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Starch biogenesis: relationship between starch structures and starch biosynthetic enzymes

Li Li
Iowa State University

Starch biogenesis: relationship between starch structures and starch biosynthetic enzymes

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Li Li

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Program of Study Committee:
Jay-lin Jane, Major Professor
Charles E. Brummer
Clark F. Ford
Martha G. James
Martin H. Spalding
Pamela J. White

Iowa State University

Ames, Iowa

2006

Graduate College
Iowa State University

This is to certify that the doctoral dissertation of

Li Li

has met the dissertation requirements of Iowa State University

Signature was redacted for privacy.

Committee member

Signature was redacted for privacy.

Committee member

Signature was redacted for privacy.

Committee member

Signature was redacted for privacy.

Committee member

Signature was redacted for privacy.

Committee member

Signature was redacted for privacy.

Major Professor

Signature was redacted for privacy.

For the Major Program

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ABSTRACT

The goal of this research is to gain understanding on starch biogenesis by studying starch granule formation and development. The objectives of this research were to modify the structure of cyanobacteria glycogen by replacing the endogenous glycogen synthase gene with a plant starch synthase and to study starch granule formation by creating a plant starch biosynthetic system in a cyanobacterium, and to investigate the development of starch granules in maize endosperm during maturation of the maize kernel.

Cyanobacterium *Synechocystis* sp. PCC6803 is used as a model system to study starch biosynthesis and starch biosynthetic enzymes. A potato starch synthase III (PSSIII) was expressed in *Synechocystis* mutants without glycogen synthase I (M1) or glycogen synthase II (M2) to produce two new mutants PM1 and PM2. The mutants PM12 and PM21, containing only PSSIII as glycogen/starch synthase, were derived from PM1 and PM2, respectively. Results indicated that the survival of *Synechocystis* required the existence of glycogen/starch synthase. The disruption of GSII and the expression of PSSIII were coupled with the increase of glycogen branching enzyme activity. Compared with WT glycogen, PM1, PM2, PM12 and PM21 produced glucans with more short chains and fewer long chains, which is consistent with the increase of the branching enzyme activity in the mutants. Results indicated that there are regulatory and cooperative interactions among plant starch synthase, glycogen synthases, and glycogen branching enzyme in the process of the biosynthesis of glucans.

To study starch structures and properties along the development of starch granules, endosperm and pericarp starches were isolated from maize (B73) kernels at different

developmental stages. Maize endosperm starch content, granule size, and amylose content increased during the kernel development. The onset gelatinization temperature of the endosperm starch increased from 61.3°C (8DAP) to 69.0°C (14DAP) and then decreased to 67.4°C (30DAP). The percentage retrogradation of the endosperm starch after 7 days at 4°C increased from 26.4% (8DAP) to 48.7% (14DAP) and then decreased to 42.7% (30DAP). The amylopectin branch chain-length of the endosperm starch on 14DAP displayed the longest average chain length and the largest percentage of the long chain (DP≥37). In contrary, there were no significant changes in size, amylose content, starch content, and thermal properties of pericarp starches. Results indicated that the endosperm starch structure was not synthesized homogenously with the maturation of kernel.

CHAPTER 1. GENERAL INTRODUCTION

Introduction

Starch is the major form of carbohydrate reserve in higher plants. Transient starch is synthesized at daytime and consumed at night for plant respiration. Storage starch is the energy reserve for the reproduction of plants and is also an important energy source for humans and animals. Starch is a valuable ingredient in the food industry, it serves not only as a nutrient source for food and feed, but also as a thickener, a binding agent, a texturizer, a filler and a film forming agent in the food industry. A selection of starch varieties for different food products depends on starch functional properties, including viscosity, shear resistance, gelatinization properties, textures, solubility, tackiness, gel stability, and retrogradation rate. These functional properties are determined by the chemical structures of starch.

The functionality of starch is the key to determining its marketing value (Zobel, 1988). To produce starch with desired functionality for selected applications, chemical modifications are widely applied to improve the functional properties of starch. Chemical modifications, however, bring great concerns of food safety and environmental pollution. Genetic modification of the starch biosynthetic pathway is an alternative approach that may avoid those concerns. On the basis of understanding of the starch biosynthetic pathway, we anticipate that starch structures can be altered with the alteration of starch biosynthetic enzymes. To generate starch with a desired structure requires profound understanding of the starch biosynthetic system.

The formation and development of starch granules have been investigated in our research. To fully understand the starch biosynthetic system, it is essential to know the basic requirements for the formation of semi-crystalline starch granules. Starch synthesis system contains multiple isoforms of AGPase, SS, BE and DBE. Glycogen biosynthesis systems are much simpler than the starch biosynthesis system with fewer biosynthetic enzymes (Ball and Morell, 2003). With expression of plant starch synthetic enzymes in the glycogen biosynthetic system, we intend to reveal the evolution of starch biosynthesis and granule formation. In this dissertation, a plant starch biosynthetic enzyme, potato starch synthase III, was expressed in cyanobacterium *Synechocystis* sp. PCC6803, and the structures of glucans produced by the mutants are presented.

Surface gelatinization of potato and maize starches by saturated neutral salt reveals that internal structures of starch granules are not homogenous (Jane and Shen, 1993; Pan and Jane, 2000). It indicates that the biosynthesis of amylose and amylopectin is regulated during granule development. In this dissertation, the development of starch granules have been investigated by using maize endosperm starches isolated at different developmental stages. Physicochemical properties of endosperm and pericarp maize starches during maize maturation are presented. This study will be useful for the understanding of how starch biosynthetic enzymes control granule growth during the development of maize kernels.

Dissertation Organization

This dissertation contains five chapters. First two chapters are general introduction and literature review. Chapter three "Characterization of cyanobacteria *Synechocystis* sp. PCC6803 mutants containing Potato starch synthase III" describes the effects of the

expression of potato starch synthase III in *Synechocystis* on its glucans structures. Chapter four "Physicochemical properties of endosperm and pericarp starches during maize development" presents the granular structures and thermal properties of maize pericarp and endosperm starch isolated at different developmental stages. The dissertation ends with chapter five "General conclusion".

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CHAPTER 2. LITERATURE REVIEW

Starch is the second largest biomass next to cellulose produced on earth. It exists in a granular form in photosynthetic eukaryotes. The starch granule is semi-crystalline and insoluble in water. Thus, a large amount of energy can be stored in a relatively small volume. The physical structure and properties of starch facilitate starch extraction from storage organs of plants. Starch is one of the most important nutrients for human food and animal feed. The biosynthesis and the physicochemical properties of starch granules have been studied extensively. We know that specific combinations of starch biosynthetic enzymes produce starch granules with characteristic structures. In turn, the starch structures determine the physical properties and functionalities.

Starch Structure

Starch functions as both transient and storage carbohydrate in plants. It exists in all tissues including leaves, seeds, fruits, stems and roots. Although starch granules are present in different organs and have different shapes and sizes, they contain the same major components: amylose and amylopectin. Amylose is mainly linear molecules containing α-1,4 linked D-glucosyl unit and a few branches of α-1,6 linkage. Amylopectin is a highly branched molecule, consisting of about 5% of α-1,6 linkages. Without amylose, amylopectin alone is sufficient to form granules and is responsible for the granular crystallinity.

Native starch granules have a semi-crystalline structure. A range of 15-45% of crystallinity is observed for many starches (Zobel, 1988). X-ray diffraction patterns of native starches correlate to the amylopectin branch chain-length distributions (Jane et al., 1999). In

general, starches of the B-type X-ray pattern consist of longer average branch chain-length than that of the A-type. The differences in branch structures between A- and B-type starches were revealed by their Naegeli dextrins (Jane et al., 1997). The A-type starches have more branch linkages in the crystalline region than the B-type starches, which are not readily susceptible to acid hydrolysis. Thus Naegeli dextrins obtained from the A-type starches contain more branched chains. In the granule, amylose and amylopectin molecules are arranged radially, growing from the hilum with their non-reducing ends towards the surface of the granule. Amylose molecules are interspersed and intertwined with amylopectin molecules (Jane et al., 1992). Surface gelatinization of native starch granules using a saturated neutral salt solution reveals that the concentration of amylose inside the granule is not homogenous (Pan and Jane, 2000). There is more amylose on the periphery of starch granules than at the core. Amylopectin molecules at the core of starch granules have longer branch chains than those at the periphery.

Starch granules are organized in three levels: amylopectin clusters, blocklets, and growth rings (Myers et al., 2000). Branch chain-length distribution profiles of amylopectin show bimodal patterns, with more abundant short chains and fewer long chains (Jane et al., 1999). The cluster model suggests that amylopectin molecules align to form alternating crystalline and amorphous lamella (French, 1984). The length of the repeating unit (9-10nm) reveals the length of clustered amylopectin side chains. The short chains (A and B1 with DP 6-24) form helices in the repeating units; the long chains (B2, B3 and longer chains) extend through two, three, or more clusters (Hizukuri, 1985). The β–limit dextrins of amylopectin molecules show that A:B chain ratio is close to one (Yun and Matheson, 1993). The amorphous lamellae consist of mostly branch linkages, which are less densely packed than

the crystalline lamellae (Myers et al., 2000). Crystalline and amorphous lamellae organize into blocklets, which can organize further to form the semi-crystalline shells of growth rings (Myers et al., 2000). The packed granules also contain some minor components, such as lipids, phosphate derivatives and proteins.

Functional Properties of Starch

Starch has been widely used in food applications, such as a thickener, a gelling agent, a water absorber, or a major nutrition source. Starch can be processed with different water content and at different temperatures. During the process, starch undergoes a series of physicochemical transitions (Jane, 1997). Although starch is composed of hydrophilic molecules, starch granules are water insoluble at room temperature. When suspended in water at room temperature, starch granules absorb a limited amount of water and swell. This is a reversible process because only the amorphous regions of the starch granule are involved in water absorption (French, 1984). When the starch granules are heated in excess water to a specific temperature, the starch crystalline structure melts and starch granules lose their birefringency. This irreversible process is called gelatinization. When continuous heating and shearing are applied, starch granules continue to swell and develop viscosity to form starch paste. Upon cooling, amylose and amylopectin molecules reassociate to form starch gel. When starch paste is stored at a low temperature, some starch molecules go back to the ordered and crystalline structure; it is termed retrogradation.

Gelatinization

Gelatinization of starch results in loss of crystallinity. The hulim of the starch granule is loosely packed with less ordered structure. The melting of crystallites always starts at this

region (French, 1984). When a starch suspension is heated to a certain temperature, loss of birefringence, loss of crystallinity, rapid swelling, and endothermic transitions are first observed in some granules (French, 1984). Other granules require higher temperatures to disrupt their ordered structures. Thus, in a granule population, gelatinization usually is completed in a temperature range of 10-15°C. With the melting of crystallites, these substances are available for further hydration and swelling. In the mean time, amylose is leaked into the solution (Eliasson and Gudmundsson, 1996).

Gelatinization temperature of starch is influenced by many factors, including starch structures, annealing, and heat-moisture treatments. The crystalline structure of starch is the determining factor of gelatinization temperature. Studies have shown that starch having shorter long-B chains displayed a lower gelatinization temperature (Shi and Seib, 1992; Yuan et al., 1993; Jane et al., 1999). Vandeputte et al. (2003) reported that the percentage of B1 chain (DP 13-24) was positively correlated with the onset gelatinization temperature of rice starches, whereas that of the short chain (DP≤12) was negatively correlated with the onset gelatinization temperature of rice starches. Starch having more short chains DP≤12 and less long B chains indicates imperfect crystalline structure, which leads to lower gelatinization temperature. Jane et al. (1999) observed that B-type starch showed lower gelatinization temperature than A-type starch when they had the same chain length. They attributed this behavior to the tightly packed crystalline structure of A-type starch. Whittam et al. (1990) reported similar melting behavior of crystalline spherulites with A and B type polymorphs of starch.

Annealing is the process where starch is treated with excess or intermediate water content at a temperature below gelatinization and above the glass transition temperature.

Heat-moisture treatment is the process where starch is treated with limited water content (18-27%) at a higher temperature. Annealing increases onset gelatinization temperature and sharpens endothermic peak of gelatinization (Fisher and Thompson, 1997; Knutson, 1990)). The decrease in swelling power and solubility of starch is observed on annealed potato starch (Erlingen et al, 1997). The annealing temperature allows limited mobility of starch molecules, thus starch molecules can be reorganized and the order of crystalline approaches perfection (Knutson, 1990). There is no observation that annealing changes the X-ray diffraction pattern. Jacob and Delcour (1998) observed the increase of X-ray peak intensity on wheat and potato starches. It indicated that annealing improves the crystalline order, but has no effect on changing the polymorphic form. Unlike annealing, heat-moisture treatment causes change of the polymorphic form, resulting from the melting and reorganizing of the crystallites under the high temperature (Eliasson and Gudmundsson, 1996).

Pasting Properties

During the pasting process, a change of viscosity is observed. At the early stage of pasting, the increase of viscosity is attributed to the leaking of amylose (Eliasson and Gudmndsson, 1996). When the temperature reaches the gelatinization temperature, the crystallites melt and intermolecular hydrogen bonds and hydrophobic force are disrupted. The starch granule is hydrated fully and the interactions develop among the swollen granules and material diffused from granules to the medium. At this stage, the viscosity continues to increase. When shearing force is applied, viscosity decrease is due to the fragmentation of the swollen granules. On cooling, a gel network forms and the viscosity increases (French, 1984). The pasting properties of starch are influenced by the amylose content, amylopectin structure and the minor components in starch granules (Jane et al., 1999). Amylose can form

a complex with lipid, thus restricting the swelling of granules. In general, waxy starch shows higher peak viscosity and lower set back viscosity than normal starch. The very long branch-chains of some amylopectins function similar to amylose, complexing with lipids and restricting the swelling. Potato starch granules contain high phosphate-monoester content, generating charge repelling force. Thus, potato starch displays a very high peak viscosity (Jane et al, 1999).

Retrogradation

Gelatinized starch undergoes reorganization from an amorphous form to a partially ordered structure when it is stored at a low temperature. This process is called retrogradation. Amylose and amylopectin contribute to the retrogradation process in different manners (Hoover, 1995). The recrystallization of amylose occurs faster than that of amylopectin (Miles e al, 1985). Crystallinity of amylose gel approaches the maximal level in two days, whereas that of amylopectin gel reaches the maximal level in about 30-40 days (Ring et al, 1987). The crystallization process may involve a double helix formation, aggregation, and crosslinked network development (Sarko and Wu, 1978). The X-ray diffraction pattern of starch gel changes from V-pattern (amylose-lipid complex) to B-pattern upon aging. The crystallites of retrograded amylopectin are completely melted by heating below 95°C (Ring, 1985), however, it takes autoclaving temperature to dissociate retrograded amylose crystallites. Starch retrogradation is greatly effected by temperature, water content, amylose/amylopectin ratio, the structure of amylopectin, and the presence of other components (Eliasson and Gudmundsson, 1996).

Starch Biosynthesis

In plants, transient starch is synthesized in the chloroplasts of photosynthetically active leaves and storage starch is synthesized in the amyloplasts. The biosynthesis pathway of starch starts from the synthesis of ADP-glucose. ADP-glucose pyrophosphorylase (AGPase) catalyzes the synthesis of ADP-glucose using glucose-1-phosphate and ATP as substrates. Then ADP-glucose is incorporated onto the non-reducing ends of glucans by starch synthases. Branch enzyme catalyzes a two-step reaction, cutting α-1,4 linkages and forming α-1,6 branches. Debranching enzymes also play roles in starch biosynthesis.

ADP-glucose pyrophosphoryase

AGPase consists of large and small subunits, which are encoded by small multigene families expressed differently among species (Ball and Morell, 2003). For example, in potato, three genes encoding large subunits and one encoding small subunit were identified (Ball and Morell, 2003). In maize, there are at least three genes encoding small subunits and two encoding large subunits (Ball and Morell, 2003). Defects on the large and small subunit genes result in producing mutants, *Shrunken*-2 and *Brittle*-2, respectively. It was believed that AGPase was only located in the plastid until cytosolic form of AGPase was discovered in cereal endosperms (Thorbjornsen et al., 1996; Denyer et al., 1996). Plastidal AGPase is a key regulating enzyme in starch biosynthesis. It is activated by 3-PGA, inhibited by inorganic phosphate (Ghosh and Preiss, 1966) and is sensitive to redox state (Tiesen et al., 2002). The cytosolic form of AGPase, however, is less sensitive to the above situations (Gomez-Casati and Iglesia, 2002). Since cereal endosperm is a relatively stable storage organ, the dramatic change of AGPase activity is not required. The cytosolic form of AGPase may adapt to this environment.

Starch Synthase

Starch synthase catalyzes the reaction adding glucosyl units from ADP-glucose to the non-reducing end of glucan. In plants, there are multiple isoforms of starch synthases. Based on their reaction patterns, they are divided into two groups. The first group, granular bound starch synthase (GBSS), binds a glucan chain and elongates the chain continuously and is required for the synthesis of amylose. The second group is soluble starch synthase, which synthesizes linear portion of amylopectin molecules. At least four isoforms of soluble starch synthases have been identified; SSI, SSII, SSIII, and SSIV (Ball and Morell, 2003).

Waxy mutants of plants produce starches without amylose as a result of GBSSI deficiency. However, GBSSI contributes not only to amylose biosynthesis in storage organs, but also to amylopectin biosynthesis. Introducing GBSSI into *Chlamydomonas* has little or no affect on amylose synthesis, but produces amylopectin with higher percentage of long chains (Delrue et al., 1992). More evidence indicated that GBSS is necessary; but not sufficient for amylose synthesis. The supplies of ADP-glucose and malto-oligosaccharide regulate amylose synthesis. The reduction of the supply of ADP-glucose by suppressing AGPase and phosphoglucomutase decreases in the amylose content of pea starch (Clarke et al., 1999). The increase in the supply of malto-oligosaccharide by knocking out glycanotransferase leads to the increase of the amylose content of *Arabidopsis* leaf starch (Critchley et al., 2001).

In waxy mutants of wheat, barley and maize, starches from leaves, pericarp, and embryo sac were stained dark-blue by iodine instead of red-brown like that from the endosperm. These suggest that the biosynthetic pathway in the endosperm is different from

that in those tissues. Vrinten and Nakamura (2000) identified new GBSS isoforms, GBSSII, from waxy wheat. GBSSII is expressed in the non-storage tissues, but not in the endosperm.

SSI is identified from many plants, maize, rice, potato, wheat and Arabidopsis. SSI expresses in the endosperm of maize, rice, and wheat (Mu et al., 1994; Nakamura, 2002; Denyer et al, 1995). SSI of maize has a C-terminal glucan binding domain (Knight et al., 1998). No maize mutant of this enzyme was obtained. Maize SSI showed high affinity to the shortest glycogen chains. Its activity decreases when it binds to longer chains (Commuri et al., 2001). Amylopectin of rice SSI deficient mutant consists of fewer short chains (DP8-12) than wild type amylopectin (Nakamura, 2002). This indicates that SSI functions to synthesize the shortest amylopectin chains. Other starch synthases, such as SSII and SSIII, are involved in synthesis of long chains.

The amylopectin of SSII-suppressed potato has more short chains (DP6-12) and fewer long chains (Edwards et al., 1999). The center of potato starch granules is cracked, and there was no big change in starch content observed. Pea mutant RUG5, with no SSII activity, however, has low starch content (Craig et al., 1998). The amylopectin of the mutant shows more short chains (DP<10), more long chains (DP>25), but fewer intermediate chains than the WT. These results indicate SSII involve more in the synthesis of long chains than SSI.

Two types of SSII, SSIIa and SSIIb, were identified in monocots. Maize SSIIa and SSIIb have different substrate specificities (Imparl-Radosevich et al., 1999). SSIIa prefers to use short-chain glycogen as the primer for elongation, while SSIIb prefers to use long-chain amylopectin as the primer for elongation. Zhang et al. (2004) report that maize SSIIa deficiency mutant (*surgary2*) produces amylopectin with increased short chains and decreased intermediate chains. A barley *sex*6 mutant lacking SSIIa activity produces starch

containing less amylopectin (Morell et al., 2003). The starch has high amylose content and its amylopectin has more very short chains (DP<15) and less long chains (DP15-45) compared with wild type amylopectin. Pleiotropic effects on other enzymes are observed in this mutant; granular bound forms of SSI, BEIIa, and BEIIb disappear from starch granules. Umemoto et al. (2002) revealed that SSIIa was responsible for the difference between *japonica* and *indica* varieties of rice. SSIIa activity was eliminated from *japonica* rice. This mutation leads to an increase in the short chains (DP<10) and a decrease of intermediate chains (DP13-22). These studies indicate that SSII and SSIIa play similar roles in starch biosynthesis, synthesizing longer amylopectin branch chains.

The elimination of SSIII activity was discovered to be responsible for the maize *dull1* mutant (Gao et al., 1998). This mutant has elevated amylose content and increased branch frequency in amylopectin. Mutant starch contains 15% of the intermediate component. These suggest that SSIII contributes to the synthesis of long chains that can extend through the clusters. This mutation has little influence on granule morphology and starch yield. The suppression of SSIII activity in potato causes changes on both the granule morphology and amylopectin structure (Edwards et al., 1999). Starch granules of the SSIII mutant have deeply cracked centers. Small granules agglomerate to form clusters frequently. The amylopectin of SSIII deficient mutant has enriched short chains (especially DP6) and decreased longer chains. From what we know, SSIII plays a role in potato amylopectin synthesis, increasing amylopectin branch chain length. Studies on *Arabidopsis* (Zhang et al., 2005) and maize (Gao et al., 1998) show that the elimination of SSIII has negative pleotropic effects on SSII and BEIIa. As mentioned above, the function of SSII is to produce longer amylopectin

branch chains. So it is difficult to conclude the role of SSIII played in amylopectin biosynthesis.

