical-scavenging standard Trolox, it was indeed several folds more potent than soybean. Another study assessed specifically the hydroxyl radical–scavenging potential and the reducing antioxidant of peptides, derived from canola meal, enzymatically hydrolyzed with Alcalase® and Flavourzyme™. The result revealed an antioxidant property, which happened to be dose dependent (Xue et al., 2009). When different fractions of these peptides were separated using Sephadex gel filtration–based on molecular weights, all of them showed antioxidative properties. Cumby et al. (2008) had previously performed a similar study and also found that canola protein from canola meal could be hydrolyzed by Flavourzyme and Alcalase to yield protein hydrolysates that show dose-dependent antioxidant activity. Moreover, they showed that using Flavourzyme (both *endo*- and *exo*-peptidase) yields an antioxidant activity superior to that of Alcalase (*endo*-peptidase). Interestingly, an additional functional property of the protein hydrolysate was suggested, which

comprised improving the water-holding capacity and consequently the cooking yield of meat.

Palm kernel protein was isolated and hydrolyzed with trypsin, while optimizing the conditions (pH, temperature, substrate concentration and substrate to enzyme ratio). The result showed a hydrolysate with improved antioxidant activity as measured by antiradical activity and reducing power (Ng et al., 2013).

Even *hemp* (*Cannabis sativa L.*) protein isolate have been evaluated, along with their corresponding hydrolysates, as potential antioxidants. Hemp protein isolate was prepared from defatted hemp meal and subsequently hydrolyzed by several different enzymes: Flavourzyme (*exo*-peptidase), Alcalase (*endo*-peptidase), Neutrase® (neutral protease), Protamex (combined protease), in addition to Trypsin and Pepsin (Tang et al., 2009). The antioxidant activity (radical-scavenging and metal-chelating ability) was found to be generally dependent on the type of hydrolysis protease used and illustrated in a somehow correlated relationship to surface hydrophobicity.

7.4 Conclusions

Although protein antioxidants have their unique set of limitations, such as potential allergenicity (soy, milk, nut, and egg), bitterness (small proteins), and even potential rheological changes at high concentrations (increased viscosity and gelation) (Elias and Decker, 2010), in many cases there are ways to overcome these.

All these proteinaceous antioxidants do hold considerable potential as natural metal-chelating and radical-scavenging compounds, although, they have not been commercialized yet. The future will bring challenges to the food industry as to balancing the demand for better quality and convenient food, with the oxidative challenges of highly unsaturated fats and long distribution channels, especially in the case of processed foods. Today's product developers could use all the help they can get in finding safe and effective natural antioxidants, and proteins and peptide can definitely become very important instruments in the toolbox of natural oxidation management solutions.

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8

Nutritional Aspects of Proteins

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8.1 Introduction

Protein represents the most important and quantitatively dominant element of all organisms and is the basic requirement for life (Wennemer et al., 2006). The world's population is increasing rapidly next to diminishing land, water and food resources; thus, it is critical to define accurately the (i) quality of protein consumed by humans and (ii) the amount of protein required to meet human amino acid needs. The optimum match between dietary protein and amino acid supply and human needs is vital to support the health and well-being of human populations. Nutritional application of proteins is governed by the knowledge of their nutritional value and of the human requirements for protein and amino acids. This chapter discusses the most relevant method for evaluation of protein quality, the biological appropriateness of the pig as a model for assessment of protein quality, and the recent development in amino acid requirement estimates for humans.

8.2 Evaluation of protein quality

The nutritional quality of a protein source for humans or animals may be described as the capacity for this protein to meet requirements for amino acids and nitrogen (Schaafsma, 2005). Protein quality differs widely among food products and is defined by its amino acid composition and digestibility (Woodward et al., 2011). For many years, protein quality was evaluated using growth bioassays in rats by comparing the growth response from feeding the test product to that of feeding a reference protein (e.g., casein); values were expressed as protein efficiency ratio (PER), net protein utilization, and

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biological value. The PER, the first adopted method and the most widely used bioassay for evaluating protein quality, is expressed as body weight gain (g) per gram protein consumed. Although rat growth bioassays are simple and relatively inexpensive, they do not accurately reflect the protein quality for humans; that is, while the amino acid composition of the test protein does not change, its postprandial amino acid utilization differs between humans and rats. For instance, the sulfur amino acid requirements of rats are 50% greater than that of humans because of fur growth (Schaafsma, 2005). The only true, valid approach for assessment of protein quality for human use is the nitrogen balance with a human volunteer, but such an approach is too expensive for routine use (Schaafsma, 2005). In 1989, the Food and Agricultural Organization (FAO) and the World Health Organization (WHO) formed an Expert Consultative Committee on Protein Quality Evaluation, which recommended the Protein Digestibility Corrected Amino Acid Score (PDCAAS) as the protein quality evaluation alternative to that of the rat growth bioassays. Despite its many shortcomings, the PDCAAS has been accepted for over the past 20 years as the routine procedure for protein quality evaluation of food products for humans.

8.2.1 Measuring protein digestibility

The nutritional value of a protein is inherently associated with both its composition and its digestibility. The rate and extent of digestibility of a protein and absorption of its amino acid constituents is the main determinant of its quality and utilization. Factors that affect protein digestibility are intrinsic and extrinsic. Extrinsic factors are somewhat independent of the protein itself and include the composition of other dietary constituents. Intrinsic factors mainly include the amino acid composition in itself (i.e., polar and nonpolar amino acid residues) and as such the degree of cross-linking and hydrogen bonding between amino acid residues, which confers the protein chemical structure. The processes that involve protein digestion and amino acid absorption are complex and relatively well understood. Utilization of dietary protein comprises numerous steps, including protein digestion in the stomach and small intestine, as well as absorption of peptides and free amino acids by the small intestine. The first step in amino acid utilization following protein digestion occurs at the luminal–apical membrane interface (i.e., the epithelial brush border), where tri- and dipeptides may be either be absorbed intact or hydrolyzed into their constituents dipeptide and single amino acids before transapical transport (Trottier and Manjarín, 2012). The intracellular fates of amino acids following brush border membrane transport include (i) metabolism, (ii) *in situ* protein synthesis (e.g., peptidases, apoproteins, and mucins), (iii) efflux back to the intestinal lumen in exchange for luminal amino acid influx, (iv) and efflux to the portal blood via the basolateral membrane (Trottier and Manjarín, 2012); hence, measurement of digestibility of a protein or its amino acids encompasses all of these steps. Measurement of protein digestibility alone—nitrogen × 6.25—is at best a crude estimation of its amino acid bioavailability or absorption. Although fundamental understanding of protein digestion and amino acid absorption is critical in order to appreciate the complexity behind the assessment of protein quality and as such of its nutritional value and application, further discussion on this subject is beyond the scope of this chapter.

Two pools exist from which protein digestibility may be derived: (i) the fecal pool, which has been routinely used in the estimation of protein digestibility and (ii) the ileal digesta pool. Although both fecal and ileal amino acid digestibility approaches have important limitations, the ileal digestibility approach offers greater value as discussed later in this chapter. The fecal protein digestibility approach is discussed in the following paragraphs.

8.2.1.1 Fecal protein digestibility Fecal protein digestibility is calculated on the basis of nitrogen output in feces relative to nitrogen intake. The fecal digestibility approach is simple and noninvasive as it only requires sampling of feces, measurement of nitrogen intake and fecal output, and the use of indigestible marker (internal or external) (Sales and Janssens, 2003). There are, however, two major arguments against the use of fecal protein digestibility: (i) the protein digestibility itself is at best a crude estimate of its post gut amino acid availability and (ii) the fecal pool composition does not accurately reflect the end result of dietary protein digestion. The massive microbial activity in the large intestine alters the amino acid composition of the ingested protein (Metges, 2000); the much greater microbial count and longer transit time in the large intestine relative to that of the small intestine (Hovgaard and Brondsted 1996; Autenrieth, 1998) favor the net production of microbial synthesis of amino acids from both nonspecific and dietary nitrogen sources (Sauer et al., 1975); thus, fecal amino acid digestibility may be either overestimated (for most of the amino acids) or underestimated depending on the diet composition and the amino acid in question and as such has led to much debate over the value of the PDCAAS approach.

8.2.1.2 The protein digestibility corrected amino acid score The PDCAAS is calculated as the product of the true fecal protein digestibility of the test protein and the *limiting* amino acid score of the test protein; the *limiting* amino acid score is derived from the ratio of the *first-limiting* amino acid in a gram of target food protein to that in a reference protein. The true fecal protein digestibility is evaluated in rats and defined as the difference between the intake of protein nitrogen and output of fecal nitrogen, with fecal nitrogen corrected for metabolic (or endogenous) fecal nitrogen. The endogenous nitrogen losses are later described in this chapter. The PDCAA equation is as followed:

$$\begin{aligned}\text{PDCAAS, \%} = {} & \text{True fecal protein digestibility} \\ & \times [(\text{mg of first limiting amino acid in 1 g of the test protein})/ \\ & \quad (\text{mg of the same amino acid in 1 g of the reference protein})]\end{aligned}$$

Several shortcomings associated with the PDCAAS method have been identified. One that is obvious is the lack of accounting for the digestibility and bioavailability of amino acids; for instance, protein digestibility does not always reflect the digestibility of individual dietary indispensable amino acids (see Table 8.1). Table 8.1 depicts the discrepancy between fecal protein and amino acid digestibility of pulses, peanut and wheat fed to growing pigs. Fecal protein digestibility value for pulses and peanut is higher than that of methionine, threonine, and tryptophan. Protein digestibility in several of the food

Table 8.1 Fecal crude protein and amino acid digestibility (%) determined in the growing pig

	Protein	Lys	Met	Cys	Thr	Trp
Pea flour	88	92	77	84	87	82
Pea (autoclaved)	83	85	62	85	78	72
Pintobean (canned)	79	78	45	56	72	70
Lentil (autoclaved)	85	86	59	75	76	63
Fababean (autoclaved)	86	85	59	75	76	63
Soyabean	90	87	82	82	84	89
Peanut	96	90	85	89	89	94
Wheat	93	83	94	97	91	96

Source: Adapted from FAO (2013).

products presented underestimates lysine digestibility, while protein digestibility of wheat grossly overestimates that of lysine. Additional critical issues remain to be addressed for improving the value of the PDCAAS (Schaafsma, 2005; FAO, 2013), including the lack of accrediting extra nutritional value to high-quality proteins, the overestimation of protein quality of food products containing antinutritional factors (Gilani and Sepehr, 2003; Gilani et al., 2005), and the overestimation of the quality of poorly digestible proteins supplemented with limiting amino acids (FAO, 2013).

Because fecal protein digestibility does not consistently reflect that of individual amino acid digestibility, a score based on individual dietary indispensable amino acid digestibility in food products rather than based on the overall protein digestibility is critically needed. Additionally, there is increasing interest in the metabolic effects of specific individual dietary amino acids; thus, accurate information on the amounts of digestible or preferably bio-available amino acids in food protein is vital. How well dietary protein can match the demand for amino acids allows prediction of dietary protein utilization. It has been recently recognized by the FAO of the United Nations Expert Consultation Committee on Dietary Protein Quality Evaluation in human nutrition that the PDCAAS does not allow for such prediction (FAO 2013). An alternative protein quality measure has been set forth as the digestible indispensable amino acid score (DIAAS).

8.2.2 The digestible indispensable amino acid score

The DIAAS differs from the PDCAAS in that instead of the true fecal protein digestibility, the digestibility values of each individual indispensable amino acid are used to calculate the digestible indispensable amino acid (DIAA) reference ratios of the food or diet of interest. The DIAA reference ratio is calculated as follows:

$$\text{DIAA reference ratio} = [(\text{mg of digestible dietary indispensable amino acid in 1 g of the dietary protein})/(\text{mg of the same dietary indispensable amino acid in 1 g of the reference protein})]$$

The lowest DIAA ratio value is selected and multiplied by 100 to obtain the DIAAS. Two critical issues remain: (i) the accuracy of the true fecal amino acid digestibility values and (ii) the dearth of amino acid digestibility values in humans. As discussed earlier, sampling from fecal pool leads to erroneous estimation of unabsorbed amino acids because of the microbial utilization and synthesis of amino acids in the large intestine. For most amino acids in the majority food products tested, amino acid digestibility values obtained by the fecal method are inaccurate in comparison to those obtained by sampling from the ileal digesta pool.

8.2.2.1 Ileal digestibility An alternative to the fecal digestibility approach is the assessment of digestibility by measuring nitrogen or the amino acid of interest in the intestinal digesta pool collected at the distal end of the ileum before its junction with the proximal large colon. Sampling at this site essentially avoids the microbial metabolism of dietary amino acids in the large intestine. Such an approach is supported by the fact that there is very little evidence for absorption and portal contribution of dietary amino acids from the large intestine (Darragh et al., 1994; Torrallardona et al., 2003; Moughan and Stevens, 2012). There are numerous data, albeit reported earlier, demonstrating the vast difference between ileal and fecal digestibility values for protein and individual amino acids; for instance, in less well-developed large intestinal segments such as that found in the nursing animal, the difference between apparent ileal and fecal digestibility is relatively smaller. In the piglet (Moughan et al., 1990) and preruminant calf (Moughan et al., 1989) fed bovine milk, apparent ileal protein digestibility was 90 and 88%, respectively, and 97 and 94%, respectively, for fecal digestibility. In contrast, in the growing pig fed meat and bone meal–based diet (Moughan et al., 1994), apparent and fecal protein digestibility values differed by as much as 22%, that is, 66 and 81%, respectively. Li et al. (1998) reported remarkable difference between ileal and fecal digestibility values of protein and individual amino acids in growing pigs fed either soybean flour containing active trypsin inhibitors or autoclaved soybean flour. Fecal digestibility of raw soybean meal flour was as high as 77% compared to 37% for ileal, a difference of as much as 40%. Autoclaved soybean flour ileal digestibility increased to 77% and that of fecal to 90%, with a relatively large difference of 13% remaining. In Table 8.2, ileal and fecal digestibility values differ for four indispensable amino acids in adult ileostomate and healthy human subjects receiving a meat, vegetable, cereal and dairy product-based diet.

The drawback of the ileal digestibility approach is its restricted achievability in human subjects; that is, the limited data available in the literature for humans have been obtained from subjects having undergone large intestinal resection. Data reported by Gaudichon et al. (2002) (Table 8.3) in human subjects comparing the nutritional value of soya to that of milk further exemplify the increased accuracy when assessing individual amino acid digestibility compared to protein alone. The protein (nitrogen $\times$ 6.25) digestibility of soya (91.7%) was lower than that of milk (95.3%). In contrast, when averaging the individual amino acid digestibility values, there was no apparent difference between soya and milk; nonetheless, digestibility values for 50% of the amino acids reported differed between soya and milk. Of the indispensable amino acids only threonine, valine, and histidine differed.

Table 8.2 Ileal and fecal digestibility amino acid values in adult ileostomate and healthy human subjects receiving a meat, vegetable, cereal, and dairy product–based diet

	Digestibility coefficient, %			
Amino acid	Ileal $n = 5$	Fecal $n = 5$	Statistical significance	Fecal–ileal difference, %
Arginine	90	93	[a]	3
Aspartate	87	90	[a]	3
Serine	87	92	[c]	5
Threonine	85	89	[b]	4
Proline	90	95	[b]	5
Glycine	72	87	[c]	15
Phenylalanine	90	91	[c]	1
Methionine	93	83	[c]	−10
Tryptophan	77	83	[a]	6

Source: From Rowan et al. (1994).

Table 8.3 Apparent ileal nitrogen and amino acid digestibility values (%) of milk and soya fed to human subjects

Amino acid	Milk $n = 7$	Soya $n = 6$
Aspartate + asparagine	94.3 ± 2.1	93.2 ± 4.0
Serine	92.0 ± 2.5	93.2 ± 3.9
Glutamate + glutamine	95.3 ± 2.0	96.6 ± 2.8
Proline	96.1 ± 2.2	92.8 ± 3.8[a]
Glycine	91.6 ± 4.0	90.1 ± 5.1
Alanine	95.9 ± 1.9	92.3 ± 2.5[a]
Tyrosine	99.3 ± 0.4	96.8 ± 1.5[a]
Threonine	93.4 ± 2.3	89.0 ± 4.9[a]
Valine	95.9 ± 1.9	92.5 ± 3.5[a]
Isoleucine	95.4 ± 1.8	93.5 ± 3.1
Leucine	95.1 ± 2.2	93.3 ± 3.0
Phenylalanine	95.6 ± 2.3	95.5 ± 2.3
Lysine	94.9 ± 2.7	95.0 ± 2.5
Histidine	94.9 ± 2.7	91.7 ± 1.7[a]
Average amino acid digestibility[b]	95.3 ± 1.8	93.8 ± 3.0
Nitrogen digestibility	95.3 ± 0.9	91.7 ± 1.8[a]

Source: Adapted from Gaudichon et al. (2002).
[a] Differs from milk at $P < 0.05$.
[b] Amino acid digestibility values were weighted by the proportion of each amino acid in milk and soya.

Clearly, dietary protein should be considered as a source of amino acids as individual nutrients and its nutritional value should reflect its ability to satisfy the metabolic needs for individual amino acids rather than for nitrogen. Furthermore, humans and animals do not have a dietary requirement for protein, but have minimum dietary requirements for essential amino acids; therefore, it is unquestionable that amino acid digestibility better reflects the amounts of amino acids absorbed and should be used in calculating DIAAS. Although amino acid digestibility does not necessarily equate bioavailability, it is the closest to its estimation; thus, for foods susceptible to damage from food processing or cooking before consumption, "reactive" rather than "total" lysine contents (Moughan and Rutherfurd, 2008) and the true ileal digestibility of reactive lysine (lysine availability) rather than that of total lysine should be determined and used in the calculation of DIAAS.

8.2.2.2 Correcting nitrogen and amino acid digestibility for endogenous nitrogen losses Another limitation in assessment of digestibility is the difficulty in accurately determining the *de novo* contribution of nitrogenous products, commonly called endogenous nitrogen losses, and delineate this pool from that of dietary origin. There are two sources of endogenous losses: (i) the basal losses that are independent of diet and mainly originate from saliva, desquamated epithelial cells, basal enzymes, and mucus; and (ii) the diet- or ingredient-specific endogenous losses that vary between foods and diet composition. If there is no correction for endogenous losses, the digestibility is overestimated, hence the term "apparent" digestibility; correcting for basal endogenous losses allows the estimation of a standardized digestibility value (Rademacher et al., 1999) and correction for both basal- and ingredient-specific endogenous losses allows the estimation of a "true" digestibility value. Because the approaches for the estimation of food ingredient–specific endogenous amino acid losses are limited, cost prohibitive, and invasive, there are currently no data available to "truly" estimate "true" protein and amino acid digestibility of a food product. The term "standardized" has yet to be adopted by the human nutrition scientific community, which still makes reference to "true" digestibility.

The FAO expert consultation committee concluded that digestibility should be based on the true ileal digestibility of each amino acid, if possible, determined in humans, but acknowledged that measurement of amino acid digestibility values may be best suited using the growing pig model with the pig being above that of the rat model (Stein et al., 2007; FAO, 2013). Current data on digestible or bioavailable amino acids in protein sources for humans are critically lagging behind that of animal models, in particular the pig.

8.2.3 The growing pig as a model for protein and amino acid digestibility measurement

The laboratory rat has long been used as a suitable animal model (Deglaire et al., 2009); however, because of the anatomical and physiological similarities of the digestive tracts,

the pig resembles the human in more ways than any other nonprimate mammalian species in its nutrient requirements, which provides a basis for the use of the pig in many human nutritional studies (Miller and Ullrey, 1987). Rates of protein synthesis in 75-kg pigs are nearly identical to those in 77-kg humans, the physiological characteristics of the gastrointestinal tract are probably due to the omnivorous diet they have evolved on, which is vastly different from carnivores, ruminants, rabbits, and rodents (Swindle and Smith, 1998). The stomachs of both the human and the pig are of the glandular type and lined with cardiac, gastric, and pyloric mucosa; however, the pig stomach is two to three times larger, and the cardiac mucosa occupies a greater portion of the stomach (Kararli, 1995). The villi that cover the luminal surface of the small intestine in both humans and pigs are "finger shaped," unlike the "tongue shape" in rats (Kararli, 1995). The topography of the pig's portal vein, mesenteric vessels, and duodenum is similar to that in man. The morphology of the colon in pigs and humans appears to be similar with all sections of the colon being sacculated. The human has a cecal region that is continuous with the colon, whereas the pig cecum is much larger (Kararli, 1995).

Functionally, both the liver and pancreas are similar to humans (Swindle and Smith, 1998). Metabolically, the liver functions similar to humans and has been used for xenoperfusion protocols for humans in hepatic comas. The pancreas of the pig is similar in color, texture, and density and has a true capsule similar to that of the human. The pancreatic drainage system and secretions rate in humans and pigs are similar (1–3 mL/min). Intestinal development of precocial species such as swine occurs mostly *in utero*, with major developments occurring before and after birth (Guilloteau et al., 2010). In both species, prenatal development in gastrointestinal function occurs mostly during the third trimester, and the small intestine is relatively mature at birth (Guilloteau et al., 2010). The porcine digestive enzymes resemble more closely human development in fetal and neonatal periods since, from a "gut point of view", pigs, like man, have a precocious mode of development. In contrast, there are large differences in gastrointestinal tract maturation around birth and weaning between rodents and man. Digesta transit times and digestive efficiencies are comparable; however, the porcine lower small intestine has a higher microbial density, so pigs are able to degrade certain indigestible carbohydrates to a higher degree. Postabsorptive metabolism is also similar in many aspects (Guilloteau et al., 2010).

Apparent and true ileal dietary amino acid digestibility values in adult human subjects and pigs given a meat-vegetable-cereal-dairy product–based diet were shown to be remarkably similar (Tables 8.4 and 8.5) (Rowan et al., 1994). When expressed on a true (i.e., standardized) basis, the number of amino acids that differ in terms of digestibility was less than when expressed on an apparent basis, emphasizing the importance of using the true values in the calculation of DIAAS. Conversely, threonine digestibility values did not differ when expressed on an apparent basis and increased above 100% in humans when expressed on a true digestible basis. Humans may secrete considerably more of mucins; mucins are endogenous proteins rich in threonine residues and not readily digestible, which leads to an increase in threonine endogenous secretion losses and the consequential overestimation of true digestibility estimate for threonine under the diet tested herein.

Table 8.4 Mean apparent ileal amino acid and nitrogen digestibility (%) for ileostomized adult humans (65 kg body weight) and growing pigs (25 kg body weight) receiving a meat-vegetable-cereal-dairy product–based diet

Amino acid	Human $n = 5$	Pig $n = 6$	SE	Statistical Significance
Lysine	93.6	92.6	0.84	NS
Arginine	90.2	92.1	0.66	*
Histidine	90.2	91.9	0.97	NS
Aspartate	87.3	90.4	0.93	**
Serine	86.5	89.9	1.06	**
Threonine	84.7	87.0	0.92	NS
Glutamate	93.6	94.6	0.44	NS
Proline	89.9	86.8	1.22	NS
Glycine	71.5	77.9	2.21	*
Alanine	88.1	88.9	0.65	NS
Valine	89.7	91.5	0.64	**
Isoleucine	90.9	92.8	0.59	**
Leucine	91.9	94.7	0.76	NS
Tyrosine	88.7	91.1	0.76	NS
Phenylalanine	89.6	91.0	0.57	**
Cysteine	85.5	82.9	1.31	*
Methionine	93.1	90.5	0.77	**
Tryptophan	76.7	80.8	2.31	NS
Nitrogen	86.9	86.0	0.72	NS

Source: Adapted from Rowan et al. (1994).
*$P < 0.05$; **$P < 0.01$.

Deglaire et al. (2009) compared the ileal nitrogen and amino acid digestibility of casein and hydrolyzed casein between humans and pigs. Overall, the nitrogen and amino acid digestibility of hydrolyzed casein was slightly but significantly lower compared to that of intact casein in both humans (94.1 vs. 92.3% for nitrogen digestibility) and pigs (97.6% vs. 95.0% for nitrogen digestibility). Good agreement was found between humans and pigs for ranking protein sources, again particularly regarding true ileal nitrogen digestibility (Deglaire et al., 2009). Table 8.6 depicts the apparent and true ileal amino acid digestibility values for mixed meals based on intact casein and hydrolyzed casein.

The FAO report of the expert consultation committee made strong suggestion as to the use of the pig as a model animal to allow routine determination of the true ileal digestibility of amino acids and nitrogen in foods for humans (FAO, 2013). The recent National Research Council (NRC) on nutrient requirements for swine has published a comprehensive compilation of amino acid values of common plant protein sources (Table 8.7) and their corresponding apparent and standardized digestibility values (Tables 8.8 and 8.9, respectively) obtained from the peer-reviewed literature (NRC, 2012). These digestibility values offer a critical source of information to calculate the amino acid pattern of the protein source (i.e., mg of amino acid per mg of protein) and the digestible indispensable amino acid reference ratio needed in the assessment of the DIAAS for humans.

Table 8.5 Mean true ileal amino acid and nitrogen digestibility (%) for ileostomized adult humans (65 kg body weight) and growing pigs (25 kg body weight) receiving a meat-vegetable-cereal-dairy product–based diet

Amino acid	Human ($n = 5$)	Pig ($n = 6$)	SE	Statistical Significance
Lysine	98.7	97.5	0.41	NS
Arginine	98.3	97.7	0.48	NS
Histidine	99.2	97.5	0.65	NS
Aspartate	99.2	97.8	0.61	NS
Serine	99.4	99.5	0.61	NS
Threonine	105.1	89.9	1.36	**
Glutamate	98.5	98.2	0.34	NS
Proline	100.9	98.1	1.13	NS
Glycine	92.4	90.5	1.28	NS
Alanine	98.9	98.0	0.48	NS
Valine	99.5	98.6	0.53	NS
Isoleucine	99.5	97.6	0.64	NS
Leucine	100.1	100.8	0.38	NS
Tyrosine	99.2	98.0	0.51	NS
Phenylalanine	99.9	97.5	0.69	*
Cysteine	100.3	92.2	1.95	***
Methionine	98.4	96.0	0.64	**
Tryptophan	99.1	96.8	2.33	NS
Nitrogen	98.0	98.7	1.17	NS

Source: Adapted from Rowan et al. (1994).
*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

Table 8.6 Apparent and true ileal amino acid digestibility (%) for meals based on intact casein fed to growing pigs and adult humans

	Apparent		True	
	Human adult	Pig	Human adult	Pig
Histidine	80.8	90.8***	94.7	99.0***
Isoleucine	83.8	92.2***	94.1	97.2
Leucine	90.0	94.3***	97.2	98.9
Lysine	91.8	95.9***	97.4	99.3**
Phenylalanine	88.9	95.7***	96.3	99.2***
Threonine	75.7	78.8*	93.3	94.5
Tyrosine	88.7	96.0***	97.2	99.4***
Valine	84.6	90.9**	93.7	96.6

Source: Adapted from Deglaire et al. (2009).
*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

Table 8.7 Crude protein and total amino acid composition of common plant protein sources (g/100 g Dry Matter)

Ingredient	CP[a]	Arg	His	Ile	Leu	Lys	Met	Phe	Thr	Trp	Val
Barley	11.33	0.53	0.27	0.37	0.72	0.40	0.20	0.53	0.36	0.13	0.52
Beans, faba	27.16	2.43	0.72	1.13	1.94	1.65	0.19	1.19	0.91	0.22	1.22
Beans, phaselous beans	22.90	1.91	0.74	1.17	2.05	1.67	0.29	1.41	1.12	0.27	1.33
Canola, full fat	22.06	1.00	0.60	0.60	1.14	1.01	0.38	0.73	0.83	0.23	0.83
Corn, yellow dent	8.24	0.37	0.24	0.28	0.96	0.25	0.18	0.39	0.28	0.06	0.38
Corn, nutridense	9.02	0.44	0.26	0.32	1.09	0.27	0.20	0.43	0.31	0.07	0.44
Kidney beans, extruded	20.03	1.28	0.19	0.94	1.90	1.51	0.25	1.35	0.94	NA[b]	1.13
Lentils	26.00	2.05	0.78	1.00	1.84	1.71	0.18	1.29	0.84	0.21	1.27
Lupins	32.44	3.61	0.92	1.39	2.31	1.58	0.21	1.34	1.20	0.26	1.32
Millet	11.90	0.57	0.29	0.49	1.22	0.37	0.28	0.55	0.45	0.17	0.66
Oats	11.16	0.73	0.24	0.41	0.79	0.49	0.68	0.52	0.42	0.14	0.63
Peanut meal, expelled	44.23	5.20	1.04	1.46	2.65	1.55	0.50	2.12	1.16	0.33	1.75
Peas, chick peas	20.33	2.25	0.84	0.91	1.61	1.41	0.30	1.23	0.91	NA	1.02
Peas, field peas	22.17	1.91	0.53	0.94	1.56	1.63	0.21	1.02	0.83	0.21	1.03
Rice	7.87	0.44	0.33	0.32	0.56	0.35	0.25	0.44	0.23	0.11	0.42
Rice bran	15.11	1.24	0.42	0.51	1.04	0.67	0.30	0.65	0.56	0.19	0.78
Rice, polished	8.00	0.52	0.18	0.34	0.67	0.30	0.18	0.39	0.26	0.10	0.49
Rice protein concentrate	67.51	5.26	1.65	2.91	5.31	2.21	1.77	3.52	2.12	0.81	4.13
Rye	11.66	0.70	0.25	0.34	0.70	0.43	0.16	0.50	0.37	0.10	0.49
Sorghum	9.36	0.36	0.21	0.36	1.21	0.20	0.16	0.48	0.30	0.07	0.46
Soybeans, full fat	37.56	2.45	0.88	1.60	2.67	2.23	0.55	1.74	1.42	0.49	1.73
Soybeans, high protein, full fat	42.77	3.16	1.07	1.51	3.34	2.50	0.57	2.25	1.57	0.48	1.76
Soy protein concentrate	65.20	4.75	1.70	2.99	5.16	4.09	0.87	3.38	2.52	0.81	3.14
Soy protein isolate	84.78	6.14	2.19	3.83	6.76	5.19	1.11	4.40	3.09	1.13	4.02
Wheat, hard red	14.46	0.60	0.34	0.47	0.91	0.39	0.22	0.64	0.40	0.17	0.58
Wheat, soft red	10.92	0.52	0.28	0.34	0.68	0.35	0.22	0.52	0.35	0.14	0.47
Wheat gluten	72.11	2.67	1.66	2.66	5.06	1.27	1.08	3.91	2.42	1.03	2.88

Source: From NRC (2012).
[a]Crude protein (CP) determined as nitrogen × 6.25.
[b]Not available (NA).

8.2.4 Reference protein amino acid pattern

Earlier, we presented and discussed in detail the tools needed to arrive at the best estimation of the *numerator part* of the DIAA reference ratio. The denominator of the DIAA ratio differs depending on the choice of the reference protein. The recommended reference proteins used for calculating protein quality for dietary assessment are based on the age of the human subject. The recommended amino acid scoring patterns and reference protein based on the FAO expert consultation committee (FAO, 2013) are as follows: for infants (i.e., birth–6 months), the pattern of amino acids in breast milk (Heine et al., 1991; Davis et al., 1994; Villalpando et al., 1998); for young children (i.e., 6 months–3 years), the whole body pattern for the 0.5 year-old infant; and for older children, adolescents,

Table 8.8 Apparent protein and amino acid digestibility of common plant sources (%)

Ingredient	CP[a]	Arg	His	Ile	Leu	Lys	Met	Phe	Thr	Trp	Val
Barley	66	75	73	72	74	66	76	76	60	73	71
Beans, faba	73	88	76	77	79	82	65	77	70	61	73
Beans, phaselous beans	49	70	NA[b]	50	52	65	52	41	50	50	49
Canola, full fat	66	81	77	73	73	70	78	73	64	66	66
Corn, yellow dent	65	75	77	73	82	60	77	78	61	62	71
Corn, nutridense	74	75	77	76	83	65	79	80	62	65	72
Kidney beans, extruded	64	84	58	66	65	82	NA	70	67	NA	58
Lentils	73	84	76	73	73	77	66	72	66	62	70
Lupins	80	92	83	83	82	82	75	82	76	78	77
Millet	79	82	85	83	87	74	72	85	75	84	81
Oats	62	85	81	73	75	70	79	81	59	59	72
Peanut meal, expelled	79	93	79	78	79	73	80	86	70	73	75
Peas, chick peas	73	87	78	76	77	82	72	77	68	NA	72
Peas, field peas	73	87	78	76	77	82	72	77	68	63	72
Rice	80	88	80	73	77	80	85	75	72	63	73
Rice bran	57	85	78	64	65	72	74	68	61	64	66
Rice, polished											
Rice protein concentrate											
Rye	69	73	71	68	71	64	76	76	59	67	67
Sorghum	63	68	64	69	78	53	74	76	54	65	66
Soybeans, full fat	74	84	78	75	75	79	75	77	71	79	73
Soybeans, high protein, full fat	82	93	88	88	87	88	88	89	78	85	84
Soy protein concentrate	85	93	89	89	89	89	90	88	82	85	87
Soy protein isolate	84	93	86	86	88	90	84	87	79	84	83
Wheat, hard red	77	83	83	82	83	72	83	85	71	82	79
Wheat, soft red		83	84	84	85	73	85	87	72	81	80
Wheat gluten	89	83	86	86	90	78	83	88	68	76	83

Source: From NRC (2012).
[a]Crude protein (CP) determined as nitrogen × 6.25.
[b]Not available (NA).

and adults, the whole body pattern for 3- to 10-year-old children (see Table 8.10). The ratio is calculated for each dietary indispensable amino acid and the lowest value designated as the DIAA score (DIAAS) and used as an indicator of dietary protein quality. The DIAAS can have values below or in some circumstances above 100%. As presented earlier in this chapter, the DIAA reference ratio is calculated as follows:

$$100 \times [(\text{mg of digestible dietary indispensable amino acid in 1 g of the dietary protein})/(\text{mg of the same dietary indispensable amino acid in 1 g of the reference protein})].$$

For the purpose of an example, the DIAA reference ratios for barley are presented in Table 8.11, except for the total sulfur amino acids and the total aromatic amino acids. The total lysine concentration in barley is 0.40% with a crude protein concentration of 11.33% (Table 8.7); thus, barley protein is composed of 3.53% total lysine

Table 8.9 Standardized crude protein and amino acid digestibility values of common plant sources (%)

Ingredient	CP[a]	Arg	His	Ile	Leu	Lys	Met	Phe	Thr	Trp	Val
Barley	79	85	81	79	81	75	82	81	76	82	80
Beans, faba	79	90	79	81	82	85	73	80	78	64	78
Beans, phaselous beans	NA[b]	72	58	54	55	68	55	44	55	55	53
Canola, full fat	64	84	80	74	76	73	81	77	70	71	71
Corn, yellow dent	80	87	83	82	87	74	83	85	77	80	82
Corn, nutridense	83	83	82	85	87	79	83	86	78	76	81
Kidney beans, extruded	76	94	66	72	71	85	NA	74	76	NA	65
Lentils	NA	86	79	77	76	79	71	75	73	68	75
Lupins	86	93	86	85	85	85	81	84	82	82	81
Millet	88	89	90	89	91	83	75	91	86	97	87
Oats	NA	90	85	81	83	76	83	84	71	75	80
Peanut meal, expelled	87	93	81	81	81	76	83	88	74	76	78
Peas, chick peas	80	90	82	81	81	85	77	80	76	NA	78
Peas, field peas	80	90	82	81	81	85	77	80	76	69	78
Rice	94	93	85	81	83	89	87	80	85	77	86
Rice bran	NA	89	87	69	70	78	77	73	71	73	69
Rice, polished											
Rice protein concentrate											
Rye	83	79	79	78	79	74	81	82	74	76	77
Sorghum	77	80	74	78	83	74	79	83	75	74	77
Soybeans, full fat	79	87	81	78	78	81	80	79	76	82	77
Soybeans, high protein, full fat	92	97	92	92	91	92	92	93	87	89	90
Soy protein concentrate	89	95	91	91	91	91	92	90	86	88	90
Soy protein isolate	89	94	88	88	89	91	86	88	83	87	86
Wheat, hard red	88	91	88	89	89	82	88	90	84	88	88
Wheat, soft red	NA	89	90	90	87	82	90	91	85	88	87
Wheat gluten	91	85	87	87	91	80	85	89	72	83	85

Source: From NRC (2012).
[a]Crude protein (CP) determined as nitrogen × 6.25.
[b]Not available (NA).

Table 8.10 Recommended amino acid scoring patterns for different human age groups, mg/g protein

Age group	His	Ile	Leu	Lys	SAA[d]	AAA[e]	Thr	Trp	Val
Infant (birth to 6 months)[a]	21	55	96	69	33	94	44	17	55
Child (6 months to 3 year)[b]	20	32	66	57	27	52	31	8.5	43
Older child, adolescent, adult[c]	16	30	61	48	23	41	25	6.6	40

Source: Adapted from FAO (2013).
[a]Indispensable amino acid profile in human milk protein (mg amino acid/g human milk protein).
[b]Amino acid profile in whole body tissue protein of 6-month-old child (mg amino acid/g whole body tissue protein).
[c]Amino acid profile in whole body tissue protein of adolescents, that is, 11–14 years old (mg amino acid/g whole body tissue protein).
[d]Sulfur amino acids (Met + Cys).
[e]Aromatic amino acids (Phe + Tyr).

Table 8.11 True ileal digestible indispensable amino acid (IAA) reference ratios for adolescents fed barley[a]

	His	Ile	Leu	Lys	Thr	Trp	Val
Concentration, g/100 g barley[a]	0.27	0.37	0.72	0.40	0.36	0.13	0.52
Concentration, mg/g barley protein[b]	24	33	64	35	32	11	46
True ileal digestibility, %[c]	81	79	81	75	76	82	80
True ileal digestible content, mg/g barley protein	19	26	51	27	24	9	37
IAA reference pattern, mg/g whole body tissue protein[d]	16	30	61	48	25	6.6	40
Digestible IAA reference ratio	1.21	0.86	0.84	0.55	0.97	1.43	0.92

[a]Values are from Table 8.7.
[b]Calculations based on crude protein concentration of 11.33%.
[c]Values are from Table 8.9.
[d]Values from Table 8.10.

(i.e., 0.40% total lysine in 11.33% crude protein) or 35 mg total lysine/g barley protein, as shown in Table 8.11. With a standardized (true) ileal lysine digestibility of 75% in barley (Table 8.9), the true ileal digestible amount of lysine in barley is 27 mg/g barley protein (i.e., 75% × 35 mg total lysine/g barley protein). The same calculations are applied to all amino acids presented in Table 8.11. The lowest value for barley is that of lysine, with a DIAA ratio of 0.55. The DIAAS for barley is thus 55%.

8.3 Protein and amino acid requirements for humans

8.3.1 Protein requirement

8.3.1.1 Recommended protein intake Protein and amino acid requirements in the United States are set by the Food and Nutrition Board, National Academy of Sciences and published as the Dietary Reference Intakes (DRI 2005). The most recent DRI was published in 2005, and definitions of key DRI terms are presented in Table 8.12. The adult protein recommended dietary allowance (RDA) is 0.8 /g/kg of body weight/day,

Table 8.12 Key dietary reference intake terms

Term	Definition
Recommended Dietary Allowance (RDA)	The average daily dietary nutrient intake level sufficient to meet the nutrient requirement of nearly all (97–98%) healthy individuals in a particular life stage and gender group.
Adequate Intake (AI)	The recommended average daily intake level based on observed or experimentally determined approximations or estimates of nutrient intake by a group (or groups) of apparently healthy people that are assumed to be adequate-used when an RDA cannot be determined.
Estimated Average Requirement (EAR)	The average daily nutrient intake level estimated to meet the requirement of half the healthy individuals in a particular life stage and gender group.

Source: Adapted from Dietary Reference Intakes (2005).

which is equal to two standard deviations of the estimated average requirement (EAR) of 0.66 /g/kg of body weight/day. For the DRI, adults are classified as males and nonpregnant females 19 years of age or older. Protein intake of 0.8 /g/kg of body weight/day is expected to give approximately 97% of the adult population adequate protein to achieve nitrogen balance. The DRI has additional life group classifications for pregnant or lactating females because these groups have additional protein requirements. The protein EAR and RDA values for all life stage groups are presented in Table 8.13. Protein

Table 8.13 U.S. estimated average requirements (EAR), recommended dietary allowance (RDA), and adequate intake (AI) for all life stage groups

Life stage group	EAR of protein (g/kg/day)	RDA or AI of protein (g/kg/day)	RDA or AI of protein[a] (g/day)
Infants			
0–6 mo		1.5*	9.1*
7–12 mo	1.0	1.2	11.0
Children			
1–3 yr	0.87	1.05	13
4–8 yr	0.76	0.95	19
Males			
9–13 yr	0.76	0.95	34
14–18 yr	0.73	0.85	52
19–30 yr	0.66	0.8	56
31–50 yr	0.66	0.8	56
51–70 yr	0.66	0.8	56
>70 yr	0.66	0.8	56
Females			
9–13 yr	0.76	0.95	34
14–18 yr	0.71	0.85	46
19–30 yr	0.66	0.8	46
31–50 yr	0.66	0.8	46
51–70 yr	0.66	0.8	46
>70 yr	0.66	0.8	46
Pregnancy			
14–18 yr	0.88	1.1	71
19–30 yr	0.88	1.1	71
31–50 yr	0.88	1.1	71
Lactation			
14–18 yr	1.05	1.3	71
19–30 yr	1.05	1.3	71
31–50 yr	1.05	1.3	71

Source: Adapted from Dietary Reference Intakes (2005).

[a]Based on gram protein per kilogram body weight for the reference body weight, for example, for adults 0.8 g/kg body weight for the reference body weight.

*$P < 0.05$.

recommendations for children less than six months of age are presented as adequate intake (AI), because an EAR has not been established. The term "AI" is used when limited scientific data is available to develop an RDA. AI values for infants were derived from studies that investigated protein requirements of infants fed breast milk during the first 6 months of life (DRI, 2005).

International protein requirements are based on the Joint WHO/FAO/United Nations University (UNU) Expert Consultation on Protein and Amino Acid Requirements in Human Nutrition (WHO, 2007). Both the U.S. and international recommendations are based on a meta-analysis of nitrogen balance studies conducted by Rand and colleagues (2003). Nitrogen balance studies have been the "gold standard" method to determine human protein requirements since the 1970s. Human adult protein requirement is defined as the intake of dietary protein required to achieve zero balance total body nitrogen in a healthy person with balanced energy (Rand et al., 2003). In general, it is when nitrogen intake is equal to nitrogen loss. It is the basal metabolic nitrogen demand and represents the sum of all obligatory nitrogenous losses by all metabolic pathways in a nutritionally adequate and stabilized nitrogen-free state (WHO, 2007). Obligatory nitrogen losses are the nitrogenous metabolites of amino acids lost in urine, sweat, and feces, as well as the body proteins lost from in hair, skin, and secretions. The basic model to describe the flow of amino acids in and out of the body is shown in Figure 8.1. Amino acids are required for protein synthesis and other metabolic processes. This demand is met via dietary protein, *de novo* synthesis, and body protein degradation. There is a considerable amount of protein turnover each day, whereby amino acids are released into the body's free-amino acid pool during protein catabolism and can be used to synthesize new proteins. Amino acids in excess of the body's requirements are removed by metabolic oxidation and are excreted as urea (Brown and Cline, 1974). Protein requirements for growth in children and adolescents account for a large fraction of the daily protein requirement and are reflected in the RDA and EAR.

8.3.1.2 Protein deficiency and malnutrition Diets deficient in protein and/or energy can result in two diseases, marasmus and kwashiorkor, which are more prevalent in developing countries. Marasmus develops when a person's body adapts to inadequate energy and protein over long periods of time (months to years). People with marasmus commonly

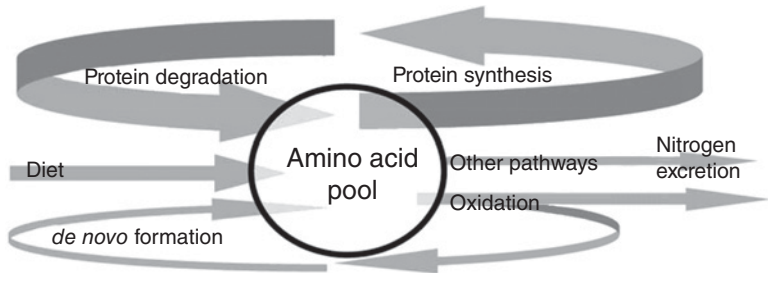


Figure 8.1 Amino acid pool. From WHO (2007)

have a starved appearance, stunted growth, and are extremely thin with wasted muscle and fat mass (Coward and Lunn, 1981). Mortality is low with marasmus, unless it is related to an underlying disease. Marasmus is treated with slow and gradual increases of protein in a dietary intervention, which helps restore normal metabolic and gastrointestinal function and reduces the risk of mortality (Collins et al., 1998).

Kwashiorkor, also known as hypoalbuminemic malnutrition, develops over shorter lengths of time (weeks) because of decreased protein intake during acute illnesses, or in people receiving adequate energy from carbohydrates, but insufficient dietary protein. People who develop kwashiorkor may have a well-nourished appearance or edema maybe present, especially in extremities like the legs. Edema is caused by low-serum concentrations of albumin and transferrin, which results in water diffusing out of the blood and into surrounding tissues (Coward and Lunn, 1981). Other clinical signs of kwashiorkor are poor wound healing, skin breakdown, hair color that changes to brown or red, and hair that can be easily pulled out. Compared to marasmus, mortality is higher with kwashiorkor, but it can be treated with a more aggressive feeding intervention (McLaren et al., 1969).

8.3.1.3 Excess protein intake Upper limits for protein and individual amino acids have not been established, and there is limited evidence in scientific literature on the adverse health effects of consuming high levels of protein or amino acids. The recommended protein intake as a percentage of total energy is 10–35%. Intake of protein in excess of 35%, common in fad diets and by weight lifters seeking additional protein, has been investigated to determine long-term health consequences. The scientific literature has not correlated high-protein diets to adverse health consequences, such as diminished renal function, cardiovascular disease, inadequate bone formation, or cancer (Eisenstein et al., 2002; Poortmans and Dellalieux, 2000). Diets with very high lean protein greater than 45% of total energy have been reported, however, by early American explorers to cause weakness, nausea, and diarrhea (McClellan and Du Bois, 1930). If high-protein diets are maintained for long periods of time, death can occur as a result of a condition called "rabbit starvation," which resulted in the deaths of early American explorers who survived by eating rabbit meat, which contains lean protein with little fat (Speth and Spielmann, 1983). Consumption of amino acids in excess, which occurs when protein is greater than 35% of total energy intake, can result in elevated levels of ammonia and urea in the bloodstream, and cause death (Bilsborough and Mann, 2006).

8.3.1.4 Protein supplements Protein supplementation is common among athletes and body builders, with the belief that it will promote muscle growth. Often athletes consume protein in extreme excess of the recommended dietary intake (RDI). Isolated whey and soy protein products are particularly popular with bodybuilders. Some studies do support that athletes may have higher protein needs (Tarnopolsky et al., 1992; Lemon et al., 1997), but the additional protein needs are usually met by the diet. Additionally, scientific experiments have not yet demonstrated protein supplements have an advantage over proteins supplied in the diet. In fact, there is evidence that dietary amino acid composition and the time at which proteins are ingested in relation to exercise are more important

Table 8.14 Indispensable and dispensable amino acids

Indispensable	Dispensable
Tryptophan	Alanine
Histidine	Asparagine
Methionine	Aspartate
Isoleucine	Glutamate
Threonine	Glycine
Valine	Serine
Phenylalanine	Arginine
Lysine	Proline
Leucine	Glutamine
	Cysteine[a]
	Tyrosine[a]

Source: Adapted from WHO (2007).
[a]Cysteine and tyrosine are conditionally indispensable for adults and indispensable for infants.

factors for stimulating muscle development. Biolo and colleagues (1995) demonstrated exercise stimulates amino acid transport, and muscle tissue is more effective at utilizing amino acids after working out; therefore, consuming an optimal proportion of amino acids after exercise may have the best opportunity to promote muscle synthesis, rather than supplementation with protein supplements.

8.3.2 Recommended amino acid intake

Amino acids are the molecular building blocks for proteins, and from a nutritional perspective they are categorized as either indispensable or dispensable. Of the 20 primary amino acids (Table 8.14), nine are indispensable because they cannot be synthesized *de novo* by humans. The indispensable amino acids (IDAAs) are tryptophan, histidine, methionine, isoleucine, threonine, valine, phenylalanine, lysine, and leucine. Conditionally, dispensable amino acids are those that can be synthesized *de novo* from other nitrogen sources in most circumstances, but may be required in the diet under certain situations such as growth or during illnesses. The remaining 11 amino acids are dispensable. These amino acids include alanine, asparagine, aspartate, glutamate, glycine, serine, arginine, cysteine, glutamine, proline, and tyrosine. The amino acids cysteine and tyrosine are classified as dispensable and are listed in Table 8.14 with the dispensable amino acids; however, it is important to note they are indispensable for infants and conditionally indispensable for adults.

The RDA and AI for IDAAs are presented in Table 8.15. These recommended values were estimated from data collected from amino acid balance, amino acid oxidation, and indicator amino acid studies, as described in the following section.

Table 8.15 Recommended dietary allowance (RDA) and adequate intake (AI) for indispensable amino acids (mg/kg/day)

	His	Ile	Leu	Lys	SAA	AAA	Thr	Trp	Val
0–6 mo, Boys and Girls	36*	88*	156*	107*	59*	135*	73*	28*	87*
7–12 mo, boys and girls	32	43	93	89	43	84	49	13	58
1–3 yr, boys and girls	21	28	63	58	28	54	32	8	37
4–8 yr, boys and girls	16	22	49	46	22	41	24	6	28
9–13 yr, boys and girls	17	22	49	46	22	41	24	6	28
14–18 yr, boys and girls	14	19	44	40	19	35	21	5	24
Adults 19+	14	19	42	38	19	33	20	5	24
Pregnant females of all ages	18	25	56	51	25	44	26	7	31
Lactating females of all ages	19	30	62	52	26	51	30	9	35

Source: Adapted from Dietary Reference Intakes (2005).
*$P < 0.05$.

8.3.3 Recent advances in determining protein and amino acid requirements

Nitrogen balance studies laid the foundation for determining human protein and amino acid requirements; however, the nitrogen balance method overestimates nitrogen intake and underestimates nitrogen excretion. This results in a model with positive nitrogen balance and underestimated protein requirements. Newer techniques have enabled researchers to more accurately estimate protein and amino acid requirements. One of these techniques is the indicator amino acid oxidation (IAAO) method (Zello et al., 1995; Elango et al., 2008). In the IAAO method, when one IDAA is deficient for protein synthesis then all other IDAAs are in excess, and the radioactively labeled–indicator amino acid will be oxidized. When the limiting amino acid is increased, oxidation of the indicator amino acid (commonly L-1-^{13}C-phenylalanine) decreases, which is a reflection of increased incorporation into protein. When plotted on a graph, the point at which the indicator reaches a plateau is call a "breakpoint" and can be analyzed by two-phase linear regression to estimate the mean or EAR of the limiting amino acid or protein. When the requirement for the limiting amino acid is met, oxidation of the indicator does not change (Elango et al., 2012). The protein and amino acid requirements obtained from this method are comparable to a more robust method called the 24-hour-indicator amino acid oxidation and balance model. Table 8.16 illustrates the differences in amino acid requirements on the basis of the IAAO and nitrogen balance methods. These newer methods have confirmed what scientists have suspected, that is amino acid requirements from newer techniques are higher than the values based on nitrogen balance studies; for example, the IAAO method estimated mean and population safe protein requirements to be 0.93 and 1.2 g/kg/day, respectively, for adults (Elango et al., 2010). In contrast, the current EAR and RDA are 0.66 and 0.80 g/kg/day, respectively. These results indicated newer methods might be more appropriate than the more traditionally used nitrogen balance method in determining protein and amino acid requirements (Humayun et al., 2007).

Table 8.16 Dietary indispensable amino acid requirements in adult humans

Amino acid	IAAO[a] estimate (mg/kg/day)	DRI[b] 2005 (mg/kg/day)
Histidine	–	11
Isoleucine	42	15
Leucine	55	34
Lysine	37	31
Methionine (without cysteine)	13	15
Phenylalanine (without tyrosine)	48	27
Threonine	19	16
Tryptophan	4	4
Valine	47	19

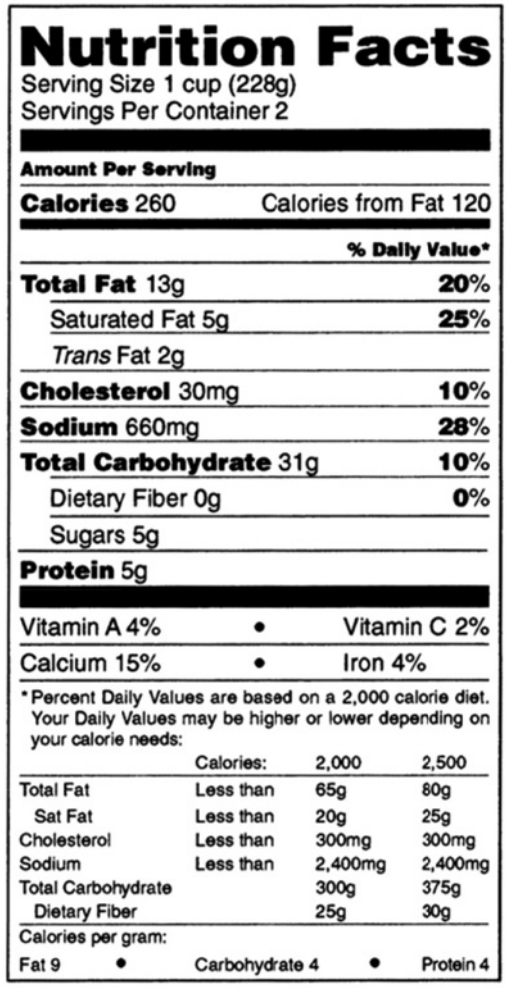
Source: Adapted from Pencharz et al. (2012).
[a]IAAO, Indicator amino acid oxidation.
[b]DRI, Dietary Reference Intake.

More recently, the IAAO method has been used to determine protein requirements for children. The IAAO method estimated mean and population safe requirements to be 1.3 and 1.5 g/kg/day, respectively, for children. In contrast, the current EAR and RDA are 0.76 and 0.95 g/kg/day, respectively (Elango et al., 2011). Children and the elderly are believed to have significantly underestimated requirements and are the focus of current studies to optimize protein nutrition, especially for the older adults (Volpi et al., 2012). Limited data have been published for these groups, but the data do suggest additional protein requirements above the current 2005 DRI recommendations.

8.3.4 Food-labeling requirements for proteins

Most food packages in the United States are required to contain a "Nutrition Facts" panel (Figure 8.2), which indicates the net content of macro and micronutrients. Proteins are required to be listed as grams of protein per serving, unless a serving contains less than 1 g of protein. Foods with less than 1 g can have the statement "less than one gram" and foods with servings containing less than 0.5 g can be listed as zero.

Foods intended for adults or children 4 years of age or older are required to express the protein quality value as a percentage if the PDCAAS is less than 20. If a food, however, is intended for children aged 1 to 4 years has a PDCAAS of less than 40, the corrected amount of protein per serving can be listed on the label or the statement "not a significant source of protein" can be made. For infant foods, when protein quality of a food is less than 40% of the reference standard (casein), which is measured by the protein efficiency ratio method, the statement "not a significant source of protein" needs to be placed next to the declaration of protein content. Manufacturers and food producers calculate protein content by multiplying the nitrogen content of food by a factor of 6.25. When foods are labeled as a percentage of the RDI, the reference value of adults and children 4 years or older is 50 g. The reference values for children less than 4 years of age, infants, pregnant women, and lactating women are 16 g, 14 g, 60 g, and 65 g, respectively (FDA, 2012).



Nutrition Facts

Serving Size 1 cup (228g)
Servings Per Container 2

Amount Per Serving

Calories 260	Calories from Fat 120
	% Daily Value*
Total Fat 13g	**20%**
Saturated Fat 5g	**25%**
Trans Fat 2g	
Cholesterol 30mg	**10%**
Sodium 660mg	**28%**
Total Carbohydrate 31g	**10%**
Dietary Fiber 0g	**0%**
Sugars 5g	
Protein 5g	

Vitamin A 4% •	Vitamin C 2%
Calcium 15% •	Iron 4%

*Percent Daily Values are based on a 2,000 calorie diet. Your Daily Values may be higher or lower depending on your calorie needs:

	Calories:	2,000	2,500
Total Fat	Less than	65g	80g
Sat Fat	Less than	20g	25g
Cholesterol	Less than	300mg	300mg
Sodium	Less than	2,400mg	2,400mg
Total Carbohydrate		300g	375g
Dietary Fiber		25g	30g

Calories per gram:

Fat 9 • Carbohydrate 4 • Protein 4

Figure 8.2 Nutrition facts panel example. From FDA (2012)

8.4 Conclusion

The amino acid score is intended to predict protein quality in terms of the potential ability of the food protein to supply the appropriate profile of dietary indispensable amino acids to match that of the reference protein. The actual capacity of the protein to satisfy amino acid needs will require the use of corrections for amino acid digestibility and availability. This concept is a significant departure from the current use of the PDCAAS, whereby the use of a single value of crude protein digestibility to correct the dietary amounts of each individual dietary indispensable amino acid for its digestibility is a significant shortcoming. There are practically important quantitative differences in the digestibility between

crude protein and individual dietary indispensable and dispensable amino acids and discrepancies between fecal and ileal digestibility values for amino acids. Rapid and efficient generation of individual amino acid digestibility values for assessment of protein quality of foods or diets for humans will depend on a consensus among the human nutrition committee to adopt the growing pig as a model. Such values will facilitate moving from the PDCAAS to the DIAAS. Current protein and amino acid requirements for growing and adult humans, estimated on the basis of the nitrogen balance and factorial methods, respectively, appear to be underestimated. Future recommendations on protein and amino acid requirements for humans will depend on more mechanistic approaches, such as the IAAO method.

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II
Plant Proteins

9
Soy Proteins

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9.1 Introduction

Soybean (*Glycine max L.*) is an annual leguminous plant that originated from northeastern China and other regions of Asia (Singh and Hymowitz, 1999). In 2011, it was planted on 75 million acres (30.3 million hectares) worldwide, producing 3.056 billion bushels (83.18 million metric tons) (Soy Stats, 2012). The United States is the number one soybean producer with 37% of the world soybean market, followed by Brazil and Argentina with 29% and 19%, respectively (Figure 9.1a); however, in 2011, Brazil was the main exporter with 41% of the world exports, followed by the United States and Argentina with 37% and 10%, respectively (Figure 9.1b) (Soy Stats, 2012).

Soybean seeds contain an average of 36–38% protein and 19% oil on a dry weight basis, although both genetic and environmental factors can strongly affect seed composition (Zarkadas et al., 2007). The soybean nutrient composition can be influenced by cultivar, breeding, growth conditions, processing, and refinement. Soybean also has phytochemical compounds, such as phytic acid, lectins, trypsin inhibitors, peroxidases, lipoxygenases, β-glucosidases, isoflavones, saponins, and enzymes, which were previously categorized as antinutrient factors (Steves et al., 2010). It is the leading edible oil source throughout the world. Although soy protein ranks higher than other vegetable proteins, its nutritional quality is not fully equivalent to animal proteins. Sulfur-containing amino acids such as cysteine and methionine are the most nutritionally limiting amino acids in soybean proteins. Soybean proteins mainly consist of globulins; made up of two subgroups known as glycinin and β-conglycinin (Manjaya et al., 2007).

Soybean and its products are economically valued because of their nutrients and presence of phytochemicals. Their low cost and associated health benefits results in the

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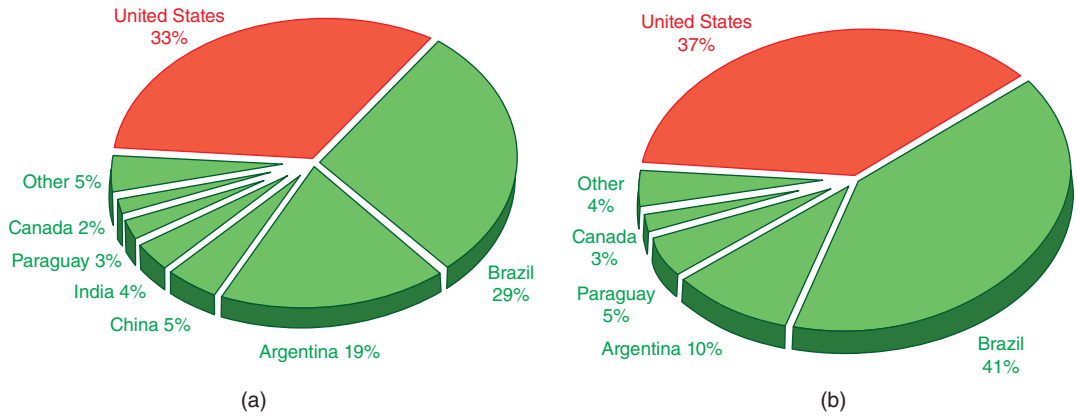


Figure 9.1 (a) World soy bean production in 2011; (b) World soybean exports in 2011. From USDA (2012)

increased use for animal and human nutrition, and these have been even reported as nutraceuticals that reduce risk factors for chronic diseases such as diabetes mellitus, cancer, cardiovascular disease, osteoporosis among others (Lee et al., 2007).

Technological approaches to produce food products have made impressive developments because of the great versatility of soy proteins. Applications range from functional ingredients in the food industry to biopolymers, biofilms, and nanocomposites. New processing technologies and chemical and enzymatic reactions produce functional changes in the protein structure. Physicochemical changes in soy proteins extend their applications in food formulations by improving sensory, textural, rheological, and nutrimental properties of food products. The structure and chemical modifications of soybean proteins depend primarily on the technology used to produce functional ingredients and new products; hence, the aim of this chapter is to address the chemical composition and principal physicochemical modifications on soybean proteins.

9.2 Soybean proteins

Soybean proteins are a complex polymorphic mixture of polypeptides, and like many species of Leguminosae, soybean contains four groups of proteins: (i) storage proteins, (ii) enzymes and enzyme inhibitors (>1% of total protein) involved in metabolism, (iii) structural proteins including both ribosomal and chromosomal, (iv) and membrane proteins (Krishnan, 2000).

9.2.1 Soybean storage proteins

The principal soybean storage proteins are globulins, which can be divided into four categories on the basis of their sedimentation rate. Major globulins are 2S, 7S, 11S, and 15S with molecular masses of approximately 25, 160, 350, and 600 kDa, respectively

(Liu, 1997). As much as 85% of these soybean storage globulins are associated with 7S and 11S globulins, which are more commonly known as β-conglycinin and glycinin, respectively.

The two major multi-subunit storage proteins of soybean are the salt-soluble glycinin (11S) and β-conglycinin (7S), which account for around 40% and 25% of the total seed endosperm protein, respectively. The composition of the storage proteins also varies with maturity, nutrient supply from the soil, fertilizer treatment, and with environmental factors. Several studies have shown that the accumulation of the sulfur-poor β-subunit of β-conglycinin is promoted by excess application of nitrogen or by sulfur deficiency, whereas the application of sulfur fertilization increases the synthesis of glycinin (Krishnan et al., 2005). In addition to these two storage proteins, there are other known proteins including Kunitz trypsin, Bowman–Birk, and related protease inhibitors, β-amylases, lipoxygenases, urease, and seed lectins, each of which accounts for about 2–5% of the total seed proteins in soybean (Liener, 1994).

The soy globulins differ in their functional properties, especially in gelation, with gels made from glycinin being harder than gels from β-conglycinin (Rickert et al., 2004). The specific subunits within glycinin and β-conglycinin contribute differentially to protein-gelling properties (Poysa et al., 2006).

9.2.2 Chemical structure of soybean proteins

9.2.2.1 Glycinin: structure and properties Glycinin is a hexamer with a molecular mass of 300–380 kDa and is formed by associating six acidic and six basic polypeptides. The acidic subunits have an isoelectric point (pI) ranging from 4.5 to 5.5 (Staswick et al., 1981), whereas the basic subunits have a pI ranging from 6.5 to 8.5 (Staswick et al., 1984b). Each subunit is composed of one acidic and one basic polypeptide connected by a disulfide linkage (Staswick et al., 1984a). It forms a hexameric complex at ambient temperature and pH 7.6 having a molecular mass of 360 kDa, which is converted into a trimeric complex of molecular mass of 180 kDa at pH 3.8 (Bradley et al., 1975). Glycinin's five subunits are further classified into group I (A1aB1b, A2B1a, A1bB2) and group II (A5A4B3, A3B4) on the basis of the extent of their homology (Nielsen, 1985). Group I subunits contain two cysteine and three cystine residues, whereas group II subunits contain two cysteine and two cystine residues (Utsumi et al., 1987).

The three-dimensional structure (3D) of proteins is important as it is the functional form of the protein. In addition, elucidation of the detailed 3D structures of proteins provides an understanding on the modifications of proteins leading into the improvement of their properties. The 3D structure determines the physicochemical properties of proteins. Studies on the 3D structures of soybean storage proteins started almost four decades ago. More recently, Fukushima (2011) used indirect methods of determining 3D structures of soybean storage proteins by using optical rotatory dispersion, circular dichroism, ultraviolet difference spectra and deuteration studies. These methods, however, provide incomplete information on the 3D structure of soybean storage proteins. To determine the complete 3D structure of soybean glycinin, the protein must be crystallized first and

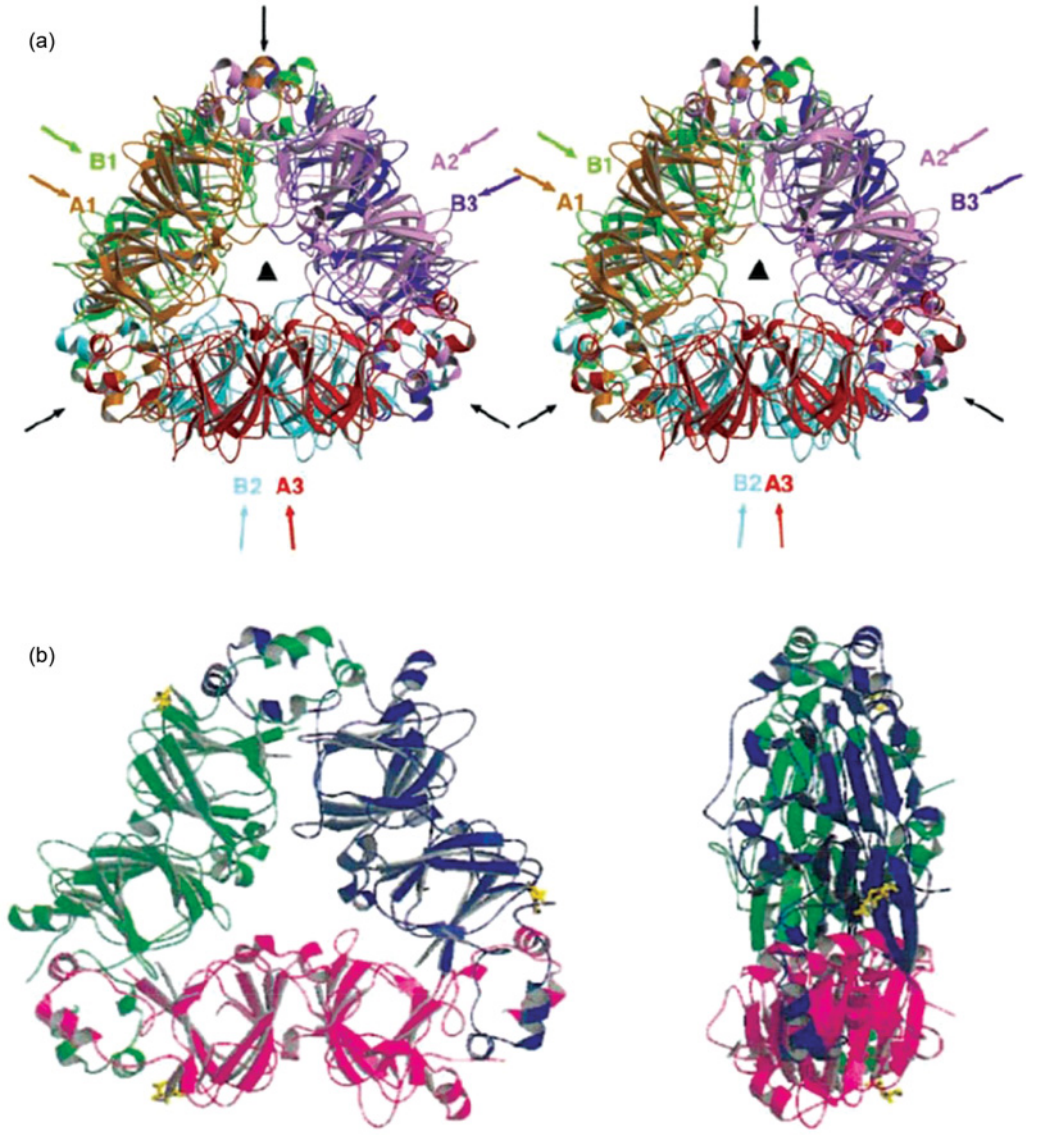


Figure 9.2 (a) Crystal structure of soybean A3B4 glycinin showing that it consists of two trimers composed of protomers A1-A3 and B1-B3. Copyright 2005 National Academy of Sciences, USA. (b) Ribbon diagram of native β-conglycinin β homotrimer. Reproduced with permission of John Wiley & Sons.

then X-ray analyzed. Because glycinin is composed of six different subunits, purification and crystallization of these subunits are difficult. Scientists have used variants of soybean that contain only one glycinin subunit to have it purified and crystallized; for example, the crystal structure of A3B4 glycinin subunit was determined using a mutant soybean cultivar containing glycinin composed of only the A3B4 subunit. Figure 9.2a shows the

crystal structure of A3B4 glycinin indicating that it has 32-point group symmetry and consists of two trimers composed of protomers A1-A3 and B1-B3 (Adachi et al., 2003). Each protomer contains 27 strands and 7 helices.

9.2.2.2 β-Conglycinin: structure and properties β-Conglycinin is a vicilin storage protein with a molecular mass of 150-200 kDa (Wang et al., 2008a). It is a glycoprotein and a trimer consisting of three subunits namely α, α′, and β with molecular masses of 68, 72, and 52 kDa, respectively (Thanh and Shibasaki, 1977). Subunits α and α′ contain one cysteine residue near the N-terminus, whereas the β subunit is devoid of any cysteine, none of these three subunits contain cystine residues (Utsumi et al., 1997). Utsumi et al. (1997), reported that these subunits are associated with each other by strong hydrophobic interaction and hydrogen bonding. β-Conglycinin undergoes the association–disassociation phenomenon depending on the ionic strength and pH; for instance, at neutral pH and ionic strength of >0.5, the storage protein is in the 7S-globulin form; this conformation is changed to an aggregated 9S form when the ionic strength is reduced to 0.2 (Thanh and Shibasaki, 1979).

The structure of the core region of the soybean β-conglycinin α′ subunit has been established. Maruyama et al. (2003) showed that the core region contains a trimer at its asymmetric unit; each monomer is divided into N- and C-terminal modules. Each module contains a β-barrel domain and extended loop domain composed of several helices. The same group of scientists reported the crystal structures of recombinant and native soybean β-conglycinin β subunit. They reported that the overall structure of β homotrimers is very consistent with phaseolin and canavalin (Maruyama et al., 2001), and each monomer is composed of N- and C-terminal modules that are very similar to each other. Each module is subdivided into a core domain that consists of β barrels and helices and a loop domain composed of several α helices that protrudes from either side of the monomer as a pair of hooks. The ribbon diagrams of the native β-conglycinin homotrimer are presented in Figure 9.2b. Structure-based alignment between the core region of the α′ and β homotrimer subunits showing that the sequences are highly homologous with an identity of 75.5% (Maruyama et al., 2003).

9.2.2.3 Separation of soybean glycinin and β-conglycinin subunits The separation of glycinin and β-conglycinin is important for two reasons; to study the chemistry and physiological activity of each subunit, and for food industrial applications. For the first purpose, high-purity preparation and appropriate storage conditions of the protein in its native state is important. Considerations as industrial application of soybean storage proteins requires less purity and less-expensive techniques, high yield, simplicity, and feasibility for large-scale industrial production; hence, the choice of additives, buffers, and technical routes for the separation is based on which purpose is being fulfilled.

On the basis of the principle that proteins will precipitate out of the solution at their respective pI, separation of glycinin and β-conglycinin can be achieved. The pI for glycinin and β-conglycinin are pH 4.85 and pH 6.4, respectively (Koshiyama, 1968; Wolf, 1977). Thanh and Shibasaki (1976) used Tris-HCl buffer with β-mercaptoethanol for the extraction of proteins from defatted soybean flour to get good product purity and keep the protein in its native conformation at pH 8. Using β-mercaptoethanol disrupts

disulfide bonds, and the use of pH 8 ensures that most of the glycinin and β-conglycinin are extracted as the pH is far from their respective pI. After removal of insoluble matters by centrifugation, the supernatant pH was adjusted to pH 6.4 to precipitate glycinin. Precipitated glycinin was then collected by centrifugation at 2–5°C. The supernatant, after centrifugation, was collected and pH adjusted to 4.8 to precipitate β-conglycinin. The precipitate containing β-conglycinin was collected by centrifugation. Both precipitates were washed and pH adjusted to 7.8 (for glycinin extraction using NaOH) and to 6.2 (for β-conglycinin extraction using HCl) to solubilize the purified fractions. The remaining precipitate was removed by centrifugation and the supernatants were called glycinin-rich and β-conglycinin-rich fractions, respectively. This method provided the basis for other methods reported later with an aim of increasing purity and yield. For instance, Nagano et al. (1992) modified the method by using deionized water as extracting solution, sodium bisulfite as reducing agent and addition of 0.25 M NaCl to increase the precipitation rate of β-conglycinin. Wu (1999) used the same conditions as Nagano et al. (1992) except for the use of higher centrifugation speed for better precipitation of the isolated fraction. Other methods used divalent cations Ca^{2+} and Mg^{2+} instead of Na^{+} to precipitate β-conglycinin more efficiently (Koshiyama, 1965; Saio et al., 1973; Deak et al., 2006). Industrial separation methods were based on these laboratory-scale methods with some modifications such as the use of ultrafiltration and reverse-osmosis technology (Wu et al., 2000) and use of decanter centrifuges for separation of components (Guo et al., 2004). Immunoaffinity chromatography based on antibody-antigen interaction (You et al., 2009), concanavalin A affinity chromatography followed by gel filtration (Hou and Chang, 2004), and use of carbon dioxide as precipitant have been reported for production of pure glycinin (Golubovic et al., 2005).

9.2.3 Soybean enzymes and enzyme inhibitors

9.2.3.1 Urease Soybean urease, EC 3.5.1.5, also known as urea amidohydrolase is a homooligomeric protein with approximately 90-kDa subunits (Mobley et al., 1995). It is rich in cysteine residues with 34 sulfhydryl groups per hexamer (Kumar and Kayastha, 2010a) with ureolytic activities dependent on a nickel metallo center active site (Carlini and Polacco, 2008). This enzyme catalyzes the degradation of urea into carbon dioxide and ammonia, enhancing the rate of reaction by 8×10^{17} units (Callahan et al., 2005), as shown in Figure 9.3a. Urease activity is present in all tissues of the soybean plants (Das et al., 2002) and has two isoforms with 87% amino acid homology (Goldraij et al., 2003). One of the isoforms called the ubiquitous urease is responsible for recycling all urea delivered to the embryo (Stebbins et al., 1991) indicating an assimilatory function for urease. The other form of urease called the seed urease has defense roles associated with its ureolytic activity, as a result high release of ammonia and concomitant increase in pH of the medium (Stebbins et al., 1991; Polacco and Holland, 1993).

In the production of soybean meal and feeds, residual urease activity is used as an index for adequate heat treatment that the product has received. Also, it is used to correlate with residual trypsin inhibitory activity. For soybean meals used for poultry feed, an acceptable range of 0.20–0.05 pH unit urease activity is suggested (Vohra and Kratzer,

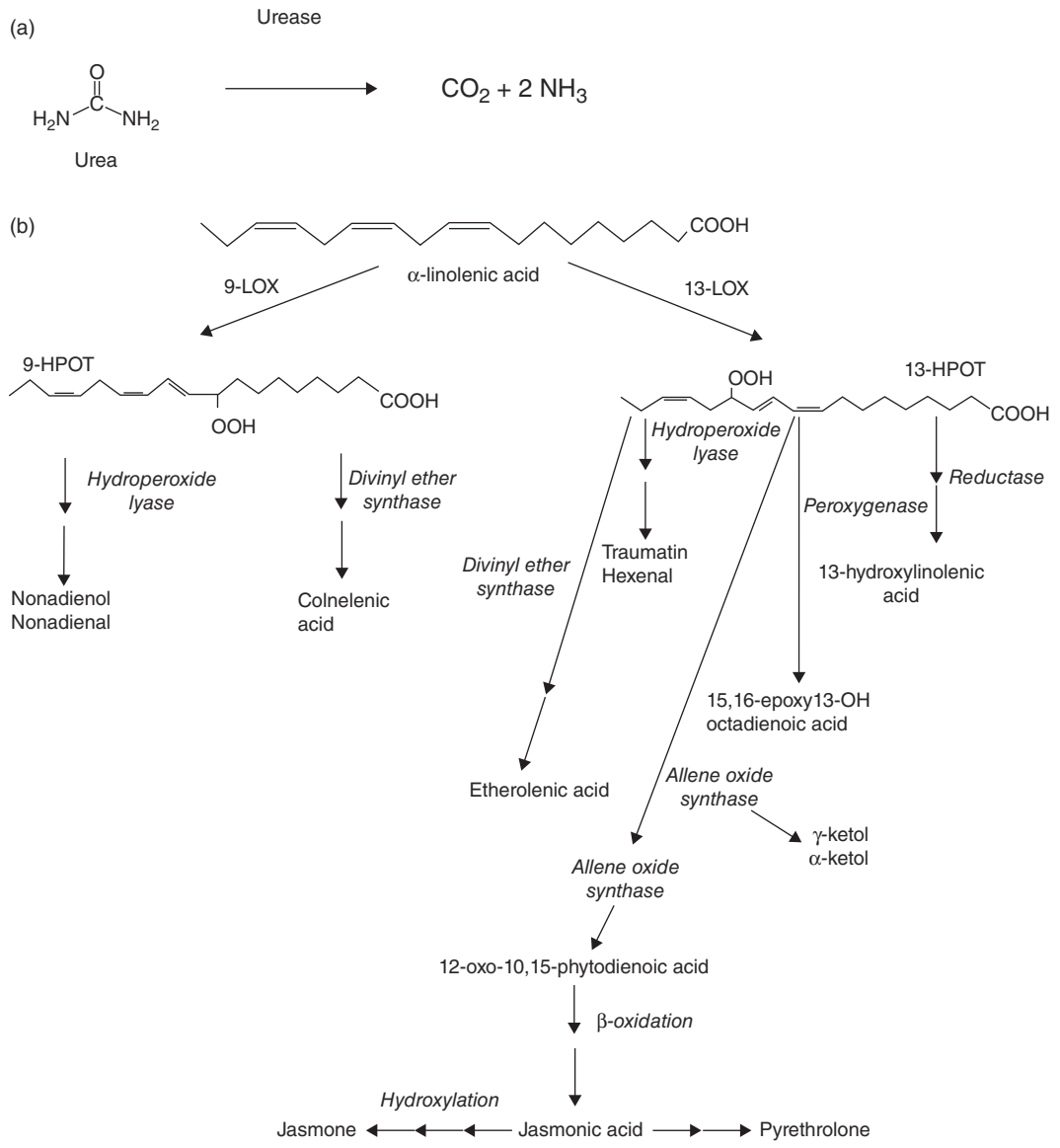


Figure 9.3 (a) Degradation of urea into carbon dioxide and ammonia. (b) The lipoxygenase pathway. The products of lipoxygenase pathway have diverse functions in plant cell and can also lead to production of undesirable flavors and odors in foods. LOX, lipoxygenase; 9-LOX, 9-lipoxygenase; 13-LOX, 13-lipoxygenase; 9-HPOT, 9-hydroxy-10,12,15-octadecatrienoic acid; 13-HPOT, 13-hydroxy-10,12,15-octadecatrienoic acid. Adapted from Porta and Rocha-Sosa (2001)

2012). In addition, residual urease activity has been used to determine the chemical composition and nutritional quality of soybean meals prepared by extruder/expeller process. Karr-Lilienthal et al. (2006) reported that extrusion of soybean meals at 121 and 135°C resulted in an underprocessed soybean meal as noted by high urease activities of 2.11 and 1.91 pH units, respectively, with corresponding lower amino acid digestibility. On the other hand, soybean meal produced by extrusion at 150°C and 160°C had higher amino acid digestibility and lower urease activity. Thin and thick layer of soybean grain toasted at 130°C for polygastric animal feed also showed effectiveness in reducing urease activity with corresponding reduction in trypsin inhibitory activity and increased protein and amino acid content (Kricka et al., 2009). A recent study also showed that soybean urease can be inhibited by NaF, Ag^+, and boric and boronic acids (Kumar and Kayastha, 2010b).

9.2.3.2 Lipoxygenase Lipoxygenase (LOX), E.C. 1.13.11.12, also known as linoleate: oxygen oxidoreductase, is a non-heme iron-containing dioxygenase that catalyzes the insertion of molecular oxygen into polyunsaturated fatty acid (Yamamoto, 1992). It accounts for as much as 2% of total soybean seed protein (Loiseau et al., 2001). In plants, LOX common substrates are linoleic and linolenic acids leading to formation of hydroxyperoxy fatty acids that are converted to different compounds as shown in Figure 9.3b. These products have diverse functions in the plant including vegetative growth, wounding, herbivore, and pathogen attack response and mobilization of storage lipids during germination (Porta and Rocha-Sosa, 2001). In mature seeds of soybean, three LOXs are present and are not associated with lipids. During germination, no substantial oxygenation of polyunsaturated fatty acids happens; suggesting that LOX is not used for lipid mobilization (Wang et al., 1999). These three LOXs are controlled by separate genes: Lox1, Lox2 and Lox3 (Lenis et al., 2010). Soybean LOX-1 is mostly α-helical that was described to be composed of 2 domains (Boyington et al., 1993) and 5 domains (Minor et al., 1996). The ribbon diagram presents the β-sandwich in the N domain, active site of iron in C domain, β-structures in the C domain and two segments of π-helix in longer helices providing 3 histidine side chains for iron ligands (Matoba et al., 1985).

In soybean-containing foods, LOX plays a role in the development of unpleasant flavors because of oxidation of polyunsaturated fatty acids; for instance, volatile degradation products of linoleic and linolenic acids in soybean oil have been described as grassy, beany, and rancid off-flavors leading to reduce consumer acceptability of soybean products. These off flavors have been associated with the production of volatile compounds such as *N*-hexanal and *cis*-3-hexenal (Matoba et al., 1985; Yukawa et al., 1995; Olias et al., 1993). Hence methods for its inactivation has been studied such as microwave heating (Wang and Toledo, 1987; Kermasha et al., 1993), pulse electric field (Li et al., 2008), acid treatment (Man and Nelson, 1989), combined pressure and temperature (Ludikhuyze et al., 1998) and use of bioactives such as catechols (Kemal et al., 1987) and isoflavans (Mascayano et al., 2011). On the other hand, the use of soybean LOX demonstrated positive effect on wheat flour properties such as the whiter color acting as a decolorant, increased stability of dough because of kneading, improved rheological properties, and improved over-all baking properties (Hoseney et al., 1980; Faubion and Hoseney, 1981; Lai et al., 1989; Nelles et al., 1998; Permyakova and Trufanov, 2011).

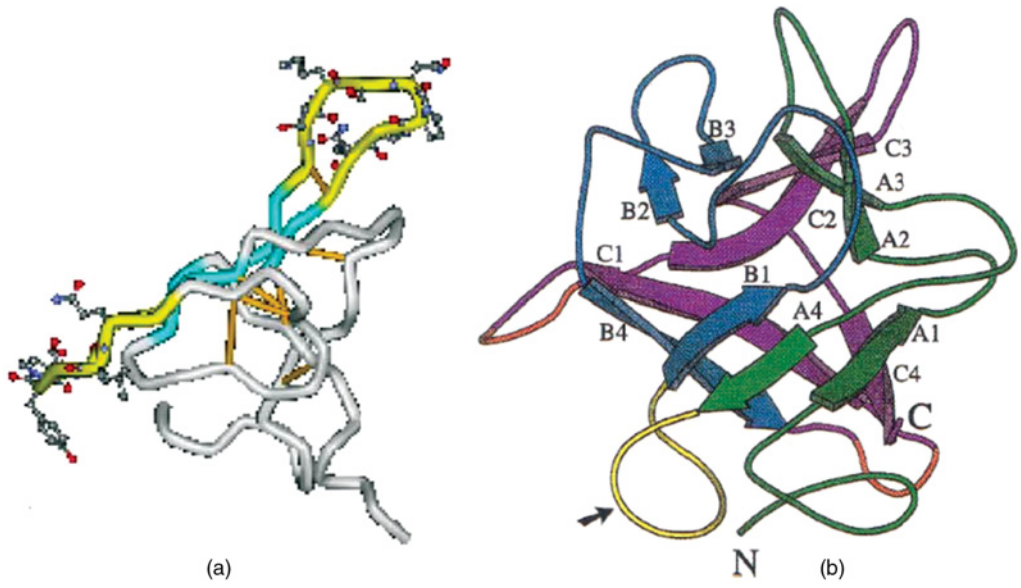


Figure 9.4 (a) Three-dimensional structure of soybean Bowman–Birk inhibitor. From Mao et al. (2005). Reproduced with permission of Springer Verlag. (b) Ribbon diagram of the over-all fold of soybean Kunitz trypsin inhibitor. From De Meester et al. (1998). Acta Crystallographica. Reproduced with permission of International Union of Crystallography. http://scripts.iucr.org/cgi-bin/paper?S0907444997015849.

9.2.3.3 Bowman–Birk inhibitor Soybean Bowman–Birk inhibitor (BBI) is composed of 71 amino acids with a molecular mass of 8 kDa (Birk, 1985; Mao et al., 2005) with a 3D structure depicted in Figure 9.4a. The isoelectric point of BBI is between pH 4.0 and 4.2 (Losso, 2008); BBI is rich in cysteine residues, accounting for as much as 20% of its total amino acids, making it very resistant to denaturation. It contains seven disulfide bonds between amino acids residues 9 and 24, 14 and 22, 8 and 62, 12 and 58, 36 and 51, 32 and 39, and 41 and 49. Wu and Sessa (1994) reported that native BBI secondary structure is composed of 61% β-sheet, 38% unordered structure, 1% β-turn and no helices, whereas its reduced form consists of 53% β-sheet, 5% β-turn and 42% unordered structure. X-ray crystallographic study showed that BBI contains two domains; one is responsible for its trypsin-inhibitory activity, whereas the other domain is responsible for inhibiting chymotrypsin (Werner and Wemmer, 1991). It forms a 1:1 complex with trypsin and chymotrypsin involving the lysine residue at position 16 and serine residue at position 17 for trypsin, and leucine residues at position 43 and serine residue at position 44 for chymotrypsin (Csaky and Fereke, 2004).

Classical identification of BBI is through gel electrophoresis by virtue of its 8-kDa molecular mass. The capability to inhibit trypsin and chymotrypsin is also a classical way of determining the presence of BBI in soybean and soybean products. The use of immunoassay method on the basis of monoclonal and polyclonal antibodies is important in quantifying the actual amount of BBI present in foods and body fluids. Wan et al. (1995) developed

an immunoassay method using four different antibodies against BBI for the detection of BBI in human urine samples. Immunoassay methods have also been used to quantify BBI in different soybean cultivars and soybean-based infant formulas (Gladysheva et al., 2000; Brandon et al., 2004). BBI can also be isolated and purified from soybean by ethanol precipitation (Odani and Ikenaka, 1973). These authors used 60% ethanol to extract BBI followed by cold acetone precipitation at pH 5.3. The precipitate was then dissolved in water and dialyzed against distilled water. Further purification was accomplished by two-stage ion-exchange chromatography at pH 4.0 using carboxymethylcellulose resin and at pH 6.5 using diethylaminoethyl resin. On the other hand, some research groups have studied the diminution of antinutrients in soybean. Dia et al. (2012) investigated the effect of germination, Alcalase (protease) hydrolysis, and their combination on the concentrations of antinutritional and bioactive compounds in Brazilian soybean. They found that a combination of germination and Alcalase hydrolysis resulted in the degradation of BBI, Kunitz trypsin inhibitor (KTI) of almost 100%.

9.2.3.4 Kunitz trypsin inhibitor Kunitz trypsin inhibitor (KTI) is a 21.5-kDa molecule consisting of 12 crisscrossing β-sheets oriented in antiparallel fashion stabilized by hydrophobic side chains (Roychaudhuri et al., 2003). The ribbon representation of the overall structure of soybean KTI is presented in Figure 9.4b with the pseudo threefold axis corresponding to the axis of the barrel (de Meester et al., 1998). It has two disulfide bonds between cysteine 39 and cysteine 86, and cysteine 136 and cysteine 145 (Tetenbaum and Miller, 2001), explaining its unusual stability against chemical and thermal inactivation. KTI has been found to have eight distinguishable isoforms namely, Tia, Tib, Tic, ti-null, Tid, Tie, Tif, and Tibi5, as reviewed by Gonzalez de Mejia and Dia (2010).

Isolation of KTI from soybean was based on its capability to precipitate out of the solution when heated in 2.5% trichloroacetic acid with minimum solubility in water at pH 4.2–4.8 (Kunitz, 1945). Combination of centrifugation, ammonium sulfate precipitation, gel filtration chromatography, and anion exchange chromatography was also used to isolate and purify KTI from soybean (Sugawara et al., 2007). A simple affinity chromatography method was also used to purify KTI from soybean. Trypsin was immobilized in an affinity chromatographic resin and an aqueous solution of defatted soybean meal was loaded into the column (Duranti et al., 2003; Shakiba et al., 2007). The principle behind this method is based on the capability of KTI to bind tightly to its substrate, trypsin. The bound KTI will then elute with diluted acid such as 0.01 N HCl. Similar to BBI, KTI is easily quantified in different soybean cultivars and products by immunoassay method (Brandon et al., 2004; Wang et al., 2008b; Brandon and Bates, 1988).

9.2.4 Proteins as allergens in soybean

Soybean is considered one of the "Big 8" most allergenic foods. The other seven are milk, eggs, fish, crustacean shellfish, tree nuts, peanuts, and wheat. Soybean allergens consist of proteins with molecular masses ranging from 7 to 71 kDa. Thirty-four reactive proteins have been identified and characterized as related to soybean allergy (FARRP, 2008; Xiang

et al., 2008). Soybean-sensitive individuals recognize 65% of the immunodominant soybean protein Gly-m-Bd 30k, or P34 (Ogawa et al., 1993, 2000). P34 is known as a soybean oil-body associated glycoprotein that consists of 257 amino acids residues. This protein is associated with α, α′, and β subunits of the globulin β-conglycinin by bi-sulfide linkage. Ogawa et al. (1993, 2000), identified three major soy allergens Gly m Bd 60K, Gly m Bd 30K, and Gly m Bd 28K. Gly m Bd 60K is an α-subunit of 7S β-conglycinin. Gly m Bd 28K is a vicilin-like glycoprotein of 473 amino acids (Xiang et al., 2004), initially isolated from soybean meal as a 28-kDa glycosylated protein. Gly m Bd 28K constitutes a minor component fractionated into 11S glycinin globulin fraction that has been recognized by soybean sensitive patients with about 25% of incidence (Ogawa et al., 2000). An important IgE-binding region was found in the C-terminal 23-kDa polypeptide, which contains an Asn-N-linked moiety with the same sugar composition as that of P34 (Hiemori et al., 2000). In addition to the list of major soy allergens, Cordle (2004) included soy hydrophobic protein (Gly m 1a), soy hull protein (Gly m 2), soy profilin (Gly m 3) (Klein-Tebbe et al., 2002), glycinin (320–360 kDa), β-conglycinin (140–180 kDa) and KTI (20 kDa) as major soy allergenic proteins. The acidic subunit of glycinin G1 (Beardslee et al., 2000) and the basic subunit of glycinin G2 (Helm and Burks, 2000) have been classified as important allergens.

Like any allergen in a food system, soy allergen reactivity is dominated by epitopes or IgE-binding sites, categorized as linear or conformational. The epitopes are not only fully characterized by their primary protein structure, but also by their tertiary structural conformations. These structures offer opportunities for different thermal and nonthermal food processing technologies to alter the nature of epitopes and thus allergen reactivity with IgE antibody (Wilson et al., 2005; Frias et al., 2008; Song et al., 2008; Wilson et al., 2008). Amnuaycheewa and de Mejia (2010) purified, characterized, and quantified soy allergen profilin (Gly m 3) in soy products. Figure 9.5a presents the electrophoretic protein profiles of most allergenic proteins present in soy protein isolate (SPI) and Figure 9.5b the steps followed to purify soy profilin.

Soybean processing, such as extrusion, may inactivate some antinutritional factors through heat treatment; however, this process may also reduce availability of amino acids, especially when the soybean product is overcooked (Danielson and Crenshaw, 1991). Extrusion is a high-temperature/short-time process in which moistened, expansive, and starchy and/or protein rich food materials are plasticized and cooked by a combination of moisture, pressure, temperature and mechanical shear, resulting in molecular transformation and chemical reactions (Castells et al., 2005). In our laboratory, the authors investigated the effect of extrusion processing parameters such as temperature, moisture, and screw speed (SS) on the reduction of soy protein immunogenicity by measuring IgE binding to human sera from allergic individuals (Yang et al., 2011). Because SPI is one of the main soy protein ingredients widely used to prepare snack foods, SPI-corn was used as an expanded model in this study. Figure 9.6a and Figure 9.6b present sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE) and Western blot comparisons among soybean samples used to prepare the extruded products. Protein profiles of products exposed to the lowest temperature, high moisture, and high SS (SPI8), and highest temperature, low moisture, and low SS (SPI1and SF1); during processing are presented.

