2.05

Genetic Modification of Plant Starches for Food Applications

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CONTENTS

5.1 INTRODUCTION

Starch is a unique natural material, valued for its uses in food, feed, and industry. It is found in higher plants, mosses, ferns, and some microorganisms where it serves as an important store of energy. In higher plants, starch is deposited as transitory starch in leaves and as storage starch in specialized storage organs such as seeds or tubers. Starch is also an important component of many fruit crops such as apple, pear, melon, banana, and tomato. Storage starch is one of the main components of cereal grain (seeds) harvested from crops like wheat, maize, oats, barley, sorghum, and rice as well as of tubers harvested from crops like cassava, yam, and potato. Whether in its native state in grain or tubers, or in isolated granular form, starch is a convenient stable material, cheap to produce, suitable for long term storage without spoilage, convenient for high volume transport, and an important source of calories, retaining functional properties for use in many potential product applications. Grain and tubers are often used directly for animal feed or human food, with little or no processing, such as cooked whole cereal grain or potatoes. Cereal grain is also ground or milled to make flour or meal, which is subsequently mixed with other ingredients and cooked to make breads and pastries. Alternatively, starch may be extracted from the storage organs and the purified starch used as a key functional ingredient added to foodstuffs such as pie fillings, puddings, soups, sauces, gravies, coatings, candies, confectionary products, yogurts, and other dairy products. Extracted starch also has many nonfood industrial uses, such as paper sizing aids, textile sizing aids, molded plastics, ceramics, dye carriers, or suspension aids. Globally, starch is an essential commodity providing most (~80%) of the worlds calories. This vital commodity supply comes from just six different plant species: three cereal crops (rice, maize, and wheat) and three tuber crops (potato, yam, and cassava).

As a result of advances in genetics and biochemistry, we have discovered much about how starch is synthesized in crop plants. Furthermore we have also unraveled the biochemical and genetic basis of some useful natural genetic variations that affect starch synthesis and consequently starch structure and functionality. Some of these variants are already commercially exploited. Examples include variants that accumulate less starch and more sugar (e.g., sweet peas, sweet corn, sweet potato) and others that cook to form clear sols rather than opaque gels (e.g., waxy corn, waxy rice, waxy wheat) and yet others that are useful industrially (e.g., amylose extender corn), and finally others valued for imparting stickiness when cooked (e.g., indica vs. japonica rice). Further progress in this area depends upon improvements in our understanding of the relationship between starch synthetic genes and enzymes, starch structure and functionality. Thus, by linking these findings with further advances in our understanding of the genes required for starch synthesis, an opportunity has appeared for us to make starches with increased usefulness and value.

This paper seeks to pull together the disciplines of biochemistry, genetics, biotechnology, and food technology of plant starches. First we will review current knowledge of starch structure and how starch is synthesized in plants. The primary focus of this review will be storage starch because this provides the main source of food starch today. Next we will summarize the effects on starch composition, physical properties, and functionality due to genetic modifications that cause changes in starch biosynthetic enzymes. Finally we will focus on food applications that might benefit from genetic modifications of crop plants and discuss future opportunities coming from traditional plant breeding and modern biotechnology.

5.2 STRUCTURE

Physically, after extraction and drying, normal starch is a white powder consisting of a mixture of amylose and amylopectin in semicrystalline granules. Starch granules are

microscopic structures approximately 0.5 to 100 μm in diameter. In shape, they are spherical, elliptical, or polyhedral. The size and morphology of starch granules is characteristic of the organ and species in which they are produced (102). Starch granules appear rather similar in size and morphology with and without amylose. Under most environmental conditions, starch granules can be considered moderately inert with little capacity to hold water. These characteristics of starch granules make them ideal vessels for storage and shipping, whether in grain or tubers or from processed isolated starch.

Chemically, starch is classified as a complex carbohydrate and is a mixture of two polymers of glucose: amylose and amylopectin. Amylose is a generally linear α-1,4 glucan which is sometimes lightly branched with α-1,6-glycosidic linkages. Amylopectin is normally higher in molecular weight than amylose. It is also an α-1,4 glucan, but is highly branched with α-1,6-glycosidic linkages. The proportions on a dry weight basis of amylose and amylopectin in starches isolated from storage tissues like potato tubers or cereal grain is normally between 20 to 30 percent amylose and 70 to 80 percent amylopectin. In addition to amylose and amylopectin, granules contain small quantities of protein and lipid. Between species there is variation in the structure of amylopectin (104), the size and structure of amylose (84,207,209,211,213), and the nature and amounts of proteins (77,78,155) and lipids (154,214). Because starch physical behavior is dependent on all of these components (55,70,104,116–119) there are specific uses of starches from different species. In addition, within a given species, rare examples have been found of grains, tubers, or roots producing starches that deviate from the typical amylose to amylopectin ratio or have altered amylopectin structure. These plants have been selected because of their unique cooking behavior due to their unusual starch composition that confers unique properties to the crop storage organ. Some of these natural variants are now cultivated on a commercial scale.

Examination of starch structure began over 60 years ago (191). The first widely accepted model shows starch as a branched structure with alternating regions of higher branching density separated by more lightly branched regions (110). A more widely accepted model shows amylopectin arranged in alternating clusters (178,179). Based on the chain length profile of debranched amylopectin and a refinement of the cluster model, the amylopectin chains were categorized into type A, B1, B2, B3, B4 and a single C chain (82). Recently, three refinements for the different modes of interconnection of the amylopectin clusters were presented (82,216) (See Figure 5.1). In starch granules, some of the chains of amylopectin are believed to be associated with one another through hydrogen bonding, forming double helices. The double helices either form higher ordered crystalline structures or may exist independently of crystalline order. The double helices are oriented radially within the granule, with the reducing ends of the chains oriented toward the center or hilum of each granule. Within the granule, crystalline regions, often referred to as growth rings, are separated in a radial fashion from each other by amorphous regions. The crystalline regions are further subdivided into amorphous and crystalline lamellae, which have a periodicity between clusters of approximately 9 nm (105). The branch points in amylopectin are believed to be the primary component of the amorphous lamellae, with the ordered amylopectin side chain double helices clustered in the crystalline lamellae. Differences in the internal chain lengths of amylopectin affect starch crystallinity (163). Important new insights into how amylopectin chain architecture may affect packing have been advocated based on small angle x-ray scattering studies and analogies with liquid crystals (230–233). Using these models it is possible to discuss the mechanisms and kinetics of interchain associations in the context of visualizing starch as a liquid crystalline polymer having different degrees of crystalline order depending on physical conditions.

Amylose contributes to the overall crystallinity of normal starch through the formation of crystalline complexes of amylose with lipids and, it is believed, through participation

