



# Resistant Starch

Sources, Applications and Health Benefits

Yong-Cheng Shi and  
Clodualdo C. Maningat

*EDITORS*

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# Resistant Starch



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# Resistant Starch

## Sources, Applications and Health Benefits

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*To my wife Lei and my son Gary – YCS*

*To my wife Josie, my daughter Barbara and my sister Susan – CCM*



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# Preface

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Since the term ‘dietary fibre’ was first coined in 1953, it has undergone several transformations with respect to its definition, composition, analytical methodology and physiological effects. Its heterogeneous composition of naturally-occurring non-starch polysaccharides, lignin and associated substances has grown to include other synthetic or novel fibres, comprising digestion-resistant dextrans and resistant starches. Because of this diverse composition, analysts are often confronted with the challenge of accurately quantifying the level of total dietary fibre of food or beverage products. Dietary fibre is now less frequently associated with bulk or regularity and is discussed much more conspicuously with its role in attenuation of glycemic/insulinemic responses, blood cholesterol lowering, satiety effects, weight management, large bowel fermentation and changes in gut microbiota composition and metabolism in regard to their impact on the general health and well-being of consumers.

Consumer demand for fibre-rich foods and beverages in the United States, Europe and Asia-Pacific is rising due primarily to the preponderance of positive epidemiological and scientific data and also an increase in consumer awareness and support from dieticians and nutritionists. Ironically, however, many Americans on average consume only about 50–60% of their recommended daily intake of 25 g of fibre.

Resistant starch (RS), in particular, has captivated leading research scientists and prominent educators, and their investigations have been featured prominently in scientific literature on fibre. Many research activities on RS highlighted its structure, composition, functionality, *in vitro* and *in vivo* studies and performance in food and beverage products. RS has five types or classes and, therefore, it provides diverse materials for research investigators. These, together with the commercial significance of RS, account for the abundance of published articles and inventions in the scientific and patent literature. Commercial sources of RS number around 30 – a substantial increase since the first RS product was introduced to the market in 1993.

The idea of writing this book was developed from the Carbohydrate Division Symposium on resistant starch and health during the 2009 IFT Annual Meeting in Anaheim, California. The symposium attracted speakers who are leading researchers and scientists from the academia and the food industry. In order to capture the important developments in RS, with emphasis on sources, applications and health benefits, the editors embarked on a project to write this book using the symposium papers plus the contribution of invited scientists and academic professionals who excel in this important area of RS.

There are 15 chapters in the book, covering various topics on RS, such as its biosynthesis, types or classes, slowly digestible starch, methodology for measurement and food applications, and also the physiological effects of RS, primarily in the area of glycemic/insulinemic control, appetite/satiety, gut microbiota metabolism and large bowel health. This book caters to a wide audience and can be a valuable resource for students, professors, research scientists, product developers and other food industry professionals, as they investigate the ever-growing area of RS and its diverse properties, numerous food and beverage applications, commercial significance and physiological effects.

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Dr. Shi received his B.S. in Chemical Engineering from Zhejiang University (Hangzhou, China) and his M.S. and Ph.D. in Grain Science from Kansas State University (Manhattan, Kansas). He is a professional member of the American Association of Cereal Chemists International and Institute of Food Technologists. He is an associate editor for *Cereal Chemistry* and a member of Advisory Board for *Starch* and *Food Digestion* journals.

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benefits of grain-derived ingredients; and research alliances with scientists and product developers in the food industry, government and academia.

Dr. Maningat received his B.S. in Chemistry from Adamson University (Manila, Philippines), his M.S. in Agricultural Chemistry from the University of the Philippines at Los Banos (Laguna, Philippines) and his Ph.D. in Grain Science from Kansas State University (Manhattan, Kansas). He is a professional member of the American Association of Cereal Chemists International, Institute of Food Technologists, American Society of Baking and American Chemical Society.

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# 1 Starch Biosynthesis in Relation to Resistant Starch

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## 1.1 INTRODUCTION

### 1.1.1 Starch components

Starch is present in amyloplasts as semi-crystalline intracellular water-insoluble granules, with alternating crystalline and amorphous layers. Starch is a glucan homopolymer composed of one-quarter amylose (molecular mass  $10^5$ – $10^6$  Da) and three-quarters amylopectin (molecular mass  $10^7$ – $10^9$  Da), along with traces of lipids (0.1–1.0%) and proteins (0.05–0.5%). Amylose is essentially a linear glucan polymer, composed of  $\alpha$ -1,4 linked glucose residues with a degree of polymerization (dp) ranging between 800 (in maize and wheat) to more than 4500 (in potato) with sparse branching (approximately one branch per 1000 residues) (Morrison & Karkalas, 1990; Alexander, 1995). Structural and functional aspects of these glucan polymers affect starch functionality and its end use.

Amylose chains are capable of forming single or double helices. On the basis of orientation of its fibres in X-ray diffraction studies, amylose can be divided into A- and B-type allomorphs (Galliard *et al.*, 1987). In B-type allomorph, six double helices are packed in an anti-parallel hexagonal mode surrounding the central water channel (36 H<sub>2</sub>O per unit cell). In A-type, the central water channel is replaced by another double helix, making the structure more compact. In this allomorph, only eight molecules of water per unit cell are inserted between the double helices (Galliard *et al.*, 1987).

Amylopectin is a highly branched glucan polymer, in which  $\alpha$ -1,4 linked glucose residues are interspersed with  $\alpha$ -1,6-glucosidic linkages (4–5%)

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which introduce branches, and a degree of polymerization ranging from  $10^5$ – $10^7$  glucose units (Myers *et al.*, 2000). Chain lengths of 20–25 glucose units between branch points are typical. The branches in the amylopectin molecule are arranged in clusters (Buléon *et al.*, 1998).

An amylopectin molecule typically consists of three types of chains, which are either located within a single cluster or connect two or more clusters (Hizukuri *et al.*, 1986; Thompson *et al.*, 2000). In amylopectin, only the C-chain has a reducing end oriented towards the centre or hilum of the granule. Attached to the C-chain with  $\alpha$ -1,6 linkages are the B-chains. These can support other B- or A-chains. The A-chains are the outermost chains, which do not support any other chains. A- and B-chains form clusters and B-chains can span and support multiple clusters. A-chains typically consist of 6–12 glucose molecules, while B-chains may contain 13–24 or up to 50 or more glucose molecules, depending on the number of clusters they span. In the section which does not contain  $\alpha$ -1,6 branch points, two neighbouring glucose chains form a double helix, and these double helices form a crystalline pattern. All of these structures are attached by hydrogen bonds. The sections where the branch points of the amylopectin are located are amorphous and contain amylose molecules.

### **1.1.2 Resistant starch**

More than 50% of calorific requirement of human diet is fulfilled by starch-based foods, and the quality and quantity of starch-based food affect overall blood glucose and homeostasis in humans. Starch digestion in humans is initialized by salivary  $\alpha$ -amylases in the oral cavity, followed by pancreatic  $\alpha$ -amylase and the intestinal brush border glucoamylases, maltase-glucoamylase, and sucrase-isomaltase (Nichols *et al.*, 2003). Brush-border enzymes convert the resultant products of digestive process into maltase-glucoamylase and sucrase-isomaltase, which enter the vascular system (Lehmann & Robin, 2007). Based on its *in vitro* enzymatic hydrolysis, the rate of glucose release and its absorption in the gastrointestinal tract, starch is classified as either readily digestible starch (RDS), slowly digestible starch (SDS) or resistant starch (RS) (Englyst *et al.*, 1992).

According to Englyst *et al.* (1992), based on *in vitro* kinetic assay, RDS is broken down into glucose molecules in  $\approx 20$  minutes, while SDS is the fraction which gets digested in  $\approx 100$  minutes. Both RDS and SDS are completely digested in the small intestine. RS is referred to that portion of starch which is not hydrolyzed until about 120 minutes have elapsed. It passes through the small intestine undigested, but is fermented in the large intestine by gut microflora. Physiological benefits of RS include hypoglycaemic effects and production of short chain fatty acids (SCFA) particularly butyrate, which

has been reported to lower lumen pH, making it a less conducive environment for cancer and other diseases (Topping & Clifton, 2001; Wei *et al.*, 2010).

A medium-to-high amount of SDS has been reported for native normal maize starch (Axelsen *et al.*, 1999), waxy starches (Weurding *et al.*, 2001), millet and sorghum (Benmoussa *et al.*, 2006) and legumes (Hoover & Zhou, 2003). A few researchers have reported a higher rate of digestibility for cereal starches than tuber starches such as potato (Fannon *et al.*, 1992; Benmoussa *et al.*, 2006). On the basis of its botanical source, physical or chemical processing, RS can be divided into four types. RS1 is physically inaccessible due to its location in the food, RS2 escapes digestion because of its granular structure, RS3 is retrograded starch and RS4 is chemically modified starch (Brown, 2004).

## 1.2 FACTORS AFFECTING STARCH DIGESTIBILITY

Starch enzymatic hydrolysis and RS are influenced by several factors, both extrinsic and intrinsic properties of starch granules. Extrinsic factors, which include starch granule surface characteristics such as porosity of granule and pit formation between the surface and centre of the granule (Fannon *et al.*, 1992), or exo-corrosion (Gallant *et al.*, 1997), affect starch digestibility. Intrinsic properties of starch granules, such as packing of amorphous and crystalline regions (Gallant *et al.*, 1992; Zhang *et al.*, 2006), or interaction of amylose with other components such as lipids (Crowe *et al.*, 2000), proteins (Escarpa *et al.*, 1997) and/or enzyme inhibitors (Bjorck *et al.*, 1987), also influence starch digestibility. Reduced digestibility of tuber starch granules has been attributed to their large and smooth surface, along with their surface properties.

The amylose to amylopectin ratio is an important determinant of starch digestibility. Amylose and amylopectin have different structural and physiological characteristics and, hence, exhibit different reactions within the body during digestion and subsequent release of glucose molecules for absorption. The amylose to amylopectin ratio is a major determinant for RS2 and RS3 (Sajilata *et al.*, 2006).

A positive correlation exists between amylose concentration and RS formation (Ito *et al.*, 1999). The straight chains of amylose limit the access of small intestine  $\beta$ -amylases to the two terminal glucose units on the amylose chain (besides, two terminal ends may not be accessible due to folding of a polymer). In contrast, the highly branched structure of amylopectin provides multiple terminal end glucose units that  $\beta$ -amylases can access readily.

During cooking, starch is gelatinized and amylose molecules are leached out of the swollen starch granules as coiled polymers which, on cooling, associate as double helices and form hexagonal networks which resist digestion. In waxy starches, instead of this network, aggregate formation occurs, and this is more susceptible to hydrolysis by amylases.

The intensity of starch digestion is also affected by the degree of polymerization and/or branching of glucan polymers, i.e. a reduction in the rate of hydrolysis with increased branching due to steric hindrance (Park & Rollings, 1994). Gamma irradiation-generated rice mutants high in RS showed increased proportion of short chains with  $DP \leq 12$ , decreased proportion of intermediate chains of  $13 \leq DP \leq 36$  and decrease in long chains with  $DP \geq 37$  (Shu *et al.*, 2007).

Another report, by Ao *et al.* (2007), mentions that  $\beta$ -amylase and maltogenic  $\alpha$ -amylase mediated partial reduction of outer branch chains of amylopectin reduces overall starch digestion rate, which was related to an increase in the amount of  $\alpha$ -1,6 linkages and decrease in  $\alpha$ -1,4 linkages. Changes in the amylopectin chain length distributions facilitated retrogradation to produce B- and V- type crystalline structures, leading to more resistant starch. It is generally believed that increased proportion of longer chains makes the starch more resistant to digestion. A possible reason could be that longer chains form longer and more stable helices, which are further stabilized by hydrogen bonds distributed over the entire crystalline region and cause decreased digestibility (Lehmann & Robin, 2007).

### **1.3 STARCH BIOSYNTHESIS**

Plants have a unique ability to capture light energy and to fix carbon dioxide and water to form triose sugars that act as precursor of simple and complex carbohydrates. Photosynthesis in the plants' chloroplast results in the production of triose-phosphates, reducing equivalents and ATP. The triose phosphates are either transported by triose-phosphate transporters to the cytosol, or are converted to phosphorylated compounds, including fructose-6-phosphate in the plastid. During the light period, chloroplasts synthesize transitory starch which, at night, is broken down into constituent sugars and transported to the storage organs. In contrast, in amyloplasts, these precursors are used to synthesize storage starch.

Analogous to chloroplasts in green tissues, storage organs contain amyloplasts which are albino plastids and devoid of internal membrane structure. These specialized plastids act as processing and storage unit for starch in plant cells. Fructose-6-phosphate in chloroplasts is used both for regeneration of



ribulose-1,5-bisphosphate and production of glucose-1-phosphate through glucose-6-phosphate. Conversion of glucose-1-phosphate and ATP to ADP-glucose by ADP-glucose pyrophosphorylase (AGPase) is the first committed step in starch synthesis.

In addition to AGPase, other enzymes involved in the starch (especially amylopectin) biosynthetic cascade include starch synthases (SS), starch branching enzymes (SBE) and debranching enzymes (DBE) (Smith *et al.*, 2001; James *et al.*, 2003; Zeeman *et al.*, 2010). Amylose is synthesized exclusively by granule-bound starch synthase-I (GBSSI). The glucose moiety from ADP-glucose is used to elongate an already existing glucan chain. Starch synthases catalyze the formation of  $\alpha$ -1,4 glucosidic linkage between the glucose units to form a linear chain. SS require a primer for elongation of glucose chain.

The initiation of glucan polymerization reaction is poorly understood. One hypothesis suggests the presence of glycogenin-like self glycosylating protein as primer for amylopectin synthesis and addition of D-glucose occurs to the non-reducing end of a growing glucan chain (Chatterjee *et al.*, 2005). Another hypothesis is the *de novo* synthesis of glucan chains mediated by a two-site insertion mechanism. Two glucose units from ADP-glucose complex with the active site of starch synthase, and are subsequently added to the reducing end of glucan chain (Mukerjee & Robyt, 2005).

Four starch synthase isoforms (SSI, SSII, SSIII, SSIV) play important role in elongating different regions of amylopectin. Therefore, alterations in SS activities would affect the amylopectin fine structure. Branches in amylopectin and amylose are introduced by SBE, which catalyze the cleavage of an  $\alpha$ -1,4 linkage and join the cleaved chain to another glucan chain through  $\alpha$ -1,6 glucosidic linkage. Two classes of SBE (i.e. SBEI and SBEII) exist, which have different substrate specificities.

Finally, debranching enzymes (isoamylase and pullulanase) act to trim the outer branches of amylopectin molecule to form ordered branch structure and packaging of the molecule into starch granules. Since multiple isoforms of starch biosynthetic enzymes exist in the endosperm and have specific functions, mutations in any of these genes would therefore lead to a change in starch content, structure and functional properties.

In addition to the core enzymes, other enzymes, such as phosphorylases, disproportionating enzymes and dikinases (glucan water dikinase, phosphoglucan water dikinase) also play important roles in starch metabolism. Starch phosphorylation involves dikinases such as glucan water dikinase (GWD, mol wt 155 kDa) and phosphoglucan water dikinase (PWD, mol wt 130 kDa), which phosphorylate the C<sub>6</sub> and C<sub>3</sub> positions of glucose units of amylopectin, respectively – an important factor in starch degradation (Fettke *et al.*, 2009).

## 1.4 STARCH BIOSYNTHESIS IN RELATION TO RS

### 1.4.1 ADP-glucose pyrophosphorylase (AGPase)

AGPase catalyzes the synthesis of ADP-glucose from ATP and glucose-1-phosphate. It is the first step in starch biosynthesis, and AGPase is also a key regulatory enzyme in the starch biosynthetic pathway. AGPase consists of two large and two small subunits, which affect allosteric and catalytic properties of the enzyme. Allosteric regulation of this enzyme plays a critical role in determining the amount of starch produced (Hannah & James, 2008). AGPase is allosterically activated by 3-phosphoglyceric acid (3-PGA) and inhibited by inorganic phosphate (Pi) in many plant tissues (Preiss *et al.*, 1996). Genetic and biochemical manipulation of its sensitivity towards Pi resulted in increase in crop productivity (starch yield) due to increased sink strength (Wang *et al.*, 2007; Sakulsingharoj *et al.*, 2004; Smidansky *et al.*, 2002). AGPase activity is also redox regulated (Hendriks *et al.*, 2003).

In general, the active form of AGPase is present in the plastids of mature cereal tissues and sink tissues of non-cereal plants. Developing cereals however, differ, with most of their AGPase activity localized mainly in the cytosol of endosperm cells. Specific transporters/ADP-glucose transporter channels are involved in the trafficking of the resultant ADP-glucose. In non-cereal plants, the sucrose to starch pathway comprises plastid import of hexose phosphates, which can be used in other biosynthetic processes in addition to starch synthesis. In contrast, in cereals, carbon entering the plastid as ADP-glucose is committed to starch synthesis (James *et al.*, 2003).

Mutations in AGPase and ADP-glucose transporters have been shown to affect the total starch content in maize, barley, pea and potato (Hylton *et al.*, 1992; Shannon *et al.*, 1998; Tjaden *et al.*, 1998; Patron *et al.*, 2004). The maize *Shrunken-2* and *Brittle-2* mutants have lesions in the large and small subunits of the cytosolic AGPase, respectively (Hannah & Nelson, 1976). *Shrunken-2* mutant kernels are deeply dented, with floury endosperm that has 25% reduced starch but is sweet due to high sucrose concentration (Hutchinson, 1921). Similarly *Brittle-2* mutant kernel germinates poorly, is dark and shrunken and has 25–34% lower starch than normal (Preiss *et al.*, 1990). A barley mutant, *Risø 16*, is associated with a deletion in the small subunit of cytosolic AGPase resulting in reduced starch concentration and seed weight (Johnson *et al.*, 2003). These changes in starch concentration have not been associated to RS (Table 1.1).

### 1.4.2 Starch synthases (SS)

Starch synthases catalyze the transfer of glucose unit from ADP-glucose to non-reducing end of an already existing glucan chain, thus forming  $\alpha$ -1,4

**Table 1.1** List of known starch biosynthetic mutants with modified starch content and structure in relation to digestibility.

Enzyme	Genus	Mutant	Starch content and structure	Digestibility	Reference
AGPase	<i>Hordeum vulgare</i>	lys5	low starch content	not reported	Patron <i>et al.</i> , 2004
AGPase	<i>Pisum sativum</i>	rb	reduced starch content	not reported	Hyllton and Smith, 1992
AGPase	<i>Solanum tuberosum</i>		≈10% decrease in amylose	not reported	Tjaden <i>et al.</i> , 1998
GBSSI	<i>Hordeum vulgare</i>		↑ amylose, ↑ DP 19–36	↓ hydrolysis	Asare <i>et al.</i> , 2011
GBSSI	<i>Ipomoea batatas</i>	wx	amylose free; lesser short chains	↑ hydrolysis	Noda <i>et al.</i> , 2002
SSI	<i>Oryza sativa</i>		amylose unaffected; ↑DP 6–7, 16–19; ↓DP 8–12	not reported	Fujita <i>et al.</i> , 2006
SSII	<i>Hordeum vulgare</i>	sex6	amylose 65–70%; ↓short chains	↓ hydrolysis	Morell <i>et al.</i> , 2003; Bird <i>et al.</i> , 2004
SSII	<i>Triticum aestivum</i>		↑ amylose 35%; ↓average CLD	resistant starch ↑100 fold	Yamamori <i>et al.</i> , 2000
SSII	<i>Pisum sativum</i>	rug5	↓ B2,B3; ↑ very short and very long chains; ↑ amylose ≈35%	not reported	Craig <i>et al.</i> , 1998
SSIII	<i>Zea mays</i>	dull1	↑apparent amylose; ↓short chains; ↓long B chains	not reported	Gao <i>et al.</i> , 1998
SBEIIa + b	<i>Hordeum vulgare</i>		amylose >65%; ↑DP <9, >15; ↓DP 9–13	↑ resistant starch	Regina <i>et al.</i> , 2010
SBEIIb	<i>Zea mays</i>	ae	↑apparent and amylose content; ↓long chains	↑ resistant starch to ≈40%	Li <i>et al.</i> , 2008
DBE	<i>Oryza sativa</i>	su-1	altered amylopectin CLD; granules shrunken, irregular and compound	not reported	Kubo <i>et al.</i> , 2005

linkage. Cereal endosperms contain at least five SS classes, based on their conserved primary amino acid sequences. SSI and SSII are present mostly in the stroma (Fujita *et al.*, 2006), whereas SSIII and SSIV are present both in the stroma and starch granule (Denyer *et al.*, 1995; Dai, 2010) and are primarily involved in amylopectin synthesis. GBSSI is bound to starch granules and is required for amylose synthesis. Recently, GBSSI has also been shown to participate in the elongation of amylopectin chains, particularly for very long branches (Yoo & Jane, 2002). The chain elongation pattern differs for each isoform and varies with plant species (Smith *et al.*, 1997). In addition to their specialized functions, some SS overlap in their functional role, while others are unique (Roldán *et al.*, 2007).

#### 1.4.2.1 Granule bound starch synthase-I

GBSSI (also known as waxy protein) present in the interior of starch granule is essential for amylose synthesis. Plants lacking GBSSI enzymatic activity produce starch without amylose, which is also called waxy starch. In wheat, GBSS has two isoforms, GBSSI and GBSSII (Nakamura *et al.*, 1998; Vrinten & Nakamura, 2000). Another isoform, GBSSIIb, exclusive to the pericarp region, has been reported in barley (James *et al.*, 2003). This is involved in transient starch accumulation, which enhances the sink strength of the young caryopsis (Patron *et al.*, 2002).

*In vitro* study using ADP[<sup>14</sup>C] glucose as precursor of starch biosynthesis in isolated starch granules showed uptake of malto-oligosaccharides of DP 2–7 by GBSSI as primers for amylose synthesis (Denyer *et al.*, 1996). GBSSI is also reported to be involved in the elongation of long chains of amylopectin (Yoo & Jane, 2002; Craig *et al.*, 1998). GBSSI elongates the glucan chains which are confined to the semi-crystalline region of the granule and cannot form branches. Consequently, the chains remain linear and are known as amylose, or long-branch chains of amylopectin (Jane *et al.*, 2010).

##### 1.4.2.1.1 Amylose in relation to RS formation

Amylose contributes to the formation of RS2 and RS3. Deficiency of GBSSI activity produces starch made of only amylopectin (waxy starch). Rate of starch digestibility is high in waxy and partially waxy starch (reduced RS) compared to normal starch from several plants (Rooney & Plugfelder, 1986; Bertoft *et al.*, 2000; Li *et al.*, 2004; Chung *et al.*, 2006; Asare *et al.*, 2011). In a recent study on starch structure and *in vitro* enzymatic hydrolysis using barley atypical amylose concentration starch (Table 1.1), Asare *et al.* (2011), using atomic force microscopy, reported high poly-dispersity indices for normal (1.4) and

increased amylose starch genotype (1.25), compared to near (partially) waxy starch genotypes (0.33). They also concluded that energy requirement for gelatinization and hydrolysis of waxy starch is lower than for normal or high-amylose starch. Waxy starches are more susceptible to hydrolytic enzymes compared to starch granules with significant amylose concentration.

Hu *et al.* (2004) investigated three types of rice cultivars with varying amylose content for *in vitro* hydrolysis and glycemic index determination. They concluded quicker, complete and significantly higher rates of starch hydrolysis for waxy and low-amylose rice than for intermediate and high-amylose rice. In a more practical approach for estimating RS contribution for amylose, Hung *et al.* (2005) substituted high-amylose wheat flour for normal wheat flour in bread-making and observed higher RS content in the substituted bread. Physical increase in amylose content through retrogradation and extended cooling after cooking can also lower digestibility (Blazek & Copeland, 2010).

#### 1.4.2.2 Starch synthase-I

In maize, SSI is responsible for extending shorter A and B1 chains up to a critical chain length, making it unsuitable for its own catalysis (Commuri & Keeling, 2001). In rice, retrotransposon Tos17 insertion mediated SSI-deficient mutant lines showed starch phenotype with decreased amylopectin chains of DP8–12, but increased chains of DP6–7 and 16–19. This suggests that SSI functions in generating DP8–12 chains from shorter chains of DP6–7 emerging from the branch point of A and B1 chains (Fujita *et al.*, 2006). Amylose synthesis was not affected by this mutation, and its effect on starch hydrolysis has not been reported.

#### 1.4.2.3 Starch synthase-II

In cereal endosperm, SSII synthesizes intermediate-length branch chains of amylopectin (see review by Jane *et al.*, 2010). Yamamori *et al.* (2000) produced triple null wheat line lacking starch granule protein-1 (SGP1), identified as SSIIa and homologous to maize SSIIa (Li *et al.*, 1999). Lack of SGP1 showed amylopectin with increased short chains of DP 6–10, a decrease in intermediate chains of DP 11–25 and a concomitant increase in apparent amylose concentration (30.8–37.4%).

In a subsequent study (Yamamori *et al.*, 2006), wheat lines lacking SGP1 showed an increase in resistant starch level (3.6%) compared to normal wheat (0.02%). In a similar approach, wheat lines deficient in SSII A and B genome polypeptides resulted in increased amylose (32%) starch, as determined by

HP-SEC analyses (Chibbar & Chakraborty 2005; Lan *et al.*, 2008). SSIIa deficient maize (*sugary2* mutation due to insertion in SSIIa) genotypes showed an increase in abundance of short (DP 6–11) and medium (DP 13–25) chains. This mutation also resulted in an increase in apparent amylose concentration from 26–40% (Zhang *et al.*, 2004). In rice, *japonica* type has a higher short to long chains ratio than *indica* type but, contrary to wheat and maize, *indica* rice has higher amylose concentration than *japonica* rice (Umemoto *et al.*, 1999, 2002).

In barley, *sex6* mutation on chromosome 7H due to G→A transition results in an early stop codon, thus inhibiting C-terminal translation of the active site of SSIIa (Morell *et al.*, 2003). The major effect of SSIIa inactivity is an increase in amylose concentration (65–70%) in the mutants, which increases RS content. In addition, a change in starch crystallinity from A-type to a mixture of B- and V-type was also reported. V-type crystallinity indicates the formation of amylose-lipid complexes, which inhibit starch swelling, and it resists digestion by amylolytic enzymes (Morell *et al.*, 2003). A barley cultivar, Himalaya-292, which has an inactive SSIIa, produces increased amylose starch and higher RS content. This RS-rich diet when fed to rats changes its bowel SCFA (Bird *et al.*, 2004).

A similar pattern of change with the SSII mutation on amylopectin fine structure and amylose content has been reported in potato (Edwards *et al.*, 1999) and pea (Craig *et al.*, 1998). SSIIa mutation in pea *rug5* decreases intermediate length amylopectin chains (B2 and B3) and produces a higher ( $\approx 35\%$ ) amylose concentration starch Table (1.1) (Craig *et al.*, 1998).

#### 1.4.2.4 Starch synthase-III

Amylopectin long B-chains are synthesized by SSIII. Mutation in maize SSIII is called *dull-1* (*du1*), which has a starch phenotype of amylopectin with decreased proportion of long B-chains, enriched short-branch chains and moderately increased amylose content (Wang *et al.*, 1993). SSIII mutation also affects SSII and SBEIIa and is capable of altering endosperm starch structure (Gao *et al.*, 1998). Ryoo *et al.* (2007) reported a mutation in rice SSIII *OsSSIIIa* (*floury*, *flo*), which produced small and round starch granules and endosperm with a loosely packed central portion, exhibiting a floury-like phenotype. In rice *flo* mutant lines, amylopectin chains with  $DP \geq 30$  were reduced, suggesting that *OsSSIIIa* has a role in the generation of relatively longer chains of amylopectin (i.e. B2 and B3 to B4). Concomitantly, a 2–4% increase in the ratio of amylose to amylopectin was also observed.

In addition to its role in extending glucan chains, SSIII influences starch structure through its association with other starch metabolizing

enzymes. *Arabidopsis* SSIII mutants AtSSIII1 and AtSSIII2 showed increased starch concentration compared to wild type, suggesting a negative regulatory role of SSIII in biosynthesis of transient starch (Zhang *et al.*, 2005). However, no report is available on the effect of SSIII mutation on starch digestibility.

#### 1.4.2.5 Starch synthase-IV

In rice, two SSIV genes, SSIVa and SSIVb, have been shown to be expressed during grain filling, both in the pericarp and the endosperm (Hirose & Terao, 2004). *Arabidopsis* SSIV mutants show a reduction in leaf starch concentration (Roldán *et al.*, 2007) and a striking reduction in leaf starch granules, which suggests a role for SSIV in starch granule initiation. Recently, it has been shown in an *in vitro* assay that SSIV has high SS activity when maltotriose is used as primer (Szydłowski *et al.*, 2009). To date, no cereal plants deficient in SSIV activity have been characterized.

### 1.4.3 Starch branching enzymes (SBE)

Starch branching enzymes cleave  $\alpha$ -1,4 linkages and transfer a free reducing C-1 to C-6 hydroxyl group of glucose-unit in another chain, forming a new  $\alpha$ -1,6 branch linkage. Since branching is an essential part of amylopectin synthesis, it will therefore be dependent on the available concentration of needed SBE.

Based on primary amino acids sequence similarity and substrate specificity, two major types of SBE (SBEI and SBEII) have been identified in cereals. *In vitro* studies in maize suggest that SBEI prefers amylose as substrate and transfers longer chains, whereas SBEII uses amylopectin as substrate and transfers shorter chains (Guan & Preiss, 1993). In wheat, SBEII is further divided into two  $\approx 85\%$  similar isoforms, SBEIIa and SBEIIb, with apparently similar molecular weight (Rahman *et al.*, 2001). In addition to this, a larger form of SBEI, SBEIc (152 kDa) has been reported in wheat (Båga *et al.*, 2000), which is preferentially associated with large A-type granules (Peng *et al.*, 2000). In dicots like pea and potato, two isoforms of SBE viz. SBEI and SBEII (or, SBE B and SBE A) have been reported (Burton *et al.*, 1995; Poulsen & Kreiberg, 1993).

In maize, mutation in SBEIIb resulting in high-amylose starch is known as *amylose-extender (ae)* (Stinard *et al.*, 1993). This results in cereal starch with high-amylose concentration ( $>50\%$ ) and amylopectin with more long branch-chains and fewer short branch-chains (Jane *et al.*, 1999). Similarly, another report suggested a higher proportion of long chains ( $DP \geq 38$ ) and a marked

reduction in short chains of  $DP \leq 17$  in *ae* rice endosperm (Nishi *et al.*, 2001). It also showed a significant increase in apparent amylose concentration from 25–35%.

The very long chains of *ae* mutant amylopectin develop B-type crystallinity (Kasemsuwan *et al.*, 1995; Hizukuri *et al.*, 1983), which favour slow enzymatic digestion. These results corroborated a similar study in maize (Li *et al.*, 2008), where *ae* mutants showed significant increase in chain lengths of amylopectin and higher apparent and absolute concentrations of amylose. Further, the mutants also showed considerably higher RS content (39.4–43.2%) compared to the parents (11.5–19.7%). A commercial product containing  $\approx 80\%$  amylose, called Hi-maize, has been derived from this mutation. Hi-maize has been added to wheat products to increase RS amount (Brown, 2004).

In a recent study, barley RNAi mediated inhibition of SBEIIa and SBEIIb activity altered starch composition and structure (Regina *et al.*, 2010). The study revealed that a reduction in expression of both SBEIIa + b to  $>80\%$  elevated the amylose content to  $>65\%$  from 28% in wild type resulting in a significant increase in RS content (Table 1.1). However, they observed minor differences when either enzyme was down-regulated. Also, reduction in expression of both SBEIIa + b showed an increase in the proportion of chains of  $DP < 9$  and  $DP > 15$  and a consequent decrease in the number of medium chains (DP9–13).

A similar trend has previously been reported in wheat, where an increase in amylose content ( $<70\%$ ) in SBEIIa mutants was observed by simultaneous inhibition of expression of both the SBE II isoforms (Regina *et al.*, 2006). In addition, decrease in proportion of amylopectin chains of DP4–12 and an increase in chains of  $DP > 12$  was also seen. *In vivo* feeding studies in rats using high-amylose wheat meal showed higher amount of RS and lower glycemic index in comparison to wild type wheat diet (Regina *et al.*, 2006). In potato, inhibition of SBE A and SBE B resulted in a very high-amylose phenotype (up to  $\approx 89\%$  by potentiometric determination), while normal high molecular weight amylopectin was absent (Schwall *et al.*, 2000). This type of starch would have lower digestibility.

Yao *et al.* (2009) studied four corn types with different doses of *amylose-extender*(*ae*) and *floury-1*(*fl1*) alleles in the endosperm. Amylose and RS contents followed a similar pattern with highest values in *aeaeaeae* (amylose = 58.3%; RS = 55.2%). They also observed higher proportion of longer branch chains with  $DP \geq 25$  in these mutants. Since *amylose-extender* mutation reduces SBEIIb activity, it results in an increase in amylose to amylopectin ratio, which in turn increases RS content.



#### 1.4.4 Starch debranching enzymes (DBE)

Final packaging of the starch granule requires the trimming of extra branches. Debranching enzymes have been postulated to play this important role in amylopectin biosynthesis (Ball *et al.*, 1996; Myers *et al.*, 2000; Nakamura *et al.*, 2002). Two different mechanisms for DBE mode of action have been proposed. The ‘preamylopectin-trimming model’ suggests that the outer branches of preamylopectin molecules are trimmed by DBE to facilitate chain elongation by SS (Mouille *et al.*, 1996; Myers *et al.*, 2000). This will form amylopectin with an ordered branch structure and allow packaging of the molecule in starch granules. In addition, glucan chains released by DBE’s action on amylopectin can be elongated by GBSSI to form the amylose fraction.

According to the ‘soluble glucan recycling model’, DBE participates in degradation of short chain glucan molecules produced either by SS or SBE action to prevent accumulation of highly branched soluble polymers at the expense of amylopectin formation (Zeeman *et al.*, 1998; Smith, 2001). Endosperms deficient in DBE activity by lesions in DBE genes result in the formation of phytoglycogen instead of amylopectin from soluble glucans (Zeeman *et al.*, 1998).

Two major DBE classes are recognized: isoamylases, which trim packed structures (like glycogen); and pullulanases, which act on more open structures (like pullulan). Three types of isoamylases have been identified in cereal endosperm (Kubo *et al.*, 2005) and in potato (Hussain *et al.*, 2003). Lack of isoamylase-1 in rice (*sugary-1*, *su-1*), and barley (*isa-1*) resulted in small but significant alteration in amylopectin chain length distribution (Kubo *et al.*, 2005). In mutant lines, starch granules were shrunken, irregular and compound (reviewed in James *et al.* (2003)).

Pullulanase type DBE mutation is termed *ZPU1* in maize. *ZPU1* is an endo-acting enzyme that cleaves only very short branch chains and it is activated by redox status and inhibited by high sugar (Dinges *et al.*, 2003). A similar report on wheat limit-dextrinase-type-DBE activity suggests its redox regulation (Repellin *et al.*, 2008). Mutations in debranching enzymes, however, have not been reported to be associated with resistant starch (Table 1.1).

### 1.5 CONCLUDING REMARKS

Starch biosynthesis is a complex process in which starch biosynthetic enzymes act in a coordinated manner to produce amylopectin, which is

architecturally conserved in starches from different botanical sources. Genetic strategies, by identifying genotypes with lesion(s) in gene(s) encoding starch biosynthetic enzymes, have revealed the role of each enzyme or its isoform in the synthesis of amylose and amylopectin constituent glucan chains and consequent alteration in starch composition and amylopectin architecture.

It has also been found that mutations in one locus in starch biosynthetic pathway affects one or more other starch biosynthetic enzymes. Maize *ae* mutant has a lesion in SBEIIb gene, but SBEI activity is reduced or absent and changes the properties of an isoamylase type DBE (Colleoni *et al.*, 2003). Conversely, genetic lesions in pullulanase (*zpu-204*) or isoamylase (*sul-si*) type DBE reduce SBEIIa activity, although SBEIIa polypeptide is not altered or reduced (James *et al.*, 1995; Dinges *et al.*, 2003). Lesions in SSII genes which reduce SSII activity also reduce/eliminate the binding of SSI, SBEIIa and SBEIIb within the granule matrix, although these enzymes have not lost their affinity to amylopectin or starch (Morell *et al.*, 2003; Umemoto & Aoki, 2005). These observations suggest that key starch biosynthetic enzymes form protein complexes (Tetlow *et al.*, 2004). Using isolated amyloplasts, starch biosynthetic enzyme complexes have been shown in wheat and maize (Tetlow *et al.*, 2004; Hennen-Bierwagen *et al.*, 2008).

In a recent proteomics study, it has been shown that phosphorylation of GBSSI, SBEIIb and Pho 1 is needed for their incorporation in to starch granules (Grimaud *et al.*, 2008). The concept of starch biosynthetic enzymes acting in a complex and its formation is dependent upon the phosphorylation status of constituent enzymes and is an additional level of control in starch biosynthesis.

There is significant interest in increasing amylose concentration in cereal and tuber starches. Increased amylose concentrations have been attributed to both SBE and SS isoforms. In addition to natural mutants in maize (*ae*) and barley (*sex6*), amylose to amylopectin ratios in starch have been manipulated by altering GBSSI and SBEII (*waxy/amylose extender*) activity in wheat (Lafiandra *et al.*, 2010; Sestili *et al.*, 2010; Regina *et al.*, 2006), in maize (Jiang *et al.*, 2010) and in rice (Wei *et al.*, 2010). In wheat and barley, very high amylose concentrations were obtained by RNAi mediated inhibition of *Sbe2a* and *Sbe2b* genes (Regina *et al.*, 2006, 2010). Recent advances in understanding starch biosynthesis, combined with innovations in genomics (Ganeshan *et al.*, 2010), can be used to develop cereal genotypes with increased amylose concentrations and alteration in amylopectin architecture which can be used to produce RS.

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## 2 Type 2 Resistant Starch in High-Amylose Maize Starch and its Development

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### 2.1 INTRODUCTION

Starch is a reserve carbohydrate widely present in seeds, roots, tubers, leaves, stems and fruits of plants. Most starch consists of two polymers – amylopectin and amylose – which are organized in a semi-crystalline granular structure (Jane, 2004). Amylopectin has highly branched structures, consisting of linear chains of  $\alpha(1 \rightarrow 4)$ -linked  $\alpha$ -D-glucopyranose units, which are connected by approximately 5%  $\alpha(1 \rightarrow 6)$  glycosidic branch linkages (French, 1984). Amylose is an essentially linear polymer of  $\alpha(1 \rightarrow 4)$ -linked  $\alpha$ -D-glucopyranose units (Takeda *et al.*, 1986, 1989, 1993).

A third component, known as intermediate component (IC), is present in maize mutants such as high-amylose and *sugary-1* maize starches. IC has branched structures with molecular weights smaller than amylopectin but similar to amylose (Baba & Arai, 1984; Klucinec & Thompson, 1998; Li *et al.*, 2008; Wang *et al.*, 1993). The IC molecules also possess iodine-binding capacities and  $\beta$ -amylolysis limits between amylopectin and amylose molecules (Kasemsuwan *et al.*, 1995). Normal maize starch contains about 30% amylose (Hasjim *et al.*, 2009). High-amylose maize starches with amylose contents of 50%, 70% and 80% have been commercialized. High-amylose maize starch with 90% amylose has also been reported (Shi *et al.*, 1998).

Starch is present in varieties of foods and is a major energy source for humans. After ingesting, starch is partially hydrolyzed by human salivary  $\alpha$ -amylase and is predominantly digested in the small intestine by pancreatic  $\alpha$ -amylase and mucosal enzymes (Sang & Seib, 2006). Starch from different botanical sources has different digestive rate and total digestibility, which results in different physiological effects on human health. Resistant starch (RS) is a portion of starch

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*Resistant Starch: Sources, Applications and Health Benefits*, First Edition.

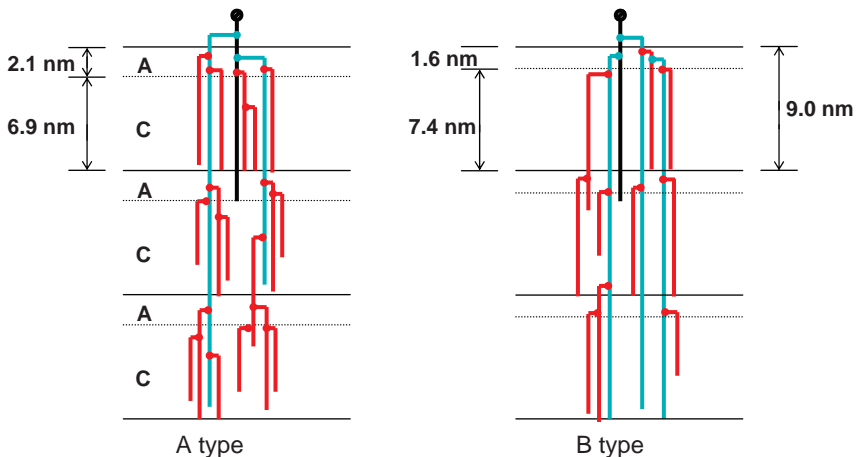
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in foods which cannot be hydrolyzed by enzymes in the small intestine and passes on to the large intestine as a prebiotic (Englyst & Cummings, 1985; Englyst & Macfarlane, 1986). RS provides many health benefits to humans, as previously reported (Behall & Howe, 1996; Behall *et al.*, 2006a, 2006b; Dronamraju *et al.*, 2009; Higgins *et al.*, 2004; Pawlak *et al.*, 2004; Robertson *et al.*, 2003, 2005; Van Munster *et al.*, 1994; Zhang *et al.*, 2007).

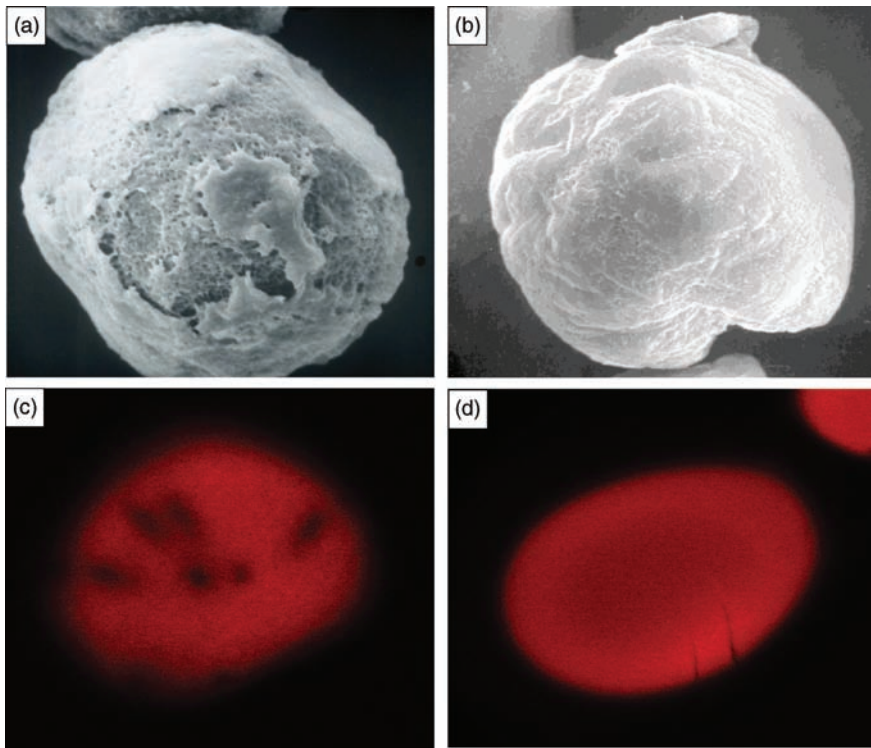
Enzymatic hydrolysis of raw starch granules is a complex process. Many characteristics of the starch determine the rate and the extent of enzymatic hydrolysis of the granules, such as granular size, structure of the amylopectin, polymorph, amylose content and lipid content. These characteristics are species dependent and are also affected by growth conditions and post-harvest treatment (Jane, 2006; Lehmann & Robin, 2007; Setiawan *et al.*, 2010; Tester *et al.*, 2006).

It is well known that starch granules with larger granular sizes are digested at a slower rate than those with smaller granular sizes, because larger starch granules have relatively smaller surface area for enzymes to attack (Tester *et al.*, 2006). The A-type polymorphic starch granules, such as normal and waxy maize, rice, wheat, and taro starch, are known to have ‘weak points’ and are more easily hydrolyzed by enzymes than are the B- and C-type polymorphic starch granules such as potato, high-amylose maize, green banana and pea starch (Jane *et al.*, 2003; Jane, 2007). This difference is attributed to the branch chain-length of amylopectin and the packing of double helices in the starch granule (Figure 2.1).



**Figure 2.1** Structure models of amylopectins for A type and B type starches. A: amorphous; C: crystalline. Reproduced from *Carbohydrate Polymers*, 49(3), Yoo S.-H. and Jane J.-I. Molecular weights and gyration radii of amylopectins determined by high-performance size-exclusion chromatography equipped with multi-angle laser-light scattering and refractive index detectors, 307–314. Copyright 2002, with permission from Elsevier.

The amylopectin of the A-type polymorphic starch has a larger proportion of short A and B1 chains but smaller numbers of long B2, B3, and B4 chains than that of the B-type polymorphic starch (Jane *et al.*, 1999). B2, B3, and B4 chains extend through multiple clusters of amylopectin and their movements are constrained by the consecutive crystalline regions in the starch granule. The short A and B1 chains, however, extend through one cluster of amylopectin and have free ends to move around within the cluster and to form closely packed monoclinic crystallites. Consequently, the A-type polymorphic starch granules consist of voids (weak points) (Figure 2.2a,c; Jane, 2006; Jane, 2007; Jane *et al.*, 2004; Pan & Jane, 2000), which are easily penetrated and hydrolyzed by



**Figure 2.2** Internal structures of starch granules. a (maize) and b (potato): scanning electron micrographs of inner part of granules remaining after removal of the chemical gelatinized granule surface. Reproduced from Jane, 2006; 2007. c and d: confocal laser-scanning micrographs of normal maize and potato starch granules, respectively; starch granules were stained using Rhodamine B dye. Reproduced from Jane, 2006.

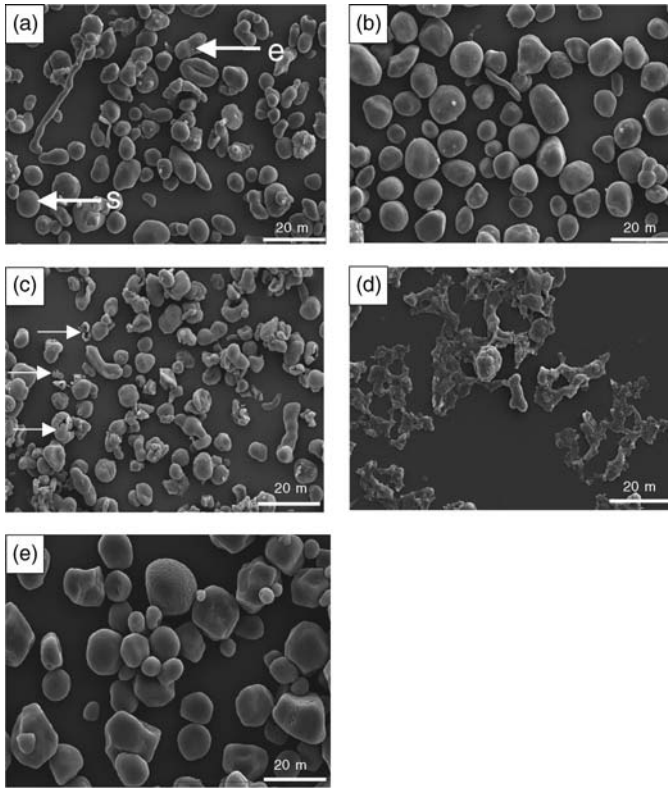
enzymes to generate pin holes and channels (Fannon *et al.*, 1992, 1993; Jane, 2006).

The B-type polymorphic starch granules, consisting of rigidly organized amylopectin branch chains (Figure 2.1) and without weak points or voids (Figure 2.2b,d) (Jane, 2006; Jane, 2007; Jane *et al.*, 2004; Jane & Shen, 1993), are normally digested slowly by erosion of starch granules from the surface (Jane, 2006; Robyt, 1998). After cooking, potato starch, however, is highly digestible. The increase in amylose content in normal starch granules (Jane *et al.*, 2003) and in high-amylose starch granules (Evans & Thompson, 2004; Jiang *et al.*, 2010b; Li *et al.*, 2008; Shi & Jeffcoat, 2001) reduce the rate of enzymatic hydrolysis of the raw starch granules. These features can be attributed to the fact that, in the normal starch granule, the amylose is more concentrated at the periphery of starch granules and interacts with amylopectin to form a hard shell (Debet & Gidley, 2007; Jane & Shen, 1993; Pan & Jane, 2000); this makes the starch granule more resistant to enzymatic hydrolysis (Tester *et al.*, 2006). In high-amylose maize starch, amylose forms crystalline structure, which is highly resistant to enzymatic hydrolysis (Evans & Thompson, 2004; Jiang *et al.*, 2010b; Li *et al.*, 2008; Shi & Jeffcoat, 2001).

In general, starch is consumed by humans after cooking. Starch gelatinization during cooking is an irreversible reaction which is associated with the dissociation of double helices and loss of birefringence viewed under a polarized light (Jane, 2004). Gelatinization properties of starch isolated from different botanical origins differ, and this relates to starch molecular structures and minor components present in the starch granule (Jane, 2004; Yoo *et al.*, 2009). The enzymatic hydrolysis of gelatinized starch (amorphous) is much faster than that of raw starch granules (semi-crystalline) (Lehmann & Robin, 2007; Tester *et al.*, 2006).

High-amylose maize (*amylose-extender*, *ae* mutant) starches with amylose contents ranging from 50–80% have been commercially available and are widely used in food and non-food applications (Ferguson *et al.*, 1994; Richardson *et al.*, 2000). Maize *ae* single-mutant starch contains about 65% apparent amylose and about 15% RS (Li *et al.*, 2008) – substantially higher than normal (non-mutant) maize starch ( $\approx 30\%$  and  $\approx 1.5\%$ , respectively) (Hasjim & Jane, 2009; Hasjim *et al.*, 2009). After cross-breeding the maize *ae*-mutant with a maize line carrying high-amylose modifier (HAM) gene, the apparent amylose content of the starch (GEMS-0067) elevates to  $\approx 85\%$  and the RS content of the starch increases to  $\approx 43\%$  (Li *et al.*, 2008).

Maize *ae*-mutant starch consists of spherical and elongated starch granules (Figure 2.3a,b), which differ from the normal maize starch in consisting of spherical and angular granules (Figure 2.3e; Hasjim *et al.*, 2009; Jiang *et al.*, 2010b; Li *et al.*, 2007; Mercier *et al.*, 1970; Perera *et al.*,



**Figure 2.3** Scanning electron micrographs of maize starches and resistant-starch (RS) residues (Jiang *et al.*, 2010b). (a) GEMS-0067 starch ( $\approx 85\%$  apparent amylose) showing spherical (s) and elongated (e) granules. (b) H99ae starch ( $\approx 65\%$  apparent amylose). (c) GEMS-0067 RS residue remaining after enzymatic hydrolysis at  $95\text{--}100^\circ\text{C}$  (AOAC Method 991.43). (d) H99ae RS residue in a gel-like form. Arrows indicate fragmented, hollowed and half-shell-like granules. (e) Normal maize starch. Reprinted from Jiang *et al.* (2010b), Copyright 2010, with permission from Elsevier.

2001; Wolf *et al.*, 1964). The elongated starch granule content of a double mutant of *ae* and HAM genes, GEMS-0067 line, increases up to 32% (Figure 2.3a; Jiang *et al.*, 2010b). These elongated starch granules retain their granular shapes after cooking at  $95\text{--}100^\circ\text{C}$  with excess water and are highly resistant to enzymatic hydrolysis (Figure 2.3c). In this chapter, formations of RS and elongated starch granules in the maize *ae*-mutant starch and how the effect of HAM gene on the RS content of the maize *ae*-mutant starch will be discussed.

## 2.2 RS FORMATION IN HIGH-AMYLOSE MAIZE STARCH

Although high-amylose maize starch has been developed for over 60 years (BeMiller, 2009; Kramer *et al.*, 1956; Vineyard & Bear, 1952; Vineyard *et al.*, 1958), interests in the RS of the high-amylose maize starch has just emerged in the past two decades, resulting from consumer awareness of health food benefits (Sajilata *et al.*, 2006). The structures and properties of the high-amylose maize starch and its RS residue have been intensively studied in order to understand the RS formation in the starch (Evans & Thompson, 2004; Jiang & Liu, 2002; Jiang *et al.*, 2010b; Li *et al.*, 2008; Shi *et al.*, 1998; Shi & Jeffcoat, 2001). It has been reported that molecular weight and branch chain length distributions of RS residues remaining after pancreatic  $\alpha$ -amylase hydrolysis are similar to that of native starch counterparts (Evans & Thompson, 2004; Shi & Jeffcoat, 2001).

After enzymatic hydrolysis of high-amylose maize starches at 95–100 °C (AOAC Method 991.43), the RS residues display semi-crystalline structures with the B-type polymorph (Jiang *et al.*, 2010b). The onset gelatinization temperatures of the RS residues are above 100 °C, indicating the long-chain double-helical crystallites are present in the RS residues. Gel-permeation chromatograms show that the RS residues consist of large molecules with degrees of polymerization (DP) 840–951 and small molecules with DP 59–74 (Jiang *et al.*, 2010b). This differs from molecular weight distributions of RS residues remaining after pancreatic  $\alpha$ -amylase hydrolysis of the native high-amylose maize starches (Evans & Thompson, 2004; Shi & Jeffcoat, 2001).

The molecular weight distributions of the debranched RS residues remaining after enzymatic hydrolysis of the high-amylose maize starches at 95–100 °C show that the RS residues consist mostly of linear molecules and are mainly derived from amylose/IC molecules (Jiang *et al.*, 2010b). Thus, it is concluded that the RS residues of high-amylose maize starches consist of mainly semi-crystalline structure of long-chain double-helices of amylose/IC (Jiang *et al.*, 2010b). It is likely that the amylose/IC crystallites in native high-amylose maize starch are present in blocks, which prevent the starch granule from swelling at 95–100 °C, maintain the semi-crystalline structure and protect the bulk of the amylose and IC and a small portion of amylopectin molecules from enzymatic hydrolysis (Jiang *et al.*, 2010b).

The presence of amylose double-helical crystallites in high-amylose maize starch is also supported by the structure and properties of the Naegeli dextrin, which is produced by hydrolyzing the starch at 38 °C using 15.3% sulphuric



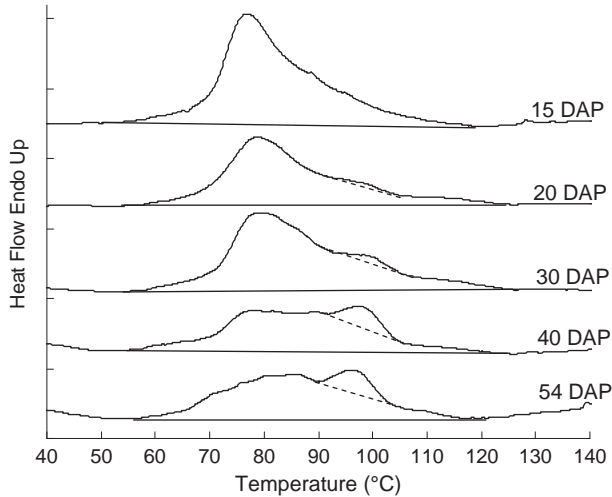
acid (Jiang *et al.*, 2010e). The Naegeli dextrans of high-amylose maize starches consist of double helices with chain lengths of  $DP \leq 12$  (8.6–11.2%),  $DP 13-24$  (38.7–54.1%),  $DP 25-36$  (23.6–30.6%) and  $DP \geq 37$  (13.1–22.1%). Because the longest detectable chains of the Naegeli dextrin of *ae* waxy maize starch are  $DP 25$  (Jane *et al.*, 1997), the long-chain double helices of  $DP \geq 25$  present in the Naegeli dextrin are attributed to crystalline fragments of amylose double helices (Jane & Robyt, 1984; Jiang *et al.*, 2010b, 2010e). The thermal properties of the Naegeli dextrans of the high-amylose maize starches display very high peak (113.9–122.2 °C) and conclusion gelatinization-temperatures (148.0–160.0 °C), which also support the presence of amylose double helices (Jiang *et al.*, 2010e; Sievert & Pomeranz, 1989, 1990).

Although high-amylose maize starches contain approximately 0.2–0.7% lipids (Jiang *et al.*, 2010b; Morrison, 1992, 1993, 1995; Morrison *et al.*, 1993), the lipids present in the starch granules also reduce the enzyme digestibility of the starch at 95–100 °C (Jiang *et al.*, 2010b). After the lipids are removed from the starch granules, the RS contents of the high-amylose maize starches reduce from 10.6–43.4% to 9.0–28.9%. The effects of lipids on the RS content of the high-amylose maize starch are discussed in other chapters of this book.

### 2.3 RS FORMATION DURING KERNEL DEVELOPMENT

Although RS and amylose/IC double-helical crystalline structures are found in mature high-amylose maize starch granules after harvesting and drying (Jiang *et al.*, 2010b), it is not known whether the RS and the amylose/IC double-helical crystalline structure are formed during kernel development or are induced during post-harvest processing and boiling/ $\alpha$ -amylase hydrolysis.

Jiang *et al.* (2010a) studied RS formation during kernel development of GEMS-0067 line, a maize double-mutant of *ae* and HAM genes. Gelatinization thermograms of the starches harvested at 15, 20, 30, 40, 54 (mature) days after pollination (DAP) display a major thermal transition (first peak) with the peak temperature between 76.6–81.0 °C and an additional thermal transition (second peak) with the peak temperature  $\approx 97.1$  °C (Figure 2.4). The second peak first appears as a shoulder on 20 DAP and gradually increases into a significant peak on 30, 40, and 54 DAP (Jiang *et al.*, 2010a). The relative intensity of the first peak (76.6–81.0 °C) decreases with kernel maturation and a decrease in the amylopectin content of the starch, indicating that this peak corresponds to the dissociation of amylopectin crystallites (Jiang *et al.*, 2010a,



**Figure 2.4** Differential scanning calorimetry (DSC) thermograms of native GEMS-0067 starches harvested at different developmental stages (Jiang *et al.*, 2010a). DAP: days after pollination. The peak area above the dashed line indicates melting of amylose-lipid complex. Reprinted with permission from Jiang *et al.* (2010a). Copyright 2010 American Chemical Society.

2010b; Kasemsuwan *et al.*, 1995; Li *et al.*, 2008). The increase in the size of the second peak correlates with the lipid content of the starch, and this peak disappears after removal of lipids from the starch, indicating that this additional peak is the melting of amylose-lipid complex (Jiang *et al.*, 2010a; Tufvesson *et al.*, 2003a, 2003b).

After removal of lipids, the conclusion gelatinization-temperature of the starch does not change. The conclusion gelatinization temperature, however, increases with kernel maturation, from 105.0 °C on 15 DAP to 117.8–122.2 °C on later dates (Jiang *et al.*, 2010a). The percentage of the enthalpy change of the thermal transition at temperature range above 95 °C, which corresponds to the melting of long-chain double-helical crystallites of amylose/IC, increases with the kernel maturation and significantly correlates with the RS content of the starch (Jiang *et al.*, 2010a). The RS content of the high-amylose maize starch increases with kernel maturation and directly correlates with the amylose/IC content of the starch ( $r=0.99$ ,  $p<0.001$ ) (see Table 2.1). Thus, the authors conclude that the long-chain double-helical crystallites of amylose/IC in the high-amylose maize starch develop with the kernel maturation, which results in an increase in the RS content of the starch (Jiang *et al.*, 2010a).

**Table 2.1** Resistant starch (RS) and amylose/intermediate component (IC) contents of GEMS-0067 starches harvested at different kernel-developmental stages (Jiang *et al.*, 2010a). Reprinted with permission from Jiang *et al.* (2010a). Copyright 2010 American Chemical Society.

Sample	RS <sup>a</sup> (%)	Amylose/IC <sup>b</sup> (%)
15 DAP <sup>c</sup>	9.0 ± 1.2	55.2 ± 0.5
20 DAP	26.4 ± 0.1	78.4 ± 0.2
30 DAP	29.6 ± 0.8	81.9 ± 0.3
40 DAP	32.0 ± 0.1	88.6 ± 1.3
54 DAP	32.1 ± 0.3	87.6 ± 0.4
Coefficient between RS and amylose/IC content		0.99 <sup>d</sup>

<sup>a</sup>RS content of starch was determined using AOAC method 991.43 for total dietary fibre.

<sup>b</sup>Amylose/IC content of starch was determined using Sepharose CL-2B gel-permeation chromatography followed by the total carbohydrate (phenol-sulphuric acid) determination.

<sup>c</sup>DAP: days after pollination.

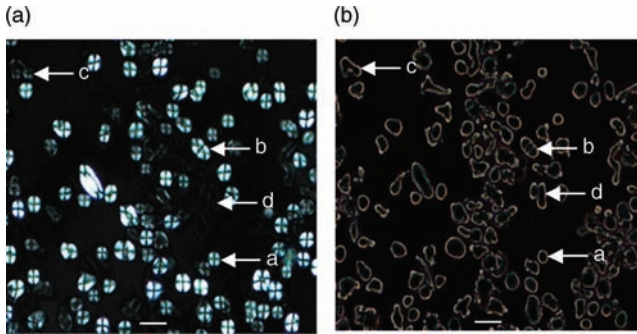
<sup>d</sup> $p < 0.001$ .