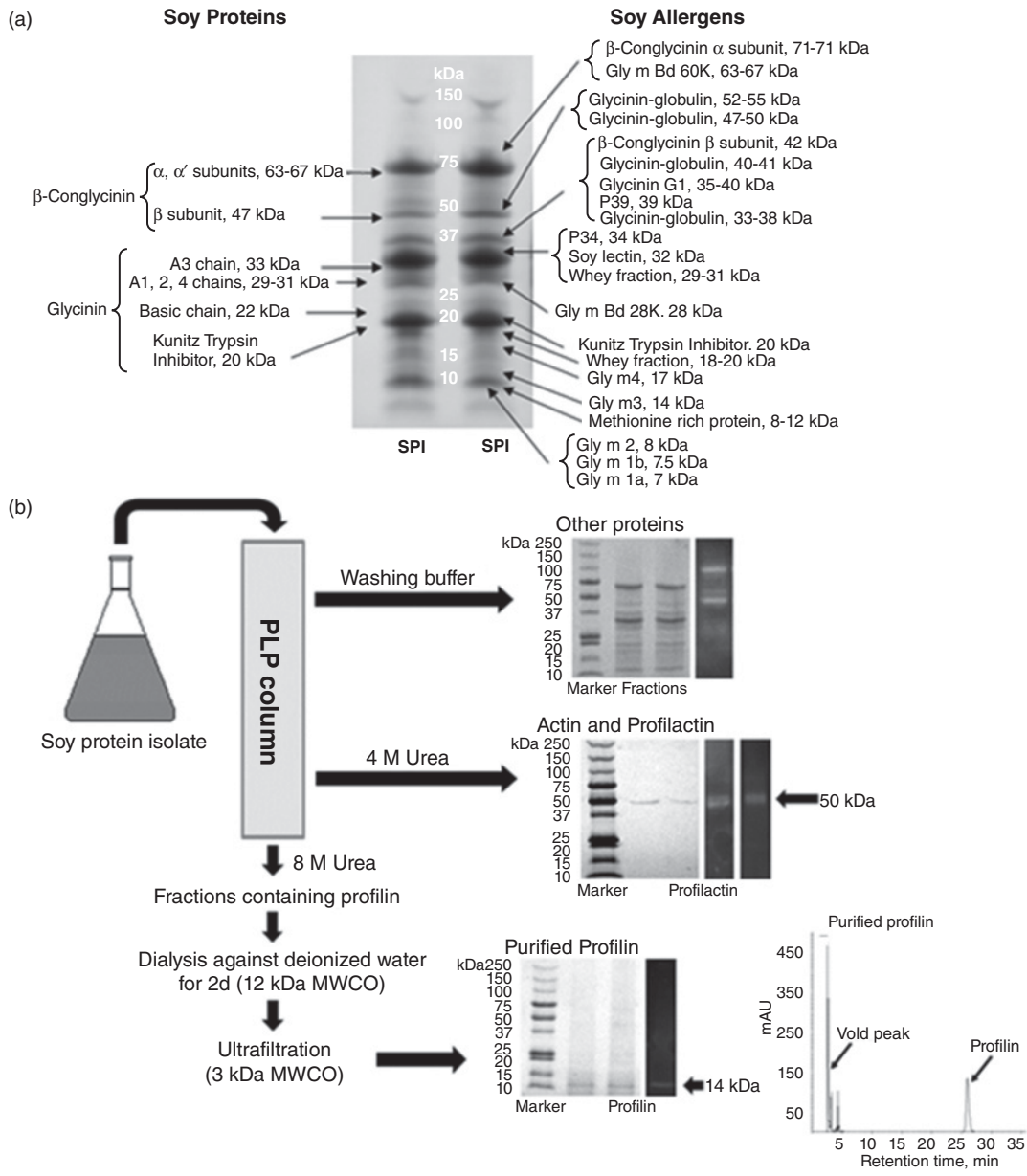
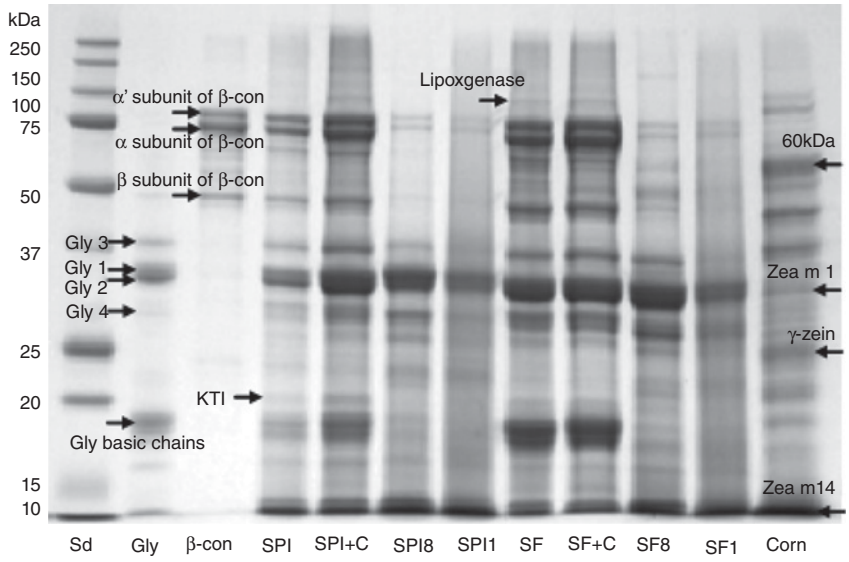


Figure 9.5 (a) Electrophoretic protein profile of soy protein isolate indicating the soy allergens. (b) Soy profilin purification steps showing native electrophoretic and immunoblotting profiles. Proteins in the PVDF membrane were incubated with 1:1000 goat profilin polyclonal antibody for 16 h at 4°C and then incubated with 1:1000 mouse anti-goat IgG HRP for 2 h and detected using chemiluminescence. A C12 RP-HPLC column (Jupiter 4u Proteo 90A column, 250 mm × 4.6 mm) was used at a flow rate of 1 mL/min with a linear gradient of solvent A (5% aqueous acetonitrile containing 0.008% TCA) and B (95% aqueous acetonitrile containing 0.1% TCA). B% from 0 to 15% in 30 min, and from 15% to 60% for 10 min, then hold at 60% for 5 min and decreased to 0% in 10 min. SPI, soy protein isolate; PVDF, polyvinylidene difluoride; IgG HRP, horseradish peroxidase-conjugated goat anti-mouse IgG; RP-HPLC, reverse phase high pressure liquid chromatography; TCA, trichloroacetic acid (Amnuaycheewa and de Mejia, 2010)



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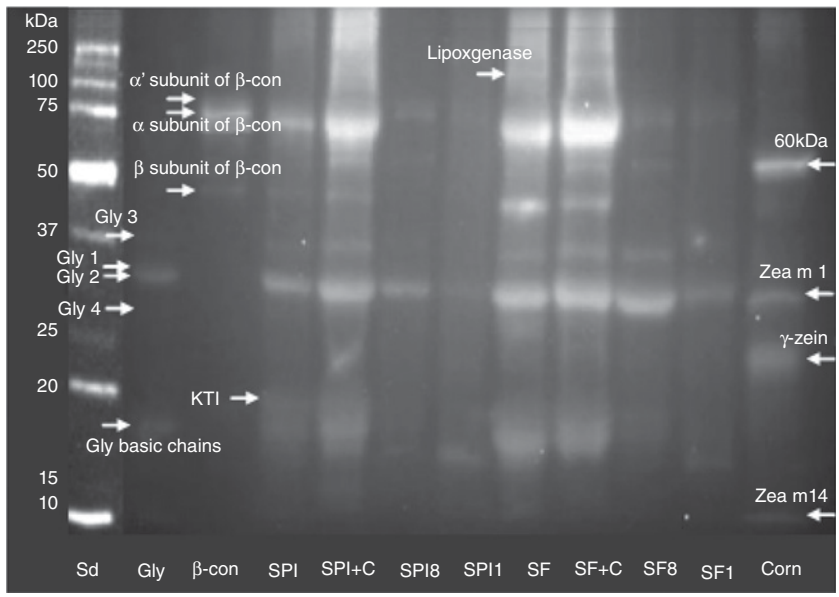


(b)

Figure 9.6 (a) SDS–PAGE electrophoretic profiles of SPI, SF, corn and their extrudates. (b) Western blot profiles of SPI, SF, corn and their extrudates. Comparison of the immunoreactivity of selected extrudates with materials extracted at pH 8.2; Sd, molecular mass standard of proteins. Gly, glycinin; ß-con, ß-conglycinin; KTI, Kunitz trypsin inhibitor; SPI, soy protein isolate; SF, soy flour; SDS–PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis (Yang et al., 2011)

Purified glycinin, ß-conglycinin, and corn meal were used as a reference. The SDS–PAGE and Western blot results of purified glycinin indicated that the acid chain is composed of Gly1, Gly2, Gly3, Gly4, and Gly5 that corresponds to 35, 32, 38, 30, and 10 kDa, respectively, and the basic chain corresponds to 18–19 kDa. As shown in Figure 9.6 the purified ß-conglycinin is composed of α, α′, and ß subunits that correspond to 75, 67, and 50 kDa proteins, respectively. Other proteins identified as allergenic proteins in SPI were 22-kDa KTI and 17-kDa 2S globulin. The allergenic protein LOX (102 kDa) was not found in SPI. For the corn meal, Zea profilin, Zea m 1, γ-zein, and Zea m 14 were identified as allergenic proteins which corresponded to 60, 35, 27, 9 kDa proteins, respectively (Weichel et al., 2006; Krishnan et al., 2010, 2011; Lee et al., 2005). Western blot profiles showed that allergens in soybean extrudates decreased significantly as compared to SPI initial material used to prepare the extrudates. Western blot results illustrate that very low intensities, almost none, of the allergen bands were observed for the SPI-corn extrudates especially SPI1. The parameters used in sample 1 decreased the immunoreactivity much more than the processing conditions used in sample 8.

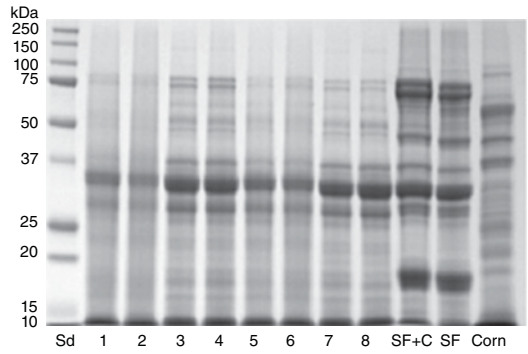
Figures 9.7a and 9.7b show SDS–PAGE and Western blot protein profiles of SPI-corn initial materials and extrudates, respectively. Different parameters influenced the immunoreactivity in the samples tested. Figure 9.7c presents the conditions used in the processing of the different extrudates; for instance, conditions used in processes 3, 4, 7, and 8 corresponding to a moisture of 40% gave higher immunoreactivity than processing conditions used in 1, 2, 5, and 6 with 20% moisture. Regarding the allergens present in corn, 60-kDa protein was completely eliminated after processing at low moisture content; no other parameter had an effect on the presence of this allergen. For γ-zein all processing conditions facilitated its elimination. In general, SPI products showed low immunoreactivity. Glycinin was more stable than β-conglycinin after different processing conditions were used. Low moisture also helped eliminate KTI more effectively. SS did not affect the elimination of KTI. If, for instance, the production of snacks uses soy flour, it is important to determine the effect of moisture and temperature on the final presence of allergens. In this study, the authors observed an effect of moisture and temperature on the remaining of β-conglycinin and glycinin in the final products.

Products 1, 2, 5, and 6 were more expanded and crispier than products 3, 4, 7, and 8. Extrusion process at lower temperatures and higher moisture content produced samples that were very hard in texture (products 7 and 8). The quick loss of moisture after expansion at higher temperature may have resulted in crispier samples.

9.3 Health aspects of soybean proteins

9.3.1 Nutritional value of soy proteins

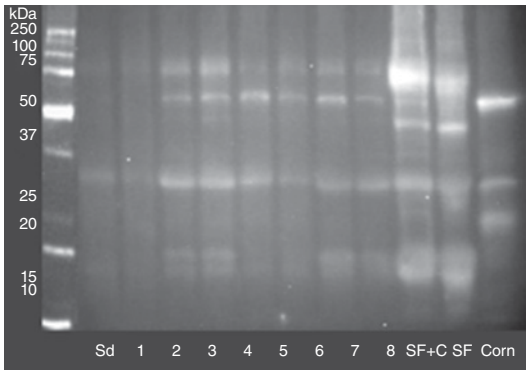
The quality of food proteins is determined by their amino acid composition, in general their ability to supply all essential amino acids. Evaluation of protein quality is necessary to determine the capacity of food proteins to satisfy the amino acid and nitrogen requirements for metabolism. Prediction of protein quality is based on the protein digestibility and amino acid composition score (PDCAAS). PDCAAS is defined as the protein



(a)

Sample code	Temperature(°C) inlet				die	Moisture (%)	Screw Speed (rpm)
1	30	75	120	130	140	20	200
2	30	75	120	130	140	20	400
3	30	75	120	130	140	40	200
4	30	75	120	130	140	40	400
5	30	75	105	105	110	20	200
6	30	75	105	105	110	20	400
7	30	75	105	105	110	40	200
8	30	75	105	105	110	40	400

(c)



(b)

(d)

Figure 9.7 (a) SDS–PAGE electrophoretic profiles and (b) Western blot profiles of soy protein isolate (SPI), corn (C) and their extrudates. SPI + C, blend before extrusion; SPI-1 to SPI-8, SPI-corn extrudates, Number 1 through 8 represent the product number processed using the different extrudate conditions; Sd, molecular mass standard of proteins; Gly represents glycinin; ß-con represents ß-conglycinin; KTI represents Kunitz trypsin inhibitor. (c) Extruded conditions and (d) pictures of final products of SPI-corn indicating the product numbers 1–8 that represent the number of the product processed using an experiment with three factors and two levels (3 × 2 designs) with two replicates. The parameters studied were barrel temperature (T) (the five temperature values represent the temperatures of five zones along the barrel from inlet to die); feed moisture concentration (MC), and screw speed (SS) (Yang et al., 2011)

digestibility multiplied by amino acid score. Protein digestibility refers to the portion of food protein absorbed, whereas amino acid score measures the effectiveness with which absorbed dietary nitrogen can meet the essential amino acid requirements at a safe level of protein intake. The amino acid score is determined by comparing the level of the limiting amino acid in the test protein with its content in the requirement pattern (WHO, 2007). Table 9.1 shows the concentration of essential amino acids from different protein sources and the suggested human amino acid requirements (Friedman, 1996). A wide range of different edible soy proteins are used for production of different products for human consumption including soy protein concentrates (SPC), isolates, full-fat, and defatted flour

Table 9.1 Essential amino acid (EAA) concentrations of different food sources and suggested human requirements, mg/g protein

						FAO/WHO requirement			
EAA	Casein	Beef	Egg white	Soy protein	Wheat flour	1 y/o	2–5 y/o	10–12 y/o	Adult
Threonine	46.4	42.1	46.8	38.4	29.3	43	34	28	9
Cys + Met	34.9	32.7	66.4	68.1	38.7	42	25	22	17
Valine	68.5	45.4	67.8	49.1	42.7	55	35	25	13
Isoleucine	53.6	41.8	52.8	47.1	33.4	46	28	28	13
Leucine	101.6	77.5	87.6	85.1	68.5	93	66	44	19
Tyr + Phe	125.4	70.2	90.8	96.6	77.8	72	63	22	19
Histidine	29.7	32.0	22.5	25.4	21.9	26	19	19	16
Lysine	84.4	79.4	69.8	63.4	26.6	66	58	44	16
Tryptophan	13.1	9.9	14.6	11.4	11.2	17	11	9	5

Source: Adapted from Friedman, 1996.
EAA, essential amino acid; FAO, Food and Agriculture Organization; WHO, World Health Organization; Cys, Cysteine, Met, Methionine, Tyr, Tyrosine; Phe, Phenylalanine.

and grits. Soybean proteins have a PDCAAS of 0.90–0.99, which depends on the processing that the soy protein has received, indicating that it is able to meet the protein needs when consumed as the only source of protein at a level of 0.6 g protein/kg body weight (Young, 1991). Although processed soy proteins are well utilized by humans, their nutritional value is still deficient when compared to milk protein. Processing such as germination can enhance the nutritional quality of soybean seeds by increasing their protein content (Mariotti et al., 1999).

9.3.2 Soy proteins and health

Lower rate of certain chronic diseases in Asian countries compared to that in the United States provided an impetus for the possible correlation between soy product consumption and reduced risk of developing chronic diseases. This purported health benefit of soy can be attributed to biologically active constituents present in soybean including isoflavones, saponins, and biologically active proteins and peptides. Biologically active proteins and peptides in soybean can either be naturally present or the result of hydrolysis either by fermentation or enzymatic treatment; for instance, consumption of soy protein has been associated with appetite regulation, thereby, possibly reducing the incidence of obesity. A previous study showed that intake of SPI and its hydrolysate can be used to reduce obesity in dietary obese and genetically obese rodents (Aoyama et al., 2000). The study compared the effect of soy protein and casein in treating obesity in obese rodents and found that rodents fed with soy protein had lower apparent absorbability of dietary energy and fat and lower body fat content in mice, and lower plasma cholesterol and glucose levels in rats compared to rodents fed with casein. It was concluded that the storage protein β-conglycinin may be responsible for the antiobesity property of soybean proteins. Nishi et al. (2003a) showed that β-conglycinin peptone inhibited food intake in

a dose-dependent manner associated with increased release of cholecystokinin (CCK) from the isolated mucosal cells of rats' jejunum. CCK is a physiologic mediator regulating satiety and gastric emptying. Further study demonstrated that the part of β-conglycinin responsible for this biological activity was fragment 51–63 showing that intraduodenal infusion of this fragment led to inhibition of food intake and marked increased in portal CCK concentration (Nishi et al., 2003b). Screening of soy and milk peptides showed that peptides from gastrointestinal digestion of soybean β-conglycinin had the capability to demonstrate CCK1 receptor activity indicating that bioactive peptides with a satiogenic effect can be used as an effective therapeutic strategy for obesity (Staljanssens et al., 2012). *In vitro* studies showed that β-conglycinin hydrolysates inhibited lipid accumulation with corresponding increase in the expression of adiponectin protein in 3T3-L1 adipocytes (Martinez-Villaluenga et al., 2008). In addition, hydrolysates from β-conglycinin downregulated expression of genes associated with obesity such as lipoprotein lipase and fatty acid synthase (Martinez-Villaluenga et al., 2009). Identification of the peptides in β-conglycinin hydrolysates showed that peptides EITPEKNPQLR and RKQEEDEDEEQQRE bound to the thioesterase domain of human fatty acid synthase suggesting that the inhibitory capacity of β-conglycinin peptides is through interaction with the thioesterase catalytic domain (Martinez-Villaluenga et al., 2010). In humans, consumption of 3 g of β-conglycinin hydrolysates resulted in enhanced fullness and reduced hunger sensation when compared with whole soy protein hydrolysates (Hira et al., 2011).

There are also naturally occurring peptides in soy products, such as lunasin. It is composed of 43 amino acid residues with cell adhesion motif arginine–glycine–aspartic acid followed by 8 aspartic acid residues at its carboxylic acid end (Galvez and de Lumen, 1999). Earlier studies have shown the capability of this peptide to inhibit cell mitosis (Galvez and de Lumen, 1999) and suppress transformation of cells initiated oncogene (Lam et al., 2003). Studies have also shown the capability of this peptide to inhibit the process of carcinogenesis. In a mouse model of skin cancer, exogenous application of lunasin reduced skin tumor incidence by 70% (Galvez et al., 2001). This anti-carcinogenic activity of lunasin may be associated with its capability to inhibit histone acetylation both in mouse fibroblast and human breast cancer cells (Galvez et al., 2001; Hernandez-Ledesma et al., 2011). Characterization of lunasin naturally isolated and purified from soybean showed that lunasin was able to inhibit colony formation induced by oncogene as well as inhibit histone acetylation in vitro (Jeong et al., 2003; Seber et al., 2012). Studies on chemically induced carcinogenesis showed that dietary lunasin was able to inhibit mammary carcinogenesis in mouse promoted by dimethylbenz(a)-anthracene (Hsieh et al., 2010a). Also, lunasin was able to inhibit the growth of MDA-MB-231 human breast cancer cells in a xenograft model of mammary carcinogenesis in mouse (Hsieh et al., 2010b). Just recently, Pabona et al. (2012) discovered the role of lunasin on mammary epithelial cells via induction of tumor suppressor PTEN. Aside from the role of lunasin in breast cancer, studies also showed the capability of lunasin to promote cell death of human colon cancer cells. Lunasin was able to inhibit the growth as well as promoted programmed cell death of different human colon cancer cells being most effective in highly metastatic cancer cells (Dia and de Mejia, 2010; Dia and de Mejia, 2011a). An *in vivo* model of colon cancer cell

metastasis showed that intraperitoneal injection of lunasin prevented the spread of colon cancer cells from the spleen to the liver of the mouse. In addition, lunasin was able to potentiate the effect of chemotherapeutic drug, oxaliplatin, in preventing colon cancer cell metastasis (Dia and de Mejia, 2011b). Possible explanation of the anticancer property of lunasin is its capability to inhibit the process of inflammation. Some *in vitro* cell culture studies have shown the capability of lunasin to prevent lipopolysaccharide (LPS)-induced inflammation in both mouse and human macrophages (Hernandez-Ledesma et al., 2009; Dia et al., 2009b; Cam and de Mejia, 2012). The mechanism involved in the anti-inflammatory property of lunasin was through inhibition of the NF–κB signaling (Cam and de Mejia, 2012; de Mejia and Dia, 2009). In addition to its anticancer property, studies have also shown that lunasin can also modify the expression of genes in different cell lines. In KM12L4 human colon cancer cells, lunasin treatment modified the expression of genes associated with extracellular matrix and cell adhesion (Dia and de Mejia, 2011a). It also modified the expression of genes in LPS-stimulated and nonstimulated mouse macrophage (Dia and de Mejia, 2011c) as well as in tumorigenic and nontumorigeneic prostate epithelial cells (Galvez et al., 2011). A feeding study in humans showed that the lunasin peptide can be found in the blood of men fed with 50 g of soy proteins, suggesting that lunasin is bioavailable and can reach target tissues and organs to exert its health-promoting effect (Dia et al., 2009a).

Other health-promoting properties of soy proteins include their roles in blood pressure, vascular function, inflammation, menopause, bone health, diabetes, and insulin resistance (Hira et al., 2011).

9.4 Soy protein analysis

The characterization of the molecular mass, isoelectric point, charges, and disulfide-bonds in subunit associations, fingerprinting, and identification of variants of storage proteins in soybean is complex (Krishnan et al., 2005). Soybean genotypes frequently differ in the number and properties of their storage protein polypeptides, the extent of this complexity has only been revealed by the application of high-resolution two-dimensional (2-DE) SDS–PAGE. The major advantage of using 2-DE SDS–PAGE, is that it provides the highest resolution in analytical separations (Agrawal et al., 2005). High resolution of 2-DE, could be increased by a prefractionation step, which is usually carried out to reduce the complexity of the sample, to minimize protein degradation as a result of the presence of proteases, and to remove interfering compounds such as salts, lipids, nucleic acids, polyphenols, alkaloids, pigments, terpenes, organic acids, and other compounds (Gorg et al., 2004). Protein degradation attenuation and removing interfering compounds could be improved using trichloroacetic acid/acetone. The use of immobilized pH gradient strips for isoelectric focusing followed by 2-DE PAGE has improved the separation of proteins up to pH 12 (Agrawal et al., 2005). Soybean proteins identification could be performed with sample solubilization and fractionation according to protein isoelectric point using multiple narrow ranges of pH gradient strips, as an important step in developing a data base for assessing soybean protein quality (Zarkadas et al., 2007).

9.5 Physicochemical modifications of soy proteins by technological approaches

The use of new processing technologies, enzymatic and chemical reactions in the modification of soybean proteins have been increasing. Soy protein ingredients allow new and sophisticated applications in the food industry; however, improved functional properties of these ingredients are obtained through physicochemical changes in the structure and size of the protein. Table 9.2 presents some of the latest research and the use of technology on the modification of soy protein and their physicochemical changes to improve their functional properties. The process could induce desired physical or chemical modifications that affect protein behavior in a food matrix. The treatments cover physical technologies such as ultrafiltration/diafiltration, microfluidization, ultrasound and high hydrostatic pressure (HHP), to chemical reactions as acid treatment, acylation, oxidation and phosphorylation; also, enzymatic hydrolysis and the combination of microbial enzymes with Maillard reaction.

SPI and SPC are the most common soy protein commercial ingredients. These products are often used as food ingredients because of their nutritional and functional characteristics. SPI (≥90% protein) is typically manufactured by extracting protein in an alkali solution, followed by acid precipitation (pH 4.5), washed, and dried (Malhotra and Coupland, 2004). The main components of SPI are glycinin (11S protein) and β-conglycinin (7S protein), which represent 34% and 27%, respectively, of the proteins occurring in the isolate (Tang et al., 2009). SPC is obtained after soy whey (soluble carbohydrates) is removed from defatted soy flour. Dry SPC is a rigid material and it contains both soy protein and insoluble soy carbohydrates (Jong, 2006). Commercial SPC are prepared by removing alcohol-soluble nonprotein compounds from defatted meal with 60–80% aqueous alcohol. SPC protein content is around 80% (Mounts et al., 1987).

9.5.1 Ultrasound treatment

Ultrasound treatment is a physical technology that is attracting the attention of the food industry. It is based on mechanical waves at a frequency above the human hearing and can be divided into two frequency ranges—high and low. Tang et al. (2009), Jambrak et al. (2009) and Hu et al. (2013) used ultrasound to modify functional properties of commercial soy proteins. The modifications in the protein structure as a consequence in its functionality occur because of different mechanisms. The mechanisms depend on the frequency, time, medium, homogenization, and soy protein source. The improved new soy proteins could be used in products as gels, smoothies, cheese alternatives, soy cream–based soups, spreads, and creamy dressings. The action of ultrasonic treatment in the formation of soluble aggregates is because the treatment makes small unstable aggregates in homogenized dispersions oscillate violently and thus remarkably slow down the association of them. This might accelerate the process of new balanced interactions between an unstable aggregate and other protein components present in the dispersion. The stable structure of formed soluble aggregates is maintained by noncovalent and covalent interactive

Table 9.2 New technologies that improve functionality by soy protein modification

New technology	Objective	Protein modification	Functionality/applications	Reference
Membrane Technologies: Ultrafiltration/ Diafiltration	Evaluate the impact of the extraction conditions to obtain SPI with low phytic acid content	Fewer protein-divalent cation-phytic acid ternary complexes were formed making the phytic acid removal more efficiently	Improvement of solubility of SPI	Lai et al., 2013
Microfluidization	Evaluate the rheological properties and microstructure of gel-like emulsions from native and preheated SPI, under well-controlled conditions, produced by microfluidization emulsification	Colloidal destabilization, especially droplet aggregation through strong attractive interactions between emulsion droplets	Improve the emulsification efficiency and thus can exhibit more potential to be applied to prepare the gel-like emulsions	Tang and Liu, 2013
Ultrasound	Characterize the formation of soluble aggregates and the changes on physical properties of soy proteins	Formation of small aggregates that interact and balance energy forces with other protein components, through non-covalent and covalent interactions. Rapid molecule movement due to cavitation and microstreaming, produce unfolding of protein chains	Gels and creams formed from SPC, SPI could be used in smoothies, cheese alternatives, soy cream based soups, spreads and creamy dressings. Improved solubility and gelling properties of commercial SPI extend the practical applications in the food industry	Hu et al., 2013 Jambrak et al., 2009 Tang et al., 2009
Acid treatment	Obtain a food ingredient with foaming properties using acid treatment followed by neutralization	Proteins unfold, exposing hydrophobic patches and become more flexible; favors their adsorption in the interface and decreases the surface tension	Improved surface and foam properties	Ventureira et al., 2012
Acylation with saturated fatty acids	Effects of acylation on the emulsifying properties of soy proteins using a variety of saturated fatty acids	Promote unfolding and increase the dissociation of the subunits from the quaternary structures, as well as shifting the isoelectric point to lower values	Functional properties (emulsifying) of soy proteins were successfully improved	Matemu et al., 2011

Enzymatic hydrolysis	Study the effects of combined extrusion pre-treatment and controlled enzymatic hydrolysis using pancreatin on the emulsifying properties of SPI	Proteases have specificity to peptide bonds, cleaving them, exposing hidden hydrophobic residues and reducing the molecular size	Produced prominent benefits in improving emulsifying capability of SPI and the stability of their emulsions.	Chen et al., 2011
Oxidation by peroxyl radicals	Characterize the effects of acrolein on soy protein structure	Acrolein reacted with the imidazole group of histidine residues, the 3-amino groups of lysine residues and the sulfhydryl groups of cysteine residues in soy protein to form covalent adducts, which contributed to protein carbonylation, degradation of protein sulfhydryl groups and cross-linking formation	Oxidation by peroxyl radicals affect protein structure; modifying SPI functional properties	Wu et al., 2010
High hydrostatic Pressure	Investigate the effects of HP treatment on the aggregation and structural conformations of the proteins in SPI	The treatment led to different changes in protein solubility, surface hydrophobicity, free SH content and secondary structure and unfolding. The unfolded proteins tend to aggregate mainly via hydrophobic interactions	Improvement of emulsifying activities due the formation of protein aggregates including those insoluble and soluble	Tang and Ma, 2009
Transglutaminase and Maillard cross- linking	Evaluate the effects of mTGase pre-incubation and ribose-induced Maillard cross-linking in the SPI behavior	Catalyzing an acyltransfer reaction between the γ -carboxyamide group of peptide bound glutamine residues (acyl donors) and variety of primary amines (acyl acceptors), including the e-amino group of lysine residues to form an ε-(γ -glutamyl) lysine isopeptide bond and reducing sugars and amino groups of amino acids and proteins to produce cross-linked protein in Maillard reaction	Obtain higher G′ values in combined cross-linked samples	Gan et al., 2009
Phosphorylation	Evaluate the degrees of phosphorylation and the best conditions of phosphorylation of SPI	Phosphorylation made the repulsion stronger than gravitation and the exposure of hydrophobic groups in the phosphorylated SPI favored the diffusion and the rearrangement of protein in the oil–water interface	Change functional properties as emulsification, solubility and viscosity	Zhang et al., 2007

SPI, soy protein isolate; HP, high pressure; SPC, soy protein concentrates; G′, storage modulus; SH, sulfhydryl; mTGase, microbial transglutaminase.

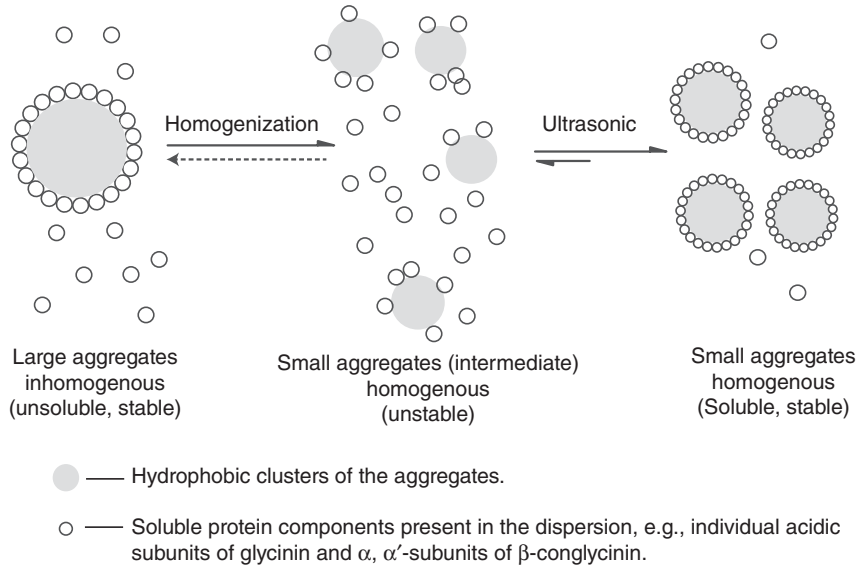


Figure 9.8 Proposed mechanism for the formation of soluble aggregates from insoluble precipitates from commercial soy protein isolate (SPI) by means of ultrasonic treatment. From Tang et al. (2009) Reproduced with permission of Copyright © 2013, Elsevier

forces, including hydrophobic and electrostatic interactions, as well as disulfide bonds. The formation mechanism of soluble aggregates from insoluble precipitates of commercial SPI, by means of combined homogenization and ultrasonic treatment, is proposed in Figure 9.8 (Tang et al., 2009). Hu et al. (2013) reported the increase of protein solubility after ultrasonic treatment following the partial unfolding and reduction of intermolecular interactions by increasing free sulfhydryl groups and surface hydrophobicity. Protein modification is also produced because of cavitation and microstreaming, causing unfolding of protein chains, leading to hydroxyl radical development of water hydrolysis and radical chain reaction leading to polymerization. Jambrak et al. (2009) obtained a gel from SPC after ultrasound treatments, and a creamer from SPI.

9.5.2 High hydrostatic pressure

HHP has been widely applied to modified soy proteins to improve their functional properties. Tang and Ma (2009) studied its effect on SPI. HHP treatment significantly increased aggregate formation. The aggregation may be because of HHP-induced unfolding and denaturation of proteins; unfolded or denatured proteins would associate to form higher molecular mass protein products via hydrophobic and other weak interactions. Unfolded and denatured proteins formed during initial stages of HHP treatment may undergo re-association or aggregation. Because of hydrophobic or other interactions, to refold the damaged secondary structure during the later stages of HHP treatment, or after the release of HHP allowed the unfolded protein to partially rebuild its secondary structure. This change in the aggregation is related with the improvement of emulsifying properties.

Changes in solubility and gelling also are attributed to differences in aggregation unfolding/refolding extents of proteins (Wang et al., 2008c). Alvarez et al. (2008) also reported increase in hydrophobicity, as well as the relative proportion of random coil, whereas the β-sheet content decreased enhancing viscoelastic behavior of SPC.

9.5.3 Membrane technologies

Membrane separation processes stand out as alternatives to conventional processes for the chemical, pharmaceutical, biotechnological, and food industries. In many cases the low energy consumption, reduction in number of processing steps, greater separation efficiency and improved final product quality are the main attractions of these processes. One important application in soy industry is the reduction of phytic acid as an undesirable molecule that affects functional properties, and nutritional value of soy proteins (Ali et al., 2010). Lai et al. (2013) studied the utilization of ultrafiltration/diafiltration in the reduction of phytic acid from SPI. They obtained SPI with low phytic acid content by considering pH adjustments between the ultrafiltration step and diafiltration step and also considering the KCl concentration. The total phosphorus content was diminished, indicating that pH and solvent were factors that significantly influenced the total phosphorus content. It was partly because of the monovalent potassium ions provided by KCl, which could bind to proteins as pH increased. The monovalent potassium ions should not be able to ionically bind to phytic acid after binding to the proteins. They hypothesized that fewer protein-divalent cation-phytic acid ternary complexes were formed making the phytic acid removal more efficient with KCl than with water. Ali et al. (2010) also decreased phytic acid content of SPI using different pH and salts by membrane technologies.

9.5.4 Microfluidization

Microfluidization is one of state-of-the-art homogenization techniques. It uses high pressure to force the fluid into microchannels of a special configuration and initiates emulsification via a combined mechanism of cavitation, shear, and impact, thus exhibiting excellent emulsification efficiency (Shen and Tang, 2012). This could greatly improve the emulsification efficiency and thus exhibit much more potential to be applied to prepare the gel-like emulsions. The instability of protein in emulsions is affected by many variables, such as pH and ionic strength, the presence of other compounds, protein concentration, and oil volume fraction, thermal denaturation, and/or aggregation of the proteins. A key variable determining the rheological and microstructural properties of the emulsions is the volume fraction of the dispersed oil phase. The mechanism of the gel-like structure formation is due to colloidal destabilization, especially droplet aggregation through strong attractive interactions between emulsion droplets (Figure 9.9) (Tang and Liu, 2013).

9.5.5 Acid treatments

Hydrochloric acid addition is a chemical treatment to reduce the pH of protein solutions without adding other chemicals; upon acidification the protein molecules unfold, expose more hydrophobic patches and become more flexible. This favors their adsorption in the

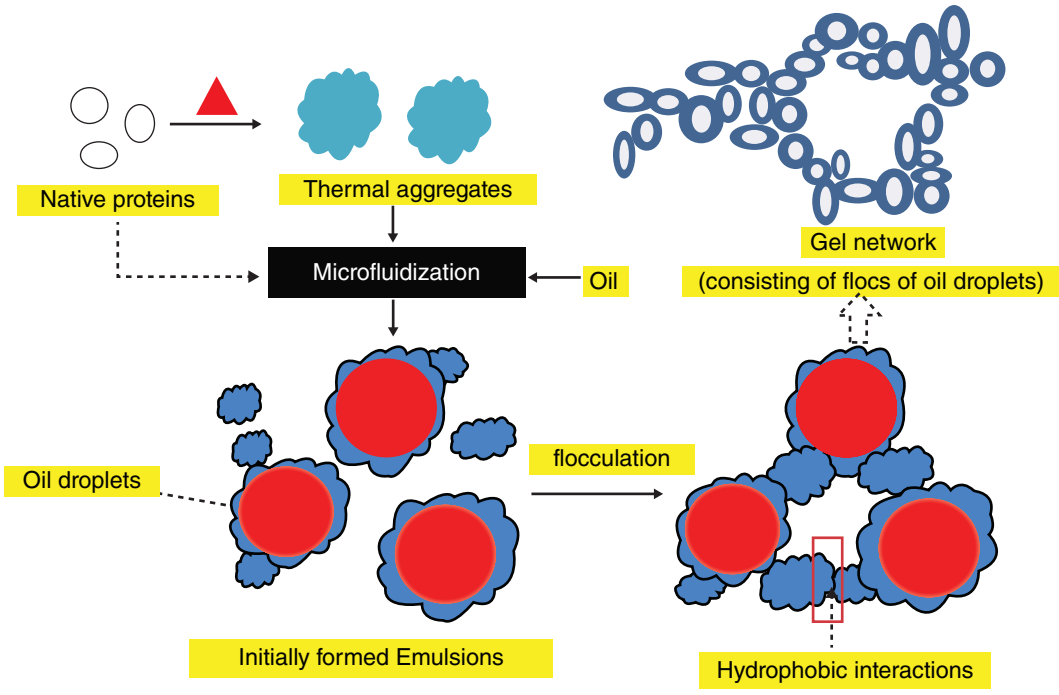


Figure 9.9 Scheme for the formation process of gel-like emulsion from preheated soy proteins, obtained by microfluidization. From Tang and Liu (2013) Reproduced with permission of Copyright © 2013, Elsevier

interface and decreases the surface tension. Surface tension reduction is probably the result of the smaller size of proteins after treatment, caused by either subunits dissociation or by the hydrolysis of proteins. Although these characteristics favor foam formation, foam stability is poor when completely unfolded proteins are used. Partial unfolding may be reached by mild acid treatments or by acid treatment followed by neutralization. It was demonstrated that after this pH-shifting process, soy proteins adopted a molten globule-like conformation that led to a markedly improved emulsifying activity and emulsion stability. Ventureira et al. (2012) reported that acid pH exposure disorganized the globular structure, which was not recovered after raising the pH to almost neutral values, thus allowing improving their foaming capacity. The authors concluded that the mixture of cereals and soybean proteins subjected to acid treatment could create an ingredient with improved surface and foam properties compared with nontreated components.

9.5.6 Acylation

Acylation of free amino groups on a polypeptide chain involves the utilization of reagents that react covalently at the amino sites of the proteins. The mechanism, depending on the nature of the modifying agent, can affect the charge balance of the molecule in three different ways—by preserving the positive charge on the amino groups, by abolishing the

charge and bringing it to neutrality, or by imposing a negative charge as a substitute for the original positive charge. The acylation of the ε-amino groups of the lysine residues, particularly with succinyl groups, markedly enhances several properties of proteins, including their emulsifying properties; water- and oil-binding and surface hydrophobicity (Mirmoghtadaie et al., 2009). Improving physicochemical functionalities of soy proteins will provide an insight into functional food formulation and processing. Matemu et al. (2011) found that the emulsifying properties of soy protein were enhanced as a result of acylation and this could be attributed to the increased surface hydrophobicity following unfolding. This process altered protein conformation by promoting unfolding, and increasing the dissociation of the subunits from the quaternary structures, as well as shifting the isoelectric point to lower values. The ability of acylated soy proteins to form and stabilize emulsions is influenced by the unfolding of protein polypeptides, charged moieties, length of fatty acids, size of protein and re-arrangement at the protein-oil interface, in contrast with the native protein. There are many factors including molecular size, molecular flexibility, and molecular charge, besides the balance between a hydrophilic and a lipophilic, which participate in determining the emulsifying properties of proteins.

9.5.7 Phosphorylation

One of the most important modifications of proteins is phosphorylation (Li et al., 2010). Phosphate groups can be attached to the oxygen of seryl, threonyl, aspartyl (β-carboxyl), and tyrosyl residues and via nitrogen to lysyl (ε-amino) and histidyl (1 and 3) residues. The major advantages of phosphorylation of food proteins are the increase in solubility and the decrease in pI of the proteins, thereby changing the functional properties, especially near the pI of the original proteins. Kunsheng et al. (2007) reported that at low pH levels, the solubility of modified proteins were greater than that of native proteins. This might result from the attached phosphate groups in the modified protein molecules, which increased the electrostatic repulsions between protein molecules so that protein molecules could dissolve even in acidic conditions. The emulsifying activities of modified proteins improved with the increase of pH. It is mostly because the phosphorylation that made the repulsion stronger than gravitation and the exposure of hydrophobic groups in the phosphorylated SPI favored the diffusion and the rearrangement of protein in the oil–water interface. To some extent, the emulsification was improved. The change of viscosity showed the change of molecule layers from the combination of protein molecules and $PO^3{}_4{}^-$. The $PO^3{}_4{}^-$ changed the shape of protein molecules and surface charges, which had great effect on the hydration layers and the interactions of proteins.

9.5.8 Enzymatic hydrolysis

Enzymatic hydrolysis has been widely used to improve protein functionalities. The physicochemical properties of hydrolysates mainly depend on the degree of hydrolysis (DH)

and type of proteases used. Controlled enzymatic hydrolysis of globular proteins can improve their emulsifying properties, by improving overall protein solubility, exposing hidden hydrophobic residues, and reducing the molecular size (Tsumura, 2009). This process requires regulating the hydrolysis with a combination of temperature, time and enzyme concentration for a desirable DH value. Chen et al. (2011) studied the effect of combined extrusion pre-treatment and controlled enzymatic hydrolysis using pancreatin on the emulsifying properties of SPI. Pancreatin has broad specificity to peptide bonds. This enzyme access cleavage sites more easily for treated SPI than for nontreated SPI. Mechanical pretreatment, such as extrusion, improved the accessibility of globular proteins to enzymatic hydrolysis. The treatment improved protein solubility by decreasing molecular mass and increasing charged groups, increasing the emulsifying capability and stability of SPI.

9.5.9 Oxidation

Protein oxidation is the covalent modification of a protein induced either directly by reactive oxygen species or indirectly by reaction with secondary by-products of oxidative stress. Proteins are susceptible to oxidative damage as a result of their abundance in foods and high rate constants for reaction. Oxidative modification has been shown to induce a number of structural changes in amino acid residue side-chains and protein polypeptide backbone, resulting in protein fragmentation, cross-linking, unfolding, and conformational changes (Davies, 2005). The oxidative modification of protein structure has been implicated in quality deterioration of protein-based food and bioavailability of the protein. The factors affecting functional properties of soy protein are complex, and oxidative modification of protein structure has been implicated in the alteration of gelling properties of soy protein. Protein oxidation and lipid peroxidation (LPO) are usually interdependent of each other (Huang et al., 2006). Soy protein is one of the potential targets to be modified by LPO products during processing and storage. LPO is a complex free radical reaction with lipid hydroperoxides (LHP) as major initial products and eventual formation of reactive aldehydes. There is abundant evidence that LPO products, which include free radicals, LHP and reactive aldehyde derivatives, are capable of modifying proteins.

Compared with free radicals and LHP, the LPO-derived reactive aldehydes are stable and can diffuse away from their site of generation; thus, transport the oxidant function to other target sites. Because of their high reactivity and relative longevity, reactive aldehydes formed during peroxidation of polyunsaturated fatty acids may play a significant role in the process of LPO product mediated-protein oxidation. Wu et al. (2010) used acrolein as a reactive aldehyde to investigate the mechanism of soy protein modification by reactive aldehydes. Acrolein exhibits the greatest reactivity with proteins through either reaction of Michael addition or formation of Schiff base-type adducts, and thereby influencing the overall functional properties and nutritional quality of the target proteins. Acrolein reacted with the imidazole group of histidine residues, the 3-amino groups of lysine residues and the sulfhydryl groups of cysteine residues in soy protein to

form covalent adducts; these contributed to protein carbonylation, degradation of protein sulfhydryl groups and cross-linking formation. Acrolein caused loss of α-helical structure and increase of the β-sheet structure content in soy protein. In summary, acrolein showed high reactivity with soy proteins and affected their structure, modifying SPI functional properties.

9.5.10 Cross-linked reactions

The application of transglutaminase enzyme in protein modification has attracted attention of the food industry. Microbial transglutaminase (mTGase) (protein–glutamine:amine γ-glutamyltransferase, E.C. 2.3.2.13) works by catalyzing an acyltransfer reaction between the γ-carboxyamide group of peptide bound glutamine residues (acyl donors) and a variety of primary amines (acyl acceptors), including the ε-amino group of lysine residues, to form an ε-(γ-glutamyl)lysine isopeptide bond (Motoki and Seguro, 1998). This treatment has been used in food products in enhancing the texture and functional properties (Gan et al., 2009). Another potential modification method, which is the Maillard cross-linking or Maillard reaction comprises the reaction between reducing sugars and amino groups of amino acids and proteins, has been shown to produce cross-linked protein and improved protein gel strength and texture of protein-based products (Md Yasir et al., 2007). To enhance the functionalities of cross-linked protein such as gelling capacity and water holding capacity, it is possible for technologists to use a combination of two cross-linking treatments; however, there could be situations when the cross-linked products are subjected to heat treatments (cooking, retort cooking, blanching or drying) in the presence of reducing sugars or other ingredients capable of undergoing the Maillard reaction. Gan et al. (2009) studied the effects of mTGase pre-incubation in the behaviors of SPI particles prior to ribose-induced Maillard cross-linking on the gelling capacity, rheological properties and the network/non-network protein formed. The rheological study showed different gelling behaviors of mTGase pre-cross-linked SPI during the heating in the presence of sucrose or ribose, and gave higher G' (storage modulus) values in combined cross-linked samples.

9.6 New trends of soy proteins in food applications

The application of soybean proteins in food products is increasing because of its availability, low cost, and nutritional value. There are several research groups working on the development of new functional products based on soy protein. These ingredients are based on new characteristics and functional properties of the soy protein or mixes with other ingredients to improve the quality of gels, emulsions, foams, dough, among others (Table 9.3). Commercial products available in the market, developed by the most important soybean processing companies, are described in Table 9.4 as well as their applications in food products. Current information about the soybean industry can be found in the Soya and Oilseed Bluebook 2013 (Soyatech, LLC).

Table 9.3 Soy proteins: applications as food ingredients

Physical properties	Components of mixture	Objective	Applications in foods	Reference
Emulsifying properties	Soy whey protein isolate	Study the suitability of SWPI-fenugreek gum conjugates prepared by controlled dry heating of soy whey protein isolate-fenugreek gum mixtures	Application of SWPI-fenugreek gum conjugates showed an improvement in emulsion stability at high salt concentration compared to SWPI alone.	Kasran et al., 2013
Emulsifying and gelling	Acid-induced soy protein-stabilized	Rheological properties of acid-induced soy protein-stabilized emulsion gels as affected by previous heat treatment	Emulsifiers and gelling agents	Li et al., 2012
Physical properties	Soy protein-pectin complexes	Study the interactions between high methoxy-pectin and a commercial SPI and the effect of thermal processing	SPI-rich beverage at pH 4 using pectin	Jaramillo et al., 2011
Cold-set hydrogels as carriers	Soy protein	Produce a device that provides effective protection of molecules against digestive enzymes and low gastric pH plus prolonged release under intestinal conditions	Functional foods containing nutraceutical molecules to be delivered in the colon.	Maltais et al., 2010
Thermosetting gelation	Soy protein isolates/ Gluten	Evaluate whether addition of SPI to gluten-rich mixes affect the gluten thermosetting gelation	Gluten/soy thermoset gel could be used in bread baking and processing texturized vegetable protein products	Bainy et al., 2010
Emulsifying and gelling	Soy protein isolate/Oils	Studied the effects of SPI heated and emulsified with different oil types at neutral pH on glucono-δ-lactone induced emulsion gel	Development of soy cheeses	Gu et al., 2009
Foaming	Soy protein hydrolysates	Study the impact of soy protein hydrolysis on foaming and interfacial properties and their relationship	Foam precursors and emulsifiers	Martinez et al., 2009
Oil-in-water emulsions	Soy protein isolates	Observe the behavior of soy proteins upon adsorption at the oil-in-water interface as a function of heating	Emulsifiers and gelling agents	Keerati-u-rai and Corredig 2009

Stability and particle size distribution of soy milk	Soy protein	Effect of protein composition on the structure of soymilk made from different soybean genotypes and the effect of heating and homogenization on the stability and particle size distribution	Soy milk	Malaki et al., 2009
Aggregation and phase separation	Soy protein aggregate/ dextran mixtures	Study the effects of both ionic strength and colloid size on the phase separation of soy protein and dextran mixes	Emulsifying and gelling	Li et al., 2009
Structural changes	Soy protein/meat	Study the changes in protein structure produced in meat batters by soy protein addition and thermal treatment	Formulations of healthier meat products with added soy protein.	Herrero et al., 2008
Gelation characteristics	Corn starch/soy protein concentrate	Study the gelation characteristics of corn starch/soy protein concentrate composite and the structural changes	New starch/soy ingredient with optimal gelation characteristics	Li et al., 2007
Quality of pasta product	Sweet potato/ soy protein	Developed new pasta from alkaline treated sweet potato flour fortified with soy proteins	Sweet potato-soy protein pasta	Limroongreungrata and Huang, 2007
Interfacial behavior	Soy protein/ polysaccharides	Studied the interfacial behavior of mixed soy proteins + polysaccharides systems and their interactions at the air–water interface	Foaming and emulsifiers	Martinez et al., 2007

SWPI, soy whey protein isolate; SPI, soy protein isolate; pH, potential of hydrogen.

Table 9.4 Commercially available soy protein ingredients and their applications

Company	Product category	Description	Application	Reference
Cargill	Soy Flour	Is derived from dehulled soybeans, which offers 50% protein by weight. Its many functional attributes include emulsifying, flavor-binding, fat repulsion, water absorption and binding properties	Bakery, processed meats, snacks, cereals, bars, pharmaceutical, industrial, seasonings, flavors	[a]
Cargill	Texturized soy flour	High quality soy protein to replace traditional protein in beef, chicken and fish products. Excellent fat binding and water absorption that results in increased yields and profits	Processed meats, snacks, cereals, cereal bars.	[a]
Cargill	Flavored soy flour	A textured soy flour base flavored to mimic bacon bits offers a lower fat, lower cost alternative to traditional bacon bits	Salad bars, pizza toppings, bakery	[a]
Cargill	Texturized soy protein	Shape of a diced chunk or crumble and available in different colors and sizes. It hydrates quickly in warm water and is convenient to use to replace meat in existing recipes. Neutral taste and absorbs the inherent flavors	Soups, stir-fry entrees, meat, fillings, meat salads, prepared meals	[a]
Solae	Soy protein isolate	90% protein on a dry weight basis and virtually carbohydrate and fat-free. Cholesterol- and lactose-free. Protein ingredient for meat-based and dairy-based products	Nutritional, functional and economical alternative to traditional proteins in food bars, beverages, baked goods, breads and cereals	[b]
Solae	Soy protein concentrate	Retain most of the fiber from the original soybeans and must contain at least 65% protein on a moisture-free basis	Processed meat products, and the main applications of the granulated protein are ground meat formulations	[b]
Solae	Texturized soy protein	52% percent protein on a dry basis. Textured soy proteins are fundamental ingredients in the food industry	From meatballs to pastry fillings. Protein source in vegetarian meat alternatives	[b]
Solae	Structured soy protein	Combination of soy protein and other vegetable ingredients. The key attribute of these proteins is their fibrous structure, which can be beneficial in a variety of food systems	Functional, nutritional and/or economic benefits can result from the use of structured vegetable protein products in processed meats, vegetarian goods and snacks	[b]

ADM	Soy protein isolate	Clean-flavored isolated soy proteins with minimum protein levels of 90% on a moisture-free basis, readily dispersible, low viscosity, highly soluble low flavor, low odor	Beverages, bars, dairy alternatives, extruded snacks and cereals, emulsified meats, sauces, gravies, and soups, processed dairy foods, milk replacer, infant formulas	[c]
ADM	Clarisoy	Specially processed for use in beverage systems with pH levels of less than 4.0, 100% soluble and transparent in beverages,	Best used in sports nutrition beverages, citrus-based drinks, fruit-flavored beverages, lemonades, powdered beverage mixes, fruit juice blends, and fortified waters	[c]
ADM	Soy protein concentrate	Minimum protein content of 65% on a moisture-free basis and a fiber content of 20%, Available both as a powder and as textured pieces	Meat, poultry & fish, meat analogs, beerbread, ice cream, novelties, dairy replacements, cream soups, nutrition bars and cereal	[c]
ADM	Soy flour/grit	Protein levels of 50% on a moisture-free basis are available in a variety of granulations	Fermentation, ground meat systems, cookies and crackers, specialty breads, gluten-free baking, doughnuts and sweet goods	[c]
ADM	Texturized soy protein	Feature a fibrous meatlike texture with outstanding moisture retention and provide excellent mouth feel, making them perfect for meat and vegetarian applications	Ground meat and poultry, formed meat products, vegetarian and analogs, nutrition bars, cereals & snacks	[c]
ADM	Soy Protein Crisps	60% or 80% protein and as an industry leading 85% protein. Excellent choice for protein fortification, delivering the nutrition, great texture, and bland flavor formulators love to work with	Snack bars, confectionary items, cereals and snacks, ice cream toppings and baked goods	[c]

Authors declared there is no conflict of interest with any of the companies cited in this table.
ADM, Archer Daniels Midland Company
[a] http://www.cargillfoods.com/na/en/products/protein/soy-proteins/index.jsp
[b] http://www.solae.com/Soy-Ingredients/Solae-Ingredients.aspx
[c] http://www.adm.com/en-US/products/food/proteins/Pages/default.aspx

9.6.1 Emulsions

Proteins in general act as macromolecular emulsifiers in the formation and stabilization of many food dispersions. Because of their amphiphilic nature, proteins tend to place themselves at the air–water and the oil–water interface, preventing coalescence of foams and emulsions, respectively (Santiago et al., 2008). Soy proteins functional properties such as gelling ability and aggregation behavior are a consequence of their surface properties as well as their emulsifying capacity. These are greatly affected by variations in pH, salt content, and heat treatment, among others. In this sense, ingredients and processing technology in foods affect protein behavior due to interactions with other food components.

Fat plays an important role in the texture of many foods, imparting viscosity and mouthfeel to milk, cheese, soy cheese, ice cream, cakes, and processed meats (Gu et al., 2009; Renkema et al., 2000). On the other hand, protein–polysaccharide conjugates combine the emulsification properties of the protein with the stabilizing effect of the polysaccharide getting synergistic emulsifying properties, through covalent coupling to form protein polysaccharide hybrid molecules or noncovalent interaction through electrostatic interactions (Neirynck et al., 2004).

Gu et al. (2009) found that fat and oil have different effects on emulsifying properties and rheological characteristics of heat induced SPI because of the difference in crystalline character. At neutral pH, the palm stearin emulsions showed higher stability than the sunflower and soy oil emulsions. Also, thermal treatment of soy protein emulsions induces droplet flocculation and a marked increase in viscosity and shear thinning behavior of the emulsions. Heat on soy proteins solutions or emulsions causes protein–protein interactions, leading to changes in the supramolecular structure of the aggregates at the interface; related directly to temperature and homogenization. (Keerati-u-rai and Corredig, 2009). Other interesting application to optimize emulsion properties is the combination of soy whey protein isolate (SWPI) with fenugreek gum. Kasran et al. (2013) found that the emulsifying properties were greatly increased by heating the SWPI-fenugreek gum conjugates before emulsification. Application of SWPI–fenugreek gum conjugates also showed an improvement in emulsion stability at high salt concentration compared with control.

9.6.2 Gels

Gelling is one of the most important properties of SPI. This property can be affected or improved by adding acids and salts; however, mixtures with polysaccharides (PS) also modify functional characteristics of soy proteins. Li et al. (2012) reported that acid-induced soy protein stabilized emulsion gel had a longer storage time and a shorter gelation time. Gelation time decreased with the increasing protein concentration, acidification temperature and oil volume fraction. Li et al. (2009), studied a soy protein and dextran mix model and found that the presence of NaCl led to a significant increase in soy protein aggregation as shown by an increase in turbidity and aggregate fractions, as well as an increase in radius of gyration; thus, segregated phase separation of soy protein aggregates/dextran was favored by the addition of salts.

Li et al. (2007) studied the gelation characteristics of corn starch/soy protein concentrate and found that the gelatinization of starch granules played an important role on the rheological properties of the composite during heating. It was observed that starch granules lost integrity and formed a gel with SPC at the temperature of maximum G' (Storage modulus).

9.6.3 Foaming

Foam formation is influenced by the adsorption of the foaming agent at the air–water interface and its ability to rapidly reduce surface tension; however, foam stabilization requires different surface properties such as the formation of a cohesive viscoelastic film via intermolecular interactions (Martinez et al., 2009). PS are used in admixture to proteins mainly to enhance stability of dispersed systems. Most high-molecular-weight polysaccharides, being hydrophilic, do not have much tendency to adsorb at the air–water interface, but they can strongly enhance the stability of protein foams by acting as thickening or gelling agents (Dickinson, 2003).

The study of the impact of soy protein hydrolysis on foaming and interfacial properties and their relationship demonstrated that a low DH (2–5%) would be enough to improve the surface activity of soy protein, decreasing foam drainage and maintaining a considerable viscoelasticity of the surface films to retard foam collapse (Martinez et al., 2009). Martinez et al. (2007) used hydroxypropylmethyl cellulose as surface-active polysaccharides and *lambda* carrageenan (λC) and locust bean gum as non–surface-active polysaccharides to study the interaction of these polymers at the air-water interface. They suggested that in spite of their role as thickeners, PS had a direct influence on the air–water interface allowing the improvement of film properties when used in admixture with soy proteins so that they potentially could control and improve the stability of soy proteins-based dispersed food products.

Suppavorasatit et al. (2011) studied the effects of enzymatic deamidation by protein-glutaminase (PG) on the functional properties of SPI. The results showed that solubility of deamidated SPI was enhanced under both acidic and neutral conditions. SPI with higher deamination degree showed better emulsifying properties and greater foaming capacity than SPI, while foaming stability was decreased. It is possible to modify and potentially improve the functional properties of SPI by enzymatic deamidation using PG.

9.6.4 Hydrogel

The effectiveness of biomedical and pharmaceutical polymer-based delivery systems for entrapping sensitive nutraceutical molecules and protecting them until they reach the intestine has been studied. The development of natural hydrogels made from food biopolymers and especially proteins showed a good possibility. Maltais et al. (2010) developed soy protein hydrogels using cold gelation process and studied the effectiveness and protection of molecules against digestive enzymes and low gastric pH plus prolonged release under intestinal conditions. Soy protein cold-set hydrogels appear to be potentially useful for developing gelled functional foods containing nutraceutical molecules to be delivered in the colon.

9.6.5 Solubility

The functionality of soy proteins are related to their protein solubility. Poor protein solubility greatly limits the practical application of commercial SPI in the food industry (Tang et al., 2009). In this sense, some research groups have worked in improving soybean protein solubility using new technologies. Tang et al. (2009) found that the homogenization combined with ultrasonic treatment could improve the protein solubility of commercial SPI. Li et al. (2007) used pulsed electric fields treatment to increase protein solubility. This treatment induced polarization of SPI, dissociation of subunits and molecular unfolding; which caused hydrophobic groups and free sulfhydryl inside the molecule to expose. On the other hand, interactions with other polymers could also change the functionality of proteins. Pectin has the property to form complexes with soy proteins. Furthermore, high methoxy pectin form more stable complexes with soy protein than low methoxy pectin and better stabilizes the solution against precipitation. Jaramillo et al. (2011) studied the interactions between high methoxy pectin and commercial SPI over a wide range of pH (3–7) and the effect of thermal processing on these complexes. The results demonstrated that soy protein-pectin interactions at pH values close to the isoelectric point with added pectin improved solubility by preventing protein aggregation. Complexes formed resisted thermal processing, albeit with some modifications to their properties. Consequently it is possible to thermally treat ingredients to ensure microbial stability and yet have them remain functional in the finished beverage and the stability of the product enhanced by homogenization.

9.6.6 Meat products

Several non-meat animal and plant-based vegetable proteins have been commonly used as ingredients in meat products as binders and extenders, reducing formulation cost and improving composition and nutritional value. The addition of soy proteins increases firmness, texture, and succulence of meat products while also reducing the amount of purge (Feiner, 2006), but the effect depends both on the characteristics of the soy protein and on the formulation conditions. The type of interactions that occur between myofibrillar and soy proteins, promote the formation of a gel network structure. Herrero et al. (2008) studied the changes in protein structure produced in meat batters by soy protein addition and thermal treatment. The protein structural changes suggested the formation of a stronger gel network as a result of soy protein addition and thermal treatment of meat batters, related to enhancement of β-sheet and turn structures. Further, the addition of soy protein to meat batter and subsequent thermal treatment generates stronger hydrogen bonds as a result of water–soy protein interactions.

9.6.7 Bakery products

Soy and gluten proteins are complementary in terms of amino acid composition. Soy proteins contain low levels of sulfur-containing amino acids, which are relatively abundant in

wheat gluten, but they are rich in lysine, a limiting essential amino acid in gluten proteins; therefore, formulations containing both proteins yield products with high protein quality in terms of nutrition. It is, however, known that the presence of soy protein weakens the formation of gluten gels (Apichartsrangkoon, 2002). Bainy et al. (2010) found that SPI limits gluten protein interactions during thermal treatments, as the gluten protein denaturizes and gelation was clearly affected. Different sources of isolates with different level of protein denaturation showed similar behavior. Additionally, there was a cooperative effect between soy and gluten proteins during heating for all the samples containing soy protein with gluten compared to gluten alone. This suggests that the addition of soy proteins could improve the properties for these mixed systems at certain levels. SPI impairs the normal thiol/disulfide interchanges that are known to occur between gluten proteins during thermal treatments.

9.6.8 Soymilk

Physicochemical changes occurring in soymilk depend on the soybean protein composition, as well as processing and environmental conditions. New soybean varieties with different seed compositions have been developed, including lines containing low levels of antinutritional components, such as trypsin inhibitor, and lines with improved flavor following reduced LOX levels. The ratio of glycinin to β-conglycinin has an important effect on the quality of soymilk and soy protein–based products. Malaki et al. (2009) demonstrated that genotypic changes in protein subunit composition strongly affect the particle size distribution and most likely the stability of soymilk. Heat treatment and homogenization also have a significant effect on physicochemical properties of soymilk, and improved the particle size distribution.

9.6.9 Pasta products

Limroongreungrat and Huang (2007) developed new pasta from alkaline-treated sweet potato flour fortified with soy proteins. The results obtained indicated that alkaline-treated sweet potato flour (ASPF) could be an alternative ingredient for production of pasta with nutritious composition and natural yellow-orange color. Pasta fortified with 15% defatted soy flour (DSF) or 15% SPC contained approximately five times higher protein content than pasta made from 100% ASPF. The products also contained higher β-carotene than recommended by RDA. These products also had cooking quality, stickiness, cohesiveness, and springiness similar to control.

9.7 Other applications of soy proteins

There are a great number of applications for soybeans proteins; complete classification of the uses of these products could be divided in industrial uses and edible uses (Figure 9.10).

Figure 9.10 Edible applications and soybeans products. From Soy Stats (2012)

9.7.1 Biofilms

Plastics are one of the most important materials used daily in human life; however, environmental pollution from their use has become a serious issue, particularly when they are used as one-time use packaging materials (Guerrero et al., 2011). Biodegradable

materials derived from natural products offer an outlet for overflowing solid waste streams. Biopolymers produced from natural resources are regarded as an alternative for petroleum-based products because they are abundant, renewable, inexpensive, ecofriendly, and biodegradable (Yu, 2009). SPI has been considered as a promising biodegradable plastic with the advantages of low cost and easy availability (Hernández-Izquierdo and Krochta, 2008).

Soy protein could be used for food packaging purposes because it meets food grade standards. Protein structure determines the ability of protein chains to interact with each other as well as with other components of the formulation. Soy protein plastics without any additive have a brittle behavior making processing difficult. Addition of plasticizers is an effective way to obtain flexible SPI–based films. Biopolymeric films are usually plasticized by hydroxyl compounds (Cao et al., 2009). Glycerol has a high boiling point and good stability and is regarded as one of the most efficient plasticizers for soy protein plastics. Glycerol-plasticized soy protein possesses good processing properties and mechanical performance (Rhim et al., 2006). Blending can endow polymers with enhanced properties by conventional processing techniques. It should be emphasized that additives should be compatible with SPI and have the ability to form films (Su et al., 2012).

Several typical intra- and intermolecular interactions, such as hydrogen-bond, disulfide-bond, dipole-actions, charge–charge, and hydrophobic interactions, in soy protein are characteristic of natural proteins. According with the amino acid composition of SPI, hydrogen-bonding occurs among –NH2 (in arginine and lysine), –NH– (in proline and histidine), –OH (in tyrosine, threonine, and serine), –COOH (in glutamic acid), and peptide bonds. It seems that the density and strength of the interactions are greatly different at specific locations in SPI molecules and, as a result, soy protein molecules contain different regions with distinct abilities to accept glycerol molecules (Knubovents et al., 1999). The use of plasticizers to break intermolecular linkage stabilizes the protein in the native structure. The orientation and restructuring of the chains as well as the formation of new intermolecular linkage stabilize the 3D network formed.

Guerrero and de la Caba (2010) obtained films with best mechanical properties at basic pH, by using glycerol-plasticized soy protein and freeze-drying and compression.

Kokoszka et al. (2010) studied the relationship between protein and glycerol concentration on the thermal and wetting properties, and water vapor permeability of SPI-based edible films. The targeted application was to maintain the weight of cheese during ripening and commercialization, but also to prevent off- flavor dissemination from very odorant products. A synergistic effect of both glycerol and protein content was observed on the water vapor permeability. Film barrier properties are much better for the lowest SPI (6%) and glycerol (40%) content.

Blending of SPI with other polymers to improve biofilms characteristics is also widely studied. The use of poly(vinyl alcohol) improves the moisture barrier properties and decreases the water sorption ability of SPI films (Su et al., 2010). SPI based films containing calcium caseinates (CaCas) and lipids provoked an increase in mechanical properties: elastic modulus and tensile strength at break, mainly because of CaCas (Monedero et al., 2010). Guerrero et al. (2011) improved the mechanical properties of SPI-based films by the addition of gelatin, especially when 15% of bovine gelatin with a 200 bloom index

was added in the system plasticized with 30% of glycerol, showing an increase in tensile strength, with the maintenance of elongation at break. The blend of food grade carboxymethyl cellulose and SPI were successfully employed to fabricate novel edible cast films. These films are suitable for low-moisture foods and pharmaceutical products (Su et al., 2010). One interesting application in the development of soy protein films is the use of genapin as a cross-linker, which could improve mechanical properties of soy protein films with small amounts of genapin (González et al., 2011).

9.7.2 Nanotechnology

A new class of materials represented by bio-nanocomposites (biopolymer matrix including proteins reinforced with nanoparticles such as montmorillonite) has proven to be a promising option in improving mechanical and barrier properties of biopolymers. The bio-nanocomposites consist of a biopolymer matrix reinforced with particles (nanoparticles) having at least one dimension in the nanometer range (1–100 nm) and exhibit much improved properties because of the high aspect ratio and high surface area of nanoparticles (Zhao et al., 2008).

Bio-nanocomposites can be obtained by several methods which include in situ polymerization, solution exfoliation, and melt intercalation. The most common class of materials used as nanoparticles are layered clay minerals such as montmorillonite (MMT), hectorite, sapnotite, and laponite. These clay minerals have been proven to be very effective due to their unique structure and properties (Zeng et al., 2005). Flexible bio-nanocomposites with improved properties can be obtained by reinforcing the SPI-plasticizer system with suitable filler materials such as MMT. Because of their renewability and bio-degradability, bio-nanocomposites, by dispersing nanoparticles in a biopolymer matrix, have attracted much attention as novel packaging materials for substituting petroleum-derived plastics (Dang et al., 2010); however, properties of these bio-nanocomposites, for example, poor moisture barrier properties resulting from the hydrophilic nature of many proteins, still require substantial improvement to compete with petrochemical-based plastics (Kumar et al., 2010a).

Kumar et al. (2010b) worked with bionanocomposite films based on SPI and MMT using melt extrusion; Figure 9.11 shows the procedure. They reported a significant improvement in mechanical (tensile strength and percent elongation at break) and dynamic mechanical properties (glass transition temperature and storage modulus), thermal stability, and water vapor permeability of the films with the addition of MMT.

Jin and Zhong (2012) studied the elastic properties of the SP hydrogels as impacted by different concentrations of sodium chloride and mTGase, with the presence or absence of SP-coated MMT. They reported that, the dynamic rheological properties of SP dispersions with SP-coated MMT were highly affected by variables during cross-linking by mTGase and were further strengthened by subsequent heating and cooling processes. The approach and conditions established in this work can be used to manufacture biodegradable nanocomposite materials with improved functional performances.

Jin et al. (2012) tried to improve the mechanical strength of composite hydrogels with constant soy protein and MMT concentrations by studying crosslinking variables of glutaraldehyde (GA) concentration, pH, and temperature. As a result, the incorporation

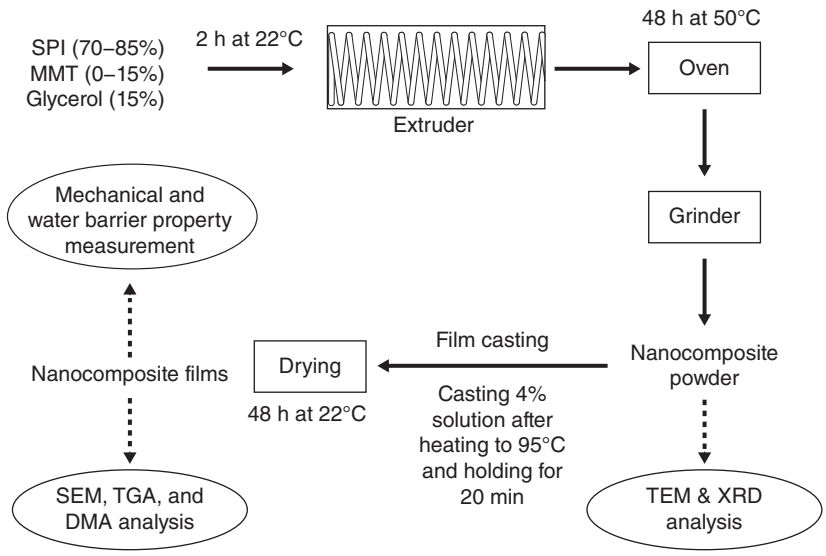


Figure 9.11 Process flow diagram for the preparation and characterization of protein isolate – montmorillonite (SPI-MMT) bio-nanocomposite films. DMA, dynamic mechanical analysis; SEM, scanning electron microscopy; TGA, thermogravimetric analysis; TEM, transmission electron microscopy; XRD, X-ray diffraction. From Kumar et al. (2010a)

of the intercalated MMT significantly promoted the development of the elastic modulus of nanocomposite hydrogels. The GA concentration, pH, and cross-linking temperature all played important roles in the network formation and mechanical strength of hydrogels.

9.7.3 Flavor

In the development of soy products with acceptable flavor quality is imperative to understand the nature of the interactions of soy proteins with the mixture of compounds present in the flavor ingredients. Depending on the nature and the strength of the binding, each aroma compound could be released at a different rate from SPI, which could have an impact on flavor suppression or alteration of flavor profiles in the final food products due to changes in the aroma balance. On the other hand, due to the problematic retention of indigenous off-flavor arising from the action of LOX in soybean is important to investigate the interaction of soy protein with those compounds. Moon and Li-Chan (2007) studied the changes in aroma attributes at different ratios of simulated beef flavor (SBF) and SPI and the effect of some ingredients as ascorbic acid and polyethylene glycol. The results of this study clearly showed that interaction of SPI with odor-active compounds in SBF was affected by addition of different ingredients. Gas chromatography analysis showed higher peak areas of individual beefy attribute related odor-active compounds in the mixture of SBF and SPI containing ascorbic acid alone or ascorbic acid with polyethylene glycol, compared with SBF–SPI without any additional ingredients. Those compounds can induce conformational changes in SPI structure mainly through disulfide bond reduction,

resulting in increased surface hydrophobicity and unordered structure on SPI; releasing the volatile compounds responsible of flavor.

9.8 Conclusion

Soybean is an extraordinary source of high-quality plant proteins that offer not just technological benefits for several food applications but also a great source of phytochemicals with positive impact to animal and human health. This chapter presented an overview on soybean proteins, their physicochemical properties and commercial utilization. Soy proteins subunits ratio and structure modification have a significant effect on their functional properties and applications by the food industry. Soybean proteins availability and functionality make them a great source of biopolymers. Extending their applications from the classical feeding purposes to production of innovative green materials used to replace the petroleum-based products.

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10

Canola/Rapeseed Proteins and Peptides

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10.1 Introduction

Canola is a variety of rapeseed that was developed in Canada. It is a rapeseed variety characterized by low erucic acid (<2%) and glucosinolate content (<30 μmol/g) in the oil and meal, respectively (Canola Council of Canada, 1990). Seeds of canola contain around 40% oil and 17–26% protein. The canola meal that results as a by-product of oil extraction can contain up to 50% protein determined on a dry basis. The nutritional profile and functional properties of canola proteins make them good candidates as ingredients for use in the food and beverage industries; however, a number of antinutritional components (e.g., glucosinolates, phenolics, phytates, tannins, sinapine and high fiber) in the canola meal have limited their food use. They also contribute to their inferior physicochemical properties, digestibility, color, and taste (Wu and Muir, 2008). Much research has been conducted to remove or reduce the amount of these undesirable components that have included investigating extraction procedures, processing methods and transgenic approaches (Tan et al., 2011b). Research focused on improving extraction technologies or modifying canola proteins has provided proteins with desirable functional properties with potential use in foods (Lonnerdal et al., 1977; Sosulski, 1979; Ismond and Welsh 1992; Vioque et al., 2000). Transgenic approaches have not been as successful.

Canola meal proteins have polypeptides with molecular weights ranging from 12 to 80 kDa (Aluko and McIntosh, 2001). Storage proteins napin and cruciferin are the major proteins that make up canola meal. Oleosin is a structural protein found in lesser amount and is associated with the oil fraction (Uppstom, 1995). This chapter discusses the

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characteristics of canola and low erucic acid rapeseed proteins as well as their functional and health-related properties.

10.2 Canola production

Canola is an oilseed crop, whose production has grown rapidly over the past 40 years. Currently, canola is the second largest oil crop and source of edible oil in the world (USDA, 2012). At one time rapeseed oil was used as lamp oil and a lubricant rather than food use because of the high level of erucic acid naturally present. In 1956 the U.S. FDA banned rapeseed oil for human consumption because of concerns erucic acid toxicity. Demand for rapeseed meal was also low because of the presence of high levels of glucosinolates, which has been shown to depress animal growth when consumed in high doses. In early 1970s, plant breeders developed low-erucic acid rapeseed (LEAR) varieties, which also had low glucosinonalate content. In 1978, these varieties were registered as "canola" for marketing purposes by the Western Canadian Oilseed Crusher's Association. In the following 10 years, European seed producers also developed LEAR varieties, referred to as "double-zero." In 1985, U.S. FDA provided Generally Regarded as Safe (GRAS) status to oil produced from LEAR varieties.

Canola meal is the second largest protein meal produced in the world after soybean meal. In 2009 30.8 million metric tons canola meal was produced. Canola meal has lower protein content than soybean meal (approximately 34–38% *vs.* 44–49%). Traditionally canola meal was used for animal feed; however, today canola meal is viewed as a valuable source for isolating proteins that can be used as ingredients in the food and beverage industry, as a potential alternative to soybean derived and other food proteins.

10.3 Storage proteins of canola and rapeseed

Canola is bred from *Brassica napus* L. spp. *Oleifera*. Similar to other plants, canola plants store proteins primarily as seed storage proteins. Canola proteins and peptides are extremely complex; however, approximately 80% of storage proteins in mature canola seeds are composed of cruciferin (12S globulin) and napin (2S albumin). The amount of cruciferin is about three times that of napin constituting ~60 and 20% of total mature seed protein, respectively (Hoglund et al., 1992). These are synthesized during seed development and stored in protein storage vacuoles (PSVs) as compact protein complexes. Schwenke (1994) presented a detailed review of the important storage proteins in rapeseed focusing mainly on their structural and physicochemical properties. The data is still limited with respect to the type and composition of canola proteins (Lonnerdal et al., 1977; Dalgalarrondo et al., 1986; Hoglund et al., 1992; Schwenke, 1994) as the extraction and purification methods used greatly influence these properties.

The structure of cruciferin and napin are quite different from each other. Criciferin is a high-molecular-weight (~420 kDa) complex with an octomeric barrel-like structure, composed of several polypeptide chains, whereas, napin is a low-molecular-weight protein consisting of two polypeptides linked by a disulfide bond. Wu and Muir (2008) examined

the major bonds involved in the stabilizing canola proteins. Cruciferin was mainly stabilized by noncovalent bonds, whereas napin was stabilized primarily by disulfide bonds. In addition, the endothermic peak for both proteins differed with cruciferin exhibiting one major peak at 91°C, whereas that for napin was at 110°C. The proposed disulfide bridge stabilization of the polypeptides in napin was previously reported by several researchers (Dalgalarrondo et al., 1986; Monsalve and Rodriguez, 1990; Schwenke, 1994; Krzyzaniak et al., 1998). Isoelectric focusing revealed that many of the proteins in the canola varieties ranged in pI from pH 4.6 to 8.3 (Tan et al., 2011a).

The amino acid composition of cruciferin is quite distinct from that of napin, with major differences in their secondary and tertiary protein structures responsible for their diverse functions and properties. Several differences in molecular properties of cruciferin and napin were reported by several researchers (Krause and Schwenke, 2001; Malabat et al., 2001) to be crucial to their utility as functional molecules. The large size, number of polypeptide chains, and the polypeptide composition of cruciferin is much more complex than the 2S globulin of napin. Cruciferin is an oligometric protein with a minimum of six subunits, each composed of two polypeptide chains, with the molecular weight ranging from 230 to 300 kDa (Schwenke, 1994). SDS–PAGE analysis of the cruciferin indicated the presence of around 10 polypeptide bands (Wu and Muir, 2008**)** which was similar to that reported previously by Dalgalarrondo et al. (1986). The minor bands observed in cruciferin were attributed to the presence of protein contaminants.

In sharp contrast, napin's 2S-albumin proteins are grouped under the prolamin protein superfamily (Shewry et al., 1995). They exhibit a high degree of polymorphism which is mainly attributed to the coding by a multigene family and different proteolytic processing sites. Gehrig et al. (1998) reported a high degree of homology between the amino acid sequences of small and large napin chains. Even though a large number of studies have been reported on plant protein secondary structures, there are few studies that deal with the molecular structures of canola proteins. Zirwer et al. (1985) reported a low content of α-helical (10%) and a high content of β-sheet (50%) structures in cruciferin, whereas the secondary structure of napin is reported to have high content of α-helical structures (40–46%) and low content of β-sheet conformation (12%) (Schwenke, 1994). Napin isolated from *B. napus* has a secondary structure consisting of 25% α-helix and 38% β-sheet arrangement that is stable over the pH range of slightly acidic to neutral (Krzyzaniak et al., 1998). Low hydrophobicity and net positive charge at neutral pH make napins hydrophilic proteins with a weakly folded structure from S–S linkages (Jyothi et al., 2007a) and thermal stability up to 75°C (Jyothi et al., 2007b).

Other proteins that are found in lesser amounts in canola seeds include structural proteins, oleosins or oil body proteins, and metabolic proteins such as lipid transfer proteins (LTP), protease inhibitors, calcium-dependent calmodulin-binding proteins and dehydrins (Wanasundara, 2011).

Both cruciferin and napin are potential sources of bioactive peptides (Marczak et al., 2003; Wu et al., 2003; Wu et al., 2008, Wu et al., 2009). Phe-Leu and Val-Ser-Val peptides with angiotensin converting enzyme inhibitory activity have been extracted from canola. Wu et al. (2008) located these peptides in the primary structure of both canola napin and cruciferin native proteins. Bioactive peptides form canola proteins will be discussed in a

later section. It is not known whether these biological activities are contributed equally by cruciferin and napin.

Isolation and further concentration of plant proteins is done to provide the full potential of that protein as an ingredient in foods. The isolation and concentration processes described in the literature for canola proteins are directly adopted from soy processes, and include production of a meal, flour, concentrate, and an isolate listed in increasing protein content. Although much research has been done on the functional properties of canola proteins, they are not yet used commercially; although, they are comparable to other plant proteins such as soy in their functionality (Wanasundara, 2011).

10.4 Important functional properties of canola/rapeseed proteins

Functional properties of proteins are important in their use as ingredients in the food and beverage industries. Tan et al. (2011b) reviewed the functional properties of canola or rapeseed meals and their proteins including emulsification, foaming, and gelling properties. Aachary and Thiyam (2012) more recently also discussed the gelation, emulsification, thermal, and water-holding capacities of crude and pure proteins and peptides isolated from canola meal. The following sections will provide a review of the important factors affecting the functional properties of canola proteins and peptides.

10.4.1 Gelation

Gelation by globular proteins (e.g., canola proteins) is primarily exploited in the food industry for various applications especially protein gel formation. Various factors have been reported that affect the nature of the protein gel (Arntfield et al., 1990a, 1990b, Arntfield et al., 1991; Arntfield and Murray, 1992; Hines and Foegeding, 1993; Arntfield, 1994; Aguilera, 1995; Rubino et al., 1996; Savoie and Arntfield, 1996; Uruakpa and Arntfield, 2005a, 2005b, 2006a, 2006b; Pinterits and Arntfield, 2008). The major one is the composition/properties of the protein itself which includes hydrophobicity, charge potential, level of sulphydryl groups, electrolytes, phytochemicals (phenolic and phytic acids), processing conditions (heating and cooling rates), extent of denaturation, and concentration. In addition, various environmental factors also affect protein gel formation including pH, ionic strength, and mineral content. Figure 10.1 provides a schematic representation of factors affecting the gelation property of canola/rapeseed proteins and peptides.

Sosulski et al. (1976) reported poor gelation properties for rapeseed flours, concentrates, and isolates. Gill and Tung (1978), however, observed gelation of 12S glycoprotein fraction of rapeseed at protein concentrations as low as 4.5%, with measurable thickening at 1% protein. Leger and Arntfield (1993) found canola protein isolate (CPI) had lower elastic strength than egg albumin. The gel strength of CPI gels was improved by the addition of polysaccharides. Guar gum was suggested by various researchers as one of the most suitable polysaccharide candidates for studying its interaction with CPI

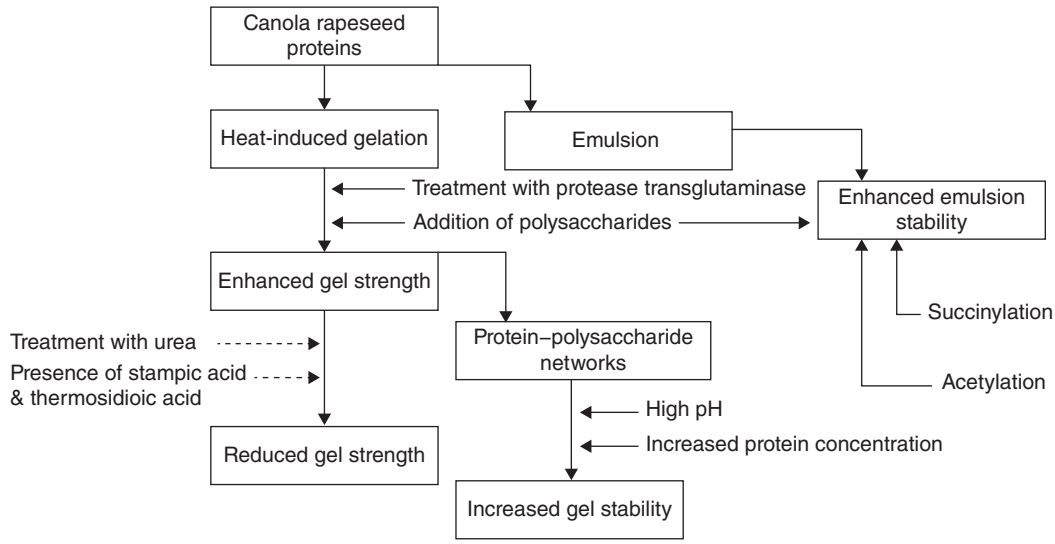

Figure 10.1 A schematic representation of factors affecting the gelation and emulsion properties of canola/rapeseed proteins and peptides

(Arntfield and Cai, 1998; Uruakpa and Arntfield, 2005b). Whistler and BeMiller (1997) pointed out that guar gum, a neutral molecule, was compatible with most other food substances, including other polymers. Further work by Arntfield and Cai (1998) observed an improvement of CPI networks when guar gum was added in small amounts; however, the study concluded that the incompatibility of the biopolymers was not the exclusive reason for the improvement in gel strength, as similar interaction data for methyl cellulose did not result in the same improvement in gelation properties.

Uruakpa and Arntfield (2005b) examined the effect of protein concentration and pH on the formation of networks and concluded that both these factors affected the strength and structure of CPI–guar gum interactions. Gels made up of commercial canola 12S globulin mixed with guar gum had both covalent and noncovalent interactions with the strength of the canola protein gel increasing directly with increase in the concentration of CPI. Leger and Arntfield (1993) and Arntfield and Cai (1998) previously observed that structural development in canola protein systems directly correlated with increase in protein concentrations; however, protein concentration had no significant effect on the gelation properties of a CPI–κ–carrageenan mixed system (Uruakpa and Arntfield, 2004).

In addition to protein concentration, Uruakpa and Arntfield (2005b) also showed that pH had a significant effect on the strength and structure of CPI–polysaccharide gel systems. The random aggregation and the degree of interaction among polypeptides in the CPI–guar gum system was reduced by increased electrostatic repulsion because of a high pH of 10; however, the improved networks formed at the higher pH were mainly the result of the balance between protein–protein and protein–solvent interactions (Paulson and Tung, 1989a). These relate to the extension of polypeptide chains and the creation

of homogeneous structures. Further studies indicated the heterogeneous nature and compact molecular nature of CPI–guar gum gels prepared at low pH with aggregation of these molecules tend to form regions of high- and low-protein concentrations (Uruakpa and Arntfield, 2005b). The overall gel strength was reduced because of the presence of regions with low protein concentrations. The importance of pH in the CPI–κ–carrageenan system has been discussed elsewhere (Uruakpa and Arntfield, 2004, 2006b). In the CPI–κ–carrageenan system, loosely cross-linked CPI networks and tightly cross–linked structures were obtained by varying the pH. At pH 6, loosely cross–linked CPI networks with large empty pores were formed while at pH 10, tightly cross–linked structures were obtained.

The gelling properties of CPI are also influenced by polysaccharide concentration, salt concentration, addition of enzymes, extent of proteolysis and the presence phenolics (Rubino et al., 1996; Uruakpa and Arntfield, 2004, 2006b; Pinterits and Arntfield, 2007, 2008). NaCl and urea concentrations had a significant effect on CPI-κ-carrageenan gel formation (Uruakpa and Arntfield, 2004, 2006b). The structures of CPI–κ–carrageenan gel are disorganized by urea treatment because of its hydrogen bond–blocking ability resulting in the complete unfolding of biopolymer molecule. Both noncovalent and disulfide interactions are important factors in determining the structural features of CPI–κ–carageenan networks as the formation of noncovalent interactions might have been prevented so that any network formed under these conditions may be because of disulfide bonds.

The physicochemical properties of heat-induced CPI gels can be further enhanced by enzymatic modification with transglutaminase. This enzyme is of particular interest as it induces gelation of rapeseed/canola proteins (Hyun and Kang, 1999; Pinterits and Arntfield, 2008). A direct correlation was evident between the gelation of canola protein and concentration of CPI and transglutaminase. An increase in gel strength observed might be attributed to the cross-linking of subunits occurred through transglutaminase treatment. The efficacy of transglutaminase treatment has been improved by a pretreatment of proteins with protease enzyme (Pinterits and Arntfield, 2007) resulting in the formation of harder gels compared to transglutaminase treatment alone.

The presence of phenolic compounds and other components in the protein isolate also affect its quality attributes. Rubino et al. (1996) reported deterioration of heat-induced gels obtained from canola protein due to the possible presence of either sinapic acid or thomasidioic acid. Selective control of sinapic acid and protein interactions may be possible through pH manipulation if thomasidioic acid could be eliminated during protein purification.

The rheology of CPI gels has been extensively investigated (Paulson and Tung, 1989b; Uruakpa and Arntfield, 2004, 2005b). It was found that the responses of translucent and opaque CPI gels to rheological tests were not identical (Paulson and Tung, 1989b) as gel microstructure is a determining factor. The incompatibility between CPI and polysaccharides, such as guar gum, may result in less elastic gels (Uruakpa and Arntfield, 2005b). The structural aspects of guar gum plays an important role. Whistler and BeMiller (1997) suggested that the side-chain galactosyl units along the main chain of guar gum may prevent intermolecular association necessary for junction zone formation. In contrast, Uruakpa

and Arntfield (2004) observed that the addition of κ–carrageenan improved gel strength and network structure when mixed with CPI gels.

10.4.2 Thermal properties

The data on the thermal properties of canola proteins are limited. Protein structure and amino acid composition, binding of metals and other prosthetic groups, protein–protein contacts, intramolecular interactions and linkages are major stabilizing structural factors affecting the thermal stability of proteins. The endothermic temperature of most globulins falls between 88 and 97°C with cruciferin exhibiting an endothermic temperature of 91°C (Marcone et al., 1998), which was reduced significantly following the addition of β-mercaptoethanol (Wu and Muir, 2008) to 76°C. This observation was consistent with previous reports on other globulins, such as soy and oat globulins (Townsend and Nakai, 1983). Although a decrease in the endothermic temperature was induced with the addition of β-mercaptoethanol, there was no change in enthalpy of denaturation of cruciferin. This was attributed to the greater structural stability of cruciferin compared to other globulins. These results suggested that noncovalent interactions are more important in stabilizing the protein conformation of cruciferin than disulfide bonds.

The data on the thermal properties of napin are also very limited (Schwenke, 1994; Krzyzaniak et al., 1998; Wu and Muir, 2008). Schwenke (1994) pointed out the importance of inter- and intrachain disulphide linkages to the high thermal stability of canola 2S protein (napin). These interactions also accounted for the relative high denaturation temperature of napin (Krzyzaniak et al., 1998). Similar to that observed for cruciferin, addition of β-mercaptoethanol also significantly reduced the thermal stability of napin by breaking the disulfide interactions (Wu and Muir, 2008). They also reported that purified cruciferin and napin showed higher endothermic peak temperatures compared to canola protein where the presence of non-protein and protein components reduced the heat stability of the crude proteins (Marcone et al., 1998).

10.4.3 Emulsion properties and water-holding capacity

Emulsions and foams are two phase systems in which one of the phases is dispersed in an aqueous continuous phase. Both are commonly found in food systems, and their formation is significantly affected by protein surface activity. Emulsifiers or foaming agents decrease the interfacial tension and facilitate formation of stable oil–water and air–water interfaces. Vioque et al. (2000) found the emulsifying properties of rapeseed/canola protein to be very weak; however, addition of hydrocolloids, guar gum, or κ-carrageenan (1%, w/v) significantly increased the emulsification properties of CPI (Uruakpa and Arntfield, 2005a). Hydrophobic interactions, hydrogen bonds, and disulfide linkages all greatly influence the stability of emulsions as they are reduced by the addition of sodium salts and denaturants.

Several researchers investigated the role of hydrophobicity on the emulsifying properties of proteins (Townsend and Nakai, 1983, Uruakpa and Arntfield, 2006a). As discussed previously, canola proteins have poor emulsifying properties because of their higher

hydrophobicity, which is affected by NaCl, hydrocolloids and pH. Addition of hydrocolloids at high pH appeared to increase exposure of hydrophobic amino acid residues on the protein surface as Uruakpa and Arntfield (2006a) observed an increase in surface hydrophobicity of CPI. With this increase in hydrophobic patches on the protein surface, the potential for higher and rapid adsorption at an oil–water interface (which is required for emulsion formation) becomes more likely.

Napin, a basic protein with a high percentage of basic amino acids, enhances the sites of electrostatic interactions (Schwenke, 1994). This explained the reduced emulsifying property of canola proteins observed by Wu and Muir (2008). The mean particle size of emulsions prepared with napin was unstable compared with the corresponding emulsions prepared from CPI or cruciferin. These differences were probably the result of the presence of large flocs and/or coalesced aggregates, which are normally correlated with the much lower emulsion stability (Aluko and McIntosh, 2001).

Paulson and Tung (1988) and Gruener and Ismond (1997) studied the effects of acetylation and succinylation on improving protein solubility, foaming, emulsification and thermal stability properties. Paulson and Tung (1988) found succinylation increased emulsification activity and emulsion stability of canola proteins, but extensive succinylation did not result in any significant improvement of these properties. In addition, protein solubility, apparent viscosity of protein dispersions, zeta potential, and difference in density between the aqueous and oil phases had some effects on the emulsion stability. Gruener and Ismond (1997) later showed that acetylation enhanced the foaming capacity and emulsifying activity of an isolated canola 12S globulin. On the basis of this information, CPI had potential as a stabilizing ingredient in food emulsions and could be utilized in baked goods or imitation meat products.

A concentration-dependent increase in the water-holding capacity of meat products and improvement in cooking yields were reported by Cumby et al. (2008) following the addition of canola protein hydrolysates. This was also influenced by the enzymes employed during the hydrolysis process.

10.5 Biological activity

Canola/rapeseed proteins and peptides exhibit a number of potential biological effects (Figure 10.2). These effects result from the formation of bioactive compounds, some of which are generated enzymatically.

10.5.1 Angiotensin-converting enzyme (ACE) inhibition

An important area in peptide research is the ability of bio-peptides derived from food proteins to inhibit the activity of the angiotensin-converting enzyme (ACE). Angiotensin 1–converting enzyme is a dipeptidyl carboxypeptidase involved in the regulation of blood pressure. It functions by being converted to the powerful vasoconstrictor angiotensin II and the inactivation of the vasodilator peptide Bradykinin (Belova, 2000; Hollenberg, 2000; Unger, 2002). Angiotensin II has been implicated as one of the important causative

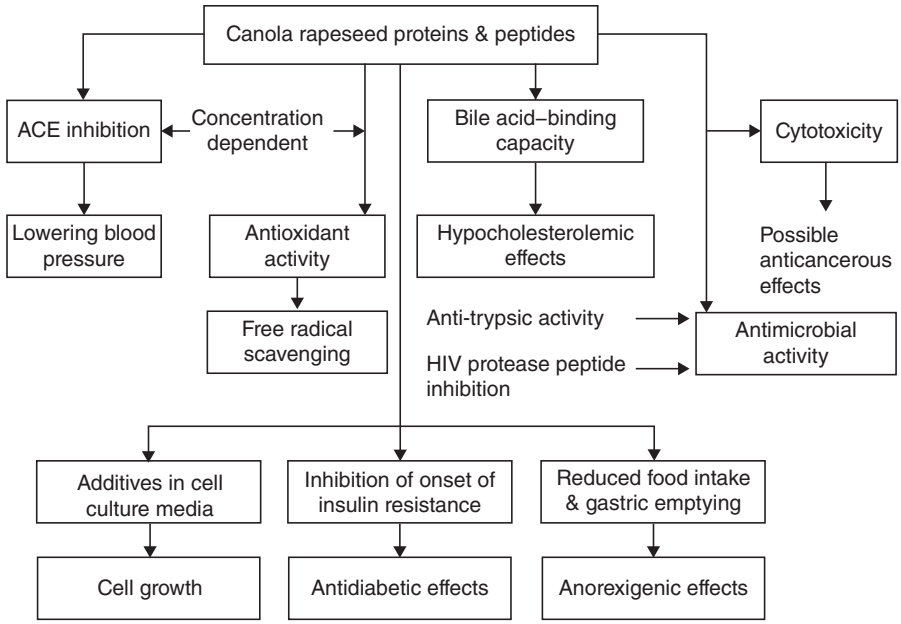

Figure 10.2 A schematic representation of various bioactive properties of canola/rapeseed proteins and peptides. ACE, angiotensin-converting enzyme

factors of various forms of cardiovascular diseases. Hypertension and related target-organ damage could be successfully prevented by ACE-inhibitors (Zaman et al., 2002), even though a few side effects have been reported with the use of such ACE-inhibitory drugs (Seseko and Kaneko, 1985). Adam et al. (2002) suggested, however, that these side effects could be significant and life threatening for some hypertensive patients who have a lifelong requirement for these medications. To avoid or minimize such drug-related side effects, Lee et al. (2004) suggested the development of natural ACE inhibitors as alternative to synthetic compounds. Subsequently, a large number of food proteins were identified as potential sources of ACE-inhibitory peptides, including milk (Meisel, 2004), fermented milk (Nakamura et al., 1995), alfalfa (Kapel et al., 2006), soybean (Mallikarjun Gouda et al., 2006; Wu and Ding, 2002), corn (Suh et al., 2003), bovine skin gelatin (Kim et al., 2001), mung bean (Li et al., 2006), buckwheat (Li et al., 2002), and rapeseed (Marczak et al., 2003).

In their study on rapeseed, Marczak et al. (2003) identified four potent ACE inhibitory peptides from the protein that were effective in reducing blood pressure in spontaneously hypertensive rats. Only a few reports are available on the production of ACE inhibitory peptides from canola proteins in which defatted canola meal was subjected to enzymatic hydrolysis for production of bioactive peptides (Wu et al., 2003, 2008, 2009); nevertheless, the efficiency of proteases in the release of ACE-inhibitory peptides from canola proteins should be studied further.

Wu et al. (2003) identified two peptides, Val-Ser-Val and Phe-Leu, from the canola hydrolysates, exhibiting ACE inhibitory activities. Compared with other ACE-inhibitory peptides (Val-Thr-Pro-Ala-Leu-Arg, Lys-Leu-Pro-Ala-Gly-Thr-Leu-Phe, and Lys-Asp-Tyr-Arg-Leu) reported by Li et al. (2006), Val-Ser-Val and Phe-Leu both had greater ACE-inhibitory potencies (Wu et al., 2008). A further study by Wu and Muir (2008) compared the ACE inhibitory activities of hydrolysates from cruciferin and napin. A direct relationship was found between peptide concentration and efficacy of ACE inhibition. This study indicated that other protein components may also contribute to ACE inhibition as the canola protein hydrolysate was a more potent inhibitor than either cruciferin or napin hydrolysates. Moreover, with respect to ACE inhibitory activity of rapeseed protein hydrolysate (Marczak et al., 2003), canola protein hydrolyzate showed potent inhibitory activity attributed to difference in protein extraction purification methods and analyses used.