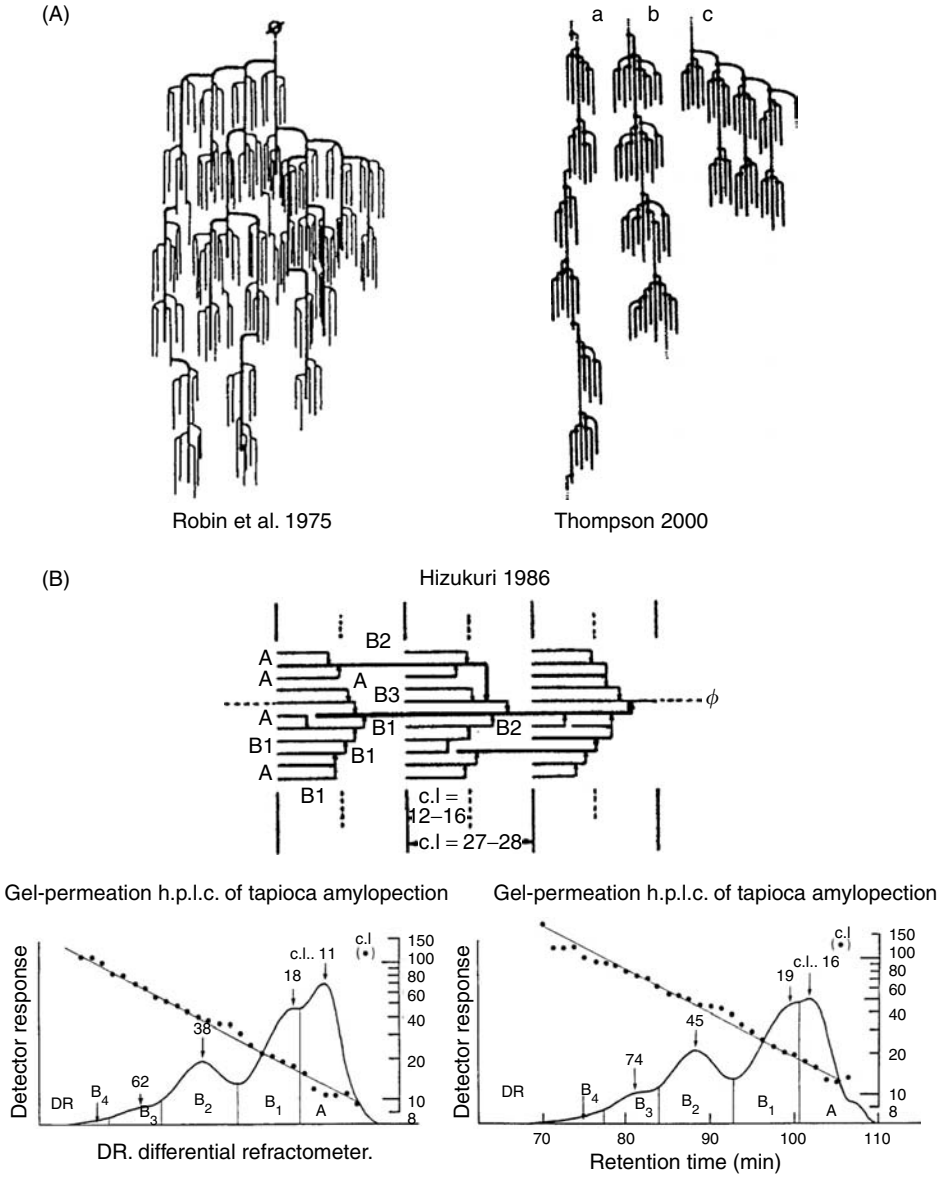

Figure 5.1 (A) Mode of Interconnection of clusters (B) Cluster models of amylopectin

in some starch double helices. However, although amylose readily forms double helices in solution, it readily leaches out of hydrated starch at granule gelatinization temperatures (68). This observation shows that amylose in starch granules does not associate in the same manner inside granules as it does outside of the granule. This observation additionally raises some intriguing questions about the nature of amylose synthesis and the kinetic trapping of amylose within starch granules.

While normal starch can be readily separated into two components, amylose and amylopectin, studies of high amylose starches have revealed what some authors describe as a third starch component, sometimes termed intermediate starch. For normal starches containing ~25% amylose, the results from quantification by iodine inclusion complex formation

(11,139,181,127), or precipitation in the presence of complexing agents (191,192,126,240,241) are in general agreement. However, for high amylose starches, these methods give different results. Thus, from detailed observations, high amylose starches were initially considered to contain either normal amylopectin and short chain linear amylose (1,2,75) or longer chain amylopectin compared with amylopectin from normal starch (148,149). Liquid chromatographic studies (140) have confirmed the latter. Additional studies of high amylose starches by differential precipitation with 1-butanol combined with Sepharose 2B chromatography (245) revealed an inability to clearly separate amylose from amylopectin. Further fractionation revealed high molecular weight material in what traditionally was the amylose fraction (8,9,209). Later studies showed that this material can be removed by repeated precipitation with 1-butanol and was most likely contaminating amylopectin (209,213), while removal of the low molecular weight material in the amylopectin fraction using differential precipitation techniques has not been successful. In summary, starch from high amylose mutants appears to contain a significant amount of an amylopectin-like component having an altered architecture. This intermediate starch component is characterized by:

1. An inability to precipitate with 1-butanol
2. An ability to eluete within the same molecular size range as amylose
3. An ability to bind iodine and having a lambda maximum between amylose and amylopectin

Estimates of intermediate material defined in this way have exceeded 55% (w/w) of the total starch of high amylose mutants (10).

Other workers (116,209,239) have defined intermediate starch to be the material that has the ability to precipitate in the presence of some complexing agents (e.g., 2-nitropropane) or mixtures of complexing agents (1-butanol with isoamyl alcohol) but not others (e.g., 1-butanol, 1-nitropropane). This type of intermediate material obtained by differential precipitation is a relatively small proportion of normal starches ($<10\%$) (116,209,220,239) irrespective of the amylose content of the high amylose starch. The molecules have been considered by some as amylopectin molecules with long external chains and a limited capacity to form clathrate complexes and precipitate (116,209,220,239). It has also been suggested (209) that this type of intermediate material could be a mixture of amylopectin and a small amount of contaminating amylose, which might be the case if the amylopectin from high amylose starch has an overall molecular size similar to that of amylose from normal starch (116).

Amylopectin molecules within granules are believed to be organized radially, with the long C-chain innermost (84). As a result of high magnification microscopy studies it was proposed that the radially oriented amylopectin clusters are organized into super helices, which may relate to the blocklets seen in microscopy studies (166). In turn the super helices may be responsible for the formation of concentric spherical rings. Although these growth rings are a characteristic of all starch granules, the mechanisms determining their formation are still not well understood (60,170). Recently, Bertoft proposed a bidirectional backbone model, where the super helix could be organized so that the longer amylopectin chains are oriented in line with the super helix, while the amylopectin clusters may be oriented radially (18) (Figure 5.2).

X-ray crystallography has shown that there are three distinct types of crystalline order in starch: A-type, B-type and V-type. V-type crystallinity is often associated with crystalline packing of amylose lipid complexes. The A-type and B-type starches differ in the organization of helices: A-type crystals are densely packed hexagonal arrays of double helices, B-type crystals, though also double helices packed in a hexagonal array, are unlike A-type

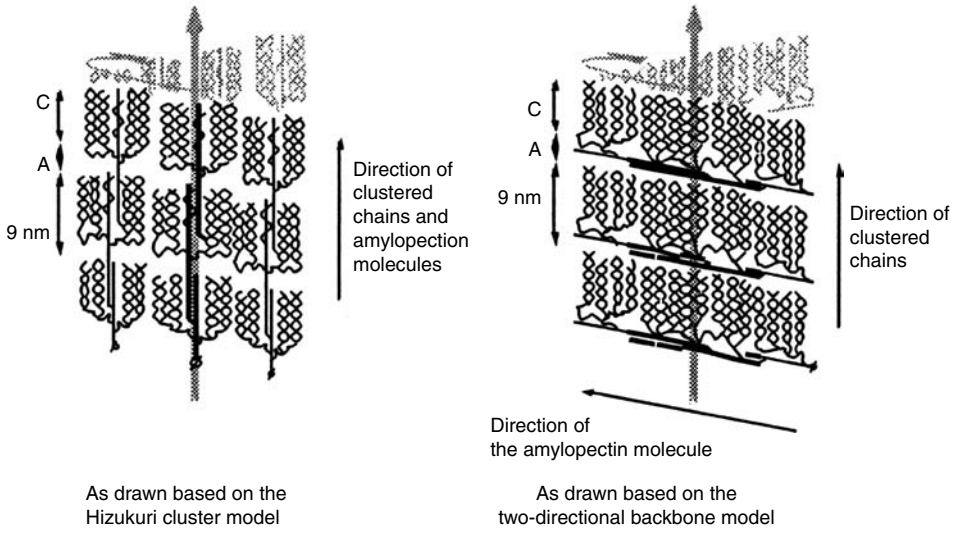

Figure 5.2 The super helix model as drawn in the Hizukuri cluster model and modified based on the two-directional backbone model.

crystals because they have an open central cavity which results in an increased water content (92). C-type starches are a mixture of A-type and B-type crystallinity (22,27,69).

5.3 SYNTHESIS

For an overview of starch synthesis, the reader is referred to recent reviews (99,151,218). In general terms, it is important to note that storage organs are composed of individual starch storing cells and each cell contains several subcellular compartments: including particularly the cytosol and amyloplast compartments. Sucrose, made in the leaves, is transported to the storage organ where it is imported into the cytosolic compartment of each cell. A well characterized pathway (see Figure 5.3) of starch synthesis achieves the enzymatic conversion of sucrose to starch. The first part of this pathway is localized in the cytosol, while the final steps of starch synthesis are located in a specialized subcellular compartment called the amyloplast.

In the cytosol, the glucosyl and fructosyl moieties of sucrose are converted into sugar phosphates. One of these types of hexose phosphates, glucose-1-phosphate is converted into ADP-glucose by the enzyme ADP-glucose pyrophosphorylase (AGPase), the first committed step in starch synthesis. In the endosperm of monocot crops, AGPase is located predominantly in the cytosol, whereas in the storage organs of dicot crops, AGPase is located predominantly in the amyloplast. Thus in monocot crops, ADP-glucose is transported into the amyloplast, while in dicot crops hexose phosphate is imported to support ADP-glucose synthesis inside the amyloplast. The conversion of ADP-glucose to starch is performed by several enzymes, which include, but may not be limited to, soluble starch synthase (SS), granule bound starch synthase (GBSS), starch branching enzymes (SBE), and isoamylase (ISA). Other enzymes that also may be involved in starch synthesis include phosphorylase (PHO), pullulanase (PU) and disproportionating enzyme (DisPE). GBSS is involved primarily in amylose biosynthesis and SSs, SBEs, and ISAs are involved in the biosynthesis of amylopectin.