## 2.4 ELONGATED STARCH GRANULES OF HIGH-AMYLOSE MAIZE STARCH

### 2.4.1 Structures of elongated starch granules

Different from normal maize starch, which contains starch granules with spherical and angular shapes (Figure 2.3e; Hasjim *et al.*, 2009; Perera *et al.*, 2001; Wongsagonsup *et al.*, 2008), high-amylose maize starch contains elongated granules in addition to spherical granules (Figure 2.3a,b; Boyer *et al.*, 1976; Jiang *et al.*, 2010b; Mercier *et al.*, 1970; Shi & Jeffcoat, 2001; Sidebottom *et al.*, 1998; Wolf *et al.*, 1964). Starch of maize double mutant GEMS-0067 consists of up to 32% elongated granules, which is substantially greater than that of the maize *ae* single-mutant starch ( $\approx 7\%$ ) and normal maize starch (0%) (Campbell *et al.*, 2007; Hasjim *et al.*, 2009; Jiang *et al.*, 2010b; Li *et al.*, 2007; Perera *et al.*, 2001; Wongsagonsup *et al.*, 2008). The percentage of elongated starch granules increases with kernel maturation and the increase in amylose/IC content of the starch (Jiang *et al.*, 2010a, 2010b, 2010d; Mercier *et al.*, 1970). Elongated starch granules are mostly found in mature endosperm cells located in the central part of the crown region of the high-amylose maize kernel (Boyer *et al.*, 1976).

Starch molecules in the granule are present in semi-crystalline structures of double helices. The birefringence pattern of a starch granule observed under a polarized light microscope reflects the arrangement of starch molecules in the granule (French, 1984). Normal maize starch granules display a typical



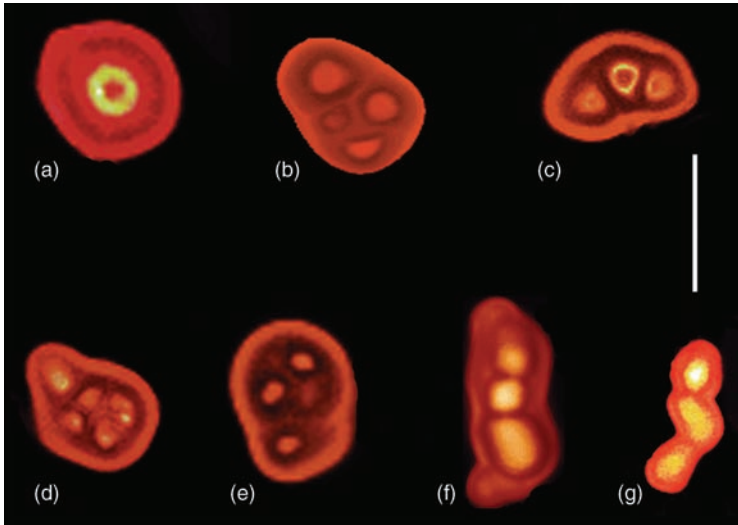
**Figure 2.5** Polarized (a) and phase contrast (b) light micrographs of high-amylose maize (GEMS-0067) starch. Arrows indicate birefringence patterns of: (a) one granule displaying one Maltese cross; (b) Maltese crosses overlapping in one granule; (c) a single granule consisting of one or more Maltese crosses and weak/no birefringence on the rest part of the granule; (d) granule showing weak/no birefringence. Bar = 20  $\mu\text{m}$ .

Maltese cross birefringence pattern (Wongsagonsup *et al.*, 2008), reflecting that starch molecules in the granule are aligned radially from the hilum, which are oriented perpendicular to the granule surface.

Most spherical granules of high-amylose maize starch exhibit a normal Maltese cross birefringence (Figure 2.5, granule a), similar to the granules of normal maize starch. This birefringence pattern is consistent with the confocal laser-scanning micrograph (CLSM) of the 8-aminopyrene-1,3,6-trisulfonic acid (APTS)-derivatized spherical granule, which shows a hilum with a bright colour at the centre of the granule (Figure 2.6a; Glaring *et al.*, 2006; Jiang *et al.*, 2010c). These findings indicate that the spherical granule is developed from one single granule nucleus.

Elongated starch granules, however, display three different types of birefringence patterns (Figure 2.5). The type 1 birefringence pattern displays several Maltese crosses overlapping in one granule (Figure 2.5, granule b), while the type 2 birefringence pattern shows one or more Maltese crosses in one part of the granule and weak or no birefringence on the rest of the granule (Figure 2.5, granule c). The type 3 birefringence pattern exhibits a granule displaying weak or no birefringence (Figure 2.5, granule d; Jiang *et al.*, 2010c; Wolf *et al.*, 1964).

The differences in birefringence patterns of elongated starch granules indicate that arrangements of starch molecules in the granule differ between elongated granules, and even between different parts of one elongated granule. This feature is consistent with the CLSM images of APTS-stained elongated granules, which show multiple regions of different fluorescence intensity in one elongated granule (Figure 2.6b–g) (Glaring *et al.*, 2006; Jiang *et al.*, 2010c).

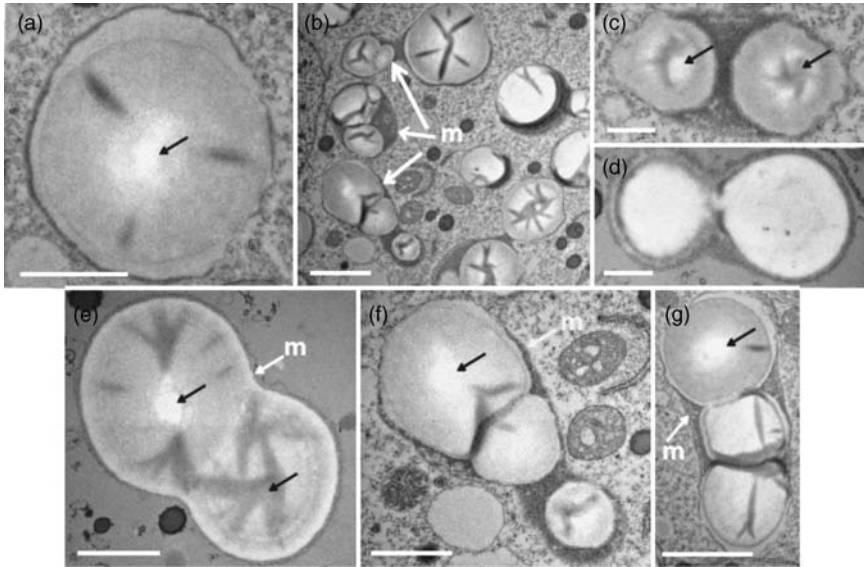


**Figure 2.6** Confocal laser-scanning microscope images of high-amylose maize (GEMS-0067) starch granules (Jiang *et al.*, 2010c). Starch granules were labelled using 8-aminopyrene-1,3,6-trisulfonic acid APTS). (a) Spherical granule showing bright colour around the hilum; (b), (c), (d), (e), (f), and (g) show elongated starch granules displaying multiple regions with intense fluorescence. Bar = 10  $\mu\text{m}$ . Reprinted from Jiang *et al.* (2010c), Copyright 2010, with permission from Elsevier.

#### 2.4.2 Formation of elongated starch granules

Starch granule formation in the amyloplast of high-amylose maize endosperm at an early stage of kernel development (20 DAP) has been studied using transmission electron microscopy (TEM) (Jiang *et al.*, 2010c). Some amyloplasts at the subaleurone layer of high-amylose maize endosperm contain only one spherical granule showing normal growth rings and a hilum (Figure 2.7a), which are similar to amyloplasts in normal maize endosperm. The majority of amyloplasts, however, contain two or more small starch granules (Figure 2.7b).

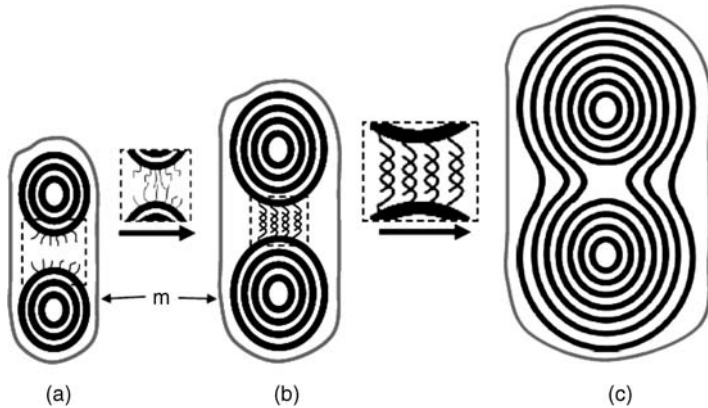
These small granules in the amyloplast, which develop at the early stage, fuse and grow into an elongated starch granule (Figures 2.7c–g; Jiang *et al.*, 2010c). This fusion is likely the result of interactions between the amylose of adjacent granules, because high-amylose maize starch contains a large concentration of amylose. The amylose molecules of one granule can contact that of an adjacent granule and form anti-parallel double helices between the



**Figure 2.7** Transmission electron micrographs of high-amylose maize (GEMS-0067) endosperm tissue harvested on 20 days after pollination (Jiang *et al.*, 2010c). (a) Spherical starch granule with a hilum at centre of granule and growth rings. (b) Overview of endosperm tissue. (c) Two starch granules initiated in one amyloplast at early stage of starch granule development. (d) Initial fusion of starch granules. (e) Two fused starch granules forming an elongated starch granule. (f) Two connected starch granules and a third starch granule in the amyloplast protrusion. (g) Three small granules fused into one granule, with one small granule at head showing hilum and growth rings and the other two granules displaying no hilum or growth rings. m: membrane boundary of amyloplast. Black arrow indicates hilum. Bars = 1  $\mu\text{m}$  on A, C, D, F and 2  $\mu\text{m}$  on B, E, G. Reprinted from Jiang *et al.* (2010c), Copyright 2010, with permission from Elsevier.

two granules (Figure 2.7d; Jiang *et al.*, 2010a, 2010b; Li *et al.*, 2008), which bind the two adjacent granules and prevent the amyloplast from dividing. Eventually, the two granules grow into one elongated granule (Figure 2.7e).

A proposed mechanism of the elongated starch granule formation is shown in Figure 2.8. The mechanism is consistent with the fact that the number of elongated starch granules increases with the increase in the amylose content of the starch (Jiang *et al.*, 2010a, 2010b). In the normal maize starch, amylose content is low ( $\approx 30\%$ ) and branch chains of amylopectin are short comparing with amylose. The short branch chains of amylopectin cannot form stable anti-parallel double helices with branch chains of an adjacent granule in a normal maize amyloplast during granule development. Thus, the small starch granules in the normal maize amyloplast are separated with the division of



**Figure 2.8** Proposed formation mechanism of elongated starch granule in the amyloplast (Jiang *et al.*, 2010c). (a) Two starch granules, each with hilum and growth rings, are initiated in amyloplast at an early stage of granule development. (b) Two granules begin fusion through amylose interaction by forming anti-parallel double helices, which prevent amyloplast division. (c) Fused granules have integrated outer layer growth rings. m: membrane boundary of amyloplast. Reprinted from Jiang *et al.* (2010c), Copyright 2010, with permission from Elsevier.

amyloplast (Jiang *et al.*, 2010c). Most amyloplasts in normal maize endosperm consist of a single starch granule.

### 2.4.3 Location of RS in the starch granule

The RS residues of GEMS-0067, consisting of about 85% amylose, are found mostly in elongated granular structures (Figure 2.3c), whereas that of the *ae* single-mutant, consisting of approximately 65% amylose, are present in a gel form (Figure 2.3d; Jiang *et al.*, 2010b). This is attributed to a greater content of amylose/IC double-helical crystallites present in the granules of GEMS-0067, particularly elongated granules (Jiang *et al.*, 2010b).

The hollowed and half-shell-shaped granules and the gel-like starch (Figure 2.3c,d) observed in the RS residues are the remnants of the outer layer of spherical granules (Jiang *et al.*, 2010b). These structures can be attributed to the fact that the amylopectin molecules around the hilum are loosely packed and can be promptly gelatinized and hydrolyzed by thermally stable  $\alpha$ -amylase at 95–100 °C. Amylose molecules, however, are more concentrated at the periphery of the spherical granules (Jane & Shen, 1993; Jiang *et al.*, 2010a; Jiang *et al.*, 2010b; Li *et al.*, 2007; Pan & Jane, 2000). The amylose molecules at the periphery of the starch granules interact between themselves and with amylopectin molecules to form a hard shell

which is difficult to disperse and, thus, is resistant to enzymatic hydrolysis (Debet & Gidley, 2007; Gray & BeMiller, 2004; Huber & BeMiller, 2001; Jane & Shen, 1993; Jiang *et al.*, 2010a, 2010b; Li *et al.*, 2007; Pan & Jane, 2000).

## 2.5 ROLES OF HIGH-AMYLOSE MODIFIER (HAM) GENE IN MAIZE *ae*-MUTANT

The role of HAM gene and its dosage effects on the physicochemical properties of maize *ae*-mutant starch have been studied and reported (Jiang *et al.*, 2010d). Maize endosperm is a triploid tissue, resulting from the fertilization of two maternal nuclei and one paternal nucleus (Birchler, 1993). Thus, endosperms with 0, 1, 2, and 3 doses of HAM gene are produced by self- and inter-pollination of an *ae* single-mutant line and the *ae* and HAM double-mutant line, GEMS-0067. The increase in HAM gene dosage in the maize *ae* mutant increases the apparent amylose content of the starch (see Table 2.2; Jiang *et al.*, 2010d). After including three doses of HAM gene to the maize *ae*-mutant, the starch has longer branch chain-lengths of IC molecules but slightly shorter branch chain-lengths of amylopectin molecules than maize *ae* single-mutant starch (Li *et al.*, 2008). Two or three doses of HAM gene in maize *ae*-mutant substantially increase the RS content of the maize *ae*-mutant starch. One dose of HAM gene, however, has little effect on the RS content.

Morphological properties of starch granules are also influenced by the dosage of HAM gene (Jiang *et al.*, 2010d). The greater the HAM gene dosage in the starch, the greater the number of elongated granules is found in maize *ae*-mutant starch. With the presence of three doses of HAM gene, many maize *ae*-mutant starch granules display weak or no birefringence,

**Table 2.2** Amylose and resistant starch (RS) contents of native maize starches isolated from kernels of self- and intercrossed lines GEMS-0067 (G) and H99ae (H) (Jiang *et al.*, 2010d). Reprinted with permission from Jiang *et al.* (2010a). Copyright 2010 American Chemical Society.

Sample	HAM gene doses	Amylose <sup>a</sup> (%)	RS <sup>b</sup> (%)
G/G	3	69.9 ± 0.0	35.0 ± 0.5
G/H	2	64.9 ± 0.4	28.1 ± 0.8
H/G	1	61.1 ± 0.1	12.9 ± 0.2
H/H	0	56.3 ± 0.4	15.7 ± 0.2

<sup>a</sup>Amylose content of starch was determined using iodine-colorimetric method.

<sup>b</sup>RS content of starch was determined using AOAC method 991.43 for total dietary fibre.



indicating changes in molecular organization of the granules. The increase in HAM gene dosage decreases the percentage crystallinity of starch, which is consistent with the decrease in amylopectin content of the starch (Jiang *et al.*, 2010d). This is attributed to amylopectin having short branch chains which are free to move and interact with one another and, thus, facilitate the formation of double-helical crystallites.

Maize *ae*-mutant starches display multiple thermal-transitions during gelatinization (Jiang *et al.*, 2010a, 2010b, 2010d). The temperature of the thermal transition range increases with the increase in the HAM gene dosage. The first thermal transition peak, with the peak temperature at  $\approx 82.9^\circ\text{C}$ , corresponds to the melting of amylopectin crystallites (Jiang *et al.*, 2008; Kasemsuwan *et al.*, 1995; Li *et al.*, 2008), which decreases with an increase in HAM gene dosage (Jiang *et al.*, 2010d). The second thermal transition peak, with the peak temperature at  $\approx 99.1^\circ\text{C}$ , corresponds to the dissociation of amylose-lipid complexes (Jiang *et al.*, 2010a; Li *et al.*, 2008; Tufvesson *et al.*, 2003a, 2003b), which increases with an increase in HAM gene dosage (Jiang *et al.*, 2010d). The conclusion gelatinization-temperature of the starch is above  $100^\circ\text{C}$  and increases with the increase in HAM gene-dosage (Jiang *et al.*, 2010d), indicating the formation of amylose/IC crystallites (Jiang *et al.*, 2010a).

These findings indicate that the formation of amylose/IC crystallites and amylose-lipid complex increase with the increase in HAM gene dosage, which is consistent with the increase in the RS content of the starch (Jiang *et al.*, 2010d).

## 2.6 CONCLUSIONS

Long-chain double-helical crystallites of amylose/IC in high-amylose maize starch are developed during kernel development and are concentrated in the elongated granules and at the outer layer of the spherical granules. The amylose/IC crystallites, having onset gelatinization temperature above  $100^\circ\text{C}$ , restrict starch granules from swelling and dispersion at  $95\text{--}100^\circ\text{C}$  and protect the starch molecules from enzymatic hydrolysis at  $95\text{--}100^\circ\text{C}$ . Lipids present in the granule also protect starch granules from enzymatic hydrolysis at  $95\text{--}100^\circ\text{C}$ . The formation of elongated starch granules in high-amylose maize starch results from amylose interactions between adjacent small granules in the amyloplast during granule development. The inclusion of HAM gene(s) to the maize *ae*-mutant increases the crystallites of amylose/IC in the starch, which results in the increase in RS content of the starch.

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## 3 RS4-Type Resistant Starch: Chemistry, Functionality and Health Benefits

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### 3.1 INTRODUCTION

The statistics on obesity are alarming; more than one-third of adult Americans and almost 17% of youth were classed as obese in 2009–2010 (Ogden *et al.*, 2012). One television news report disturbingly labelled overweight youth as ‘coronary time bombs’, as they are likely to develop heart disease when they grow into adulthood. As a potential consequence, billions of dollars will be spent in weight-related bills. The high price tag is simply because obesity is not a singular medical condition. Being overweight or obese is also associated with Type 2 diabetes, cardiovascular disease, high cholesterol, hyperlipidaemia, hypertension, stroke, cancer and other diseases. About one-third of Americans have hypertension – a major risk factor for heart disease and stroke if left untreated.

Resistant starch (RS), as a component of dietary fibre, is not only beneficial but can be considered essential to the general health and well-being of consumers worldwide. It demonstrates numerous positive physiological effects and, therefore, plays a critical role as a food ingredient in addressing the widespread metabolic syndrome that afflicts the general population. This chapter specifically highlights the properties of RS belonging to the RS4 classification and underscores its effectiveness in alleviating diet and lifestyle-related diseases.

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### 3.2 HISTORICAL ACCOUNT OF STARCH INDIGESTIBILITY

From the early 1900s into the 1930s, scientists explored the *in vitro* and *in vivo* amylase digestibility of raw and gelatinized starches from different botanical sources (Fofanow, 1911; Thorpe, 1913; Daniels & Strickler, 1917; Langworthy & Deuel, 1920, 1922; Katz, 1934) (Table 3.1). Those researchers succeeded in developing two of the concepts that we know today about RS: that several raw starches, especially raw potato starch, are resistant to digestion; and that retrograded starch, which was then termed as ‘amylocoagulose’ (Katz, 1934), resisted digestion.

The linkage between dietary fibre and starch as a dietary source of fibre was established in 1982, when British researchers discovered the presence of starch ‘resistant’ to digestion in the residue of cooked and cooled potatoes recovered after amylase hydrolysis during the determination of non-starch polysaccharides (Englyst *et al.*, 1982). A decade after Englyst’s work, a formal definition of resistant starch as ‘the sum of starch and products of starch degradation not absorbed in the small intestine of healthy individuals’ was established (Asp, 1992). Then, in 2001, a revised definition of dietary fibre, which included RS under analogous carbohydrates, was introduced by the American Association of Cereal Chemists (2001).

Subsequently, other organizations and countries followed suit (Institute of Medicine, 2001; European Food Safety Authority, 2007) and, finally in 2009, the Codex Alimentarius Commission adopted a new dietary fibre definition that included RS under carbohydrate polymers obtained from food raw material by physical, enzymatic or chemical means (Codex Alimentarius Commission, 2009). In 2012, Health Canada, after several years of deliberation, issued a new dietary fibre definition that is consistent with the definition adopted by Codex Alimentarius Commission, and includes RS under novel fibres (Health Canada, 2012).

RS4-type resistant starch belongs to one of the five types or classes of RS. Englyst & Macfarlane (1986), citing the work of Englyst (1985), reported on the classification of starch for nutritional purposes into readily digestible starch and resistant starch, which was further classified into RS1, RS2, RS3a, and RS3b. The preceding two types of RS3 were differentiated by their composition as being mainly staled amylopectin and mainly retrograded amylose, respectively.

Englyst & Cummings (1987) proposed a classification of starch based on its digestibility that can distinguish readily digestible starch in freshly cooked foods, partially resistant starch in raw potato and banana starch, and resistant



**Table 3.1** Chronology of events leading to resistant starch, its classification to RS1-RS5 and its inclusion in the definition of dietary fibre.

Year	Events	References
1911	Raw potato starch was less digested in human subjects, but raw wheat, oat and rice starches were practically assimilated.	Fofanow, 1911
1917	Taka-diestase indigestible starch was found in bakery products.	Daniels and Strickler, 1917
1920–1922	Raw corn, wheat, cassava, rice and taro root starches were completely digested <i>in vivo</i> , but raw potato, arrowroot and canna starches were less digestible.	Langworthy and Deuel, 1920, 1922
1934	'Amylocoagulose', a retrograded material in annealed starch gels, was not digested by malt extract.	Katz, 1934
1953	The word 'dietary fibre' was first used to describe plant cell walls in the diet.	Hipsley, 1953
1961–1962	Raw high-amylose corn starch was resistant to <i>in vitro</i> and <i>in vivo</i> digestion.	Ackerson, 1961; Leach & Schoch, 1961; Borchers, 1962; Sandstedt <i>et al.</i> , 1962
1969–1971	Chemically modified starch was resistant to digestion by pancreatin.	Janzen, 1969; Leegwater & Luten, 1971
1971–1973	Prevalence of 'Western' diseases in developed societies, but not in Africa, was attributed to inadequate dietary fibre (non-starch polysaccharides) intake.	Burkitt, 1971, 1973a,b; Trowell, 1972
1977	Raw banana starch resisted <i>in vitro</i> digestion by $\alpha$ -amylase.	Fuwa, 1977
1981	Protocol for measuring glycemic index was established.	Jenkins <i>et al.</i> , 1981
1982	The term 'resistant starch' was coined for retrograded starch found in cooked and cooled potatoes.	Englyst <i>et al.</i> , 1982
1984	Retrograded amylose was resistant to digestion by $\alpha$ -amylases and yielded resistant fragments with degree of polymerization of 43–50.	Jane and Robyt, 1984
1986	Starch was classified for nutritional purposes into readily digestible starch and resistant starch, which was further classified into RS1, RS2, RS3a and RS3b.	Englyst & Macfarlane, 1986
1992	Official definition for 'resistant starch' was established as 'the sum of starch and products of starch degradation that are not absorbed in the small intestines of healthy individuals'.	Asp, 1992

(continued)

**Table 3.1** (Continued)

<b>Year</b>	<b>Events</b>	<b>References</b>
1992	The <i>in vitro</i> Englyst method to measure rapidly digestible starch, slowly digestible starch and resistant starch (by difference) was established.	Englyst <i>et al.</i> , 1992
1992	RS1, RS2 and RS3 classification of resistant starch was introduced.	Englyst <i>et al.</i> , 1992
1993	Hi-Maize™, the first commercially available resistant starch ingredient from high-amylose maize starch, was released.	Brown <i>et al.</i> , 1995
1993	Chemically modified starch was alluded to as another form of resistant starch.	Eerlingen <i>et al.</i> , 1993
1995	Chemically modified starch was independently reported by two laboratories to represent RS4 classification.	Eerlingen and Delcour, 1995; Brown <i>et al.</i> , 1995
1995–1996	The Englyst method for <i>in vitro</i> assay of resistant starch was validated <i>in vivo</i> .	Silvester <i>et al.</i> , 1995; Englyst <i>et al.</i> , 1996
2000–2001	Low prevalence of ‘Western’ diseases among Africans was associated with adequate consumption of resistant starch, rather than the non-starch polysaccharide components of dietary fibre.	Ahmed <i>et al.</i> , 2000; Topping & Clifton, 2001
2001	American Association of Cereal Chemists issued a revised definition of dietary fibre to include resistant starch under analogous carbohydrates; Institute of Medicine of the National Academy of Sciences issued a new definition for dietary fibre, functional fibre and total fibre, with resistant starch falling under the classification of functional fibre.	American Association of Cereal Chemists, 2001; Institute of Medicine, 2001
2002	Direct method for quantifying resistant starch was developed for RS1, RS2 and RS3.	McCleary <i>et al.</i> , 2002
2006	RS5 classification was proposed to represent amylose-lipid complexes organized in the V-type polymorph.	Brown <i>et al.</i> , 2006
2009	Codex Alimentarius Commission issued a new definition for dietary fibre to include resistant starch under carbohydrate polymers obtained from food raw material by physical, enzymatic or chemical means.	Codex Alimentarius Commission, 2009
2010–2011	Methods to determine soluble, insoluble, and total dietary fibre (Codex definition) were developed.	McCleary <i>et al.</i> , 2010, 2011
2012	Health Canada issued a new definition for dietary fibre to include resistant starch under novel fibres.	Health Canada, 2012

starch formed as a result of food processing. RS was classified into three types (RS1, RS2 and RS3) as discussed by Englyst *et al.* (1992):

- RS1 pertains to physically-entrapped starch present in whole or coarsely-ground grains and pulses where the starch granules are encapsulated within a cell wall so that amylase enzymes are prevented or delayed from having access to the glycosidic bonds.
- RS2 is raw starch with inherent enzyme resistance by virtue of its crystalline structure.
- Recrystallized or retrograded amylose represents the RS3 type.

Eerlingen *et al.* (1993) alluded to the presence of another type of RS in food, which they described as chemically modified starch to represent starch that is rendered indigestible to amylase enzymes by virtue of their chemical modification. Chemically modified starches can inhibit or reduce starch digestibility by their functional groups blocking access of amylase enzymes, or by the presence of atypical linkages such as (1 → 2)- and (1 → 3)-glycosidic bonds that are not recognized by amylase enzymes.

A fourth type of RS (i.e. RS4) was introduced independently by Eerlingen & Delcour (1995) and Brown *et al.* (1995) into the existing three types or classes of RS. Brown *et al.* (2006) then added a fifth type of RS (i.e. RS5) to represent amylose-inclusion complexes with lipids that exist as the V-type crystals. It is worth noting that guest molecules such as emulsifiers and surfactants can also form helical complexes with amylose and, to a lesser extent, amylopectin (Krog, 1971; Evans, 1986; Eliasson, 1998; Faergemand & Krog, 2006).

### **3.3 STARCH MODIFICATION YIELDING INCREASED RESISTANCE TO ENZYME DIGESTIBILITY**

Chemically modified food starches are traditionally used as ingredients in the food industry to enhance the processing performance, physical attributes, sensory properties and storage stability of consumer packaged food products. Production of modified starches for food use includes acid treatment, oxidation, esterification and etherification, or combinations of modification (Huber & BeMiller, 2010; Mason, 2009).

Starch granules are penetrable by low molecular weight, water-soluble solutes (or chemical reactants), but molecules larger than 1000 Daltons are effectively excluded (Brown & French, 1977). The pathway of entry is probably through the pores and channels located on the granule surface

(Huber & BeMiller, 2000; Kim & Huber, 2008; Han *et al.*, 2006). These granular features and other factors influence the chemical reactions occurring in the granule.

Huber & BeMiller (2001, 2010) summarized the intrinsic factors (botanical source, granule morphology, molecular structure, composition, starch properties) and extrinsic factors (reaction medium, reaction temperature, amount of chemicals, type of chemicals, addition of salts, presence of catalysts) that influence the chemical modification of starch. Biliaderis (1982) stated that factors influencing the rate and selectivity of starch modification reactions at the macromolecular level consisted of the reactivity of the starch hydroxyl groups, specific area and organization of the starch granule, diffusion rate and reactivity of the co-reactant, and steric factors due to bulkiness of the substituent groups.

Other researchers discovered that cereal and legume starches are less susceptible to structural modification than root and tuber starches (Singh *et al.*, 1993). Corn and amaranth starches displayed differences in optimum reaction conditions during the preparation of sodium carboxymethyl derivatives (Bhattacharya *et al.*, 1995), while maize starches with a differing amylose/amylopectin ratio exhibited variations in the distribution of hydroxypropyl groups on the glucan chains (Azemi & Wooton, 1995).

Even though a combination of factors appears to dictate the rate and selectivity of the modification reaction, Biliaderis (1982) and Hood & Mercier (1978) suggested that the nature of a modifying agent is more important in determining the substituent distribution in starch chains than the physico-chemical characteristics of the granule. When the degree of substitution (DS) increased to a relatively high value (e.g. DS = 0.85–2.89), a disorganization of the granule crystal structure occurred (Chi *et al.*, 2008). Small-angle X-ray scattering measurements of acylated starches revealed that a longer substituent chain like butyrate is accommodated easily within the crystalline lamellar structure, compared to acetate and propionate chains, leaving the nanostructure practically unchanged (Lopez-Rubio *et al.*, 2009).

At the molecular level, substituent groups are distributed unevenly (both between starch molecules and over the length of molecules), indicating that starch granules react heterogeneously (Huber & BeMiller, 2001, 2010). The DS of the amylose fraction was much higher than that of the amylopectin fraction, suggesting a difference in reactivity of amorphous and crystalline regions (Huang *et al.*, 2007).

Hoover & Sosulski (1986) hypothesized that derivatization sites for phosphorus oxychloride (POCl<sub>3</sub>) occurred in the amorphous regions of the granule, because no difference was observed between the x-ray diffractions of the cross-linked and unmodified starches. Because POCl<sub>3</sub> (or its first

hydrolysis product, HOPOCl<sub>2</sub>) is highly reactive, it reacts with the first starch molecules it encounters near the surface of the granules.

The location of modifying groups was proposed earlier by Biliaderis (1982) when he introduced two possible structures for chemically modified amylopectin. Fast-reacting reagents such as acetic anhydride or POCl<sub>3</sub> tended to react at or near the surface of a granule, where the tips (or non-reducing ends) of the amylopectin cluster chains are located. Slow-reacting reagents like propylene oxide tended to penetrate deeper to react not only near the tips of amylopectin cluster chains, but also in the amylopectin region, where there is dense branching. The location of phosphate cross-links may be assumed to be uniformly distributed near the non-reducing ends and the branch points in amylopectin, as noted by Manelius *et al.* (2000) for the reaction of a cationic epoxide with potato starch. Both locations comprise the amorphous region of the starch granule.

According to Huber & BeMiller (2000, 2001), POCl<sub>3</sub> reaction with potato and sorghum starches occurred at the granule surfaces, channels and cavities, and likely within the granule matrix, as revealed by scanning electron microscopy with compositional backscattered electron imaging. However, POCl<sub>3</sub> reacted with potato starch largely on the surface of the granule, because potato starch granules lack pores and channels. It was observed that the penetration of POCl<sub>3</sub> through the perimeter surface of the granules inward was somewhat impeded by a highly associated 'outer shell'.

A number of researchers employing *in vitro* and *in vivo* techniques reported that chemical modification of starches restricted to various degrees the hydrolytic action of amylase enzymes. For example, modified raw or gelatinized starch with varying levels of cross-linking and/or substitution had differential reduction of susceptibility to amylase action (Bjorck *et al.*, 1989; Hoover & Sosulski, 1986; Wolf *et al.*, 1999; Wooton & Chaudhry, 1979; Conway & Hood, 1976; Janzen, 1969; Ackar *et al.*, 2010; Klaushofer *et al.*, 1978; Xie & Liu, 2004; Xie *et al.*, 2006; Wepner *et al.*, 1999; Kim & Huber, 2008; Leegwater & Luten, 1971; Azemi & Wooton, 1995; Hood & Arneson, 1976; Hahn & Hood, 1980; Annison *et al.*, 1995, 2003; Bajka *et al.*, 2007; Wolf *et al.*, 2001; Wang *et al.*, 2001; Woo & Seib, 2002).

On the basis of the above-mentioned studies, the mechanism of amylase resistance of RS4 resistant starch can be summed up as follows. The substituent groups along the  $\alpha$ -1,4 D-glucan chains hinder enzymatic attack and also make neighbouring glycosidic bonds resistant to degradation. The presence of cross-linked polymer chains inhibits granular swelling and also provides steric hindrance to the approach of the active site of amylase enzymes. Moreover, cross-linking of starch may restrict the movement of  $\alpha$ -amylase through the granule surface pores and channels. Unusual

glycosidic bonds, such as  $\alpha$ -1,2,  $\alpha$ -1,3 or  $\beta$ -1,6 bonds, which form during pyrolysis reaction on starch, are not substrates for amylases.

### 3.3.1 Cross-linked RS4 starches

Depending on the level of modifying chemicals, cross-linking of starch granules from different botanical sources had the general effect of maintaining granular integrity, restricting swelling, resisting mechanical shear and reducing paste viscosity (Huber & BeMiller, 2010; Mason, 2009). The degree of cross-linking of starches used as thickeners in food products is too low to elicit significant resistance to  $\alpha$ -amylase hydrolysis (Wurzberg & Vogel, 1984; Ostergard *et al.*, 1988; Woo & Seib, 2002). Numerous cross-linking agents have been reacted with starch, such as  $\text{POCl}_3$ , sodium trimetaphosphate (STMP), epichlorohydrin, adipic anhydride, citric acid and glutaric acid. These inorganic and organic reagents are said to have bi- or poly-functional reactive groups. That is, they react with two or more hydroxyl groups on the starch molecular chains.

Early descriptions in the patent literature of cross-linking starch were those of Felton & Schopmeyer (1943), utilizing 0.005–0.25%  $\text{POCl}_3$  at pH 8–12, and Van Patten & Powell (1969), who improved the thickening ability of starch by adding trisodium phosphate prior to the cross-linking reaction with  $\text{POCl}_3$ .  $\text{POCl}_3$ -treated starch has a monoester:diester ratio of 1:3, based on the thallium:phosphorus ratio assayed by inductively coupled plasma-atomic emission spectroscopy (Koch *et al.*, 1982). Chemical modifications such as phosphate cross-linking inhibit alpha-amylase hydrolysis, as reported by different researchers (Bjorck *et al.*, 1989; Hoover & Sosulski, 1986; Wolf *et al.*, 1999; Wooton & Chaudhry, 1979; Conway & Hood, 1976; Janzen, 1969).

The presence of cross-linked residues across the glucan chains provides steric hindrance to the action of amylase enzymes. Analysis of cross-linked granular corn starch by a combination of gel permeation chromatography, enzymatic hydrolysis and  $^{31}\text{P}$ -NMR confirmed that amylose was cross-linked with amylopectin, no cross-linkages among amylose molecules existed, and that amylose molecules were molecularly interspersed inside the granule (Jane *et al.*, 1992; Kasemsuwan & Jane, 1994).

Low levels of cross-linking of potato starch by 0.05–0.10%  $\text{POCl}_3$  had no effect on pancreatin digestion of the gelatinized starch (Janzen, 1969), but considerable inhibition of starch hydrolysis by pancreatin was displayed at higher levels of  $\text{POCl}_3$  treatment (0.5–1.5%). Digestibility of raw or gelatinized starch to pancreatic  $\alpha$ -amylase was very slightly reduced (1–3%) by low level cross-linking ( $\approx 0.0825\%$   $\text{POCl}_3$ ) of lentil, faba bean and field pea starches (Hoover & Sosulski, 1986). Using epichlorohydrin as a cross-linking

agent at 0.1%, 0.3% and 0.5% levels, starch from two Croatian wheat varieties (Ackar *et al.*, 2010) yielded low levels of resistant starch (0.31–2.25%) compared to the respective native wheat starches (0.48–0.58%). By comparison, 0.06–0.12% STMP treatment of waxy maize starch slightly reduced the four-hour percent hydrolysis (13.0–13.7%) of the gelatinized starch by hog pancreatic  $\alpha$ -amylase compared to the gelatinized unmodified waxy maize starch (15.8%) (Hahn & Hood, 1980).

Wheat starch cross-linked with 0.1%  $\text{POCl}_3$  or 0.3% epichlorohydrin did not generate significant RS when assayed for total dietary fibre by AOAC Method 991.43 (Woo & Seib, 2002). However, reaction with 1–2%  $\text{POCl}_3$  or 1–2% epichlorohydrin yielded 52.7–85.6% total dietary fibre and 57.4–75.8% total dietary fibre, respectively. The cross-linking conditions were 33% starch solids, 15%  $\text{Na}_2\text{SO}_4$ , pH 11.5, reaction temperature of 25 °C, and one hour reaction time. The use of 12% of a 99:1 blend of STMP and sodium tripolyphosphate (STPP) as modifying agents at approximately the same reaction conditions as above, except for 10%  $\text{Na}_2\text{SO}_4$ , reaction temperature of 45 °C, and three hours reaction time, resulted in a phosphorylated cross-linked RS4 product with low swelling power ( $\approx 3$ ), 0.32% phosphorus and 75.7% total dietary fibre. At a 10% level of 99:1 ratio of STMP/STPP, the level of phosphorus is 0.32% and total dietary fibre is 75.6%, but other ratios (25:75, 50:50, or 75:25) of STMP/STPP generated lower phosphorus incorporation (0.13–0.29%) and diminished total dietary fibre (21.6–63.7%).

General cross-linking conditions of 0–20%  $\text{Na}_2\text{SO}_4$ , 10–19% STMP/STPP (99:1 ratio), pH 11.5–12.3, reaction temperature of 25–70 °C and reaction time of 0.5–12 hours were utilized for preparation of different phosphorylated cross-linked RS4 wheat starches with 0.32–0.35% phosphorus and 75.7–88.1% total dietary fibre. Phosphorylated cross-linked RS4 starches from normal wheat, waxy wheat, normal corn, waxy corn and potato showed 0.32%, 0.32%, 0.34%, 0.33% and 0.32% phosphorus and 76%, 80%, 35%, 58% and 73% total dietary fibre, respectively.

Four phosphorylated cross-linked RS4 wheat starches with phosphorus contents of 0.13–0.38% and total dietary fibre levels of 14.0–93.4% (Woo & Seib, 2002) were analyzed for the amount of RS by the Englyst method (Englyst *et al.*, 1992). The resulting RS levels by this method, before and after gelatinization of the samples, correlated directly with total dietary fibre using AOAC Method 991.43. Actual RS values ranged from 35.5–66.1% for the ungelatinized samples and 2.6–15.2% for the gelatinized samples. Corn starch cross-linked with 4%, 8%, and 12% of a 99:1 mixture of STMP/STPP introduced 0.14–0.37% phosphorus to the granules, which was directly proportional ( $R^2 = 0.9971$ ) to the amount of RS (24.5–81.6%), as measured by AOAC Method 991.43 (Chung *et al.*, 2004).

Woo *et al.* (2006) reported on a pregelatinized, phosphorylated, cross-linked RS4 starches from wheat, potato and tapioca that exhibited resistance to  $\alpha$ -amylase digestion, possessed fat replacement properties and demonstrated stability to freeze-thaw processing. This particular pregelatinized RS4 starch was formulated in a number of reduced-fat foods, namely bakery products, instant mashed potato, sausages, salad dressings, desserts, ice cream, yogurt and cr me filling.

Although Kerr & Cleveland (1957) pioneered the cross-linking of starch at alkaline pH using STMP, its combination with small levels of STPP during the reaction formed the basis for patenting a phosphorylated cross-linked food-grade starch resistant to  $\alpha$ -amylase and the method of preparing the same product (Seib & Woo, 1999). Analysis of phosphodextrins from  $\alpha$ -amylase and glucoamylase digestion of phosphorylated cross-linked RS4 wheat starch by proton-decoupled  $^{31}\text{P}$  NMR (Sang *et al.*, 2007) showed four types of phosphate esters: cyclic-monostarch monophosphate (cyclic-MSMP), monostarch monophosphate (MSMP), monostarch diphosphate (MSDP), and distarch monophosphate (DSMP), with signals centred at  $\delta$  15.9 ppm,  $\delta$  3–5 ppm,  $\delta$  –5 and –10 ppm, and  $\delta$  –1 to 1 ppm, respectively. Phosphorylation of wheat starch with STMP/STPP produced  $\approx 37\%$  MSMP and 63% DSMP, whereas the use of  $\text{POCl}_3$  changed the proportion to  $\approx 20\%$  MSMP and 80% DSMP (Sang *et al.*, 2007).

Starch hydroxyls have ionization constants between pKa 12.5–13.0 at 45 °C (Lammers *et al.*, 1993). Less than 1% of hydroxyl groups is ionized at pH 10.5, and about 5–10% are ionized at pH 11.5. Depending on the pH used during the reaction with STMP, the ratio of DSMP/MSMP can vary from 41 : 22 for pH 10.5 to approximately 65 : 35 for higher pH (11.5–12.5). The lower-reaction pH of 10.5 also produced 18% MSDP and 19% cyclic-MSMP.

The proposed reaction mechanism between starch and STMP at alkaline pH involves the initial attack by a starch alcoholate ion, resulting in ring-opening of STMP and formation of a monostarch triphosphate intermediate. This is followed by the reaction of another starch alcoholate ion with the intermediate, forming DSMP and pyrophosphate. The formation of MSDP occurs by losing the  $\gamma$ -phosphoryl group on the monostarch triphosphate by the peeling mechanism. Cyclic-MSMP and MSDP are reactive at high alkalinity (pH 11.5 for 3 hours), which causes the disappearance of cyclic-MSMP and the reduction of MSDP from 17% to 11%. The DSMP content of phosphorylated cross-linked RS4 wheat starch correlated positively ( $r = 0.96$ ;  $P = 0.02$ ) with RS content (as determined by the method of Englyst *et al.* (1992)) and with total dietary fibre content ( $r = 0.90$ ;  $P = 0.05$ ) as assayed by AOAC Method 991.43.



Sang *et al.* (2010) reported a phosphorylated cross-linked RS4 wheat starch (0.37% phosphorus, 88.8% total dietary fibre) exposed to NaOH at pH 9–11 and 40 °C for four hours that did not significantly change the phosphorus content (0.35–0.37%), even with the elevated pH treatment. A much higher alkalinity of pH 12 at the above time-temperature combination reduced the phosphorus content to 0.29%, which indicates that about 22% of covalently-bound phosphate was removed from the starch. In addition, cyclic-MSMP and MSDP disappeared, as confirmed by the absence of  $^{31}\text{P}$  NMR signals, which suggests the instability of those two types of phosphate esters at pH 12. However, the cross-linked phosphate ester, DSMP, increased to  $\approx 18\%$  after exposure at pH 12 and 40 °C for four hours, indicating that new DSMP was formed from the reaction of MSDP or cyclic-MSMP with hydroxyl groups on another starch chain. Total dietary fibre content was little changed (88.8% vs. 86.5%) upon exposure to pH 12, even though the total phosphorus decreased by 22%. The 18% increase of cross-linked phosphate esters (DSMP) compensated for the 22% loss of total phosphorus. The integrated NMR intensities of DSMP and MSMP indicated that they contained 0.17% phosphorus (or 46% of total phosphorus) and 0.077% phosphorus (or 21% of total phosphorus), respectively.

Starch citrate is the nomenclature used in the literature for the cross-linked product derived from high-temperature treatment of a low-moisture mixture of starch and citric acid, a six-carbon tricarboxylic acid. The reaction mechanism involves dehydration of citric acid to anhydride form, and this functional group consequently forms cross-linking bonds with starch molecules. Klaushofer *et al.* (1978) developed a cross-linked starch using citric acid as the primary modifying agent. The process involved drying to 5–20% moisture an aqueous mixture of starch and citric acid (5–40%, starch basis) and heating for 1–5 hours at 110–140 °C to yield starch citrate. This product exhibited decreased *in vitro* amylase digestibility with an increasing degree of esterification. In addition, the esterified starch showed improved stability to shear and freeze-thaw processing conditions.

Xie & Liu (2004) reported that for a corn starch/citric acid weight ratio of 5 : 2, pH 3.5 and a reaction temperature range of 120–150 °C for 3–9 hours, the DS varied from 0.09–0.12 and the level of RS ranged from 41.1–78.8%. An improvement of RS content to 92.9% was achieved when a reaction temperature of 150 °C was employed. On the other hand, Wepner *et al.* (1999) obtained 45.9–57.5% RS from starch citrates prepared from potato, pea, corn, and wheat starches using the following reaction conditions: starch/citric acid weight ratio of 5 : 2, pH 3.5 and five hours at 140 °C. The DS expressed as percent esterified citric acid ranged from 12.2–14.4%.

Citric acid-treated starch containing slowly digestible and resistant starch fractions was described by Shin *et al.* (2007). The preparation procedure consisted of a two-step process of autoclaving (121 °C for 30 minutes) an aqueous mixture of starch-citric acid, followed by heat treatment as high as 130 °C for up to 24 hours. The resulting product has a dextrose equivalent (DE) of 10.2, DS of 0.027, cold water solubility of 55.2%, slowly digestible and resistant starch fractions of 54.1%, and low blood glucose response in mice.

Other researchers reacted glutaric acid, a five-carbon dicarboxylic acid, with adlay starch in preparing cross-linked RS4 resistant starch and characterizing its properties (Kim *et al.*, 2008). Starch glutarate with high RS content of 65–66% was prepared from adlay starch using the following reaction conditions: 30–40% glutaric acid (starch basis), 115–130 °C, and 6–7.5 hours of reaction time. The modified product displayed a carbonyl absorption band at 1730 cm<sup>-1</sup> by FT-IR and 173 ppm and 32 ppm CP-MAS <sup>13</sup>C NMR peaks assigned to carbonyl and methylene groups, respectively, suggesting cross-linking between starch chains by esterification. It was observed that formation of β-1,6-glycosidic bonds may have also occurred during the preparation of starch glutarate.

### 3.3.2 Substituted RS4 starches

Food-grade substituted starches used for technological effects are normally treated with appropriate modifying agents (for example, acetic anhydride or propylene oxide) to yield a DS or a molar substitution (MS) of ≈0.1 (Mason, 2009). These esterified or etherified starches have the general properties of increased swelling, elevated viscosity and enhanced clarity, and their pastes have improved stability to room temperature, as well as refrigerated or freezer storage.

Pancreatin digestibility of gelatinized hydroxypropylated potato starch decreased with increasing DS (Leegwater & Luten, 1971). A logarithmic plot of DS (0.02–0.45) and reducing power (2.6–61.0 mg glucose per mmol chain unit) demonstrated that the digestibility decreased exponentially with increasing DS. In a related observation, Klaushofer *et al.* (1978) reported declining *in vitro* amylase digestibility with an increasing degree of esterification with citrate. Furthermore, an increasing degree of hydroxypropyl substitution among cereal starches resulted in a diminished *in vitro* enzymatic hydrolysis (Azemi & Wooton, 1984).

Hahn & Hood (1980) showed that gelatinized hydroxypropylated waxy maize starch (MS = 0.095–0.131) was digested to 11.4% after four hours of hydrolysis with hog pancreatic α-amylase, compared to 15.8% hydrolysis of

the gelatinized unmodified waxy maize starch. In another study, the digestibility of gelatinized wheat starch by porcine pancreatic  $\alpha$ -amylase was reduced by 13.3–23.4% by substitution with hydroxypropyl (DS = 0.06) or acetyl groups (DS = 0.07), while cross-linking with phosphate (0.05%  $\text{POCl}_3$ ) had a minor lowering effect (2.5%) on its digestibility (Wooton & Chaudhry, 1979). Etherification with hydroxypropyl groups lowers the digestibility of wheat starch to a larger extent (23.7–25.2%) than esterification with acetyl groups (10.8–13.3%), most likely due to the larger influence of the more bulky hydroxypropyl group on enzyme digestion.

The relative degree of hydrolysis to hog pancreatic  $\alpha$ -amylase (0.0175–0.30% enzyme concentration based on starch) was 75.3–80.1% for gelatinized tapioca hydroxypropyl distarch phosphate (1.6% hydroxypropyl) and 50.6–58.2% for gelatinized hydroxypropylated tapioca starch (4.2% hydroxypropyl), compared to 100% for gelatinized unmodified tapioca starch (Conway & Hood, 1976). These digestibility values are comparable to other studies conducted on hydroxypropylated starches by Leegwater & Luten (1971) and by Hood & Arneson (1976).

Highly hydroxypropylated (DS = 0.12), lightly cross-linked (0.000085%  $\text{POCl}_3$ ) waxy starch showed decreased digestibility (34.4%) to  $\alpha$ -amylase/glucoamylase compared to unmodified waxy starch (98.0%) (Wolf *et al.*, 1999). Moderately hydroxypropylated (DS = 0.07), moderately cross-linked (0.00037%  $\text{POCl}_3$ ) *dull* waxy starch displayed decreased digestibility (68.7%) compared to unmodified *dull* waxy starch (90.6%).

Due to their beneficial health effects, acylated starches that possess much higher DS compared to traditional food-grade modified starches were suggested for possible use in food products (Annison *et al.*, 2003; Bajka *et al.*, 2010). Acylated starches as a form of substituted RS4 starch was borne out of a strategy to deliver short chain fatty acids directly to the large bowel (Annison *et al.*, 1995). The 2-, 3- or 4-carbon short-chain fatty acids represented by acetic acid, propionic acid or butyric acid, respectively, can be attached to starch by an esterification process. Annison *et al.* (2003) reported on the preparation of these esters with a DS of approximately 0.20 by reacting 600 g starch with 115 ml, 180 ml or 230 ml of acetic anhydride, propionic anhydride or butyric anhydride, respectively.

Butyrate esters of corn starch tended to increase starch resistance to enzymic hydrolysis *in vitro* and to intestinal amylolysis *in vivo* (Annison *et al.*, 1995, 2003). Butyrate is released in the large bowel by the action of microbial esterases and lipases, leaving the starch backbone available for fermentation (Bajka *et al.*, 2006, 2007). A butyrylated starch product with a DS of approximately 0.3 was exceedingly resistant to amylolysis, but was still capable of liberating butyrate when exposed to faecal microflora (Bajka *et al.*,

2007). Less butyrate is released when the DS is greater than 0.3, which may be explained by decreased solubility or increased steric obstruction to the action of microbial esterases.

Waxy maize starch esterified with 3% octenyl succinic anhydride showed 28.3% slowly digestible starch, which rose to 42.8% after the succinylated starch was heat-moisture treated at 120 °C for four hours and 10% moisture (He *et al.*, 2008). Cooked 1-octenyl succinylated corn starch (DS = 0.07; DE  $\approx$  3) decreased the extent of 15-hour *in vitro* hydrolysis (70%) by  $\alpha$ -amylase/amyloglucosidase (Wolf *et al.*, 2001) compared to cooked, unmodified corn starch (99.9%). Cooking of butyrylated high-amylose corn starch increased *in vitro*  $\alpha$ -amylase/amyloglucosidase hydrolysis from 6% to 43% (Bajka *et al.*, 2006).

### 3.3.3 Pyrodextrinized RS4 starches

Pyrodextrins are conventionally produced by treatment of granular native starch at low moisture contents and elevated temperatures with varying amounts of mineral acids and, sometimes, carboxylic acids (Wurzberg, 1986; Tomasik *et al.*, 1989; Wang *et al.*, 2001; Laurentin *et al.*, 2003; Huber & BeMiller, 2010). These conditions destroy the original starch backbone structure and promote hydrolysis, transglucosidation and repolymerization. As a result, pyrodextrins have low molecular weights, possess atypical glycosidic linkages (i.e. (1  $\rightarrow$  2)-, (1  $\rightarrow$  3)-glycosidic bonds) and contain new branched structures.

Using dextrinization conditions of pH 2.5–2.6, a temperature of 170 °C, and a three-hour reaction time, Wang *et al.* (2001) demonstrated, by reducing sugar analysis, that hydrolysis occurred during the first hour, followed by transglucosidation and repolymerization in the remaining two hours. This result agreed with the low content of enzyme resistant dextrins during the first hour of pyrodextrinization, followed by a rapid increase of enzyme-resistant dextrins in the next two hours.

Pyrodextrinization, followed by amylase treatment, was the basis for a number of patents to produce soluble dietary fibres whose nomenclature may fall under different names, such as indigestible dextrins, resistant dextrins, resistant maltodextrins or digestion-resistant maltodextrins (Ohkuma *et al.*, 1994, 1995, 1997). A product of this type dissolves into a clear and stable aqueous solution with low viscosity. It has a DE of 10–12, an average molecular weight of 2000 Da, and its structure is composed of (1  $\rightarrow$  2)-, (1  $\rightarrow$  3)-, (1  $\rightarrow$  4)-, and (1  $\rightarrow$  6)-glycosidic linkages and levoglucosan (Ohkuma & Wakabayashi, 2001; Okuma & Kishimoto, 2004). It is approximately 90% indigestible, with a low caloric count (0.5 kcal/g). Resistant

maltodextrins that are available commercially vary in total dietary fibre content, ranging from 52–90%, and they are being offered either as a hydrogenated version, in agglomerated form, in admixture with honey solids and stevia glycosides, or in liquid corn syrup version (75% solids).

Another manufacturing process for resistant dextrins involves controlled dextrinization, wherein the starch undergoes a degree of hydrolysis followed by repolymerization (Lefranc-Millot *et al.*, 2010). This process converts starch into dietary fibre through the formation of non-digestible glycosidic bonds that deter cleavage by amylase enzymes. When analyzed by AOAC Method 2001.03, the resistant dextrin obtained in this process contains 85% total dietary fibre, which consists of 50% insoluble fibre in ethanol and 35% resistant oligosaccharides. In another study, lightly, moderately and highly converted dextrins from common corn starch displayed digestibilities of 90.1%, 84.2% and 63.8%, respectively, when treated with a mixture of  $\alpha$ -amylase and glucoamylase, whereas raw common corn starch has 97.9% digestibility (Wolf *et al.*, 1999).

Formation of enzyme-resistant fractions during pyrodextrinization was shown to be affected by the botanical source of starch (Laurentin *et al.*, 2003). These researchers also revealed that granule morphology of native starch was similar to that of pyrodextrinized starch, and that the latter exhibited no endotherm by differential scanning calorimetry (DSC). Extensive depolymerization of pyrodextrinized starches occurred as evident in the gel filtration profile, and the amount of starch hydrolysable by the combined action of Termamyl  $\alpha$ -amylase and amyloglucosidase was significantly decreased.

### 3.4 PHYSICOCHEMICAL PROPERTIES AFFECTING FUNCTIONALITY

The chemical name of a phosphorylated cross-linked RS4 starch produced by treatment with a mixture of STMP and STPP at a 99 : 1 ratio (Seib & Woo, 1999) is phosphated distarch phosphate (E-Number 1413 and CAS No. 977043-58-5). The use of these two phosphate-modifying agents is regulated to yield a food-grade product with no more than 0.4% phosphorus, as specified in the section on Food-Starch Modified of the US Code of Federal Regulations Title 21 Part 172.892 (Code of Federal Regulations, 2010). Commercial-scale production yielded a product that delivered a minimum of 85% (dry basis) total dietary fibre with an average of  $\approx$ 94% (dry basis) total dietary fibre by AOAC Method 991.43 (Woo *et al.*, 2009). The fibre exists primarily as insoluble fibre. A phosphorylated cross-linked RS4 wheat starch was

described in a US patent publication to contain 93.4% total dietary fibre and 0.38% phosphorus (Seib & Woo, 1999).

Phosphorylated cross-linked RS4 starches from wheat, corn, potato and rice are practically insoluble in dimethyl sulphoxide or 1 M KOH (Woo & Seib, 2002; Shin & Seib, 2004). Scanning electron microscopy showed the same shape and smooth surface as their parent starches (Seib & Woo, 1999). Pasting curves at 8% starch solids did not rise above the baseline when heated from 30 °C to 95 °C. Wide-angle x-ray diffraction showed phosphorylated cross-linked RS4 starches from cereal, and potato sources gave A- and B-type polymorphic crystal patterns, respectively.

When analyzed by DSC, the transition temperatures of phosphorylated cross-linked RS4 potato starch were slightly affected. In contrast, the other cross-linked RS4 starches from normal wheat, waxy wheat, normal corn and waxy corn gave elevated  $T_o$ ,  $T_p$ , and  $T_c$  by 4.3–8.4 °C, 5.4–8.3 °C, and 3.6–10 °C, respectively (Woo & Seib, 2002). Enthalpy of gelatinization tended to be lower for all the RS4 starches, except for RS4 potato starch. It was surmised that annealing of starch occurred during exposure to the cross-linking reaction conditions, and that cross-linking could inhibit cooperative melting of crystals in starch granules (Woo & Seib, 2002; Jacobs *et al.*, 1995).

No significant differences in DSC transition temperatures and enthalpies of gelatinization were found after a phosphorylated cross-linked RS4 wheat starch was held at pH 9.0–12.0 for four hours at 40 °C (Sang *et al.*, 2010). This alkaline pH treatment did not alter its typical A-type x-ray diffraction pattern or its degree of crystallinity.

The phosphorus content of phosphorylated cross-linked RS4 corn starch modified with 4–12% of a 99:1 blend of STMP/STPP was inversely proportional ( $R^2 = -0.9537$ ) to the swelling volume of the cross-linked product (Chung *et al.*, 2004). Cross-linking has the added effect of increasing the glass transition temperature of corn starch in excess water by nearly 1 °C, which was explained as a reduction in chain mobility, such that a higher temperature ( $T_g$ ) is required to induce movement. On the other hand, the glass transition temperature of the cross-linked starches measured at 15% moisture decreased by  $\approx 1$  °C, which was attributed to internal plasticization of starch by the ionic phosphate groups. Furthermore, the retrogradation enthalpy after one week of storage of the gelatinized starches (67% moisture) at 4 °C was decreased by cross-linking.

Cross-linking of banana starch with 11.9% STMP and 0.1% STPP raised the DSC peak temperature of gelatinization to 86.6 °C, compared to 79.2 °C for the parent native banana starch, while the enthalpy of gelatinization decreased to 9.36 J/g from 25.1 J/g for the parent native banana starch (Aparicio-Saguilan *et al.*, 2008). Autoclaving at 121 °C for one hour, followed

by storing at 4 °C for 24 hours, decreased the RS content (total dietary fibre assay) of phosphorylated cross-linked banana starch from 94.68% to 85.35%. As a result of autoclaving and cooling treatments, the peak temperature and enthalpy of gelatinization decreased to 68.5 °C and 1.5 J/g, respectively.

When a phosphorylated cross-linked RS4 wheat starch was heated in a cooking viscometer (8% solids) from 30 °C to 95 °C, no pasting curve (i.e. flat line) was generated, which is indicative of the highly restricted swelling of the cross-linked starch granules (Woo & Seib, 2002; Xie & Liu, 2004). The granules remained intact after heating, and it was observed that disintegrated or convoluted granules were not evident, and neither were granule ghosts. DSC analysis of phosphorylated cross-linked RS4 wheat starch demonstrated elevated initial, peak, and conclusion temperatures (4.3–10.5 °C higher), but a slight reduction in enthalpy of gelatinization (0.9 J/g lower) compared to the parent native wheat starch.

When heated from 35 °C to 60 °C in excess water, the granules of a commercial phosphorylated cross-linked RS4 wheat starch maintained their structures essentially unchanged (Ratnayake & Jackson, 2008). Above 65 °C and up to 85 °C, the granules became increasingly swollen. Coinciding with this observation, the DSC enthalpies of gelatinization did not change during the 35–60 °C treatment, but gradually disappeared between 60 °C and 85 °C. X-ray crystallinity gradually decreased within the same temperature range of 60–85 °C, but the A-type polymorph was preserved up to 70 °C. Consistent with previous studies (Seib & Woo, 1999; Woo & Seib, 2002), Ratnayake & Jackson (2008) found that phosphorylated cross-linked RS4 wheat starch did not completely dissolve in 90% dimethyl sulphoxide.

A phosphorylated cross-linked RS4 wheat starch with 72.9% total dietary fibre exhibited a low swelling power of 2.8 g/g and low solubility in water of 0.5% at 95 °C (Woo & Seib, 2002). Compared to other cereal starches, it displayed similar water vapour sorption and desorption isotherms at 25 °C and at water activities below 0.8 (Shin *et al.*, 2003).

Lefranc-Millot *et al.* (2010) described a resistant pyrodextrin that has a clean, neutral taste, possesses no sweetness and is compatible with sugar-free claims. The commercial product dissolves rapidly in aqueous systems and has limited impact on viscosity. It is stable to high temperature, variable pH and high shear processing. The resistant dextrin is officially recognized as a soluble dietary fibre, with suggested ingredient labelling of 'dextrins' in food product packages.

Consistent with heavily cross-linked starches, corn starch citrates resisted granular swelling as exhibited by the flat RVA pasting curve (i.e. 0 RVU) when heated from 50 °C to 95 °C at 8% starch solids (Xie & Liu, 2004). Heating starch citrates in water (7% solids) at 100 °C for 30 minutes resulted in

9.8–16.8% loss in RS content, indicating intermediate thermal stability of this product. The DSC enthalpy of gelatinization tended to decrease in starch citrates (0.4–3.0 J/g), compared to the native corn starch (6.7–15.6 J/g).

Analysis by DSC demonstrated that adlay starch glutarates with RS content of 30–66% exhibited lower  $T_o$  (46.0–49.9 °C),  $T_p$  (60.5–65.6 °C),  $T_c$  (73.1–73.9 °C), and  $\Delta H$  (5.7–9.5 J/g) compared to control starch ( $T_o$ , 60 °C;  $T_p$ , 71.3 °C;  $T_c$ , 82.6 °C;  $\Delta H$ , 13.3 J/g), presumably because of the disruption of molecular (double helical) and crystalline orders during the preparation of the glutarates (Kim *et al.*, 2008). Hot water solubility at 80 °C of the glutarate derivatives was drastically reduced to 0.71–1.52%, compared to 81.2% for the control starch. When subjected to  $\alpha$ -amylase and amyloglucosidase treatments, gelatinized starch glutarate lost its granular shape and its granules cohered together. Its good stability to heat treatment in excess water was demonstrated when the RS content before heating of starch glutarate was shown to be similar to that after heating.

### 3.5 PHYSIOLOGICAL RESPONSES AND HEALTH BENEFITS

Dietary fibre sources, including RS, promote a number of physiological benefits in humans, which include, but are not limited to, the following: decreased intestinal transit time; enhanced satiety; reduced postprandial blood glucose and/or insulin levels; diminished blood total and/or low-density lipoprotein cholesterol concentrations; and fermentability by colonic microbiota to give short-chain fatty acids (SCFA) (Baghurst *et al.*, 1996; Topping & Clifton, 2001; Nugent, 2005; Topping, 2007; Witwer, 2008; Buttriss & Stokes, 2008; Bird *et al.*, 2009).