The study on SSIV is primitive. This enzyme was identified in an expressed sequence tag data base. The gene product and the function of this enzyme group have been little studied. Recently, two genes encoding rice SSIV were cloned and expressed. SSIV-1 is mainly expressed in the endosperm at the later developmental stage whereas SSIV-2 is mainly expressed in leaves (Dian et al, 2005).

Branching Enzyme

Branching enzymes are divided into two groups based on their sequence similarities: BEI and BEII. They transfer glucan chains with different lengths to form branches. There are two types of BEII in monocots: BEIIa and BEIIb. The different branching enzyme isoforms show different expression patterns. BEI is expressed in leave and endosperm of maize (Gao et al., 1997). BEIIb is only present in the endosperm of maize. BEIIa is found in both leaves and endosperm of maize. BEI is expressed at high levels in the potato tuber, while BEII expression level is low in the potato tuber (Jobling et al., 1999).

Maize BEI displays high affinity to amylose and prefers to transfer longer chains with minimal chain length of DP15 compared with BEII (Guan and Preiss, 1993). However, the inhibition or elimination of BEI has only minor effects on starch synthesis, amylose content, and amylopectin structure of maize and potato (Blauth et al., 2002; Safford et al., 1998). The rice BEI mutant displays altered amylopectin structure with significant increase in shortest chains (DP<10) and decrease in chains of DP12-21 and long chains (DP>37) (Satoh et al., 2003). Antisense inhibition of BEII increases the apparent amylose level in the tuber starch and altered amylopectin structure with increased average chain length in potato (Jobling et

al., 1999). When both branching enzymes were inhibited, starch with very high amylose content was produced (Schwall et al., 2000). These suggest that the role of some branching enzymes (BEI) in starch synthesis can be compensated partially by other branching enzymes. Maize BEIIa and IIb show higher affinity to amylopectin and prefer to transfer chains that are shorter than BEI (Guan and Preiss, 1993). They require a minimal chain length of DP12 for the branching reaction. Maize endosperm starch from BEIIa deficient mutant has no difference from wild type, it suggests that BEIIa's primary role isn't biosynthesis of endosperm starch (Blauth et al., 2001). BEIIb mutant is known as *amylose-extender* mutant. In both maize and rice, the mutants contain large amylose content in the endosperm. Their endosperm amylopectins display altered structures with significantly decreased short chains (DP8-12) (Nakamura, 2002). These suggest BEIIb plays a special role in endosperm starch biosynthesis; its function cannot be compensated completely by other branching enzymes.

Debranching Enzyme

When debranching enzyme (DBE) mutant lines have been constructed in many species, the importance of DBE on starch synthesis was revealed. DBEs are classified into isoamylase and pullulanase on the basis of their substrate specificities. Isoamylase-type DBE mutants accumulate water-soluble polysaccharides and have low starch content. The amylopectin of maize *isoamylase*1 mutant has more short chains (DP3-12) and less intermediate chains (DP12-30) than that of wild type (Dinges et al., 2001). Similar changes of amylopectin structures are observed from maize *sugary*-2 mutant (Perera et al., 2001). The elimination of isoamylase 1 in rice leads to the production of phytoglycogen and *sugary*-amylopectin (contains more short chains DP<12) (Wong et al., 2003). A glucan trimming model was proposed to explain how DBE involves in starch biosynthesis (Ball and Morell,

2003). This model suggests that DBE trims the excess branches on the surface of "preamylopectin". Bustos et al. (2004) propose that isoamylase controls starch granule initiation. They discovered that potato isoamylase I and II mutants only produced small amount soluble glucans. The starch yield and amylopectin structure of mutants show no big difference from the control lines. The mutants, however, produce large numbers of tiny starch granules in the tuber. The inhibition of isoamylase activity in barley also alters starch granule initiation in the endosperm. Barley mutants, *Risϕ7* and *Nortch*-2, accumulate larger amount of starch granules than the wild type (Burton et al., 2002). In addition, they lose the second wave of granule initiation. A mutant study on maize pullulanase (*ZPU*1) indicates that it plays a role in starch degradation. It is also involved in starch biosynthesis by compensating isoamylase function (Dinges et al., 2003).

Starch biosynthesis requires the cooperation of a group of enzymes. Pleiotropic effects observed in some mutants suggest that there is protein-protein interaction among enzymes. For instance, barley SSIIa deficient mutant loses the granule binding form of SSI, BEIIa and BEIIb (Morell et al., 2003). Maize *dull1* mutant exhibits lower SSII and BEIIa activity in the endosperm (Gao et al., 1998). Another interesting topic in starch biosynthesis is that some starch biosynthesis enzymes are regulated by phosphorylation. The phosphorylation state regulating enzyme activity was observed in *Arabidopsis.* The reduction of the production of protein 14-3-3 leads to an increase in leaf starch accumulation (Chung et al., 1999, Sehnke et al., 2001). Protein 14-3-3 functions in phosphorylation-related regulation by binding phosphoserine/threonine. Tetlow et al. (2004) report that phosphorylation states affect catalytic activities of wheat BEI, BEIIa, and BEIIb.

With the increasing number of starch biosynthetic enzymes identified and mutants with deficiency of different isoforms of starch biosynthesis enzymes characterized, we gain more understanding on the functions of each enzyme isoform on the formation of starch granules. Since each starch biosynthetic enzyme exists in several isoforms, which express in different plant tissues with different expression patterns, starch synthesized in different tissues at different times should have different structures. There is lack of understanding, however, on detailed structures of starch at different developmental stages and in different tissues. Thus, it is necessary to investigate the starch structure through development.

Glycogen Structure and Biosynthesis

The structure of glycogen resembles that of amylopectin, but with larger branch frequency. In general, glycogen consists of 7-10% branches, about 2 folds of that of amylopectin (Manners, 1991). Glycogen mainly exists in a soluble, amorphous form in microorganisms and animals. Glycogen forms β particles with diameters of 40-60 nm in cyanobacteria, yeasts, and mammals (Chao and Bowen, 1971; Takeshige et al., 1992; Calder, 1991). Glycogen structure is more dispersed than starch because of its larger branch percentage and shorter average chain length. The branch chain-length distribution profile of glycogen is unimodal and lacks the long B chain. It does not have a cluster structure (Yoo et al., manuscript in preparation).

The mechanism of glycogen biosynthesis is much simpler than that of starch. It usually involves one ADP/UDP glucose pyrophosphorylase, one or two glycogen synthases and one branching enzyme (Ball and Morell, 2003). *E. coli* contains only one glycogen synthase (Guan et al., 1995), while cyanobacteria contain two (Kaneko et al, 1996). One

branching enzyme was found in *E. coli* (Guan et al, 1995) and cyanobacteria (Keneko et al., 1996). Glycogen debranching enzyme is involved in glycogen degradation, but does not play a role in glycogen biosynthesis. In mammals and fungi, a protein named glycogenin was proposed as the primer of glycogen synthesis (Alonso et al., 1995). Glycogenin contains a protein that is covalently bound to an α-1,4 linked glucan. It is not known, however, if a primer participates in bacteria glycogen biosynthesis. Ugalde et al. (2003) suggested that glycogen synthase served as both the primer for the glycogen synthesis and the synthase for glycogen elongation in *Agrobacterium tumefaciens*. They extracted GS from a mutant that can't synthesize glucose nucleotides. Thus, the crude extract contained no α-glucans primers. With the supply of only ADP-glucose as substrate, the enzyme alone can synthesize α-glucans, but it is not known if this applies to other bacteria glycogen biosynthesis.

Cyanobacteria Model System

Microbial systems are widely used to study the functions and activities of all kind of proteins and enzymes because they provide simple model systems and are easy to be manipulated. *E. coli* and yeast are used as host species to study starch biosynthesis and granule development. Guan et al. (1995) introduce maize BEI and BEIIb into *E. coli* species without endogenous BE, glycogen-like polysaccharides were produced. Maize BEs were introduced into brewer's yeast by Seo et al (2002). They obtain soluble glucans with increased size and more long chains (DP18-36).

Cyanobacteria are photoautotrophic organisms capable of photosynthesis (Van Liere and Walsby, 1982). The photosynthesis system of cyanobacteria displays significant similarity to that of eukaryotic plants. Cyanobacteria are considered as the ancestor of plant

chloroplasts. Although the intracytoplasmic membrane of cyanobacteria doesn't packs as tight as chloroplast membranes of higher plants (Golecki and Drews, 1982), they contain similar membrane lipids including monogalactosyl diglyceride, digalactosyl diglyceride and sulphoquinocosyl diglyceride. Both glycogen and starch granules are formed closely to the thylakoid membrane. Thus, cyanobacteria may provide a similar environment for the synthesis of starch. From the evolution point of view, cyanobacteria are more closely related to plants than *E. coli.* Yeasts use UDP-glucose as the substrate for the glycogen synthesis, which is different from the substrate of starch biosynthesis, while cyanobacteria use ADP-glucose (Greenberg and Preiss, 1964). So, cyanobacteria may serve as a better system for the study of plant starch biosynthetic enzymes.

Cyanobacteria *Synechocystis* sp. PCC6803 is a mutant that can survive in the situation with the supply of high concentration of glucose and continuous light. As a host for the study of plant starch biosynthetic enzyme, *Synechocystis* sp. PCC6803 has some advantages. First, the genome of this species is completely sequenced (Keneko et al., 1996). Based on genome sequence analysis of *Synechocystis* (Keneko et al., 1996), the following deduced genes that are related to glycogen metabolism were identified: two deduced glycogen synthases (*sll*0945 and *sll*1393), one glycogen branching enzyme (*sll*0158), two isoamylase-type glycogen debranching enzymes (*slr*0237 and *slr*1857), one disproportional enzyme (*sll*1676), two glycogen phosphorylase enzymes (*sll*1356 and *slr*1357) and one pullulanase-type glycogen debranching enzymes (*sll*0842). Since the endogenous enzymes involved in glycogen metabolism are identified, it is much simpler to study the changes in the glycogen metabolic system. Second, foreign DNA can be taken into *Synechocystis* very

easily. This species can be spontaneously transformed by incubating with foreign DNA, which is integrated into the host genome by homologous recombination.

Starch biosynthetic system contains multiple isoforms of AGPase, SS, BE and DBE. The lack of one or two enzymes has no influence on the formation of the starch granule. But the basic requirement for the granule formation is still a mystery. It was proved that the replacement of *E. coli* BE by maize BEI and BEIIb didn't produce amylopectin (Guan et al., 1995) in *E. coli.* Thus, other starch biosynthetic enzymes may be required for further modification on the structure of bacteria glycogen.

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CHAPTER 3. CHARACTERIZATION OF CYANOBACTERIA SYNECHOCYSTIS SP. PCC 6803 MUTANTS CONTAINING POTATO STARCH SYNTHASE III

Li Li, Shayani D. N. Perris, Martin H. Spalding and Jay-lin Jane*

Department of Food Science and Human Nutrition, Iowa state University, Ames, Iowa 50011(L.L., J.J.); Department of Genetics Development & Cell Biology (S.D.N.P., M.H.S.), Iowa State University, Ames, Iowa 50011

*Corresponding author:

Fax: 515-294-8181; E-mail address: jjane@iastate.edu

Abstract

Cyanobacterium *Synechocystis* sp. PCC6803 is used as a model system to study the function of starch biosynthetic enzymes and starch biosynthesis. A potato starch synthase III (PSSIII) was expressed in *Synechocystis* mutants that were missing glycogen synthase I (M1) or glycogen synthase II (M2) to produce two new mutants PM1 and PM2. The remaining glycogen synthase gene in PM1 and PM2 was disrupted to generate mutants PM12 and PM21, containing PSSIII as the only glycogen/starch synthase. Results indicated that the survival of *Synechocystis* required the existence of glycogen/starch synthase. The disruption of GSII and the expression of PSSIII were accompanied by an increase of glycogen branching enzyme activity. The branching structures of mutant α-glucans were analyzed by

using high-performance anion exchange chromatography (HPAEC). The unimodal distribution of the branch chains of the soluble α-glucan suggested that the glucan was synthesized in the glycogen form in mutants. Compared with WT glycogen, PM1, PM2, PM12 and PM21 produced glucans with more short chains and fewer long chains. This is consistent with the increase in the branching enzyme activity in the mutants. There is no significant difference between the structure of PM12 and PM21 glucans. Results indicated that there are regulatory and cooperative interactions among plant starch synthase, glycogen synthases, and glycogen branching enzyme in the process of the biosynthesis of glucans.

Introduction

Starch can be a transient or long-term storage carbohydrate in plants. Native starch granules have semi-crystalline structure, containing two main components: amylose and amylopectin. Amylopectin is primarily responsible for the crystallinity of starch granules. Glycogen, a storage carbohydrate in animals and microorganisms, mainly exists in a soluble, amorphous form. Glycogen structure is very similar to that of amylopectin, but contains up to two times as many (7-10%) branch linkages as found in amylopectin (~5%) (Manners, 1991).

The roles of starch biosynthetic enzymes on starch structure have been widely studied by the elimination of single genes (Blauth et al., 2001; Craig et al., 1998; Gao et al., 1998; Safford et al., 1998; Umemoto et al, 2002). In addition, *E. coli* and yeast were used as model systems to study the functions of maize branching enzymes (Guan et al., 1995; Seo et al., 2002) because of the similarities between glycogen and starch biosynthetic pathways. The

glycogen synthetic pathway usually involves one ADP/UDP glucose pyrophosphorylase, one or two glycogen synthases and one branching enzyme (Ball and Morell, 2003). Only one glycogen synthase has been found in *E. coli* genome (Guan et al., 1995), while two have been found in some cyanobacteria genome (Kaneko et al, 1996). Only one branching enzyme has been found in *E. coli* (Guan et al, 1995) and in cyanobacteria genomes (Keneko et al., 1996). In contrast, the starch biosynthetic system is much more complex than that of glycogen. In general, two isoforms of ADP-glucose pyrophosphorylase, four or more starch synthases, three branching enzymes and two groups of debranching enzymes are involved in starch biosynthesis.

Cyanobacteria are photoautotrophic organisms capable of photosynthesis (Van Liere and Walsby, 1982). From the evolutionary point of view, cyanobacteria are more closely related to plants than is *E. coli*, because they are considered to be the ancestors of plant chloroplasts. The intracytoplasmic membrane organization of cyanobacteria is very similar to that of chloroplast membranes of higher plants (Golecki and Drews, 1982). Cyanobacteria thylakoid membrane contains three major lipids: monogalactosyl diglyceride, digalactosyl diglyceride and sulphoquinocosyl diglyceride, which are also found in the chloroplast membrane of high plants. Both glycogen and starch granules are formed closely to the thylakoid membranes. Thus, cyanobacteria may provide an environment similar to that of the starch biosynthetic system. Yeasts use UDP-glucose as the substrate for glycogen synthesis, while cyanobacteria use ADP-glucose (Greenberg and Preiss, 1964), which is the same substrate used in starch biosynthesis. On the basis of these known factors, cyanobacteria may serve as a better host for the study of the functionality of starch biosynthetic enzymes.

The cyanobacteria *Synechocystis* sp. PCC6803 was selected as the host species for this study. This species can survive in an environment with only glucose as the carbon source or under continuous light. The reasons we chose this species are the following: First, the genome of this species is completely sequenced (Keneko et al., 1996), thus the endogenous enzymes related to glycogen metabolism can be identified. Second, this species can be spontaneously transformed by incubating it with foreign DNA, which is integrated into host genome by homologous recombination.

With expression of plant starch synthetic enzymes in cyanobacteria, we intended to study the function of individual starch synthetic enzymes in starch/glycogen biosynthesis and to explore the evolution of starch biosynthesis. Yoo et al. (2006) disrupted *Synechocystis* GSI and GSII genes to generate mutants, M1 and M2, respectively. In this study, we expressed a potato starch synthase III in *Synechocystis* mutants M1 and M2 to produce mutants PM1 and PM2. We disrupted the remaining glycogen synthase gene in PM1 and PM2 to produce mutants containing PSSIII as the only starch/glycogen synthase. The structures of glucans produced by mutants were determined by high performance anion exchange chromatography (HPAEC).

Materials and Methods

Materials and strains

Restriction enzymes were purchased from Promega Biotech (Madison, WI). Chemicals used in BG11 medium were purchased from Fisher Scientific (Pittsburgh, PA).

Other chemicals were purchased from Sigma (St. Louis, MO). The vector pBluescript II KS + was purchased from Stratagene (La Jolla, CA). The Chloramphenicol resistant cassette was amplified from vector pBD-GAL4 Cam (Stratagene, La Jolla, CA). The potato starch synthase III cDNA vector, pRAT, was a gift from Dr. Alison M. Smith (John Innes Centre, Colney Lane, Norwich NR4 &UH, UK). The *E. coli* strain DH5α was cultured at 37°C in LB medium. The cyanobacterial strain *Synechocystis* sp. PCC6803 was cultured at room temperature in BG11 medium (Rippka et al., 1979) with flurescent lights (μ40 moles photons m^{-2} s^{-1}) (Yoo et al, 2006). For glycogen isolation and enzyme assays, *Synechocystis* was grown in BG11 medium for 7-8 days, transferred into BG11 medium with limiting nitrogen supply and then grown for 3 days. Cells were collected by centrifugation at 6,000*g* for 10 min (Yoo et al, 2006).

Mutant construction

To express PSSIII in *Synechocystis*, a recombinant vector was designed to integrate the PSSIII gene into the psbAII coding sequence (Lagarde et al., 2000). The recombinant vector was designed as follows. A 500 bp SacI-SacII fragment at the upstream of psbAII gene coding region and a 500bp EcoRI-XhoI fragment at its downstream border were amplified. First, the psbAII upstream fragment (SacI-SacII) was inserted into pBluescript KS vector and then full length potato SSSIII gene (SacII-XbaI) was inserted to form the pUP vector. Second, a psbAII downstream fragment (EcoRI-XhoI) was inserted into another pBS KS vector and then a spectinomycin resistance gene (XmaI-EcoRI) from pHP45Ω (Prentki and Krisch, 1984) was inserted between the XmaI and EcoRI digestion sites to form the pDS vector. The spectinomycin resistance gene and the downstream fragment of psbAII gene

were cut from pDS by XmaI and XhoI and inserted into pUP to construct the vector pUSD (Figure 1). Sequencing results showed that there is 100% identity between amino acids of PSSIII in pUD and pRAT. The pUSD plasmid was transformed into mutants M1 and M2 to create new mutants PM1 and PM2, respectively (Williams, 1988).

In order to eliminate the remaining glycogen synthase activity from PM1 and PM2, a chloramphenicol resistance gene was used to disrupt the remaining glycogen synthase gene. The disruption vectors were constructed from vectors pSHK945 and pSHK1393 (Yoo et al, 2006). The spectinomycin resistance gene in those two vectors was replaced by a chloramphenicol resistance gene. The vector structure is shown in Figure 2. With the disruption of the remaining glycogen synthase gene, mutants PM12 and PM21 were generated from PM1 and PM2, respectively.

RT-PCR

Total RNA was extracted by using RNeasy Mini Kit (Qiagen, Valencia, CA). The DNA contamination was removed by using RNase-free DNase Kit (Qiagen, Valencia, CA). An aliquot of total RNA was amplified by using OneStep RT-PCR kit (Qiagen, Valencia, CA). The primers were designed based on the sequence of potato starch synthase III gene: the sense primer 5'-ACTGTCTCAGAAGCATGTAG-3' and the antisense primer 5'-CAAACAGTCATACTTAGGTAAG-3'. The reaction started with cDNA synthesis at 50 °C for 30 min and DNA polymerase activation at 95 °C for 15 min. PCR amplification was performed for 35 cycles with 94 °C denaturation for 30s, 55 °C annealing for 30s and 72 °C extension for 1 min. PCR product was loaded in 0.7% agarose gel with ethidium bromide for the electrophoresis.

Zymogram analysis of glycogen synthase activity

Cells were harvested by centrifugation on the third day after being transferred into nitrogen-limited medium. The cell pellet was resuspended in 1 ml of extraction buffer (1mM Tris-HCl pH 7.0, 1 mM EDTA) containing 1mM dithiotheritol (DTT), 1 mM benzamidine, and 0.2 mM phenylmethyl sulfonylfluoride (PMSF). The cell suspension with an equal volume of glass beads (100-150 μm) added, was agitated 6 times at 5000 rpm for 20 seconds in a Mini-beadbeater (Biospec Products, Bartlesville, OK). The crude enzyme extract was obtained by centrifugation at 10,000*g* for 15 min. The zymogram analysis was performed following the method of Yoo et al (2006). The crude enzyme extract (about 20 μl) was separated in 7.5% native polyacrylamide gel at 90V for 2 hours. After washing twice with ice-cold distilled water, the gel was immersed in 25 ml of substrate solution containing sodium citrate buffer (pH7.0, 50mM), 20 mg glycogen and 15 mg ADP-glucose. The reaction was carried out at room temperature overnight with gentle shaking. The active protein bands were detected by staining the gel with iodine solution (I_2/KI=0.01/0.1%, w/v) in 0.1M sodium acetate buffer (pH5.0). The crude enzyme extract was also separated in 6% native polyacrylamide gel containing 0.3% rabbit liver glycogen by using electrophoresis. The gel was incubated with the substrate solution containing 0.1M Bicine (pH8.5), 0.5 sodium citrate, 0.5mg.ml BSA, 10gml rabbit liver glycogen, 133mM $(NH_4)_2SO_4$, 7mM $MgCl_2$, 25mM 2-mercaptoethanol, and 5mM ADP-glucose at 30°C overnight with gentle shaking (Zhang et al., 2004). The detection method used was the same as the one in the method of Yoo et al. (2006).