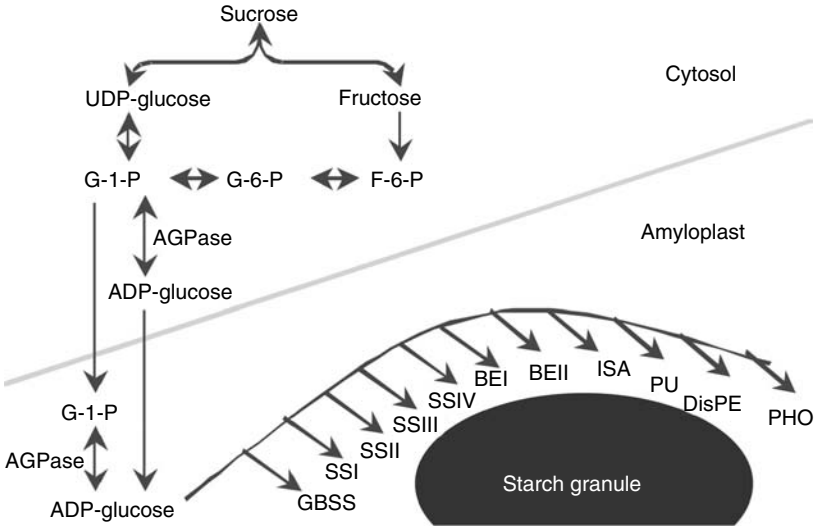

Figure 5.3 Pathway of starch biosynthesis

Across species, well conserved families of genes encode the enzymes in the pathway. Thus, diverse plant species possess similar sets of multiple forms (isoforms) of each enzyme. The conservation of isoforms suggests that each has a unique role in the process of starch synthesis. This idea has been largely confirmed in several plant species by studies of genetic modifications in which particular isoforms have been overexpressed or reduced.

5.3.1 Granule Bound Starch Synthase

Granule bound starch synthase (GBSS) is a member of the glycosyltransferase class of enzymes more generally known as starch synthases. GBSS is more formally named ADPglucose:1,4-α-D-glucan-4-α-D-glucosyltransferase (E.C. 2.4.1.21). In storage organs there are several starch synthase proteins associated with starch. However, only one isoform (GBSSI) is found predominantly associated with starch, and based on studies of amylose free mutants which lack the GBSSI protein, this isoform appears to be exclusively responsible for amylose synthesis in maize (200) and other plant species (44,88). There is a similar isoform in leaves (229). GBSSI has been identified, mapped and cloned from several species (218). The activity of GBSSI is correlated with the product of the waxy, lam, and amf locus of cereals, pea, and potato respectively (44,88,89,160,200).

As well as associating with starch granules, another key attribute of GBSSI is its ability to elongate a growing glucan chain processively. This means that the enzyme does not necessarily dissociate from the glucan chain after the addition of the first glucose but can remain associated with it to add further glucose units before finally dissociating. Soluble starch synthases enzymes in comparison add single glucose units per encounter with the glucan chain (distributive elongation). This processive mechanism of elongation by GBSSI is consistent with the proposed role of GBSSI in amylose synthesis (45,46). One of the more intriguing aspects of the function of GBSSI is that in isolated granules, its amylose synthesizing activity appears to be dependent on the presence of low concentrations of maltooligosaccharides (45,46). These experiments with isolated granules also showed that GBSSI can elongate glucan chains within amylopectin, a finding that may explain why mutants lacking GBSSI have altered amylopectin structure as well as lacking amylase (83,172,251).

5.3.2 Soluble Starch Synthase

Soluble starch synthase (SS) isoforms are all members of the glycosyltransferase class of enzymes. SS is more formally named ADPglucose:1,4-α-D-glucan-4-α-D-glucosyltransferase (E.C. 2.4.1.21). Like GBSSI, the SS enzymes are also associated with starch granules, but are also present in the stroma of the amyloplast, leading to their classification as soluble starch synthase. SS catalyses the transfer of glucose from ADP-glucose to an acceptor glucan chain, by a mechanism that has been described as distributive (46).

Four SS forms have been identified, mapped and cloned from several species (218). Although the various isoforms are believed to be present in all starch synthesizing cells, they appear to have different relative activities in different species and tissues. SS is believed to be primarily responsible for amylopectin synthesis, and there is evidence that each isoform is responsible for the synthesis of different chain lengths. SSI has been cloned and mapped from several species, but the only known mutant type described so far is in rice (159). SSI appears to be primarily responsible for synthesizing shorter chains of amylopectin based on biochemical studies of the isolated enzyme (38) and structural studies of the rice mutant (159). The SSII and SSIII isoforms appear to be involved in synthesizing the intermediate chains of amylopectin, as evidenced by changes observed with mutant and transgenic rice (40,63,221). The precise role of SSIV remains to be elucidated, and it may even be revealed that SSIV is a subclass of the SSIII isoform. All of these classifications are consistent with evidence obtained from starch isolated from potatoes transformed with antisense constructs to the potato enzymes (51,68).

5.3.3 Branching Enzyme

Starch branching enzyme (SBE) is a member of the glycosyltransferase class of enzymes. The branching enzymes are more formally and collectively named 1,4-α-D-glucan:1,4-α-D-glucan 6-α-D-(1,4-α-D-glucano)-transferase (E.C. 2.4.1.18). Like the SS enzymes, the SBEs appear to have a weak association with the starch granule as evidenced by the fact that these enzymes may be found entrapped inside starch granules. SBEs catalyze the interchain cleavage of a glucan chain with subsequent bonding of the cleaved portion to the parent glucan chain. SBEs act by cutting an α-1,4 linkage forming a new linkage between this cleaved glucan chain and an adjacent glucan chain via an α-1,6 linkage. There appear to be at least two major classes of highly conserved SBEs across plant species (SBEI and SBEII). Thus, like the soluble starch synthases, SBEs appear to have a distinct role in the mechanism of amylopectin structure development. The SBEs must also play some role in amylose synthesis, as amylose is lightly branched. All known SBEs have been mapped, cloned, and sequenced from several plant species. SBEIIb appears to be primarily responsible for transferring longer chains of amylopectin based on biochemical studies of the isolated enzyme (76,212) and structural studies of the maize and rice mutant (162,205,212). The role of SBEI and SBEIIa is less clear as mutants of SBEI in rice have reduced intermediate and long chains (159)while in maize the chain lengths are unaffected in mutants of SBEI and SBEIIa (20,21).

5.3.4 Debranching Enzyme

Debranching enzymes are members of the amylase class of enzymes. There appear to be at least four debranching enzymes in the storage organs: three isoforms of Isoamylase (ISA) and one Pullulanase (PU). Although ISA is known to catalyze the selective cleavage of α-1,6 linkages in branched glucans, their precise catalytic role in starch granule biosynthesis remains unresolved. Plants lacking ISAI accumulate higher levels of sucrose, more starch granules and phytoglycogen and have reduced amounts of starch, strongly indicating that ISAI is required for normal starch biosynthesis (28,29,59,122). Some studies have highlighted the involvement of ISA in starch granule initiation, probably mediated by degradation

of soluble glucans which would otherwise initiate granule formation (28,29,122). The role of ISAII and ISAIII in starch biosynthesis remains unresolved at the present time, as no mutants are known to exist. What little is known about their catalytic properties has led to the proposal that each isoform has retained a specific role (90). Studies in maize of the limit dextrinase/pullulanase (PUI) class of debranching enzymes has successfully identified a mutant, which on its own appears to have minimal effects on starch deposition (13,47).

5.4 MODIFICATIONS

Most of the maize starch mutants affecting starch structure were discovered before the genes and enzymes responsible for starch synthesis were known. Their names are therefore based upon the phenotypic changes observed in that storage organ or on the properties of the starch, rather than that of the gene or enzyme affected by the mutation. For example, numerous mutant phenotypes have been reported for maize (73,36) and several phenotypes (e.g., waxy, amylose extender, dull, shrunken, sugary-2, and sugary) have been described extensively with regard to their effects on carbohydrate composition and response to genetic background, allelic dosage, or interaction with other mutations (42,64,65,87). Examination of maize kernels with differing starch phenotypes has been instrumental in determining which enzymes are required for starch synthesis in this storage organ (24,194). Mutations that are responsible for most of the abnormal starch phenotypes have been located in genes encoding starch synthetic enzymes. Related isoforms, for which there are as yet no mutants available, have also been identified and characterized. Table 5.1 summarizes the enzymes

Table 5.1
Summary of individual isoforms of enzymes linked with specific mutants and the effects of these mutations on starch structure (similar genetic modifications affecting these starch biosynthetic enzymes exist in other species).