Different types of RS can elicit significantly different glycemic responses, as demonstrated by a comparison between RS2 high-amylose corn starch and phosphorylated cross-linked RS4 wheat starch in human subjects (Haub *et al.*, 2010). The glucose response and the incremental area under the curve of phosphorylated cross-linked RS4 wheat starch were significantly decreased, compared with RS2 high-amylose corn starch and dextrose (control).

Aqueous dispersions of two RS4 potato starches (38 grams in 296 ml of water), when consumed as a drink (Haub *et al.*, 2012), elicited a significantly decreased glycemic response compared to dextrose (control). There were no blood glucose response differences and no satiety effects when the same doses of RS4 potato starches were added to dextrose and compared with dextrose alone. Rice kernels coated with RS4 wheat starch using agar and locust bean



gum had lower *in vitro* starch digestibility, a decreased glucose response in rats and a slower rate of blood glucose decrease, compared to uncoated rice and rice mixed with RS4 wheat starch (Choi *et al.*, 2010).

Using the glycemic index protocol, nutritional bars formulated with 34 weight percent of phosphorylated cross-linked RS4 wheat starch displayed attenuated postprandial blood glucose and insulin levels, when compared to a control glucose drink and to another nutritional bar in which the above RS4 wheat starch was replaced by an equivalent amount of puffed wheat (Al-Tamimi *et al.*, 2010). Incremental areas under the glucose curves were 140, 84 and 28 mmol/l  $\times$  2 hour for the glucose control drink, puffed wheat bar and cross-linked RS4 bar, respectively. The corresponding incremental areas under the insulin curves were 17 575, 8758, and 3659 pM  $\times$  2 hour, respectively. The results are noteworthy, as the bar formulas contain high levels of glycemic carbohydrates, in the form of 11% brown sugar and 20% corn syrup.

Other researchers compared the properties *in vivo* of phosphorylated cross-linked RS4 wheat starch and RS2 high-amylose corn starch in order to gauge their impact on gastrointestinal microbiota composition and metabolism in human volunteers (Martinez *et al.*, 2010). During the three-week feeding period, both types of resistant starch were well tolerated by the subjects when fed daily with 100 grams of snack crackers (containing 33 grams of dietary fibre contributed by the resistant starch ingredient). Pyrosequencing of faecal samples demonstrated that, at the phyla level, RS4 crackers significantly decreased Firmicutes and increased Bacteroidetes and Actinobacteria. These changes were associated with a decrease in the family Ruminococcaceae and increases in the genera Parabacteroidetes and Bifidobacterium.

Denaturing gradient gel electrophoresis revealed that RS4 crackers induced a swift and reversible increase in band intensity of *Bifidobacterium adolescentis*, whereas RS2 crackers caused a gradual rise. Quantitative enumeration of bifidobacteria by qRT-PCR confirmed the significant increase in cell numbers during consumption of RS4 and RS2 crackers, compared to the control crackers (no resistant starch). The total cell numbers of bifidobacteria increased more than three-fold with RS4 crackers, while RS2 crackers doubled the cell numbers.

*In vitro* digestion of phosphorylated cross-linked RS4 wheat starch and phosphorylated cross-linked RS4 potato starch by successive treatments with pepsin and pancreatin-bile yielded 82% and 74% of indigestible residues, respectively (Thompson *et al.*, 2011). Incubation of these indigestible residues with fresh human faecal inoculum produced gases that increased linearly over the 24-hour fermentation period, with the two RS4 starches exhibiting similar gas production rates as well as similar rates of production of total short-chain fatty acids. Acetic acid was the major SCFA produced, along with relatively

higher amounts of butyric acid than propionic acid. The fractional molar ratios of the above acids (acetic : propionic : butyric) are 0.586 : 0.186 : 0.228 and 0.577 : 0.200 : 0.223 for RS4 wheat starch and RS4 potato starch, respectively. Caloric contribution was calculated to be one-third lower for the two RS4 starches, compared to unmodified (non-resistant) starch.

In an animal feeding trial, hamsters on a phosphorylated cross-linked RS4 wheat starch diet consumed less feed and gained less weight than those on a cellulose diet (Seib & Woo, 1999). Total serum cholesterol in the animals was not significantly different between the two diets, but the concentration of high-density lipoprotein cholesterol was significantly higher in the RS4 diet. Consequently, the calculated levels of low- and very low-density lipoprotein cholesterols were less in the serum of animals consuming the RS4 diet than the cellulose diet. Analysis of fermentation products from the caecum of hamsters showed elevated levels of SCFA (in particular butyric acid) in the RS4 diet, compared to the cellulose diet.

In mice fed for six weeks with a high-fat diet, supplemental addition at a 15% level of phosphorylated cross-linked RS4 corn starch and phosphorylated cross-linked RS4 high-amylose corn starch lowered body weight gain as well as total lipid, triglyceride and total cholesterol in the serum and liver, compared to the corresponding unmodified starches (Lee *et al.*, 2012). Rats fed for four weeks with a diet containing 5% phosphorylated cross-linked RS4 corn starch (92.6% total dietary fibre) exhibited lower serum levels of total cholesterol (12.6% decrease) and low density lipoprotein plus very low density lipoprotein cholesterol (24.3% decrease) than rats fed with a diet containing 5% cellulose (Song *et al.*, 2010). In addition, this RS4 corn starch can potentially ameliorate allergic inflammation in the mesenteric lymph nodes of rats, because of its impact on elevating serum immunoglobulin A levels, as well as the CD4<sup>+</sup> T cell population and the ratio of CD4<sup>+</sup>/CD8<sup>+</sup> T cells.

Annisson *et al.* (2003) summarized the strategies to elevate SCFA in the large bowel as follows:

- a) Increase the consumption of foods high in RS content.
- b) Consume SCFA directly in foods or beverages.
- c) Consume foods that contain starches esterified with specific SCFA.

The rat feeding study by these researchers confirmed that starches esterified with specific SCFA through reaction with acetic, propionic or butyric anhydride resisted digestion in the small intestines and delivered those SCFAs into the large bowel, where they were released by the action of the microflora. The selective increase of individual SCFA was observed even only after three days,

which indicates that the rat microflora can readily adapt. Esterified starches with a DS of around 0.20–0.25 have the potential to improve the nutritional properties of foods through delivery of SCFA directly to the large bowel.

Ingestion by ileostomists of starches esterified with acetate, propionate or butyrate with DS of 0.23–0.25 resulted in the recovery of 73–76% of the esterified acid at the terminal ileum, which suggests that SCFA delivery to the large bowel could occur in humans with an intact gastrointestinal tract (Clarke *et al.*, 2007). Free faecal butyrate concentrations were increased in human subjects after consumption of butyrylated high-amylose corn starch, and about 27% of total esterified butyrate was absorbed in the small intestine, 15.8% was recovered in the faeces, and about 57.2% was released in the large bowel (Clarke *et al.*, 2011a). The population of *Parabacteroides distasonis* was also increased by the above-mentioned butyrylated starch derivative.

Reduction in DNA single-strand breaks was twice that in rats fed butyrylated high-amylose corn starch compared to unesterified high-amylose corn starch, which was interpreted as the greater ability of the butyrylated starch to deliver butyrate to the large bowel, thereby raising the concentration of this particular SCFA (Bajka *et al.*, 2008). High-protein diets increased colonocyte DNA single-strand breaks in rats, but this effect was opposed by inclusion in the diet of butyrylated high-amylose corn starch (Conlon *et al.*, 2012). Butyrylated high-amylose corn starch protects colonocytes in rats from genetic damage by high dietary protein (Bajka *et al.*, 2008), which may be explained by increased caecal butyrate pools.

Bajka *et al.* (2006) studied the effects of feeding high-amylose corn starch and butyrylated high-amylose corn starch in rats and the effect of cooking on resistance to *in vitro* and *in vivo* amylolysis. Cooking of butyrylated high-amylose corn starch increased *in vitro*  $\alpha$ -amylase/amyloglucosidase hydrolysis from 6% to 43%. Cooked butyrylated high-amylose corn starch delivered significantly greater amounts of esterified butyrate in the rats' large bowel, compared to raw or cooked high-amylose corn starch. The caecum is the main site of bacterial fermentation of carbohydrates in rats. By comparison, ingestion of butyrylated low-amylose corn starch by rats resulted in lower digesta pH in the large bowel and elevated butyrate concentrations in the caecal, proximal and distal colon, as well as in the portal vein (Bajka *et al.*, 2010).

Butyrylated high-amylose corn starch (DS = 0.25) affected the faecal microbiota composition of rats treated with azoxymethane, a carcinogenic agent (Abell *et al.*, 2011). It increased propionate and butyrate concentrations in distal colonic digesta and was associated with the appearance of *Parabacteroidetes distasonis*, *Lactobacillus gasseri* and *Phascolarctobacterium faecium*. Following acute exposure to azoxymethane, rats fed with butyrylated

high-amylose corn starch had higher caecal tissue and digesta weights and lower pH in the large bowel, compared with rats fed with unmodified low-amylose and high-amylose corn starches (Clarke *et al.*, 2011b). Morphological assessment of the rats' distal colonic epithelium showed that butyrylation has a positive impact, as indicated by an elevated apoptotic index. Butyrate concentrations in hepatic portal plasma, as well as in caecal and proximal and distal colonic digesta, were higher in azoxymethane-treated rats fed with butyrylated high-amylose corn starch, compared to non-acylated low-amylose and high-amylose corn starches (Clarke *et al.*, 2008). This butyrate effect is associated with reduced tumour incidence, number and size in rats.

Net incremental area under the blood glucose curve (Wolf *et al.*, 2001) was lower ( $P < 0.05$ ) after fasting healthy adult subjects consumed 25 g of 1-octenyl succinylated starch (107 mmol.min/l) than that for 25 g of glucose (127 mmol.min/l). A blunted glycaemic response was displayed by 1-octenyl succinylated starch, as indicated by the calculated relative glycaemic response of 93.8% compared to that of glucose.

Digestibility in the small intestine of a resistant pyrodextrin (Lefranc-Millot *et al.*, 2010) is in the range of 8.7–19%, which results in a low glycaemic response ( $\approx 25$ ) and low insulinemic response ( $\approx 13$ ). The low insulinemic response is reported to induce a high satiety feeling and to reduce postprandial hypoglycaemia. When the resistant pyrodextrin was formulated in pasta, beverages and biscuits, consumption of these food products displayed low glycaemic responses (Lefranc-Millot *et al.*, 2006).

In a cross-over feeding study, 20 volunteers consuming 300 g rice with 5.7 g of resistant maltodextrins displayed significantly lower postprandial blood insulin and glucose levels, compared to rice without resistant maltodextrins. When 13 volunteers ate a fast-food restaurant meal with 10 g of resistant maltodextrins, the insulin level at the 30-minute postprandial period was significantly reduced in comparison to the same meal without resistant maltodextrins (Gordon, 2007).

Other researchers confirmed *in vivo* the blunting effect of resistant maltodextrins on postprandial blood glucose and insulin levels, coupled with a decrease in intestinal glucagon (Okuma & Kishimoto, 2004; Wakabayashi *et al.*, 1992; Tokunaga & Matsuoka, 1999). Co-ingestion of resistant maltodextrins with monosaccharides or disaccharides can moderate increases in postprandial blood glucose levels, resulting in reduced insulin requirements and preventing fat accumulation (Ohkuma & Wakabayashi, 2001). Among Type 2 diabetics, 10–20 g of resistant maltodextrins fed at every meal for three months significantly reduced fasting blood glucose levels, improved glucose tolerance and decreased HbA1C (Nomura *et al.*, 1992; Fujiwara & Matsuoka, 1995).

Body fat was significantly reduced when 12 overweight males with hyperlipidaemia were fed 30 g of resistant maltodextrins each day for three months (Gordon, 2007). Long-term feeding of diets with added resistant maltodextrins to patients with hyperlipidaemia had the general effect of decreased low-density lipoprotein cholesterol and triglycerides, but increased high-density lipoprotein cholesterol (Okuma & Kishimoto, 2004).

Supplementation of resistant maltodextrins in the diet at the rate of 7.5–15 g/day for three weeks was well tolerated by human subjects and tended to increase the faecal Bifidobacterium population (Fastinger *et al.*, 2008). There was also a shift in the molar proportions of SCFA towards butyrate. In an earlier study, resistant maltodextrins increased faecal bulk and decreased symptoms of constipation (Satouchi *et al.*, 1993).

Flickinger *et al.* (1998) determined that a dextrinized corn starch with mixed glycosidic linkages was hardly digested in the small intestine of ileally cannulated dogs. This dextrin has an estimated caloric value of 2.2 kJ/g. Another mixed-linkage resistant dextrin (non-viscous soluble dietary fibre) described by Lefranc-Millot *et al.* (2010), and made either from wheat or corn starch, has a caloric value of 2 kcal/g (Vermorel *et al.*, 2004), which is in agreement with the above reported caloric content.

### 3.6 PERFORMANCE IN FOOD AND BEVERAGE PRODUCTS

Product developers and designers have an arsenal of conventional dietary fibres and resistant starch ingredients to boost the fibre content of food and beverage products for nutrient and calorie labelling claims (Baghurst *et al.*, 1996; Erickson, 2005; Topping, 2007; Witwer, 2008; Bird *et al.*, 2009). Resistant starches from different sources and belonging to RS1–RS5 types are traditionally white in colour and possess fine particle size and neutral flavour. However, they have distinct differences in water-holding capacity that connote formulation changes because of the impact on water absorption and processing parameters of bakery foods (Woo *et al.*, 2009).

A number of bakery, pasta, noodle, dairy, snack and confectionery products were formulated with a phosphorylated cross-linked RS4 wheat starch to enhance fibre content, lower net available carbohydrates and reduce caloric counts (Maningat *et al.*, 2005, 2008). A white pan bread (Yeo & Seib, 2009) containing one part of phosphorylated cross-linked RS4 wheat starch blended with nine parts of wheat flour had a slightly lower specific volume (5.96 cc/g) and slightly firmer texture (427.8 g), compared to a native wheat starch formula (6.07 g/cc; 475.2 g) and a control wheat flour formula (6.28 g/cc;

406.9 g). In the same study, sugar-snap cookies were used to compare the performance of phosphorylated cross-linked RS4 wheat starch with RS2 potato starch. Both starches produced cookies with top grain and spread factors comparable to the control flour. However, cookies made with RS4 wheat starch exhibited similar snapping force, but RS2 potato starch demonstrated significantly lower snapping force when compared to the control cookie.

In a related study, Erickson (2005) formulated an oatmeal chip cookie by total replacement of cookie flour with RS4 resistant starch. The usage level of 12.78% RS4 resistant starch in the cookie formula delivered a 'good source of fibre' claim (3 g of dietary fibre per 30 g serving size).

High-protein, high-fibre white or whole wheat bread doughs formulated with 11.6% of phosphorylated cross-linked RS4 wheat starch (based on total formula weight) had 4–14% higher water absorption, 3–5 minute shorter mixing time, 17–23 minute shorter proof time, and a baking time about four minutes longer than the respective control doughs (Maningat *et al.*, 2005). The high-protein, high-fibre breads displayed greater loaf volume (260–325 cc higher) and higher moisture (3.2–4.6% higher), protein (6.2–9.0% higher), and dietary fibre (12.4–15.7% higher), along with a  $\approx 27\%$  average reduction in calories (by calculation), compared to the respective control doughs.

Flour tortillas formulated with 5%, 10%, and 15% of phosphorylated cross-linked RS4 wheat starch generated doughs that were characteristically soft, extensible and easy to spread, with minimal shrink-back after pressing (Alviola *et al.*, 2010). The diameters of tortillas containing 10% and 15% RS4 wheat starch were significantly larger ( $P < 0.05$ ), but the thickness tended to decrease, compared to the control tortillas. The calculated specific volume tended to increase as the dosage of RS increased. Flour tortillas with 15% RS wheat starch had 14.3% dietary fibre and produced significantly higher ( $P < 0.05$ ) overall acceptability scores than the control tortillas (dietary fibre = 2.8%).

A pregelatinized version of phosphorylated cross-linked RS4 resistant wheat starch showed a significant ( $P < 0.001$ ) and linear ( $R^2 = 0.97$ ) trend of increasing flour tortilla weight with an increased level of RS substitution (Alviola *et al.*, 2010). This increased water requirement results in competition with gluten for water yielding doughs that were less extensible (resisted spreading). The resulting tortillas were less puffed and denser than the control tortillas, but had significantly increased tortilla weight by up to 6.2% and dietary fibre by 13.6% at a 15% level of substitution.

The addition of RS2 and RS4 high-amylose corn starches in pasta at 2.5–10.0% levels significantly decreased water absorption and swelling index,

compared to control pasta (Bustos *et al.*, 2011). As the level of RS4 starch increases, the hardness of pasta tends to increase, but springiness, cohesiveness and chewiness tend to decrease.

Corn starch cross-linked with 4–8% of a 99 : 1 mixture of STMP and STPP produced fried batters with improved crispiness and hardness and reduced oil uptake, as indicated by the higher TA.XT2 peak number and peak force, along with lower oil content compared to unmodified corn starch (Han *et al.*, 2007). The phosphorylated cross-linked RS4 corn starch may have formed a rigid matrix in the fried batter that increased batter hardness and inhibited oil absorption during frying.

To measure the textural contribution and test the integrity or survivability of phosphorylated cross-linked RS4 wheat starch under adverse processing conditions, it was subjected to a Wenger TX-57 twin-screw extrusion process during the preparation of a ring-shaped breakfast cereal containing 0%, 5%, 10%, 15% and 20% of the RS4 wheat starch (Miller *et al.*, 2011). Analysis of total dietary fibre (AOAC Method 991.43) of the extruded breakfast cereals showed that RS4 wheat starch retained about 78–89% of its fibre content after extrusion, with an average fibre retention of around 83%. Product density increased as the level of RS4 wheat starch increased, but internal cell wall thickness and size or porosity were not affected. Higher addition of levels of 15% and 20% RS4 wheat starch decreased cereal ring diameter, but increased initial (dry) cereal crispness and extended bowl life.

While not considered a food-grade starch in the USA and other countries, starch citrate was evaluated for its performance in pasta and bakery products by Wepner *et al.* (1999). Starch citrates from different botanical sources (potato, pea, wheat, and corn), with 12.2–14.4% esterified citric acid and with RS content ranging from 45.9–57.5%, were formulated in toast bread by replacing 10% of the flour in the formula. Toast breads enriched with starch citrate appeared more yellow in colour, exhibited coarser crumb structure and possessed slightly lower loaf volume than the control bread. Approximately 60–85% retention of RS content was observed during the preparation of toast bread.

In another type of baked product, wafers prepared by replacing 7.5–15.0% of flour with starch citrate displayed a lower percent retention (around 50%) of RS content compared to toast bread. The sensory attributes of the starch citrate-fortified wafers were described as harder in texture, lighter in colour, friable and more brittle, compared to the control wafer.

The addition of 7.5–15.0% starch citrate in a pasta formula caused a reduction in quality, as demonstrated by reduced firmness of the cooked pasta and increased cooking loss (Wepner *et al.*, 1999). Twin-screw extrusion of starch citrates at low shear force resulted in retention of 52–85% of the

theoretical RS content. By contrast, extrusion at high shear force showed lower retention (29–65%) of the theoretical RS content. The extrudates in both treatments had a comparable expansion index, water absorption index and water solubility index, but extrusion at a low shear force caused a higher breaking force (13.66–33.28 N) compared to extrusion at a high shear force (7.79–23.40 N).

### 3.7 CONCLUSIONS AND FUTURE PERSPECTIVES

Physical activity, portion control and wholesome foods define a person's healthy lifestyle. While the first two items are behavioural in nature, the third can be designed in foods. With health and wellness soundly resonating with consumers, food developers are constantly challenged to design consumer food products that not only have superior taste and texture, but are also nutritious.

Resistant starch ingredients, in general, provide an easy solution to assist food scientists in tailoring foods with remarkable end-use qualities and that address health concerns. Since Hi-Maize<sup>TM</sup> was released in 1993 as the first resistant starch ingredient, the growth of RS has been phenomenal, with at least 30 RS ingredients now sold and marketed worldwide. As discussed in this chapter, a range of RS4-type resistant starches, in addition to the other categories of RS, are available in the tool box of food designers. Prevailing evidence confirms a host of physiological benefits and food product functionalities of RS4-type resistant starches. There are opportunities for research scientists and food technologists to identify additional useful attributes of commercially-available resistant starch ingredients other than for caloric reduction or as a fibre source.

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## 4 Novel Applications of Amylose-Lipid Complex as Resistant Starch Type 5

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### 4.1 INTRODUCTION

Amylose is known to form single helical complexes with many other substances, including iodine, monoglycerides, lysophospholipids, fatty acids and alcohols. Amylose-lipid complex is commonly found in native starch granules and processed starch (Becker *et al.*, 2001; Morrison *et al.*, 1993, 1984). The hydrocarbon chain of the lipid interacts with the hydrophobic moiety of the amylose chain and fills the central cavity of the amylose single helix (Jane & Robyt, 1984; Jane *et al.*, 1985; Morrison *et al.*, 1993). The number of glucose units per helical turn ranges from six to eight, depending on the size of the cross-section of the complexing agent (Jane & Robyt, 1984; Shogren *et al.*, 2006).

Amylopectin can also form a single helical complex with lipids. Cold-water soluble starch prepared by heating normal maize starch in aqueous alcohol solution shows the V-type X-ray diffraction pattern associated with starch-lipid complex (Jane *et al.*, 1986a, 1986b). The degree of crystallinity of the cold-water soluble maize starch is higher than its amylose content, which implies that amylopectin is involved in the complex formation with alcohols. The presence of amylopectin-lipid complex also restricts the reassociation of amylopectin branches into double helices, as observed in the decrease of the degree of starch retrogradation during storage (Eliasson & Ljunger, 1988; Huang & White, 1993).

Amylose-lipid complex is resistant to amylytic enzyme hydrolysis (Holm *et al.*, 1983; Jane & Robyt, 1984; Kitahara *et al.*, 1996; Seneviratne & Biliaderis, 1991). It has been proposed as a new source of resistant starch

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(RS, type 5) in addition to the four earlier proposed sources (i.e. physically inaccessible starch (RS type 1), native B- and some C-type polymorphic starch granules (RS type 2), retrograded amylose (RS type 3) and chemically modified starch (RS type 4)) (Eerlingen & Delcour, 1995; Englyst *et al.*, 1992).

Amylose-lipid complex has been shown to reduce postprandial glycemic and insulinemic responses (Hasjim *et al.*, 2010; Holm *et al.*, 1983; Takase *et al.*, 1994). Removal of lipids from native high-amylose maize starch (RS type 2) also results in a decrease in the enzyme resistance of the starch (Jiang *et al.*, 2010). The advantages of amylose-lipid complex as RS comparing with other types of RS are that:

1. amylose-lipid complex is more heat stable than most native B- and C-type polymorphic starch granules (RS type 2) such as potato and banana starches;
2. the production of amylose-lipid complex requires less extensive physical and/or chemical processing than that of retrograded amylose (RS type 3) and chemically modified starch (RS type 4);
3. amylose-lipid complex restores its complex structure spontaneously during cooling after being heated above its dissociation temperature (Biliaderis & Galloway, 1989; Hasjim *et al.*, 2010).

## **4.2 ENZYME DIGESTIBILITY OF AMYLOSE-LIPID COMPLEX**

Amylose-lipid complex is more resistant to amyolytic enzyme hydrolysis than amorphous amylose and most A-type polymorphic starches. The enzyme resistance of amylose-lipid complex depends on the molecular structures of the complexing lipid and the amylose (Gelders *et al.*, 2005; Kitahara *et al.*, 1996) and on the crystalline structure of the amylose-lipid complex (Seneviratne & Biliaderis, 1991). In starch granules, amylose-lipid complex has been shown to retard granule swelling during heating in excess water, which reduces the enzyme accessibility to hydrolyze the starch granules (Cui & Oates, 1999; Tester & Morrison, 1990).

In a recent study, cooking of tapioca, normal maize and high-amylose maize starches in the presence of corn oil, soy lecithin, palmitic acid (PA), stearic acid (SA), oleic acid (OA) or linoleic acid (LA) was shown to reduce the susceptibility of the starch to enzyme hydrolysis (Ai *et al.*, 2013). The same phenomenon, however, was not observed with waxy maize starch. The results indicate that the amylose-lipid complex formed during the cooking of non-waxy starch and lipid was responsible for the decrease in the enzyme digestibility of starch. The presence of amylose-lipid complex was later

confirmed from the endothermic peak of the dissociation of amylose-lipid complex by differential scanning calorimetry (DSC) for starch samples cooked with soy lecithin, PA, SA, OA, and LA. The amylose-lipid complex in the cooked starch-corn oil mixture was confirmed by  $^{13}\text{C}$  nuclear magnetic resonance (NMR) spectroscopy.

#### 4.2.1 Effects of lipid structure on the enzyme resistance of amylose-lipid complex

The properties of amylose-lipid complex, including the dissociation temperature and the susceptibility to enzyme hydrolysis, are affected by the molecular structure of the complexing lipid. In general, the dissociation temperature of amylose-lipid complex increases with the hydrocarbon-chain length of the fatty acid (FA) (Biliaderis & Galloway, 1989; Raphaelides & Karkalas, 1988; Tufvesson *et al.*, 2003), which reflects the stability of the amylose-lipid complex, i.e. the hydrophobic interaction between amylose and the hydrophobic chain of the FA. Short-chain and some medium-chain FAs (up to 10 carbon units) are not as effective complexing agents as long-chain FAs (12 carbon units and above). This is due to the greater water solubility of the short- and medium-chain FAs, allowing the FAs to participate in the aqueous solution instead of complexing with amylose (Tufvesson *et al.*, 2003; Yotsawimonwat *et al.*, 2008).

The dissociation temperature of amylose-lipid complex also decreases with the increase in the number of *cis* double bonds in the FA chain of complexing lipid (Karkalas *et al.*, 1995; Raphaelides & Karkalas, 1988; Tufvesson *et al.*, 2003). The kink structure of *cis* double bond disrupts the alignment of the FA in the cavity of amylose single helix. The dissociation temperature of amylose complexed with an unsaturated FA (e.g. OA and LA) is lower than that of amylose complexed with a saturated FA of the same hydrocarbon chain-length (e.g. SA; Tufvesson *et al.*, 2003). Consequently, a saturated long-chain FA produces a more enzyme-resistant amylose complex than a shorter or an unsaturated FA (Kitahara *et al.*, 1996).

The low water solubility and the high melting temperature of long-chain FAs, however, reduce the dispersion of these FAs in water and reduces their availability in the aqueous solution to form complex with amylose (Yotsawimonwat *et al.*, 2008). The effectiveness of the complex formation between amylose and long-chain FA can be improved by increasing the solubility of the long-chain FA at the pH above the  $\text{pK}_a$  of the FA. Long-chain FAs are more soluble in aqueous solution when they are in salt form, carrying a negative charge (Tufvesson *et al.*, 2003; Yotsawimonwat *et al.*, 2008).

### **4.2.2 Effects of crystalline structure on the enzyme resistance of amylose-lipid complex**

On the basis of crystallinity, there are two forms of amylose-lipid complex. The amorphous (Form I) complex is produced by mixing amylose and lipid at a lower temperature (25–60 °C), whereas the crystalline (Form II) complex is produced at a higher temperature (90–100 °C) (Gelders *et al.*, 2005; Seneviratne & Biliaderis, 1991; Tufvesson *et al.*, 2003). Consequently, the amorphous complex has a lower dissociation temperature (<100 °C) than does the crystalline counterpart (>100 °C). Amorphous amylose-lipid complex can be converted to crystalline complex through isothermal annealing at a temperature above the dissociation temperature of the amorphous complex and below that of the crystalline complex.

The enzyme resistance of amylose-lipid complex can be attributed to the collapsed helical conformation of the starch-lipid complex, preventing the dispersion of amylose molecules and interfering with the binding of amylose molecules by enzyme for hydrolysis (Jane & Robyt, 1984). In addition, the crystalline structure of amylose helical complex protects the bulk of the amylose in the crystallites and enhances the enzyme resistance of the amylose-lipid complex. Hence, the crystalline amylose-lipid complex (Form II) is more resistant to amylolytic enzyme hydrolysis than the amorphous complex counterpart (Form I) (Gelders *et al.*, 2005; Seneviratne & Biliaderis, 1991).

### **4.2.3 Effects of amylose-lipid complex on the enzyme resistance of granular starch**

Amylose-lipid complex is commonly found in native cereal starch granules (Morrison *et al.*, 1993, 1984). The native lipids in cereal starch granules, including maize, rice, barley, oat, millet, and sorghum, are mostly free FAs and lysophospholipids, and the content of lipids in cereal starch granules is positively correlated with the amylose content of the starch (Morrison *et al.*, 1984). These lipids are found on the surface as well as in the inner part of starch granules (Morrison, 1981). Amylose-lipid complex of granular starch usually shows a dissociation temperature ranging between 70–108 °C, as analyzed using DSC in the presence of excess water (Kugimiya *et al.*, 1980; Li *et al.*, 2008).

The physicochemical properties of native starch granules are greatly affected by the presence of amylose-lipid complex, including the decreases in granule swelling, molecular leaching and enzyme digestibility. The formation of amylose-lipid complex with a helical conformation enhances the

entanglements between amylose and amylopectin molecules, restricting amylose leaching and retarding the swelling of starch granules during heating in excess water (Becker *et al.*, 2001; Cui & Oates, 1999; Tester & Morrison, 1990). Consequently, it reduces the accessibility of enzyme to hydrolyze the starch (Cui & Oates, 1999). Furthermore, a decrease in RS content was observed when native high-amylose maize starch was defatted using methanol solution (Jiang *et al.*, 2010).

### **4.3 PRODUCTION OF RESISTANT GRANULAR STARCH THROUGH STARCH-LIPID COMPLEX FORMATION**

A technology of producing RS by increasing the amount of amylose-lipid complex (RS type 5) in granular starch has been recently developed. The process includes a treatment of swollen starch granules with a debranching enzyme (isoamylase or pullulanase) to remove the branch linkages of amylopectin. The resulting free long branch-chains of amylopectin can function in a similar way to amylose molecules; thus, they can effectively complex with FA. High-amylose maize starch VII (HA7) was chosen for this technology because it consists of large proportions of amylose and long branch-chains of amylopectin (Jane *et al.*, 1999; Li *et al.*, 2008).

A suspension of HA7 is pre-heated at an elevated temperature and is then incubated with a debranching enzyme – either isoamylase (ISO) or pullulanase (PUL). The ISO- or PUL-treated-HA7 suspension is then mixed with FA at a temperature above the melting temperature of the FA with stirring for 30 minutes to allow the amylose-lipid complex formation. The complex is recovered by centrifugation, and the excess FA is removed by washing with 50% ethanol. FAs are effective complexing agents for making RS and are economically favourable, because FAs are often the by-products of edible oil refining process. The resulting HA7-FA complex products have RS contents up to 75%, measured using the AOAC Method 991.43 for total dietary fibres, in which the each complex product was hydrolyzed for 30 minutes with a thermally stable  $\alpha$ -amylase in a boiling water bath (Horwithz, 2003; see Table 4.1). The effects of debranching and complex formation with FA on the RS contents are significant at  $p < 0.01$ .

#### **4.3.1 Effects of fatty-acid structure on the RS content**

The increase in the RS content of HA7 after complex formation with FA (Table 4.1) is attributed to the increased amount of amylose-lipid complex (RS type 5). PA and SA are preferred for the production of RS type 5 because of

**Table 4.1** Resistant starch contents of high-amylose maize starch VII after pre-heating at 80 °C, treatment with debranching enzyme and complexing with fatty acid at 80 °C.<sup>1</sup>

Treatments <sup>2</sup>	Resistant starch content (%) <sup>3</sup>
Pre-heated HA7 control	36.7 ± 0.2
HA7-SA complex	59.8 ± 2.8
HA7-PA complex	58.3 ± 1.7
ISO-treated HA7	57.8 ± 0.1
PUL-treated HA7	46.0 ± 1.6
(ISO-treated HA7)-SA complex	74.8 ± 1.5
(ISO-treated HA7)-PA complex	74.3 ± 2.4
(PUL-treated HA7)-SA complex	71.6 ± 0.3
(PUL-treated HA7)-PA complex	69.9 ± 2.2
(PUL-treated HA7)-MA complex	62.7 ± 3.0
(PUL-treated HA7)-BA complex	44.8 ± 0.8
(PUL-treated HA7)-NaPr complex	48.1 ± 2.9
(PUL-treated HA7)-OA complex	63.2 ± 0.6

<sup>1</sup>Values are means ± standard deviations.

<sup>2</sup>HA7 = high-amylose maize starch VII, SA = stearic acid, PA = palmitic acid, ISO = isoamylase, PUL = pullulanase, MA = myristic acid, BA = butyric acid, NaPr = sodium propionate, and OA = oleic acid.

<sup>3</sup>Resistant starch content was analyzed following the AOAC Method 991.43 for total dietary fibres using thermally stable  $\alpha$ -amylase in a boiling water bath.

their long hydrocarbon chains and straight molecular structures, which form stable inclusion-complexes with amylose (Tufvesson *et al.*, 2003; Yotsawimonwat *et al.*, 2008).

When short-chain FAs, such as sodium propionate and butyric acid (BA), are used to form helical complexes with HA7, the products display lower RS contents than those made with PA and SA (Table 4.1). The differences in the RS contents are attributed to the greater water solubility of short-chain FAs, preventing the formation of stable helical complexes with amylose. As a result, the order of the RS contents of the complex products is: SA complex > PA complex > myristic acid (or MA) complex > BA complex.

Unsaturated FA with *cis* double bond(s) (e.g. OA, which has a kink in the hydrophobic chain), is less effective for amylose-lipid complex formation (Karkalas & Raphaelides, 1986; Tufvesson *et al.*, 2003) and produces a complex product with a lower RS content (63.2%) than the complex product of saturated FA with the same carbon number (SA, 71.6%) (Table 4.1).

The RS contents of pre-heated HA7 control and (PUL-treated HA7)-FA complex products are substantially reduced after they are defatted using 85% methanol solution in a Soxhlet extractor for 24 hours (Table 4.2). These results support the fact that amylose-lipid complex contributes to the enzyme



**Table 4.2** Resistant starch contents of high-amylose maize starch VII-fatty acid complex products after defatting.<sup>1</sup>

Treatments <sup>2</sup>	Resistant starch content (%) <sup>3</sup>		
	Before defatting	After defatting	Difference
Pre-heated HA7 control	36.7 ± 0.2	30.2 ± 1.3	6.5
(PUL-treated HA7)-PA complex	69.9 ± 2.2	38.2 ± 0.7	31.7
(PUL-treated HA7)-SA complex	71.6 ± 0.3	50.9 ± 0.4	20.7

<sup>1</sup>Defatting was carried out using 85% methanol solution in a Soxhlet extractor for 24 hours. Values are means ± standard deviations.

<sup>2</sup>HA7 = high-amylose maize starch VII, PUL = pullulanase, PA = palmitic acid, and SA = stearic acid. HA7 starch was pre-heated at 80 °C before treatments with PUL and fatty acids.

<sup>3</sup>Resistant starch content was analyzed following the AOAC Method 991.43 for total dietary fibres, using thermally stable  $\alpha$ -amylase in a boiling water bath.

resistance of the control HA7 starch and the (PUL-treated HA7)-FA complex products. The RS contents of the defatted (PUL-treated HA7)-FA complex products are, however, higher than those of the pre-heated HA7 control, with and without defatting, which suggests that the FA in the (PUL-treated HA7)-FA complex products cannot be completely removed by the defatting process.

These results further support the complex formation between the PUL-treated HA7 starch and FA because the FA in starch-lipid complex is less extractable than uncomplexed FA (Karkalas & Raphaelides, 1986). In addition, the defatted (PUL-treated HA7)-SA complex product shows a substantially higher RS content than the defatted (PUL-treated HA7)-PA complex product, which could be attributed to the lower extractability of the longer-chain FA (SA) in the starch-lipid complex than the shorter one (PA).

### 4.3.2 Effects of debranching on the RS content

The RS contents of the HA7 starch and the HA7-FA complex products increase after the HA7 starch is debranched with ISO or PUL (Table 4.1). Debranching enzyme (ISO or PUL) hydrolyzes the  $\alpha$ -(1 → 6) glycosidic linkages and releases linear chains from amylopectin and also from amylose. The free linear chains have greater mobility to associate between themselves to form double helices, resulting in the increase of RS content as shown in the ISO-treated and PUL-treated HA7 starch products (without complexing with FA).

A combination of the debranching reaction and the complex formation with FA further increases the RS content of HA7 starch; examples are

(ISO-treated HA7)-FA and (PUL-treated HA7)-FA complex products (Table 4.1). These results show a synergistic effect of the two treatments, in which the linear chains produced from the debranching reaction can form complex more efficiently with FA than can the highly branched amylopectin molecules.

The complex formation between FAs and relatively short linear dextrans produced from the debranching of amylopectin in waxy rice starch has been reported (Yotsawimonwat *et al.*, 2008). Because most high-amylose maize starches, including HA7 starch, have amylopectin with long branch-chains (Jane *et al.*, 1999; Li *et al.*, 2008), some linear long chains released from the debranching of HA7 amylopectin are expected to function in a similar fashion to amylose. This is supported by the ability of the starch from double-mutant *ae*-waxy maize (which consists of only amylopectin, with similar structure to the HA7 amylopectin) to form complex with iodine and give a blue colour equivalent to 34.5% apparent amylose content (Jane *et al.*, 1999).

The highly branched structure of amylopectin molecules creates steric hindrance, which prevents the formation of highly ordered amylopectin-lipid complex. Thus, the long-branch chains of amylopectin, after being released from the parent molecules through the debranching reaction, can form helical-complex with lipids more effectively.

#### **4.4 APPLICATIONS OF THE RS TYPE 5**

The (ISO-treated HA7)-FA and (PUL-treated HA7)-FA complex products contain RS up to 75% after enzyme digestion at 100 °C in excess water following the AOAC Method 991.43 (Table 4.1). The results indicate that RS type 5 in the (ISO-treated HA7)-FA and (PUL-treated HA7)-FA complex products are thermally stable and, thus, are suitable for food processing that requires heating.

In addition, the heat stability of an (ISO-treated HA7)-PA complex product has been tested in bread making. In a white bread recipe, 72% (dry basis, db) of wheat flour was replaced with (ISO-treated HA7)-PA complex product (60%, db) and wheat gluten (12%, db) (Hasjim *et al.*, 2010). The analytical result of the RS content of the bread containing RS type 5, determined using the AOAC Method 991.43, was 34.4% (db), which was similar to the value calculated (33.6%, db) from the RS contents of the wheat flour, (ISO-treated HA7)-PA complex product and wheat gluten used to make the bread (Table 4.3). Although bread made with such a high content of RS type 5 was not intended for a desirable texture, the result proves that RS type 5 is heat stable and that the enzyme resistance remains in the bread after baking.

**Table 4.3** Resistant starch content of bread made from RS type 5. Reprinted from Hasjim *et al.*, 2010.

Samples	Resistant starch (%)	
	Analytical <sup>1</sup>	Calculated <sup>2</sup>
Wheat flour	2.1	–
(ISO-treated HA7)-PA complex	52.7	–
Wheat gluten	11.0	–
Control white bread <sup>3</sup>	3.3	2.0
RS type 5 bread <sup>4</sup>	34.4	33.6

<sup>1</sup>Resistant starch analyzed using the AOAC Method 991.43.

<sup>2</sup>Resistant starch calculated on the basis of resistant starch contents of wheat flour, (ISO-treated HA7)-PA complex product and wheat gluten.

<sup>3</sup>Control white bread contained 96% wheat flour.

<sup>4</sup>Enzyme-resistant bread contained 24% wheat flour, 60% (ISO-treated HA7)-PA complex product and 12% wheat gluten.

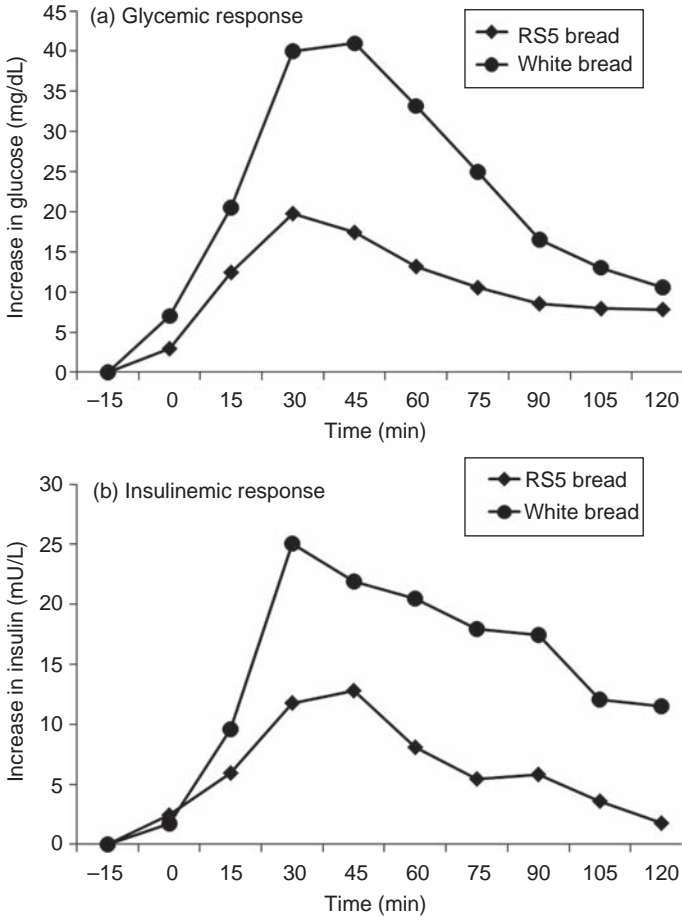
## 4.5 HEALTH BENEFITS OF RS TYPE 5

The high contents of RS type 5 in the (ISO-treated HA7)-FA and (PUL-treated HA7)-FA complex products from *in vitro* studies (Tables 4.1 and 4.2) suggest that they have potential health benefits in controlling postprandial glycemic and insulinemic responses and preventing colon cancer (Higgins, 2004; Nugent, 2005). Human and animal studies have been conducted to investigate the health benefits of the RS type 5 (Hasjim *et al.*, 2010; Zhao *et al.*, 2011).

### 4.5.1 Glycemic and insulinemic control

A human feeding study was conducted to understand the effects of RS type 5 on postprandial glycemic and insulinemic responses (Hasjim *et al.*, 2010). The two types of breads used in the study were control white bread and RS-type-5 bread containing 60%, db, (ISO-treated HA7)-PA complex product (Table 4.3). The results showed that the total areas under the curves of the postprandial glycemic and insulinemic responses were reduced to 55% and 43%, respectively, when the human subjects ingested the bread containing RS type 5, compared with the control white bread (as 100%) (Figure 4.1). Similarly, reduced postprandial insulinemic response was also observed from the rats fed with starch-mono-stearoylglycerol complex, which was accompanied by declined lipogenesis in the adipose tissue and liver (Takase *et al.*, 1994).

The results from the *in vivo* studies indicate that RS type 5 can be used to prevent the occurrence of hyperglycaemia and hyperinsulinaemia, which also



**Figure 4.1** (a) Average postprandial glycemic and (b) insulinemic responses of 20 male human subjects after ingesting control white bread and bread containing 60% (ISO-treated HA7)-PA complex product (RS type 5). Reprinted from Hasjim *et al.*, 2010.

prevents the hyperglycaemia-induced hypoglycaemia and the constant feeling of hunger associated with it (Cryer, 1999; Weinger *et al.*, 1995). Furthermore, the results showed that the insulinemic response was reduced to 2 mU/L (close to the baseline) 120 minutes after ingesting the bread containing RS type 5, versus about 12 mU/L after ingesting the white bread (Figure 4.1; Hasjim *et al.*, 2010). This suggests that RS type 5 may prevent the development of insulin resistance, an abnormality caused by persistent hyperinsulinaemia from frequent consumption of high glycemic-response foods (Byrnes *et al.*,

1995; Higgins, 2004). Thus, RS type 5 has a potential for interventions of metabolic syndrome including type 2 diabetes, obesity, hypertension, lipid abnormalities and heart disease, which have been associated with repeated hyperinsulinaemia and insulin resistance (Higgins, 2004; Ludwig, 2002; Takase *et al.*, 1994).

#### **4.5.2 Colon cancer prevention**

Potential gut health benefits of RS type 5 were studied using an animal model, and the (PUL-treated HA7)-SA complex product was used as the source of RS type 5 (Zhao *et al.*, 2011). Normal maize starch (NMS) and HA7 were used as negative and positive controls, respectively. The starches were heated in excess water (1 g starch in 3 g water) on a stove until they were boiling or formed consistent starch paste. After cooking, the starches, at 55% (db) of the total weight, were mixed with other ingredients to prepare NMS, HA7 and RS-type-5 diets following the recipe of AIN-93 Rodent Diets (Reeves, 1997) without the addition of cellulose.

Three groups of five-week-old male Fischer-344 rats were injected with two doses (20 mg/kg body weight) of azoxymethane (AOM) in one week apart and fed with NMS diet until three days after the second injection, followed by a treatment diet (NMS, HA7, or RS-type-5 diet). After eight weeks of feeding on the treatment diet, the rats were sacrificed, and the occurrence of mucin-depleted foci (MDF) and aberrant crypt foci (ACF) in the colon as well as the caecum digesta weight and pH were analyzed. MDF and ACF are commonly considered as the precursor lesions of chemically induced colon cancer (Arakaki *et al.*, 2006; McLellan *et al.*, 1991).

It was found that the average number of MDF in the colons of the rats fed with the RS-type-5 diet was significantly lower than those in the colons of the rats fed with the NMS and HA7 diets (Table 4.4). The average number of ACF was also reduced by the RS-type-5 diet, although the number was not significantly different from those of the other diets. A significantly larger amount of caecal digesta and a lower caecum pH were observed in the group fed with RS type 5, compared with those in the other two groups (Table 4.4). Lowering the pH of digesta may create an environment that suppresses the growth of pathogenic organisms in the colon (Kleessen *et al.*, 1997; Silvi *et al.*, 1999). No significant differences were found in the average daily food intake and body weight gain among the rats fed with different diets in this study.

In another study using the same animal model and diets, it was found that a significantly larger amount of faeces was discharged by the rats fed with RS type 5 than by those fed with the other two diets (Table 4.5). In addition, the

**Table 4.4** Mucin-depleted foci, aberrant crypt foci and caecum digesta weight and pH from rats injected with azoxymethane as colon cancer inducer (Zhao *et al.*, 2011).<sup>1</sup> Adapted from Zhao *et al.*, 2011. Copyright 2011 American Chemical Society.

Diet <sup>2</sup>	Mucin-depleted foci (MDF)	Aberrant crypt foci (ACF)	Caecal digesta weight (g)	Caecum digesta pH
Cooked normal maize starch	3.5 ± 1.8	47.5 ± 13.4	3.29	7.5 ± 0.1
Cooked HA7	1.8 ± 1.4*	39.6 ± 18.0	13.59*	6.6 ± 0.6*
Cooked (PUL-treated HA7)-SA complex	0.3 ± 0.5*	30.9 ± 25.0	18.67*	5.7 ± 0.2*

<sup>1</sup>Values are means ± standard deviations.

<sup>2</sup>Each diet contained 55% (db) starch.

\*Significant at  $p < 0.05$  compared with cooked normal maize starch diet.

faeces from the rats fed with RS type 5 contained significantly greater amounts of starch and lipid than those from the other two groups fed with NMS and HA7. Polarized-light micrographs of the faeces collected from the rats fed with RS type 5 showed starch granules with birefringence (Figure 4.2). The results of gas chromatography showed that SA was the major component of the lipids extracted from the faeces of the rats fed with RS type 5, indicating that a proportion of the (PUL-treated HA7)-SA complex product was not digested in the small intestine and was not fermented in the colon (Table 4.5). Therefore, SA was not absorbed in the digestive tract and was discharged as starch-lipid complex. The results also suggest that 15% (db) of

**Table 4.5** Daily weight, total starch content, and lipid content of faeces collected from rats fed with different diets.<sup>1</sup>

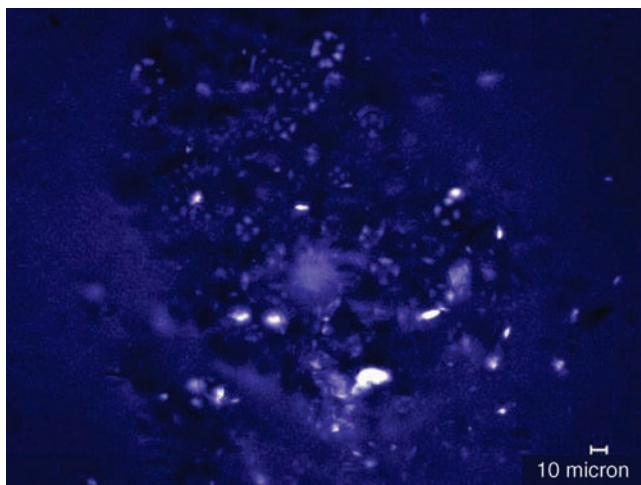
Diet <sup>2</sup>	Faeces weight (g/day)		Starch content of faeces (%) <sup>3</sup>	Lipid content of faeces (%) <sup>4</sup>
	As is	Dry		
Cooked normal maize starch	0.23 ± 0.07 a	0.20 ± 0.06 a	0.2 ± 0.0 a	16.8 ± 0.4 ab
Cooked HA7	0.55 ± 0.31 a	0.45 ± 0.25 a	16.8 ± 1.0 b	11.7 ± 0.3 a
Cooked (PUL-treated HA7)-SA complex	2.04 ± 0.76 b	1.65 ± 0.62 b	44.0 ± 0.0 c	22.0 ± 2.2 b

<sup>1</sup>Values are means ± standard deviations. Different letters on the same column represent significant difference at  $p < 0.05$ .

<sup>2</sup>Each diet contained 55% (db) starch.

<sup>3</sup>Starch content was analyzed using Megazyme Total Starch Assay Kit following the AOAC Method 76-13.

<sup>4</sup>Lipid content analyzed using the AOAC Method 996.06.



**Figure 4.2** Polarized-light micrograph of starch isolated from faeces of rats fed with (PUL-treated HA7)-SA complex product.

the consumed RS type 5 was not utilized by the rats and the microbes residing in the digestive tract of the rats. The inferior digestibility of the RS type 5, however, did not affect the body weight gain of the rats.

The results from these two *in vivo* rat studies suggest two possible mechanisms of RS type 5 in reducing the amount of MDF in the colon. One is the greater amount of the faeces discharged from the colon. The larger, bulky amount of faeces can purge carcinogen (e.g. the injected AOM) out from the colon. The other is the FA present in the starch-lipid complex, which may have the affinity to absorb carcinogen and to remove it from the colon.

## 4.6 CONCLUSION

Amylose-lipid complex has been proposed as RS type 5, because of its resistance to enzyme hydrolysis. The presence of amylose-lipid complex in starch granules also increases their enzyme resistance by restricting the granule swelling during cooking. The enzyme resistance of amylose-lipid complex depends on the molecular structure of the lipid and the crystalline structure of the single helices. FA with a longer hydrocarbon chain length produces amylose-lipid complex with greater enzyme resistance, whereas FA with a greater degree of unsaturation produces amylose-lipid complex with

lower enzyme resistance. Furthermore, a crystalline form of amylose-lipid complex has greater enzyme resistance than its amorphous counterpart.

A technology has been recently developed to produce RS using amylose-lipid complex by treating granular HA7 starch with debranching enzyme, followed by complex formation with FA. The RS contents of the complex products were significantly higher (up to 75%). Feeding human subjects with this RS type 5 reduced postprandial glycemic and insulinemic responses compared with the control diet of white bread, which suggests that RS type 5 has potential to intervene in metabolic syndromes including type-2 diabetes, obesity, hypertension, lipid abnormalities and heart disease, as well as other complications associated with repeated hyperinsulinaemia and insulin resistance. RS type 5 also shows the ability to reduce colon cancer development, as observed by lowering the amount of MDF in the colons of AOM-treated rats.

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## 5 Digestion Resistant Carbohydrates

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### 5.1 INTRODUCTION

The rates of obesity, diabetes, cardiovascular and other diseases have increased dramatically over the last century, which has led to increased consumer awareness of the importance of nutrition in the development and progression of these diseases. As a result of this awareness, and increased scientific research in this area, demand for 'healthier options' is high. Consumers understand the value and potential health benefits of dietary fiber, so one top demand is for fiber-containing foods that taste good. For food ingredient companies, this presents both an opportunity and a challenge to supply the market with new and nutritionally improved carbohydrate ingredients, including starch and fiber.

To develop fiber-containing ingredients, it is important to understand the enzymes involved in food digestion, as well as the properties of a food that make it either highly digestible or highly enzyme-resistant and, therefore, high in dietary fiber. In the case of starch, the structures responsible for enzyme resistance can be either physical or molecular. This chapter examines the physical and molecular structures of starch that influence enzyme digestibility, and describes examples of commercially available resistant starch ingredients and their source of enzyme resistance related to starch structure.

### 5.2 STARCH DIGESTION

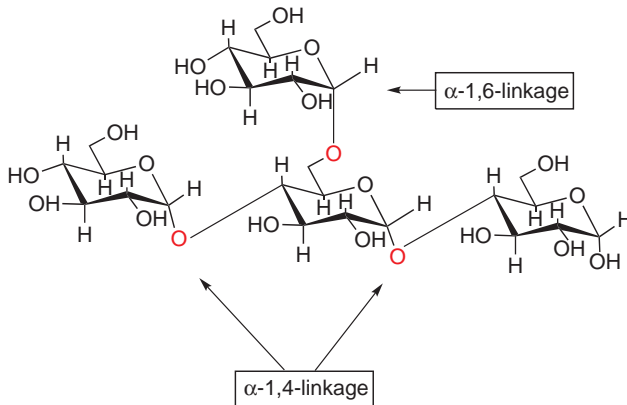
Starch is the major source of carbohydrates in the human diet. Starch can be obtained from fruit, vegetables, roots and grains. Starch and starch derivatives are a nutritive, abundant and economical food source. Today, starch is consumed unprocessed in the form of raw fruits and vegetables, or in the

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**Figure 5.1** Structures found in starch molecules.

form of more shelf-stable processed foods (Moore *et al.*, 1984). The processing of starch evolved in the last 80 years from a simple isolation process to the production of diversified lines of sweeteners and starch products (O'Dell, 1979). Food starches contribute to the characteristic viscosity, texture, mouth-feel and consistency of many food products.

Starch is a glucose polymer composed of highly branched amylopectin ( $\alpha$ -1,4 and  $\alpha$ -1,6-linkages) and essentially linear amylose molecules (mainly  $\alpha$ -1,4-linkages) (Figure 5.1).

Starch digestion in the human body is mainly done by  $\alpha$ -amylases. Salivary  $\alpha$ -amylase is the first amylase that comes in contact with the food in the mouth. It catalyzes the hydrolysis of amylose to maltose, maltotriose and maltotetraose, and the hydrolysis of amylopectin to the same products plus two  $\alpha$ -limit dextrins (Robyt, 1984). The salivary  $\alpha$ -amylase quickly passes from the mouth to the stomach, together with the food starch. The pH in the stomach is about 2, which inactivates the salivary  $\alpha$ -amylase. After some resident time in the stomach, the partially hydrolyzed starch passes into the small intestine, where it is neutralized. The main hydrolysis of the starch is then accomplished by pancreatic  $\alpha$ -amylase, secreted from the pancreatic duct, via a multiple attack mechanism (Mazur & Nakatani, 1993).

Two other enzymes are necessary to convert the hydrolysis products of the  $\alpha$ -amylases into glucose, which can be actively transported across the small intestine membrane. These two enzymes are the brush border glucogenic enzymes maltase-glucoamylase and sucrase-isomaltase (Swallow *et al.*, 2001; Nichols *et al.*, 2003). Maltase-glucoamylase converts maltose, maltotriose, and maltotetraose into D-glucose by successive action from the non-reducing

end (Robyt, 1984). Sucrase-isomaltase hydrolyses the  $\alpha$ -1,6-linkages. The resulting D-glucose can then actively be transported across the luminal membrane of the small intestine and thence into the blood via the sodium-glucose cotransporter (SGLT-1), which is located at the luminal surface of enterocytes (Pencek *et al.*, 2002).

Starch digestion is influenced by factors that affect enzyme activity and the susceptibility of the starch substrate to the hydrolytic enzymes (Tester *et al.*, 2004). Enzyme activity may depend on quantity of the enzymes present, enzyme isoform and enzyme inhibitors either present in the consumed food or produced during enzyme hydrolysis of the food. While brush border enzymes are capable of cleaving both  $\alpha$ -1,4 and  $\alpha$ -1,6 glycosidic bonds as well as other glycosidic bonds (e.g.  $\alpha$  1,2), the main hydrolysis of starch is performed by the  $\alpha$ -amylases. Starch digestion by  $\alpha$ -amylases requires a series of steps where the enzymes first need to diffuse to the starch matrix into the food, then bind to the substrate, then finally cleave the  $\alpha$ -1,4-glycosidic linkages (Brayer *et al.*, 2000).

### 5.3 PHYSICAL STRUCTURES OF STARCH

To understand factors that may influence the susceptibility of a particular starch substrate to  $\alpha$ -amylase hydrolysis, four aspects of hydrolysis should be considered: the diffusion of the enzyme towards the substrate; the porosity of the starch substrate; the adsorption of the enzyme on the substrate; and the catalytic event (Colonna *et al.*, 1992). Porosity has to be considered at two levels of scale: at the macro (1–100  $\mu\text{m}$ ) and at the micro molecular level (<10 nm) (Colonna *et al.*, 1992). The physical form of the starch also influences enzyme binding. Physical entrapment of starch in the food matrix, starch granular structure, and also smaller scale structures such as crystallinity and helicity, may influence the binding of enzymes to the starch substrate.

For native starch granules, the limiting factor for the hydrolysis has been shown to be the penetration of the enzyme into the granules by successive formation of pits and larger pores (Gallant *et al.*, 1973).

Before a substrate can be hydrolyzed, adsorption of the enzyme onto the substrate is crucial. Any factors that influence the binding of the  $\alpha$ -amylases to the starch substrate can slow down or limit starch digestion. The hydrolysis products maltose and maltotriose have been shown to act as substrate inhibitors for  $\alpha$ -amylase (Seigner *et al.*, 1985).

For an enzyme to be able to hydrolyze a substrate, proper enzyme-substrate recognition is necessary. Substrate binding by means of the enzyme sub-sites has to be energetically favoured. For the catalytic action of the enzyme to

occur, the substrate to be hydrolyzed has to be oriented properly inside the active side of the enzyme. Only the part of the starch substrate that can fit into the active site cavity of the  $\alpha$ -amylase can be hydrolyzed. Structures such as double helices are too big to fit into the active site cavity and can only be hydrolyzed in the unwound state.

### 5.3.1 Starch helices

Amylose is known to form single helices in solution. The addition of complexing ligands such as iodine, fatty acids, alcohols, emulsifiers or flavour compounds to aqueous starch solutions induces the formation of single helices (Heinemann *et al.*, 2003). V-amylase is the generic term for amylose obtained by complexation, and the compounds used to obtain the single helices may or may not be present in the final V-type starch (Buleon *et al.*, 1998). The amylose single helix has been described as a left-handed helix with six glucose residues per turn, with a pitch height of about 0.7 nm (Buleon *et al.*, 1998). The outer surface of the single helix is thought to be mostly hydrophilic, while the centre channel of the helix is hydrophobic. Single helices are stabilized by 2-O . . . O-6 hydrogen bonds between spatially close glucose residues of the next spiral turn (intrahelical hydrogen bonds). The structure of the single helices is further cooperatively strengthened by 2-O . . . O-3 hydrogen bonds between adjacent glucose units (Immel & Lichtenthaler, 2000).

The formation of single helices from branched material has been little studied. Formation of single helices from amylopectin with complexing agents has been shown (Heinemann *et al.*, 2003). Considering the structure of amylopectin molecules, it would be expected that external chains that are in close proximity to each other may easily form double helices.

The external chains of amylopectin and amylose chains are known to be able to form double helices within starch granules and in non-granular form (Wu & Sarko, 1978a, 1978b, 1978c). Evidence and models for both right-handed (Mueller *et al.*, 1995) and left-handed double helices (Imberty *et al.*, 1988) have been provided. The left-handed form is the energetically favoured form and is commonly accepted as the form of starch double helices (Imberty *et al.*, 1988; Buleon *et al.*, 1998). The starch double helix has a pitch of 2.13 nm with six glucose units per turn (Imberty *et al.*, 1988, Buleon *et al.*, 1998). Starch double helices are stabilized by hydrogen bonds between 2-O . . . O-6 of glucose units on different strands of the double helix (interhelical hydrogen bonds). The outer surface of the double helix has an irregular distribution of hydrophobic and hydrophilic areas (Immel and Lichtenthaler, 2000).

### 5.3.2 Crystalline structures

Single helices formed by non-granular starch can arrange themselves to form crystalline entities. If the crystalline arrays are large enough to diffract x-rays, an x-ray diffraction pattern can be observed. The characteristic structure of crystalline V-amylose has been extensively studied (Rappenecker & Zugenmaier, 2001; Godet *et al.*, 1995). The double helices formed by external chains of amylopectin or amylose molecules can also form crystalline entities.

Double helical starch can form different crystalline polymorphs. Native starch granules can be found with A-type crystallinity (cereal starches) and B-type crystallinity (tubers, high-amylose maize starches) (Banks & Greenwood, 1975; Zobel, 1988). The double helical structures within the two polymorphic forms are essentially identical (Imberty *et al.*, 1991), but the packing of the helices within the crystalline structure is different. In general, A-type crystals are less hydrated and more densely packed than B-type crystals (Imberty *et al.*, 1987; Imberty & Perez, 1988).