Glucan extraction and yield

Cyanobacterial pellets were resuspended in distilled water, and an equal volume of glass beads was added. Samples were disrupted for 6 times at 5000 rpm for 20 seconds in a Mini-beadbeater (Biospec Products, Bartlesville, OK) with the tubes were cooled on ice between each treatment. The supernatant was collected by centrifugation and boiled for 15 min to inactivate enzymes. The concentration of α-glucans was measured by using Glucose Diagnostics Kit (Sigma, St. Louis, MO) after amyloglucosidase digestion.

Glycogen synthase activity assay

The crude enzyme extract was prepared as described in zymogram method. The glycogen synthase assay was following the method of Yoo et al (2006) with modifications.The glycogen synthase activity was measured by incorporation of ^{14}C from ADP-glucose into glycogen. The crude extract (20 µl) was mixed with 50 mM glycine (pH 9.0), 100 mM $(NH_4)_2SO_4$, 5 mM β-mercaptoethanol, 5 mM $MgCl_2$, 0.5 mM BSA, 10 mg/ml rabbit liver glycogen, 4 mM ADP-glucose and 0.04 µCi ADP[U-^{14}C]glucose in a final volume of 200 µl. The reaction was carried out at 30°C. An aliquot (30µl) of the reaction mix was taken out at 0, 5 10, 20, and 40 min, and the reaction was terminated by adding 1 ml of 75% ice-cold ethanol. The precipitate was collected by filtering through a Whatmann 934-AH filter. The dried filters were emerged in 5 ml liquid scintillation cocktail and the radioactivity was measured by liquid scintillation spectroscopy.

Branching enzyme assay

Branching enzyme activity was measured by the phosphorylase-stimulation method (Boyer and Preiss, 1978) with modifications. The enzyme extract was prepared following the method for zymogram assay. The enzyme extract was centrifuged at 50,000g for 1hour at 4 °C and the supernatant was used for the assay. The crude extract was mixed with 1 mM AMP, 0.1 mg/ml rabbit-liver phosphorylase A, 0.1 M sodium citrate (pH7.0), and 45 mM glucose-1-phosphate in a final volume of 200 µl. The reaction was carried out at 30 °C. An aliquot (25 µl) of reaction mix was collected at 0.5, 5, 10, 30, 60, and 90 min and boiled for 10 min to stop the reaction. The released phosphate was measured by using the M-G assay (Baykov et al, 1988).

Branch chain-length distribution

Glycogen (3mg) was dispersed in 3ml of 10mM acetate buffer (pH3.5), and *Pseudomonas amyloderamosa* isoamylase (120 units) was added to debranch the sample. The reaction was carried out at 40°C with shaking at 120 strokes per minute for 24 hours. The digested sample was adjusted to pH 6-7 by adding NaOH and boiled for 15 min to inactivate isoamylase. The branch chain-length distribution of amylopectin was analyzed by using high performance anion-exchange chromatography (Dionex-300, Sunnyvale, CA) equipped with an amyloglucosidase post-column and an ED50 electrochemical detector (Dionex, Sunnyvale, CA) (HPAEC-ENZ-PAD). The debranched samples were separated by a PA-100 anion-exchange analytical column (250×4 mm) and a guard column ((Dionex, Sunnyvale, CA), following the same condition reported by McPherson and Jane (1999) described. The data were analyzed by using Chromeleon software (Dionex, Sunnyvale, CA).

Statistical analysis

The results were analyzed by using the *t* test. When the P-value was less than 0.05, the difference was considered statistically significant at the 0.05 level.

Results

Expression of potato starch synthase III in Synechocystis mutants

The construct, vector pUSD, contained 0.5 kb fragments of upflanking region and downflanking region of the psbAII gene for homologous recombination (Figure 1A). It also contained a spectinomycin resistant gene as a marker for the selection of new mutants. The construct was transformed into M1 (strain with GSI eliminated) and M2 (strain with GSII eliminated) genomes to produce two new mutants PM1 and PM2. *Synechocystis* contains more than one set of genome copies (Ermakova-Gerdes and Vermass, 1999). In order to obtain complete segregation, transformants were selected under antibiotic selection for several generations. The complete segregation was confirmed by PCR analysis (Figure 1B). A 1.3 kb PCR product was amplified from PM1 and PM1 genomic DNA using forward primer from the up flanking region of psbAII (PrC1) and reverse primer from internal region of PSSIII (PrC2). No PCR product was amplified from WT genomic DNA. When the forward primer PrC1 and the reverse primer from the internal region of psbAII were used, no PCR product was amplified from PM1 and PM2 genomic DNA (data not shown).

Construction of Synechocystis mutant without endogenous glycogen synthase

Yoo et al. (2006) intended to create a double mutant without both glycogen synthases, but PCR amplification of genomic DNA revealed that all putative double-mutant strains contained copies of a WT GS gene even after many generations of antibiotic selection. These results suggested that the double mutant were not segregated completely and that the elimination of both glycogen synthases was lethal to *Synechocystis*. These observations also confirmed that GSI and GSII are the only glycogen synthases in *Synechocystis*.

With the expression of PSSIII in mutants PM1 and PM2, the elimination of the remaining glycogen synthase could be achieved. The rest of the glycogen synthase gene in the PM1 and PM2 genome was disrupted with a chloramphenicol resistance gene cassette construct (Figure 2A) to produce either mutant PM12 or PM21. The complete segregation of PM12 and PM21 was confirmed by PCR analysis (Figure 2B). PCR product of WT showed the band with the original size of glycogen synthase gene (~1.3 kb) using primers (PrA1, PrA4, PrB1, and PrB4) that were shown in Figure 2A. Insertion of the chloramphenicol resistant gene into the glycogen synthase gene resulted in 2.2 and 2.3kb PCR products, respectively, for PM12 and PM21.

To confirm the expression of PSSIII, total RNAs from WT and mutants were subjected to RT-PCR using PSSIII internal primers PrD1 and PrD2 (Figure 1A) to demonstrate the existence of PSSIII mRNA. A 0.5kb PCR product was amplified by using total RNA of PM1, PM2, PM12, and PM21 as a template, but not for WT, M1 and M2 (Figure 2C). The result indicated that the transcript of PSSIII was present in mutants PM1, PM2, PM12, and PM21.

A zymogram analysis was employed to detect the glycogen/starch synthase activity in cyanobacterial mutants by using the method of Yoo et al. (2006) (Figure 3). Iodine staining

revealed glycogen/starch synthase as brown bands on the native gel. The two brown bands detected in WT disappeared in PM12 and PM21, indicating both glycogen synthase genes were disrupted. However, no band corresponding to PSSIII was detected. In order to detect the PSSIII activity, the method of Zhang et al. (2004) was used to perform the zymogram analysis. The addition of rabbit liver glycogen in the native gel slowed the migration of glycogen synthases in native gel. One brown band appeared at the same position in the PM1, PM2, PM12 and PM21 lanes in Figure 4B, which was missing in Figure 4A. This result indicated that PSSIII was actively expressed in mutants PM1, PM2, PM12 and PM21. One unknown brown band appeared in all samples in both Figure 4A and 4B, which was not influenced by the mutation on either GS or PSSIII.

Total glucan synthase activity and branching enzyme activity

Total glucan synthase activities of WT and mutants were found to be 0.084, 0.083, 0.138, 0.073, 0.222, 0.012 and 0.010 μmol glucose $min^{-1}mg^{-1}$ protein for WT, M1, M2, PM1, PM2, PM12, and PM21, respectively (Table 1). The rates of [^{14}C] glucose incorporation for M1 and PM1 were similar to that of WT whereas the rates for M2 and PM2 were higher than WT. The mutants PM12 and PM21 displayed relatively low glucan synthase activity.

The glycogen branching enzyme activities of WT and mutants are shown in Table 1. M1 showed similar branching enzyme activity to WT and mutants M2 showed higher branching enzyme activity than M1and WT, which agreed with previously reported (Yoo et al., 2006). It is also noteworthy that mutants M2, PM2 and PM21 showed higher branching enzyme activity than M1, PM1 and PM12, which suggests that either the order of the disruption of GS I and GSII has different effects on branching enzyme activity or additional,

undetected mutations affecting glycogen branching enzyme activity were introduced into M2. Another effect on branching enzyme activity coincided with the expression of PSSIII, with PM1 and PM2 showing higher branching enzyme activity than M1 and M2.

The soluble glucan yields of WT and mutants are shown in Table 1. By T-test, there is no significant difference among WT and other mutants at 0.05 level of probability. Although mutants PM12 and PM21 displayed only 14% and 12% total glycogen synthase activity of WT, they produced a similar amount of glucans to WT. The insoluble glucans also were extracted and quantified, but the amount of insoluble glucan produced in WT and the mutants was negligible comparing with the soluble portion.

Chain-length distribution of mutant glucans

Branch chain-length distributions of polysaccharides from WT and the mutants are shown in Figure 5. The branch chain-length profiles showed a unimodal distribution, with peak chain lengths at DP6 or DP8. The branch structures of M1 and M2 are similar to those reported previously (Yoo et al., 2006). Glucans from M1 showed fewer short chains (DP4-7) and long chains (>DP19) and more intermediate chains (DP8-18) than that from WT. The branch chain-length distribution of glucans from M2 was similar to that from WT, but with more abundant short chains (DP4-11). PM1 produced glucans with more short chains (DP 4-12) and fewer long chains than M1. PM2 produced glucans with more intermediate-length chains (DP 7-18) and fewer very short and long chains than M2. Compared with WT glycogen, PM1 and PM2 both produced glucans with more short chains and fewer long chains (Figure 6). These data are consistent with the increase of the branching enzyme activity in the mutants. PM1 and PM2 had greater branching enzyme activity than WT, M1

and M2, which would explain the generation of more short branches in PM1 and PM2 glucans.

The branch chain-length profiles of PM12 and PM21 glucans were very similar (Figure 7F), except PM21 glucans had slightly more short chains (DP 4, 5, and 7) than PM12 glucans. Comparing with WT and other mutants (Figure 7), PM12 and PM21 glucans contained more short chains and fewer long chains. The debranching patterns of PM12 and PM21 glucans showed more similarity to that of PM1 than to those of M2 and PM2.

Discussion

Glycogen synthases in Synechocystis sp. PCC6803

On the basis of similarity in gene sequence, two glycogen synthases, GSI and GSII, were identified in the cyanobacterium *Synechocystis* sp. PCC6803 genome. GSII was more similar to other bacteria glycogen synthases, whereas GSI was more similar to other cyanobacteria glycogen synthases and some eukaryotic starch synthase isoforms IV and V. However, the roles GSI and GSII play in the biosynthesis of glycogen structure are not known. GSII is the only active glycogen synthase in mutant M1, and the changesion the branch chain-length distribution of glycogen suggests that GSII may play a role on the synthesis of intermediate size branches (DP 8-18) (Yoo et al., 2006). Compared with WT, M2 glycogen contained more short branches (DP4-11), which might be the result of an increase in branching enzyme activity in M2 (Yoo et al., 2006). Thus, it is difficult to conclude the extract role of GSI in the glycogen biosynthesis.

Results of previous studies on disruptions of GSI and GSII gene (Yoo et al., 2006) showed that the double mutant lacking both endogenous glycogen synthases apparently could not be constructed. Studies on yeast and E. coli show that mutants in these two organisms totally lacking glycogen synthase activity are viable (Farkas et al., 1991; Edwards et al., 1996). The glycogen yields of *Synechocystis* mutants reported earlier and in this study remained at similar levels to the wild type. Taken together, these data indicate that glycogen deposition is necessary for the survival of *Synechocystis* sp. PCC6803. One possible explanation for the importance of glycogen deposition is because that *Synechocystis* sp. PCC6803 is grown in glucose containing medium and/or in continuous lighting, conversion of glucose to glycogen may be necessary for maintaining glucose homeostasis and minimizing excess glucose supplies in the medium and/or produced by photosynthesis.

The expression of PSSIII in Synechocystis sp. PCC6803

Potato starch synthase III (PSSIII) is the major starch synthase in potato tuber, accounting for 80% of the soluble starch synthase activity in the tuber (Marshell et al., 1996). The PSSIII gene was introduced into M1 and M2 by replacing the *psbA*II gene in the genome. The replacement of *psbA*II with PSIII was confirmed at the DNA level, and the expression of PSSIII was confirmed in mRNA and enzyme activity level in PM1 and PM2. However, the PSSIII activity band was only detected by using the zymogram method described by Zhang et al. (2004), not by the one described by Yoo e al. (2006). This suggested that the reaction condition is important in the measurement of glucan synthase activity. The PSSIII activity was not only clearly demonstrated by the zymogram analysis, but also supported by the synthesis of glucans in the absence of GSI and GSII.

Although both PM12 and PM21 showed low total glycogen synthase activities, their glycogen yields were similar to the WT and other mutants. This result was consistent with other reports, suggesting that starch synthase activity is not always the determined factor for starch (glucan) yield. When the soluble starch synthase activity was reduced to 20% of the WT in potato tubers by antisense suppression of soluble starch synthase II and III, the starch content of the tubers remained unchanged (Edwards e al., 1999). A similar situation was observed in maize *Dull*1 mutants, in which the disruption of maize starch synthase III had little impact on the starch yield of mature kernels (Gao et al., 1998). A unicellular eukaryote, *Saccharomyces cerevisiae*, contains two glycogen synthases, GSY1 and GSY2. Mutants with GSY1 or GSY2 disrupted had 85% and 10%, respectively, of the GS activity but 100% and 40%, respectively, of the glucan yield of WT (Farkas et al., 1991). These reports all suggest that starch/glycogen synthase activity is not proportional to starch/glycogen yield. Although PSSIII activity in *Synechocystis* mutants was low, it was sufficient to support substantial glycogen synthesis. Another possibility is that our experimental condition was not optimum for detecting the activity of PSSIII, and the PSSIII activity *in vivo* is much larger than that detected and was adequate for the necessary glycogen synthesis.

Starch/glycogen synthase and glucan structures

The biosynthetic pathway of starch is similar to that of bacterial glycogen. The products generated by these two pathways share certain similarities, except that glycogen has a higher branch frequency than amylopectin, the major component of starch, which gives glycogen and starch distinct features. Starch exists in a semi-crystalline form, whereas glycogen exists in a soluble form. Glycogen biosynthesis requires only one or two glycogen synthases and one branching enzyme, but starch synthesis apparently requires a much more

complex enzyme system. Starch biosynthesis involves at least four or more types of starch synthases, three branching enzymes and two types of debranching enzymes. Each enzyme has its own distinct function and can not be fully replaced by others.

As the major soluble starch synthase in potato, PSSIII contributes to amylopectin synthesis and structure determination. With the antisense suppression of PSSIII, potato mutants produce amylopectin with more very short chains (DP6) and fewer long chains (Edwards et al., 1999). Thus we expected that the introduction of PSSIII in *Synechocystis* would produce glucans having fewer short chains. The change in branch chain-length distribution was rather complex in PM1 and PM2. The expression of PSSIII was accompanied by an increase in branching enzyme activity, although the reasons for these changes are not clear. Studies on maize (Gao et al., 1998) and Arabidopsis (Zhang et al., 2005) showed that the elimination of SSIII had negative pleiotropic effects on SSII and BEIIa. The function of SSII apparently is to elongate very short chains of DP 6 to10 to intermediate chains of DP11 to 24. The accumulation of very short chains (DP6) in a potato mutant with antisense suppression of PSSIII may be the result of both the suppression of PSSIII and the decrease in SSII. Thus, the role of PSSIII in amylopectin structure is not yet fully understood. PSSIII may not function to elongate short glycogen branch chains. Nielsen et al. (2002) report the different branching behaviors between *Arabidopsis* WT and a mutant without plastidic isoamylase (*dbe*) during glucan biosynthesis. The branching of WT starch occurs rapidly to newly formed branch chains with DP12-19 and longer chains are less accessible to branching because of the protection of crystalline structure. The branch chains of phytoglycogen from *dbe* mutant are available for branching in a longer time period due to the amorphous structure of phytoglycogen. Thus, even if PSSIII elongates short branch

chains, the elongated chains of glucans that are not in a crystalline structure may serve as substrates for glycogen branching enzyme, leading to altered branching patterns and an increase in short chains.

Mutants PM12 and PM21 contain only one starch synthase, PSSIII. These two mutants should be exactly the same except that they are constructed from different mutant strains. PM21, however, displayed a higher branching enzyme activity than PM12. Their branching profiles were very similar except PM21 glycogen having slightly more short chains (DP 4, 5, and 7) than PM12 glycogen. As we mentioned above, disrupting GSII first had a pleiotropic effect on the glycogen branching enzyme. Pleiotropic effects have been observed also in starch biosynthetic systems. Morell et al. (2003) report that SSI, BEIIa and BEIIb lose the ability to bind starch granules when SSII activity is eliminated in barley (*sex*6 mutant). This mutant produces amylopectin with more short chains. Similarly, the elimination of SSIII in the maize *dull*1 mutant reduces BEIIa activity significantly in endosperm tissue. These reports suggest that there are interactions among starch biosynthetic enzymes. Tetlow et al. (2004) demonstrate by co-immunoprecipitation under phosphorylated conditions that wheat SBEI and SBEIIb form a complex. Cannon et al. (1994) report that point mutations in GBE result in the loss of glycogen accumulation or the production of glycogen with altered structure in *Saccharomyces cerevisiae*, and they suggest that there is a protein-protein interaction between glycogen synthase and GBE in yeast. It is also possible that there are specific interactions between glycogen synthases and glycogen branching enzyme in *Synechocystis*. Further studies on glycogen biosynthetic enzymes are needed to reveal if there are interactions and regulations among starch/glycogen synthase and GBE.

Although a plant starch synthase, PSSIII was expressed in *Synechocystis* and *Synechocystis* mutants still produced soluble α-glucan instead of semi-crystalline starch. So the expression of a single plant starch synthase is not sufficient for the formation of semi crystalline starch structure in cyanobacteria. Previous efforts have been made to try to synthesize amylopectin-like glucans in bacteria and yeast by introducing plant starch biosynthetic genes. Guan et al. (1995) expressed maize BEIIa and BEIIb in *E. coli* without endogenous BE. Only glycogen-like glucans were synthesized. Seo et al. (2002) expressed different combinations of maize BEI, BEIIa and BEIIb in yeast without endogenous BE. Mutants with BEIIa and BEIIb produced glucans with larger molecular weight, but the branch chain length profiles of those glucans still showed a unimodal distribution. These studies indicate that semi-crystalline amylopectin structure cannot be created by the simple replacement of one type of bacteria glycogen biosynthetic enzyme with a corresponding starch biosynthetic enzyme. Multiple isoforms of each starch biosynthetic enzyme are found in all starch-synthesizing organisms. Although semi-crystalline amylopectin structure exists with the elimination of one or two starch synthases or branching enzymes in some plants, a change in the starch structure is always observed. This suggests that multiple isoforms with their specific, distinct functions and a certain level of functional redundancy are necessary for the formation of the semi-crystalline structure.

Synechocystis is a useful model system for the study of starch biosynthesis. This study provides more valuable knowledge for understanding starch/glycogen biosynthesis. With the expression of starch biosynthetic enzymes in *Synechocystis*, we will gain more understanding on its role involved in glycogen synthesis in *Synechocystis*.

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Figure legends

Figure 1. The construction to express PSSIII in psbAII coding region (A). The primers PrC1 and PrC2 were used in PCR analysis to examine if the replacement of psbAII with PSSIII by homologous recombination was successful. The primers PrD1 and PrD2 were used in RT-PCR analysis for the transcription of PSSIII gene. (B) PCR analysis on PM1 and PM2 using PrC1 and PrC2 as primers. 1. 1kb plus DNA ladder, 2. WT, 3. PM1, 4. PM2.

Figure 2. The construction for disrupting the remaining glycogen synthase gene in PM1 and PM2. (A) The center of GSI and GSII was replaced with a chloramphenicol resistant (CRP^R) cassette. The arrows show the positions of primers, PrA1, PrA4, PrB1, and PrB4, for PCR analysis. (B) PCR confirmation on the disruption of the remaining glycogen synthase gene in mutants PM12 and PM21. 1.1kb plus DNA ladder; 2, 3, and 4: PCR amplification on PM12, PM21, and WT genomic DNA using prA1 and prA4 as primers. 5, 6, and 7: PCR amplification on PM12, PM21, and WT genomic DNA using prB1 and prB4 as primers. 8, 9, 10, 11: PCR amplification on PM12, PM21, WT and plasmid pUSD using PrC1 and PrC2 as primers. (C) The expression of PSSIII mRNA revealed by RT-PCR using PSSIII gene internal primers (PrD1 and PrD2). 1. 1kb plus DNA ladder, 2. WT, 3. M1, 4. PM1, 5. M2, 6. PM2, 7. PM12, 8. PM21.

Figure 3. Zymogram analysis of glucans synthase activity from wild type and mutants. Soluble extracts (20 μl) were separated in a native PAGE by electrophoresis. The native gel was incubated in sodium citrate buffer (50 mM, pH7.0) containing ADP-glucose and rabbit-

liver glycogen for 24 hours and then stained with iodine solution. 1. WT, 2. M1, 3. M2, 4. PM1, 5. PM2, 6. PM12, 7. PM21.

Figure 4. Zymogram analysis of glucan activity from wild type and mutants. Soluble extracts (20 μl) were separated in a native PAGE containing 0.3% glycogen by electrophoresis. (A) The native gel was incubated in sodium citrate buffer (50 mM, pH7.0) containing ADP-glucose and rabbit-liver glycogen for 24 hours and then stained with iodine solution. (B) The native gel was incubated in 0.1M Bicine buffer (pH8.5), 0.5 sodium citrate, 0.5mg.ml BSA, 10gml rabbit liver glycogen, 133mM $(NH_4)_2SO_4$, 7mM $MgCl_2$, 25mM 2-mercaptoethanol, and 5mM ADP-glucose at 30°C overnight for 24 hours and then stained with iodine solution. 1, WT, 2. M1, 3. M2, 4. PM1, 5. PM2, 6. PM12, 7. PM21.