There are some reports where ACE-inhibitory peptides failed to show potent hypotensive activity *in vivo*, but exhibited powerful ACE-inhibitory activity *in vitro* (Fujita et al., 2000). This difference in the efficacy is likely because the peptides may be (i) susceptible to hydrolysis by ACE under physiological conditions and (ii) destroyed by gastric enzymes. Studies have suggested evaluating the susceptibility of the active protein hydrolysate to simulate gastric digestion before *in vivo* tests. Wu et al. (2008) showed that there was not much change in the ACE inhibitory activity of canola meal protein hydrolysate even after incubation with ACE. This indicated that peptide fraction from canola hydrolyzate is not an ACE substrate (Wu et al., 2009); therefore, theoretically this fraction could resist the digestion in the gastrointestinal tract, and that means they could potentially exert certain desirable physiological effects.

Most bioactive peptides are composed of short-chain peptides, which is important because peptide size affects biological properties. Previous work by Matthews (1977) observed that di- and tripeptides were absorbed more efficiently than either amino acids or intact proteins because of their lower osmolarities compared to free amino acids. Since major portions of the canola protein hydrolysates are composed of peptides less than 1300 Da (Wu and Muir, 2008), with most of these peptides being di- and tripeptides, they could survive the gastrointestinal tract and exert *in vivo* antihypertensive activity; however, this assumption must be confirmed *in vivo* using an appropriate animal disease model such as spontaneously hypertensive rats to establish possible therapeutic applications (Wu and Muir, 2008).

10.5.2 Antioxidant activities

Depending on protein composition and the degree of hydrolysis, it is reported that hydrolysis generally increases the solubility of proteins to varying extents so that such products may have novel physicochemical and bioactive properties not found in the original proteins (e.g., antioxidant capacity and water-holding capacity). At least one study indicates that antioxidant capacity of a peptide depends on its composition (Saito et al., 2003), as different combinations of amino acids in tripeptide chains were found to exhibit different

antioxidant activities. Studies have also shown that the presence or absence of aromatic amino acids would affect its scavenging capacity and may be responsible for variation in radical-scavenging activity found in different polypeptide hydrolysates. It was evident that antioxidant activity of peptides can be changed by using either endo or exo proteases (Adler-Nissen, 1986; Hamada, 2000) for the hydrolysis of canola proteins. Consequently, the antioxidant activity of canola protein hydrolysates appeared very dependent on the hydrolysis treatment used for their production (Cumby et al., 2008) as well as on their concentration. Similarly, the reducing power of canola protein hydrolysates appeared to be concentration-dependent and increased with increasing concentration; however, there are some limitations for these studies. Because these protein hydrolysates were not pure extracts, it is possible that other components, such as some phenolics, might contribute to the results of the antioxidant assay; therefore, research should be conducted on purified protein hydrolysates to avoid any interference from phenolic constituents.

10.5.3 Hypocholesterolemic effect and bile acid–binding capacity

A dietary plant protein, such as soy protein, has been shown to be hypocholesterolemic (De Schrijver, 1990). This involves the reduction in low-density lipoprotein (LDL) cholesterol in blood vessels, which is generally strongly associated with the development of heart disease; however, hypercholesterolemia can be reversed by a combination of exercise, a healthy diet, together with hypolepidemic agents known as bile acid sequestrants (Anderson and Siesel, 1990). Yoshie-Stark and Wasche (2004) attributed the hypocholesterolemic effects of plant proteins to their bile acid–binding capacity. Although little data exist on the hypocholesterolemic effects of canola proteins, a recent study by Yoshie-Stark et al. (2008) observed a higher bile acid–binding capacity for a protein isolates from rapeseed perhaps because of the higher concentration of fiber; however, it exhibited a lower bile acid–binding capacity and ACE inhibition capacity than de-oiled soybean. Data on the bile acid–binding capacity of rapeseed/canola protein isolates is of particular importance because bile acid–binding promotes the utilization of cholesterol in the liver.

10.5.4 Antidiabetic, anticancer, and antimicrobial properties

It is well known that the insulin sensitivity is mainly associated with the type of energy macronutrients in the diet (lipids and carbohydrates) (Colagiuri and Brand Miller, 1997; Daly, 2003; Reaven, 2005; Carpentier et al., 2006; Feldeisen and Tucker, 2007). Some studies, however, have also focused on the effects that dietary proteins have on insulin resistance (Blouet et al., 2007, and Lavigne et al., 2000). Both these studies indicated that some types of dietary protein could specifically prevent insulin resistance in rats fed a high-sucrose diet, without any modifications to body weight or composition. Lavigne et al. (2001) attributed this observation to the amino acid pattern of dietary protein sources. Rapeseed protein, is a rich source of cysteine as well as other amino acids such as the nitric oxide precursor arginine (Scherrer and Sartori, 2000; Chan and Chan, 2002). As a result, it

has the potential for preventing insulin resistance and related metabolic or physiological abnormalities associated with metabolic syndrome. When rapeseed protein was substituted for milk, Mariotti et al. (2008) reported that it inhibited the onset of insulin resistance in rats fed a high-saturated, high-sucrose diet. More studies are warranted, however, to understand the underlying mechanisms and to evaluate the efficacy of rapeseed/canola protein on later endpoints of metabolic syndrome.

Xue et al. (2009) investigated the cytotoxicity of rapeseed peptides on cell proliferation using human cervical cancer Hela cell lines and reported a dose- and time-dependent inhibition of cell proliferation by rapeseed peptides. Treatment with different concentrations of rapeseed peptides changed the morphology of Hela cells, which included cell shrinkage, nuclear fragmentation, and chromatin condensation. These changes were characteristics of apoptotic cells and suggest that further studies be carried out.

Studies also demonstrated the antimicrobial properties of canola proteins; for example, napins exhibited antimicrobial properties on various bacterial strains, which was attributed to their high content of positively charged amino acids and the high proportion of α-helix in their secondary structure (Neumann et al., 1996a, 1996b; Terras et al., 1993a, 1993b). Antagonistic effects of calmodulin and an antitrypsic activity of napins have also been reported (Neumann et al., 1996a, 1996b; Terras et al., 1993b). Nioi et al. (2012) recently reported that napins isolated from rapeseed meal exhibited antimicrobial activity against *Fusarium langsethiae*. Such antimicrobial activity points to the potential application of rapeseed meal in the area of food safety.

Lee and Maruyama (1998) reported plant peptides as HIV protease inhibitors; however, their use as therapeutic agents was limited by their difficulty in penetrating cell membranes. The generation of HIV protease peptide inhibitors by peptides released from rapeseed protein by a food grade endoprotease (alcalase) was described by Yust et al. (2004). Interestingly, these peptides were detected by their ability to enter the cells of *E. coli* expressing the HIV protease, inhibiting the HIV protease, and improving cell growth. These results illustrate a new approach in the research of HIV protease inhibitors from rapeseed and related (canola) proteins.

10.5.5 Effects on cell growth in serum-free medium and anorexigenic effects

The use of rapeseed protein hydrolysates as cell culture medium supplements have also been reported (Deparis et al., 2003; Farges-Haddani et al., 2006, 2008; Chabanon et al., 2008). Rapeseed protein hydrolysates were shown to enhance Sf9 cell growth in serum-free medium (Deparis et al., 2003). The potential use of rapeseed protein hydrolysates as additives in animal cell culture media avoids the risks associated with ingredients of bovine origin (Farges-Haddani et al., 2006). A mixture of rapeseed peptides was better able to influence Chinese Hamster Ovary cell kinetics than the total rapeseed protein hydrolysate. These peptides exerted a strong reduction in cell death rate, an elevation of recombinant γ-interferon production, and enhancement of cell adaptation to serum-free conditions. The positive effects of these low-molecular-weight peptides on Chinese

Hamster Ovary cell growth was later demonstrated by Chabanon et al. (2008). In another study, a rapeseed peptide fraction strongly increased the Chinese Hamster Ovary cell growth and its survival and showed an increase in the quantity of IFN-g produced and its specific production rate (Farges-Haddani et al., 2008). All these studies demonstrate the positive effects of the rapeseed hydrolysates on cell growth, which is attributed to two factors: (i) the small peptides serve as nutrients for cell growth and (ii) certain peptides exhibit growth or survival factor effects. This makes them a promising substitute to other commonly used proteins such as transferrin, albumin, and insulin in the cell culture medium to enhance the cell growth.

Marczak et al. (2006) reported that a bioactive tripeptide, Arg-Ile-Tyr, from rapeseed protein reduced food intake and gastric emptying in mice when administered orally. It is well known that CCK, an endogenous peptide localized in the gastrointestinal tract and the brain, decreases food intake and gastric emptying (Moran and Kinzig, 2004; Strader and Woods, 2005). The two receptor subtypes for CCK are CCK1 and CCK2 receptors. Of these, it is the CCK1 receptor that mediates the inhibitory effect on food intake and gastric emptying. Marczak et al. (2006) later reported that peripheral injection of lorglumide (a CCK1 receptor antagonist) significantly blocked the Arg-Ile-Tyr-induced suppression in food intake and gastric emptying. The latter suggested that the activity of this tripeptide was mediated by the CCK1 receptor; however, this peptide did not show any affinity for either CCK1 or CCK2 receptors suggesting that orally administered Arg-Ile-Tyr suppressed feeding and gastric emptying through the stimulation of CCK secretion and not binding to the CCK1 receptor. On the other hand the dipeptide, Ile-Tyr, did not decrease food intake after oral administration, although it had a higher ACE inhibitory activity compared to Arg-Ile-Tyr (Marczak et al., 2006). It was, therefore, concluded that the anorexigenic effect of Arg-Ile-Tyr was independent of its ACE inhibitory activity and completely attributed to absorbed Arg-Ile-Tyr.

10.6 *In vivo* dietary toxicity

Even though, the use of canola or rapeseed protein as human food has been suggested since the early 1980s (Sosulski, 1983), it was limited by the high level of fiber and such anitinutritional factors as tannins, sinapine, and phytates. Over the years, genetic selection of varieties with no erucic acid and low glucosinolates (<20–50 μmol/g dry matter) improved the quality of low erucic acid rapeseed or canola meal. A further decrease in fiber, sinapine, and tannins was achieved by dehulling and incorporating high temperatures and organic solvents during oil extraction (Mawson et al., 1995; Higgs et al., 1996). Burel et al. (2000c) noted that further thermal treatment improved the digestibility of rapeseed meal when fed to rainbow trout and turbot. Bos et al. (2007) found that rapeseed proteins had a low real ileal digestibility (RID) in humans compared with other plant proteins; it, nevertheless, had a high nutritional potential for humans.

Toxicological studies with rapeseed products low in glucosinolates and erucic acid have been conducted in various mammals such as dogs (Loew et al., 1976), rats (Plass et al., 1992; Mejia et al., 2009a, 2009b). Loew et al. (1976) reported that the level of serum thyroxine concentration, thyroid weights and histology in rats and dogs fed high protein

concentrate flours made from rapeseed, containing low levels of glucosinolates, remained normal. Although there were no treatment-associated abnormalities observed in dogs, the serum glutamic–pyruvic transaminase activity decreased and packed cell volume and erythrocyte counts increased slightly in rats. The antithyroid effects observed in this study were substantially lower than that reported in previous studies using other rapeseed products, which probably contained high levels of glucosinolates. Plass et al. (1992) also found similar effects in a subacute feeding study of rapeseed products in rats. It is interesting to note that neither of these studies had a significant overall toxicological impact.

Most of the aforementioned studies have been focused primarily on determining the feasibility of using maximum tolerable limits of rapeseed meal or protein concentrates in different animal species such as poultry, swine, ruminants, and fish (Burel et al., 2000c). Mejia et al. (2009a) recently demonstrated that cruciferin-rich CPI was safe when fed to rats up to a level of 20% for a period of 13 weeks. The level of glucosinolate/g in cruciferin-rich CPI was significantly too low to evoke alterations in thyroid weight and morphology, which contrasted with the negative effects of a high intake of glucosinolates and glucosinolate-derived compounds reported by Tripathi and Mishra (2007) when fed to animals. This was a level lower than the upper limit of 0.5 μmol glucosinolates/g diet reported in the rat without adverse effects when using a diet corresponding to 20% inclusion of rapeseed meal containing 2.5 μmol glucosinolates/g (Bjerg et al., 1989). A 13-week toxicological study by Mejia et al. (2009b) concluded that Sprague Dawley rats well tolerated daily administrations of napin-rich CPI at three dose levels (5%, 10%, and 20% w/w) suggesting that the napin-rich CPI could be considered safe.

10.7 Role in aquaculture

The potential use of canola meal and protein as substitute for fish meal has been reported for rainbow trout (Yurkowski et al., 1978; Hilton and Slinger, 1986; McCurdy and March, 1992; Gomes et al., 1993), Chinook salmon (Higgs et al., 1982), tilapia (Jackson et al., 1982; Davies et al., 1990), channel cat fish (Webster et al., 1997) and shrimp (Lim et al., 1997). In relation to supply, the quality of canola/rapeseed protein that is potentially available for inclusion in aquatic animal diets surpasses the global amount of fish meal protein that is produced each year (Higgs et al., 1996). Riche and Brown (1999) observed an increase in apparent phosphorus availability and true phosphorous availability in rainbow trout when the protein source in the feed was replaced with CPI; however, the nutritional value of canola meal is influenced by the presence and concentration of glucosinolates and residual oil. In this context, Burel et al. (2001) suggested that care should be taken so that glucosinolates do not impair circulating thyroid hormone levels and/or feed intake. Moreover, high level of glucosinolates may lower the feed intake and plasma thyroid hormone levels, in addition to an enlarged thyroid gland and occasional abnormalities in liver and kidney occasionally organ (Van Etten and Tookey, 1983). Although 38.04% canola meal reduced growth in juvenile hybrid tilapia, diets in which 30% of soya bean meal replaced with 19.02% canola meal and supplemented with lysine yielded the best growth and feed utilization in juvenile hybrid tilapia (Zhou and Yue, 2009).

Most of the *in vivo* studies indicated that the relative importance of nutrient composition and levels of antinutritional factors in the diet influences the digestibility of canola proteins (Aachary and Thiyam, 2012). The digestibility and nutritional values of canola and rapeseed meals and concentrates by various aquatic species has been thoroughly reviewed (Higgs et al., 1982, 1983; Mwachireya et al., 1999; Forster et al., 1999; Kissil et al., 2000; Burel et al., 2000a, 2000b, 2001; Glencross et al., 2004). A marked improvement in protein and energy digestibility was observed when canola meal with reduced levels of fiber, glucosinolates, sinapine, phytate and carbohydrate was used in the feed of rainbow trout (McCurdy and March, 1992; Hajen et al., 1993a, 1993b; Higgs et al., 1995; Mwachireya et al., 1999).

The extent of antinutritional factors in the diet and the relative significance of nutrient composition are the major factors contributing to the digestibility of canola proteins. In the experiments with rainbow trout, Mwachireya et al. (1999) showed an increase in protein digestibility when using CPI contained low level in all antinutritional factors and indigestible carbohydrates. A comparison with other plant protein product or fishmeal, showed the protein digestibility coefficient of CPI was one of the highest in salmonoid fishes (Cho et al., 1982; Hajen et al., 1993a; Anderson et al., 1997). Although the digestibility of the energy in CPI was lower than that reported by Cho et al. (1982) for herring meal, it is noteworthy that the digestible energy contents of these protein sources are comparable for trout (Mwachireya et al., 1999). Studies by Sosulski and Thacker (1993) and Buchanan et al. (1997), however, showed that nutrient digestibility improved with the application of enzymes in terrestrial and aquatic species. Additionally, a lower organic matter discharge into the environment was reported when canola protein concentrates and isolates were used in diets for trout in comparison to any other laboratory-processed meals (Higgs et al., 1996; Mwachireya et al., 1999).

Glencross et al. (2004) recorded an increase in total digestible energy in red sea bream (*Pagrus auratus*) when an Australian variety of canola protein concentrate was used in the feed. They also reported a reduction in the amount of total digestible protein relative to the expeller canola meal. At the same time, Allan and Booth (2004) conducted two experiments investigating the effects of processing on the apparent digestibility coefficients of legumes and oilseeds for juvenile silver perch, *Bidyanus bidyanus.* They reported that extrusion delivered negative effects to the digestibility of canola. Dephytinized canola protein concentrates was also found to have the potential to replace substantial levels of fishmeal in carnivorous fish diet without affecting performance (Thiessen et al., 2004). The apparent protein digestibility for canola protein concentrates was similar to that reported by Higgs et al. (1995). Higgs et al. (1994) and Mwachireya et al. (1999) both showed that the protein fraction in concentrated canola protein products was highly available to salmonid fish.

Thiessen et al. (2004) found that the individual amino acid availability values for a dephytinized canola protein concentrate (CPC) fed to rainbow trout ranged from 870 to 950 g kg^{-1}; however, Newkirk et al. (2003) showed that the lysine digestibility of canola meal fed to broiler chickens varied from 655 to 857 g kg^{-1} and that the high temperature processing of canola meal was responsible for the relatively low digestibility of lysine. The high lysine digestibility of 935 g kg^{-1} reported by Thiessen et al., 2004, however, suggested

that the processing method used to produce CPC did not damage the protein fraction of this product.

Some researchers have focused on the effect of rapeseed/canola meal/protein on growth (Yurkowski et al., 1978; Teskeredzic et al., 1995; Forster et al., 1999; Thiessen et al., 2004). When 750 g kg^{-1} of fishmeal was replaced with canola protein concentrate, Thiessen et al. (2004) observed almost no difference in feed intake, weight gain, feed conversion ratio and efficiency of protein by rainbow trout. This contrasted with an earlier study by Yurkowski et al. (1978) who found a significant reduction in rainbow trout feed intake when fishmeal was replaced with rapeseed protein concentrate. A later study by Forster et al. (1999), however, showed that rainbow trout fed four canola protein concentrate diets, irrespective of their phytase or phosphorus levels, resulted in growth rates, feed efficiencies, and protein utilization similar to the control fish fed anchovy meal.

10.8 Role in ruminant nutrition

In North America and Europe canola meal is used mainly in dairy cow feeds. It is highly valued because of its palatability and ability to enhance milk production (Arntfield and Hickling, 2011). The high level of rumen-degradable protein in canola meal stimulates microbial protein production increasing the amount of absorbable amino acids for the lactating cows. Physical factors and chemical agents can enhance the rate and extent of ruminal degradation of canola meal proteins (Khorasani et al., 1993; McAllister et al., 1993; Moshtaghi Nia and Ingalls, 1992, Moshtaghi Nia and Ingalls, 1995; McKinnon et al., 1995;, Sadeghi and Shawrang, 2006). These treatments, however, can adversely affect the protein digestibility of the final product in the small intestine. Studies with Holstein dairy cows by Shawrang et al. (2008) reported an increase in intestinal protein digestibility and a decrease ruminal protein degradation when gamma irradiated canola meal was used.

10.9 Food applications of canola proteins

Proteins perform a variety of functions in food systems; however, there are relatively few studies on the food applications of canola protein hydrolysates. Available literature shows the potential of these proteins for improving quality attributes of various kinds of foods. Canola protein hydrolysates produced by treatment with Flavourzyme, Alcalase, or with a combination of both enzymes, significantly improved the water-holding capacity of a meat system (consisting of 8.5 g of ground pork, and 0.5 and 1% (w/w) canola protein hydrolysates) and thus enhanced cooking yield (Cumby et al., 2008). Interestingly, this effect was found to be concentration-dependent as well as on the type of enzymes used for protein hydrolysis. Protein hydrolysates prepared by Alcalase were less effective in reducing the drip volume, compared to Flavourzyme-produced hydrolysates or hydrolysates prepared by combination of Alcalase and Flavourzyme. The effectiveness of the Flavourzyme-produced hydrolysates was attributed to the lower-molecular-weight peptides produced by this enzyme as the water holding capacity of these peptides was greater than the larger-size peptides. The production of peptides through enzymatic

hydrolysis has other implications. A study by Guo et al. (2010) reported that the enzymatic hydrolysates of *Brassica* sp. protein can be used as the primary ingredient for the production of thermal processing flavors with meatlike characteristics evaluated by a sensory panel.

Application of canola protein hydrolysates as stabilizers have also been reported (Aluko et al., 2005) with the results showing that partial replacement of egg yolk with unmodified or enzymatically modified canola proteins increased particle size, reduced viscosity, and increased discoloration of the mayonnaise samples. In addition, the limited hydrolysis improved the incorporation of canola proteins into mayonnaise compared to unhydrolyzed proteins. The use of canola protein concentrates and isolates have also been examined in baked foods (Kodagoda et al., 1973; Mansour et al., 1999). A decrease in the loaf volume by 10–20% was observed when wheat flour was replaced with 5% (w/w) rapeseed protein concentrate/isolate; however, replacing wheat flour protein of bread dough with canola protein concentrate even up to 18% did not changed dough and loaf quality attributes (Mansour et al., 1999). Incorporation of such proteins enhances the level of essential amino acids in the final products (Alireza-Sadeghi and Bhagya, 2008). Previous reports showed that the addition of rapeseed protein concentrate enhanced the liquid retention of beef patties, peelability of wieners and improved whipping properties in meringues (Thompson et al., 1982). Canola proteins also effectively partially replaced the meat content of bologna (up to 3 % weight) resulting in high water-holding capacity and improved cook yield (Mansour et al., 1996).

10.10 Conclusion

Incorporation of canola proteins in the human diet is not only based on their nutritional quality but also on their functional properties. This chapter discussed the functional properties of canola derived protein hydrolysates and/or concentrates as natural sources of cruciferin and napin and their bioactive peptides. The effects of processing on the functional properties of canola proteins, and their improvement through modification, are also discussed. Because of their various functional properties and potent bioactive attributes, canola protein concentrates can be used to develop products with desirable properties. These are further discussed in the industrial exploitation of canola derived proteins and peptides to enhance health and prevent disease. While there is a need for functionally active protein food ingredients, the nutritional quality of cruciferin, napin and their corresponding peptides cannot be ignored as they could contribute, in a significant way, to the daily intake of dietary protein.

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11
Wheat Proteins

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11.1 Introduction

The wheat seed is a wheat plant's way of producing another wheat plant. The protein of the wheat seed is the combination of storage protein (mainly water insoluble), plus the residue of many metabolic proteins (mainly water soluble) that have been needed by the developing grain. All these proteins provide the supply of amino acids needed to carry life on into the next generation of the wheat plant, after the germination process has commenced in the moistened wheat seed.

A wheat grain is the miller's and baker's way of giving us our "daily bread." Critical to this processing is the grain's storage protein (mainly water insoluble). For the baker's purposes, it is more than merely storage protein. When the grain is milled and mixed with water, the water-insoluble storage protein forms a dough due to the unique rheological properties of the gluten proteins so formed. This dough is capable of retaining gas bubbles, so that flour from the wheat grain alone is suited for making the many wheat-based food products that sustain millions around the world—bread in its many diverse forms, including chapatti and roti; a wide range of noodles and pasta; cakes, croissants and cookies; pastries and pizza; doughnuts and bagels; and many other related foods, as well as animal feed and industrial uses.

The reasons for the success of the wheat plant around the world lie in this combination of properties, namely, the world's need for foods that can be supplied only by the wheat

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grain and the ability of the wheat plant to thrive in a wide range of environments, including every continent, except Antarctica. Thus, the rheological properties of wheat gluten can be seen as the reason why some 10^{14} wheat plants are grown annually worldwide, producing over 600 million tonnes of grain, the yield averaging about 3 tonnes per hectare (Wrigley, 2009). Although all this wheat grain approximates 250 grams per day per person worldwide, many of the world's people do not receive this theoretical distribution, as many regions of production are distant from populated areas in developing countries, and because a significant amount of wheat goes to animal feed and to industrial uses.

Gluten was identified as the basis of wheat's unique dough-forming properties long ago. It was one of the first few proteins to be isolated in reasonably pure form. This achievement is attributed to the Italian chemist, Beccari (Bailey, 1941). In 1728, he reported that he had made a dough and kneaded it under a stream of water, thereby washing out the starch to leave a small cohesive ball of gluten. A century later (in 1838), the Swedish chemist Berzelius suggested the name "protein" (meaning primary substance) for a small collection of substances—casein, blood fibrin, egg albumin, eye-lens crystallin, and gluten (Hartley, 1951; Wrigley, 2012). However, it was not a simple task for these early chemists to determine that this growing diverse collection of compounds was unified by the same chemistry—polymers of amino acids.

Since those early times, the word "gluten" has come into popular use; perhaps it is a result of its everyday use that misunderstandings have arisen. To illustrate, there is the story of a food-science lecturer who had a favorite question for his students: "What is the percentage of gluten in wheat?" Eventually, he gave his version of the answer, which was "Zero%." Gluten, he explained, is formed after wheat is milled to flour and after the flour is mixed with water to form a dough. Before this processing, the gluten-forming proteins are referred to as storage protein. Wheat gluten may be defined as a protein–lipid–carbohydrate complex formed as a result of specific covalent and non-covalent interactions from flour components during dough making as the components are hydrated and as energy from mechanical input from the mixing process is provided (Islam et al., 2011). A century ago, wheat chemists had a simplistic view of the composition of gluten. In contrast, our present view of wheat-grain proteins, described in this chapter, is a picture of great complexity but also a wondrous picture of emerging harmony of interacting components.

11.2 The protein classes of wheat endosperm

11.2.1 Nomenclature

Wheat seeds comprise three main parts, a protective seed shell, a small embryo, and a starchy endosperm. Due their importance in everyday life and their easy solubility wheat proteins have been of particular interest for the analysis using different molecular and proteomic approaches. The classic nomenclature of wheat-seed proteins is based on their solubility using the methodology originally described by Osborne (1924). The term "Osborne fractions" covers four groups of proteins, namely the water-soluble albumins, the salt-soluble globulins, the alcohol-soluble gliadins and the alcohol-insoluble glutenins.

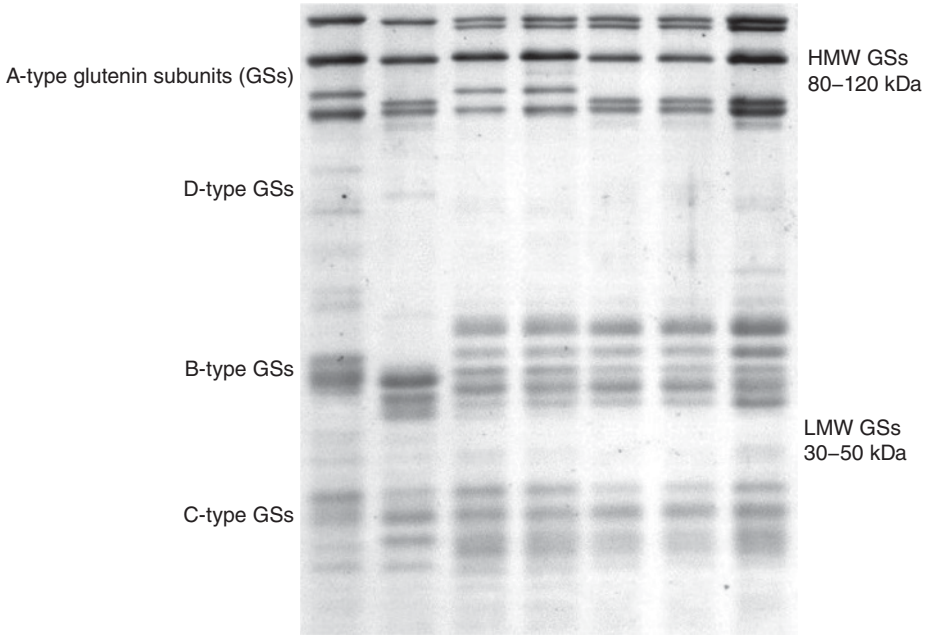

Figure 11.1 Classification of reduced gluten polypeptides based on the mobility of each component on SDS-polyacrylamide gel electrophoresis

The protein composition of wheat endosperm consists about 80% of storage proteins, which serve as deposits of nitrogen and sulfur for germination and have a significant impact on human nutrition. These gluten proteins function as building blocks for the formation of one of the plant kingdom's largest protein networks, wheat gluten (Wrigley, 1996). The gluten proteins of wheat belong to two of the Osborne fractions, the gliadins and the alcohol-insoluble glutenins. Although Osborne fractionation is still used by seed biochemists, the nomenclature of seed proteins and of wheat-seed proteins in particular has evolved toward a more complex classification which is mainly based on their genetic composition, structural features and molecular function.

One of the classification methods is based on the observation of electrophoretic mobility of each single protein in SDS-polyacrylamide gel electrophoresis (SDS-PAGE) gels (Figure 11.1). This method partitions the reduced protein fractions of wheat prolamins into four groups: The A subunits correspond to the high molecular weight (HMW) glutenin subunits with an apparent molecular weight (MW) range of 80–120 kDa (Payne and Corfield, 1979). The majority of reduced prolamins belong to the B subunits. This group mainly consists of more basic low molecular weight (LMW) glutenin subunits with an MW range from 42 to 51 kDa. The minor C subunits seem to be a mixture of proteins belonging to either α-/β- and γ-gliadins or LMW glutenin subunits. One of the most important characteristics of this group of proteins is that all of them contain an odd number of cysteine residues (Payne and Corfield, 1979; Payne et al., 1986; Thompson et al.,

1994, Shewry and Tatham, 1997). They have a wide range of isoelectric points and MWs between 30,000 and 40,000 Da. The remaining group, the D subunits, has lower mobility than the B and C subunits and they are one of the most acidic groups of endosperm proteins (Payne et al., 1986). They are cysteine-mutant forms of ω-gliadins in which the presence of at least one free cysteine residue allows them to establish inter-molecular disulfide bonds and to be incorporated into the glutenin polymer (Payne et al., 1987; Masci et al., 1993).

The classification method of Shewry et al. (1999) is based much more on the genetic similarities, dividing the prolamin groups into three distinct groups of proteins, namely the S-rich, S-poor and HMW prolamins (Shewry et al., 1999). Evolutionarily, all the prolamins of *Triticeae* can be grouped into one of the above groups, showing significant conserved domains. In addition, the number of cysteine residues is conserved in their sequences (Shewry and Halford, 2002). The conserved domains clearly demonstrate that HMW glutenin subunits evolved in a different way compared to the S-rich and sulfur-poor prolamins. While HMW glutenin subunits share similarities to rice glutelins (which belong to globulin type of proteins), S-rich and sulfur-poor prolamins have developed due to some duplication events (Clarke and Appels, 1998). In wheat, the monomeric and cysteine-mutant ω-gliadins comprise the S-poor prolamin fraction. Generally they do not contain cysteine residues, except for the mutant forms, in which point mutations have led to the formation of a single cysteine residue. The S-rich prolamin fraction consists of both monomeric (α-/β- and γ-gliadins) and polymeric proteins low molecular weight glutenin subunits (LMW-GS). The number of cysteine residues generally varies between 6 and 8. The polymeric HMW-glutenin subunits (HMW-GS) correspond to the HMW prolamins. The literature on wheat endosperm proteins has been discussed in detail by a number of reviews. A recent summary of knowledge has been published by Shewry et al. (2009), focusing not only on protein characteristics but also on genetics and expression studies, on the biological function of wheat endosperm proteins and on their molecular function in end-use quality.

11.2.2 High molecular weight glutenin subunits

The HMW-GS of wheat endosperm belong to the protein group that has been studied in most detail, although they represent a minor group in terms of their expressed amount. The HMW glutenin fraction represents only about 17% of the total gluten protein (Seilmeier et al., 1991). They have a crucial effect on the development of the gluten network and they make important contributions to end-use quality due their significant effect on dough strength and dough stability. The HMW glutenins are encoded by *Glu-1* genes on the respective long arms of homoeologous chromosomes 1A, 1B and 1D (Figure 11.2). Each HMW glutenin locus harbors two adjacent genes, an x-type and a y-type gene. The polypeptides coded by the x-type genes have larger molecular weights. Due to the presence of inner stop codons, as well as gene inactivation events, the numbers of HMW glutenin subunits that are actually expressed vary from 3 to 5. In hexaploid wheat, the y-type subunit coded at chromosome 1A is generally absent (Harberd et al., 1987;

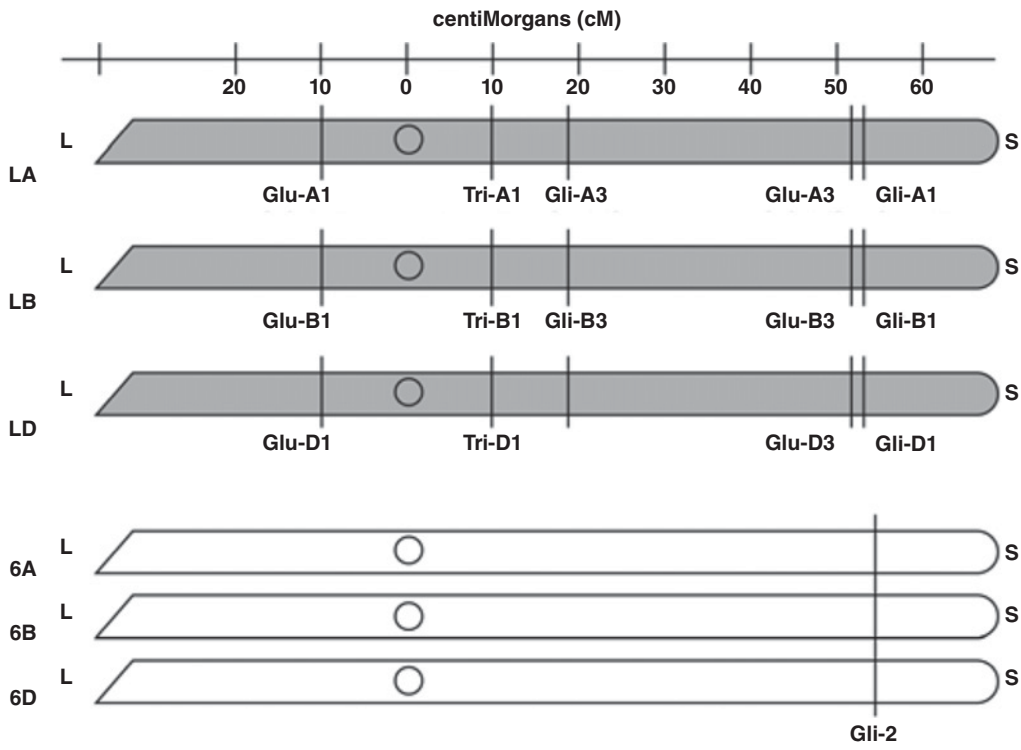

Figure 11.2 Chromosomal locations of genes coding for wheat prolamins. Reprinted from Gianibelli et al. (2001b). *Cereal Chemistry*, 78:635–646

Halford et al., 1989; Gu et al., 2004b). However, Margiotta and co-workers have identified some Swedish wheat lines in which all six HMW glutenin subunits are expressed (Margiotta et al., 1996). Similarly, *Glu-A1* y-type subunits have been identified in diploid wheat species (Gianibelli et al., 2001a).

Extensive polymorphisms have been detected at all three *Glu-1* loci. The degree of polymorphism is still growing with the analysis of different landraces, wild species and wheat relatives (Gregova et al., 1999, 2004; Wan et al., 2000; Gianibelli et al., 2001b). The present number of identified alleles based on the Grain Genes 2.0 database is 22 for *Glu-A1*, 52 for *Glu-B1,* and 36 for *Glu-D1*. The effects on dough quality of the most frequent *Glu-1* alleles have been assessed for the Payne scoring system developed by Payne and Lawrence (1983).

Usually the amount of expressed subunits shows some conserved pattern in the different genotypes, where Bx-type subunits are expressed in the highest amount (Larroque et al., 1997). Generally, the ratio of x-type to y-type subunits varies between 1.7 and 3.2, depending on the genotype characterized (Wieser and Kiefer, 2001). Although the overall expressional variation of different x- and y-type subunits is rather low in hexaploid germplasm, some unusual exceptions have been identified. The expressional variation

of different alleles of subunit Bx7 has been characterized in detail by several groups (Marchylo et al., 1992; D'Ovidio et al., 1997; Juhász et al., 2003a, 2003b; Rampitsch et al., 2000; Butow et al., 2003a, 2004; Vawser and Cornish, 2004). These studies have differentiated allelic variants of subunit 7 with rather similar molecular weights but different expressional profiles. Possible origin and dissemination patterns of the over-expressing Bx7 variant (Bx7oe) have been identified by Butow et al. (2004). The subunit 7 variants identified in several genotypes (e.g., the Canadian cv. Glenlea, the Argentinian cv. Klein Universal, the Australian cv. Chara, and the Hungarian cv. Bankuti 1201) show about 150% overexpression compared to its most closely related normal-expressing variant identified from cv. Chinese Spring (Ragupathy et al., 2008). On the other hand, an under-expressing variant of Bx7 has been identified from several old Hungarian landraces (Juhász et al., 2003a, 2003b). The molecular background of these over-expressing genotypes has been characterized by several groups (Butow et al., 2003a, 2004; Radovanovic and Cloutier, 2003; Ragupathy et al., 2008). In cultivars Red River 68 and Glenlea, the phenomenon has been due to a gene duplication event (D'Ovidio et al., 1997; Lukow et al., 1992; Cloutier et al., 2001). However, in most of the over-expressing gene variants, a duplicated matrix-attachment region has been identified in the distal promoter region (Butow et al., 2003b; Juhász et al., 2003b; Radovanovic and Cloutier 2003). The positive effect of these different expressional patterns on dough properties have been discussed in detail (Marchylo et al., 1992; Lukow et al., 1992; D'Ovidio et al., 1997; Juhász et al., 2002; Butow et al., 2003a, 2003b). A new, over-expressing Bx7 variant with an additional cysteine residue in its amino acid sequence has been identified recently in the Australian genotype H45 (Gao et al., 2012). The effect of this novel allelic variant on dough properties has been characterized and the expected level of its advantageous effect lagged behind that of the subunit Bx7oe identified from cv. Glenlea.

Studies targeting HMW-GS started in the 1970s (Bietz and Wall, 1972). Using SDS-PAGE their molecular weights were estimated to cover a range of 80–130 kDa. However these estimates have since been shown to be over-estimates, based on amino acid sequences (Anderson and Green, 1989). The phenomenon of over-estimation by SDS-PAGE might be due to their unusual amino acid content, based on which HMW-GS bind less SDS than other proteins. Following this phenomenon, there are some further characteristics such as their stability against heat treatment when in solution, their specific amino acid content with high percentages of segments rich in proline, glutamic acid (glutamine), the amounts and distribution of the hydrophobic residues, the large number of repeats composed from several amino acids or their behavior in near-UV-, far-UV-circular dichroism and NMR experiments based on which they show high similarities to intrinsically unordered proteins (IUPs). Most general definitions for IUPs cover a large group of proteins which lack a unique folded structure, either entirely or in parts, when alone in solution (Dunker et al., 2001; Wright and Dyson, 1999). Like HMW glutenin, some elastic proteins such as elastin, spider silk, and abductin possess longer intrinsically unordered regions and thus belonging to the IUP group. With recent advances in protein biochemistry, it has become clear that frequency of disorder is about 19% in Eukaryotes and is over 16% in Arabidopsis (Ward et al., 2004).

Similarly to other prolamins, HMW glutenin polypeptides show unusually high levels of proline, glutamine and glycine in their sequences. Their secondary structure reveals short N- and C-terminal domains with high cysteine content, α-helical structure and a large repetitive region with frequent repeats in the sequences. Cysteine residues enable the formation of intra- and inter-molecular disulfide bonds which are responsible for the formation of the polymeric glutenin structure. Repeats of the repetitive domain of x-type and y-type sequences show special conserved patterns (Shewry et al., 1999) resulting in a structure built from reverse β-spirals (Tatham et al., 1985; Miles et al., 1991).

11.2.3 Low molecular weight glutenin subunits

The LMW-GS are controlled by genes at the *Glu-A3*, *Glu-B3,* and *Glu-D3* loci on the short arms of chromosomes 1A, 1B, and 1D, respectively (Figure 11.2). Based on their N-terminal sequences they fall into three main groups. These groups were originally distinguished by the first amino acid of their mature peptide sequences: either isoleucine (LMW i-type), serine (LMW s-type) or methionine (LMW m-type) at their N-terminal, as identified by N-terminal sequencing (Kasarda et al., 1988; Tao and Kasarda, 1989; Lew et al., 1992; Masci et al., 1995). Sequences starting with SHIPGL were supposed to belong to the s-type sequences. However the deduced amino acid sequences of their encoding genes started either with IENSHIPGLEK, MENSHIPGLEK, or MENSHIPGLER, suggesting a possible post-translational modification due the effect of the asparaginyl peptidase at position N (D'Ovidio and Masci, 2004). M-type sequences were described as mature peptides starting with MET, including types with METRCIPGLER, METSCIPGLER, METSCISGLER, METSRVPGLEK, METSHIPGLEK, METSHIPSLEK, and MDTSCIPGLER. LMW i-type genes are considered as gene types completely lacking their N-terminal region and starting directly with the repetitive domain. This type includes sequences starting with ISQQQQPPPFS, ISQQQQPPLFS, ISQQQQQPPFS, ISQQQQAPPFS.

Although i-type genes are solely expressed in the A genome of wheat, most of the gene types express at all A, B, and D genomes, respectively. Groupings made, based on the N-terminal sequences and the positions of cysteine residues, have resulted in more detailed information. Based on these characteristics, Ikeda et al. (2002) have identified 12 distinct groups and further 5 groups were identified by Juhász and Gianibelli (2006). The number of expressed proteins varies between 11 and 20, resulting in alleles with different numbers and combinations of subunits at all three loci (Ikeda et al., 2002; Ikeda et al., 2006). However the number of identified alleles is 6 at locus *Glu-A3*, 12 at *Glu-B3,* and 5 at *Glu-D3* (Grain Genes 2.0, see Anon, 2009).

Most LMW-GS consist of between 250 and 300 amino acid residues, and they have a clear three-domain structure (Wieser, 1995; Cassidy et al., 1998; Ikeda et al., 2002). The signal sequences are quite conserved among all the LMW-GS, although their lengths differ slightly. Kreis et al. (1985) and Colot et al. (1989) reported that the signal sequence terminates with Ala. The N-terminal sequence in LMW-GS is shorter than that reported for HMW-GS (Shewry et al., 1992, 2003) and is composed of 13 amino acid residues in most

of the LMW-GS m- and s-types. In general, the variability in length of sequences of the glutenin genes is mainly due to differences in the numbers of repetitive motifs in the repetitive region. The overall residue length of the repetitive domain is between 137 and 175. Lee et al. (1999b) studied the LMW-GS controlled by the *Glu-A3* locus of diploid species relatives of wheat. LMW-GS genes possess repetitive domains that are shorter and contain different motifs than those observed in HMW-GS. While the LMW glutenin repetitive structure is much more similar to other S-rich prolamins and is composed mainly of Pro, Gln, and Phe residues, the HMW repetitive domains contain more Gly and Tyr residues, along with high proportions of Pro and Gln. The overall Pro- and Gln-rich composition enables the formation of a loose spiral structure (Shewry et al., 1999). Kreis et al. (1985) and Shewry and Tatham (1990) suggested that the repeats had a consensus of PQQPPPFS and QQQQPVL. Cassidy and Dvorak (1991) suggested a peptide repeat pattern of "PPFSQQQ".

The C-terminal domains of all the S-rich prolamins contain three main regions, called A, B, C, which are conserved among the LMW-GS gene family, as well as among the gliadins and seed storage proteins in rye and barley (Kreis et al., 1985; Colot et al., 1989). These regions are separated and flanked by intermediate regions. Regions I_1 and I_3 contain stretches of poly-glutamines, making them higher in glutamine content than regions A, B or C. The intermediate region I_2 is conserved among the LMW-GS genes but it has diverged from the corresponding regions of the gliadins, secalins, and hordeins (Kreis et al., 1985; Colot et al., 1989). Region I_4 corresponds to the carboxyl terminal end of the protein, which is also conserved within the LMW-GS gene family, but is different from the termini of other families of seed storage proteins. There are some characteristic differences in the length and composition of the C-terminal domains of i-, s-, and m-types. Usually, the LMW-GS have a C-terminal of 180–185 residues, except the s-type LMW-GS, which have a C-terminal domain shorter by 10–15 residues. The secondary structure of LMW-GS, except for the D subunits, has an overall similarity with the structure of the S-rich gliadins (Tatham et al., 1987; Thompson et al., 1994; D'Ovidio et al., 1995). All the S-rich prolamins possess at least one disordered region bordered by regions composed from α-helices and β-turns. This structure results in compact, tightly folded molecules and an unusual stability to thermal denaturation, partly due to the presence of intra-molecular disulfide bonds (Shewry et al., 1999). Repeated sequences account for about 30–50 mol% of these proteins, in contrast to the more extensive repeats in the S-poor gliadins and HMW-GS. The γ-gliadins contain a single conserved motif and the distribution of β-turns may be sufficiently regular to form a loose spiral structure, leading to a slightly extended structure for the whole molecule. A similar structure is characteristic of LMW-GS. The short N-terminal domains are rich in α-helix and appear to be more compact (Thomson et al., 1992; Shewry et al., 1999).

11.2.4 Gliadins

Gliadins form the most diverse group of wheat prolamins. Gliadins are usually characterized as monomeric and alcohol-soluble proteins with a molecular range between 30 and 75 kDa (Gianibelli et al., 2001b). Initially, based on their electrophoretic mobility on

acid-PAGE analysis, they have been differentiated into four groups, α-, β-, γ-, and ω-gliadins, with α-gliadins as the fastest group and ω-gliadins as the slowest group. However, based on their primary sequences α- and β-gliadins share common characteristics; therefore they are often grouped together (Kasarda et al., 1987). The ω- and γ-gliadins are controlled by several tightly linked genes present at the *Gli-1* loci (*Gli-A1, Gli-B1, Gli-D1*) on the short arms of the homoeologous group 1 chromosomes, whereas the α-gliadins are controlled by the *Gli-2* loci (*Gli-A2, Gli-B2, Gli-D2*) present on the short arms of the group 6 chromosomes (Figure 11.2) (Payne, 1987; Shewry et al., 2009).

11.2.4.1 γ-Gliadins γ-Gliadins share similarities in their sequence and molecular size to LMW glutenin polypeptides. They contain eight cysteine residues, all of which are involved in the formation of intra-molecular disulfide bonds. Their structure consists of a short (12 amino acids long) N-terminal domain, a relatively large repetitive domain with 78–161 residues, and a large C-terminal domain of over 130 residues. The C-terminal domain contains all the cysteine residues grouped into a cysteine-rich and a cysteine-poor region dispersed by a glutamine-rich region. The γ-gliadins differ from α-gliadins in their amino acid compositions, possessing higher amounts of aspartic acid, methionine, tryptophan, and lower amounts of tyrosine and phenylalanine (Shewry et al., 2009).

11.2.4.2 α-Gliadins The estimated number of genes encoding α-type gliadins ranges from 25 to 150 copies (Anderson et al., 1997) and it has been suggested that gene amplification and deletion events as well as retrotransposon insertion have been the major molecular mechanisms responsible for the large number of α-gliadin genes and for their uneven distribution in the *Gli-2* region (Gu et al., 2004a). Despite the large number of genes, only 16 α-gliadin proteins have been identified (Lafiandra et al., 1984) using the high-resolution method of two-dimensional PAGE. A similar number of these proteins has been identified in wheat flour of cv. Butte 86 by Dupont et al. (2011). The anomaly observed between the large gene number and smaller number of expressed proteins is mainly due to the unusually large number of pseudogenes present (Anderson et al., 1997).

The secondary structure of gliadins contains a small, five-residue long N-terminal domain, followed by a roughly similar long repetitive domain and a C-terminal domain. This structure provides the α-gliadin molecules with a globular structure. The six cysteine residues present in α-gliadins are concentrated in the C-terminal domain, forming three intra-molecular disulfide bonds. Like γ-gliadins, the C-terminal domain has its own compartmentalization. The cysteine-rich region possesses four of the six cysteine residues. This is followed by a glutamine-rich region and then by a third region with the rest of the cysteine residues. Cysteine-mutant variants have been identified in both γ- and α-gliadin families, resulting in modified numbers of cysteine residues (Lew et al., 1992; Ferrante et al., 2006). This phenomenon enables these mutant gliadins to partition in polymer formation either as chain extenders or chain terminators (Kasarda, 1989).

11.2.4.3 ω-Gliadins The number of genes encoding ω-gliadins is the smallest among the gliadins, with about 15 genes in hexaploid wheats and 5–10 genes in durum wheats. The secondary structure of ω-gliadins shows significant differences from α- and γ-gliadins.

The structure is composed from a short N-terminal with about 11 residues, a large repetitive domain with more than 200 amino acid residues, and a short C-terminal region of 12 residues. ω-gliadins tend to have molecular weights slightly larger than those of the LMW glutenins. They differ in amino acid composition from other gliadins. Due to the absence of the majority of the C-terminal domain, they do not have cysteine residues. They can be characterized by high levels of glutamine (+ glutamate) (40–50 mol%), proline (20–30 mol%), and phenylalanine (7–9 mol%) (Tatham and Shewry, 1995). Based on their amino acid composition, they are the most hydrophobic group of wheat prolamins. The cysteine-mutant types of ω-gliadins possess a single cysteine residue and these variants are called LMW-D glutenins (Masci et al., 1993, 1999). Due to their single cysteines, they can participate in polymer formation as chain-terminator molecules.

The individual *Gli-1* and *Gli-2* loci exhibit extensive polymorphism in different bread and durum wheat cultivars, with a consequent high diversity of gliadin patterns as detected by electrophoretic and chromatographic techniques, thus providing the basis for distinguishing different wheat cultivars (Bietz and Huebner, 1995; Lookhart and Wrigley, 1995). The number of alleles identified at the *Gli-1* and *Gli-2* loci varies as follows: *Gli-A1* 18, *Gli-B1* 16, *Gli-D1* 12 and *Gli-A2* 24, *Gli-B2* 22, *Gli-D2* 19.

11.2.4.4 Other gliadin loci Although the genes encoding most of the ω- and γ-gliadins are tightly clustered at the *Gli-A1*, *Gli-B1,* and *Gli-D1* loci on the distal ends of the short arms of chromosomes 1A, 1B, and 1D (Figure 11.2) (Payne, 1987), a few gliadin components and LMW subunits have been shown to be encoded by additional, dispersed genes which have been mapped on the short arms of chromosomes 1A, 1B, and 1D, tightly linked to one or another of the major *Gli* loci. *Gli-5* is a minor locus tightly linked to *Gli-1* and it encodes several ω-gliadin genes (Pogna et al., 1993). Their encoding loci *Glu-2*, *Gli-3*, *Gli-5,* and *Gli-6* have been characterized by Pogna et al. (1993), Felix et al. (1996), and by Sobko (1984).

11.2.5 Minor prolamins

11.2.5.1 Wheat α-amylase and protease inhibitors Wheat α-amylase and serine/trypsin protease inhibitors are salt-soluble proteins that inhibit various insect proteases and amylases. Like other members of the prolamin superfamily they contain large numbers of conserved cysteine residues (10) which are able to form intra-molecular disulfide bonds. The wheat α-amylase inhibitors (WAI) are present in three distinct forms, in monomeric (WMAI), dimeric (WDAI), and tetrameric (WTAI) forms. Their tertiary structure consists of four major α-helices and a one-turn helix arranged in an up-and-down manner (Oda et al., 1997). The monomeric inhibitors are about 12 kDa in size and they are encoded by genes on the short arms of group 6 chromosomes. The WDAI group consists of 24 kDa homodimers and are encoded on group 3 chromosomes. The hetero-tetrameric α-amylase inhibitors (WTAI), 60 kDa in size, are composed of one copy of the CM2 proteins encoded at chromosomes 7B and 7D, plus three proteins (one copy of CM16 or CM17 and two copies of CM3, encoded by genes on 4B and 4D) (Altenbach et al., 2011). The expression of these proteins occurs almost exclusively in the starchy endosperm and

some members show a level of expression similar to that of several gluten proteins. Their amino acid composition is more balanced compared to the gluten proteins and they also serve as a reservoir of nutrients (thus they are "storage proteins") due their relatively high level of accumulation. As a result, these proteins compensate in part for the deficiencies in essential amino acids of the gluten proteins, thereby contributing important reserves for both seedling growth and human nutrition (Altenbach et al., 2011).

Trypsin protease inhibitors share structural similarities to the α-amylase inhibitors but are active against specific proteases. The wheat trypsin inhibitors (CMx proteins) are encoded by genes on the group 4 chromosomes and their expressed forms have been identified from flour extracts of cv. Butte 86 by Altenbach et al. (2011). Another class of putative protease inhibitors, referred to as serpins or wheat chymotrypsin inhibitors (WCI), may inhibit chymotrypsin. They were identified in cv. Butte 86 and also in an Italian cultivar, San Pastore (GenBank: AJ422078). Most members of the seed-inhibitor class are allergenic components in wheat flour, causing severe symptoms to patients suffering from baker's asthma.

11.2.5.2 Wheat farinins and purinins In addition to gliadin and glutenin, wheat flour contains a number of minor storage protein types, some of which are close to the traditional prolamins in composition and sequence. One minor type was given the name avenin-like proteins or more recently farinins based on the sequence similarities to both oat avenins and γ-gliadins (Dupont et al., 2011). Another group of minor prolamins called purinins or LMW gliadins consists of proteins with molecular weights less than 20 kDa (Salcedo et al., 1979; Anderson et al., 2001; Clarke et al., 2003; Dupont et al., 2011). The purinin proteins all contain 14 cysteine residues, 8 of which correspond to the characteristic conserved cysteine skeleton of the prolamin superfamily. The controlling genes are located on chromosomes 7A, 7D, and 4A (Clarke et al., 2003). Both purinins and farinins are prolamin-like in composition but have lower contents of glutamine and proline than the γ-gliadins, whose amino acids are related to the absence of extensive repetitive sequences.

11.2.5.3 Puroindolines and grain-softness proteins Friabilin, a protein fraction of roughly 15 kDa, has been identified from the surface of starch granules isolated from soft wheats. Friabilin comprises more than 10 closely related components, of which puroindoline a, puroindoline b, and the grain-softness proteins (GSPs) are the main components (Giroux and Morris, 1997). Genes for both puroindolines and GSPs are located at the hardness locus (*Ha*) on the short arms of group 5 chromosomes. The *Ha* locus is the major locus controlling grain texture in hexaploid wheat. In durum and hexaploid wheats, genomic deletion during polyploidization and several chromosomal rearrangements have led to the absence of puroindoline genes on chromosomes 5A and 5B (Chantret et al., 2005). However, they are present in diploid and tetraploid *Aegilops* and *Triticum* species (Gautier et al., 2000). The phenotypic appearance of the different puroindoline proteins results in softer grain texture compared to durum wheats. In hexaploid wheats, several allelic variations of puroindoline genes occur, from which the combined presence of wild

alleles of puroindoline a and puroindoline b genes (*Pin-a* and *Pin-b*) results in soft-grain characteristics. The mean molecular weight of the various puroindoline proteins is 12.8 kDa (Pasha et al., 2010) and they belong to the class of cysteine-rich prolamins. The secondary structure of puroindonlines involves a relatively large ratio (40%) of unordered structure combined with 30% α-helix regions and 30% β-sheet. Under high ionic strength and low pH, puroindoline genes form small aggregates, in which a special region rich in amphiphilic tryptophan seems to play a key function. Mutations in any of the puroindoline alleles are consistently associated with hard-kernel texture. In hexaploid wheat, 8 *Pin-a* alleles have been identified; there is greater polymorphism in the *Pin-b* alleles, with 18 different allelic variants identified (Bhave and Morris, 2008). Further polymorphisms have been found in different *Aegilops* species for both *Pin-a* and *Pin-b* genes (Chen et al., 2005; Bhave and Morris, 2008). Detailed characterization of the different puroindoline alleles has been described by Bhave and Morris (2008). Studies on hardness genes and end-use quality have been discussed in detail by Morris (1992), Bettge and Morris (1995), and Miller et al. (1997). These issues are summarized later in this chapter.

The GSP, GSP-1, is closely related to the puroindolines and is a member of the same prolamin "superfamily" that includes α-amylase/trypsin inhibitors, the "CM" proteins, and non-specific lipid-transfer proteins (ns-LTPs) (Gautier et al., 1994). Three orthologous loci for GSP-1 exist in hexaploid wheat. These genes are located on the distal end of the homoeologous group 5 chromosomes. The GSP-1 locus on 5DS is closely linked to the puroindoline genes and thus is linked to grain texture. The term GSP has been used interchangeably with friabilin. However in sequence comparison, GSP-1 shows only about 40% homology to puroindoline a and it shares 50% of sequence homology with some of the oat avenins (Blochet et al., 1993; Fabijanski et al., 1985). Some studies have investigated how allelic variants of GSP-1 affect kernel texture properties (Jolly et al., 1993, 1996; Tranquilli et al., 2002). Based on our most recent knowledge, GSP does not have a direct effect on grain-texture properties, seeming to be irrelevant for grain hardness compared to the puroindolines (Morris, 2002).

11.2.5.4 Lipid-transfer proteins The ns-LTPs are small, basic proteins that are characteristic of different tissues of higher plants. They are generally 7–15 kDa in size and are basic proteins. Like other members of the prolamin superfamily, they contain eight cysteine residues which are able to form four intra-molecular disulfide bonds (Douliez et al., 2000a, 2000b). One of the characteristics of this group of proteins is that, in contrast to most of the lipid-binding proteins, the ns-LTPs do not possess any Trp residues (Shewry et al., 2009). The ns-LTPs can be classified into two major groups, ns-LTP1 and ns-LTP2. However, the classification system of Jang et al. (2007) has distinguished five groups and further subfamilies have been identified based on the position of cysteine residues and their distance to some specific residues characteristic to the individual groups (Boutrot et al., 2008). The tertiary structure of both ns-LTP1 and ns-LTP2 shows four α-helices separated by flexible loops held together by four disulfide bridges (Gincel et al., 1994; Douliez et al., 2000a, 2000b). The helical structure is considered important for lipid binding, with a hydrophobic cavity formed in the C-terminal half comprising the lipid-binding

site. The cavity formed by the four α-helices is able to bind a wide range of lipids and other hydrophobic compounds. Unlike ns-LTP1, ns-LTP2 is able to bind sterols, the binding tunnel being able to open up to accommodate the sterol ring system (Cheng et al., 2004). Based on structural and sequence data analyses, they can fulfill a diverse range of functions. However many of them are generally involved in plant defense mechanisms and pathogen responses (van Loon and van Strien, 1999). The binding cavity of ns-LTP1 is less flexible and consequently can only bind linear lipids. It has been proposed that association with sterols, a constituent of fungal cell membranes, is a requirement for ns-LTP2 molecules to interact with plant receptors involved in plant defense responses and may relate to the *in vivo* role of these proteins.

11.2.5.5 Thionins Thionins comprise a group of small (~5 kDa) Cys-rich peptides that also occur in cereal species (Stec et al., 2004). They are generally 45–47 amino acids long and are classified into five subclasses (Type I–Type V). Purothionins, or Type I thionines, first isolated from wheat endosperm, have been identified in three variants: that is, α1, α2, and β (Fernandez de Caleya et al., 1972). Their genes are controlled by loci on the long arms of the group 1 chromosomes (*Pur-A1*, *Pur-B1*, *Pur-D1*) (Fernandez de Caleya et al., 1976). They are basic polypeptides, only 45 amino acids long, containing 8 cysteine residues. Other thionin members have been isolated from leaf tissues, seeds, and nuts of various monocotyledon and dicotyledon species (for review see Shewry et al., 2009). The three-dimensional structure of purothionins is comprised of two α-helices (which form a helix-turn-helix motif), a C-terminal coil and two short β-strands (that form a β-sheet). The tertiary structure is held together by four disulfide bonds, salt bridges, and intra-molecular hydrogen bonds (reviewed in Stec et al., 2004).

Thionins are highly toxic to a wide range of organisms including bacteria (Stuart and Harris, 1942; Fernandez de Caleya et al., 1972) and fungi (Balls and Harris, 1944; Nose and Ichikawa, 1968). Many hypotheses have been proposed regarding the mechanisms of cell lysis by various thionins, for example, ion channels (Hughes et al., 2000; Llanos et al., 2006), lipid rafts (Giudici et al., 2004) and solubilization of membrane phospholipids (Stec et al., 2004). Recent studies with model membranes (Majewski and Stec, 2010) showed solubilization to be the cause of membrane leakage. Most interestingly, Oard (2011) has proposed the formation of water channels, similar to aquaporins, by α-hordothionin, wherein the highly conserved Cys and Tyr residues form the pore wall, leading water molecules to the centre of the phospholipid bilayer, thus causing local membrane disruption (Bhave and Methuku, 2011).

11.2.6 Water-soluble proteins

11.2.6.1 The 3S and 7S globulins Several types of globulins are also present among the flour proteins. Proteins termed globulin-1, 3S, or α-globulin are encoded at the highly conserved *Glo-2* locus between the loci for the x- and y-type HMW-GS on chromosome 1 (Gu et al., 2010; Xu and Messing, 2009). Globulin-2 or 7S globulins are members of the cupin superfamily, similar to known food allergens. Analysis of wheat endosperm cDNA libraries shows that α-globulin transcripts are present and encode a mature protein of

about 19,000 Da. A 22 kDa *Glo-2* protein has been identified from cv. Butte 86 using a proteomic approach (Altenbach et al., 2011).

The genes encoding 7S globulins are encoded at two gene copies on chromosome arms 4AL, 4BS, and 4DL (Millan et al., 1992; Loit et al., 2009). They are expressed in protein bodies of wheat aleurone cells but not in starchy endosperm cells. Gene variants *Glo-3A*, *Glo-3B,* and *Glo-3C* have been identified recently from cv. Glenlea (Loit et al., 2009) and expressed proteins were identified from cv. Butte 86 (Altenbach et al., 2009). 3S α-globulins and 7S γ-globulins are predominant in the globulin fraction of wheat seed compared to oat where 11S globulins largely predominate (Robert et al., 1985).

11.2.6.2 Triticins The triticins of wheat endosperm are legumin-like 11S globulin storage proteins encoded at *Tri-A1, Tri-B1,* and *Tri-D1* on the short arms of chromosomes 1A, 1B, and 1D (Singh and Shepherd 1985; Singh et al., 1993). They account for about 5% of the total seed protein and form small polymeric proteins, consisting of hetero-tetramers linked by disulfide bonds. The triticin locus *Tri-B1*, identified on chromosome 1B, seems to be an inactive locus (Dubcovsky et al., 1997). The *Tri-A1* and *Tri-D1* loci are loosely linked to the gliadin genes at *Gli-1*. Although they contain cysteine residues, enabling disulfide bond formation, the triticins are not considered to be part of the gluten-forming complex of proteins. Compared to prolamin proteins, they possess higher amounts of sulfur, threonine, and lysine. When considering their solubility properties, they behave as globulins. No important link with bread-making quality has been established.

11.2.6.3 HMW albumins These non-prolamin proteins of ~60 kDa, revealed by SDS-PAGE fractionation of reduced extracts, are mostly enzyme related and water soluble (Gupta et al., 1991). Certain HMW-albumin bands (65, 63, and 60 kDa) occur as both disulfide-linked oligomers and monomeric forms in their native state. They are β-amylases controlled by chromosome arms 4DL, 4AL, and 5AL (β-*Amy-1* loci). HMW albumins are absent from protein bodies (Payne et al., 1986; Forsyth and Koebner, 1992). A limited amount of allelic variation in these bands has been observed. A fourth HMW albumin with faster mobility and MW of ~45,000 Da was distinct from the β-amylase group according to immunoblotting analysis. HMW albumins tend to be present either in monomer form or they form small aggregates, stabilized through disulfide bonds (Gupta et al., 1991).

11.2.6.4 LMW albumins LMW albumins share similar characteristics to HMW albumins with a lower molecular mass of 45 kDa. They show low polymorphism on SDS-PAGE. The amounts of them depend strongly on sulfur availability during the grain development process (MacRitchie and Gupta, 1993).

11.2.7 Glutenin polymers

Because of their strong relationships with the bread-making properties (see in detail in Section 5.2), the polymeric proteins have received intense research attention (as recently reviewed by Wieser et al. (2006) and by Hamer et al. (2009)).

A minor portion (~20%) of alcohol-soluble gliadin is the class of oligomeric proteins (MW ~100–500 kDa) linked by interchain disulfide bonds. This fraction, called HMW gliadin, aggregated gliadin, or ethanol-soluble glutenin (Shewry et al., 1983; Huebner and Bietz, 1993), has been shown to consist of α- and γ-gliadins (probably with an odd number of cysteines) and LMW-GS.

In contrast, the glutenin fraction contains alcohol-insoluble polymeric proteins of varying size with molecular weights ranging from about 500 kDa to more than 10 million Da. They are mainly formed from LMW- and HMW-GS linked by interchain disulfide bonds, also stabilized by non-covalent bonds.

Glutenin polymers are amongst the most complex protein aggregates in nature due to their wide range of molecular weight, the high number of different subunits incorporated and the different covalent and non-covalent bonds forming and stabilizing the polymeric structure. The composition of the polymers varies widely according to genotype, growing conditions, and technological processing. The major protein components of the polymers are the HMW- and LMW-GS that are able to form interchain disulfide bonds. The predominant protein types present in the polymeric glutenin fraction are LMW-GS; their proportion amounts to ~60%. The second type is HMW-GS constituting about 30%. The remaining ~10% is contributed by gliadins having odd numbers of cysteine residues (one Cys in ω-gliadins, five or seven in α-gliadins, and seven or nine in γ-gliadins). They are covalently bound to glutenins via an interchain disulfide bond and act as "terminators" to glutenin polymerization (Kasarda, 1989).

Studies of disulfide structures indicate that HMW-GS generate linear polymers by end-to-end disulfide bonds. Additional interchain disulfide bonds lead to branching of the HMW-GS backbone. LMW-GS form polymers, too, which are branched. Terminators of polymerization are gliadins with an odd number of cysteines and LMW thiol compounds. Disulfide-bonded polymers are stabilized by non-covalent hydrogen, ionic, and hydrophobic bonds.

Disulfide bonds play a key role in determining the structure and properties of gluten proteins. They are formed between the sulfhydryl groups of cysteine residues either within a single protein (intrachain) or between proteins (interchain). They are important in stabilizing the conformation of proteins or protein aggregates and determine the sizes of glutenin polymers. Disulfide formation starts rapidly after synthesis of proteins within the lumen of the endoplasmatic reticulum as an integral part of protein folding and this may be assisted by the enzyme disulfide isomerase (Shewry, 1999). It is likely that intrachain disulfide bonds form more rapidly than interchain disulfide bonds. But glutenin subunits, as synthesized, cannot combine all of their cysteine residues to form intrachain disulfide bonds; some remain as free thiols or must form interchain disulfide bonds (Kasarda, 1989).

After residing in the protein bodies, glutenins undergo redox changes during the development and maturation of the grain. Free thiol groups become oxidized during grain dehydration which coincided with high MW polymers (Rhazi et al., 2003). A few cysteine residues of glutenins, however, can be found in a free thiol form even in mature flours (Antes and Wieser, 2000).

Tilley et al. (2001) have reported that glutenin also contains dityrosine cross-links formed between pairs of adjacent tyrosine residues present in the nonapeptide repeat

motifs of the HMW subunits (i.e., Gly.Tyr.Tyr.Pro.Thr.Ser.Pro/Leu.Gln.Gln). Dityrosine cross-links differ from disulfide bonds in that they are not broken by reducing agents. Hanft and Koehler (2005) demonstrated that between 1 in 1000 and 1 in 10,000 of the total number of tyrosine residues in gluten are involved in dityrosine bonds in dough mixed with improver and in flour, respectively. They also calculated that the maximum abundance was 1 dityrosine cross-link to 142 disulfide bonds.

Glutenin polymers are the major determinants of the rheological properties of wheat dough. Their amount in flour and their ratio to monomeric gliadins are important quantitative factors for dough quality. The molecular weight distribution (MWD) of glutenin has been recognized as one of the main determinants of physical dough properties (for review see Southan and MacRitchie, 1999). The proportion of polymers with the highest MW glutenin macropolymers (GMP) has been demonstrated to be highly correlated with dough properties and bread-making quality. The largest polymers termed "unextractable polymeric protein" (UPP) or "GMP" make the greatest contribution to dough properties and their amount in flour (≈20–40 mg/g) is strongly correlated with dough strength and loaf volume (Southan and Mac Ritchie, 1999; Weegels et al., 1996); Mac Ritchie, 1999): (1) the ratios of HMW-/LMW-GS and x-/y-type HMW-GS; (2) the allelic variation of HMW-GS; and (3) the proportion of terminators. The presence of oxidants during dough mixing is additionally important for the generation of HMW polymers.

In spite of the extreme difficulties, a few research groups have tried successfully to identify the positions of disulfide bonds in glutenin polymers, as reviewed by Lutz et al. (2012). A range of molecular models has been developed to explain the structure and functionality of glutenins. The "linear glutenin hypothesis" of Ewart (1977) suggested that glutenin subunits form linear polymers stabilized by interchain disulfide bonds with little, if any, branching; HMW- and LMW-GS are proposed to be randomly linked. Other models agree on the importance of interchain disulfide bonds, but include variable ratios of linear and branched linkages (reviewed by Ewart, 1990). Graveland et al. (1985) proposed a model in which x- and y-type HMW-GS are linked head-to-tail to form the backbone of glutenin polymers. This is in agreement with the model of linear concatenations as proposed by Ewart, with the addition of strings of LMW-GS branched off from the y-type HMW-GS. The experimental findings available until now support Graveland's model.

With respect to quantitative data on the subunit composition of glutenin (ratio of LMW-GS to HMW-GS ~2:1 and of x- to y-type ~2.5:1) and to the molecular weight of subunits, the basic molecular "double unit" of glutenin polymers might consist of 2 y-type HMW-GS, 4 x-type HMW-GS, and around 30 LMW-GS covalently linked by interchain disulfide bonds. The molecular weight of this unit amounts to about 1.5 million Da (Wieser et al., 2006) (Figure 11.3).

A comprehensive model including covalent and non-covalent bonds has been proposed by Hamer and van Vliet (2000). Above the molecular level, the mesoscopic level describes further aggregation of polymers by entanglements stabilized by non-covalent bonding and additional disulfide bonds. Inspired by polymer theory, Singh and MacRitchie (2001) suggested that highly polymerized glutenins can best be described as an entangled polymer network. The authors use a point entanglement model in which these are small regions where the polymers interact separated by extended, non-interacting regions. The amount

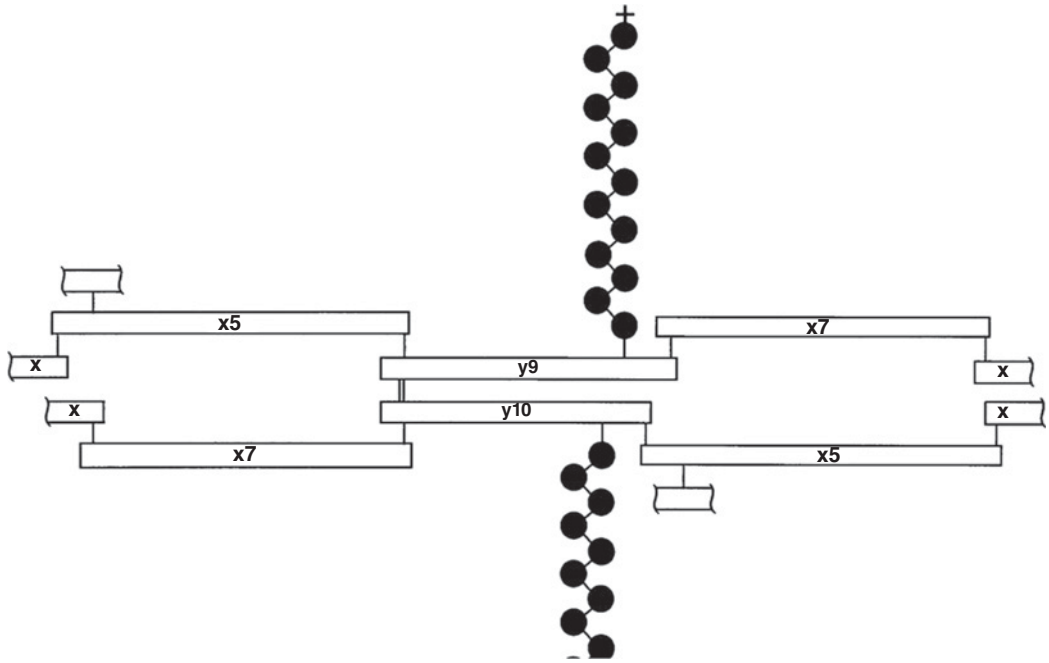

Figure 11.3 The basic molecular "double unit" model for the interchain disulfide structures of low-molecular-weight glutenin subunits (LMW-GS) (●) and high-molecular-weight glutenin subunits (HMW-GS) (□) to form polymeric glutenin. From Wieser et al., 2006. Reproduced with permission from AACCI

of entanglements is proposed to be related to the structure, size, and concentration of the polymers. If the polymers are of a moderate length, their entanglements with one another can be readily disentangled by slippage. By contrast, the disentanglement of high MW polymers is much more difficult; therefore, resistance to extension and mixing time would be greater and extensibility lower. The size of such aggregates will be <100 μm. It is expected that this mesoscopic aggregation will occur in the kernel and in dough during the first phase of resting.

Mesoscopic particles can aggregate further to a third macroscopic level by the formation of entanglements between particles. Aggregates of this level (>100 μm size) are thought to be predominantly influenced by process conditions (shear, stress) and other particles interfering with the formation of entanglements (e.g., hard fat and arabinoxylans).

11.3 Proteins of non-endosperm tissues

With the increasing demand for healthy cereal products, wheat germ and wheat bran have become a more valuable food source, especially in the development of dietary products or products for patients with special nutritional needs. Due to the very efficient commercial milling process, the separation of endosperm from other parts, such as germ or bran is routine.

11.3.1 Germ proteins

The proteomic composition of wheat germ is of nutritional significance and helps to elucidate processes that occur during germination, especially during pre-harvest sprouting.

Protein composition of wheat germ has been characterized in detail by Mak and coworkers (2006) using gel-based two-dimensional proteomics tools. In total, 612 protein spots have been characterized by peptide mass fingerprint analysis. The majority of the proteins identified were in functional categories associated with processes involved in embryonic development and activating growth, including different transcriptional, translational processes, energy and general metabolism, transport, cell division, and signaling processes. A major class of proteins present in the germ fraction represents different stress-associated proteins, from which only a few protein types express in the endosperm. About 25% of the protein spots identified belonged to protein groups of either unclassified function or represent proteins what were considered to be hypothetical proteins in the genomic database. Membrane proteins comprise about 20–40% of the total proteins in germ cells; however their poor solubility and inherent hydrophobicity require special treatments during extraction (Stevens and Arkin, 2000). Increased solubilization of denatured membrane proteins was achieved by the use of surfactants such as SDS, which is however incompatible with isoelectric focusing (the first step of the analysis) (Nouwens et al., 2000). Therefore, this type of protein may be under-represented in the germ proteome maps. Approximately 20% of the proteins identified in the germ were enzymes, which represent in all six Enzyme Commission (EC) classification groups: transferases, isomerases, lyases, oxydoreductases, hydrolases, and ligases.

The protein composition of germinating wheat seeds has been analyzed in the first three days of germination (Mak et al., 2009; Michalcova et al., 2012). All identified proteins involved in transcription, transporter activities, or secondary mechanisms have shown significant decreases in their abundance. Decreasing levels of expression were also identified for some proteins involved in energy-related mechanism, protein synthesis, and degradation, as well as proteins of signal transduction, stress-related mechanisms and metabolism. The proteins that increased in abundance during the germination period have diverse roles including energy, protein degradation, protein folding, and in the cytoskeleton.

11.3.2 Bran proteins

Bran has an important function as a protective barrier for the grain. The grain is a potential food for insects, fungi, and bacteria, and it is also exposed to many environmental stresses, thus the bran characteristics need to assure a protection against all these factors. Proteomic analysis of wheat bran and bran tissue fractions has been reported by Jerkovicz et al. (2010) and Laubin et al. (2008), while spatial and temporal expression of peripheral layer proteins were followed by Tasleem-Tahir et al. (2011). Due to the strong bonds present between various tissue layers and the endosperm tissue in mature grain, the possibility for collecting bran tissue fractions in quality is limited. Thus, a method to obtain bran layers free from contaminants, such as adjacent tissue and endosperm, is required to provide a sample suitable for proteomic analysis. The bran, the outermost component

of the grain is composed of about seven distinct tissue layers. The outermost layers of the bran are collectively called the pericarp. The pericarp consists of the epidermis, hypodermis, cross-cells, and finally the tube cells. The next three tissue layers are the testa, nucellar, and aleurone. The aleurone tissue is in contact with the endosperm and it is the only bran tissue layer that is still alive and functional at the cellular level in mature grain and is critical during germination (Antoine et al., 2003, 2004). Proteomic analysis of bran tissue fractions from wheat grain revealed the location and distribution of many common plant defense-related proteins, which appear to be specific to certain tissue layers within the bran. Around 80% of proteins identified within the outer layers fulfill a general defensive role and they provide resistance to fungal and bacterial attack. Defense- and stress-related proteins were also identified in the inner fraction; however they comprised only around 5% of the protein array. The major protein in the inner fraction was 7S globulin storage protein, which is likely to be involved in defense against oxalate-secreting fungi and also to serve in protecting itself during grain development when there is potentially a high oxalate level (Dunwell et al., 2000). Some of the major classes of defense proteins are termed pathogenesis-related proteins (PR), and include PR-1 (antifungal proteins), PR-2 (1, 3-β-glucanases), PR-3 (chitinases), PR-4 (wheatwin), and PR-5 (thaumatin-like proteins) (Selitrennikoff, 2001; Desmond et al., 2006).

Studies of the aleurone layer of the seed have been stimulated by its nutritional and health benefits. The aleurone (AL) provides proteins rich in lysine and also contains several minerals (P, K, Mg, Mn, and Fe) and vitamins (B1, B2, B3, B6, B9, and E). Although biochemical studies have already been conducted to distinguish and characterize the different layers of wheat bran (Antoine et al., 2004), until recently this was not the case for the protein composition of the aleurone layer.

Developmental processes of peripheral layers have also been monitored by Tasleem-Tahir et al. (2011). More than 200 proteins of grain peripheral layers (inner pericarp, hyaline, testa, and aleurone layer) were identified and classified into 16 different functional categories. Study of the protein expression over time allowed identification of five main profiles and four distinct phases of development. Wheat grain peripheral layers at early stages are the site of metabolic activity, photosynthesis and all the metabolic pathways link to the reactive oxygen species (ROS) production and detoxification. Composite expression curves indicated that there was a shift from metabolic processes, translation, and transcription and ATP interconversion toward storage and defense processes. Protein synthesis, protein turnover, signal transduction, membrane transport, and biosynthesis of secondary metabolites were the mediating functions of this shift.

11.4 Functional roles of wheat proteins

11.4.1 Milling—the puroindoline story

The miller's goal is to separate the botanical parts of the wheat kernel—germ and bran from endosperm—and then to reduce the endosperm to flour-sized particles. This separation is accomplished by exploiting the differences in inherent material properties among

the various botanical parts. Bran tends to be leathery and resistant to breakage when hydrated, while germ tends to be more plastic and therefore deformable.

Milling properties are mostly determined by grain hardness, which is (with protein content) of greatest importance for wheat utilization (Osborne et al., 2007). The hardness trait was described by Greer and Hinton (1950) who proposed that soft wheats tend to fracture along the starch–protein interface. However in hard wheats, where there is greater adhesion at the starch–protein interface, fracture occurs preferentially along cell boundaries and even right through starch granules.

The level of starch damage in flour depends on the hardness of the grain from which it was milled. Starch damage refers to starch granules that have been physically altered from their native granular form during the milling process. As starch granules are loosely attached to the protein matrix, soft wheat crushes easily, producing largely intact starch granules and fine flour. On the other hand, the starch granules of hard wheat are tightly bound to the protein matrix, requiring greater milling energy and producing coarser flour with higher levels of starch damage.

Importantly, smaller particles hydrate more easily during dough preparation. Normally, starch granules absorb one-third their weight in water; but when damaged, that absorption increases to 2–3 times their weight. The level of starch damage therefore significantly affects Farinograph water absorption, as well as dough extensibility and resistance measured in the Alveograph. Damaged starch granules are very susceptible to attack by α-amylase enzymes; thus damage provides a further supply of sugars to the yeast during fermentation. On the other hand, too much starch damage can result in poor loaf volume, heavy texture, and excessively colored crust.

Standard methods are available for the measurement of wheat hardness. These include high-throughput methods such as near infra-red spectroscopy (NIRS) and the single-kernel characterization system (SKCS). Research on wheat-grain rheology aims to define and measure hardness in terms of mechanical properties. For example, Turnball and Rahman (2002) defined hardness as a measure of the resistance to deformation under applied stress. Hardness and vitreosity are separate traits linked by overlapping quality trait loci under genetic control at the 5D *Ha* locus (Turnbull and Rahman, 2002). The growth environment affects the expression of both hardness and vitreosity, but it does not change the ranking of cultivars with respect to these attributes. Modifying genes account for the range of hardness differences within hard or soft classes. Among these genes, the *pin* genes, coding for puroindoline proteins (introduced earlier in Section 12.2.5.3.) are the most important. It is the expression of these genes that is thought to be important in determining milling quality.

The most comprehensive review on wheat proteins published in recent years (Shewry et al., 2009) characterizes the discovery of the puroindolines and the GSPs as well as describing the elucidation of their roles in determining grain texture as one of the most fascinating stories in cereal chemistry. As was mentioned earlier in this chapter (Section 11.2.5.3), the puroindolines are encoded by genes at two loci on the short arm of chromosome 5D: *Pina-D1* (two alleles, *a* and *b*) and *Pinb-D1* (many alleles, *a-g*, *l*, *p*, *q*) (Morris, 2002; Jones et al., 2006; Wanjugi et al., 2008). Bread wheat generally contains two types of puroindolines differing slightly in size. Soft wheat possesses both puroindoline a and puroindoline b, due to the allele combination *Pina-D1a* and *Pinb-D1a*.

The whole "puroindoline story" reviewed by Shewry et al. (2009) consists of more than a hundred research publications, extending from the first publication of Greenwell and Schofield (1986) until the *in vivo* proof experiments introducing the *pin* gene into rice (Krishnamurthy and Giroux., 2001) and into wheat (Beecher et al., 2002; Hogg et al., 2005). The intervening papers describe the detailed analysis of the *Ha* locus, the allelic variation of puroindolines and their relationship to grain softness, the structure and lipid-binding action of puroindolines, finally, the mechanisms causing grain softness.

The puroindolines are basic lipid-binding proteins, rich in cysteine, of about 13 kDa (Gautier et al., 1994; Morris, 2002; Jones et al., 2006). They belong to the 2S albumin superfamily of proteins. Le Bihan et al. (1996) reported detailed studies of their secondary structures, using IR, Raman, and fluorescence spectroscopies. They showed that the secondary structure comprises about 30% α-helix, 30% β-sheet, and 40% unordered structure at pH 7 and that Pin a and Pin b had similar structures. Pin a also tended to aggregate under conditions of low pH and high ionic strength, and a tryptophan-rich domain appeared to be involved in this phenomenon. They proposed a secondary structure assignment with a protein fold similar to that found by Douliez et al. (2000a) in ns-LTPs. This comparison also showed that the tryptophan-rich sequence is present in an inserted region that is not present in the LTPs and that this region may form a loop stabilized by an additional disulfide bond (between Cys 28 and Cys 48 in Pin a, and between Cys 29 and Cys 48 in Pin b).

It has been known for some time that hardness is associated with decreased free polar lipids in wheat flour (Panozzo et al., 1993). The mechanistic explanation for this observation is not clear, but it may be related, either directly or indirectly, to the fact that Pins are able to bind polar lipids. Dubriel et al. (1997) showed that Pin a was able to bind tightly to both wheat phospholipids and glycolipids, while Pin b bound negatively charged phospholipids tightly but formed loose lipoprotein complexes with glycolipids. Pin a was also more efficient at preventing the destabilization of foams by wheat polar lipids, indicating that Pins may play a role in the formation and stabilization of foams in bread dough. The difference in the lipid-binding behavior of Pin a and Pin b is consistent with spectroscopic studies (fluorescence emission and near-UV CD), which showed that the tryptophan-rich loop region is involved in binding the zwitterionic lipids *n*-hexadecylphosphoglycol and hexadecyltrimethyl ammonium bromide (Kooijman et al., 1997). These studies together provide convincing evidence for a role of the tryptophan-rich loop in lipid binding by Pins *in vitro*. However, they do not prove that lipid binding occurs *in vivo* or, if so, that the mechanism is the same. It is also possible that the tryptophan-rich loop binds to the surface of the starch granules. If so, any *in vivo* lipid binding must be at a different site, perhaps into an internal pocket as in ns-LTPs (Shewry et al., 2009).

Jolly et al. (1993) reported the characterization of a 15 kDa protein that clearly differed from Pins in partial sequences determined for the N-terminus and several peptides released by enzymatic digestion. They also showed that the total amount of this protein (which they called GSP) was greater in soft cultivars and in soft near-isogenic lines. Furthermore, although GSP was present on the surface of starch granules from soft and hard cultivars, the amount was greater on the former.

Jolly et al. (1996) also showed that polymorphic variation of genes of a similar protein (GSP-1) isolated by Rahman et al. (1994) (*GSP-1*) co-segregated with *Ha* alleles in the progeny of a cross between hard and soft lines. This result appeared to establish a role for

GSP in determining grain texture. However, this has not been borne out by more recent studies. In particular, Tranquilli et al. (2002) compared the effects of different dosages of *GSP-1* genes on grain texture, using isogenic substitution and deletion lines of wheat cv. Chinese Spring. They showed that deletion or allelic variation in the *GSP-A1* and *GSP-B1* genes did not have any significant effect on grain texture. Furthermore, recent work by van den Bulck et al. (2002, 2005) has shown an interesting homology that may indicate that *GSP-1* has an entirely different function as the precursor for an arabinogalactan peptide (AGP).

The AGP of wheat has an MW of about 22 kDa and comprises a short peptide backbone with several arabinogalactan chains attached to hydroxyproline residues (Fincher et al., 1974). van den Bulck et al. (2002) showed that this peptide comprised 15 amino acid residues, including 3 hydroxyprolines. Furthermore, the amino acid sequence of the peptide is identical to residues 21–36 of the protein encoded by *GSP-1*, corresponding to a region starting at or close to the predicted N-terminus of the mature protein. Hence, the GSP-1 protein appears to be the precursor for the AGP of wheat. It is, of course, possible that the remaining part of the GSP-1 ultimately binds to the starch granule, but it is perhaps more likely that the GSP proteins reported by Jolly et al. (1993) and GSP-1 proteins cloned by Rahman et al. (1994) are related but have different functions. The protein encoded by the *Ha* locus clearly corresponds to GSP-1, so the genetic control of the other GSP components remains unknown. *GSP-1* homologues are also encoded by genes on chromosomes 5A and 5B of durum and bread wheats (Chantret et al., 2005) and are present in all members of the Triticeae so far studied, including barley, rye, and a range of *Aegilops* and *Triticum* species (reviewed by Shewry et al., 2009).

11.4.2 Functional roles of gluten proteins in baking properties

11.4.2.1 Dough and gluten formation; the uniqueness of wheat The fundamental basis of utilizing wheat flour as one of the most important food sources around the world is its unique property of forming dough and developing gluten when wheat flour is mixed with water. The usual chemical composition of untreated gluten formed during this process is ~75% protein, 6% fat, 15% carbohydrate, and 0.85% ash (Sarkki, 1980). The protein of wheat flour is not only "gluten protein," but it also includes 8–10% soluble proteins (albumins and globulins). Removal of gluten from bread formulations often results in a liquid batter, rather than a dough system during the pre-baking phase; the resulting baked bread has crumbling texture, poor color, and other quality defects (Gallagher et al., 2004). Indeed, gluten is the main structure-forming protein present in wheat dough, and plays a major role in bread-making functionality by providing visco-elasticity to the dough, good gas-holding properties, and good crumb structure for many baked products (Gallagher et al., 2004; Moore et al., 2004).

Wheat varieties at the same protein level have been found to differ in their bread-making quality, giving the first indication that "protein quality," as well as protein amount, is important for good bread-baking quality (Finney and Barmore, 1948). The gluten proteins contribute 80–85% of the total wheat protein and are the major storage proteins of wheat. They belong to the prolamin class of seed storage proteins (Shewry and Halford,

2002). Gluten proteins are largely insoluble in water or dilute salt solutions. Two functionally distinct groups of gluten proteins can be distinguished: monomeric gliadins and polymeric (extractable and unextractable) glutenins (Lindsay and Skerritt, 1999). Gliadins and glutenins are usually found in more or less equal amounts in wheat. Gluten has a unique amino acid structure, with Glu/Gln and Pro accounting for more than 50% of the amino acid residues (Eliasson and Larsson, 1993). The low water solubility of gluten is attributable to its low content of Lys, Arg, and Asp residues, which together amount to less than 10% of the total amino acid residues. About 30% of the amino acid residues in gluten are hydrophobic, and these residues contribute greatly to its ability to form protein aggregates by hydrophobic interactions and by binding lipids and other non-polar substances. The high contents of glutamine and hydroxyl amino acids (~10%) are responsible for its water-binding properties. In addition, hydrogen bonding between glutamine and hydroxyl residues of gluten polypeptides contributes to its cohesion–adhesion properties. Cysteine and cystine residues account for 2–3% of the total amino acid residues, and during the formation of dough, these residues undergo sulfhydryl–disulfide interchange reactions, resulting in extensive polymerization of gluten proteins.

The great extent of polymorphism of wheat prolamins results in a special effect in relation to the overall functional properties of wheat dough. During dough formation when prolamin proteins are hydrated to form the gluten network, the numerous structurally similar but slightly different proteins produce a mass in which several characteristics (such as size, polarity, charge distribution, solubility, and viscosity) show a continuous distribution over relatively large intervals. This structural feature provides a unique characteristic for gluten proteins that is unique and distinct from any other protein system.

It is generally accepted that the bread-making quality of wheat is related to the presence and properties of the two gluten protein classes. Many studies have attempted to relate wheat proteins to bread-making quality (Bushuk, 1985; Hoseney and Rogers, 1990; MacRitchie, 1992; Borneo and Khan, 1999; Toufeili et al., 1999; Uthayakumaran et al., 1999; Wooding et al., 1999; Khatkar et al., 2002a, 2002b). Gliadin proteins have little resistance to extension and are mainly responsible for the cohesiveness of dough, whereas glutenin proteins give dough resistance to extension (Dimler, 1965; Hoseney, 1992; Uthayakumaran et al., 2000c). The gliadin fraction has been reported to contribute to the viscous properties and dough extensibility of wheat dough (Don et al., 2003a, 2003b). The glutenin fraction of wheat gluten has long been considered to have a prominent role in the elasticity and strengthening of dough (MacRitchie, 1980, 1992; Wooding et al., 1999; Xu et al., 2007). The relative proportions of gliadin and glutenin found in dough affect the physical properties of dough, with higher relative proportions of glutenin imparting greater dough strength (MacRitchie, 1987). However, there is still debate as to the roles of the various protein classes on bread-making parameters such as water absorption, mixing, loaf volume, and crumb grain. For example, gliadin proteins have been reported to be highly related to loaf volume by many researchers (Hoseney et al., 1969; Finney et al., 1982; Branlard and Dardevet, 1985; Weegels et al., 1994; Fido et al., 1997; Khatkar et al., 2002a, 2002b). Others, however, have observed that gliadin proteins have an insignificant effect on loaf volume and that the glutenin proteins are the major components responsible for loaf volume (MacRitchie, 1978, 1985; MacRitchie et al., 1991;

Gupta et al., 1992; Borneo and Khan, 1999; Toufeili et al., 1999; Uthayakumaran et al., 1999, 2002b; Labuschagne et al., 2004).

Individual dough-property parameters describe only certain essential elements of dough properties. Depending on the final product, different levels of these attributes are required to contribute superior processing quality. For example, the balance of dough strength and extensibility are believed to be the most important factors governing the suitability of a flour to make good bread (Bushuk and Békés, 2002). However, for different types of breads, and even for different types of processing technologies, a diversity of dough strength and extensibility values may provide the optimum balances needed in each case (Oliver and Allen, 1992). For most traditional uses, wheat quality derives mainly from two interrelated characteristics: grain hardness and protein content with each end-use requiring a particular type of "protein quality." These aspects of quality are mainly determined by the protein molecular structure which, in turn, controls the interactions of the proteins during the bread-making process (Bushuk, 1998; Shewry et al., 1999).

11.4.2.2 Balances on different structural levels The complexity of relating protein composition to quality derives from the fact that the question can (and has to be) investigated on different levels of protein composition (Wrigley et al., 2006c) namely, protein content, the ratio of polymeric to monomeric protein, the ratio of HMW to LMW glutenin subunits (and which specific subunits are present), and the proportions of x- and y-type HMW glutenin subunits. These various parameters can be determined for a specific flour sample to see if there is a "good balance" between the various components in the sample, thereby to satisfy quality-related criteria. The polymeric glutenin is mostly responsible for the elasticity of the dough, whereas the monomeric gliadins are the extensibility-related characters in the system (Hoseney, 1986). Thus, the ratio of polymeric to monomeric proteins (the glutenin-to-gliadin ratio) can be directly related to the balance of dough strength and extensibility of the sample.

To investigate these relationships, there are important requirements for the experiments to be considered to be valid. Because dough properties are significantly dependent of protein content, the balance of glutenin-to-gliadin ratio can best be compared among samples with similar protein contents. Meanwhile, the composition of both the glutenin and gliadin proteins has to be taken into account because, for example, at the same glutenin-to-gliadin ratio, the balance of HMW-to-LMW glutenin subunits in the polymeric fraction can significantly alter dough strength and extensibility (Uthayakumaran et al., 1999). Clear examples have been reported to demonstrate the extremes in dough properties that result from drastic changes in HMW-to-LMW ratio. For example, dough strength systematically decreased, while extensibility increased, as a result of decreasing the HMW-to-LMW subunit ratio for sibling lines of an Olympic × Gabo cross (Lawrence et al., 1988), providing single-, double- and triple-null lines for HMW subunit-coding genes (Uthayakumaran et al., 2002a; Beasley et al., 2002). On the other hand, the increased number of copies of the genes coding for Dx5 subunits in transgenic wheat samples resulted in doughs so strong that it was not possible to mix them with traditional equipment (Rooke et al., 1999; Popineau et al., 2001). Further manipulation

of protein balance in dough can be achieved by systematically changing the ratio of x- to y-type HMW glutenin subunits (Butow et al., 2003a), whilst maintaining equivalent levels for protein content, glutenin-to-gliadin ratio and HMW-to-LMW subunit ratio. The presence and the relative levels of individual polypeptides can also be related to quality attributes. For example, the "imbalance" in glutenin-subunit composition caused by the over-expression of subunit Bx7 in certain wheat varieties around the world (Glenlea, Red River, Bankuti 1201, Chara, Kukri) provides extra dough strength and better overall bread-making quality, compared to samples with comparable protein content and glutenin-to-gliadin ratios (Butow et al., 2003a; Juhász et al., 2003b).

11.4.2.3 Polymer formation of the glutenin subunits: *in vitro* studies The molecular structure of individual glutenin polypeptides determines their different capabilities to form polymers. The application of special reconstitution techniques is needed for such investigations. For example, the "base flour method" involves systematically altering the composition of a flour by supplementing bulk protein isolates or individual polypeptides and using a reversible reduction–oxidation procedure to incorporate glutenin subunits into the partially reduced then re-oxidized polymeric glutenin during micro-mixing. This approach has revealed the structural features of the glutenin subunits that are important in polymer formation in terms of the extent to which dough properties are modified (Gras and Békés, 1996; Békés and Gras, 1999; Gras et al., 2001).

Like the effects of gliadin supplementation, the addition of monomeric glutenin subunits to a base flour would effectively reduce the average molecular weight of the protein in the composite flour. In such an *in vitro* experiment, the added HMW-GS would not be expected to display the effects to be found in an *in vivo* experiment because they would not form part of the extended disulfide-linked glutenin structure. Meaningful estimates of the effects of added glutenin subunits on dough properties can be made only if they can be chemically incorporated into the glutenin polymer, as they would be in an *in vivo* experiment.