Once starch has lost its granular structure, it can be crystallized into one of the two crystalline polymorphs, depending on the starch structure and crystallization conditions. The A-type polymorph is the more thermodynamically stable form and is preferably formed by shorter chains, higher temperature and lower starch concentrations. The B-type polymorph is formed by longer starch chains, lower temperatures and higher starch concentrations (Gidley & Bulpin, 1987). B-type crystallites are commonly believed to be more enzyme resistant, while A-type polymorphs are more heat stable compared to B-type formed from chains with similar molecular weight (Whittam *et al.*, 1990; Le Bail *et al.*, 1995).

### 5.3.3 Starch granule structure

Starch granules are the naturally occurring storage form of starch. Different levels of organization exist in native starch granules. At the lowest such level, the starch granules consist of amorphous and semi-crystalline shells that are often called growth rings. At a higher level of structure are the so-called 'blocklets' (Gallant *et al.*, 1997). One blocklet is thought to contain several amorphous and crystalline lamellae that are, in turn, created by the chain organization of amylopectin molecules. The side chains of amylopectin can form double helices and, due to the close proximity of the chains to each other, the double helices can easily form crystalline arrays. These crystalline arrays, and the non-crystalline zones between them that have been shown to contain the amylopectin branch points (Jenkins *et al.*, 1993), are organized into lamellae.

Heating of starch granules can lead to partial or complete melting of the crystallites and helices present in the granules. Depending on the heat treatment conditions used, the heat treatment can be classified as gelatinization, annealing or heat-moisture treatment. Gelatinization of starch is defined as the complete melting of granular structures in the presence of excess water. Annealing and heat-moisture treatment are both heat treatments that only partially melt structures in the starch granules by using temperature/moisture combinations below the melting point of the treated starch.

Annealing is a term used in polymer chemistry to describe a treatment that uses heat and moisture to increase the order of polymer chains. In the case of starch, the term 'annealing' is used to describe a heat treatment that employs temperatures below the melting point of the starch at moisture contents above 40%. Heat-moisture treatment of starch is described as a heat treatment with temperatures below the melting point of starch, at moisture levels below 35% (Stute, 1992; Jacobs & Delcour, 1998). These treatments are generally used to 'perfect' crystalline order in starch granules.

## 5.4 RESISTANT STARCH DUE TO PHYSICAL STRUCTURE

Resistant starch has been defined as 'the starch and products of starch digestion that are not absorbed in the small intestine of healthy individuals' (Asp, 1992). Based on the source of the enzyme resistance, resistant starch has been classified into five different types (Table 5.1). In type 2, type 3 and type 5 resistant starches, the enzyme resistance is due to the physical structure of the starch molecules.

The physical structures responsible for formation of type 2 resistant starch are part of the natural organization of starch granules. Examples of starches high in type 2 resistant starch are green banana, potato and high-amylose

**Table 5.1** Classification of resistant starch (RS).

RS type	Source of enzyme resistance	Examples
1	Whole grains	Seeds, grains
2	Raw starch granule structure	Green banana, high-amylose, potato
3	Structures formed on re-association or retrogradation of starch after heat treatment	Cooked pasta, cooked potato
4	Chemical modifications of the starch	Modified starch
5	Starch lipid complex	Amylose containing starch



maize. Several commercial type 2 resistant starch ingredients are available. These are usually high-amylose starches that might undergo heat-moisture treatment to enhance their inherent RS content (US Patent 5593503).

Type 3 resistant starch is formed by reassociation of starch chains after heat treatment. Different factors, such as amylose/amylopectin ratio, chain length, lipid content and processing conditions, influence the amount and quality of the type 3 RS (Eerlingen & Delcour, 1995). Annealing, heat moisture treatment and gelatinization of starch all melt crystalline structures present in native starch granules either fully or partially. Upon cooling, linear amylose molecules, as well as linear regions of amylopectin molecules, organize into a mix of amorphous areas as well as helices and crystallites, with varying degrees of enzyme resistance and, therefore, varying levels of resistant starch. In the case of gelatinization, this reassociation of starch chains upon cooling is called retrogradation.

The resulting resistant starch often exhibits B-type crystallinity, but A-type crystalline RS can also be obtained if the retrogradation or crystallization occurs at high temperatures or involved starch molecules with short chain length. Formation of crystalline structures that can form RS 3 take place above the glass transition temperature (and below the melting temperature), and any components present that influence the glass transition temperature can, therefore, be expected to influence the formation (yield and quality) of the formed type 3 RS.

Amylose content has been positively correlated with RS yield (Sievert & Pomeranz, 1990) and the formation of type 3 RS is strongly related to the crystallization process of amylose (Eerlingen & Delcour, 1995). The yield of RS formed is dependent on the water content and temperature used. At high starch concentrations, the starch chains interact more easily, leading to increased crystal and RS formation. Water does act as a plasticizer in the system, and a minimum water content is necessary to achieve the chain mobility needed to form crystalline structure resistant to enzyme digestion. The presence of lipids has been shown to decrease the formation of type 3 RS, due to formation of amylose lipid complexes (type 5 RS) (Czuchajowska *et al.*, 1991).

Annealing and heat-moisture treatment can be used for the manufacture of resistant starch, since the more 'perfect' structures formed often lead to an increase in enzyme resistance of the starch (Jacobs & Delcour, 1998; Chiu *et al.*, 1999; Brumovsky & Thompson, 2002).

Some commercial examples of type 3 resistant starch are Hi-maize 330 (National Starch and Chem. Co) and Promitor Resistant Starch 60 (Tate & Lyle). Both of these ingredients are manufactured from high-amylose maize starch. In both cases, the starch is first gelatinized and then cooled to

retrograde the starch chains. Heat-moisture treatments are then applied to the retrograded starch to increase the molecular order and, therefore, the enzyme resistance of the starch (US5281276; US7189288). Type 3 resistant starches have also been produced by a process in which the starch is first treated with a debranching enzyme to increase the amount of linear chains, followed by controlled reassociation or crystallization of the linear chains to form enzyme resistant starch. One commercially available example of type 3 resistant starch produced in this fashion is Actistar<sup>TM</sup> RM (Cargill Inc.), which is produced by debranching and crystallization of tapioca maltodextrin (Patent EP 0846704 B1).

Tate & Lyle recently described a resistant starch process in which a starch is first treated with a glucanotransferase enzyme to elongate the external chains of amylopectin, followed by debranching and then crystallization of the linear chains (US Patent Application 2007/0059432 A1).

The enzyme resistance in Type 5 resistant starch is due to the molecular structure of amylose lipid complexes that can either be present in the native starch or formed by controlled reactions using non-granular starch and lipids to form the resistant amylose lipid complex (Brown *et al.*, 2006; Haşjim *et al.*, 2010).

## 5.5 MOLECULAR STRUCTURE OF STARCH

Starch is comprised of two polymers. Amylose is an essentially linear polymer, in which glucose monomers are linked by  $\alpha$ -1,4-glycosidic bonds. Amylopectin is a highly branched polymer that, in addition to the linear  $\alpha$ -1,4-linkages, also contains  $\alpha$ -1,6-linked glucose monomers that create branch points.

Certain processing treatments alter the molecular structure of starch and may create additional linkage types. The amount and distribution of  $\alpha$ -1,6-branch points can be altered by enzymatic treatment of starch. Debranching enzymes such as isoamylase or pullulanase can be used to decrease the branch points, and branching enzymes can be used to increase the amount of  $\alpha$ -1,6-linkages in starch. Transferase enzymes can alter the distribution of branch points in starch.

In addition to altering the amount and distribution of linkages present in native starch, processing treatments can be used to create new types of linkages not found in native starch molecules. When heat is applied to starch at low moisture and low pH conditions, dextrinization of the starch occurs (Wurzburg, 1986). During the initial stages of dextrinization, acid-catalyzed hydrolysis occurs. This is followed by a recombination of the fragments to

form branched structures. The dextrinization process converts a portion of the normal  $\alpha$ -1,4-,  $\alpha$ -1,6-linkages to random  $\alpha$ -1,4-,  $\alpha$ -1,6-,  $\alpha$ -1,2-,  $\alpha$ -1,3 and even  $\beta$ -type linkages (Wurzburg, 1986). Figure 5.2 shows a representative molecular structure of a dextrin.

Chemical modification of starch is a process widely used in the food industry to alter the texture and processing stability of starch. A wide range of properties can be achieved, depending on the botanical origin of the starch, amylose and amylopectin structure, and the amount and type of modification agent. Starch modification can be achieved through derivatization such as etherification, esterification and cross-linking of starch (Figure 5.3). Chemical modification involves the introduction of functional groups into the starch molecules, resulting in significantly different properties. Starches with altered gelatinization, pasting and retrogradation behaviour can be produced through chemical modification (Singh *et al.*, 2007).

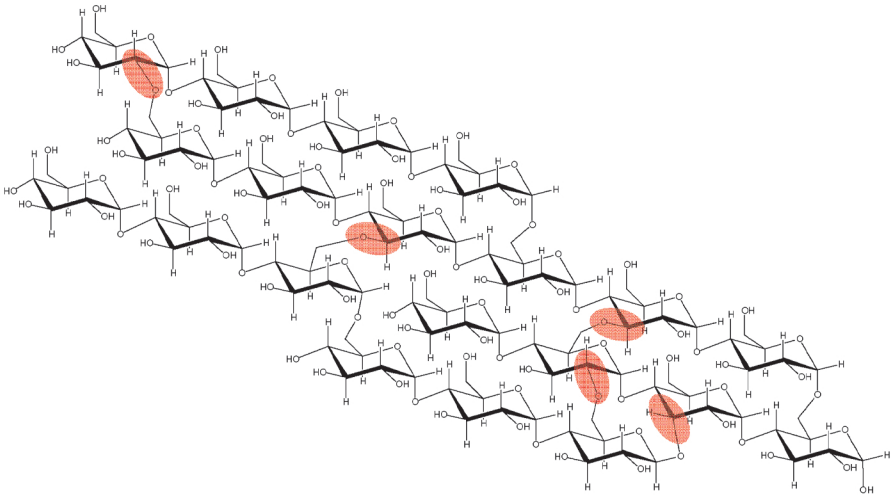
## 5.6 ENZYME RESISTANCE DUE TO MOLECULAR STRUCTURE

The linear regions of the starch molecules are easily digested by human pancreatic  $\alpha$ -amylase. The small sugar and oligosaccharide digestion products of  $\alpha$ -amylase can then be digested further by the intestinal brush border enzymes.

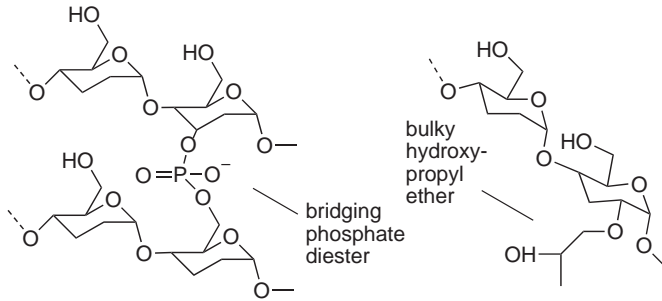
Branch points can create a steric hindrance for enzyme digestion, irrespective of the type of glycosidic linkage that creates the branching of the starch chain. For  $\alpha$ -amylase hydrolysis to occur, a linear portion of the starch chain has to fit in the enzyme's active site. This linear portion has to be long enough to create energetically favourable binding. Glucose units close to the branch points have less favourable binding energy with the sub-sites of the enzyme than glucose units further down the chain; sub-site binding therefore affects the efficiency with which the enzyme hydrolyses a linkage close to a branch point. Linkages directly adjacent may not be hydrolyzed at all, leading to the formation of limit dextrans (Shannon *et al.*, 1984; Colonna *et al.*, 1992).

Chemical modification of starch can be viewed as a modification that creates chain irregularities or branches in the starch chains. Cross-linking of starch covalently links two starch chains together, in effect creating a branch point on both chains. Chemical substitution introduces a bulky side group to the starch chains. The introduction of these chemical groups may create a steric hindrance to one or more human digestive enzymes.

Several type 4 resistant starch ingredients are available commercially, with total dietary fiber values of up to 90%. These type 4 products, from different



**Figure 5.2** Representative molecular structure of dextrin. The highlighted linkages represent glycosidic linkages that can be found in dextrans and lead to reduced enzyme digestibility.



**Figure 5.3** Structures found in modified starch. Left: structure of chemical cross-link (cross-linking with  $\text{POCl}_3$ ). Right: structure of chemical substitution (substitution with propylene oxide).

botanical origins, contain high amounts of resistant starch due to a high level of chemical cross-linking (US 2006/0188631 A1). Substituted starches are not currently produced as resistant starch ingredients.

Dextrinization of starch leads to the formation of potentially indigestible linkages. The glycosidic linkages in dextrans are usually predominately  $\alpha$ -1,4-, but  $\alpha$ -1,6-,  $\alpha$ -1,2-,  $\alpha$ -1,3 and, possibly, even  $\beta$ -type linkages can be found (Wurzburg, 1986). The  $\alpha$ -1,4- and  $\alpha$ -1,6-bonds are digestible by human pancreatic  $\alpha$ -amylase and brushborder enzymes, respectively. However, while some of the  $\alpha$ -1,2-bonds might also be digestible by brushborder enzymes, other bonds formed during dextrinization are not digestible by human enzymes.

Commercially, the technique of dextrinization is used by several companies to create dietary fiber ingredients that (depending on their resulting molecular size) can be classified as resistant starch or resistant maltodextrins. The Nutriose<sup>®</sup> line of dietary fiber ingredients is marketed by Roquette (Roquette Frères). Nutriose<sup>®</sup> soluble fibres are food dextrans made from wheat or maize starch (US5620871). Fibersol 2 is produced and marketed by a joint venture between ADM (Archer Daniels Midland Company) and Matsutani (Matsutani LLC). It is classified as resistant maltodextrin in the USA (US5358729) and is produced by dextrinization of starch followed by a proprietary enzymatic molecular weight reduction of the dextrans. Tate & Lyle's Promitor<sup>™</sup> Soluble Corn Fiber ingredients are produced by heat treatment of starch hydrolysis products at low pH (US7608436). They can be classified as corn syrup or maltodextrin, depending on the molecular weight of the resulting product.

## 5.7 CONCLUSION

Carbohydrates play an important role in the human diet for a number of reasons. The World Health Organization recommends that at least 55% of the energy of a diet be derived from carbohydrates. Starch is the major source of carbohydrates in the human diet. Many carbohydrate-containing foods also contain important micronutrients and phytochemicals.

Dietary fiber is a class of carbohydrates that are important in overall health. Today, food ingredient companies have the knowledge and expertise to maximize the health-giving potential of starch ingredients by modulating not only the texture and sensory properties of commercial starches, but also the digestibility and physiological effect of a starch ingredient.

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## 6 Slowly Digestible Starch and Health Benefits

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### 6.1 INTRODUCTION

Starch is the major glycaemic carbohydrate of foods, and its nutritional property is related to its rate and extent of digestion and glucose absorption in the small intestine. A classification of starch into rapidly digestible starch (RDS), slowly digestible starch (SDS) and resistant starch (RS) has been used to specify the nutritional quality of starch. SDS is the most elusive fraction of the three, perhaps due to its somewhat transient nature, and it is certainly less studied than RS. Yet, the physiological consequences of SDS may be just as profound as those of RS, in terms of regulating glucose delivery to the body and its associated metabolic effects, triggering gut hormones to affect gastrointestinal tract motility and, perhaps, even reducing appetite and increasing satiety. This chapter covers the current understanding of SDS, including its concept and potential health benefits, the starch digestion process, structure and mechanism of SDS and approaches to making SDS.

On one level, available carbohydrates are an important part of the diet and should provide 45–65% of total caloric intake as recommended in the Dietary Guidelines for Americans (U.S. Department of Agriculture, 2010). However, quantity alone is not an accurate assessment of nutritive quality and rate – the extent of digestion and absorption of glycaemic carbohydrates is an important parameter (Jenkins *et al.*, 1981; Englyst *et al.*, 2003).

Over thirty years ago, Jenkins *et al.* (1981) proposed the concept of glycaemic index (GI), which is represented by area under the *in vivo* glycaemic response curve (AUC) of a tested food compared to a reference glucose or white bread containing the same amount (50 g) of available

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carbohydrate. Glycaemic response is a broader concept that includes the nature and shape of the postprandial blood glucose profile. The digestion property of glycaemic carbohydrates is the main determinant of the GI value of different foods, although factors that affect glucose transport through the gut enterocytes, as well as other human factors, also impact rate of rise of blood glucose levels (Wolever *et al.*, 2006).

Brand *et al.* (1991) categorized foods based on their GI values as high GI (GI > 70), low GI (GI < 55) and intermediate GI foods. Since the advent of the GI concept, there have been extensive clinical and nutritional investigations on dietary carbohydrates, particularly regarding potential health benefits of low-GI foods (Wolever, 2003), and an extensive list of different foods have been tested and compiled by Foster-Powell *et al.* (2002).

There exists a controversy that has developed within both scientific and industrial groups regarding the use of the GI concept in the context of a tool to affect public health. This is in large part due to a body of studies with varying results on the relationship between low-GI food and health (Howlett & Ashwell, 2008), even though this includes carefully conducted studies with mechanistic underpinnings. Slowly digestible glycaemic carbohydrates is one way to achieve low glycaemic response in foods, but it is not the only way. A better understanding of the structural and mechanistic basis of low glycaemic response foods will provide important information to this field of study and to the development of low glycaemic response products.

## **6.2 SDS AND POTENTIAL BENEFICIAL HEALTH EFFECTS**

Slowly digestible starch offers the possibility of moderated glucose delivery to the body, which can be beneficial to glucose homeostasis and related processes, as well as distal small intestinal glucose release that may affect motility of food through the gastrointestinal tract and, perhaps, fullness and satiety. Due to its importance as the central metabolic fuel, levels of glucose are tightly controlled in the body through a series of mechanisms. Although glucose may be supplied internally through glycogenolysis and gluconeogenesis, dietary derived glucose is critical for growth and development and for energy-consuming organs such as the brain and central nervous system (Peters *et al.*, 2004).

Glucose is also a signal molecule in energy metabolism that stimulates insulin secretion, governs glucose utilization and inhibits endogenous glucose production. Starch, generally being the most abundant glycaemic

carbohydrate consumed, is the major source of dietary glucose. Importantly, the manner of digestion of starch and subsequent release of glucose in the digestive system is the starting-point of the glucose homeostasis regulatory cascade, and its timing and location of release controls glucose delivery of foods.

### 6.2.1 Potential health benefit of SDS relative to RDS

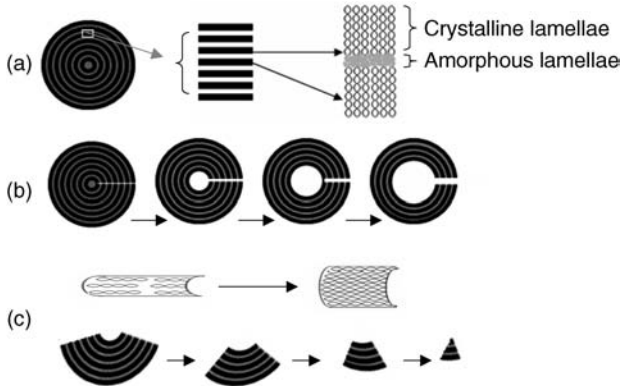
The nutritional property of starch has been ascribed to relative amounts of RDS, SDS and RS. RDS results in a large fluctuation in blood glucose that can generate stress on the regulatory system of glucose homeostasis (Ludwig, 2002), and this may even be associated with cell, tissue and organ damages under long-term stress (Brownlee, 2001). In commonly consumed starchy foods that are processed, RDS is usually dominant. High RDS correlates with high value of GI and glycaemic load (GL,  $GI \times \text{amount}$ ). Moreover, the high postprandial glycaemic response elicited by RDS coincides with high levels of plasma insulin, the principal hormone to regulate postprandial blood glucose levels.

RS, that starch which escapes digestion by host enzymes, is ascribed to a number of positive physiological effects and benefit to the colon microbiota (Annison & Topping, 1994; Ferguson *et al.*, 2000; Topping *et al.*, 2008). Most of the health benefits ascribed to SDS are deduced from low-GI foods which may have a similar glycaemic response as SDS. However, not all low-GI foods have a slow digesting component. The property of SDS has not been studied clinically to a significant degree.

When considering possible physiological effect/differences between chronic consumption of foods high in RDS versus SDS, one might first look at the level of the gut itself. RDS releases glucose quickly after leaving the pyloric valve of the stomach, and is absorbed in the duodenum or proximal jejunum. The exact fate of SDS is somewhat less clear. While most SDS-containing foods still have a rapidly digesting component, certainly some portion of the starch or its intermediate degradation products is digested and releases glucose into the distal jejunum and ileum.

Such differences in starch digestion rate have, in recent years, been documented to cause changes at the level of the gut enterocytes in terms of gene expression that lead to changes in levels of the mucosal  $\alpha$ -glucosidases (Mochizuki *et al.*, 2010a, 2010b), glucose transporters (Shimada *et al.*, 2009) and gut hormones (Wachters-Hagedoorn *et al.*, 2006; Shimada *et al.*, 2008), all of which could affect human physiology and health.

Seal *et al.* (2003) showed that, when fed to human subjects, normal uncooked corn starch, which is a good source of SDS, resulted in a slow and prolonged ( $\approx 5$  hours) postprandial release of glucose, as well as low insulin levels throughout



**Figure 6.1** Depiction of slowly digesting side-by-side mechanism and resulting inside-out digestion pattern (Zhang *et al.*, 2006a). Reprinted with permission from Zhang *et al.*, 2006a. Copyright 2006 American Chemical Society.

the postprandial phase (Figure 6.1). Unlike RDS, there was a lack of a hypoglycaemic episode with SDS ingestion and there was a shift in peak blood glucose to a longer time. SDS consumption also extended exogenous glucose oxidation and resulted in a lower level of plasma-free fatty acids.

In the study by Wachters-Hagedoorn *et al.* (2006), ingestion of normal corn starch resulted in elevated and prolonged increase in the incretin hormones glucagon-like peptide-1 (GLP-1) and glucose-dependent insulintropic polypeptide (GIP) – the former up to 300 minutes postprandial. Apart from its insulin stimulation role, GLP-1 has been implicated in decreasing gastric emptying and improvement in insulin sensitivity and glycaemic response in diabetics. This suggests that slowly digesting carbohydrates (SDC) in general may be beneficial on energy homeostasis and regulation. SDC may have additional benefits in areas of cognitive/mental performance (Benton *et al.*, 2003; Lang *et al.*, 2003) and satiety (Sparti *et al.*, 2000; Araya and Alviña, 2004). The review of Lehmann & Robin (2007) discuss these other areas of possible benefit.

Thus, SDS is associated with positive health outcomes that may include moderated postprandial glycaemia, circulating free fatty acids and oxidative stress (Jenkins *et al.*, 2002). Harbis *et al.* (2004) showed that slowly available glucose, the *in vivo* counterpart to SDS, when provided to obese and insulin-resistant individuals, resulted in some improved metabolic profiles, including lower postprandial insulinaemia and circulating triacylglycerols. Moreover, high levels of SDS-containing legumes are considered beneficial to diabetic control (McCrary *et al.*, 2010), and it has been shown that a bedtime meal containing SDS (corn starch) improved glucose tolerance for the second

morning meal (Axelsen *et al.*, 1999). SDS-containing foods may be a dietary intervention to curb or delay the prevalence of metabolic syndrome and related diseases such as cardiovascular disease and diabetes (Cook *et al.*, 2003; Hu *et al.*, 2004; Giugliano *et al.*, 2006).

## 6.3 THE PROCESS OF STARCH DIGESTION

Starch is digested in the mammalian gastrointestinal tract first by salivary and pancreatic  $\alpha$ -amylases which produce small linear and branched  $\alpha$ -limit dextrins, which are further digested to glucose by the  $\alpha$ -glucosidases, maltase-glucoamylase (MGAM) and sucrase-isomaltase (SI) at the mucosal brush border of the small intestines (Swallow *et al.*, 2001; Nichols *et al.*, 2003). The resulting glucose is then absorbed by the sodium-glucose active transporter (SGLT-1) found in the apical membrane of the enterocytes (Pencek *et al.*, 2002). This is a very efficient system of reducing starch to glucose, though the rate can be influenced by a number of intrinsic factors related to the starchy food (food matrix, starch molecular form (dispersed, retrograded, crystalline), natural enzyme inhibitors) (Tester *et al.*, 2004). Gelatinized starches that are completely dispersed are particularly susceptible to enzyme action.

A significant, though often overlooked, factor in determining the rate of starch digestion and glucose absorption is the transit time of food through the upper gastrointestinal tract. This is related principally to gastric emptying time, which is controlled by gut hormones as triggered by macronutrients or short chain fatty acids generated by fermentation of dietary fibre, including resistant starch, as well as food form (solids vs. liquids, viscosity). Viscosity itself can delay starch digestion by limiting access of enzymes to substrate. It is possible that these factors can be used to generate a slow starch digestion effect.

### 6.3.1 Enzyme action

Salivary and pancreatic  $\alpha$ -amylases (E.C. 3.2.1.1) are  $\alpha$ -1,4 endo-glucosidases. It is generally considered that starch is only slightly digested by salivary amylase, with the bulk of hydrolysis occurring by the action of pancreatic  $\alpha$ -amylase. There are five sub-sites for starch binding for human  $\alpha$ -amylase (Brayer *et al.*, 2000), and cleavage of the  $\alpha$ -1,4 glycosidic linkages uses a multiple attack mechanism (Mazur & Nakatani, 1993) to produce  $\alpha$ -limit dextrins. On the other hand, the brush border  $\alpha$ -glucosidases hydrolyze both  $\alpha$ -1,4 and  $\alpha$ -1,6 glycosidic bonds and produce glucose. Regarding kinetics of bond cleavage,  $\alpha$ -1,6 glycosidic bonds hydrolyze more slowly

compared to  $\alpha$ -1,4 bonds (Kerr *et al.*, 1951), and this approach has been used to slow starch digestion rate (Ao *et al.*, 2007).

While pancreatic  $\alpha$ -amylase plays a pivotal role in starch digestion (Englyst *et al.*, 1992), the mucosal  $\alpha$ -glucosidases are a recognized control point for glucose generation and, consequently, are of equal importance in strategies to slow glucose delivery from starch. Even the  $\alpha$ -amylase degradation products of maltotriose, maltotetraose, and maltopentose have been shown to have some inhibitory action on the most active of the four  $\alpha$ -glucosidases, the glucoamylase (Quezada-Calvillo *et al.*, 2007, 2008).

Mucosal MGAM (E.C. 3.2.1.20 and 3.2.1.3, encoded by the gene MGAM, located on chromosome 7q34) and SI (E.C. 3.2.1.48 and 3.2.1.10, encoded by the gene SI, located on chromosome 3q26) (Nichols *et al.*, 1998, 2003) are complexes of two enzymes each that belong to the glucohydrolase Family 31. All four enzyme subunits have  $\alpha$ -1,4 exo-glycosidic activity from the non-reducing ends of maltooligosaccharides. Though commonly termed maltase, glucoamylase, isomaltase and sucrase, these enzymes all have maltase activity and other activities overlap into different subunits. This lack of specificity of terminology has led to the use of terms associated with their location relative to the anchor point on the brush border enterocytes. Thus, N-terminal MGAM is maltase, C-terminal MGAM is glucoamylase, N-terminal SI is isomaltase and C-terminal SI is sucrase. The main debranching enzyme is Nt-SI although Nt-MGAM also has some isomaltase activity (Sim *et al.*, 2010). Ct-MGAM has higher hydrolytic property for  $\alpha$ -glucans with longer chains (Sim *et al.*, 2008), and can even digest native starch amylopectin (Lin *et al.*, 2012).

## 6.4 STRUCTURAL AND PHYSIOLOGICAL FUNDAMENTALS OF SDS

SDS is not a defined entity of starch, as is RS with its subcategories of RS 1-4 types, and it is more of a concept than a specific material; it is an *in vitro* measurement of starch digestion between 20 and 120 minutes when using the enzymes and conditions as stated by Englyst *et al.* (1992). It corresponds to the *in vivo* concept of slowly available glucose (SAG), although SAG can come also from other slowly digestible glycaemic carbohydrates. Accordingly, knowledge of starch structure and the various ways that SDS can be generated is necessary to develop strategies for SDS product development. In our view, there are four basic mechanisms for generating SDS:

1. physical or food matrix structures that decrease enzyme accessibility;
2. chemical structures that limit rate of hydrolysis;



3. other food factors that decrease digestion rate (e.g. viscosity, inhibitors);
4. physiological control of food motility (i.e. gastric emptying).

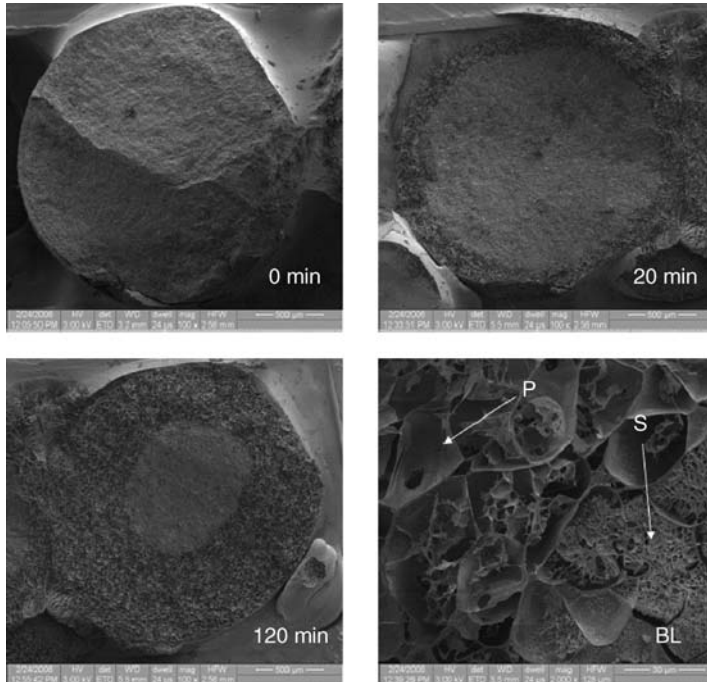
#### 6.4.1 Physical or food matrix structures related to SDS

Processed foods, such as pasta, often have structures related to food matrices that lead to SDS. These are generally where starch in the food is less accessible due to other surrounding food components, such as the dense protein matrix of pasta (Fardet *et al.*, 1998) or hard-to-digest protein matrix in sorghum porridges (Zhang & Hamaker, 1998).

It is raw starch itself, with its natural semi-crystalline granular structure comprised of concentric layers of amorphous and crystalline regions, that is perhaps the best example of SDS physical structure. Amylopectin fine structure differences form the basis of the semi-crystalline A, B, and C x-ray diffraction patterns (Thompson, 2000). Due to a combination of crystallite packing and accessibility of the granule to enzymes afforded by surface pores leading to interior channels, raw cereal starches naturally have a high amount of SDS material (Seal *et al.*, 2003).

Gérard *et al.* (2001), Jane *et al.* (1997), and Planchot *et al.* (1997) have shown that B-type tuber starches are resistant to enzyme digestion, while A-type cereal starches are mostly slowly digestible. While amorphous regions of the starch granule have been thought to be more easily digested than crystalline regions (Gallant *et al.*, 1992), Zhang *et al.* (2006a, 2006b) revealed that degree of crystallinity did not change after amylolysis, indicating that amorphous and crystalline regions are digested simultaneously. The proposed side-by-side digestion mechanism starts from the channels (see Figure 6.1) and proceeds to the observed inside-out digestion pattern. Apparently, this is caused by tight linkages between amorphous and crystalline layers. The layer-by-layer crystalline (A-type) and amorphous regions are the structural basis for the slow digestion property of native cereal starches, providing a strategy for developing starch-based structures that are slowly digestible.

As mentioned, supramolecular level food matrices, such as exist in cooked pasta with protein networks, may cause slow starch digestion due to limited accessibility of starch-degrading enzymes to starch (Fardet *et al.*, 1998). To better control starch digestion rates for experimental purposes, we made starch-entrapped microspheres that consist of starch in a quick-setting gel (sodium alginate in calcium chloride) (Venkatachalam *et al.*, 2009; Figure 6.2). The porous polysaccharide structure permits entrance of amylase to gelatinized starch and the rate of digestion is controlled by pore size. This provides a strategy to achieve SDS.

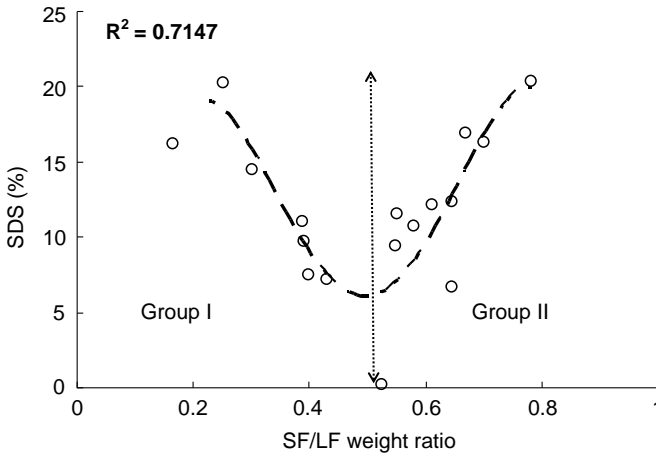


**Figure 6.2** Starch-entrapped microspheres in a gelled porous alginate matrix digested to 120 minutes with the *in vitro* Englyst assay. Reproduced with permission from Venkatachalam *et al.*, 2006.

#### 6.4.2 Starch chemical structures leading to SDS

Two main factors regarding starch structure affect its digestion rate: the degree and extent of retrogradation; and the amount and placement of  $\alpha$ -1–6 branch points. Certainly, most starchy foods that are consumed are cooked. In this process, when water content is sufficient, starch gelatinizes, with a concomitant loss in starch crystalline structure and a large increase in rate of starch digestion. Generally cooked processed foods have very little SDS component.

Regarding retrogradation of starch, gelatinized amylose retrogrades rapidly and forms double helices in the range of 40 to 70 DP long that align into higher-ordered crystalline structures that become resistant in nature (Leloup *et al.*, 2004). It is not clear whether a strategy of controlling amylose retrogradation into more slowly digesting structures is possible, but this could be a way to obtain SDS. A less well known effect of retrogradation on digestion rate is that of amylopectin. Retrogradation of the external chains



**Figure 6.3** A parabolic relationship between SDS percentage and the weight ratio of short-chain (SF) to long-chain fraction (LF) (Zhang *et al.*, 2008a). Reprinted with permission from Zhang *et al.*, 2008a. Copyright 2008 American Chemical Society.

slows rate of digestion (Zhang *et al.*, 2008b). Along this line, retrogradation of partially debranched amylopectin after isoamylase treatment has been used to make SDS (Shi *et al.*, 2003; Shin *et al.*, 2004).

In our own investigations (Zhang *et al.*, 2008a, 2008b), we showed that amylopectin fine structure differences can drive the SDS amount. Using a range of maize starch mutants, two paths towards increase were found, represented by a parabolic relationship between SDS content and the weight ratio of amylopectin short chains ( $DP < 13$ ) to long chains ( $DP \geq 13$ ) (Figure 6.3). Amylopectin with either a high amount of short chains (higher branch points and shorter external chains), or a higher proportion of long internal chains, had a higher content of SDS (Figure 6.3).

Two mechanisms were found to drive slow digestion of gelatinized starches: inherently high branch density somewhat slows digestion due to the slower kinetics associated with digestion of  $\alpha$ -1,6 linkages, while high long chains drives faster retrogradation, as has been noted by others (Klucinec & Thompson, 1999; Matalanis *et al.*, 2009). In the latter retrograded type, the SDS property appeared to be transient in nature, as increased storage time converted some SDS to RS (Zhang *et al.*, 2008b). Regarding the effect of branched density, another  $\alpha$ -glucan structure with high  $\alpha$ -1,6 linkages, though not in a branched structure like amylopectin, is pullulan, and digestion was likewise affected (Wolf *et al.*, 2003). Thus, amylopectin seems associated with the SDS property.

### 6.4.3 Other food factors that decrease digestion rate

Much focus on decreasing starch digestion rate has been on food viscosity, as well as  $\alpha$ -amylase and  $\alpha$ -glucosidase natural inhibitors, and other factors such as organic acids. These have been associated mostly with changes in glycaemic response or GI.

Water-soluble polysaccharides that impart significant viscosity effect have been shown to result in slower digestion and absorption of glycaemic carbohydrates, and also reduced postprandial glycaemic and insulinaemic responses (Jenkins *et al.*, 1976, 1977). Common fibres in this category include  $\beta$ -glucans, psyllium and guar gum, and these have been shown to moderate glycaemic response even when incorporated into food products (Wood *et al.*, 1990; Yokayama *et al.*, 1997; Jenkins *et al.*, 2002). At a mechanistic level, viscosity appears to cause a slower gastric emptying as well as lower accessibility of enzymes to substrates (Leclère *et al.*, 1994; Slavin, 2005), accompanied by a reduced diffusion rate of digested carbohydrates to the mucosal surface (Briani *et al.*, 1987).

Natural  $\alpha$ -amylase and  $\alpha$ -glucosidase inhibitors can moderate starch digestion to blunt both glycaemic and insulinaemic responses. Strong inhibition can result in an increase in resistant starch, though partial inhibition can produce a slowly digestible effect. Acarbose, a modified tetrasaccharide formed by the genus *Actinoplanes*, is one of the best studied of such inhibitors, with most specific inhibition of the C-terminal  $\alpha$ -glucosidases (Hiele *et al.*, 1992; Eskandari *et al.*, 2011). Due to its blunting of postprandial glycaemic response, it has been used in treatment of diabetes (Chiasson *et al.*, 1994; Conniff *et al.*, 1994). A well-studied  $\alpha$ -amylase inhibitor comes from Great Northern beans (phaseolamin) and has been shown to promote glucose homeostasis (Boivin *et al.*, 1988).

There is a significant literature on natural inhibitors of both  $\alpha$ -amylase and  $\alpha$ -glucosidases. A number of phenolic compounds, such as those found in green tea extract (Konishi *et al.*, 2006), inhibit enzyme activity and lower glycaemic response. As a strategy to slow glycaemic carbohydrate digestion and/or absorption, use of natural inhibitors in foods necessitates more study to determine physiologically relevant levels and other potential unintended consequences.

Lower glycaemic and insulinaemic responses have been noted with consumption of organic acids, such as found in sourdough fermentation (Liljeberg and Björck, 1996, 1998; Liljeberg *et al.*, 1995). For example, sodium propionate added to bread decreased postprandial blood glucose and insulin responses and also increased satiety compared to control breads. This was attributed to delayed gastric emptying. Vinegar added to a starchy meal had a similar effect (Liljeberg and Björck, 1998).

#### **6.4.4 Physiological control of food motility**

Slow digestion of starch can perhaps be best achieved by control of transit time of food through the upper gastrointestinal tract, and this is mainly through control of the rate of gastric emptying. Both the ileal break and the colonic break have the effect of slowing gastric emptying. The former relates to sensing of macronutrients (fat, protein, carbohydrate) in the ileum, through triggering of gut hormones and the nervous system that causes longer retention of food in the stomach (Van Citters & Lin, 2006). The colonic break relates mainly to dietary fibre, including RS, fermentation that also triggers the gut hormone peptide YY and, perhaps, GLP-1, which slows gastric emptying (Massimino *et al.*, 1998; Cuche *et al.*, 2000).

Starch that is portioned out of the stomach to the small intestine over an extended period of time is, in essence, a 'slowly digestible starch'. Dietary fibre fermentation also can cause the 'second meal effect', as noted by Jenkins *et al.* (1982), Wolever *et al.* (1988) and Brighenti *et al.* (2006), which moderates glycaemic and insulinaemic responses of the subsequent meal. Resistant starch has specifically been shown to have this effect (Björck *et al.*, 2000).

### **6.5 APPLICATION-ORIENTED STRATEGIES TO MAKE SDS**

Currently, there are very few approaches to make commercially viable SDS. Practically, at this time strategies can be reduced to two areas: starch molecular structures that reduce digestion rate; and food matrices with slow starch digestion. A central question that remains, however, is what degree of slow digestion effect, and what amount consumed, is necessary to obtain a desired physiological, health-related outcome. This is one of the main unanswered questions that impedes SDS development of commercial products.

#### **6.5.1 Starch-based ingredients**

As mentioned above, starch amylopectin structures with high branching and short external chains, or with a high proportion of long chains, have higher SDS contents. Additionally, enzymatic, physical, and chemical modifications of starch have been attempted, with the aim of decreasing digestion rate.

Modification of starch molecular structures through genetic manipulation of synthesizing enzymes, or through enzyme modification by processing,

is an approach with good potential for the creation of SDS products. Main strategies would be to produce highly branched structures with short external chains or structures that retrograde to slowly digestible crystallites.  $\beta$ -amylase has been used to shorten non-reducing external chains of amylopectin to increase branch density and branching enzymes to add branches (Hamaker & Han, 2006; Ao *et al.*, 2007). The patent of Backer & Saniez (2005) showed a method to produce a highly branched soluble  $\alpha$ -glucan using an enzymatic approach. Shi *et al.* (2003), in their patent, showed a method of making retrograded debranched linear chains that are slowly digestible.

Physical modification of starch to slow starch digestion rate has been achieved by heat-moisture treatment, temperature cycling and storage conditions. Hydrothermal treatment of sweet potato starch resulted in A-type crystalline structure that coincided with a large increase in SDS over native granules (Shin *et al.*, 2004). Using waxy potato starch, Lee *et al.* (2011) increased SDS content to 42% by heat-moisture treatment and gave a favourable glycaemic response in mice. Temperature-cycling retrogradation of waxy rice starch also resulted in a high SDS value of 52% (Zhang *et al.*, 2011).

Regarding chemical modification, digestion properties of starch can be changed through the addition of functional groups to the linear chains of starch. Many modification methods make some degree of RS, such as oxidation, acetylation, cross-linking and etherification (Wolf *et al.*, 1999). Octenyl succinic anhydride (OSA) esterification, in particular, appears to increase SDS content (Han & BeMiller, 2007), and Wolf *et al.* (2001) found modified *in vivo* glycaemic response of OSA-modified starch. OSA-modified starch molecules slow enzyme digestion through an uncompetitive inhibitor mechanism (He *et al.*, 2008).

### **6.5.2 SDS generation in a food matrix**

Another approach to making SDS products is through use of food processing technologies to create matrices that slow access to starch and to limit the extent of starch gelatinization, which also slows digestion. Dense food matrices, such as dense pastas, are normally associated with SDS, though the interactions of specific food components with starch can also slow starch digestion. The most recognized interaction is the inclusion complex between amylose and lipid.

Lower digestibility has been noted in foods that have amylose-monoglyceride complexes (Holm *et al.*, 1983; Seneviratne & Biliaderis, 1991; Murray *et al.*, 1998). Saturated monoglycerides with long aliphatic tails cause slower digestion (Eliasson & Krog, 1985). Patil *et al.* (1998)

showed that amylose-lipid complexes mixed in the diet of dogs somewhat lowered glycaemic and insulinaemic responses. Starch conjugated through the Maillard reaction with protein or amino acids has also been shown to reduce digestibility (Yang *et al.*, 1998). Legume or pulses, particularly when cooked as a whole grain, have a comparably high content of SDS that is at least partly due to physical entrapment in cell walls (Englyst *et al.*, 1996; McCrory *et al.*, 2010).

## 6.6 CONSIDERATIONS

There is growing evidence that the rate and location of starch digestion in the upper gastrointestinal tract has physiological and metabolic consequences that relate to health. This applies not only for starch, which is the major glycaemic carbohydrate in most diets, but also for other slowly digestible carbohydrates. While SDS is distinctly different from RS in that it is digestible and generates glucose directly for absorption in the small intestine, starch digestion rate is impacted by RS and its effect on gastric emptying. Additionally, glucose release in the ileum of the small intestine also likely slows gastric emptying. When considering the wide range of time of postprandial digestion of starches in foods, perhaps control of gastric emptying has the largest single effect on rate of starch digestion, and SDS may itself lengthen emptying time through feedback mechanisms.

However, even considering that some methods exist to make SDS ingredients or foods, there is a lack of knowledge as to the amount and manner of delivery that is needed to produce a desired effect. Further understanding of consequences of SDS that might include energy balance, metabolic diseases, cognitive and physical performance and satiety, are needed. New technologies also must be identified to make robust and more defined SDS materials for use in the food industry.

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# 7 Measurement of Resistant Starch and Incorporation of Resistant Starch into Dietary Fibre Measurements

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## 7.1 INTRODUCTION

Resistant starch (RS) is that portion of the starch that is not broken down and absorbed in the small intestine of humans. It enters the large intestine, where it is partially or wholly fermented. The presence of a starch fraction resistant to enzymic hydrolysis was first recognized by Englyst *et al.* (1982) during their research on the measurement of non-starch polysaccharides.

Several *in vivo* approaches have been adopted for the measurement of resistant starch, including: the hydrogen breath test; direct collection of ileal effluent from patients (ileostomy patients) who have had the colon removed; and direct collection of the ileal effluent from healthy subjects using a long triple lumen tube (Champ *et al.*, 2001). Of these, the ileostomy model is considered to be the best, but not necessarily perfect.

It is generally accepted that any *in vitro* method used to measure resistant starch must give values in line with those obtained with ileostomy patients. Berry (1986) modified the *in vitro* method of Englyst *et al.* (1982) to mimic physiological conditions more closely. Incubations were performed at 37 °C. Pancreatic  $\alpha$ -amylase and pullulanase was again employed, but the initial heating step at 100 °C was omitted. Using this method, the measured resistant starch contents of samples were much higher than those previously obtained. This was subsequently confirmed by Englyst & Cummings (1985, 1986, 1987) through studies with healthy ileostomy subjects.

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These authors (Englyst *et al.* 1992) also divided RS into three classes, namely:

- RS1: physically trapped starch as found in coarsely ground or chewed cereals, legumes, and grains.
- RS2: resistant starch granules or non-gelatinized starch granules which are highly resistant to digestion by  $\alpha$ -amylase until gelatinized, e.g. uncooked potato, green banana and high-amylose starch).
- RS3: retrograded starch polymers (mainly amylose), which are produced when starch is cooled after gelatinization or during heat-moisture treatment on annealing of starch granules (during which the starch is not gelatinized).

A fourth type of resistant starch (RS4; chemically modified starch) was introduced by Brown *et al.* (1998). This starch, unlike RS1, RS2 and RS3, contains additional chemical groups. Englyst *et al.* (1992) also reported on a method for the measurement of readily digested starch (RDS), slowly digested starch (SDS) and resistant starch (RS). In this method, resistant starch is calculated by subtracting the sum of RDS plus SDS from total starch. Although the method can yield useful information, it is very laborious and gives poor reproducibility without extensive training of the analyst (Champ *et al.*, 2001). Accuracy is severely hampered by the fact that, with samples containing high levels of starch with low resistant starch content, one large analytical value is subtracted from another large value. In fact, the errors in the measurement may be as large as the resistant starch value, e.g. materials with approximately 70% starch and 2% resistant starch.

By the early 1990s, the physiological significance of RS was fully realized. Several new or modified methods for its measurement were developed during the European Research Program, EURESTA (Englyst *et al.*, 1992; Champ, 1992). The Champ (1992) method, was based on modifications to the method of Berry (1986), and gave a direct measurement of RS. Basically, sample size was increased from 10 mg to 100 mg, the sample was digested with pancreatic  $\alpha$ -amylase only, and incubations were performed at pH 6.9 (pH 5.2 was used by Englyst *et al.* (1982, 1992) and Berry (1986)). RS determinations were performed directly on the pellet.

Muir & O'Dea (1992) developed a procedure for RS in which samples were chewed, treated with pepsin and then with a mixture of pancreatic  $\alpha$ -amylase and amyloglucosidase (AMG) in a shaking water bath at pH 5.0, 37°C for 15 hours. The residual pellet (containing RS) was recovered by centrifugation and washed with acetate buffer by centrifugation, and the RS was digested by a combination of heat, DMSO and thermostable  $\alpha$ -amylase treatments.



Other methods for RS determination were developed by Faisant *et al.* (1995), Goni *et al.* (1996), Akerberg *et al.* (1998) and Champ *et al.* (1999). These modifications included changes in enzyme concentrations employed, types of enzymes used (all used pancreatic  $\alpha$ -amylase, but pullulanase was removed, and in some cases replaced by AMG), sample pre-treatment (chewing), pH of incubation and the addition (or not) of ethanol after the  $\alpha$ -amylase incubation step (Champ *et al.*, 1999).

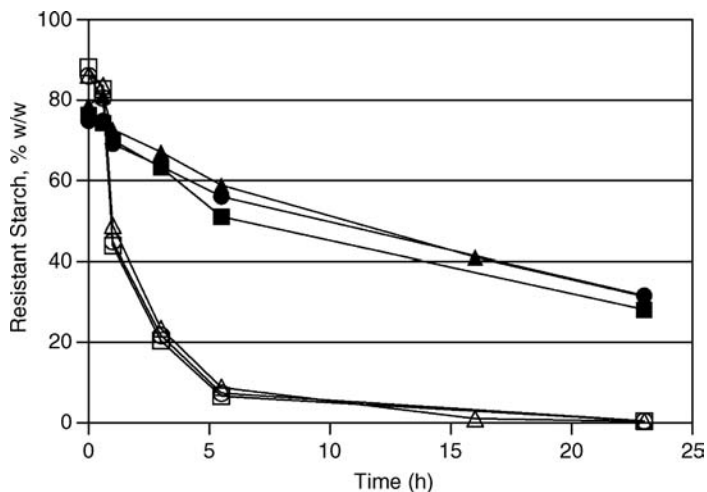
## **7.2 DEVELOPMENT OF AOAC OFFICIAL METHOD 2002.02**

While significant steps were made in the development of *in vitro* methods for the measurement of resistant starch during the 1990s, none of these methods were successfully subjected to interlaboratory evaluation. This prompted McCleary & Monaghan (2002) to look at each of these methods in detail, to evaluate all of the parameters involved and to identify sources of variability. The ultimate aim was to develop a procedure that gave values in line with those obtained with ileostomy patients, but also a method that could survive the rigors of interlaboratory evaluation. Parameters investigated included:

- a) incubation conditions (shaking/stirring, pH, temperature, time);
- b) level of pancreatic  $\alpha$ -amylase employed;
- c) level of AMG employed;
- d) the importance of protease pre-treatment;
- e) procedures for recovery of resistant starch;
- f) method for the dissolution of RS; and
- g) glucose determination procedure.

Incubations were performed at physiological temperature (37 °C) in both a shaking water bath and in an arrangement in which the contents of the tubes were continually stirred at different speeds. Incubations were allowed to proceed for up to 24 hours, and the RS values obtained for a set of samples were compared to values obtained from ileostomy studies. Typical results obtained for regular maize starch (RMS) and high-amylose maize starch (HAMS) are shown in Figure 7.1.

These incubations were performed in the presence of optimal levels of AMG and at a pH of 6.0. This pH was chosen as a compromise to allow for the different pH optima of  $\alpha$ -amylase (pH 6.9) and AMG (pH 4.5) and the determined stability of the two enzymes on extended incubation at different pH values. At pH 6.0, pancreatic  $\alpha$ -amylase has approximately 80% of the



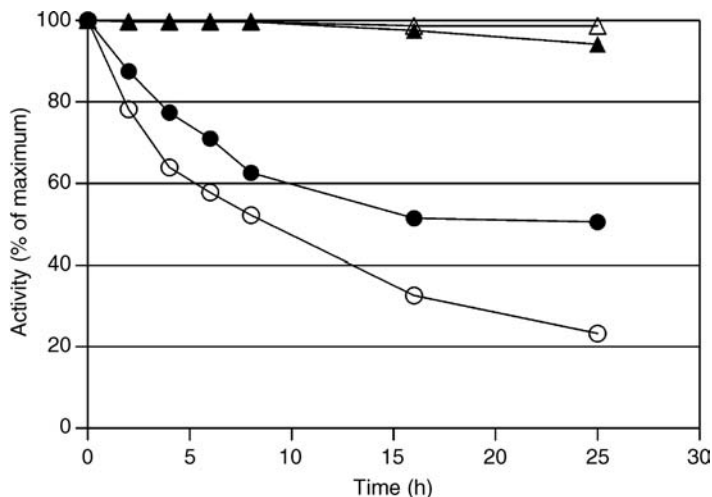
**Figure 7.1** The effect of the concentration of pancreatic  $\alpha$ -amylase and incubation time on the determined RS value of regular maize starch (RMS) and high-amylose maize starch (HAMS) at pH 6.0 and 37 °C for up to 25 hours. HAMS with pancreatic  $\alpha$ -amylase concentrations of (black triangle) 15, (black circle) 30 and (black square) 60 U/mL; RMS with pancreatic  $\alpha$ -amylase concentrations of (open triangle) 15, (open circle) 30 and (open square) 60 U/mL.

activity at the optimal pH of 6.9, and AMG has 20% of the activity at the optimal pH of 4.5. The level of each enzyme is adjusted to optimize the assay.

AMG is very stable at both pH 6.0 and 6.9, losing less than 5% of initial activity on incubation at 37 °C for 16 hours. Pancreatic  $\alpha$ -amylase is less stable, however; even at pH 6.0, approximately 30% of activity remains on incubation of the enzyme under assay conditions of 16 hours at 37 °C (compared to 50% at pH 6.9) (Figure 7.2).

There was considerable flexibility in the concentration of pancreatic  $\alpha$ -amylase used. RS values obtained for RMS and HAMS varied little, with enzyme concentrations ranging from 15–60 units/ml of incubation mixture (see Figure 7.1).

AMG in the incubation mixture had a considerable effect on the determined RS values (McCleary & Monaghan, 2002); this is considered to be due to the known inhibitory effect of maltose on pancreatic  $\alpha$ -amylase. The AMG removed the maltose by hydrolyzing it to glucose, which has no inhibitory effect. The effect of protease on determined RS values was studied by including a pre-treatment with pepsin at pH 2. Results obtained indicated that the protease pre-treatment had no significant effect on determined RS values (McCleary & Monaghan, 2002). This may be due, in part, to the presence of an active protease in the pancreatic  $\alpha$ -amylase preparation used.



**Figure 7.2** Stability of pancreatic  $\alpha$ -amylase and AMG on incubation at pH 6.0 and 6.9 and 37°C for up to 25 hours. AMG at pH 6.0 (closed triangle) and 6.9 (open triangle); pancreatic  $\alpha$ -amylase at pH 6.0 (open circle) and 6.9 (closed circle).

Resistant starch is crystalline and is difficult to dissolve. Solvents used to dissolve this material include dimethyl sulphoxide (DMSO) and 2–4 M potassium hydroxide. For all samples studied, the RS was completely dissolved by stirring the RS containing pellet in 2 M KOH in an ice/water bath for 20 minutes (conditions used by several authors). On neutralization, it was essential to hydrolyze this starch rapidly to avoid re-crystallization (which would again render the starch resistant to hydrolysis by AMG. To achieve this and to simplify neutralization, a concentrated sodium acetate buffer (1.2 M, pH 3.8) was added, followed immediately by AMG (320 units/test; one unit of AMG activity is the amount of enzyme required to release one micromole of glucose from soluble starch per minute at pH 4.5 and 40°C).

This method for the measurement of RS was subjected to evaluation under the auspices of AOAC International and AACC International, to determine the interlaboratory performance statistics. The materials used in the study represented food materials containing RS (cooked kidney beans, green banana and cornflakes) and a range of commercial starches, most of which naturally contain (or were processed to contain) elevated RS levels. Thirty-seven laboratories tested eight pairs of blind duplicate starch or plant material samples, with RS values between 0.6 (regular maize starch) and 64% (fresh weight basis). For samples excluding regular maize starch, RSD<sub>r</sub> values

ranged from 1.97–4.2% and  $RSD_R$  values ranged from 4.58–10.9%. The range of applicability of the test is 2–64% RS.

The method was not suitable for samples with less than 1% RS (e.g. regular maize starch; 0.6% RS). For such samples,  $RSD_T$  and  $RSD_R$  values are unacceptably high. On the basis of this evaluation, the method was accepted as AOAC Official Method 2002.02 and AACC Recommended method 32–40.01 (McCleary *et al.*, 2002).

### 7.3 DEVELOPMENT OF AN INTEGRATED PROCEDURE FOR THE MEASUREMENT OF TOTAL DIETARY FIBRE

Hipsley (1953) coined the term *dietary fibre* to cover the non-digestible constituents of plants that make up the plant cell wall (known to include cellulose, hemicellulose and lignin) with the aim of defining some property of the constituent of the food that could be related to physiological behaviour in the human small intestine. This definition was broadened by Trowell *et al.* (1976) to become primarily a physiological definition, based on edibility and resistance to digestion in the human small intestine. Thus, the definition included indigestible polysaccharides such as gums, modified celluloses, mucilages and pectin, and non-digestible oligosaccharides (NDO).

Methods which were developed to meet this analytical requirement focused on the use of enzymes to remove starch and protein. The enzymes employed require a defined level of activity and must be devoid of contaminating enzymes active on dietary fibre components such as pectin,  $\beta$ -glucan, arabinoxylan and other hemicelluloses. Following extensive international collaboration, the method that evolved was AOAC Official Method 985.29, 'Total dietary fibre in foods; enzymatic-gravimetric method' (Prosky *et al.*, 1985, 1994) This method was subsequently extended to allow measurement of total dietary fibre (TDF), soluble dietary fibre (SDF) and insoluble dietary fibre (IDF) in foods (AOAC Official Method 991.43) (Lee *et al.*, 1992) Other modifications to these methods for fibre analysis have also been approved by AOAC International (Theander & Aman, 1982).

In concurrent research in the UK, methods were developed for the measurement of non-starch polysaccharides (NSP) (Englyst *et al.*, 1982; Englyst & Cummings, 1984, 1985; Englyst & Hudson, 1987), based on the original work of Southgate (1969) and Southgate *et al.* (1978). These NSP procedures measure only NSP; RS and NDO are excluded. Starch in the sample is completely dissolved in hot dimethyl sulphoxide (DMSO), diluted in buffer and depolymerized with thermostable  $\alpha$ -amylase, followed by a mixture of

pancreatin and pullulanase. The NSP recovered is acid hydrolyzed to monosaccharides, which are measured by high-performance liquid chromatography (HPLC), gas-liquid chromatography (after derivatization) (Englyst & Cummings, 1984) or colorimetrically (Englyst & Hudson, 1987). These methods have not been successfully subjected to international interlaboratory evaluation.

A survey of scientists initiated in 1993 (Lee & Prosky, 1995) showed that 65% of the respondents favoured the inclusion of NDO and 80% favoured inclusion of RS in the definition of dietary fibre. This led to the development of methods for measurement of RS (AOAC Method 2002.02) and for a number of NDO, including fructo-oligosaccharides (AOAC Methods 997.08 and 999.03), polydextrose (AOAC Method 2000.11), resistant maltodextrins (AOAC Method 2001.03), and galacto-oligosaccharides (AOAC Method 2001.02).

In 1998, the American Association of Cereal Chemists began a critical review of the current state of dietary fibre science, including consideration of the state of the dietary fibre definition. Over the course of the following year, the committee held three workshops and provided an international website, available to all Web users worldwide, to receive comments. All interested parties were provided with additional opportunity for comment. After due deliberation, an updated definition of dietary fibre was delivered to the AACC Board of Directors for adoption in early 2000 and published (Anon, 2001) namely:

*'Dietary fibre is the edible parts of plants or analogous carbohydrates that are resistant to digestion and absorption in the human small intestine with complete or partial fermentation in the large intestine. Dietary fibre includes polysaccharides, oligosaccharides, lignin, and associated plant substances. Dietary fibres promote beneficial physiological effects including laxation, and/or blood cholesterol attenuation, and/or blood glucose attenuation.'*

Several definitions of dietary fibre have appeared over the past ten years. The Food Nutrition Board of the Institute of Medicine of the National Academies (USA) (2002) defined dietary fibre as follows: *'Dietary fibre consists of nondigestible carbohydrates and lignin that are intrinsic and intact in plants. Added fibre consists of isolated, nondigestible carbohydrates that have beneficial physiological effects in humans. Total fibre is the sum of dietary fibre and added fibre.'*

At the 30th session of the CODEX Committee on Nutrition and Foods for Special Dietary Uses (CCNFSDU; 2008), the Committee agreed on the following definition for dietary fibre:

*'Dietary fibre is carbohydrate polymers<sup>1</sup> with ten or more monomeric units,<sup>2</sup> which are not hydrolyzed by the endogenous enzymes in the small intestine of humans and belong to the following categories:*

- *Edible carbohydrate polymers naturally occurring in the food as consumed.*
- *Carbohydrate polymers which have been obtained from food raw material by physical, enzymatic or chemical means and which have been shown to have a physiological effect of benefit to health as demonstrated by generally accepted scientific evidence to competent authorities,*
- *Synthetic carbohydrate polymers which have been shown to have a physiological effect of benefit to health as demonstrated by generally accepted scientific evidence to competent authorities.*

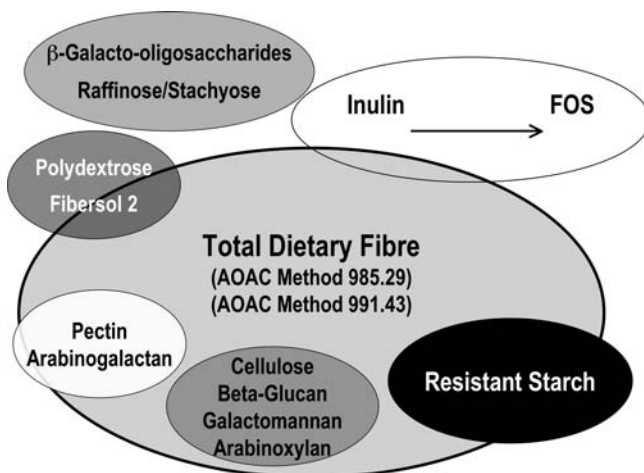
The fact that a single method to measure all dietary fibre components is needed has been known for some time. While it is possible to measure many individual fibre components with specific and non-specific methods, total dietary fibre cannot simply be calculated by adding the values for these specific components to the determined value of high molecular weight dietary fibre, as measured with AOAC Official Methods 985.29 or 991.43. Since these latter methods also measure some of the RS and various NDO in food materials, summation leads to 'double counting' of this material (Figure 7.3; McCleary *et al.*, 2009).