Figure 5. Branch chain-length distribution profiles of glucans from WT and mutants. The glucans from WT and mutants were completely debranched and separated by using HPAEC system.

Figure 6. Debranching patterns of the glucans from WT and mutants. A. PM1-WT, B. PM2-WT, C. PM1-M1, D. PM2-M2.

Figure 7. Debranching patterns of the glucans from WT and mutants. A. PM21-WT, B. PM21-M1, C. PM21-M2, D. PM21-PM1, E. PM21-PM2, F. PM21-PM12.

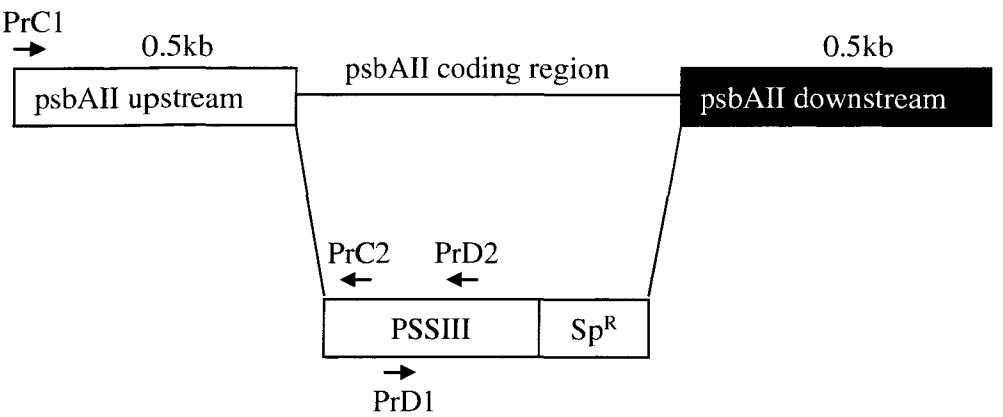
Table 1. Total glucan synthase activity, glucan branching enzyme activity, and glycogen yield of WT and mutants.

Strain	GS activity	BE activity	Yield
	^{14}C incorporation rate	P_i releasing rate	
	μmol glucose min^{-1} mg^{-1} protein	mM P_i min^{-1} mg^{-1} protein	mg glycogen g^{-1} wet cell mass
WT	0.084±0.005	0.21±0.01	51.1±3.1
M1	0.083±0.001[a]	0.25±0.02[a]	46.7±4.0[a]
M2	0.138±0.009[b]	0.41±0.05[b]	42.2±3.3[a]
PM1	0.073±0.002[a]	0.37±0.04[b]	46.8±1.2[a]
PM2	0.222±0.006[b]	0.91±0.01[b]	47.7±1.0[a]
PM12	0.012±0.001[b]	0.35±0.01[b]	39.8±5.2[a]
PM21	0.010±0.001[b]	0.75±0.01[b]	49.1±1.4[a]

[a]Not significantly different

[b]Significantly different at the P=0.05 level of probability

A

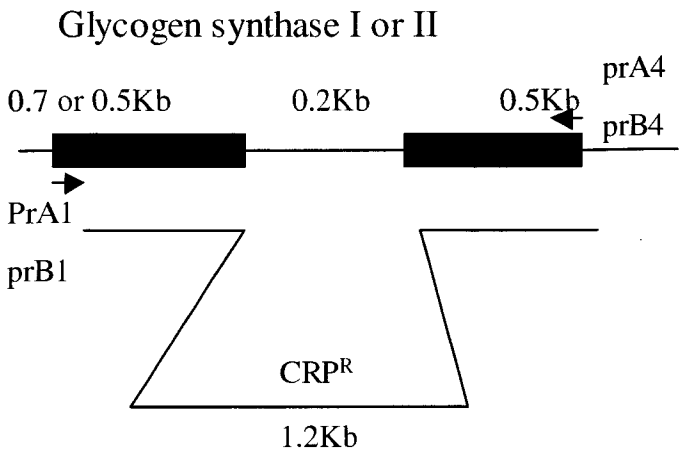

PrC1
0.5kb
psbAII upstream
psbAII coding region
0.5kb
psbAII downstream
PrC2
PrD2
PSSIII
Sp^R
PrD1

B


1 2 3 4

Figure 1.

A



B

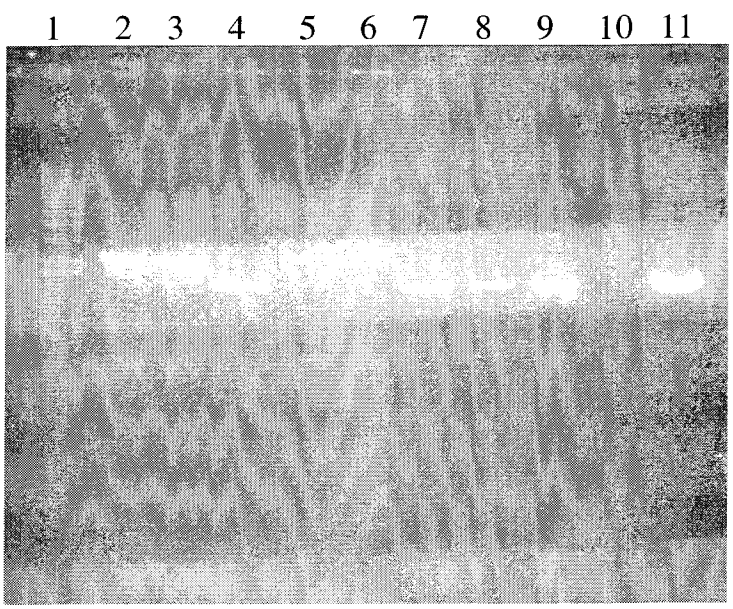


Figure 2A and 2B.

C

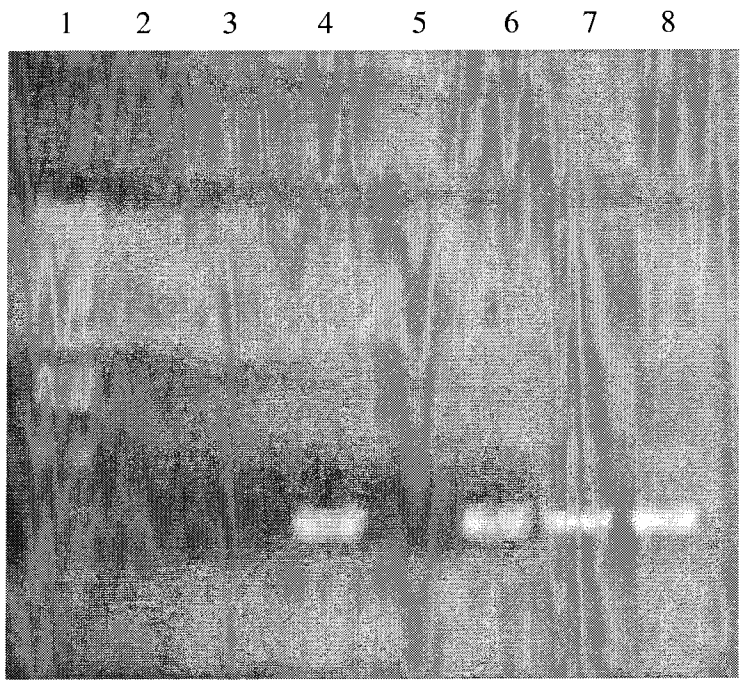


Figure 2C.

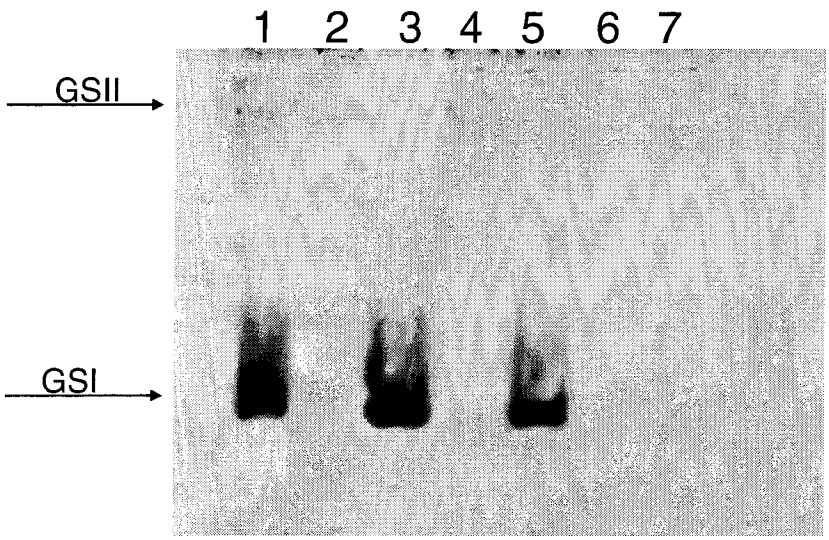


Figure 3.

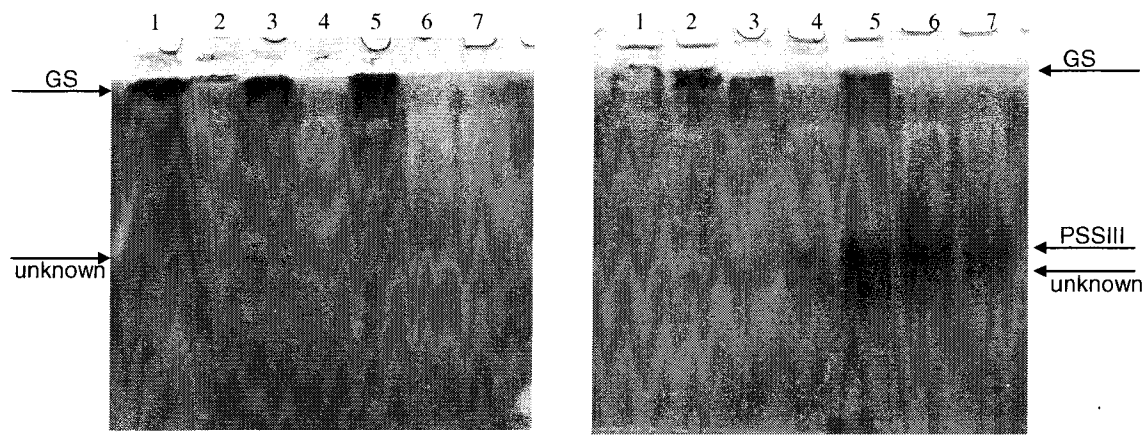


Figure 4.

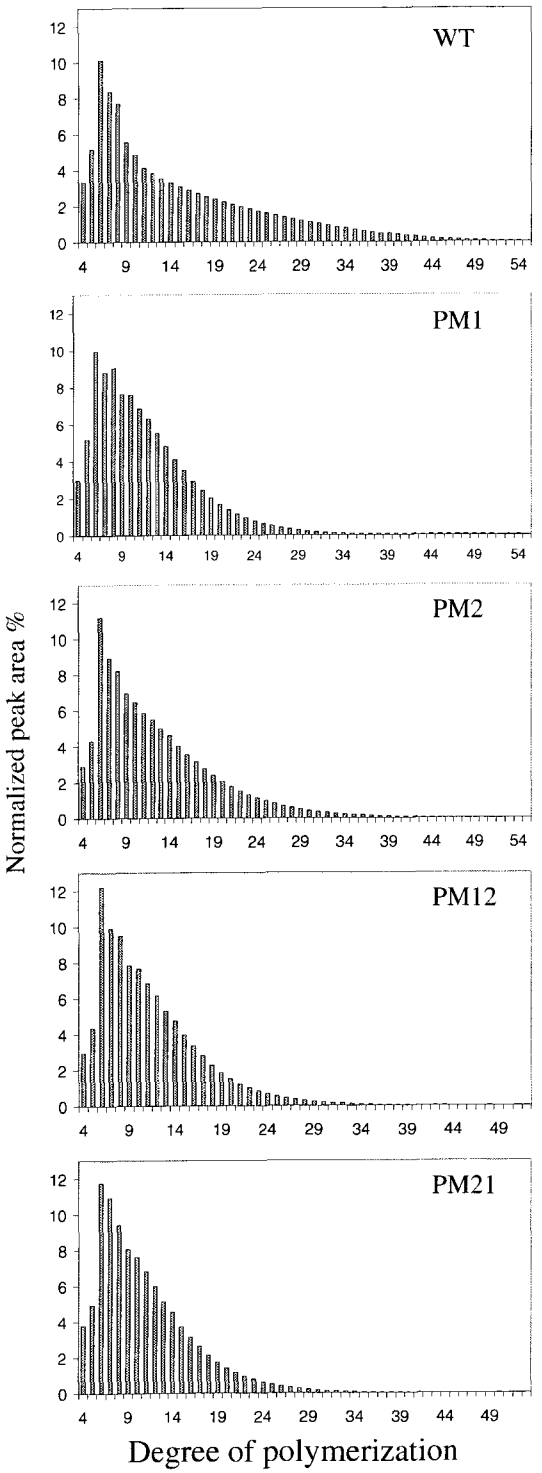

WT
PM1
PM2
PM12
PM21
Normalized peak area %
Degree of polymerization

Figure 5.

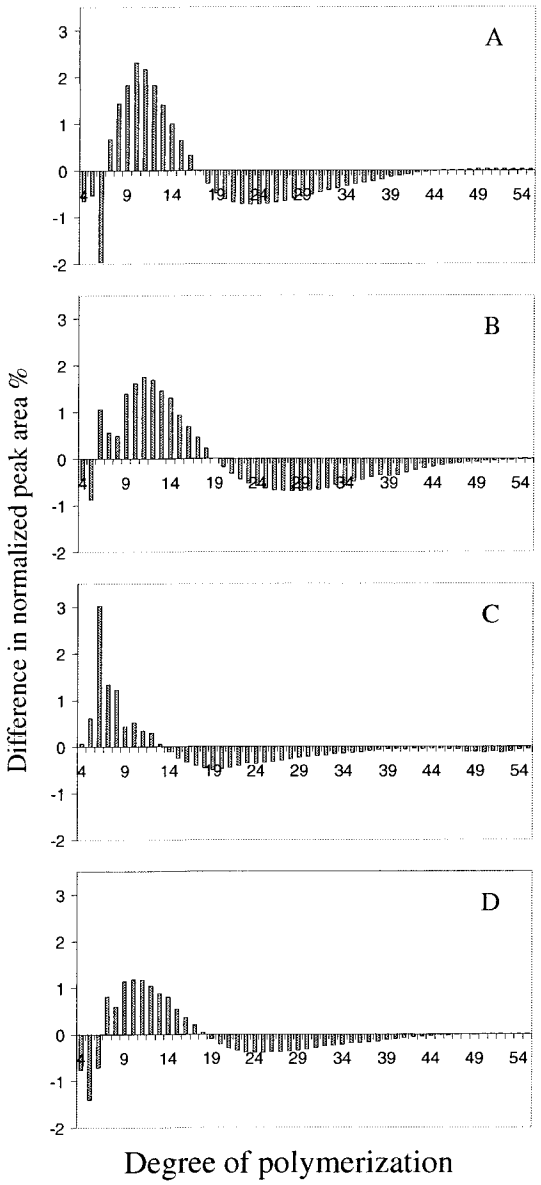

A
B
C
D
Difference in normalized peak area %
Degree of polymerization

Figure 6.

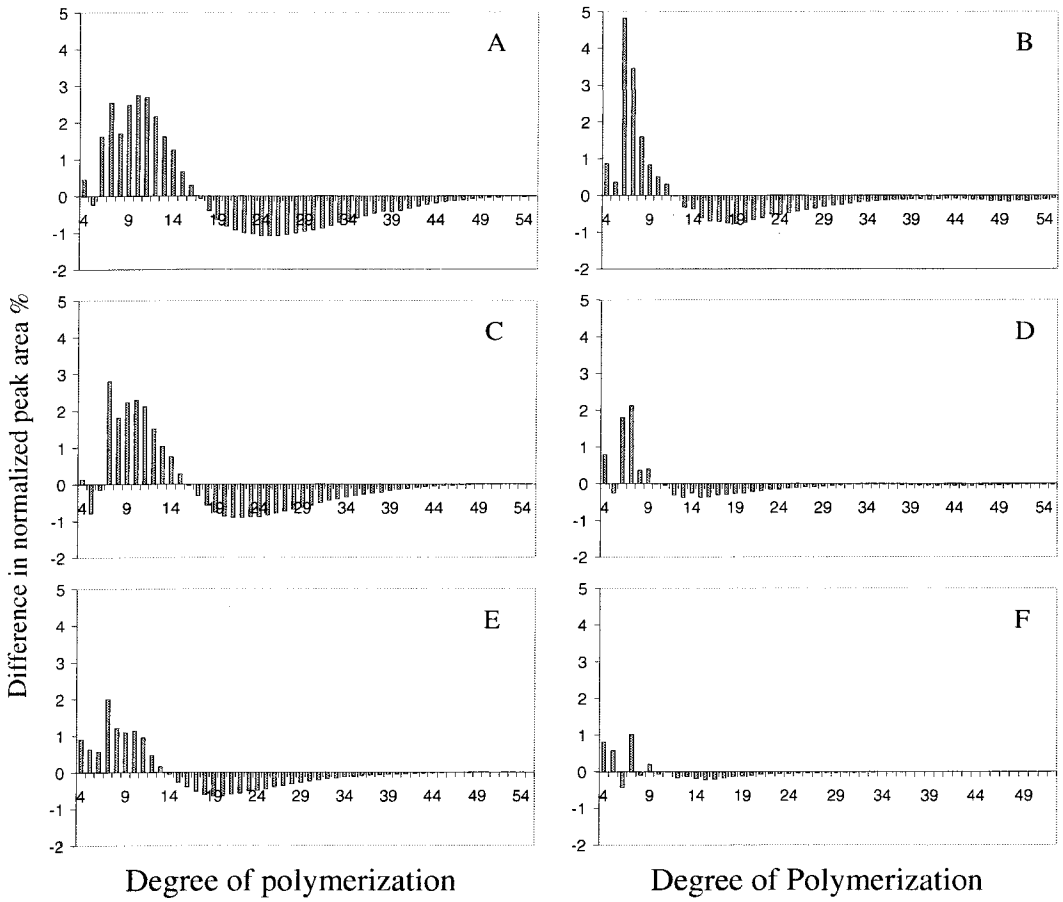

A
B
C
D
E
F
Difference in normalized peak area %
Degree of polymerization
Degree of Polymerization

Figure 7.

CHAPTER 4. PHYSICOCHEMICAL PROPERTIES OF ENDOSPERM AND PERICARP STARCHES DURING MAIZE DEVELOPMENT

Li Li[1], Michael Blanco[2], and Jay-lin Jane[1]*

[1]Department of Food Science and Human Nutrition, Iowa state University, Ames, Iowa 50011; [2]USDA-ARS/Plant Introduction Station, Ames, Iowa 50011

*Corresponding author:

Fax: 515-294-8181; E-mail address: jjane@iastate.edu

Abstract

Endosperm starch and pericarp starch were isolated from maize (B73) kernels at different developmental stages. Starch granules, with small size (2-4 μm diameter), were first observed in the endosperm on 5 days after pollination (DAP). The size of endosperm starch granules remained similar until 12DAP, but the number increased extensively. A drastic increase in granule size was observed from 14DAP (diameter 4-7 μm) to 30DAP (diameter10-23 μm). The starch content of the endosperm was little before 12DAP (less than 2%) and increased rapidly from 10.7% on 14DAP to 88.9% on 30DAP. The amylose content of the endosperm starch increased from 9.2% on 14DAP to 24.2% on 30DAP. The amylopectin branch chain-length of the endosperm starch increased from DP23.6 on 10DAP

to DP26.9 on14DAP and then decreased to DP25.4 on 30DAP. The onset gelatinization temperature of the endosperm starch increased from 61.3°C on 8DAP to 69.0°C on 14DAP and then decreased to 62.9°C on maturation. The percentage retrogradation of the endosperm starch after 7 days at 4°C increased from 26.4% on 8DAP to 48.7% on 14DAP and then decreased to 42.7% on 30DAP. Results indicated that the endosperm starch structure was not synthesized consistently with the maturation of kernel. The pericarp starch, however, showed similar granule size, starch content, amylose content, amylopectin structure and thermal properties at different developmental stages of the kernel.

Introduction

Maize is one of the most important crops extensively cultivated in the US. Mature kernels consist of up to 78% starch (Watson, 2003). Mature endosperm starches of maize and other cereal crops have been extensively studied for their compositions, structures, and properties. Starch structures and properties during kernel development, however, are not fully understood. Although the activities of many starch biosynthetic enzymes have been analyzed within the developing kernels of cereals, there is no integrated understanding of the relative roles of the expression of starch biosynthetic enzymes to starch structures at different developmental stages.