Studies of the effects of a range of reductants and oxidants on the functionality of gluten proteins during dough mixing showed that it was possible to effectively destroy dough functionality with a reductant, and then to recover it by subsequent oxidation (Békés et al., 1994b). Although several reductants were tested, dithiothreitol was found to be the only one whose action on dough mixing properties could be readily reversed. Careful selection of the oxidant, its concentration, and reaction conditions allowed essentially complete recovery of the original dough-mixing properties. For the oxidation step, bromate was the oxidant of choice, performing better than iodate, permanganate, or hydrogen peroxide. The parameters of this reduction–oxidation procedure needed to be optimized to ensure that subsequently there would be less than 5% difference in mixing time, peak dough resistance and dough stability (resistance breakdown) between a treated and untreated dough. Under these conditions, no significant difference in the size distribution of the proteins isolated from the two samples could be detected (Békés et al., 1994b).

The application of the reduction/oxidation procedure to doughs produced for extensibility measurement required different conditions compared to those for mixing studies,

presumably because of continuing slow oxidation of dough components during the long relaxation required before stretching. The differences may offer further insight into the nature of the changes taking place between mixing and extension testing. Formulation and the reduction/oxidation conditions also had to be modified if the resulting dough was to be baked, because of the toxicity of dithiothreitol to yeast. Nevertheless, it has been possible to develop protocols in which functionality is maintained (Uthayakumaran et al., 2000a, 2000b; Gras et al., 1997).

The reduction/oxidation procedure, with its mild reducing conditions, has been shown not to alter the mixing properties of mixtures made from flour and isolated gliadins (Murray, 1999), indicating that the presence of intra-molecular disulfide bonds in gliadins do not seem to interfere with the reduction/oxidation of glutenin.

This reduction/oxidation procedure has since been applied to the incorporation of a wide range of partially purified fractions or individual purified HMW (Sapirstein and Fu, 1996; Veraverbeke et al., 1999) and LMW (Sissons et al., 1997; Lee et al., 1999a) glutenin subunits into the polymeric phase that we refer to as "native glutenin" as reviewed by Békés and Gras (1999) and by Anderson and Békés (2011). Most of these experiments have been carried out with bacterially expressed wheat polypeptides (reviewed by Tamás and Shewry, 2006). This powerful technique has been applied to genetically modified glutenin genes that have produced altered sets of novel mutants of Dx5 glutenin subunits (Buonocore et al., 1997; Anderson et al., 1996a, 1996b; Anderson and Békés, 2011) and mutants of an ω-gliadin analogue C-hordein (Tamás et al., 1997, 2002; Greenfield et al., 1997) with different lengths of the central repetitive domain and with different numbers and locations of cysteines in the proteins.

The results show a significant positive correlation between subunit size and both mixing time and peak resistance, as well as a significant negative relationship of subunit size with resistance breakdown (Békés et al., 1994a). Data obtained by mixing studies of naturally occurring glutenin polypeptides of very similar size but differing in their cysteine contents indicate that protein functionality is related not only to the size of the subunits, but also to the number and availability of cysteine groups, through which the inter-molecular disulfide bonds are formed. Incorporation of x–y pairs of subunits showed responses much higher than expected from their calculated average molecular weights (Figure 11.4). This synergistic effect was at a maximum when equimolar ratios of either subunits 5 and 10 or subunits 2 and 12 were used, indicating a special interaction between the x- and y-type 1D subunits. Under the same experimental conditions, subunits coded by the genes on chromosomes 1A and 1B showed the same relationships between mixing properties and the size of subunits as those coded by the 1D chromosome. However, synergistic effects between x- and y-type subunits were only observed for subunits coded by chromosome 1B when flours of lines null for *Glu-D1* and *Glu-B1* were used as the base flour (Uthayakumaran et al., 2000b).

The limitation of the "base flour" method is that the extent of incorporation of the polypeptides depends on the base flour used. The resulting changes in rheological properties of the wheat dough are partly dependent on the direct contribution of the incorporated protein and partly on the protein's interaction with other prolamin-type proteins present in the base flour. A novel approach, introducing incorporation into rice flour

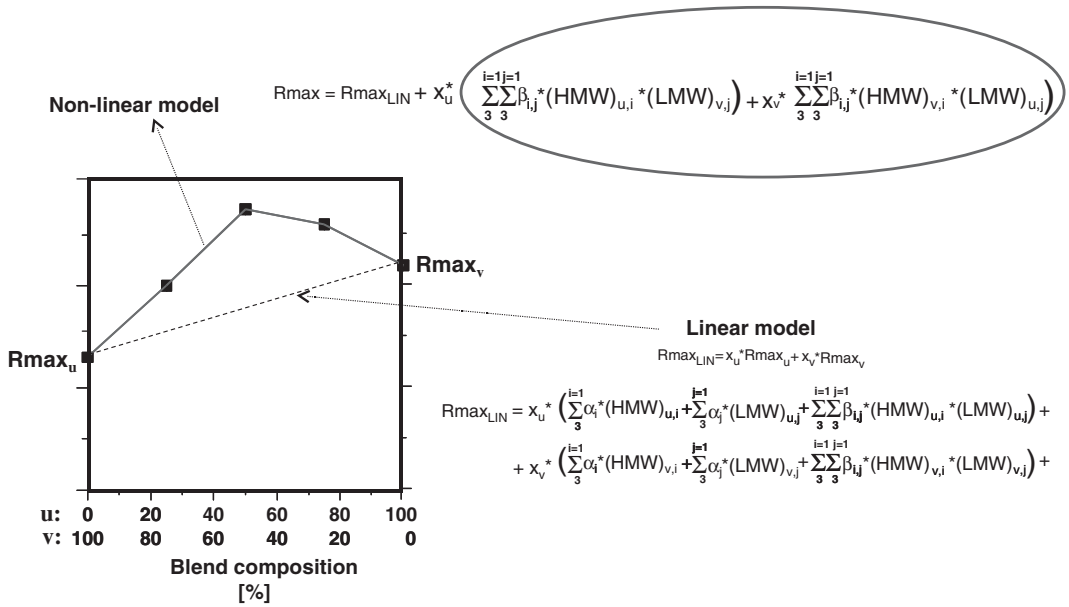

Figure 11.4 Linear and non-linear Protein Scoring System (PSS) model prediction equations describing dough strength measured as Rmax for a two component blend, (containing flours **u** and **v**) as the function of blend composition. The difference between the linear and non-linear model (circled) contain only expressions describing interactions of alleles of flour **u** with those of flour **v**. Adapted from Békés (2011)

dough can now clearly monitor only the direct effects of individual polypeptides on mixing properties while the same experiments using wheat flour doughs result in direct interactive effects (Oszvald et al., 2011). Rice flour does not contain any wheat prolamin-type proteins; therefore in principle it can provide a new way to investigate the functional properties of wheat proteins. By incorporating these proteins into rice flour dough, the direct effect of the proteins on the functional properties of the rice dough can be investigated in the absence of any interactions with the other prolamin-type proteins.

A reduction–oxidation procedure has been developed, optimized for rice flour, which is suitable for incorporating purified wheat protein fractions into rice dough and monitoring their effects on the mixing properties of rice dough. It was also confirmed that dough with reasonable strength and stability can be made from rice flour supplemented by wheat gluten (Oszvald et al., 2008a, 2008b). Initial results of incorporating different wheat storage proteins in different proportions into both rice and wheat flour doughs demonstrated the potential of using rice flour as a model system to characterize the functional properties of wheat storage proteins (Oszvald et al., 2011).

11.4.2.4 The role of size distribution of polymeric glutenin The hydrated network in developed dough contributes to its rheological properties. Dough strength (Rmax) and extensibility (Ext) are determined by the protein composition and are thus under genetic control. Knowledge of the relationship between protein composition and Extensograph

parameters can therefore be utilized to solve processing problems as well as to form a sound basis for manipulating dough properties in breeding programs.

Orth and Bushuk (1972) made the pioneering finding that dough strength is related not to the total amount of polymeric glutenin proteins but especially to a certain fraction with different extractability. This finding led firstly to a realization of the importance of the size distribution of polymeric proteins in relation to functional properties and then to different prediction procedures.

In a study of factors governing flour properties, it was found by Gupta et al. (1992) that Rmax correlated best with the percentage of polymeric protein in the total protein (PPP), whereas Ext showed an even higher correlation with the percentage of polymeric protein in the flour (FPP). A subsequent study (Gupta et al., 1993) showed that for certain cultivar sets, Rmax did not always correlate well with PPP but correlated with the percentage of polymeric protein unextractable by SDS-buffer solution (UPP). The UPP was taken to be a simple relative measure of MWD based on the well-known inverse relationship between solubility and molecular size.

Several similar methods like UPP have been developed to quantify the large polymeric proteins in wheat flour. The procedure of Sapirstein and Fu (1998) is based on the same principles but using spectrophotometric measurements instead of HPLC separation, while Bean et al. (1998) used nitrogen combustion analysis to determine residue protein, followed by repeated extraction with 50% 1-propanol (1 mL) for 5 min. Measurement of the molecular size distribution of polymeric glutenin and its relevance to dough quality is reviewed by Southan and MacRitchie (1999), who include related methodologies, from the simplest (multi-stacking SDS electrophoresis) (Khan and Huckle, 1992) to the most sophisticated (field-flow fractionation, FFF) (Stevenson and Preston, 1996; Daqiq et al., 2007).

The results of UPP analysis can be interpreted to mean that only molecules above a critical molecular size contribute significantly to dough strength. This critical value may correspond to the critical size for effective entanglements, a concept well established in polymer science for relating physical properties of polymers (Ferry, 1961). Evidence for the importance of the larger-sized glutenin polymers in explaining dough strength has been presented in the work of Popineau et al. (1994), in studies by Weegels et al. (1996) on what they term the GMP, and in numerous publications relating dough strength and product quality to glutenin size distribution measurements (Park et al., 2006; Dowell et al., 2008; Tsilo et al., 2010; Ohm et al., 2010).

While the estimation of dough strength is achievable using these procedures in practical applications, the predictability of extensibility seems to be a significantly more problematic task. The best estimate for this quality trait is related to the amount of polymeric protein in the flour (percentage of glutenin multiplied by the protein content of the flour) but this simple relationship does not have the capability to successfully predict extensibility in practical terms. Bangur et al. (1997) aimed at investigating whether there are critical molecular weight cut-off points governing Extensograph parameters. For Rmax, a maximum correlation was obtained for estimated molecular weights greater than 250 kDa, while the highest correlation for extensibility was found for proteins larger than 52 kDa. The different dependencies of Rmax and extensibility on protein composition provide

us with the capacity to manipulate these parameters in predictable ways by selecting for appropriate composition in breeding lines, thus opening the way for the production of cultivars with properties that meet specific requirements.

11.4.2.5 The dual film hypothesis In the light of the recent results of Sroan et al. (2009) and Sroan and MacRitchie (2009) finally we have an observation-based hypothesis about the complex nature of wheat dough and the gas cells in it.

The stability of the gluten–starch matrix, which is the primary factor to stabilize expanding gas cells against disproportionation and coalescence, depends on its tendency to strain harden. The phenomenon of strain hardening appears to depend on the balance between strength and the extensibility of the entangled network of polymeric proteins. Extensibility ensures slippage of the maximum number of statistical segments between entanglements (Singh and MacRitchie, 2001), whereas strength prevents disruption of the entangled network of polymeric proteins. Thus, to ensure the stability of gas cells, the dough needs to be sufficiently extensible to respond to gas pressure but also strong enough to resist collapse. Differences in gluten quality, as demonstrated in this study can significantly affect bread-making potential. Strength is conferred by the fraction of polymeric proteins having a molecular weight greater than 250kDa whereas the fraction of gluten protein smaller then 250kDa may counter the strength by acting as diluents. The optimum balance seems to exist when the relative proportions of polymeric proteins greater and smaller than 250kDa are roughly 60:40, respectively. Shift in the balance to either side will decrease loaf volume. Increase in smaller proteins (less than the 250kDa) may decrease the stability of the gluten-starch matrix due to a lesser number of entanglements per chain. On the other hand, an increase in strength-conferring proteins may prevent sufficient expansion of the gluten–starch matrix required to increase loaf volume due to reduced slippage of gluten polymers through entanglement nodes as a result of an increase in the number of entanglements per chain. The secondary stabilizing mechanism involves thin liquid lamellae stabilized by adsorbed surface-active compounds (lipids and proteins) at the gas–liquid interface. Liquid lamellae prevent coalescence and disproportionation of gas cells when they come in close contact with each other during the late proving and early baking stages of bread making, that is, when discontinuities begin to appear in the gluten–starch matrix. Flour lipids at their natural levels do not influence the rheological properties of the gluten–starch matrix surrounding the gas cells, as measured by the dough inflation system. Nevertheless, the small amounts in which these lipids are naturally present are sufficient to influence surface properties.

To study this secondary stabilizing mechanism, different lipid fractions have been added incrementally to defatted flours. No effects were observed on the rheological properties of the dough. However, large effects on loaf volume were measured. Polar lipids had positive effects on loaf volume, while non-polar lipid, linoleic and myristic acids had negative effects. The different effects of the lipid fractions were thought to be related to the type of monolayer that is formed. Polar lipid and palmitic acid form condensed monolayers at the air–water interface whereas non-polar lipid, linoleic and myristic acids form expanded monolayers. This study for the first time demonstrates that the lipids at their natural levels do not affect dough rheology and gas cell stabilization by the gluten–starch matrix.

Nevertheless, their ability to modify loaf volume and crumb structure supports the dual film hypothesis of Gan et al. (1990, 1995). It suggests the presence of liquid lamellae, providing an independent mechanism of gas cell stabilization. The effects of different surface-active components may be explained by the type of monolayer that they form.

11.4.2.6 Beyond the gluten proteins As is obvious from the dual film hypothesis, bread-making quality cannot be described by considering only the gluten proteins. Based on a large statistical analysis carried out by Gupta et al. (1994), the gluten proteins are responsible for around 70–72% of the variation of bread-making quality; the remaining more than a quarter of the variation is related to certain soluble proteins, lipids, starch and non-starch polysaccharides. In relation to the role of lipids and non-starch carbohydrates, excellent reviews have been published recently by Pareyt et al. (2011) and Wang (2003), respectively, while progress in improving our understanding of starch-related quality research is reviewed by Wrigley et al. (2009) and Park et al. (2009).

11.4.2.7 Predicting dough properties from wheat protein composition Relating protein composition to certain quality traits by statistical means is a frequently used methodology to relate structure composition to functionality in cereal science. The classic tool used by breeders is the Payne score (Payne, 1987; Payne et al., 1987)

$$Q = \sum_{13}^{i=1} \alpha_i^* (q_H)_i \qquad (1)$$

where **Q** is the Payne score, estimating the dough strength of the sample; $\boldsymbol{\alpha_i}$ the contribution of individual HMW-GS alleles while the value of $\boldsymbol{(q_H)_i}$ is either one or zero depending on whether the actual allele is present or not.

Since the significant success of using the Payne score in breeding programs as a single number to estimate dough strength from the HMW-glutenin allelic composition, there have been several attempts in addition to involve the LMW-glutenin alleles in similar mathematical formulas (Gupta et al., 1991; Cornish et al., 2006; Békés et al., 2006; Eagles et al., 2002). By the application of sophisticated statistical approaches, the Wheat Simulator of Eagles et al. (2002), and the Protein Scoring System (PSS) of Békés et al. (2006) are capable of describing the effects of both HMW- and LMW-GS alleles on dough strength and extensibility, both individually and considering pair-wise interactions among the alleles. In the latter case, developing an incident matrix from the allelic composition of the samples, a complex multiple regression type of statistical analysis has been carried out providing factors to describe the contribution of each of the HMW- and LMW-glutenin alleles and their pair-wise interactions with dough strength and extensibility. The PSS model has two different types of applications. Applications of the models indicate that the approach of relating allelic composition to quality attributes is possible with careful data selection and applying robust mathematical tools. Secondly, the genetic potential of a line, with a certain combination of alleles on the six glutenin coding loci, can meaningfully

be predicted where both the contribution of the individual alleles and their pair-wise interactions play equally important roles (Békés et al., 2006; Cornish et al., 2006; Branlard et al., 2003b; Baracskai et al., 2011)

$$Q = \sum_{17}^{i=1} \alpha_i^* (q_H)_i + \sum_{16}^{j=1} \alpha_i^* (q_L)_j + \sum_{17}^{i=1} \sum_{16}^{j=1} \beta_{i,j}^* (q_H)_{j*} (q_L)_j \quad (2)$$

where $\boldsymbol{\alpha_i}$ and $\boldsymbol{\beta_i}$ describe the individual and interactive contributions of the alleles to **Q** quality parameters (dough strength or extensibility), while $\mathbf{(q_H)}_i$ and $\mathbf{(q_L)}_i$ indicate the presence of a certain HMW- or LMW-glutenin allele. (Only 3 $\mathbf{(q_H)}_i$ and 3 $\mathbf{(q_L)}_i$ values are equal to 1 in an ordinary wheat sample, representing the *Glu-1* and *Glu-3* gene products on the A, B, and D loci.)

The coefficients for the different alleles and allele–allele interactions provide a basis to select parents for crossing with the aim of a specific dough strength–extensibility combination. One of the most important conceptual findings from evaluating such models is the realization that, because of the large contribution of allele–allele interactions, the different allelic combinations (rather than the individual glutenin alleles) should be targeted in breeding situations to develop new lines with certain quality attributes (Békés et al., 2006).

The effects of allele–allele interactions recently became independently measurable by applying the incorporation methodology described above in Section 11.4.2.3 using wheat or rice as the base flour. The effects of the individual alleles are best determined using rice flour, while the wheat flour measurement indicates the superposed individual and interactive effects (Oszvald et al., 2011).

The original version of the PSS model is capable of predicting the genetic potentials for dough strength and extensional properties for dough from a flour with 12% protein content and with the ratios of glutenin-to-gliadin and HMW-to-LMW-GS of 1.0 and 0.2, respectively. The further version of the model (Békés et al., 2006) is capable of considering the effects of the expression levels of the different storage protein genes, so that the actual dough parameters can be predicted. The input of this model is the allelic composition and the quantitative protein composition (including UPP%), while the output provides a good estimate of the actual dough strength and extensibility of a given sample ($r^2 > 0.85$ and $r^2 > 0.75$, respectively).

Further applications of the models are also useful in blending mill streams and in predicting the bread-making potential of flour samples. The interactive effects of the alleles present in commercial flour blends are responsible for a well-known industrial problem, namely, dough properties (dough strength and extensibility) are not simply additive characteristics. They usually show non-linear relationships with blend formulation (Békés et al., 1998). Applying equation (2) to describe the dough strength (Rmax) of a blend with two (**u** and **v**) components (Figure 11.4), it is clear that the difference between the non-linear and linear models contains only interactive elements; non-linearity is the function of the interactions of alleles of component **u** with those of component **v**. If the allelic

composition of the components as well as the α_i and $\beta_{i,j}$ parameters are known, the quality attributes of blends can be estimated, providing an efficient way to use samples in stock (Békés et al., 1998), and even to develop non-linear optimization models for blend formulation (Békés et al., 2002; Békés, 2011).

Recent research has sought high-throughput methodologies to provide quantitative information about protein composition for these predictions. MALDI-TOF and PCR-based analyses of wheat proteins (Liu et al., 2009) provide the required sensitivity and reproducibility for application in basic and applied research for these predictions.

The genetic makeup up the gluten proteins, together with information about the allelic composition of glutenin and gliadin alleles, has thus become essential information in wheat breeding. Large databases containing wheat varieties grown around the world are available to search for certain combinations of alleles holding specific quality attributes (Metakovsky et al., 2006; Wrigley et al., 2006a, 2006b).

11.4.2.8 Predicting dough properties from wheat protein composition: water absorption The above models however are only suitable for the estimation of dough properties. Other parameters, such as water absorption, cannot be predicted on the basis of gluten proteins alone. Water absorption—the amount of water needed to hydrate flour components to produce a flour with optimum consistency—can be described as functions of protein content, the amounts of pentosans, and the level of starch damage in the sample (Bushuk and Békés, 2002). Experiments carried out with flours supplemented with different protein classes resulted in the observation that while mixing requirement, dough strength and extensibility significantly depend on the glutenin-to-gliadin ratio, water absorption is not sensitive to this ratio (Uthayakumaran et al., 1999). However, the ratio of the amount of gluten proteins to soluble proteins can significantly affect water absorption (Tömösközi et al., 2002). Supplementing wheat flour with soluble proteins of different origin and polarity has shown that polarity and hydrophobicity, as well as the charge distribution of albumins and globulins, are the key features changing the amount of water needed for hydration (Tömösközi et al., 2004).

A significantly high positive correlation ($r^2 > 0.8$) was obtained for 63 straight-run flours between water absorption and damaged starch content compared to a value of $r^2 = 0.5$ for protein content alone (Tara et al., 1972). Predicting equations using multiple linear models with protein content and starch damage have been developed and it was found that more than 90% of the variation in water absorption is covered by these two parameters. It was also concluded that the remaining variation is mostly related to the pentosan content of the flours. The relationships between pentosan composition and water absorption indicate that arabinoxylans play the major role (the largest effect being caused by the soluble, small- to medium-sized arabinoxylans), while the large polymers do not have marked effects on water absorption (Primo-Martin and Martinez-Anaya, 2003). So, the compositional data needed to estimate water absorption are the composition of the water-soluble components of wheat flour, qualitative and quantitative aspects of albumin/globulin content, as well as pentosan composition together with the overall protein content, and the level of starch damage (produced by the milling procedure).

11.5 Gluten as an industrial commodity

11.5.1 Gluten isolation—now a big industry

Recognition that the gluten complex is the basis of wheat's unique bread-making ability leads to the obvious next step: isolation of gluten for trade in its own right. The process of gluten isolation is simple, as was originally illustrated by Beccari centuries ago (Bailey, 1941). The process is so simple that it can readily be carried out in the kitchen or classroom (Wrigley, 2012). In the past half century, Beccari's method has been developed into an efficient commercial process. As a commodity, gluten may be defined as the "cohesive, visco-elastic proteinaceous material prepared as a by-product of the isolation of starch from wheat flour" (Maningat et al., 1994; Day et al., 2006). At times when the price of gluten is high, starch may be regarded as the by-product. In its dried state, gluten is traded as "Vital Wheat Gluten," which can be regenerated into functional gluten by adding water for use in a wide range of food and non-food products. For the food industry especially, gluten is today a significant ingredient that is an important item of world trade (Krishnakumar and Gordon, 1995; Boland et al., 2005).

11.5.2 The term "gluten"

The word "gluten" can be erroneously used to indicate the protein of grains other than wheat, especially the protein from corn (maize) after washing out its starch (Patil, 2003, 2004). In this case, the distinguishing term "corn gluten" should be used, especially because corn gluten does not have the properties of vital wheat gluten. Another source of confusion for the term "gluten" relates to its role (sometimes a presumed role) in causing dietary problems. For people with celiac disease (Feighery, 1999; Murray, 1999), the offending "gluten" relates not only to the storage protein of wheat, but also that of rye, triticale, barley, and possibly oats (Kasarda, 2001). For these people, and probably for others with less well-defined intolerances, the phrase "gluten-free foods" refers to food products with less than 20 mg/kg of protein from these cereal grains (http://www.codexalimentarius.org/).

11.5.3 The history of gluten as a commodity

The first commercial use of gluten washing was designed to produce only the starch component of dough. In1840, Isaac Reckitt (in Hull, England) made "a mash of the wheat after soaking in water for many days, and when soft enough, putting the mash through sieves, the wheat starch passing through, leaving the bran in the sieves to be cast aside for pigs" (Anon, 1912, Wrigley, 2002a). Reckitt's Hull site has continued as an operation center for the multinational Reckitt-Benckiser.

The potential value of the gluten fraction of dough was appreciated and developed commercially in separate enterprises in North America and in New Zealand–Australia (Dubois, 1996; Wrigley, 2000). In the early 1900s, the Jenks family in Lake Huron,

Michigan, washed dough to produce starch, which was sold for laundry use to stiffen clothes after ironing. In the 1920s, they heat dried the gluten residue, using this "gum gluten" to make low-carbohydrate bread for diabetics and for Kelloggs to produce the breakfast cereal "Special K" (Thompson and Raymer, 1958). The crude gluten fraction was also subjected to acid hydrolysis to make monosodium glutamate. In the late 1950s, the gluten-drying method was altered to the "new type of drying process used in Australia" (quoted from Dubois, 1996), based on the "ring" or "flash" drying process. The gluten dried by this process retained its functional properties, being readily sought by bakers to improve loaf volume and crumb texture.

In parallel with these developments, a New Zealand pastry cook, Harry Maltwood Williams, found in the 1920s that bread of increased volume and improved texture was produced by augmenting his dough with gluten extracted from flour using fermentation and water washing (Wrigley, 2000). He patented the gluten-enriching process worldwide, using the trademark "Procera" for the resulting bread. He exploited the patent in New Zealand, Australia, and later Britain by charging the baker a royalty of 0.1 penny per loaf. By 1932, the marketing package involved the use of a band of paper to go around each loaf of "Procera" bread, thereby operating as a franchising company. However, the protein-enriching Procera method was still confined to individual bakeries, which were left with the problem of locally disposing of the starch-rich effluent. Subsequently, the Procera method was converted into an industrial-scale process in which dehydrated gluten was produced for the bakers and the washed-out starch was dried for food use (Wrigley, 2000). Thus began the Australian starch–gluten industry of Fielders Mills, based in 1938 in Tamworth, NSW. A significant development was the "ring" or "flash" drier in which wet gluten pieces were rolled in dry gluten and subjected to heat drying (Knight, 1965; Batey, 2004). From its beginnings for the bread industry, a significant export industry of dried gluten commenced for Australia, later expanding to a worldwide industry with dried gluten as a major trade commodity.

11.5.4 Industrial production of dry gluten

Gluten is mainly produced on a commercial scale in Australia, Europe, and North America (Table 11.1). The main commercial procedures are the batter process (largely

Table 11.1 Production of gluten and wheat starch worldwide

Year	Gluten (in thousands of tons)	Starch (in thousands of tons)
1993	404	2120
1996	450	2360
2003	480	2520
2004	594	3120
2005	750	3940
2006	808	4240
2007	890	4670

Source: Adapted from BeMiller and Whistler (2009).

batch wise) and the Martin process (largely continuous) (Maningat et al., 1994; Batey, 2004). Using the latter method, a dough is formed and it is water washed while passing through a cylindrical agitator. Starch passes through small holes in the cylinder while the gluten strands are retained. For the batter process, flour is mixed with water to form a thick suspension rather than a dough. As this flour–water batter is stirred slowly for a few hours, the starch separates from the gluten protein and settles to pass down through a sieve. Further water washing is needed to optimize the starch–gluten separation. In related versions, centrifuges and hydrocyclones are used to accelerate the process. Methods have also been developed using whole grain as a starting material, thereby avoiding the expensive preliminary step of dry milling grain to flour. Nevertheless, most gluten–starch processing involves flour as the starting material (Batey, 2004; van der Borght et al., 2005; BeMiller and Whistler, 2009).

In some situations, it may be possible to provide the wet gluten (~70% moisture) directly for further utilization, but generally it is dried for convenience and for food safety during transport and storage. As mentioned previously in relation to gluten's history, the drying step is critical as functional properties can easily be heat damaged. On the other hand, some applications of gluten require its properties of insolubility and water-binding capacity but not its visco-elasticity. For this "devitalized gluten," drum drying and other severe heat treatments may be used.

Just as the early days of starch–gluten washing were dogged by effluent problems, such issues still create problems, even though efforts are made to recover all fractions for further use. All forms of the process involve relatively large volumes of water; consequently all fractions are very wet and the effluent contains considerable amounts of water-soluble materials (especially proteins, damaged starch, sugars, pentosans, and fiber) in solution and in fine suspension. Effluent disposal may involve drying for industrial and feed uses, fermentation to reduce the amount of solutes, or (least desirable) discharge of effluent into the sewerage system, depending on local regulations and costs.

Inevitably, some of these non-protein materials are also captured within the gluten matrix. As a result, commercial dry gluten is generally only about 75% protein, depending on the procedure used to determine protein content. For wheat grain and flour, the factor of 5.7 is conventionally used as a factor to convert nitrogen content to protein content, compared to the factor of 6.2 used for proteins generally. This difference is because of the unusually high level in the gluten proteins of nitrogen-rich amino acids such as glutamine. The remaining composition of gluten consists of moisture (about 8%), small starch granules, lipids, and fiber.

11.5.5 Quality control for isolated gluten

The major use of vital dry gluten is to augment the baking quality of flour, especially low-protein flour, for conventional bread production. Thus, the buyer needs to be able to test for relevant aspects of gluten quality. Desirable color is cream; excess heat is likely to cause darkening. A quantitative estimate of color is usually obtained with a meter such as the Minolta Color Analyzer, particularly L values (darkness, 0 = black, 100 = white) and b values (yellowness (+), blueness (–)).

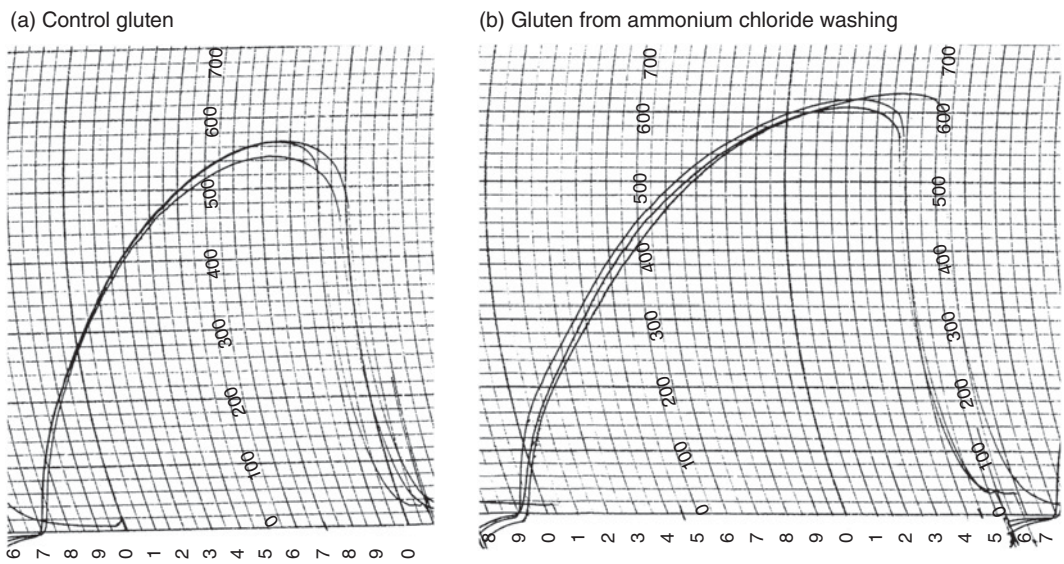

Figure 11.5 Extensograph curves (in triplicate, superimposed) comparing gluten washed out in (a) water only and (b) 0.5% ammonium chloride solution, both from the same flour. Adapted from Day et al. (2009)

Odor is another quality attribute; any rancid odor is obviously undesirable.

Good strength and extensibility is needed in the gluten for bakery use and several other applications. Equipment used for dough testing is often poorly suited to testing the rheology of wet gluten due to its strength being greater than normal dough. To provide a more normal test system, gluten can be mixed with wheat starch for dough testing often in the proportion of one part of dry gluten to six or so parts of starch (Day et al., 2009). Figure 11.5 shows mixing curves obtained with the Extensograph. Dry gluten (40 g) was mixed with wheat starch (260 g) in the mixing bowl of the Farinograph. Water (up to about 200 mL) was then added with mixing to give a mean dough viscosity of 400±20 Brabender Units at the end of 5 minutes. The resulting dough was molded into 150 g pieces, rested for 45 minutes and stretched in the Extensograph. The heights of the curves in Figure 11.5 indicate the strength of the gluten just before rupture; the lengths of the curve at the base indicate the extensibility contributed by the gluten samples.

The lipid content of the gluten is a further quality issue. Flour lipid is strongly bound to the protein following gluten washing, and its presence tends to reduce gluten quality, causing darkening of the gluten, a rancid odor, and reduced rheological quality. The bound lipid also interferes with ongoing processing for some uses in foods.

11.5.6 Gluten modification

11.5.6.1 Improving gluten's attributes Improvement in the above qualities of gluten may be obtained by modifying the initial washing procedure. For these purposes, there has been a traditional industrial "secret"—to add sodium chloride at about 2% to the water

used for dough make-up and for washing; the resulting gluten has thus been stronger with a lower lipid content. Day et al. (2009) reported that similar advantages could be obtained with ammonium chloride as the salt at a concentration of only 0.5%. One advantage of this substitution is that this salt is volatile; thus it is readily removed during gluten drying. Further advantages included lower lipid content for the resulting gluten, lighter gluten color, better gluten yield and improved rheological properties for the gluten (Figure 11.5). These improvements were most marked for low-grade flour streams, such as the "H/J" stream, with a high proportion of non-endosperm material and high ash content, this stream being otherwise most undesirable for starch–gluten production.

11.5.6.2 Modifying gluten's attributes Gluten is cheaper than other food proteins, although costs vary with availability. Alternatives include soy protein isolate, sodium caseinate, and whey protein, at about 2×, 3×, and 5× the cost of gluten, respectively (Day et al., 2006). Gluten's natural attributes prevent it from substituting directly for these alternatives, but such substitution would be possible by modifying gluten. The main modification applied to gluten is solubilization. Gluten is soluble, or at least dispersible, in a variety of solvents including urea solutions, mild acid, soaps and detergents, strong alkali, 70% ethanol, and 2-chloroethanol. Many of these are incompatible with food products, but for non-food purposes, there are few limitations other than cost, safety, and environmental concerns. Solubilization by chemical deamidation (with acid or alkali) is the major method of modification currently applied (Batey and Gras, 1981). Removal of the amide group of glutamine, for example, increases its solubility, but acidic deamidation risks peptide hydrolysis. This risk is less in alkali solutions, but alkali may attack the disulfide bonds of the critical amino acid cystine, with the formation of lysinoalanine (Batey and Gras, 1981). Nevertheless, the deamidation of gluten provides easy dispersal, for emulsification or foam stabilization. It has been used in meat products, non-dairy coffee whiteners, beverages, and milk puddings. Acid treatment has been reported to greatly increase the water-binding capacity of gluten (Maningat et al., 1994). Enzymic rupture of peptide bonds can solubilize gluten using commercially available proteases such as papain, bromelain, subtilisin, trypsin, and pronase, thereby reducing the HMW of the gluten molecules. However, small peptides may also be produced, giving a bitter taste. Intentional heat treatment, to form "devital gluten," produces a product that lacks visco-elasticity, but retains the water-binding character of gluten. Alternatively, extrusion technology produces gluten with a fibrous structure that simulates meat fibers (Maningat et al., 1994, 1999). High-pressure treatments have been applied to create various modifications, depending on the degree of pressure (Day et al., 2006). Gluten has proved to be a suitable material for casting into thin films, generally by using solubilization in ethanol followed by irradiation. The resulting films have good tensile strength (Rhim et al., 1999).

11.5.7 Applications of gluten in foods

11.5.7.1 Bakery uses Bread manufacture is the major commercial use of vital gluten (Table 11.2). Its unique visco-elastic properties improve dough strength, mixing tolerance, and handling properties. Its film-forming ability provides gas retention and controlled

Table 11.2 Uses of gluten in different regions (as percentages of total usage for each region)

Uses	North America	European Union	Australia	Japan
Baking	68	45	84	30
Flour fortification	11	41		
Pet foods	13	8	6	
Meat as binder			5	
Breakfast cereals	2	1	2	
Meat/fish analogues	1			25
Animal feed		1		
Aquaculture feed			1	
Noodles				10
Sausages				12
Other uses	5	4	2	23

Source: Derived from Maningat et al. (1994).

expansion for improved volume, uniformity, and texture; its thermosetting properties contribute necessary structural rigidity and bite characteristics; and its water absorption capacity improves baked product yield, softness, and shelf-life.

The gluten–flour proportions for bakery applications vary widely, depending on the required texture and shelf-life of bakery products (Maningat et al., 1994). For example, addition of about 1% of gluten to flour reduces pretzel breakage in the finished product, but the addition of too much gluten may produce pretzels that are too hard to eat. Gluten is used at ~2% in pre-sliced hamburger and hot-dog buns to improve the strength of the hinge and provide desirable crust characteristics when buns are stored in a steamer. It can also be used to strengthen pizza crust, making it possible to produce both thin and thick crusts from the same flour. The incorporation of gluten provides crust body and chewiness, and reduces moisture transfer from the sauce to the crust.

11.5.7.2 Uses beyond the bakery Outside the bakery, gluten also serves as a water-binding agent or protein enhancer for food products, such as breakfast cereals, meats, cheese, snack foods, and texturized meat analoges (Day et al., 2006). Table 11.2 illustrates the diversity of these further uses of gluten preparations in foods. However, the extent of these uses poses a potential problem for those who need to pursue a gluten-free diet as gluten (native or modified) may be used in food products that would not traditionally have posed a problem in this case. Helpful to gluten-sensitive individuals are lists of foods that may be gluten containing (Wrigley, 2004). Food-labeling laws provide further assistance in identifying such products (Jones and Russell, 2004).

11.5.8 Non-food uses of gluten

The list of possible uses of gluten in non-food situations provided by Bietz and Lookhart (1996) includes those already in routine use as well as others in the research phase. The adhesive properties of gluten make it suitable for many industrial products, including

pressure-sensitive medical bandages and adhesive tapes. Feed uses are prominent, especially as pet food and for aquaculture. Gluten is used to facilitate slow-release encapsulation of flavors, colors, medicines, pest- or weed-control agents. Gluten is incorporated into building materials to provide light-weight, frost-resistant concrete. Cross-linked gluten is used to remove heavy metals from solution.

Compared with alternative commercial protein products, gluten has a lesser biological value (due to its unique amino acid composition), but economic considerations rank it above milk- or soy-derived products. Gluten's functional properties give it a unique place among the various protein products. However gluten, modified gluten and its fractions need to compete on price and fitness-for-purpose with other protein ingredients if wheat proteins are to be successful in the wider industrial markets.

11.6 Nutritional aspects of wheat proteins

The three major cereal grains, wheat, rice and maize, are a major source of our dietary protein. Applying the FAO production figures for the years 2001–2005 (http://faostat.fao.org) and considering a conservative estimate of 10% protein content, over 200 million tonnes of cereal-grain protein is harvested each year, with a substantial proportion of this being consumed by humans.

The diets consumed in developed countries usually contain various sources of dietary protein (cereals, legume seeds, meat, etc.) and the compositions of individual dietary components are of little real importance in relation to nutritional requirements. However, this is not the case in some less developed countries in which a single cereal may account for a major part of the total protein intake. In this case, the nutritional quality (i.e., content of essential amino acids) of the protein as well as the amount may be important.

Similarly, the protein composition is important when formulating feed for livestock in developed countries, particularly fast-growing monogastric animals such as pigs and poultry. Improving the quality for these animals has therefore been the major driver for much of the work on cereal protein quality in developed countries.

Research and breeding strategies to improve protein content and composition largely depends on the way the different cereals are utilized. Whereas the major emphasis in barley has been on low protein for malting, the emphasis in wheat has been on high-protein wheats for nutritional enhancement and improved processing performance.

11.6.1 Grain protein content (GPC) of wheat

Because of their different production systems and levels of fertilizer use, it is difficult to obtain comparative values for the protein contents of different cereals. However, consideration of values reported indicates that relatively small differences exist within and between species and that these are amplified by environmental factors. Initial screening of the USDA World Wheat Collection showed that the protein content of 12,600 lines varied from about 7% to 22% (Vogel et al., 1978), with the genetic component accounting for about a third of this variation (i.e., about 5% in protein content). Johnson et al. (1985) have provided an excellent overview of earlier studies of sources of high protein

in wheat while recent developments in the area are reviewed by Shewry (2007). Hence, the greater part of variation is due to non-genetic factors and this strong environmental impact has made breeding for high protein difficult.

Nevertheless, genetic sources of high protein have been identified with Atlas 50 and Atlas 66 being derived from the South American cultivar Frondoso (Johnson et al., 1985) and Nap Hal from India (www.indiaresource.org). These sources appear to have different "high-protein genes" and crosses between Atlas 66 and Nap Hal showed transgressive segregation for protein content. Both lines were used extensively for crosses in the Nebraska wheat breeding program and the Atlas 66 genes were successfully transferred into the hard red winter wheat Lancota, which has good quality and agronomic performance.

Wider sources of genes for high protein have also been identified and exploited by crossing with *Aegilops* (Finney, 1978) and wild emmer (*Triticum turgidum* var. *dicoccoides*) (Avivi, 1978). Joppa and Cantrell (1990) assigned the locus controlling this trait to chromosome 6B using substitution lines of emmer chromosomes into the durum wheat cv. Langdon. The precise gene has since been identified, mapped, and designated *Gpc-B1* (Distelfeld et al., 2004, 2006; Olmos et al., 2003). The quantitative trait locus (QTL) associated with this locus appears to account for about 70% of the variation in GPC in crosses (Chee et al., 2001; Joppa et al., 1997). Recent work has shown that the *Gpc-B1* gene encodes a transcription factor that accelerates senescence, resulting in increased mobilization and transfer of nitrogen and minerals (zinc, iron) to the developing grain (Uauy et al., 2006). Hence, lines expressing this allele contain higher amounts of iron and zinc in their grain as well as higher amounts of protein (Distelfeld et al., 2006; Uauy et al., 2006).

Other studies have identified QTLs for grain protein on chromosomes 5A, 5D, 2D, 2B, 6A, 6B, and 7A of bread wheat (Blanco et al., 2002; Groos et al., 2003; Snape et al., 1993; Turner et al., 2004; Worland and Snape, 2001) and on chromosome 5B as well as 6B of emmer wheat (Gonzalez-Hernandez et al., 2004). Worland and Snape (2001) suggested that less than 30% of the variation in protein content of UK winter wheats can be explained by known genes and suggested that this applied to wheat in general. The only major exception to this may be the *Gpc-B1* QTL which can account for up to 70% of the variation in crosses.

An important limitation to develop commercial wheat varieties with high protein content is the inverse correlation between yield and protein content which is well established for all cereals (reviewed by Simmonds, 1995). Consequently, there is almost always a yield penalty when growing high-protein cereals, which means that a higher price must be guaranteed to ensure support from farmers.

Although the theoretical basis for this inverse correlation has been debated (Miflin, 1980), the practical outcome is that high-protein cereals are unlikely to be commercially successful without financial incentives to growers.

11.6.2 Nutritional quality

Protein nutritional quality is determined by the content of essential amino acids. These amino acids cannot be synthesized by animals and hence must be provided in the diet.

Although only nine amino acids (lysine, isoleucine, leucine, phenylalanine, tyrosine, threonine, tryptophan, valine, and methionine) are strictly essential, cysteine is often included since it can be synthesized only from methionine, which is itself essential. Hence, a combined value for cysteine and methionine is usually presented. Similarly, combined values for the related aromatic amino acids tyrosine and phenylalanine are also presented.

The contents of amino acids in whole cereal grains are largely determined by the starchy endosperms which typically comprise about 80% of the grain dry weight. The starchy endosperm is the major storage tissue of the grain, storing starch and protein. The latter is predominantly prolamins in all cereals except oats and rice in which the major storage proteins are related to the 11S globulins ("legumins") of legumes and other dicotyledonous plants, with the prolamins being only minor components. The essential amino acid compositions of protein fractions of wheat are shown in Table 11.3.

Comparison of the amino-acid composition of wheat flour with the levels of essential amino acids recommended by the Food and Agriculture Organization of the United Nations (FAO) for infants and adults shows that wheat is severely deficient in lysine for infants, and, to a lesser extent, in threonine (Table 11.3). The aleurone and embryo tissues of grains do contain higher contents of essential amino acids (~4.8% and 8.3 g% lysine, respectively, in wheat) (Jensen and Martens, 1983) but these are often not available for human nutrition as they are removed by milling. These deficiencies also apply when wheat is used as feed for rapidly growing monogastric livestock such as pigs and poultry. This deficiency results from the low contents of these essential amino acids in the gluten proteins. Furthermore, because the accumulation of gluten proteins is differentially increased by the application of N fertilizer, the deficiencies are exacerbated when N is applied to increase the yield and protein content of the grain.

Attempts have been made over the last 50 years to increase the content of lysine in cereals, but these have met with little success due to the association of the improved nutritional quality with undesirable traits. Studies of diploid cereals (maize, barley, and sorghum) have identified a number of high-lysine mutants (Shewry, 2006). However, in most cases, this trait resulted from a decrease in prolamins and was associated with low yield and poor agronomic performance. Little progress has been made with wheat, although Singh et al. (2000) identified a 37 kDa protein (now known to be fructose 1–6-biphosphate aldolase) that correlated with lysine content in diploid relatives of wheat with the A genome. The development of genetic engineering technology allows the development of high-lysine lines by transferring genes for specific lysine-rich proteins (as reviewed by Sun and Liu, 2004 and Shewry, 2006, 2007). The compensatory increases in other nitrogenous fractions which occur in a number of high-lysine mutants include increases in free (i.e., non-protein) amino acids. Thus this fraction has also been a target for genetic engineering studies but this has not yet been applied to wheat.

It is clear from the small number of studies carried out till now that there is potential to use genetic manipulation to improve grain nutritional quality. However, none of the transgenic approaches have so far been shown to produce lines which are competitive with conventionally bred cultivars in their yield and agronomic properties (Shewry, 2007). It is also necessary to establish that such new lines are substantially equivalent in terms of grain composition and that the proteins expressed pose no risk to health in terms

Table 11.3 Proportions and amino acid compositions (g/100 g of protein) of the major protein fractions of flour

			Soluble proteins		Gluten proteins			Essential amino acids: FAO[a]	
								Recommendations	
Extracting solvent	Wheat –	Flour –	Albumin Water	Globulin 0.5M NaCl	Gliadin 70% ethanol	Glutenin Acetic acid	Residue protein –	Adult	Infant
% amino acid[b]:		100	3–5	6—10	40–50	30–40	6–10		
Amino acid[c]									
Asp	4.7	3.7	5.8	7.0	1.9	2.7	4.2	4.3	0.9
Thr	2.4	2.4	3.1	3.3	1.5	2.4	2.7		
Ser	4.2	4.4	4.5	4.8	3.8	4.7	4.8		
Glu	30.3	34.7	22.6	15.5	41.1	34.2	31.4		
Pro	10.1	11.8	8.9	5.0	14.3	10.7	9.3		
Gly	3.8	3.4	3.6	4.9	1.5	4.2	5.0		
Ala	3.1	2.6	4.3	4.9	1.5	2.3	3.0		
Val	3.6	3.4	4.7	4.6	2.7	3.2	3.6	5.5	1.3
Met	1.2	1.3	1.8	1.7	1.0	1.3	1.3	4.2	1.7
Cys	2.8	2.8	6.2	5.4	2.7	2.2	2.1		
Ile	3.0	3.1	3.0	3.2	3.2	2.7	2.8	4.6	1.3
Leu	6.3	6.6	6.8	6.8	6.1	6.2	6.8	9.3	1.9
Tyr	2.7	2.8	3.4	2.9	2.2	3.4	2.8	7.2	1.9
Phe	4.6	4.8	4.0	3.5	6.0	4.1	3.8		
His	2.0	1.9	2.0	2.6	1.6	1.7	1.8	2.6	1.6
Lys	2.3	1.9	3.2	5.9	0.5	1.5	2.4		
Arg	4.0	3.1	5.1	8.3	1.9	3.0	3.2		
Trp	1.5	1.5	1.1	1.1	0.7	2.2	2.3	1.7	0.5
NH3	3.5	3.9	2.5	1.9	4.7	3.8	3.5		

[a] Data from FAO/WHO (1985).
[b] Data from Konzac (1977).
[c] Data from Bushuk and Wrigley (1974).

of toxicity or allergenicity. Although such studies have been carried out on transgenic plants engineered for other traits, including wheat with altered seed protein composition for improved processing quality (reviewed by Shewry, 2007), they have not so far been reported for transgenic cereal lines with improved nutritional quality.

11.7 Health-related aspects of wheat proteins

The terms "wheat-related intolerance" (sensitivity) and "wheat allergy" are often used interchangeably. However, it is generally accepted that wheat sensitivity is specifically defined as the negative reaction in individuals to wheat with clinical manifestations such as chronic urticaria, gastrointestinal problems (i.e., irritable bowel syndrome, eosinophilic colitis, ulcerative colitis), depression, eczema and low blood-iron levels. These problems may sometimes take days to be manifest after ingestion or contact. In contrast, wheat allergy is a negative reaction where symptoms appear rapidly following exposure to macromolecules such as proteins. Wheat sensitivity affects many more people (about 15% of the human population) than wheat allergy (about 1–4% of the population), such as acute anaphylactic hypersensitivity and baker's asthma (Houba et al., 1998) as well as the autoimmune enteropathy, celiac disease (van Heel and West, 2006).

Studies on plant food sensitivity and allergy have demonstrated clearly that plant proteins are the primary allergens (Mills et al., 2004). Celiac disease (CD) affects 1% of Caucasians and South Asians (Maki et al., 2003; Fasano et al., 2003; Dube et al., 2005; Green and Cellier, 2007). In addition to nutrient deficiencies if CD is not treated, it can result in an increased risk of other autoimmune diseases and malignancy (Lohi et al., 2007; Rubio-Tapia et al., 2009). Gluten allergy is well described (Rasanen et al., 1994; Sicherer, 2000). Gluten intolerance and fructose malabsorption cause symptoms associated with irritable bowel syndrome, which may affect 30% of Westerners (Skoog and Bharucha, 2004).

Because wheat sensitivity can be chronic and its symptoms diverse, the underlining immunological mechanisms are ill-defined. The abnormal responses associated with wheat sensitivity include both the cellular and humoral immune system as the development of sensitized T cells leads to circulation of specific antibodies such as IgG or IgE, cytokines (e.g., IL13), and interferons (Eigenmann et al., 1998; Prescott et al., 2006).

In consideration of celiac disease, "gluten" must be considered as the storage protein of rye and barley as well as wheat. CD affects about 1% of the general population and causes significant health problems. Adverse inflammatory reactions to gluten are mediated by inappropriate T-cell activation leading to severe damage of the gastrointestinal mucosa, causing atrophy of absorptive surface villi. Gluten peptides bind to the chemokine receptor, CXCR3, and induce release of zonulin, which mediates tight-junction disassembly and subsequent increase in intestinal permeability. Proinflammatory cytokine IL-15 also contributes to the pathology of CD by driving the expansion of intra-epithelial lymphocytes that damage the epithelium and promote the onset of T-cell lymphomas.

This is in clear contrast to the well-defined IgE-mediated acute anaphylactic response for severe food allergy, where the specific circulating IgE molecules bind to the IgE

receptors on basophils and mast cells upon re-exposure of allergens, inducing the immediate release of histamine, tryptase, and arachidonate. The release of these molecules results, within minutes, in inflammatory responses such as gastrointestinal symptoms (nausea), respiratory symptoms (breathing difficulty), and/or cutaneous symptoms (rash or urticaria) (Lemke and Taylor, 1994). The molecular mechanisms for celiac disease are also well studied and reviewed (Anderson and Wieser, 2006; Catassi and Fasano, 2008). The disease is triggered by a genetic predisposition (HLA class II haplotype DQ2 and DQ8) and the ingestion of dietary gluten from wheat, barley, or rye (van Heel and West, 2006). Extensive studies have proved that immunogenic sequential peptides are the causative allergenic epitopes in wheat sensitivity, allergy, and celiac disease (van Heel and West, 2006).

11.7.1 Toxic peptides

Short amino acid sequences composed mainly of proline and glutamine are important elements of the primary structure of prolamins, and are related to their allergenic properties. Allergies are related to the pentapeptide Gln-Gln-Gln-Pro-Pro, and celiac disease to the tetrapeptides Gln-Gln-Gln-Pro and Pro-Ser-Gln-Gln. In the literature, these are referred to as "toxic peptides."

The pentapeptide Gln-Gln-Gln-Pro-Pro has the specific ability to bind to IgE antibodies in serum of patients with allergy symptoms (Watanabe et al., 1995). When gluten was digested with chymotrypsin and the resulting peptides separated by chromatography, several allergenic fractions were obtained, as revealed by ELISA using serum of persons having atopic dermatitis. The most allergenic component, a tridecapeptide, was purified and sequenced. It contained four characteristic Gln-Gln-Gln-Pro-Pro type sequences. This is actually the shortest sequence necessary for IgE binding; the N-terminal glutamine residue plus two proline residues are of prime importance, while other amino acids play a secondary role in IgE binding (Tanabe et al., 1996).

Evidence for activity of short peptides in celiac disease has been presented by De Ritis et al. (1988), who expressed peptide activity as a function of intestinal mucosa enterocyte cell enlargement in *in vitro* cultures. When the 266-residue of A-gliadin was cleaved with cyanogen bromide, three peptides (CB1, CB2, and CB3) resulted. CB1 and CB2 caused growth of enterocytes from persons with celiac disease, while CB3 was inactive. CB1 was then digested with chymotrypsin, yielding three smaller fragments (Ch1.1, Ch1.2, and Ch1.3). Only Ch1.1 was active.

It was again digested with chymotrypsin, yielding "toxic" peptides Ch1.1.1 and Ch1.1.2. All peptides found to be active in celiac disease contained the sequences Gln-Gln-Gln-Pro and Pro-Ser-Gln-Gln; inactive peptides contained no such sequences. These sequences are thus of special importance for disease development (Marsh, 1992). Most "toxic" tetrapeptides (3 of the 5 identified) came from the region of repeatable sequences near A-gliadin's N-terminal region (residues 1 to 55); thus, this region is considered the most "toxic" within the protein molecule. Only two "toxic" tetrapeptides were found in the region of specific sequences, and A-gliadins C-terminal region showed no "toxicity." The immunological activity of Gln-Gln-Gln-Pro-Pro in allergies and Gln-Gln-Gln-Pro

and Pro-Ser-Gln-Gln in celiac disease was confirmed by others (Maruyama et al., 1998; Wieser, 1996). The toxicity of these peptides was also shown by substitution of individual amino acids; such modifications caused total loss of peptide immunoreactive properties (Kocna et al., 1991).

It is unclear why Gln-Gln-Gln-Pro-Pro, active in allergies, is not active in celiac disease, even though it contains the motif Gln-Gln-Gln-Pro (Maruyama et al., 1998). It maybe that these peptides cannot cause disease symptoms by themselves since they are too small to effect release of histamine from basophils, and can only function as antibody-binding epitopes. It is thus hypothesized that Gln-Gln-Gln-Pro-Pro is an epitope indispensable for IgE binding, and the release of histamine results from a high concentration of tridecapeptides.

It is notable that the sequence Pro-Ser-Gln-Gln also occurs in capsule proteins of the adenovirus Ad12, which infects the alimentary tract. When a person with celiac disease is so infected, resulting symptoms are similar to those that appear after consumption of gluten-containing products. This is further evidence that "toxic" sequences cause the disease. However, antibodies against Ad12, which bind synthetic α-gliadin peptides, do not link with peptide CB1, containing two Pro-Ser-Gln-Gln sequences (Marsh, 1992). Consideration of other differences in structures of Ad12, synthetic α-gliadin and peptide CB1 suggests that the specific "toxic" properties of Pro-Ser-Gln-Gln depend on the structure of the whole protein molecule, on combination with other amino acids, on conformation, on the degree of folding, and/or on the configuration of higher-order structures.

11.7.2 Mechanisms of wheat-related disorders

In spite of significant differences between allergy and celiac disease, both health disorders result from improper functioning of the immunological system. In celiac disease, the enzyme tissue transglutaminase (tTG), occurring in various mammalian tissues and systemic fluids, is of special importance for disease development. This enzyme catalyzes the formation of isopeptide bonds between glutamine and lysine. Gliadin (containing much glutamine), introduced into a sensitive person, undergoes modification in which monomeric polypeptides are transformed to HMW gliadin–gliadin or gliadin–tTG complexes. They are especially bound by the main histocompatibility complex proteins (of HLA DQ2 type), and are then recognized by gliadin-specific T lymphocytes. These reactions initiate inflammatory processes having symptoms characteristic of celiac disease, such as intestinal villus atrophy or small-intestine mucosa damage.

Only 50 years ago, gliadin was declared the main antigen in celiac disease (Dicke et al., 1952). Modern criteria for diagnosing the disease were established by the European Society for Pediatric Gastroenterology and Nutrition (ESPGAN) in 1989. Celiac disease is now recognized when, after including gluten in a diet, clinical symptoms typical of that disease occur, and when in a patient's serum-specific anti-endomysium (EmA) or antireticulin (ARA) antibodies are identified in the IgA class (or when IgA is lacking, in the IgG class). All four gliadin fractions can produce symptoms of celiac disease in sensitive persons; α-gliadins, especially A-gliadin, are most active. A-gliadin, coming in contact with

the small intestinal surface, causes characteristic changes in the epithelium. This has been confirmed by an *in vitro* study of intestine surface fragments from patients (Wieser, 1996). Pathological changes were so severe in this procedure that at first, allergenicity was related only to A-gliadins. Further studies, both *in vivo* and *in vitro*, suggest that in celiac disease all the gliadin groups can be active (Howdle et al., 1984). For example, the ability of intestinal T cells from persons with celiac disease to recognize γ-gliadin epitopes indicates their "toxicity" (Varjonen et al., 2000). Such a possibility is also suggested by the presence of Gln-Gln-Gln-Pro and Pro-Ser-Gln-Gln tetrapeptides in β-, γ-, and ω-gliadins. Nevertheless, the problem is still under discussion, while the "toxicity" of A-gliadin is not questioned.

Another type of immunological activity of wheat storage proteins occurs in allergy. Sensitized persons produce excessive amounts of IgE antibodies against gluten proteins and their analogs from rye, barley, and oats. Linkage of antigens with antibodies results in the release of histamine from basophils (i.e., cells especially rich in histamine which, as a result of the immunological reaction, comes out), which damage organs in various ways. There is a relationship between gliadin polymorphism and the type of disease symptoms they produce; in some cases, certain groups of these proteins are stronger allergens than they are at other times.

LMW α-gliadins, in addition to causing celiac disease, can also cause symptoms of dermatitis (Varjonen et al., 2000). The ~14 kDa fraction, like IgA and IgG antibodies in persons with celiac disease, can also bind IgE antibodies in patients with atopic dermatitis. β-Gliadins, which are important allergens in those with celiac disease showing symptoms of atopic dermatitis, have similar properties. This is proven by the ability of IgE, IgA, and IgG antibodies, present in patients' serum, to bind polypeptides of molecular weight 30–43 kDa.

γ-Gliadin can cause anaphylaxis symptoms. Studies using immunoblotting showed that chromatographically purified γ-gliadin forms strong bonds with antibodies of patients' serum (Palosuo et al., 1999). ELISA proved that sensitive persons' serum showed a significantly increased level of IgE against that fraction. Other studies of patients with symptoms of anaphylactic shock showed that γ-gliadins can inhibit the immunological reaction between the gluten protein complex and serum IgE (Morita et al., 2001). Thus, in the case of the anaphylactic shock, γ-gliadins are a much stronger allergen than other gluten proteins. Considering dermal symptoms, the γ fractions (like β fractions) can be a cause of urticaria, but they do not induce atopic dermatitis. Varjonen et al. (2000) observed IgE antibodies against ethanol-soluble prolamins in patients with urticaria symptoms. The molecular weight of these proteins was 30–50 kDa, making it possible to classify these proteins as β- and γ-gliadins. Dermatological symptoms caused by food allergens can result from the ability to deposit immunological complexes in the subcutaneous layer (Vainio et al., 1983). Another cause of that phenomenon can be the non-specific cross-reaction of gliadin with reticulin protein in skin.

ω-Gliadins cause allergy symptoms in children. One chromatographically purified ω-5 fraction (about 77 kDa) is an allergen responsible for breathing trouble, food allergy, and atopic dermatitis. Serum of children aged about 2.5, who showed severe symptoms of these diseases, contained much IgE antibody against ω-5 gliadin (Palosuo et al., 2001). The same

protein can also be an important allergen in anaphylactic shock in adults. An increased level of IgE, as well as of IgA and IgG antibodies, and increased release of histamine from basophils in patients accentuates the role of ω-5 gliadin in this disease (Lehto et al., 2003).

Although the harmful action of gliadins in allergic persons intolerant of gluten raises no serious doubts, the role of glutenins as food allergens has not been clearly explained. Some reports imply that an immunological reaction involving glutenins and anti-glutenin IgE antibodies is possible; others do not connect them to allergy symptoms. Sandiford et al. (1997) found IgE antibodies against HMW glutenin subunits in serum of persons with bakers' asthma.

The immunoreactive properties of these glutenin subunits were comparable to those of α- and ω-gliadins. However, in bakers' asthma, the strongest allergens are albumins and globulins. Sandiford et al. (1997) accepted a hypothesis that the observed effect results from structural similarity of epitopes in the above protein groups. Sutton et al. (1982) observed strong bonds in children with asthma and eczema between serum IgE antibodies and a glutenin fraction, which may prove the allergenic properties of glutenin polypeptides in these diseases. Kushimoto and Aoki (1985), by digesting HMW glutenins with pepsin, obtained polypeptides of molecular weight 15–100 kDa. These peptides exhibited allergenicity in patients showing urticaria symptoms. In the authors' opinion, digestion of glutenins with pepsin and trypsin can cause increased allergenicity by releasing epitope-containing peptides directly bound to IgE antibodies. Simonato et al. (2001) found that allergenicity of glutenins occurs when the conformation of proteins in the gluten matrix is altered and as a result of processes during baking. Allergenic properties of lower molecular weight proteins then disappear, and emerge in higher molecular weight fractions.

Gluten proteins are, however, not the only allergenic components of the wheat seed. Albumins and globulins also exhibit strong allergenic properties, especially in asthma and bakers' asthma (Sanchez-Monge et al., 1992; Garcia-Casado et al., 1994, 1996; Armentia et al., 2002). Other important allergens include LTP, causing urticaria and angioedema symptoms (Breiteneder and Ebner, 2000; Pastorello et al., 2001).

It is noteworthy that the proteins discussed above, in addition to causing alimentary and respiratory diseases (these being the main routes in which antigens are introduced), can also cause conditions such as skin diseases, cerebellar ataxia, or psychosomatic complications. This indicates that complexes of proteins with specific antibodies can go from blood to other systems, causing damage.

11.7.3 Treating wheat-related disorders

Consumption of gluten-containing food causes disease for a significant minority of people who consume foods derived from wheat, rye, barley, and oats. Commercial activity related to "gluten toxicity" is largely focused upon "gluten-free" food. The fact is however, that in several types of diseases related to the consumption of gluten-containing cereals, the trigger compounds are not components of the gluten. There is a certain lack of related knowledge about the causes of different symptoms or diseases and about the terms "gluten," "prolamins," "gliadin," and "glutenin" not only in the minds of the consumer but also in medical practice.

The general public in most Western countries is now aware of the potential adverse effects of cereals containing "high calorie," "toxic," and "allergic" gluten with stories and books appearing in the lay press (Braly and Hogganm, 2002; Ford, 2008; Wangen, 2009; Davis, 2011) promoting gluten-free diets, many without drawing attention to the importance of appropriate diagnosis or defining what gluten "intolerance" an individual may have. Such adverse publicity poses a significant threat to the grain industry.

"Gluten-free" substitutes attract a premium over standard gluten-containing products, yet in only a few countries are patients with gluten-related disease assisted with the costs of following a gluten-free diet. In addition, standards for "gluten-free" labeling vary between countries. Most gluten-free products are not labeled as such, and substitutes for gluten-containing foods (e.g., bread) are seldom as palatable or as versatile in cooking. Frequently, the gluten-free diet is further complicated by the need to treat co-existent diabetes, fructose or lactose intolerance, which may compromise the utility of many "gluten-free" substitutes.

While the number of publications (i) about gluten-related health disorders (ii) about the causative agents for celiac disease and other different illnesses (such as wheat intolerance, gluten intolerance, irritable bowel syndrome) and (iii) about the development of gluten-free food products is far beyond 1000 in the last 20 years, there are not many works written in a style and language to be understandable for all the three related professions (medicine, plant biology, and the food industry). A mutual understanding and collaboration is essential in the future among them to satisfy the customer's need and to avoid the unnecessary and often unfounded rejection of cereal products.

One of the recent exceptions in the scientific literature which aims to make bridges among the related parties is the review of Islam et al. (2011). Based on excellent reviews of the specific areas, cited here, this work defines the different health-related disorders in a "clear to all" but scientifically exact style. So too do other reviews (Anderson et al., 2000; Anderson and Wieser, 2006; Armentia et al., 1990; Catassi and Fasano, 2008; Kushimoto and Aoki, 1985; Lauriere et al., 2006). Up-to-date knowledge about the wheat proteins containing toxic and allergenic epitopes is provided by Amano et al. (1998); Mills et al. (2004); Mittag et al. (2004); Tatham and Shewry (2008) as well as about the non-protein causative components of wheat such as fermentable oligo-, di-, and monosaccharides and polyols (FODMAPS) (Gibson, 2007).

Finally, Chavez and Barca (2010) provide a good overview on research and development in the food industry in relation to gluten-free products. This area involves the analytical and labeling issues in relation to gluten content of food (Hischenhuber et al., 2006; Wieser, 2008; Haraszi et al., 2011), the activities to find formulations with functionality similar to wheat gluten (Arendt et al., 2008; BeMiller, 2008; Rosell and Marco, 2008; Schoenlechner et al., 2008), looking for less harmful wheat varieties and wheat relatives (Klockenbring et al., 2001; Nakamura et al., 2005; Spaenij-Dekking et al., 2005; van Herpen et al., 2006) and methods and technologies to reduce the level of harmful compounds in wheat-based products such as the use of different enzymes (Berti et al., 2007; Mitea et al., 2008; Renzetti et al., 2008; Stenman et al., 2009), and/or sour-dough technology (Ehren et al., 2008; Gobbetti et al., 2008; Goesaert et al., 2008; Marco et al., 2008).

11.8 Wheat proteomics—new perspectives

11.8.1 Applications of proteomics

Proteome mining mainly deals with identification and characterization of proteins from different seed organelles or sub-proteomes utilizing the differences observed in their structure and solubility. Analysis of sub-proteomes, mainly those of cereal endosperms, peripheral layers, or germ-protein fractions serve as an important base for studies that relate to different aspects of end-use quality studies, immunogenic analyses, or studies on changes due to abiotic or biotic stresses.

Proteomic approaches used in cereal seed science can be classified based on several rules, which include the approaches used, the targeted analyses, the raw material analyzed Proteomic approaches suitable for the analysis of the wheat-seed proteomes are presented in Figure 11.6.

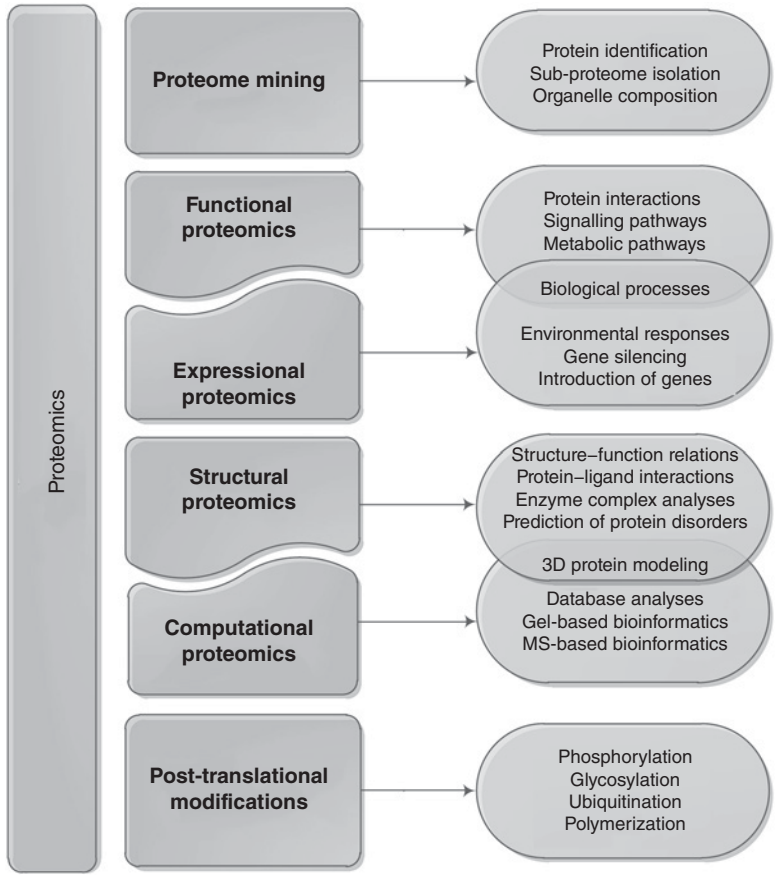

Figure 11.6 Proteomic approaches and their possible use in wheat grain protein analyses

Proteomic research activities on wheat and the possible and/or already existing application areas of proteomics in wheat research have been reviewed by Skylas et al. (2005). They included dough quality, starch properties, puroindoline, chromosomal location of coding genes, effects of growth environment, insect damage to wheat grains, genotype identification. More recent reviews have been published summarizing the different techniques of proteomics both in plant biology and food science and are provided in Table 11.4.

The classical methodologies used in wheat-seed proteomics include two-dimensional gel electrophoresis (2D-GE) for relatively quantitative analysis for comparing small numbers of samples. One of the first proteomic analyses of developing and mature grain was undertaken by Skylas et al. (2000) using N-terminal amino acid sequencing for protein identification.

The combination of 2D-GE and mass spectrometry analyses, such as MALDI-TOF-MS or ESI-MS, can resolve the seed proteome up to over a thousand spots (Skylas et al., 2000; Mak et al., 2009; Dupont et al., 2011; Lesage et al., 2012; Yang et al., 2011; Tasleem-Tahir et al., 2011). Total protein extracts of flour milled from a single cultivar, Butte 86, were analyzed in detail by Dupont and co-workers (2011) using MS-coupled 2D-GE analysis. All members of the prolamin superfamily were identified, including variants of α- and γ-gliadins and different sorts of globulin-type proteins. Similarly, a single cultivar, cv. Recital, was used to monitor expression profiles of metabolic proteins of the starchy endosperm soluble under a low salt concentration (Tasleem-Tahir et al., 2011). Expression profiles of 487 proteins have been identified at 21 different developmental stages. Albumin/globulin and amphiphilic proteomes of hard and soft kernel were compared in the analysis of Lesage et al. (2011, 2012). The gel-based approach combined with mass spectrometry can also identify many post-translational modifications, by comparing protein sequence with physical properties.

In wheat, the knowledge of starch biosynthesis has led to developing wheat varieties with better nutritional properties to produce lower-calorie cereal-based foods. The understanding of the interrelationships among metabolic pathways helps to break the inverse relationship between harvest yield and protein content, in order to maintain a high level of production with better quality source material for the industry. The improved knowledge about the protective mechanism of the plant and the effects of biotic stresses on the protein composition has direct relationships to food safety. The better understanding of the effects of genetic and environmental factors on the expression and deposition of different proteins should lead directly to the production of wheat varieties with better end-use quality, with higher nutritive value, and with less harm for sensitive individuals.

The development of techniques to improve extraction, separation, and identification of proteins and peptides is facilitating functional proteomics and the analysis of sub-proteomes from small amounts of starting material, such as seed tissues (reviewed in Jorrin et al., 2007; Miernyk and Hajduch, 2011; Finnie et al., 2011). The combination of proteomics with structural and functional analysis is increasingly applied to target subsets of proteins. These “next-generation” proteomic studies differentiate between spatial sub-proteomes and functional sub-proteomes, thus contributing to our understanding of

Table 11.4 Proteomic approaches to published studies of wheat-based foods

Approach	Applications	Examples for applications in wheat-based food proteomics	References
Proteome mining	Protein identification	Cultivar identification, polymorphism studies Wheat lines with chromosome deletions Primitive wheats as gene sources	Cornish et al. (2001); Skylas et al. (2001); Wrigley (2002b); Wrigley et al. (2003); Wu et al. (2011); Yahata et al. (2005); Dupont et al. (2011); Islam et al. (2002, 2003a, 2003b); Wu et al. (2011)
	Sub-proteome isolation (embryo, endosperm, bran, aleurone)	Hardness, milling properties Dough properties Starch properties	Amiour et al. (2002, 2003); Branlard et al. (2003a); Lesage et al. (2012) Liu et al. (2009, 2010); Dworschak et al. (1998); Ghirardo et al. (2005); Muccilli et al. (2005); Salt et al. (2005); Hurkman et al. (2008) Tetlow et al. (2008)
	Organelle composition (amyloplast, protein bodies, plasma membrane, cell wall)	Starch granule development	Andon et al. (2002); Balmer et al. (2006); Bechtel (2003)
Functional proteomics	Protein interactions	Identification of allergic proteins from wheat Food safety applications in wheat allergies, celiac disease	Akagawa et al. (2007); De Angelis et al. (2006, 2008, 2010), Larré et al. (2011); Ruoppolo et al. (2003); Sotkovsky et al. (2008); Yang et al. (2011); Kasarda et al. (2008); Gobbetti et al. (2008); Haraszi et al. (2011); Heick et al. (2011); Matsuo et al. (2004)
	Signalling pathways		
	Metabolic pathways	Starch synthesis analysis	Balmer et al. (2006); Dupont (2008)
Expression proteomics	Biological processes (seed development, dormancy, germination)	Manipulation of wheat grain development for improved yield, quality Germination studies, pre-harvest sprouting	Altenbach et al. (2011) Kamal et al. (2009); Guo et al. (2011)

(*continued*)

Table 11.4 (*Continued*)

Approach	Applications	Examples for applications in wheat-based food proteomics	References
	Environmental responses (abiotic/biotic stresses, defense responses)	Heat, drought, frost studies Farming practice/fertilization Virus, rust, powdery mildew effects on grain quality	Finnie et al. (2004); Horvath-Szanics et al. (2005) Hurkman et al. (2009); Laino et al. (2010); Majoul et al. (2004); Miernyk and Hajduch (2011); Hashiguchi et al. (2010); Neilson et al. (2010); Sancho et al. (2008); Skylas et al. (2002, 2005) Altenbach et al. (2011); Flate et al. (2005) Antoine et al. (2003); Blein et al. (2002); Desmond et al. (2006); Dunwell et al. (2000); Gane et al. (1998); Gorlach et al. (1996); Kav et al. (2007); Laino et al. (2010); Mak et al. (2006); Mellersh et al. (2002); Niranjan-Raj et al. (2006); Selitrennikoff (2001); Wang et al. (2005); Zhou et al. (2005)
	Gene silencing Introducing genes into the genome	GMO detection	Debiton et al. (2011); Gobaa et al. (2007); Guo et al. (2011); Horvath-Szanics et al. (2006); Islam et al. (2003b); Di Luccia et al. (2005)
Structural proteomics	Structure–function relations	Droplet evaporation method for quality and vitality analysis	Kokornaczyk et al. (2011)
	Plant–pathogen interactions	Grain mycotoxin identifications	Berthiller et al. (2005); Bhatnagar et al. (2008)
	Enzyme complex analysis		
	Ligand–protein interactions	Development of wheat sources with improved dietary content (functional foods)	Pollet et al. (2010); Laubin et al. (2008); Merlino et al. (2009)
	Prediction of protein disorders		
	3D protein modeling	Allergen epitope Ig antibody interactions	

Computational proteomics	3D protein modeling	Allergen epitope Ig antibody interactions	
	Database analysis	Quality-related proteins and their expression Food safety applications	Juhász et al. (2011); Altenbach et al. (2011); Heick et al. (2011)
	Gel-based bioinformatics	Search for diagnostic proteins for organic wheat farming Identification wheat allergens	Zörb et al. (2006, 2009); Akagawa et al. (2007); Denery-Papini et al. (2007) Larré et al. (2011); Yang et al. (2011)
	MS-based bioinformatics	Determination of wheat quality based on MS analysis of wheat seed	Gottlieb et al. (2002); Herrero et al. (2011); Altenbach et al. (2011); Ghirardo et al. (2005); Dupont et al. (2011)
Post-translational modifications	Phosphorylation	Biotic stress signalling, defence signalling Starch branching enzyme activity analyses	Rampitsch et al. (2010); Bykova et al. (2011) Tetlow et al. (2004)
	Glycosylation	Modification of glutenins	Bollecker et al. (1998)
	Ubiquitination	Growth, development, stress response analyses	Wilson et al. (2005); Wong et al. (2004)
	Polymer formation, proteolytic cleavage	Gluten polymer analyses	Beasley et al. (2002); Gao et al. (1992) Lindsay et al. (2000); Weegels et al. (1997)

the processes controlling cereal-grain development, nutritional, and processing characteristics as well as the changes caused by biotic (Kav et al., 2007) and abiotic (Hashiguchi et al., 2010) stresses. The importance of abiotic stresses caused by global climate change has particular significance in the future of agriculture and food industries. Advances in crop proteomics provide the basis for interpreting changes in the grain as a result of climatic factors including photosynthetic stress, air pollutants, thermal stress including heat and cold, and osmotic stress (drought, salt, flooding), and metal stress to help to eliminate or to reduce the negative effects of these factors on the food production chain (Finnie et al., 2011).

Reviews on the use of proteomics in wheat-related food technology (Carbonaro, 2004; Mamone et al., 2009; Pischetsrieder and Baeuerlein, 2009; Gaso-Sokac et al., 2010) focus on applications related to the characterization and standardization of raw materials, including areas important to selecting parents for crossing in breeding (glutenin-allele identification) or regulating the entire wheat chain (variety identification), process development, and detection of batch-to-batch variations and quality control of the final product. Aspects of food safety, especially regarding biological and microbial safety and the use of genetically modified foods are also important targets.

11.8.2 Functional proteomics

A more complex area of proteomics, functional proteomics, aims to characterize signaling and metabolic pathways, function of protein complexes and protein activities during developmental processes such as seed development, dormancy, or germination in order to interpret environmental and chemical affects on grain composition. Spatiotemporal analyses of expressed proteins form a sub-group of the functional proteomics studies, relying especially on the outputs from DNA- and RNA-based studies. Expression studies enhance our understanding of seed responses to different abiotic stresses, pathogen attack, or symbiotic interactions, including the effects of gene silencing or the introduction of additional genes.

The major challenge of structural proteomics is to assign predicted or in silico modeled protein structure data to protein sequences obtained from genome and transcriptome sequencing projects. Structural proteomic studies of cereal seeds are used to predict the presence of epitopes important to food products, plant–pathogen interactions, to identify receptor functions by the analysis of ligand–protein interactions, or to analyze enzyme complexes. Structural proteomics builds on computational proteomics, utilizing the potential of data analysis and 3D modeling. The collection and evaluation of data from protein databases combined with gel-based, MS-based, or immunoanalytical computational analyses can deliver outputs in drug discovery, personalized medical treatments, and food safety analyses.

11.8.3 Proteins with post-translational modification—emerging proteomic techniques

The analyses of post-translational modifications, which modulate the activity of many seed proteins and the modified "sub-proteomes" that result, such as glycosylated,

phosphorylated proteins, or modified membrane proteins, can now be studied using mass spectrometry. Post-translational modifications, leading to polymer formation through inter-molecular disulfide binding, are especially important in the structure–function relationship analysis of wheat gluten for food products. Methodologies used in wheat-seed proteomics include 2D-GE-based proteomics for relatively quantitative analysis to compare small numbers of samples. The combination of 2D-GE and mass spectrometry analyses, such as MALDI-TOF-MS or ESI-MS, can resolve the seed proteome into 3000 polypeptides. This approach can also identify many post-translational modifications, by comparing protein sequence with physical properties.

Proteins with extreme hydrophobicity, mass, or pIs can be under-represented in standard approaches because of solubility (for review, see Santoni et al., 2000; Görg et al., 2004) or other technical limitations. To identify these extreme proteins, gel-free proteomic approaches, such as multidimensional protein identification technology (MudPIT) (Link et al., 1999; Washburn et al., 2001) have been explored. MudPIT separates peptides by a strong cation exchange phase in the first dimension followed by reversed-phase chromatography in the second dimension. Comparisons of MudPIT and 2D-GE indicate that these technologies are complementary (Koller et al., 2002; Katavic et al., 2006). Another gel-free MS approach is the isotope-coded affinity tags (Gygi et al., 1999), which is used for quantitative comparison of proteomes. Seed tissues contain a large number of diverse proteins, with different chemical characteristics which have resulted in the development of special extraction and purification approaches that retain the quality of proteins for analysis on 2D gels or by MS (Branlard and Bancel, 2007. For cereal grains, the major storage components are starch and various carbohydrates which have a detrimental effect on the extractability of proteins, as a result of seed proteins being present as aggregates or coupled to other compounds such as starch granules or cell wall elements. Compared to other plant tissues, the extraction of seed proteins first requires that the hard structure of the mature seed must be broken using grinding or milling. Solubilization of proteins may depend on the particle size and the homogeneity in the size distribution of the ground material. Extraction methods in seed proteomics can be divided into two main types: (1) using universal protocols for the main protein groups. These include classes of water- or salt-soluble proteins, moderately hydrophobic proteins, enriched hydrophobic proteins, and membrane proteins; and (2) specific protocols that target certain cereal proteins, namely, those soluble in water, salt solutions, dilute alcohol, and acidic or basic solutions (Görg et al., 2004).

Fresh, developing seed tissues need the use of liquid nitrogen during isolation to rapidly freeze the tissues before grinding. The presence of some contaminating compounds, such as phenolics, phosphatases, lipids, salt ions, nucleic acids, insoluble materials, and proteolytic enzymes, requires the use of special chemicals. Proteolytic enzymes are intrinsically involved in many aspects of plant physiology and development, ranging from the mobilization of storage proteins during seed germination to the initiation of cell death and senescence programs (Loukas, 2002; Creemers, 2002; Schaller, 2004). Post-translational modification of proteins, for protein assembly, for the activation of prohormones to hormones, and for the maturation and control the activity of enzymes are processes carried out by grain components present in small amounts. Different mixtures of protease inhibitors, trichloroacetic acid (TCA) precipitation, or boiling in anionic detergent solution are often

used to avoid protein degradation in seed tissue extracts. Loss of function of enzymes or membrane proteins can be avoided using native, non-denaturing conditions during the extraction (Fido et al., 2004). The presence of phenolic compounds generally has a detrimental effect on the separation of proteins, resulting in horizontal and vertical streaks on 2D-GE. Precipitation of proteins with TCA, followed by rinsing with ice-cold acetone plus TCA, will help to eliminate phenolic compounds present in fresh tissues. Starch or complex carbohydrates can also seriously reduce protein extraction, and is a primary problem in the proteomic analyses of maize or rice seeds.

Although peptide mass fingerprinting is a sensitive high-throughput method, it is not always easy to identify the plant proteins or genes by fingerprinting because the complete nucleotide sequence of the genome of various species, including wheat is not yet available and the protein database does not have enough information for the identification of unknown proteins. In addition, plant proteins are often post-translationally modified which means peptide mass fingerprinting needs to initially deal with a subpopulation of peptides that are not modified. Improved sample preparation methods for peptide mapping are important to develop for MS analysis of wheat (Woo et al., 2002). Due to the high proline and glutamine content observed in grain storage proteins, the usual enzymatic digestion protocols do not result in sufficient accuracy to differentiate members of the prolamin protein families, such as HMW or LMW glutenins and gliadins. However, using different combinations of proteinases, more distinctive peptide mass fingerprint datasets can be generated (Vensel et al., 2005; Dupont et al., 2011). Peptides that are distinctive for specific proteins are the key to acquiring validating information for the expression of a gene that has been annotated in the genome (Futcher et al., 1999; Gygi et al., 1999; Appels et al., 2012). In order to analyze proteome-based protein expression on a large-scale, mass spectroscopy technologies are generally considered to be the most appropriate (Woo et al., 2002; Skylas et al., 2005; Appels et al., 2012).

11.9 Future prospects

Over a century of cross-breeding has brought remarkable improvements to wheat, both for agronomic performance and for grain quality. Breeders have moved from almost blind selection for phenotype to the present use of marker-assisted selection, using knowledge of specific genes or at least using suitable marker genes. The growing demand for high quality wheat-based raw materials under diverse environmental conditions, as well as the urgent need to reduce chemical use in agronomy, has led to the emergence of a number of potential targets for analysis by wheat scientists. These include various aspects of complex mechanisms such as seed development, stress tolerance, nutrient uptake, and utilization. To study these mechanisms, projects involving multidisciplinary approaches, such as genomics, proteomics, metabolomics, and bioinformatics are needed.

Further advances in intelligent manipulation will flow from the completion of the whole wheat genome, which brings with it a new era in wheat-protein research. This information has wide-ranging implications for research on structure–function relationships, thereby greatly expanding on the descriptions, in this chapter, of grain proteins and their roles for the grain itself and for our utilization of it.

The growing source of genetic information will greatly facilitate the ability of genetic engineering, in conjunction with conventional breeding, to introduce improvements that could not be considered previously. Research of this nature will clarify the roles played by specific proteins for agronomic and quality traits, thereby helping to fill gaps in our understanding of the complex interactions of genotype with growth environment. Irrespective of the nature of these intermediate mechanisms, the bottom line is still seen in the phenotypic outcome—the resulting grain and its utilization potential.

11.10 Conclusion

As mentioned at the outset of this description of wheat proteins, the wheat seed is the means by which a wheat plant reproduces itself. In contrast, the wheat seed (as an edible grain) is the means by which a baker produces a loaf of bread. The grain proteins are essential in both these processes. Storage proteins, laid down by the ripening grain as a source of amino acids to fuel the germinating seedling, become the protein complex that makes wheat uniquely suitable for bread making.

Why does the maturing wheat grain produce such a diverse array of proteins during its development? One answer is that many of these proteins have served essential metabolic purposes, such as catalyzing the synthesis of starch, lipids, cell walls, and many other parts of the grain. The second answer is less clear. The "diverse array" description is even more relevant to the grain's storage proteins, as they appear to serve no specific purpose other than to be a source of amino acids for the germinating seed. If this is their sole purpose, why is such a diverse array of proteins synthesized under such predictably discrete genetic control? We can only presume that the biochemical principles governing protein synthesis apply equally to metabolic and storage proteins. In the case of the metabolic proteins, such as specific enzymes, a mutation that alters the conformation of amino acids around an enzyme's active site may prevent it from carrying out its enzymic function; thus the mutation would be lethal and the mutant would not survive. On the other hand, it is unlikely that such mutations would be lethal for proteins whose sole function is storage, except perhaps if the resulting protein became much more soluble and more likely to be lost by leaching during germination. As a result, successive mutations continue to be preserved in the storage proteins.

The "diverse array" description for the wheat storage proteins appears to be relevant to the unique bread-making properties of wheat, as has been explained in this chapter.

When the storage proteins of wheat, unlike those of other cereal grains, are mixed with water they produce a diverse array of interacting monomeric and polymeric proteins covering a wide range of molecular sizes. It is this broad range of macromolecules that gives gluten its unique dough-forming qualities.

This characteristic is illustrated by the following contrast. Most naturally occurring protein mixtures produce "pretty" electrophoretic patterns on gel electrophoresis—discrete well-resolved bands against a clear background, such as the subunits released from glutenin by rupturing disulfide bonds (Figure 11.1). On the other hand, native glutenin produces an "ugly" smear throughout the mobility range—not a satisfying result (Wrigley et al., 1993)—indicating a continuous wide range of size distribution. This unresolved

streak of protein is the "beauty" of disulfide-cross-linked glutenin polymers—the secret of wheat gluten.

This understanding of gluten's secret leads to some final questions. Why do we not have a clear structural model for gluten proteins, especially the involvement of the HMW-GS, which are believed to serve as a backbone to the gluten network? What genetic mechanism controls the disulfide cross-linking of the glutenin polypeptides? What interactions are involved between genotype and growth conditions in the synthesis of gluten proteins? How may we manipulate these controls to improve wheat quality?

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12
Rice Proteins

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12.1 Introduction

Rice (*Oryza sativa* L.) is one of the major cereal crops in the world, feeding a large section of its population. In many Asian countries and other areas where rice is the staple food, it is considered the most important crop. Rice provides a large proportion of the daily nourishment of their people because the bulk of the caloric intake is derived from this undeniably essential commodity. When food protein sources are scarce, rice also supports the protein requirements of these consumers.

12.1.1 Production and consumption of rice

Rice is widely cultivated in the world because it is produced in almost all continents and in over 100 countries. Table 12.1 shows the harvested area, paddy yield, and production of paddy and milled rice in 2011 (IRRI, 2013). A total of 164 million hectares were devoted to rice production worldwide, and the average yield per unit hectare was 4.4 metric tons. The total paddy and milled rice production were about 723 and 482 million metric tons, respectively. The highest paddy production was obtained from Asia with 653 million metric tons, whereas the lowest was gathered from Southern Africa with 2620 metric tons only.

Total consumption of milled rice, per capita consumption, calorie intake, and protein intake figures for 2011 are indicated in Table 12.2 (IRRI, 2013). Total consumption of milled rice was highest in Asia (397 million metric tons) and lowest in Oceania (450,000 metric tons). The highest per capita consumption was obtained from Southeast Asia (129 kg/year) and, not surprisingly, the highest calorie and protein intake as well.

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Table 12.1 Harvested area, paddy yield, and production of rice in 2011

Continent/Region	Harvested area (000 ha)	Paddy yield (t/ha)	Paddy production (000 t)	Milled rice production (000 t)
Africa	11,168.79	2.38	26,531.82	17,687.88
Americas	6880.43	5.50	37,874.08	25,249.39
Asia	145,270.27	4.50	653,240.40	435,493.56
Caribbean	438.99	2.84	1248.65	832.43
Central America	335.28	4.20	1408.74	939.16
Central Asia	196.22	3.60	706.81	471.20
East Asia	33,312.30	6.60	219,852.27	146,568.19
Eastern Africa	3542.03	2.35	8339.44	5559.63
Eastern Europe	264.06	5.15	1359.43	906.28
Europe	725.37	6.03	4375.88	2917.25
Middle Africa	801.33	0.95	764.79	509.86
Northern Africa	602.97	9.48	5714.25	3809.50
Northern America	1059.48	7.92	8391.87	5594.58
Oceania	80.13	9.21	738.15	492.10
South America	5046.68	5.32	26,824.82	17,883.22
South Asia	62,072.37	3.62	224,788.23	149,858.81
Southeast Asia	49,522.47	4.17	206,754.25	137,836.16
Southern Africa	1.04	2.51	2.62	1.75
Southern Europe	436.30	6.61	2884.45	1922.97
Western Africa	6221.42	1.88	11,710.72	7807.15
Western Asia	166.91	6.82	1138.81	759.21
Western Europe	25.00	5.28	132.00	88.00
World	**164,124.98**	**4.40**	**722,760.30**	**481,840.20**

Source: From IRRI (2013).

These data clearly indicate that rice is mostly produced and consumed in developing countries in Asia, where it is the dietary staple.

12.1.2 Structure of the rice grain and milling fractions

The structure of the rice grain was described in detail by Juliano (2007). Rice is harvested in the form of rough rice or paddy. Its outermost covering is a tough siliceous hull that provides protection to the caryopsis or brown rice (BR) and also helps deter fungal infestation of the rice grain.

BR is made up of bran, the embryo, and the endosperm. Bran is composed of different components. The embryo or germ, which is found on the ventral side at the base of the grain, is bounded on the outside by a single aleurone layer and by the fibrous cellular remains of the pericarp, seed coat, and nucellus. The starchy endosperm is divided into the subaleurone layer and central region. It contains starch granules and protein bodies. There are different chalky regions in the endosperm of some nonwaxy rices. Depending on location, they can either be white core (extending to the center of the endosperm and

Table 12.2 Rice consumption and calorie and protein intake in 2011

Continent/Region	Total consumption milled rice (000 t)	Per capita consumption (kg/year)	Calorie intake (% total/d)	Protein intake (% total/d)
Africa	23,118.00	21.10	8.24	6.31
Americas	24,282.00	20.00	6.33	4.30
Asia	397,525.00	77.20	28.71	19.39
Caribbean	2004.00	47.60	17.94	14.16
Central America	1727.00	10.40	3.46	2.39
Central Asia	477.00	7.00	2.49	1.54
East Asia	145,149.00	74.60	25.90	15.41
Eastern Africa	5723.00	14.40	6.80	5.32
Eastern Europe	1293.00	4.00	1.21	0.72
Europe	4409.00	5.20	1.58	0.98
Middle Africa	910.00	14.90	6.74	4.84
Northern Africa	3906.00	16.20	5.49	3.71
Northern America	4608.00	8.50	2.43	1.43
Oceania	450.00	13.70	4.08	2.54
South America	15,942.00	31.40	10.78	7.62
South Asia	130,855.00	70.90	29.46	22.41
Southeast Asia	117,566.00	129.10	47.27	36.77
Southern Africa	796.00	13.80	4.67	3.20
Southern Europe	1365.00	7.70	2.35	1.41
Western Africa	11,786.00	34.20	12.63	10.76
Western Asia	3480.00	16.70	5.29	3.51
Western Europe	1122.00	4.70	1.41	0.86
World	**449,659.00**	**53.30**	**18.93**	**12.74**

Source: From IRRI (2013).

the edge of the ventral side), white belly (middle of the ventral side), or white back (a long white streak on the dorsal side).

The first stage of the milling process is dehulling, where the rice hull is removed from the rough rice. The resulting BR (unpolished or dehulled rice) is then subjected to abrasive milling, which eliminates the bran to yield white rice (WR) (polished or milled rice). Boiled or steamed WR is the form commonly consumed as staple food. Different by-products or co-products from rice processing include hull, bran, polish, and broken grains.

12.2 Rice proteins

Proteins are present in the different milling fractions of rice: rough rice or paddy (5.6–7.7%), rice hull (2.0–2.8%), BR (7.1–8.3%), rice bran (11.3–14.9%), and milled rice (6.3–7.1%) (Juliano, 2007). Valuable rice proteins are considered healthy for human consumption because of important nutritional and health properties. Aside from being uniquely hypoallergenic (Helm and Burks, 1996), they have a desirable amino acid profile with particularly good amounts of the essential amino acid lysine, hypocholesterolomic

properties, and antioxidant activities. The quality of rice protein is nutritionally superior to wheat and corn.

12.2.1 Classification

There are four types of rice proteins according to the protein solubility classification of Osborne (1907). These are albumins, globulins, prolamins, and glutelins, which are soluble in water, salt solution, alcohol solution, and alkali solution, respectively.

Albumins constitute about 3.8–8.8% of total endosperm protein (Cagampang et al., 1966) and about 35% of the rice bran (Adebiyi et al., 2009). Their molecular weight (MW) ranges from 14 to 16 kDa, with isoelectric point between 6 and 8 (Juliano, 2007). Albumins are water soluble because of the presence of enough net electric charge and the absence of extensive disulfide cross-linking (Hamada, 1997; Shewry and Casey, 1999).

Globulins are present in rice endosperm at 9.6–10.8% (Cagampang et al., 1966) and about 15–26% in rice bran (Krishnan et al., 1992). They have a wide range of MW as evidenced in the four types of globulins reported, namely, α-, β-, γ-, and δ-globulins, with apparent MWs of 25.5, 15, 299 kDa and higher, respectively (Morita and Yoshida, 1968). Using SDS–PAGE, 25 kDa and 16 kDa globulin polypeptides were identified (Krishnan et al., 1992). Globulins contain high amounts of sulfur-containing amino acids (cysteine and methionine) but have low levels of lysine. They are salt soluble because of the presence of net electrical charge (Hamada, 1997).

Albumins and globulins are known as metabolic proteins. Their isolation is not as simple because they are co-extracted with water because of the presence of some salts in rice grains (Juliano, 2007). To isolate these proteins separately, repetitive precipitation (Iwasaki et al., 1982), ultracentrifugation (Mawal et al., 1987), and dialysis (Hamada, 1997; Juliano, 2007) must be conducted.

Prolamins comprise 2.6–3.3% (Cagampang et al., 1966) and about 4% (Fabian and Ju, 2011) in rice endosperm and bran, respectively. They are the hydrophobic protein fractions localized in protein bodies I (PBI). The MW of prolamins ranged from 12 to 17 kDa (Padhye and Salunkhe, 1979). Three polypeptide subunits with apparent MWs of 10, 13, and 16 kDa were identified (Ogawa et al., 1987). Prolamins contain high levels of glutamic acid or glutamine, alanine, glycine, and arginine (Shyur and Chen, 1994). They are readily solubilized in alcohol.

Glutelins are the major storage protein in rice. The endosperm contains 66–78% (Cagampang et al., 1966), whereas the bran has about 11–27% (Mitsuda et al., 1967; Sawai and Morita, 1968). They were found to have high MW, ranging from 45 to 150 kDa (Hamada, 1997), and are heterogeneous. Glutelins are composed of two major polypeptide subunits, namely, the α-(acidic) and β-(basic) subunits with apparent MWs of 30–39 and 19–25 kDa, respectively (Juliano, 2007). They are difficult to be hydrolyzed because of hydrophobic, hydrogen and disulfide bonding (Juliano, 2007), extensive aggregation (Sugimoto et al., 1986), and glycosylation (Wen and Luthe, 1985). Glutelins are extremely insoluble in water but are readily soluble in acidic (pH < 3.0) or alkaline (pH > 10.0) (Shih, 2004) conditions.