Enzyme and Specific Isoform	Mutant (Maize)	Effect on Starch
Granule Bound Starch Synthase		
GBSSI	Waxy (*wx*)	Low amylose content.
Soluble Starch Synthase		
SSI	None known	Not known
SSII	Sugary2 (*su2*)	Lacks intermediate glucan chains in amylopectin
SSIII	Dull1 (*du*)	Lacks longer glucan chains in amylopectin
SSIV	None known	Not known
Branching Enzyme		
BEI	BEI (*be1*)	None/Minimal
BEIIb	Amylose extender (*ae*)	High amylose content.
Debranching Enzyme		
ISAI	Sugary1 (*su1*)	Forms compound granules and phytoglycogen
ISAII	None known	Not known
ISAIII	None known	Not known
PUI	Pullulanase1 (*pu1*)	None/Minimal

required for normal starch synthesis in maize kernels, the associated mutant loci and the starch phenotypes of these mutants.

As a result of the great progress made in genetics in the last decade, in addition to the more traditional modifications used in plant breeding, it has become possible to modify or engineer starch synthesis enzymes using modern biotechnology. Collectively this is opening an oppurtunity to consider using all the tools of breeding, genetics and biotechnology to genetically control starch deposition in plants so as to enhance starch yield and starch quality. Thus, instead of crudely controlling enzyme activity by selecting certain mutants or selecting among different alleles of starch synthesis enzymes using plant breeding, it is possible to more precisely reduce the expression or activity of certain enzymes (such as by using antisense or RNAi technology). Alternatively the enzymes may be overexpressed: an opportunity that is unavailable using conventional plant breeding. What is additionally exciting about enzyme overexpression is that novel enzyme isoforms can be selected in order to act in concert with the existing starch pathway enzymes. With full sequencing of many homologous genes from different plants, we have begun to be able to identify domains within enzymes, which impart particular catalytic properties. This reclassification of enzymes into domain classes is an important new facet of our time, and is generating new understandings of the origins, mode of action and potential for genetic modification of different enzymes. The starch created from such work has the potential to be extremely novel and valuable. However, while the opportunities seem limitless, we are constrained by our limited understanding of the links between starch structural changes and functionality. Furthermore we are significantly constrained by our understanding of what determines the specific catalytic properties of the enzymes.

In the following sections, we will expand upon the properties and roles of the best studied of the enzymes of starch synthesis and describe the effects on the properties of starch of genetic modifications that eliminate or modify the genes encoding them.

5.4.1 Modification of GBSSI Activity

Modifications in granule bound starch synthase activity result in changes in amylose content or amylose structure. In maize, the GBSSI mutant phenotype was named waxy because the intact seed has a waxy phenotypic appearance, unlike normal seed that has a shiny and glossy appearance (194,236). No other enzyme appears to be involved in determining amylose content, as the waxy mutants of cereals (96,157), the amf mutants of potato (88), the lam mutants of pea (44), and GBSSI antisense lines of potato (4,228) show either a reduction or elimination of GBSS activity and a specific reduction of amylose in starch from tubers. Some studies have attempted to restore the production of amylose in amylose free potato plants by transforming the plants with genes for GBSSI enzymes produced by other plants. Between 3.5% and 13% amylose was restored to amylose free mutants of potato by transformation with the cassava GBSSI enzyme (183). Similarly, amylose free potatoes transformed with pea GBSSI isoforms resulted in potatoes with amylose contents of between 0.8% and 1%. Like, the other low amylose potatoes and pea starch, heterogeneity in amylose and amylopectin content was observed within the granules: the granules stained with iodine revealed amylose in concentric rings or having blue-staining granule cores (52).

Extensive work, initially in Japan, has identified wheat mutants lacking GBSSI activity (143,144,145,156,158,255). Miura and Sugawara (144) showed that substitution of genes producing functional GBSSI enzyme with the null alleles could result in starches with a 22 to 23% amylose content rather than the 25.5% amylose content of the normal control. Likewise, Miura et al. have shown that elimination of an active GBSSI enzyme at 2 of the 3 loci in wheat endosperm results in a wheat starch that has an amylose content

of 16% to 21% (143). Thus, the presence of a functional GBSSI enzyme from a single locus pair is sufficient to produce a starch with an amylose content of at least 16%. Others (164) have shown that low amylose wheat starches having amylose content between 14.1 and 16.7% can be created through ethyl methanesulphonate (EMS) mutagenesis of the seeds. Amylose free wheat starches were created using triple null combinations of GBSSI mutants (255) and using mutagenesis of a double null wheat known as Ike to generate a nonnull wheat (WO09815621) which stained red when stained with iodine.

Two functional Wx alleles of rice exist: Wx^a, which produces a large amount of amylose, and Wx^b, which produces a smaller amount of amylose. Studies of the effects of the two alleles on the gene expression at the waxy locus in rice (187) showed that the Wx^b allele resulted in an ineffective production of GBSSI enzyme and amylose in japonica rice, while the Wx^a allele produced larger quantities of GBSSI enzyme and amylose in indica rice (186). On a specific activity basis, other authors have shown that the Wx^a allele was less effective in the production of amylose than the Wx^b allele based on analysis of 40 rice varieties (226). It was observed that for two wild-type rice alleles, Wx^a and Wx^b, Wx^b had a GBSS expression tenfold lower than Wx^a at the protein and mRNA levels (97). The decrease in the expression of Wx^b compared to Wx^a was the result of a point mutation within the genetic sequence for the normal rice enzyme (Wx^a allele). The Wx^b allele resulted in the synthesis of a 3.4 kilobase pair mRNA transcript compared to a 2.3 kilobase pair mRNA transcript for Wx^a as a result of the inclusion of an intron into the mRNA sequence as a result of the point mutation (97). Amylose produced from rice plants was related to the ability of the plant to excise the intron 1 from the mRNA sequence (235). Plants expressing high levels of mature mRNA (without intron 1) and no pre-mRNA (containing intron 1) produced the highest levels of GBSSI protein and the highest levels of amylose (20.0 to 27.8% amylose). These were all indica species. With more balanced expression of mature and pre-mRNA, lower levels of GBSSI protein and amylose were observed (6.7 to 16.0% amylose). Both indica and japonica species were within this group. When all of the mRNA contained intron 1 and no mature mRNA was observed, no GBSSI protein was observed and no amylose was detected (235). This pattern relating amylose content to mature mRNA with properly excised intron 1 could be applied across 31 different rice cultivars (235). Thus, based on extensive studies (97,198,235), low amylose rice appears to be the result of a decrease in the amount of normal GBSSI through a mutation which results in problems with mRNA processing rather than due to a mutation in the mature mRNA sequence. However, some differences in the behavior of Wx^a and Wx^b may be present in different rice species.

In recent years a number of patents have been filed covering genetic modifications of granule bound starch synthase in plants, utilizing knowledge gained after cloning, sequencing and transforming plants with the GBSSIs. Examples include WO9211376, US5365016, WO09827212, WO028052, WO02018606, and WO04078983.

5.4.2 Modification of SS Activity

Modifications in starch synthase activity result in changes in amylopectin content or amylopectin structure. Starch synthase may have either a subtle or profound impact on the starch, depending on the activity or inactivity of a specific isoform on the structure and composition of the starch. Although SSI is a relatively minor isoform in potato, it is the predominant isoform of SS in the cereals. Of the starch synthases, the effects of SSIIa and SSIII on starch structure and composition are the best elucidated, especially in maize. Mutants lacking the putative SSIV isoform have not yet been reported. The first reported SSI mutant was in rice (159) and no mutants have yet been reported in other plant species. In rice, the SSI mutant has only a minor effect on starch content and quality. In maize, SSIIa maps to a locus known as Sugary-2 which when mutated produces the sugary-2

mutant. Starch from mutants lacking SSIIa lack certain intermediate amylopectin chains in maize, pea and rice (31,40,221). Wheat, null for the SSIIa enzyme in each of the A, B, and D genomes, has an increase in starch chains with a DP below 10, an elevated amylose content, and a poor x-ray diffraction pattern (246). Barley null for the SSIIa enzyme produces a starch with an apparent amylose content between 50 and 70% of the dry weight of the starch, with a concurrent increase in the proportion of short chains through a DP of 35 (150). The structural gene for SSIII in maize is known to be the Dull1 locus which when mutated gives the dull1 mutant. Starch from the dull1 mutant is relatively lacking in longer amylopectin chains (62). In potato the simultaneous antisense inhibition of SSII and SSIII resulted in a grossly modified amylopectin (51,136), with yet further changes in structure if GBSSI as well as SSII and SSIII are inhibited (108).