Starch biosynthesis requires a series of enzymes working coordinately, including ADP-glucose pyrophosphorylase, granule-bound starch synthase (GBSS), soluble starch synthases, branching enzymes (BE) and debranching enzymes (DBE). Studies on starch

biosynthesis have shown that each starch biosynthetic enzyme exists in several isoforms. The expression of certain isoform is tissue-specific. For example, ADP-glucose pyrophosphorylase mostly exists as an extra-plastidial form in the cereal endosperm, whereas it is in a plastidial form in other cereal tissue (James et al., 2003). In waxy wheat grains, GBSSI was knocked out from the endosperm tissue, but GBSS II was found in the pericarp (Nakamura et al., 1998). Maize BEIIb was only found in the endosperm and the reproductive tissue (James et al., 2003). Even in the same tissue, the expression patterns of enzyme isoforms are different. For example, BEIIa is expressed at the maximal level (5-7 days after flowering, DAF) earlier than BEI and BEIIb (7-10 DAF) in rice endosperm (Mizuno et al., 2001). Rice starch synthase (SS) III-1, SSIII-2, and SSIV-1 are expressed to the maximum level at the early, mid, and late developmental stages of the endosperm, respectively (Dian et al., 2005). In addition, these isoforms function differently in the starch biosynthesis. For example, studies of maize branching enzyme isoforms suggested that maize BEI catalyzes the transfer of longer chains than BEII (Guan and Preiss, 1993). Studies on kinetic properties of maize starch synthase isoforms suggests that maize SSI and SSIIb prefer using shorter chains as the primer for elongation than SSIIa (Imparl-Radosevich et al., 1999). A maize mutant with a disruption of the SSIII gene (*dull1*) produced starch containing 15% of intermediate material with the size and structures between amylopectin and amylose (Gao et al., 1998). These findings indicate that the disruption of SSIII results in the decrease of the production of the long chains that can extend though amylopectin cluster. Thus, SSIII functions in biosynthesis of long chains.

Starch is synthesized in both storage form and transit form. As mentioned above, the expression patterns of starch biosynthetic enzymes are different in storage organ and other

tissues. It suggests that starches produced in different tissues at different times have different structures. Differences in structures of starches produced in different tissues have been reported in waxy cereals. Starch in endosperm, embryo sac and pollen of waxy kernel is stained brown-red by iodine, whereas starch from leaves and pericarp is stained black-blue (Hixon and Brimhall, 1968). In the storage organ of maize and potato, the amylose content increased with the increase in size and the radial distance from the hilum (Jane and Shen, 1993; Pan and Jane, 2000). Chemical gelatinization of normal maize and potato starch granule reveals that the amylose content is greater at the periphery of the starch granule than that at the core, and amylopectin had shorter long-branch-chains at the periphery than at the core (Jane and Shen, 1993; Pan and Jane, 2000). These results suggest that starch structures change with the development of granules. However, there is lack of understanding on detailed structures of starch at different developmental stages and in the different tissues.

In this study, we investigated starch granule morphology, the amylose content, amylopectin branch chain-length distribution and thermal properties of maize endosperm and pericarp starches isolated at different developmental stages. This information will be useful for the understanding of how enzymes catalyze starch biosynthesis and granule growth during the maturation of maize kernel.

Materials and Methods

Starch isolation

Maize kernels of self pollinated inbred B73 were harvested on 0, 5, 6, 8, 10, 12, 14, 20, and 30 days after pollination (DAP) and kept at -20°C until starch extraction. The pericarp was separated from the endosperm by hand. Separated endosperms were combined and soaked in absolute ethanol to inactivate the enzymes, so were pericarps. The steeped sample was milled in a microblender 3×1min. The ground sample was filtered through a nylon screen with a pore size of 53μm and washed with excess of absolute ethanol. The residue portion was ground again with additional absolute ethanol until no more starch was released. Starch was collected by centrifugation, resuspended in 0.1M aqueous NaCl solution containing 10% toluene and stirred one hour using magnetic stirrer at a high speed to remove protein. This step was repeated until the toluene layer contained no protein. The purified starch was washed three times with water and rinsed twice with ethanol and dried at 30°C for 48 hours. Mature B73 maize starch was isolated from kernels collected on 45DAP and dried (provided by USDA-ARS/Plant Introduction Station).

Starch content of the endosperm and the pericarp

The starch contents of the endosperm and the pericarp were determined by using the Total Starch Kit (Megazyme, Co. Wicklow, Ireland). The separated endosperm and pericarp samples were freeze-dried before analysis. These samples were ground and then washed with 80% ethanol to remove any glucose residues. The sample was then digested with heat stable α-amylase and amyloglucosidase, mixed with GOPOD reagent (Megazyme, Co. Wicklow, Ireland), and measured at 510nm. The sample was analyzed in duplicate. The moisture content of the sample was determined by drying the sample in an oven at 110°C for 3 hours.

Scanning electron microscopy and light microscopy

Starch granules and cracked maize kernels were placed on the surface of a brass disk on double sided adhesive silver tape and coated with gold/palladium (60/40) and viewed by using scanning electron microscope (JOEL model 1850, Tokyo, Japan) at Bessey Electron Microscopy facility, Iowa State University. Micrographs of each starch sample were taken at 1,500× or 5,000× magnification. Fresh maize endosperms harvested on 6, 8, 10, 12 and 14 DAP were crashed, stained by iodine solution, and observed by using light microscope (Zeiss, Jena, Germany) under bright field and polarized light.

Amylose content of starch

The amylose content of starch was determined by using gel permeation chromatography (GPC), following the method of Jane and Chen (1992). Starch (15 mg) was dispersed in 90% DMSO, precipitated with ethanol, and then re-dispersed in distilled water (5 ml). Starch dispersion (2 ml) was injected into a Sepharose CL-2B gel permeation (Pharmacia, Piscataway, NJ) column (1 cm ID × 48 cm) and eluted by using an eluent containing 25 mM NaCl and 1 mM NaOH at a flow rate of 0.7 ml/min in a descending mode. Fractions of 1.0 ml each were collected and analyzed for total carbohydrate (Phenol-sulfuric acid method) (Dubois et al., 1956) and blue value (iodine staining) (Juliano, 1971) at 490 and 630nm, respectively. The sample was analyzed in duplicate. The amylose content was calculated by dividing the total carbohydrate content of the amylose peak by the sum of that of amylopectin and amylose peaks.

Amylopectin branch chain-length distribution

Amylopectin was separated using GPC. The fractions under the amylopectin peak were combined, evaporated, and used for branch chain-length distribution analysis. The amylopectin was dispersed in a DMSO solution (90%) with stirring to disperse the molecules. The amylopectin was precipitated by adding 3 volumes of absolute ethanol and centrifuged at 8000 g for 20 min. The amylopectin (3mg) was re-dispersed in hot water (2.7 ml) and stirred in a boiling water bath for 20 min, cooled down to room temperature and digested with 60-120 units isoamylase in acetate buffer (0.01N, pH3.5) containing 0.02% sodium azide at 40°C and 120 strokes per minute for 24 hours. The digested sample was adjusted to pH 7 by adding NaOH and boiled for 15 min to inactivate the isoamylase.

The branch chain-length distribution of amylopectin was analyzed by using high performance anion-exchange chromatography (Dionex-300, Sunnyvale, CA) equipped with an amyloglucosidase column and an ED50 electrochemical detector (Dionex, Sunnyvale, CA) (HPAEC-ENZ-PAD). The debranched samples were separated by using a PA-100 anion-exchange analytical column (4×250 mm) and a guard column ((Dionex, Sunnyvale, CA). The operating condition was the same as that described by McPherson and Jane (1999). The data were analyzed by using Chromeleon software (Dionex, Sunnyvale, CA). The sample was analyzed in duplicate.

Molecular weight and gyration radius of amylopectin

The weight-average molecular weight (M_w) and z-average gyration radius (R_z)' of amylopectin were determined by using high-performance size-exclusion chromatography equipped with a multi-angle laser-light scattering (Dawn DSP-F, Wyatt Tech., Santa Babara, CA) and a HP 1047A refractive index detectors (Hewlett Packard, Valley Forge, PA)

(HPSEC-MALLS-RI). A Shodex OH pak KB-G guard column and KB-806 and KB-804 analytical columns were used to separate the sample. The sample was analyzed in duplicate. The operating condition was the same as that described by Yoo and Jane (2002).

Differential scanning calorimetry (DSC)

Thermal properties of native starch were determined by using a differential scanning calorimeter (DSC-7, Perkin-Elmer, Norwalk, CT), following the method of Song and Jane (2000). The starch sample (about 3mg, dry starch basis (dsb)) with excess water (1:3) was heated at 10°C/min from 25-110°C in sealed aluminum pans, using an empty pan as the reference. The sample was analyzed in triplicate, and the data were analyzed by using Pyris software (Perkin-Elmer, Norwarlk, CT). The gelatinized starch sample was stored at 4°C for 7 days and then analyzed following the same procedure to determine the properties of retrograded starch.

Results and Discussion

Starch granule development in the kernel

Starch granules were first observed in the endosperm on 5 days after pollination (Figure 1). Scanning electron micrographs (SEM) revealed that few granules were present in the center of the endosperm and the sizes of the starch granules were small (1-4 μm) (Figure 1B). Starch granules of 1-4 μm diameters didn't show maltese cross under a polarized microscope, and they were stained blue with iodine solution (Figure 2). Up to 12DAP, the size of starch granules remained similar and small. A significant increase in starch granule

size was observed on 14DAP (diameter about 7 μm) (Figure 1G). The size of some starch granules reached 23 μm on 30DAP, which is similar to the size of mature endosperm starch granules. It appeared that spherical granules observed on 20DAP (Figure 1H) became polygonal shape on 30DAP (Figure 1I), resulting from space restriction. Starch granules located at the periphery of the endosperm had a smaller size than those at the center and maintained spherical shape. SEM of isolated endosperm starches are shown in Figure 3. Compared with starch granules isolated before 30DAP, more pinholes were observed on the surface of mature endosperm starch granules (Figure 4).

Development of starch granules have been studied in many plant species. Those studies show either that starch granules remain similar in size or increase in size with the development of roots or endosperms. Increases in the size of starch granules during the maturation have been reported in rice (Murugesan and Hizukuri, 1992), barley endosperms (Duffus and Cochrane, 1993), yam (Sugimoto et al., 1988), potato (Liu et al., 2003), and arrowhead tubers (Sugimoto et al., 1988). Starch granules of taro (Sugimoto et al., 1987), however, display similar sizes and shapes throughout the development of the root. In this study, an increase in granule size was observed after 12DAP. It has been proposed that the number of starch granules in the rice endosperm is determined at the early developmental stage (Briones et al., 1968). Our results indicated that the initiation of starch granules was the major focus before12 DAP in the maize endosperm, and the size of starch granules increased and filled up the space of the endosperm during later developmental stage. This was in agreement with that reported by Briones et al. (1968).

Pericarp starch granules, however, maintained a similar size during kernel development. The shape of pericarp starch granules was spherical at the early stage of the

kernel development, but became polygonal and irregular at the later stage of the kernel development (Figure 5). The pericarp was evolved from the ovary wall after pollination. The size increase in the pericarp was accompanied by its cell division and enlargement during the kernel development (Watson, 2003). The pericarp starch could be an energy source for the growth of the pericarp, which was not controlled by pollination.

Starch content of the endosperm and pericarp

The starch content of maize endosperm and pericarp are shown in Table 1. The starch content of maize endosperm increased from 1.0% on 8DAP, 2% on 12DAP, 10.7% on 14DAP, and 88.9% on 30DAP. These results were in agreement with the activities of starch biosynthesis related enzymes in the developing maize endosperm (Tsai et al., 1970), who reported that the activities of UDP-glucose pyrophosphorylase, ADP-glucose pyrophosphorylase, hexokinase, granule bound starch synthase and soluble starch synthase increased rapidly after 12DAP and reached the maximum on 20-22DAP. The results of these two studies suggest that the fast deposit of starch in the endosperm after 12DAP result from the rapid increase of starch biosynthetic enzyme activities. There was no significant difference observed in pericarp starch content during the kernel development except the low starch content of unpollinated ovary.

Amylose content of endosperm and pericarp starches

The amylose content of endosperm starch and pericarp starch are shown in Table 2. The amylose content of the endosperm starch increased from 9.2% on 12DAP to 24.2% on 30DAP. The mature endosperm starch had similar amylose content as the starch isolated on

30DAP. This suggested that small granules contained less amylose than large granules. Kidney bean starch (Yoshida et al., 2003), potato starch (Jane and Shen, 1993; Liu et al., 2003), and maize starch (Pan and Jane, 2000) show the same trend for starch of different granule sizes. This result was consistent with the fact that the outer layer of starch granules, separated by chemical surface gelatinization, contains greater amylose content than the inner part of the granules (Pan and Jane, 2000; Jane and Shen, 1993) using surface gelatinization method. It is known that starch granule is synthesized from the hilum towards the periphery (Jane et al., 2003). Thus, the core of the granule corresponds to the small granules initiated at the early stage of the starch granule development.

Granule bound starch synthase I (GBSSI) is the primary enzyme for amylose biosynthesis in storage organ (Vrinten and Nakamura, 2000). In pea and potato storage organs, the level of GBSSI expression increases in the later developmental stage (Dry et al., 1992). The mRNA of rice (Dian et al., 2005) and maize (Gao et al., 1996) GBSSI reached the maximal level in the late stage of endosperm development. Thus, the increase in GBSSI expression is likely responsible for the increase in the amylose content of starch granules during the maize kernel maturation.

Unlike that of endosperm starch, the amylose content of pericarp starch didn't increase with the kernel development but remained similar. Using iodine staining, Hixon and Brimhall (1968) report that leaf and pericarp starches of the waxy mutant of maize stain black-blue, while endosperm and pollen starches of the same mutant stain brown-red. Similar results are also obtained in rice (Igaue, 1964) and wheat (Nakamura et al., 1998). Vrinten and Nakamura (2000) report that different isoforms of granule-bound starch synthases are present in the endosperm and the pericarp tissues of wheat, and it is known that starch biosynthesis system

of the endosperm is different from that of the pericarp, which may explain the amylose contents of endosperm and pericarp starches vary (Table 2).

Amylopectin branch chain-length distributions and molecular weights

The branch chain-length distributions of endosperm and pericarp amylopectins are shown in Tables 3 and 4, respectively. The branch chain-length profiles showed a bimodal distribution with peak chain lengths at DP14 and DP47-50 (Figure 6). The shortest branch chain detected is DP4 on 12DAP. The short chains (DP≤12) of endosperm amylopectins decreased from 21.1% on 10DAP to 16.7% on 20DAP and then increased to 17.4% on 30DAP. Branch chains of DP13-24 decreased from 48.3% on 10DAP to 42.4% on 12DAP and then increased to 47.5% on 30DAP. The long chains (DP≥37) of endosperm amylopectins increased from 16.3% on 10DAP to 22.5% on 14DAP and then decreased to 20.6% on 30DAP. The same trend was also observed on the average chain length of endosperm amylopectins. Compared with amylopectin from 30DAP endosperm, the mature amylopectin contained more short chains (DP≤12), less branch chains of DP13-24 and shorter average chain length. Amylopectin of endosperm starch harvested on 14DAP showed the longest average chain length. These results indicated that the structures of amylopectin molecules were not homogenous in starch granules and changed with the development of the endosperm. BEI and BEIIb in maize W64A displays different expression pattern during the kernel development (Gao et al., 1996). The ratio of BEI to BEIIb relative transcript level was higher at the early developmental stage (until 12DAP). Guan and Preiss (1993) have reported that maize BEI transfers longer branches than BEII in vitro. The increase of relative expression level of BEIIb may result in the production of amylopectin with shorter average

branch chain-length after 14DAP. At the late developmental stage (after 20DAP), the average chain length and the percentage of long chains decreased. It is in agreement with that long B chains of amylopectin at the periphery of maize and potato granule are shorter that those at the core of the granules reported by Jane and Shen (1993) and Pan and Jane (2000). The branch chain-length distributions of pericarp starch are shown in Table 4. There was no obvious correlation observed between the branched structure of pericarp amylopectin and the kernel development.

The M_w and gyration radii of endosperm amylopectins are shown in Table 5. The M_w of endosperm amylopectins decreased from 3.7×10^8 on 12DAP to 1.3×10^8 on 14DAP and then increased to 2.0×10^8 on 30DAP. The gyration radii of endosperm amylopectins decreased from 234nm on 12DAP to 135nm on 14DAP and then increased to 185nm on 30DAP. It appeared that the M_w of amylopectin decreased as the average branch chain length increased.

Thermal properties

Thermal properties of endosperm starch measured by using DSC are shown in Table 6. The onset gelatinization temperature of endosperm starches increased from 61.3°C on 8DAP and 61.5°C on 10DAP to 69.0°C on 14DAP and then decreased to 67.4°C on 30DAP and 62.9°C on maturation. The low gelatinization temperature of starch produced on 10DAP agreed with its shortest average branch chain length of amylopectin, the smallest amount of the long chains (DP≥37), and the largest proportion of short chains (DP≤12). Studies have shown that starch having more short chains DP≤12 and less long B chains displayed lower gelatinization temperature (Shi and Seib, 1992; Yuan et al., 1993; Jane et al., 1999).

Vandeputte et al. (2003) have reported that the percentage of B1 chain (DP 13-24) is positively correlated with the onset gelatinization temperature of rice starches, whereas that of the short chain (DP≤12) is negatively correlated with the onset gelatinization temperature of rice starches. The increase in the onset gelatinization temperature from 63.0 to 69.0°C from 12 DAP to 14DAP can be attributed to the decrease in short branch chains (DP≤12) from 21.1% to 18.3% and the increase in branch chains of DP 13-24 from 42.2% to 45.6% (Table 4). The enthalpy change increased from 13.0 J/g on 8DAP to 15.6 J/g on 14DAP, indicating less crystallinity in small granules than large ones. This result was consistent with the observations that the hilum of a granule was loosely packed with less ordered structure (Pan and Jane, 2000; Baker et al., 2001). The onset gelatinization temperature of endosperm starch decreased from 69.0°C on 14DAP to 67.4°C on 30DAP. It agreed with the decrease in amylopectins long B chain from 22.5% on 14DAP to 20.6% on 30DAP.

The retrogradation rate of endosperm starches increased from 26.4% on 8DAP to 48.7% on14DAP (Table 7). Increasing amylose content of the starch and increasing branch chain length of amylopectin were attributed to the increase in retrogradation rate. It is known that amylose molecules and long-branch chains of amylopectin are retrograde relatively quickly. The gelatinization temperature and the retrogradation rate of pericarp starches did not change significantly with the development of kernels (Table 8 and Table 9).

Conclusions

Maize endosperm starch content, granule size, and amylose content increased during the kernel development. Branch chain-length distribution of endosperm amylopectins showed that amylopectin had shorter average chain length (DP 23.6) on 10DAP, increased to the

maximum (DP26.7) on 14DAP, and then decreased to DP 25.4 on 30DAP. The onset gelatinization temperature of endosperm starches increased from 61.3°C on 8DAP to 69.0°C on 14DAP and then decreased to 67.4°C on 30DAP. The retrogradation rate of endosperm starches increased from 26.4% on 8DAP to 48.7% on 14DAP and decreased to 42.7% on 30DAP. In contrast, there were no significant changes in size, amylose content, starch content, and thermal properties of pericarp starches during the development of maize kernel.

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Figure legends

Figure 1. Starch granules at the center of endosperms in the B73 maize kernels harvested at the different developmental stages (1,500×). A: Without pollination. B: 5DAP. C: 6DAP. D: 8DAP. E: 10DAP. F: 12DAP. G: 14DAP. H: 20DAP. I: 30DAP.

Figure 2. Light micrographs of starch granules in endosperms of kernels harvested on 6, 8, 10, 12, and 14 DAP. Samples were viewed under bright field, polarized light. Iodine stained endosperm samples were viewed under light field. The magnification was set at 100×. The arrows point to iodine stained small starch granules.

Figure 3. SEM of endosperm starches isolated at different developmental stages (1,500×). A: 8DAP. B: 10DAP. C: 12DAP. D: 14DAP. E: 20DAP. F: 30DAP.

Figure 4. SEM of endosperm starch isolated at 30DAP and mature endosperm starch (5,000×). A: 30DAP. B:Mature.

Figure 5. SEM of pericarp starches isolated at different developmental stages (1,500×). A: Ovary (without pollination). B: 6DAP. C: 8DAP. D: 10DAP. E: 12DAP. F: 14DAP. G: 20DAP. H: 30DAP.

Figure 6. Branch chain-length distributions of maize amylopectins isolated at different developmental stages.

Table 1. Starch contents of the endosperm and the pericarp at different developmental stages of maize

Days after Pollination	Endosperm (% Dry weight)	Pericarp (% Dry weight)
0	N. D.	4.1±0.0 (Ovary)
8	1.0±0.1	11.1±0.8
10	1.5±0.8	9.9±0.1
12	2.0±0.5	10.8±1.2
14	10.7±1.7	8.2±2.8
20	68.3±4.9	11.3±0.0
30	88.9±5.1	10.9±0.4

N. D. ; Not detectable

Table 2. Amylose contents of endosperm and pericarp starches at different developmental stages of maize

Days after Pollination	Endosperm (%)[a]	Pericarp (%)
0	nd[b]	19.6±0.8
6	nd	19.7±1.9
8	nd	19.0±1.3
10	nd	14.7±0.9
12	9.2±0.8	14.4±1.4
14	11.1±0.6	16.2±3.5
20	21.4±0.9	18.3±1.2
30	24.2±0.8	19.3±2.3
Mature	24.4±0.7	nd

[a] Values given are means ± standard deviation obtained from two replicates.
[b] Not determined.

Table 3. Branch chain-length distributions of endosperm amylopectins

Samples	Percent distribution				Average CL
Days after pollination	DP≤12	DP13-24	DP25-36	DP≥37	
10	21.1±1.6[a]	48.3±0.2	14.2±0.0	16.3±1.8	23.6±0.9
12	21.1±0.1	42.2±0.1	15.8±0.4	20.9±0.2	24.8±0.1
14	18.3±0.1	45.6±0.2	13.7±0.5	22.5±0.7	26.9±0.2
20	16.7±0.0	46.2±0.1	15.1±0.3	22.1±0.3	26.3±0.1
30	17.4±0.5	47.5±0.9	14.5±0.3	20.6±1.6	25.4±0.6
Mature	19.4±0.0	46.3±0.8	13.4±0.3	20.8±1.0	24.9±0.5

[a]Values given are means ± standard deviation obtained from two replicates.