12.2.2 Protein bodies

Rice storage proteins are located in specialized structures called protein bodies found in the embryo, aleurone layer, and endosperm (Juliano, 2007). They differ drastically in gross composition (Juliano, 1985). The embryo and aleurone protein bodies have globoids containing phytic acid potassium magnesium salts. The protein bodies in the embryo contain 98% albumin, whereas those in the aleurone layer have 66% albumin, 7% globulin, and 27% prolamin, plus glutelin (Juliano, 2007).

Rice endosperm accumulates two types of protein bodies: (i) the spherical PBI rich in prolamin and (ii) the irregular-shaped PBII rich in glutelin and globulin (Juliano, 2007). The ratio of PBI to PBII was estimated to be 1:1.6 and the total proteins were approximately 53% glutelin, 35% prolamin, and 12% globulin (Krishnan and White, 1995).

12.2.3 Rice bran proteins

Rice bran is a major by-product or co-product of the rice milling process. It is the outer component of BR and is composed of the bran layers, germ, and a small portion of the endosperm. Bran, which is obtained when BR is polished to produce WR, comprises 8.8–11.5% by weight of BR (Connor et al., 1976). It is produced in large quantities, with an estimated amount of 60 to 68 million metric tons (66 to 74 million tons) available globally (Ryan, 2011).

Rice bran has good nutritional value and is rich in protein, lipid, dietary fiber, vitamins, and minerals. Early reports on the composition of rice bran indicate the presence of 8–12% moisture, 10–16% protein, 15–22% lipids, 7–11.4% fiber, 6.6–9.9% ash, and 34.1–52.3% carbohydrates (Juliano, 1985; Saunders, 1990). Recent data on the proximate composition of rice bran is shown in Table 12.3.

Table 12.4 presents the fractions of albumins, globulins, prolamins, and glutelins in rice bran. The protein in rice bran has desirable amino acid profile (Table 12.5). Its lysine content is approximately 3–4%, which is higher than the proteins from rice endosperm, other

Table 12.3 Proximate composition (%) of rice bran

Rice bran	Moisture	Protein	Fat	Crude fiber	Ash	Carbohydrates	Reference
Full-fat	10.5	12.6	12.8	2.8	14.5	46.8	Shih et al., 1999
Full-fat	8.5	12.6	21.1	5.6	9.0	43.1	Jiamyangyuen et al., 2005
Full-fat	11.2	15.4	19.9	13.7	7.9	–	Cao et al., 2009
Full-fat	6.5	13.7	22.5	10.5	9.8	37.2	Sadawarte et al., 2007
Defatted	12.4	13.9	1.9	6.0	10.1	55.6	Jiamyangyuen et al., 2005
Defatted	8.4	16.8	4.0	11.0	15.2	45.4	Sadawarte et al., 2007
Defatted	10.3	20.8	–	16.7	14.9	–	Bandyopadhyay et al., 2008
Defatted Sieved	11.0	24.1	–	13.9	11.3	–	Bandyopadhyay et al., 2008
Defatted	6.6	14.7	3.5	11.7	12.5	50.7	Yadav et al., 2011
Defatted	10.3	19.3	0.9	14.4	12.4	41.1	Yeom et al., 2010

Table 12.4 Protein fractions (%) in rice bran

Protein	Cagampang et al., 1966	Hamada, 1997	Cao et al., 2009	Adebiyi et al., 2009
Albumins	37	34	43	37
Globulins	36	15	12	31
Prolamins	5	6	3	2
Glutelins	22	11	40	27

cereals, or legumes (Yeom et al., 2010). In addition, rice bran proteins are hypoallergenic. The properties and food uses of rice bran proteins were extensively reviewed by Prakash (1996). Fabian and Ju (2011) discussed the properties and extraction methods of rice bran proteins, whereas Ali et al. (2010) summarized their processing and functionality.

Table 12.5 Amino acid composition (g/16 g N) of rice bran

Amino acid	Houston et al., 1969	Connor et al., 1976	Wang et al., 1999	Juliano 2007
Alanine	6.15	6.18	6.1	6.92
Arginine	8.28	7.19	8.9	7.55
Asparagine	–	–	–	
Aspartic acid	9.09	9.56	8.0	9.33
Cysteine	2.32	2.24	1.6	–
Glutamic acid	13.58	13.09	12.5	13.22
Glutamine	–	–	–	–
Glycine	5.47	5.27	5.4	6.24
Histidine	2.71	2.42	2.9	2.53
Isoleucine	3.94	3.53	3.9	4.05
Leucine	6.91	6.72	7.4	7.29
Lysine	4.81	4.41	4.7	4.64
Methionine	2.32	1.83	2.2	2.18
Phenylalanine	4.47	4.20	4.6	4.53
Proline	4.23	3.79	-	4.76
Serine	4.68	4.52	4.1	4.72
Threonine	3.78	3.69	3.7	3.95
Tryptophan	–	–	1.2	0.77
Tyrosine	3.13	2.54	3.3	–
Valine	6.00	5.85	6.3	6.28

12.2.4 Rice endosperm proteins

Although majority of rice proteins can be found in the bran, the endosperm also contains a small portion. Rice endosperm proteins can be obtained from milled rice, broken rice, or rice flour. Broken rice, which is one of the major by-products or co-products from the milling process, contains about 8% protein (Hou et al., 2010).

Proteins in rice endosperm are found in the form of discrete particles known as protein bodies, which accumulate as storage protein to provide a nitrogen source for seed

germination (Kumagai et al., 2006). Type I protein body consists of prolamins, whereas type II protein body has glutelins and globulins (Tanaka et al., 1980). The protein fractions in rice endosperm and amino acid composition are indicated in Tables 12.6 and 12.7.

Table 12.6 Protein fractions (%) in rice endosperm

Protein	Cagampang et al., 1966	Cao et al., 2009
Albumins	5	6
Globulins	9	6
Prolamins	3	7
Glutelins	83	79

Table 12.7 Amino acid composition (g/16 g N) of milled rice

Amino Acid	Houston et al., 1969	Juliano 2007
Alanine	5.51	5.51
Arginine	8.28	7.93
Asparagine	–	–
Aspartic acid	9.05	8.95
Cysteine	2.52	–
Glutamic acid	17.74	18.54
Glutamine	–	–
Glycine	4.54	4.34
Histidine	2.25	2.24
Isoleucine	4.63	4.11
Leucine	8.04	7.86
Lysine	3.51	3.44
Methionine	2.88	2.24
Phenylalanine	5.20	5.09
Proline	4.42	4.47
Serine	5.12	5.01
Threonine	3.53	3.40
Tryptophan	–	1.11
Tyrosine	4.86	–
Valine	6.45	5.81

12.3 Extraction of rice proteins

Rice proteins are extremely complex owing to the wide range of molecular weights and different types of proteins present. Albumins, globulins, prolamins, and glutelins are soluble in the solvents water, salt, alcohol, and alkali solutions, respectively. They have different degrees of hydrogen, hydrophobic, and disulfide bondings that link the polypeptides together. This complexity poses a great challenge in the extraction and separation of rice proteins.

12.3.1 Rice bran proteins

12.3.1.1 Alkali extraction The conventional method in isolating rice bran proteins is alkali extraction followed by precipitation at the isoelectric pH. Many researchers have used this method in obtaining proteins from full-fat, defatted, or stabilized rice bran. The various extraction conditions employed resulted in different yields and protein content (Table 12.8).

Table 12.8 Yield and protein content of rice bran proteins extracted using the alkali method

Sample	Yield (%)	Protein content (%)	Reference
RBPC[a]	13.8–20.5	22.8–31.3	Connor et al., 1976
RBPC	–	41.2	Bera and Mukherjee, 1988
RBPC	8.06	69.16	Jiamyangyuen et al., 2005
RBPC	–	52–59	Chandi and Sogi, 2007
RBPC	–	36.5	Sadawarte et al., 2007
RBPI[b]	–	86.2	Bandyopadhyay et al., 2008
RBPI	42.6	69.3	Yeom et al., 2010
RBPC	13.2	37.6	Yadav et al., 2011

[a]RBPC, Rice bran protein concentrate.
[b]RBPI, Rice bran protein isolate.

Defatted rice bran was extracted using alkaline method, which yielded 82% protein at pH 12.0 and 33% at neutral pH (Chen and Houston, 1970). Connor et al. (1976) described a mild alkaline extraction or rice bran using dilute NaOH at 24°C. Acid or heat precipitation produced protein concentrate from full-fat rice bran with 23–31% protein (Connor et al., 1976).

Rice bran protein concentrate (RBPC) was prepared from defatted bran from three rice cultivars (Basmati 370, Basmati 386, and HBC 19) using alkaline extraction (Chandi and Sogi, 2007). The protein contents were 54.08%, 58.92%, and 52.46%, respectively.

Bran was obtained from rice bran oil extraction plant, defatted and passed through an 80-mesh sieve (Bandyopadhyay et al., 2008). Rice bran protein isolate (RBPI) was then prepared by alkaline extraction and recovered by isoelectric point precipitation. Its protein content was 86.2%.

The proximate composition of rice bran proteins extracted using this method is indicated in Table 12.9.

Table 12.9 Proximate composition (%) of rice bran proteins extracted using the alkali method

Sample	Moisture	Protein	Fat	Crude fiber	Ash	Carbohydrates	Reference
RBPC[a]	5.6	37.6	1.4	1.7	6.5	50.7	Yadav et al., 2011
RBPC	6.2	36.5	1.5	1.5	5.5	49.4	Sadawarte et al., 2007
RBPI[b]	–	86.2	–	1.8	0.3	–	Bandyopadhyay et al., 2008
RBPI	3.8	77.6	0.7	1.5	5.0	10.3	Yeom et al., 2010

[a]RBPC, Rice bran protein concentrate.
[b]RBPI, Rice bran protein isolate.

12.3.1.2 Enzymatic extraction Aside from alkali extraction, an alternative method for the extraction of rice bran proteins is the use of enzymes. This is especially advantageous for food applications. Enzymes such as carbohydrases and proteases can help facilitate the release of proteins by different actions.

Carbohydrases are carbohydrate-hydrolyzing enzymes that attack the cell wall components. These carbohydrases, which include cellulase, hemicellulase, and xylanase, therefore, aid in the liberation of proteins. Because most of the carbohydrates in rice bran are cellulose and hemicellulose, the most commonly used carbohydrases are cellulase and hemicellulase (Ansharullah et al., 1997; Shih et al., 1999). On the other hand, proteases are enzymes that hydrolyze protein to peptides. According to Hamada (1999), two classes of protease exist. Exoprotease splits single amino acids from either ends of the peptide chain, whereas endoprotease attack peptide bonds in the interior of the polypeptide chain.

12.3.2 Rice endosperm proteins

12.3.2.1 Alkali extraction Since most of the endosperm proteins are glutelins, alkali extraction is an effective method in isolating them. In fact, a maximum of 97% of the protein was extracted using 0.1 N NaOH or KOH (Cagampang et al., 1966).

12.3.2.2 Enzymatic extraction Starch-hydrolyzing enzymes such as α-amylase, glucoamylase, and pullulanase are commonly utilized to remove the starch in rice endosperm to facilitate the separation of the proteins (Shih, 2003). Protein isolate with more than 90% protein content was produced from rice flour treated with heat-stable α-amylase at 97°C for 2 hours. (Morita and Kiriyama, 1993).

12.4 Functional properties of rice proteins

Proteins from plant sources have long been used as ingredients in the food industry. The success in the utilization of a specific protein as food ingredient depends greatly on its solubility. This critical parameter affects the protein's behavior and functionality.

12.4.1 Rice bran proteins

RBPCs were prepared from defatted bran from three rice cultivars (Basmati 370, Basmati 386 and HBC 19) using alkaline extraction (Chandi and Sogi, 2007). Their functional properties are presented in Table 12.10. RBPCs of Basmati 386 and HBC 19 had comparable solubility and significantly higher than that of Basmati 370. Foaming properties were affected by pH, salt, and sugar concentration. Emulsifying capacity ranged from 24 to 74%, and the study showed that these RBPCs were stable under various conditions of pH, salt, and sugar; thus, they have the potential to be utilized in the food industry as good emulsifiers.

The foaming capacity and foaming stability of RBPC extracted by alkaline method were 11.0% and 90.6%, respectively. It had emulsion activity of 40.0% and emulsion stability of 44.9% (Yadav et al., 2011).

Table 12.10 Nitrogen solubility index and foaming properties of RBPC from three rice cultivars

Functional property	Basmati 370	Basmati 386	HBC 19
Nitrogen solubility index (%)	47.69	72.67	73.14
Foaming capacity (%)			
pH 5	0.20	0.40	5.20
7	5.20	8.70	8.10
9	4.80	10.10	10.03
Salt 0.5%	9.09	11.20	11.10
1.0%	6.25	8.78	9.20
1.5%	4.80	8.83	10.02
Sugar 5%	6.25	7.93	12.23
10%	4.80	7.63	12.13
15%	5.76	4.41	9.21
Foam stability (half-life)			
pH 5	0	0.65	4.30
7	2.50	1.67	4.0
9	1.33	2.50	3.67
Salt 0.5%	2.50	3.38	8.81
1.0%	7.91	5.83	11.27
1.5%	9.99	13.38	2.99
Sugar 5%	4.0	3.83	3.33
10%	34.20	30.70	24.0
15%	42.60	38.40	29.60

Source: From Chandi and Sogi (2007).

RBPI from defatted rice bran meal was prepared by alkaline extraction followed by isoelectric point precipitation (Bandyopadhyay et al., 2008). It was then hydrolyzed with 0.1% papain at pH 8.0 and 37°C for 10, 20, 30, 45, and 60 minutes to determine the effects on its functional properties (Table 12.11).

12.4.2 Rice endosperm proteins

Cao et al. (2009) compared the functional properties of proteins in BR and WR extracted from the same rice variety. The isoelectric point of these rice proteins was found to be pH 4.5. Because the solubility of protein in aqueous solutions is dependent on pH, the lowest solubility obtained was at pH 5, and it increased at higher and lower pH values. The substantial increase in solubility under acidic (pH < 4) and alkaline (pH > 7) conditions is the result of protein denaturation and hydrolysis. When the pH value was over 7 or below 4, the solubility of WR proteins was higher than those from BR.

Proteins in BR and WR had the lowest foaming capacity at pH 5. The foaming capacity of BRP and WRP increased 3.65- and 5.52-fold, respectively, when the pH was increased from 5 to 11.

Maximum emulsifying volume of 44.32% was obtained from BRP and 47.06% from WRP at pH 11. WRP, which is mainly composed of glutelins, were better emulsifiers than

Table 12.11 Functional properties of rice protein isolate (RPI) and different rice protein hydrolysates (RPH) modified by papain

Functional property	RPI	RPH10	RPH20	RPH30	RPH45	RPH60
Solubility (%)						
pH 3	28.5	29.8	33.4	39.1	44.4	44.9
5	54.1	59.6	60.1	60.3	61.1	61.5
7	79.6	83.6	84.7	86.2	86.6	87.0
9	84.5	86.3	86.9	91.6	91.7	94.8
Emulsion activity index (m^2/g)	108.9	149.6	150.8	156.9	167.1	185.4
Emulsion stability index (min)	36.9	46.9	47.3	45.9	46.9	48.0
Foam capacity (%)	70.0	117.6	120.2	133.4	140.6	151.2
Foam stability (mL)						
10 min	71.0	68.0	67.0	65.0	63.0	60.0
30 min	65.0	60.0	60.0	59.0	57.0	55.0
45 min	59.0	57.0	58.0	57.0	55.0	53.0
60 min	56.0	54.0	54.0	53.0	52.0	52.0

Source: From Bandyopadhyay et al. (2008).

the other protein in strong acid or alkali conditions. Emulsifying capacity was also found to be inversely related to salt concentration (Cao et al., 2009).

The effects of controlled glycosylation, deamidation and enzymatic hydrolysis on solubility and emulsifying properties of rice endosperm proteins extracted by alkaline method were studied (Paraman et al., 2007). Glycosylation renders the proteins more hydrophilic while deamidation increases the negative charge and hydration (Schwenhe, 1997). The highest solubility (68%), emulsion activity (0.776), and emulsion stability (24 minutes) were obtained from the rice protein modified by controlled alkali-deamidation (Table 12.12). Glycosylation had positive effects on the emulsion activity (0.721) and stability (26.8 minutes) of the protein. Enzymatic hydrolysis only slightly improved the solubility (33%), emulsion activity (0.468), and emulsion stability (17.5 minutes); therefore, glycosylation and deamidation were more effective than hydrolysis by Alcalase.

Table 12.12 Solubility and emulsifying properties of modified rice protein isolates (RPIs)

RPIs	Solubility (%)	Emulsion activity (A_{500})	Emulsion stability (min)
[a]RP control	18.0	0.266	14.7
RP alcalase	33.3	0.468	17.5
RP glucose	39.7	0.721	26.8
RP xanthan gum	38.6	0.661	26.1
RP deamidation	68.3	0.776	24.0
[b]RP IEP	14.7	0.282	15.5
RP UF	37.2	0.376	22.6
RP UF-DF	40.1	0.414	21.4

[a]Paraman et al. (2007).
[b]Paraman et al. (2008).

Paraman et al. (2008) improved the functional properties of protein concentrate from rice endosperm by using ultrafiltration, which is an alternative method in recovering the proteins isolated by alkali extraction. Its solubility was higher than the protein isolate recovered by the conventional isoelectric precipitation by 37%. The improved solubility contributed to better emulsion activity and stability of the protein concentrate.

12.5 Applications in foods

Protein energy malnutrition remains to be a problem in the developing countries. Because traditional protein sources are usually expensive and not always available, there is an increasing demand for alternative economical sources of proteins; therefore, interest in plant proteins surges because they are relatively cheap and widely available. These proteins can be utilized by the food industry to increase the nutritional value of food products at low cost.

Interest in rice as a potential source of inexpensive high-quality proteins has increased recently. Rice proteins have unique nutritional properties such as well-balanced amino acid profile and hypoallergenicity compared with other cereals and legume proteins; therefore rice protein concentrates and isolates can serve as valuable ingredients in many food applications. Ali et al. (2010) summarized some of the applications of rice proteins in foods.

12.5.1 Infant formulas and baby foods

The unique nutritional and hypoallergenic properties of rice proteins make them suitable ingredient for infant food formulations (Helm and Burks, 1996). In fact, rice bran protein has been used as hypoallergenic milk replacers in infant formulas (Landers and Hamaker, 1994). Rice proteins were also utilized in infant foods (Hansen et al., 1981). They could also replace allergenic cereal foods and add variety to the restricted diets of children with food allergies (Helm and Burks, 1996).

12.5.2 Bakery products

Jiamyangyuen et al. (2005) explored the potential of alkali-extracted RBPC in the preparation of wheat bread. The optimum extraction conditions of pH 11 and 45 minutes yielded 69.16% protein content and 8.06% yield. Adding RBPC at 1–5% levels increased the protein content up to 12.10% and corresponding increase in fiber content (Table 12.13). On the basis of sensory evaluation, bread with 1% RBPC had similar color, taste, flavor, texture, and overall acceptability as the control, which was made from 100% wheat flour (Table 12.14).

RBPC prepared by alkaline method was again used to enrich bread (Sadawarte et al., 2007). The optimum extraction conditions (pH 11, 60°C, 60 minutes) produced a maximum protein yield of 36.5%. The levels of substitution for refined wheat flour were 5%, 10%, and 15% protein concentrate, respectively. The protein content of the bread increased from 9% for the control (100% wheat flour) to 12.3%, 16.5%, and 21.1%,

Table 12.13 Proximate composition (%) of bread and biscuits added with rice bran protein concentrate (RBPC)

Sample	Moisture	Protein	Fat	Fiber	Ash	Carbohydrates
[a]Bread with added RBPC						
Control	28.53	9.01	7.53	0.49	0.69	53.86
1.0	29.74	9.84	7.67	1.68	0.71	50.25
2.0	30.55	10.16	7.71	2.00	0.70	49.03
3.0	28.82	10.52	7.63	2.19	0.69	50.07
4.0	27.81	11.59	7.95	2.50	0.67	49.65
5.0	27.17	12.10	8.31	2.59	0.70	49.08
[b]Biscuit with added RBPC						
Control	2.5	7.3	33.1	1.4	1.3	58.3
5.0	3.5	9.6	34.0	1.5	1.6	53.7
10.0	3.8	12.4	34.1	1.5	1.7	44.4
15.0	3.9	15.4	34.6	1.5	2.0	37.1

[a]Jiamyangyuen et al. (2005).
[b]Yadav et al. (2011).

respectively. The samples were subjected to sensory evaluation for color, taste, flavor, texture and overall acceptability. Bread with 5% rice bran protein concentrate was comparable with the control in terms of all the sensory attributes considered. Sensory quality of bread was affected negatively when the level of substitution was beyond 5%.

Aside from bread, RBPC prepared by wet alkaline extraction method was also incorporated in biscuits (Yadav et al., 2011). The yield and protein content of RBPC were 13.2%

Table 12.14 Sensory properties of bread and biscuits added with rice bran protein concentrate (RBPC)

Sample	Color	Appearance	Odor	Flavor	Taste	Texture	Overall liking/Acceptability
[a]Bread with added RBPC							
Control	7.35	–	6.75	–	7.47	6.95	7.55
1.0	6.25	–	5.75	–	6.70	6.85	6.80
2.0	6.10	–	5.85	–	6.07	6.15	5.90
3.0	6.05	–	5.20	–	6.20	5.85	6.10
4.0	5.60	–	5.25	–	5.90	5.80	5.80
5.0	6.35	–	5.50	–	6.42	6.00	6.25
[b]Biscuit with added RBPC							
Control	2.5	7.8	–	8.7	8.3	7.8	8.2
5.0	3.5	7.7	–	7.5	7.8	7.2	7.6
10.0	3.8	6.8	–	7.5	7.2	7.5	7.1
15.0	3.9	6.2	–	7.0	6.8	6.3	6.6

[a]Jiamyangyuen et al. (2005).
Nine-point Hedonic scale: 1 = very undesirable, 2 = undesirable, 3 = moderately undesirable, 4 = slightly undesirable, 5 = neither undesirable nor undesirable, 6 = slightly desirable, 7 = moderately desirable, 8 = desirable, 9 = very desirable.
[b]Yadav et al. (2011).
Nine-point Hedonic scale: 1 = dislike extremely and 9 = like extremely.

and 37.6%, respectively. Biscuits were added with 5%, 10%, and 15% RBPC. As shown in Table 12.13, the protein content of the biscuits increased from 7.3% (control) up to 15.4% (with 15% RBPC). The biscuits with 5% RBPC were evaluated to be similar in color, taste, flavor, texture, and overall acceptability as the control (Table 12.14).

12.5.3 Other products

A 10% substitution of rice bran with high amounts of protein was reported to be suitable in the preparation of pork meatballs (Huang et al., 2005). This resulted in decreased hardness, gumminess, and chewiness as shown by the textural profile analysis.

Rice products have been used as ingredients in gels, puddings, and ice creams (Chrastil, 1992). Rice proteins have found applications in breakfast cereal (Bakar and Hin, 1985), snack foods (Capanzana et al., 1984) and edible films (Shih, 1996; Adebiyi et al., 2008). Protein concentrates from rice bran have been incorporated in the preparation of beverages, pasta and confections (Saunders, 1990) as well as in soups, sauces, gravies, meat products, and other savory applications (Giese, 1994; Weir, 1986).

Similar to hydrolysates from other protein sources, rice bran protein hydrolysates may also be used as nutritional supplements, functional ingredients, and flavor enhancers in foods, coffee whiteners, confectionary, and in the fortification of soft drinks and juices (Fabian and Ju, 2011).

12.6 Conclusions

The importance of rice as major food crop is unquestionable. It is the staple food of a significant portion of the world's population and provides majority of their carbohydrate and protein requirements. Boiled or steamed milled rice is the most commonly consumed form.

Reduced food supply causes the persisting problem in protein–energy malnutrition. This results in the continuing pursuit to explore alternative sources of food proteins. Because rice proteins offer unique nutritional properties, they can help address the nutritional status of humans. Rice proteins can be extracted from rice bran and broken rice, which are the major by-products of rice processing; however, the complexity and insolubility of rice proteins limit their application as ingredients in the food industry. To address these concerns, various investigators have devoted time and resources to optimize the extraction methods and modification of rice proteins. Consequently, they have been utilized in infant formulas, baby foods, baked goods, meat products, and other items.

Although numerous efforts have been made to improve the extraction efficiency and functionality of rice proteins, continuous research must be conducted to maximize the nutritional benefits from rice proteins. Hypoallergenicity, an interesting amino acid profile, and nutritional and health benefits are good enough reasons to fully explore the potential of rice proteins as value-added ingredients not only in the food industry but also in the pharmaceutical, cosmeceutical and other major industries.

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13
Sorghum and Millet Proteins

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13.1 Introduction

Sorghum ranks fifth worldwide in terms of production among the cereal grains, and pearl millet, one of the many species lumped into the classification of "millets," ranks sixth. These rankings tend to underestimate the importance of these grains, however, as sorghum and millet provide the major source of energy and protein for roughly 1 billion people across various parts of Africa and Asia in the semi-arid tropics (Belton and Taylor, 2004). Developing countries account for the vast majority of the land under sorghum and millet cultivation; land which is typically in very dry conditions with poor soils (Taylor and Belton, 2002). The majority of sorghum and millet produced in the semi-arid tropics is used for human consumption, whereas in developed countries, sorghum and millet are considered mainly a feed grain with sorghum increasingly used for biofuel production.

13.2 Taxonomy

Sorghum is a member of the grass family Poaceae and all cultivated sorghum is now classified as *Sorghum bicolor* ssp. *bicolor* (Moench) (Dahlberg, 2000). Harlan and de Wet (1972) divided *Sorghum bicolor* into five major races – *bicolor, guinea*, *caudatum*, *kafir*, and *durra*. They also described 10 intermediate races that are combinations of the major

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races (Harlan and de Wet, 1972; Dahlberg, 2000). For a complete discussion of the classification of sorghum, readers are directed to Dahlberg (2000).

The term "millet" can refer to any one of several members of the Poaceae family of grasses. Millets have been grouped into two broad categories: (i) pearl millet [*Pennisetum glaucum* (L.) R. Br.] and (ii) the small or minor millets, which includes proso millet [*Panicum miliaceum* (L.)], foxtail millet [*Setaria italica* (L.) P. Beauv.], finger millet [*Eleusine coracana* (L.) Gaertn.], barnyard millet [*Echinochola crusgalli* (L.) P. Beauv.], and teff (*Eragrostis tef*) among others (Obilana and Manyasa, 2002; Amadou et al., 2011).

13.3 Production and distribution

Sorghum is grown around the world for a variety of uses. Regionally, Africa was the major producer of sorghum during 2000–2010, accounting for 39% of the world's sorghum production, followed closely by the Americas as the second largest producing region (Figure 13.1). Sorghum production by country for this same time period shows the United States produced the most sorghum (10.6 M tonnes), followed by Nigeria (7.85 M tonnes) and India (7.28 M tonnes) (Figure 13.2). Within the United States, sorghum is grown primarily in the central Great Plains, with Kansas typically the top sorghum producing state, followed closely by Texas (Figure 13.3).

Considering all types of millets together as one class of grain, the leading millet producing region in the world was Africa, followed closely by Asia (Figure 13.4). India was by far the top millet producing country for this time span, averaging 10.7 M tonnes (Figure 13.5).

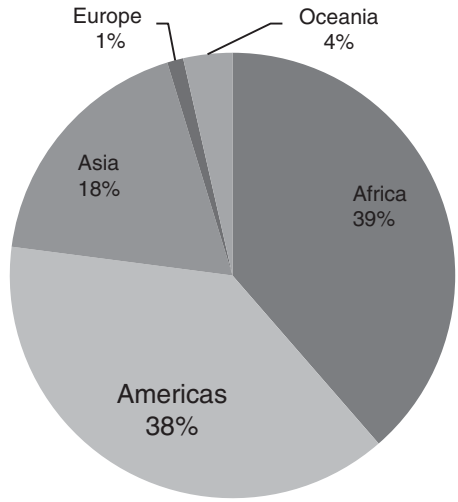

Figure 13.1 Percent sorghum production by world region for 2000–2010. Data from http://faostat3.fao.org/home/index.html

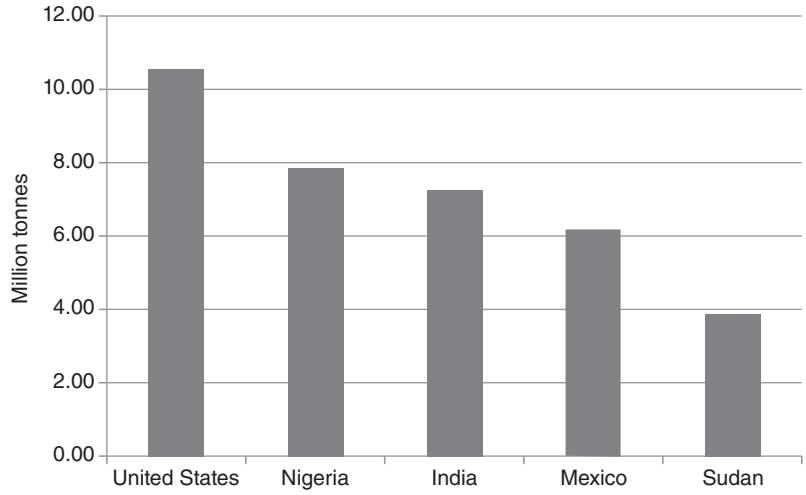

Figure 13.2 Sorghum production (millions tonnes) of the top five sorghum-producing countries between 2000 and 2010. Data from http://faostat3.fao.org/home/index.html

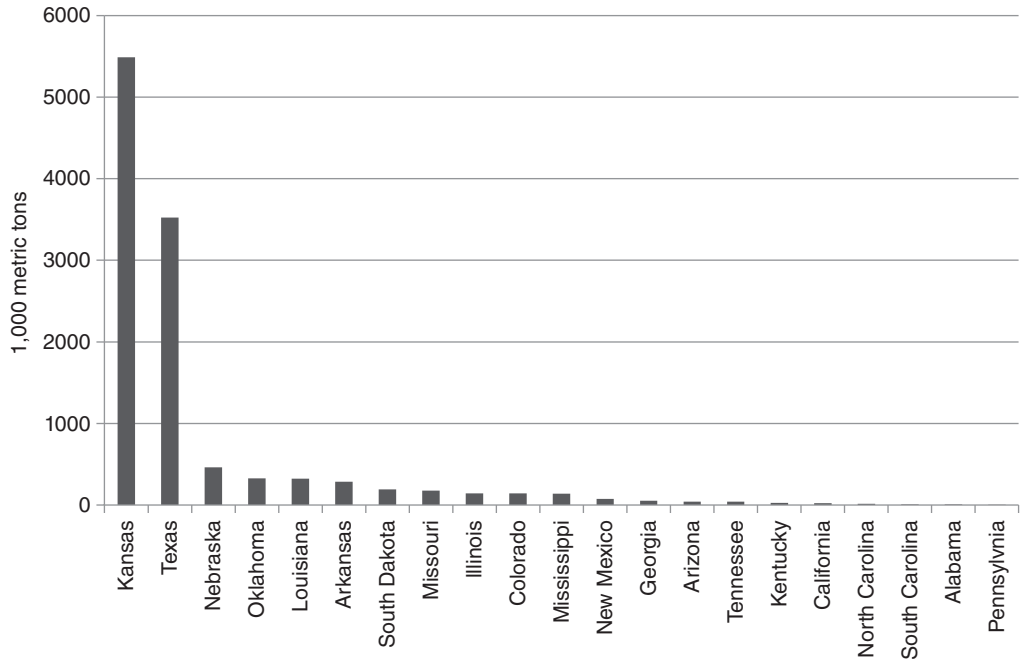

Figure 13.3 Average grain sorghum production for the United States ranked by state for 2007–2009. Data from http://www.nass.usda.gov/Publications/Ag_Statistics/index.asp

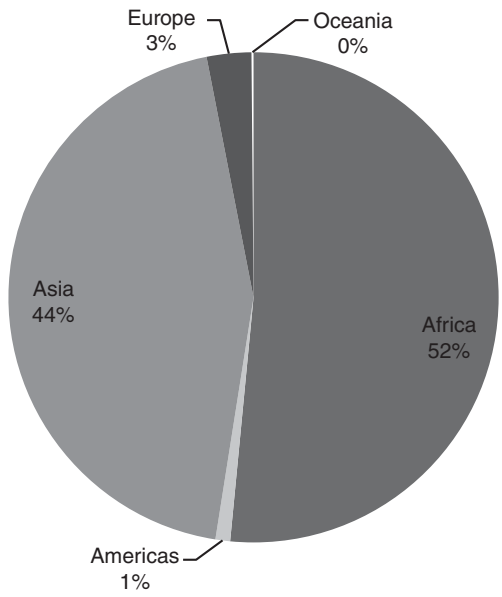

Figure 13.4 Percent total millet production by world region for 2000–2010. Data from http://faostat3.fao.org/home/index.html

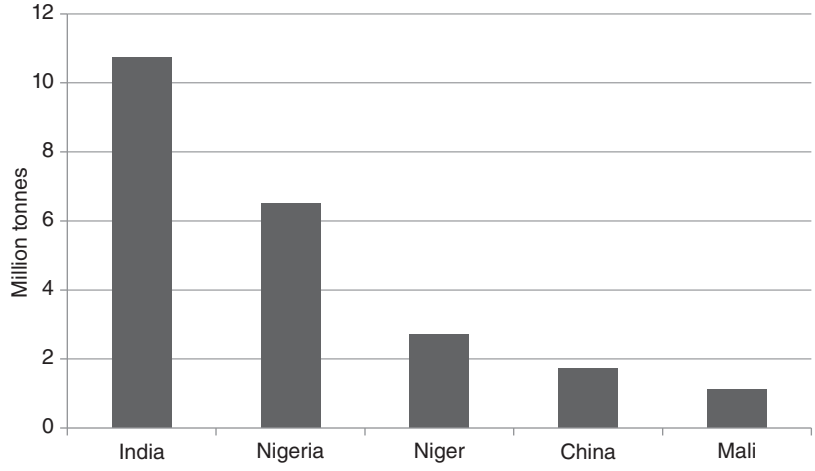

Figure 13.5 Millet production (millions tonnes) of the top five millet-producing countries between 2000 and 2010. Data from http://faostat3.fao.org/home/index.html

13.4 Physicochemical properties of sorghum and millet proteins

13.4.1 Sorghum grain structure

A brief review of sorghum kernel anatomy is necessary to provide a context to describe the distribution of sorghum proteins within the grain. Great variation exists in the phenotypic expression of the many cultivars of sorghum grain; however, in general, the sorghum kernel may be described as a spherical, naked caryopsis–containing a kernel germ and endosperm. Kernel weights varying from between 16 and 30 mg (Lásztity, 1996; Bean et al., 2006a) to 3 and 80 mg (Waniska, 2000) have been reported, the former range being typical of commercial US varieties. The sorghum caryopsis includes a protective outer covering called the "pericarp" that exists in a variety of colors, including white, yellow, brown, red, black, and many subtle permutations of these colors. In sorghum, a testa layer resides beneath the pericarp and may or may not be pigmented. The presence of a pigmented testa layer is indicative of so-called tannin sorghums that contain proanthocyanidins (Waniska, 2000; Waniska et al., 1992). As not all sorghums contain a pigmented testa, not all sorghums contain tannins—a common misperception about sorghum (Dykes and Rooney, 2006). Tannins are powerful antioxidants and may have unique human health benefits (Awika and Rooney, 2004; Dykes and Rooney, 2006) as well as being beneficial from an agronomic standpoint by aiding in reduction of grain losses from crop pests and microflora; however, numerous studies have shown the presence of condensed tannins have a negative impact on the nutritional availability of sorghum protein (Duodu et al., 2003; Taylor et al., 2007). An example of the relative proportions of the major constituents of a typical sorghum kernel were described by Hoseney (1994), and reported to compose 7.9% pericarp, 9.8% germ, and 82.3% endosperm. The endosperm to germ ratio of a typical sorghum kernel is 8.4:1 (FAO, 1995a). Figure 13.6 provides the physical perspective on the distribution of the various kernel parts previously described.

13.4.2 Endosperm protein

Apart from genotype, multiple environmental factors, including soil conditions, water availability, fertilizer application, and growing temperatures, affect the protein content of sorghum grain. In studies of the distribution and relative percentages of total protein within sorghum grain, the largest percentage was found in the endosperm and ranged from 80% to 84% of total protein. The germ accounted for 9.4–16% of grain protein, and the pericarp contained an additional 3–6.5%(Serna-Saldivar and Rooney, 1995; Taylor and Schössler, 1986).

The starchy endosperm is the largest single kernel component tissue, and represents the greatest value of the typical cereal grain (Evers and Millar, 2002). During seed development nutrients accumulate in the form of starch granules and protein bodies embedded in a protein matrix and are stored for use as nutrient sources for the germinating embryo. A cross section of a sorghum kernel (Figure 13.6) reveals the nonuniform appearance of the starchy endosperm, which is divided into two major regions differing in physical and

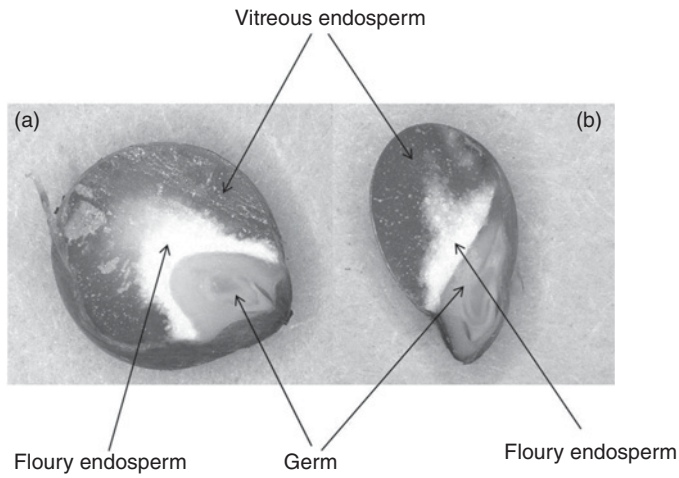

Figure 13.6 Cross section (a) and longitudinal section (b) of a sorghum grain

chemical characteristics. The peripheral or outer endosperm layer has been variously referred to as the hard, vitreous, corneous, or glassy endosperm. Similarly, the central or inner endosperm layer is frequently referred to as the soft, floury, or opaque endosperm. Numerous studies using electron microscopy provide details of the physical differences that exist within the vitreous and floury endosperm layers (Figure 13.7) (Hoseney et al., 1974; Seckinger and Wolf, 1973; Shapter et al., 2008; Shull et al., 1990). The vitreous endosperm is characterized by tightly packed polygonally shaped starch granules surrounded by a continuous intergranular protein matrix containing embedded protein bodies. The density of this packing arrangement results in a translucent "glassy" appearance. The floury endosperm, in contrast, contains relatively spherical, loosely packed starch granules with visible intergranular air spaces within which a thin or discontinuous layer of protein matrix and protein bodies reside. The presence of air spaces within the floury endosperm causes a diffraction of incident light resulting in the distinctive opaque appearance.

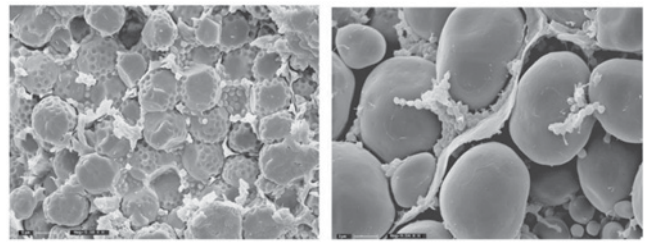

Figure 13.7 Scanning electron micrographs of (a) the outer layer (vitreous) endosperm and (b) soft (floury) inner endosperm layer of sorghum. Reproduced with permission of Elsevier

13.4.3 Protein bodies and matrix

As mentioned previously, the kernel endosperm is by far the major repository of sorghum kernel protein. Nutrient availability and functional attributes of sorghum grain protein are greatly affected by the form in which this protein exists within the endosperm. Seed storage proteins evolved as a means of storing nitrogen over potentially long periods of time for later use during plant reproduction and development, and in mature cereal grains may represent from 50% to more than 80% of total protein (Hamaker et al., 1995; Shewry and Halford, 2002).

One mechanism for achieving nitrogen storage in the seeds of plants was the development of specialized organelles known as protein bodies (Shewry et al., 1995). Amino acids needed for growth at germination can be stored for years, protected in the membrane-bound protein bodies (Möntz, 1998). Formed in the endoplasmic reticulum (ER), the protein bodies consist almost entirely of prolamin proteins (Möntz, 1998; Herman and Larkins, 1999; Seckinger and Wolf, 1973; Taylor et al., 1984a). The protein body prolamins develop within the ER in the form of large oligomeric aggregates (Herman and Larkins, 1999). Proteins in the interior of the protein bodies are predominantly composed of α-kafirin along with smaller quantities of β- and γ-kafirin (Shull et al., 1992). The periphery of the protein body on the other hand, is thought to contain an abundance of the β- and γ-kafirins. Containing high levels of cysteine, it is postulated the β- and γ-kafirins could form a cross-linked shell around the α-kafirins in the interior of the protein body (Belton et al., 2006). Protein bodies located in the vitreous endosperm were described by Shull et al. (1992) as 0.3 to 1.5 μm spheroids. Likewise, the floury endosperm protein bodies were similar if somewhat smaller in size, but exhibited somewhat irregular shape.

Also deriving from the ER, the endosperm matrix protein appears to provide a connecting structure within which the protein bodies and starch granules reside (Chandrashekar and Mazhar, 1999). In addition to protein storage, the matrix probably also functions as an enzyme source for starch and protein hydrolysis (Wu and Wall, 1980; Taylor et al., 1984b). On a quantitative basis, the protein matrix was considered the second most important endosperm fraction in a study by Taylor and Schössler (1986). The primary composition of the protein matrix appears to be in the form of glutelins based on solubility characteristics and amino acid composition (Taylor et al., 1984b; Taylor and Schössler, 1986).

13.4.4 Endosperm proteins and kernel hardness

Sorghum proteins also appear to play a crucial role in the relationship of endosperm type and kernel hardness. Shull et al. (1990) examined the differences in proteins of the hard and soft endosperm over the course of development of sorghum varieties varying in endosperm texture. They observed the presence of a continuous protein matrix in the vitreous endosperm as well as faster development of a protein matrix correlated with the harder sorghum varieties. In a review concerning grain strength in sorghum and maize, Chandrashekar and Mazhar (1999) advanced this concept further by concluding protein bodies were formed at earlier stages of development and the production of matrix was favored in vitreous versus floury grains. In a similar study, Mazhar and Chandrashekar

Table 13.1 Crude protein and essential amino acid content of sorghum[a]

					S	g/100g protein				
Reference	% protein[b]	Ile	Leu	Lys	Met	Phe	Thr	Trp	Val	His
Khalil et al., 1984	15.6	4	13.7	2.6	1.4	5.2	3.2	0.9	4.8	1.8
Pontieri et al., 2010	11.3	2.06	13.3	2.67	3.62	6.37	3.34	n/a	3.61	2.44
Ejeta et al., 1987	12.0	3.8	13.1	2.5	1.7	5.2	3.3	0.9	5.1	2.2
Ebadi et al., 2005	11.1	4.01	12.53	1.79	1.43	4.68	3.22	2.24	5.28	2.27
Ahmed et al., 1996	9.0	4.1	13.5	2.4	1.9	4.7	4.3	n/a	7.6	3.1
Subramanian et al., 1990	12.1	4.28	14.61	2.44	0.86	4.5	3.32	η a	5.77	2.07
Solsuski and Imafidon, 1990	11.3	2.26	9	1.32	9	3.02	1.96	0.75	3.64	1.38
da Silva et al., 2011b	10.4	3.75	14.8	2.08	1.65	4.74	2.73	n/'a	4.79	1.95
Youssef, 1998	13.3	3.65	13.2	2.11	1.36	5.62	3	n/a	4.55	1.82

[a]Data adapted from listed references.
[b]Values represent averages when study used multiple samples.

(1993) noted the presence of greater cross-linking within kafirins among predominately hard endosperm cultivars.

13.4.5 Amino acid composition

Table 13.1 provides a cross section of whole kernel crude protein contents and essential amino acid profiles from a variety of studies to show how these parameters vary across an array of sorghum varieties and types. Similar to maize proteins, sorghum is low in lysine and threonine (Wall and Paulis, 1978). In fact, sequence analysis of cDNA clones from sorghum mRNA indicated extensive homology between kafirin and zein (DeRose et al., 1989). Multiple amino acid repeat regions in zein found to be conserved in kafirin imply similarities in formation of three-dimensional rodlike structures as proposed for zein (DeRose et al., 1989; Argos et al., 1982).

13.4.6 Sorghum protein classes

Cereal proteins are commonly divided into four major classifications on the basis of their solubility in defined solvent systems. The major cereal protein classes consist of the albumins, globulins, prolamins, and glutelins (Hoseney, 1994; Branlard and Bancel, 2006). Despite significant overlap among the solubility classes, classification by solubility differences persists as a fundamental method for the characterization of cereal proteins. Various researchers through the years have modified solubility-based classification schemes and such schemes have been improved by considering protein functionality, structure, and sequence homology as well as solubility (Shewry et al., 1986).

Originally based on the work of T.B. Osborne (Osborne, 1907, 1924), the solubility classification of cereal proteins has been the subject of numerous modifications intended for optimizing recoveries and increasing selectivity (Landry and Moureaux, 1970; Jambunathan et al., 1975; Taylor et al., 1984c; Hamaker et al., 1995; Landry, 1997). Comparison of the relative distribution of different sorghum protein classes is complicated by

differences in study extraction protocols. A more in-depth discussion of sorghum protein solubility differences, extraction methods, and resulting implications follows later in this chapter.

The water-soluble albumins and salt-soluble globulins of sorghum predominate in the kernel germ and are reported to make up from 10 to more than 30% of total grain protein (Taylor et al., 1984c; FAO, 1995b; Wall and Paulis, 1978). The reported molecular mass ranges between 14 and 67 kDa (Taylor and Schössler, 1986). Nutritionally the albumin and globulin classes offer a more desirable balance of amino acids than the sorghum prolamins, particularly with respect to higher lysine contents (Taylor and Schössler, 1986; Lásztity, 1996).

Of the four major classes, the prolamins form the bulk of sorghum storage protein (Taylor, 1983). In sorghum, the prolamins are known as kafirins, and these terms will be used interchangeably in succeeding discussions of sorghum proteins. Studies by Taylor et al. (1984c) and Hamaker et al. (1995) showed kafirins contributing from 48 to more than 70% of total protein on a whole grain flour basis. Typically kafirins have been extracted using aqueous alcohol and aqueous alcohol plus reducing agents and have been found to exist almost exclusively in the form of protein bodies (Taylor et al., 1984c; Taylor et al.,1985; Shull et al., 1992; Chandrashekar and Mazhar, 1999; Shewry and Halford, 2002). The need for reducing agents is indicative of the disulfide cross-linking potential represented in this protein class. Also unique to prolamins is the abundance of proline and glutamine subunits, which can contribute over 30% of the total amino acid residues present in this fraction (Belton et al., 2006). The name "prolamin" derives from this fact.

There has been little research conducted to determine the secondary structure of kafirin, but analysis of isolated kafirins and sorghum protein bodies has revealed that α-helix is the predominant structure present. The structure of individual kafirins, especially the α-kafirins, has been speculated to be very similar to that of α-zein and an excellent discussion of this can be found in Belton et al. (2006).

An early study by Beckwith (1972) isolated glutelins by enzymatic degradation of starch from defatted globulin, albumin, and prolamin pre-extracted flour. The resulting glutelin fraction was said to make up more than 50% of the total protein. In contrast, using modifications of the Landry–Moureaux procedure (Landry and Moureaux, 1970), other studies have reported relative average glutelin percentages of 27.7% (Taylor et al., 1984b), 31.0% (Subramanian et al., 1990), and 4.5–34.3% (Nucere and Sumrell, 1979). Using a nonconventional approach, Hamaker et al. (1995) first tried extracting all the kernel proteins with a pH 10 borate buffer containing sodium dodecyl sulfate and β-mercaptoethanol, followed by precipitation of non-kafirin proteins by addition of aqueous ethanol. Using this extraction method, non-kafirin proteins were reported to only make up from 5.3% to 14.6% of total protein in whole grain samples. The glutelins appear to function as structural elements in the hard and soft endosperm matrix and may also be a source of enzymes for starch and protein reserve hydrolysis (FAO, 1995b; Taylor et al., 1984b).

13.4.7 Kafirin subclasses

The prolamins of cereals are composed of a homogenous mixture of monomeric, oligomeric, and polymeric protein species (Belton et al., 2006). As the major protein

component of grain sorghum, understanding the organizational composition of kafirin helps provide a rationale on which to consider the potential nutritional and functional contributions of sorghum grain. Current consensus regarding the kafirin subclass nomenclature follows from studies of maize prolamins (zeins). Kafirin and zein similarities based on solubility, molecular weight, structure, and amino acid sequence have been confirmed using comparisons of isolated fractions using SDS–PAGE and immunological cross reactivity (Shull et al., 1991; Shull et al., 1992; Mazhar et al., 1993). The resulting kafirin subclass groups are known as the α-, β-, and γ-kafirins. An additional kafirin subclass category known as δ-kafirin has been proposed using homology of DNA sequences with δ-zein (Belton et al., 2006).

The α-kafirins form the majority of the kafirins and make up approximately 70–80% of total kernel prolamin protein content (Watterson et al., 1993). The α-kafirins predominate in both the vitreous and floury endosperm, and are reported to contain approximately 1 mol% cysteine (Shull et al., 1992). Utilizing differential solubility, SDS–PAGE, and immunological techniques, Shull et al. (1991), described the α-kafirins as comprising two bands of Mr 23,000 and 25,000. Others (Mazhar et al., 1993) have reported molecular weights of 28,000 and 22,000 using similar techniques, and subdivided α-kafirin nomenclature into α1- and α2-kafirins, respectively. Although SDS–PAGE reveals only two major α-kafirin bands, it has been recently reported that up to 19 α-kafirin genes may be expressed in sorghum (Xu and Messing, 2008). Figure 13.8 shows the amino acid structure of two α-kafirins and their alignment with two α-zeins.

The γ-kafirins have been characterized on the basis of differential solubility, SDS–PAGE, and immunological techniques and have been reported to comprise 9–12% of vitreous endosperm and 19–21% of opaque endosperm by (Shull et al., 1991; Watterson et al., 1993). Once reduced, the γ-kafirins are soluble in water and in aqueous organic solvents that span a wide range of polarity such as 10–80% tert-butanol (Shull et al., 1991), despite being the most hydrophobic of the kafirins on the basis of free energy of hydration (Belton et al., 2006). The odd solubility of the γ-kafirins may reflect the influence of other noncovalent factors such as electrostatic repulsion from high histidine content (Belton et al., 2006). Cross reactivity with γ-zein for a migration band at Mr 28,000 provided confirmation of the molecular weight of γ-kafirin (Shull et al., 1991). The cross-linking potential of γ-kafirin is indicated by the relatively high cysteine content, having been reported as 7 mol% (Duodu et al., 2003). The extensive participation of γ-kafirin in the formation of kafirin oligomers and polymers has been corroborated in studies by El Nour et al. (1998) and Nunes et al. (2005). Figure 13.9 shows the amino acid structure of two γ-kafirins and their alignment to a γ-zein.

The β-kafirins have also been characterized on the basis of their solubility in 10–60% tert-butanol plus a reducing agent in addition to cross reactivity with β-zein antibodies and amino acid sequence, and three different Mr components have been identified as β-kafirins (16 kDa, 18 kDa, 20 kDa) (Shull et al., 1991; Shull et al., 1992). Later studies by Chamba et al. (2005) using molecular cloning techniques identified a single gene encoding for a mature Mr 18,745 β-kafirin species. Containing 5.8 mol% cysteine and ten cysteine residues, this even number suggests β-kafirin may be involved in intra- and interchain disulfide bonding (El Nour et al., 1998). These same researchers found evidence for

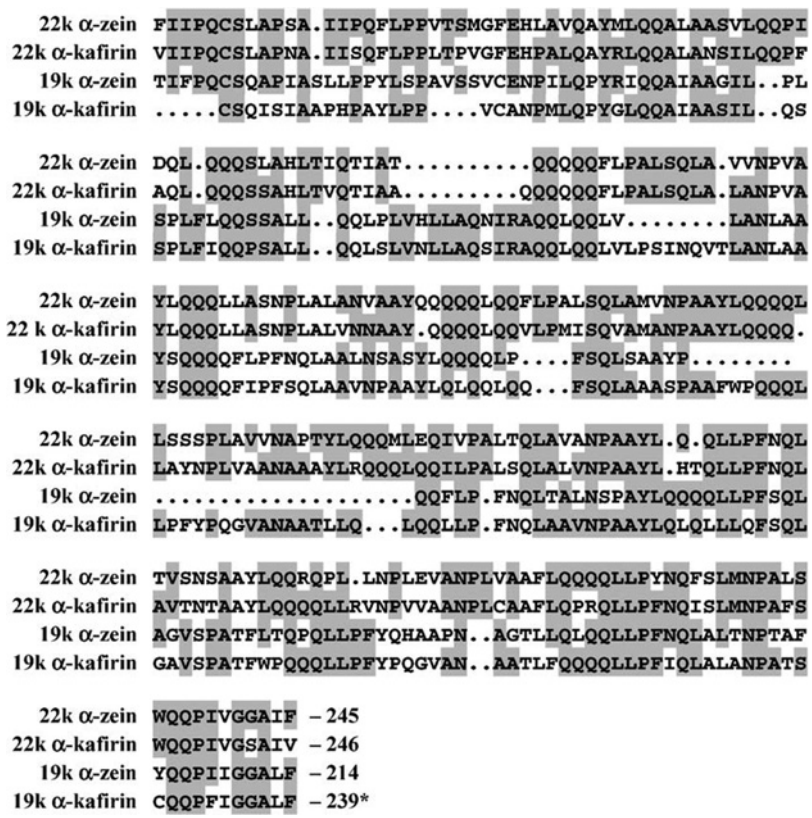

Figure 13.8 Amino acid sequence of two α-kafirins aligned with those from two α-zeins with conserved residues shaded. Reproduced with permission of Elsevier

β-kafirin acting as an oligomer chain extender in predominantly higher molecular weight polymers. The vitreous endosperm has been reported to contain 7–8% β-kafirin versus 10–13% β-kafirin in the opaque endosperm (Watterson et al., 1993). Figure 13.10 shows the amino acid structure of a β-kafirin and its alignment to a β-zein.

Possibly the least characterized of the kafirin subclasses, especially at the protein level, are the δ-kafirins. By employing molecular cloning experiments of cDNA encoding for δ-kafirin, Izquierdo and Godwin (2005) were able to describe a 147 amino acid polypeptide (Mr 16,000) rich in methionine. Two δ-kafirin DNA sequences have been described by Belton et al., 2006, GENPEPT AAK72689 and AAW32936, that showed extensive homology with Mr 14,000 δ-zein. Total seed storage protein in mature sorghum grain is thought to be made up of less than 1% δ-kafirin (Laidlaw et al., 2010).

13.4.8 Physicochemical properties of millet proteins

As mentioned previously, the term "millet" has been applied to grasses in a number of different genera. When categorized according to worldwide agricultural importance, the

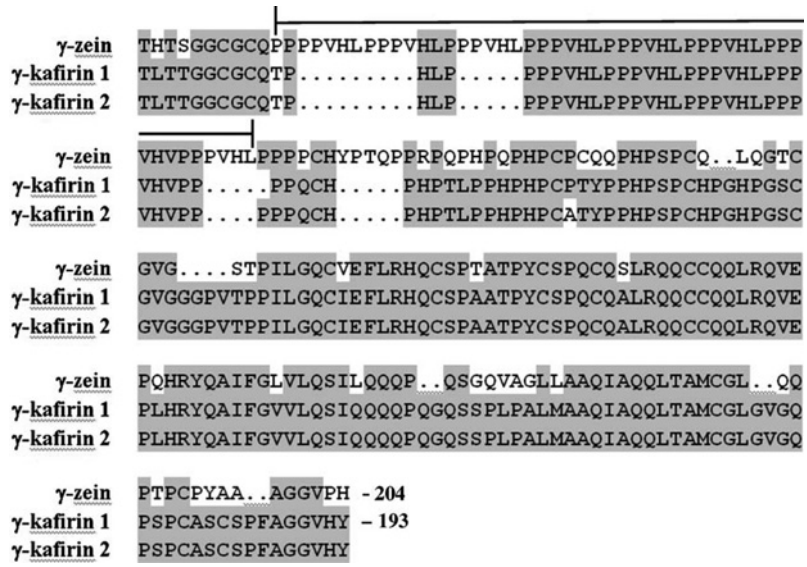

Figure 13.9 Amino acid sequence of two γ-kafirins aligned with that from a γ-zein with conserved residues shaded and the line showing repeated peptides. Reproduced with permission of Elsevier

most predominant millets are pearl, proso, finger, and foxtail millets. Other varieties of millet confined to production in more limited geographic areas include fonio, kodo, and teff (Dendy, 1995); therefore, descriptions concerning the physical and chemical properties of millets will focus on the four predominant millet varieties. Similar to sorghum, the many varieties of millet exhibit a great deal of variation from one another in their physical kernel qualities.

13.4.9 Grain structure

Kernel weights of pearl millet average about 8.9 mg (3–15 g/1000 kernels) (Hoseney, 1994; Serna-Saldivar and Rooney, 1995). The tear-shaped kernels of pearl millet exhibit

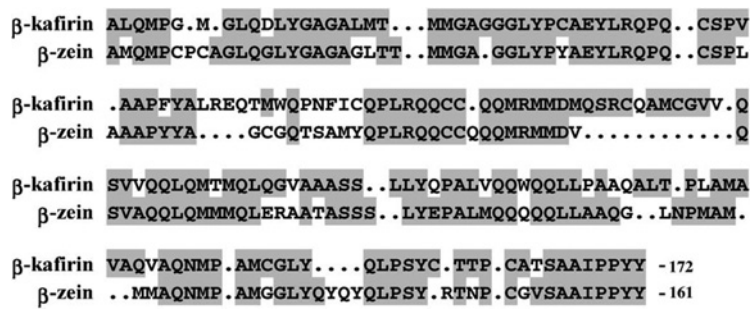

Figure 13.10 Amino acid sequence of β-kafirin aligned with that from β-zein with conserved residues shaded. Reproduced with permission of Elsevier

variations in color, but are most commonly slate gray (Hoseney, 1994). Pearl millet kernels contain a testa layer that is occasionally pigmented. Some finger millets have a pigmented testa layer containing condensed tannins, whereas proso millet does not (Dykes and Rooney, 2006). The presence of tannins in pearl millet has not been confirmed (ibid). Proportions of the major kernel constituents as described by Abdelrahman et al. (1984) were 7.2–10.6% pericarp, 15.5–21% germ, and 71–76.2% endosperm. Zelaznak and Varriano-Marston (1982) estimated the germ to comprise up to one third of the pearl millet caryopsis. A distinguishing characteristic of pearl millet is that the germ makes up a larger proportion of the endosperm than seen in sorghum, with an endosperm to germ ratio reported from 2.5:1 (Serna-Saldivar and Rooney, 1995) to 4.5:1 (FAO, 1995a), and germ protein content of about 20% (Obilana and Manyasa, 2002). Apart from a seed coat that is present in all varieties of millets, the endosperm contains both vitreous and floury regions similar to that of sorghum (Badi et al., 1976). The pericarp of pearl millet is strongly attached to the seed (caryopses), whereas in proso, finger, and foxtail millets the pericarp is attached at only one point on the seed (utricle) (Dykes and Rooney, 2006).

13.4.10 Endosperm protein

The protein content of millets, as in sorghum, is influenced by a variety of factors including variety, environment, and agronomic practices (Lásztity, 1996). Reported values for the protein content of pearl millet vary depending on source, and range from 8% to 19% (Nambiar et al., 2011) up to 5% to 23% (Lásztity, 1996). In comparison, the protein content of proso millet ranges from 11.3% to 12.7%, and is comparable to maize (Kalinova and Moudry, 2006). Distribution and relative percentages of total protein within the pearl millet grain consisted of 59.5% in endosperm, 31.2% in the germ, and an additional 9.4% in the pericarp (Serna-Saldivar and Rooney, 1995). The higher germ to endosperm ratio of pearl millet compared to sorghum results in higher protein content, as well as higher percentages of the albumin and globulin protein classes. This also favorably impacts the amino acid composition from a nutritional standpoint, especially in terms of lysine and tryptophan content.

13.4.11 Protein bodies and matrix

The prolamins of millets, as in sorghum, are found in protein bodies (Jones et al., 1970; Leite et al., 1999). Protein bodies present in the vitreous endosperm of pearl millet range in size from 1 to 2.5 μm, and are embedded in a protein matrix covering the tightly packed polygonal starch granules (Zeleznak and Varriano-Marston, 1982; Badi et al., 1976; Jones et al., 1970). In contrast, the floury endosperm contains loosely packed, spherical starch granules, and no protein bodies appear to be present (Badi et al., 1976).

13.4.12 Endosperm proteins and kernel hardness

Grain hardness is highly influenced by the proportions of vitreous and floury endosperm. Hard kernel millets have more vitreous endosperm relative to floury endosperm. These

Table 13.2 Crude protein and essential amino acid content of millet[a]

Reference	species	Number of varieties	% protein[b]	g/100 g protein He	Leu	Lys	Met	Phe	Thr	Trp	Val	His
Ejeta et al., 1987		2	10.6	4.3	11.2	3.0	2.6	5.4	4.3	n/a	6.0	2.4
Malleshi and Desikachar, 1986	pearl	1	12.6	n/a	n/a	3.7	2.6	n/a	3.7	1.5	n/a	n/a
Badi et al., 1976		1	12.3	3.9	9.8	3.6	1.9	5.0	4.1	n/a	5.2	2.6
Burton et al., 1972		1	16.0	4.1	9.6	2.5	4.1	4.7	3.1	n/a	1.2	2.3
Jones et al., 1970	proso	1	12.5	4.1	12.2	1.5	2.2	5.5	3.0	0.8	5.4	2.1
Ravindran, 1992		6	14.4	4.9	14.0	1.7	4.1	6.3	4.1	n/a	6.4	2.4
Barbeau and Hilu, 1993		10	9.8	4.6	11.5	2.3	2.9	6.3	4.4	n/a	6.7	2.6
Malleshi and Desikachar, 1986	finger	1	8.2	n/a	n/a	3.5	3.6	n/a	3.2	1.3	n/a	n/a
Ravindran, 1992		3	9.8	5.2	11.7	3.1	4.5	6.1	5.2	n/a	8.2	2.8
Ravindran, 1992	foxtail	4	15.9	5.1	16.0	1.9	4.0	6.2	4.5	n/a	6.3	2.3
Malleshi and Desikachar, 1986		1	11.4	n/a	n/a	3.0	2.1	n/a	3.1	1.1	n/a	n/a
Montiero et al., 1982	Italian	14	13.6	4.2	16.5	2.6	2.0	5.1	3.8	0.6	5.4	3.0

[a] Data adapted from listed references.
[b] Values represent averages when study used multiple samples/varieties.

proportions vary widely in pearl millet and reflect the corresponding variety of kernel hardness seen in this grain. Proso and finger millets exhibit intermediate hardness, and foxtail millet kernels are hard because of the predominance of vitreous endosperm texture (FAO, 1995a).

13.4.13 Amino acid composition

Table 13.2 provides a cross section of whole kernel crude protein contents and amino acid profiles from numerous millet studies to show how these parameters vary across an array of millet varieties and types. As mentioned previously, the higher germ to endosperm ratio of pearl millet results in a larger albumin and globulin fraction than for sorghum. As a result, the higher levels of lysine and tryptophan present in those protein fractions results in an improved amino acid composition (Serna-Saldivar and Rooney, 1995).

13.4.14 Millet protein classes

Compared with sorghum, the prolamin fraction in pearl millet is smaller ranging from 8% to 19%. Considered using the Landry–Moureaux solubility scheme, the relative proportions of the protein classes in pearl millet are albumins/globulins 22–28%, prolamin and prolamin-like 22–35%, and the glutelin and glutelin-like 28–32% (Nambiar et al., 2011). Using the same extraction method, Ejeta et al. (1987) compared the true prolamin and prolamin-like fractions of pearl millet to normal maize and sorghum. They found the true

prolamin fraction of millet to be comparable to that of maize, and higher than that in sorghum. The prolamin-like fraction, that is the fraction requiring aqueous alcohol plus a reducing agent to solubilize (i.e., cross-linked prolamins), was six times higher in sorghum than in pearl millet. This suggests the prolamins of pearl millet may be less prone to cross-linking and more nutritionally available. For comparison, protein fractions obtained by Dharmaraj and Malleshi (2011) for finger millet were albumins 8.14%, globulins 4.07%, prolamin and prolamin-like 46.6%, and a glutelin and glutelin-like protein content of 41.17%. A study by Kumar and Parameswaran (1998) examined how the storage protein class in foxtail millet varied over the course of seed maturation from 5 through 36 days after anthesis. Prolamin made up over 50% of total protein during all stages of development. The synthesis of prolamin polypeptides started 5 days after flowering (DAF) and was complete by 10 DAF, after which only changes in prolamin quantity occurred. Comparing various millets, Parameswaran and Thayumanavan (1995) found that while prolamin formed the major storage protein of foxtail millet, in proso millet glutelin performs this function. Similarly, the glutelins of kodo millet are the predominant protein fraction, constituting 40.4–52.1% of total protein (Sudharshana et al., 1988).

13.4.15 Extraction and isolation of sorghum and millet protein

Successful analytical characterization of cereal protein is frequently dependent on the prior application of appropriate extraction and isolation procedures. Considering their heterogeneous nature, the significant physical and chemical variations present in sorghum and millet proteins present challenges to complete and efficient extraction and isolation. From an analytical perspective, accuracy in chemical characterization often benefits by obtaining the various protein forms in states altered as minimally as possible to reflect their true *in vivo* nature. Industrial extractions, on the other hand, are concerned with acquiring protein in a form possessing those attributes most appropriate for a targeted end use, functionality, or product. In such instances, alterations or modifications stemming from the extraction process itself may actually serve to enhance or add value to the resultant component. Additionally pertinent to seed protein analysis, Branlard and Bancel (2006), stressed the importance of considering confounding factors to successful extraction procedures such as interference from cell wall material and other non-protein components, including starch granules and phenolic compounds, as well as the necessity for application of uniform and appropriate sample size reduction techniques. Successful extraction and isolation procedures continue to evolve and be modified. A recent review by de Mesa-Stonestreet et al. (2010) provides a thorough discussion, including historical as well as current research regarding sorghum extraction and isolation techniques. A brief discussion of a cross section of reported methods may serve as catalyst for new ideas, however, and is included subsequently.

13.4.16 Laboratory extraction procedures

Cereal protein extractions intended for laboratory characterization frequently utilize solubility fractionation for resolving protein classes and subclasses, as well as isolation from

non-protein components. Osborne (1907, 1924) developed methodology for extracting four major protein classes that included the albumins, globulins, prolamins, and glutelins of seed proteins using sequential extractions with water, salt solution, alcohol, and dilute acid or base solution, respectively. Johns and Brewster (1916) introduced an early modification to Osborne's procedure by using heated alcohol solutions, which were claimed to enhance the solubility of kafirin versus the maize prolamin zein. Numerous studies of cereal protein chemistry have relied on and confirmed the utility of Osborne's fractionation method (Virupaksha and Sastry, 1968; Skoch et al., 1970; Elkhalifa and El Tinay, 1994). Taylor et al. (1984c), systematically investigated changes to the original Osborne method to obtain a method specifically optimized for sorghum grain. Factors studied included pre-extraction defatting, NaCl concentration, temperature effect on prolamin extractions, and various changes in the extraction sequence order. Application of the optimized method to a total of 41 sorghum varieties allowed investigators to assign average protein class distributions represented by 50% prolamins, 25% albumins/globulins/low-molecular-weight nitrogen, and less than 30% glutelins (Taylor et al., 1984c).

Building on Osborne's techniques, Landry and Moureaux (1970) increased the number of fractions obtained by following the Osborne initial alcohol extraction with a reduced alcohol extraction step, and adding an additional final extraction using reduced basic buffer containing the detergent sodium dodecyl sulfate (SDS). In this scheme, cross-linked prolamins were isolated with the reduced alcohol (fraction III) and a previously unextractable glutelin fraction (fraction V) was solubilized by the detergent containing reduced base. The relationship between Osborne and Landry–Moureaux protein solubility fractions is presented in Table 13.3. The application of the original Landry–Moureaux extraction method and various method modifications and iterations has been successfully applied in numerous studies of sorghum proteins (Chibber et al., 1978; Guiragossian et al., 1978; Landry and Moureaux, 1980; Hibberd et al., 1985; Hamaker et al., 1986) that serve as testament to the usefulness of this fractionation technique. Table 13.4 illustrates how the Landry–Moureaux protein fractionation of a variety of sorghums aids in understanding the comparative impact of factors like high tannin content or high lysine expression in different sorghum lines; for example, the increased tannin levels of the high tannin varieties noted in Table 13.4 seems to correlate with the presence of a higher proportion of fraction V (true glutelin) that likely represents additional unextractable high-molecular-weight

Table 13.3 Relationship between Osborne and Landry–Moureaux sorghum protein classifications based on solubility fractionation

Osborne type Extraction solvent	Classification Protein fraction	Landry–Moureaux type Extraction solvent	Classification Protein fraction
saline	albumin + globulin	saline	I
aqueous alcohol	prolamin	aqueous alcohol aqueous alcohol + β-ME	II (true prolamin) III (prolamin-like)
alkaline/acidic solution	glutelin	alkaline + SDS alkaline + SDS + β-ME	IV (glutelin-like) V (true glutelin)

ME, mercaptoethanol; SDS, sodium dodecyl sulfate.

Table 13.4 Landry–Moureaux protein fractions of a variety of sorghums[a]

Reference	Number of varieties	% protein[b]	Fraction (% of N)[b]					
			I	II	III	IV	V	Residual
Jambunathan and Mertz, 1973	3	12.2	16.1	14.6	17.3	5.6	34.9	n/a
	3 high tannin	9.1	6.1	4.9	11.0	13.5	53.8	n/a
Vivas et al., 1992	1	11.9	16.1	20.0	44.0	n/a	14.7	8.9
	1	12.0	9.0	25.1	25.1	6.8	34.0	n/a
Guiragossian et al., 1978	2 high lysine	10.5	25.8	10.3	17.1	4.4	42.2	n/a
	1 high tannin	9.4	6.2	10.2	18.7	9.4	55.5	n/a
Hamaker et al., 1986	1	n/a	16.6	17.3	24.5	4.8	27.2	11.5
Hamaker et al., 1994	1	n/a	8.0	20.0	33.0	<0.1	20.0	14.0
Gorinstein et al., 1999	1	11.0	14.0	30.1	13.4	10.9	19.4	12.3
Subramanian et al., 1990	6	9.7	17.6	9.6	18.4	3.7	34.3	9.5
	2 high lysine	19.3	23.4	8.5	16.2	3.2	21.0	17.9

[a] Data adapted from listed references.
[b] Values represent averages when study used multiple samples.

protein complexes thought to be promoted by tannin–protein interactions (Guiragossian et al., 1978). The presence of significantly higher levels of fraction I (albumins + globulins) in the high-lysine sorghum varieties reflects the presence of higher lysine content in these proteins, and helps account for the enhanced nutritional value of these lines. Table 13.5 shows Landry–Moureaux protein fractions of various millets for comparison with sorghum.

Table 13.5 Landry–Moureaux protein fractions of a variety of millets[a]

Reference	Species	Number of varieties	% protein[b,c]	Fraction (% of N)[b]					
				I	II	III	IV	V	Residual
Okoh et al., 1985		7	14.3	22.5	40.3	6.3	9.6	21.2	n/a
Ejeta et al., 1987	pearl	3	12.7	26	40	4	8	16	n/a
Vivas et al., 1992		1	9.8	25.4	47.9	11.4	12.9	3.3	n/a
Nwasike et al., 1979		1	14.3	22.3	41.4	6.8	9.3	20.9	n/a
Kumar and Parameswaran, 1998		1	10.5	17.1	50.0	10.7	3.2	16.0	4.6
Kumar and Parameswaran, 1998	foxtail	1 high protein	11.2	12.4	51.7	12.4	3.1	15.5	4.8
Kumar and Parameswaran, 1998		1 low protein	9.2	13.4	56.0	8.9	3.4	13.4	4.8
Montiere et al., 1982		14	13.63 (w.b.)	17.1	56.1	7.3	9.2	6.7	n/a

[a] Data adapted from listed references.
[b] Values represent averages when study used multiple samples.
[c] Dry basis unless otherwise noted.
n/a, data not available.

Isolating seed storage protein organelles (protein bodies) before chemical fractionation offers opportunities for observing the physical characteristics, interactions, and other changes related to the application of controlled processing or degradation conditions. This was the procedure applied in two studies involving sorghum protein body composition and ultrastructure, as well as details about how protein bodies degrade during germination (Taylor et al., 1984a,b). The protein body isolation method involved wet-milling defatted flour that had been previously soaked in metabisulfite solution to reduce cross-linked proteins. Centrifugation allowed collection of a crude protein preparation that was further purified using density centrifugation through linear sucrose gradients. Protein body preparations consisting of 89.6% protein were obtained by this method, and provided material for detailed examination of the changes occurring over the course of seed germination. Using a different method of protein body isolation, Elkhalifa et al. (2009) isolated sorghum protein bodies by solubilizing the gelatinized starch in cooked whole grain flour/water preparations with α-amylase. Subsequent centrifugation, washing, and lyophilization allowed collection of protein body preparations used in testing for pharmaceutical applications in tablet formation.

As opposed to Osborne or Landry–Moureaux type selectively sequential extractions, Hamaker et al. (1995) developed a procedure whereby an initial extraction using a pH 10 buffer containing 1% sodium dodecyl sulfate and 2% β-mercaptoethanol extracts essentially all of the protein from decorticated and degermed sorghum or maize flour. Subsequent precipitation of non-prolamin proteins is accomplished by addition of the appropriate concentration and type of alcohol, resulting in selective differentiation of prolamins and non-prolamins. Purity of the prolamin fractions was tested using SDS–PAGE and determined to be pure. Prolamins obtained using this method reflected higher levels than those obtained using the conventional Landry–Moureaux procedure. Simplicity and accuracy were cited as primary advantages of this method. Park and Bean (2003) investigated ways to optimize the extraction factors and conditions of the Hamaker et al. (1995) method with the intent to reduce the time requirement and obtain optimal extraction reproducibility. Factors studied included the role of pH, detergent concentration and type, sample to solvent ratio, reducing agent type and concentration, and extraction time.

Exploring alternatives to protein extraction limitations imposed by methods relying only on manipulation of pH and organic solvent content, a number of studies have investigated the beneficial effects of solution additives in the form of detergents, chaotropes, and soaps, as well as extraction process energy input from sonication. Already discussed, Park and Bean (2003) identified the superiority of the anionic detergent SDS versus other cationic and zwitterionic detergents for sorghum protein extractions. Kang et al. (2008) provide an informative discussion on the mechanism of solubilization of hydrophobic protein complexes by detergents. Using only shorter chain length soaps (sodium salts of fatty acids) in distilled water, Fliedel and Kobrehel (1985) demonstrated up to 95% of sorghum flour proteins could be solubilized. A particular advantage noted by these researchers was that covalent bonds are not disrupted, thereby making further characterization of intact proteins possible. Application of sonication energy for solubilizing intact polymeric proteins from isolated hard and soft sorghum endosperm was used by Ioerger et al. (2007) to

characterize differences in protein cross-linking in those tissues. By extracting unreduced intact protein polymers for subsequent analysis, this technique allowed determination of molecular weight distribution differences in hard and soft endosperm. Extracting proteins from mashed and unmashed sorghum meal using sonication provided Zhao et al. (2008) with information with which to predict ethanol fermentation quality.

Understanding the relationships between cereal grain proteins and nutritional and functional qualities will require improvements in our understanding of protein structure components and how respective kafirin subclasses interact. Esen (1986) devised a method for isolating the subclass proteins of zein, the prolamin of maize similar to sorghum kafirin, into three fractions differing in polypeptide composition. Two isolation schemes were developed that took advantage of the differential solubilities of the three subclass fractions in various concentrations of 2-propanol plus reducing agent, and sodium acetate solution at pH 6.0. This method allowed determination of the relative amounts of each subclass component in zein as well as respective molecular weight contribution of each by SDS–PAGE analysis. A different approach was taken by Mazhar et al. (1993) for isolating α-, β-, and γ-kafirins. In this method, α-kafirin was isolated by extracting endosperm flour with 40% t-butanol to remove β-kafirins, followed by extraction of α-kafirins from the residue with 90% t-butanol. Subsequent lyophilization and washing with 5% β-mercaptoethanol to remove residual γ-kafirin left the purified α-kafirins. The β-kafirins were extracted from a fresh batch of endosperm flour with 30% t-butanol. This supernatant was lyophilized, the residue redissolved in 60% t-butanol and then centrifuged to remove undissolved non-prolamin contamination. Isolation of the γ-kafirin was taken from Watterson et al. (1990), and consisted of direct extraction of flour with 20% 2-mercaptoethanol that had previously been extracted with 0.5 M NaCl.

13.4.17 Industrial extraction

Methods developed for the commercial extraction of maize protein were optimized for use extracting sorghum proteins to find how initial protein extraction conditions relevant to industrial processes affected the products of subsequent isolation procedures (Bean et al., 2006b). Information on how specific groups of proteins might be targeted for production by manipulation of those conditions was also determined. Extraction conditions employed included nonreduced 70% ethanol, ultrasound, and using food-safe reducing agents. Isolation methods for protein precipitation that were applied to extracted proteins included lowering ethanol concentration and/or lowering pH, or addition of sodium chloride. They found that purity and yield of isolated protein was a function of initial extraction conditions, and led Bean et al. (2006b) to conclude that extraction manipulations of this type were feasible for the production of proteins for specific industrial applications.

In contrast to traditional prolamin extraction methods based on alcohols, Taylor et al. (2005a) used an alternative method for obtaining sorghum protein for industrial or food use. Acetic acid can solubilize hydrophobic proteins because of its low dielectric constant. After presoaking sorghum grain in a solution of food-compatible reducing agent (sodium metabisulfite), subsequent extraction with glacial acetic acid produced kafirin yields of 80–90%. Temperature control was necessary to prevent excessive polymerization compared

to alcohol extraction, but the resulting protein was postulated suitable for such industrial applications as edible bioplastic films or nut and fruit coatings.

Protein concentrates from sorghum and millet may be useful as emulsifiers as well as raw material for extruded foods. An extraction scheme developed by Wu (1978) to obtain protein concentrates from normal and high-lysine varieties of whole grain sorghum was based on extraction with alkali. Two extractions with 0.1–0.15 N sodium hydroxide at pH 11.8–11.9 followed by pH adjustment to mildly acidic conditions resulted in a 48–60% protein concentrate that was 90% soluble at pH 8.7–10.8. Lysine remained available and appeared not to be negatively affected by the harsh extraction conditions. Emulsifying activity and emulsion stability were evaluated in a simple soybean oil and water system. In a comparison of high-lysine sorghum varieties to commercial soy protein isolate, emulsifying activity of the sorghum concentrates (54% and 53%) was 8–9% better and emulsion stability (47% and 40%) averaged the same as the soy protein isolate.

Gao et al. (2005) conducted a study to find a kafirin extraction method that could be applied industrially for production of bioplastic films with desirable characteristics. A procedure using 70% ethanol with 0.5% sodium metabisulfite and 0.35% sodium hydroxide at 70°C resulted in protein extracts with properties most favorable in terms of yield, film forming properties, and food compatibility. Protein aggregation was minimized by maximizing extraction of kafirin with native like α-helical structure, and this resulted in films with more desirable properties.

Using sorghum as a wheat substitute for production of gluten free food products will require tapping the functional potential for kafirin–protein interactions. Schober et al. (2011) conducted an investigation on the effects of different isolation procedures on subsequent kafirin or zein aggregation characteristics with the goal of finding conditions conducive to formation of true dough systems. The application of successful zein extraction conditions proved insufficiently hydrophobic for obtaining kafirin extract with viscoelastic properties. Use of 83% isopropanol to obtain kafirin provided material that produced partially viscous aggregate when mixed in warm water with added reducing agent. Study results provided indications of the impact subtle changes in extraction and isolation conditions have on the potential industrial end-use properties of sorghum proteins.

For commercial extraction of any protein, a suitable starting material must be used. Although sorghum or millet flour is an obvious starting source for commercial isolation of these proteins, flour is generally low in protein (see section 13.4.2 on protein content). da Silva and Taylor (2004) investigated sorghum bran as a starting material for industrial (commercial) extraction of kafirin. Bran could be a coproduct from flour milling or decortication of sorghum and the bran produced in this research contained higher protein than whole grain flour because of the bran containing some outer layers of the endosperm, which are relatively rich in protein. Pure kafirin was able to be isolated from bran, but yields were lower than when using flour as a starting material, partially because of the bran containing high levels of fat, phenolics, and other compounds that could interfere with protein extraction. Bioplastic films made from proteins extracted from bran had different properties from films made from proteins extracted from sorghum flour (da Silva and Taylor, 2005).

Wang et al. (2009) investigated the use of distillers dried grains plus solubles (DDGS) as a source of starting material for kafirin isolation. As a by-product of ethanol fermentation, DDGS are typically much higher in protein than flour (35% for the DDGS used in this study). Three different extraction solvents were tested for their effectiveness in extracting protein from DDGS, acetic acid, aqueous ethanol–containing NaOH, and aqueous ethanol–containing HCl. Using acetic acid as solvent as described in Taylor et al. (2005a) resulted in highly purified protein (99%). Extraction yield was 24–57% depending on the solvent used.

13.5 Functional properties of sorghum and millet proteins

13.5.1 Functionality of isolated proteins

Compared to other food proteins, little research has been conducted on the functionality of sorghum and millet protein isolates. Babiker and Kato (1998) reported on the functionality of isolated sorghum proteins conjugated to polysaccharides. In this work, isolated sorghum proteins were mixed with either dextran or galactomannan, freeze dried and then heated at 60°C for 7 days. Extracted proteins were also cross-linked together using microbial transglutaminase. Conjugation of the sorghum proteins to either dextran or galactomannan greatly improved protein solubility across all pH ranges and improved solubility after heating. Likewise, conjugated proteins had improved emulsifying properties. Proteins cross-linked with transglutaminase showed only minor improvements in emulsifying properties and little change in solubility except for at pH 4. Although the conjugated proteins had improved solubility and emulsifying properties that could be beneficial in food systems, no characterization of the sorghum proteins extracted for this work was conducted. The use of a mild alkaline buffer (pH 8) would not be expected to extract significant amount of protein from sorghum (de Mesa-Stonestreet et al., 2010). The extraction protocol used by Babiker and Kato (1998) was adapted from that of Wu (1978); however, the original work of Wu used buffers at pH 11.8–11.9, which would likely extract proteins much more effectively than at pH 8. It would be valuable to repeat the work of Babiker and Kato (1998) using more efficient extraction procedures for the initial extraction of the sorghum proteins. Mohamed et al. (2010) used a combination of proteolysis and cross-linking with transglutaminase to modify the functional properties of sorghum proteins. In this work, sorghum proteins extracted using a pH 8 buffer were subsequently digested with chymotrypsin. The hydrolysates were then treated with transglutaminase and their functionality evaluated. Minor improvements in emulsifying properties were reported when the hydrolysates were treated with transglutaminase; however, improvements were also noted when the undigested proteins were also treated with transglutaminase. Emulsion stability was impacted to a much greater degree when either the undigested proteins or hydrolysates were treated with transglutaminase.

Musigakun and Thongngam (2007) conducted limited functionality studies on isolated kafirins. In this study, kafirins were extracted from decorticated grain from two different

sorghum lines using 70% ethanol containing 0.5% sodium metabisulfite and 0.35% sodium hydroxide. Water and oil binding capacity were evaluated with the isolated kafirin having higher oil binding capacity than water binding capacity, which is not surprising given the hydrophobic nature of kafirins. Interestingly, the isolated kafirin used in this project was found to form spherical structures and interacted tightly together when imaged by SEM.

In addition to the aforementioned functionality research on sorghum proteins, some studies have also been conducted on the functionality of isolated proteins from millets. Bailey and Sumrell (1980) used solutions of 70% isopropanol, alkaline extraction buffers and acidic extraction buffers in various orders and combinations to produce protein isolates from pearl millet. Hassan et al. (2007) extracted pearl millet proteins using a pH 8 buffer and hydrolyzed the isolated proteins with chymotrypsin. The hydrolysates were then modified by conjugation to galactomannan or modified by cross-linking with transglutaminase. As found with sorghum proteins (discussed above), the conjugated pearl millet hydrolysates had improved solubility across a wide pH range and improved emulsifying properties. Kamara et al. (2009) prepared protein isolates from two varieties of foxtail millet and evaluated their functionality. The protein isolates were prepared using an alkaline buffer at pH 9.5 and consisted of polypeptides ranging in molecular weight from around 14–66 kDa with minimum solubility between pH 4–5. Emulsifying and foaming properties were compared to that of soy protein isolate; the foxtail millet protein isolates were found to have a higher emulsifying capacity than and roughly equal foam capacity as that of the soy protein. This research was later expanded to look at the functional properties of foxtail millet protein hydrolysates (Kamara et al., 2010). In this research, defatted foxtail millet flour was digested with commercially available proteases including Protamex, papain, Alcalase, Favourzyme, and Neutrase. The use of Alcalase resulted in the highest amount of protein hydrolyzed in this study and hydrolysates produced using Alcalase had higher emulsifying and foam capacity than undigested foxtail millet protein isolates. Kamara et al. (2011) also investigated the use of macroporous adsorption resin to debitter foxtail millet hydrolysates.

The studies referenced earlier provide some insight into the functionality of sorghum and proteins from millets and their hydrolysates, though their application in food systems needs further investigation. Modification of isolated sorghum and proteins from millets and hydrolysates may prove beneficial to increasing the utilization of these proteins. Much of the work described earlier has used proteins extracted with weakly alkaline aqueous buffers. Because the majority of the proteins in sorghum and millets are the alcohol-soluble prolamins, research investigating the isolation, functionality, and modification of these proteins would prove valuable. Using proteases to digest sorghum/millets flour would appear to be a promising method for improving the utilization of both crops as this may avoid the need to first extract the proteins. The work of Kamara et al. (2010) demonstrated that commercial proteases could effectively digest proteins directly in flour. Freeing proteins either through extraction or hydrolysis is an important aspect to consider regarding improving the functionality of sorghum and millets as the majority of the protein in sorghum and millet is found in protein bodies and not accessible for interactions in foods.

13.5.2 Functionality in bioplastic film formation and encapsulation

An interesting use of isolated sorghum proteins in a food related area has been in the production of films and encapsulating agents. Buffo et al. (1997) first used laboratory isolated kafirins and compared their film-making potential to that of commercial zein. Taylor et al. (2005b) evaluated several different solvents for casting kafirins films including: 70% ethanol, isopropanol (55%, 70%, and 88%), lactic acid, glacial acetic acid, glycerol, and acetone (70% and 80%). Solvents were tested for their ability to solubilize isolated kafirin at various temperatures. Glacial acetic acid and lactic acid were found to be the best solvents for dissolving kafirin followed closely by 55% isopropanol and 70% ethanol. The use of glacial acetic acid as solvent for kafirins produced films with similar quality attributes as those cast from 70% ethanol; however, the use of glacial acetic acid had the added advantage that lower casting temperatures could be used (Taylor et al., 2005b). Gao et al. (2005) examined how the extraction conditions used to initially isolate kafirin impacted the protein's structure and film forming abilities. The best films were formed from kafirin that had been extracted using 70% ethanol with 0.5% sodium metabisulfite and 0.35% sodium hydroxide. It was reported that kafirins containing mainly α-helical structures with as little β-sheet as possible were preferred for film formation. A critical factor in the preparation of kafirin for film formation was how the protein was dried; applying heat during drying was deleterious to film forming properties (Gao et al., 2005). The effect of microwave heating isolated kafirin before its use in forming films was investigated by Byaruhanga et al. (2005). Heating of kafirin in a dry state had no impact on film properties; however, heating kafirin that had been wetted resulted in films with different properties from those made with unheated kafirins. Kafirins wet before microwave treatment produced films with lower digestibility, increased biodegradability, and reduced water vapor permeability. It should be noted that kafirins subjected to microwave heating had to be initially extracted from flour at room temperatures to minimize any heat-induced denaturation for effects to be seen (Byaruhanga et al., 2005). Research was also conducted to investigate the effect of applying microwave heating to cast kafirin films (Byaruhunga et al., 2007). Microwave heating improved the tensile strength of the films, and this effect was amplified when plasticizers were included in the films. The effects seen when heat was applied to the films was attributed to heat-induced disulfide bond formation between proteins (Byaruhunga et al., 2006).

Film properties were also modulated by incorporating condensed and hydrolysable tannins with kafirins (Emmambux et al., 2004, Byaruhunga et al., 2006). Addition of either condensed or hydrolysable tannins decreased the oxygen permeability and water absorption of kafirin films and altered their physical properties making the films "stiffer but less plastic" (Emmambux et al., 2004). The impact of condensed tannin addition on kafirin film properties was attributed to the effects of hydrogen bonding between tannins and kafirins (Byaruhunga et al., 2006).

More recently, isolated kafirins have been used to form protein microparticles (Taylor et al., 2009a, b). Kafirin microparticles were prepared by dissolving isolated kafirin in glacial acetic acid, lactic acid, or propionic acid. After an equilibration period of 16 hours,

water was added to the protein–organic acid mixture upon which microparticles were formed. Protein microparticles made from acetic acid were larger and had internal openings, whereas those made from 70% ethanol were smaller and had smooth continuous surfaces with few openings (Taylor et al., 2009a). Taking advantage of the porous nature of the particles made using acetic acid, Taylor et al. (2009b) demonstrated that kafirin microparticles could encapsulate catechin and condensed tannins. The encapsulated compounds were released from the microparticles over time demonstrating that the microparticles could be used in time release applications such as controlled delivery of antioxidants (Taylor et al., 2009b). In a related application, Elkhalifa et al. (2009) demonstrated that isolated kafirin could be used to form tablets for possible pharmaceutical use. Release of caffeine as a model drug from kafirin tablets was investigated and sustained release was observed with some pH dependence; release was somewhat faster at pH 1.3 than higher pH levels.

13.5.3 Applications of sorghum and millet proteins in foods

Proteins from sorghum and millets do not appear to play a major functional role in food products typically made directly from flour, that is, foods made from flour, not foods with isolated proteins added. Only a few studies have reported correlations to protein content or composition and food quality. Subramanian and Jambunathan (1982) reported that the amount of water-soluble proteins in sorghum flour was positively correlated with the quality of *roti* (a type of flat bread). Cagampang and Kirleis (1984) also found that the amount of water-soluble protein in sorghum was correlated to the adhesion of cooked sorghum paste, cooked grain texture and gel stiffness as was the amount of kafirins present. The water-soluble proteins of sorghum and millets are not found in protein bodies in the flour and thus are able to interact with other flour components.

Chandrashekar and Kirleis (1988) reported that sorghum protein bodies surrounding starch granules provided a "rigid cover" that prevented complete gelatinization of the sorghum starch. Zhang and Hamaker (2005) later found that proteins in sorghum flour formed complexes with starch and lipids, which influenced starch-pasting properties. Free fatty acids were involved in this complex and increases in free fatty acid levels during the storage of sorghum flour could result in changes to the functionality of the flour with regard to the production of food items such as porridges.

Hugo et al. (2003) reported that fermentation of sorghum flour resulted in deactivation of amylases and increased the viscosity of sorghum flour. This fermented flour was found to improve the quality of wheat–sorghum composite breads relative to the use of nonfermented sorghum flour. These authors speculated that fermented sorghum flour may have potential in a sourdough composite bread formula. Schober et al. (2007) investigate the use of sorghum sourdough breads for the production of wheat-free breads. Sourdough fermentation was found to degrade flour proteins, which may have improved the strength of the starch gel formed during baking, leading to an improvement in the wheat-free sorghum bread quality. Renzetti and Arendt (2009) investigated the use of a commercial protease (Neutrase) in the production of sorghum bread and found that the use of the protease resulted in decreased bread quality despite limited hydrolysis of the sorghum proteins.

The breads of Renzetti and Arendt (2009) were produced without the addition of hydrocolloids, whereas in the work of Schober et al. (2007) a hydrocolloid was added to the bread formula, making it difficult to directly compare results between these two studies. As noted earlier, hydrolysis of sorghum and millet proteins, either with fermentation or added enzymes, and its effect on flour functionality is an area where more research is needed as hydrolysis could be one avenue to disrupt the protein bodies and make proteins/peptides available for interaction with other flour components.

Recently, there has been interest on the functionality of sorghum proteins with regard to viscoelastic dough formation. This has been driven in part by the use of isolated corn proteins (zein) to produce wheatlike dough. Because the initial report of Lawton (1992) that demonstrated that isolated zein could form dough when mixed at elevated temperatures, there have been several reports further investigating this property of zein (Bugusu et al., 2001; Mejia et al., 2007; Oom et al., 2008; Schober et al., 2008, 2010, 2011; Erickson et al., 2011). As zein and kafirin appear to be similar in many aspects, isolated kafirins may also form dough when mixed under similar conditions. Oom et al. (2008) compared the viscoelastic properties of commercial zein isolates to laboratory prepared kafirins isolates. The isolated kafirins used by Oom et al. (2008) were found to have viscoelastic properties similar to that of the commercial zein isolates when mixed into resins; however, the kafirin-based resin quickly stiffened and after 2.5 hours at room temperature did not retain the same functionality as the zein (Figure 13.11). It was hypothesized that the stiffening of the kafirin resin was owing to the cross-linking of the kafirins over time in the resin. The kafirin isolates used in this study contained both γ- and α-kafirins, whereas the commercial zein isolate contained only α-zein. The γ-kafirins are known to contain high levels of cysteine and could, therefore, readily form disulfide cross-links. Such cross-links would not be expected in the commercial zein isolate as the α-zeins contain little cysteine. Johansson and Stading (2008) later demonstrated that kafirin resin formed with isolates with reduced levels of the β- and γ-kafirins was softer and aged more slowly than that made from kafirin isolates containing higher levels of the β- and γ-kafirins. Schober et al. (2011) investigated how extraction procedures influenced the viscoelastic properties of zein and kafirin. Zein and kafirin isolates that contained pure α-prolamins were found to have the best viscoelastic properties. Using more nonpolar extraction solvents (83% isopropanol vs 70% ethanol) to isolate the kafirins was found to be necessary to produce kafirins that had the some degree of viscoelastic properties as that of zein. This was attributed to the production of kafirin isolates with less β-kafirin when more nonpolar extraction solvents were used. The β-kafirins are known to have high levels of cysteine and would readily form disulfide cross-links. Collectively, the work of Oom et al. (2008), Johansson and Stading (2008), and Schober et al. (2011) indicate that the formation of disulfide cross-links is deleterious to the viscoelastic properties of kafirin (and zein).

The target of much research in the gluten-free food area has focused on simply cross-linking proteins with the idea that protein cross-links would impart gluten-like functionality to non-wheat proteins. It is apparent that not all protein cross-linking and network formation is equal, that is, simply forming cross-linked proteins does not automatically insure functionality similar to the large gluten polymers found in wheat.