An integrated method for the measurement of total dietary fibre was published in 2007 (McCleary, 2007). This method allows the accurate measurement of total high molecular weight dietary fibre (HMWDF), which includes IDF (including RS) and higher molecular weight soluble dietary fibre which precipitates in the presence of 76% aqueous ethanol (SDFP), as well as lower molecular weight soluble dietary fibre which remains soluble in the presence of 76% aqueous ethanol (SDFS). Details of this procedure are outlined in Figure 7.4.

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<sup>1</sup> *When derived from a plant origin, dietary fibre may include fractions of lignin and/or other compounds when associated with polysaccharides in the plant cell walls and if these compounds are quantified by the AOAC gravimetric analytical method for dietary fibre analysis: Fractions of lignin and the other compounds (proteic fractions, phenolic compounds, waxes, saponins, phytates, cutin, phytosterols, etc.) intimately 'associated' with plant polysaccharides in the AOAC 991.43 method. These substances are included in the definition of fibre insofar as they are actually associated with the poly- or oligo-saccharidic fraction of fibre. However when extracted or even re-introduced in to a food containing non digestible polysaccharides, then they cannot be defined as dietary fibre. When combined with polysaccharides, these associated substances may provide additional beneficial effects.*

<sup>2</sup> *Decision on whether to include carbohydrates of 3 to 9 monomeric units should be left up to national authorities.'*

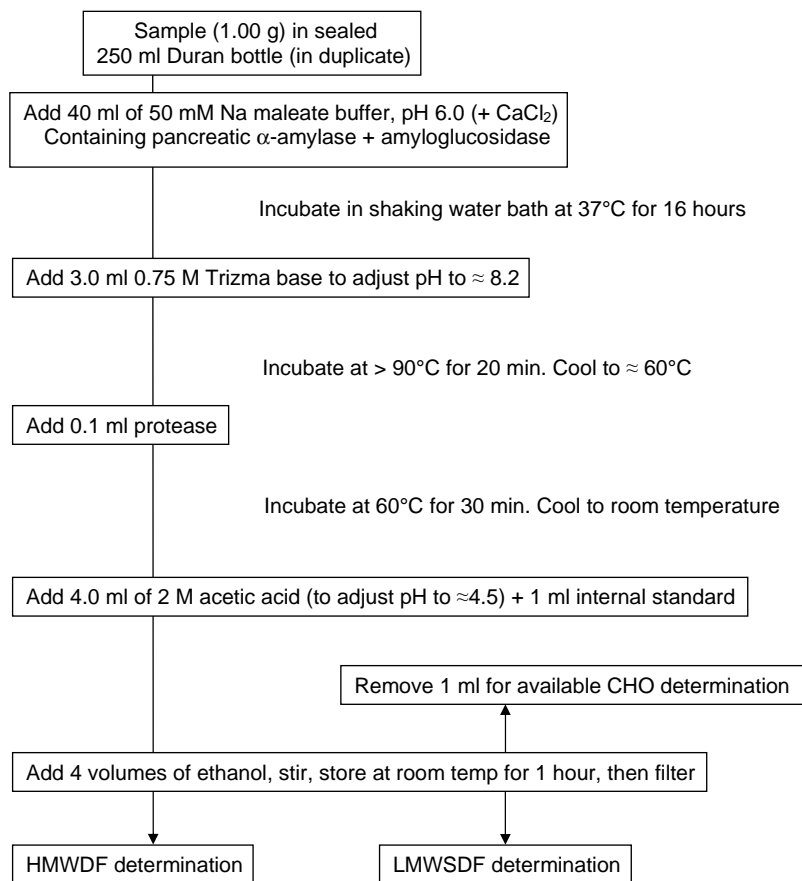


**Figure 7.3** Schematic representation of dietary fibre components measured, and not measured, by AOAC Official Methods 985.29 and 991.43. Also depicted are the problems of partial measurement of RS, Polydextrose® and resistant maltodextrins by current AOAC total dietary fibre methods. Most of the SDFS (galactooligosaccharides, fructooligosaccharides, etc) are not measured. The currently described integrated total dietary fibre procedure measures all components shown, with no double counting.

The use of pancreatic  $\alpha$ -amylase more closely simulates digestion in the human digestive tract and yields RS values in line with those obtained with AOAC Official Method 2002.02, and with results from ileostomy patients. For most food and ingredient samples analyzed, the RS values obtained with AOAC Method 2009.01 were higher than those obtained with AOAC Method 985.29. The notable exception is for phosphate cross-linked starch (RS4), where values obtained with Method 2009.01 are much lower than those obtained with Method 985.29. The physiological significance of these results will be discussed separately (McCleary *et al.*, 2013).

This method was successfully subjected to interlaboratory evaluation (McCleary *et al.*, 2009) and accepted as AOAC Method 2009.01. In this study, total HMWDF and SDFS were measured.

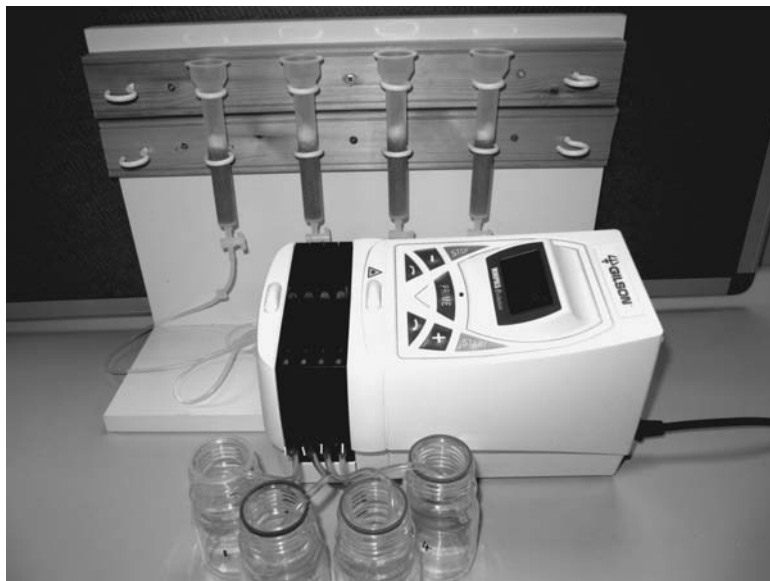
In an AOAC/AACCI interlaboratory study recently completed, the method was evaluated for the measurement of IDF, SDFP and SDFS. IDF and SDFP are measured using the standard gravimetric procedures, with allowance for ash and non-digested protein. SDFS is analyzed by HPLC using D-sorbitol as internal standard and a Waters Corporation Sugar Pak® chromatographic column. To obtain resistant starch values in line with those obtained *in vivo* with ileostomy patients, incubations are performed either in a shaking water



**Figure 7.4** Schematic representation of the integrated TDF assay procedure, also showing where samples can be removed for determination of available carbohydrates.

bath in orbital motion, or with a VarioMag<sup>®</sup> magnetic stirrer at 37 °C for 16 hours. The pH is adjusted, and  $\alpha$ -amylase and AMG are inactivated and protein denatured by heating the sample to about 100 °C. Incubation with protease is followed by pH adjustment and filtration to separate IDF and soluble fibres. SDFP is precipitated with ethanol and recovered and dried. After weighing, IDF and SDFP residues are analyzed for residual protein and ash. The SDFS fraction (in the ethanolic filtrate) is concentrated by rotary evaporation, re-dissolved and adjusted to pH 4.2–4.7 and incubated with AMG to remove completely any traces of higher molecular weight





**Figure 7.5** Arrangement for the deionization of samples using a mixed bed resin in a Bio-Rad, Econo-Pac® disposable chromatographic column (cat. No. 732-1010) with an Alltech One-Way Stopcock (cat. No. 211524). Also shown is a Gilson Minipuls® Evolution pump and collection bottles.

maltodextrins. The solution is then either desalted by passage through a column of mixed cation and anion ion exchange resins (Figure 7.5) or, alternatively, is desalted on line using a de-ashing pre-column.

Quantitation is greatly simplified by including an internal standard. The preferred internal standard is D-sorbitol and this is added to the sample just prior to adding ethanol to precipitate the SDFP. A number of compounds were evaluated as potential internal standards, including 1,5-pentanediol, diethylene glycol and triethylene glycol. Of these, diethylene glycol appeared best. However, on closer study, some of this is lost when the SDFS fraction is rotary evaporated, presumably by adsorbing to the glass rotary evaporator flask. This was not observed with D-sorbitol. When D-sorbitol is rotary evaporated with a range of sugars and NDO, the ratio of the components remains the same. Results of this interlaboratory study have been published (McCleary *et al.*, 2012).

A major advantage of the described method for the measurement of total dietary fibre is that it allows the separate measurement of IDF, SDFP and SDFS. There is some international debate as to whether NDO (SDFS) should be included in the dietary fibre measurements. Until there is agreement, this

oligosaccharide material can be measured and simply reported as NDO. With minor modification, the method can also be adapted to measure digestible carbohydrates (fructose, glucose, sucrose, maltodextrins and non-resistant starch, and the glucose component of lactose; McCleary, 2007).

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## 8 *In Vitro* Enzymatic Testing Method and Digestion Mechanism of Cross-linked Wheat Starch

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### 8.1 INTRODUCTION

Early studies have shown that a fraction of some starches consumed by humans escape the small intestine (Anderson *et al.*, 1981; Stephen *et al.*, 1981; Englyst & Cummings, 1985; Asp *et al.*, 1987). The total amount of starch and products of starch degradation that resist digestion in the small intestine of healthy people is termed resistant starch (RS) (Asp, 1992). RS is further categorized into five classes: physically inaccessible starch known as RS1; granular starch known as RS2; cooked and retrograded starches known as RS3; chemically modified starches known as RS4; and amylose-lipid complex (Brown *et al.*, 2006; Hasjim *et al.* 2010). The degree of starch digestibility is affected by the structure of starch granules, the physical characteristics of food, the amylose and amylopectin ratio, retrogradation of amylose and the presence of other nutrients and anti-nutrients (Sharma *et al.*, 2008; Bird *et al.*, 2009).

Chemical modification of starch has been proved to affect the extent and rate of digestibility in the small intestine (Wolf *et al.*, 1999), based on starch source, the type and degree of modification, extent of granule gelatinization and the source of enzyme used (Filer, 1971). Cross-linked (CL) starch is one of the most highly produced and utilized chemically modified starches in the food industry (Wurzburg, 1986). The highest cross-linking levels that are acceptable as food starch with 0.4% phosphorus (P) add-ons are achievable by

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using 12% sodium trimetaphosphate (STMP) and sodium triphosphate (STPP) as phosphorylating agents (Woo & Seib, 2002).

Several *in vitro* methods have been suggested to quantify RS in food, all of which involve enzymatic digestions on starch, removal of the digested starch and quantitation of remaining RS. Quantitation of RS can be carried out using direct methods or indirect methods. While direct methods determine RS in the remaining starch after removing the digested portion (Berry, 1986; Champ, 1992; Faisant *et al.*, 1993; McCleary *et al.*, 2002), the indirect methods measure RS by subtracting the digested starch from the total starch (Englyst, *et al.*, 1992; Tovar *et al.*, 1990). Determination of RS is achieved by solubilizing RS and quantifying with colorimetric methods (Berry, 1986; McCleary *et al.*, 2002), or by isolating RS gravimetrically after ethanol precipitation (Lee *et al.*, 1992; McCleary *et al.*, 2010).

Most methods use  $\alpha$ -amylase (Englyst *et al.*, 1992) or a cocktail of  $\alpha$ -amylase and amyloglucosidase (McCleary *et al.*, 2002, 2010) to hydrolyze the RDS and SDS fractions. Enzyme cocktails are used to avoid possible inhibition of  $\alpha$ -amylase by maltose and maltotriose (Sharma *et al.*, 2008). The extrinsic factor (i.e., extent of chewing, rate of orocecal transit), incubation temperature, incubation time and enzyme source may affect the *in vitro* yield of these assays (Englyst *et al.*, 1992).

Table 8.1 shows a comparison of *in vivo* and *in vitro* RS content using Englyst, AOAC Method 991.43, AOAC Method 2002.02 and AOAC Method 2009.01 in several samples. The RS content of each sample either differed slightly or significantly when compared to the *in vitro* methods used. Several samples determined by the Englyst method (raw potato starch, cornflakes and raw green banana) and AOAC Method 2002.02 (raw potato starch and raw green banana) showed results consistent with the *in vivo* data. Although AOAC Method 991.43 was reported to have consistency with ileostomy patients fed with bean and potato flakes (Schweizer *et al.*, 1990), the RS reported in Table 8.1 revealed that none of the samples except cornflakes had similar *in vivo* compatibility with the RS determined by AOAC Method 991.43. The significantly lower RS (determined by AOAC Method 991.43) of raw potato starch, high-amylose corn starch and raw green banana was likely due to a higher incubation temperature (100 °C).

The most current *in vitro* method, namely AOAC Method 2009.01, claims to effectively determine non-starch polysaccharides, RS and non-digestible oligosaccharides content in samples (McCleary *et al.*, 2010). The latter method applies key features of AOAC Method 985.29, AOAC Method 991.43, AOAC Method 2001.03 and AOAC Method 2002.02 (McCleary

**Table 8.1** Comparison of resistant starch (RS: % total starch) content in raw potato starch, high-amylose corn starch, corn flakes and raw green banana determined by *in vitro* methods (Englyst Method, AOAC Method 991.43, AOAC Method 2002.02 and AOAC Method 2009.01) and *in vivo* method (ileostomy model).

Source of starch	<i>In vitro</i> RS (%)				<i>In vivo</i> RS (%)
	Englyst	AOAC 991.43	AOAC 2002.02	AOAC 2009.01	
Raw potato starch	66.5 <sup>a</sup>	0.9 <sup>b</sup>	64.9 <sup>b</sup>	56.8 <sup>b</sup>	67.9 <sup>c</sup>
High-amylose corn starch	71.4 <sup>a</sup>	25.6 <sup>b</sup>	50.0 <sup>b</sup>	49.3 <sup>b</sup>	43.7 <sup>c</sup>
Corn flakes	3.9 <sup>a</sup>	3.3 <sup>e</sup>	2.2 <sup>b</sup>	2.4 <sup>b</sup>	3.1–5.0 <sup>d</sup>
Raw green banana	54.2 <sup>b</sup>	7.5 <sup>b</sup>	51 <sup>b</sup>	38 <sup>b</sup>	55.3 <sup>c</sup>
Cross-linked wheat starch*	81.7	82.3	–**	23.9	–

<sup>a</sup>Englyst *et al.* (1992).

<sup>b</sup>McCleary (2007).

<sup>c</sup>Langkilde & Andersson (1995).

<sup>d</sup>Muir and O'Dea, (1992).

<sup>e</sup>Pendlington (1999).

\* RS content was determined by *in vitro* methods in Kansas State University lab.

\*\* Unable to determine RS in cross-linked wheat starch due to insolubility of cross-linked starch in 2 M potassium hydroxide.

*et al.*, 2010). The starch digestion conditions in AOAC Method 2009.01 were similar to those used in AOAC Method 2002.02, which involve the incubation of a sample with a pancreatic  $\alpha$ -amylase and amyloglucosidase cocktail for 16 hours at 37 °C. Although the digestive enzyme concentration in AOAC Method 2009.01 was significantly higher, results produced by AOAC Method 2009.01 and AOAC Method 2002.02 were claimed to be similar for most samples (McCleary, 2007).

The RS content by AOAC Method 2009.01 in the samples listed in Table 8.1 showed no consistency with *in vivo* data. Based on a comparison of *in vitro* methods (Englyst Method, AOAC Method 991.43, AOAC Method 2009.01) on CL wheat starch, AOAC Method 2009.01 provided a significantly lower RS content (Table 8.1). AOAC Method 2002.02 was not included in the latter comparison, because CL wheat starch is not able to solubilize in 2 M potassium hydroxide for determination of glucose content using the colorimetric procedure.

The findings of RS content in CL wheat starch raise questions as to the efficiency of AOAC Method 2009.01 to quantify RS and the compatibility with *in vivo* results. As opposed to the Englyst Method, AOAC Method 2009.01 differs in enzyme concentration, buffers, measurement method, incubation time and enzyme sources, but both methods employ the same

incubation temperature (37 °C). The digestion kinetics, specifically the mechanism of enzyme action and incubation time in AOAC Method 2009.01, was our major interest. We predicted that 16 hours of incubation time, with a relatively high amount and concentration of digestive enzyme (40 ml  $\alpha$ -amylase (50 U/ml) and amyloglucosidase (3.4 U/ml)), would be too harsh of a condition for CL wheat starch. Hence, the objectives of this research were to study the *in vitro* digestion behaviour of CL wheat starch at various time intervals, the progressive changes of CL wheat starch granules during the *in vitro* digestion and the mechanism of the digestive enzymes during the digestion period in AOAC Method 2009.01.

## 8.2 MATERIALS AND METHODS

### 8.2.1 Materials

CL wheat starch (Fibersym<sup>®</sup> RW) and native wheat starch (Midsol<sup>™</sup> 50) were obtained from MGP Ingredients, Inc<sup>®</sup> (Atchison, Kansas). The integrated total dietary fibre assay kit (catalogue no. INTDF 06/12) was purchased from Megazyme International Ireland Ltd. (Wicklow, Ireland). All chemicals were reagent grade.

### 8.2.2 General methods

P content was assayed using the procedure of Smith & Caruso (1964.) Moisture content was obtained according to AACC Method 44-15 (AACC 2000).

### 8.2.3 Conversion of CL wheat starch to phosphodextrins and <sup>31</sup>P NMR spectra of the phosphodextrins

The treatment was based on the method described by Sang *et al.* (2010). Starch (1.0 g db) was weighed into a 50 ml centrifuge tube. The starch was slurried with 30 ml 2.0 mM calcium chloride at pH 8.2, and heat-stable  $\alpha$ -amylase (100  $\mu$ l) was then added. The mixture was heated in a boiling water bath for 30 minutes and 100  $\mu$ l of heat stable  $\alpha$ -amylase was added into the mixture again. After cooling to room temperature, the pH of the mixture was adjusted to 4.5, and amyloglucosidase (200  $\mu$ l) was incorporated for one hour to incubate at 60 °C. The mixture was adjusted to pH 7.0, centrifuged (1500  $\times$  g, 10 min), and the supernatant was freeze-dried.



The freeze-dried starch digest (1.0 g, db) was dissolved in deuterium oxide (1.0 ml) containing 20 mM EDTA and 0.002% sodium azide. The pH of the solution was adjusted to 8.0 by adding 0.1 M sodium hydroxide. The proton-decoupled  $^{31}\text{P}$  NMR spectra were obtained using a 11.75 Tesla Varian NMR System (Varian Inc., Palo Alto, CA). Using a method by Sang *et al.* (2007), the  $^1\text{H}$  NMR was operated at 499.84 MHz and  $^{31}\text{P}$  at 202.34 MHz. The  $^{31}\text{P}$  NMR data collection was carried out at 25 °C using a delay of six seconds between pulses, with a pulse width of 15.0  $\mu\text{s}$  and a sweep width at 12,730 Hz. The samples were run for eight hours for  $^{31}\text{P}$  spectra detection. The obtained spectra were processed and analyzed using Varian software VNMRJ Version 2.2C. Chemical shifts were reported in  $\delta$  (ppm) from the reference signal of an 85% phosphoric acid external standard.

### **8.2.4 Digestibility of CL wheat starch**

Digested starch of native and CL wheat starches were determined at 0, 1, 2, 4, 6, 8, 16 and 24 hours, using the  $\alpha$ -amylase and amyloglucosidase incubation procedure from AOAC Method 2009.01. To collect undigested starch for characterization, CL wheat starch was weighed to 1.0 g (db), wet with ethanol (1.0 ml), and a pancreatic  $\alpha$ -amylase/amyloglucosidase mixture (40 ml) was added to a 250 ml glass bottle. The pancreatic  $\alpha$ -amylase/amyloglucosidase mixture contained 50 units (U)/ml of  $\alpha$ -amylase and 3.4 U/ml amyloglucosidase. 1 U of  $\alpha$ -amylase at pH 5.8 and 37 °C was defined as the amount of enzyme required to release one micromole of D-glucose per minute from soluble starch. 1 U of amyloglucosidase at pH 4.5 and 40 °C was defined as the amount of enzyme required to release one micromole of D-glucose per minute from soluble starch.

The bottle was capped and placed in a water bath at 37 °C with a continuous (170 rpm) stirring via a stir bar. Fourteen bottles were incubated for each sample, and two bottles were taken out after 1, 2, 4, 6, 8, 16 and 24 hours. After cooling to 25 °C, the pH of the mixture was decreased to 2.5 to inactivate the enzyme activity with the addition 1.0 M hydrochloric acid. The mixture was held at pH 2.5 for one hour before increasing the pH to 6.0 via the addition of 1.0 M sodium hydroxide. Subsequently, the mixture was centrifuged (1500  $\times$  g, 10 min), supernatant decanted, pellet washed (two times) with water and centrifuged (1500  $\times$  g, 10 min), and the pellet was then oven-dried at 37 °C for 5 hours. The dried pellet (undigested portion of the CL wheat starch) was gently ground using a pestle and mortar and was stored at room temperature in an airtight container.

### 8.2.5 Thermal properties

The gelatinization temperatures and the enthalpy of digested CL wheat starch were measured by differential scanning calorimetry (DSC) (TA Instruments Q100, TA Instruments, New Castle, DE). The total solids content of samples were 33.3% (w/w, dry basis). After hydration for one hour at 25 °C, 30 mg of well-stirred sample suspensions were weighed into 40  $\mu$ l aluminium pans and hermetically sealed immediately to prevent moisture loss. Scans were performed from 10–130 °C at a constant heating rate of 10 °C/min. A sealed empty pan was used as a reference and the DSC was calibrated using indium. The gelatinization enthalpy ( $\Delta H$ ) and transition temperatures, namely the onset temperature ( $T_o$ ), peak temperature ( $T_p$ ) and conclusion temperature ( $T_c$ ), were determined on the basis of the first-run DSC heating curves. The  $\Delta H$  was evaluated.

### 8.2.6 Microscopic observation

CL wheat starch digested after 1, 2, 4, 6, 8, 16 and 24 hours of incubation were placed on microscope slides and observed under an Olympus BX-51 microscope (Olympus, Tokyo, Japan) with a 40 $\times$  objective. CL wheat starch before digestion was also observed under a microscope for comparison with the assayed CL wheat starch.

### 8.2.7 Scanning electron microscope (SEM)

The samples were sprinkled lightly onto a carbon double-sided adhesive tape on metal specimen stubs, which were then coated with gold-palladium (60:40 ratio) under vacuum with a Desk II Sputter/Etch Unit (Denton Vacuum, LLC, Moorestown, NJ). Micrographs of the samples were obtained at 1000 $\times$  and 5000 $\times$  magnifications by SEM (S-3500N, Hitachi Science Systems, Ltd, Japan) at an accelerating potential of 20 kV using an X-ray Detector-Link Pentafet 7021 (Oxford Instruments Microanalysis Limited, Bucks, England).

### 8.2.8 Statistical analysis

All data were statistically analyzed by analysis of variance (ANOVA) using Statistical Analysis Software (SAS) (version 9.2, SAS Institute, Cary, NC), and the values are expressed as means  $\pm$  standard deviations from two replicates, unless stated otherwise.

## 8.3 RESULTS AND DISCUSSION

### 8.3.1 Effects of $\alpha$ -amylase/amyloglucosidase digestion on P content and chemical forms of the phosphate esters on starch

The P content of CL wheat starch after different incubation times with  $\alpha$ -amylase/amyloglucosidase is reported in Table 8.2. The initial P content of CL wheat starch (at 0 hours) was 0.36%. The P content of CL wheat starch remained constant for the first two hours but started to increase at four hours. The P content progressively increased with incubation time, until it reached 0.56% at 24 hours, indicating that the molecules with higher P density were more resistant to digestion. The regions with no or low bound P were selectively removed, thus elevating the concentration of P in CL wheat starch residue.

The  $^{31}\text{P}$  NMR spectra of phosphate derivatives of CL wheat starch and their digestive residues after various  $\alpha$ -amylase/amyloglucosidase incubation periods are also tabulated in Table 8.2. The effect of digestive enzymes

**Table 8.2** Phosphorus content and levels of different phosphate esters of cross-linked wheat starch and their digestive residues after various  $\alpha$ -amylase/amyloglucosidase incubation periods.

Digestion time (h)	Total phosphorus (%) <sup>a</sup>	Phosphorus content (% dry basis) in the form of:			
		Cyclic-MSMP <sup>1</sup>	MSMP <sup>2</sup>		DSMP <sup>3</sup>
			C3	C6	
0	0.36 ± 0.01 <sup>d</sup>	0.000	0.078	0.054	0.228
1	0.36 ± 0.02 <sup>d</sup>	0.000	0.078	0.052	0.230
2	0.36 ± 0.00 <sup>d</sup>	0.000	0.080	0.052	0.229
4	0.38 ± 0.02 <sup>cd</sup>	0.004	0.090	0.057	0.229
6	0.40 ± 0.01 <sup>c</sup>	0.004	0.089	0.061	0.245
8	0.41 ± 0.00 <sup>c</sup>	0.004	0.099	0.062	0.246
16	0.47 ± 0.01 <sup>b</sup>	0.004	0.104	0.069	0.292
24	0.56 ± 0.00 <sup>a</sup>	0.005	0.136	0.080	0.338

<sup>a</sup>Values within columns bearing the same small letter superscript/s do not significantly differ at ( $p < 0.05$ ).

<sup>1</sup>Cyclic-MSMP, cyclic-monostarch phosphate.

<sup>2</sup>MSMP, monostarch monophosphate positioned at C-3 and C-6.

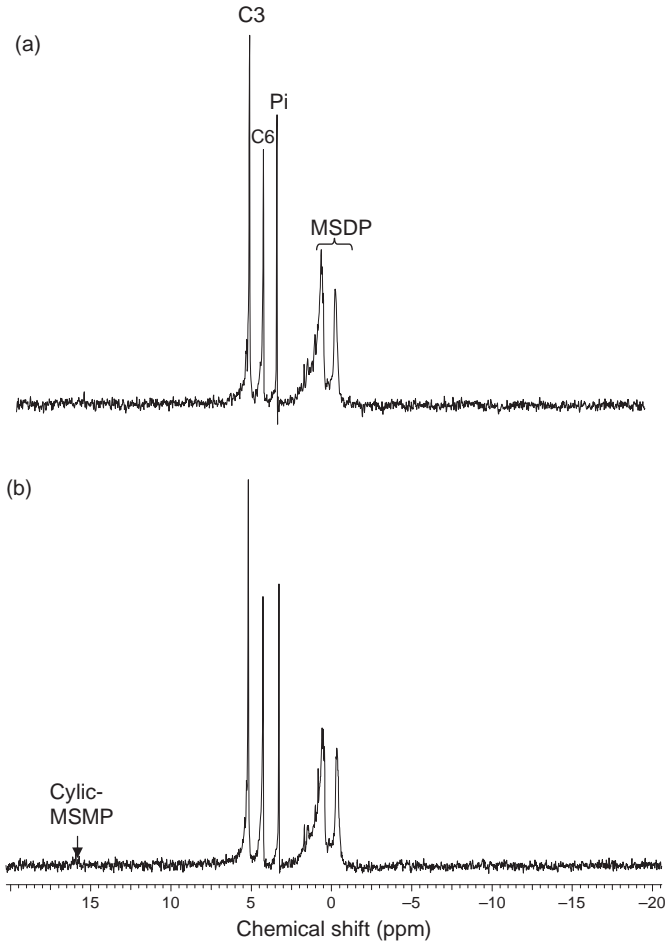
<sup>3</sup>DSMP, distarch monophosphate.

on the CL wheat starch started after four hours of incubation, causing an increase in the cyclic-monostarch monophosphate (cyclic-MSMP) and monostarch monophosphate (MSMP). The distarch monophosphate (DSMP) of CL wheat starch started to increase drastically after six hours of incubation. The remaining periods of incubation showed a significant increase in MSMP and DSMP, whereas cyclic-MSMP remained constant until the digestion was terminated.

The results suggest that cyclic-MSMP and other phosphate esters increased due to hydrolysis of starch molecules that may not be linked to P molecules, leaving the remaining starch granules that were cross-linked to P molecules.  $^{31}\text{P}$  NMR spectra of CL wheat starch after 16 hours of digestion, as shown in Figure 8.1b, has signals of cyclic-MSMP, MSMP positioned at C3 and C6, inorganic phosphate (Pi) and DSMP at  $\delta$  15.4 ppm,  $\delta$  1–3 ppm,  $\delta$  3.5 ppm and  $\delta$  0 to –1 ppm, respectively. The cyclic-MSMP had stronger peak detection as compared to the spectra before digestion (Figure 8.1a), which was consistent with the quantified cyclic-MSMP (Table 8.2).

### 8.3.2 Thermal properties

The gelatinization transition temperatures, consisting of  $T_o$ ,  $T_p$ , and  $T_c$  as well as  $\Delta H$  of CL wheat starches at different times of  $\alpha$ -amylase/amyloglucosidase incubation, are reported in Table 8.3. Gelatinization of all starches took place between 72 °C (for  $T_o$ ) to 94 °C (for  $T_c$ ), and an endothermic peak was observed in the DSC curves of all samples. The data was slightly higher than that reported by Woo & Seib (2002), presumably due to higher P content (0.36%) in the commercial CL wheat starch in our study as opposed to the laboratory-prepared CL wheat starch (0.32%). There was a slight increase in the  $T_p$  and  $T_c$  for digested CL wheat starch after 16 and 24 hours incubation. The  $\Delta H$  for CL wheat starch at all incubation times was comparable to the  $\Delta H$  before digestion, which was inconsistent with potato starch (Jiang & Liu, 2002) and maize starch (Shresta *et al.*, 2012). An amylose-lipid complex peak was absent for CL wheat starch at all incubation times. Similar values of the thermal parameters imply that the remaining CL wheat starch residual after the  $\alpha$ -amylase/amyloglucosidase digestion was likely to be intact, with little or no effect on starch granule crystallinity. Native wheat starch (Colonna *et al.*, 1988), native barley starch (Lauro *et al.*, 1999) and native maize starch (Brewer *et al.*, 2012) had been reported to have decreased crystallinity after enzymatic digestion. The current result showed that cross-linking of wheat starch aided in the retention of starch crystallinity up to 24 hours amylolysis.



**Figure 8.1**  $^{31}\text{P}$  nuclear magnetic resonance spectra of dextrans prepared from cross-linked wheat starch before (a) and after 16 hours of  $\alpha$ -amylase/amyloglucosidase digestion (b). Cyclic-MSMP: cyclic-monostarch monophosphate; C-3: monostarch monophosphate positioned at C3; C-6: monostarch monophosphate positioned at C6; MSDP: monostarch diphosphate; Pi: inorganic phosphate.

### 8.3.3 Starch granular morphology before and after enzyme digestion

Figures 8.2 and 8.3 represent light microscopic and SEM micrographs respectively of CL wheat starch after various  $\alpha$ -amylase/amyloglucosidase incubation periods. The SEM results were correlated with the microscopic

**Table 8.3** Thermal properties of cross-linked wheat starch and their digestive residues after various  $\alpha$ -amylase/amyloglucosidase incubation periods.

Digestion time (h)	Temperature ( $^{\circ}$ C)			$\Delta$ H (J/g)
	$T_o$	$T_p$	$T_c$	
0	72.0 $\pm$ 0.2 <sup>a</sup>	76.2 $\pm$ 0.2 <sup>c</sup>	89.8 $\pm$ 0.7 <sup>bc</sup>	10.7 $\pm$ 0.3 <sup>bc</sup>
1	72.2 $\pm$ 0.1 <sup>a</sup>	76.1 $\pm$ 0.1 <sup>c</sup>	90.4 $\pm$ 0.2 <sup>c</sup>	10.8 $\pm$ 0.2 <sup>bc</sup>
2	72.2 $\pm$ 0.1 <sup>a</sup>	76.1 $\pm$ 0.1 <sup>c</sup>	90.7 $\pm$ 0.3 <sup>c</sup>	11.8 $\pm$ 0.1 <sup>b</sup>
4	73.2 $\pm$ 0.4 <sup>a</sup>	77.3 $\pm$ 0.5 <sup>b</sup>	89.3 $\pm$ 0.6 <sup>bc</sup>	11.5 $\pm$ 0.1 <sup>b</sup>
6	72.3 $\pm$ 0.3 <sup>a</sup>	76.6 $\pm$ 0.3 <sup>c</sup>	91.5 $\pm$ 0.5 <sup>b</sup>	12.2 $\pm$ 0.1 <sup>a</sup>
8	72.3 $\pm$ 0.1 <sup>a</sup>	77.6 $\pm$ 0.6 <sup>b</sup>	91.7 $\pm$ 0.5 <sup>b</sup>	12.2 $\pm$ 0.3 <sup>a</sup>
16	73.2 $\pm$ 0.1 <sup>a</sup>	78.3 $\pm$ 0.3 <sup>ab</sup>	93.6 $\pm$ 0.4 <sup>a</sup>	11.3 $\pm$ 0.4 <sup>b</sup>
24	73.7 $\pm$ 0.1 <sup>a</sup>	78.9 $\pm$ 0.0 <sup>ab</sup>	93.8 $\pm$ 0.3 <sup>a</sup>	11.6 $\pm$ 0.4 <sup>b</sup>

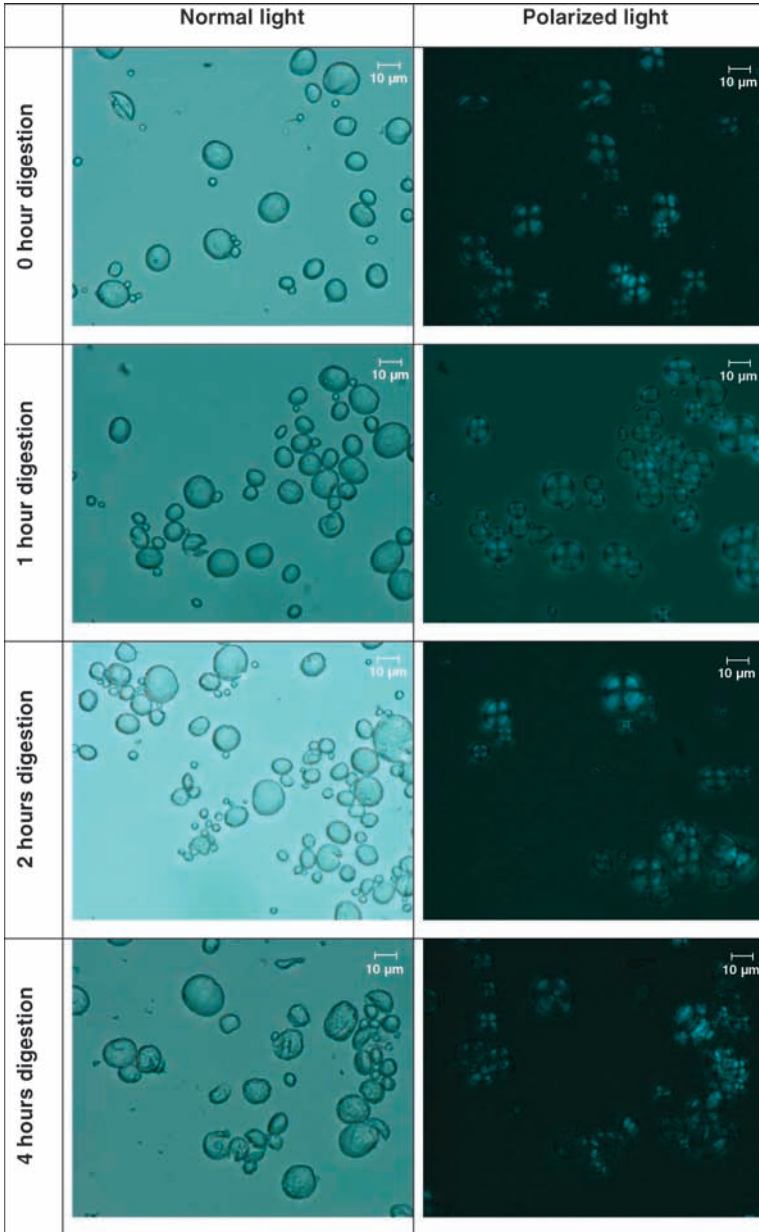
Values within columns bearing the same small letter superscript(s) do not significantly differ at ( $p > 0.05$ ).

evidence. However, the SEM micrographs provide a better observation of the morphological changes of CL wheat starch granules after an incubation period ranging from 0 to 24 hours.

At the start (0 hours), the CL wheat starch granules had a smooth surface, with round, shallow indentations of flower petal-like patterns which are restricted to only some of the granules. In the first hour of digestion, mild erosion on the surface of most starch granules was observed, indicating the susceptibility of CL starch granules early during the incubation.

The progression of starch granule corrosion continued to be observed after incubation from 1–4 hours. Scattered erosion on the surface of starch granules increased and deepened with time. Most starch granules still, however, remained intact, with the retention of the Maltose Cross pattern. After incubation for six hours and eight hours, surface erosion of CL wheat starch granules intensified, causing severe damage and the disappearance of granular identity of some starch granules. The severity of damage on CL wheat starch granules was more prominent after incubation for 16 hours and 24 hours, when smaller fragments of broken starch granules were evident. However, some starch granules remained intact and retained crystallinity, as depicted by the Maltose Cross. This observation indicated that hydrolysis of CL wheat starch granules by an  $\alpha$ -amylase/amyloglucosidase cocktail was progressing continuously and did not stop until the 24 hours incubation was terminated.

CL wheat starch showed unequal granule degradation by digestive enzymes at all incubation times. From 1–24 hours incubation, some of the starch granules remained intact, while others had mild to extensive



**Figure 8.2** Microscopic photos (40× objective) of cross-linked wheat starch at different incubation times of  $\alpha$ -amylase/amyloglucosidase digestion.

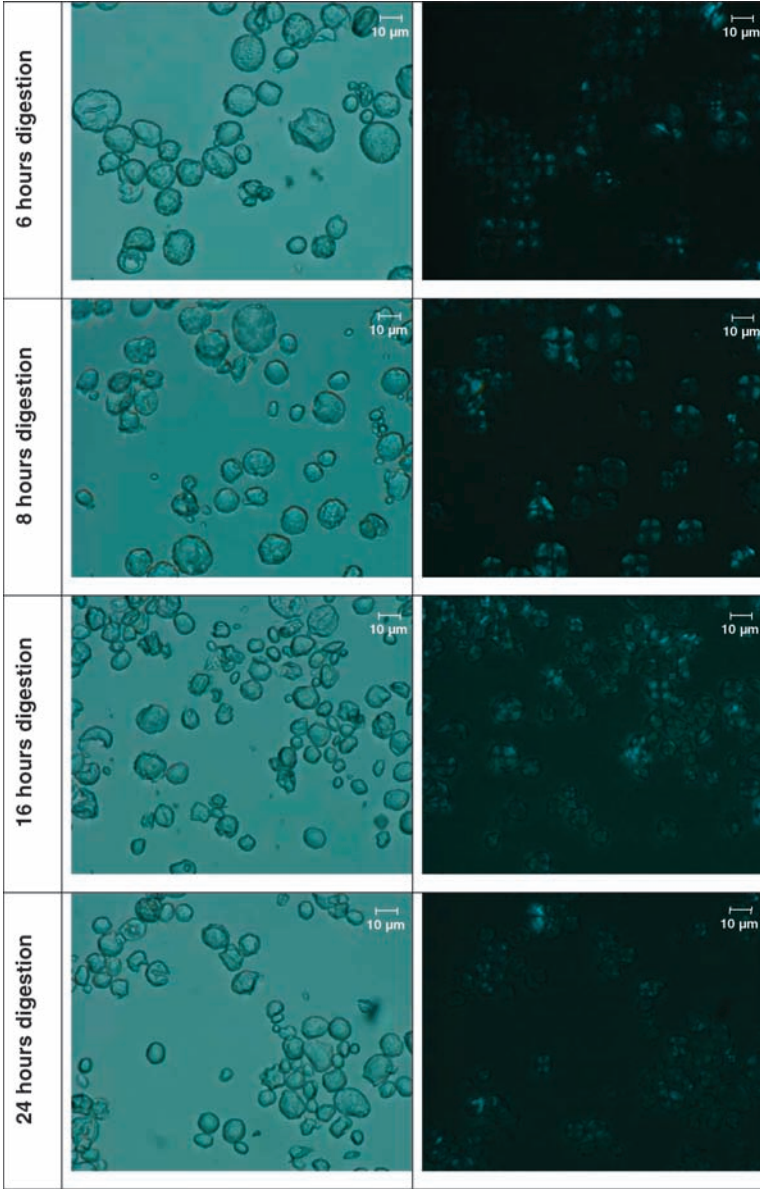
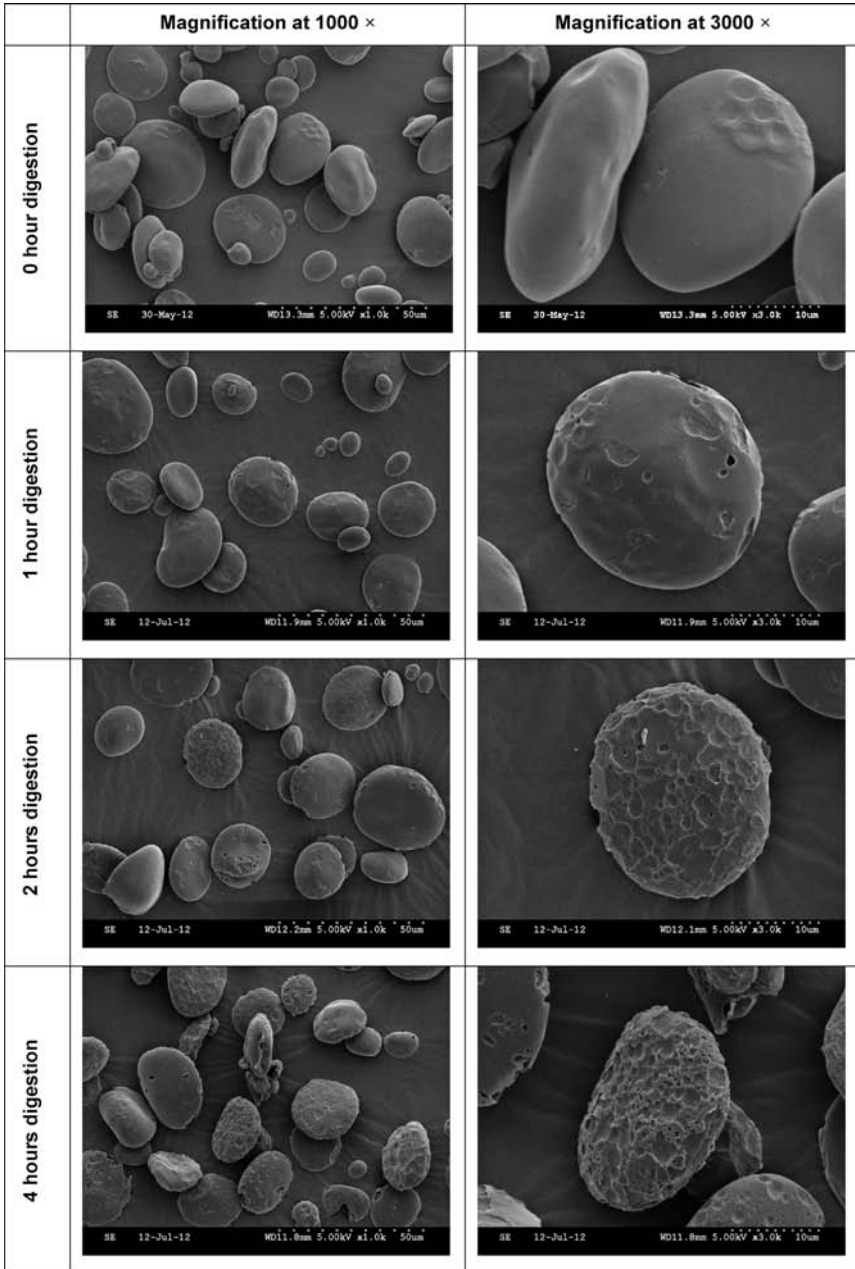


Figure 8.2 (Continued)





**Figure 8.3** Scanning electron micrographs (1000× and 3000× magnifications) of cross-linked wheat starch at different times of  $\alpha$ -amylase/amyloglucosidase digestion.

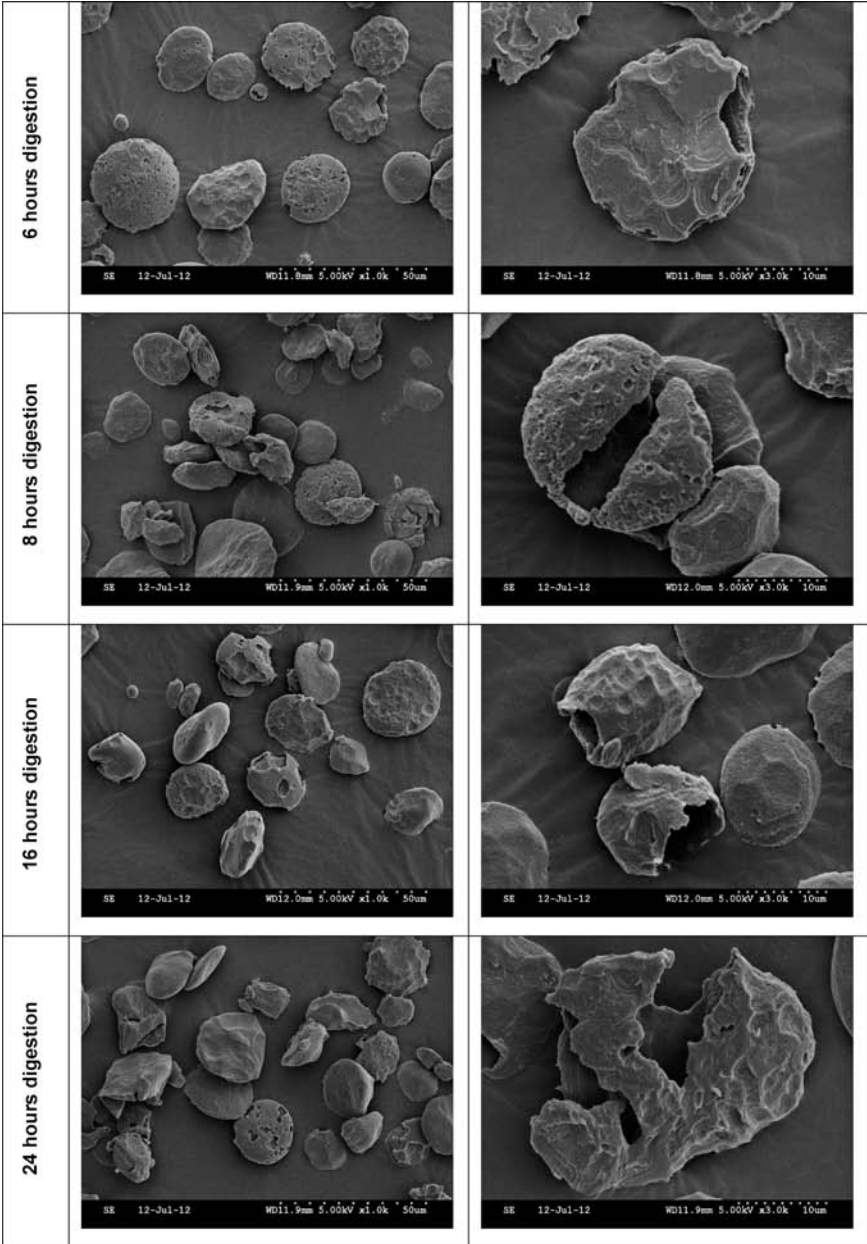
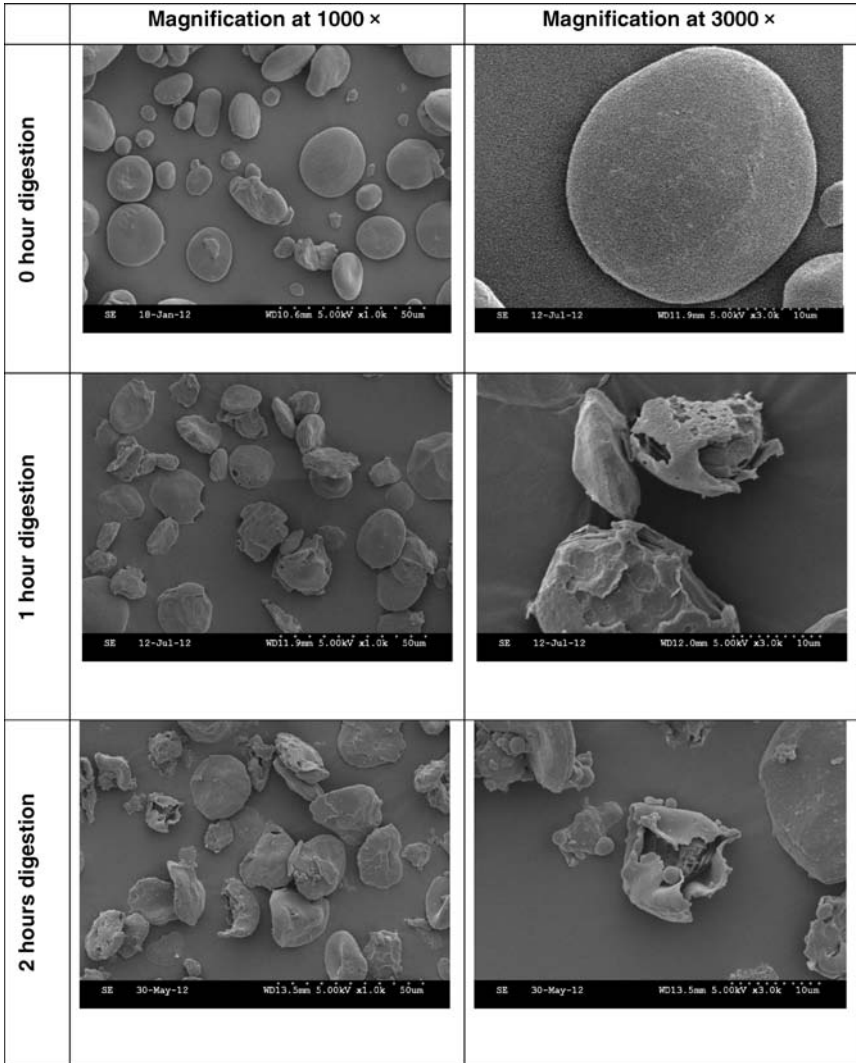


Figure 8.3 (Continued)



**Figure 8.4** Scanning electron micrographs (1000× and 3000× magnifications) of native wheat starch at one hour and two hours of  $\alpha$ -amylase/amyloglucosidase digestion.

degradation. The same phenomenon was observed on the native wheat starch granules (Figure 8.4 ), although the starch damage was more prominent at one and two hours of digestion. This could be attributed to heterogeneous action of the digestive enzymes on the wheat starch granules. Starch granules are also

described as not equally susceptible to enzymatic hydrolysis, greatly depending on the adsorption manner of amylases on the granule and the method of isolation of the starch (Colonna *et al.*, 1988; Oates, 1997).

Colonna *et al.* (1988) had a similar observation of unequal amylase digestion on native wheat starch, in which some granules had minimal pitting and a few were severely cratered. However, all large granules were completely degraded.

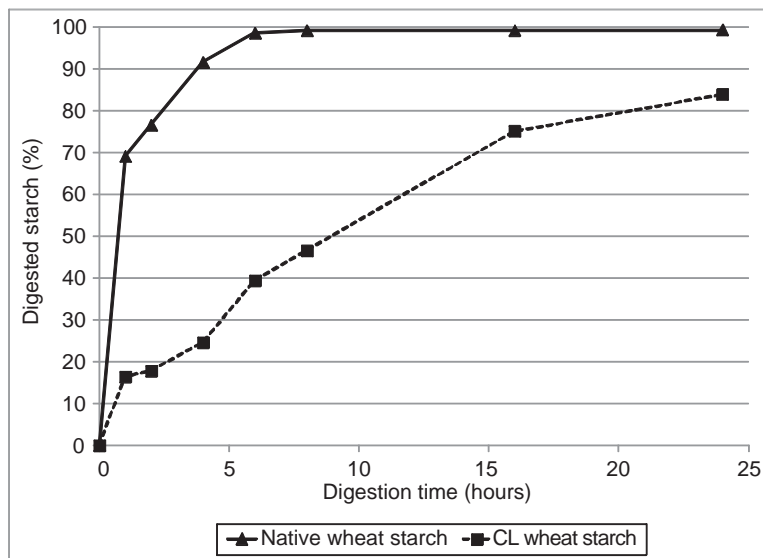
The mechanism of enzyme attack on starch granules is due to exo-corrosion (Shresta *et al.*, 2012), endo-corrosion or a combination of both (Manelius *et al.*, 1997; Apinan *et al.*, 2007). The microscopic and SEM results suggest that the starch hydrolysis by amylase progressed, starting from the surface and proceeding inside of native wheat and CL wheat starch granules by exo-corrosion. In addition, the starch granules were most probably digested by the digestive enzymes using the side-by-side mechanism, as observed by Zhang *et al.* (2006) and Shresta *et al.* (2012), in which the enzyme digested on the amylose and amylopectin as well as the amorphous and crystalline regions (Zhang *et al.*, 2006).

Unlike digested maize starches, which are reported to have holes or tunnels deepening into the interior of granules, or ‘Swiss cheese’ shell appearance (Robyt, 2009; Lauren *et al.*, 2012), CL wheat starch granules (Figure 8.3) and native wheat starch (Figure 8.4) had a roughened superficial surface and exposure of layered internal structures that intensified with increased incubation time, causing the formation of cavities on some starch granules. In addition, CL wheat starch was theoretically obstructed by phosphate groups within the granule surface pores and channels, inhibiting the diffusion of amylase molecules (Thompson *et al.*, 2011).

### 8.3.4 Digestibility

The extent of digestibility of CL wheat starch at different incubation times (up to 24 hours) was determined using the AOAC Method 2009.01. Wheat starch was also assayed using the same method as control, since wheat starch is easily hydrolyzed by digestive enzymes (Björck *et al.*, 1986). The amount of digested starch after each incubation period is shown in Figure 8.5. Both starches showed rapid digestion at one hour, and the rate of digestion slowed until the end of the incubation period. The latter observation is in agreement with that of Bertoft & Manelius (1992), which showed two stages of starch digestion, the first being the initial rapid hydrolysis, followed by a slower and more constant rate of hydrolysis.

For wheat starch, the amount of starch digested was close to 70% at one hour of incubation and 99% at six hours, indicating a high susceptibility of



**Figure 8.5** Digested starch of cross-linked (CL) wheat starch and native wheat starch at different incubation times of  $\alpha$ -amylase/amyloglucosidase digestion.

wheat starch granules to hydrolysis. The SEM results (Figure 8.4) showed the severity of starch hydrolysis at the early stage of digestion (one hour and two hours). Our result is different from that reported by Colonna *et al.* (1988), who observed 74% and 91% starch hydrolysis after incubation for ten hours and 21 hours, respectively. The difference could be due to enzyme source. Colonna *et al.* (1988) used *Bacillus subtilis*  $\alpha$ -amylase (17.5 Phadebas Unit/ $\mu$ g protein), whereas the current study used a combination of pancreatic  $\alpha$ -amylase (50 U/ml) and amyloglucosidase (3.4 U/ml). Pancreatic  $\alpha$ -amylase had been suggested to be the most effective enzyme to digest native starch, followed by barley, bacterial and fungal amylases (Kimura & Robyt, 1995).

When subjected to a cocktail of  $\alpha$ -amylase and amyloglucosidase at 37 °C for 24 hours, CL wheat starch was digested at a slower rate than native wheat starch and, by 24 hours, 84% of the CL starch was digested. The cross-linking treatment decreased the susceptibility of starch granules to digestive enzymes due to the stabilizing effect of granules with a high degree of cross-linking (Seib & Woo, 1999; Woo *et al.*, 2009), therefore causing a slower rate of digestion. The amount of digested CL wheat starch was negatively correlated with P content, but had little effect on starch crystallinity.

O'Brien & Wang (2009) proposed that amylopectin could be more reactive in the cross-linking process, due to higher retained phosphate salts in the branched structure of the polymer. Since amylopectin is comprised of amorphous (tightly-spaced branches) and crystalline regions (parallel glucans) (Oostergetel & van Bruggen, 1989), phosphate groups may stabilize both amorphous and crystalline regions by promoting stronger interactions between the two regions. Therefore, the undigested portion of the residual starch had increased stability and was less susceptible to digestive enzymes, as depicted by the intact, yet highly roughened starch granule surfaces observed in SEM micrographs (Figure 8.3). Increased P content with increased incubation time showed that phosphate groups were retained in the undigested residuals, reflecting the incapability of digestive enzyme to cleave bonds close to a glucose linked to a phosphate group.

Previous RS quantitation of CL wheat starch, using the Englyst Method and AOAC Method 991.43, showed 83% and 76%, respectively (Woo & Seib, 2002; Yeo & Seib, 2009; Thompson *et al.*, 2011). While the Englyst Method (incubation at 37 °C) and AOAC Method 991.43 (incubation at 100 °C) use 120 minutes and 35 minutes of incubation time, respectively, AOAC Method 2009.01 incubates samples for 16 hours at 37 °C, yielding 25% RS content for CL wheat starch.

The significantly lower RS content of CL wheat starch, as determined by AOAC Method 2009.01, compared to the values obtained by the Englyst Method and AOAC Method 991.43, raised questions about the validity of AOAC Method 2009.01 to measure accurately RS content that is consistent with *in vivo* human conditions. At 24 hours, RS content of CL wheat starch further decreased to 16%, indicating that the digestive action of the  $\alpha$ -amylase and amyloglucosidase cocktail on CL wheat starch was still progressing.

In humans, the transit time of food in the intestine takes 3–4 hours (Perera *et al.*, 2010). Long-term hydrolysis may lead to an increase in substrate surface availability caused by amylase attack (Colonna *et al.*, 1988). At 16 hours incubation time in AOAC Method 2009.01, a much lower yield of RS content resulted, as indicated in the current study, and may not reflect *in vivo* response, which is important in the food industry.

## 8.4 CONCLUSIONS

CL wheat starch was assayed using the digestive enzymes and incubation conditions of AOAC Method 2009.01, and samples were collected at predetermined times. The digestion of CL wheat starch continued to

increase up to 24 hours. The RS value obtained at 16 hours was lower than the RS content measured by the Englyst Method and AOAC Method 991.43. Microscopic and SEM results of the indigestible residues collected at the predetermined times showed progressive degradation of CL wheat starch granules. However, some starch granules remained intact, which explained the comparable DSC data for CL wheat starch at all incubation times. The mechanism of enzyme attack on the CL wheat starch was exo-corrosive, as identified by the surface erosions and the non-existence of pinholes on the granules.

Future studies will include assaying CL starches from different botanical sources in order to understand further the mechanism of enzyme attack as affected by cross-linking.

## **8.5 ACKNOWLEDGEMENTS**

We thank MGP Ingredients, Inc. for donating the starch samples and Dr. Susan Sun for the use of DSC. This is Contribution no. 13-233-J from the Kansas Agricultural Experiment Station.

## **8.6 ABBREVIATIONS USED IN THIS CHAPTER**

$\Delta H$ , gelatinization enthalpy; ANOVA, analysis of variance; CL, cross-linked; DSC, differential scanning calorimeter; P, phosphorus; RDS, rapidly digestible starch; RS, resistant starch; SDS, slowly digestible starch; SEM, scanning electron microscope; STMP, sodium trimetaphosphate; STPP, sodium triphosphate;  $T_c$ , conclusion temperature;  $T_o$ , onset temperature;  $T_p$ , peak temperature.

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## 9 Biscuit Baking and Extruded Snack Applications of Type III Resistant Starch

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### 9.1 INTRODUCTION

Enzyme-resistant starch may be defined as the sum of starch and products of starch degradation not absorbed in the small intestine of healthy individuals (Eerlingen, 1994). It may be classified into four types, designated I to IV.

Physically inaccessible starch, locked in the plant cell, is classified as type I. This type can be found in partially milled grains and seeds and legumes.

Native granular starch found in uncooked ready-to-eat starch-containing foods, such as in bananas, is classified as type II resistant starch. Enzyme susceptibility of type II resistant starch is reduced by the high density and partial crystallinity of the granular starch.

The amount of type I and type II resistant starch is generally less than about 12% by weight, after grain milling and cooking, based on the amount of starch contained in the starch source and in the formula. Type I and type II resistant starches have low melting points and do not survive a baking process where temperatures are substantially above 100°C and there is sufficient moisture in the formula to gelatinize the starch (e.g. >20%). Type I and type II resistant starch do not survive high shear processing such as extrusion, which would disrupt granular integrity.

Starch may undergo a treatment process to obtain an indigestible starch fraction. Depending upon the type of treatment, a type III resistant starch or a type IV resistant starch may be produced. An indigestible starch fraction which forms after certain heat-moisture treatments of the starch may be present in, for example, cooked, cooled potatoes; this is type III enzyme-resistant starch.

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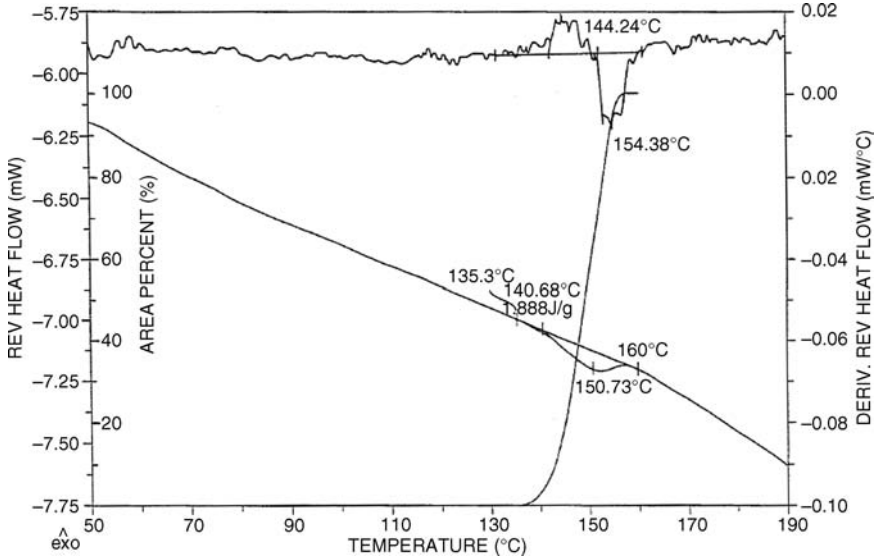
In type IV resistant starch, the enzyme resistance is introduced by chemically modifying or thermally modifying the starch. For example, glycosidic bonds other than  $\alpha$ -(1-4) and  $\alpha$ -(1-6) bonds may be formed by heat treatments. Other glycosidic bonds may reduce the availability of starch for amylolytic enzymes. In addition, the digestibility of starch may be reduced by chemical cross-linking (Woo & Seib, 2002). Production and use of such ingredients may be subject to legal limitations imposed by the US Food and Drugs Administration.