Table 4. Branch chain-length distributions of pericarp amylopectins

Samples	Percent distribution				Average CL
Days after pollination	DP≤12	DP13-24	DP25-36	DP≥37	
0	20.5±0.7[a]	45.7±1.1	13.8±0.1	20.0±1.9	25.1±1.1
6	18.0±0.7	44.2±0.7	14.0±0.3	23.8±1.1	26.8±0.5
8	19.6±0.2	44.8±0.4	13.6±0.0	22.0±0.6	25.9±0.3
10	20.2±0.5	45.9±0.5	13.6±0.3	20.4±0.8	25.3±0.4
12	21.3±0.0	43.4±0.4	11.1±0.0	23.8±0.5	28.3±0.1
14	20.0±0.2	46.5±0.1	14.1±0.1	19.2±0.2	24.7±0.1
20	21.0±0.3	46.1±1.1	13.9±0.2	19.1±1.3	24.5±0.6
30	21.8±0.1	47.0±0.6	12.9±0.0	18.2±0.8	24.1±0.3

[a]Values given are means ± standard deviation obtained from two replicates.

Table 5. Molecular weights and gyration radii of endosperm amylopectins

Days after Pollination	Mw ($\times 10^8$)[a]	R_Z (nm)[b]
12	3.7±0.3[c]	234 ± 9
14	1.3 ± 0.0	135 ± 4
20	2.0 ± 0.2	169 ± 8
30	2.0 ± 0.2	185 ± 4

[a]Weight-average molecular weight

[b] *z* -average radius of gyration

[c] Values given are means ± standard deviation obtained from two replicates.

Table 6. Thermal properties of native endosperm starches

Samples[a]	Native starch			
Days after pollination	T_0 (°C)	T_p (°C)	T_c (°C)	ΔH (J/g)
8[b]	61.3	66.5	73.2	13.0
10[b]	61.5	66.9	73.3	13.3
12	63.0±0.2[c]	67.8±0.3	74.8±0.5	14.5±0.2
14	69.0±0.2	72.9±0.2	77.8±0.2	15.6±0.5
20	67.5±0.0	71.9±0.0	77.7±0.1	15.6±0.3
30	67.4±0.1	71.0±0.2	75.3±0.1	14.2±0.3
Mature	62.8±0.1	68.0±0.1	72.8±0.1	13.8±0.1

[a] Samples (~3.0 mg,dsb) and deionized water (~9.0 mg) were used for the analysis; T_0, T_p, T_c and ΔH are onset, peak, conclusion temperature, and enthalpy change, respectively.
[b] Value was measured once.
[c] Values were calculated form three replicates; ±Standard deviation

Table 7. Thermal properties of retrograded endosperm starches

Samples[a]	Retrograded starch				R (%)[b]
Days after pollination	T_0 (°C)	T_p (°C)	T_c (°C)	ΔH (J/g)	
8[c]	40.7	55.0	62.0	3.4	26.4
10[c]	41.0	54.7	61.2	3.3	24.8
12	41.1±0.8	52.9±0.8	62.8±0.5	6.7±0.3	46.1±2.3
14	40.3±0.6	52.0±0.5	63.0±0.5	7.6±0.6	48.7±3.3
20	40.7±0.7	52.3±0.5	63.9±0.2	6.7±0.2	42.9±2.3
30	40.2±0.9	52.0±0.8	62.7±0.8	6.1±0.5	42.7±4.4
Mature	36.3±0.7	49.1±0.3	61.0±0.3	7.9±0.3	57.4±2.1

[a] After storage at 4°C for 7 days

[b] Retrogradation (%)=$\Delta H_{retro}/\Delta H_{native}\times 100$

[c] Value was measured once. Other values were calculated form three replicates; ±Standard deviation

Table 8. Thermal properties of native pericarp starches

Samples[a]	Native starch			
Days after pollination	T_0 (°C)	T_p (°C)	T_c (°C)	ΔH (J/g)
0	55.9±0.8[b]	64.5±0.5	72.9±0.7	15.2±0.2
6	63.5±0.3	69.1±0.3	75.3±0.4	14.9±0.6
8	61.2±0.6	67.0±0.8	74.2±1.0	14.2±0.4
10	61.6±0.6	67.3±0.5	74.4±1.3	14.9±0.9
12	60.8±0.4	66.2±0.5	71.0±3.1	14.4±0.4
14	60.8±0.2	66.6±0.4	75.2±0.9	15.3±0.7
20	59.4±0.4	65.8±0.5	72.7±0.9	14.3±0.3
30	62.6±0.5	68.6±0.5	75.9±0.7	14.7±0.7

[a] Samples (~3.0 mg,dsb) and deionized water (~9.0 mg) were used for the analysis; T_0, T_p, T_c and ΔH are onset, peak, conclusion temperature, and enthalpy change, respectively.
[b] Values were calculated form three replicates; ±Standard deviation

Table 9. Thermal properties of retrograded pericarp starches

Samples[a]	Retrograded starch				R (%)[b]
Days after pollination	T_0 (°C)	T_p (°C)	T_c (°C)	ΔH (J/g)	
0	42.8±1.0	53.1±0.4	61.3±0.1	4.3±0.1	28.1±0.2
6	42.1±0.4	55.2±1.3	63.0±0.7	4.6±0.7	30.9±3.0
8	42.5±0.5	55.6±1.6	63.0±0.9	4.4±0.3	31.0±3.1
10	43.0±1.2	54.8±0.4	63.7±0.4	4.6±0.4	31.1±4.0
12	42.0±1.3	55.4±1.1	63.9±0.7	4.0±0.3	27.7±3.1
14	42.9±0.7	54.3±0.2	63.3±0.3	4.8±0.7	31.6±4.7
20	40.6±0.2	54.3±0.4	63.7±0.4	3.9±0.4	27.1±3.2
30	40.4±0.9	54.1±0.3	62.7±0.3	4.1±0.4	27.8±3.4

[a] After storage at 4°C for 7 days

[b] Retrogradation (%)=$\Delta H_{retro}/\Delta H_{native}\times 100$

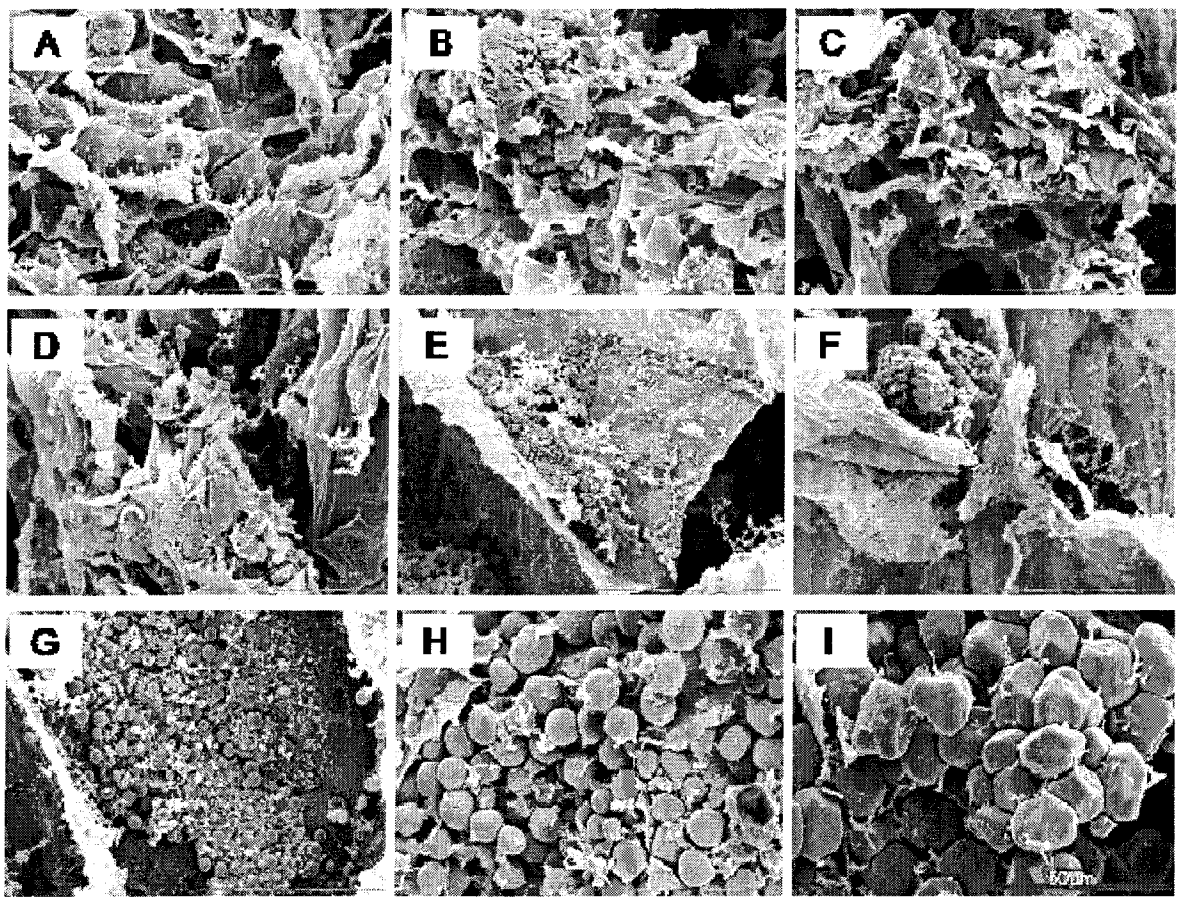

Figure 1.

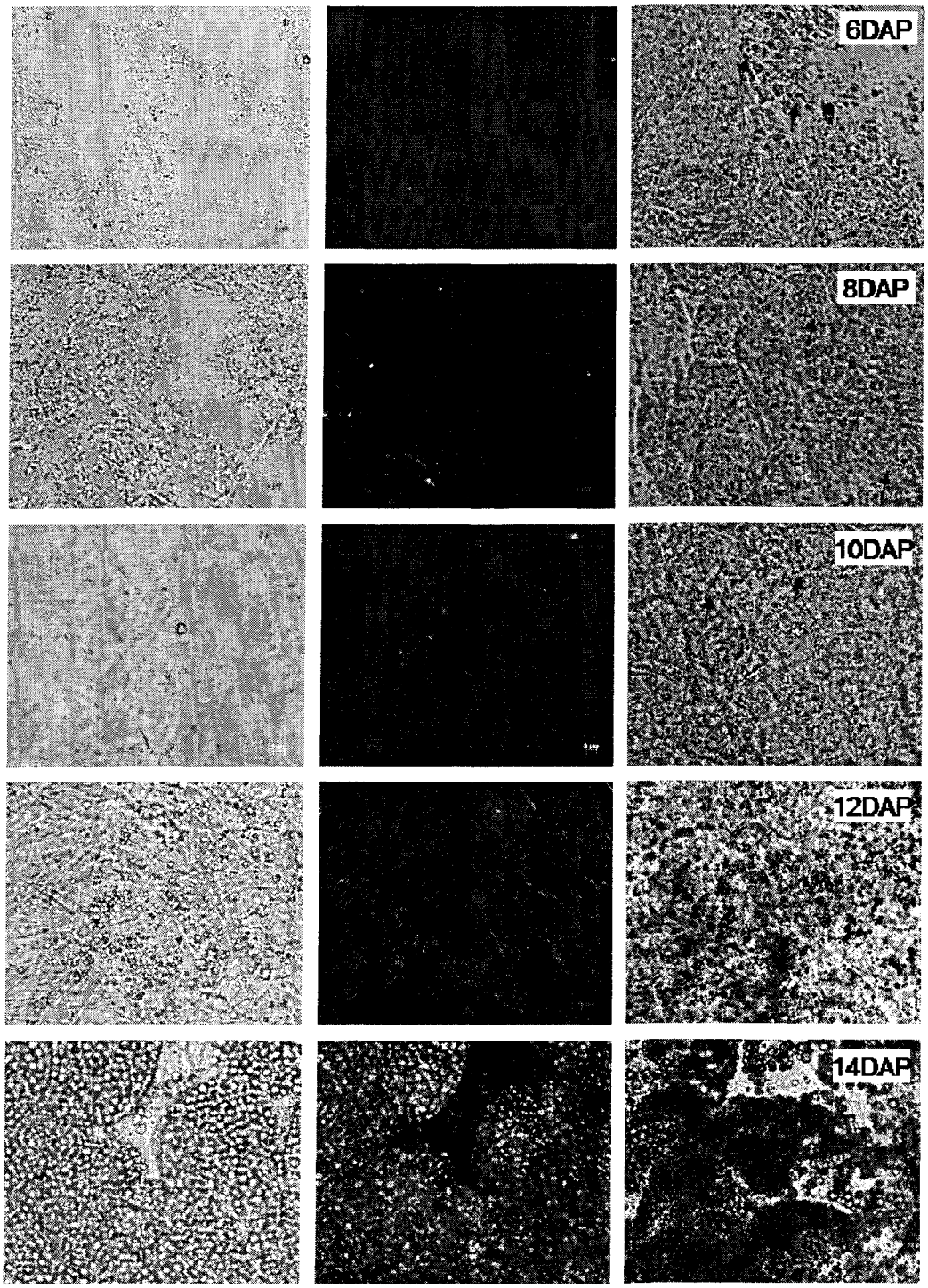
6DAP
8DAP
10DAP
12DAP
14DAP
Bright field
Polarized light
Iodine stained

Figure 2.

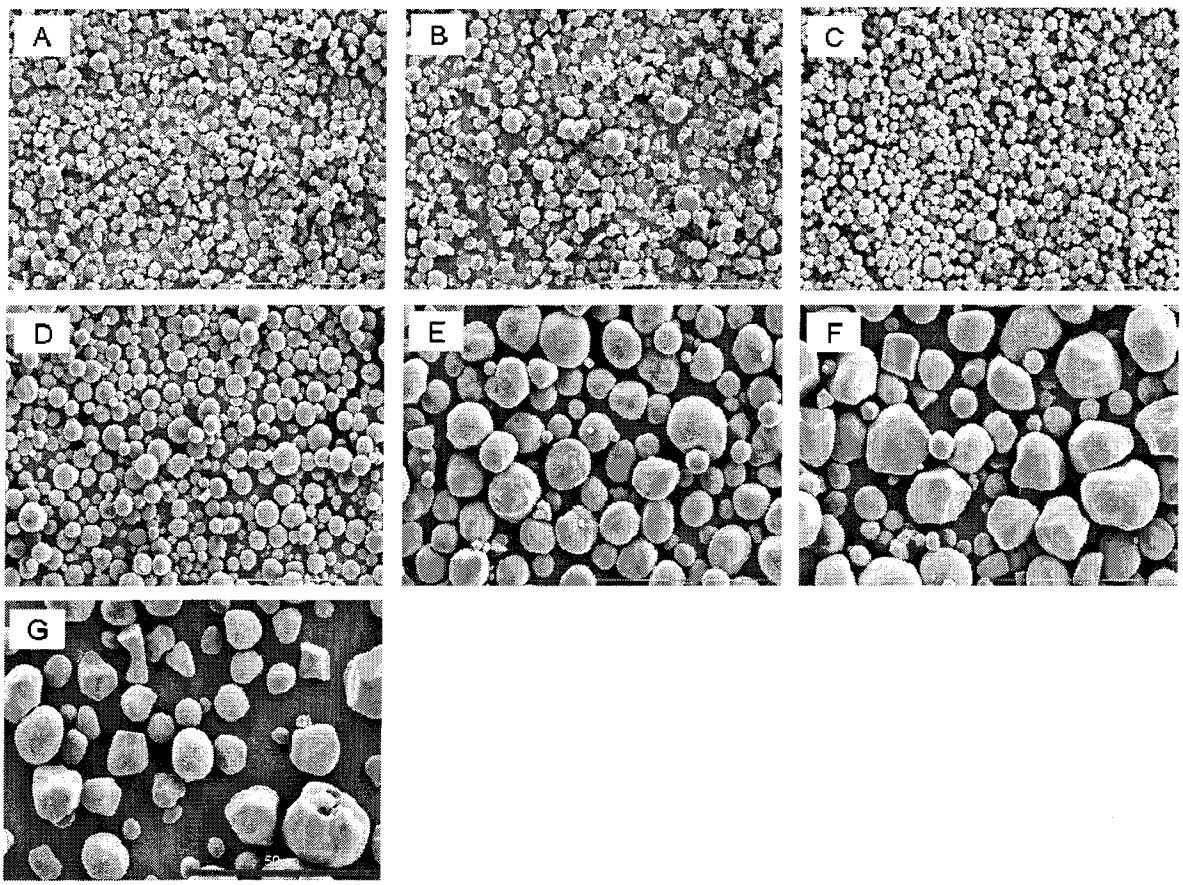

Figure 3.

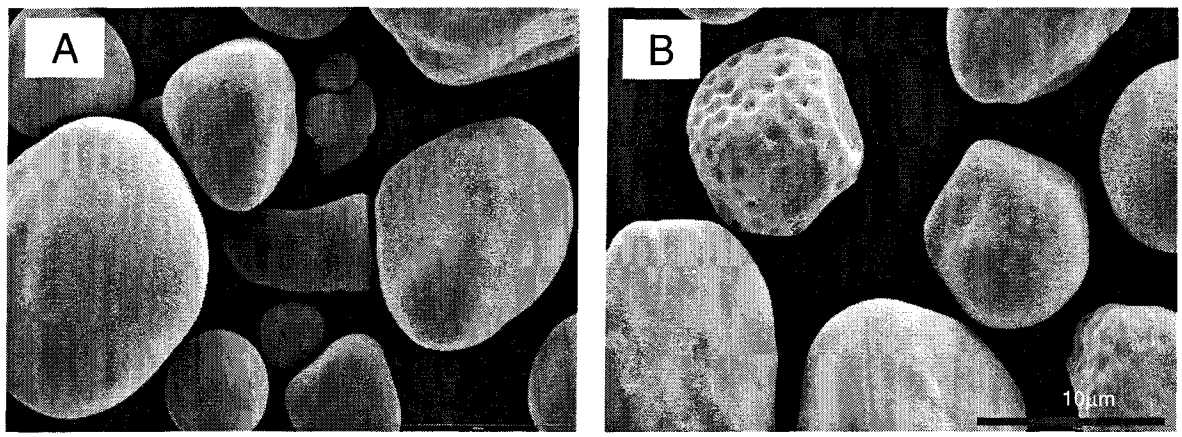

Figure 4.

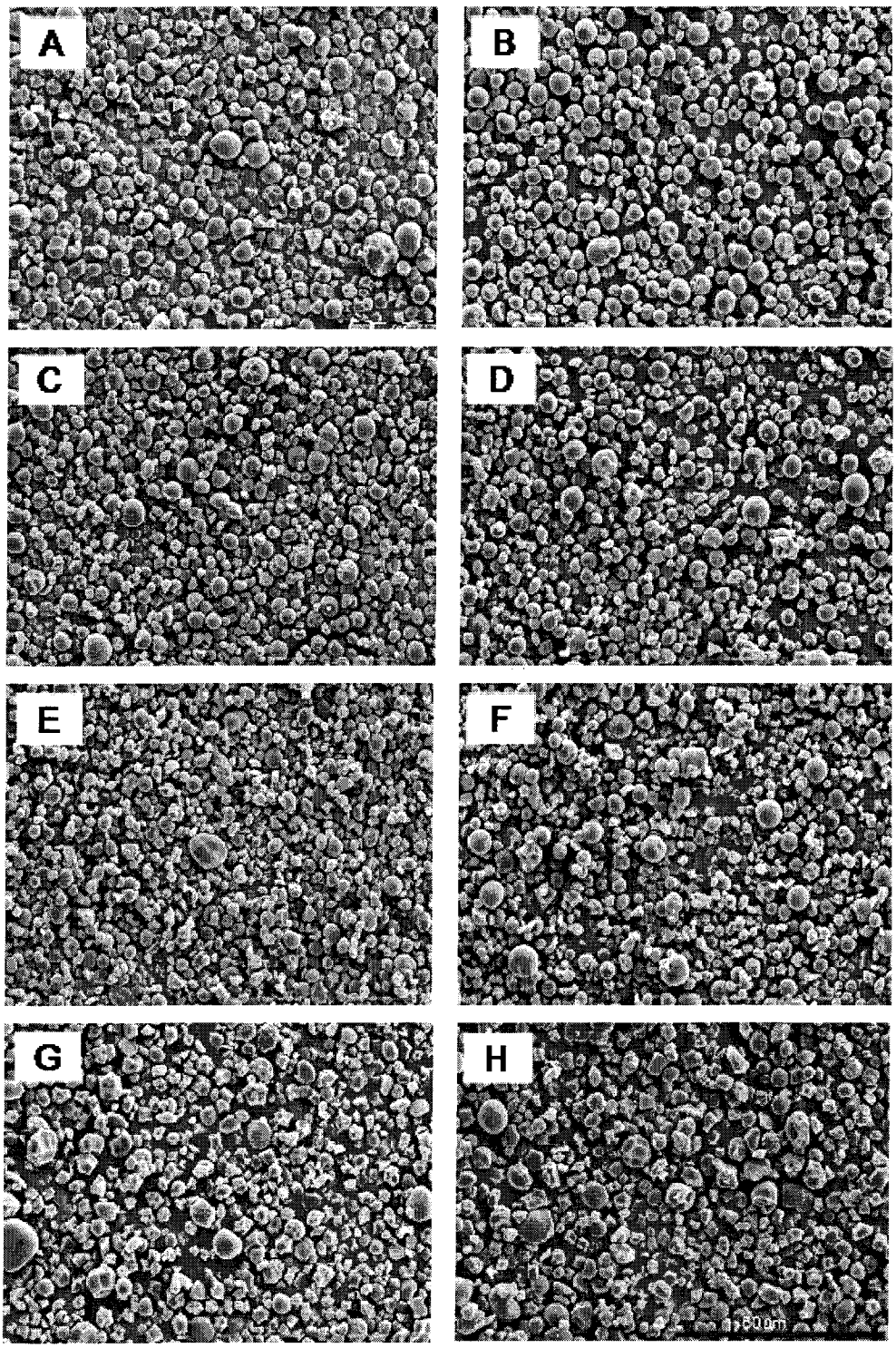

Figure 5.