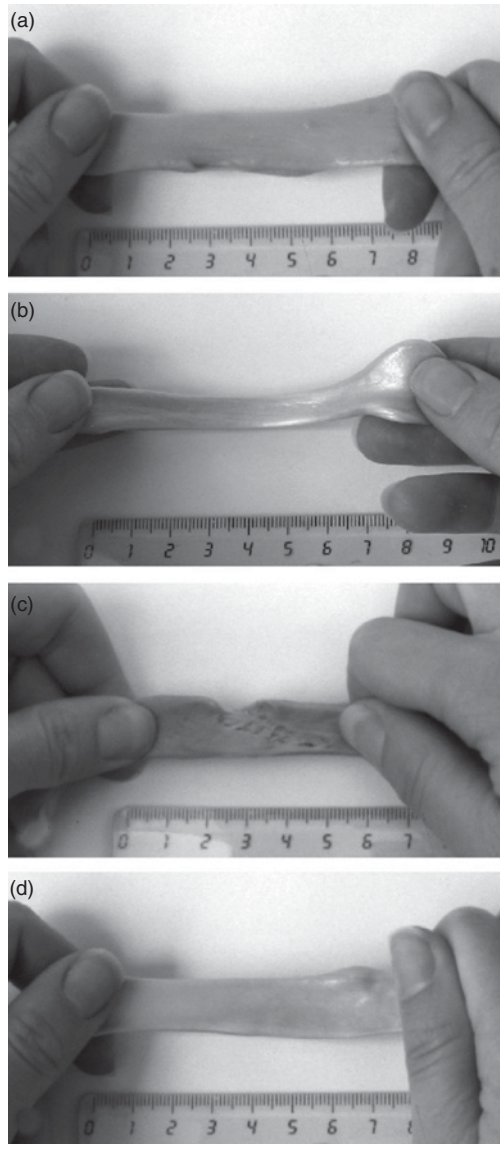

Figure 13.11 Freshly prepared and stretched resins of kafirin and zein: (a) kafirin resin freshly prepared (b) zein resin freshly prepared (c) kafirin resin stretch after 2.5 hours (d) zein resin stretched after 2.5 hours. Reproduced with permission of Elsevier

As can be seen from the limited research on the role of sorghum and millet proteins on foods, proteins play a relatively minor role in the functional properties of these flours. This is most likely because the majority of sorghum and millet proteins are found encapsulated in protein bodies and individual proteins are, for the most part, not free to interact with other flour components; however, it is interesting to note as discussed previously

Figure 13.12 Flour from normal sorghum (left) mixed with 18% vital wheat gluten, and flour from a high digestible sorghum (right) mixed with 18% vital wheat gluten. Reproduced with permission of Elsevier

that isolated sorghum and maize protein (and thus removed from protein bodies) do have some wheat-like viscoelastic properties when mixed under the right conditions, suggesting that freeing the proteins from body proteins may lead to improved functionality of sorghum as suggested by Bugusu et al. (2001). Interestingly, high digestible sorghum lines that have misshapen protein bodies have been found to have improved functionality when mixed with vital wheat gluten compared to sorghum lines containing normal protein bodies (Goodall et al., 2012). Sorghum flour from the high digestible sorghum was able to form dough when mixed with gluten, whereas sorghum flour from a normal sorghum did not (Figure 13.12). To create a more functional sorghum or millet flour by altering the properties of the proteins, scientists must take into account the intrinsic macromolecular structures of the proteins found in the flour itself. More research is needed that target methods to disrupt protein bodies in sorghum and millet flour followed by protein modifications to improve their functionality. Section 13.5.1 on the functional properties of isolated proteins from sorghum and millets provides evidence that the functional properties of these proteins can be modified, provided they are available to be modified. Modifying proteins directly in the flour by first disrupting the protein bodies could provide flours with improved functional properties for millions of people living in regions where sorghum and millets are basic food staples.

13.5.4 Effect of processing on protein structure and functionality

Just as the methods for extracting proteins from flour can modify protein structure and functionality, processing of flour into food products can also influence the properties of the proteins present in the flour. Vivas et al. (1992) investigated the impact on proteins when sorghum and pearl millet were made into acidic or alkaline porridges. Protein solubility was decreased for water and salt-soluble proteins and for prolamins extracted without use of a reducing agent. Solubility of the cross-linked prolamins and glutelins was increased however. Sorghum protein solubility has also been found to be modified during extrusion processing (Hamaker et al., 1994). Solubility of cross-linked prolamins was found to increase during extrusion processing along with an increase in protein digestibility. The amount of nonextractable protein greatly increased during extrusion, however.

These results suggest that extrusion processing of sorghum may have rendered some kafirins more soluble while, at the same time, decreasing the solubility of glutelin, presumably through aggregation and cross-linking. This change in solubility of the glutelins was seemingly not related to digestibility as digestibility increased during extrusion.

Vivas et al. (1987) used a sequential extraction procedure to examine changes in solubility of sorghum and maize proteins during tortilla production (using an alkaline processing procedure). The process of tortilla production altered protein solubility decreasing the solubility of water and salt-soluble proteins while increasing the solubility of the residual proteins. Changes in the molecular weight of some proteins were also noted. Interestingly, protein digestibility was decreased in the tortillas relative to the original unprocessed flour. Sorghum proteins were found to be more impacted by the processing than the maize proteins.

AwadElkareem and Taylor (2011) investigated the production of a fermented flatbread (*kisra*) from tannin and non-tannin sorghums and the effects on protein quality. Tannins are known to bind strongly to kafirins (Taylor et al., 2007) and *kisra* made from the tannin containing sorghum "had a more dense and brittle texture" than did the one made from the non-tannin sorghum (AwadElkareem and Taylor, 2011). This led AwadElkareem and Taylor (2011) to speculate that proteins must play a role in determining the quality of *kisra* and, presumably, tannin–protein interactions interfered with the sorghum proteins functional role during *kisra* making.

Both high-pressure (Vallons et al., 2010) and hydrothermal treatment (Dharmaraj and Malleshi, 2011) has been applied to sorghum and finger millet to alter their functionality. Protein extractability was greatly decreased when finger millet grain was hydrothermally processed with large decreases in the solubility of the albumins, prolamins, and glutelins observed. After the hydrothermally treated grain was decorticated, however, an increase in protein solubility was observed (Dharmaraj and Malleshi, 2011). No explanation was provided for these differences, but it is possible that the hydrothermal treatment impacted the outer layers of the grain more than the interior. High-pressure treatment (200 MPa) of sorghum batter resulted in batters with properties different from those of untreated batters. These differences were attributed to depolymerization of protein structures in the sorghum flour; however, no improvement in sorghum bread quality was observed when treated batters were added into sorghum bread formulas (Vallons et al., 2010).

One of the most important aspects of "processing" sorghum flour with regard to protein structure and function is cooking. It has long been known that the protein digestibility of sorghum decreases upon cooking (Duodu et al., 2003). While several factors have been put forth as the cause of sorghum proteins' unique behavior with regard to digestibility, the major cause appears to be an increase in disulfide cross-links when sorghum is cooked in the presence of water (Duodu et al., 2003; da Silva et al., 2011a). Wet-cooking sorghum was found to cause a decrease in kafirin solubility (Hamaker et al., 1986) and the formation of disulfide resistant oligomers (Duodu et al., 2002; Nunes et al., 2004; Ezeogu et al., 2005) that appear to be at least one reason why cooked sorghum has lower digestibility than cooked maize. The secondary structure of proteins in wet-cooked sorghum and maize protein bodies changes to include more antiparallel β-sheet structures with slight

losses in α-helix structures; such changes were greater in the kafirin proteins than in the maize proteins, which may also play a role in the differences in the digestibility between wet-cooked sorghum and maize (Duodu et al., 2001). This work is significant in that isolated protein bodies were used in these experiments and that changes to secondary structures were still observed, which indicated that changes to sorghum proteins during cooking could impact not only "free" proteins but also those in the protein bodies. Proteins in dry-cooked (popped) sorghum and maize showed similar changes to those caused by wet cooking, but to a lesser extent (Duodo et al., 2001). Popping sorghum does not lead to a decrease in protein digestibility (Parker et al., 1999), but the disruptive process of popping may alter grain structure to such a degree that protein digestibility is not hindered by changes to protein structure (Parker et al., 1999; Duodo et al., 2001). Dry-heating of sorghum flour (without popping) has been reported to result in only minor changes to protein digestibility relative to that of raw flour, and protein solubility was unchanged between the dry and raw flours (Correia et al., 2010).

Interestingly, data from Hamaker et al. (1987) showed that adding a reducing agent during cooking greatly increased the digestibility of the cooked flour, whereas the digestibility of the reduced cooked flour did not quite reach that of the raw flour. It has been hypothesized that some disulfide cross-links in cooked sorghum may form in the hydrophobic regions of proteins and be inaccessible to reducing agents (Duodo et al., 2003). Cysteine residues of the kafirin subclasses have been predicted to be located in the hydrophobic regions of proteins, especially in the β-kafirins (Schober et al., 2011). It is also possible that additional types of cross-links form during the cooking of sorghum (Duodo et al., 2003).

If the cross-linking of sorghum proteins (or protein bodies) into weblike structures could be controlled to produce protein networks that had some functional properties, this could be an important avenue to improving the functionality of sorghum and flour from millets; however, such research should not be geared toward simply producing protein cross-links because these already exist in excess (at least in heated sorghum flour). Rather, research that focuses on forming the right kinds of protein networks, that is, those that have some functional properties needs to be conducted. This may require disruption of the protein bodies or interfering with intrinsic networks that already exist in the cooked sorghum flour.

13.6 Conclusion

Sorghum and millets are an important protein source for a large number of people living in Africa and Asia and of growing importance for people elsewhere. Although considerable research has been conducted on the proteins of these grains, especially with regard to protein composition and nutritional quality, comparatively little research has been conducted on the functionality of sorghum and millet proteins in foods. Additional research is needed to discover ways to improve the functionality of sorghum and millet proteins, as well as their isolated proteins in foods.

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III
Animal Proteins

14
Muscle Proteins

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14.1 Introduction

Meat as a food source has been consumed for most of human existence, and the trend will most likely continue as long as humans inhabit the earth. Meat provides human beings with a variety of nutrients for maintaining good health such as proteins, lipids, iron, zinc, selenium, glutathione, taurine, B-complex vitamins, and retinol (an active form of vitamin A) (Higgs, 2000). Therefore, animal production on farms, appropriate processing at plants, and reliable delivery to consumers are critical to meet the demands of a growing human population. The US meat and poultry industries provide contributions in four important areas: (1) production (feeding 307 million Americans), (2) economy (feeding US economy), (3) nutrition (feeding essential nutrients), and (4) trade (feeding the world). When an animal is raised, harvested, and distributed, a series of economic activities is stimulated through animal farms, slaughterhouses, packers, distributors, suppliers, and operators. In the United States, agriculture is the third largest sector of the US economy, and the meat and poultry industry is the largest sector of agriculture with $143 billion in annual sales, 500,000 employees and a growing list of customers overseas (The Market Works, 2012). The United Nations predicts that the world population will reach 9.2 billion by the year 2075, with 36% of human-consumed protein coming from meat, milk, eggs, and fish. Due to the population growth and increased demand for animal products, it is important to understand and improve the efficiency of animal growth, harvesting, processing, muscle protein quality control, and value-added techniques. This chapter will primarily focus

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on several key factors affecting raw meat production, structure and function of skeletal muscle, muscle protein utilization, protein quality control, and value-added processing.

14.2 World livestock for muscle protein production

During ancient times, people actively hunted animals for food, clothing, weaponry, and other utilities. As agriculture developed, people started to raise livestock for a reliable food source (meat and dairy products), power, fertilizer, and by-products such as fur and leather. In those times, due to poor preservation and lack of transportation, meat and meat products were used locally, whereas today it is shipped to the markets of the world. Meat production is reported to grow at an average rate of 1.8% per year through 2020, with most (78%) of the growth being primarily from poultry and pork in developing countries (Table 14.1).

Demand for animal products is influenced by income capacity. In general, per capita consumption of meats is high among high-income groups, such as individuals who are affluent or countries where a strong economic growth has been achieved. People in industrialized countries receive more than 40% of their dietary protein from food of livestock origin (FAO, 2006). In Asia, the total protein supply from livestock for human diets was significantly increased by 140%, followed by 32% in Latin America.

Meat production in developing countries exceeded that of developed countries around the year of 1996, with an estimated increase in their share of production to two-thirds by the year 2030 (FAO, 2003). Food and Agriculture Organization of the United Nations (FAO) expects that global meat consumption will rise to 460 million tons in 2050 and additional increase (65%) is expected within the next 40 years (Steinfeld et al., 2006). Over the last few decades, the increased demand for animal protein has been largely met by the worldwide growth in livestock production, particularly poultry. The production of poultry

Table 14.1 Meat growth projections to 2020

		2008–2010	2020	(Increased)	
		× 1000 metric tons			%
Poultry meat	Developed	39,923	47,459	7536	18.88
	Developing	55,096	74,952	19,856	36.04
Pork	Developed	41,216	44,457	3241	7.86
	Developing	65,072	82,842	17,770	27.31
Beef/veal	Developed	29,893	31,864	1971	6.59
	Developing	35,565	42,263	6698	18.83
Sheep meat	Developed	3085	3245	160	5.19
	Developing	9747	12,429	2682	27.52
All meats	Developed	114,116	127,023	12,907	11.31
	Developing	165,480	212,486	47,006	28.41
World total	All	279,596	339,509	59,913	39.72

Source: Adapted from WATT executive guide to world poultry trends (2011).
Volumes shown in ready-to-cook weight for poultry meat, but in carcass weight equivalent for pork, beef, and sheep meat.

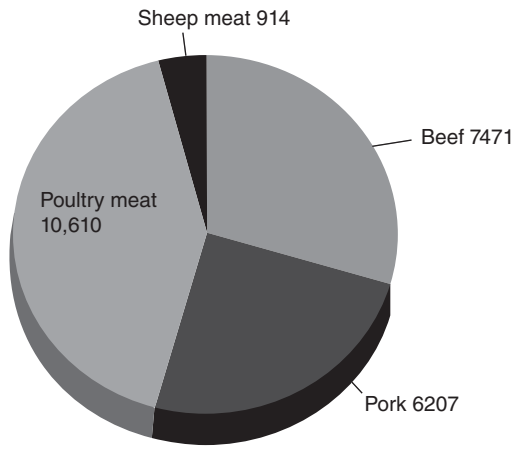


Figure 14.1 Share of world meat exports in 2011 (×1000 metric tons). Adapted from WATT executive guide to world poultry trends (2011)

meat has increased from 9 million tons in the year 1960 to 68 million tons in the year 2000, overtaking the production of beef (60 million tons in the year 2000) (Speedy, 2003). Now, poultry meat is traded between countries, more than any other meats (Figure 14.1).

Today, all eyes are on the major emerging economies of the world such as Brazil, Russia, India, China, and South Africa (BRICS), with the prediction of average annual economic growth of 5.7% through 2015 and 4.57% through 2020. The rapid rising of middle class especially in China and India would help drive the BRICS' economic development and expansion of the global economy. By the year 2020, the middle class population of the BRICS' countries is expected to be more than double that of the developed G7 (The United States, Japan, Britain, Canada, Germany, Italy, and France) economics (Wilson et al., 2010) (Figure 14.2).

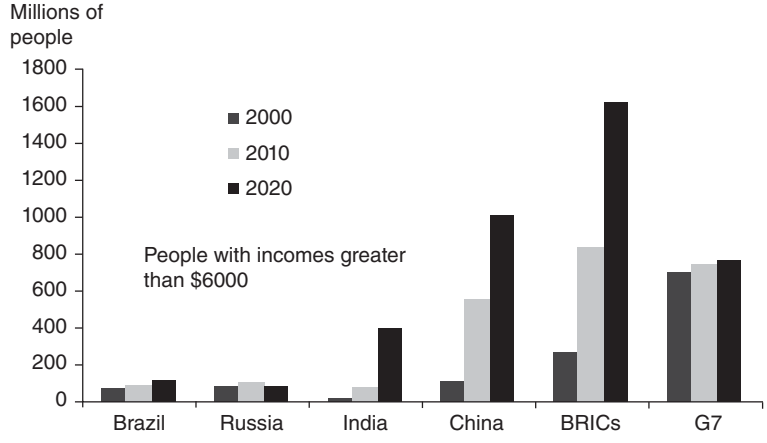


Figure 14.2 Income comparison of BRIC countries to that of G7. Adapted from Wilson et al. (2010)

14.3 Emerging markets: meat production and consumption by BRICS countries

14.3.1 Brazil

Brazil accounts for 15.5% of world's broiler production, the second largest beef producer after the United States, and 3% of world's pork production (Ferraz and de Felicio, 2010). In 2004, Brazil became the biggest exporter of poultry meat with a share of 33.2% in total world poultry meat exports followed by the United States with a share of 32.1% (Yasushi, 2006). The most consumed meat in Brazil is poultry with per capita annual consumption at 12.2 kg followed by pork and beef/veal with per capita annual consumption of 12.1 and 6.66 kg, respectively (OECD-FAO 2011–2020). The higher efficiency of poultry production over beef and pork leads to lower poultry meat cost and higher per capita consumption than other meats in Brazil (Aguiar and da Silva, 2002).

14.3.2 Russia

The most highly produced and consumed meat in Russia in 2010 was poultry with a total production of 2.9 million tons and consumption of 3.3 million tons. Pork was the second most produced (2.4 million tons) and consumed (3.3 million tons) meat followed by beef/veal with 1.7 million tons production and 2.6 million tons consumption (OECD-FAO 2011–2020). The wide gap between production and consumption is obviously due to the high demand for meat in the country without sufficient production, which is why Russia is one of the major meat importers. Looking at the income level of people, we can see that meat consumption in Russia is relatively high as compared to other nations. Per capita meat consumption (including beef/veal, pig, poultry, and sheep) in the year 2010 was around 53.5 kg. Russians are clearly meat eaters with a good appetite for red meat, which has always been associated with high status in Russian culture. Although women prefer fish, men mostly do not feel satisfied without red meat in their diets (Honkanen and Voldnes, 2006). However, due to economic restraints, red meat is now increasingly being replaced by poultry and fish.

14.3.3 India

In 2009, beef/veal and poultry production in India was 2.8 and 2.6 million tons, respectively. Although the FAO database indicated more beef/veal production than poultry in 2009, per capita consumption of poultry was more than all other meats in India. Per capita poultry consumption was 1.93 kg over 1.27 kg of per capita beef/veal consumption in 2009 (OECD FAO 2011–2020). This can be explained by the fact that most of the beef/veal produced in India is exported to Africa and the Middle East because Indians, with the exception of Muslim population, do not eat beef/veal but rather consume practically poultry, goat, lamb, and pig. The low meat consumption rate is explained by the fact that 20% of the Indian population is strict vegetarian. Less than 30% of the population consumes meat regularly due to religious and economic restraints (USDA, 2001). India has been ruled by

Hindu, Muslim, and British rulers in the past, which influenced modern Indian cuisines. Hindus comprise approximately 80% of the population and do not eat beef, particularly cow. Muslims comprise approximately 19% of the population and do not eat pork due to religious reasons. This leads to low beef and pork consumption in India. Only two states, Kerala and West Bengal, allow the slaughter of cows and bulls, and the meat sector in India is not well organized.

14.3.4 China

Pork is the major meat produced and consumed in China, with about 50% of the world pork production followed by the European Union (25 countries of Europe), United States, Brazil, and Canada (USDA, 2006). Pork also plays the biggest role in Chinese rural nutrition although Muslim followers do not consume pork and blood-related products. According to the USDA (2012), Chinese annual pork production and consumption in 2010 were 51 and 51 million tons, respectively. The second most consumed meat in China in 2010 was poultry followed by beef/veal with a consumption of 12 and 5.6 million tons, respectively. Per capita meat consumption, excluding fish, in China was around 45 kg compared to 93 kg in the United States and 65 kg in the European Union (OECD FAO 2011–2020). These figures in combination with the growing Chinese economy indicate a sharp increase in meat consumption in the near future. The increased meat production and consumption presumably boost the Chinese meat processing industry which is still unstable and inefficient as compared to the more developed nations (AAFC, 2010). In China, food and medicine are closely related. Pork is believed to have medicinal properties for diabetes, dry cough, gastritis, and constipation, whereas beef is consumed to increase muscle strength and blood production in the body. It is often consumed by people with low blood pressure (Zhao and Ellis, 1998). Poultry is consumed to assist blood circulation and overcome problems like anemia, slow lactation, leucorrhea, and liver/kidney malfunction. Chicken soup having herbs like ginger, ginseng, dried dates, wine, and sesame oil is commonly consumed by patients, with their belief to recover. In south China, women consume chicken stewed with sesame oil, rice wine, and ginger roots in the early months of pregnancy.

14.3.5 South Africa

South Africa is the largest economy sector in Africa and the most consumed meat is poultry with the consumption of 1.2 million tons, followed by 0.8 million tons of beef/veal and 0.3 million tons of pig in 2009 (OECD-FAO 2011–2020). In native South African tribes, no village is without a poultry house but tribes do not grow poultry for eggs due to the small size of eggs, which are about half the size of an ordinary European hen's egg (Junod, 2003). Interestingly, per capita consumption of beef and veal has decreased from 23.95 kg in 1965/1966 to 12.32 kg in 1999/2000. In fact, the portion of red meat consumption has decreased by around 50% while white meat has increased by more than 50% from 1960s to late 1990s (Poonyth et al., 2001). South Africa has been a country with a rich heritage of wilderness and game animals. Game animals contributed to a significant portion of the

red meat consumed by native people from the earlier times. Presumably, restriction of wild animals and modernization of civilization shifted their consumption habits toward more white meat like poultry. South Africa is a country in transition, improving economic environment and consuming more nutritious foods (Vorster et al., 2005).

14.4 Meat processing for various utilizations

Meat processing is defined as "preparation of meat for human consumption" or "subjecting meat to a series of operations to produce desired changes" in Britannica Online Encyclopedia (2013) and Medical dictionary (2013). In general, any actions or preparations of animal carcasses and meat products for human consumption are considered meat processing. It can range from a basic preparation such as washing, cutting, salting, or sun-drying in prehistoric times to today's advanced technology such as rapid freezing, irradiation, high-pressure processing, controlled fermentation, etc. Several meat processing and preservation techniques have been largely developed due to the military needs: (1) canning process during the Napoleonic war in France, (2) large scale meat-freezing during World War I, (3) antibiotic preservation during World War II, and (4) irradiation/sterilization/freeze drying for space race or travel. The development of meat processing techniques is further moving forward due to the demands of various consumer groups for convenience (ready-to-eat products), diet (low fat/low sodium), health (natural, organic, minimal processing), fortification (vitamin, mineral), young/aged groups (soft/tender meat), and ethnic (halal/kosher) requirements.

14.5 Structure and chemical composition of skeletal muscle

There are more than 600 muscles in an animal's body. These muscles are highly structured and specialized for various kinds of activities such as movement, chemical-to-mechanical energy conversion, heart-beat synchronization, etc. (Figure 14.3). The properties of muscle structure and their activity/functions are interconnected with meat tenderness and final product quality after the animal is slaughtered. Once the animal is harvested, the muscles convert into meat. Lean skeletal meat consists of approximately 19% protein, 3% fat, 75% moisture, and 1% ash, and proteins in specific locations have various functions (Table 14.2).

Skeletal muscle consists of membrane-bound cells and is multinucleated on the surface. Each muscle is covered by a thick sheath of connective tissue, called the epimysium, which linked to bones via a network of tendons. The muscle, composed of muscle bundles (or fasciculi), is surrounded by another sheath of connective tissue, named the perimysium. Within the muscle bundle, individual muscle fibers are shielded by a thin layer of connective tissue, called endomysium located just outside the muscle cell membrane called sarcolemma (Bailey and Light, 1989) (Figure 14.3). In a cross section of muscle fiber, there are over 1000 myofibrils (1–2 μm diameter). Under observation with a light microscope, regular transverse striations can be seen with alternating light (less dense I-band) and dark

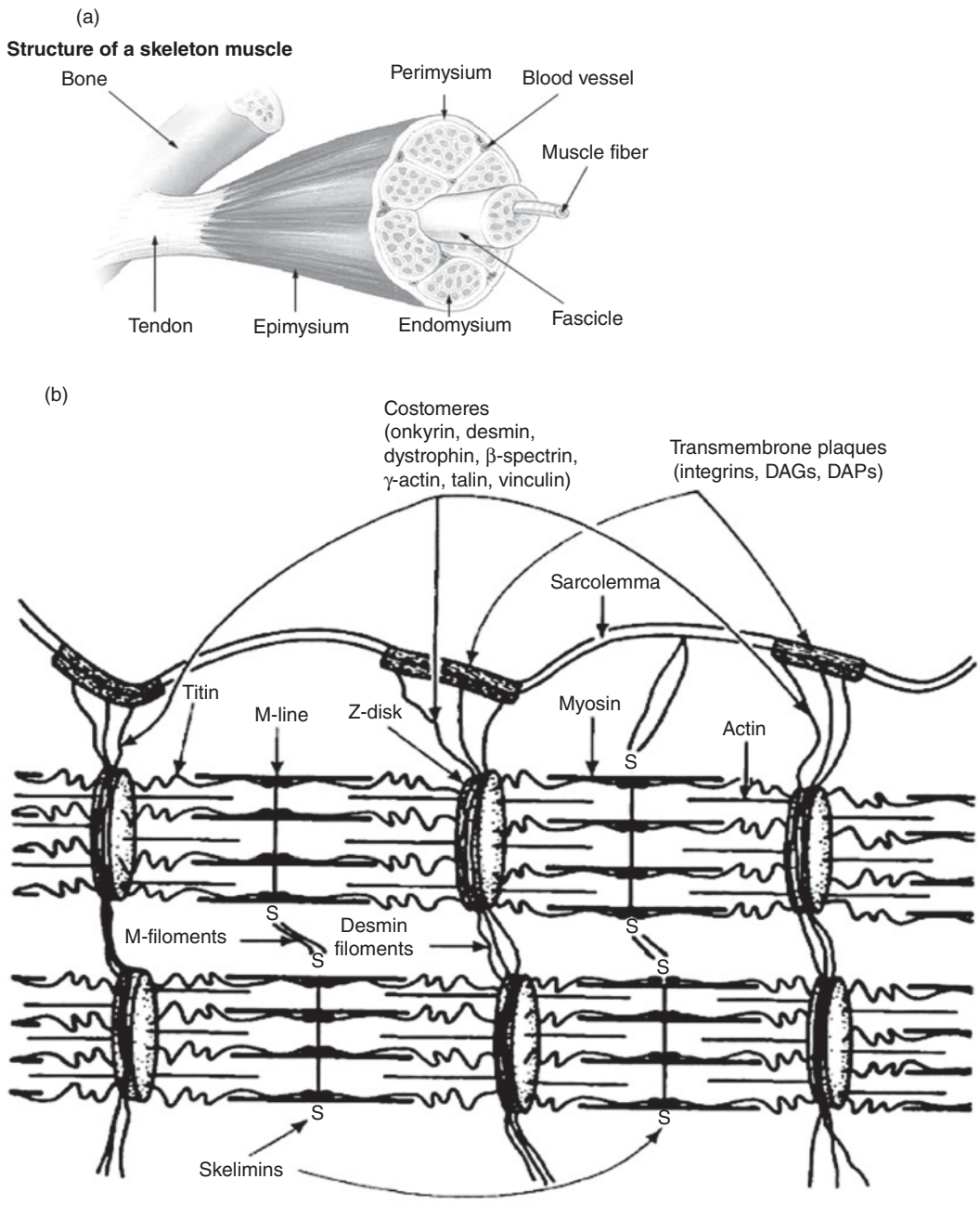


Figure 14.3 Protein interactions in muscle foods. (a) Adapted from SEER Training Modules at National Cancer Institute (2012), (b) Adapted from Taylor et al. (1995)

Table 14.2 Composition and function of skeletal muscles

Component (range)	%
Water (65–80)	
Protein (16–22)	18.5

	MW (kDa)	Percentage of total protein	Location	Functions
Contractile proteins				
Myosin	480	26	Thick filament	Molecular motor of muscle contraction
Actin	42	13	Thin filament	Molecular motor of muscle contraction
Regulatory proteins				
Tropomyosin	70	3	Thin filament	Regulation of muscle contraction
Troponin	80	3	Thin filament	Regulation of muscle contraction
Tropomodulin	40	<1	Thin filament	Regulation of muscle contraction
Cytoskeletal proteins				
Titin	3700	5	Sarcomere	Myofibrillar assembly and structure; links thick filaments, anchored to M-line
Nebulin	800	2	Thin filament	Anchors thin filaments to Z-line, regulates thin filament length
C-protein	135	1	Thick filament	Maintains thick filaments in bundles of 200–400 molecules
Myomesin	185	1	M-line	Anchors myosin to titin elastic filament system, maintains thick filament lattice
M-protein	165	<1	M-line	Anchors myosin to titin elastic filament system, present only fast (type II) fibers
Desmin	52	<1	Z-line, costameres	Intermediate filament, connects adjacent myofibrils at Z-line
Filamin	500	<1	Z-line, costameres	Binds to actin
Vinculin	116	<1	Z-line, costameres	Helps connect peripheral myofibrils to sarcolemma, binds to α-actinin
Synemin	220	<1	Z-line	Associated with desmin/vimentiin intermediate filaments
Creatine kinase	86	3	Sarcoplasm	Glycolytic enzyme
α-actinin	95	1	Z-line	Z-line integrity, anchors thin filaments
H-protein	69	<1	Thick filament	Associated with myosin and C-protein
Skelemin	195	<1	M-line	May facilitate attachment of desmin filaments to M-line

Cap Z	68	<1	Z-line	Caps Z-line end of actin filament, may interact with titin and nebulin	
Paranemin	280	<1	Z-line	Associated with desmin/vimentin intermediate filaments	
Destrophin	427	<1	Z-line, costameres	Anchors actin filaments to sarcolemma	
Talin	270	<1	Z-line, costameres	Helps connect peripheral myofibrils to sarcolemma, binds actin	
Sarcoplasmin proteins					
Soluble sarcoplasmic and mitochondrial enzymes					
Myoglobin	16.8	1	Sarcoplasm	Oxygen storage and transport	
Hemoglobin	68	<1	Sarcoplasm	Oxygen storage and transport	
Cytochrome c	12	<1	Mitochondria	Electron transport, oxidative phosphorylation	
Stromal proteins					
Collagen				Extracellular muscle support, provide framework for muscle movement, transmit force of muscle contraction to the skeleton	
Type I and III	300	6	Epymisium Perimysium Endomysium	Endomysial network structure, binds to fibronectin, laminin and haparan sulfate	
Type IV	420	<1	Endomysium	Main constituent of elastic fibers	
Elastin	74	<1	Vascular system		
Lipids (1.5–13.0)					3.0
Neutral lipids					
Phospholipids					
Cerebrosides					
Cholesterol					
Non-protein nitrogenous substances					1.5
Creatin and creatin phosphate					
Neucleotides					
Adenosine triphosphate					
Adenosin diphosphate, etc.					
Free amino acids					
Peptides					
Anserine, carnosine, etc.					

(*continued*)

Table 14.2 (*Continued*)

Component (range)	MW (kDa)	Percentage of total protein	Location	Functions	% 18.5
Other nonprotein substances Creatin, urea, inosine, monophosphate (IMP), Nicotiamide adenine dinucleotide (NAD), Nicotiamide adenine dinucleotide phosphate (NADP)					
Carbohydrates and non-nitrogenous substances (0.5–1.5)					1.0
Glycogen					
Glucose					
Intermediates and products of cell metabolism Hexose and triose phosphate, lactic acid, citric acid, Fumaric acid, succinic acid, acetoacetic acid, etc.					
Inorganic constituents					1.0
Potassium					
Total phosphorous Phosphate and inorganic phosphorous					
Sulfur (including sulfate)					
Chlorine, sodium					
Others (including magnesium, calcium, iron, cobalt, etc.)					

Source: Adapted from Aberle et al. (2001) and Tarte and Amundson (2006).

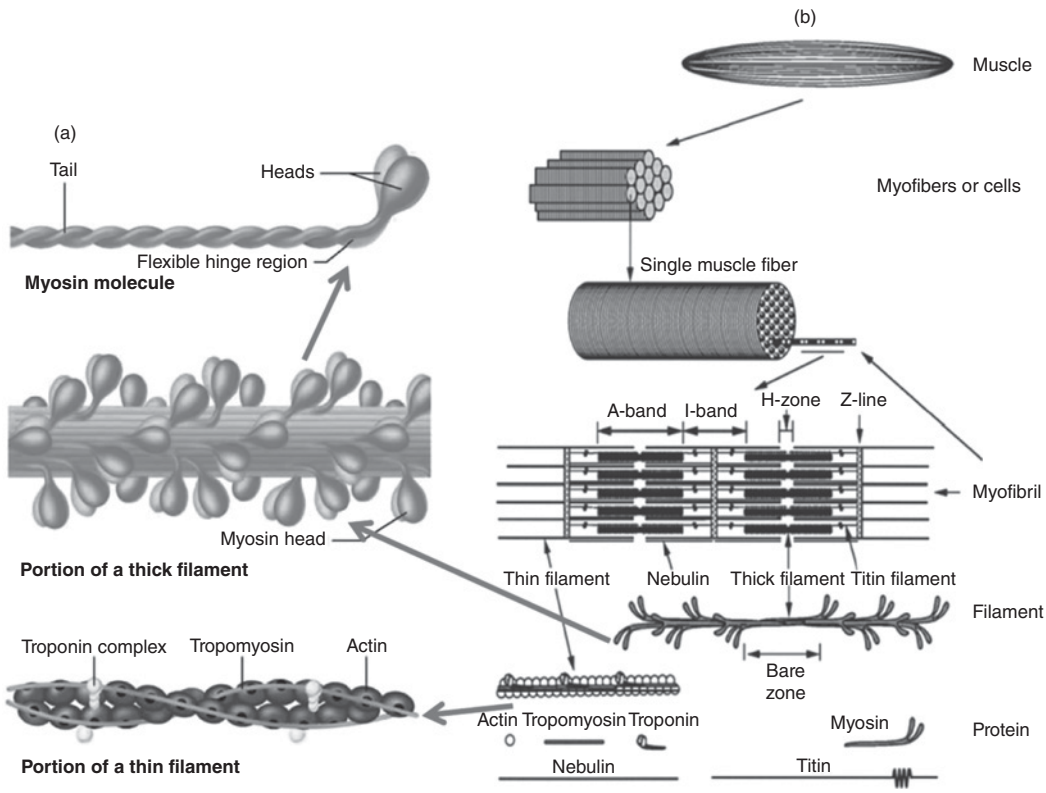


Figure 14.4 Muscle ultrastructure. (a) Adapted from Corey (2001), (b) From Greaser and Pearson (1989)

(less dense A-band) bands where two major proteins of thick (myosin, 15 nm diameter) and thin (actin, 6–8 nm diameter) filaments exist, respectively (Figure 14.4). Based on the functional properties, muscle proteins are classified into three groups: (1) myofibrillar protein, (2) sarcoplasmic protein, and (3) stromal protein (Table 14.2).

14.5.1 Myofibrillar proteins

Myofibrillar proteins are responsible for 60% of total muscle protein, with 90% of the total myofibrillar proteins being composed by six major proteins: myosin (26%), actin (13%), titin (5%), tropomyosin (3%), troponin (3%), and nebulin (2%), all of which compose over 52% of the total protein. Myofibrillar proteins are further classified into contractile, regulatory, and cytoskeletal proteins based on their function. Myosin and actin are two major contractile proteins making up 43% and 22% of the total myofibrillar proteins, respectively. Myosin is a fibrous protein in an elongated rod shape in thick filaments while actin is a globular-shaped molecule (G-actin), and forms a fibrous actin (F-actin) by linking 400 G-actin molecules in a helical shape in thin filaments (Greaser and Pearson, 1989; Corey, 2001) (Figure 14.4a and b).

Tropomyosin and troponin are the two most abundant regulatory proteins comprising 5% of myofibrillar protein for each. A strand of tropomyosin binds along the length of the major groove in the actin filaments. Tropomyosin and troponin complex regulate the interaction of actin and myosin in muscle contraction and relaxation. When calcium (Ca^{2+}) concentration increases in the myofilament space, tropomyosin goes through a conformational change and facilitates actin–myosin interactions (Hitchcock-DeGregori et al., 2002; Muthuchamy et al., 1997). Troponin attaches to tropomyosin and lies within the grooves of the actin filaments. Troponin has a complex structure containing three subunits of troponin I (inhibits the actin–myosin interaction), troponin T (binds to tropomyosin), and troponin C (binds to calcium ions).

Titin (>3000 kDa) and nebulin (800 kDa) are two giant proteins, which are believed to play major roles in the assembly and function of sarcomeres by linking and anchoring the cell's contractile components. Titin molecule is more than 1 μm long and spans half the sarcomere, with the N-terminus in the Z-line and the C-terminus in the M-line. It connects the end of the thick filament to the Z-line, as well as in the Z- and M-line regions. Nebulin makes up 2–3% of the myofibrillar protein. A single nebulin molecule spans the thin filament with its C-terminus anchored at the Z-disk and its N-terminal region directed toward the thin filament pointed end (Tarte and Amundson, 2006).

14.5.2 Sarcoplasmic proteins

Sarcoplasmic proteins are found in the sarcoplasm or in the fluid surrounding myofibrils. They are also known as water-soluble proteins due to their extractability in water or low ionic strength (0.06) salt solutions. The proteins comprise about 29% of muscle protein, most of which includes glycolytic enzymes to control aerobic and anaerobic glycolysis. Other sarcoplasmic proteins include cytochromes, flavin nucleotides, lysosomal enzymes, heme in myoglobin pigments, and mitochondrial oxidative enzymes. The heme group is responsible for fresh meat/processed meat color with oxygen binding, oxidation and nitrite binding during curing (Tarte and Amundson, 2006).

14.5.3 Stromal proteins

Stromal protein or connective tissue is the primary component of the extracellular matrix and provides a supportive frame by connecting or holding various parts of the animal body. It comprises 25–30% of the total protein, with being present in all tissues such as bone, skin, tendons, cartilage and muscle. Connective tissue proteins primarily include collagen, elastin, and reticulum.

Collagen is the single most abundant protein in rod-shape, 300 nm long and 1.5 nm in diameter. Collagen has a unique amino acid composition which is rich in glycine (33%), proline and hydroxyproline (23%), alanine (11%) as well as a small portion (less than 1%) of tyrosine, histidine, and sulfur-containing amino acids (Bechtel, 1986). Collagen has an additional unique property of intramolecular cross-links, which increase with age, resulting in greater toughness of the meat from old animals.

Elastin is the major protein component of ligaments and walls of major arteries. Elastin is extremely unreactive, heat stable (to 150°C), resistant to most solvents and proteolytic enzymes, and very stretchable and recoiling (Bandman, 1987). It does not contain any measurable hydroxylysine, but has two unique amino acids of desmosine and isodesmosine, which condense with one lysine residue to yield either desmosine or isodesmosine cross-links (Davis and Anwar, 1970).

14.6 Conversion of muscle to meat

Once an animal is harvested, the muscle becomes a highly nutritious and palatable food source through a series of post-mortem (PM) changes. The quality of meat and meat products is primarily influenced by physical and biochemical modifications during the PM period in addition to the handling of live animals prior to slaughter. During the first few hours after exsanguination, animal tissues are subjected to dramatic changes in environmental and biochemical conditions. In this new environment, cells in the carcass make every effort to maintain physiological conditions in body such as optimum temperature, pH, oxygen concentration, and energy supply. The conversion of muscle to meat is complex but is a systematic and predictable process.

14.6.1 Rigor development and meat tenderness

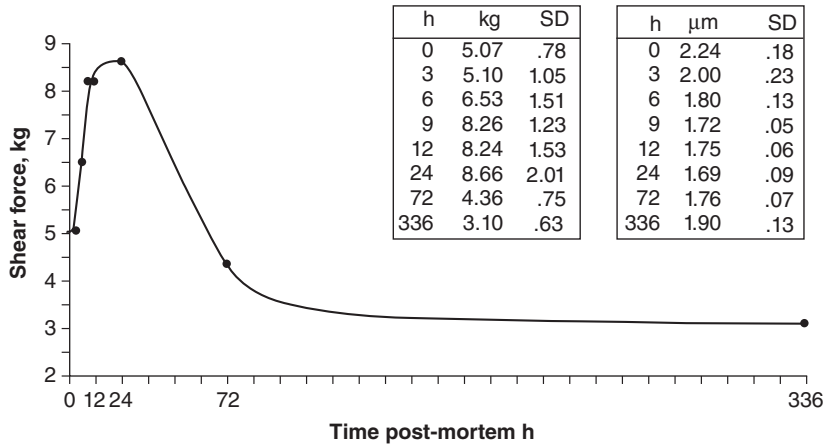
Different animals require different amounts of time for rigor onset (Table 14.3) with a negative relationship between sarcomere length and meat toughness (Wheeler et al., 2000). Rigor onset begins at low ATP concentration (0.1 μM/g wet tissue) (Hamm, 1977, 1981; Honikel et al., 1981) and continues through the completion of rigor or prior to resolution. During the extended storage up to 336 hours at 4°C, lamb *longissimus* muscle showed an intermediate tenderness (5.07 kg Warner–Bratzler shear force) at the point of slaughter, toughness (8.66 kg) at 9–24 hours PM, and tenderness (3.10 kg) thereafter (Figure 14.5). Generally, shear force decreases as PM storage and sarcomere length increase.

However, the relationship between sarcomere length and shear force was poor when muscles were stored at high temperatures due to the proteolytic activity overcoming the sarcomere shortening. Hwang et al. (2004) reported that the beef muscles, *M. semitendinosus*, excised soon after death and held at different temperatures had similar shear force

Table 14.3 Delay time before onset of rigor mortis

Species	Hours
Beef	6–12
Lamb	6–12
Pork	$^1/_4$–3
Turkey	<1
Chicken	$<^1/_2$
Fish	<1

Source: Adapted from Aberle et al. (2001).



h	kg	SD
0	5.07	.78
3	5.10	1.05
6	6.53	1.51
9	8.26	1.23
12	8.24	1.53
24	8.66	2.01
72	4.36	.75
336	3.10	.63

h	μm	SD
0	2.24	.18
3	2.00	.23
6	1.80	.13
9	1.72	.05
12	1.75	.06
24	1.69	.09
72	1.76	.07
336	1.90	.13

Figure 14.5 Changes in Warner–Bratzler shear force and sarcomere length of lamb longissimus during post-mortem storage. Adapted from Wheeler and Koohmaraie (1994)

values, although the sarcomere length (1.42 μM) of muscle at 15 and 36°C was shorter than the length (1.76 μM) of muscle at 15°C. Physical stretching of major muscles, until the completion of rigor mortis, has been adapted to improve the tenderness by modifying carcass suspension known as tender stretch (Macfarlane et al., 1974) (Figure 14.6).

14.6.2 Water-holding capacity and meat tenderness

During the PM conversion of muscle to meat, water distribution in muscle cells is one of the most important events. Any water loss in muscle leads to an adverse effect on the product appearance, juiciness, and tenderness (Lawrie, 1998; Oeckel et al., 1999; Bertram et al., 2000). The amount of natural water in lean meat is up to 75%, which is held by either entrapment within its structures and/or immobilization due to chemical interaction.

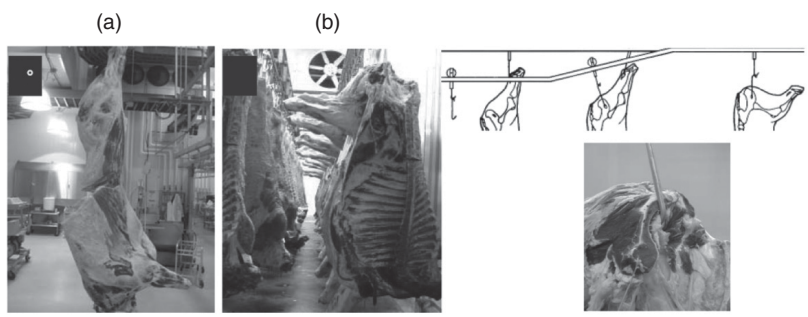


Figure 14.6 Two types of beef carcass hang. (a) Vertical hanging by Achilles tendon. (b) Horizontal hanging by aitchbone (obturator foramen). Adapted from Tenderstretch (2006)

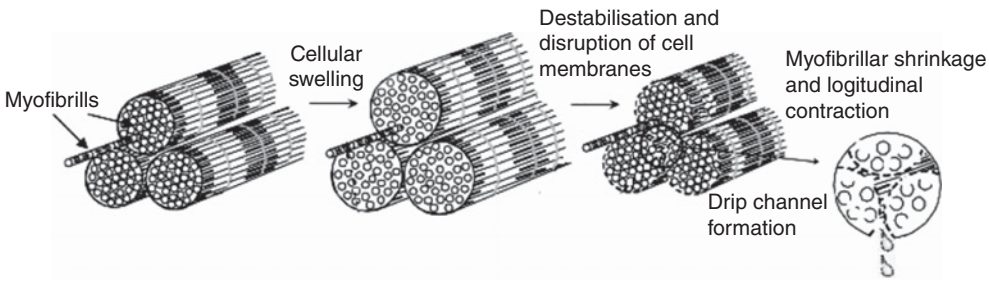


Figure 14.7 Illustration of the mechanisms of early post-mortem events of importance for changes in water distribution within muscles. Adapted from Bertram et al. (2004)

Regarding the structural entrapment, moisture is held in the space between thick and thin filaments, between fiber bundles, between fibers, and through electrostatic attraction in proteins (Bond et al., 2004) (Figure 14.7). At the initial PM stage, muscle cells start to swell likely due to the increased intracellular osmolarity, resulting in increased intracellular water volume. At the later PM stage, the water is pressed from the myofibrillar matrix due to the combination of longitudinal and lateral shrinkages in myofilament fibers resulting from pH drop (Bertram et al., 2004) (Figure 14.7).

In terms of chemical interaction, myofibrillar proteins such as actin and myosin have ionic charges in their side groups, and these charges (either positive or negative) attract a certain amount of water because water is a dipolar molecule. The rate of PM pH fall is approximately 0.005 pH units per minute in beef and sheep although this rate is affected by the muscle temperature (Person and Young, 1989; Poso and Puolanne, 2005). From the pH (about 5.3) of muscle isoelectric point, there is a net increase of negative charges in protein as muscle pH increases and a net increase of positive charges as muscle pH decreases (Figure 14.8). The isoelectric point is the pH where muscle proteins have the least amount of bound water, resulting more drip loss and tougher meat. Pale, soft, and exudative meat (PSE) is characterized by pale color, soft muscle texture, and wateriness (exudative) on the muscle surfaces (Figure 14.9). This condition is resulted from the combination of a rapid pH drop (5.4–5.6) and elevated muscle temperature after slaughter, resulting in protein denaturation (Goutefongea, 1971). This type of meat is not fully utilized by butchers or meat processors due to the reduced protein functionality and visual defect. On the other hand, the muscle of high post-rigor pH (5.9–6.5) develops dark cutting muscle that is known as dark, firm, and dry (DFD) meat. This meat has less appeal to consumers due to its dark color and dry texture (Figure 14.9). Unlike the low pH of PSE, the high muscle pH promotes bacterial growth and becomes more susceptible to spoilage. DFD meat occurs when animals are stressed in a long period or injured before slaughter so that their skeletal muscles have low glycogen content. This condition is found more in cattle or sheep and less in pigs and turkeys.

The PSE problem in pork industry reportedly results in an annual loss of $200 million (Schultz-Kaster and Hill, 2006) and over $200 million loss from the turkey industry with a flock-to-flock variation (1–29% PSE meat) in the United States (Owens et al., 2000;

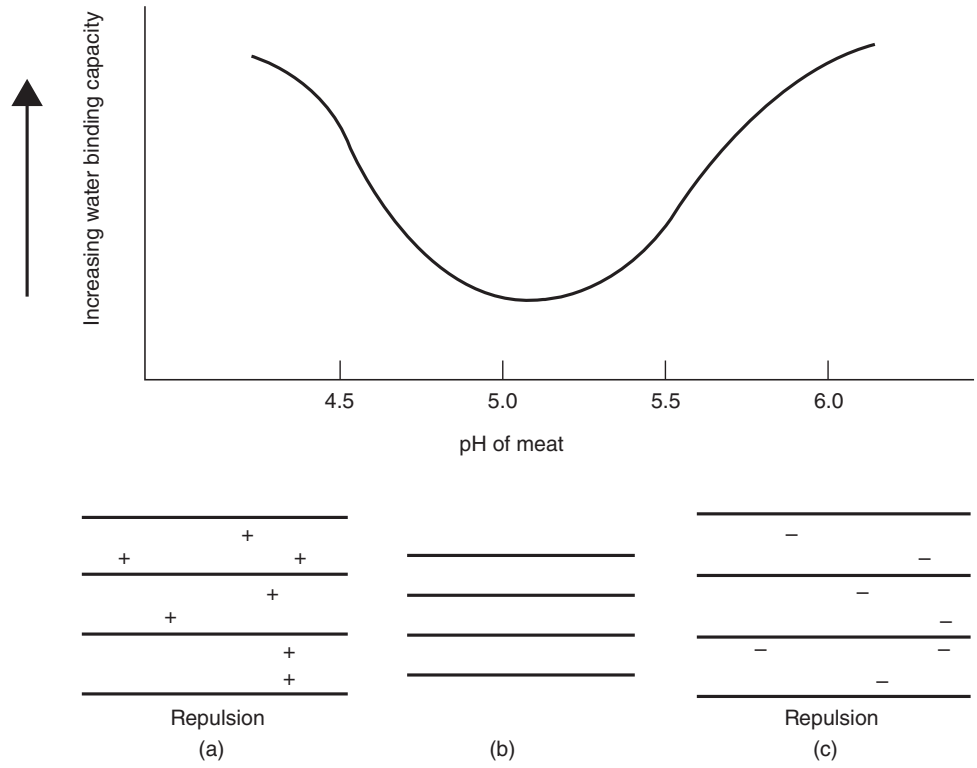


Figure 14.8 Effect of pH on the amount of immobilized water. Adapted from Wismer-Pedersen (1971)

Strasburg and Chiang, 2009). PSE is associated with major gene defects (e.g., halothane sensitivity and Rendement Napole (RN)), resulting in the animal's inability to tolerate stress (Le Roy et al., 1990; Otsu et al., 1991; Sellier, 1998). PSE is also related with severe ante-mortem stressors and PM handling including environmental temperature, pre-slaughter treatment, transportation stress, and carcass chilling regimes (McKee and Sams, 1997). After harvest, rapid chilling with no delay can reduce the incidence of PSE (Aberle et al., 2001). During further processing, the quality of PSE meat was improved with the addition of functional ingredients such as modified starch, carrageenan, polyphosphates, transglutaminase or normal meat (Motzer et al., 1998; Milkowski and Sosnicki, 1999; Torley et al., 2000). Improved ante-mortem and post-harvest management strategies have also been implemented while identification and utilization of genetic markers, associated with the stress response, have been studied to aid in genetic selection as one of long-term solutions.

14.6.3 Aging and structural changes of skeletal muscle

After the completion of rigor mortis, muscles become tenderized during extended storage. Ultra-structural changes in bovine longissimus muscle were observed by electron

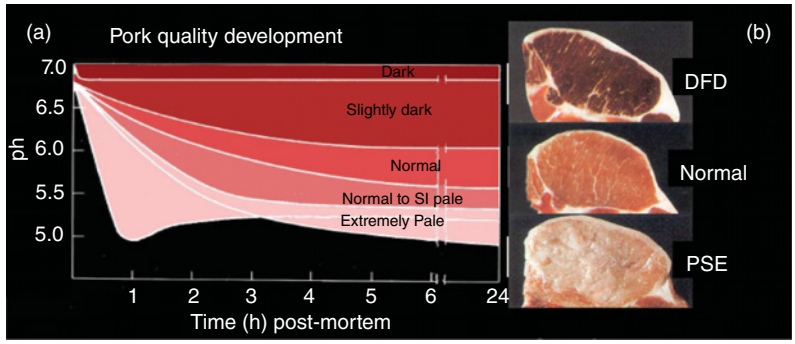


Figure 14.9 (a) Variations in post-mortem pH patterns. Adapted from Briskey (1964). (b) Visual appearance of muscle cut at different pHs. Adapted from FAO (2001). DFD, dark, firm, and dry; PSE, pale, soft, and exudative.

photomicrographs during PM aging (Figure 14.10). No discernible Z-disc degradation (fiber degradation between adjacent sarcomeres) was detected at 1 hour PM beef muscle while torn or fragmented myofibrils were found at the junction of I-band (thin) filaments and the Z-disc at 48 hours PM muscle.

For maximum benefit of the rigor resolution and meat tenderization, beef should be stored for 10–14 days, lamb for 7–10 days, pork for 5 days, and poultry for 0.17 (or 4 hours)–1 day (Koohmaraie, 1996; Owens et al., 2010). The resolution of rigor mortis is not from the breaking of actomyosin bonds but from the proteolytic degradation of specific myofibrillar proteins and their linkage to the sarcolemma, including troponin-I, troponin-T, desmin, vinculin, *meta*-vinculin, dystrophin, nebulin, and titin (Taylor et al., 1995).

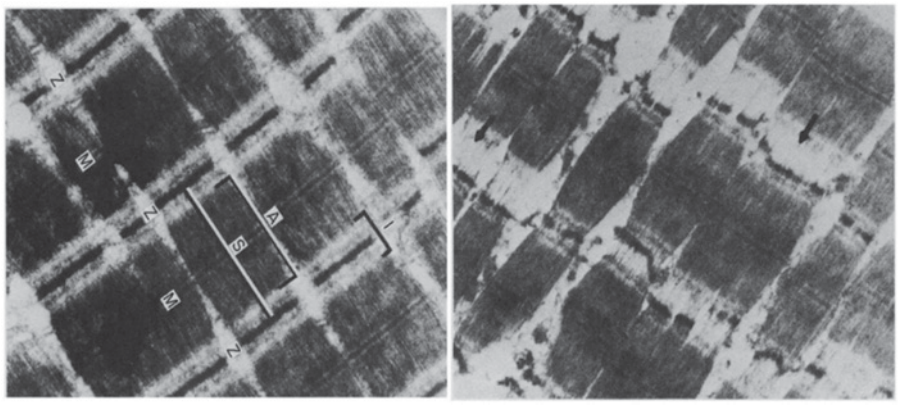


Figure 14.10 Electron micrographs of bovine longissimus myofibers at 1 hour post-mortem and Z-disc degradation at 48 hours post-mortem. A, A-band; I, I-band; Z, Z-disc; S, Sarcomere; M, M-line. Adapted from Gann and Merkel (1978)

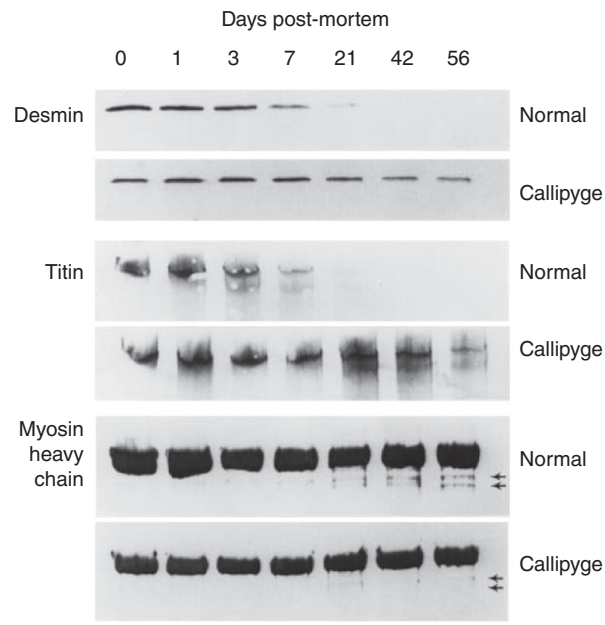


Figure 14.11 Western blot analysis of desmin, titin, and myosin heavy chain degradation during post-mortem storage of normal and callipyge biceps femoris. Adapted from Geesink and Koohmaraie (1999)

In muscle protein degradation, three major cytoskeletal structures are primarily involved: (1) Z-line-to-Z-line attachments by intermediate filaments (mostly composed of desmin), (2) Z-line and M-line attachments to the sarcolemma by costameric proteins (vinculin, spectrins, ankyrin, talin, and gamma actin), and (3) elastic filament protein titin (Taylor et al., 1995). During PM storage, Koohmaraie and Geesink (2006) compared the ultrastructure of normal and callipyge lamb which generally produces tough meat with over 40% of chops considered unacceptable (Clare et al., 1997). Desmin (Mw 53 kDa) forms one of the intermediate filaments, encircles the Z-disks and connects adjacent myofibrils at the Z-disk level. The protein was degraded at a slower rate in muscle from callipyge lambs than in normal muscle during the PM period (Figure 14.11). Titin, the largest protein ($\geq$3000 kDa) in nature, spans half the length of a sarcomere and connects Z-lines and M-lines in the striated muscle. Robson et al. (1997) indicated that titin degradation might play an important role for PM tenderization due to its huge size and structural role in the myofibril. However, myosin heavy chain (200 kDa), the largest subunit of myosin (480 KDa) in thick filaments, showed almost no PM degradation during PM storage although a few breakdown products were detected after extended storage. These results indicated that PM tenderization is not due to the proteolysis of myosin molecule.

14.6.4 Role of proteolytic enzymes in meat tenderization

As early as the turn of the twentieth century, meat tenderization was suggested to be the result of enzymatic activity during cool storage (Lehmann, 1907; Hoagland et al., 1917).

Numerous enzymes in living tissues were then believed to remain active after animal slaughter and the extended PM period. Subsequently, a great deal of research has been conducted over the past decades on proteolytic enzyme systems in skeletal muscles and their involvement in meat tenderization (Koohmaraie, 1992, 1996; Etherington, 1984; Kemp et al., 2010). Of the various protease candidates, three groups of enzymes playing major roles are: (1) calpains/cathepsins system, (2) cathepsin peptidases, and (3) proteasomes—multicatalytic proteinase complex.

14.6.4.1 Calpain and calpastatin Calpain, calcium-dependent neutral proteases, is a large family of 14 cystein proteases which are expressed ubiquitously. In skeletal muscle, the calpain system consists of three proteases: micromolar Ca^{2+}-requiring protease (μ-calpain), millimolar Ca^{2+}-requiring protease (m-calpain), and calpain 3 (or p94). In addition, calpastatin is associated with the calpain system as a calpain-specific endogenous inhibitor. The general properties of μ-calpain, m-calpain, and calpastatin are summarized in Table 14.4.

Proteolytic calpain activity is widely accepted as a major contributor to meat tenderization during muscle aging (Koohmaraie and Geesink, 2006). The enzyme activity is affected by many factors including pH (7.2–7.8), temperature (25°C), and calcium concentration (3–800 μM for calpain I or II) (Kanawa et al., 2002). The calcium concentration in a live resting muscle is about 0.2 μM, but free calcium reaches 100 μM in a cell after slaughter (Jeacocke, 1993; Goll et al., 2003).

Huff-Lonergan et al. (1996) reported that myofibrils were degraded by μ-calpain at a pH of 5.6, a temperature of 4°C, and a calcium chloride level of 100 μM, which are similar to the conditions of meat during aging. According to Dransfield (1994a, 1994b), calpain I in beef muscle is activated around 6 hours after slaughter as the calcium ions are released from the sarcoplasmic reticulum and mitochondria with the muscle pH of 6.1–6.3. At 16 hours or later PM, calpain II becomes activated as the number of calcium ions rises further and the muscle is tenderized more. Regarding the relationship between meat tenderness and PM degradation, Huff-Lonergan et al. (1996) suggested the role of calpain on breaking down several key proteins. The PM muscles having lower shear force

Table 14.4 Some general properties of three well-characterized members of the Ca^{2+} dependent proteinase (calpain) system, μ-calpain, m-calpain, and calpastatin

Protein type	Polypeptides	$[Ca^{2+}]$ for half-maximal activity
Proteases		
μ-calpain	80, 28 kDa	3–50 μM
m-calpain	80, 28 kDa	400–800 μM
Inhibitor		
Calpastatin	Variable, 46 kDa 70–76 kDa 87 kDa	Requires Ca^{2+} to bind to the calpains; [Ca2] requirement depends on the calpain

Source: Adapted from Goll et al. (2003).

exhibited faster degradation of presumably key proteins than the muscle having higher shear force. Both nebulin and titin bands in the lower shear force muscles were absent at 3 and 7 days PM, respectively, and both desmin and troponin-T in the lower shear force samples were degraded more rapidly than those in higher shear force samples.

The level of calpains and calpastatin were reported to be not the same in different species (beef, pork, sheep, and poultry) and muscles (Northcutt et al., 1998). It is also known that the activity of calpain is mostly regulated by calpastatin, and a greater proportion of variation in beef tenderness is influenced by the calpastatin activity during aging (Shackelford et al., 1994). Having higher amounts of calpastatin, callipyge sheep showed a reduced rate of PM proteolysis and less tenderization (Koohmaraie et al., 1995). Comparing messenger RNA (mRNA) expression of μ-calpain, m-calpain, p94, small subunit (calpain-4; 28 kDa), and three types of calpastatin isoform in 10 skeletal muscles of Holstein cattle, Muroya et al. (2012) reported that both calpain and calpastatin expressions are regulated by muscle types, especially by muscle fiber types. Subsequently, the higher extent of PM proteolysis in longissimus thoracis and psoas major muscles than lingual muscle was contributed to the differences in calpains/calpastatin ratios.

During the aging processing of muscle, myofibrillar proteins are oxidized and some amino acid residues (e.g., histidine) are converted to carbonyl derivatives, causing intra and/or inter cross-links of protein disulfides (Martinaued et al., 1997). Having both histidine and SH- cysteine residues at active sites, calpain enzymes might be susceptible to PM oxidization and inactivation of their activity. When muscle tissue was exposed to irradiation during the early PM period, reduction of proteolysis in the irradiated samples was detected compared with non-irradiated samples (Figure 14.12).

Regarding the calpain activity and muscle oxidation, Rowe et al. (2004b) showed that the muscle fed with α-tocopherol showed increased proteolysis of troponin-T over the muscle with no α-tocopherol. Irradiation accelerates the production of free radicals in muscle although it is a commercially viable tool for food safety. Nam and Ahn (2003) reported that irradiation changed oxidative potential in meats, thereby influencing the color of fresh meat. When beef steaks were exposed to irradiation, those steaks had lower L values (more dark in color) and higher protein carbonyl contents than non-irradiated steaks, with a positive correlation between the carbonyl contents and Warner–Bratzler shear force values (Rowe et al., 2004a).

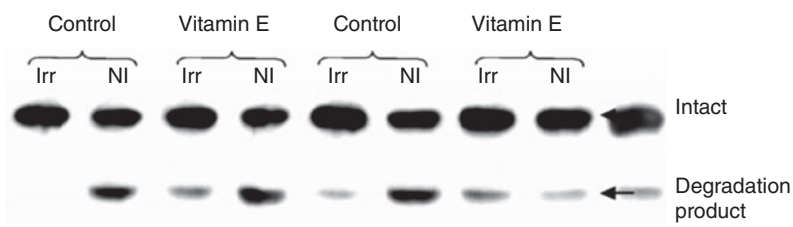


Figure 14.12 Western blot of desmin in purified myofibrils from irradiated (Irr) and non-irradiated (NI) beef longissimus dorsi aged 7 days post-mortem. Adapted from Huff-Lonergan and Lonergan (2005)

14.6.4.2 Cathepsin enzymes Cathepsins comprise a family of proteinases that exist in lysosomes and the sarcoplasmic reticulum of muscle cells (Decker et al., 1980). These enzymes are responsible for the intracellular protein degradation that is physiologically and pathologically important in living tissue (Kirschke and Barrett, 1987). They are usually categorized by their amino acids at their active sites—cysteine (cathepsins B, H, L, X), serine (cathepsin G), and aspartic (cathepsins D, E) peptidase families (Sentandreu et al., 2002). Out of 15 lysosomal cathepsin enzymes known to exist, only eight (B, L, H, S, F, K, D, and E) have been found in skeletal muscle fibers (Sentandreu et al., 2002). Four (B, D, H, and L) of the eight cathepsin enzymes in skeletal muscle have been found to degrade some proteins during PM aging (Sentandreu et al., 2002). Mikami et al. (1987) reported that, in addition to actin and myosin, cathepsin L hydrolyzed the largest number of myofibrillar proteins, including troponin T, I, and C, nebulin, titin, and tropomyosin. Although it appears to be unlikely that cathepsins play a major role in meat tenderization, their participation should be considered as membrane integrity is not maintained with decreasing ATP, temperature, and pH during PM storage.

14.6.4.3 Proteasomes Proteasomes (26S) are very large protein complexes, consisting of a 19S regulatory subunit and a 20S multicatalytic structure for proteolytic enzyme activities (Dahlmann et al., 2001). Proteolysis by the proteasome is a ubiquitin-mediated process, which requires the attachment of at least four ubiquitin molecules to lysine residue on the target substrate (Goll et al., 2003). The poly-ubiquitinated proteins are then recognized by the 19S proteasome, which subsequently removes the ubiquitin chain and degrades the substrate (Taillandier et al., 2004). These proteasomes were reported to hydrolyze nebulin, myosin, actin, and tropomyosin in bovine myofibrils (Taylor et al., 1995; Robert et al., 1999). However, Koohmaraie (1992) and Koohmaraie and Geesink (2006) reported that proteasomes were not involved in the initial steps of myofibril disassembly, and different degradation patterns were found between the myofibrillar proteins incubated with 20S proteasome and those in PM aging.

14.6.5 Electrical stimulation

Post-mortem electrical stimulation (ES) on animal carcasses was first developed and commercially implemented in the 1950s in New Zealand. ES subsequently became widely spread to the red meat industry in the 1970s (Chrystall and Hagyard, 1976; Chrystall and Devine, 1985). The electricity passing through a freshly slaughtered animal carcass accelerates pH fall and rigor onset via muscle contraction (Figure 14.13). In 1979, Cross suggested three hypotheses for the effect of ES on meat tenderization: (1) prevention of cold shortening by rapid ATP depletion while muscle temperature is still warm, (2) enhancement of endogenous protease activity due to the low muscle pH and high muscle temperatures—favorable conditions for lysosomal enzymes to degrade muscle proteins, and (3) physical disruption of the muscle fiber through the strong muscle contraction—less muscle filament overlap and less resistance during chewing.

Today, ES has been globally implemented to increase sensory panel tenderness ratings by 20–26% and decrease shear force values by 22–23% mostly in beef and lamb carcasses

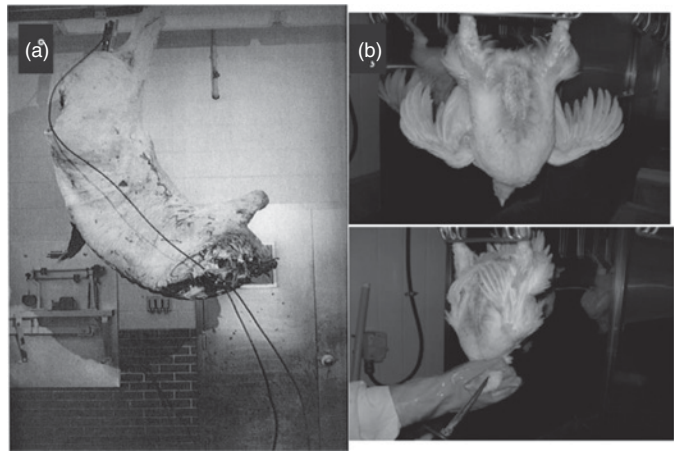


Figure 14.13 (a) Electrical stimulation of beef carcass and (b) broiler carcass. Adapted from Aberle et al. (2001)

(Stiffler et al., 1982). A variety of electrical stimulators are available for packing plants of 1 to 10 heads per day, 10 to 40 heads per hour, or 200 to 300 heads per hour. ES is applied at various processing steps such as after bleeding, after dehiding/before evisceration, or after evisceration/carcass splitting (Stiffler et al., 1982). The ES has been tested from low (40 V) to high (1130 V) voltage, with the common voltage systems of high (440, 550, or 700 V) and low (35 to 70 V). The high voltage ES is more effective on muscle tenderness than the low voltage ES (King et al., 2004), although the low voltage is safer to use. The current challenge with ES technology is to develop systems that optimize the activation of enzyme activity through the alternation of muscle cell structure and reduction of cold-induced shortening by manipulating rigor mortis at optimal carcass temperature.

ES has also been adapted to poultry commercial processing lines. The purpose of ES in broiler carcasses is to increase through-put of boneless broiler processes by reducing carcass aging time. Years of research indicated that broiler carcasses need to be aged at refrigerated temperatures (<4°C) for 4–6 hours before deboning (Lyon et al., 1985). Currently, carcasses are aged for 2.5–4.5 hours in refrigerated storage before deboning because broiler carcasses are usually chilled for 1.5 hours in water immersion. When carcasses were subjected to high-current ES for 15 seconds and deboned after 1.5–2 hours PM chilling, the resulting meat was considered "slightly to moderately tender" to consumers (Hirschler and Sams, 1997). Comparing product yield, Hirschler and Sams (1997) reported that deboning breast fillets at 2 hours PM would increase meat yield by 3.4% compared to deboning at 11 hours PM.

14.7 Pre-rigor/hot boning technology

It is always desirable for meat packers to increase the speed of muscle-to-meat conversion with no quality loss. Pre-rigor or hot-boning processes accelerate the speed of the meat

conversion and generate high-quality muscle protein. Pre-rigor meat is the muscle that is removed from animal carcasses prior to rigor mortis development. Hot-boned meat, a synonym of pre-rigor meat, is the muscle removed from animal carcasses before chilling or substantial reduction of the body heat. Hot-boned meat may include muscle that has entered the onset of rigor, depending on the time of muscle harvest. In general, hot-boned meats have greater water-holding capacity, high protein extractability, and better emulsifying capacity than cold-boned meats (Hamm, 1981; Claus et al., 1998; Claus and Sorheim, 2006).

Currently, the hot-boning technique is utilized in many countries such as Australia, New Zealand, Norway, South Africa, and Sweden. The advantages of hot-boning are energy efficiency, labor reduction, high meat yield, shorter processing time, less drip loss in vacuum packs, faster cure penetration, and greater color development (Kastner, 1977; Cuthbertson, 1980; Troy, 2006). In the case of beef, hot-boning prevents evaporation loss by 1–2.2% during cooling, drip loss by 0.1–0.6% during storage, cooler-space loss by 50–55%, and labor loss by 20% due to the rapid meat turnover (Pisula and Tyburcy, 1996).

Despite these advantages, the technique has not been widely implemented due to several issues such as high initial investment, staff training, meat cuts with unconventional shapes, synchronization difficulty between slaughter and deboning lines, grading difficulty of hot carcasses, and unfamiliarity of the meats. In addition, the protein superiority disappears as rigor mortis develops with no carcass restraint. Therefore, the meat needs to be properly handled to preserve the protein functionality. Although pre-rigor meats maintain their functional properties when frozen quickly (below −20°C), the meats become very tough (thaw-rigor toughening) when thawed at room temperature. Recently, Lee et al. (2007) reported that both higher tenderness and less muscle shortening were observed when hot-boned and rapidly frozen beef was thawed at −2°C over the muscle thawed at 2 and 18°C. When mixing ground-pre-rigor muscles with sodium chloride (2–4%), glycolysis is inhibited due to a denaturation of glycolytic enzymes with high ionic strength. As a result, the salted pre-rigor meat maintained high water-holding capacity due to a strong electrostatic repulsion between adjacent protein molecules, high pH, and ionic strength (Hamm, 1977).

14.7.1 Hot-boning and physical stretch

Hopkins and Geesink (2009) indicated three basic factors in meat tenderness: (1) background toughness, (2) toughening phase, and (3) tenderization phase. The last two of the three factors can be modified to a certain level by altering muscle shortening during PM storage, although the background toughness is difficult to modify at processing plants (Hopkins and Thompson, 2001).

Application of ES to animal carcasses prevents cold-shortening by accelerating the rigor onset before the muscle temperature reaches the cold-shortening zone (Eikelenboom et al., 1985). An additional approach to improve the tenderness of hot-boned meat is to physically restrain the muscle until the rigor mortis is completed (Macfarlane et al., 1974). The concept of physical stretch started with a modified carcass suspension (called tender stretch) for stretching major muscle groups (Figure 14.6), which was adopted to

Figure 14.14 Beef topside muscle of before (28 cm) and after (46 cm) SmartStretch™ stretch. Adapted from Taylor et al. (2011)

excised muscles by clamping, and further advanced to the Pi-Vac Elasto-Pack System, using tubes of elastic film and released pressure (Meixner and Karinitzschky, 2001).

Currently, SmartStretch™ technology (Figure 14.14) is performed on excised hot-boned individual muscle while tenderstretch is applied to whole carcasses. The SmartStretch™ caused 46% and 38% reduction in the shear force of hot-boned sheep topsides over the non-stretched control at 0 and 5 days aging, respectively (Toohey et al., 2008). When beef *longissimus thoracis* muscle was stretched to 20, 40, and 60%, the 20% stretch produced a significantly lower shear force than the non-stretched control, but no further reduction was seen from the 40- and 60% stretched samples (Simmons et al., 1999).

14.8 Conclusions

Proteins are the building blocks of life, which must be consumed in abundance to meet the body's need for tissue synthesis and biological functions. Proteins are found in both animal (meats, milk, fish, and eggs) and plant sources (soy, beans, legumes, nut butters, and grains), but the former contains amino acids that are more similar, in proportion, to the need of our body. Animal production, converting forages and crop by-products to high-quality human foods, makes important contribution to agricultural economics throughout the world and to food security. The total demand for animal-origin meats is expected to be more than double by the year 2020 in developing countries, with a continuous request in developed countries for more than 40% of their dietary protein. Subsequently, animal agriculture will continuously to be an important part of food-producing sectors, which needs to be maximized through research and development in livestock production system, innovation in animal processing, and adequate global food supply to feed a growing population.

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15
Seafood Proteins and Surimi

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15.1 Introduction

The most common seafood protein commercially utilized is surimi (a raw material) used for surimi seafood (a finished product). Surimi, like the muscle proteins of other animal species (egg white, plasma, and milk), forms thermo-irreversible gels upon heating, which do not melt with further temperature change. Consequently, heat-induced gelation is the primary property of surimi that makes it useful as a food ingredient (Lanier et al., 2005).

Surimi is stabilized myofibrillar proteins obtained from mechanically deboned fish flesh that is washed with water and blended with cryoprotectants. Surimi is an intermediate product used in a variety of products ranging from the traditional kamaboko products of Japan to various surimi seafood products including crabstick. Before 1960, surimi was manufactured and used within a few days as a refrigerated raw material because freezing deteriorated muscle proteins and induced protein denaturation, which resulted in poor functionality. However, with the discovery of cryoprotectants, the surimi industry was able to tap into previously unexploited resources (Park and Lin, 2005).

This chapter will briefly cover world production, distribution, and utilization of surimi. It will review the chemistry and rheology of muscle proteins in regards to surimi gelation, as affected by various factors associated with the manufacturing of surimi and surimi

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seafood. Biochemical and immunochemical assay used for proteins, differentiation of fish species, and allergen identification will also be discussed.

15.2 World production, distribution, and utilization of surimi

Modern surimi production started with the Japanese fish processing industry. Early developments, however, have expanded the industry into the United States, South Korea, and Southeast Asia. With increased surimi production in the United States, the involvement of Japan in world surimi production decreased. The annual US production of surimi reached about 150,000 to 220,000 metric tons (MT) (1990–2005). However starting in 2006, the US surimi production volume changed significantly based on the global supply and demand: tropical surimi started to play a major role as Alaska pollock and Pacific whiting had more value as fillets and headed and gutted fish (H&G). The world surimi production over the last 20 years ranged between 420,000 and 800,000 MT (Guenneugues and Ianelli, 2014). The surimi industry mainly utilized Alaska pollock for surimi production, which covered 50–70% of total surimi, but its proportion has been continuously reduced beginning in the 1990s. Since 1991, efforts to use other species have also been successful through technical and marketing advances in Japan. Currently more than 60% of surimi is produced from tropical fish, 30% from Alaska pollock, and 10% from other cold water species.

As for the production of surimi seafood, it started with chikuwa in Japan based on the product concept derived from a cattail plant 900 years ago. There are four traditional types for surimi seafood in Japan based on cooking methods: kamaboko (steamed), chikuwa (grilled), satsuma-age (fried), and hanpen (boiled) (Park, 2005). Until the development of a new generation product called crabstick (crab leg) in Japan in 1974–1975, surimi and surimi seafood was not known outside of Japan. The development of crabstick became a corner stone for the globalization of surimi and surimi seafood.

The world surimi seafood market was estimated at over 2 million MT in 2010, corresponding to a usage of about 750,000 MT of frozen surimi. The consumption has grown by 2–3% per year, particularly in Asia. Surimi seafood market in Japan is approximately 570,000 MT with the usage of 320,000 MT of surimi representing nearly 40% of the world surimi market. The production of surimi seafood in other regions include: South Korea at 300,000 MT, Southeast Asia at 160,000 MT, China at 1,000,000 MT, Europe/Russia at 210,000 MT, and North America at 90,000 MT (Guenneugues and Ianelli, 2014).

In Japan, the production of surimi seafood is seasonal. The industry produces more surimi seafood for the winter season as a result of the increased consumption during the New Year holidays in Japan. Like in South Korea, Japanese consumers also enjoy hot served products [oden (Japan), ahmook and hot bar (South Korea)] in winter. However, the market in the United States is exactly the opposite and surimi seafood is consumed largely during the summer as a salad ingredient. Since the late 1970s, crabstick has become increasingly popular in North America. The consumption of surimi seafood has proliferated mainly in cold salads with an annual growth rate of 10–100% in the 1980s. Then the market has been matured since is about 90,000 MT.

15.3 Surimi production

15.3.1 Conventional surimi

Surimi is stabilized myofibrillar proteins refined from mechanically deboned fish flesh that is washed with water and blended with cryoprotectants (Park and Lin, 2005). Conventional surimi processing (Figure 15.1) from white flesh fish utilizes typically 25–30% of the body mass, including recovered insoluble protein particles from wash water. Fish flesh is ground into small particles (3–4 mm diameter) first before going through rigorous washing and dewatering using batch washing and rotary screens, respectively. Washing is an essential step in removing water-soluble proteins (primarily sarcoplasmic proteins) and other impurities that reduce product quality. Sarcoplasmic proteins exist in the fluids within and between muscle fibers, and include many metabolic enzymes that diminish the stability of functional proteins during storage. Myofibrillar proteins, the primary components with the ability to form a three-dimensional gel network, constitute approximately 70% of the total proteins in minced fish meat. Removing water-soluble proteins, in turn, concentrates myofibrillar proteins. Thus it is known to enhance the functional property of surimi.

A proper washing process is vital in achieving high-quality surimi with high recovery. An insufficient washing process could result in substantial loss of gel quality during frozen storage. However, overwashing could cause substantial loss of fine particles and excessive moisture content. Maintaining water temperature near 5°C or below is critical for cold water species, such as Alaska pollock and Pacific whiting to maintain protein quality (Park and Lin, 2005).

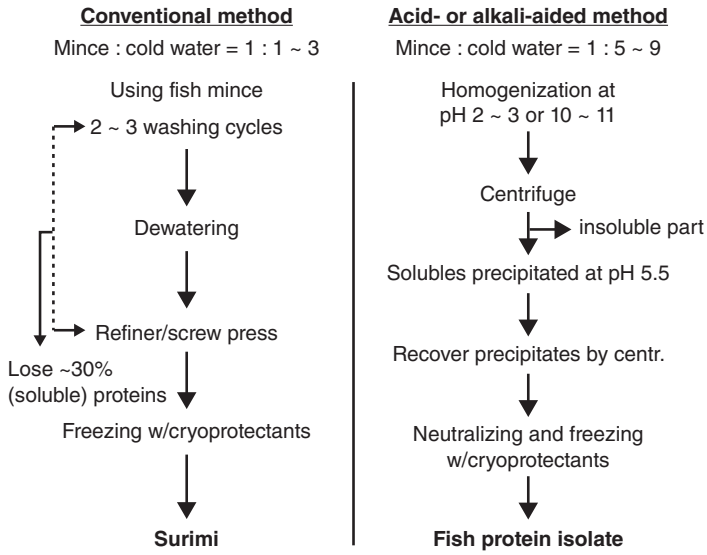

Figure 15.1 Manufacturing flow chart for surimi and fish protein isolate. Reproduced with permission of John Wiley & Sons

Once washing is completed, washed meat will go through mechanical refining, which removes scales, pin bones, and connective tissues. Then refined meat will be subjected to mechanical screw press to reduce the moisture content to 83–85%. This dewatered meat is then mixed with cryoprotectants to maintain frozen stability of myofibrillar proteins. It is typical that cryoprotectants consist of 4–5% sorbitol, 4% sugar, and 0.2–0.3% sodium tripolyphosphate. However, a reduced level of cryoprotectants (6% sugar and 0.2% sodium polyphosphate) is used for tropical surimi because myofibrillar proteins from tropical fish possess much higher thermal stability. Surimi packed in a 10-kg block is then frozen using a contact plate freezer before storing at –20°C or lower for a 2-year shelf life.

15.3.2 Fish protein isolate

A fish protein isolate (FPI) process (Figure 15.1) using acid or alkali extraction followed by isoelectric precipitation provides extremely high yields (35–45%) with the inclusion of sarcoplasmic proteins and it also demonstrates good functional properties (Hultin and Kelleher, 2000). This process consists of homogenizing fish tissue, solubilizing in acid (pH <3.0) or alkali (pH >10.0), recovering proteins by centrifugation (10,000 × g) after adjusting the pH to 5.5 using 1–2 N NaOH or 1–2 N HCl, respectively, and neutralizing with 1–2 N HCl or 1–2 N NaOH, respectively, before freezing with cryoprotectants. The two approaches (surimi and FPI) in isolating fish proteins have a distinctive difference in their processing chemistry. Conventional surimi processing avoids any possible denaturation to prevent protein damage and maintain protein quality. In contrast, the FPI process induces chemical denaturation by adjusting pH to an acid or alkali condition and neutralizing by NaOH or HCl, respectively. Two acidic and alkaline chemicals used during processing are basically neutralized as NaCl and H_2O. Therefore these chemicals can be categorized as a processing aid, but the resultants (salt and water) have to be labeled. Depending on the pH of fish (raw material) and the pH of FPI (end product), it should also be noted that unused sodium ions can be retained in FPI, possibly resulting in higher sodium content (Park, 2009).

Several groups in the United States have led numerous research on functional FPI made from various species (Pacific whiting, herring, catfish, Pacific sardine, Atlantic croaker, tilapia, jack mackerel, menhaden, rockfish, trout, krill, and giant squid) since 2003 following the original patent by Hultin and Kelleher (2000).

Alkaline extraction appears to give better gelling functionality (Kim et al., 2003; Yongsawatdigul and Park, 2004; Kristinsson and Liang, 2006; Chen and Jaczynski, 2007; Thawornchinsombut and Park, 2007). According to Yongsawatdigul and Park (2004), the gelling properties of rockfish proteins at various pH extraction conditions were clearly demonstrated with a descending order of alkaline extraction, water washing, and acid extraction (Figure 15.2). It should be noted a distinct protein with MW of 120 kDa was found in acid-extracted paste, but it disappeared when made into a gel (Figure 15.3). This protein band presumably resulted from degradation of myosin heavy chain (MHC) during acid extraction and probably disappeared to interact with other proteins during gelation. In alkali-extracted gel, protein bands with 120 and 42 kDa almost disappeared. Therefore gelation of alkali-extracted FPI could have been completed through interaction of myofibrillar and sarcoplasmic proteins via disulfide linkages. They also demonstrated the

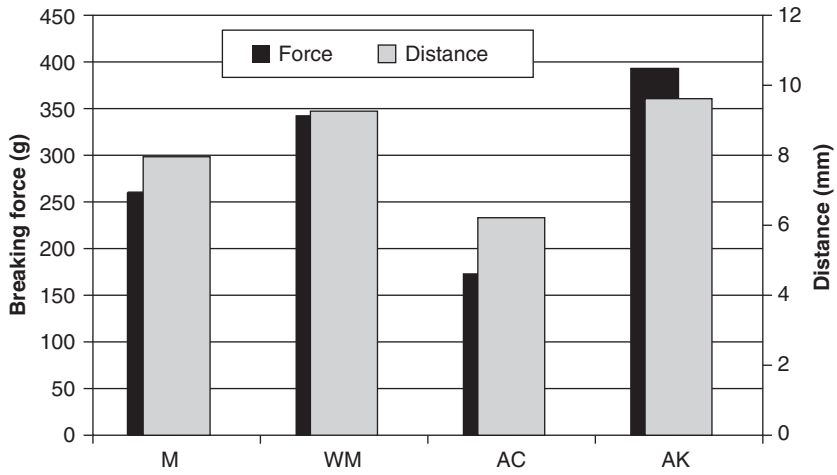

Figure 15.2 Breaking force and deformation of rockfish muscle proteins prepared by various treatments. Reproduced with permission of John Wiley & Sons. M, mince; WM, washed mince (surimi); AC, acid-extracted; AK, alkali-extracted

significant reduction of total sulfhydryl content as FPI formed a gel. It should also be mentioned that other interactions, such as hydrophobic interactions, have been reported to play an important role in gelation of fish muscle (Park et al., 1994b), which could contribute to the rheological properties of acid-extracted and alkali-extracted FPI. Kristinsson and Hultin (2003a) reported an increase in surface hydrophobicity of cod myosin when it was treated in acidic or alkaline pH conditions, indicating the role of hydrophobic interactions in gel formation.

Yongsawatdigul and Park (2004) observed that actin bands of acid- and alkali-extracted FPI, under sodium dodecyl polyacrylamide without β-mercaptoethanol, were less intense than those of mince or washed mince (surimi), indicating actin may favorably interact with MHC of acid- and alkali-extracted FPI through disulfide linkages (Figure 15.3). Kristinsson and Hultin (2003a, 2003b) found that alkali- and acid-treated cod myosin had more exposed reactive SH groups, presumably promoting myosin head-to-head aggregation.

Kristinsson and Hultin (2003b) extensively examined the unfolding of acid- or alkali-treated myosin and gelling at a lower temperature, suggesting a less stable conformational structure of the refolded proteins. Improved functional properties were due to partial unfolding of myosin by acid or alkali. Thawornchinsombut and Park (2007) reported that salt solubility of proteins from FPI did not contribute significantly to their gelation properties. FPI prepared at pH 3 or 11 with NaCl could be partly refolded at pH 7.0. Nevertheless, some myosin fragments and actin did not refold. Kristinsson and Hultin (Kristinsson and Hultin, 2003b) confirmed that the earlier onset of gelation correlated well with thermal unfolding/aggregation behavior of myosin. This is consistent with thermal unfolding being a prerequisite for gelation. Development of gel strength on heating has been found to correlate with increased turbidity (Samejima et al., 1981; Gill et al., 1992) and hydrophobicity (Wicker et al., 1986; Xiong, 1997).

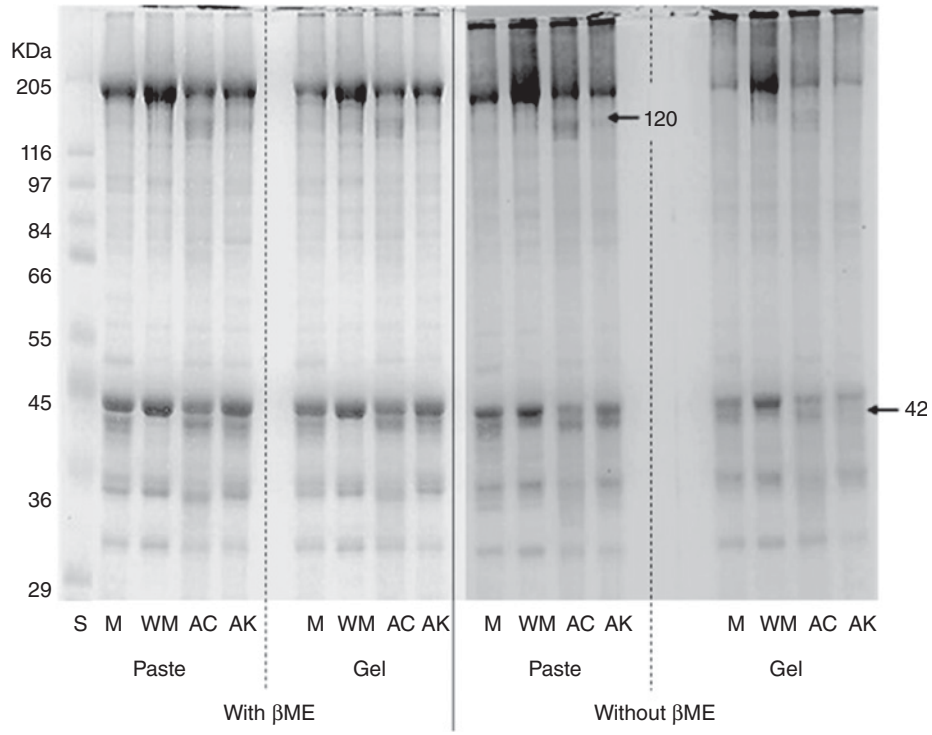

Figure 15.3 Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) patterns of rockfish muscle proteins prepared by various treatments solubilized in buffer with and without β-mercaptoethanol (βME). Reproduced with permission of John Wiley & Sons. S, standard molecular weight; M, mince; WM, washed mince (surimi); AC, acid-extracted; AK, alkali-extracted

15.4 Fish proteins

15.4.1 Myofibrillar proteins

Myofibrillar proteins consist primarily of myosin, actin, tropomyosin, m-protein, α-actinin, β-actinin, c-protein, troponin T, I, and C as well as other minor proteins associated with the myofibril, but which are present in very small quantities. Surimi consists mainly of concentrated myofibrillar proteins, which typically have a high degree of functionality for gel formation when cooked. Myosin is the major muscle protein that is found in fish and comprises approximately 55–60% of the myofibrillar proteins (Lanier et al., 2005). Myosin and actin together account for 65% of the total muscle protein; tropomyosin and the troponins each contribute an additional 5% and the remaining 25% is composed of the other regulatory and structural proteins. Myofibrillar proteins, composed mainly of myosin and actin, are also known as "salt-soluble proteins" due to their ability to be solubilized in solutions of neutral salts.

15.4.1.1 Myosin Of the myofibrillar proteins, myosin is known to be the most significant for the gelling properties of surimi. Myosin is a large asymmetric molecule that has a long α-helical coiled-coil tail and two globular heads with an approximate weight of 500 kDa (Hodge and Cope, 2000). The basic body plane of myosin consists of an N-terminal head or motor domain, a light chain-binding neck domain, and a class conserved, C-terminal tail domain. It has been categorized into over 20 different classes (Mooseker and Foth, 2008). The head or motor domain has a core sequence that is highly conserved in all myosin classes, and it contains the ATPase active site (Holmes, 2008). The neck region, also known as the lever arm, consists of a long α-helix of variable length and a tail region that is extremely variable in sequence, length, domain composition, and organization. The molecular weight of MHC when dissociated in strong denaturing solutions is approximately 220 kDa (Lanier et al., 2005).

In order to better understand the important role that myosin plays in the gelation of myofibrillar proteins, extensive studies have been performed on purified myosin from a variety of fish species. Myosin from many fish species used in the production of surimi has been characterized. These include Alaska pollock (Fukushima et al., 2003, 2005), Pacific whiting (Yongsawatdigul and Park, 1999), cod (Kristinsson and Hultin, 2003b; Brenner et al., 2009), and threadfin bream (Toyohara and Shimizu, 1988; Yongsawatdigul and Park, 2003; Hemung et al., 2008). Due to the importance of myosin it has also been studied in many other fish that are not used specifically for surimi, such as chum salmon, herring, carp, Japanese stingfish, Pacific sardine, and catfish (Chan et al., 1993; Bouraoui et al., 1997; Kakinuma et al., 1998; Nagai et al., 1999; Iwami et al., 2002; Park et al., 2008; Raghavan and Kristinsson, 2008).

By studying the physical and biochemical components of myosin, a better understanding of the gelling capability of myosin from a specific species is possible. Takahashi et al. (2005) studied myosin from carp, rainbow trout, tilapia, yellowtail, pink salmon, brown sole, witch flounder, atka mackerel, and walleye pollock. They found in their study that the characteristics of myosin were species-specific and highly dependent upon the habitat of the fish. The major differences seen between fish species were due to the water temperature of the fish's natural habitat. They found that the myosin from cold water species had a tendency to denature and form gels at a lower temperature than their warm water counterparts.

Skeletal myosin can be broken up into six polypeptide chains, two heavy chains, and four light chains. Myosin light chains typically range from 17 to 25 kDa. These amino acid chains are non-covalently attached to the myosin head (Lanier et al., 2005). Myosin can also be broken into fragments by proteolysis. When myosin is exposed to proteolytic enzymes, trypsin, or α-chymotrypsin, fragmentation occurs in the middle of the tail yielding heavy meromyosin (HMM, molecular weight about 350 kDa) and light meromyosin (LMM, molecular weight about 150 kDa). In addition to the mechanism of trypsin and α-chymotrypsin digestion being similar, Margossian and Lowey (1982) showed that using trypsin or α-chymotrypsin produced similar proteolysis fragments. The HMM contains the head group and a short tail and can be further fragmented by the use of papain into subfragment 1 (S1, molecular weight 110 kDa) and subfragment 2 (S2, molecular weight 240 kDa).

There are two types of myosin light chains: the regulatory light chains and the essential light chains. The regulatory light chains are known for their role in phosphorylation and dephosphorylation (Sobieszek, 1988). The regulatory light chains can be selectively dissociated from myosin by 5,5′-dithiobis-2-nitrobenzoic acid (DTNB); thus they are often referred to as the DTNB light chains (Wagner, 1982). The role of phosphorylation and dephosphorylation corresponds to the contraction and relaxation of smooth muscle. The essential light chains are often referred to as alkali light chains because they are dissociated from myosin under alkaline conditions (Weeds and Pope, 1977). Each myosin head unit contains one DTNB subunit and one alkali unit. This means that there are four myosin light chains per myosin molecule. However, it is interesting to note that when whole myosin is analyzed using SDS-PAGE techniques, there are typically only three bands found for the myosin light chains. This occurs because each head unit has one light chain 2 (LC 2), the DTNB light chain, and then one essential light chain, which can be either light chain 1 or light chain 3 (Bechtel, 1986). Due to the repetition of LC2 in both head units, the SDS-PAGE protein pattern shows only three myosin light chains.

MHC can be enzymatically cleaved into HMM and LMM. It has been disputed as to which portion of the myosin molecule relates to which role in the thermal aggregation of myosin. Gill and Conway (1989) studied the chymotryptic cleavage of thermally aggregated cod myosin and came to the conclusion that the tail portion (LMM) of myosin was involved first in thermal aggregation rather than the head region (HMM). Sano et al. (1990) examined HMM and LMM and found evidence that the initial stages of gel formation was indeed due to the gelation of LMM between 30 and 44°C.

Using differential scanning calorimetry (DSC) methods, Reed and Park (2011b) studied differences in myosin, HMM, and LMM purified from tilapia. Purified myosin showed three major peaks at 17.5, 41.9, and 49.9°C, with enthalpies of denaturation −0.0022, 0.0400, and 0.0146 J/g, respectively. HMM showed one endothermic peak at 43.0°C and one major exothermic peak at 67.1°C, with enthalpies of denaturation of 0.0143 and −0.0517 J/g, respectively. LMM showed three endothermic peaks at 40.4, 51.7, and 69.0°C, with enthalpies of denaturation of 0.0459, 0.0593, and 0.0047 J/g, respectively (Figure 15.4).

Liu et al. (1996) showed thermal transition points at 35.3, 48.1, 49.9, and 67.0°C for chicken breast myosin (0.6 M NaCl, 50 mM phosphate buffer pH 6.0). When studying rabbit myosin head (subfragment 1) in 50 mM TRIS buffer (pH 8.0), 0.6 M KCl, along with HMM in 0.1 M KCl, Shriver and Kamath (1990) found exothermic peaks at 48 and 65°C. They also found that the exothermic peak was attributed to aggregation and precipitation of the unfolded protein. Park and Lanier (1988) found that this exothermic peak near 50°C was a likely indicator of fish myosin in the pre-rigor state. They explained that it was likely due to a rapid rise in ATP hydrolysis induced by rising temperatures. Like their tilapia study, we also used myosin prepared from pre-rigor tilapia and obtained an exothermic peak near 67.1°C in the HMM fraction.