Details of a patented process and flour replacer performance in a cookie formula have been published (Haynes *et al.*, 2002), utilizing a process comprised of gelatinization, nucleation/propagation and heat treatment stages for an *amylose extender* corn starch (*aeWx VII*). The characteristics of a thermal-stable, shear-stable enzyme-resistant starch type III RS (X150) and its use in biscuit baking and extrusion applications, without the formation of glycosidic bonds other than  $\alpha$ -(1-4) and  $\alpha$ -(1-6) glycosidic bonds, are described.

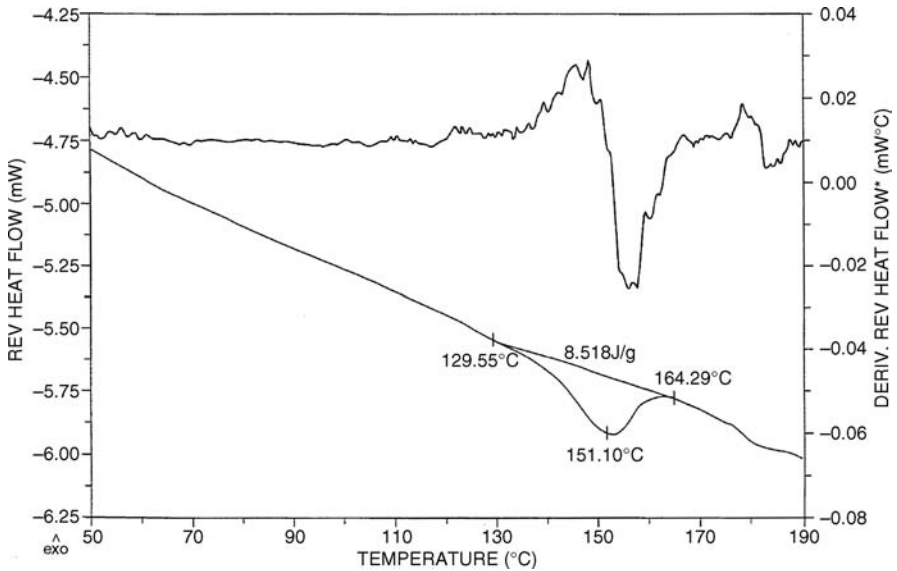
## **9.2 THERMAL CHARACTERISTICS OF HEAT-SHEAR STABLE RESISTANT STARCH TYPE III INGREDIENT**

The starch-based ingredient is comprised of about 60% by weight enzyme-resistant starch type III, which has a melting point of about 150°C and which is formed by gelatinization, followed by at least one cycle of crystal nucleation and crystal growth or propagation. In the critical cooling step, the gelatinized starch is cooled to a crystal nucleating temperature of >60°C, above the melting point of amylopectin starch such that the nucleation of amylolipid crystalline complex is not favoured. A crystal propagating temperature of about 130°C is used to maximize the formation of the heat-stable RS III, which has a thermal melt temperature of 150°C. A debranching enzyme, such as pullulanase may be used to increase the yield of the high-melting enzyme-resistant starch type III.

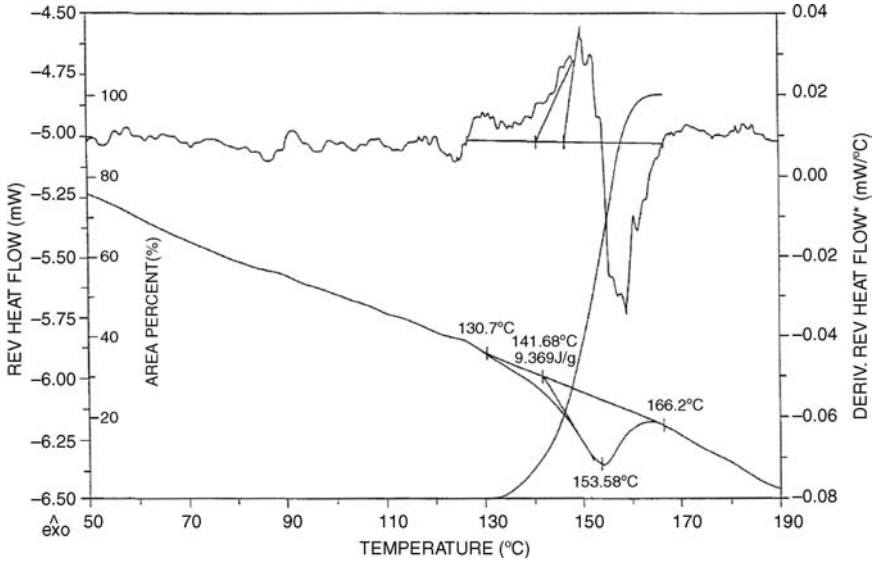
Shown in Figures 9.1 and 9.2 are the MDSC curves for enzyme-resistant starch type III ingredient and isolate. In the MDSC technique, the material being analyzed is heated at a steady rate with a programmed saw-tooth pattern of heating and cooling imposed upon the steady rate. The amplitude of the fluctuation in temperature allows a more precise analysis of the equilibrium melting point because it separates overlapping thermal events such as irreversible decomposition. In Figure 9.1, a single crystalline melt is observed at about 150°C for resistant starch type III bulk ingredient. Enzyme-resistant



**Figure 9.1** Modulated differential scanning calorimetry (MDSC) curve for an enzyme-resistant starch type III ingredient (X150) or bulking agent, obtained from a single nucleation temperature of about 70°C and a propagation temperature of about 130°C.



**Figure 9.2** MDSC curve for the isolated, heat-treated enzyme-resistant starch type III bulking agent.



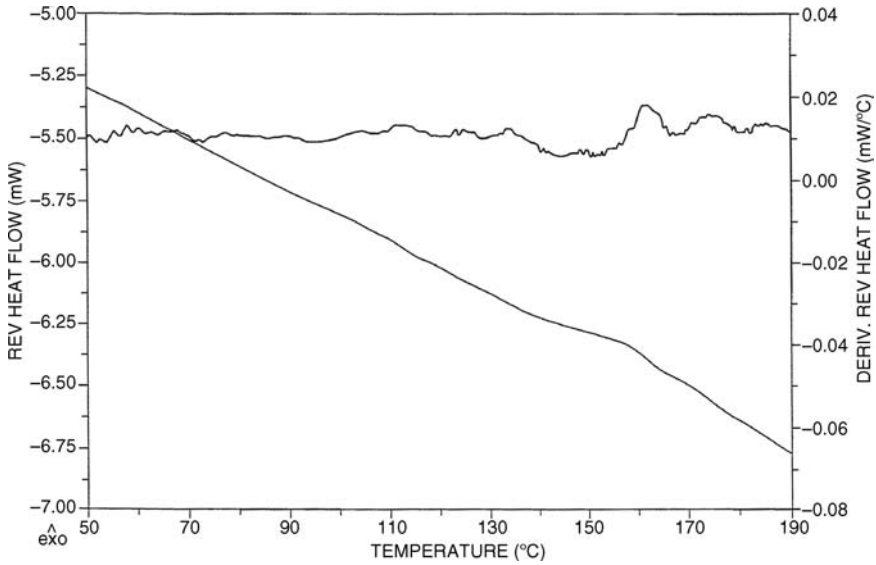
**Figure 9.3** MDSC curve for an isolated enzyme-resistant starch type III ingredient (X150) obtained from the bulk.

starch can be isolated from the bulk ingredient by a method adopted and modified from the Prosky method for Total Dietary Fibre in baked goods set forth in AOAC Method 991.43.

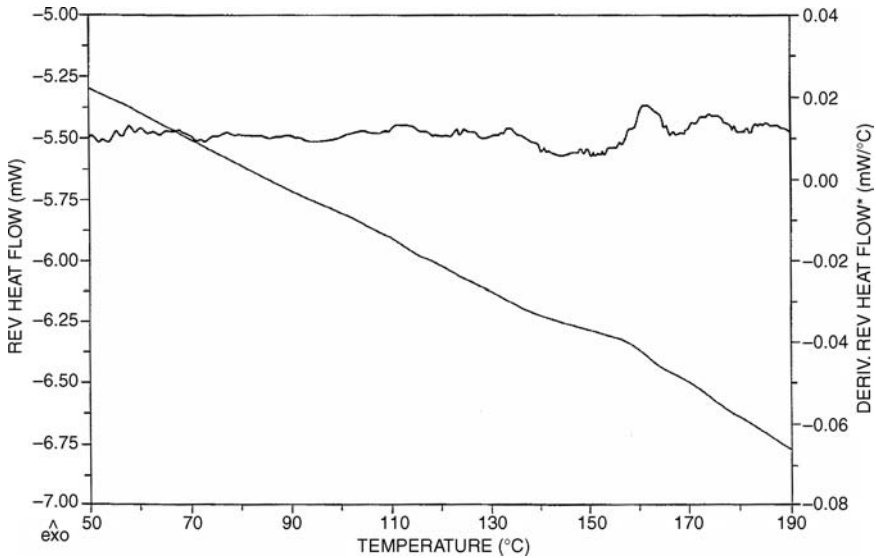
Shown in Figure 9.2 is the MDSC thermal profile of enzyme-resistant type III starch isolated from the bulk ingredient.

The second stage nucleation/propagation temperature cycling is followed by a third stage involving heat treatment of the enzyme-resistant starch type III product. The heat treatment is conducted at a temperature of about 130°C for about one hour at a moisture content of 18%. Figure 9.3 shows a MDSC curve for the isolated, heat-treated enzyme-resistant starch type III bulking agent. The heat treatment increases the total dietary fibre content and improves baking functionality. Corresponding MDSC curves for type II resistant starch isolate (Figure 9.4) and type II resistant starch bulk ingredient after heat treatment (Figure 9.5) show no high temperature melting.

An enzyme-resistant starch type III bulking agent produced from gelatinization and recrystallization at high temperatures, followed by heat treatment, has about 60% total dietary fibre and is resistant to enzymes such as  $\alpha$ -amylase,  $\beta$ -amylase, amyloglucosidase and pancreatin, and it provides a reduced calorie or low calorie, highly functional ingredient for replacing flour in baking or extruded applications. As a result of the very high melting point of



**Figure 9.4** MDSC curve for isolated, heat-treated enzyme-resistant granular starch type II obtained by heat-treating granular type II (Novelose 240) bulking agent at 130°C for one hour, followed by isolation of the RS from the heat-treated bulking agent. (Novelose 240).



**Figure 9.5** MDSC curve for heat-treated enzyme-resistant granular starch type II, obtained by heat treating an enzyme-resistant granular starch type II (Novelose 240) bulking agent.

150°C, the enzyme-resistant starch ingredient is extremely heat-stable. The resistant starch content of the type III resistant starch bulking ingredient survives the high shear conditions of extrusion, enabling its use as a low calorie, high fibre flour replacer in baking and extrusion applications.

### 9.3 APPLICATION TO BISCUIT BAKING: COOKIES

Cookies are baked products which generally contain three major ingredients, i.e. flour, sugar and fat. Cookie quality attributes are their size and tender bite (Miller & Hosenev, 1997). Also, cookies baked from soft wheat flour display not only a large spread but also a uniform surface cracking pattern (Miller *et al.*, 1997). In cookie production, mixing disperses ingredients evenly and promotes water absorption, rather than developing a true dough structure (Huebner *et al.*, 1999). Due to the high levels of fat and sugar, the development of the gluten network is limited (Slade & Levine, 1994). Sugar is pre-dissolved in water at a concentration in the formula water which exceeds 35% weight per weight, usually in the presence of formula fat. The dough moisture is typically about 17–18%. The total solvent, sugar plus formula water is about 64% (wt per cwt flour).

The cookie baking method used to evaluate resistant starch ingredient performance is a standard wire-cut cookie baking method (AACC 10–53) designed at the Nabisco Biscuit Company for the evaluation of ingredient functionality and predictive correlation between sensory and mechanical texture analysis (mechanical texture by Instron three-point bend or puncture test). The AACC 10–53 was adopted as the approved method of the American Association of Cereal Chemists after collaborative testing in 1992. Shown in Table 9.1 is the formula used in the AACC 10–53 wire-cut cookie baking method.

Cookie geometry and moisture bake-out is critical in the industrial setting, which demands high through-put and automated packing of the product. Correct cookie baking results in proper colour, taste and texture which, in turn, influences consumer acceptance of the finished product. Achieving the right geometry, colour and moisture balance is highly dependent upon ingredients used in the formula.

In order to obtain a significant reduction in calories or an increase in total dietary fibre per serving, usually 25% to 50% of the flour must be replaced. At this level, solvent binding properties of flour replacers can disturb the colour and moisture balance in the baked product. The water holding capacity of conventional un-gelatinized wheat flour may be about 0.6 grams of water per gram of dry flour. The bulking agent solvent holding properties ideally would



**Table 9.1** AACC 10–53 wire-cut cookie formula.

Ingredient	Weight (g)	Per cwt	Ingredient moist	Ingredient dry weight (g)
Low trans shortening	90	40.00	0.20%	89.82
Sucrose, fine gran.	94.5	42.00	0.50%	94.03
Salt	2.81	1.25	0.50%	2.8
Non-fat dry milk	2.25	1.00	4.00%	2.16
High fructose corn syrup	3.38	1.50	29.00%	2.4
Ammonium bicarbonate	1.13	0.50	99.50%	0.01
Sodium bicarbonate	2.25	1.00	50.00%	1.13
Flour	225	100.00	13.00%	195.75
water	49.5	22.00	100.00%	0
<b>Total weight</b>	<b>470.82</b>		<b>Dry solids</b>	<b>388.08</b>
<b>Total moist</b>	<b>17.57%</b>			

approximate that of the flour that is being replaced. Typically, gelatinized starch used as a baking ingredient has an extremely high water holding capacity of about 10 g of water per gram of starch (Pomeranz, 1964). The resistant starch type III ingredient bulking agent, ground to a particle size distribution the same as that of conventional wheat flour, has a water holding capacity of less than 3 g of water per gram of dry matter. The lower water holding capacity generally results in lower viscosity dough and has a beneficial effect upon spread and baking characteristics. The water holding is substantially unaltered by baking.

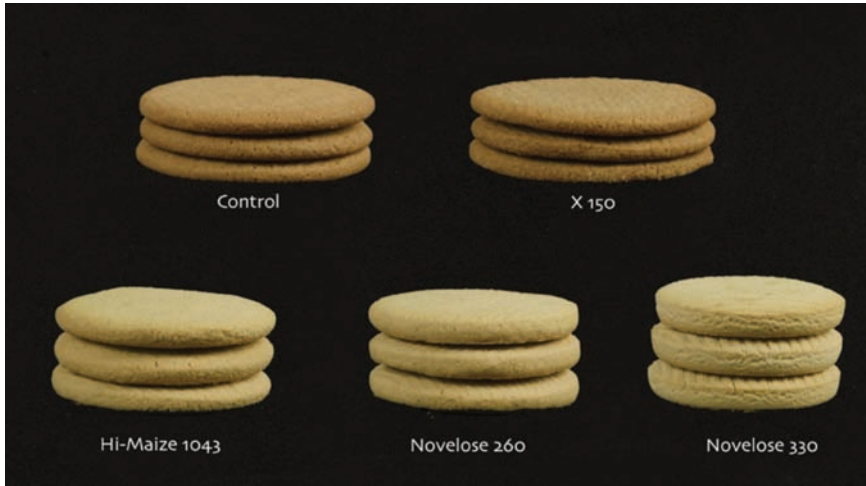
Baking performance of the resistant starch ingredients is tested by replacing 50% by weight of the wheat flour in the standard dough formulation with enzyme-resistant starch ingredient to obtain a blend. For each of the resistant starch compositions, the amount of wheat flour replacement, the ingredient type, the enzyme-resistant starch content or yield, the AOAC total dietary fibre content, the cookie width and the MDSC enthalpy are set forth in Table 9.2. Also in Table 9.2 are the  $L^*a^*b^*$  colour measurements.

As shown in Table 9.2 and in Figure 9.6, the heat-treated RS Type III (X150) ingredient exhibits a superior baking characteristic of greater cookie spread. Along with cookie geometry, the product colour and moisture are closest to control in the cookie made with RS type III and are likely to deliver optimum commercial production efficiencies.

The enzyme resistance and low caloric value of the very high melting enzyme-resistant type III starch ingredient component is substantially unaltered by baking. The pure (or 100% by weight) enzyme-resistant starch

**Table 9.2** Characteristics of AACCC 10–53 wire cut cookies made with 50% of flour replaced with resistant starch ingredient.

Baking Functionality of RS Ingredients Used To Replace Wheat Flour										
Sample	Starch ingredient	Wheat flour replacement (% by weight)	Processing	RS content	AOAC total dietary fiber	Cookie width	Cookie color			MDSC enthalpy @ >140° C, bulk ingredient
							L'	a'	b'	
Control 1	Wheat Flour	0	None	0%	3%	33.0 cm	72.7	6.9	31.8	0 J/g
	Non-Heat Treated RS III Ingredient	50%	Stage 1 & Stage 2, oven dried	32%	37%	32.4 cm	–	–	–	1.9 J/g
2	Heat Treated RS III Ingredient	50%	Stage 1 & Stage 2 & Stage 3	47%	63%	35.5 cm	64.6	8.0	32.6	2.0 J/g
Comparative 1 3	Novelose 240	50%	granular starch	34%	64%	29.3 cm	82.4	2.1	27.5	0 J/g
	Heat Treated Novelose 240	50%	granular starch + stage 3	46%	70%	29.2 cm	–	–	–	0 J/g
Comparative 2 4	Novelose 330	50%	Retrograded starch	28%	33%	26.3 cm	80.7	2.4	18.1	0 J/g
	Heat Treated Novelose 330	50%	Retrograded starch + stage 3	43%	57%	28.3 cm	–	–	–	0 J/g



**Figure 9.6** AACC 10–53 wire cut cookies made with 50% replacement of flour with resistant starch ingredient.

type III has a caloric value of essentially zero, or less than about 0.5 calories/gram even after baking. The caloric value for starch which is not resistant starch is about 4 calories per gram. Thus, a resistant starch type III ingredient or bulking agent with at least a 30% yield of RS type III will exhibit a caloric value of less than about 2.8 calories/gram ( $0.70 \times 4 \text{ cal/g} + 0.30 \times 0 \text{ cal/g} = 2.8 \text{ calories/gram}$ ). The resistant starch type III ingredient or bulking agent exhibits excellent baking characteristics in terms of oven spread, edge contour, oil release, surface cracking, odour, colour or browning, mouth-feel and texture. It may be used alone or in combination with other fibres or whole grain flour to produce a healthy, wholesome product.

## 9.4 CRACKER BAKING

Chemically leavened cracker dough formulas differ from cookie formulas in two important aspects. First, the level of sugar in a cracker formula, measured as wt sugar/wt sugar + formula water, is lower at about 28%. Second, the total solvent (sugar + water) per cwt of flour is lower at about 48% (wt per cwt flour), most of which is water. The cracker formula moisture is about 35% of formula, significantly higher than cookie formulas. The cracker formula conditions of low sugar and higher formula moisture allow for the development of wheat flour gluten, such that a cohesive, elastic dough can be formed

**Table 9.3** Model formula for chemically leavened cracker baking test.

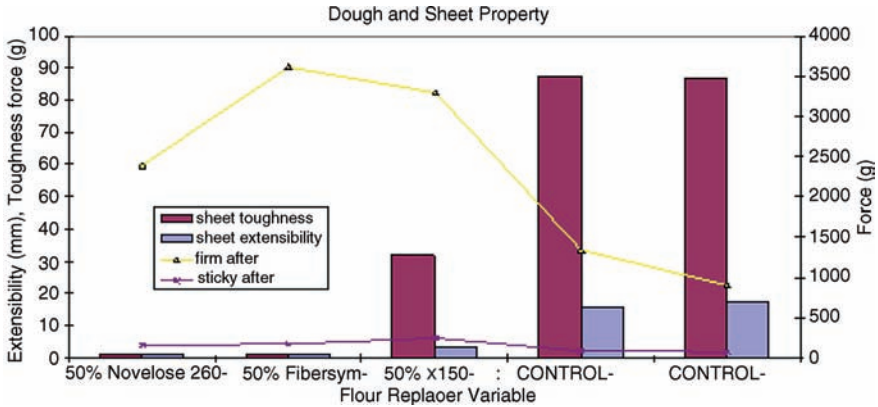
	<b>Ingredients</b>	<b>Total weight</b>	<b>Per cwt</b>
Stage 1	Pre-dissolved sucrose	6.12	11.08
Stage 1	Corn starch	3.1	5.61
Stage 1	Salt	0.61	1.10
Stage 1	Mono calcium phosphate	0.31	0.56
Stage 1	Sodium bicarbonate	0.16	0.29
Stage 2	Low trans oil	2.75	4.98
Stage 2	Tap water (110°F)	18.87	34.17
Stage 2	High fructose corn syrup	1.84	3.33
Stage 3	Flour	55.23	100.00
	<b>Total weight</b>	<b>88.99</b>	<b>161.13</b>
	<b>Total % moist</b>	<b>0.3532</b>	

into a thin sheet from which cracker dough pieces are cut. Ingredients with high water absorption are undesirable, because a tough, dry, less elastic dough results. To compensate, more formula water is added, which can contribute to longer bake time, requiring greater amounts of heat energy to remove the excess moisture.

Type II granular resistant starch, Type III resistant starch (X150), made from preferred process as described (gelatinization, nucleation/propagation and heat treatment stages), and Type IV chemically modified resistant starch (Fibersym<sup>®</sup> 70 or its old name Fiberstar 70) are evaluated in a cracker formula. Table 9.3 shows the formula amounts for sugar, which calculates to 24% (weight sugar divided by weight of sugar plus formula water), total solvent (sugar + syrup + water) per cwt of flour of about 48% and the total formula moisture of about 35%. The cracker dough is sheeted and dough pieces cut and weighed and baked.

As a result of the lower solvent holding capacity of the high melting point, thermally stable type III resistant starch (X150), formula water requirements are lower and superior baking characteristics are achieved. Although there is sufficient moisture to gelatinize starch, due to the high melting point of Type III RS made by the preferred process there is no loss of enzyme resistance upon baking. Differences in dough sheet toughness and extensibility, compared to control flour, were noted for all dough made with resistant starch. Reducing the flour content of the formula reduces the amount of flour gluten protein available for dough development. Figure 9.7 shows the sheeting property of dough when flour is replaced at the 50% level.

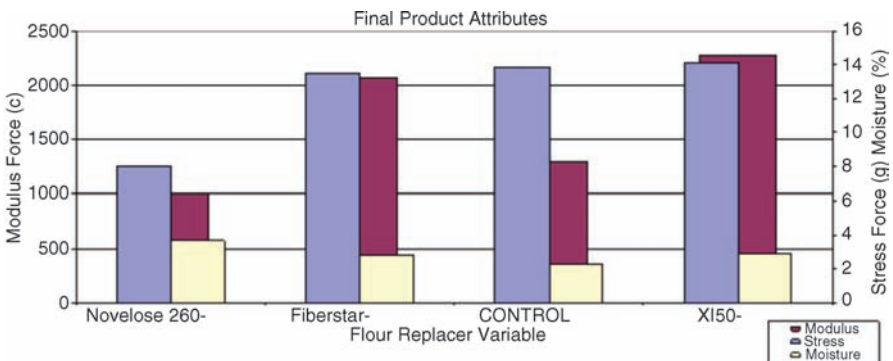
Replacement of flour with resistant starch makes the dough less tough/cohesive and less extensible. Using stronger flour with the resistant starch does help to improve dough toughness, although there is still a notable



**Figure 9.7** Force required to stretch dough and distance stretched before breaking are shown for resistant starch ingredient used to replace flour before and after dough resting. Note: additional water (19.5g) is added to the Novelse sample because dough did not develop with formula amount.

decrease from control. Addition of vital wheat gluten shows increase in toughness and extensibility of the dough sheet, as vital wheat gluten level increases, toughness and extensibility also increase.

The finished product is baked for the same length of time and the moisture measured by percentage weight loss. Resistant starch type II (Novelse 260) requires more water to form dough, so current bake time results in out-of-specification cracker moisture. Tougher dough tends to result in crackers with higher moisture. Type III resistant starch (X150) at a 50% level demonstrates a mechanical break force closest to control, as shown in Figure 9.8.



**Figure 9.8** Finished product modulus and stress force for cracker made with resistant starch replacing 50% of regular flour. Final moisture contents of finished products are also shown.

**Table 9.4** Results of AOAC method 991.43 fibre analysis in finished crackers.

Product	Flour replacer	Product flour	Flour replacement	% Dietary fibre (theoretical)	% Dietary fibre (actual analysis)	% Fibre retention after processing
Cracker, unoiled	Control	75.8%	0%	2.2	2.4	109%
	X150	75.8%	50%	19	17.3	91%
	Novelose 260	75.8%	50%	22.7	19.9	88%

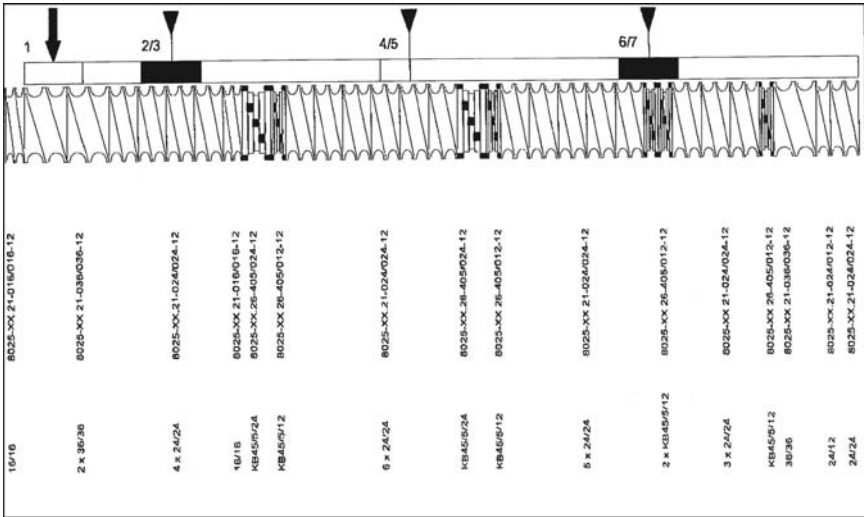
Retention of enzyme resistance during baking was confirmed by AOAC 991.43 fibre analysis of the finished cracker product. The results of fibre analysis are shown in Table 9.4.

## 9.5 EXTRUDED CEREAL APPLICATION

Ready-to-eat (RTE) cereals are typically constituted by a flour blend (wheat flour, ground cereal fines, barley malted flour), which constitutes approximately 96% by weight of the solids, 2% granulated sugar, 1.25% fine granular salt and 0.5% of other minor ingredients. Enzyme-resistant starch type III (X150) has been used to replace up to 50% of the flour blend in RTE cereals.

In the processing of RTE cereals, the solid ingredients are extruded using a twin-screw extruder at a moisture content of approximately 9% moisture (w.b.) under medium-shear screw profile, configured as shown in Figure 9.9. The extrudate expands at the exposure to atmospheric pressure just outside of the die, referred to as ‘flashing off’ of moisture.

The very-high-melting, high-shear-surviving enzyme-resistant starch is substantially unaltered by extrusion, i.e. it remains substantially enzyme resistant and exhibits a reduced calorie value of less than about 1.6–2.0 kcal/gram (60–50% by weight RS type III, having a melting point or endothermic peak temperature of at least 140°C, as determined by MDSC), as determined by fibre analysis. Enthalpy values for the high-melting enzyme-resistant starch in the cereal are typically in the order of 6 Joules/g (based on grams of resistant starch in the flour blend), at a temperature of from 130°C to about 165°C. This is highly advantageous for producing reduced-calorie extruded cereals because, if the crystal structure that provides enzyme-resistance is destroyed or melts during extrusion, and if the crystal recrystallizes into a lower-melting form which is not enzyme-resistant, then calorie reduction will not be achieved in the extruded product. For example, when



**Figure 9.9** Medium-shear screw profile used in extruder.

using a type II or type IV resistant starch, the calorie reduction would be less, since these types of resistant starch do not survive high temperature or high shear processing, such as extrusion.

The RS type III ingredient gives the extruded RTE cereal excellent extrusion characteristics in terms of density, moisture content, texture (brittleness) and cell structure.

### 9.5.1 Preparation of extruded RTE cereal and analysis

The extrusion functionality of an enzyme-resistant starch type III prepared in accordance to the above procedure was compared to the extrusion functionality of a commercially available, enzyme-resistant starch type II ingredient (Hi-Maize™ 1043, produced by National Starch and Chemical Co.) and a commercially available, enzyme-resistant starch type IV ingredient (Fibersym® 70, produced by MGP Ingredients) using conventional, non-heat-treated wheat flour as Control.

Hi-Maize™ 1043 has the following characteristics, as claimed by National Starch and Chemical Co.:

- Moisture: 13% maximum;
- Total dietary fibre (AOAC method 991.43): 60% minimum (dry basis);
- Calories: approx. 1.6 kcal/g.

Fibersym<sup>®</sup> 70 (also known by its old name as Fiberstar 70) has the following characteristics, as claimed by MGP:

- Moisture: 10.6% typical;
- Total dietary fibre (AOAC method 991.43): 70% minimum (dry basis);
- Calories: approx. 3.6 kcal/g (not corrected for insoluble fiber)

Extrusion functionality was evaluated by measurement of density, moisture content, texture (brittleness) and cell structure. Resistant starch ingredients that resulted in cereal properties as close to the properties achieved with the wheat flour control were considered to have the best extrusion functionality. Total dietary fibre was used to determine thermal and shear stability of the resistant starch ingredients.

Control was produced following a standard formula for extruded, expanded RTE cereals. In test samples, 50% of the total flour weight in the formula (including wheat flour, ground cereal fines and barley malted flour) was replaced by a resistant starch, namely RS III (X150) (variable 1), Hi-Maize 1043 (variable 2), or Fibersym 70 (variable 3), with the objective of testing their functionality as flour replacers.

#### 9.5.1.1 *Formulas*

The formulas are shown in Table 9.5.

#### 9.5.1.2 *Extrusion*

The operating conditions used in the extruder to produce the cereal samples are shown in Table 9.6.

#### 9.5.1.3 *Results*

As shown in Table 9.7, the bulk density of the three test samples was lower than that of the control, in the following descending order: Fibersym 70 > RSIII > Hi-Maize 1043. Bulk density is an important parameter when dealing with packing of a standard weight of cereals in a standard volume.

Also as shown in Table 9.7, resistant starch raw materials RSIII, Hi-Maize 1043 and Fibersym 70 had MDSC enthalpies of 8.39 J/g, 0 J/g and 0 J/g, respectively, at temperatures above 140°C. Hi-Maize 1043 presented an endotherm at 101.6°C and an enthalpy of 4.4 J/g, while Fibersym 70 presented an endotherm at 74.9°C and an enthalpy of 11.71 J/g, representing low-melting resistant starches.



**Table 9.5** Cereal formulas.

Ingredient	Control		Variable 1: Type III RS		Variable 2: Hi-Maize™ 1043		Variable 3: Fibersym® 70	
	% (w/w)	Weight (lb)	% (w/w)	Weight (lb)	% (w/w)	Weight (lb)	% (w/w)	Weight (lb)
Hard red winter wheat flour	74.26	37.12	37.14	18.56	37.14	18.56	37.14	18.56
Type III RS	0.00	0.00	48.11	24.06	0.00	0.00	0.00	0.00
Hi-Maize™ 1043	0.00	0.00	0.00	0.00	48.11	24.06	0.00	0.00
Fibersym® 70	0.00	0.00	0.00	0.00	0.00	0.00	48.11	24.06
Ground cereal fines*	14.99	7.50	7.50	3.75	7.50	3.75	7.50	3.75
Barley malted flour	7.00	3.50	3.50	1.75	3.50	1.75	3.50	1.75
Granulated sugar	2.00	1.00	2.00	1.00	2.00	1.00	2.00	1.00
Fine granular salt	1.25	0.63	1.25	0.63	1.25	0.63	1.25	0.63
Trisodium phosphate dodecahydrate	0.50	0.25	0.50	0.25	0.50	0.25	0.50	0.25
Total	100.00	50.00	100.00	50.00	100.00	50.00	100.00	50.00

\* Granulation: on US#18: 1% max.; on US#40: 20% max.; on US#100: 40% max.

**Table 9.6** Operating conditions used in a Werner & Pfleiderer ZSK 25 extruder.

Processing parameter	Control		Variable 1: RSIII		Variable 2: Hi-Maize <sup>TM</sup> 1043		Variable 3: Fibersym <sup>®</sup> 70	
	Set point	Actual	Set point	Actual	Set point	Actual	Set point	Actual
Barrel temp. zone 1 & 2 (°C)	80.0	79.0	80.0	81.0	80.0	80.0	80.0	80.0
Barrel temp. zone 3 & 4 (°C)	110.0	110.0	110.0	110.0	110.0	110.0	110.0	110.0
Barrel temp. zone 5 & 6 (°C)	125.0	125.0	125.0	125.0	125.0	126.0	125.0	127.0
Screw speed (rpm)	200.0	203.0	200.0	203.0	300.0*	301.0*	350.0*	352.0*
Powder feed rate (lb/h)	40.0	40.0	40.0	40.0	30.0*	30.0*	42.0*	42.0*
Water feed rate (lb/h)	3.2	3.2	3.2	3.2	2.8*	2.8*	2.5*	2.5*
Torque (%)		45		49		30		36
Die pressure (psig)		801		465		118		246
Die temperature (°C)		148		151		153		166

\* Hi-Maize<sup>TM</sup> 1043 and Fibersym<sup>®</sup> 70 extrusion operated at different conditions to prevent caking at the feeding port.

**Table 9.7** Final product property measurements.

	<b>Bulk density (g/cm<sup>3</sup>)</b>	<b>Moisture (%)</b>	<b>MDSC enthalpy @ &gt;140°C (J/g)*</b>	<b>RS melting peak temperature (°C)</b>	<b>L*, a*, b*</b>
RSIII, raw material	–	–	8.39	151.2	–
Hi-Maize <sup>TM</sup> 1043, raw material	–	–	0	101.6	–
Fibersym <sup>®</sup> 70, raw material	–	–	0	74.9	–
Control cereal	0.452	9.73	0	–	70.61, 3.81, 25.33
RSIII cereal, 50% flour replacement	0.309	9.99	9.05	151.2	68.49, 4.62, 25.59
Hi-Maize <sup>TM</sup> 1043 cereal, 50% flour replacement	0.195	10.02	4.35	144.7	75.29, 2.47, 22.26
Fibersym <sup>®</sup> 70 cereal, 50% flour replacement	0.365	7.81	0	122	73.39, 3.21, 26.74

\* Joules per g of flour, g of RSIII in flour blend, g of Hi-Maize<sup>TM</sup> in flour blend, or g of Fibersym<sup>®</sup> in flour blend, respectively.

The control did not present a MDSC enthalpy above 140°C, indicating that no enzyme-resistant starch was present and that none was created during processing. Cereal samples containing RSIII presented a MDSC enthalpy of 9.05 J/g (based on g of RSIII in the flour blend), comparable to that of the RSIII raw material, indicating that the RSIII ingredient was stable during extrusion. In addition, it presented a high melting point of 151.2°C, corresponding to enzyme-resistant starch ingredients. Since it remained unchanged during extrusion, RSIII was very identifiable in the final product.

Cereal samples containing Hi-Maize 1043 presented a MDSC enthalpy of 4.35 J/g (based on g of Hi-Maize 1043 in the flour blend) at 144.7°C, value, which was equivalent to the enthalpy exhibited by the Hi-Maize 1043 ingredient, but presented a shift in temperature of about 45°C with respect to the original melting temperature of 101.6°C in the Hi-Maize 1043 ingredient. Thus, generation of crystalline resistant starch, as expected from high temperature/moisture processes, occurred during extrusion and/or DSC analysis. A smaller enthalpy than that for RSIII represented a sample with less degree of crystallinity and, thus, less content of enzyme-resistant starch. In addition, the generated enzyme-resistant starch had a lower melting point than that of RSIII.

Cereal samples containing Fibersym 70 presented a MDSC enthalpy of 1.2 J/g (based on g of Fibersym in the flour blend) at 122°C, a value that was

**Table 9.8** Fibre content of extruded, RTE cereals.

	<b>% total dietary fibre, theoretical<sup>a</sup></b>	<b>% AOAC total dietary fibre<sup>b</sup></b>	<b>% fibre in RS ingredient after processing<sup>c</sup></b>	<b>% fibre retention in RS ingredient<sup>d</sup></b>
RSIII ingredient	50	–	–	–
Hi-Maize™ ingredient	62	–	–	–
Fibersym® 70 ingredient	70, min.	80	–	–
Control cereal	5.58	7.7	–	–
RSIII cereal, 50% flour replacement	23.3	22.4	48	96
Hi-Maize™ 1043 cereal, 50% flour replacement	28.9	19.4	42	67
Fibersym® 70 cereal, 50% flour replacement	37.3	12.60	27	34

<sup>a</sup>Calculated based on fibre content of ingredient declared in specifications.

<sup>b</sup>As measured by AOAC 991.43.

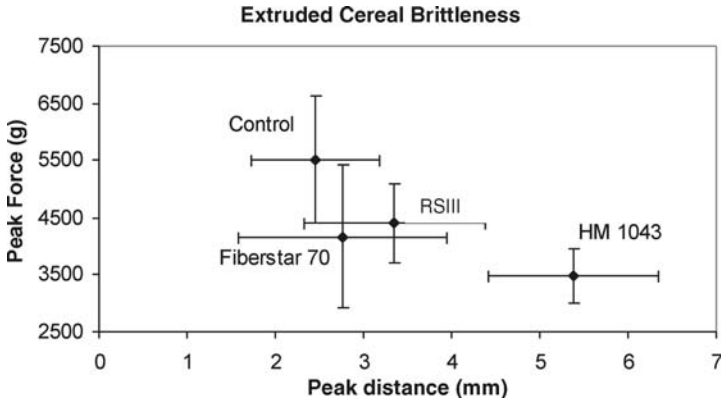
<sup>c</sup>Calculated based on measured AOAC TDF in final product.

<sup>d</sup>Calculated based on % total dietary fibre in RS ingredient, declared in specifications, vs. % fibre in RS ingredient after processing.

very different in both magnitude and temperature from the original Fibersym 70 raw material. Since Fibersym 70 is a cross-linked starch, as opposed to granular and retrograded starches in the case of Hi-Maize 1043 and X-150, enthalpy might not correlate well with the amount of enzyme-resistant starch. A better measurement would correspond to amount of total dietary fibre, as will be discussed later.

As shown in Table 9.8, the theoretical total dietary fibre content in the resistant starches corresponded to: RSIII = 50%; Hi-Maize 1043 = 62%; Fibersym 70 = 70% (although it assayed at 80% using the AOAC 991.43 method), based on fibre content of ingredient declared in specifications.

Although cereal samples containing Hi-Maize 1043 had a higher theoretical dietary fibre content than cereal samples containing RSIII, calculated based on fibre content of the ingredient declared in the specifications, the measured AOAC total dietary fibre for cereal samples containing RSIII was higher than that for cereal samples containing Hi-Maize 1043. The calculated % fibre retention (i.e. fibre that survived processing plus that being generated during processing) corresponded then to 96% for RSIII cereals and only 67% for Hi-Maize 1043 cereals.



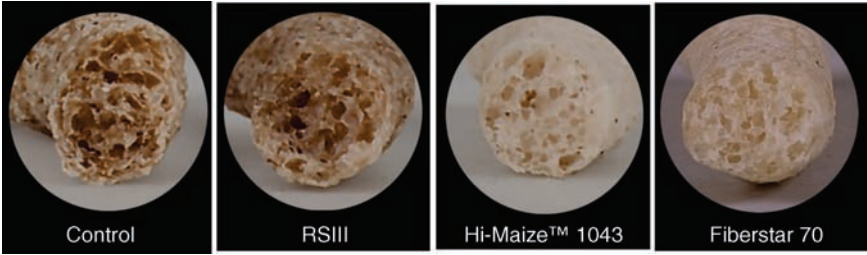
**Figure 9.10** Extruded cereal brittleness.

Although cereal samples containing Fibersym 70 had the highest theoretical dietary fibre content than the rest of the samples, calculated based on measured fibre content of the ingredient (80%), the measured AOAC total dietary fibre for cereal samples containing Fibersym 70 was the lowest of all the samples. The calculated percentage fibre retention corresponded to only 34%, which suggests that Fibersym 70 did not completely survive high temperature and high shear conditions present during extrusion.

Cereal brittleness is presented in Figure 9.10. A high peak force and low peak distance would correspond to a brittle sample, while a low peak force and high peak distance would correspond to a less brittle sample. The *x*- and *y*-axis error bars represent  $\pm 1$  standard deviation, while the diamonds represent averages. Overlapping error bars represent samples that were not significantly different from one another. In this case, control, X-150 and Fibersym 70 samples did not present a significantly different peak force or a significantly different peak distance, indicating that they possessed a similar brittleness. Hi-Maize 1043 samples showed a larger peak distance than the rest of the samples, indicating a lower brittleness. Hi-Maize 1043 samples did not present a significantly different peak force than RSIII or Fibersym 70 samples, but they were significantly softer (lower peak force) than the control.

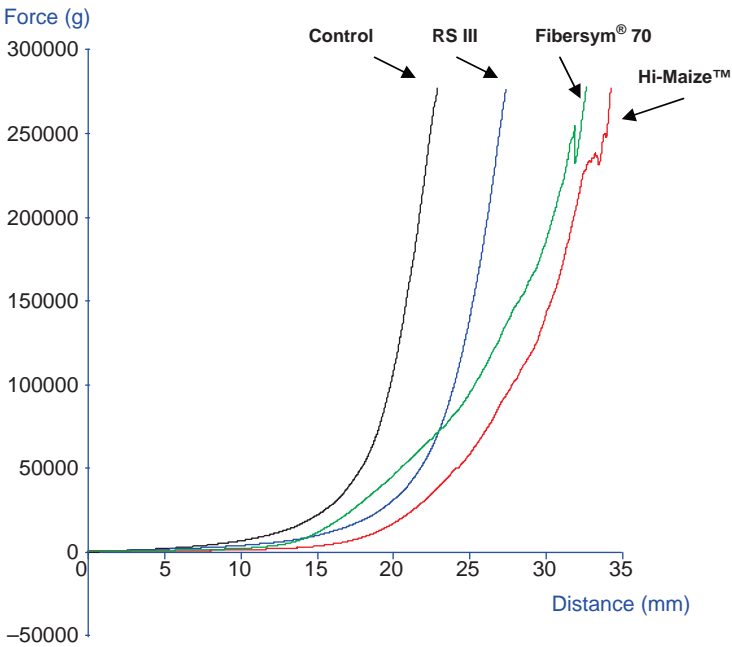
As shown in Figure 9.11, air cells were more numerous and larger in the control than in the rest of the samples, a characteristic which can be correlated to bulk density. Cell structure is the result of expansion during extrusion, governed by moisture content, moisture flash-off and flour blend (matrix) physicochemical properties.

As shown in Figure 9.12, cereal samples containing RSIII showed superiority in bowl life when compared to the other two resistant starch-containing

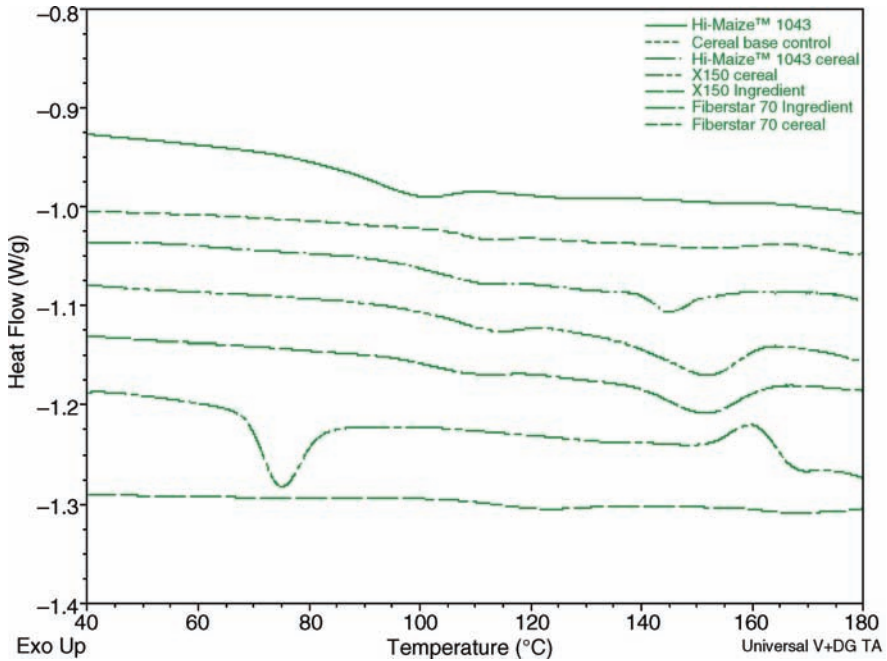


**Figure 9.11** Cell structure of extruded cereals.

samples (i.e. RSIII samples force vs. distance (deformation) behaviour after soaking in 8°C water for 30 minutes was closer to the control than that of Fibersym 70 and Hi-Maize 1043). For example, if a distance of 10 mm was chosen on the force vs. distance plot, it can be seen that the control was harder (higher force) than the rest of the samples, and that RSIII was harder (higher force) than Hi-Maize 1043 and Fibersym 70. The same behaviour was



**Figure 9.12** Extruded cereal bowl life.



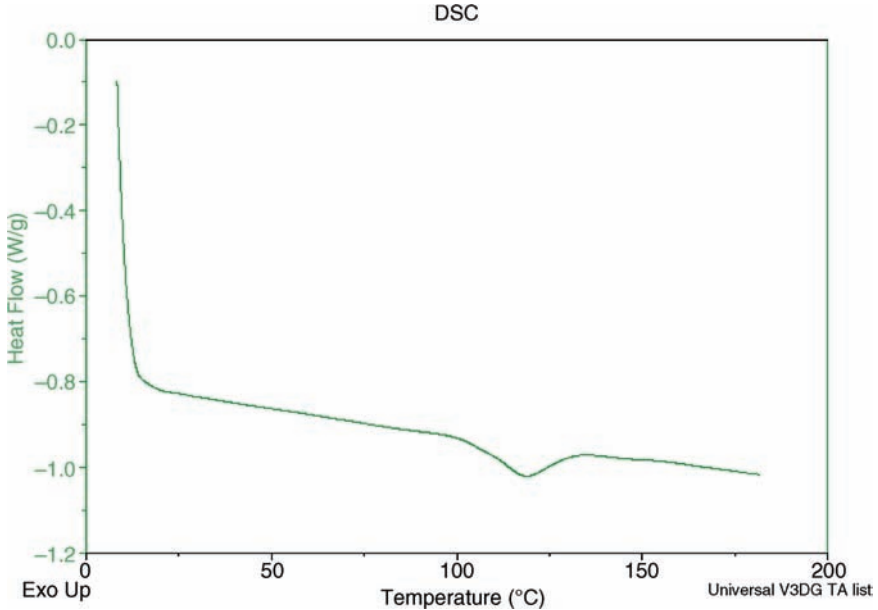
**Figure 9.13** MDSC curves for Hi-Maize™ 1043 (RSII), RSIII (X150) and Fibersym® 70 (RS IV) ingredients, and for cereals containing these raw ingredients. 1:1 dilution in distilled water.

observed at deformations above 23 mm. Between 15–23 mm of deformation, Fibersym 70 presented a higher force than both Hi-Maize 1043 and RSIII, but its force vs. deformation curve behaved in a very different manner than the control.

In summary, and as shown by the results discussed above, samples containing RSIII had a superior extruding functionality and dietary fibre content compared to Hi-Maize™ 1043 and Fibersym® 70.

The melting profile or thermal characteristics of the resistant starches Type II, III and IV, as well as the cereals containing these ingredients, were determined by modulating differential scanning calorimetry (MDSC). Results of the MDSC analysis for Hi-Maize 1043, RS III (X150) and Fibersym 70 are shown in Figure 9.13.

- For Hi-Maize 1043, the onset of melting occurs at about 93°C, the endothermic peak or melting point is about 101°C and the endpoint of melting occurs at about 112°C.



**Figure 9.14** MDSC curve for Novelose 330 (RSIII). 1:1 dilution in distilled water.

- For RSIII, the onset of melting occurs at about 135°C, the endothermic peak or melting point is about 151.2°C and the endpoint of melting occurs at about 165°C.
- For Fibersym 70, the onset of melting occurs at about 68°C, the endothermic peak or melting point is about 75°C and the endpoint of melting occurs at about 95°C.

The software calculates the enthalpy of the endothermic peak in J/g.

Results of the MDSC analysis for cereals containing resistant starch ingredients are also shown in Figure 9.13.

- For Hi-Maize-containing cereal, the onset of melting occurs at about 135°C, the endothermic peak or melting point is about 145°C and the endpoint of melting occurs at about 155°C.
- For RSIII-containing cereal, the onset of melting occurs at about 135°C, the endothermic peak or melting point is about 152°C and the endpoint of melting occurs at about 165°C.
- For Fibersym 70-containing cereal, the onset of melting occurs at about 107°C, the endothermic peak or melting point is about 122°C and the endpoint of melting occurs at about 133°C.



MDSC results for Novelose 330 (National Starch & Chemical Co., Bridgewater, NJ), another commercially available type III resistant starch, are shown in Figure 9.14. The onset of melting occurs at about 104°C, the endothermic peak or melting point is about 118.2 and the endpoint of melting occurs at about 135°C. Thus, even though this is a type III resistant starch, it melts at a lower temperature than RSIII, indicating that it would not survive high-temperature extrusion conditions such as those used in breakfast cereal manufacture, having very little functionality as a fibre.

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# 10 Role of Carbohydrates in the Prevention of Type 2 Diabetes

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## 10.1 INTRODUCTION

The main objectives of this chapter are to review basic facts about diabetes mellitus (definition, types, prevalence and risk factors) and to understand how dietary carbohydrates affect the risk of developing type 2 diabetes and some of the mechanisms by which dietary carbohydrates may be able to prevent type 2 diabetes, namely reduced postprandial glucose responses and increased colonic fermentation.

## 10.2 BACKGROUND

### 10.2.1 Definition of diabetes

Diabetes is a condition in which the body either cannot produce insulin, or cannot use the insulin it produces, resulting in a rise in the blood glucose concentration. Therefore, the diagnosis of diabetes is made based on the finding of high blood glucose. For men and non-pregnant women, diabetes is present if any one of the following three conditions is met (Canadian Diabetes Association Clinical Practice Guidelines Expert Committee, 2008):

- fasting plasma glucose equal to or greater than 7.0 mmol/l (126 mg/dl);
- random (i.e. at any time of the day) plasma glucose equal to or greater than 11.1 mmol/l (200 mg/dl); or
- a plasma glucose equal to or greater than 11.1 mmol/l (200 mg/dl) two hours after consuming 75 g glucose (oral glucose tolerance test).

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### 10.2.2 Types of diabetes

There are three main types of diabetes: type 1, type 2 and gestational diabetes:

- Type 1 diabetes, formerly known as insulin-dependent diabetes or juvenile onset diabetes, is an autoimmune disease in which the insulin-producing cells of the pancreas are destroyed, so that the body no longer produces any – or only a very small amount of –insulin. Type 1 diabetes usually develops in children or adolescents, affects about 5–10% of people with diabetes and is treated with lifelong injections of insulin.
- Type 2 diabetes, formerly known as noninsulin-dependent diabetes or maturity onset diabetes, occurs when the pancreas cannot produce enough insulin to meet the body's needs and/or the body is unable to respond normally to the actions of insulin (insulin resistance). Type 2 diabetes usually develops in middle-aged or elderly adults (although it can occur in children), affects about 90–95% of people with diabetes and is treated by diet and exercise and, if necessary, oral medications and/or insulin.
- Gestational diabetes is high blood glucose that develops during pregnancy. It affects about 2–4% of pregnant women and is treated by diet and, if necessary, insulin. Blood glucose levels usually return to normal after delivery.

### 10.2.3 Complications of diabetes

Diabetes has serious complications, including heart disease, kidney disease, eye disease, impotence and nerve damage, which are caused by high blood glucose. Treatment of diabetes to reduce blood glucose levels is known to prevent, delay the onset of or reduce the severity of these complications.

### 10.2.4 Prevalence of diabetes

Diabetes is a major public health issue because of the increased morbidity and mortality associated with it, the high cost of treatment, and the fact that its prevalence is rising rapidly in all parts of the world. In 1998, it was estimated that there were 135 million people in the world with diabetes and that this number would more than double to 300 million by 2025 (King *et al.*, 1998). However, the prevalence of diabetes is increasing faster than previously expected; it is now estimated that there are 285 million people worldwide who are affected by diabetes, and that this number is increasing by seven million per year to reach 390 million by 2025 (Canadian Diabetes Association, 2010).

### 10.2.5 Risk factors for type 2 diabetes

Since most people with diabetes have type 2 diabetes and since diet plays a larger role in the prevention of type 2 than type 1 diabetes, the rest of this chapter is concerned with type 2 diabetes. Consideration of dietary strategies to prevent type 2 diabetes is particularly important for people at high risk of developing the condition. Major factors associated with increased risk for type 2 diabetes include (Canadian Diabetes Association Clinical Practice Guidelines Expert Committee, 2008):

- being overweight or obese (body mass index over 25 kg/m<sup>2</sup>);
- age over 40 years; family history of diabetes in a first degree relative (parent or sibling);
- being a member of a high risk population (Aboriginal, African, Hispanic, Asian or South Asian);
- having previously had gestational diabetes or high blood glucose (impaired fasting glucose which is fasting glucose between 5.6–6.9 mmol/l or 100–125 mg/dl or impaired glucose tolerance, which is blood glucose two hours after oral glucose tolerance test between 7.8–11.0 mmol/l or 140–199 mg/dl);
- having high serum triglycerides or low serum high-density lipoprotein (HDL) cholesterol;
- the presence or history of peripheral-, coronary- or cerebro-vascular disease;
- the presence or history of schizophrenia.

## 10.3 CARBOHYDRATES AND RISK OF TYPE 2 DIABETES

With respect to the prevention of type 2 diabetes, there is strong evidence that the quality of dietary carbohydrate is more important than the quantity. There is little evidence that the amount of carbohydrate consumed influences risk for type 2 diabetes. In six out of seven recent prospective studies in which it was examined, the amount of carbohydrate consumed had no significant effect on type 2 diabetes (see Table 10.1).

### 10.3.1 Markers of carbohydrate quality

The quality of dietary carbohydrate has traditionally been assessed by considering simple versus complex carbohydrates. However, there is no evidence that a high intake of simple carbohydrates causes diabetes (Janket

**Table 10.1** Relative risk of type 2 diabetes for 5th vs. 1st quintiles of intake of total carbohydrate, cereal fibre, glycemic index and glycemic load.

	<b>Total carbohydrate</b>	<b>Cereal fibre</b>	<b>Glycemic index</b>	<b>Glycemic load</b>
Salmeron <i>et al.</i> , 1997a	ns	0.70	1.37	ns
Salmeron <i>et al.</i> , 1997b	ns	0.72	1.37	1.47
Meyer <i>et al.</i> , 2000	ns	0.64	ns	ns
Schulze <i>et al.</i> , 2004b	ns	0.63	1.59	ns
Hodge <i>et al.</i> , 2004	ns	ns	1.23	ns
Zhang <i>et al.</i> , 2006	ns	0.77	1.30	1.61
Villegas <i>et al.</i> , 2007	1.28	–	1.21	1.34
Krishnan <i>et al.</i> , 2007	–	0.82	1.23	1.22

Values are relative risks which are significantly different from 1 for fully adjusted models; ns = non-significant effect; '–' is shown for studies where no result is given.

*et al.*, 2003; Hodge *et al.*, 2004), although consumption of sugar-sweetened beverages may increase diabetes risk (Schulze *et al.*, 2004a).

Measures of carbohydrate quality which do influence risk for type 2 diabetes include cereal fibre and glycemic index (Table 10.1). Cereal fibre is considered here to be a qualitative indicator of carbohydrate intake, because it represents a small proportion of total carbohydrate (usually less than 10%) and is a marker of the types of carbohydrates consumed. In six out of seven studies in which it was examined, a high intake of cereal fibre was associated with a significant reduction in the relative risk of developing type 2 diabetes; the mean reduction in risk was about 30%.

The other marker of carbohydrate quality is glycemic index (GI). GI is defined as  $100 \times F/R$ , where F is the incremental area under the glycemic response curve (AUC) elicited by a portion of food containing 50 g available carbohydrate, and R is the mean AUC elicited by 50 g available carbohydrate from the reference food or glucose tested 2–3 times by the same subject. The GI is the mean of these values for a group of subjects (typically  $n = 10$ ) (Brouns *et al.*, 2003; Wolever, 2006). In seven out of eight studies in which it was examined, a high GI diet significantly increased risk for type 2 diabetes, with the mean increase in risk being about 30%.

A marker of both carbohydrate quality and quantity is glycemic load, defined as  $GI \times g$ , where GI is the food glycemic index and g is the amount of available carbohydrate in the food. Glycemic load is considered by many to be

a more complete indicator of the physiological impact of dietary carbohydrate. However, it is not a consistent predictor of diabetes risk, with a significant effect only found in four out of the eight studies in which it was examined (Table 10.1).

## 10.4 PATHOGENESIS OF TYPE 2 DIABETES

Prospective studies suggest that the quality of dietary carbohydrate (high cereal fibre intake and/or low GI) is more important than the quantity consumed for preventing type 2 diabetes. To determine if there are plausible mechanisms by which high cereal fibre and low GI diets reduce the risk for type 2 diabetes, it is necessary to understand the pathophysiological mechanisms which cause type 2 diabetes.

Many factors influence blood glucose, but perhaps the most important is insulin. Insulin controls the blood glucose concentration via a classical feedback loop. A rise in blood glucose stimulates the secretion of insulin from the beta-cells of the pancreas, and the resulting rise in blood insulin stimulates muscle and adipose tissue to increase glucose uptake, hence causing blood glucose to fall. The ability of a rise in plasma glucose to stimulate insulin secretion is termed insulin secretion, and the ability of insulin to stimulate glucose uptake from the blood is termed insulin sensitivity. In normal subjects, there is a hyperbolic relationship between insulin secretion and insulin sensitivity (Clausen *et al.*, 1996). Thus, people with high insulin sensitivity (or low insulin resistance) have low insulin secretion in response to a rise in blood glucose, while those with low insulin sensitivity (or high insulin resistance) have high insulin secretion. The product of insulin secretion and insulin sensitivity is termed disposition index, which is a marker of beta-cell function.

It is commonly thought that diabetes develops because of decreased insulin sensitivity (or increased insulin resistance). However, the relationship between insulin secretion and insulin sensitivity suggests that blood glucose remains normal as long as changes in insulin sensitivity are compensated for by changes in insulin secretion. Type 2 diabetes develops when the disposition index falls – that is, when insulin secretion cannot be increased enough to compensate for reductions in insulin sensitivity (Kahn, 2003).

Most studies investigating the role of diet on the pathogenesis of type 2 diabetes only measure insulin sensitivity, but this does not necessarily indicate risk for type 2 diabetes, because the deleterious effects of reduced insulin sensitivity or the beneficial effects of increased insulin sensitivity on

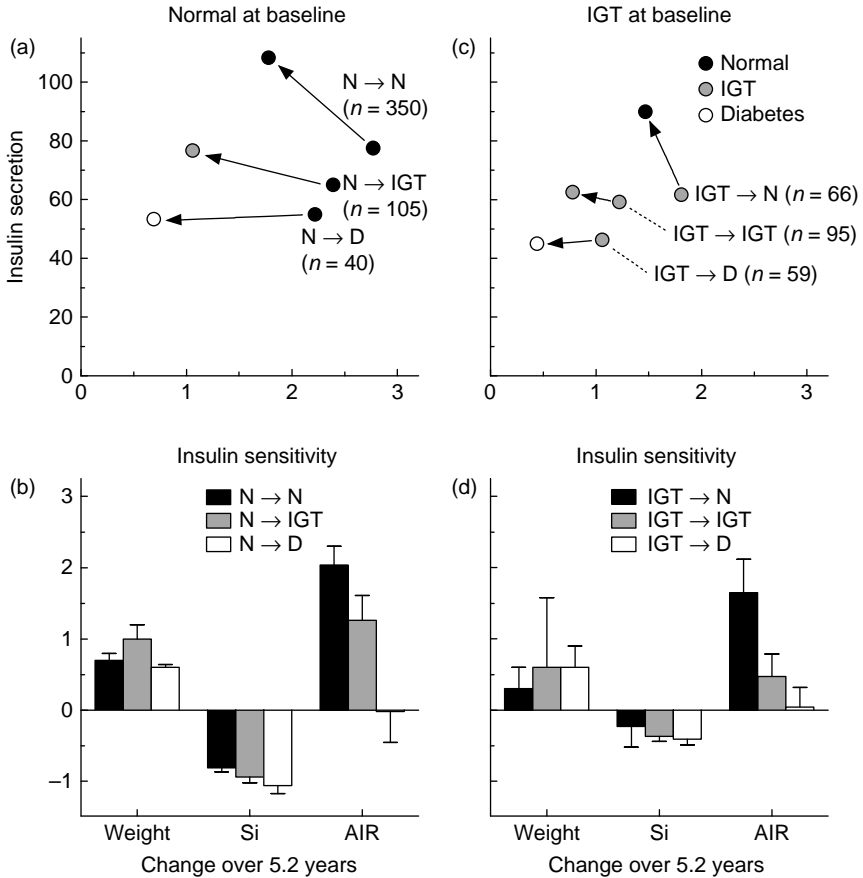
blood glucose could be compensated for by compensatory changes in insulin secretion. Indeed, studies in which both insulin secretion and insulin sensitivity have been measured have shown that factors which increase insulin sensitivity, such as exercise (Kahn *et al.*, 1990), are accompanied, at least in the short term, by a compensatory reduction in insulin secretion. Conversely, factors which cause a reduction in insulin sensitivity, such as pregnancy (Catalano *et al.*, 1993) or use of nicotinic acid (Kahn *et al.*, 1989) are associated with compensatory increases in insulin secretion, such that the disposition index did not change.