In recent years a number of patents have been filed covering genetic modifications of starch synthase in plants, utilizing knowledge gained after cloning, sequencing and transforming plants with the starch synthase enzymes. Examples include WO9409144, US6013861, US5824790, JP06070779, US6130367, WO9720936, EP779363, WO09726362, WO09744472, WO09745545, WO9844780, WO9924575, WO9966050, WO006755, WO031274, and WO112826.

5.4.3 Modification of SBE Activity

Modifications in branching enzyme activity result in changes in apparent amylose content which are believed to be due to an increase in amylose and to changes in amylopectin structure. The first naturally high amylose starch was reported 50 years ago when Vinyard and Bear successfully found a maize endosperm mutant termed *amylose extender* (*ae*) (12,227). Despite extensive research, kernels containing exclusively amylose as the reserve starch polysaccharide have never been found (197,201). The relationship between the Ae mutation and SBE activity was proposed nearly 30 years ago (25). We now know that the structural gene for SBEIIb is the *Ae* locus (205), which when mutated produces *ae* mutant starch having an amylose content of up to 50%. Although the biochemical basis of maize starches having amylose contents above 50% clearly requires the *ae* mutant (SBEIIb), the biochemical basis of the additional increases over 50% amylose is not clear at the present time (201). Furthermore, it does not appear as though SBI is involved, as SBI mutants do not have increased amylose alone or in combination with SBEIIb mutants (20,250). Similarly, SBEIIa mutants (21) had no detectable effects on seed starch structure.

Irrespective of the means of defining or partitioning *ae* starch into amylose and amylopectin components, it is clear that the relatively clear demarcation between amylose and amylopectin existing for normal starch is blurred for *ae* starch, giving rise to a third type of starch termed intermediate starch. Additional evidence for this comes from material collected as amylopectin from *wxae* intermediate starch which has no GBSSI activity and hence produces no true amylose, and has reduced amylopectin branching (61,104,116,135,196,254). Further, the beta-amylolysis limits for *wx* starch, *wxae* starch, and the amylopectins from normal maize and *ae* starches are all between 55 and 61% (259, 260). Thus, both the average exterior chain length and average interior chains length are proportionally longer for the amylopectin from *ae*-containing starches and the double mutant *wxae* starch than for *wx* starch and the amylopectin for normal maize starch. The combination of the absent GBSSI and BEIIb activity in the *wxae* double mutant produces an amylopectin which has sufficient linearity to have apparent amylose content approximately 20% as measured by iodine binding (25).

SBEIIb mutants also exist in rice (162) but there appear to be pleiotropic effects that complicate directly linking SBE activity with increased amylose. Rice SBEI mutants have

also been found (189) which, like maize, do not have increased amylose content and do not further increase amylose when combined with SBEIIb mutants. High amylose rice starches appear to have amylose contents between 30 and 50% of the weight of the starch (226). However, with the variability of amylose content of what may be considered normal rice varieties (19,173,210) and the recent implication that other starch biosynthetic enzymes affect the amylose content of such normal rice starch (221) with a few exceptions (146,147,225), it is difficult to know whether deficiencies in SBE are explicitly responsible for some of these high amylose rice starches. In such instances, the rice starch granules have the characteristic changes in starch properties, amylose properties, and amylopectin structure seen with high amylose maize starches (6,225,249). Similar to rice, high amylose barley starches with an amylose content between 30 and 45% of the weight of the starch clearly exist (184,203,252), although it is unknown whether these starches are a result of down regulation of SBE activity or are a result of changes in the expression and activity of other enzymes.

Reports of high amylose potato starch obtained through transgenic down regulation of multiple starch branching enzymes have been published recently (85,107,182,193). Work on development of a high amylose potato starch has been occurring for at least the past 10 years. Amylose contents as high as commercially available maize starches have been obtained in potato with a decrease in the overall molecular weight distribution.

In recent years a number of patents have been filed covering genetic modifications of branching enzymes in plants, utilizing knowledge gained after cloning, sequencing and transforming plants with the SBEs. Examples include US6013861, WO9211375, WO9214827, WO09507355, WO9634968, WO9722703, WO9720040, WO9820145, WO9837214, WO9837213, WO9914314, WO9964562, WO015810, WO031282, WO022140, JP0621767, JP06098656, JP05317057, and JP04088987.

5.4.4 Modification of DeBE Activity

Modifications in debranching enzyme activity can result in significant changes in starch granule structure. ISAI mutants accumulate starch in compound instead of simple starch granules (compound refers to amyloplasts containing many small granules, while simple refers to amyloplasts containing one major granule) and sometimes also accumulate phytoglycogen (a highly branched nongranular storage product). Mutants known in ISAI include the sugary1 locus in maize and sugary of rice (100,122) and in barley by lines named *Riso17* and *Notch-2* (28). Similar results were observed using antisense technology to reduce ISAI activity in rice (59). In potato where antisense constructs for ISAI and ISAII were combined, the tubers accumulated large numbers of small granules (28,29,122). Mutations in PUI have been identified (47), but effects on starch content are minimal. Modifications in the other isoamylase (ISAII and ISAIII) have not yet been identified, although there is some evidence that they each play distinct roles in starch synthesis (90). The maize sugary mutants are important because they are one of the main sources of producing sweet corn.

In recent years a number of patents have been filed covering genetic modifications of debranching enzymes in plants, utilizing knowledge gained after cloning, sequencing and transforming plants with the ISA and PU enzymes. Examples include WO9202614, WO09504826, WO09619581, US5750876, WO9603513, US5912413, WO09732985, WO09742328, WO9850562, WO09906575, WO9912950, WO09958690, and WO0001796.

5.4.5 Modification of Multiple Pathway Enzymes

By eliminating multiple starch biosynthesis enzymes, other alterations of the starch biosynthetic pathway can occur resulting in even more novel starches. Several patents exist

on the creation and use of such starches (US4428972, 4615888, 4767849, 4789557, 4789738, 4801470, 5009911, and 5482560). More recently, several patents and published applications have described the production and utilization of heterozygous combinations of mutations in the starch biosynthetic pathway to obtain commercially useful starches (WO9535026, US5356655, US5502270, and 5516939). The production of many of these starches involves the use of double or triple mutant plants. Due to the number of mutations required to sufficiently alter the starch (at least 2 or 3 within a single plant) many of these starches are difficult and costly to produce commercially, so many of these starches from plants with mutations in the starch biosynthetic pathway are uncompetitive with chemically modified starches. Further, these combinations of two or more mutations, whether they are combined homozygously or heterozygously in the plant endosperm, rely on the alteration of the structure of amylopectin from normal or waxy starch.

5.5 FUNCTIONALITY

The origin of wide angle x-ray scattering (WAXS) diffraction patterns from amylose fibers was determined to be due to the crystalline packing of double helices of starch chains (242,243). This work was further developed to explain the crystallinity of starch granules. When starch granules are heated sufficiently in excess water, the WAXS diffraction pattern is lost (257) and an endothermic transition is observed (39). Double helical order is additionally lost during this endothermic event, and it is this loss and not the loss of crystalline order that is believed to be responsible for the endothermic event (39). This loss of granular, crystalline, and double helical order is called gelatinization, and it is irreversible with respect to the three dimensional granule organization (7). Subsequent to gelatinization, starch chains of both amylose and amylopectin may organize into new double helical and crystalline structures in a process called retrogradation (7,70,176).

In its native granular form mixed with water, starch makes a high solids mixture, useful in making batters and doughs. During heating, starch granules swell, associating with water up to 30-fold its dry weight, to form a hydrated starch. This process is reversible provided temperatures and pressures are lower than those that lead to disruption of the organized intramolecular glucan chain associations within the granule. Above these temperatures and pressures, the granules will undergo irreversible changes and glucan chains will dissociate, permitting further swelling. With additional heating and applied shear forces, swollen hydrated granules will eventually collapse to form an unorganized paste of starch molecules. Such hydration, with heating, results in a thickening effect, imparting texture and structure. In functional terms, this process of starch granule swelling and dissociation is known as gelatinization (7,18,214,238). The glucan polymers of a gelatinized starch are able to associate with each other or other components of food to impart additional character to the food system including stickiness, tackiness or a rubbery texture (258). In addition, amylose readily leaches out of the granule during gelatinization and interacts with the other food components (17,53,165). Under mild cooking conditions, amylopectin does not so readily leach from the granule and hence is enriched inside the gelatinized granule. However, in more extreme cooking conditions, the amylopectin is much more dispersed, resulting in products with a tacky or sticky character.

