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 α-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_2O 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 α-1,4 linked glucose residues are interspersed with α-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 α-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 α-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 α-amylases in the oral cavity, followed by pancreatic α-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 ≈20 minutes, while SDS is the fraction which gets digested in ≈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 β-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 β-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 β-amylase and maltogenic α-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 α-1,6 linkages and decrease in α-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 α-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 (Mukerjea & 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 α-1,4 linkage and join the cleaved chain to another glucan chain through α-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_6 and C_3 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 α-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	*Hordeum vulgare*	lys5	low starch content	not reported	Patron *et al.*, 2004
AGPase	*Pisum sativum*	rb	reduced starch content	not reported	Hylton and Smith, 1992
AGPase	*Solanum tuberosum*		≈10% decrease in amylose	not reported	Tjaden *et al.*, 1998
GBSSI	*Hordeum vulgare*		↑ amylose, ↑ DP 19–36	↓ hydrolysis	Asare *et al.*, 2011
GBSSI	*Ipomoea batatas*	wx	amylose free; lesser short chains	↑ hydrolysis	Noda *et al.*, 2002
SSI	*Oryza sativa*		amylose unaffected; ↑DP 6–7,16–19; ↓DP 8–12	not reported	Fujita *et al.*, 2006
SSII	*Hordeum vulgare*	sex6	amylose 65–70%; ↓short chains	↓ hydrolysis	Morell *et al.*, 2003; Bird *et al.*, 2004
SSII	*Triticum aestivum*		↑ amylose 35%; ↓average CLD	resistant starch ↑100 fold	Yamamori *et al.*, 2000
SSII	*Pisum sativum*	rug5	↓ B2,B3; ↑ very short and very long chains; ↑ amylose ≈35%	not reported	Craig *et al.*, 1998
SSIII	*Zea mays*	dull1	↑apparent amylose; ↑short chains; ↓long B chains	not reported	Gao *et al.*, 1998
SBEIIa + b	*Hordeum vulgare*		amylose >65%; ↑DP <9, >15; ↓DP 9–13	↑ resistant starch	Regina *et al.*, 2010
SBEIIb	*Zea mays*	ae	↑apparent and amylose content; ↑long chains	↑ resistant starch to ≈40%	Li *et al.*, 2008
DBE	*Oryza sativa*	su-1	altered amylopectin CLD; granules shrunken, irregular and compound	not reported	Kubo *et al.*, 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, GBSSIb, 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[^{14}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 GBSS1 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, s*ex6* 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 (≈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 ≥ 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 malto-triose is used as primer (Szydlowski *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 α-1,4 linkages and transfer a free reducing C-1 to C-6 hydroxyl group of glucose-unit in another chain, forming a new α-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 ≈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 ≥ 38) and a marked

reduction in short chains of DP ≤ 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 ≈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 ≈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 ≥ 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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