It has been shown that the thermal stability of fish myosin increases as the species adapts to increases in environmental temperatures (Davies et al., 1988). When carp was acclimatized to different holding temperatures (10, 20, 30°C) DSC thermograms demonstrated a significantly increased thermal stability of myosin and LMM from carp

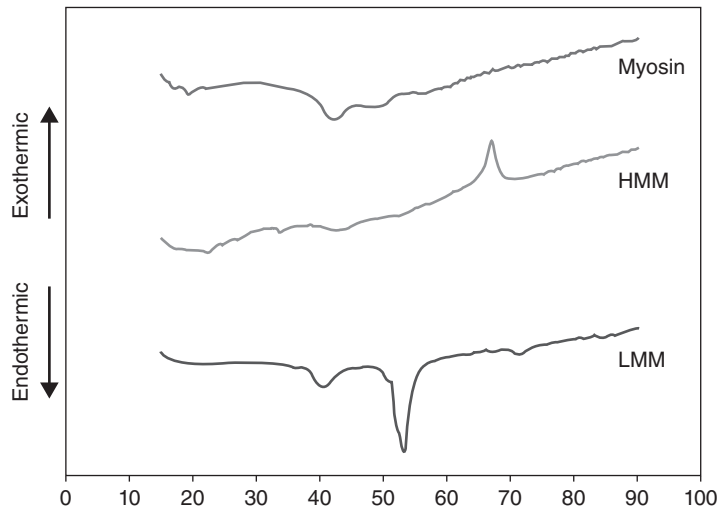

Figure 15.4 Differential scanning calorimetry thermogram of tilapia myosin, HMM, and LMM heated from 10 to 90°C at 1°C/min. From Reed and Park (2011b) with permission

stored at 30°C (Nakaya et al., 1997, 2002). They showed thermal transition temperatures at 35.9, 39.7, and 49.1°C for the carp acclimatized to 30°C, whereas those for the 10°C carp were lower at 32.8, 34.9, and 49.1°C. Togashi et al. (2002) studied myosin and LMM from Alaska pollock and they found that for myosin there were two major endothermic peaks at 33.7°C and 41.3°C. The endothermic peaks for our tilapia study were shown at higher temperature of 41.9 and 49.9°C which may be explained by the habitat of the fish based on the fact that Alaska pollock is cold water fish while tilapia is a warm water fish (Park, 2005). When studying the differences in the gel-forming ability of tilapia and Alaska pollock, Klesk et al. (2000) found that there were significant differences in the optimal cooking temperatures for surimi produced from each fish. Due to its warm water habitat, tilapia showed increased shear stress and shear strain values when the surimi was set at 40°C for 1 hour before cooking at 90°C for 15 minutes. These values were higher than those of Alaska pollock surimi gels. Our results coincided with setting temperature of 40°C, with endothermic peaks appearing at 41.9, 43.0, and 41.1°C for myosin, HMM, and LMM, respectively.

15.4.1.2 Physicochemical properties of fish myosin The functional properties of myofibrillar proteins are extremely important in many muscle food products with myosin playing a significant role in the gel-forming ability (Toshiyuki et al., 1961; Macfarlane et al., 1977). Purified myosin is soluble in salt water, but will form aggregates when heated and if the myosin concentration is high enough, it can lead to gel formation (Gill et al., 1992; Chan et al., 1993; Lin and Park, 1998; Yongsawatdigul and Park, 1999; Fukushima et al., 2003; Lefevre et al., 2007). However, purified fish myosin is very unstable and particularly sensitive to thermal denaturation by heating or freezing. The stability of fish myosin

has also been found to vary between fish species with some evidence of fish species habitat playing a role (Davies et al., 1988; Ogawa et al., 1993).

Heat is one contributing factor of protein denaturation. A variety of chemical testing can be performed to understand the gelation process of myosin. The denaturation of proteins by heating can be monitored and studied by monitoring the degree of surface hydrophobicity of the protein. By measuring the surface hydrophobicity of fish myosin as affected by heat treatment, protein denaturation can be determined (Lin and Park, 1998; Visessanguan et al., 2000; Thawornchinsombut and Park, 2004). Hydrophobic bonding is thought to play a significant role in gel formation as well and typically increases the gel strength as it is heated with a maximum increase in strength up to approximately 60°C (Gilleland et al., 1997; Lanier et al., 2005). The three-dimensional structure of native myosin has a hydrophobic core, which allows hydrophobic amino acids, such as valine, leucine, isoleucine, methionine, phenylalanine, tryptophan, and cysteine, to exclude water and to strengthen their interaction (Betts and Russell, 2003). However, as the protein begins to denature, its native structure is disrupted, which exposes the hydrophobic portions of the protein to interactions with water and other substances (Benjakul et al., 2003). Therefore, studying the hydrophobicity of fish myosin can provide critical insight into the denaturation process. (Wicker and Knopp, 1988; Chan et al., 1993; Lin and Park, 1998; Visessanguan et al., 2000; Yongsawatdigul and Park, 2003; Thawornchinsombut and Park, 2004).

Measuring the amount of reactive sulfhydryl (SH) groups can also serve to help understand the denaturation of myosin (Gilleland et al., 1997). When studying the amount of reactive sulfhydryl content, there are two ways to approach: one measures the surface-reactive sulfhydryl groups (SR-SH) and the other measures the total reactive sulfhydryl groups (TSH). It is useful to test for the SR-SH groups using a non-denaturing buffer to measure the amount of SH groups found on the surface of the native myosin. The SR-SH content of the protein can be compared to the TSH groups when the sample is tested using both a denaturing and non-denaturing buffer (Hoffmann and Van Mil, 1997; Hsu et al., 2007). Many of the SH groups are buried inside of the native myosin protein due to the protein conformation, and cannot react when tested for the SR-SH content. When 8 M urea is used, the myosin is completely denatured, thereby exposing the buried SH groups and allowing for their quantification (Riddles et al., 1979). When the myosin is heated, it will begin to denature and the exposed sulfhydryl groups will begin to spontaneously form disulfide linkages as the temperature approaches 50°C and above.

When studying physicochemical properties of fish myosin, turbidity measurements of a protein solution is useful in understanding the denaturation and aggregation of the particular protein. Turbidity is a measure of the cloudiness or haziness created by the aggregation of suspended particles in a solution. As the sample is heated, the proteins begin to denature and with prolonged heating, they can begin to form larger myosin aggregates (Chan et al., 1993) thereby increasing the optical density (OD) at 320 nm. This change in OD is due to the aggregation of the suspended particles of myosin, which according to Yongsawatdigul and Park (1999) can be species specific. The OD of myosin measured in a linear heating pattern can help provide information about the denaturation and aggregation characteristics of fish myosin.

15.4.2 Sarcoplasmic proteins

Sarcoplasmic proteins consist of myoglobin, hemoglobin, cytochrome proteins, and a wide variety of endogenous enzymes associated with the citric acid cycle, and the electron transport chain (Forrest et al., 1975). These proteins constitute 30–35% of the total muscle protein. Sarcoplasmic proteins are soluble in solutions of low salt concentration and in water. Myoglobin consists of a globular protein portion (globin) and a non-protein portion called a heme ring. The heme portion of the pigment plays a special role in meat color determined by the oxidation state of iron within the heme ring.

The proteins in the sarcoplasmic fraction are excellently suited to distinguishing fish species, as each species has a characteristic band pattern when separated by the isoelectric focusing (IEF) method. Hsieh et al. (1997) found that sarcoplasmic proteins were well suited for identification of cooked red snapper using IEF. Other species such as perch (Berrini et al., 2006), and seven species of puffer fish (Chen and Hwang, 2002) were also identified using sarcoplasmic proteins.

All fish enzymes are sarcoplasmic proteins. Protease and trimethyleamine oxide demethylase degrade myofibrillar proteins and denature/aggregate them during frozen storage, respectively. Endogenous transglutaminase (eTGase) is unique to fish and functions to link glutamic acid and lysine into non-disulfide covalent bonds. These enzymes are largely removed from surimi through washing/dewatering, but their small quantity is still strong to affect the quality of surimi.

15.4.2.1 Enzymes in fish muscle Softening of fish muscle can be induced by cooking, especially when slow heating is applied. It is characterized by rapid and severe degradation of myosin. Several heat-stable proteinases have been shown to contribute to the thermal softening of fish muscle, that is, lysosomal proteinases (primarily cathepsins), alkaline proteinase, calpain, and collagenase (Wasson, 1993). Likewise, extremely high proteolytic activity is detected during the relatively slow heating processes used in the manufacture of certain large diameter or relatively thick surimi gels or surimi seafood. The presence of proteolytic enzymes is most notable in surimi manufactured from Pacific whiting, arrowtooth flounder, and almost all tropical fish. Low-grade pollock surimi also contains significant levels of proteolytic enzymes.

Fish endopeptidases may be classified into four main subgroups of serine, cysteine, aspartic, and metallo-proteinases, according to the chemical group of their active site (though histidine-linked proteinases have also been reported) (Choi et al., 2005). Only serine and cysteine (also termed thiol or sulfhydryl) proteinases appear to be involved in surimi gel degradation during slow heating. From a practical standpoint, fish muscle proteinases may also be grouped according to the optimum pH of their activity on muscle proteins, such as acid, neutral, and alkaline proteases.

Kinoshita et al. (1990) classified 12 fish species based on the extractability characteristics of the proteinase from the muscle (i.e., sarcoplasmic or myofibrillar-associated), the optimum temperature (50 or 60°C) for MHC degradation, and the sensitivity of the proteinase activity to *n*-butanol. Among the 12, 6 species had proteinases that were easily

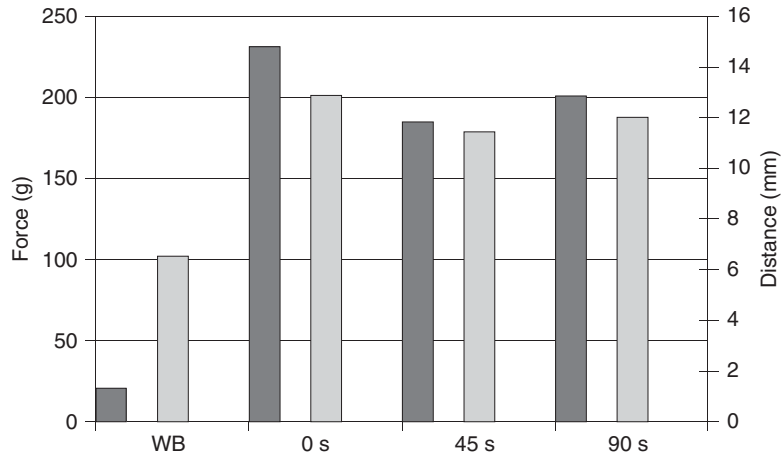

Figure 15.5 Gelling properties of Pacific whiting surimi as affected by slow (WB, water bath) and rapid (ohmic at 250 V) heating. Black bar indicates gel hardness (breaking force) while grey bar gel cohesiveness (penetration distance). 0 s, 45 s, and 90 s indicate that surimi paste was heated to 90°C using 250 V and gels were held at 0, 45, and 90 seconds, respectively

extractable in the sarcoplasma, 3 possessed proteinase tightly associated with the myofibrils, and 3 others possessed proteinases of both types.

Pacific whiting is a good example of a species that contains proteolytic enzymes of both types (easily extractable and tightly associated). While cathepsins B, H, and L present in this species show comparable activities in fish mince, a large portion of cathepsin B and almost all of cathepsin H are removed during the washing process of surimi manufacture, while cathepsin L is not washed out, indicating that it alone is tightly associated with the myofibrils.

Control of proteolytic enzymes can be done either using food grade enzyme inhibitors or cooking rapidly. Plasma proteins (beef and pork) most effectively inhibit these proteases, while dried egg white (DEW) and whey protein concentrate (WPC) also moderately inhibit them. The most effective and economic strategy to control these proteolytic enzymes is to cook enzyme-laden surimi rapidly (Figure 15.5). Pacific whiting surimi's gelling properties increased significantly when rapidly cooked: more than 10 times for breaking force and more than 2 times for breaking distance. This indicates that surimi quality typically measured based on the slow cooking water bath method is underestimated and enzyme-laden surimi is sold at a significantly lower value. However, enzyme-laden surimi performs well in fast-cooked products (i.e., crabstick and fried surimi seafood). We have teamed up with Kami Steel (Seattle, WA) to introduce a rapid cooking gel preparation machine, RAPSA to the global surimi industry. It certainly helped the industry obtain accurate gel texture information for protease-laden surimi.

Trimethylamine oxide (TMAO), a water-soluble nitrogenous compound used by fish for osmoregulation is present in most marine species. TMAO demethylase is an enzyme,

which is especially prevalent in gadoid (cod-like) species, such as whiting, hoki, and pollock, degrades TMAO to formaldehyde (FA) and dimethylamine (DMA) during frozen storage. FA is a strong protein denaturant and thus, the gelling properties of surimi or minced fish can be deteriorated rapidly if this enzyme system is active and present at a sufficient concentration (Lanier et al., 2005).

Much of the TMAO demethylase activity resides in organ tissues, such as kidney, liver, and pyloric caeca. If these are thoroughly removed from the fish before deboning of the meat, the problem is somewhat alleviated. Fortunately, if properly conducted, the leaching process acts to remove the majority of the TMAO from the meat, and seems to inactivate or remove the demethylase enzyme as well (Holmquist, 1982). However, as surimi production tries to squeeze more meat out of fish, it is somewhat unavoidable to have a part of kidney tissue entering the deboning machine. When quality is rapidly reduced during frozen storage, it is most likely that residual TMAO has lead to the development of FA and denaturation of surimi proteins.

The presence of eTGase and subsequent setting ability are the most unique properties of fish protein when compared to other proteins. It is generally water soluble, but its small residual concentration is still effective to be functional. Sufficient calcium ions must be present for the eTGase to be active and induce setting. Setting, which is suwari in Japanese, is a result of non-disulfide covalent bond between glutamic acid and lysine as transglutaminase mediates the reaction. Unlike eTGase, microbial transglutaminase (mTGase by Ajinomoto) is calcium independent.

High variability in the TGase activity of surimi is common and is due to a combination of factors. First, TGase is a water-soluble enzyme and its content can vary greatly with the type and extent of purification process employed during surimi manufacture (Lanier et al., 2005). Nowsad et al. (1995) showed that the sarcoplasmic fraction of fish can actually enhance the gelling ability when added back to surimi due to its higher TGase activity. Yongsawatdigul et al. (2002) indicated that the first washing cycle removed a large portion of the endogenous TGase from threadfin bream muscle. Only about 44% of the original TGase activity was retained in the final surimi.

As demonstrated in Figure 15.6, the uniqueness of fish proteins is that they can set at 5–40°C without heating when all samples were comminuted with 2% NaCl. Cold water fish Alaska pollock surimi set well at 5, 25, and 40°C. Temperate water fish Pacific whiting set relatively well at 5 and 25°C. However, tropical fish set only at 40°C. This indicates eTGase activity is highly dependent on fish thermal stability as affected by fish habitat temperature. Red meats demonstrated no sign of change, when subjected to low temperature setting. It is also interesting to see the significant quality reduction at 40°C for Pacific whiting surimi as compared to other setting temperatures. This is probably related to the competition between eTGase and proteolytic enzyme (cathepsin L). The latter dominated the former.

Calcium addition enhances the eTGase-mediated setting reaction resulting in a significant improvement in gel texture compared to surimi gels cooked without setting (Lee and Park, 1998). Calcium addition to surimi before freezing, however, potentiates greater denaturation during frozen storage of surimi; therefore it is best to add calcium only during surimi seafood manufacturing (Saeki, 1996).

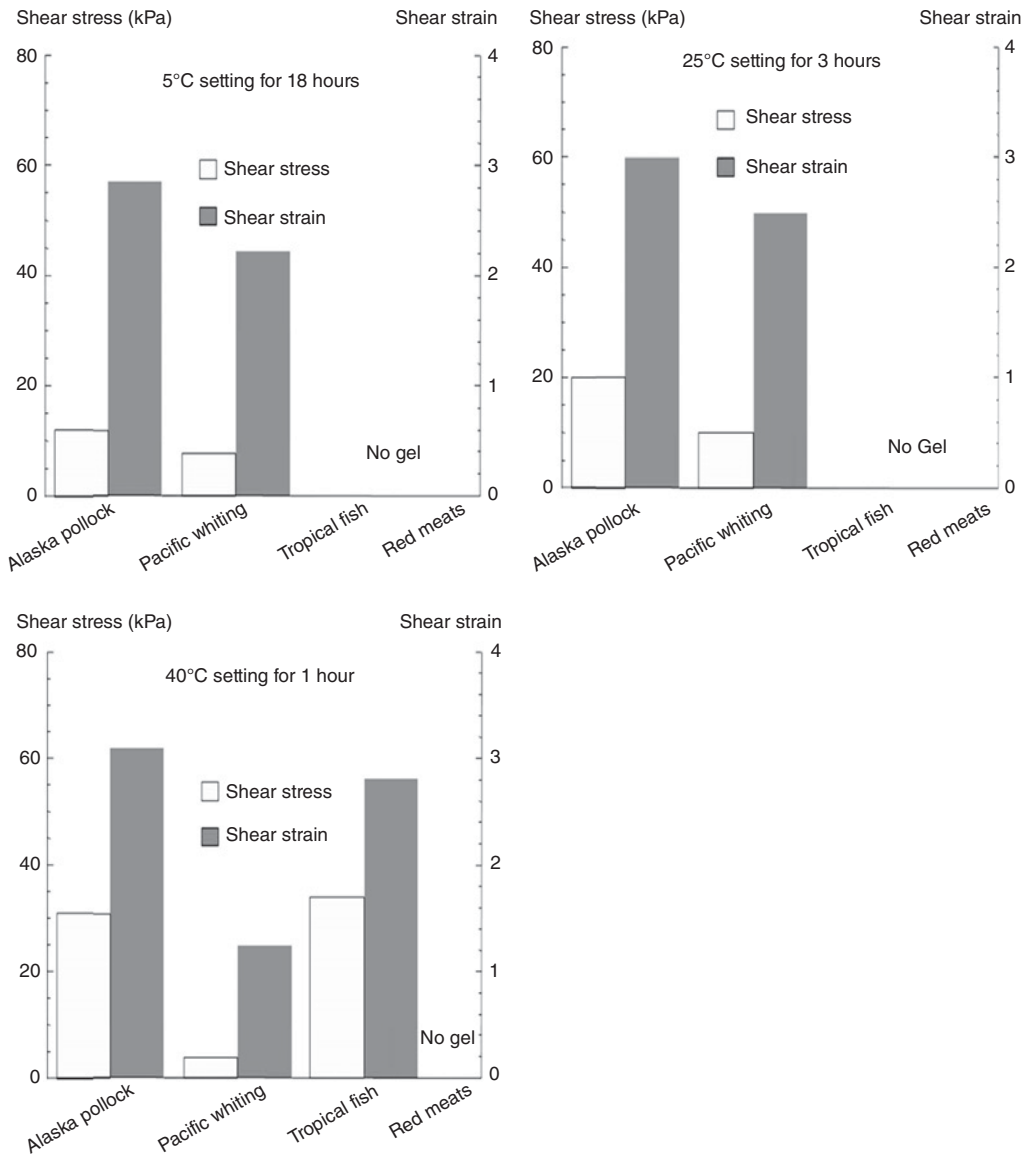

Figure 15.6 Uniqueness of fish proteins setting at 5, 25, or 40°C, depending on the thermal stability of fish species. Fish paste set gels while red meats still demonstrated no changes. Paste was prepared using 2% NaCl. Reproduced with permission of John Wiley & Sons

15.4.3 Stroma proteins

Stroma proteins are connective tissue proteins, consist primarily of collagen and elastin, but also includes lipoproteins of the cell membrane and sarcoplasmic reticulum. In muscle, the connective tissue is composed mainly of collagen and serves as an extracellular

support for the fibers. Compared to red meats, the concentration of connective tissue in fin fish is extremely low: beef (16–28%) versus cod (3%) (Lanier et al., 2005). The physicochemical properties of collagen are different in tissues such as skin, swim bladder, and the myocommata in muscle. In general, collagen fibrils form a delicate network structure with varying complexity in the different connective tissues in a pattern similar to that found in mammals. However, the collagen in fish is much more thermo labile and contains fewer, but more labile, cross-links than collagen from warm-blooded vertebrates. Different fish species contain varying amounts of collagen in body tissues. This has led to a theory that the distribution of collagen may reflect the swimming behavior of the species. Furthermore, the varying amounts and varying types of collagen in different fishes may also have an influence on the textural properties of fish muscle.

During surimi processing almost all connective tissues are removed through deboning and refining.

15.5 Methods used to determine physicochemical properties of fish proteins

15.5.1 Dynamic rheology

Dynamic rheology is a powerful tool that can be used for characterization of gelling properties and aggregation of fish proteins, such as purified fish myosin. Many fish such as Alaska pollock, Pacific whiting, Southern blue whiting, Northern blue whiting, threadfin bream, lizardfish, and croaker are used to produce surimi (Guenneugues and Morrissey, 2005). In order to better understand the role that myosin plays in the gelation of surimi, many commonly utilized species have been studied using dynamic rheology. Fukushima et al. (2003) studied the rheological changes and differences for Alaska pollock, white croaker, and rabbit myosin. In their studies, they found that the thermal denaturation of myosin was species specific and yielded information on the stabilities of each species myosin. They also reported that the difference in the temperature at which myosin denatured and formed a gel was an indication of their overall gel-forming capability.

Not only can dynamic rheology be useful when studying different species, it can also provide valuable information about myosin that is obtained from different muscle portions in the same fish. Lefevre et al. (2007) studied the aggregation properties of salmon myofibrils and myosin purified from white and red muscles. When they compared myofibrils and myosin from white and red muscles, they found that the rheological profile for the red muscle proteins correlated to an increasing shift to a higher temperature in denaturation and aggregation. The gel rigidity for both red- and white-muscle myosin gels was similar, however the denaturation demonstrated through dynamic rheology showed distinct differences in the proteins from the two muscle types.

Reed and Park (2011a) studied purified myosin from Chinook salmon as affected by constant heating rate. In their study they looked at the rheological and biochemical changes of myosin. Salmon myosin was subjected to dynamic rheological measurements in order to better understand the gelling capability of purified myosin. G' is known as the storage modulus and is used to describe the elastic component of myosin gel, which is the

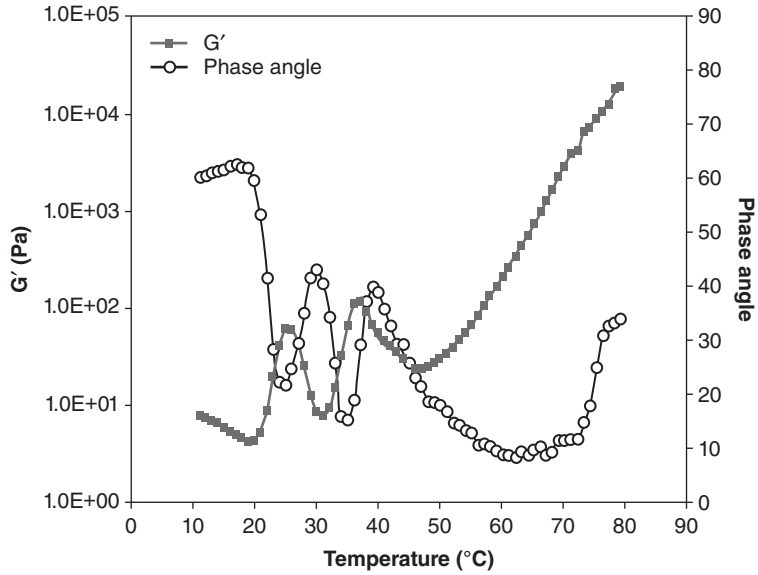

Figure 15.7 Changes in storage modulus and phase angle of salmon myosin during temperature sweep (2°C/min). Reproduced with permission of Elsevier

energy that is applied to the sample and stored as elastic energy. G″ is the loss modulus, which describes the amount of energy dissipated to the gel as heat and is used to describe the viscous component of myosin gel. When the salmon myosin is heated from 10 to 80°C, it goes from a more viscous material to a more elastic material. This property of viscoelasticity is described in the form of the phase angle. The phase angle is given when G′ and G″ are examined together and expressed as the tan δ (G″/G′) (Rao, 1999). This phase angle can be used to help determine the nature of the sample, that is to say, where the sample may be in terms of its viscoelastic properties at a given temperature. The rheogram of salmon myosin heated at 2°C/min shows five distinctive transition points. Transition point five shows that G′ decreased as the temperature increased from 36.8 to 46.9°C, after which G′ increased as temperature increased to a final temperature of 80°C (Figure 15.7).

The gelation point is often defined as the crossover of the storage (or elastic) modulus G′ and the loss (or viscous) modulus G″, which is the point at which log G′ = log G″ (Winter and Chambon, 1986; Winter, 1987; Friedrich and Heymann, 1988). The gelation point for the 2°C/min myosin sample was approximately 31.1°C. As shown in Figure 15.8, the first crossover at 21.7°C was likely due to the transformation of myosin subunits into a semi gel (gel-like), where the balance became more elastic mode than viscous mode. The second crossover was probably due to disruption of the semi gel, where the balance was more viscous mode than elastic mode. The third crossover was probably due to re-orientation of unfolded proteins for the formation of a stronger gel. The phase angle of the salmon myosin heated at 2°C/min (Figure 15.8) was in agreement with studies done using Pacific whiting and cod myosin (Yongsawatdigul and Park, 1999) vicilin and

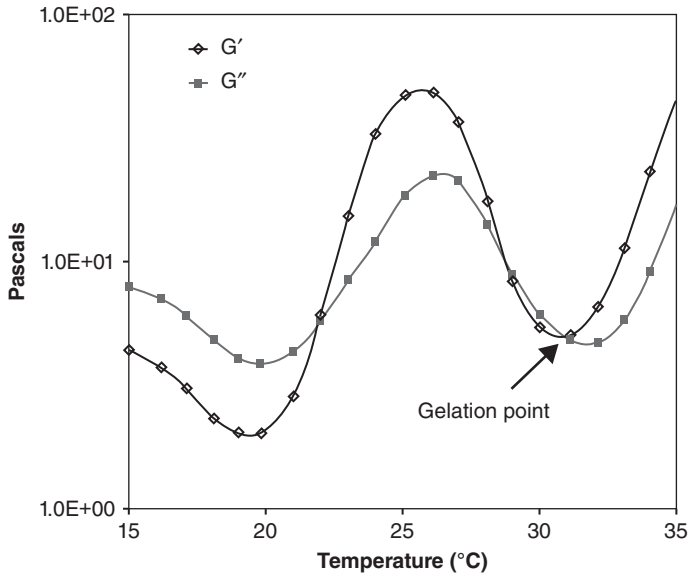

Figure 15.8 Relationship between G′ and G′′ of salmon myosin during heating at 2°C/min. The gelation point is defined as the point at which log G′ = log G′′. Reproduced with permission of Elsevier

ovalbumin (Arntfield and Murray, 1992) and whey protein (Li et al., 2006). The phase angle presented a pattern that corresponds inversely to the changes in G′.

15.5.2 Differential scanning calorimetry

DSC is a method of physicochemical thermal analysis and can be used to determine the energy changes in substance under thermal treatments. The principle of the method is based on measuring temperature and spontaneous or compensating heat fluxes (Bershtein and Egorov, 1994). The main application of DSC is to study the phase transitions, such as melting, glass transitions, or exothermic decompositions. These transitions involve energy changes that can be detected by DSC with great sensitivity.

The information and data that can be obtained from DSC is of great importance in many areas of research, such as thermal behavior of proteins, and more specifically in the thermal behavior of muscle proteins. Myofibrillar proteins are the most important fish proteins in the gelation of surimi and surimi seafood. Of the myofibrillar proteins, myosin is known to be of key importance for the gelling properties of surimi (Carvajal et al., 2005). Many key surimi fish species have been studied using DSC, such as Alaska pollock (Wang and Kolbe, 1991; Park, 1994a; Fukushima et al., 2003), Pacific whiting (Thawornchinsombut and Park, 2007), and threadfin bream (Yongsawatdigul and Park, 2003).

Beas et al. (1990) studied the maximum temperatures of denaturation enthalpies of hake (*Merluccius hubbsi*) using DSC. They found that whole muscle free from

connective tissue showed two distinct transitions. When they studied muscles that had the sarcoplasmic proteins removed, the enthalpy of the second transition was diminished. Yongsawatdigul and Park (2003) studied the effect of thermal denaturation and aggregation of threadfin bream actomyosin. They found that threadfin bream actomyosin exhibited three major transitions at 38.4, 51.0, and 80.7°C with onset temperatures of 36.5, 47.0, and 76.2°C, respectively. Enthalpies of denaturation of each major transition were 0.152, 0.169, and 0.251 J/g, respectively. The gelation properties of fish proteins make surimi useful for surimi seafood; however, the effects of freezing and frozen storage can have a significant impact on the gelation properties of surimi fish proteins. Moosavi-Nasab et al. (2005) studied the effect of freezing methods and storage on the gelation properties of actomyosin from Alaska pollock surimi and fish mince. They found a shift in the DSC endothermic peak of actin for both fish mince and surimi to a lower temperature. This provides an indication that the fish proteins are destabilized through freezing and frozen storage.

Thermal stability of fish myosin was studied and compared to that of rabbit myosin (Ogawa et al., 1993) using DSC methods. The researchers found the structure of fish myosin was much more unstable than rabbit myosin. By further studying fish myosin, they were able to determine that instability of fish myosin was due to the myosin rod moiety.

Park et al. (2008) found that the endothermic peaks of purified sardine myosin could be affected by pH and ionic strength. Testing myosin at pH 2, 7, and 10 they found that at pH 2 the thermogram was not typical and that the myosin had been denatured. However, at pH 7 and 10, sardine myosin exhibited the typical three endothermic peaks of fish myosin: 31, 51, and 65°C for pH 7; 31, 41, and 61°C for pH 10. Thawornchinsombut and Park (2004) studied the effects of pH and ionic strength on the number of endothermic peaks of acid- and alkali-treated Pacific whiting FPIs. In their study they found that the control sample, FPI only with 25 mM NaCl added, demonstrated five endothermic peaks. However, all other FPI with ionic-strength-controlled treatments exhibited only three endothermic peaks. They also found that the actin peak completely disappeared. It appears that pH and ionic strength play an important role in the irreversible protein denaturation as shown by the endothermic peaks. Yongsawatdigul and Park (2004) found that acid-induced and alkali-induced denaturation of rockfish muscle occurred as confirmed by DSC thermograms. They found that there was no net enthalpy that appeared in the acid-induced and alkali-induced samples indicating FPI was chemically denatured before thermal treatment.

Results obtained by DSC are extremely useful in identifying key components to protein denaturation. Kakinuma et al. (1998) investigated the thermal unfolding of three acclimation temperature-associated isoforms of carp LMM expressed by recombinant DNAs. In the study they used DSC techniques to study recombinant forms of LMM from 10 and 30°C acclimatized carp. They found by studying the thermograms that the 10 and 30°C endothermic peaks were at different temperatures. Liu et al. (1996) showed thermal transition points at 35.3, 48.1, 49.9, and 67.0°C for chicken breast myosin; however, none of the transitions were exothermic. When studying rabbit myosin head (subfragment 1) in 50 mM TRIS buffer (pH 8.0), 0.6 M KCl, along with HMM in 0.1 M KCl, Shriver and Kamath (1990) found exothermic peaks at 48 and 65°C.

15.6 Methods for protein identification and species differentiation

15.6.1 Sodium dodecyl polyacrylamide gel electrophoresis

Electrophoretic techniques have proven to be useful in visualizing and identifying food protein components. Researchers have had success in identifying a variety of fish species using sodium dodecyl polyacrylamide gel electrophoresis (SDS-PAGE) and IEF (Hsieh et al., 1997; Piñeiro et al., 1999; Etienne et al., 2000; Etienne et al., 2001; Chen and Hwang, 2002). SDS-PAGE works on the basic principle that as proteins are heated in the presence of SDS, an anionic detergent, they are denatured and evenly coated with SDS, which imparts a uniform negative charge to the protein that overwhelms the intrinsic protein charge (Nelson and Cox, 2000b). The denaturing effects of SDS remove all secondary, tertiary, and quaternary protein structures and the addition of β-mercaptoethanol disrupts any disulfide bonds. When the denatured protein is loaded on the polyacrylamide gel and an electrical current is passed through the gel, the proteins begin to migrate away from the negatively charged cathode and toward the positively charged anode. The discontinuous SDS-PAGE gel is made up of two distinct polyacrylamide gel sections. The first section the protein enters is known as the stacking gel. The purpose of the stacking gel is to concentrate, or "stack" the proteins in a tight group so that all of the proteins enter the resolving gel at the same time. This is accomplished by the use of a Tris-HCl glycine buffer with a pH of 6.8 and a lower concentration of acrylamide, which is less impeding on the movement of the proteins. Glycine is a zwitterion, a molecule that carries both a negative and a positive charge, and at pH 6.8 the neutral form is favored. As the electrical field is applied, the negatively charged chloride ions have a higher electrophoretic mobility than the neutral glycine ions and the mobility of the proteins is between that of the chloride and glycine. As the electrophoresis of the sample continues, the proteins are trapped in a very tight band between the chloride ions and the glycine ions, which allow for the proteins to be stacked and arrive at the resolving gel at the same time. As the protein sample enters the resolving gel, the pH is changed from 6.8 to 8.8, at which point the glycine ions are then changed predominantly to the negatively charged glycinate form. The glycinate mobility is increased and they pass the proteins in the gel and they migrate through PAGE gel, which acts as a molecular sieve. The electrophoretic mobility of the protein will increase as well; however, the resolving gel has smaller pore sizes than that of the stacking gel so the movement of the proteins is decreased. The separation at this point is due to the size of the protein as smaller proteins may move faster through the gel than larger ones, allowing for a separation based solely on molecular weight. Due to the nature of SDS-PAGE, it allows for consistent protein patterns to be separated, which can then be used for the comparison of standard proteins and the molecular size of the protein can be estimated accurately (Weber et al., 1972). The visualization of the separated protein bands is typically done by staining and then destaining the gels using a dye such as Coomassie brilliant blue.

As for the preparation of surimi or surimi gel samples for the SDS-PAGE, there is a challenging issue based on whether the sample is surimi (uncooked) or surimi gel/surimi

seafood (cooked). Due to the nature of the gel matrix formed when surimi is cooked, the total extraction of proteins is simple. In their paper, Reed and Park (2008) attempted to quantify the amount of Alaska pollock surimi used in crabstick using SDS-PAGE techniques. In order to completely and consistently extract the proteins, they mixed a homogenized gel sample in a heated (90°C) extraction solution of 5% (w/v) SDS solution and then heated the mixture for 1 hour in a 90°C water bath. By using this technique they were able to consistently extract the protein from crabsticks and quantify the amount of Alaska pollock used in the crabstick. In addition to the extraction method, Reed and Park found that the use of a thicker SDS-PAGE gel (0.75 vs. 1.0 mm) produced a clearer and easier image to be interpreted.

Species identification, primarily using sarcoplasmic proteins as the target proteins has been well documented in SDS-PAGE studies (An et al., 1989; Piñeiro et al., 1999; Etienne et al., 2001). However, an SDS-PAGE method for the quantification of fish used for surimi has not been studied as intensely as species identification. Fish species identification in surimi is not easy because almost all sarcoplasmic proteins are removed during surimi production. Claeys et al. (1995) have shown a linear relationship between densitometric readings and protein content of myofibrillar proteins in beef. In addition, Jin and Park (1996) have shown that densitometric readings can be used for quantification in goat milk cheese and the electrophoretic pattern can be used to differentiate it from cheese made of cow milk. Kolster and others (1992) have also shown that the high-molecular-weight wheat proteins of glutenin subunits are quantified using densitometry and Coomassie brilliant blue staining. Furthermore, Chen and Hwang (2002) have shown that using the low-molecular-weight region ($\leq$30.0 kDa) of myofibrillar extracts of seven puffer fish species it was possible to identify the species.

SDS-PAGE has been used for fish species identification as well as endeavoring to quantify the amount of protein found in other food products made from fish. Reed and Park (2008) have used SDS-PAGE along with densitometry to quantify the amount of Alaska pollock surimi used in crabstick manufacture. However, one major obstacle that has proven difficult to overcome when using SDS-PAGE as a means for identification is the overlapping of protein bands when migrating through the SDS gel matrix (Figure 15.9). Reed and Park (2008) found that when attempting to identify and quantify the amount of DEW used in crabstick manufacture the issue was complicated due to the similar molecular weights of ovalbumin from DEW and actin from the Alaska pollock surimi. Ovalbumin is approximately 54% (Stevens, 1991) of the total protein found in DEW and has a molecular weight of 45 kDa, while actin comprises 15–30% (Lanier et al., 2005) of the myofibrillar protein and has a molecular weight of 43 kDa. This large quantity of proteins of similar molecular weight can cause the bands to overlap and not be resolved. Due to the limitations of SDS-PAGE for qualification and quantification in complex food mixtures, other methods have also been explored out for protein identification.

15.6.2 Isoelectric focusing

Amino acids are the building blocks for proteins and each amino acid has a characteristic isoelectric point (pI). The pI is the pH at which the amino acid has an overall net

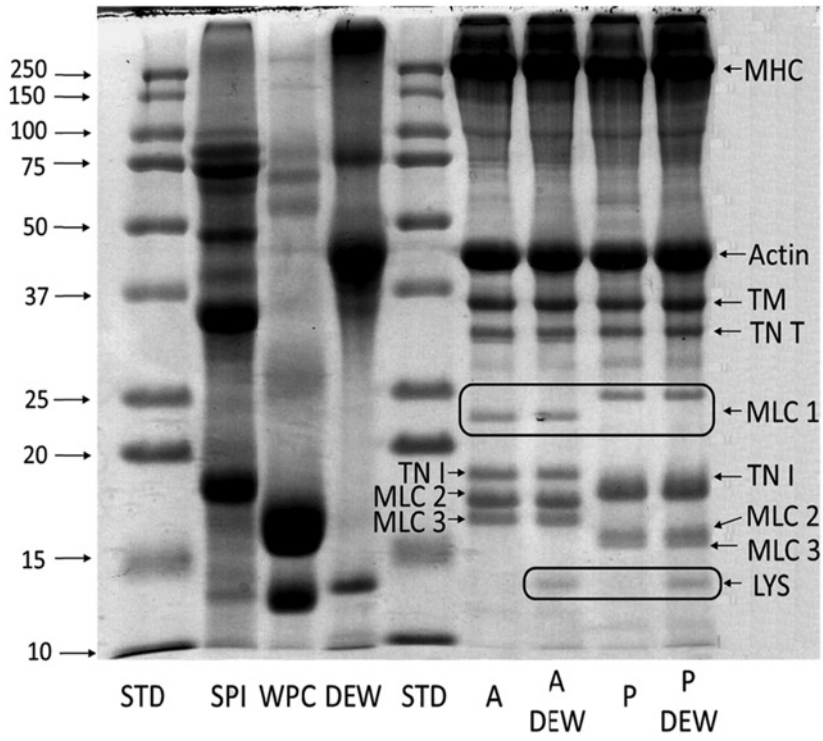

Figure 15.9 SDS-PAGE pattern of protein sources. STD, kaleidoscope protein standard (kDa); SPI, soy protein isolate; WPC, whey protein concentrate; DEW, dried egg white; AP, Alaska pollock; A DEW, Alaska pollock crabstick with DEW; PW, Pacific whiting; P DEW, Pacific whiting crabstick with dried egg white. Reproduced with permission of Elsevier

charge of zero. The pI of amino acids can range from 2.77 for aspartic acid to 10.76 for arginine (Nelson and Cox, 2000a). When amino acids are linked together through peptide bonds they form proteins. The amino acid sequence imparts an overall characteristic pI for that individual protein, that is to say each protein will have a characteristic pH at which its overall net charge is zero. IEF is an electrophoretic technique that takes advantage of the characteristic pI of individual proteins. When using IEF, a pH gradient is created between the anode and the cathode by allowing a mixture of low-molecular-weight organic acids and bases, known as ampholytes, to distribute themselves in an electric field that is generated across the gel (Gaál et al., 1980). The protein sample is then added to the gel and the electrical field is then reapplied and the proteins move along the pH gradient until they reach their pI at which point they have no net charge and they cease to move.

The Regulatory Fish Encyclopedia (FDA, 2009) contains standardized isoelectric focus plates for 94 different commercially important fish species for North America. In 1990, the Association of Official Analytical Chemists adopted IEF as the official method for raw fish species identification (AOAC, 1990). Hsieh et al. (1997, 1998) demonstrated the

ability of species identification using IEF for the commercially important fish species of red snapper (*Lutjanus campechanus*). Using IEF Hsieh et al. (1995) tested 121 retail market snapper fillets for compliance with labeling regulations. Samples were tested against 12 authentic snapper species. They found that of the 81 samples labeled as red snapper, only 24 (30%) were confirmed through IEF to be true red snapper. This meant that 57 (70%) of the fillet samples labeled as red snapper were actually other fish species. It was also reported that the majority of the fish species substituted as red snapper were actually scarlet snapper (*Lutjanus sanguineus*). This type of fish substitution can have a severe economic impact on the consumer. Species substitution has also been detected using IEF with a variety of other fish species such as European sea bass (*Dicentrarchus labrax*), spotted sea bass (*Dicentrarchus punctatus*), common pandora (*Pagellus erythrinus*) (Colombo et al., 2000), European perch (*Perca fluviatilis*), Nile perch (*Lates niloticus*), European pike perch (*Stizostedion lucioperca*), and sunshine bass (*Morone chrysops* × *Morone saxatilis*) (Berrini et al., 2006).

15.6.3 DNA-based identification

DNA testing methods have been used extensively in commercial fish and seafood species (Rasmussen and Morrissey, 2008). The use of DNA methods allows for a wide range of fish and fish products to be identified. Using restriction fragment length polymorphism (RFLP), Chakraborty et al. (2007) were able to differentiate between two species of hairtail fillets (*Trichiurus japonicus* and *Trichiurus sp.*) sold at Japanese markets under the same name of Tachiuo. Along with identification of fish species (Bossier, 1999; Horstkotte and Rehbein, 2003; Espiñeira et al., 2008), DNA methods have also been used for identification in various products such as caviar (Ludwig et al., 2002; Rehbein et al., 2008), smoked fish products (Carrera et al., 2000; Rehbein, 2005; Smith et al., 2008), bivalve species (Espiñeira et al., 2009), sterile fish mixtures (Asensio et al., 2004), and surimi seafood (Pepe et al., 2007).

DNA has certainly proven itself in the realm of species identification but it falls short when the need arises to quantify the amount of the identified species. Lockley and Bardsley (2000) have developed a DNA method by which they can discriminate between tuna (*Thunnus thynnus*) and bonito (*Sarda sarda*) but would require additional steps if quantification of the fish was desired.

Due to the highly processed nature of surimi it is impossible to identify the species used for its production by the morphological characteristics of the fish. Surimi is mixed with other ingredients such as starch, egg white, WPCs, and other flavors for the production of crabsticks, which further complicates the identification of fish species used in its production. In order to maintain quality and comply with government labeling standards, it is imperative that fish species and protein additives in crabsticks be identified and quantified. Pepe et al. (2007) studied 19 different cooked surimi-based products using polymerase chain reaction and direct sequence analysis of the cytochrome b gene. They found that of the 19 products labeled as Alaska pollock (*Theragra chalcogramma*) 84.2% were shown to be prepared with species different than the one declared.

15.6.4 Immunochemical analysis

Even though DNA methods have been used for species identification, it lacks the ability to quantify the amount of protein present. Immunochemical methods, such as enzyme-linked immunosorbent assays (ELISA), utilize the interaction between antigens and antibodies and can be used to identify fish species (Cespedes et al., 1999; Asensio et al., 2003a). They can also be set up to quantify the amount of protein of interest. ELISA assays are extremely sensitive, with detection limits down to the nanogram level. Also, they are often much easier to perform.

Crabsticks made with Alaska pollock surimi support an extremely important commercial fishery in the United States. It is one of the world's largest fisheries and during 2006 made up 71.4% of the average ground fish catch off the coast of Alaska (Noaa, 2006). By having a well-managed fishery, such as the Alaska pollock fishery, has aided in sustained growth for the use of Alaska pollock. However there is some abuse of Alaska pollock that comes mainly from the substitution of other cheaper and often lower-quality fish that masquerade as Alaska pollock (Jacquet and Pauly, 2008). Most substitution of pollock comes from the surimi industry where Alaska pollock is considered the premium species for surimi production (Guenneugues and Morrissey, 2005). Consequently, crabstick quality is directly related to the amount of fish protein from raw surimi that is used. A high-quality crabstick product typically contains surimi (mid–high grade) at 40% or higher. The protein content and quality of raw surimi from different species and of different grades, though, varies.

Huang et al. (1995) were able to identify red snapper *(L. campechanus)* using monoclonal antibodies. Asensio et al. (2003b) were able to unequivocally identify grouper (*Epinephelus guaza*), wreck fish (*Polyprion americanus*), and Nile perch (*L. niloticus*) fillets using an indirect ELISA using polyclonal antibodies that were raised against species specific proteins. ELISA has also been used for quantification of a variety of food products including fish and fish products (Medina, 1988; Panheleux et al., 2000; Ochiai et al., 2001; Vidal et al., 2005; Werner et al., 2007).

Reed and Park (2010) created a competitive ELISA to detect the myosin light chain 1 (MLC 1) from Alaska pollock. A key component to the success of this competitive ELISA was the ability of the anti-pep-AP antibody to recognize the denatured form of the MLC 1. The MLC 1 was extracted under denaturing conditions (SDS, β-ME) and was also separated out using denaturing SDS-PAGE methods. In order to have a relevant assay they chose to use a range of surimi content from 25% to 45%, which allows for the coverage of most common commercial crabsticks. Using the appropriate ELISA assay format was critical in creating a successful ELISA assay. According to Reed and Park (2008), the MLC 1 of Alaska pollock comprises approximately 1% of the total protein found in surimi. By purifying the MLC 1 from Alaska pollock surimi and using it as the coating protein they were successful in designing an assay that could detect and quantify the MLC 1 from cooked and processed crabsticks. Figure 15.10 shows the accuracy of the competitive ELISA when using MLC 1 detecting antibody.

In addition to species identification, ELISA has proven to be extremely useful in the detection of many of the common allergens found in fish and seafood such as the

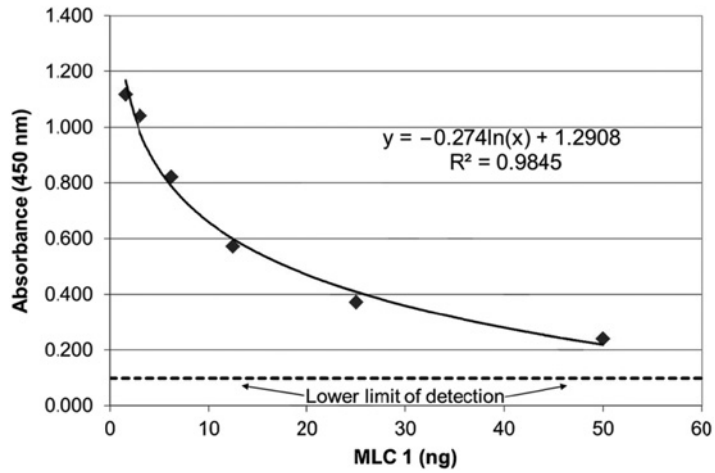

Figure 15.10 Response of the competitive ELISA using purified MLC 1 from Alaska pollock in a serial dilution. Reproduced with permission of Elsevier

β′-component, which is a fragment of vitollegenin in salmonid roe (Shimizu et al., 2009), tropomyosin from crustaceans (Fuller et al., 2006), and parvalbumin from fish (Kawase et al., 2001; Chen et al., 2006; Fæste and Plassen, 2008; Ma et al., 2008; Yoshida et al., 2008; Gajewski and Hsieh, 2009). Fish allergens are discussed in more detail in the next section.

The crabstick industry does not have a standard of identity established for protein additives, such as DEW and WPC. A lack of standard of identities can lead to increased use and/or abuse of protein additives in the crabstick formulation, which decreases surimi quantity and correlates to lower-quality product if the usage is not properly optimized. Surimi is comminuted with other ingredients, such as starch, DEW, WPC, and other flavors in the production of crab-flavored seafood (crabsticks). DEW has been used extensively as a functional food ingredient because of its gelling and foaming properties (Mine, 1995). Beta-lactoglobulin (β-LG) is largely responsible for the physicochemical properties as well as the functional behavior of food products that contain whey protein (Foegeding et al., 2002). Burgarella et al. (1985) showed that DEW and WPC can be used in low concentrations to provide an additive effect of increased gel strength and deformability in surimi seafood.

15.6.5 Chromatographic detection

Although it is not relevant to a chapter on fish proteins it is important to mention that in recent years chromatographic methods, such as gas chromatography (GC) coupled with mass spectroscopy, have been used for authentication of meat products (Sivadier et al., 2008). Chromatographic methods, including GC, thin-layer chromatography (TLC), high-performance liquid chromatography (HPLC), are commonly used in food analysis. GC has been used to detect volatile compounds in fish sauce (Sanceda et al., 1983) as well as being used to predict sensory qualities of cold smoked salmon (Jónsdóttir et al., 2008).

Morrison and others (2007) used capillary GC and the isotopic composition of fish oil to distinguish between farmed and wild gilthead sea bream.

15.7 Fish allergens

The adulteration of food products may be objectionable for health or religious reasons (Jacquet and Pauly, 2008) However there may be more serious issues involving allergens. A variety of testing methods are available for the detection of food contamination with known food allergens, most of which are directed toward proteins or glycoproteins. Techniques, such as immunochemical detection and amplification of DNA, allow for the detection and subsequent labeling of the offending allergens. The consumption of products containing undeclared constituents may cause serious health risks and problems such as an allergy in sensitized individuals (Mackie, 1996). Fish is one of the most common causes of IgE-mediated food hypersensitivity. The detection of many of the common allergens found in fish and seafood such as the β′-component, which is a fragment of vitollegenin in salmonid roe (Shimizu et al., 2009), tropomyosin from crustaceans (Fuller et al., 2006), and parvalbumin from fish (Kawase et al., 2001; Chen et al., 2006; Fæste and Plassen, 2008; Ma et al., 2008; Yoshida et al., 2008; Gajewski and Hsieh, 2009) can be done using ELISA. Extensive studies were done in the early and mid-1970s to try and determine the major cause of food hypersensitivity to fish. Elsayed and coworkers. (Elsayed and Aas, 1971; Elsayed and Bennich, 1975; Elsayed and Apold, 1983) determined that parvalbumin was the culprit for the majority of food hypersensitivity to fish. Even a minute intake of the fish allergen is sufficient to cause a severe reaction in those with hypersensitivity. Due to the potential danger for those with fish allergies many studies have been performed with the intent of identifying fish allergens and the detection of those fish allergens in food (Esteve-Romero et al., 1996; Kawase et al., 2001; Poulsen et al., 2001; Chen et al., 2006; Fæste and Plassen, 2008; Ma et al., 2008; Yoshida et al., 2008; Gajewski and Hsieh, 2009; Sun et al., 2009). Along with fish, shellfish are also common cause of allergies than can be of concern for those with shellfish hypersensitivity (Fuller et al., 2006; Lu et al., 2007).

15.8 Conclusion

Surimi is refined fish myofibrillar proteins stabilized with cryoprotectants and used as a raw material for various surimi seafood products. Refining process of fish muscle is to concentrate desired myofibrillar proteins while removing undesired components such as lipid, stroma proteins, sarcoplasmic proteins, and other impurities. Refined fish myofibrillar proteins, which are virtually surimi, have unique gelling ability to transform fish meat into a wide range of surimi seafood. Unlike other proteins that form a gel, fish myofibrillar proteins have setting ability that forms a gel by the function of eTGase if salted paste is held at a certain temperature (5–40°C) depending on fish's own thermal stability. Its gelling and setting uniqueness has played a major role in the development of numerous surimi seafood products and contributed positively to the successful market position of these products.

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16
Milk Proteins

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16.1 Introduction

Milk proteins contribute ~25% of the daily protein intake of the U.S. diet, suggesting the importance of milk proteins in human nutrition. Milk proteins include caseins (CN), whey proteins, milk fat globule membrane (MFGM) proteins and enzymes that naturally occur in milk. The classification of milk proteins according to American Dairy Science Association (ADSA)'s nomenclature (Farrell et al., 2004) is as follows: Caseins are phosphoproteins that precipitate from milk at pH 4.6 at 30°C. Caseins represent about 78–80% proteins in bovine milk and comprise α_{s1}-, α_{s2}-, β-, γ-, and κ-CN, in the approximate ratio of 0.45:0.12:0.33:0.10. Quantitatively, whey proteins represent ≈20% of the total milk proteins and consist mainly of α-lactalbumin (α-LA), β-lactoglobulin (β-LG), serum albumin (SA), immunoglobulins (Igs), proteose peptone fraction (derived from proteolysis of β-CN), and lactoferrin (LF). Also included in the family of milk proteins are indigenous milk enzymes and MFGM proteins (Eigel et al., 1984; Kobylka and Carraway, 1972, 1973; Mather and Keenan, 1975; Patton and Keenan, 1975). The distribution of proteins in milk is shown in Figure 16.1.

16.2 Caseins

16.2.1 Caseins structure

Knowledge of casein structure is important to understanding of dairy processing and technology, and their functional properties in dairy products, food systems and non-food

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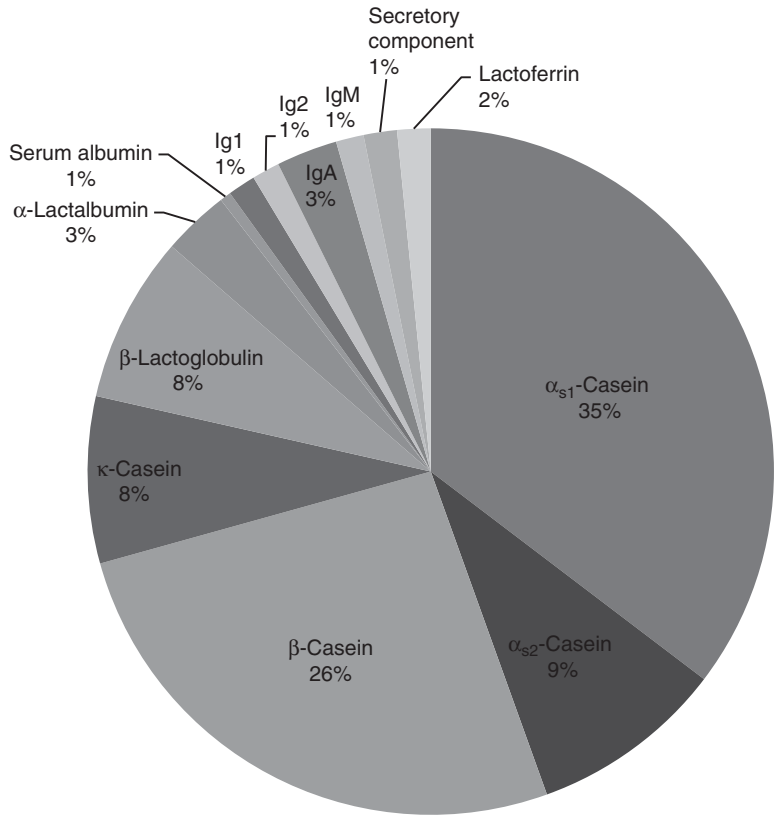

Figure 16.1 Distribution of proteins in milk

applications. The concentration of caseins in bovine milk is ~2.5 g/100 g—the levels of which depends mostly on the breed of cow, lactation and season. Milk from Jersey breed is typically higher in casein content (~80% of the total protein) than milk from other breeds such as Holsteins, in which caseins represent ~77–78% of the total protein. Caseins are unique phosphoproteins that are in suspension in milk as colloidal particles. The major caseins exist in strong association with themselves and with each other in the form of spherically shaped colloidal particles, ~50–500 nm diameter (mean ~120 nm), and known as "casein micelles" (Fox and Brodkorb, 2008; McMahon and Oommen, 2008). For a historical perspective on the development and use of the term, "casein micelles," the reader is referred to Fox and Brodkorb (2008).

Several models have been used to describe casein micelles to help provide a better understanding of the properties of caseins. Earlier models were proposed by Waugh (1958) and Schmidt (1980). More recent and generally accepted models include the following:

In the *submicelle model* (Slattery and Evard, 1973; Walstra, 1990, 1999) the casein micelle is roughly spherical and built of smaller units, called submicelles. The submicelles

vary in composition, consisting of α_s- and β-CN or α_s- and κ-CN that are linked together by small calcium phosphate clusters bridges. The submicelles aggregate to form a micelle with κ-CN at the outside such that the C-terminal of κ-CN protrudes from the micelle surface, forming a "hairy" layer that causes steric and electrostatic repulsion, preventing further aggregation of submicelles. The hairy layer is also held responsible for the stability of the micelles against flocculation. Rollema (1992) suggested that micelles consist of substructures held together by both hydrophobic bonds and colloidal calcium phosphate (CCP) bridges. Early scanning electron microscopy studies seemed to indicate the presence of submicelles, but McMahon and McManus (1998) reported that the structures are likely to be artifacts of fixation during sample preparation.

In the *dual bonding model* (Holt, 1992), the micelle is thought to be a mineralized, cross-linked, and tangled network of polypeptide chains, consisting of hydrophobic regions of α_s-, and β-CN cross-linked at their hydrophilic phosphoserine (SerP) clusters by CCP nanoclusters. The model also suggests κ-CN molecules located at the surface of the micelle interact through hydrophobic bonding with the other caseins (α_s- or β-) and orient their highly hydrophilic regions (hairs) into the serum. According to Dalgleish et al. (2004), this model does not have a well-defined hairy structure but rather a layer of decreasing protein density at the micellar surface. Also, SEM studies suggest that the surface is not smooth but contains gaps between the substructures (Dalgleish et al., 2004).

The *interlocked lattice model* (McMahon and Oommen, 2008) suggests an interlocked lattice consisting of CCP nanoclusters spaced ~18.6 nm apart (de Kruif et al., 2012) bound to SerP groups of α_{s1}-, α_{s2}-, or β-CN, or combinations thereof to form casein polymer chains that are linked together to maintain the integrity of casein micelles. The hydrophobic ends of the caseins orient away from the CCP nanoclusters, thereby allowing interactions and binding to each other. Besides the strong CCP linkages between caseins, weak interactions—such as hydrophobic interactions, hydrogen bonding, ion bonding, Van der Waals attraction, and other factors—contribute to self-association of casein micelles.

In all the models, the arrangement of casein within the micelle is such that the interior is occupied mainly by the calcium-sensitive caseins (α_{s1}-, α_{s2}- and β-CN), whereas κ-CN is located principally at the surface, with its hydrophilic C-terminal region (caseinomacropeptide), containing 14 carboxylic acid groups, oriented outward in the serum phase as protruding negatively charged hairs, which create an electrokinetic potential of ~−20 mV, giving stability to the micelle by electrostatic repulsion, Brownian movement, and steric repulsion (de Kruif and Holt, 2003; Horne and Banks, 2004).

The C-terminal region of κ-CN protruding from the micelle surface is considered to be an extended polyelectrolyte brush (de Kruif, 1999) in the milk serum, which has a high ionic strength (~0.08 M) because of the presence of various ions (e.g., potassium, sodium, chloride, phosphate, and citrate). This region of κ-CN is also glycosylated (Saito and Itoh, 1992; Mollè and Leonil, 1995; Fox and McSweeney, 1998; Mollè et al., 2006)—containing galactose, *N*-acetylgalactosamine (GalNAc) and *N*-actetylneuraminic (sialic) acid (NANA) (Dziuba and Minkiewicz, 1996). Glycosylation of κ-CN further enhances its ability to increase micelle stability by steric impedance and electrostatic repulsion through their contribution to water binding (owing to the carbohydrate moieties) and to negatively-charged carboxylic groups (on the NANA molecule). The level of

glycosylation of κ-CN and its surface hydrophobicity increases with micelle size (O'Connell and Fox, 2000).

Casein micelles are highly solvated and sponge-like. The micelles bind ~4 g water/g, of which ~15% is bound to the protein while the remainder is occluded within the colloidal particles (de Kruif and Holt, 2003; Farrell et al., 2003).

Casein micelles dissociate at high temperature (>40°C) and at ethanol concentrations >40% (Zadow, 1993) owing to a change in solvent quality as temperature increases (O'Connell et al., 2001). In addition, pH, urea addition or sequestration of calcium destabilizes casein micelles. Urea disrupts hydrophobic bonds and high concentrations (~8 M) disintegrate the micelle. Removal of calcium from the micelles by the addition of sequestrant (e.g., EDTA, citrate, or oxalate) disintegrates the micelles. Reducing the milk pH solubilizes the CCP (Dalgleish and Law, 1988) and at pH 5.2, most, if not all, the CCP is rendered soluble. Milk contains ~7% CCP on a dry matter basis (de Kruif et al., 2012). Increasing the pH may be expected to be the reverse of the dissolution process and to favor the formation of calcium phosphate species. Low temperature (<15°C) storage of milk weakens hydrophobic interactions and causes dissociation of β-casein from the micelles (Dalgleish and Law, 1988), possibly some of which is not bound in the core of the micelle. Raising the temperature to its initial value reverses the process and the β-casein is reincorporated into the micelle.

Initial studies of casein structure by optical rotary dispersion suggested that caseins lacked α-helix structure (Farrell, 1988). Hence, it was first believed that caseins are random-coil proteins; however, circular dichroism and Raman spectroscopy studies show some secondary structure in caseins (Swaisgood, 2003). The amino acid composition of caseins is shown in Table 16.1. Caseins are rich in Glu (~20%) and Pro (~12%). Collectively, the caseins contain very little Cys (all the Cys is contributed by κ-CN and is zero in α_s-CN and β-CN) because of which they lack the –S–S– bonding as in whey proteins, which contain ~3% Cys residues.

The interactions between casein, Ca^{2+}, and CCP are necessary for the formation and maintenance of casein micelles. The anionic clusters of SerP residues are the primary binding sites for Ca^{2+}. Consequently, solubility of caseins as a function of Ca^{2+} concentration correlates with the number of SeP clusters per molecule; thus, the order of solubility is α_{s2}- < α_{s1}- < β- < κ-CN, which contain 3, 2, 1, and 0 SerP clusters, respectively. This makes κ-casein least susceptible to Ca^{2+} precipitation, requiring over 8 mM calcium for precipitation. The sensitivities of caseins to Ca^{2+} are given in Table 16.2.

Adding 25 mM Ca^{2+} has no effect on β-CN fluorescence but increases the turbidity of α_s-CN in solution and results in a sixfold increase in fluorescence of α_s-casein, suggesting fibril-like structure of casein aggregates. Transmission electron microscope of casein micelles showed the presence of short (7–10 nm) fibers, resembling protofibrils, that are cross-linked by dense aggregate junction zones (Lencki, 2007).

16.2.1.1 α_{s1}-Casein Total α_s-CN (i.e., α_{s1}-CN plus α_{s2}-CN) comprises 44–55% of the total casein in milk; α_{s1}-CN represents ~80% of the total α_s-CN. The α_{s1}-CN molecule (MW, 23,644 Da) contains 199 amino acids and eight SerP groups and no Cys residues. Its

Table 16.1 Amino acid composition in skim milk, whole milk, casein, and whey proteins

Amino acid	Milk (g AA/100 g)[a,b]	Milk (g AA/100 g protein)[b]	Casein (g/100 g protein)[c]	Whey Protein (g AA/100 g protein)[d]
Alanine	0.11	3.0	3.4	5.5
Arginine	0.11	3.3	4.1	3.3
Aspartic acid	0.26	7.8	7.4	11.0
Cystine	0.02	0.6	0.4	3.0
Glutamic acid	0.77	23.2	23.2	15.5
Glycine	0.06	1.8	2.1	3.5
Histidine	0.10	3.0	3.2	2.4
Isoleucine	0.14	4.2	6.6	7.6
Leucine	0.29	8.7	10.0	11.8
Lysine	0.27	8.1	8.1	7.2
Methionine	0.06	1.8	3.2	2.4
Phenylalanine	0.16	4.8	5.4	4.2
Proline	0.32	9.6	11.8	4.4
Serine	0.16	4.8	6.6	5.5
Threonine	0.15	4.5	4.3	8.4
Tryptophan	0.04	1.5	1.3	2.4
Tyrosine	0.15	4.5	5.8	4.2
Valine	0.16	4.8	7.5	7.2

[a] USDA Nutritional Data (2012).
[b] Csapó-Kiss et al. (1995); Swaisgood, H.E. (1995).
[c] Gallagher et al. (1997).
[d] Belitz et al. (2009).

Table 16.2 Properties of milk proteins

Property	α_{s1}-B 8P	α_{s2}-A 11P	β-A2 5P	κ-B 1P	α-LA B	β-LG B	Serum albumin
Molecular weight (Da)	23,614	25,230	23,983	19,023	14,171	18,363	66,267
No. of amino acids	199	207	209	169	123	162	582
No. of Proline	17	10	35	20	2	8	34
No. of Cysteine	0	2	0	2	8	5	35
Intra molecular —S—S— bonds	0	0	0	0	4	2	17
Phosphorus content	0.9	1.1	0.6	1.4	0	0	0
Carbohydrates	0	0	0				0
Hydrophobicity (kJ/residue)	4.9	4.7	5.6	5.1	4.7	5.1	4.3
Mol % residue	34	36	23	21	28	30	34
Net charge per residue	−0.10	−0.07	−0.06	−0.02	−0.02	−0.04	−0.02
Distribution	Uneven	Uneven	Very uneven	Very uneven	Even	Even	
A280	10.1	14.0	4.5	10.5	20.9	9.5	6.6
Sensitivity to calcium	6 mM	2 mM	3 mM	>8 mM			
Sensitivity to Na	28 mM						
pI	4.4–4.8		4.8–5.1	3.7–4.2	5.1	5.2–5.3	

1													**(Section 14–26 deleted in variant A)**					
Arg	Pro	Lys	His	Pro	Ile	Lys	His	Gln	Gly	Leu	Pro	Gln	**(Glu**	**Val**	**Leu**	**Asn**	**Glu**	**Asn**	**Leu**
									30										**40**
Leu	**Arg**	**Phe**	**Phe**	**Val**	**Ala)**	Pro	Phe	Pro	Gln	Val	Phe	Ply	Lys	Glu	Lys	Val	Asn	Glu	Leu
									50			**(ThrP in variant D; Ala in variant B)**							**60**
Ser	Lys	Asp	Ile	Gly	**SerP**	Glu	**SerP**	Thr	Glu	Asp	Gln	**(Ala)**	Met	Glu	Asp	Ile	Lys	(Glu)	Met
									70										**80**
Glu	Ala	Glu	**SerP**	Ile	**SerP**	**SerP**	**SerP**	Glu	Glu	Ile	Val	Pro	Asn	**SerP**	Val	Glu	Gln	Lys	His
									90										**100**
Ile	Gln	Lys	Glu	Asp	Val	Pro	Ser	Glu	Arg	Tyr	Leu	Gly	Tyr	Leu	Glu	Gln	Leu	Leu	Arg
									110										**120**
Leu	Lys	Lys	Tyr	Lys	Val	Pro	Gln	Leu	Glu	Ile	Val	Pro	Asn	**SerP**	Ala	Glu	Glu	Arg	Leu
									130										**140**
His	Ser	Met	Lys	Gln	Gly	Ile	His	Ala	Gln	Gln	Lys	Glu	Pro	Met	Gly	Val	Asn	Asn	Gln
									150										**160**
Glu	Leu	Ala	Typ	Phe	Tyr	Pro	Glu	Leu	Phe	Arg	Gln	Phe	Tyr	Gln	Leu	Asp	Ala	Tyr	Pro
									170										**180**
Ser	Gly	Ala	Trp	Tyr	Tyr	Val	Pro	Leu	Gly	Thr	Gln	Tyr	Thr	Asp	Ala	Pro	Ser	Phe	Ser
									190		**(Gly in variant C)**							**199**	
Asp	Ile	Pro	Asn	Pro	Ile	Gly	Ser	Glu	Asn	Ser	**(Glu)**	Lys	Thr	Thr	Met	Pro	Leu	Trp	OH

Figure 16.2 Primary sequence of bovine α_{s1}-casein B. The amino acids in parenthesis are the sites that are different in genetic variants A, C, and D. Phospho serine residues (SerP) are shown in bold

amino acid sequence is illustrated in Figure 16.2. The segment, α_{s1}-CN (f45–89), contains all the SerP groups, making α_{s1}-CN a highly charged protein. In addition, this segment contains 12 carboxyl groups and two ε-amino groups, resulting in a net charge of −24 for α_{s1}-CN at pH 6.7, leaving the rest of the molecule with a net charge of −1 at the pH of milk. Isolated α_{s1}-CN is very sensitive to Ca^{2+}; it begins to precipitate at 0.007 M and precipitates completely at 0.02 M, when eight Ca^{2+} are bound. Calcium binding reduces the net charge of α_{s1}-CN from −24 to −8, which is not enough to prevent aggregation. Recent evidence indicates that the binding of Ca^{2+} to α_{s1}-CN causes a conformational change in the protein, which causes more hydrophobic groups to become exposed to the solvent. α_{s1}-CN has an average hydrophobicity of 1170; it has three very hydrophobic regions at f1–44, f90–113 and f132–199. The molecule contains 17 Pro residues that are almost randomly distributed within the hydrophobic regions, which effectively precludes the presence of secondary protein structure in α_{s1}-CN.

There are four known genetic variants of α_{s1}-CN, with variant B being the most common. Variants C and D differ from variant B by only one amino acid. Variant A contains 13 less amino acids (f14–26) than the other α_{s1}-CN variants, suggesting gene deletion. α_{s1}-CN has a highly charged 46 amino acid segment (f45–89) that maximizes its contact with water. One end of the segment is highly negatively charged and the other end contains little charge, and is largely apolar.

16.2.1.2 α_{s2}-Casein α_{s2}-CN comprises 9–10% of the total casein in bovine milk and represents ~20% of total α_s-CN (i.e., α_{s1}-CN + α_{s2}-CN). The primary sequence for α_{s2}-CN variant A is illustrated in Figure 16.3. It contains 207 amino acids with an average MW of ~25,100 Da. It has a dipolar structure with a concentration of positive charges near the C-terminus and negative charges near the N-terminus. It contains two Cys residues

1									**10**									
Lys	Asn	Thr	Met	Glu	His	Val	**SerP**	**SerP**	**SerP**	Glu	Glu	Ser	Ile	Ile	**SerP**	Gln	Gln	Thr	Thr
21									**30**									
Lys	Glu	Glu	Lys	Asn	Met	Ala	Ile	Asn	Pro	Ser	Lys	Glu	Asn	Leu	Cys	Ser	Thr	Phe	Cys
41									**50**									
Lys	Glu	Val	Val	Arg	Asn	Ala	Asn	Glu	Glu	Glu	Tyr	Ser	Ile	Gly	**SerP**	**SerP**	**SerP**	Glu	Glu
61									**70**									
SerP	Ala	Glu	Val	Ala	Thr	Glu	Glu	Val	Lys	Ile	Thr	Val	Asp	Asp	Lys	His	Tyr	Gln	Lys
81									**90**									
Ala	Leu	Asn	Glu	Ile	Asn	Glu	Phr	Typ	Gln	Lys	Phe	Pro	Gln	Tyr	Leu	Gln	Tyr	Leu	Tyr
101									**110**									
Gln	Gly	Pro	Ile	Val	Leu	Asn	Pro	Trp	Asp	Gln	Val	Lys	Arg	Asn	Ala	Val	Pro	Ile	Thr
121									**130**									
Pro	Thr	Leu	Asn	Arg	Glu	Gln	Leu	**SerP**	Thr	**SerP**	Glu	Glu	Asn	Ser	Lys	Lys	Thr	Val	Asp
141									**150**									
Met	Glu	**SerP**	Thr	Glu	Val	Phe	Thr	Lys	Lys	Thr	Lys	Leu	Thr	Glu	Glu	Glu	Lys	Asn	Arg
161									**170**									
Leu	Asn	Phe	Leu	Lys	Lys	Ile	Ser	Gln	Agr	Thr	Gln	Lys	Phe	Ala	Leu	Pro	Gln	Tyr	Leu
181									**190**									
Lys	Thr	Val	Tyr	Gln	His	Gln	Lys	Ala	Met	Lys	Pro	Trp	Ile	Gln	Pro	Lys	Thr	Lys	Val
201						**207**													
Ile	Pro	Tyr	Val	Arg	Tyr	Leu	OH												

Figure 16.3 The primary sequence of bovine α_{s2}-casein. Serine residues identified as phosphorylated (SerP) are indicated in bold. From Brignon et al. (1977) as modified by Farrell et al. (2004)

and has 10–13 phosphates. α_{s2}-CN binds calcium strongly and is more sensitive to Ca^{2+} precipitation than α_{s1}-CN, with almost complete precipitation occurring in 2 mM Ca^{2+} at pH 7, whereas precipitation of α_{S1}-CN requires 6 mM Ca^{2+} (Aoki et al., 1985). It self-associates at neutral pH in the presence of Ca^{2+}. Segment, f164–207, is reported to have antibacterial activity against *Listeria innocua* (McCann et al., 2005).

16.2.1.3 β-Casein β-Casein represents ~25–35% of the total caseins in bovine milk. It consists of 209 amino acids with a MW of ~23,980 Da. The primary sequence is illustrated in Figure 16.4. β-CN contains five SerP groups, no Cys, and 17% Pro (35 Pro residues) that are randomly distributed throughout the protein. The N-terminal segment (f1–42) of β-CN contains all the phosphate groups, contributing all of its net charge of −13 at pH 6.7. The remaining 80% of β-CN has no net charge and is very hydrophobic; the average hydrophobicity of β-CN is 1335.

β-CN precipitates in the presence of Ca^{2+} depending strongly on temperature. β-CN is completely soluble in the presence of Ca^{2+} at low temperatures (0–5°C). At 30°C, 80% of β-CN precipitates in 8–10 mM Ca^{2+}. The solubility of β-CN in 0.01 M $CaCl_2$ is 0.3 g L^{-1} (Zittle and Walter, 1963).

Removal of the very hydrophobic C-terminal tripeptide, —Ile—Ile—Val, prevents association of β-CN. At 4°C, β-CN dissociates from the casein micelles. As temperature increases, β-CN becomes less soluble and associates with other casein molecules. Intrinsic viscosity data suggest that at 4°C, β-CN is highly asymmetric and may even exist as a random coil. The viscosity does not increase when the molecule is placed into 6 M guanidine HCl, suggesting a lack of secondary structure. At temperatures >13°C, there is evidence

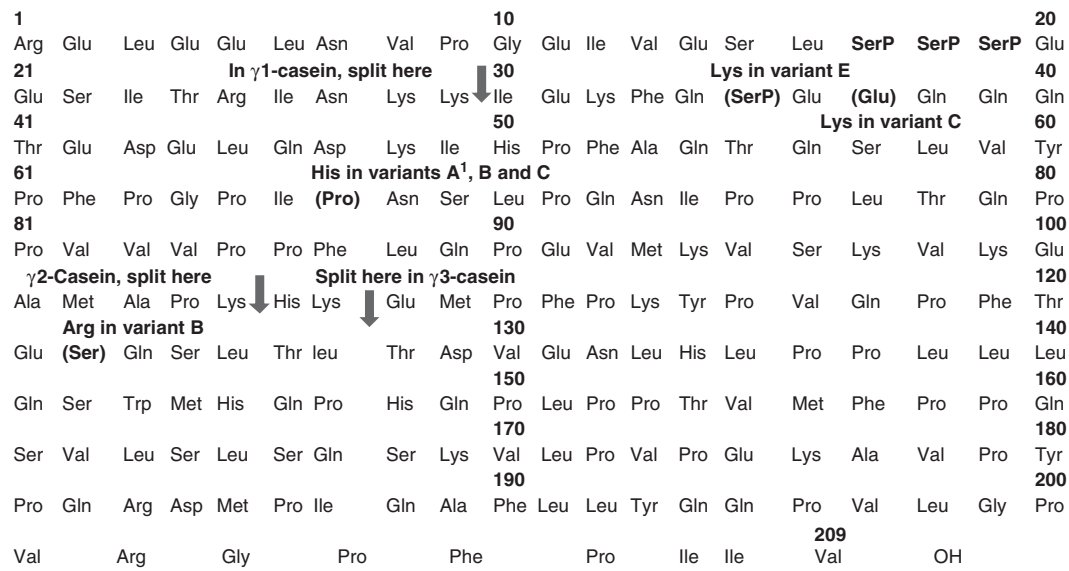

Figure 16.4 Primary sequence of bovine β-casein A. Amino acid substitutions are indicated for genetic variants B, C, and E. Arrows indicate points of hydrolysis to yield γ-casein

(Creamer et al., 1981) for the existence of small amounts of α-helix (10%) in the structure of β-CN. There are five genetic variants of β-CN, all of which are the result of one amino acid change.

16.2.1.4 γ-Casein The γ-caseins, representing ~5% of the casein, are derived by limited proteolysis of β-CN by the indigenous milk proteinase, plasmin, which apparently is transmitted in small quantities into milk from blood. The nomenclature for the γ-caseins is: γ_1-CN (β-CN f29–209, MW ≈20,600 Da); γ_2-CN (β-CN f106–209, MW ≈11,800 Da); γ_3-casein (β-CN f108–206, MW ≈11,600 Da). The γ-caseins have very low net charges and are extremely hydrophobic. These molecules can be solubilized by ethyl alcohol and other solvents of low dielectric constant. The N-terminal fractions of β-CN resulting from proteolysis are called proteose peptones components 5, 8-fast, and 8-slow, which are the respective complementary peptides for γ_2-CN, γ_1-CN, and γ_3-CN (Farrell et al., 2004).

16.2.1.5 κ-Casein κ-CN consists of 169 amino acids and has a molecular weight of 19,005 Da. The protein can contain from 0 to 5 trisaccharide units composed of N-acetylneuraminyl (2→6) β-galactosyl (1→3 or 6) N-acetylgalactosamine. It contains one SerP group and two Cys residues. There are two genetic variants of κ-CN. The primary sequence of κ-CN is presented in Figure 16.5.

The average hydrophobicity of κ-CN is 1205. Unlike α_s-CNs and β-CN, κ-CN has charged sections at both ends of the molecule. The 53 C-terminal amino acids have a net charge of −11, contain the only SerP group and all of the carbohydrate of κ-CN. This

1										**11**									
Glu	Glu	Gln	Asn	Gln	Glu	Gln	Pro	Ile	Arg	Cys	Glu	Lys	Asp	Glu	Arg	Phe	Phe	Ser	Asp
21										**31**									
Lys	Ile	Ala	Lys	Tyr	Ile	Pro	Ile	Gln	Tyr	Val	Leu	Ser	Arg	Tyr	Pro	Ser	Tyr	Gly	Leu
41										**51**									
Asn	Tyr	Tyr	Gln	Gln	Lys	Pro	Val	Ala	Leu	Ile	Asn	Asn	Gln	Phe	Lue	Pro	Tyr	Pro	Tyr
61										**71**									
Tyr	Ala	Lys	Pro	Ala	Ala	Val	Arg	Ser	Pro	Ala	Gln	Ile	Leu	Gln	Trp	Gln	Val	Leu	Ser
81										**91**									
Asp	Thr	Val	Pro	Ala	Lys	Ser	Cys	Gln	Ala	Gln	Pro	Thr	Thr	Met	Ala	Arg	His	Pro	His
101				**105**	**106**					**111**									
Pro	His	Leu	Ser	Phe	Met	Ala	Ile	Pro	Pro	Lys	Lys	Asn	Gln	Asp	Lys	Thr	Glu	Ile	Pro
121										**131**				**Ile in variant B**					
Thr	Ile	Asn	Thr	Ile	Ala	Ser	Gly	Glu	Pro	Thr	Ser	Thr	Pro	(Thr)	Thr	Glu	Ala	Val	Glu
141							**Ala in variant B**												
Ser	Thr	Val	Ala	Thr	Leu	Glu	(Asp)	SerP	Pro	Glu	Val	Ile	Glu	Ser	Pro	Pro	Glu	Ile	Asn
161								**169**											
Thr	Val	Gln	Val	Thr	Ser	Thr	Ala	Val											

Figure 16.5 Primary sequence of bovine κ-casein B. Amino acids substitutions for genetic variant B and point of hydrolysis by chymosin are indicated

carbohydrate further increases the net negative charge of this portion of the molecule. The remainder of the molecule is very hydrophobic and has a net positive charge at pH 6.7.

Most of the net charge of the molecule is not derived from serine phosphate groups and thus κ-CN is soluble in the presence of Ca^{2+}. It can also interact with α_s-CNs and β-CN and stabilize them in the presence of Ca^{2+}. There is a relationship between the amount of κ-CN present and the size of casein micelles. Large micelles tend to have less κ-CN than small micelles. Addition of κ-CN to a suspension of micelles decreases the average micelle diameter, suggesting that κ-CN limits the size of casein micelles by interacting at micellar surfaces.

When milk is treated with milk-clotting enzymes (e.g., chymosin) the casein micelles are destabilized resulting in coagulation. Chymosin cleaves the Phe105–Met106 bond in κ-CN, resulting in the liberation of the soluble C-terminal portion, glycomacropeptide (GMP; f106–169; MW ~6,800 Da), which contains the only serine phosphate and carbohydrate groups in κ-CN. The macropeptide portion of κ-CN is also devoid of Phe and hence has been marketed in supplements and diets for infants with the metabolic condition, phenylketonuria (van Calcar and Ney, 2012).

The N-terminal portion of κ-CN (f1–105), called para-κ-CN (MW ~12, 271 Da) with a net positive charge at pH 6.7 remains part of the curd during cheesemaking because it is extremely hydrophobic and insoluble (Figure 16.5).

16.2.2 Caseins, caseinates, and milk protein products rich in casein

There are three, perhaps four, distinct types of commercially available caseins—acid casein, rennet casein co-precipitates, and micellar casein—depending on the method used

Table 16.3 Chemical composition of milk protein products

Product	Protein (%)	Fat (%)	Lactose (%)	Ash (%)	Moisture (%)	pH
NFDM	36.0	0.8	52.0	8.0	3.2	
MPC56	56.0	1.2	30.0	8.0	4.8	6.0–8.0
MPC70	70.0	1.4	16.8	7.6	4.2	6.0–8.0
MPC80	80.0	1.5	5.8	7.4	4.8	6.0–8.0
MPI	90.0	2.0	1.0	2.0	4.3	6.7–7.2
Micellar casein	85.0	2.0	<0.5	8.0	5.0	6.7–7.1
Acid casein	85.2	2.0	<0.5	2.8	10.0	4.2–5.2
Rennet casein	84.0	2.0	1.6	4.2	10.0	4.6–5.1
Co-precipitate	89–94	1.5	1.5	4.5	5.0	~6.8
Sodium caseinate	89.0	2.5	1.0	5.0	4.0	6.6–7.3
Potassium caseinate	89.0	2.5	1.5	5.0	4.0	5.6–6.9
Calcium caseinate	88.0	2.5	1.0	6.0	4.0	6.6–7.3
Ammonium caseinate	90.0	2.0	1.0	2.5	4.0	6.0–6.5

Source: From USDEC (2006).

to extract them from milk. Acid casein is obtained by precipitation of casein from skim milk at its isoelectric point of pH 4.6 at ~40°C using preferably HCl, H_2SO_4 or *in situ* production of lactic acid by lactic acid bacteria. Rennet casein is obtained by the coagulation of skim milk with rennet (chymosin), whereas native micellar casein (NMC) is essentially a whey–protein–free milk protein concentrate obtained by microfiltration and diafiltration of milk followed by spray-drying (Schuck et al., 1994). Nonmicellar caseins are caseins found in milk serum. Co-precipitates are casein products obtained by acid + heat coagulation of milk, containing both caseins and whey proteins. Other milk protein products high in casein include nonfat dry milk (NFDM), milk protein concentrates (MPC), and milk protein isolates (MPI). Table 16.3 shows the chemical composition of various milk protein products.

16.2.3 Functional properties of casein products

This section deals with the functional properties of whole milk proteins and casein products. The functional properties of whey protein products are dealt with in a later section. The functional properties, that is, properties of proteins that influence its function in food systems are affected by the physicochemical and conformational characteristics of the protein molecules (Damodaran, 1994; Kinsella, 1977). Functional properties include, but are not limited to, solubility, foaming, whipping, gelation, water absorption, heat stability, film formation, and emulsification. Proteins also impart dispersibility, wettability, thickening, texture, viscosity, coagulation properties to foods. In addition, some proteins form protective films and glasses in food systems. Proteins also contribute to color and although bland tasting, hydrolyzed proteins are important contributors to flavor in foods. These functional properties are manifestations of hydrodynamic and surface-related properties of the proteins (Damodaran, 1994; Fox and Mulvihill, 1983). Milk proteins may also be

subjected to various chemical modifications to achieve unique properties. For a review of the chemical modifications, the reader is referred to Imafidon et al. (1997).

The open and flexible nature of the caseins makes them unusual proteins. Although caseins may have a preferred secondary and tertiary structure, they are often in other conformations which result in the exposure of hydrophobic groups to contact with water. Casein micelles are highly solvated, binding ~4 g water per gram of protein. The structures attained by the caseins can accommodate this contact. For most proteins, unfolding and exposure of hydrophobic groups to water results in unstable structures. The proteins must refold to lower the contact with water and precipitation often results. Because caseins exist in open structures, they are not as sensitive to structural alterations; for example, caseins are very stable to heat treatment. They may be exposed to boiling for extended periods of time and remain completely soluble, an extremely useful property for food products subjected to severe heat treatment (e.g., UHT processing). Caseins find application in food products where flexibility is required for functionality.

Most proteins that contain significant amounts of strong secondary and tertiary structures require time to unfold at air or oil interfaces. The time required depends on the flexibility of the protein. The most stable air cells and lipid droplets result from proteins that are able to rearrange their structures quickly and lower the interfacial free energy.

Traditional milk protein products include skim milk, condensed skim milk, whole milk powders, skim milk powders (nonfat dry milk), whey powders, whey protein concentrates (WPCs) and isolates (WPIs), buttermilk powders, caseins, and caseinates. More recently, milk protein products containing both caseins and whey proteins at the ratio (80:20) similar to that occurring in milk are obtained by membrane filtration technologies. Such products include milk protein concentrates identified by the numerical protein content (e.g., MPC70 or MPC 85—containing 70% or 85% protein, respectively) and MPI—containing ≥90% protein.

16.2.3.1 Solubility For a protein to be used as an ingredient in foods or other products, solubility is essential. Several terms have been used to describe the solubility of food proteins for commercial purposes. These include, water-soluble protein (WSP), water-dispersible protein (WDP), protein dispersibility index (PDI), nitrogen solubility index, NSI (Kinsella, 1984). Traditionally, the solubility of milk powders is expressed as the volume of insoluble material in the powder.

Caseins are insoluble in the pH range 4.5–5.0 but soluble above pH 5.5 and below pH 3.5. Caseins are made soluble by converting to caseinates by the addition of NaOH, KOH, $Mg(OH)_2$, $Ca(OH)_2$, or NH_4OH to form, respectively, Na caseinate, K caseinate, Mg caseinate, Ca caseinate, or NH_4 caseinate. Na and K caseinates are completely soluble at neutral pH, whereas Ca caseinates disperse in water to form a milk-white colloidal dispersion instead of solutions.

The solubility of MPC85 ranges from 32% to 98% and is influenced by manufacturing conditions, storage temperature, and time (Havea, 2006). The insoluble material consists of large particles (~100 nm) of casein micelles containing predominantly α- and β-caseins fused by protein–protein interactions. MPC insolubility may also be due to protein cross-linking at powder surfaces (Anema et al., 2006) and powder microstructure and chemical

state of the components (Murrieta-Pazos et al., 2011) and heat treatment during powder drying.

The solubility of micellar caseins ranges from 58% to 83% (Schokker et al., 2011). Poor solubility and ease of reconstitution can be improved by increasing the amount of non-micellar casein (serum casein) during spray-drying. Adding non-micellar casein prevents micelle–micelle interactions. Also, solubility can be enhanced by the addition of Na caseinate to the concentrate before drying. NaCl may also be added to improve the reconstitution of high-casein powders as a result of partial solubilization of calcium from the casein micelles, leading to a loosening of the casein micelle structure (Baldwin, 2010; Carr, 2002; Schuck et al., 1994). Improved reconstitution can also be achieved by using chelating agents (Bhaskar et al., 2001).

16.2.3.2 Heat stability Ca, Na, and K caseinates are heat stable (140°C for 15 min at pH 7), making them suitable in retort applications, soups, gravies, coffee creamers, and nutritional application. At 4%, Ca caseinate dispersions are heat stable at 120°C for 15 min above pH 7 (Kinsella, 1984). Casein also exhibits melting properties that are unique among proteins. After limited proteolysis, casein becomes thermoplastic and flows on heating. A similar effect is achieved by chelation of some of the Ca^{2+} present in caseins. These phenomena are the basis for the melting of natural cheeses and the production of process or imitation process cheese.

Meltability is owing to changes in the structure of caseins obtained by precipitation with calcium, acid, or coagulation with milk-clotting enzymes. Caseins do not form thermal gels and have little functionality in applications requiring temperature set. High heat stability and the ability to melt are the two properties of caseinates that make them difficult to replace in many food applications (e.g., pizza) where flow of melted protein is desired.

16.2.3.3 Coagulation and gelation Coagulation and gelation of caseins are the basis for the manufacture of cheese and structure development in cultured dairy products owing to their ability to form three-dimensional casein networks. When milk-clotting enzymes, for example, chymosin, is added to milk, it hydrolyzes κ-CN at the Phe105–Met106 bond to give para-κ-CN that remains with the curd and glycomacro peptide (GMP), which is lost in the whey. The physicochemical effect resulting from the addition of small quantities of chymosin to milk is an increase in viscosity, which can be taken advantage of in the manufacture of high-viscosity sour cream and cottage cheese dressing. Concentrated Ca caseinate dispersions form a gel when heated. At high-salt concentrations, Na caseinate solutions form gels in the cold that will liquefy on heating.

16.2.3.4 Viscosity High-solution viscosity is a result of the very open, nearly random, structures of casein molecules. The viscosity of Na caseinate increases with concentration. Because of the formation of aggregates, Ca caseinate is less viscous, whereas rennet casein is more viscous than Na caseinate. Because Na caseinate is more viscous than comparable dispersions of Ca caseinate, and forms gels above 17% solution (Kinsella, 1984), Na caseinate is often used in products requiring high viscosity. The lack of solubility in the presence of calcium changes the behavior of casein in its presence. When calcium is

added to a solution of Na caseinate, aggregation of casein into structures that resemble micelles occurs. As the aggregates increase in size and number, the viscosity of the solution decreases. The solution will also become turbid as the particles become large enough to scatter light. If a clear solution with high viscosity is required, Na caseinate is a good choice; however, Ca caseinate is preferred when a solution of relatively low viscosity and high turbidity (milky appearance) is desired. The viscosity of Ca caseinate increases with heat treatment suggesting formation of colloidal casein aggregates; however, viscosity of Na caseinate decreases on heating (Schmidt, 1978). The viscosity of caseinate solutions is influenced by pH. Generally, as pH increases, viscosity rises then falls (MAF Quality Management, 1996).

16.2.3.5 Film formation Milk proteins, such as caseins and caseinate films are attractive for use in foods as surface coatings (e.g., bakery glazes) because they are transparent, flexible and bland. Similarly, non-food applications, e.g., paper products, leather coloring and glues use caseins for their film-forming attributes (USDEC, 2006). Na caseinate can easily form films from aqueous solutions owing to the random coil structure of caseins and their ability to form extensive intermolecular hydrogen electrostatic and hydrophobic bonds, resulting in an increase of the interchain cohesion (Avena-Bustillos and Krochta, 1993; McHugh and Krochta, 1994). Caseinate films are stable over a wide range of pH, temperature, and salt concentrations (Kinsella, 1984); hence they resist thermal denaturation and/or coagulation.

16.2.3.6 Emulsification The emulsifying properties of milk proteins were reviewed by Dickinson (2003). Caseins and caseinates play important roles in the emulsification of fats or oils in products such as coffee creamers, whipped toppings, cheese analogues, soups, gravies, and meat products. Sodium caseinate is a more effective emulsifier than calcium caseinate. The two major caseins, α_{s1}-casein and β-casein, are distinctly amphiphilic and have strong tendencies to adsorb at oil–water interfaces and stabilize oil-in-water emulsions (Singh, 2011).

16.2.3.7 Foaming and whipping Foaming and whipping applications for caseins and caseinates include whipped toppings, mousses, frothy beverages like Frappuccino and foamed confections like marshmallows. The foaming properties of milk has been reviewed by Huppertz (2010) who reported that skim milk foams formed at 40–50°C are very stable; however, the presence of lipids (phospholipids, free fatty acids, and partial glycerides) strongly impairs foaming and foam stability, particularly at a temperature, for example, 20°C, where milk fat is partially crystalline. Heating Na caseinate at 132°C reduced significantly its viscosity and foaming and emulsifying capacity, but its foam stability appeared to be improved by heating (Guo et al., 1996).

Na caseinates have better foaming properties than Ca caseinates. The poor foaming of Ca caseinate is explained by the rectification process occurring along the foam column causing the protein to reach a two phase region and a lower capability to reestablish the surface concentration limiting the adsorption to the newly created surfaces (Abascal and Gracia-Fadrique, 2009).

16.2.4 Bioactive peptides from caseins

Milk proteins are rich in amino acid sequences that directly influence nutritional, immunological, neurological, gastrointestinal, behavioral and hormonal responses and are now being exploited as health-promoting ingredients in food systems. These bioactive peptides are released as a result of hydrolytic reactions catalyzed by proteolytic enzymes. For a review of bioactive peptides derived from milk proteins, see reviews by Clare and Swaisgood (2000) and Mills et al. (2011). Bioactive peptides are also discussed in Chapter 6.

16.3 Whey proteins

16.3.1 Whey proteins properties

Whey (or serum) proteins are the group of milk proteins that remain soluble after precipitation of the caseins by acid (at pH 4.6 and 30°C). Whey derived from acid precipitation or rennet coagulation is, respectively, called acid or rennet whey. Milk contains ~0.6–0.7% whey proteins, which represent ~20% of total milk proteins. Whey proteins comprise four major fractions and six minor fractions, with the major proteins being β-lactoglobulin, β-LG (~50%), α-lactalbumin, α-LA (~25%), immunoglobulin, Ig (~9%) and serum albumin, SA (~6%). Although GMP is not a natural component of milk serum, it is released from κ-CN during rennet action on cheese milk and is present as a part of whey components. Traditionally β-LG, α-LA, SA, Ig, and proteose–peptone (PP) fractions have been the most characterized components of whey proteins. Recently, considerable attention has been paid to biologically active components such as LF, lactoperoxidase, insulin-like growth factor, and other minor proteins. Approximate concentrations of proteins in whey are provided in Table 16.4.

Serum proteins are divided into heat-labile proteins (e.g., α-LA, β-LG, and Ig), which precipitate at pH 4.6–4.7 and the PP fractions, which remain in solution after heating milk to 85°C × 30 minutes. The PP fraction precipitates from milk after treatment with 12%

Table 16.4 Approximate concentrations of proteins in whey

Component of whey	Amount (mg/L milk)
β-Lactoglobulin	3.2
α-Lactalbumin	1.2
Glycomacropeptide	1.2
Proteose peptone	1.1
Immunoglobulin G	0.7
Serum albumin	0.4
Lactoferrin	0.06
Imuunoglobulin A	0.04
Immunoglobulin M	0.04
Lactoperoxidase	0.03
Lysozyme	0.0004

trichloroacetic acid (TCA). The heat-labile serum proteins can be separated by salting out with saturated solutions of $MgSO_4$.

Normal pasteurization of milk (72°C × 15 seconds) denatures <3% of whey proteins that contain –SH groups, hence, cooked flavor is not obvious in pasteurized milk. The susceptibility of whey proteins to denaturation (i.e., loss of solubility at pH 4.6) in the pH range 5.2–8.8 is in the order Igs > SA/LF > β-LG > α-LA. The rates of denaturation of Igs and SA/LF decreases with increasing pH, whereas rates of denaturation of β-LG > α-LA increases with pH (Law and Leaver, 2000).

Whey is rich in several nutritional components. Nutritionally, whey proteins are considerably superior to plant proteins and are considered to be one of the highest quality proteins in the food industry. Whey proteins contain higher levels of branched-chain amino acids (BCAA, i.e., Leu, Ile, and Val) than caseins. Leu is a regulator of whole body and skeletal muscle protein metabolism in humans (Nair et al., 1992). The proportion of essential amino acids in whey, including BCAA, is similar to the amino acid needs of the human body. BCAAs play an important role in muscle redevelopment and recovery from sports and stress fatigue (Rieu et al., 2007). Leu plays a key role in initiating the transcription of protein synthesis. The amino acid profile of whey proteins is very similar to that of the skeletal muscle, and whey protein supplements generally provide a higher dose of the essential amino acids than other protein sources. Whey proteins are beneficial for bodybuilding as they are digested very rapidly and help return the postworkout body from a catabolic state to an anabolic state. When Leu is ingested in high amounts such as in WPI or WPC, there is greater stimulation of protein synthesis, which may speed recovery and adaptation to stress such as exercise.

Whey proteins are rich in the sulfur-containing amino acid, Cys, which can be used to make glutathione. Glutathione is an antioxidant that defends the body against free radical damage and might reduce the risk of cancer.

Whey proteins also contain a range of biologically active peptides, which are encrypted within the sequence of the parent proteins, and can be released by enzymatic hydrolysis such as during gastrointestinal digestion or during processing (McIntosh et al., 1998). Developments in new processing technologies such as membrane filtration and ion exchange chromatography have stimulated commercialization of some of the biologically active components from whey. Concentrates of these biologically active whey proteins have now found applications in functional foods, dietary supplements, nutraceuticals, sports, and infant and medical foods.