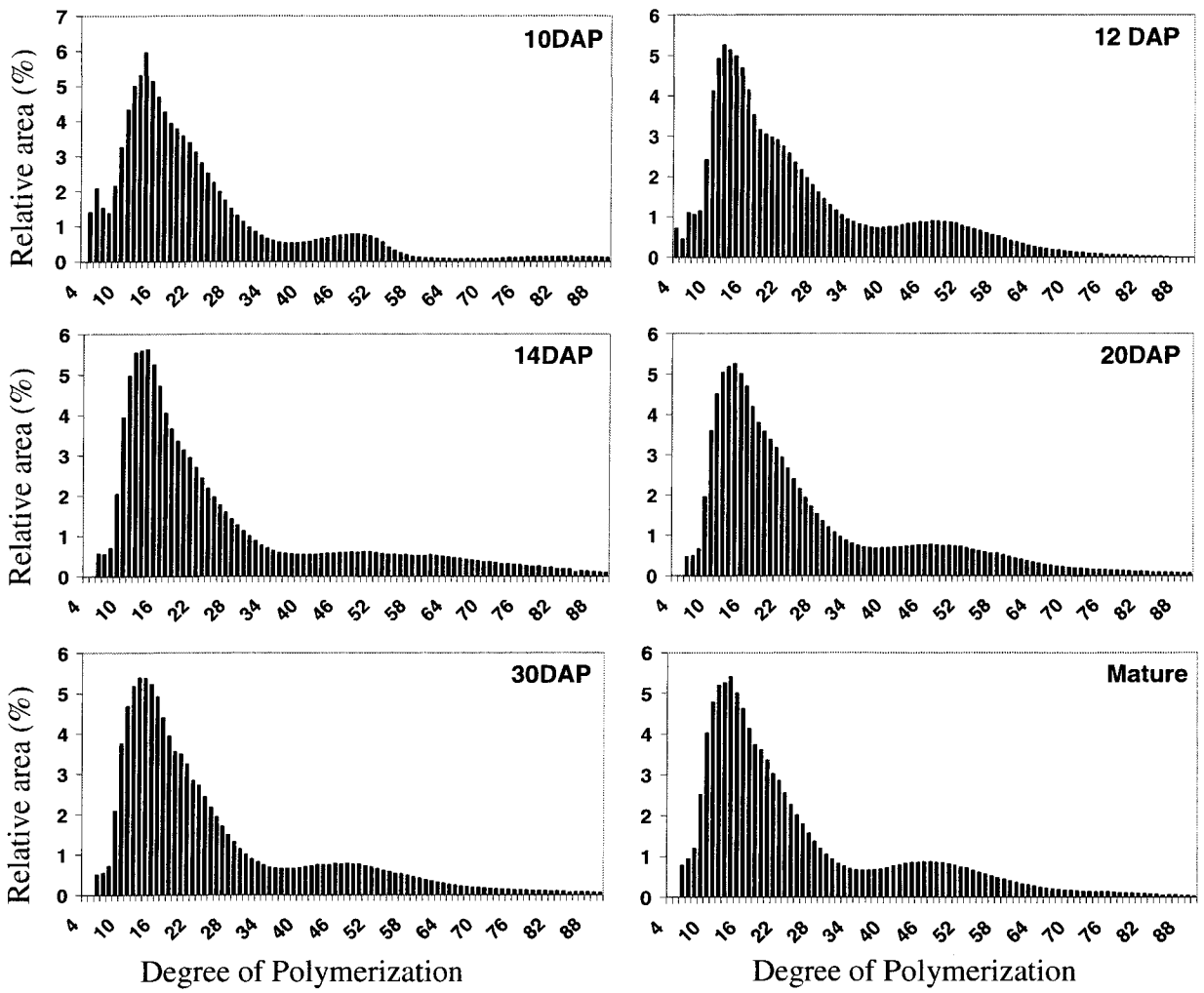
10DAP
12 DAP
14DAP
20DAP
30DAP
Mature
Relative area (%)
Degree of Polymerization

Figure 6.

CHAPTER 5. GENERAL CONSLUSIONS

The goal of this research is to gain understanding on starch biogenesis by studying starch granule formation and development. Starch granule formation was investigated in a cyanobacteria model system by replacing endogenous glycogen synthase genes with a plant starch synthase gene, and the development of starch granule was investigated by using maize endosperm starches isolated at different developmental stages.

Chapter 3 described the changes on glycogen synthetic enzyme activities and glucans structures had been caused by the expression potato starch synthase III in cyanobacterium *Synechocystis* sp. PCC6803 ant its mutants. The mutant only survived when at least one glycogen/starch synthase was present. Results indicated that the survival of *Synechocystis* required the existence of glycogen/starch synthase. The elimination of glycogen synthase II and the expression of PSS III were accompanied by the increase of glycogen branching enzyme activity. Compared with WT, the mutants containing PSSIII produced glucans with more short chains and less long chains, which is consistent with the increase of the branching enzyme activity in the mutants. Branch chain length distributions of mutant glucans show a unimodal distribution, which indicates that semi-crystalline amylopectin structure cannot be created by the simple replacement of one type bacteria glycogen biosynthetic enzyme with corresponding starch biosynthetic enzyme. Results suggested that there were regulative and cooperative interactions among plant starch synthase, glycogen synthases and glycogen branching enzyme in the process of glucans biosynthesis.

Chapter 4 described physicochemical properties of maize endosperm and pericarp starches isolated at different developmental stages. Maize endosperm starch content, granule size increased significantly after 14DAP. Our results indicated that the initiation of starch granules was completed before12 DAP in the maize endosperm, and the size of starch granules increased and filled up the space of the endosperm on the later developmental stage. Branch chain-length distribution of endosperm amylopectins showed that amylopectin had shorter average chain length (DP 23.6) on 10DAP increased to the maximum (DP26.7) on 14DAP, and then decreased to DP 25.4 on 30DAP. The onset gelatinization temperature of endosperm starches increased from 61.3°C on 8DAP to 69.0°C on 14DAP and then decreased to 67.4°C on 30DAP. Results indicated that the endosperm starch structure was not consistently growing with the kernel development, which reflects the change of the expression patterns of starch biosynthetic enzymes. The pericarp starch, however, showed similar granule size, starch content, amylose content, amylopectin structure and thermal properties at different developmental stages of the kernel. It indicates that starch biosynthesis system of the endosperm is different from that of the pericarp.

APPENDIX: BRANCHING ENZYME ACTIVITY, STRUCTURES AND PROPERTIES OF A TRANSGENICALLY MODIFIED MAIZE STARCH

L. Li[1], B. Mou[1], K. Wang[2], M. James[3], J. Jane[1] and C. Jansson[4]

[1]Dept. of Food Science and Human Nutrition; [2]Plant Transformation facility; [3]Dept. of Biochemistry, Biophysics and Molecular Biology, Iowa State University, Ames 50010; [4]Dept. of Plant Biology, The Swedish University of Agricultural Sciences, Uppsala, Sweden

Abstract

A barley branching enzyme IIa was introduced into Hi II maize by using a biolistic gun, the maize was then crossed to Oh43 and Oh43*aeae* inbred lines to produce maize starch with altered structures. Kernels were collected on 20 day-after-pollination (DAP) for the analysis of branching enzyme activity. Thermal properties of the starches isolated from mature kernels were determined by using differential scanning calorimetry. Some mature kernels displaying different phenotypes from normal maize kernel indicated 1:1 segregation of normal and transgenic maize kernels. Branching enzyme activities of 20 DAP kernels varied from 5 to 200%. Onset and peak gelatinization temperatures of transgenic maize starch were in range of 61.6-70.1°C and 74.3-103.0°C, respectively. Onset dissociation temperature of retrograded starch ranged from 37.9-78.8°C. Retrogradation rate varied from 4.5 to 100% after 7days storage at 4°C. Starches from maize kernels with a lightly shrunken appearance showed wide gelatinization temperature ranges, high percentages of

retrogradation and high amylose content. Results suggested that some transgenic corns might have suffered silencing of maize branching enzyme gene, producing starch with high retrogradation rate, whereas others expressed barley branching enzyme and displayed lower gelatinization temperature and retrogradation rate.

Introduction

Starch is one of the most important energy reserves of higher plants. The basic building unit of starch is glucose, which is linked by α(1-4) linkages to form linear chains and by α(1-6) linkages to form branches in the molecule. Chemical structures of starch molecules determine the functional properties of starch, and functional properties of starch affect its market value (Zobel, 1988). By modifying the structure of starch to improve its property, one may create a product with increased market value.

Studies have shown that starch containing amylopectin with a greater proportion of very short chains displays a low gelatinization temperature and a better paste stability with a slow retrogradation rate (Jane et. al., 1999). Starch with these properties requires less energy for processing and cooking, also produces food products with longer shelf life. Although both have A-type crystalline structure, barley starch displays a lower gelatinization temperature and retrogradation rate than normal maize starch (Jane et. al., 1999). The branch chain-length profiles show that barley amylopectin contains a greater proportion of short chain of DP6-12 and less branch chains of DP13-24 than maize amylopectin. The branch chains of DP18-21 matches the length one cluster of amylopectin crystalline (Cameron and Donald, 1992). The larger proportion of short chains of DP6-12 and the smaller proportion of

chains of DP13-24 indicate imperfect crystalline structure, which leads to a lower gelatinization temperature (Jane et. al., 1999).

Starch biosynthesis is carried out by ADP-glucose pyrophosphorylase, starch synthases, starch branching enzymes and starch debranching enzymes. Starch branching enzyme (SBE) is the enzyme that catalyzes the formation of α (1-6) branch linkages. It cleaves α(1-4) linkages in linear glucosyl chains and transfers the cleaved chains to form α (1-6) linkages. Different BE isoforms have different specificities on chain transferred. For example, maize contains three BEs, including, BEI, BEIIa and BEIIb. Maize BEI prefers to transfer longer chains than BEIIa and BEIIb (Gao et. al. 1996). In this study, a barley branching enzyme (BBE) IIa was introduced into maize using a biolistic gun-mediated delivery method. BE activities of positively transformed plants were measured and the structure and thermal properties of starches isolated from mature kernels were analyzed.

Materials and Methods

Approaches used to express barley branching enzyme IIa gene in maize.

A full-length barley SBEIIa gene was inserted into pUC19 as shown in Figure 1. A gamma zein promoter, the first intron of maize *adh1* gene and a *nos* terminator were used for the expression of barley SBEIIa. A herbicide resistance gene (*bar*) was constructed into another vector to form pBAR vector for liberty herbicide selection. The pMO12 vector and pBAR vector were introduced into callus tissues of maize Hi-II line by using a biolistic gun-mediated delivery method. The clones were selected by using liberty herbicide. Positive clones were further selected by using PCR to amplify barley SBEIIa sequence. Transgenic

plants were grown in a greenhouse (Iowa State University) and pollinated with Oh43 inbred and Oh43 *ae ae* mutant. Kernels on 20 days after pollination (DAP) were used in branching enzyme assay. Mature kernels were harvested for thermal property analysis.

Starch isolation from single kernel

Mature kernel was socked in 0.45% sodium metabisulfite solution for 24 hour at room temperature. The pericarp and germ were removed by hand. The endosperm was homogenized with 10ml water by using a Tekmar Vortex type tissue homogenizer (Ultra-TurraxT24, 600W, Cincinnati, OH) at 20,500 rpm for 1min. The homogenized sample was filtered through a 53μm nylon screen with several washes. The starch was purified by sedimentation of starch suspension with excess water for three times.

BE activity of transgenic maize kernels

Branching enzyme activity was measured using phosphorylase-stimulation method of Boyer and Preiss (1978) with modifications. The protein sample (20mg) was mixed with 1 mM AMP, 0.1 mg/ml rabbit-liver phosphorylase A, 0.1 M sodium citrate (pH7.0), and 45 mM glucose-1-phosphate in a final volume of 200 μl. The mixture was incubated at 30 °C for 80 min and boiled for 10 min to stop the reaction. The released phosphate was determined by using the malachite green assay and measured the absorbance at 630 nm after 10 min incubation at room temperature (Baykov et al, 1988).

Thermal properties of transgenic maize starch

Gelatinization and retrogradation properties of starch were determined by using a differential scanning calorimeter (DSC-7, Perkin-Elmer, Norwalk, CT), following the method of Song and Jane (2000). The starch sample (about 3mg, dsb) from single kernel with excess water (1:3) was heated at 10°C/min from 25-110°C in aluminum pans, using an empty pan as the reference. The sample was analyzed in triplicate. The data were analyzed by using Pyris software (Perkin-Elmer, Norwarlk, CT). The gelatinized starch sample was stored at 4°C for 7 days and then analyzed following the same procedure to determine the properties of retrograded starch.

Molecular mass distribution of transgenic maize starch

Starch samples were analyzed by gel permeation chromatography (GPC) using Sepharose CL-2B gel, following the method of Jane and Chen (1992). Starch dispersion (1%) was injected into a Sepharose CL-2B gel permeation (Pharmacia, Piscataway, NJ) column (1cm ID×48cm height) in a descending mode. The fractions were collected and analyzed for total carbohydrate (Phenol-sulfuric acid method) (Dubois, et al., 1956) and blue value (iodine staining) (Juliano, 1971) at 490 and 630nm, respectively. The sample was eluted with an aqueous solution of 25mM NaCl and 10mM NaOH. Fractions of 0.8ml were collected for the analysis of total carbohydrate and blue value.

Results and Discussion

Branching enzyme activities of transgenic kernels

Transgenic plants containing barley SBEIIa gene were pollinated with pollen from wildtype Oh43 and homozygote Oh43 *aeae* lines. Four immature kernels were collected at 20 DAP from each plant. Protein was extracted from kernels for branching enzyme activity analysis. The branching enzyme activity of each kernel was shown in Table 1. Branching enzyme activities of transgenic maize kernels varied from 0.02 nmol/min/mg to 1.09 nmol/min/mg (equivalent to 4%-214% of activities of the controls). The control maize (Hi II without BBE) crossed to Oh43 *aeae* produced seeds with larger absolute branching enzyme activities (0.93 nmol/min/mg) than that crossed to Oh43 (0.43 nmol/min/mg). Both enhancement and suppression of branching enzyme activity were observed in transgenic maize.

Appearance of mature kernels

Half of the transgenic plants only produced kernels with normal appearance. The remaining plants produced kernels with two different appearances, normal and shrunken. The ratio of normal kernels to shrunken kernels was 1:1, because the transgenic plants were crossed with Oh43 and Oh43 *aeae* lines. Mature kernels of transgenic maize with different phenotype from normal maize kernels are shown in Figure 2. Kernels show different levels of shrunken appearances.

Thermal properties of transgenic starches

Based on the results of BE assay and kernel appearance, kernels with normal or shrunken appearance were selected from the cobs with normal, enhanced and suppressed BE activities for starch isolation. Onset and conclusion gelatinization temperatures of transgenic

maize starches were in ranges of 61.6-70.1°C and 74.3-103.0°C, respectively, whereas control maize starches showed onset temperatures of 67.6-68.2°C and conclusion temperature of 70.8-72.7°C (Table 2). Onset melting temperatures of retrograded transgenic maize starches ranged from 38.3-74.6°C, whereas control maize starches showed onset temperatures of 37.9-41.7°C. Retrogradation rate varied from 4.5% to more than 100%, whereas control maize starches showed retrogradation rates of 55.4-64.0% (Table 3). Most starches isolated from kernels with shrunken appearance showed abnormal onset gelatinization temperature, temperature range and percentages of retrogradation. The low gelatinization temperature and low retrogradation rate were observed on starch isolated from shrunken kernel of p2p90-8-1 X Oh43 *aeae*. The suppression of BE activity was detected from 20DAP kernels from the same cob. The starches isolated from light shrunken kernels from p2p90-4-10 X Oh43 and p2p90-4-7 X Oh43 *aeae* showed wide gelatinization ranges (33.6-37.5°C) and high retrogradation rate. Both enhanced and suppressed BE activity were detected from 20DAP kernels from those two lines. The starches isolated from light shrunken kernels from p2p90-8-2 X Oh43 showed similar gelatinization and retrogradation properties compared to the control. The BE activity from this line was similar to the control line. The results indicates that the change of BE activity leads to the change of thermal properties of transgenic starches.

Molecular weight distributions of transgenic starches

Since starch was isolated from single kernels, we didn't have enough material to perform gel permeation chromatography on each sample that was studied by using DSC. The GPC profiles of control and transgenic starches are shown in Figure 3 and Figure 4.

Amylopectin eluted earlier than amylose because of its large molecular weight. The starches isolated from lightly shrunken kernels displayed greater amylose peaks than the control starches. Some starches isolated from kernels with normal appearance (p2p90-2-2XOh43 and p2p92-15-3XOh43*aeae*) also showed greater amylose peak. Comparing with Hi II NegXOH43 and Hi II NegXOh43*aeae* (controls), these transgenic maize starches have higher amylose content (Table 4), up to 69.5%. They also showed relatively larger R values for amylopectin than control maize starches, indicating amylopectin with longer branch chain length. The decrease in SBE activity was observed in 20DAP kernels of these transgenic plants, which may be responsible for the changes on amylose content and amylopectin structure. The disruption of SBEI (*r* locus) causes the similar change in amylose content in pea (Bhattacharyya et al, 1990). The amylose content increases from 30% to 70% in the mutant. It is well known that maize *ae* mutant (the deficiency of SBEIIb) produces high amylose starch containing amylopectin with reduced branch points.

The decrease in SBE activity was widely observed in transgenic maize kernels. This may result from the co-suppression of barley SBE and maize endogenous SBE. Gene-silencing by co-suppression of introduced gene and homologous host gene has been observed in many plant species (Cogoni and Macino, 2000; Napoli etal., 1990). It is known that this phenomenon results from RNA interference (Guo and Kempheus, 1995; Fire et al., 1998).

Conclusions

Results obtained from this study suggested that some transgenic maize could have suffered silencing of maize branching enzyme gene, producing starch with more amylose and long-branch chains of amylopectin and displaying larger retrogradation rates, whereas others

expressed barley branching enzyme and displayed shorter branch chain length, lower gelatinization temperatures and retrogradation rates. This study will enable us to gain better understandings of starch biosynthesis and starch structure effects on its functions.

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Table 1. Branching enzyme activity from single kernel. The each genotype of transgenic plant was obtained one isolated transformation events.