The dynamic relationship between insulin secretion and insulin sensitivity makes teleological sense, because it allows blood glucose control to be maintained despite changes in insulin sensitivity that normally occur throughout life due to physiological factors such as puberty, pregnancy and changes in body composition that occur during aging, and also by environmental factors such as short-term changes in activity and the quality and quantity of food intake.

The relative importance of changes in insulin secretion and changes in insulin sensitivity in causing the development of type 2 diabetes has been shown in two studies in which both were measured longitudinally in the same subjects (Weyer *et al.*, 1999; Festa *et al.*, 2006). These studies show that a reduction in insulin sensitivity is not necessarily associated with the development of diabetes but, rather, it is the change in insulin secretion that determines whether an individual develops diabetes or not. In the larger study of this kind, oral glucose tolerance was assessed, and insulin secretion and insulin sensitivity were measured, using a frequently sampled intravenous glucose tolerance test in nearly 800 subjects at baseline and after a mean period of 5.2 years follow-up.

Figures 10.1a and 10.1c show the results for subjects who had normal glucose tolerance at baseline divided into three groups: those who remained normal at follow-up; those who developed impaired glucose tolerance (IGT); and those who developed diabetes. All three of these groups of subjects gained weight and became more insulin resistant (less insulin sensitive) with time. What distinguished them was that subjects who remained normal increased insulin secretion more than those who developed impaired glucose tolerance, while those who developed diabetes had no change in insulin secretion. Similar results are seen for subjects who had impaired glucose tolerance at baseline (Figures 10.1b and 10.1d). Remarkably, some subjects with impaired glucose tolerance at baseline became normal at follow-up, despite the fact that they became more insulin resistant; the increase in insulin secretion was large enough to overcome the reduction in insulin sensitivity.





**Figure 10.1** Changes in body weight, insulin sensitivity (Si) and insulin secretion (AIR, area under the insulin response curve after intravenous glucose) over 5.2 years in subjects who had normal glucose tolerance at baseline (a and c) and subjects who had impaired glucose tolerance at baseline (b and d), and whose glucose tolerance at follow-up had remained, or become, normal (N), impaired (IGT) or diabetic (D). Adapted from Festa *et al.* (2006).

## 10.5 EFFECT OF ALTERING SOURCE OR AMOUNT OF DIETARY CARBOHYDRATE ON INSULIN SENSITIVITY, INSULIN SECRETION AND DISPOSITION INDEX

The glycemic impact of the diet can be reduced by changing the source of dietary carbohydrate (i.e. using low-GI foods) or by reducing the amount of

carbohydrate consumed. Since epidemiological studies suggest that carbohydrate quality is associated with risk for diabetes, but that carbohydrate quantity is not (Table 10.1), we hypothesized that, on weight-maintaining diets, reducing diet GI without reducing carbohydrate intake would have a more beneficial effect on insulin sensitivity and insulin secretion than a moderate reduction in carbohydrate intake.

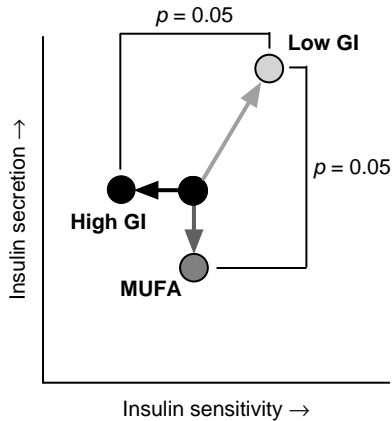
To test this, we studied 35 subjects with IGT, whom we identified by screening subjects with risk factors for type 2 diabetes using a 75 g OGTT (Wolever & Mehling, 2002). They were randomly assigned to consume one of three diets for four months:

1. a diet containing 50–55% carbohydrate using at least one high-GI food at every meal (high-GI,  $n = 11$ );
2. a diet containing 50–55% carbohydrate using at least one low-GI food at every meal (low-GI,  $n = 13$ ); or
3. a diet in which carbohydrate intake was reduced to 40–45% of energy and monounsaturated fat (MUFA) intake was increased (MUFA,  $n = 11$ ).

Subjects were provided with high- and low-GI foods and food sources of MUFA to use in their diets. Insulin sensitivity, insulin secretion and disposition index (the product of insulin sensitivity and insulin secretion) were measured using a frequently-sampled intravenous glucose tolerance test at baseline and after four months on the diet. To compare the effects of the three diets on blood glucose, insulin and free-fatty acid (FFA) responses while consuming the study diets, subjects also underwent eight-hour metabolic profiles. At baseline, all subjects consumed breakfast and lunch meals reflecting the high-GI diet. At four months they consumed breakfast and lunch meals reflecting the diet to which they had been randomized; subjects on high-GI consumed the same meals as at baseline, subjects on low-GI consumed meals of the same energy and carbohydrate content but using low-GI instead of high GI foods, and subjects on MUFA consumed meals with the same energy as at baseline but containing less carbohydrate and more MUFA.

The results showed that disposition index tended to deteriorate on the high-GI and MUFA diets, but significantly increased on the low-GI diet. The change in disposition index on the low-GI diet was significantly different from the changes on both the high-GI and MUFA diets (Figure 10.2).

Similar effects have been seen in other studies. For example, 72 subjects with metabolic syndrome were randomly assigned to receive a diet containing oat, wheat and potatoes (foods shown to elicit high glucose and insulin responses) or a diet containing whole rye breads and pasta (low glucose and



**Figure 10.2** Changes in insulin sensitivity and insulin secretion in subjects with impaired glucose tolerance after four months on a high-carbohydrate, high-glycemic index (GI) diet (high-GI), a high-carbohydrate, low-GI diet (low-GI) and a low-carbohydrate, high-monounsaturated fat (MUFA) diet. Adapted from Wolever and Mehling (2002).

insulin responses). Beta-cell function improved significantly on the rye-pasta diet compared to the oat-wheat-potato diet (Laaksonen *et al.*, 2005). These studies suggest that low-GI foods reduce diabetes risk by improving beta-cell function.

## 10.6 MECHANISMS BY WHICH LOW-GI FOODS IMPROVE BETA-CELL FUNCTION

Mechanisms by which low-GI foods may increase beta-cell function include reduced glucose toxicity, reduced serum FFA and increased levels of incretin hormones such as GLP-1.

### 10.6.1 Glucose toxicity

Glucose toxicity refers to damaging effects of high blood glucose concentrations *per se* on body tissues and regulatory processes due to a variety of mechanisms, including increased flux through the polyol and glucosamine pathways, increased non-enzymatic glycation products and glycosylation of proteins, activation of diacylglycerol and protein kinase C and increased oxidative and carbonyl stress (Brownlee, 2005).

Glucose toxicity has been implicated in the pathogenesis of diabetes by reducing beta-cell function (Leahy *et al.*, 1988). However, if glucose toxicity was the only mechanism involved, any method of reducing blood glucose should have a beneficial effect on beta-cell function. That this is not the case is shown by the fact that, in subjects with IGT, reducing diet GI improved beta-cell function, but reducing the amount of carbohydrate consumed did not, despite the fact that both diets reduced mean eight-hour blood glucose compared to the high-GI diet and did so to an equivalent extent (Wolever & Mehling, 2003). Similarly, in subjects with type 2 diabetes a low-GI diet reduced serum c-reactive protein as a marker of chronic inflammation, while a lower carbohydrate, high MUFA diet did not (Wolever *et al.*, 2008). Both of these studies suggest that reducing glucose toxicity is not the only mechanism by which low-GI foods improve beta-cell function.

### **10.6.2 Reduced serum free fatty acids (FFA)**

The beta-cell secretes insulin in response to a rise in blood glucose, and it has been proposed that the intracellular mechanism by which glucose stimulates insulin secretion is via intracellular long-chain fatty acids. A rise in blood glucose increases glucose entry into the beta-cell and, hence, increases flux through the glycolytic pathway and glucose oxidation. Increased glucose oxidation within the beta-cell inhibits the oxidation of fatty acids, causing the intracellular concentration of fatty acids to rise and this, in turn, stimulates the release of insulin from the cell (Prentki & Corkey, 1996). This is confirmed by studies showing that the addition of fatty acids to the medium of cultured beta-cells increases insulin secretion (Zhou & Grill, 1994) and, in humans *in vivo*, acutely increasing the serum FFA concentration increases insulin secretion (Carpentier *et al.*, 1999).

However, in the presence of chronically high concentrations of intracellular fatty acids and glucose, the glucose signal is lost. Prolonged exposure of cultured beta cells to high levels of FFA and glucose reduces insulin secretion (Zhou & Grill, 1994). Similarly, in humans, a prolonged increase in serum FFA inhibits insulin secretion (Carpentier *et al.*, 1999). For this reason, we measured serum FFA concentrations after four months on the low-GI, high-GI and MUFA diets and showed that 0–8 hours mean serum FFA concentration on the low-GI diet was significantly lower than on high-GI, whereas the levels of FFA on the MUFA diet were similar to those on high-GI (Wolever & Mehling, 2003). This suggests that it may be necessary to reduce both glucose and FFA in order to improve beta-cell function.

### 10.6.3 Increased GLP-1 secretion

GLP-1 (glucagon-like polypeptide-1) is an incretin, secreted from the L-cells in the lower end of the small intestine, which has multiple biological functions, including delaying gastric emptying, increasing satiety, increasing insulin sensitivity and increasing insulin secretion (Drucker, 1998). The latter is thought to be achieved not only by increasing the amount of insulin secreted by each beta-cell, but also by increasing the number of beta-cells. GLP-1 is secreted in response to the presence of nutrients in the distal small intestine and also via a neuroendocrine loop, whereby GIP secretion in the upper intestine sends a signal via the vagus nerve and the brain to the L-cells (Brubaker & Anini, 2003).

Animal studies suggest that GLP-1 secretion is also stimulated by the short-chain fatty acids (SCFA) produced upon fermentation of dietary fibre and resistant starch in the large intestine (Reimer & McBurney, 1996). This might be a mechanism by which diets high in cereal fibre reduce diabetes risk. The cereal fibre most commonly consumed in North America is wheat bran; however, studies lasting 4–12 weeks have shown no significant effect of wheat fibre on blood glucose control or oral glucose tolerance (Munoz *et al.*, 1979; Kestin *et al.*, 1990; Jenkins *et al.*, 2002; Costabile *et al.*, 2008).

One way to reconcile discrepancy between epidemiological studies showing that cereal fibre is a strong predictor of diabetes risk (Table 10.1), and long-term clinical trials showing that wheat bran has little or no effect on glucose metabolism, might be that the clinical trials lasted only four weeks to three months, which is not long enough for the colonic bacteria to adapt to the change in fibre intake. The colon is a very complex ecosystem, containing hundreds of species of bacteria. If an input into an established ecosystem (here the input is the amount of fibre entering the colon) is changed, the output of the ecosystem becomes unstable and varies with time until a new equilibrium is reached. The more complex the ecosystem, the longer it takes for a new equilibrium to be established (Feng & Chai, 2008).

Studies in animals demonstrate this concept. For example, in a four-month study, when the resistant starch content of the diet of rats was increased, the SCFA content of the caecum began to increase and continued to increase over entire period of the study (Le Blay *et al.*, 1999). This suggests that it takes the colonic ecosystem more than four months to equilibrate after a change in fibre intake. Thus, we hypothesized that cereal fibre, in the form of All-Bran<sup>®</sup> cereal would significantly increase serum SCFA concentrations and GLP-1 secretion, but that this would only be apparent after a long adaptation period of at least six months.

To test this, we randomly assigned 28 hyperinsulinemic subjects to consuming two cups of a low fibre cereal per day, or one cup of All-Bran<sup>®</sup> daily for one year (Freeland *et al.*, 2010). At baseline, and after every three months, subjects underwent a metabolic profile in which the glucose, insulin, FFA, SCFA and GLP-1 responses elicited by the diet were measured. The results showed significant time  $\times$  treatment interactions for serum acetate, butyrate and GLP-1; that is, the difference between the diets depended upon the time. Acetate increased temporarily after nine months on the diet, butyrate began to increase at three months and reached a plateau after nine months, and GLP-1 tended to gradually increase with time to reach a level significantly higher than the control after 12 months. These results supported the hypothesis that cereal fibre may reduce diabetes risk by increasing GLP-1 secretion mediated by colonic short chain fatty acids, but that these events take many months to occur, as the colonic ecosystem gradually adapts to the increased fibre intake.

## 10.7 CONCLUSIONS

The quality of dietary carbohydrates may be an important factor in determining risk for type 2 diabetes. The results of recent studies suggest that a deterioration in beta-cell function may be the critical pathophysiological event which causes type 2 diabetes. Evidence has been presented here that low-GI carbohydrates may preserve beta-cell function by reducing postprandial glucose fluctuations and, hence, glucose toxicity, while at the same time reducing serum FFA concentrations. Cereal fibre may preserve beta-cell function, at least in part, by increasing colonic fermentation and up-regulating GLP-1 secretion, but these effects may not occur until the colon has adapted to the increased intake of fibre – a process which may take 6–9 months or more.

However, whether low-GI foods or cereal fibre will actually prevent type 2 diabetes in humans remains to be shown.

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# 11 Resistant Starch on Glycemia and Satiety in Humans

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## 11.1 INTRODUCTION

There is a major public health effort to decrease the prevalence of obesity-related health outcomes. Given the role of diet and nutrition on the aetiology of obesity, it is imperative that dietary options should be targeted to assist with preventing these comorbid conditions such as type 2 diabetes, dyslipidaemia and other cardiometabolic complications. While resistant starch (RS) has been studied by cereal chemists and food companies for several years, studies conducted by clinical scientists have been limited, but these are increasing and providing interesting results indicating a strong evidence that RS may provide significant metabolic health benefits. The majority of studies indicates a capacity to lower glycemia and improve insulin sensitivity. Other data, while less conclusive, indicate the potential to attenuate satiety and decrease subsequent food intake. Thus, there seems to be an inverse relationship between glycemic response and food intake following the consumption of RS or foods containing adequate amounts of RS. Moreover, while the data are limited, evidence seems to indicate that different types of RS can elicit different metabolic responses.

This review will focus on the glucoregulatory aspects of RS, with discussions on outcomes from primarily human clinical trials relating to satiety, food intake and potential to address the obesity issue present in most industrialized societies.

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## 11.2 DIET AND RESISTANT STARCH

Diabetes, obesity and cardiovascular disease are interrelated. Diet is one factor that links them all together. Specifically, the current obesity epidemic has been suggested to be a result of increased carbohydrate intake (Gaesser, 2007), even though dietary fat has frequently been touted as the primary dietary culprit of many deleterious metabolic conditions. The fact is that we, as a nation, actually increased carbohydrate consumption – perhaps to address the public health message to avoid dietary fat – during the latter years of the past century, just as the obesity epidemic began to surge (Gaesser, 2007). Thus, the national response to decrease fat may have inadvertently led to overconsumption of carbohydrates. From one perspective, the public health effort to decrease the percent of energy derived from fat in our diets was moderately successful; however, total energy intake and attaining a weight-maintaining energy balance was not affected by those behaviours.

So, while it is laudable to try to change the behaviour of individuals (to choose other foods), it might be more effective to address this nutritional issue by providing bioactive compounds that ‘behave’ like traditional starch, yet elicit a more favourable metabolic profile. In other words, let people continue to choose the foods they prefer, but simply make those foods healthier by incorporating bioactive ingredients and/or ingredients that affect energy metabolism. This might seem to be an achievable goal, but some past efforts have not proven to be acceptable to consumers.

To address this issue, RS shows promise at successfully improving glucoregulation in a consumer-acceptable fashion, while eliciting side effects that are similar to typical dietary fibres (e.g. bran) that have had difficulty being accepted by a most consumers. Compared with typical monosaccharides and disaccharides, RS elicits minimal glucose and insulin excursions and can be incorporated easily into regular food items with general acceptance by consumers, which is a barrier to many dietary fibres. That said, there is a paucity of evidence, especially from humans, to illustrate the glucose-lowering mechanisms. The common theory is based on the glucose-fatty acid cycle, whereby increasing fat oxidation allows for increased insulin sensitivity and glucose disposal (Randle *et al.*, 1963).

Testing this theory in human clinical trials will increase our understanding of the mechanisms through which RS, and other modified starches with similar properties, elicit their benefits. This will help food and pharmaceutical scientists to develop and/or enhance products to better prevent, manage and treat metabolic conditions. Currently, evidence indicates a glucoregulatory benefit, but how this occurs is not well established.

## 11.3 RESISTANT STARCH AND INSULIN SENSITIVITY

RS is a dietary carbohydrate that has been frequently studied. Most RS research, however, has focused on fermentation characteristics, while few studies have been designed to investigate mechanisms involved with increasing insulin sensitivity. Most of the mechanistic studies have used rodents and other small animals rather than using a human clinical trial.

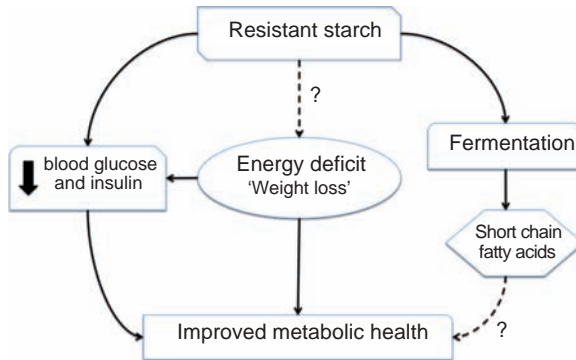
Basing RS mechanisms on animal study outcomes is problematic, given the coprophagic tendency of some species, which makes it difficult to interpret the results and apply those potentially confounded results to humans. Moreover, humans prefer eating a varied diet throughout the day and over time, while animal studies test ingredients with the animals eating the same food at every meal, every day. An additional research design and translational science issue is that we do not fully understand how to relate animal outcomes to humans, given that the dose and duration of treatment given to animals may not apply to typical human scenarios.

There has been one human clinical trial (Robertson *et al.*, 2005) that specifically investigated the effects of resistant starch on insulin sensitivity using the standard euglycaemic-hyperglycaemic clamp. They observed that individuals consuming 30 g/day of RS over four weeks elicited significant increase in glucose disposal and decreased insulin area under the curve during a meal tolerance test. However, while they observed increased insulin sensitivity, they were unable to discern clearly how RS elicited this response.

Results from other human clinical trials (Yamada *et al.*, 2005; Robertson *et al.*, 2003; Haub *et al.*, 2010; Behall *et al.*, 2006; Al-Tamimi *et al.*, 2010), support this notion that RS decreases postprandial glucose and insulin responses. While that evidence is fairly strong and consistent, the long-term benefits of RS are still not well established. What is less clear is the mechanism through which long-term RS ingestion exerts its benefits, and whether RS consistently affects health and lifestyle outcomes (e.g. obesity, satiety, and food intake) other than glucose metabolism in humans.

## 11.4 CURRENT THEORETICAL MECHANISM

A testable theory to explain the metabolic regulation by RS pertains to the formation of short chain fatty acids via fermentation, which subsequently increases fat oxidation (centrally and peripherally). While this theory has not formally been tested relative to RS, it is supported by the observation that increasing fat oxidation is a key factor to increase insulin sensitivity in those with insulin resistance (Figure 11.1).



**Figure 11.1** This figure depicts means through which RS is suggested to elicit metabolic benefits. It has been established that RS decreases blood glucose and insulin, which decreases risk for type 2 diabetes over time. It has, also, been shown to be fermented by gut microbiota, thereby changing the gut ecology and producing products (short chain fatty acids) that have been suggested to improve metabolic health. To date, however, it is not known whether some of the changes attributed to RS are due to changes in energy balance as RS may decrease energy intake acutely and/or chronically. Thus, some benefits attributed to RS may be indirect due to decreased energy being absorbed, and/or different ratios of macronutrients due to conversion of some RS into fatty acids by the gut microbiota.

Moreover, it was reported by Higgins *et al.* (2004) that the ingestion of RS leads to acute increases in lipid oxidation, which may lead to decreased fat accumulation and/or accretion over time. While the role of short chain fatty acids in this process is plausible, inducing an energy deficit also increases fat oxidation and insulin sensitivity by decreasing the inhibition on fat oxidation elicited by insulin and other glucoregulatory hormones. Thus, since RS only yields half the energy of regular starch ( $\approx 2$  kcal/g), it is plausible that a lower energy state of the cell or tissue may increase fat oxidation.

Specifically, based on the outcomes of Robertson *et al.* (2003), it was suggested that the increased insulin sensitivity arises from alterations in substrate oxidation due to the increased availability of short chain fatty acids derived from fermentation of RS (Figure 11.1). However, these researchers failed to include a treatment that only increased short chain fatty acids, thereby limiting their capacity to better understand how RS elicits the 'second meal effect' or whether other factors, such as an energy deficit, were contributing factors.

However, in a study by Johnston *et al.* (2010), the RS treatment (40 g/d) over 12 weeks elicited increased insulin sensitivity without a concomitant change in body weight or intracellular lipid content. These data indicate that

RS may even improve glycemia and insulinaemia, regardless of energy intake or lipid metabolism.

## 11.5 SATIETY

One unique effect of RS that seems to be developing is the impact that these starches seem to have on satiety. With lifestyle modification – specifically reducing energy intake – being a regular recommendation to prevent and treat various metabolic diseases and conditions (Poirier *et al.*, 2006; Jakicic *et al.*, 2001), this bioactive effect could be extremely beneficial from a public health perspective.

The means through which RS has been shown to induce satiety and lead to decrease energy intake (may not decrease volume or weight of food consumed) seems likely to involve alterations in PYY and other neuroendocrine components (Zhou *et al.*, 2008). Zhou *et al.* (2008) administered RS to mice over 32 days and observed that active forms of PYY and GLP-1 increased. The mice eating RS also gained less fat mass and had a lower ratio of body fat to body weight, while eating the same weight of food.

Similarly, So *et al.* (2007) fed mice high and low RS diets and measured numerous body composition and metabolic parameters after the eight-week intervention. They observed several favourable outcomes following the high RS treatment, compared with the low RS:

1. decreased intracellular fat deposition;
2. decreased body weight and adiposity;
3. smaller adipocytes;
4. their brain imaging results indicated greater satiety despite decreased energy intake.

While those studies were completed in mice, the satiating effect of RS has been reported in humans. Although hormone levels were not reported, Willis *et al.* (2009) observed satiety and feelings of fullness were evident longer following trials with RS and corn bran, compared with lower fibre bleached oat bran,  $\beta$ -glucan, and polydextrose. In support with those satiety results, outcomes from Anderson *et al.* (2010) indicate that foods containing 40–70% RS seem to reduce food intake during meals later in the day. In a study out of our lab (Haub *et al.*, 2012), RS4 from potato starches elicited similar ratings of satiety when ingested with an energy dense beverage (dextrose solution) or ingested with water. Taken together, the mechanisms for the reduced intake and ratings of satiety are not well understood, as gut hormone responses have either not been studied or did not elicit consistent results.

## 11.6 FERMENTATION AND GUT MICROBIOTA

A new area of research has been better in understanding how gut microbiota (especially *lactobacilli* and *bifidobacteria*), fermentation, and short chain fatty acids impact human health (Roberfroid *et al.*, 2010), with a few of these aspects depicted in Figure 11.1. The food industry has been actively placing prebiotic and probiotic foods on the market to provide consumers with foods to try to meet this health outcome. Specific to RS, Martinez *et al.* (2010) reported changes to gut microbiota following the ingestions of RS2 and RS4 by human volunteers. They observed that RS intake significantly altered gut microbiota in as little as one to three weeks, and that RS2 and RS4 elicited different changes in microbiota.

Moreover, short chain fatty acids (SCFA) formed from the fermentation of dietary fibre and RS sources have been selected as potential factors for increased satiety. However, a study using oligosaccharides (Hess *et al.*, 2010), reported that increased fermentation does not necessarily alter satiety in men or women. Collectively, RS and the by-products of fermentation may interact synergistically to elicit feelings of fullness. This is an area requiring more research to understand better how the effects of gut changes in bacteria and alterations in the fermentation process affect human health acutely and over time.

## 11.7 EFFECT OF RS TYPE

An area of RS research that needs more investigation is comparing the forms and types of RS currently available. Specifically, there is a paucity of data comparing RS compounds of different types (e.g. Type 2 vs. Type 3) within the same trial to determine similarities and differences on *in vivo* outcomes. This information is imperative for consumers and others interested in selecting RS to incorporate into foods and diets.

To our knowledge, there is only one peer-reviewed paper to have compared commercially available RS types from different companies on glycemia. Haub *et al.* (2010) compared the glucose lowering effects of a commonly tested type of RS (RS2) with a less studied cross-linked type (RS4). It was observed that the RS4 elicited a greater reduction in capillary blood glucose than RS2. This study clearly indicated that the type of RS used in clinical studies needs to be accounted for, since different types can elicit significantly different responses.

Together with the results from Martinez *et al.* (2010), it can be erroneous to conclude that all RS types will elicit the same physiological or psychological



responses. Further research investigating the differences in RS types will help illustrate how much of each RS is necessary to elicit a desired response. This outcome is critical when developing recipes and/or budgets for individual consumers or companies.

## 11.8 SUMMARY

Over the past two decades, data from animal and human intervention studies demonstrate that RS seems to elicit favourable changes in glucoregulatory outcomes and factors relating to gut health. The results from animal studies indicate a clearer effect on body composition and satiety, while the human data are not as conclusive in that regard. More studies designed to test body composition effects are needed to enhance our understanding of RS as a potential ingredient to significantly affect obesity-related outcomes. Likewise, while it is apparent that RS, as with other dietary fibres, does elicit changes in metabolic and gut health outcomes, the appropriate dose and/or RS-containing foods that elicit desired outcomes and significant health effects are still not well known.

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## 12 The Acute Effects of Resistant Starch on Appetite and Satiety

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### 12.1 APPETITE REGULATION

Appetite is defined as the desire or physical craving to eat and is typically separated into hunger, satiation and satiety. In a review by Mattes *et al.* (2005), hunger is defined as '*sensations that promote food consumption*', satiation as '*sensations that govern meal size and duration*' and satiety as '*sensations that determine the inter-meal period of fasting*'. Therefore, all are important for determining energy intake. Appetite is difficult to quantify, as it varies greatly between individuals. It is closely regulated by physiological processes, but it can also be influenced by many other aspects, including psychological factors (e.g. learned habits) and emotional factors (e.g. as a response to stress), as well as by external cues (e.g. accessibility of food, the presence or absence of other individuals or the hedonic properties of the food itself).

The hypothalamus plays a vital role in appetite regulation and the subsequent control of food intake; it receives peripheral signals from the digestive tract (gut peptides) and adipose tissue (through signalling molecules, including leptin and insulin) (Murphy & Bloom, 2004; Wynne *et al.*, 2005). While the hypothalamus is composed of many areas (nuclei), the arcuate nucleus (ARC) is thought to be the most important in appetite regulation and is essential for interpreting these peripheral signals (Wynne *et al.*, 2005; Dhillon, 2007). The ARC has an incomplete blood-brain barrier, so peripheral signals are able to cross the barrier, interact with receptors and consequently cause the release of neuropeptides that subsequently regulate ingestion (Neary *et al.*, 2004).

There are two classes of neurons in the ARC: those that stimulate and those that inhibit food intake. The neurons that stimulate appetite (and, therefore, promote food intake) are neuropeptide Y (NPY) and

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agouti-related peptide (AgRP) (Wynne *et al.*, 2005). NPY causes an increase in food intake through activation of the G-protein-coupled receptors Y1 and Y5, while AgRP causes an increase in food intake through antagonism of the melanocortin-4 (MC4) receptor and, therefore, the inhibition of  $\alpha$ -melanocyte-stimulating hormone ( $\alpha$ -MSH) (Neary *et al.*, 2004; Wynne *et al.*, 2005). The neurons that inhibit food intake are pro-opiomelanocortin (POMC) and cocaine- and amphetamine-regulated transcript (CART). These neurons stimulate  $\alpha$ -MSH, which interacts with the MC4 receptor (Murphy & Bloom, 2004; Neary *et al.*, 2004; Wynne *et al.*, 2005) that promotes subsequent anorexigenic activity. Once the peripheral signals are interpreted in the ARC, this can both inhibit and stimulate the different classes of neurons whether to promote food intake or to prevent further intake.

The peripheral signals from the digestive tract are hormones that are both anorexigenic (increase satiety) and orexigenic (promote hunger). The hormone ghrelin is unique in being orexigenic, and it is often termed the 'hormone of hunger'. Anorexigenic hormones are released postprandially in response to ingested nutrients; they act by both direct and indirect (via the vagus) mechanisms and promote feelings of satiety. These hormones include glucagon-like peptide-1 (GLP-1) and peptide YY (PYY).

## 12.2 MEASUREMENT OF APPETITE IN HUMANS

Appetite is particularly difficult to directly measure in humans, as it is a subjective sensation that can be influenced by many factors. Human studies, therefore, have used different indirect measures in order to assess appetite sensations (Mattes *et al.*, 2005). The most frequently used indirect methods are recording food intake, using questionnaires regarding appetite feelings and measuring biomarkers (Mattes *et al.*, 2005).

Records of food intake rely on participants accurately recording all they have to eat and drink over a certain time period, as well as on accurate interpretation by the investigators. As such, food records may not always provide precise results. Food intake can also be assessed at weighed and controlled *ad libitum* meals. While these do provide accurate measures of food intake, they might not show habitual intakes, as they are often completed in artificial environments.

Questionnaires relating to subjective appetite feelings are often used in human studies. These questionnaires rely on those filling out the questions reporting on their thoughts and feelings relating to hunger, satiation and satiety. Typically, there are two main types of questionnaire that are used – category scales and visual analogue scales (VAS). These are quick and easy to

complete and have been proven to be reproducible and valid in appetite research.

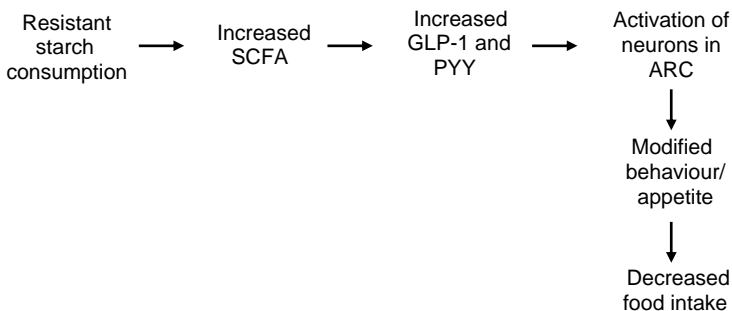
Biomarkers of appetite are also often measured in human studies, and include changes to gut hormone concentrations. These are often a preferred measurement of changes to appetite, as they are less likely to be influenced by other factors.

### 12.3 PROPOSED MECHANISMS FOR AN EFFECT OF RESISTANT STARCH ON APPETITE

Although the exact mechanism as to how resistant starch (RS) may affect appetite is not known, various mechanisms have been proposed. These mechanisms may be specific to RS or may be ubiquitous for all dietary fibres.

As RS is not digested in the small intestine but is fermented in the colon, the most likely mechanism behind an effect on appetite is due to fermentation and the subsequent increase in production of short-chain fatty acids (SCFA). The SCFA may increase production of GLP-1 and PYY which, in turn, may activate the neurons in the hypothalamus, therefore affecting behaviour and causing changes to food intake (Figure 12.1).

The cells that secrete GLP-1 and PYY are primarily located in the ileum and colon. GLP-1 receptors have been located in the hypothalamus (Murphy & Bloom, 2004; Holst, 2007), therefore suggesting a role for GLP-1 in directly affecting food intake. This has been shown in rodent models, where central infusion of GLP-1 reduced food intake (Turton *et al.*, 1996). GLP-1 may also indirectly affect food intake via the vagus nerve, as studies in rodents



**Figure 12.1** Possible pathway by which resistant starch consumption affects appetite and food intake.

have shown that, following a vagotomy, the anorexic effects of GLP-1 are lost (Dhillon, 2007). Studies where GLP-1 has been infused in humans provide mixed results, with some studies showing an effect (Flint *et al.*, 1998; Naslund *et al.*, 1999) and others none (Long *et al.*, 1999; Naslund *et al.*, 1998). However, a meta-analysis (which included the above studies in addition to others) showed a dose-dependent reduction in food intake following GLP-1<sub>7-36</sub> (biologically active form) peripheral administration in humans (Verdich *et al.*, 2001).

Similarly, studies that have infused PYY in rodents and humans have found a reduction in food intake (Batterham *et al.*, 2002; Challis *et al.*, 2003). In one instance, the decrease in energy intake was 30% following peripheral infusion of the active form (PYY<sub>3-36</sub>) in humans (Batterham *et al.*, 2003). PYY may also be released due to neural reflex, potentially mediated by the vagus nerve (Fu-Cheng *et al.*, 1997).

It has also been shown that while these hormones can reduce food intake independently, when they are administered together, the effect on reducing food intake is additive (Neary *et al.*, 2005; Steinert *et al.*, 2010).

Therefore, as these two hormones have been shown to reduce food intake, they are likely to be the most affected by RS consumption due to fermentation in the colon.

## 12.4 RODENT DATA

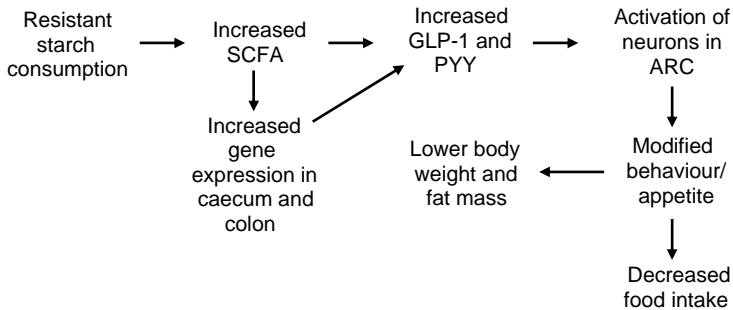
Results from rodent studies have shown promising effects of RS intake on appetite and food intake, as well as on gut hormone regulation. However, these rodent studies have not been acute (10–65 days), so therefore the translation of the data from these experiments to acute effects in humans is not possible. As these studies do provide some evidence for potential mechanisms, they will be discussed briefly below and are summarised in Table 12.1.

Based on the potential pathway by which RS may affect appetite and food intake described above (Figure 12.1), there is evidence in rodent models for each of the steps as shown in Figure 12.2 and discussed in more detail below.

A few experiments in rodents have shown increased concentrations of SCFA following RS consumption in studies relating to appetite work, as well as effects of the RS consumption on changes to gene expression. Keenan *et al.* (2006) proved significantly higher caecal SCFA concentrations following a RS diet compared to a non-fermentable cellulose energy controlled diet; they also observed increased PYY and proglucagon (the precursor molecule for GLP-1) gene transcription in the caecum and large intestine following RS compared to the control. Zhou *et al.* (2006) showed that, after a RS diet,

**Table 12.1** Summary of rodent studies.

<b>Authors</b>	<b>Methods</b>	<b>Results</b>
Aziz <i>et al.</i> (2009)	46 rats (split into four groups). Fed high-amylose (70% amylose) or high amylopectin (100% amylopectin) diet for four weeks (freely or restricted).	High-amylose = lower energy intake, weight gain, fat pad mass. High-amylose = higher GLP-1 and PYY.
Keenan <i>et al.</i> (2006)	Three experiments (1st energy, not fibre). 2nd – 30 rats, fed: control (5% fibre, 3.6 kcal/g), RS (39.9% fibre, 3.2 kcal/g) or non-fermentable methylcellulose (37.5 g fibre, 2.5 kcal/g) for 32 days. 3rd – 30 rats, fed RS (3.3 kcal/g, 36.3 g fibre) or non-fermentable cellulose energy control (3.3 kcal/g, 15.7 g fibre).	2nd – Lower body weight and fat mass in treatment groups compared to control. RS = higher PYY, GLP-1, proglucagon gene transcription and PYY transcription gene. No difference in energy intake, ghrelin or CCK. 3rd – RS = lower body weight and fat mass and higher SCFA and PYY. Food intakes similar.
Shen <i>et al.</i> (2009)	52 rats. Fed RS or energy control diet for 65 days.	No effect on food intake. RS group had decreased body fat and leptin and increased GLP-1, PYY and POMC gene expression in ARC.
So <i>et al.</i> (2007)	40 mice. High RS or rapidly digestible starch diet for eight weeks.	Same body weight. RS = lower energy intakes, total fat, subcutaneous fat, visceral fat, liver fat, leptin, adiponectin and smaller adipocytes.
Zhou <i>et al.</i> (2008)	Five different studies investigated different outcomes. 1st – 100 rats, fed RS (30%) or control diet for 10 days. 2nd – 30 rats, fed RS, control or non-fermentable methylcellulose for 32 days. 3rd + 4th – <i>in vitro</i> work. 5th – diabetic mice, fed RS or control.	Changes in neuronal activity suggested satiety effects of RS. RS = increased GLP-1 and PYY and lower body fat. Food intakes similar.
Zhou <i>et al.</i> (2006)	Rats. Fed control or RS diet for four weeks.	Fermentation (in the colon and caecum) and subsequent SCFA production was associated with increased proglucagon and PYY gene expressions. RS-fed diabetic mice had better glucose tolerance. PYY and proglucagon mRNA expression upregulated in the caecum and colon of the RS-fed rats.



**Figure 12.2** Possible pathway by which resistant starch consumption affects food intake and behaviour in rodents.

compared to control, PYY and proglucagon mRNA expression were significantly unregulated in the caecum and colon. Further work by Zhou *et al.* (2008) also showed *in vitro* that the fermentation of RS increased the concentrations of SCFA, and that the SCFA (when at concentrations similar to those observed in the caecal content in the study by Keenan *et al.* (2006)) directly stimulated proglucagon gene expression.

Several studies in animals have shown increased GLP-1 and PYY concentrations following RS consumption. The study by Keenan *et al.* (2006) found greater serum PYY and GLP-1 concentrations following the RS diet, but no effect on serum ghrelin or cholecystokinin (CCK) concentrations. Plasma PYY and GLP-1 concentrations were also increased in the study by Shen *et al.* (2009). Zhou *et al.* (2008) found RS resulted in significantly higher plasma GLP-1 and PYY concentrations throughout the day. Aziz *et al.* (2009) compared high-amylose with high amylopectin diets and found that the high-amylose diets resulted in higher plasma GLP-1 and PYY concentrations.

There is also evidence from rodent studies for an effect of RS consumption on activation of neurons in the hypothalamus. Shen *et al.* (2009) found that, following a high RS diet, POMC expression in the ARC was significantly upregulated, but there was no effect on NPY or AgRP. So *et al.* (2007) conducted manganese-enhanced magnetic resonance imaging during *ad libitum* food intake to monitor neuronal activity in the areas of the hypothalamus involved in appetite regulation and found that there appeared to be a satiating effect of the RS diet compared to the rapidly digestible starch.

Most of these studies, in the course of looking at possible mechanisms behind an effect of RS on appetite, have also reported changes to food intake and weight, with mixed findings. Aziz *et al.* (2009) reported that the high-amylose diets, when consumed *ad libitum*, resulted in lower energy intakes, weight gain and fat pad mass. Similarly, So *et al.* (2007) also reported lower



energy intakes in the RS group and found that, although body weight was the same in both groups, the RS group had lower total, subcutaneous, visceral and liver fat, and also smaller adipocytes. However, the studies by Keenan *et al.* (2006), Zhou *et al.* (2008) and Shen *et al.* (2009) found similar food intakes between groups, but lower body weights and fat mass in the RS group.

From these studies in rodents, it is clear that high RS intakes result in increases in both GLP-1 and PYY (satiety hormones), most likely due to increased production of SCFA from fermentation. Although, in some of these studies, RS appears to decrease both body weight and fat mass, the effects on actual energy and food intake are less clear.

In many of these animal experiments, the amount of RS given would be much greater than would normally be consumed by a human. Similarly, the length of time (normally days to weeks) that the rodents were fed the starch is difficult to compare to the same time span in humans. The gut physiology of rodents is also very different (greater large bowel length) from that of humans and, therefore, fermentation effects in a rodent can not be directly translated into similar effects to be observed in humans.

While the evidence for an effect of RS on appetite in rodent studies is positive, these results have yet to be demonstrated fully in humans.

## **12.5 HUMAN DATA**

Compared to the data from rodent studies, the acute effects (up to 48 hours) of RS on appetite are less clear in humans.

There are few human studies that have directly investigated the effects of RS on appetite and food intake, with these studies providing mixed findings (summarised in Table 12.2). Direct comparisons are hampered by the use of different protocols and different types of RS (the majority of these studies used type 2 or 3). Some early studies have also compared the different effects of amylose and amylopectin on appetite and food intake. As RS is high in amylose, the results from these studies are included.

Based on the potential pathway by which RS may affect appetite and food intake described above (Figure 12.1), there is only some evidence in humans for some of the steps, as shown in Figure 12.3 and discussed in more detail below.

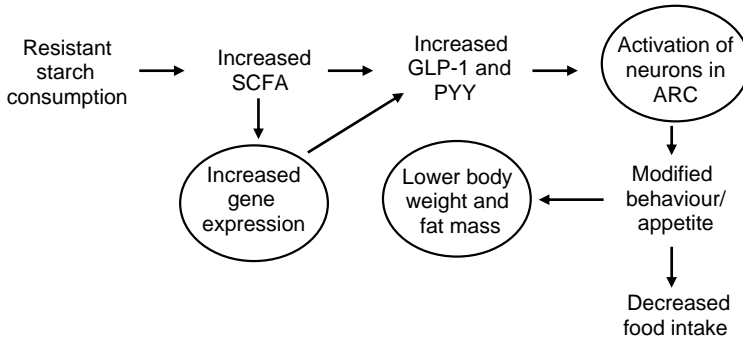
Very few human studies have reported the effects of RS on GLP-1 and PYY. In the study by Raben *et al.* (1994) it was found that there were lower plasma GLP-1 concentrations with RS, compared with their placebo. Bodinham *et al.* (2013) also found lower GLP-1 concentrations following a breakfast containing RS compared to a placebo. A study by Klosterbuer *et al.* (2012) found no effect of RS on GLP-1 concentrations. Although a study by

**Table 12.2** Summary of human studies.

Authors	Methods	Results
Bodinhham <i>et al.</i> (2013)	30 healthy males. 24 hour study. 48 g RS compared to energy and carbohydrate matched placebo.	RS = significantly lower. GLP-1 after breakfast.
Bodinhham <i>et al.</i> (2010)	20 healthy males. 24 hour study. 48 g RS compared to energy and carbohydrate matched placebo.	RS = lower energy intake at test meal and over 24 hours. No effect on subjective appetite ratings.
Granfeldt <i>et al.</i> (1994)	Ten healthy males and females. Consumed at breakfast and effects measured up to three hours afterwards. Six types of barley products and a white bread. Amylose content varied depending on the grain type used (7–44% starch).	All barley products had higher satiety than white bread. High-amylose product had the greatest satiety.
Holt & Brand Miller (1995)	Nine healthy males and females. Puffed rice cakes that were high (1: 2.6) and low (1: 4) in amylose. Consumed at breakfast and two-hour effects assessed.	Satiety AUC 50% higher with high-amylose. Food intake (in grams) 1.4 times higher with low-amylose than high immediately after and over 24 hours. No difference in energy intake.
Kendall <i>et al.</i> (2010)	22 healthy males and females. 120 minute postprandial study. Control (no RS) and three levels of RS (5 g, 10 g, 15 g).	RS = least palatable. Short term increase in satiety. No significant differences in <i>ad libitum</i> intake.
Klosterbuer <i>et al.</i> (2012)	20 healthy males and females consumed at breakfast and effects measured up to three hours afterwards 25 g RS compared to control and other fibre test meals.	RS = no effect on subjective appetite ratings or food intake or GLP-1 concentrations.

Liljeberg <i>et al.</i> (1999)	Ten healthy males and females. Consumed at breakfast and effects at lunch assessed. Seven different products (barley/wheat) – different GI and RS (0.1–11.2 g) contents.	Higher satiety observed in the higher RS meals.
Nilsson <i>et al.</i> (2008)	15 healthy males and females. Consumed at evening meal then tested after following breakfast. Different grains (barley/wheat) in breads – containing natural and added RS (1.3–30.9 g).	Increased satiety with the barley oats high in $\beta$ -glucan (also had the highest RS content).
Raben <i>et al.</i> (1994)	Ten healthy males. Five-hour postprandial study. 50 g pregelatinized (0 g RS) or 50 g raw potato starch (27 g RS) mixed into syrup.	RS = lower GLP-1 and gastric inhibitory peptide (GIP). Lower satiety and fullness.
Robertson <i>et al.</i> (2003)	Ten healthy males and females. 60 g RS or energy and carbohydrate matched placebo consumed for 24 hours before the study.	No effect of starch on GLP-1.
van Amelsvoort & Weststrate (1992)	22 healthy males. Consumed at lunch and effects measured for six hours. High (45:55) or low (1:100) amylose to amylopectin ratios	Immediately after meal high-amylose least palatable and most satisfying. High-amylose had highest ratings of fullness.
Weststrate & van Amelsvoort (1993)	22 healthy males. High-amylose or low-amylose meals Served at breakfast (high = 15%E, low = 2%E) and lunch (high = 17%E, low = 10.7%E). Consumed at breakfast and lunch, effects measured for four hours after each meal.	No significant effects on subjective appetite ratings.
Willis <i>et al.</i> (2009)	20 healthy males and females. 180 minutes. 8 g RS compared to other fibre muffins and low-fibre muffin.	RS and corn bran most satiating. RS least palatable.

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**Figure 12.3** Possible pathway by which resistant starch consumption affects food intake and behaviour in humans. Circles show areas where there is no acute evidence in human work.

Robertson *et al.* (2003) was not investigating the effects of RS on appetite, one of their outcomes was the effects of RS consumption on GLP-1 concentrations, and this also showed no effect. The lack of evidence for an effect on gut hormones in humans could be due to the neuronal effects of GLP-1 and PYY which would not be measured in the general circulation. Alternatively, as GLP-1 is rapidly broken down in the circulation by dipeptidyl peptidase IV (DPP-IV) and both hormones are quickly cleared from the blood via the liver (Holst, 2007), the hormones may not appear in peripheral blood at raised concentrations, but there may be raised concentrations in splanchnic blood.

Several studies have measured the effects of RS on modifying appetite using changes to subjective questionnaires. These studies have also reported mixed findings:

- three studies have reported no differences between RS/high-amylose and a control (Weststrate & van Amelsvoort, 1993; Bodinham, *et al.*, 2010; Klosterbuer *et al.* 2012);
- one study reported lower satiety with RS compared to a control (Raben *et al.*, 1994);
- several studies have reported greater satiety with RS (Kendall *et al.*, 2010; Willis *et al.*, 2009; Nilsson *et al.*, 2008; Liljeberg *et al.*, 1999; Holt & Brand Miller, 1995; Granfeldt *et al.*, 1994; van Amelsvoort & Weststrate, 1992).

However, some of these studies also found that the RS supplement was less palatable than the control used (Kendall *et al.*, 2010; Willis *et al.*, 2009; van Amelsvoort & Weststrate, 1992), which could account for the effects observed.

Only a few studies have investigated the effects of RS on actual food intake. Two of these studies (Kendall *et al.*, 2010; Klosterbuer *et al.*, 2012) found no

significant differences between treatments for energy intake at the *ad libitum* meal. However, two other studies (Bodinham *et al.*, 2010; Holt & Brand Miller, 1995) found a reduced intake with RS. The study by Bodinham *et al.* (2010) found the reduced energy intake at both an *ad libitum* test meal (seven hours postprandially) and over a 24-hour period. Holt & Brand Miller (1995) found that intake (in grams) following the high-amylose rice cake was significantly lower after two hours and over the rest of the day, compared to the low-amylose rice cake. However, the differences in energy were not significant.

Based on the evidence from the studies using RS and differences in amylose/amylopectin ratios, there is an acute effect of RS on appetite. In the few studies where GLP-1 concentrations have been measured (Raben *et al.*, 1994, Robertson *et al.*, 2003) in human studies, RS does not appear to have an effect. As the data in rodents suggest that effects on satiety hormones may be a mechanism by which RS affects appetite, satiety and food intake, further investigations in humans would be required. Further studies with similar study designs are also required to determine the dose that is needed for an effect to occur, as well as other possible mechanisms behind the effects.

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## 13 Metabolic Effects of Resistant Starch

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Resistant starch (RS) is currently defined as the sum of starch and products of starch degradation not absorbed in the small intestine of healthy individuals (Asp, 1992). As a consequence, RS includes not only fractions which are resistant to endogenous enzyme digestion, but also starch and dextrins which are potentially digestible but which, due to too short interaction with endogenous alpha-amylase, arrive in the colon. RS, whatever its bioavailability, is submitted to digestion and fermentation by colonic microbiota. Most of the RS disappears in the colon and is metabolized into short-chain fatty acids (acetate, propionate and butyrate) and gases (CO<sub>2</sub>, H<sub>2</sub> and CH<sub>4</sub>).

Metabolic effects of resistant starch could *a priori* be classified according to:

1. the type of metabolic effect of RS or of its metabolites; or
2. the mechanism which is involved – it can potentially be ‘direct’, due to a matrix effect as shown for soluble (and viscous) dietary fibre, or mediated by the short chain fatty acids produced from RS fermentation by the colonic microbiota.

A third category is a subject of controversy. Indeed, it is linked to the effect of the substitution of digestible starch by resistant starch. As a consequence, it is not the metabolic effect of RS *per se*, but the decrease of the ‘digestible’ part of the starch, which is mostly responsible for the metabolic effect that can be observed.

In most investigations of metabolic studies with RS, the mechanism(s) involved has not been elucidated fully, and/or two different mechanisms can be simultaneously responsible for the physiological effects which have been described. As a consequence, the first classification will be adopted all along the review, but mechanisms involved will be discussed.

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In order to conclude to a metabolic effect of RS through its intrinsic properties, the design of the study has to be faultless, particularly with regard to the formulation of the experimental diet. Indeed, with most RS sources containing digestible starch, the ‘true’ RS concentration in the food/ingredient (as eaten) has to be determined prior to the clinical trial in order to adapt the level of incorporation of the food/ingredient to this concentration. Unfortunately, this statement is neglected in several studies, leading to a misinterpretation of the results. Moreover, physiological or metabolic effects can be misattributed to RS when it is present in a diet/food/ingredient which also has a low glycemic index.

This review will mostly refer to metabolic impact of resistant starch. Broader overviews on the physiological effects of resistant starch have been published earlier (Champ, 2004; Champ *et al.*, 2003).

### **13.1 FERMENTATION OF RS AND ITS IMPACT ON COLONIC METABOLISM**

RS fermentation is characterized by a significant ratio of butyrate among SCFAs produced by its fermentation, compared to most dietary fibres (see Tables 13.1 and 13.2).

Most of the data available on RS fermentation are issued from *in vitro* fermentation of RS by animal or human faecal inocula (see Table 13.1). Studies made with effluents from ileostomy from human patients are *a priori* the most accurate (Langkilde *et al.*, 2002; Silvester *et al.*, 1995). However an appropriate (validated procedure) predigestion of an RS-containing food can be considered as a relevant method to obtain the samples which have to be submitted to *in vitro* fermentation (Fässler *et al.*, 2006). Finally, many studies have been performed on rodents or other animal species, but their microbiota are significantly different from human microbiota and fermentation profiles cannot be extrapolated to humans even if, in some cases, a similar feature is observed between species (Wang *et al.*, 2004; Murray *et al.*, 2001).

Several data of concentration of SCFAs in human stools are available in the literature (Hylla *et al.*, 1998; Jenkins *et al.*, 1998; Noakes *et al.*, 1996; Cummings *et al.*, 1996; Phillips *et al.*, 1995; van Munster *et al.*, 1994). These are a partial reflection of fermentation, as most SCFA are absorbed in the colon. However, they probably reflect both the intensity and the profile of the fermentation (Table 13.2).

Recent study from Verbeke *et al.* (2010) demonstrated higher concentration of butyrate in both plasma and urine from volunteers who ingested  $^{13}\text{C}$  enriched intact barley kernels (containing RS and non starch polysaccharides) compared to barley porridge (containing only non starch polysaccharides). The investigation of RS fermentation in pigs by quantification of SCFA in portal blood (Martin *et al.*, 2000) raised the question of the significance of the measurement of butyrate out of the site of production. Indeed, butyrate uptake by the colonic mucosa seems to vary according to fermentation rate of RS with a higher uptake when fermentation is slow compared to fast fermentation. As a consequence, quantification of butyrate in blood, urine or faeces only reflects part of the production of butyrate.

Butyrate is the main fuel of the healthy colonocyte and is consumed by these cells, explaining why only minor amounts of butyrate can be found in the peripheral blood. Its depletion is thought to induce damage to the colonic epithelium. As a consequence, dietary fibres inducing butyrate production, such as RS, are described as ingredients that have the potential to prevent colonic diseases such as colon cancer (Le Leu *et al.*, 2009, 2007a, 2007b; Clarke *et al.*, 2008; Bauer-Marinovic *et al.*, 2006; Perrin *et al.*, 2001; Wollowski *et al.*, 2001; Hylla *et al.*, 1998; Young & Gibson, 1995).

Butyrate would also be of interest in the healing of ulcerative colitis (Vernia *et al.*, 2003), even if most recent data only partly confirm earlier findings (Hamer *et al.*, 2010). Most studies, however, confirm that butyrate oxidation by the colonic mucosa is impaired in various situations of inflammation (de Preter *et al.*, 2009; Jacobasch *et al.*, 1999).

It is now admitted that RS can be a good substrate for butyrate production in the colon (Le Leu *et al.*, 2009, 2007a). The restoration of the integrity of rat caeco-colonic mucosa in chemically induced colitis by RS, but not by fructo-oligosaccharides (Moreau *et al.*, 2003), probably reveals that fermentation profile and kinetic of fermentation are determinant factors of the benefits of dietary fibres on colonic diseases (in its broadest definition; Howlett *et al.*, 2010).

According to several authors, RS would improve colonic mucosal integrity in healthy animals and/or reduce colonic and systemic immune reactivity for which health benefits in inflammatory conditions are likely to be associated (Nofrariás *et al.*, 2007). It would confirm the interest of RS in a prevention context of several colonic diseases.

Apart from butyrate, RS fermentation induces production of acetate and propionate. Both SCFAs are absorbed, but part is metabolized by the liver, whereas mostly acetate can be found in the blood and used by peripheral organs (Wong *et al.*, 2006).

**Table 13.1** *In vitro* fermentation of resistant starches.

Sample		Specie	Total SCFA		Acetate		
			mmol/L	mmol/g ileal subst.	mmol/L	mmol/g ileal subst.	molar ratio
Raw banana (flour)	Ileal effluents	Human (ileostomy)	120 ± 2.8	5.5 ± 0.1	62.3 ± 3.0	2.9 ± 0.1	55 ± 1
vs.							
Cooked banana (flour)			107 ± 3.1	7.0 ± 0.2	54 ± 2.0	3.5 ± 0.1	55 ± 1
High RS	Ileal effluents	Human (ileostomy)	69.6 ± 6.9	4.9 ± 0.4	44.1 ± 4.5	3.2 ± 0.3	66 ± 1.6
vs.							
Low RS			40.9 ± 3.9	4.8 ± 0.3	27.9 ± 2.5	3.3 ± 0.2	72 ± 1.1
					mmol	mmol/L	molar ratio
Raw potato starch	Ileal effluents	Pig (ileal cannulation)			13.3	20	53.6
vs.							
Control					12.3	17.5	52.3
			mmol/g OM		mmol/g OM		
Barley	Native	Ileal effluents	Dog	3.61	2.66		
	HT extrusion		(ileal cannulation)	6.08	4.27		
Corn	Native			3.13	2.26		
	HT extrusion			3.68	3.08		
Potato starch	Native			1.64	1.46		
	LT extrusion			6.86	4.56		
Rice	Native			5.45	3.82		
	HT extrusion			7.45	5.74		
Sorghum	Native			3.70	2.67		
	HT extrusion			6.47	4.13		
Wheat	Native			2.85	2.17		
	HT extrusion			8.13	5.38		
				mmol			molar ratio
Preparation RS3	<i>in vitro</i> predig. (ba)			161			31
(retrograded long chain maltodextrin)	<i>in vitro</i> predig. (dy)			217			35
	Ileal effluents	Human (ileostomy)		189			34
Preparation RS2	<i>in vitro</i> predig. (ba)			150			32
(native high amylose corn starch)	<i>in vitro</i> predig. (dy)			87			23

ba: batch; dy: dynamic.

Propionate			Butyrate			Reference	
mmol/L	mmol/g ileal subst.	molar ratio	mmol/L	mmol/g ileal subst.	molar ratio		
17.0 ± 0.5	0.8 ± 0.0	14.0 ± 0.5	34.8 ± 1.1	1.6 ± 0.1	31.0 ± 0.9	Langkilde <i>et al.</i> , 2002	
18.3 ± 0.6	1.2 ± 0.1	18.0 ± 4	27.0 ± 0.7	1.7 ± 0.1	27.0 ± 0.9	Silvester <i>et al.</i> , 1995	
11.8 ± 1.3	0.8 ± 0.1	17 ± 0.7	11.1 ± 1.1	0.8 ± 0.1	17 ± 1.1		
6.3 ± 0.9 mmol	0.7 ± 0.1 mmol/L	16 ± 1.1 molar ratio	4.5 ± 0.6 mmol	0.5 ± 0.1 mmol/L	12 ± 0.7 molar ratio	Wang <i>et al.</i> , 2004	
6.1	8.2	22.5	4.2	5.7	15.5		
5.9	7.8	23.2	3.2	4.5	13.5		
mmol/g OM			mmol/g OM				
0.59			0.36			Murray <i>et al.</i> , 2001	
0.85			0.96				
0.45			0.42				
0			0.60				
0.11			0.07				
1.42			0.88				
0.92			0.71				
1.53			1.18				
0.67			0.36				
1.61			0.73				
0.52			0.16				
1.50			1.25				
		molar ratio	mmol			molar ratio	Fässler <i>et al.</i> , 2006
		48	32.6			20	
		49	35.5			16	
		57	17.0			9	
		43	35.8			25	
		51	22.5			26	

**Table 13.2** Concentration of SCFA in stools from subjects after resistant starches consumption.

	Sample	Total SCFA	Acetate	Propionate	Butyrate	Reference
		mM			mM	
Control	Stools	87.8			9.1	van Munster <i>et al.</i> , 1994
RS2		89.6			10.4	
Oat bran	Stools	101.1			23.5	Noakes <i>et al.</i> , 1996
Low-amylose starch		100.6			20.1	
High-amylose starch		119.2			31.1	
		mmol/kg wet wt			mmol/kg wet w	
RS = SDS	Stools	98.9			15.0	Cummings <i>et al.</i> , 1996
Bran		77.1			15.8	
Potato RS2		99.7			18.4	
Banana RS2		97.5			15.2	
Wheat RS3		83.4			17.0	
Corn RS3		85.7			14.6	
		mM			mM	
Low RS	Stools	79.0			19.0	Phillips <i>et al.</i> , 1995
High RS		99.5			26.2	
Low fibre	Stools	102.8			19.2	Jenkins <i>et al.</i> , 1998
Wheat bran		107.9			21.3	
RS2 & 3		108.1			22.7	
			24 hr $\mu\text{mol}/100\text{ mg substrate (\%)}$			
Glucose $n=2$	Stools		457 (62)	118(16)	158 (22)	Weaver <i>et al.</i> , 1989
Corn starch $n=2$			526 (61)	82(10)	170 (22)	
				$\mu\text{mol (\%)}$		
Corn starch	Stools		470 (69)	60 (9)	136 (20)	Weaver <i>et al.</i> , 1992
Cabbage fibre			448 (73)	91 (15)	57 (9)	

## 13.2 RESISTANT STARCH, GLYCEMIA, INSULINAEMIA AND GLUCOSE TOLERANCE

When substituting digestible starch, RS obviously reduces glycemic load of the food/diet and, consequently, reduces insulin secretion (Raben *et al.*, 1994). When comparing raw potato starch (50 g containing 54% RS) to pre-gelatinized potato starch (50 g), postprandial plasma concentrations of glucose and insulin were lower with the RS diet, compared to the control diet. Ranganathan *et al.* (1994) showed no alteration of postprandial glucose response to a 50 g glucose load when lintnerized starch (30 g of lintner from maize starch containing 70% amylose) was added to the experimental meal. A lintner is a starch which has been treated with acidic solution and is poorly fermented by the microbiota. The conclusion of the study was that the acute effect of the ingestion of a lintnerized starch on the measured metabolic indexes is similar to that of cellulose (insoluble and poorly fermentable fibre).

Resistant starch (RS3, Promitor<sup>TM</sup> RS, Tate & Lyle Inc, IL, USA) (from 5–25 g in a meal) has been shown to have no effect on incremental area under the curve (iAUC) for glucose or insulin. However, there was a decrease of the incremental blood glucose and insulin levels after a meal containing 25 g RS at 90 and 120 minutes after the meal ( $p = 0.004$  and  $0.001$  for glucose,  $p = 0.043$  and  $p = 0.042$  for insulin, respectively) (Kendall *et al.*, 2010).

The comparison of 70% amylopectin to 70% amylose corn starch (probably RS2) in normal and hyperinsulinaemic men (14-week intervention cross-over study) revealed that the magnitude of the response in carbohydrate and fat oxidation was blunted in hyperinsulinaemic subjects consuming excess levels of the amylose diet (Howe *et al.*, 1996). The hypotheses of the authors were that this was due to:

1. an improvement in overall insulin response; or
2. a change in available substrates for oxidation resulting from microbial fermentation.