16.3.1.1 β-Lactoglobulin β-Lactoglobulin (β-LG) is the most prevalent protein in whey and is precipitated by saturation with $MgSO_4$ or 50% saturation of ammonium sulfate. It comprises 10% of the total milk protein or about 58% of the whey protein. It contains 162 amino acids with a MW of ~18,300 Da. The primary sequence of β-LG showing four genetic variants is given in Figure 16.6. β-LG contains two –S–S– and one free –SH groups and no phosphorus. One of the –S–S– bonds is between Cys 66 and Cys 160. The other –S–S– bond is dynamic, occurring between Cys 106 and sometimes Cys 121 and sometimes with Cys 119. Thus, half of the Cys 119 and half of the Cys 121 exist as a free –SH group.

1 **11**
Leu Ile Val Thr Gln Thr Met Lys Gly Leu Asp Ile Gln Lys Val Ala Gly Thr Thr Trp
21 **31**
Ser Leu Ala Met Ala Ala Ser Asp Ile Ser Leu Leu Asp Ala Gln Ser Ala Pro Leu Arg
41 **Gln in variant D** **51** **His in variant C**
Val Tyr Val Glu Glu Leu Lys Pro Thr Pro Glu Gly Asp Leu Glu Ile Leu Leu Gln Lys
61 **Gly in variants B, C** **71**
Asp Glu Asn Asp Glu Cys Ala Gln Lys Lys Ile Ile Ala Glu Lys Thr Lys Ile Pro Ala
81 **91**
Val Phe Lys Ile Asp Ala Leu Asn Glu Asn Lys Val Leu Val Leu Asp Thr Asp Tyr Lys
101 **111** **Ala in variants B, C**
Lys Thr Leu Leu Phe Cys Met Glu Asn Ser Ala Glu Pro Glu Gln Ser Leu Val Cys Gln
121 **131**
Cys Leu Val Arg Thr Pro Glu Val Asp Asp Glu Ala Leu Glu Lys Phe Asp Lys Ala Leu
141 **151**
Lys Ala Leu Pro Met His Ile Agr Leu Ser Phe Asn Pro Thr Gln Leu Glu Glu Gln Cys
161 **162**
His Ile OH

Figure 16.6 Primary sequence of bovine β-lactoglobulin A. The locations of the amino acid substitutions in the genetic variants are indicated. Disulfide bonding occurs between Cys 66 and Cys 160; Cys106 and Cys 119; or Cys 121

The pI for β-LG is 5.23, β-LG is 5.3 and pooled β-LGAB is 5.28 (Treece et al., 1964). Below pH 3.5 and above pH 7.5, β-LG exists as a monomer. Between pH 3.1 and 5.1 at low temperatures and high protein contents, it associates to form an octamer. This polymerization seems to be the result of the action of carboxyl groups and thus variant A forms better octamers than variant B. At other pH values, including the pH of milk (6.6–6.8), β-LG occurs as a dimer. β-LG dimers are spherical with a diameter of about 18 Å.

Irreversible denaturation occurs at a pH above 8.6, as well as by heating or at higher levels of Ca^{2+} (Belitz et al., 2009). β-LG undergoes a reversible pH-induced conformational change at about pH 7, known as the Tanford transition (Tanford et al., 1959). Denaturation of β-LG A by hydrostatic pressure (Valente-Mesquita et al., 1998).

β-LG is synthesized in the mammary gland for inclusion in milk also occurs where its role is unknown. All ruminant milk contains β-LG, whereas most nonruminant milk does not. Although biological functions of β-LG have been speculated, to date, none has been fully accepted. The molecule has a very hydrophobic area that is quite effective in binding retinol. Some authors speculate that the binding of Vitamin A may have a regulatory role in the mammary gland. Because of its prevalence in bovine milk, to a large extent the properties of WPCs are in effect, are influenced by the properties of β-LG.

β-LG is a rich source of essential amino acids, especially BCAA, which play an important role in muscle redevelopment and recovery from sports and stress fatigue. Besides being a source of essential and BC amino acids, this protein has the potential to modulate lymphatic responses; thus, it plays an important role in immune functions in protecting the body against the infections and spread of tumors.

Arg in variant A

1																		
Glu	Gln	Leu	Thr	Lys	Lsy	Glu	Val	Phe	Gln	Glu	Leu	Lys	Asp	Leu	Lys	Gly	Tyr	Gly	Gly
21										**31**									
Val	Ser	Leu	Pro	Glu	Trp	Val	Cys	Thr	Thr	Phe	His	Thr	Ser	Gly	Tyr	Asp	Thr	Glu	Ala
41										**51**									
Ile	Val	Glu	Asn	Asn	Gln	Ser	Thr	Asp	Tyr	Gly	Leu	Phe	Gln	Ile	Asn	Asn	Lys	Ile	Trp
61										**71**									
Cys	Lys	Asn	Asp	Gln	Asp	Pro	His	Ser	Ser	Asn	Ile	Cys	Asn	Ile	Ser	Cys	Asp	Lys	Thr
81										**91**									
Leu	Asn	Asn	Asp	Leu	Thr	Asn	Asn	Ile	Met	Cys	Val	Lys	Lys	Ile	Leu	Asp	Lys	Val	Gly
101										**111**									
Ile	Asn	Tyr	Trp	Leu	Ala	His	Lys	Ala	Leu	Cys	Ser	Glu	Lys	Leu	Asp	Gln	Trp	Leu	Cys
121		**123**																	
Glu	Lys	Leu	OH																

Figure 16.7 Primary sequence of bovine α-lactalbumin B. The position of the amino acid substitution that occurs in genetic variant A is indicated. Disulfide bounds are formed between the following pairs of Cys residues: 6 and 120, 28 and 111, 61 and 77 and 73 and 91

16.3.1.2 α-Lactalbumin α-Lactalbumin (α-LA) exists in true solution in milk. It is the second most predominant protein in whey and represents ~2% of the total milk protein and ~13% of the total whey protein. α-LA consists of 123 amino acids and has a molecular weight of 14,146 Da. Its primary structure is shown in Figure 16.7. α-LA is stabilized by four —S—S— bonds. It does not contain a free —SH group (Brew and Grobler, 1992) and hence is relatively heat stable; it survives high-temperature short-time (HTST) pasteurization of milk while 2.4% is denatured after heating to 90°F × 30 seconds. One of the —S—S— bonds (Cys6-Cys120), however, unlike most proteins that exhibit increased heat sensitivity in the presence of Ca^{2+}, is more sensitive to reduction than the other three (Kuwajima et al., 1990). α-LA is more stable to heat in the presence rather than the absence of Ca^{2+}. This is probably owing to the ability of calcium to promote the formation of ionic intermolecular cross-links with most proteins. These cross-links hold the molecules in proximity, thereby, increasing the likelihood of aggregation on heating. α-LA, on the other hand, uses calcium to form intramolecular ionic bonds that tend to make the molecule resistant to thermal unfolding. Under favorable conditions of Ca^{2+} and pH, α-LA remains soluble when heated to 100°C.

α-LA is a rich source of Trp, which has been shown to improve sleep quality, cognitive performance under stress, and mood under stress through the formation of the neurotransmitter serotonin. Supplementation with α-LA-enriched whey protein has been shown to increase the ratio of plasma tryptophan to other large neutral amino acids and improve the cognitive ability (evaluated by memory tests) of high stress-vulnerable individuals.

α-LA plays an important role in the biosynthesis of lactose. In the presence of α-LA, galactosyl transferase adds UDP-galactose to *N*-acetyl glucosomine groups that are attached to proteins. It transfers UDP galactose to glucose (Brew et al., 1968). The affinity of the transferase alone for glucose is low (Km ≈2 mM), resulting in a slow reaction. The affinity increases 1000-fold in the presence of α-LA.

Under acidic conditions, human α-LA forms a complex with oleic acid form a complex, termed "HAMLET" (Human Alpha-Lactalbumin Made LEthal to Tumor cells), which has been shown to inhibit a wide array of tumors through an apoptosis-like event (Svanborg et al., 2003). The bovine counterpart of HAMLET, termed "BAMLET," was recently shown to display potent cytotoxic activity against eight cancer cell (Rammer et al., 2010).

α-LA can also facilitate the absorption of minerals and exert antibacterial and immune-modulatory effects. Clinical trials have demonstrated significant reductions in depression after an intake of α-LA. Trials have also shown improved sleep patterns by increasing sleepiness, reducing sleep latency and improving morning alertness. α-LA has also been shown to be capable of undergoing structural changes to induce apoptosis (programmed cell death) in a cancer cell line, under conditions similar to the stomach. α-LA is rich in essential and conditionally essential amino acids and is the dominant protein in human milk. In infant formula products, adding α-LA provides a number of nutritional and physiological advantages. The high content of Cys in α-LA is also valuable in boosting the immune system and promoting wound healing (Ha and Zemel, 2003).

When the amino acid sequences of α-LA and lysozyme are compared, about 40% of the residues are identical, including all the Cys residues. Also, there are structural similarities in another 20% of the residues for both proteins. α-LA helps to synthesize the same linkage that lysozyme cleaves suggesting that both proteins are closely related; thus, knowledge of the three-dimensional structure of lysozyme has been utilized to predict the three-dimensional structure of α-LA. Despite their similarity, they do not work on the same substrates and are not related antigenetically. The site of synthesis of α-LA, like β-LG, is the mammary gland. α-LA binds divalent cations (e.g., Ca^{2+}, Zn^{2+}) and may facilitate the absorption of essential minerals (Stănciuc and Râpeanu, 2010).

16.3.1.3 Bovine serum albumin Bovine milk SA is identical to the blood serum protein; thus, SA is not synthesized in the mammary gland, but passively leaks into the milk from blood. SA is a single polypeptide of 582 amino acid residues with a molecular weight of 66,433 Da (Hsia et al., 1984). It contains no phosphorus, 17 –S–S– and 1 –SH group, Cys34 (Carter and Ho, 1994). It is characterized by an overall oblate shape and consists of three domains (I, II and III), each stabilized by an internal network of disulfide bonds (Carter and Ho, 1994), with a molecular structure consisting of nine loops. The secondary structure is composed of 76% helix, 10% turn, and 23% extended chain, and no β-sheet (Carter and Ho, 1994; Gelamo et al., 2002; Gelamo and Tabak, 2000; Reed et al., 1975; Wetzel et al., 1980). In blood plasma, bovine SA is a carrier of fatty acids. The molecule has specific binding sites for hydrophobic molecules and may bind them in milk as well.

16.3.1.4 Immunoglobulins The Igs comprise at least 2% of the total proteins and ~10% of the whey proteins in milk. Three classes of Igs are found in milk: lgG, lgA, and lgM. All of the Igs have similar basic structures, composed of two identical light chains with a molecular weight of 20,000–25,000 Da and two identical heavy chains, with a molecular weight of 50,000–70,000 Da. The four chains are joined together by disulfide bonds; thus, the complete Ig or "antibody" molecule has a molecular weight of ~180 kDa

(Korhonen et al., 2000). Bovine IgG molecule occurs predominantly in two subclasses, IgG1 and IgG2.

The Igs, except IgM, are not synthesized in the mammary gland (Butler, 1969). They are selectively transported from the serum into the mammary gland, as a result of which, the first colostrum contains very high concentrations (~40–200 mg/mL) of Igs (Korhonen et al., 2000). Elfstranda et al. (2002) reported that colostrum samples obtained from the first milking of Swedish Friesian cows contained ~90 mg/mL IgG1, 2.8 mg/mL IgG2, 1.6 mg/mL IgA and 4.5 mg/mL IgM with concentrations declining by 92%, 87%, 93%, and 84%, respectively, in mature milk. They enter milk from the mammary gland. Igs are antibodies that are synthesized by mammals in response to antigenic or immunogenic stimuli such as bacteria and viruses, thereby providing protection against microbial infections. Bovine colostrum is a rich source of Igs, which confers passive immunity to the newborn until it synthesizes its own antibodies (Mehra et al., 2006).

HTST pasteurization of milk results in the loss of ~34–41% of the Ig present in raw milk with no apparent effect of homogenization, skimming or standardization to 1% or 2% fat contents. (Li-Ghan et al., 1995).

16.3.1.5 Proteose-peptones This fraction of milk has been defined as those proteins that remain in solution after milk is heated at 95°C for 20 minutes followed by acidification to pH 4.7. The proteose peptones (PP) in milk are precipitated by 12% TCA and represent ~1.1% of the total milk protein and about 10% of total whey proteins (Innocente et al., 1998). There are four major PPs: designated component 3 (PP3), component 5 (PP5), component 8 fast (PP8f), and component 8 slow (PP8s) corresponding to their mobilities in free-boundary electrophoresis. In addition, there are ~38 ill-defined minor PP components (Sørensen and Petersen, 1993).

PP3 (also known as lactophorin) is found only in whey and is not derived from casein. It is a small phosphoglycoprotein (MW ~20,000), containing over 17% carbohydrate, which is exclusively expressed in the lactating mammary gland (Pedersen et al., 2012). Antibodies to PP3 cross-react with the MFGM, suggesting that it may originate from the MFGM (Brunner and Kester, 1982). PP3 has good emulsifying properties and may be responsible for the foaming of milk (Girardet and Linden, 1996).

PP5 (molecular weight 13,000 Da) is present in both the whey and casein fractions of milk. PP5 contains phosphorus; it represents β-CN (f1–105) resulting from plasmin proteolysis. PP8f (molecular weight 3900 Da) represents β-CN (f1–28), whereas PP8s (molecular weight 9900 Da) represents β-CN (f29–105). The PPs are heat resistant and very surface active partly because of their low molecular weights and also to the carbohydrate present in PP3.

16.3.1.6 Glycomacropeptide GMP is a hydrophilic peptide, κ-CN (f106–169), that provides stability to the casein micelles in milk. When chymosin acts on κ-casein during the manufacture of cheese, GMP is released into the whey. GMP makes up about 15–20% of the whey proteins and is often found in WPCs. Recent advances in fractionation technologies have allowed separation of GMP from cheese whey into commercial GMP-enriched ingredients. Because of the highly negative charge of GMP at low pH,

where whey proteins are positively charged, an ion exchange process can be used to isolate GMP.

GMP is unique is a glycoprotein containing an oligosaccharide chain. It also is unique because it does not contain Phe, Trp, or Tyr and also contains high levels of the BCAAs Leu, Ile, and Val. This feature of GMP gives it some unique characteristics that can be exploited in a variety of nutritional applications; for example, individuals with the medical condition phenylketonuria (PKU) and are unable to digest Phe and GMP is one of the few peptide sources PKU patients can tolerate (van Calcar and Ney, 2012). Along with whey proteins, GMP is reported to enhance the growth of bifidobacteria in probiotic fermented milk (Janer et al., 2004).

16.3.1.7 Lactoferrin Lactoferrin (LF) was first identified in bovine milk by Groves et al. (1963). It is an iron-binding glycoprotein present in colostrum and milk at concentrations of 1.5 mg/mL and 0.02–0.35 mg/mL, respectively (Levay and Viljoen, 1995). Human milk contains ~4 mg/mL (Madureira et al., 2007). For a recent review of the structure, function and application of LF see Gonzalez-Chavez et al., (2009). Bovine LF is a single polypeptide chain containing ~689 amino acid residues and five potential glycosylation sites (Pierce et al., 1991). It has a molecular weight between 76 and 80 kDa, varying because of the degree of glycosylation. It is folded into two homologous globular units called N- and C-lobes, referring to the N-terminal and C-terminal parts of the molecule, respectively. Each lobe consists of two sublobes or domains, N1, N2, C1, and C2 having one Fe^{3+}-binding site and one glycosylation site. In bovine LF, N1 corresponds to sequences 1–90 and 251–233, N2 for 91–250, C1 for 345–431 and 593—676, and C2 for 432–592. The two lobes are hinged by a short, 10–15 α-helical peptide (Baker and Baker, 2005) with three turns, sequence 334–344 (Steijns and van Hooijdonk, 2000). Bovine LF has two iron-binding sites; each unit is able to bind 1.4 mg of iron per gram. In addition to iron, bovine LF also binds copper or other metals. Bovine LF has ~70% similarity in amino acids composition with human LF (Pierce et al., 1991).

LF is one of the components of the immune system of the body and may interact with DNA and RNA. The antimicrobial activity (bacteriocidal and fungicidal) is the result of the iron-binding ability of LF. LF is positively charged in whey at pH 7.0; it has an isoelectric pH of ~pH 8.7. The main biological function of bovine LF is binding and transport of iron ions. Bovine LF also has antioxidant, immune modulation, antibacterial, antiviral, antiparasitic, anticancer, and antiallergic properties (Farnaud and Evans, 2003; Marshall, 2004; Pan et al., 2006). The iron-binding properties of bovine LF deprive bacteria of an element necessary for their growth. Bovine LF can also bind to the lipopolysaccharides of bacterial cell walls and its oxidized iron component oxidizes bacteria through the formation of peroxides that affect membrane permeability, resulting in cell lysis. Bovine LF not only disrupts the membrane, but even penetrates the cell. This is associated with a specific cationic peptide corresponding to residues 17–41 in the N-lobe and is released by hydrolysis by pepsin (Tomita et al., 1992).

Bovine LF is also reported to penetrate the cell of viruses and can suppress virus replication (Harmsen et al., 1995). Many viruses have the tendency to bind to lipoproteins of cell membranes and then penetrate the cell. Bovine LF binds to the same lipoproteins,

thereby preventing the viruses from binding and penetrating the cell. Bovine LF also binds viruses, such as hepatitis viruses. The antiviral effect of LF is the result of its ability to affect natural killer cells, granulocytes, and macrophages (Madureira et al., 2007).

Bovine LF is also reported to decrease bone resorption and increase bone formation. This is shown by a decrease in bone resorption markers such as deoxypyridinoline and *N*-telopetide and an increase in bone formation markers such as osteocalcin and alkaline phosphatase (Naot et al., 2012).

16.3.1.8 Lactoperoxidase Lactoperoxidase [EC 1.11.1.7] is an enzyme present in colostrum, milk and whey, with a MW of ~77.5 kDa. Bovine colostrum and milk contain about 11 to 45 mg/L and 13 to 30 mg/L of lactoperoxidase, respectively. In whey, lactoperoxidase constitutes ~0.5% of total whey proteins. For a review of the structure, functions, and application of bovine milk lactoperoxidase, see de Wit and van Hooydonk (1996); Reiter and Harnulv (1984).

It functions as a natural antibacterial agent. The oxidized products produced through the action of this enzyme have potent bacteriocidal activities. This reaction involves two cofactors, hydrogen peroxide and thiocyanate ions, which together with lactoperoxidase constitute the lactoperoxidase system (LPS). Activation of the enzyme results in the formation of hypothiocyanite ions, which are responsible for the antimicrobial action. The LPS plays an important role in the innate immune system by killing bacteria in milk and mucosal secretions. Lactoperoxidase, as part of the LPS, has a broad spectrum of antibacterial activity, having a bacteriostatic effect against Gram-positive bacteria and a bactericidal effect against Gram-negative microorganisms, e.g. pseudomonads, coliform, salmonella and Listeria.

Commercially, lactoperoxidase is isolated from either skim milk or whey using an ion-exchange process similar to that used for isolation of LF. Lactoperoxidase has a pI of 9.0–10.0 and is positively charged at the normal pH of whey and can be bound to cation exchange resins and fractionated from the other whey proteins. The lactoperoxidase system is reported to be used for gingivitis and paradentosis (Poulsen, 1988). Lactoperoxidase has been used in toothpaste and mouth rinse to kill oral bacteria (Tenovuo, 2002) and in contact lenses cleaning solutions (McKee and Sills, 2003).

16.3.2 Whey protein products

16.3.2.1 Whey protein concentrates and isolates Whey proteins can be processed to WPCs or isolated and purified to WPIs using membrane ultrafiltration or ion exchange chromatography.

WPCs contain 35–80% proteins with correspondingly reduced amounts of lactose and minerals (Table 16.5). For WPC containing a high level of protein, such as WPC80, which contains 80% protein, the retentate is diluted with water (diafiltration) and reconcentrated by ultrafiltration. WPCs containing ≥90% protein are generally known as WPI.

WPI is manufactured using ion exchange chromatography followed by concentration and spray drying or microfiltration of whey followed by ultrafiltration and spray drying.

Table 16.5 Composition of some whey protein products, dry matter basis

Component	Sweet whey	Acid whey	WPC34	WPC80	WPI
Moisture (%)	4.5	4.5	4.0	4.0	4.5
Fat (%)	1.0	0.5	3.0	5.0	1.0
Protein (%)	12.0	12.0	34.0	80.0	92.0
Lactose (%)	73.0	68.0	50.0	4.0	0.4
Ash (%)	8.0	11.0	6.0	7.0	0.2

For the manufacture of WPI by ion exchange, the pH of clarified whey is lowered to 3.0–3.5 and passed through an ion exchange resin, where most of the proteins are adsorbed, subsequently eluted and the pH readjusted. The resulting protein solution is then concentrated by evaporation, ultrafiltration or reverse osmosis, and finally spray dried.

16.3.2.2 Whey protein hydrolysates Milk protein is hydrolyzed by proteolytic enzymes to produce protein ingredients with improved nutritional, dietetic, and medicinal values. The degree of hydrolysis can be varied and proteins are hydrolyzed into peptides of different sizes, as well as free amino acids.

To prepare whey protein hydrolysates, a proteolytic enzyme is added to a protein solution (substrate) at a specific enzyme: substrate ratio. Under controlled conditions, the enzyme cleaves the peptide bonds and produces the desired level of proteolysis. The hydrolyzed protein is clarified, followed by flavor reduction, concentration and spray drying.

16.3.3 Functionality and application of whey products in foods

As mentioned earlier the functional properties of milk proteins, for example, solubility, foaming and emulsification, are affected by the physicochemical and conformational characteristics of the protein (Damodaran, 1994; Kinsella, 1977). Proteins also impart dispersibility, wettability, thickening, texturization, viscosity, water-binding, and gelation properties to food, in addition to forming films and glasses. They also contribute to the color and flavor of foods. These functional properties are manifestations of hydrodynamic and surface-related properties of the proteins (Damodaran, 1994; Fox and Mulvihill, 1983) and the heat stability of the proteins in various food systems. Milk proteins are also responsible for the stability of milk fat emulsion in the aqueous phase of milk.

Because purified individual milk proteins exhibit better functionality than in their native protein mixtures, there is great interest in developing methods to prepare pure casein and whey proteins on a large scale (Cayot et al., 1991; Migliore-Samour and Jollès, 1988; Murphy and Fox, 1991). The use of newer technologies, for example, high-pressure processing in combination with heat treatment influence the behavior of whey proteins (Considine et al., 2007).

16.3.3.1 Solubility At the pI of a protein, the numbers of positive and negative charges are equal and protein–protein interactions are most favored. For this reason,

many proteins are insoluble at their isoelectric point. Many food proteins have a pI close to 4.5, making it difficult to use them in solutions around this pH. Whey proteins, however, are soluble at their isoelectric points and can be utilized at these pH values. This makes WPCs uniquely applicable for addition to acidic beverages. There has been considerable work regarding the fortification of fruit juices and soft drinks with WPC. The level of protein fortification has generally been low because of the presence of lactose and salts. Utilization of WPCs would allow for fortification at a higher level. While protein-fortified soft drinks have not caught on with consumers, they provide a potentially lucrative market for WPC. It has been estimated that if 1% of the soft drinks sold in the United States were fortified at the 3% protein level with 35% WPC, there would be an increased demand for 40 million kg of WPC per year (Mangino, 2007).

WPCs may also be used in beverages produced at pH values closer to neutrality. In these products they can be used for a variety of reasons including to function as emulsifiers, nutritional supplements, to increase viscosity or to provide turbidity. In these applications it is especially important that the WPCs have high solubility and a bland flavor.

The functionality of WPCs in beverages has been described by Rittmanic (2006). The percentage of WPC in the beverage depends on the processing and packaging options. Because whey proteins are stable and soluble over a wide pH range, it is important to consider their natural gelation abilities in determining the heat treatment given to the beverage and the concentration of whey protein desired. Concentrations of sugars and mineral ions in the beverage solution also influence whey protein behavior during processing and throughout storage.

16.3.3.2 Gelation Protein gels can be formed by the addition of salts, the action of enzymes, changes in pH, or by the application of heat; hence, whey protein gels are obtained by heating. This discussion will be limited to the mechanisms of heat-induced protein gelation.

The effects of composition on the strength and texture of WPC gels have been studied. At low calcium concentrations, weak gels were formed. Gel strength increases as calcium concentration is increased up to about 11 mM, but at calcium concentrations above this value gel strength decreases. In the presence of high levels of Ca^{2+}, heating causes protein aggregation before protein unfolding and a three-dimensional network cannot be formed and chelation of calcium would increase gel strength. For a review of physical and chemical methods to induce protein gelation, see Totosaus et al. (2002).

The sulfhydryl content of WPCs influences the strength and textural characteristics of gels. As with calcium, an optimum concentration for maximum gel strength has been reported. The content of β-LG has been shown to be correlated with the sulfhydryl content of WPCs and thus can be correlated with the strength of the gels formed. It has been noted that the appearance and strength of the gels formed from WPCs is dependent on the pH of gelation. At low pH values, gels were rather soft and opaque with characteristics of coagulated milk. At higher pH values, the gels became more elastic, transparent, and have increased gel strengths, which make them suitable for use in different food products. The relationship of protein hydrophobicity and gelation characteristics of WPCs have been described (Voutsinas et al., 1983; Shimada and Cheftel, 1989). As with calcium and

sulfhydryl content, there appears to be an optimal level of hydrophobicity beyond which gel strength is weakened. In commercially available WPCs, this level has not been reached and there is a direct positive correlation between gel strength and protein hydrophobicity.

The gelation properties of WPCs can be important to their inclusion in a number of food products. In some cases, the proteins are present to help in water binding and in others they actually add to the gel matrix. Gelation can also be important in other functional properties such as foam stability. WPCs can form excellent foams, but the stability of these foams is generally poor. In products such as cakes and meringues, if the protein does not denature at a low enough temperature, the foam will rupture during heating and collapse.

In general, textural attributes/properties of foods are influenced by gelation of proteins, hence, consumer acceptability of food is correlated with the heat treatment received by the whey proteins during processing. Relatively mild heat treatments (64°C × 15 seconds) of retentates for WPC production cause protein unfolding, whereas high heat treatments (72°C × 15 seconds) cause excessive unfolding and denaturation resulting in reduced gel strength in gels produced from solutions containing 12.7% WPC. (Marushige and Mangino, 1989). This suggests that WPCs can be produced to form heat-induced gels with different texture attributes under different conditions. The gelation properties of WPCs are one of their most important and marketable functional characteristics.

16.3.3.3 Emulsification The lack of reliable standardized methods to study the emulsifying properties of proteins has resulted in a body of literature that is confusing and often contradictory. The emulsifying properties of whey proteins were reviewed by Dickinson (2003). More recent aspects of milk protein-stabilized emulsions have been reviewed recently (Singh, 2011).

Emulsifying capacity is the amount of oil that can be made into an emulsion by a given quantity of protein. Emulsifying capacity is a property not only of the protein under study but also of the emulsion system, the equipment being used, and the method employed in emulsion formation. This method does not address the stability of the emulsion system and tends to measure at protein to lipid ratios that are far removed from those found in food systems.

Emulsifying capacity does not vary much in WPCs. In acid WPCs with solubility ranging from 25 to 82%, the emulsifying capacity ranges from 38 to 52 mL of oil per gram of protein. It was reported that the contents of potassium, phosphorus, and magnesium were the factors most closely related with the emulsifying capacity of these samples (Liao and Mangino, 1987). The emulsifying capacity of sweet WPCs ranged between 52 and 53 mL oil per gram of protein. Kim et al. (1987) reported that although the range of values was limited, a significant correlation between emulsifying capacity and the soluble β-Lg content existed. In general, results of emulsifying capacity measurements do not correlate well with performance in food systems.

Other methods used to study emulsions attempt to measure emulsion stability. A number of procedures are based on an estimate of the average fat globule diameter on the assumption that the more efficient emulsion formation the smaller the average diameter of the fat globules. One of the most popular of these procedures is the so-called emulsion activity index. In this procedure, light scattering by an emulsion is related to total surface

area of the emulsion. Purified β-LG is more efficient emulsion formation than other whey proteins and better emulsions are formed with β-LG than with WPCs (Kim et al., 1987). It is hypothesized that α-LA actually inhibits emulsion formation.

16.3.3.4 Foaming Undenatured whey proteins are excellent foaming agents, whereas heating of WPCs increases foam stability (DeVilbiss et al., 1974). Mild heating may cause a partial unfolding of the protein molecules, which makes intermolecular interactions necessary for stable foam formation easier. Heating WPCs at a temperature of 50–60°C causes reversible improvement in their foaming properties. Cooling of protein suspensions before whipping reverses the observed improvement in foaming properties. Heating disrupts protein–lipoprotein complexes. Storage WPCs at 4°C reduces its foaming properties which are attributed to association of β-LG at low temperatures. This effect is completely reversible by mild heat treatment (50–60°C); however, the importance of such complexes to protein functionality in foods in the pH range 6–7 has been questioned (Morr (1979) because heat treatment of electrodialyzed WPC did not increase foaming compared to heat treatment of other types of WPCs. This may be owing to an inhibition of denaturation of β-LG and α-LA by the salts and lactose present.

Most studies of foaming in WPCs have been performed in dilute aqueous solutions that often do not resemble food products. A high-fat foam product that contains 6% protein, 30% fat, and simulates a commercial whipped topping has been described (Liao and Mangino, 1987). For acid WPCs, protein hydrophobicity and the content of sulfhydryl groups were the factors most important in determining whipped topping overrun. In commercial WPCs, the sulfhydryl content was an important factor in whipped topping overrun. The content of native β-LG was strongly correlated with the whipped topping overrun of eight 75% WPCs. Pasteurization of the ultrafiltration retentate used to produce WPCs has been shown to have a significant negative effect on whipped topping overrun.

16.4 Conclusions

Milk proteins compared to other food proteins have unique nutritional and structural–functional properties that make them increasingly important as food ingredients. The versatility of milk proteins as food ingredients is as a result of their bland taste; hence, these do not impart undesirable flavors. Because they are soluble at various pH values, they can be used under acidic and alkaline conditions. Caseins have good emulsifying and fat-binding properties. They are also surface active and form stable foams allowing for their use in whipped toppings and frothy beverages. Surface active properties have also been exploited in the formation of films and coatings. Caseins are heat stable allowing for their use in highly heated and retorted foods. Acidification of caseins under quiescent conditions results in the formation of weak gels that are desirable in products such as yogurt; they can be coagulated into cheese by the action of milk clotting enzymes. The viscoelastic properties of caseins are exploited in products such as pizza in which cheese proteins become molten on heating and can be pulled to align and stretch the cheese. The flow properties of caseins have led to caseins being spun into fibers.

Milk proteins have been suggested as nano delivery vehicles for nutraceuticals (Abd el-Salam and El-Shibiny, 2012) possibly because of their water-binding and fat-binding properties and bland taste. Hydrolyzed caseins impart flavor to cheese. In addition, several bioactive peptides have been identified in hydrolyzed caseins but have fully not been commercially exploited.

Whey proteins and their hydrolysates provide a range of physiologically functional components that offer several health benefits. Many of the whey protein ingredients with health benefits have been successfully commercialized.

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17
Egg Proteins

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17.1 Introduction

The eggs of avian species have long been recognized as an excellent source of proteins and other nutrients for humans. Because their highly nutritive value (Watkins, 1995) and unique functional properties, hen egg proteins and components remain among the most commonly used ingredients in the food industry. The chemical composition of eggs has been and continues to be the object of intensive investigations, which can be traced back to the sixties (Bolton, 1961; Parkinson, 1966) or even earlier (Burns and Ackerman, 1955). Earlier references on this topic are reviewed in several monographs and reviews that describe in detail the chemical composition of eggs (Li-Chan and Nakai, 1989; Huopalahti et al., 2007; Li-Chan and Kim, 2008). Similarly, the molecular basis of the functional properties of egg protein has been the object of multiple scientific reviews (Li-Chan and Nakai, 1989; Mine, 1995, 2002; Campbell et al., 2003; Lomakina and Mikova, 2006; Anton, 2013). Understanding egg functional properties and the chemistry behind these properties is important for the application of hen eggs in the food industry to develop novel food products or improve the quality of existing products. The food industry also endeavors to prolong the shelf life of eggs, protect the nutritional value of eggs during the storage period, and incorporate egg products into other marketable products such as cake and ice cream via food processing (Burley and Vadehra, 1989).

This chapter aims to provide detailed information about egg proteins, their chemistry, and their functionality to help improve the understanding of the most recently acquired knowledge on the functional properties of egg components.

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17.2 World production of avian eggs

Domestication of the first fowl has been suggested to be around 3200 B.C. in India. Ancient Egyptian and Chinese records provide evidence of egg-laying fowl for human consumption as early as 1400 B.C. Throughout history, eggs have been an essential part of the diet as well as an effective ingredient in food preparation.

Today, the world egg production has expanded from 51.2 million tons to almost 65 million tons. There are estimated to be 4.93 billion egg-laying hens in the world. Approximately 800–1,000 million laying hens are in China, 276 million in the United States, 290 million in the 15 member states of the European Union, 133 million in India, 115 million in Mexico. Today's laying hens are each capable of producing over 300 eggs per hen per year. China is the world's largest egg producer (20.25 million tons during 2012). Annual egg consumption per capita varies from country to country. In 2012, egg consumption per capita in the highest egg-consuming countries was as follows: Mexico, 321; United States, 255; France, 248; Portugal, 186; and India, 40. In the United States, approximately two-third of eggs are consumed as shell eggs, whereas one-third as egg products (IEC, 2012).

17.3 Structure and chemical composition of the egg

Hen egg is composed of the three main components: the eggshell, egg white (albumen) and egg yolk. The egg yolk is located in center of the egg and is surrounded by the egg white or albumen, which is then enclosed by the eggshell. There is also a layer of eggshell membrane in the interval between the albumen and the eggshell. (Kovacs-Nolan et al., 2005). Eggshell, which includes the shell membrane, makes up 9–11% of the egg, the egg white or albumen makes up 60–63%, and the egg yolk about 28–29% (Table 17.1).

17.3.1 The eggshell

The eggshell, which is the non-edible portion of the egg and makes up 9–11% of the total weight of the egg, has a polycrystalline structure that includes a natural porous cuticle layer, a calcite layer, and two shell membranes. It contains 7,000–17,000 pore canals per egg for exchange of gases. The eggshell is composed of two main structures:

Table 17.1 Approximate composition of whole egg, eggshell, albumen, and yolk

	Proximate composition, % (w/w)				
Egg component (% of total)	Moisture	Protein	Lipid	Carbohydrate	Ash (minerals)
Whole egg (100%)	66.1	12.8–13.4	10.5–11.8	0.3–1.0	0.8–1.0
Eggshell (9–11%)	1.6	6.2–6.4	0.03	trace	91–92
Albumen (60–63%)	87.6	9.7–10.6	0.03	0.4–0.9	0.5–0.6
Yolk (28–29%)	48.7	15.7–16.6	31.8–35.5	0.2–1.0	1.1

Source: Adapted from Li-Chan and Kim (2008).

the external inorganic mineral matrix (eggshell *per se*) and the internal organic portion (shell membranes). The eggshell is made up of mostly calcium carbonate (98.4%), as well as minor amounts of magnesium carbonate and calcium phosphate (both ~0.8%). The eggshell also consists of ~6.4% protein. These proteins are found as protein fibers and are interwoven with the calcium carbonate crystals. They serve an important role in the mineralization process and in providing structure to the egg shell. The two shell membranes are found between the inner surface of the shell and the albumen. The membrane proteins are primarily protein–polysaccharide fibers and keratin. Their entangled threadlike structures are important in providing protection to the interior of the egg from microbial contamination.

The color of the egg shell is directly influenced by the breed of the hen. Approximately 50% of eggs produced in the world are brown and 50% are white (IEC, 2012).

17.3.2 Egg white proteins

The egg white, or albumen, makes up approximately 63% of the whole egg. Albumen is actually opalescent in raw egg, but becomes white on heating or beating. Egg albumen is made up of four alternating distinct layers of thick and thin consistencies. About 23.3% of albumen is made up of a thin outer layer attached to the shell's inner membrane, whereas the majority of albumen (57.3%) is composed of a viscous or thick white layer. Around 16.8% of albumen is constituted by the next inner thin white layer, and 2.7% of is composed of a chalaziferous layer (Burley and Vadehra, 1989; Li-Chan and Kim, 2008). The viscosity of thick albumen is much higher than that of thin albumen because of its high content of ovomucin. In fresh eggs, thick albumen covers the inner thin albumen and the chalaziferous layer, holding the egg yolk in the center of the egg. Egg white proteins have an important dietary role because of their high biological value. They also have important functional properties in foods.

Egg white has over 40 different proteins, some of which are still uncharacterized; however, the main proteins of egg albumen include ovalbumin (54% of dry matter), ovotransferrin (or conalbumin) (12–13%), ovomucoid (11%), lysozyme (3.4–3.5%), G_2 and G_3 ovoglobulins (2%), and ovomucin (1.5–3%). The physicochemical and biological functions of these proteins are presented in Table 17.2. Other egg white proteins, including ovostatin, ovoflavoproteins, avidin, and also enzymes (α-mannosidase, β-galactosidase, β-glucuronidase, β-*N*-acetylglucosaminidase, catalase, mono- and diphosphoesterases, peptidases, and α-amylase) (Vadehra and Nath, 1973; Li-Chan and Nakai, 1989).

17.3.2.1 Ovalbumin Ovalbumin (OVA) is the most abundant constituent of egg white proteins. It is a globular phosphoglycoprotein with a molecular mass of 45 kDa isoelectric point (pI) of 4.5. It is composed of 386 amino acid residues and constitutes more than half of the egg white proteins (54% w/w). (Nisbet et al., 1981). It belongs to the serpin (**ser**ine **p**rotease **in**hibitor) superfamily; however, it lacks inhibitory activity. In spite of a number of investigations, its biological function remains largely unknown (Huntington and Stein, 2001) although it has been suggested to be a storage protein. OVA contains one carbohydrate unit, up to two phosphoserine residues. Fothergill's group (1970) reported that

Table 17.2 Physicochemical properties of proteins found in egg white

Protein	% of albumen proteins	Isoelectric point	Molecular weight, kDa
Ovalbumin	54	4.5	45
Ovalbumin Y		(5.1–5.3)	(42.4)
		(5.3–5.5)	(53.4–54.3)
Ovotransferrin	12	6.1	76
		(6.2–6.7)	(85–75)
Ovomucoid	11	4.1	28
		(5.0–5.3)	(37.2–43.1)
Ovomucin	3.5	4.5–5.0	5500–8300
Lysozyme	3.4	10.7	14.3 (15)
Ovoglobulin		(6.1–5.3)	
G_2 globulin	4.0	5.5	30–45
G_3 globulin	4.0	4.8	nd[a]
Ovoinhibitor	1.5	5.1	49
		(6.2–6.4)	(69.5–63.6)
Ovoglycoprotein	1.0	3.9	24.4
		(5.0–5.4)	(37.2–43.1)
Ovoflavoprotein	0.8	4.0	32
		(5.0–5.2)	(37.4–40)
Ovomacroglobulin	0.5	4.5	769
Cystatin	0.05	5.1	12.7
		(6.1)	(17)
Avidin	0.05	10	68.3
TENP[b]	nd[a]	(5.9–6.3)	(48.9–50.2)
Clusterin	nd[a]	(6.1–6.6)	(33–32.4)
Ch21[c]	nd[a]	(5.7)	(21)
VMO-1[d]	nd[a]	nd[a]	(17.6)

Source: Adapted from Li-Chan and Kim (2008).
[a]nd = not determined.
[b]protein with strong homology with bacterial permeability increasing protein.
[c]quiescence-specific protein or extract fatty acid binding protein.
[d]vitelline membrane outer layer protein 1.

OVA contained six cysteine residues, two of them involved in disulfide bond formation and the other four are found as free sulfhydryl (SH) groups. The amino acid sequence of OVA has been determined (Nisbet et al., 1981), and a 3-D configuration has been proposed by Stein and others (1991) on the basis of X-ray crystallography at 1.95 Å resolution, indicating three β-sheets and nine α-helices (Stein et al., 1991). Half of OVA's residues are hydrophobic and one-third is represented by charged residues, of which the majority is acidic, conferring to the protein as acidic isoelectric point (pI) of 4.5 (Li-Chan et al., 1995). The denaturation temperature of OVA is close to 84°C. OVA is the main constituent responsible for the gelling properties of egg white (Mine, 1995).

Purification of OVA is usually carried out using precipitation under specific conditions of pH and salts concentrations (e.g., saturated ammonium sulfate), followed by ion exchange chromatography (Croguennec et al., 2000). In its native form, OVA has been

reported to be resistant to trypsin digestion, but become susceptible to its action after heat denaturation or acid treatment (Ottesen and Wallevik, 1968).

As result of denaturation, OVA can be transformed into a more heat-stable *S*-OVA through an intermediate state (Lechevalier et al., 2007). High pH and temperature both have been reported to contribute to increasing the rate of conversion of OVA to *S*-OVA. The appearance of *S*-OVA can also be enhanced by storage time of eggs; which has been also associated with the loss of its nutritional value (Huang et al., 2012). The conversion of OVA to *S*-OVA during storage has been attributed to increase in pH because of the release of carbon dioxide through the eggshell. The *S*-OVA conversion may reach 81% in an egg after 6 months storage at low temperatures (Vadehra and Nath, 1973). About 2–5% loss of α-helices and increase of antiparallel β-sheet were attributed to the conformational changes in *S*-OVA (Huntington and Stein, 2001). Transformation to *S*-OVA could be a serious problem in food processing, because egg white that contains large amount of *S*-albumin does not produce heat induced gels with good strength. Recently, Huang et al. (2011) reported that *S*-OVA can be used as an indicator to estimate egg freshness.

It is important to note that OVA is one of the important egg allergens (Mine and Yang, 2007).

17.3.2.2 Ovotransferrin Ovotransferrin (OVT), formerly known as conalbumin, is a 686 amino acid-glycoprotein that accounts for 12% of the egg white proteins. It has a molecular mass of 77–80 kDa and pI of 6.0. It belongs to the transferrin family, a class of proteins characterized by their strong capacity to reversibly bind ferric (Fe^{3+}) ions. OVT is folded to form two lobes. Each lobe consists of two distinct alpha/beta domains of similar size with a single binding site, as well as 15 disulfide bridges and no free SH groups. The disulfide crosslinks provide stability to the tertiary structure of the protein (Li-Chan and Kim, 2008; Superti et al., 2007). The iron-binding ability of OVT provides for bacteriocidial and bacteriostatic properties of this protein. OVT has been shown to have antimicrobial activity against various Gram-negative, Gram-positive bacteria, fungi, and viruses (Williams, 1982). Iron binding ability and thus antimicrobial activity of OVT is pH dependent. It decreases rapidly below pH 6.0. OVT is more heat labile and less susceptible to surface denaturation than OVA. It's use as a nutritional ingredient in iron-fortified products has been suggested (Superti et al., 2007).

It is important to note OVT is also a significant egg allergen (Walsh et al., 1988, 2005).

17.3.2.3 Ovomucoid Ovomucoid (OVM) is a heat-stable glycoprotein of 186 amino acids with an approximate molecular mass of 28 kDa and contains 20–25% of carbohydrates attached to the polypeptide chain at asparaginyl residues (Kato et al., 1987c; Li-Chan and Nakai, 1989). It also contains 0.4–4% sialic acid. OVM is unique due to its resistance to heat coagulation. OVM represents 11% of total egg white proteins and belongs to the Kazal family of protease inhibitors (Li-Chan and Nakai, 1989). It comprises three homologous domains (domains I, II and III) cross-linked by intradomain disulfide bridges, with a total of nine disulfide bonds and no free sulfhydryl groups. OVM contains six potential glycosylation sites (recognition sequence Asn-X-Thr/Ser), but only five of them are glycosylated (Réhault, 2007). The fifth Asn residue in ovomucoid domain III can be either glycosylated or unglycosylated (Li-Chan and Nakai, 1989). It is characterized

by a high resistance to heat- and urea-8M-induced denaturation under acidic conditions, as opposed to its sensitivity to heat denaturation in alkaline environment (Deutsch and Morton, 1956, 1961; Li-Chan and Nakai, 1989). OVM is a trypsin inhibitor. The active site is only found in domain II, which is attributed to inhibiting trypsin activity in the chicken egg white (Li-Chan and Nakai, 1989). Other protease inhibitors found in egg white include ovostatin, ovoinhibitor, and cystatin.

Of clinical importance, ovomucoid is the primary egg allergen in humans (Rupa and Mine, 2006; Rupa et al., 2007).

17.3.2.4 Lysozyme Hen egg white lysozyme (HEL), previously referred to as ovoglobulin G_1, is a highly basic protein of 129 amino acids and a molecular mass of 14.3–14.6 kDa. It makes up approximately 3.4% of egg white proteins. HEL is also known as *N*-acetylmuramoyl hydrolase, it is a small enzyme capable of hydrolyzing the β-1,4-linkage between muramic acid and *N*-acetyl glucosamine of mucopolysaccharides present in the bacterial cell wall of especially Gram-positive bacteria (Li-Chan and Nakai, 1989). HEL has four disulfide linkages and no free SH groups. It has basic character with an isoelectric point (pI) of 10.7, which is much higher than other albumen proteins. Because of its basic character, lysozyme can bind to ovomucin, OVA and OVT (Li-Chan and Kim, 2008). Antibacterial activity of HEL has led to its widespread use as a preservative agent in the food industry. It is also incorporated into pharmaceuticals, and has medical applications. HEL remains one of the most widely investigated globular proteins for its structural and antigenic properties.

HEL has also been documented as an important egg allergen (Walsh et al., 1988, 2005).

17.3.2.5 Ovomucin Ovomucin is a sulfated glycoprotein with a very high molecular weight. It accounts for 2–4% of egg white proteins. Ovomucin is composed of two subunits differing in their carbohydrate contents and solubility. (Li-Chan and Kim, 2008). Carbohydrate poor and less soluble α-ovomucin has 2087 amino acid residues and a molecular mass of 230–250 kDa (Watanabe et al., 2004). The carbohydrate rich and more soluble β-ovomucin consists of 872 amino acids and has a molecular mass of about 400–720 kDa (Hammershøj et al., 2008; Hiidenhovi, 2007; Itoh et al., 1987). Insoluble ovomucin plays a key role in forming the gel-like insoluble fraction of the thick albumen in egg white, whereas soluble ovomucin is mainly distributed in the outer and inner albumen (Burley and Vadehra, 1989; Li-Chan and Kim, 2008). In foods where egg albumen is used as an ingredient, ovomucin contributes to foaming properties and stability of the foam.

Ovomucin is usually insoluble at neutral pH in non-denaturating solvents; unless it is solubilized by mechanical treatments such as homogenization and sonication in mild alkaline conditions, or chemical treatments including denaturing solvents and reducing agents (Kato et al., 1985). Earlier studies have shown that ovomucin could be dissociated into smaller subunits by various treatments without any degradation of its chemical composition (Hayakawa and Sato, 1976). Today ovomucin is still one of the less-defined egg proteins because of difficulties in its purification owing to its large size and poor solubility after isolation.

17.3.2.6 Ovoglobulin G_2 and G_3 Earlier studies documented the presence of three ovoglobulins, G_1, G_2, and G_3 in egg white (Longsworth et al., 1940). G1 globulin was later identified as HEL. Ovoglobulins G_2 and G_3 have molecular weights of 36 and 45 kDa, respectively. These proteins resemble albumen in that they are coagulated by heat and are soluble in mild saline solutions (Messier, 1991). They each represent 4% of egg white protein content (Li-Chan and Nakai, 1989); however, few studies have been conducted to thoroughly characterize them. Reports of their importance in the foaming properties of egg white (Sugino et al., 1997) has triggered new attention, which is discussed in later sections on functional properties.

17.3.2.7 Minor egg white proteins There are over 40 proteins that make up egg white proteins (Mine, 2002). In spite of many efforts to develop efficient separation and purification procedures (Desert et al., 2001; Guérin-Dubiard et al., 2005), the full characterization of all the hen egg white proteins, especially the ones found in minor amounts, still remains to be completed. The presence of a mixture of major and minor proteins with a wide range of molecular weights requires high-resolution procedures.

Some of the better known minor egg white proteins include avidin, cystatin, and ovoflavoprotein. Avidin is a basic protein that strongly binds biotin (α–B complex vitamin). Although it is found in trace (0.05%) amounts in egg white, it has been of interest because it has been used in immunoassays and diagnostic tests. Cystatin has been shown to have antiviral activity. Ovoflavoprotein has been shown to bind riboflavin (also α–B complex vitamin).

17.3.3 Egg yolk proteins

Egg yolk makes up 28–29% of the whole egg and consists of 52–53% of dry matter, of which proteins represent about one-third (31%), lipids about two-third (65%) and the remaining being carbohydrates, vitamins and minerals (Li-Chan et al., 1995).

Egg yolk represents a natural oil-in-water emulsion made of lipid protein particles (granules) in suspension in a clear yellow fluid (plasma). Granules are made up of insoluble protein aggregates, whereas the plasma fraction contains low-density lipoproteins (LDL) and soluble proteins. Granule and plasma fractions can be separated by mild centrifugation (10,000 g for 30 min) (Anton, 2013). Egg yolk plasma has higher content of lipids, whereas granules contain approximately three times more protein than plasma (Li-Chan et al., 1995). Figure 17.1 summarizes the main components of egg yolk and Table 17.3 summarizes fresh yolk composition.

Egg yolk granules are circular complexes approximately 0.3 μm–2 μm in size. They are made up of high-density lipoprotein (HDL, 60%) and phosvitin (16%) linked together by phosphocalcic bridges, and low-density lipoproteins (LDL, 12%). Yolk plasma is extremely rich in low-density lipoproteins (LDL, 85%) but also contains livetins (15%) (Anton, 2007a).

17.3.3.1 Low-density lipoproteins (LDL) Low-density lipoproteins (LDL) are micellar structures mainly found in yolk plasma, with a small proportion present in the yolk

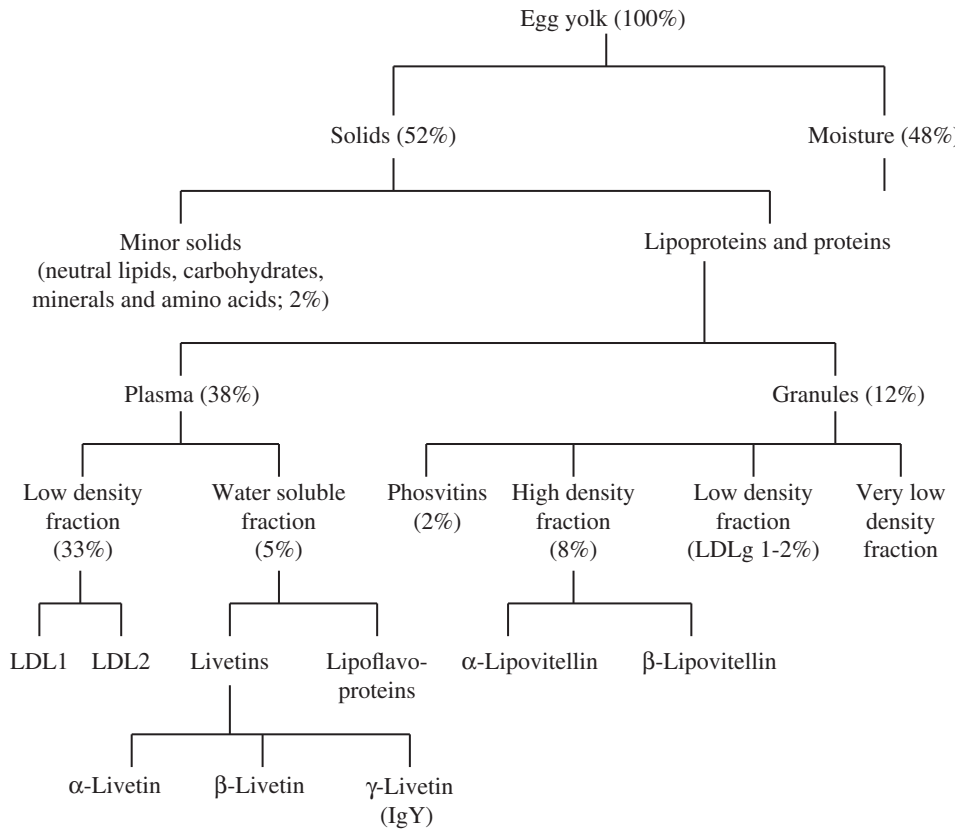

Figure 17.1 Composition of hen egg yolk. Adapted from Kovacs-Nolan et al., 2005

granules (LDLg,) and are also commonly known as the plasma fraction. The LDLs are composed of about 14% protein and 86% lipid, which include 74% neutral lipid and 26% phospholipid (Martin et al., 1964). LDLs consist of apoproteins and phospholipids; they have amphiphilic properties and can be dispersed at the oil–water (O/W) interface; thus, LDLs are the essential components responsible for the emulsifying properties of egg yolk. There are six types of apoproteins in the LDLs of egg yolk with molecular weights between 15 and 130 kDa and a pI range of 6.3–7.5. Apoprotein I constitutes about 70% of total apoproteins and is less soluble in water. Apoprotein II contains a high proportion of amphipathic α-helix chains that contribute to dispersion at the O/W interface (Anton et al., 2003). The remaining apoproteins are in less significant amounts. Very low-density lipoproteins (VLDL)s are precursors of egg yolk LDL and are transferred from the hen blood into the ovary.

17.3.3.2 High-density lipoproteins (HDL) High-density lipoproteins (HDL) consist of α- and β-lipovitellins, which differ in their amino acid composition as well as bound

Table 17.3 Proteins and lipids in egg yolk

Constituent	Major components	Relative %
Proteins	Apovitellenin I–VI	37.3
	Lipovitellin apoproteins	
	α-Lipovitellin	26.7
	β-Lipovitellin	13.3
	Livetins	
	α-Livetin (serum albumin)	2.7
	β-Livetin (α_2 glycoprotein)	4.0
	γ-Livetin (γ-globulin)	2.7
	Phosvitin	13.3
	Biotin-binding protein	trace
Lipids	Triglyceride	65
	Phosphatidylcholine	26
	Phosphatidylethanolamine	3.8
	Lysophosphatidylcholine	0.6
	Cholesterol	4
	Sphingomyelin	0.6

Source: Adapted from Li-Chan and Kim (2008).

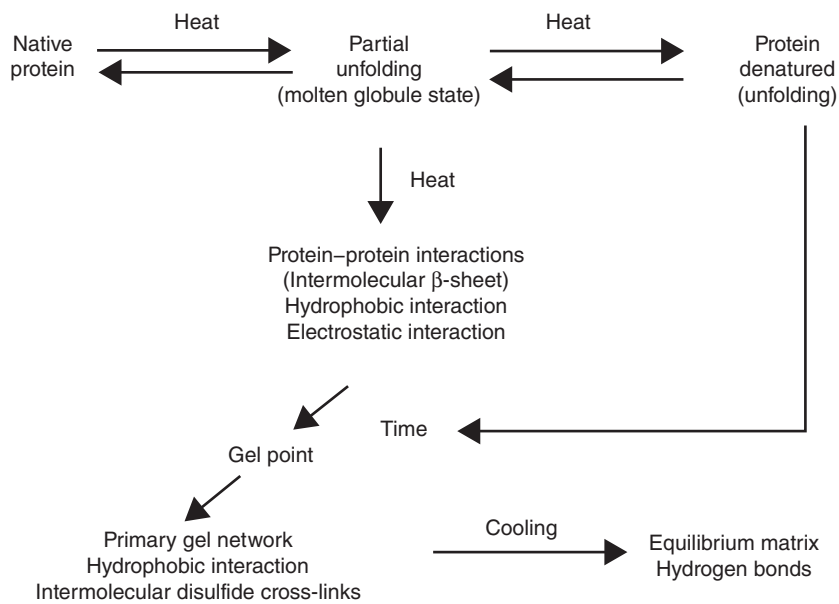

Figure 17.2 Schematic representation of heat-induced gelation of egg white proteins. Adapted from Mine, 1995

phosphorus and carbohydrate content (Li-Chan and Kim, 2008). Lipovitellins are composed of about 80% proteins and 20% lipids, divided into phospholipids (60%, primarily lecithins) and triacylglycerols (40%). They also contain small amounts of cholesterol, sphingomyelin, and other lipids (Burley and Vadehra, 1989). Unlike LDLs, HDLs do not have a micellar structure, but resemble globular proteins by forming pseudomolecular complexes of two monomers (Anton, 2007b). Both α- and β-lipovitellins are glycoconjugates featuring mannose, galactose, glucosamine, and sialic acid; however, α-lipovitellin contains much higher sialic acid content than does β-lipovitellin, explaining its relatively acidic nature. The apoprotein present in lipovitellins, sometimes is referred to as *vitellin*, is present as a dimer and the delipidation of lipovitellin was reported to result in a loss of its solubility (Juneja and Kim, 1997). Five distinct apoproteins have been accounted in the structure of HDL. Five disulfide bridges as well as a number of ionic and hydrophobic interactions are responsible for the maintenance of HDL structure. Complexes formed between HDL and phosvitin via phosphocalcic bridges constitute the basic element of egg yolk granules (Anton, 2007b). At low ionic strength, HDL–phosvitin can form insoluble complexes rendering the granules structure very compact, weakly accessible to enzymatic digestion, and resistant to thermal denaturation or heat gelation (Anton, 2007b).

17.3.3.3 Phosvitin Phosvitin represents about 11% of egg yolk total proteins (Anton et al., 2007) and is a glycoprotein that consists of two polypeptides (α- and β-phosvitins). It is composed of 10% phosphorus, which makes it one of the most highly phosphorylated proteins in nature. Phosvitin is a strong chelator, a natural metal-binding biomolecule, thought to be the strongest metal-binding biomolecules in nature. Almost half of the amino acids in phosvitin are serine residues. The 90% phosphorylation, provide a central hydrophilic area, which is surrounded by two hydrophobic areas at the N- and C-termini. Two subtypes of phosvitins, α- and β-phosvitin, are found in chicken egg yolk and account for 80% of the phosphorus-binding proteins in yolk (Li-Chan and Kim, 2008). The higher content of phosphoserine residues and special conformational structure of phosvitin contribute to its resistance to heat denaturation and proteolytic cleavage (Anton et al., 2000; Juneja and Kim, 1997).

17.3.3.4 Livetins The livetin fraction of egg yolk contains water-soluble proteins that account for 30% of the plasma proteins. It is composed of α-livetin (serum albumin), β-livetin (α_2-glycoprotein), and γ-livetin (IgY) occurring in the ratio 2:5:3, respectively (Schade and Chacana, 2007; Li-Chan and Kim, 2008). The α-livetin has a molecular mass of 70 kDa and a pI value between 4.3 and 5.7 and has allergenic activity to induce type I hypersensitivity (Williams, 1962; Schade and Chacana, 2007). The β-livetin has a molecular weight of 45 kDa and contains 7% hexose. The molecular weight of γ-livetin is approximately 167 kDa. Egg yolk α-livetin and chicken serum albumin were reported to be identical (Schade and Chacana, 2007). Information on β-livetin remains scarce. The γ-livetins or γ-globulins in yolk are referred to as immunoglobulin Y (IgY) to distinguish them from mammalian IgG. Immunoglobulins Y have been the object of intensive investigations for the production of polyclonal antibodies (Behn et al., 2001) and as a means of passive immunization (Kovacs-Nolan and Mine, 2004, 2012).

17.3.3.5 Other egg yolk proteins A number of enzymes including cholinesterase, acid phosphatase, acid proteases, amylase, cathepsin-D, and peptidase, as well as sialylglycopeptides have also been reported in egg yolk (Li-Chan and Kim, 2008).

17.4 Functional properties of egg proteins

In food systems, the term "functionality" has been defined as "any property aside from nutritional attributes that influences an ingredient's usefulness in foods" (Kinsella, 1976; Pour-El, 1981; Boye et al., 1997; Culbertson, 2012). The functional properties of a protein are primarily related to their physical, chemical, and conformational characteristics, such as size, shape, amino acid composition and sequence, net charge, and charge distribution. These parameters will in turn determine their hydrophilicity *vs.* hydrophobicity, the architecture of their secondary (e.g., α-helix, β-sheet, and random structures), tertiary and quaternary structures, the presence of inter- or intrasubunit bonds (e.g., disulfide crosslinks), as well as their flexibility on exposure to environmental changes (Damodaran, 1997a), and ultimately reflect the nature of their functional properties.

Understanding of the unique functional properties of egg components has important consequences on the consumption of eggs and their targeted use in the food industry. The three most acknowledged functions imparted by eggs are their gelation (e.g., cakes and quiches), their foaming properties (e.g., baked goods and meringues), and their emulsifying components (e.g., batters and mayonnaise), all of which contribute to the textural properties of egg-containing food products (Table 17.4).

17.4.1 Denaturation of egg proteins

The folding of a protein from a linear primary structure to a tertiary or quaternary structure is mainly driven by noncovalent interactions, such as van der Waals, electrostatic, and hydrophobic interactions as well as hydrogen bonding, and in some cases covalent bonds such as disulfide bridges. Under normal conditions of pH and temperature, each polypeptide assumes a specific conformation, referred to its "native" state. It corresponds to a thermodynamically stable and organized status characterized by a minimal free energy

Table 17.4 Functional properties attributed to egg proteins in food systems

Functions	Underlying mechanisms	Examples
Water binding	Hydrogen bonding and ionic hydration	Cakes and bread
Gelation	Water entrapment and immobilization, network formation	Gels, cakes, bakeries
Cohesion, adhesion	Hydrophobic, ionic, and hydrogen bonds	Pasta, baked goods
Emulsification	Adsorption and film formation at interface	Cakes, dressings
Foaming	Adsorption and film formation at interface	Whipped toppings, ice cream, cakes, desserts
Aroma—flavour binding	Hydrophobic bonds, entrapment	Low-fat bakery products, doughnuts

Source: From Stadelman and Cotterill (1973).

(Anfinsen, 1973). The lowest state of free energy usually results from maximal interactions of polar groups with water and minimum interactions of nonpolar groups with water. A number of studies, however, have shown that egg proteins, in particular OVA, could exist in a stable intermediate folded state known as a "molten globule state" (Hirose, 1993; Mine, 1995). "Globule" refers to the native compactness, and "molten" refers to the increased enthalpy and the entropy of transition from the native structure to the new state (Ohgushi and Wada, 1983). The molten globule state, therefore, may be defined as a stable partially folded conformation that can be distinguished from either the native or the fully denatured forms (Mine, 1995).

Denaturation is commonly defined as a process during which a major change is induced in the native structure of a protein, which does not alter the primary amino acid sequence (DeMan, 1999) and usually leads to variations in the protein physicochemical or functional properties. Denaturation can be induced by a variety of physicochemical agents including heat, pH, salts, and surface effects. As will be described in the following paragraphs, the denaturation of a food protein is often a prerequisite in the exhibition of any functional property. The extent to which proteins unfold and the conformation they assume on denaturation affects the functional and nutritional quality of a food system (Boye et al., 1997; Culbertson, 2012).

17.4.2 Gelation

Egg whites or yolks are often used as ingredients to enhance the water-holding capacity of food products or their gel strength. Egg white is often preferred over egg yolk for its gelling ability owing to its lower lipid content. Also, it is colorless and milder in flavor. The textural and rheological properties of many food products such as meringues, angel food cakes are dependent on the heat coagulation or gelation properties of egg proteins, in particular their irreversible heat coagulation. A thermally irreversible gel is a viscoelastic solid formed on heat application, which does not revert to a viscous liquid on reheating.

17.4.2.1 Mechanism of gel formation A gel is a material containing a continuous solid network resulting from the assembly of particles or polymers, and embedded in an aqueous solvent (Smith, 1994; Culbertson, 2012). Gels can be described by their capacity to immobilize liquids (water holding capacity in food products), their macromolecular structure, their texture, and by their rheological properties (Phillips et al., 1994). In case of globular proteins, the formation of a heat-induced gel network classically involves the following: (i) a denaturation process, the extent of which depends on the heating time, the temperature, and the nature of the protein, followed by (ii) an aggregation of the unfolded molecules into high-molecular weight complexes, leading to the formation of a coagulum or a gel depending on the conditions (Raikos et al., 2007).

The terms "coagulation" and "gel" have often been used interchangeably; however, earlier reports have distinguished *coagulum*, resulting from formation of a disorganized protein cluster, from *gel*, which rather results from an ordered polymerization of protein molecules (Hayakawa and Nakai, 1985). Electron microscopy is a powerful technique for

studying the architecture of solid gels (Clark et al., 1981; Tani et al., 1995). Using this technique, studies revealed that a transparent gel was primarily composed of a network of linear aggregates of heat-denatured molecules, whereas, heat-denatured molecules gathered into random agglomerates were found to form a turbid gel or coagulum. The conditions of protein denaturation are determining in the formation of either type of structures; for example, changing pH and ionic strength conditions can form a variety of gel-like structures that range from highly ordered to randomly aggregated structures (Hermansson, 1988; Doi, 1993).

The mechanism of gelation involves the partial unfolding of egg white proteins induced by physical (i.e., heat), mechanical (agitation), or chemical means (acids, salts, or denaturing agents such as urea). Complete denaturation of the protein is usually not recommended for gelation because extensive hydrogen bonding or hydrophobic interactions between unfolded chains may then lead to the formation of insoluble precipitates. Under favorable conditions, the partially unfolded egg proteins are capable of forming complexes that result in formation of a coagulum (in the case of a random interaction between egg proteins) or a gel (formation of a 3-Dnetwork exhibiting certain degree of order) (Doi and Kitabatake, 1997). It is believed that the unfolding of protein molecules leads to the exposure of buried hydrophobic groups and protein–protein hydrophobic interactions are, therefore, the main cause of subsequent aggregation (Campbell et al., 2003). Disulfide and hydrogen bonding, as well as ionic interactions, however, have also been shown to be involved in the cross-linking of aggregates from denatured proteins. Studies investigating the heat-induced aggregation of OVA molecules, have reported the existence of an intermediate conformational state commonly known as "molten globule" (Hirose, 1993; Mine, 1995; Tani et al., 1995). Molten globule conformation state refers to a native-like structure in which conformation of heat-denatured OVA at the secondary structure level is not very different from that of the native molecule, but some of the buried reactive/hydrophobic sites become exposed on heating.

Conditions for the formation of a transparent gel usually fall between a narrow range of pH and ionic strength; however, this range can be broadened using a two-step heating procedure (Kitabatake et al., 1987). Studies show that the initial heating of an OVA solution produces a clear sol under salt-free conditions. When this sol is reheated after mixing with salt, the second heating yields a transparent gel even at high salt concentrations, whereas a turbid gel is formed after only a single heating. The basic unit of a transparent gel in a clear sol was later identified as linear aggregates (Koseki et al., 1989a, 1989b; Doi and Kitabatake, 1989).

The ability of certain proteins to form intermolecular disulfide bonds during heat treatment may be a prerequisite for their coagulation and gelation. Heat treatment can result in cleavage of existing disulfide bond structure or "activation" of buried sulfhydryl groups through unfolding of the protein. These newly formed or activated sulfhydryl groups can form new intermolecular disulfide bonds, essential for the formation of aggregate structures, through a process known as "disulfide–sulfhydryl interchange reactions" (Mine et al., 1991; Mine, 1995). The disulfide-exchange mechanism has been shown to be critical in the formation and stabilization of heat-induced gel structure with globular proteins such as OVA (Mine, 1996).

17.4.2.2 Factors affecting gelation properties of egg proteins Egg white proteins are the main actors in the gelation properties of eggs. The formation of a protein-based gel will be favored on the basis of protein flexibility, such as their ability to denature and give extended chains, as well as the protein ability to form extensive networks by cross-linking (Oakenfull et al., 1997). Earlier investigations on thermally induced changes of major egg proteins have been reviewed in excellent reports (Li-Chan and Nakai, 1989).

Gel strength and cohesiveness are minimal at pH values close to isoelectric point where net charge is minimal, that is, in the range of pH 6–7 in egg white (Woodward, 1990). Egg white OVA has long served as a model to study the process of denaturation in heat-induced gels. Because OVA makes up more than half of the albumen protein content, its behavior dominantly affects the formation of a gel. Research has shown that the properties of heat-induced OVA gels depend on factors such as pH, ionic strength, and protein concentration. They were shown to produce either transparent, opaque, or turbid OVA gels (Hatta et al., 1986). The intermolecular interactions between heat-denatured OVA molecules, which are still in a globular shape, are controlled by both the attractive hydrophobic and repulsive electrostatic interactions. When the electrostatic repulsion is relatively strong and the attractive hydrophobic interaction is restricted, the denatured OVA molecules form soluble linear aggregates; these ordered aggregates look like strings of beads. At high protein concentration, these soluble linear aggregates are cross-linked and form a 3-D gel network. At low protein concentration, the soluble linear aggregates do not form a gel network, but rather a viscous transparent sol; however, when the electrostatic repulsion is repressed either by adjusting the pH too close to the isoelectric point and/or by increasing the ionic strength, the denatured protein molecules aggregate randomly, resulting in a turbid gel or suspension, depending on the protein concentration. Analyses by far-UV circular dichroism (CD) spectrum of heated OVA confirmed this model (Koseki et al., 1989b). Among these gel types, the transparent and opaque/translucent gels exhibit higher gel strength and water-holding capacity (Mine, 1995; Campbell et al., 2003).

OVT was shown capable to form an opaque gel on incubation at near-neutral pH and at room temperature with a thiol reagent, such as 2-mercaptoethanol, or glutathione (Hirose et al., 1986; Oe et al., 1986). The thiol-induced gelation was shown to be pH-dependant, with hard gels formed at range of pH between 7 and 9 only; however, under very acidic or alkaline conditions, hard gels were also formed even in the absence of mercaptoethanol. The gel formation was explained by the cleavage of some disulfide bonds, accompanied by an increase in surface hydrophobicity, and followed by aggregation of the denatured molecules through intermolecular hydrophobic interactions.

Lysozyme does not exhibit significant functional properties in food systems, that is, foaming, gelling, or emulsification; however, because lysozyme is a very basic protein, it easily interacts with other proteins or components in food systems, and may, therefore, significantly influence its textural properties. Studies have indeed shown that the properties of heat-induced OVA gel are affected by the addition of lysozyme (Arntfield and Bernatsky, 1993). These effects were shown to be pH-dependant: the gel network formation of OVA gel was not affected by addition of lysozyme at pH 5.5, whereas network strength was significantly increased at pH 7.0 and 8.5. As with OVT, the reduction of

lysozyme (disulfide bridges) can increases the flexibility of the protein and was shown to further improve its gelling properties (Hayakawa and Nakamura, 1986; Tani et al., 1993). Earlier studies determined that major proteins such as ovomucoid and ovomucin were not heat-coagulable proteins (Johnson and Zabik, 1981b, 1981c).

Yang and Baldwin (1995) have summarized the multiple factors that may influence the coagulation of eggs. The influences include parameters such as temperature, protein concentration, pH conditions, ionic strength, and pH conditions, and some of them have been discussed in the previous paragraphs; however, the effects of combined influences on the gelling properties of egg white proteins have not been widely investigated. This was addressed in a study in which the authors investigated the combined effects of pH, sugar, and monovalent salts on the gelling properties of whole egg, as well as egg white and egg yolk, as separate entities (Raikos et al., 2007). The study determined that NaCl had a more significant inhibiting effect on egg protein gel formation when compared with sugar, as shown by the increase in the thermal transition temperature of egg proteins. Three distinct pH values—acidic pH 2, alkaline pH 8, and pH close to the isoelectric point of numerous egg proteins pH 5—were selected to investigate the influence of protein charge on the egg gelation process. A linear relationship between firmness of heat-induced gels and pH was observed for whole egg and egg yolk gels, while egg white gels exhibited the highest values of gel strength at pH neighboring pI values. At pH 5 and pH 8, addition of sugar (3%) and salt (3%) seemed to produce a synergistic effect and led to formation of stronger gels with whole egg and egg yolk, while egg white gels were firmer in the absence of sugar and salt (Raikos et al., 2007).

Heat-induced gelation of egg yolk has also been reported and represents also an important functional property in the preparation of food products such as creams, cakes and confectioneries (Kiosseoglou, 2003). Because egg yolk does not represent a solution of pure proteins, but rather a dispersion of particles that are LDL micelles and HDL granules, the molecular mechanisms of egg yolk gelation have been more difficult to elucidate; however, it has been documented that the apolipoproteins of LDL micelles appear to dominate the gelation process (Anton et al., 2001; Kiosseoglou, 2003). Other studies tend to suggest that the yolk lipid molecules are also critically involved in gel structure formation (Paraskevopoulou et al., 2000).

17.4.2.3 Chemical and physical modifications of gelation properties The supplementation of eggs with chemical agents such as aluminum sulfate, lactic acid and hydrogen peroxide, has initially been reported (Li-Chan and Nakai, 1989). Subsequently, Kato et al. (1989) reported that the use of heating process on egg products in a dry state could significantly improve their functional properties. These findings revolutionized egg processing methods employed in the food industry and the approach has since flourished in a number of ways to improve the functional properties of egg components; for example, the relationship between protein structure and aggregation, as well as heat-induced gelling properties, of seven dried egg white (DEW) products was investigated (Handa et al., 2001). Strong correlations were found between hydrophobicity, surface –SH groups and average molecular weight of DEW, and physical properties of the gels obtained from DEW products.

These data indicated that controlling the aggregation of DEW proteins in the dry state was crucial to controlling the gelling properties of DEW.

17.4.3 Foaming properties of egg proteins

Foams represent colloidal systems in which air bubbles are dispersed within a liquid or solid continuous phase (Davis and Foegeding, 2007). On application of mechanical forces (by blender or whipping apparatus) or by sparging gas through a protein solution, the egg proteins come in contact with the air–water interface, adsorbed at the interface, and start unfolding. The egg proteins tend to expose their hydrophobic groups to the air phase, while their hydrophilic part remains in contact with the liquid phase (water). A good foaming agent often imparts properties that are similar to that of an emulsifier. The film formed at the air–water interface traps air to form bubbles, and a stable foam occurs (Nakamura and Doi, 2000).

The best foaming agents found in the food industry are food-derived proteins, in particular egg white proteins. The lightness of angel and sponge cakes, foamy omelets, meringue, soufflés, and mousses can be attributed to the foaming properties of egg white. In all of these products, egg white proteins are the main surface-active agents that help in the formation and stabilization of the dispersed gas phase. Recent reviews can be found on the investigations conducted on the foaming properties of egg white (Li-Chan and Nakai, 1989; Murray and Ettelaie, 2004; Lomakina and Mikova, 2006; Murray, 2007).

17.4.3.1 Mechanism of foaming Earlier studies have determined that a good foaming agent was one that has the ability to rapidly adsorb at the air–water interface during whipping or bubbling, unfold and quickly reorient at the interface, to form a stable interfacial film around the air bubbles, which can resist gravitational and mechanical stresses (Johnson and Zabik, 1981a; Mine, 1995; Damodaran and Xu, 1996).

The two most important features defining the quality of foam are its volume and its stability. Foam volume depends on the ability of the foaming agent to adsorb at the interface and rapidly reduce interfacial tension, as well as during the energy input such as whipping, whereas foam stability depends on the ability of the foaming agent to form a stable interfacial film, usually by forming a viscous continuous phase (Damodaran, 1997b).

On whipping, egg white proteins denature at the surface and interact with one another to form a stable, viscoelastic interfacial film (Mine, 2002). Some egg white proteins are commonly associated with carbohydrates. When these glycoproteins adsorb at the surface, the hydrophilic carbohydrate moieties bind to the aqueous phase, thereby increasing viscosity, reducing drainage, and contributing to foam stability. Addition of ingredients such as sugar will also increase viscosity and favor the foam stability, whereas addition of carbohydrates sucrose, lactose, and dextrose is not recommended during the initial phase of whipping, as they inhibit foam formation (Yang and Baldwin, 1995).

During excessive whipping, decreased elasticity occurs around the air bubbles, due to excessive insolubilization of proteins at the interface (Nakamura and Sato, 1964a; Johnson and Zabik, 1981c; Lomakina and Mikova, 2006) and lead to unstable foams. Unless

heated, a protein-based foam will tend to collapse with time. A protein-based foam will mainly collapse due to either (i) lamellae rupture, as the attractive and repulsive forces causes bubbles to coalesce, or (ii) water drainage, in which proteins are removed from the interfacial film, thereby decreasing its strength and causing air bubbles to coalesce (Lomakina and Mikova, 2006).

Foam stability is assured by a multitude of forces, including the viscosity of the liquid phase as well as the electrostatic and steric forces between the proteins. On the other hand, destabilizing forces such as electrostatic attractions or repulsions (in highly charged proteins) and hydrophobic attractions between the molecules, will tend to minimize foam formation and break down the foam (Walstra, 1996; Kristinssen, 2006).

A number of studies pertaining to the interfacial behavior of isolated proteins, such as OVA, have been reported and have been very useful to establish interfacial model of mechanisms; for example, the formation of disulfide linkages during the foam formation and the foaming properties of OVA were investigated in a study (Doi et al., 1989), which concluded that the essential factor to a stable foam formation of OVA was not the disulfide linkages formation, but rather the network formed by noncovalent interactions. The role of disulfide bonding was, however, reported to contribute to the stabilization of the protein structure, by constraining the molecular unfolding and preventing the total exposition of hydrophobic regions (Li-Chan and Nakai, 1991).

Earlier studies determined the importance of protein flexibility on the foaming properties of egg white proteins such as OVA and lysozyme (Kato et al., 1986). With respect to their foaming and whipping properties, egg white proteins have been classified in order of importance as globulins, OVA, OVT, lysozyme, ovomucoid, and ovomucin (Johnson and Zabik, 1981a, 1981c).

Yang and Baldwin (1995) described that the presence of multiple proteins in egg white accounted for its good foaming ability in a way that each protein accomplishes a different function (e.g., globulins contribute to foam formation) whereas ovomucoid and lysozyme contribute to foam stability. More specifically, the authors reported that meringues and egg white cakes could be made out of ovomucins and ovoglobulins alone; nevertheless, the cake mass would collapse during the beating period in the absence of OVA. When OVA is used alone in an angel cake mixture, however, longer beating period was required for foaming and a thicker texture was obtained (MacDonnel et al., 1955).

Ovomucin was considered for a long time as an important component of foam stabilization among egg white components. Earlier studies demonstrated, however, that ovomucin was not sufficient to ensure satisfactory formation of egg white foam (Forsythe and Bergquist, 1951). Subsequent studies reported that the foam stability of egg white was markedly increased by the addition of ovomucin, while its foaming power could be slightly decreased (Nakamura and Sato, 1964a). The authors reported that ovomucin played a major role in foam stability but not in foam formation.

Ovoglobulins were shown to contribute to high viscosity and hence to inhibit the drainage of liquids from the foam (Alleoni, 2006). The strong protein–protein interactions of ovomucins were suggested to be responsible for its viscous nature (Kato et al., 1985). Soluble ovomucin, α-ovomucin, and β-ovomucin were, therefore, compared for their foaming properties (foaming power and foam stability) in relation to their viscosity.

The foaming properties of ovomucin were shown to decrease proportionally to decreases in viscosity (Kato et al., 1985).

Interestingly, it has been reported that egg yolk can also be whipped into stable foam, using an optimum temperature of 72°C. Above that temperature threshold, foam volume drops and leads to protein coagulation, unless the preparation is acidified (acetic acid). This process is often used in the manufacturing of highly stable sauces (Belitz et al., 2004a, 2004b).

17.4.3.2 Factors affecting foam formation Protein concentration, film thickness, ionic strength, pH, temperature, and presence of other components in the food systems, in addition to physical–chemical properties of proteins, are all parameters affecting foaming properties; for example, it is well known that any cross-contamination of egg white with egg yolk lipids greatly reduces foaming ability (Kim and Setser, 1982; Wang and Wang, 2009). In addition, the increase of the protein concentration generally causes the formation of a thick lamellar film, which yields more stable foam (Phillips et al., 1994; Hammershøj and Qvist, 2001).

The quality of the foam is also a function of the initial quality of the egg albumen (i.e., ratio of thick *vs.* thin albumen), storage conditions, age of the eggs, and the hen genetic background. The importance of the hen's genetic strain, the age of the egg, albumen height on whipping volume have been investigated. Hammershøj and Qvist (2001) reported that the foam overrun of thin albumen significantly decreased with the hen's age, whereas the foam overrun of thick albumen was not significantly affected. Whipping volume and albumen height were negatively correlated during increased storage time (Silversides and Budgell, 2004). Similarly, Alleoni and Antunes (2004) observed that increasing content of *S*-OVA (during storage) yielded increased volume of drained liquid from the egg white foam and decreased foam stability.

Structure–function relationship between protein conformational changes and foam properties were explored. Foaming properties were reported to be primarily affected by (i) surface hydrophobicity of the protein: increased surface hydrophobicity usually results in better foam ability, (ii) protein charge density and charge distribution: excessive charge leads to excessive repulsion and, therefore, poor foam stability, and (3) protein flexibility: increased flexibility leads to more rapid foam formation (Damodaran, 1997b). These physiochemical properties are themselves highly influenced by the environmental conditions to which the proteins are exposed. Indeed the same parameters that determine the structure and flexibility of a protein (e.g., electrostatic, hydrophobic interactions, and the disulfide linkages) will also determine the interfacial behavior of the protein (Phillips et al., 1994).

The surface activity of a protein has been assessed on the basis of the irreversibility of its adsorption, and its resistance to displacement from the interface by other surface-active proteins/peptides or low molecular mass surfactants (Halling, 1981).

Pasteurization of egg white results in longer whipping time to attain foam comparable to the foam from unpasteurized albumen with regard to specific gravity. This was attributed to the irreversible denaturation of the ovomucin–lysozyme network (Lomakina and Mikova, 2006). A solution to increase the denaturation temperature of egg white

protein network and to maintain their foaming properties was the addition of metallic ions (Fe, Cu, Al, or other), and salts of phosphoric and citric acids upon pasteurisation (Hatta et al., 1997).

Kato et al. (1994) reported that the heating of egg white in a dry state (7.5% moisture, at 80°C for 10 days) could improve its foaming power and foam stability by fourfold without any loss of solubility. Analyses by the same author showed that an increase in molecular flexibility and surface hydrophobicity could explain the faster unfolding and increased intermolecular interaction, contributing to the formation of a strong cohesive film (Kato et al., 1990a).

Studies have explored the behavioural changes of egg foams under various pH conditions. Earlier investigations had reported that addition of minute amounts of 1N H_2SO_4 or NaOH to liquid egg white could alter its foaming properties (Nakamura and Sato, 1964b). A number of studies have recently demonstrated that pH variations could induce moderate unfolding and refolding regime, which could significantly improve the foaming properties of egg white (i.e., foaming capacity, stability, and rheological properties) (Liang and Kristinsson, 2005; Mleko et al., 2007). The improvements in foaming properties were attributed to the partial unfolding of egg albumen proteins (before foaming), as well as the interactions between egg albumen proteins through disulfide and/or hydrophobic groups (Liang and Kristinsson, 2005). The same authors reported that foaming capacity of egg albumen was high near the isoelectric points of its major proteins (pH 4–5) and decreased as the pH went up. They recently reported that high quality foams were typically produced at pH values ranging from 4 to 5 and from 8 to 9 (Kristinssen, 2006). More specifically, the study suggested that the controlled acid and alkali denaturation of egg white, followed by pH readjustment to renaturing conditions, could significantly improve its foaming properties. The pH conditions used in these latter studies were claimed to allow tailored modifications of egg protein conformations, characterized by both increased hydrophobicity and flexibility (Kristinssen, 2006).

17.4.3.3 Modifications of egg foaming properties To improve their foaming properties, investigations have considered the chemical modification of DEW (Ma et al., 1986). Although succinylation of spray-dried egg white solids significantly reduced both foam ability and foam stability, carboxylation tended to improve the foaming properties (Table 17.5). Effect of enzymatic hydrolysis on the foaming properties of egg white was also explored using papain (Lee and Chen, 2002) or protease–peptone (Phillips et al., 1987; Lomakina and Mikova, 2006).

Similar to the gelation properties of egg, a common approach that has been used to improve the foaming properties of egg proteins has been the use of heat-induced denaturation. Several studies have demonstrated that foaming properties of proteins could be improved when heated either in a dry state (Kato et al., 1981; Kato et al., 1989; Mine, 1997; Gauthier et al., 2001) or in solution, above their denaturation temperature (Zhu and Damodaran, 1994; Du et al., 2002).

The effect of irradiation on the foaming properties of egg white was reviewed (Ma et al., 1994). Irradiation doses of 0.97 kGy on shell eggs did not significantly alter egg white

overrun, but doses of 2.37 and 2.98 kGy did enhance the overrun. Furthermore, increasing doses led to increases in the time for 50% drainage (Table 17.6a). Similarly, foaming properties of spray-dried egg white were significantly improved on irradiation. In contrast, irradiation of frozen egg white led to a reduction in the overrun without affecting foam stability (Table 17.6b).

Table 17.5 Foaming properties of spray-dried egg white solids after succinylation and carboxylation

	Foaming ability (%)	Foam stability[a] (%)
Unmodified	200 ± 10	33 ± 2
Succinylated (24.5%)[b]	145 ± 5	23 ± 2
Succinylated (91.6%)	140 ± 5	20 ± 1
Carboxyl-modified (25.2%)	210 ± 10	35 ± 2
Carboxyl-modified (68.5%)	225 ± 15	37 ± 1

Source: Adapted from Lomakina and Mikova (2006).
[a]foam remaining after 60 min.
[b]modification.

Table 17.6 Foaming properties of albumen upon irradiation treatment of shell eggs and egg products

Shell eggs[a]		
Dosage (kGy) (m^2/g)	Overrun (%)	Time for 50% drainage (min)
0	1146	30
0.97	981	35
2.37	1354	42
2.98	1446	52
SEM[2]	91.3	3.4
Frozen egg white		
Dosage (kGy) (m^2/g)	Overrun (%)	Time for 50% drainage (min)
0	815	40
1	870	35
2.5	779	42
4	666	42
SEM[b]	19.3	1.8
Dosage (kGy) (m^2/g)	Overrun (%)	Time for 50% drainage (min)
0	627	27
2	848	29
5	953	30
8	1105	34
SEM[b]	22.0	0.70

Source: Adapted from Lomakina and Mikova (2006).
[a]Average of two or three determinations.
[b]SEM-standard error of the mean.

Knorr et al. (2004) reported that the combined use of ultrasound and high pressure could efficiently increase the foam ability (percentage overrun) of liquid whole egg, owing to a more even distribution of protein and fat particles. Earlier reports suggested that the addition of metallic ions, in particular copper Cu^{2+}, could improve the foaming properties of egg white. Addition of copper ions to spray-dried egg white submitted to heat treatment (Cotterill et al., 1992; Lomakina and Mikova, 2006) led to increase in foam volume, explained by the protective effect of the metallic ions on the heat denaturation of proteins such as OVT. It was indeed demonstrated that OVT in egg albumen can interact with Cu^{2+} and Fe^{3+} to form OVT–metal complex and result in more stable foams (Nakamura and Doi, 2000).

A comparative study finely investigated the effect of heat denaturation on the interfacial properties of OVA molecules (Croguennec et al., 2007). OVA molecules were heat-denatured in solution (10 g/L, pH 7, NaCl 50 mM) under controlled conditions (5–40 min at 80°C). The application of heat treatment led to a more open structure, exhibiting higher hydrophobicity and increased exposure of sulfhydryl groups when compared to unheated OVA. This more open structure favored a fast adsorption rate of aggregates at the air–water interface, in spite of their larger hydrodynamic size. Establishment of rapid contacts between the aggregates was observed as evidenced by faster increase of both surface pressure and shear elastic constant, thus preventing a premature foam destabilization. Non-heated OVA was slower to developed intermolecular contacts and exhibited, therefore, lower foam stability (Croguennec et al., 2007).

17.4.4 Emulsifying properties of eggs

The exceptional emulsifying properties of egg yolk make it one of the most commonly used ingredients in the food industry. Some essential aspects of yolk functionality with regards to the role of plasma (LDL and livetins) and granules (HDL and phosvitin) in the stabilization of oil-in-water emulsions are described in several reviews (Kiosseoglou, 2003; Anton 2013). Even though, studies have been scarce in the past decade, recent findings have been reported on the structure of interfacial film and their formation and will be presented in this section. Emulsifying properties of egg white proteins will also be discussed.

Fluid emulsions are thermodynamically unstable mixtures of immiscible liquids such as vegetable oil and water (Mangino, 1994) and their formation, therefore, commonly requires the application of energy (i.e. homogenization). On prolonged storage, increased surface energy can lead to a rapid phase separation or coalescence, a phenomenon which can be prevented by the addition of active molecules known as "emulsifiers." An emulsifier usually is an amphiphilic compound containing both a water-soluble moiety and a nonpolar moiety. A good emulsifier has a strong propensity to adsorb at the oil-in-water interface, and form a strong film responsible for the stability of food emulsions (Anton and Gandemer, 1999). Phospholipids (i.e., lecithin) and lipoproteins found in egg yolk are examples of naturally occurring amphiphilic molecules. In fact, egg yolk plasma constituents (LDL and livetins) have been shown to be outstandingly soluble in common

ranges of pH and salt concentrations found in food emulsions (Saari et al., 1964; Sirvente et al., 2007); for instance, mayonnaise represents a typical example of oil-in-water emulsion made of 50–85% edible oil, 5–10% of egg yolk, supplemented by vinegar, salt, and seasonings (Belitz et al., 2004a).

Earlier evidence of the critical role of LDL apoproteins adsorption at interfaces was brought by studies showing that the interfacial tension reaches a minimum at pH values close to the isoelectric region of yolk proteins (Anton and Gandemer, 1999; Mel'nikov, 2002). More recent investigations unambiguously demonstrated that LDL were the main contributors to the emulsifying properties of egg yolk, in particular the protein part of LDL (Martinet et al., 2002; Anton et al., 2003; Martinet et al., 2003; Jolivet et al., 2006). The apoproteins present in LDL have been recently explored with regard to the emulsifying properties of egg yolk (Jolivet et al., 2006). Analyses by liquid chromatography-tandem mass spectrometry (LC/MS–MS) allowed identification of two particular apoproteins (apovitellenin I and apo-B) as major protein component of hen egg yolk LDL.

17.4.4.1 Mechanisms of egg protein emulsion Hypotheses on mechanisms of film formation in food emulsion suggested that the breakdown of lipoproteins at the interface was ensued by the adsorption of apoproteins, and the coalescence of neutral lipids with oil droplets (Mine, 2002).

A common approach to the study of lipid-protein film formation consists in adding lipids into an interfacial film of proteins. The displacement of the protein layer by lipids has been described as the orogenic displacement, literally meaning "mountain generating process" (Mackie et al., 1999). It is described as a three-phase mechanisms: (i) the film compression phase, in which the protein surface area decrease owing to the adsorption of lipids, but is not accompanied by any increase in film thickness; (ii) the second stage occurs when the film is no longer compressible and its thickness increase in order to compensate for the decrease in protein surface area; and (iii) a last phase in which, the protein network breaks down owing to high surface pressure and results in desorption of small molecules or protein aggregates (Dauphas et al., 2007).

We described earlier that the two main components of egg yolk, granule *vs.* yolk, have very different profiles with regard to their composition and their structure (Section 17.3.3.). These differences are also reflected in their emulsifying properties: while yolk plasma exhibit higher emulsifying activity, granules exerted better emulsion stability (Anton and Gandemer, 1997).

Effect of thermal treatment on their functional properties was also investigated. During the production of egg formulations, thermal pasteurization is routinely used to eradicate pathogenic microorganisms (e.g., Salmonella). The main goal is to design thermal procedure with minimal deleterious effects on the functional properties of egg yolk. Le Denmat et al. (1999) have shown that egg yolk granules were more resistant to heat treatment than plasma or whole yolk, as evidenced by polyacrylamide gel electrophoresis under non-denaturing conditions and assessment of their emulsifying properties (creaming rate and final oil volume fraction). The study reported a severe decrease of plasma emulsifying activity after heating at 72°C, whereas the same treatment did not alter that of egg yolk

granules. The substitution of granules for whole yolk in food emulsions has been suggested to address the incomplete eradication of microbial flora and limited shelf life of egg yolk sometimes encountered during food processing.

17.4.4.2 Factors affecting emulsifying properties Similar to gelation properties, the composition of interfacial films and properties of food emulsion formed by egg components largely depend on conditions such as pH, ionic concentration, and protein concentrations. Although egg white proteins have been reported as good emulsifying agents owing to their capacity to behave in a manner similar to surface-active agents (Mine et al., 1991), they do not surpass the emulsifying properties of egg yolk; nevertheless, isolated egg white proteins have served as useful models in the study of food emulsions. Studies sought to clarify the relationships between protein structure and lipid–water interface adsorptivity. Earlier studies have indeed determined the importance of protein surface hydrophobicity and flexibility on the emulsifying properties of egg white proteins such as OVA and lysozyme (Kato et al., 1986). Emulsifying properties of ovomucins were also shown to be dependent on surface hydrophobicity (Kato et al., 1985). Other parameters such as, oil-phase volume and presence of salts (0.2 M NaCl *vs.* 10 mM $CaCl_2$) and protein concentrations (>0.5% under given conditions of pH and salts concentrations) were also deemed to affect the emulsifying properties (emulsion activity and stability) of OVA protein.

Studies on the emulsifying properties of OVA were finely investigated (Mine et al., 1991) and authors reported that pH conditions were critical parameters affecting emulsifying properties. pH variations commonly modify the balance between electrostatic and hydrophobic interactions. Detailed analyses by circular dichroism and fluorescence measurements determined that the structural changes occurring at acidic pH (optimal pH 3.0) were responsible for an enhanced flexibility and surface hydrophobicity of the protein. Optimal pH conditions were also investigated in the formation of egg yolk emulsion. On the basis of the measurements of droplet size, protein solubility, adsorption kinetics, and interfacial protein concentration, authors suggested that egg yolk emulsification be prepared at pH values of 6 rather than pH 3 or more alkaline pH9, and pH conditions be subsequently adjusted if required (Anton and Gandemer, 1999).

To explore the influence of surface charges, the emulsifying properties of phosvitin were investigated on the basis of the chemical (alkaline treatment, calcium ions) and enzymatic (phosphatase) removal or neutralization of phosphate anionic groups (Kato et al., 1987b). The authors demonstrated that these modifications could significantly decrease the emulsifying properties of phosvitin, with a greater impact observed on the emulsion stability rather than the emulsion activity. Using an enzymatic treatment (neuraminidase), the same authors showed earlier that the repulsive forces created by the presence of sialic acid groups could have deleterious effects on the emulsifying properties of ovumucin, whereas foaming properties of ovomucin were shown to be increased by neuraminidase treatment (Kato et al., 1987a).

17.4.4.3 Modification of egg emulsifying properties Earlier studies determined that the emulsifying properties of OVA could be improved by coupling the protein to

dextran (Kato et al., 1990b) or by processes using freeze-drying and spray-drying (Kitabatake et al., 1989).

The potential to use high-pressure treatments (200, 400, and 600 MPa) for the manufacture of more stable oil–water emulsion has recently been investigated (Speroni et al., 2005). Indeed high-pressure treatment has been suggested as an alternative mean to ensure microbial safety in egg-based products since it was reported as a less denaturing and milder approach than thermal treatments. The study showed that under alkaline pH conditions (pH 8 *vs.* pH 3), high-pressure treatment of LDL suspension did not affect their adsorption capacity at the oil–water interface. The high-pressure treatment had, however, led to a significant decrease of depletion and bridging flocculation, explained by enhanced protein aggregation and denaturation. The same authors reported that mechanical treatments such as high-pressure homogenization may have led to preformation of aggregates capable of efficient adsorption at the oil–water interface (Sirvente et al., 2007), as speculated by earlier studies (Aluko and Mine, 1998; Anton et al., 2000). Interestingly, the high-pressure treatment of LDL dispersions combined with alkaline pH (pH 8) was shown to lead to more stable oil–water emulsions. At alkaline pH, aggregation and protein denaturation were shown to be enhanced, without altering the capacity of LDL adsorption at the oil–water interface, as measured by the percentage of adsorbed proteins (Speroni et al., 2005).

17.5 Conclusion

Eggs are one of nature's highest quality sources of protein (in relation to human nutrition), lipids, valuable minerals, carbohydrates, vitamins, and indeed contain many of the key ingredients for life. Eggs are also widely used in the food industry owing to their unique multifunctional properties (e.g., foaming, gelling, and emulsifying). In the last two decades, extensive studies on physicochemical characteristics have been conducted. These applications are being developed to take advantage not only of the nutritional and functional contributions of eggs in food products, but also in terms of the bioactive components that may be used as nutraceutical and functional food ingredients with potential to reduce risk of disease and to enhance human health. The better understanding of structure–function relationships of egg proteins enable us for tailoring egg protein functionalities by various method and to improve texture, food quality and develop more egg-related food products for adding value to food system.

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