Once a cooked starch is allowed to cool, the glucan chains begin to reassociate in a process resulting in a change in the functional characteristics, including a decrease in paste clarity and gelation of the paste. In functional terms, this process of reorganization is known as retrogradation (7,70,176). Normal starches are generally recognized for their ability to retrograde within hours (175) and usually form opaque gels (41). Retrogradation

of amylopectin gels occurs during days or weeks of storage. As a consequence of their different molecular weights and chain length profiles, the rates of retrogradation of amylose and amylopectin are not the same. The rapid setting of the structure of breads is believed to be due to rapid (within seconds or minutes) amylose retrogradation to form a network structure. Starch functionality is therefore a consequence of the degree of gelatinization and is influenced by retrogradation, time, temperature, concentration, and the presence of other food components or additives. In addition, modifying starch using chemical, enzymatic, or physical treatments alters and extends its functional properties.

Measuring starch functionality and applying it to food applications is problematic because the results are subject to extrapolation to systems and processes which are far more complex than laboratory testing is able to emulate. Analytical instruments (e.g., Differential Scanning Calorimetry, DSC) are frequently used to quantify the temperature range and amount of energy needed to melt crystalline starch. The amounts and molecular size of amylose and amylopectin may be measured using gel permeation or size exclusion chromatography. Granule size and shape are measured microscopically or by light diffraction techniques, and granule viscosity is measured using various rheometers. Rheological measurements may include various temperature and shear programs that attempt to mimic thermal treatments, pumping, and shearing forces that occur during food processing. Such measurements of texture provide information on adhesiveness, cohesiveness, yield stress, viscous flow, and rigidity of starch sols and gels.

5.5.1 Amylopectin Retrogradation

Because retrogradation manifests itself in many ways and over many different spatial scales, establishing relationships between the structural orders and the physical properties of the starch are complicated. Thus, a number of techniques are required for such an assessment. NMR has been used to probe the most fundamental physical order of the starch: double helical order (71), and x-ray diffraction has been used to examine the crystallinity of starches (114). However, neither of these two measures of starch order adequately represents the rheological properties of a cooked starch paste because rheology is dependent on not just the order of the starch but also the larger scale interrelationships of this order with the covalent structure of the starch molecules themselves. There are additional problems that make relationships between low level molecular order and larger scale order difficult to establish: residual granular order (41,141,153) or polymer incompatibility (49,66,112) may result in macromolecular heterogeneity in the system. To avoid these complications, many investigators interested in starch retrogradation have examined the behavior of waxy-type starches (16,30,57,134,135,144,154,175,176,195,253). Likewise, the properties of amylopectin free amylose have been studied either by preparing synthetic amylose using glucose-1-phosphate and the enzyme phosphorylase (35,68,70,180), or by studying highly pure amylose fractionated from the native starch mixtures of amylose and amylopectin (116,126,209,239). Amylopectin retrogradation has been examined by multiple techniques including DSC (57,95,137,169,196,254), WAXS (175,176,217), turbidimetry (98,176), and rheology (16,30,55,111,117,176,253).

The length of a double helix, whether in a starch granule or in a retrograded starch, is limited by the length of the external chains participating in the double helix. Linear chain lengths of amylopectin are generally shorter than the DP 40 to DP 70 lengths observed for amylose double helices (103,129,138). Amylopectin double helices are generally observed to dissociate below 70°C by DSC (39) when the starch is in excess water (<30% starch). The DSC endotherm of retrograded amylopectins containing a higher proportion of longer chains than normal amylopectin (i.e., amylopectin from some mutant starches) may extend above 100°C. The endotherms from these mutant starches are also broader than an endotherm

observed for retrograded amylopectin from normal starch, perhaps indicating that amylopectin with longer external chains has an ability to form a broader distribution of double helical lengths than amylopectin with shorter chains. The absolute lower limit for the length of a stable double helix appears to be six anhydroglucose residues (69), the length required to complete one turn of a chain in a double helix (242,243). Practically, the lower limit appears to be closer to ten anhydroglucose units, given that in systems of a pure oligosaccharide, chains with a length (or degree of polymerization, DP) less than ten anhydroglucose units could not self associate into stable double helices and crystallize (69). However, this minimum appears to be somewhat dependent on the lengths of other chains available for double helix formation, because in mixed systems of short chain oligosaccharides chains longer than a DP of 6 may form double helices with longer chains.

As with double helical and crystalline formation with oligosaccharides, the ability for amylopectin chains to retrograde is closely related to the average external chain length (ECL) of the molecules. With stubs of DP 1, 2, or 3 anhydroglucose residues as external chains, β-limit dextrins of amylopectin do not retrograde after one month of storage at 4°C (176). Shortening the average ECL by just three anhydroglucose units (from 14 to 11) by alpha-amylase was sufficient to reduce the enthalpy and solid character (assessed by DSC and pulsed NMR, respectively) to 10% of those of the native starch (12,244). Chromatography of the modified starches was instructive, because it showed that the component chains of the starch do not all need to be equally shortened to have an effect on starch retrogradation: some chains were reduce to a DP less than 5, with the remainder remaining largely unchanged (12,244).

Examination of starches with intact chains (135,196,254) has indicated that starches with high proportions of chains below a DP of 11 to 12 have a slower rate of retrogradation than those starches with fewer short chains, irrespective of concentration. The retrogradation rate of starches with higher proportions of shorter chains is also more concentration dependent than for those starches with fewer short chains (57,135). However, conclusions from these studies must be tempered by any possible confounding effects of additional changes in the fine structure of the amylopectin (e.g., branching pattern).

5.5.2 Amylose Gelation

Amylose retrogradation has been monitored by the development of supramolecular organization using turbidimetric and rheological methods (35,48,56,70,141,142). Gels of amylose have been suggested to develop within minutes through the retrogradation of amylose into double helices (35,70) or through an initial phase separation of amorphous amylose, followed by retrogradation into double helices in these concentrated amylose regions. The aggregation event is followed by an additional retrogradation event: the development of double helical crystallites within hours (91,128,141,142). Increasing the chain length and concentration of the amylose both have a destabilizing effect on amorphous amylose in solution (70).

Destabilization of amylose may lead to retrogradation in the form of gelation, precipitation, or both. The relatively long, stable double helices of amylose result in more thermally stable gels than gels of amylopectin. In the formation of amylose gels, one outcome of amylose retrogradation, gel development is understood to be related to the ability of a single molecule to interact in double helical junction zones which bind molecules together into a three dimensional network structure, and the subsequent ability for the double helices to either orient or aggregate once formed (70,128). For synthetic, completely linear amylose, the network formation is possible for amylose molecules with a DP above 100 because these molecules are able to interact with more than one additional molecule to form a network (70). Precipitation, the other outcome of amylose retrogradation, is

favored over gelation for amylose molecules below a DP of 250, gelation is favored over precipitation for amylose molecules above a DP of 1100, and both network forming (gelation) and breaking (precipitation) retrogradation phenomena are similarly favored for amylose molecules with a DP of 440 and 660 (35). The concentration of amylose also influences whether amylose will gel or precipitate. Higher concentrations of amylose result in an increased frequency for chain to chain interactions, which would both increase the rate of gel development and decrease the time required for the onset of a three dimensional network.

Amylose gels prepared in water have been described as phase separated systems consisting of a solvent rich phase and a filamentous polymer rich phase consisting of bundles of amylose chains (74,91,129,222). Portions of amylose chains that participate in the double helices residing in the crystalline regions of amylose gels have been estimated to range in length between DP 40 and DP 70 (103,128,137,138). As measured by DSC, the double helices of amylose appear to dissociate over a very broad temperature range (~50°C) with an endothermic peak temperature between 120°C and 160°C (23,37,117,129,202).

5.5.3 Gelation of Mixtures of Amylose and Amylopectin

Understanding the retrogradation behavior of normal starches, considered as mixtures of amylose and amylopectin, is even more complex than understanding amylose or amylopectin retrogradation in isolation. This is due to the possibilities for phase separation of the two types of molecules and for interactions between amylose and amylopectin to develop.

Cooked normal starches have been considered phase separated systems of swollen granules consisting of amylopectin trapped within a continuous gel matrix of leached amylose (141,153,167). In these systems, the amylose and amylopectin behaviors appear to be relatively independent of each other: the amylose forms a thermally irreversible (at $<$100°C) gel network within hours, followed by a thermally reversible increase in gel elastic modulus attributable to an increase in granule rigidity due to amylopectin retrogradation (113,141,167). In the absence of granular order, amylose and amylopectin mixtures have been shown to phase separate over time (66,111). Examination of gels of artificial mixed systems of amylose and amylopectin showed that over a small increase (10–15% w/w) in the amylose content of gels (from 25 to 40% amylose on a total starch basis), the gels developed the behavior of pure amylose gels in their rate of turbidity development (49), their elastic modulus, and their susceptibility to enzymatic and acid hydrolysis (128). Both groups suggested that a phase inversion from an amylopectin to an amylose continuous phase at an amylose to amylopectin ratio of about 30:70 or about 17:83 was the cause of the observed differences in gel characteristics at high and low amylose to amylopectin ratios. The differences may have been with the starches and concentrations used, with the methods of preparation of both the amylose and amylopectin prior to mixing, and the method of mixing the amylose and amylopectin. From examination of the fracture of gels of jet cooked high amylose maize starches prepared between temperatures of 121°C and 166°C, it was hypothesized (33)that higher processing temperatures resulted in more homogeneous mixtures of amylose and amylopectin than processing at lower temperatures, and that the more homogeneous mixtures would take longer to phase separate and gel during cooling at a constant rate. The delay in phase separation during cooling was thought to result in phase separation and gelation at lower temperatures, resulting in weaker gels due to the more rapid formation of a less perfect gel network.