The data from Brighenti *et al.* (2006) would tend to support the hypothesis of a second-meal effect of fermentable carbohydrates such as RS. Indeed, both a 'slowly digestible, partly fermentable high-amylose starch (Hylon VII) plus cellulose' meal and an 'amylopectin starch plus lactulose' meal, ingested within breakfast, improved glucose tolerance at lunch. According to these authors, a reduction of non-esterified fatty acids (NEFA) competition for glucose disposal might be a mechanism involved in this metabolic effect, even if the result is not statistically significant for the RS containing meal, whereas it is with the lactulose-containing meal (Brighenti *et al.*, 2006).

This observation has not been made by Liljeberg *et al.* (1999), who described a beneficial effect of slow absorption and digestion of starch from a breakfast meal on glucose tolerance at the second meal (lunch), whereas the content of indigestible carbohydrates (RS and dietary fibre) had no effect. But the same group (Nilsson *et al.*, 2008) demonstrated that an evening meal containing RS and dietary fibre (barley kernel bread) significantly improved glucose tolerance at a following standardized breakfast (28%, compared to white wheat bread), independently of the glycemic index of the evening meal. The benefits of RS + DF were attributed to colonic fermentation, which may be characterized by a prebiotic effect.

de Roos *et al.* (1995) compared daily supplement of 30 g RS2 (high-amylose corn starch) to RS3 (retrograded high-amylose corn starch), using glucose as a control. They determined C-peptide excretion as a measure for the 24-hour insulin secretion. They concluded that consumption of 30 g/day RS3 (but not RS2) during one week reduced the insulin secretion. This result would also suggest an impact of RS fermentation rate on insulin.

Robertson *et al.* (2005) suggested that dietary supplementation with RS had the potential to improve insulin sensitivity. Indeed, insulin sensitivity assessed by euglycaemic-hyperinsulinaemic clamp and meal tolerance test was higher after RS supplementation (30 g RS/day during four weeks) than after placebo treatment of healthy subjects. More recently, the same group (Johnston *et al.*, 2010) confirmed an improvement of insulin sensitivity of insulin resistant subjects (40 g RS/day during 12 weeks); however, this modification was not associated with changes in whole-body composition.

These results apparently contradict those obtained from studies on rodents. Indeed, a high-RS diet has been shown to decrease intra-hepatic fat storage and reduce inflammatory markers (Kabir *et al.*, 1998; So *et al.*, 2007). However, these studies on animal models have compared RS to digestible starch, which is not the case with Robertson's studies (Robertson *et al.*, 2005).

Kwak *et al.* (2012) observed that a four-week dietary treatment with rice containing resistant starch (6.51 g RS per day, compared with control composed of rice with low level of RS) reduced postprandial blood glucose and oxidative stress, measured as plasma malondialdehyde (MDA), urinary 8-epi-PGF (2 $\alpha$ ) (whereas RH-PAT index and total nitric oxide were increased) in patients with prediabetes or newly diagnosed type 2 diabetes.

### 13.3 RS CONSUMPTION AND LIPID METABOLISM

De Deckere *et al.* (1993) concluded, from a study on rats fed semi-purified diets containing a low (0.8 g/MJ) or a high (9.6 g/MJ) amount of RS, that



dietary RS can reduce serum total cholesterol and triacylglycerols concentration and fat accretion (epididymal fat pads).

Cheng & Lai (2000) observed reduced serum and hepatic cholesterol in rats fed a diet containing 63% rice starch (richer in RS than corn starch), compared to animals receiving corn starch as a control. The authors attributed this effect to the significant increase of propionate in rats fed on rice starch. Propionate may, indeed, be involved in the control of hepatic cholesterol synthesis. However, the experimental diets had different amount of digestible starch and it cannot be ignored that the impact on cholesterol concentrations could be explained by the different insulin responses to the digestible starch fractions of the diets.

Moreover, a study on germ-free rats (Sacquet *et al.*, 1983) also demonstrated a reduction of plasma cholesterol with RS (RS2: high-amylose starch). In this study, the hypothesis of an effect of SCFA cannot explain the beneficial metabolic effect. Ten years later, the same group (Mathé *et al.*, 1993) described a reduction of plasma cholesterol in genetically obese (fa/fa) and lean (fa/-) rats when fed a high-amylose corn starch diet. Among the possible mechanisms, the authors suggested an increased biliary secretion of bile acids linked to an accelerated catabolism of lipoproteins by the liver. A second hypothesis, which has been mentioned earlier, is the involvement on insulin (glucagon/insulin ratio) secretion, which would explain the reduced plasma lipid and lipoprotein levels.

The impact of RS consumption in healthy human subjects is controversial. Three of the studies (Stewart *et al.*, 2010; Heijnen *et al.*, 1996; Behall & Howe, 1995) failed to demonstrate any favourable effect of RS consumption on lipaemia or cholesterol concentration, whereas two earlier studies (Behall *et al.*, 1989; Reiser *et al.*, 1989a, 1989b) concluded to a significant decrease of total cholesterol and triacylglycerol when the subjects received, respectively, 247 g and 183 g per day of high-amylose corn starch during four and five weeks (compared to high amylopectin starch and fructose as a control). Heijnen's 1996 study was a randomized, single-blind, 3 × 3 Latin-square study (total of 57 subjects), in which the subjects received 30 g of raw (RS2; Hylon VII, National Starch) or retrograded RS (RS3; Novelose, National Starch) vs. glucose during three weeks. In the 1995 study of Behall & Howe, the subjects received approximately 200 g raw high-amylose (vs. low-amylose) corn starch (RS2) per day. They observed a significant increase in total cholesterol and a decrease in triacylglycerol after the first four weeks of supplementation, whereas Achour *et al.* (1997) concluded from an acute study that retrograded high-amylose corn starch (RS3) leads to a reduction in lipolysis in the post-absorptive period.

Noakes *et al.* (1996) failed to observe any metabolic changes when high-amylose starch (Hi-Maize<sup>TM</sup>, Starch Australasia) (17 g and 25 g/day in women and men, respectively) was introduced in the diet of hypertriglyceridaemic patients, whereas oat bran had a significant effect on plasma triacylglycerol.

Results from Faisant *et al.* (unpublished personal data) indicated that supplementing a normal meal with RS3 had little or no effect on postprandial parameters of normal subjects. However, triacylglycerol metabolism was improved, especially in subjects with higher (upper limit of the 'normal' range) basal triacylglycerol levels, most probably by an improvement in clearance of postprandial chylomicron remnants.

### **13.4 RS CONSUMPTION, GIP, GLP-1 AND PYY SECRETION**

Glucagon-like peptide-1 (GLP-1) and peptide YY (PYY) are gut-secreted peptides that have been proposed as potential anti-diabetes/obesity drugs. These two hormones are also naturally secreted in response to meal ingestion, but they degrade rapidly after endogenous secretion or exogenous injection. Thus, pharmaceutical means to maintain substantial high plasma levels of GLP-1 and PYY are intensely targeted.

RS has been reported by numerous authors to increase plasma total GLP-1 and total PYY in rodents of both sexes at different ages (Keenan *et al.*, 2006; Zhou *et al.*, 2006). For instance, Keenan *et al.* (2006) observed that rats fed fermentable RS (RS2, Hi-Maize<sup>TM</sup> 260, National Starch) had (compared to methylcellulose) increased caecal weights and plasma PYY and GLP-1 and increased gene transcription of PYY and proglucagon. They conclude from their study that inclusion of RS in the diet may affect energy balance through its effect as a fibre or a stimulation of PYY and GLP-1 expression. In the same year, Zhou *et al.* (2006), using *in vivo* and *in vitro* approaches, concluded that the distal part of the gut has the ability to sense nutrients such as butyrate, resulting in the up-regulation of PYY and proglucagon gene expression.

Later on, the same group (Zhou *et al.*, 2008) concluded from a study on RS-fed rodents (Hi-Maize<sup>TM</sup> 260 as RS2) that:

1. RS stimulated GLP-1 and PYY secretion in a substantial day-long manner, independent of meal effect on changes in postprandial glycemia;
2. fermentation and the liberation of SCFAs in the lower gut are associated with increased proglucagon and PYY gene expression;

3. glucose tolerance, an indicator of increased active forms of GLP-1 and PYY, was improved in RS-fed diabetic mice.

They concluded from their study that fermentation of RS is most likely to be the primary mechanism for increased endogenous secretions of total GLP-1 and PYY in rodents. Any factor that affects fermentation should be considered when dietary fermentable fibre is used to stimulate GLP-1 and PYY secretion.

Tachon *et al.* (2013) observed that mice fed an energy-controlled diets containing 18% or 36% type 2 RS (from high-amylose maize) were colonized by higher levels of *Bacteroidetes* and *Bifidobacterium*, *Akkermansia* and *Allobaculum* species in proportions that were dependent on the concentration of the dietary fibre. The proportions of *Bifidobacterium* and *Akkermansia* were positively correlated with mouse feeding responses, gut weight and expression levels of proglucagon, the precursor of GLP-1.

To our knowledge, Raben *et al.* (1994) were the first to show that, in humans (healthy subjects), when substituting digestible starch, RS reduces insulin secretion but increases gastric inhibitory polypeptide (GIP), GLP-1 and epinephrine.

More recently, Nilsson *et al.* (2008) showed that improved tolerance was observed after a standardized breakfast when RS and fibre (both from barley) were ingested during the preceding evening meal. This was associated with decreased concentration of free fatty acids and IL-6 and increased GLP-1 and adiponectin at the time of the breakfast, thus providing evidence for a link between the gut microbial metabolism and key factors associated with insulin resistance.

### 13.5 RS CONSUMPTION, SATIETY AND SATIATION AND FAT DEPOSITION

An acute randomised, single-blind crossover study aiming at determining the effects of consumption of 48 g RS on appetite compared to an appropriate control (energy and available carbohydrate-matched placebo) recently showed a significantly lower energy intake following the RS supplement, compared to the placebo supplement at the *ad libitum* test meal (Bodinham *et al.*, 2010). However, there was no associated effect on subjective appetite measures.

According to de Roos *et al.* (1995), 30 g/day RS2 (raw high-amylose corn starch) or RS3 (extruded and retrograded high-amylose corn starch) had little influence on appetite and food intake. However, supplementation with RS2 caused significantly lower ( $p < 0.05$ ) appetite scores than did supplementation

with RS3 or glucose, though subjects paradoxically felt less full while consuming RS2. This feeling might be related to a higher hydration capacity of extruded retrograded starch compared to raw starch.

Kendall *et al.* (2010) compared the impact of lower doses of RS3 (from 5–25 g of Promitor<sup>TM</sup> RS, Tate & Lyle Inc, IL, USA) in a meal. Feelings of fullness were greater with the 5 g dose of RS compared to control, while the satiety quotient for overall appetite was significantly greater for 25 g RS in the early phase after the eating period ( $p = 0.036, 0.075$  and  $0.97$  at 45, 30 and 15 minutes after lunch).

In several studies, RS seems to have an impact on satiety which could be mediated by acetate produced during colonic fermentation. Indeed, RS reduced subjective satiety ratings in the intermediate absorptive period (Raben *et al.*, 1994; Achour *et al.*, 1997) but led, in the post-absorptive period, to higher subjective scores of satiety, which may reduce subsequent food intake during the next meal (Achour *et al.*, 1997). The lower subjective scores for satiety and fullness after the RS (RS2; raw potato starch) than after digestible starch (pregelatinized potato starch) were associated with lower postprandial plasma concentration of glucose, insulin, GIP and GLP-1 (Raben *et al.*, 1994).

Nilsson *et al.* (2008) observed the effect of an evening meal containing RS (high-amylose barley kernel) or  $\beta$ -glucan ( $\beta$ -glucan-rich barley kernel) on satiety after a subsequent standardized breakfast (ten hours after the evening meal). Satiety was positively correlated to breath hydrogen, an indicator of colonic fermentation.

Monsivais *et al.* (2010) recently showed that soluble fibre-dextrin (SFD) enhanced the satiating power of beverages, compared to soluble corn fibre, polydextrose and resistant starch. Indeed, only soluble fibre dextrin significantly suppressed energy intake ( $p = 0.023$ ). This SFD could be considered as part of RS3. The SFD is probably highly fermentable, and its satiating effect might also be mediated by SCFA produced within the four hours following the fibre intake. Similar results on resistant dextrins were obtained by Nazare *et al.* (2011).

Anderson *et al.* (2010) concluded from their recent study that estimates of starch digestibility by the *in vitro* Englyst method were able to predict food intake in young men 30 minutes and 120 minutes after consumption of a soup containing 50 g maltodextrin, whole grain, high-amylose, regular cornstarch or no added starch.

As shown by Tagliabue *et al.* (1995), RS (potato starch, in this study) seems to have no thermogenic effect and its presence does not influence the size of the thermic response to digestible starch. RS intake was, as expected followed by lower oxidation and greater fat oxidation.

Lerer-Metzger *et al.* (1996) concluded that the replacement of 570 g wheat starch/kg diet with mung-bean starch for five weeks resulted in a reduction in plasma triacylglycerol concentration and adipocyte volume in both normal and diabetic rats. Thus, the type of starch mixed into the diet may have important metabolic consequences in normal and diabetic rats.

Robertson *et al.* (2005) showed that a supplementation with RS lead to increased insulin sensitivity and a reduction in glycerol and free fatty acids across subcutaneous adipose tissue. This was suggestive of a change in adipocyte metabolism that may lead to a change in insulin sensitivity. RS and inulin have been demonstrated to increase the release of gut hormones with roles in appetite regulation and possibly, leptin release (Delzenne *et al.*, 2005; Keenan *et al.*, 2006).

Keenan *et al.* (2006) and Zhou *et al.* (2006) have shown that dietary resistant starch increased GLP-1 and PYY secretion. The same group (Zhou *et al.*, 2008) found that:

1. RS stimulated GLP-1 and PYY secretion in a substantial day-long manner, independent of meal effect on changes in postprandial glycemia;
2. fermentation and the liberation of SCFAs in the lower gut are associated with increased proglucagon and PYY gene expression;
3. glucose tolerance, an indicator of increased active forms of GLP-1 and PYY was improved in RS-fed diabetic mice.

They concluded from their study that fermentation of RS is most likely the primary mechanism for increased endogenous secretions of total GLP-1 and PYY in rodents.

In an acute, randomized double-blind, cross-over study comparing the effects of four fibre and a low-fibre treatment on satiety, Willis *et al.* (2009) observed that resistant starch (Novelose 330 and Hi-Maize<sup>TM</sup> 260, National Starch; RS3 and RS2, respectively) and corn bran had the most impact on satiety, whereas polydextrose had little effect and behaved like the low-fibre treatment.

The same group (Willis *et al.*, 2010) showed that satiety, gut hormone (ghrelin, GLP-1, and PYY (3–36)) response, and food intake at subsequent meals did not change in a dose-dependent manner after subjects consumed 0, 4, 8 and 12 g of mixed fibre in muffins for breakfast. However, despite lack of differences in satiety, gut hormone levels differed among treatments. Ghrelin was higher after the 12 g fibre dose than after the 4 g and 8 g fibre doses; GLP-1 was higher after the 0 g fibre dose than after the 12 g and 4 g fibre doses; and PYY(3–36) did not differ among fibre doses.

According to Shen *et al.* (2009), the mechanism of decreased body fat by RS would be linked to increased neuropeptide POMC gene expression in the

hypothalamus, and such an effect is independent of the involvement of visceral afferent capsaicin-sensitive neurons.

So *et al.* (2007) investigated the impact of RS on body fat patterning and central appetite regulation in mice. Mice receiving high RS (HRS; high-amylose corn starch (60% RS)) diet during eight weeks had similar body weights than those fed the low RS (LRS) diet, although their total body adiposity, subcutaneous and visceral fat, intrahepatocellular lipids, plasma leptin, plasma adiponectin and plasma insulin/glucose ratios were significantly lower than the low RS group. Adipocytes isolated from the HRS group were significantly smaller and had higher insulin-stimulated glucose uptake. Manganese-enhanced magnetic resonance imaging (MEMRI) of the ventromedial and paraventricular and paraventricular hypothalamic nuclei suggested a satiating effect of the HRS diet despite a lower energy intake. These data suggest that there may be appetite regulatory differences between animals exposed to varying amount of RS, with the LRS animals producing MEMRI data closer to fasted animals, and the HRS animal producing MEMRI data closer to that of satiated animals.

So *et al.* (2007) suggest a link between short chain fatty acids production from the fermentation of RS and the change in adipocyte morphology. Indeed, the G protein receptor (GPCR43) on adipocytes may play a role in adipocyte differentiation and proliferation; the ligands for these receptors appear to be the short-chain fatty acids, acetate and propionate (Brown *et al.*, 2003). There is recent evidence that activation of GPCR43 by acetate and propionate causes a decrease in leptin secretion and an increase in adipocyte differentiation (Hong *et al.*, 2005).

Significantly larger adipocyte size and over-insulin-stimulated glucose oxidation in rats fed diets low in RS, compared with rats fed high RS diets, had been described earlier by Kabir *et al.* (1998). So *et al.* (2007) also noticed a significant difference in plasma leptin concentrations, with the low RS group having significantly higher levels despite similar body weights. This would confirm the observation made earlier by Skurk *et al.* (2007) that leptin levels are driven by the lipid content and size of the adipocyte rather than by the overall body weight. The concomitant increase in adiponectin and leptin in the mice with low RS diet, compared to 'high RS' mice, is consistent with an induction of leptin resistance in these mice (So *et al.*, 2007).

## 13.6 CONCLUSION

Resistant starches are not homogeneous entities, as they can be totally or partly fermented in the colon and their fermentability rate differs from one to

another. These various properties probably explain some of the contradictory results described in the literature. Their fermentation produces a significant amount of butyrate but, depending on the methodology used, butyrate or propionate can be the second SCFA produced during fermentation of RS, after acetate. Butyrate, being the nutrient of the colonocyte, is usually taken up by the colonic mucosa, and only minor amounts can be found in the plasma (or in urine).

When digestible starch is substituted in the diet by RS, glycemic load and, consequently, insulin secretion is reduced. In the long term, RS seems to be able to improve insulin response in hyperinsulinaemic subjects. The impact of RS consumption at dinner on a subsequent breakfast revealed the involvement of RS fermentation on improvement of glucose tolerance and insulin sensitivity in healthy and insulin-resistant subjects.

GLP-1 and PYY, two gut-secreted peptides which have been proposed as potential anti-diabetes/obesity drugs, are also naturally secreted in response to meal ingestion. RS has been reported to increase:

1. plasma total GLP-1 and total PYY in rodents; and
2. gene expression of PYY and proglucagon, probably through its fermentation.

GLP-1 has been shown to increase in healthy subjects when part of the digestible starch was substituted by RS. This impact of RS on gut peptides has been confirmed within studies, showing an improved tolerance observed after a standardized breakfast when RS (and fibre) was ingested during the preceding dinner. It is associated to increased GLP-1 and adiponectin at the time of the breakfast. These findings provide evidence for a link between RS fermentation and the beneficial impact on insulin resistance. GLP-1 secretion would also explain the satiating effect of RS at several hours after its ingestion.

Recent works have suggested a change in adipocyte metabolism that may also lead to a change in insulin sensitivity. A link between short-chain fatty acids production from the fermentation of RS and smaller adipocytes, associated with better prognostic in metabolic syndrome, also supports the beneficial metabolic impact of some RS. There is, however, still much work to be performed in order to obtain new claims on several of the resistant starches. Indeed, the effects will have to be demonstrated on each type of RS, unless a clear correlation can be made between each fermentation pattern and corresponding metabolic effects.

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# 14 The Microbiology of Resistant Starch Fermentation in the Human Large Intestine: A Host of Unanswered Questions

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## 14.1 INTRODUCTION

The human intestinal tract carries a large and diverse community of resident microorganisms. The highest cell densities ( $10^{11}$  per gram) are found in the large intestine, where flow rates are slowest and conditions in the gut lumen become anaerobic. Some energy for microbial growth is derived from endogenous sources through secreted proteins such as mucin and digestive enzymes, and sloughed epithelial cells. In general, however, the main energy sources are carbohydrates that have remained undigested in the stomach and the small intestine (Macfarlane & Gibson, 1997).

Many plant oligosaccharides and cell wall polysaccharides are undegradable, or poorly degraded, by mammalian digestive enzymes. Perhaps surprisingly, however, for many diets it is a fraction of the potentially digestible polysaccharide starch ('resistant starch') that escapes small intestinal digestion which is estimated to provide the single largest energy source for microbial growth in the colon (Cummings *et al.*, 1996). There is increasing evidence that the fermentation of resistant starch in the human large intestine may lead to health benefits that include prevention of colorectal cancer and improved insulin responses (Hylla *et al.*, 1998; Bird *et al.*, 2000, 2008; Robertson *et al.*, 2005).

Despite extensive early cultural studies on polysaccharide utilization by human colonic bacteria (Salysers *et al.*, 1977a, 1977b), our understanding of the microbiology of resistant starch fermentation is still rather limited. One

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problem is that it is not straightforward to reproduce, under *in vitro* conditions, the state of the undigested starch particles that arrive in the large intestine (Annison & Topping, 1994). Microbiological studies on pure cultures necessarily tend to use autoclaved starch preparations, whose properties may differ from those of starch in small intestinal effluent (Englyst & Macfarlane, 1986).

A second problem lies with the microbial community, which, in the colon, is dominated by anaerobic bacteria that are not easily cultivated. Despite a rapid increase recently in sequence-based diversity studies, relatively few laboratories work routinely with anaerobes, making functional information scarce. There are, however, excellent prospects now for combining molecular and cultural approaches to obtain new insights into the impact of resistant starches on microbial populations in the human colon.

## **14.2 IDENTIFYING THE MAJOR DEGRADERS OF RESISTANT STARCH IN THE HUMAN GI TRACT**

### **14.2.1 The human colonic microbiota**

Direct sequence analysis of DNA and RNA from faecal and gut samples has revealed a more complete picture of the diversity of bacteria that colonize the human large intestine than was possible using culture-based approaches. Most studies agree that only one-third of the bacterial diversity estimated from 16S rRNA gene sequencing is currently represented by named, cultured species (Suau *et al.*, 1999; Hold *et al.*, 2002; Eckburg *et al.*, 2005). On the other hand, a much higher percentage of the numerically predominant species are available as cultured isolates, so that more than half of amplified ribosomal sequences detected in faecal samples can correspond to cultured species (Walker *et al.*, 2011).

The two most abundant bacterial phyla are the gram-positive *Firmicutes* and gram-negative *Bacteroidetes*, followed by the *Actinobacteria* and *Proteobacteria*, with small numbers of other phyla such as *Verrucomicrobia*. The molecular diversity of eukaryotic gut microbes (protozoa and fungi) has received comparatively little study to date.

### **14.2.2 Cultural studies**

Extensive early bacterial isolation work using anaerobic techniques revealed a large number of human gut bacteria that are capable of growth on starch. Salyers *et al.* (1977a, 1977b) reported finding starch utilizers within the *Bacteroidetes*, *Firmicutes* and *Actinobacteria*, with this activity being



**Table 14.1** Amyolytic activity in human gut anaerobes.

	Strains tested	Growth with:	
		amylose	amylopectin
<i>Bacteroides</i> spp.	188	72%	73%
High G + C <i>Actinobacteria</i>	52	31%	52%
Low G + C <i>Firmicutes</i>	38	16%	53%

Data from Salyers *et al.* (1977a, 1977b).

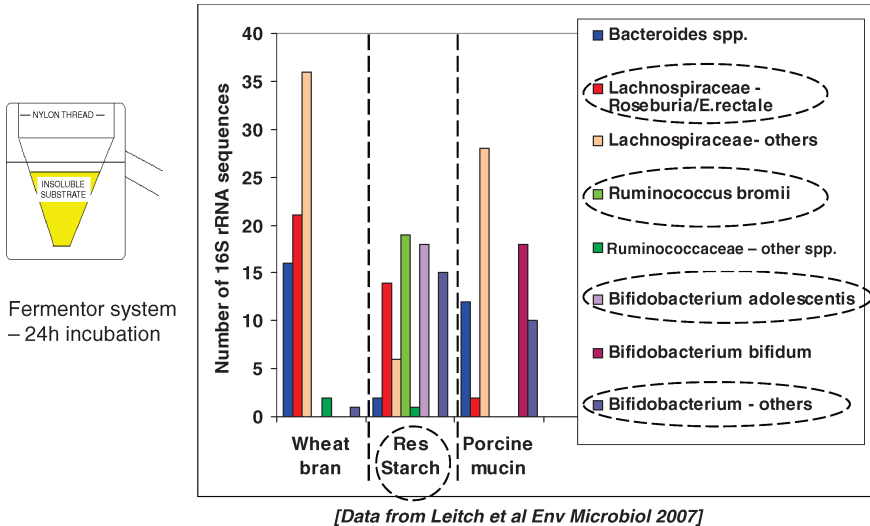
particularly common among isolates of *Bacteroides* spp. (Table 14.1). Subsequent selective isolations on media using starch as the sole energy source produced a high proportion of *Bifidobacterium* spp. (*Actinobacteria*) (Macfarlane & Englyst, 1986). The number of starch-degrading species from these early studies among what is normally the most abundant phylum, the *Firmicutes*, remained low, but it now appears that this may reflect the relatively low coverage of this phylum previously by cultured strains (Eckburg *et al.*, 2005).

### 14.2.3 16S rRNA-based studies

While 16S rRNA-based community analysis reveals the diversity of human colonic bacteria, it does not by itself provide any functional information. On the other hand, when used in conjunction with selective functional screens, it can potentially reveal uncultured starch-degrading species. Leitch *et al.* (2007) used 16S rRNA sequencing to study the colonization of insoluble resistant starch (Hylon VII) by mixed human faecal bacteria in an anaerobic fermentor system, and 80% of starch-attached sequences recovered from four different microbial communities corresponded to four species – *Eubacterium rectale*, *Ruminococcus bromii*, *Bifidobacterium adolescentis* and *Bifidobacterium breve* (Figure 14.1).

This represents a very small subset of the diversity present in the starting inocula, showing that the ability to bind to and colonize resistant starch is quite limited among human colonic bacteria. The fact that the predominant attached bacteria belong to cultured species suggests that the major colonizers may already have been identified from the earlier isolation studies.

Another elegant functional approach is provided by stable isotope probing. Kovatcheva-Datchary *et al.* (2009) incubated <sup>13</sup>C labelled resistant starch with mixed human faecal bacteria and recovered <sup>13</sup>C labelled nucleic acid, resulting from starch utilization, by density gradient centrifugation.



**Figure 14.1** Colonization of insoluble substrates by human faecal bacteria. Mixed faecal bacteria were introduced into a porous bag containing insoluble substrate (Hylon VII starch, wheat bran or porcine mucin) within an anaerobic continuous flow fermentor system. The experiment was repeated with inocula from four different healthy individuals. The predominant bacteria attached to each type of substrate were estimated by amplification and sequencing of 16S rRNA genes, as described by Leitch et al. (2007).

16S rRNA analysis again revealed that the major utilizers were bifidobacteria, *R. bromii* and *E. rectale*. The last two are low %G+C bacteria belonging to the *Firmicutes*, emphasizing that members of this phylum also play an important role in starch utilization. This is also apparent from analysis of the responses among the faecal microbiota to added dietary starch *in vivo*, discussed further below.

### 14.3 SYSTEMS FOR STARCH UTILIZATION IN GUT BACTERIA

The availability of genome sequence data for an increasing number of starch-utilizing bacteria opens up new prospects for analyzing the amylolytic gene complement of starch-degrading species. Interpretation of these sequence data, however, depends critically on the relatively small number of detailed

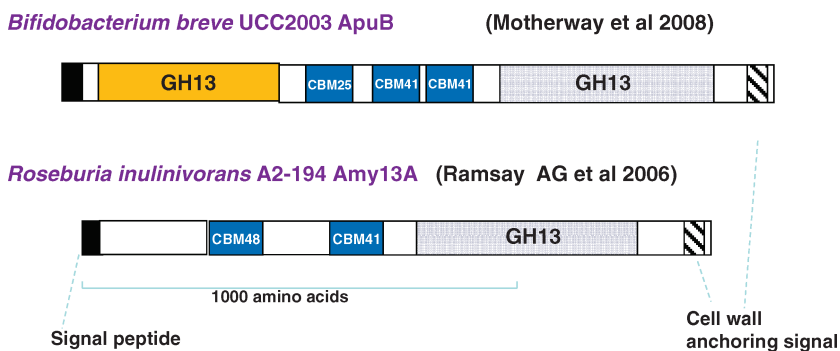
functional investigations that have been carried out in cultured gut bacteria (Flint *et al.*, 2008).

### 14.3.1 *Bacteroides* spp.

The starch utilization system of *B. thetaiotaomicron* is better understood than that of any other human gut bacterium, thanks to the detailed studies of Salyers *et al.* (reviewed Xu & Gordon, 2003; Flint *et al.*, 2008). This gram-negative species has a double cell membrane enclosing a periplasmic space. Soluble starch molecules are proposed to be trapped by a complex of Sus proteins located on the outer membrane surface. The molecules are then subject to limited hydrolysis and translocation into the periplasmic space, where the majority of the amylolytic activity is located. The major amylases are described as neopullulanases, with the ability to hydrolyse  $\alpha(1-4)$  linkages in amylopectin and pullulan as well as in amylose.

### 14.3.2 *Bifidobacterium* spp.

Several species of bifidobacteria found in the human colon – mainly *B. breve*, *B. adolescentis* and *B. pseudocatenulatum* – show amylolytic activity, and these include isolates with activity against high-amylose starches (Wang *et al.*, 1999; Ryan *et al.*, 2006). A major cell surface amylase was identified in *B. breve* that contains separate pullulanase and amylase catalytic domains, together with a C terminal sortase cell wall anchoring signal (Figure 14.2). The pullulanase domain was shown to debranch amylopectin by cleaving



**Figure 14.2** Domain structures of the major cell surface amylases from two species of Gram-positive bacteria found in the human large intestine. GH13 – glycoside hydrolase family 13 catalytic domain; CBM – carbohydrate binding module. Adapted from Flint *et al.* (2012) *Gut Microbes* 3:4, 289-306. Fig. 3A.

$\alpha(1-6)$  linkages and acts in synergy with the  $\alpha$ -amylase that cleaves  $\alpha(1-4)$  linkages. Disruption of this gene in *B. breve* eliminated the ability to grow on starch (Motherway *et al.*, 2008).

### **14.3.3 *Lachnospiraceae* - *Roseburia* spp., *Eubacterium rectale* and relatives**

Bacteria related to *Roseburia* spp. and *Eubacterium rectale* represent an important group of butyrate-producers in the human colon, accounting for around 7–10% of bacteria in human faeces (Aminov *et al.*, 2006). Most isolates from this group can utilize starch as a growth substrate (Ramsay *et al.*, 2006). *R. inulinivorans* A2-194 produces a prominent amylase that is bound to the cell surface via a sortase-mediated step. This multi-domain enzyme includes putative starch binding modules, together with an  $\alpha(1, 4)$  amylase (neopullulanase) catalytic domain that acts on pullulan and amylose. The *R. inulinivorans* amylase, therefore, has many similarities to the enzyme described from *B. breve* (Figure 14.2). These features appear consistent with extracellular hydrolysis of starch molecules combined with the uptake and processing of malto-oligosaccharides, as in *B. breve*.

### **14.3.4 *Ruminococcaceae***

This family of *Firmicutes* bacteria is numerically abundant in the human colon, but is quite poorly represented by cultured strains. *Ruminococcus bromii* has been regarded as a specialist starch-degrading species, but little if any work appears to have been done on its amylase system. Recent work, however, showed that the enzyme system of *R. bromii* was more effective in degrading several particulate resistant starches than the amylases of *B. thetaiotaomicron*, *E. rectale* or *B. adolescentis* (Ze *et al.*, 2012). This difference was most apparent when these starches were incubated without heating, or after boiling for ten minutes, whereas autoclaving increased degradability by the other species. Relatives of *R. bromii* were previously shown to be preferentially associated with particulate material in human faecal samples (Walker *et al.*, 2008).

## **14.4 METAGENOMICS**

Extraordinary recent advances in rapid sequencing capability are permitting analysis of the gene complement of complex microbial communities (e.g. Kurokawa *et al.*, 2007). This allows the frequency of amylase genes, for

example, to be monitored in the community in response to dietary change. This approach should be seen as complementing, rather than replacing, the need for organism-based studies. First, the annotation of genomic data depends crucially on functional information, which is still limited. Second, as discussed above, the possession of an amylase gene does not define the role of an organism in starch utilization, which is determined by the complete enzyme complement and the organization of the relevant catalytic activities and transport capabilities.

## **14.5 FACTORS INFLUENCING COMPETITION FOR STARCH AS A GROWTH SUBSTRATE**

It is expected that the chemical and physical structure of resistant starch entering the large intestine will influence the types of micro-organism capable of exploiting it. While it seems likely that the different types of enzyme system described above are equipped to deal with different forms of starch, there is as yet little experimental evidence to support this notion. The failure to detect *Bacteroides* among bacteria attached to resistant starch particles *in vitro* (Leitch *et al.*, 2007) perhaps suggests that *Bacteroides* are better able to utilize solubilized than particulate starches, but this possibility needs more rigorous testing. Several studies suggest that bifidobacteria are particularly active against high-amylose starches (Wang *et al.*, 1999) but, again, direct comparisons with amylolytic *Firmicutes* bacteria do not appear to have been made.

The gut environment can also play a role in determining the types of bacteria that utilize a given form of starch, and therefore which bacterial populations may be stimulated by the addition of resistant starch to the diet. A good example is the influence of gut pH. Active microbial fermentation in the proximal colon results in mildly acidic conditions, whereas pH is closer to neutrality when fermentation is less active, e.g. in the distal colon.

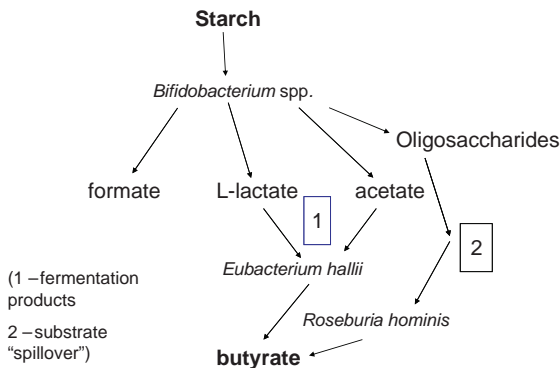
In a fermentor system supplied continuously with soluble starch and other polysaccharides at pH 5.5, the microbial community comprised approximately 25% of *E. rectale*-related butyrate-producing bacteria and 25% *Bacteroides* relatives. On switching to pH 6.5, however, *Bacteroides* spp. became dominant (80%) and *E. rectale* relatives became undetectable (Walker *et al.*, 2005; Duncan *et al.*, 2009).

Since *Bacteroides* spp. appear to be less tolerant of growth inhibition by weak acids at reduced pH than many gram-positive species, it appears that the mildly acidic pH creates the opportunity for the gram-positive species to compete successfully with *Bacteroides* for the polysaccharide substrates. The consequence of these differences in pH tolerance, via their effects on

the balance of the microbial community, was a four-fold higher butyrate concentration in the fermentor at pH 5.5, compared to that at pH 6.5 (Walker *et al.*, 2005).

## 14.6 METABOLITE CROSS-FEEDING

The anaerobic microbial communities of the rumen and large intestine are characterized by high cell densities and by extensive interspecies cross-feeding of fermentation products (Flint *et al.*, 2007). Addition of resistant starch to the diet could, therefore, lead potentially to the indirect stimulation of many groups of bacteria and their metabolic products, in addition to the primary amylolytic species. *B. adolescentis* L2-32, for example, degrades starch to produce lactate, acetate and formate. *E. hallii* cannot degrade starch, but it can convert acetate and lactate into butyrate. Co-cultures of these two bacteria convert starch largely into butyrate, with growth of both *B. adolescentis* and *E. hallii* (Duncan *et al.*, 2004; Belenguer *et al.*, 2006). Cross-feeding of partial degradation products from polysaccharide substrates is also a well established phenomenon (Belenguer *et al.*, 2006; Falony *et al.*, 2006; Figure 14.3).



**Figure 14.3** Potential contribution of metabolite cross-feeding to butyrate formation from resistant starches. Two distinct mechanisms are shown, one involving cross-feeding of fermentation products (lactate and acetate) and the other cross-feeding of partial breakdown products from starch. *E. hallii* is a lactate-utilizing bacterium that produces butyrate, while *R. hominis* is a non-lactate utilizing butyrate producer; neither species can grow by itself on resistant starch, but both grew when partnered with a starch-degrading *Bifidobacterium adolescentis* strain. Based on Belenguer *et al.* (2006).

## 14.7 IMPACT OF DIETARY RESISTANT STARCH UPON COLONIC BACTERIA AND BACTERIAL METABOLITES IN HUMANS

The application of molecular approaches to bacterial enumeration in human dietary intervention studies can reveal microbial responses to resistant starch (RS) *in vivo*. Almost invariably, these responses have to be monitored in stool samples. These can be expected to provide a good reflection of the microbiota of the colonic lumen, but the temporal relationship between changes in the proximal colon and changes seen in stool samples, of course, depends on transit time.

Abell *et al.* (2008), using 16S rRNA based DGGE profiling, detected an increase in relatives of *R. bromii* following increased RS intake. Another recent study looked at the impact of diets high in NSP (wheat bran) or high in type III resistant corn starch, each consumed for three weeks by 14 overweight males, using a cross-over design (Walker *et al.*, 2011). Bacterial groups were monitored by qPCR, and for six subjects by 16S rRNA sequencing. This analysis detected rapid increases in *R. bromii*-related bacteria and in the *Eubacterium rectale*/*Roseburia* groups on the RS diet, although responses varied markedly between individuals. Perhaps surprisingly, only one individual showed a strong response for bifidobacteria. A previous study failed to detect a response in *Eubacterium*-related bacteria in humans consuming diets high in RS (Schwiertz *et al.*, 2002), but *E. rectale* was not monitored.

Physiological effects of RS intake are also evident from human studies, in particular changes in faecal SCFA and improvements in insulin resistance (Robertson *et al.*, 2005). Evidence for protection against colorectal cancer comes largely from animal studies and is proposed to occur mainly through the butyrogenic effect of resistant starch (McIntyre *et al.*, 1993). Among the amylolytic groups known to be abundant in the human colon, only relatives of *E. rectale* are butyrate-producers. As mentioned above, however, metabolite cross-feeding might account for a butyrogenic effect of RS, even when the dominant bacteria stimulated by starch are not butyrate-producers. Changes in SCFA production rates have the potential to alter physiological functions including appetite, inflammation and gut motility through signalling to gut receptors (Brown *et al.*, 2003).

The delivery of additional fermentable carbohydrates to the colon can have numerous other consequences. Depression of colonic pH has been already discussed above, and this is known to influence  $\text{Ca}^{2+}$  availability as well as microbial competition. Another important effect is the increased incorporation of nitrogen into microbial cell protein, resulting from increased bacterial

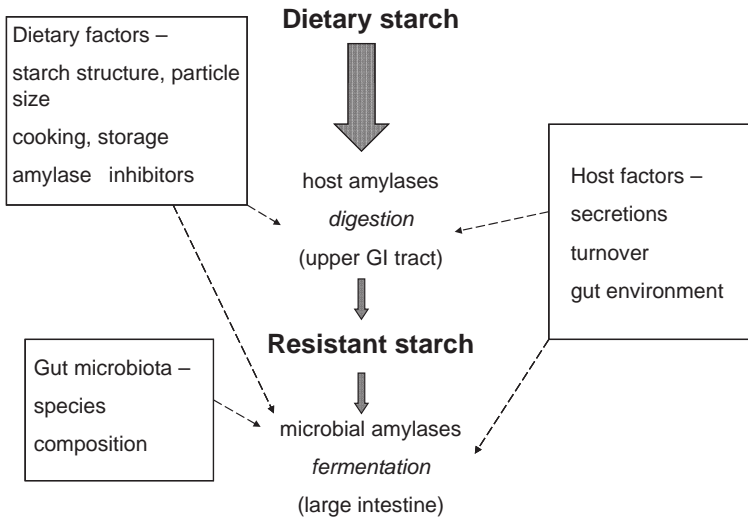
growth and biomass, since this tends to decrease the concentration of nitrogenous compounds available for catabolism to toxic or carcinogenic products in the colon (Gill & Rowland, 2002).

## 14.8 CONCLUSIONS AND FUTURE PROSPECTS

Animal studies have shown that different types of RS can differ substantially in their effects on gut metabolism and host physiology (Le Leu *et al.*, 2009). Such variation can be assumed also to occur in humans. At the same time, there is evidence that RS fermentation may vary markedly between individual humans (Walker *et al.*, 2011). Host factors, the nature of the dietary substrate and the composition of the gut microbiota are all likely to play a role in determining the fermentability of resistant starch in the large intestine (Figure 14.4).

The likely inter-dependence of these various factors is explored briefly below.

1. *RS structure will determine its rate and site of fermentation in the large intestine.* The structure of dietary starch (particle structure, association with other polymers, crystallinity, branching, retrogradation, cross-linking)



**Figure 14.4** Factors influencing the fate of starch in the intestine.



is known to determine its digestibility in the small intestine. It can be assumed that these characteristics will also determine how rapidly resistant starch is fermented in the colon, but less research has been done on this. Rapid fermentation of RS may lead to complete fermentation in the proximal colon, whereas slower rates will also support fermentation in more distal regions, with the possibility of incomplete colonic fermentation overall.

2. *Different types of RS may promote different groups of colonic bacteria.* It is possible that different forms of RS will prove to be accessible by different groups of colonic microorganisms. This could result in selective effects of starch intake upon the species composition of the colonic microbiota, as well as differential effects on gut metabolism. For example, it was suggested above that *Bacteroides* spp. may be best adapted to utilizing soluble starch molecules. It has also been proposed that certain types of RS might be more bifidogenic because of the high activity of this group on high-amylose starches. If such selective effects occur, then individuals who differ in the composition of their gut microbiota can be expected to show different responses to RS intake as, indeed, is suggested by the recent work of Martinez *et al.* (2010).
3. *Host and dietary factors can influence what fraction of starch comprises RS.* Host factors (e.g. affecting secretion of digestive enzymes or transit times) could play an important role in determining the undigested starch fraction that arrives in the large intestine and its subsequent rate of fermentation in the colon. This is also likely to be true for dietary factors (e.g. the pattern of starch ingestion over time) and the presence of amylase inhibitors of dietary origin (Wolin *et al.*, 1999). Thus, it may be that the types of starch that comprise RS will differ between individuals and also within individuals over time, driven by changes in dietary intake.
4. *Multiple factors (host genotype, diet, health, medication) have the potential to alter the amylolytic community within the large intestine.* It seems possible that a few species of amylolytic anaerobe play key roles in the initial degradation of RS in the colon, especially when the RS exists in particulate form. If so, variation in these 'keystone' species between individuals could result in individual differences in fermentability of RS (Ze *et al.*, 2012). One possibility is that certain species might be eliminated by antibiotic treatment during an individual's lifetime and may fail to re-establish, as has been proposed, for example, to explain variations in oxalate degradation between humans (Duncan *et al.*, 2002).

We have to conclude that some of the most fundamental questions about the factors that influence starch fermentation in the human colon, and its consequences for human health, remain unanswered. A combination of

cultural microbiology, microbial genomics and molecular ecology promises progress in understanding microbial fermentation of starch by colonic microorganisms. This effort will clearly need to be allied, however, to an understanding of starch chemistry, and to physiological and nutritional studies both in humans and in animal models.

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## 15 Colon Health and Resistant Starch: Human Studies and Animal Models

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### 15.1 RS CLASSIFICATION

Resistant starches (RS) are named for their resistance to digestion in the small intestine. This resistance allows them to pass into the large intestine and serve as a substrate for microbial fermentation. RS have been categorized into four types (Haralampu, 2000). Type 1 RS is physically inaccessible to digestive enzymes such as starch from coarse grains. RS type 2 is ungelatinized starch, including starch from potatoes or high-amylose corn starch, with long chains that are uniformly packed (Jane, 2006). RS type 3 is starch that has been cooked, then cooled, and is retrograded (Haralampu, 2000). It is the most thermally stable RS and is exemplified by potato starch in cold potato salad. RS4 is chemically modified starch that is, for example, cross-linked in a manner to restrict swelling of the starch granules (Jane, 2006).

### 15.2 RS AND COLON HEALTH: OVERVIEW

Colon health involves maintenance of normal function of the colon and the prevention of colon diseases. Healthy colon function permits regular bowel movement on a daily basis or somewhat more frequently, consisting of relatively soft but non-diarrhetic faeces. Colon health may be reflected in the measurement of laxation (the frequency and total amount of faeces excreted) and in the avoidance of constipation, which may be defined as bowel movement frequency less than three times per week (Marks, 2009).

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There is little data to support what daily faecal weight should be considered to be healthy, but one study of 53 Australians showed that the 16 subjects with daily faecal mass of >150 g had higher faecal butyrate concentrations and lower ammonia concentrations in faeces, which were thought to be protective against colon cancer (Birkett *et al.*, 1997).

Colon diseases include colon cancer, irritable bowel syndrome (IBS – also referred to as spastic colon), inflammatory bowel diseases or colitis (e.g. Crohn's disease, ulcerative colitis), and diverticulosis/diverticulitis. The roles of RS in preventing or protecting from each of these conditions are not yet well understood, but seem to be most closely related to improved laxation and increased short chain fatty acid (SCFA) production. The ability of RS to shift gut microbial populations to increase probiotics (i.e. beneficial microbes) and/or to decrease detrimental or pathogenic microbial species may also play a role in disease protection. The biology and chemistry of interactions between RS and the gut has been characterized to at least some extent in varying models. This work provides a basis for further investigation of RS in colon health and disease.

### **15.3 RS, GUT MICROBES AND MICROBIAL FERMENTATION**

Several innovative model systems have enriched the understanding of how RS affects gut microbial populations and their metabolic products. Germ-free F344/N rats were orally dosed with pooled human faeces from three individuals from the UK or from Italy and were fed either a high sucrose diet or 15% RS (CrystaLean<sup>TM</sup>) substituted for a portion of the sucrose for four weeks (Silvi *et al.*, 1999). Body weights of male rats fed RS were significantly greater than for sucrose-fed males, whereas body weights of female rats fed RS and bearing microbes from individuals from the UK were significantly less than in sucrose-fed females. Counts of *Lactobacilli* and *Bifidobacteria* were increased, and *Enterobacteria* were decreased by RS feeding compared with sucrose feeding. *Staphylococci* and *Streptococci* were increased by RS in rats bearing microflora from humans from the UK, whereas *Staphylococci* were decreased in rats fed RS and bearing Italian human microbes. Cecal  $\beta$ -glucosidase activity was increased and ammonia concentration was decreased by RS. Cecal  $\beta$ -glucuronidase was only decreased by RS in rats bearing microbes from humans from the UK. Cecal butyrate was increased and propionate was decreased by RS feeding (Silvi *et al.*, 1999). Butyrate enhanced tight junctions, improving the epithelial barrier function of Caco-2 cells, a widely used model for human intestinal uptake and metabolism (Peng *et al.*, 2009).



This barrier function is thought to be important for protection from colon diseases.

As reviewed by Wollowski *et al.* (2001), the above changes in human microflora-associated rats by RS to increase lactic acid bacteria (LAB; *Lactobacilli* and *Bifidobacteria*) may help to detoxify some carcinogens, lower faecal pH which is associated with colon cancer prevention, and stimulate butyrate production, which improves protection of colonocytes from oxidative stress, and maintains normal colon cell function. Note that LAB produce lactate, which is then a substrate for butyrate-forming species (such as *Clostridia* cluster XIVa microbes) (Collins *et al.*, 1994), so the interactions among gut bacteria needed for healthy colonic metabolism are complex.

Additional studies of RS feeding to female BALB/c mice showed that increasing high-amylose corn starch intake from 0–30 (or 40% of the diet) increased *Bifidobacteria* counts after 25 days (Wang *et al.*, 2002). When *Bifidobacterium* Lafti 8B was co-administered orally, the 40% high-amylose starch diet also increased faecal butyrate concentrations by about fourfold compared to control starch feeding (Wang *et al.*, 2002), lending further support to the concept that RS may benefit both gut bacterial composition and microbial metabolism.

*In vitro* anaerobic incubations of resistant starches or their residues (starch remaining after  $\alpha$ -amylase treatment to simulate human starch digestion) from various sources with human faeces also provide models for examining gut microbes and microbial metabolism. Pyrodextrin residues (heat-treated starches creating RS type 4) from potato, lentil and cocoyam increased the propionate:acetate ratio, but did not alter butyrate in human faecal incubations, compared with residues from the non-resistant parent starches (Laurentin & Edwards, 2004). An RS type 3 polymorph made from thermal processing of high-amylose cornstarch (HACS) increased butyrate production and *Bifidobacteria* counts in continuous batch fermentations with human faecal inocula over 11 days, compared with the control HACS (Lesmes *et al.*, 2008). Thus, a human faecal incubation model produces generally similar results to rodent models, regarding the ability of RS to increase butyrate and *Bifidobacteria*, two factors thought to benefit colon health. Proof of such benefits in human feeding studies remains to be determined. A few relevant and recent studies regarding some aspects of colon health are further described in the following.

### **15.3.1 RS and laxation**

Because RS provides indigestible carbohydrates to the colon, RS should be considered to be a subclass of dietary fibre. RS would therefore potentially have health benefits similar to that of dietary fibres in general. Improved

laxation was observed in 14 subjects given 25 g RS (PROMITOR™) or 25 g wheat bran fibre per day for 14 days in a randomized crossover design (Maki *et al.*, 2009). Both the RS and wheat bran increased faecal weight significantly and did not differ from each other; both increased daily faecal weight above 150 g. This suggests that colon diseases associated with lesser laxation, including diverticulosis/diverticulitis and IBS of the constipation type, may be benefited by intake of RS. Colon cancer might also be more likely to be prevented by RS intakes great enough to enhance laxation, based on the findings of (Birkett *et al.*, 1997).

### 15.3.2 RS, IBS and diverticulosis

No human studies have been performed on the role of RS in IBS to date, and there are no clearly relevant animal models of this condition; neither has the relation between RS intake and diverticulosis been studied. In an 18-month study of nine groups of a total of 1800 Wistar rats given 0–17% wheat fibre, incidence of diverticula was negatively associated with fibre intake and ranged from 9% in rats fed the greatest fibre to 47% in rats fed no fibre. However, the fibre intakes of the six treatment groups between 0–17% dietary fibre ranged only from 0.5–4.2%, with incidence of diverticula ranging from 28–41% (Fisher *et al.*, 1985).

Estimated dietary fibre intakes in the US from the Continuing Survey of Food Intakes of Individuals (1998), 10th–90th percentiles of intake ranged from 7.1–24.8 g/d (Food & Nutrition Board, 2005) or  $\approx$ 1.4–5.0% by weight of diet (based on 500 g intake). This would suggest modest variance across dietary fibre intakes in the US population in incidence of diverticula, which is a common condition associated with aging. The significance of RS as a type of dietary fibre in preventing diverticula in humans is probably scant.

### 15.3.3 RS and IBD

The ability of RS to prevent or reduce inflammatory bowel diseases (IBD – e.g. ulcerative colitis) has been better studied and seems promising. In Sprague-Dawley rats induced with colitis by 5% dextran sulphate sodium in drinking water given for seven days, subsequent intake of 4% RS (type 3) for seven days significantly improved colon histopathology scores compared to controls, and also increased SCFA concentrations in cecal contents (Moreau *et al.*, 2003).

In rats with colitis induced by trinitrobenzene sulphonate (TNBS), fed  $\approx$ 6% RS for 14 days before and 21 days after TNBS, increased SCFA concentrations were found in caecal and colon contents and healing was

accelerated compared with controls (Jacobasch *et al.*, 1999). Pigs fed 17–24% RS (raw potato starch, type 2 RS), compared with  $\approx$ 5–7% RS from corn starch, for 14 weeks, showed increased butyrate in colon contents and reduced immune system reactivity in intestinal epithelia, including reduced numbers of T-helper cells. This suggests that pigs fed more RS would be able to respond to intestinal inflammation better (Nofrarias *et al.*, 2007).

Twenty-two patients with quiescent ulcerative colitis (UC) consumed 60 g oat bran (20 g dietary fibre) for 12 weeks. Faecal butyrate was increased at four weeks, compared with baseline, but not at later time points in these subjects. They did not show an increase in gastrointestinal symptoms during this time, compared with ten control UC patients not ingesting oat bran, who showed increased symptom scores according to a questionnaire (Hallert *et al.*, 2003). This study suggests that RS, which would share fermentation characteristics with oat bran, might also be of some benefit in ulcerative colitis. However, well-controlled trials of RS in colitis patients remain to be reported. Such studies would be quite difficult to do; recruitment of sufficient subjects of similar disease state, measuring compliance with test diets and monitoring of treatments and symptoms over sufficient time periods would be challenging. The needs of patients for standard treatments, depending on disease state, might confound effects of RS.

#### **15.3.4 RS and colon cancer risk – human studies**

As with IBD, the role of RS in colon cancer risk has been studied to a very limited extent. The following review of existing data generally does not support the ability of increased RS intake to decrease colorectal cancer risk in humans. This review updates similar findings (Young, 2004). Faecal samples from 17 Native Africans (from South Africa), 17 African Americans and 18 Caucasian Americans showed that the Africans had greater faecal SCFA concentrations than either group of Americans. This finding was attributed to the diet of the Africans, assumed to consist largely of maize porridge, which could contribute 30–60 g of RS per day (O’Keefe *et al.*, 2009).

Subjects who had been treated previously for hereditary nonpolyposis colon cancer (Lynch syndrome) were given 13 g RS/d (Novelose) for 29 months or a placebo (waxy maize starch, composed entirely of amylopectin). The incidence of colon neoplasms after this time period did not differ between the two treatments; 18.7% of 358 subjects given RS vs. 18.4% of 369 subjects given the placebo (Burn *et al.*, 2008).

Twenty-four pre-operative colorectal cancer patients given the same RS as in the Burn *et al.* study showed a lesser proportion of mitotic cells in the upper half of colon crypts than did ten similar subjects given amylopectin placebos

after ingesting the starches for four weeks (Dronamraju *et al.*, 2009). The reduction in this marker of premalignancy indicated that RS may protect against colon cancer. In sporadic adenoma patients, 19 g of RS consumed daily for two months did not alter epithelial cell proliferation in colorectal biopsies (bromodeoxyuridine labelling index) compared with controls ingesting placebo starch ( $n = 28$  per treatment; van Gorkom *et al.*, 2002). After colon adenomectomy, 23 patients consumed 45 g of highly digestible maltodextrin or digestion resistant maltodextrin (28 g/d of RS type 2) for four weeks. Faecal bile acid concentrations decreased significantly in subjects given RS, but SCFAs and colorectal cell proliferation did not differ between the treatments (Grubben *et al.*, 2001).

To date, few studies have been done on RS and colon cancer risk in humans. Two studies suggest possible antineoplastic effects of RS, in suppressing colon cell proliferation (Dronamraju *et al.*, 2009) and lessening faecal bile acids (Grubben *et al.*, 2001) which may be colon tumour promoters (Nagen-gast *et al.*, 1995). Three studies (van Gorkom *et al.*, 2002; Grubben *et al.*, 2001; Burn *et al.*, 2008) did not show differences in colon cell proliferation between RS and placebo treatments in colon cancer patients. Such patients are at greater risk of colon cancer than the general population and are considered good populations to attempt dietary interventions. Because cancers develop over the lifespan and more often emerge later in life, long-term dietary intervention trials of RS in general populations or in individuals with genetic traits predisposing to colon cancer might be considered, if animal models are sufficiently supportive of a protective role for RS.

## **15.4 COLON CANCER PREVENTION – ANIMAL MODELS**

There has been considerable interest in the ability of RS to reduce the yield of colon preneoplastic lesions and colon cancers in rodent models. In most cases, RS type 1 or 2 has been fed in diets preceding and/or following treatment with a chemical carcinogen. In one study, Sprague-Dawley rats were fed diets containing 0%, 10% or 20% raw high-amylose corn starch for four weeks prior to treatment with azoxymethane (AOM), and colon cancers were assessed 25 weeks after AOM treatment. Both doses of starch inhibited colon tumour development and increased SCFA including butyrate in the distal colon (Le Leu *et al.*, 2007).

In a series of studies, the ability of RS to counteract DNA damage in the colon that was induced by high protein intakes was assessed. A high-amylose butrylated starch was compared with high-amylose starch in preventing the

DNA damage caused by a high protein diet, as measured by the comet assay in rats. Both diets were effective in protecting against DNA damage by the high protein diet, but the diet with butyrlated starch was the most effective. This protection paralleled increasing butyrate in the large intestine (Bajka *et al.*, 2008). Further, feeding 20% high-amylose corn starch for four weeks with cooked beef or chicken prevented the genetic damage caused by these meats (Toden *et al.*, 2007).

Although much of the research has focused on RS type 1 or 2, one study was conducted with a hydrothermally processed type 3 resistant starch (Novelose 330) fed for 20 weeks following treatment of Sprague-Dawley rats with 1,2-dimethylhydrazine. The RS completely prevented the development of tumours, compared to rats fed control starch. The RS-3 starch also increased apoptosis and decreased proliferation in the colon (Bauer-Marinovic *et al.*, 2006).

Since not all studies have observed reduced tumour yields in rodents fed RS, a study was designed to assess the timing of feeding RS relative to AOM treatment in Wistar rats. Raw potato starch was fed for a three-week interval, either before or after AOM treatment, and the number of aberrant crypt foci (ACF) was increased in the rats fed RS before AOM, in comparison with those fed control diet, while the yield of ACF was suppressed in a dose-dependent manner in the rats fed RS following AOM treatment (Liu & Xu, 2008). These authors concluded that RS fed following carcinogen treatment was more likely to inhibit the colon lesions induced by AOM than RS fed preceding carcinogen treatment.

SCFA, including butyrate, acetate and propionate, have been implicated as mediators of the resistant starch impact on colon carcinogenesis (Topping & Clifton, 2001). These short-chain fatty acids are produced by microbes that ferment the resistant starch that reaches the large intestine. The hypothesis generally implicates production of butyrate, because of observations that butyrate promotes differentiation of colonocytes, but data in support of this hypothesis have been mixed. For example, studies comparing cellulose and high-amylose resistant starch revealed the greatest increase in caecal butyrate with the resistant starch, but the greatest inhibition of colon tumours with cellulose (Sakamoto *et al.*, 1996). Furthermore, Nakanishi *et al.* (2003) treated rats with *Clostridium butyricum*, and with or without high-amylose starch, during treatments with AOM. Treatment with *C. butyricum* alone increased butyrate in the caecum, but it did not decrease aberrant crypt foci. However, treatment with both *C. butyricum* and high-amylose starch decreased aberrant crypt foci and increased acetate and propionate concentrations in the caecum. Elevated  $\beta$ -glucuronidase activity was also observed in the caecal contents from the rats treated with both *C. butyricum* and high-amylose starch.

Studies were conducted with AOM-treated F344 rats, and the key endpoints were ACF and mucin-depleted foci (MDF). Secondary endpoints were caecal pH, weight and SCFA concentrations (Zhao *et al.*, 2011). ACF are morphologically altered crypts, alone or in cluster, identified by microscopic examination of methylene blue-stained whole mount preparations of colonic mucosa from carcinogen-treated rodents (Bird, 1995). They are characterized by being at least two to three times larger than normal crypts, having increased pericryptal space and a slit-like opening, with thicker epithelium that stains darker with methylene blue.

In recent years, a subset of AOM-induced ACF were believed to be better predictors of cancer; these are MDF that are observed in alcian blue-stained tissue which stains mucins. MDF are identified by the absence of the deep blue staining indicative of mucins. These lesions highly correlated to tumour development in AOM-treated rats (Caderni *et al.*, 2003). More recently, these lesions were observed in humans (Femia *et al.*, 2008). AOM is selective for the colon, and only a few small bowel/stomach tumours are noted. Carcinomas are generally found approximately 30 weeks after carcinogen administration, and tumour incidence and multiplicity can be accurately titrated (Pories *et al.*, 1993). AOM produces both adenomas and carcinomas, with a predominance of lesions induced in the distal colon, consistent with human sporadic cancers.

A high-amylose starch with exceptionally high resistance to digestion was prepared by complexing high-amylose starch with lipids in a manner that was expected to make it less digestible and, thus, to pass into the large intestine and modify the gut microbial populations (Hasjim *et al.*, 2010). Studies evaluated the impact on pre-cancer lesions in the colon of rats by including this resistant starch, fed in comparison with raw high-amylose corn starch or normal corn starch, or cooked with high moisture as would be used in a pudding, in comparison with similarly cooked high-amylose corn starch or normal corn starch. The rats were fed a normal corn starch, a high-amylose starch or the processed high-amylose starch after treatment with the carcinogen AOM (Zhao *et al.*, 2011).

This study showed that the high-amylose starch and the processed high-amylose starch reduced pH and increased caecum weight, irrespective of whether they were cooked by a high-moisture method. The raw high-amylose starch reduced ACF, but ACF were not reduced by the processed starch in comparison with raw normal starch or raw high-amylose starch (Zhao *et al.*, 2011). When all three starches were cooked by boiling in water, the processed starch reduced MDF the most, high-amylose starch had an intermediate reduction and the most MDF were found in the rats fed the normal starch. Parallel observations were made with ACF, the more abundant precancerous lesion induced by AOM (Zhao *et al.*, 2011). This research supports the ability

of the processed high-amylose starch to prevent the development of colon cancer in this rat model.

## 15.5 CONCLUSIONS

Research in humans and laboratory animals suggest potential health benefits of dietary RS in protection against diseases of the colon, including irritable bowel syndrome, inflammatory bowel diseases (colitis) and colon cancer. To date, the research suggests that the type and cooking of RS, the control starch used, the time of feeding relative to the disease process and the nature of the disease may all impact the ability of RS to benefit colon health. Although the research has shown considerable promise for RS, further research is needed at all levels to provide a firm foundation for the use of RS in the maintenance of human health.

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