Sample genotype Cross to Oh43		BE activity (nmol/min/mg)	Sample genotype Cross to Oh43 *aeae*	BE activity (nmol/min/mg)
Hi II neg	1	0.41[a]	Hi II neg-2	1.01
	2	0.41		0.92
	3	0.36		1.02
	4	0.53		0.79
p2p90-2-2	1	0.04	p2p91-16-10	0.51
	2	0.19		0.98
	3	0.02		1.09
	4	0.09		0.97
p2p90-4-10	1	0.36	p2p90-2-6	0.93
	2	0.19		0.98
	3	0.91		0.91
	4	0.67		0.47
p2p90-10-2	1	0.91	p2p9-4-7	0.96
	2	0.43		0.99
	3	0.42		0.06
	4	0.56		1.03
p2p90-16-11	1	0.55	p2p90-7-4	1.00
	2	0.70		0.82
	3	0.19		0.80
	4	0.02		0.92
p2p91-6-1	1	0.49	p2p90-10-13	0.84
	2	0.51		0.83
	3	0.39		0.77
	4	0.51		0.79
p2p91-12-1	1	0.52	p2p90-11-1	0.85
	2	0.30		0.86
	3	0.61		0.77
	4	0.75		0.78
p2p91-5-2	1	0.45	p2p91-6-7	0.86
	2	0.54		0.88
	3	0.44		0.80
	4	0.53		0.86
p2p90-7-6	1	0.57	p2p91-8-10	0.91
	2	0.48		0.95
	3	0.57		0.90
	4	0.59		0.88

p2p90-8-2	1	0.50	p2p91-5-3	0.83
	2	0.36		0.60
	3	0.52		0.88
	4	0.69		0.59
p2p90-18-2	1	0.58	p2p90-8-1	0.42
	2	0.52		0.47
	3	0.52		0.48
	4	0.52		0.84
p2p90-13-1	1	0.51	p2p90-18-3	0.98
	2	0.65		0.92
	3	0.57		1.02
	4	0.56		0.95
p2p90-5-6	1	0.54	p2p90-5-5	0.98
	2	0.58		1.01
	3	0.51		0.59
	4	0.81		0.89
p2p91-8-2	1	0.63	p2p91p12-3	1.00
	2	0.53		0.92
	3	0.48		0.91
	4	0.59		0.12
p2p91-15-4	1	0.59	p2p91-15-3	0.85
	2	0.58		0.24
	3	0.66		0.88
	4	0.67		0.20
p2p91-40-7	1	0.43	p2p91-34-7	0.92
	2	0.35		0.95
	3	0.21		0.77
	4	0.35		0.90
p2p91-41-1	1	0.52	p2p91-52-4	0.98
	2	0.63		1.04
	3	0.53		0.98
	4	0.61		1.02
p2p91-34-4	1	0.49	p2p91-35-1	1.01
	2	0.61		1.08
	3	0.53		1.07
	4	0.49		0.96
p2p91-52-6	1	0.49	p2p91-45-3	0.92
	2	0.79		1.02
	3	0.50		1.06
	4	0.57		1.01

[a] Values were calculated from two replicates

Table 2. Thermal properties of native transgenic starch

Genotype		T_o °C	T_p °C	T_c °C	Range	ΔH
Hi IINegXOh43[a]	1	68.0±0.6[b]	71.7±0.7	75.7±1.2	7.7	12.0±0.3
	2	67.8±0.1	70.8±0.2	74.3±0.2	6.4	12.7±0.5
	3	68.0±0.2	71.9±0.4	76.0±0.7	7.9	12.4±0.4
	4	67.6±0.3	71.4±0.3	75.4±0.4	7.8	12.4±0.3
p2p90-2-2XOh43	1	66.9±0.0	71.7±0.0	75.1±0.0	8.2	11.9±0.1
	2	70.1±0.2	73.1±0.1	76.2±0.3	6.1	12.6±0.1
	3	69.0±0.1	74.2±0.0	82.2±0.5	13.3	3.8±0.2
	4	68.1±0.7	71.9±0.1	75.3±0.0	7.2	11.3±0.4
p2p90-4-10XOh43	1	64.0±0.2	70.4±0.1	75.0±0.2	10.9	11.9±0.2
	2	66.3±0.1	70.5±0.0	74.7±0.0	8.5	12.5±0.5
lightly shrunken	3	66.5±0.2	72.4±0.0	104.0±0.5	37.5	14.3±0.4
lightly shrunken	4	65.7±0.7	72.8±0.0	102.8±1.6	37.1	10.2±1.1
p2p90-8-2XOh43	1	67.4±0.1	71.5±0.0	77.2±0.2	9.8	12.9±0.2
	2	68.4±0.0	72.0±0.0	76.5±0.1	8.1	13.6±0.2
lightly shrunken	3	67.4[c]	72.5	79.7	12.3	8.0
lightly shrunken	4	67.0	72.7	81.0	14.0	7.5
Hi II NegXOh43aeae	1	67.5±0.2	72.7±0.0	77.3±0.3	9.8	13.6±1.0
	2	68.0±0.1	72.4±0.1	76.9±0.1	8.9	12.8±0.2
	3	68.2±0.2	72.5±0.2	76.9±0.1	8.7	12.0±0.4
	4	68.0±0.3	72.7±0.3	77.2±0.4	9.2	12.6±0.1
p2p90-4-7XOh43aeae	1	66.9±0.1	71.5±0.0	76.0±0.3	9.1	11.2±0.1
	2	66.0±0.2	72.7±0.2	77.1±0.3	11.1	12.5±0.5
lightly shrunken	3	68.1±0.6	87.3±0.8	103.0±1.0	34.9	10.3±0.2
lightly shrunken	4	68.8±1.6	88.2±0.2	102.5±1.2	33.6	8.9±1.5
p2p90-8-1XOh43aeae	1	68.7±0.0	72.3±0.1	76.3±0.2	7.6	12.8±0.5
	2	70.1±0.2	73.3±0.3	77.2±0.4	7.1	14.3±1.8
shrunken	3	61.6	70.8	74.7	13.1	12.3
p2p91-15-3Xoh43aeae	1	68.7±0.2	73.5±0.2	78.4±0.1	9.7	8.5±0.1
	2	68.8±0.2	73.4±0.1	78.1±0.3	9.3	9.6±0.2
	3	68.2±0.2	74.1±0.1	81.0±0.2	12.8	8.8±0.4
	4	68.7±0.0	73.3±0.0	78.0±0.0	9.3	9.2±0.2

[a] Samples (~3.0 mg,dsb) and deionized water (~9.0 mg) were used for the analysis; T_0, T_p, T_c and ΔH are onset, peak, conclusion temperature, and enthalpy change, respectively.
[b] Values were calculated from two replicates.
[c] Value was measured once.

Table 3. Thermal properties of retrograded transgenic starch

Genotype		T_o °C	T_p °C	T_c °C	ΔH	R%
Hi IINegXoh43[a]	1	37.9±0.4[b]	49.4±0.1	61.9±0.8	6.8±0.8	56.4[c]
	2	39.3±0.5	49.8±1.0	62.1±0.2	7.0±0.5	55.4
	3	38.7±0.4	49.5±0.5	62.3±1.0	7.3±0.7	59.2
	4	40.1±0.6	50.3±0.1	62.4±0.2	7.8±0.8	62.8
p2p90-2-2Xoh43	1	42.0±0.7	52.7±0.2	62.4±0.0	5.6±0.2	47.5
	2	42.9±0.0	54.1±0.1	63.2±0.0	6.2±0.1	49.3
	3	47.5±0.8	57.0±0.2	63.6±0.7	1.4±0.5	36.4
	4	42.5±0.9	52.7±0.5	61.5±0.6	5.6±0.1	49.3
p2p90-4-10Xoh43	1	38.3±0.1	52.2±0.5	60.9±0.0	6.2±0.3	52.0
	2	41.0±1.9	51.7±2.7	61.9±0.1	5.5±1.1	44.4
lightly shrunken	3	41.7±0.9	56.4±0.3	111.0±2.8	47.8±4.3	>100
lightly shrunken	4	39.9±0.0	54.9±0.1	104.7±2.3	22.1±6.9	>100
p2p90-8-2Xoh43	1	42.1±0.2	52.7±0.1	61.8±0.0	6.8±0.6	52.3
	2	42.1±0.3	53.1±0.1	61.6±0.1	7.3±0.1	54.0
lightly shrunken	3	44.1[d]	53.7	63.5	3.1	37.7
lightly shrunken	4	44.6	56.5	63.6	2.6	34.6
Hi II NegXoh43aeae	1	40.8±0.2	52.2±0.0	62.7±0.4	7.5±1.0	55.4
	2	41.7±0.7	52.3±0.1	62.4±0.2	7.7±0.3	60.0
	3	38.1±0.2	49.8±0.0	61.9±0.5	7.5±0.7	63.1
	4	38.8±0.3	49.8±0.4	63.0±1.0	8.0±1.2	64.0
p2p90-4-7Xoh43aeae	1	39.3±0.2	50.5±0.0	62.6±0.0	8.2±0.5	73.4
	2	40.4±0.7	52.1±0.2	62.3±0.2	7.7±0.6	61.5
lightly shrunken	3	44.3±2.2	91.0±0.0	103.7±1.0	13.3±2.9	>100
lightly shrunken	4	41.6±2.2	93.4±1.8	101.2±0.5	4.5±1.3	61.4
p2p90-8-1Xoh43aeae	1	40.6±0.2	52.3±0.2	62.4±0.0	7.9±0.7	61.8
	2	41.7±0.1	52.8±0.1	63.2±0.2	9.2±1.6	64.0
shrunken	3	44.0	53.7	62.2	1.6	4.5
p2p91-15-3Xoh43aeae	1	43.8±0.4	54.5±0.0	63.7±0.6	5.2±1.0	60.5
	2	39.8±0.3	53.8±0.4	63.5±0.8	3.5±0.4	37.1
	3	42.5±0.3	57.4±0.1	68.0±1.9	3.2±0.0	35.8
	4	42.5±0.4	54.4±0.1	64.9±1.2	4.2±0.3	45.8

[a] After storage at 4°C for 7 days

[b] Values were calculated from two replicates.

[c] Retrogradation (R%)=$\Delta H_{retro}/\Delta H_{native}\times 100$

[d] Value was measured once.

Table 4. Amylose content and R value of control and transgenic starches. R is the ratio of blue value and total carbohydrate of amylopectin peak, the amylose peak is used as the reference.

Genotype	Phenotype	R value	Amylose content (%)
Hi IINegXOh43	normal	0.21	27.2
p2p90-2-2XOh43	normal	0.20	32.6
	normal	0.29	69.5
p2p90-4-10XOh43	normal	0.23	39.8
	lightly shrunken	0.36	68.5
Hi II NegXOh43aeae	normal	0.19	33.4
p2p90-4-7XOh43aeae	normal	0.30	40.1
	lightly shrunken	0.39	55.0
p2p91-15-3Xoh43aeae	normal	0.27	53.4
	normal	0.30	48.9

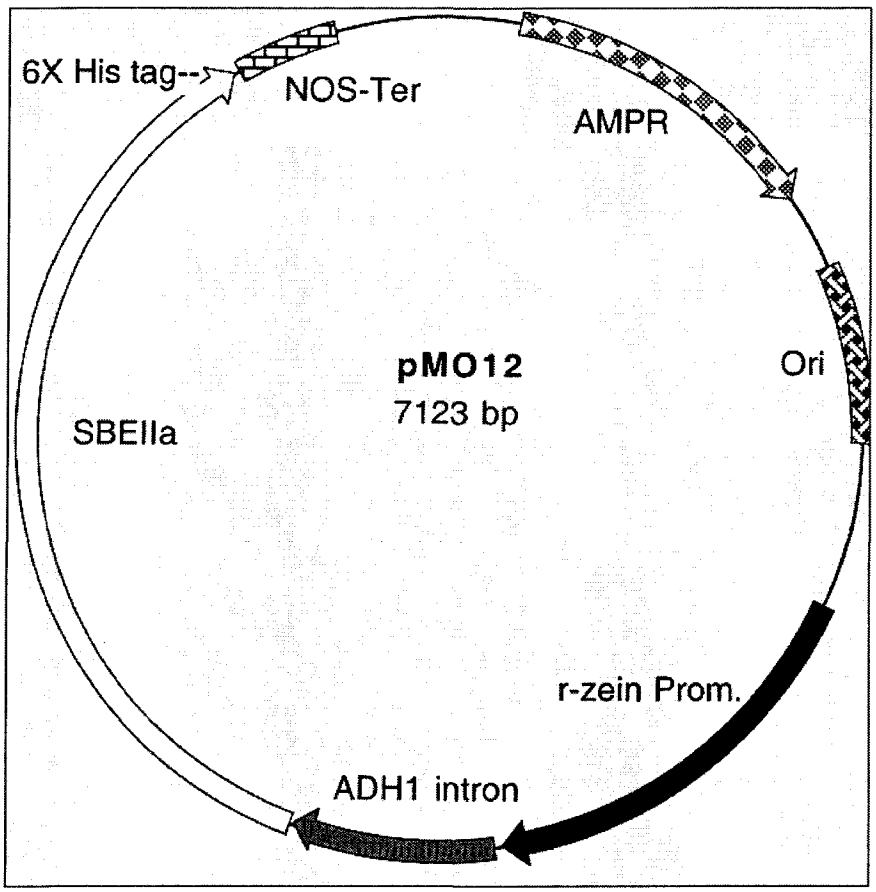

Figure 1. The construction to introduce barley SBEIIa into maize. The vector, pMO12, contains maize gamma zein promoter, the first intron of maize *adh1*gene, full-length barley SBEIIa cDNA, and *nos* terminator.

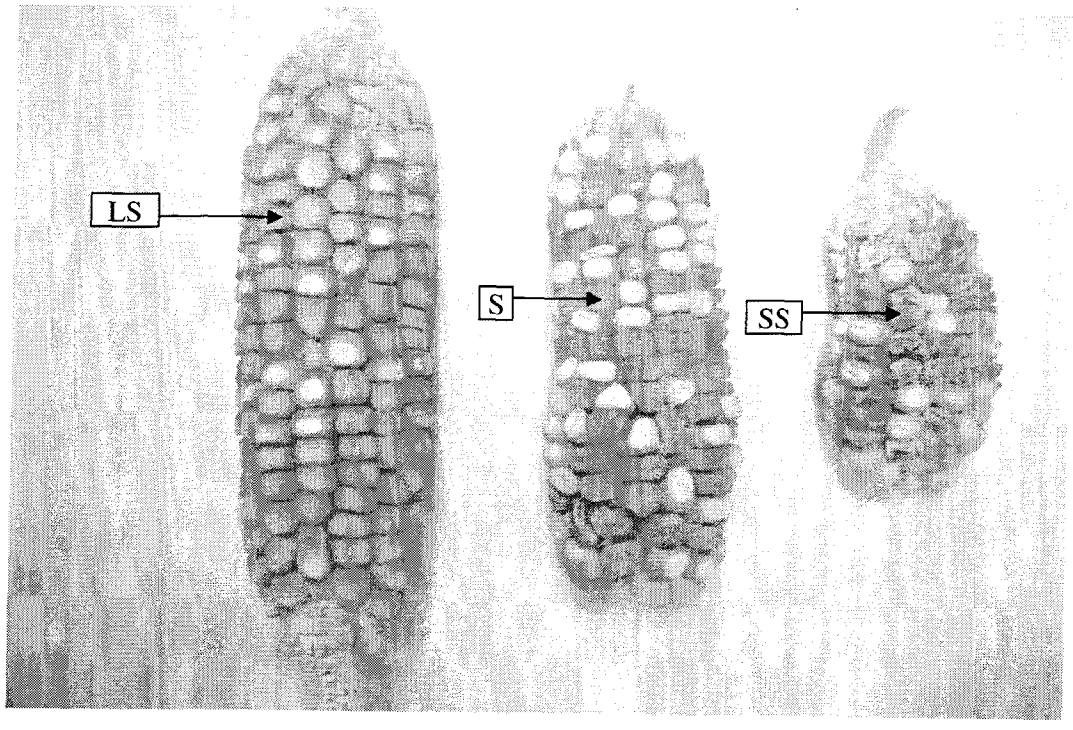

Figure 2. Transgenic maize with different kernel appearances. LS: lightly shrunken, S: shrunken, SS: severely shrunken.

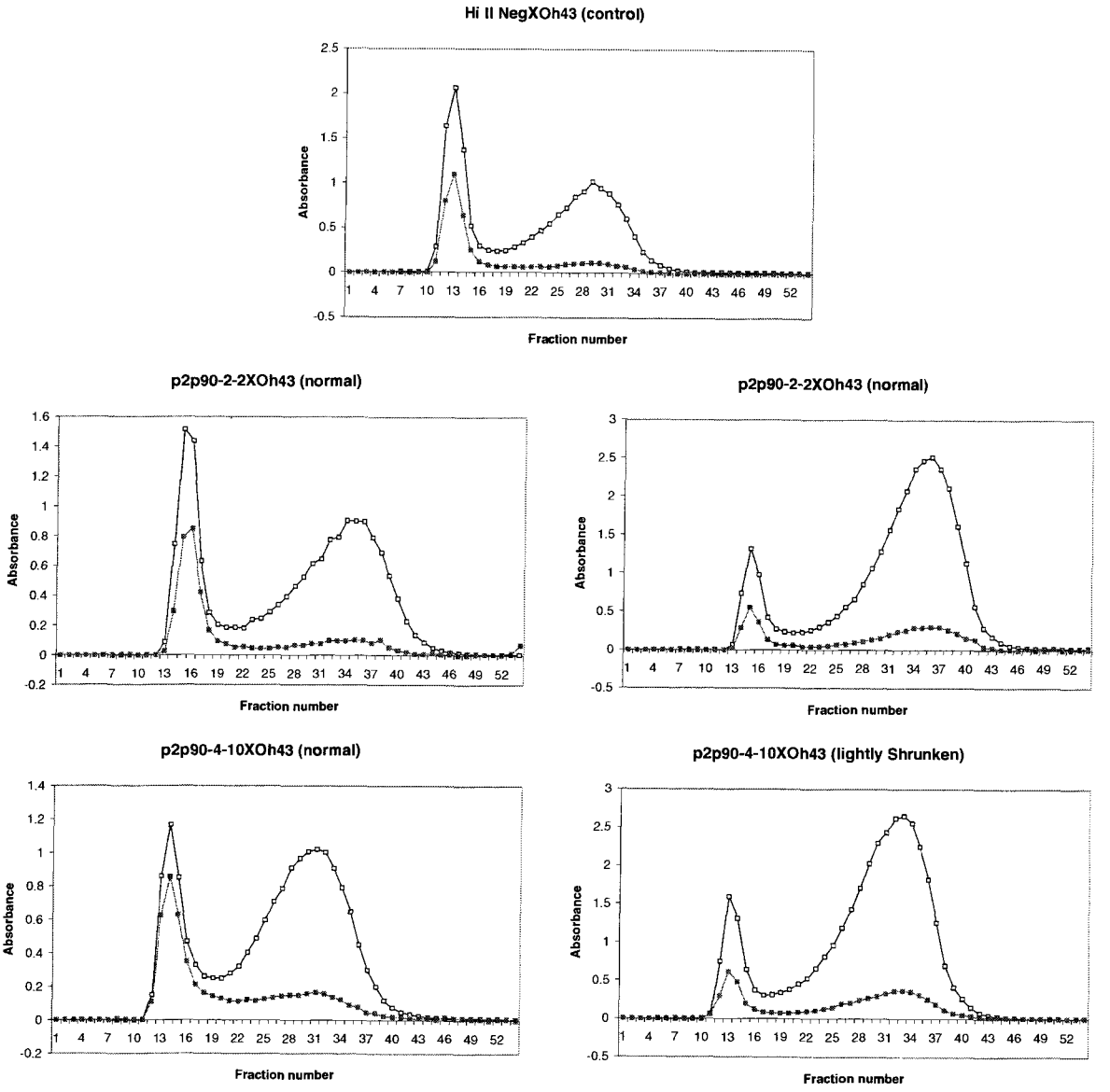

Figure 3. Gel permeation chromatographic profiles of control and transgenic maize starches isolated from transgenic kernels crossed to inbred line Oh43. Starches were separated by using sepharose CL-2B column. The Hi II NegXOh43 was used as the control. The open square represents blue value. The filled square represents total carbohydrate.

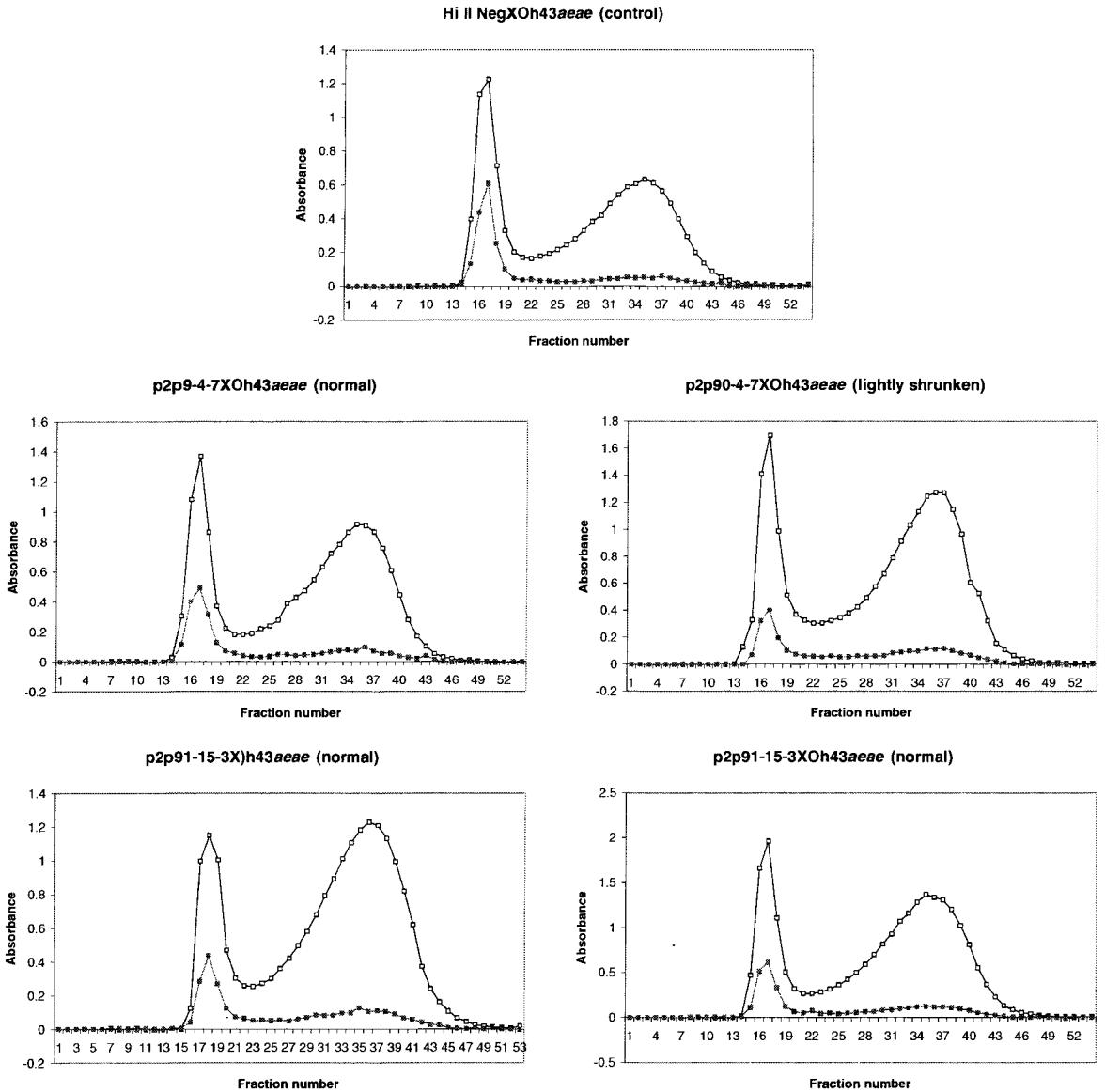

Figure 4. Gel permeation chromatographic profiles of control and transgenic maize starches isolated from transgenic kernels crossed to inbred line Oh43*aeae*. Starches were separated by using sepharose CL-2B column. The Hi II NegXOh43*aeae* was used as the control. The open square represents blue value. The filled square represents total carbohydrate.

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