Others have suggested that amylose and amylopectin interact more extensively than suggested by the relatively independent behavior of mixtures of phase separated amylose and amylopectin (101,117,168,190) and that the short chains of branched molecules interfere with the molecular association of amylose molecules (23,117). In gels prepared from reconstituted mixtures of amylose and amylopectin, the chain length distribution of the

amylopectin, the size of the amylopectin, and the amylose content appear to influence the formation and properties of starch gels. From examination of gels formed by waxy maize starch (considered amylopectin) and oligosaccharide chains with a DP of 21 or 35, linear chains as short as DP 21 appear sufficient to form junction zones with multiple amylopectin chains resulting in a gel (190). Acetylation, substitution of the hydroxyl groups of the starch with structure-inhibiting moieties, also appear to destroy the gel-forming ability of the amylopectin, and the addition of amylose instead of the oligosaccharides appears to result in stronger gels. Substitution was suggested to limit associations preventing gelation, but was not sufficiently high to prevent gelation in the presence of amylose or short, linear chains (190). Using a variety of amylopectin sources with different chain lengths and a consistent source of amylose and ratio of amylose to amylopectin ratio (20:80), the strongest gels appear to form from the mixtures containing the amylopectin with the longest chains (101). Using 25:75 or 50:50 (w/w) mixtures of amylose with either *wx* starch or small, medium, or large dextrins prepared from waxy maize starch, gels prepared with the medium sized dextrins, irrespective of amylose content, formed weaker gels than gels prepared from the other dextrins or the *wx* starch (168). Examination of starches by DSC shows that amylose appears to retrograde independently when the starch is heated to temperatures below 140°C, but does not do so when heated to temperatures above 160°C in an excess water environment (23). Further, dynamic shear rheology of these same starches indicated that the length of these double helices formed between amylose and amylopectin was dictated by the length of the external chains of the amylopectin: when gels prepared from mixtures of completely dispersed starches were heated, the decrease in the elastic modulus paralleled the decrease in the elastic modulus for the amylopectin isolated from the starch (117).

5.6 APPLICATIONS

As well as providing essential calories, starches from different crops play an important role in foods, such as improving processing, shelf life, consistency and appearance by providing texture and thickening capability for suspending solids. There are myriads of applications of starches in foods and these uses have been extended due to application work by food technologists using physical and chemical modifications (designated by E-numbers in the EU). These include oxidation (E1404, E1451), monostarch phosphorylation (E1410), distarch phosphorylation (E1412), acetylation (E1414, E1420, E1422), hydroxypropylation (E1440, E1442), and octenylsuccination (E1450). In this section, we will focus on starch applications of various genetic modifications of the major crop plants and how these modifications impact the uses of these starches in foods. In particular we will emphasize modifications in amylose and amylopectin content and molecular weight, amylopectin chain length, starch granule size and morphology, crystal structure and phosphate content. We will conclude with a discussion of new opportunities for enhanced starches resulting from new genetic modifications of plants.

5.6.1 Amylose Free Starch

The most widely used amylose free starches are obtained from waxy maize and rice and in all cases are considered non-GM natural variants of normal maize and rice. Staining starch with iodine readily identifies amylose free types: normal starch will stain blue or purple whereas the starch produced from amylose free plants will stain red or brown or brownish red in color. Amylose free maize starches are generally considered to contain zero or almost zero amylose. Amylose free rice starches have been shown to contain

between 0 and 3% amylose, though collectively these starches are referred to as amylose free or waxy rice starches (109,173,185). With these amylose free rice starches, it has been assumed that the differing cooking and paste properties are due to differences in the structure of the amylopectin of the starch rather than variations in the low levels of amylose of the starch (234). The effects of amylose and other molecular and compositional characteristics of rice starches on rice (19,34) or rice starch properties remain unclear (125).

Amylose free starches have useful functionality that has encouraged their commercial development. They are considered useful as water binders, viscosity builders, and texturizers in food as well as in industrial applications (171). However, these starches are less resistant to shear, acid, and high temperatures than are normal starches, and extended cooking results in stringy, cohesive pastes. Amylose free starches are generally recognized for their improved transparency after processing compared to normal starches (41) and have better freeze and thaw stability compared to normal starches once cooked (171,237). They are also recognized for their improved long term storage capability as they require weeks to gel if they could be considered to gel at all (15,253). To correct for some of the negative paste attributes of amylose free starch, such as poor stability to temperature, shear and acid and undesirable paste quality, most amylose free starches are chemically modified by substitution, cross linking, or both (171,238,256).

In recent years there has been significant interest in developing amylose free starches in other crops so as to take further advantage of any species specific qualities of the starch produced from that species. However, although mutants are readily found in some plant species (such as maize, rice, and barley) this is more difficult in other plant species where there are multiple copies of each gene (such as the polyploid species like potato, oats, and wheat). In the case of wheat, the advances have been made by screening for mutants (non-GM), while in potato the waxy types were made using biotechnology (GM). Applications of amylose free wheat and potato are at present still being developed. However, the most likely applications for amylose free potato include the paper, adhesive, textile, and packing industries. In the EU, certain GM varieties of modified amylose free (high amylopectin) potato have not been approved thusfar (http://europa.eu.int/comm/food/fs/sc/scp/out24_en.html), while others appear to be in the approval process (http://europa.eu.int/comm/food/fs/sc/scp/out129_gmo_en.pdf). Amylose free wheats are finding applications in foods such as noodles and baked goods including breads (5,247).

5.6.2 Low Amylose Starch

Although staining starch with iodine readily identifies amylose free starches (161), care must be taken when using this quantitatively to adequately account for the iodine binding capacity of the amylopectin (120). For example, amylopectin from an amylose free plant having an inactive GBSSI enzyme might appear to contain 5% amylose based on iodine binding, blue value, measurement (116). After carefully considering these potential problems certain low amylose starches have been identified. For example, in the early 1940s, a waxy maize mutant (wx^a) was discovered in two exotic Argentinean small seeded flint varieties that contained a starch that had an amylose content of 2.4% and stained a pale violet color with iodine (26). Additionally, the amylose content of the starch increased from 0% (full waxy) to 0.65% to 1.3% to 2.4% (full wx^a) with increasing dose of the wx^a allele (26,204). With these same crosses, the viscosity of starch pastes decreased with increasing dose of the wx^a allele. The wx^a allele was described as resulting in a 95% reduction in the amount of GBSSI protein produced and a starch with a low amylose content (174, 204,261).

Wheat starches have been produced with amylose contents of about 7.5% and 13.5% by crossing normal wheat with amylose free wheat (188). Peak viscosities of all starches

differed by less than 20% of the peak viscosity of the amylose free wheat starch, with the low amylose starches having a higher peak viscosity than both normal and waxy wheat starch. The gelatinization temperatures and enthalpy were highest for waxy wheats and decreased in the order waxy > 13.5%, amylose wheat > 7.5%, and amylose wheat > normal wheat starch. The retrogradation temperatures and enthalpy were insignificantly different for amylose free wheat, normal wheat, or any of the low amylose wheat starches. Starch granules extracted from a wheat strain derived from mutagenized Tanikei A6099 had an apparent amylose content of 1.6% and stained dark brown with dark cores compared to red-staining waxy wheat starch with 0.4% apparent amylose (115). The Tanikei A6099 mutant wheat starch had a higher initial pasting stability than an amylose free wheat starch (0.4% amylose). However, the viscosity of the low amylose starch paste decreased dramatically, to the same viscosity as the amylose free wheat, during continued cooking and remained at the same viscosity as amylose free wheat after cooking. The mutagenized Tanikei A6099 wheat is known to produce a mutant GBSSI enzyme (115,248).

By screening microtubers from plants exposed to x-ray radiation an amylose free mutant of potato was identified (88). Potato starches considered amylose free have been shown to have an amylose content varying from 0 to 7.9% (183,204,223,224). The amylose free potatoes were null for the GBSSI enzyme. Staining with iodine gave varied results: sometimes the starch stained red and other times reddish brown and blue. These results are indicative of a heterogeneous mixture of amylose free starch and amylose containing starch of unknown quality within the potato tuber. In further attempts to understand the link between function and activity of GBSSI in potato, antisense transgenic plants having amylose contents between 3.0 and 8% were produced (123,224,228). These tubers had both blue and red brown staining portions (228) again indicative of heterogeneous mixtures of amylose free starch and amylose containing starch of unknown quality. Others (123) observed additional heterogeneity at a granule level, with starch granules having blue cores surrounded by a red brown shell of starch. The size of the blue core appeared to be correlated with the amylose content of the starch**.** Starch extracted from plants produced from crosses between an amylose free potato and a normal potato had no linear correlation between GBSS activity and amylose content (58). Additionally it was observed that the swelling and rheological properties of the granules could not be clearly linked with amylose content (183).

The waxy barley starches have been shown to contain up to approximately 5% apparent amylose (215). However, this apparent amylose is due to a mixture of starch granules within the barley seeds. The amylose content of the granules typically ranges from an undetectable level up to approximately 10%, with the granules closest to the surface of the seed having the highest amylose content (3). Recent work with waxy barley starch (with amylose contents up to 6.44%) shows delayed peak viscosity development and viscosity varying during cooking under shear (132). Additionally, all of the waxy barley starches began to develop viscosity at a similar time and temperature in the cooking process.

Low amylose rice starches have been shown to have amylose contents between 7 and 15% (124). Shimada et al. (199) produced several antisense rice plants with starch having an amylose contents between 6 and 13%. The iodine staining qualities of these starch granules were not reported. Further, any cooking properties of the starches, the elastic properties and gelling abilities of pastes and the gel properties of gels produced from these low amylose rice starches produced by transgenic rice plants are unknown.

5.6.3 High Amylose Starch

Commercialized high amylose maize starch is a result of down regulation of the maize BEIIb enzyme, utilizing the *amylose extender* mutation. High amylose maize starches

bring important differentiated properties for food applications, having amylose contents between 30 and 90% of the weight of the starch. The starch imparts gelling ability to the food system, improving adhesion to water impermeable surfaces and altering product texture. These starches also have improved film forming ability and improved fat impermeability compared to the normal starch counterparts. High amylose starches provide firmness, extend cooking times and increase the crispiness of coatings.

Because of their high resistance to processing and subsequent digestion, in addition to their rapid retrogradation if gelatinization occurs, high amylose maize starches are also being utilized as a source of resistant starch in a number of food products. Reviews (43,79,93) provide an overview of resistant starch and its valued physiological benefits in foods. Commercialized resistant starches include unprocessed starches from different botanical sources (e.g., green banana, legumes, potato) as well as more highly processed forms of high amylose starches. High amylose starches from other sources are also being advocated for their resistant starch properties.

The most widely available high amylose starches originate from maize using the *amylose extender* mutation that results in a starch having 40–50% amylose. As a result of extensive breeding and selection work higher amylose contents (up to 90%) have been achieved (201). High amylose starches are also available in barley and rice.

5.6.4 Amylopectin Chain Length

As already stated, care must be taken when using the iodine binding assay for quantifying amylose content. In a more extreme example of this problem, the combination of the absent GBSSI activity and the *ae* mutation produces an amylose free maize starch that stains blue or purple and appears to have an amylose content of 15–26% (194). This is because the *ae* mutation causes a decrease in starch branching enzyme activity (24), which results in the formation of long chain amylopectin (104), which will itself stain blue with iodine. Other examples of this come from genetic modifications that affect amylopectin structure.

Loss of SSIIa activity in maize results in a starch with an amylose content near 40% of the total starch weight. This starch develops viscosity very slowly at temperatures above 90°C and forms stable gels that strengthen only very slowly compared to normal starch gels (31). The loss of SSIIa activity resulting in elevated amylose contents highlights the often observed disconnect between the viscosity development of the starch and the thermally detected gelatinization of the starch. Despite the resistance of the starch to develop viscosity, likely a consequence of the elevated amylose content, loss of SSIIa activity results in a decrease in the gelatinization temperature range of the starch from about 70°C to 80°C for normal starch to approximately 55°C to 65°C (31,21). Examination of the chain length distribution of the maize starch in combination with the absence of amylose, as a result of the inactivity of GBSSI, indicates that the starch has a elevated proportion of short component chains below a DP of 30 compared to normal starch (212) and additionally an elevated proportion of very short component chains below a DP of 10 compared to normal starch (94,134). This high proportion of very short chains imparts a decreased tendency to retrograde compared to normal starch (134,135).

Recently, potatoes have been engineered to eliminate both SSIIa and GBSSI activity, resulting in an increase in short chains below a DP of 14 compared with normal starch (108). This had the benefit of a decrease in the tendency for the starch to retrograde after cooking which may have implications for improved freeze and thaw tolerance. Potatoes engineered to have reduced SSIII activity had decreased amounts of chains longer than DP 17 (60).

The SSIIa enzyme of rice has been implicated as one of the major enzymes that affects whether the grain is of the indica-type vs. the japonica-type (221). Indica-type rices have been long known to have higher kernel integrity and higher granule stability than

japonica-type rices (131). The properties of indica rice starches have been attributed to their higher proportion of longer chains than japonica-type rice starches.

It has been known for some time that the inactivation of SSIII in the dull mutant of maize results in an elevation in the apparent amylose content to 30 to 40% (81). Beyond this, a number of chromatographic studies on dull starches, including those additionally lacking GBSSI enzyme activity, indicate that the chain distribution of maize starch is only slightly different from normal starch, with some elevation in the chains with a DP less than 30 to 50 (94,196,254). The beta-limit dextrins, starches with the exterior chains digested to stubs of maltose to maltotriose, provide a more complete picture of the changes observed with elimination of SSIII in maize. In this case the lengths of the residual chains containing all of the branching are considerably shorter when SSIII is absent during synthesis. It has been suggested (135) that this change has implications for the retrogradation of starch produced in the absence of SSIII. Thus, during gelatinization and then continued heating after gelatinization, the starch chains are less able to randomly orient themselves compared with starches having longer spacing between branch points (i.e., normal or waxy starch). Thus, the retrogradation rate of *du wx* starch is less concentration dependent than *wx* starch despite the similar chain length distributions of the two starches after debranching.

5.6.5 Novel Starches

Recent developments in biotechnology are opening ways of making novel starches in plants. Thus starches obtained from different crops having varied functionalities due to differences in composition may now theoretically be readily transferred into crops from one or another different plant species. In recent years a number of patents have been filed covering novel genetic modifications of starch in plants. Examples include WO09711188, WO008184, WO09961580, WO9814601, WO9404692, WO09601904, WO09729186, WO014246, WO9924593, WO9839460, WO047727, WO011192, WO9929875, WO9747806, WO9747807, WO9747808, and WO9822604. In general we can consider making changes in any aspect of amylose/amylopectin, phosphate, protein, phospholipids, crystallinity, gelation, and pasting characteristics, flavor and starch granule morphology. One merely has to understand the genetic basis of the differences.

One interesting example of a cross species difference that may be exploited is phosphate content in potato. Recent work (177) has shown that starch phosphate content may be attributed to a novel glucan water dikinase enzyme. This discovery opens up the possibility of creating novel genetically modified starches having varied phosphate contents. Although it is too early to conclude whether there would be useful applications in food, possibilities include changing starch digestibility or starch viscosity after cooking. Another interesting character in one species that is not available in others is associated with the genes responsible for endosperm texture in some cereal crops. In this case the puroindoline genes, described as PinA and PinB are interesting candidates (14,72,86,152). Transforming the wheat genes into rice successfully created the softness trait in the seed (121). At this time it is still too early to say how useful this character will be in food applications.

Although starch granule morphology varies extensively across species (102) and starch from different species is highly valued for certain applications, there are few specific reports that granule morphology is the attribute desired for valuable food applications. Furthermore, although there have been some reports of progress in understanding the mechanisms that control and influence starch granule size (28,29,122), for granule morphology there is little that can be defined as clear enough to arouse interest in a genetic approach. Similarly, flavor can be considered to vary extensively across species and a bland flavor of (e.g., tapioca starch) is valued over certain cereal starches that impart a mealy character. However, as with granule morphology, our understanding of the genetic components

determining this character is rather poor, making flavor a difficult target for genetic modification at the present time. Thus in these cases it is difficult to see a way of using such differences in granule morphology and flavor to exploit that character in another species.

Another novel concept has come from bifunctional domains involving cellulose and starch, which may enable developments with novel biomaterials (130). Another possibility is to explore incorporation of inulins with starch (80). Even more novel possibilities to consider include adding new functionality to starch using gene constructs containing fusion proteins (106). The fusion proteins can be proteins, which might affect starch properties or enzymes, or other bioactive proteins.

ACKNOWLEDGMENTS

The authors wish to thank Dr Kay Denyer for comments and constructive criticism of the manuscript. All starch structure diagrams kindly supplied by Dr Eric Bertoft (with permission).

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