2001

Structures, properties, and biogenesis of starch and cyanobacterial glycogen

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Structures, properties, and biogenesis of starch and cyanobacterial glycogen

by

Sang-Ho Yoo

A dissertation submitted to the graduate faculty

in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

Major: Food Science and Technology

Major Professor: Jay-lin Jane

Iowa State University

Ames, Iowa

2001

UMI Number: 3016759

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TABLE OF CONTENTS

Structures, properties, and biogenesis of starch and cyanobacterial glycogen

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Structures and properties of starches isolated from waxy, amylose-reduced (Kanto 107), and normal hard-red-winter wheat (Centura and a commercial product) grains were characterized. The absence of amylose (AM) in waxy wheat starch did not affect crystalline pattern (A-type), granule size and morphology, and gelatinization temperature, but increased the degree of crystallinity and changed pasting properties. Differences in pasting temperatures and peak viscosities between waxy and normal wheat starches were substantially greater than the differences between maize starch counterparts.

The *weight*-average molecular weight (M_w) of the wheat amylopectin (AP) displayed a negative correlation with AM content, whereas the proportion of extra-long branch-chains of wheat AP were positively correlated with AM content. The M_w of APs varied from 7.0×10^7 to 5.7×10^9, depending on botanical source. The APs of waxy starches had larger M_w and, in general, larger dispersed molecular densities than did those of normal AP counterparts. Waxy wheat AP did not contain extra-long chains (ELC) that were synthesized progressively by granule-bound starch synthase I (GBSSI) in normal wheat AP. These results suggested that the APs of waxy starches carried more branch-chains but no ELCs, which resulted in more densely packed molecules than did those of normal AP counterparts. Different

branch structures between APs of A- and B-type starches resulted in different dispersed molecular densities in dilute solutions. The APs of B-type starches had much longer but fewer branch-chains, which resulted in smaller dispersed molecular densities compared with the A-type APs.

Individual glycogen synthases (GSI and II) and glycogen branching enzyme (GBE) of cyanobacterium *Synechocystis* sp. PCC6803 were insertionally mutagenized by homologous recombination. Branch chain-length distributions of glycogens produced by GSI^- and $GSII^-$ mutants were different from each other. Prominent increases in intermediate-size chains (DP8-18) were observed in glycogen of the GSI^- mutant compared with those of the wild-type and the $GSII^-$ mutant. The results indicated that the two GS isoforms had different specificities on glycogen structure. Because of the GBE deficiency, GBE^- mutant strain produced less glucan that had negligible branch-linkages and was water-insoluble. The GBE increased overall rate of glycogen biosynthesis in cyanobacterium.

GENERAL INTRODUCTION

Introduction

Starch is the second most abundant carbohydrate reserve in plants, serving as the major storage form for carbon and energy. It also serves as main energy source in diet of most animals, including humans. Starch is found in leaves, seeds, stems, roots, and fruits of plants. Starch is semi-crystalline, water-insoluble, and has a compact granular form. Botanical sources and chemical and physical modifications of starch provide great diversity in its functional properties, which contribute to the importance of starch in food and non-food applications. Starch is widely used in various products such as adhesives, textiles, foods, papers, and animal feedstock.

Starch contains two major glucose polymers, amylopectin and amylose, and small amounts of non-carbohydrate components such as lipids, proteins, phosphate-monoesters, and ash. Contents and structures of amylopectin, amylose, and the minor components substantially affect the physical properties. Starch granules display birefringence under polarized light microscope, indicating the radial direction of molecular orientation. Birefringence disappears during gelatinization as a result of losing crystallinity. Starch granules show growth rings, which display repeating concentric layers of alternating high and low density and crystalline. There is a less organized area, known as hilum, at the organic center of the granule. The hilum is the starting point of starch biosynthesis. In spite of the nutritional and economic importance of starch, the biosynthesis of starch is still not fully understood.

Modification of starch structures to obtain desirable functionality can be achieved by using various methods available, such as physical, chemical, and genetic approaches. These modifications can change properties, such as gelatinization, pasting, gel stability, solubility, and swelling power of starch. There are, however, limitations of using physical or chemical treatments because they are costly and have potential to cause environmental pollution. In addition, the reactions are also random and the quality of products are not easily controlled. To overcome these limitations, genetic approaches have been introduced to modify starch structures. A good understanding of the molecular structure and biosynthetic pathway of starch/glycogen is important, which will enable us to use genetic engineering to produce modified starch that meets specific needs in food and industrial applications. Combining advanced analytical chemistry and starch biochemistry, we are advancing from the relatively unsophisticated uses of starches as raw materials for the food and other industries to the production of starch using biotechnology to design its structure precisely for specific end-uses. The properties of current native starches may not satisfy specific needs of end-users.

Objectives of these studies are to characterize and compare the molecular structures of starch and bacterial glycogen, and relate specific functions of individual biosynthetic enzymes on starch/glycogen structures. Structures of waxy, partial waxy, and normal wheat starches were characterized and related to their physical properties. Amylopectin molecular densities of various starches and their branch structures were reported before I proposed amylopectin structures. To understand the mechanism of starch granule formation, I selected *Synechocystis* sp. PCC6803

as a model system to study starch granule biosynthesis. I expect this research to advance our understanding of starch structure and biosynthesis, and accelerate developing new structurally modified starch, by genetic engineering, which provides the properties desirable for various applications.

Dissertation Organization

This dissertation consists of four papers. The first paper, "Structural and Physical Characteristics of Waxy and Other Wheat Starches" and the second paper, "Molecular Weights and Gyration Radii of Amylopectins Determined by High-Performance Size-Exclusion Chromatography Equipped with Multi-Angle Laser-Light Scattering and Refractive Index Detectors", have been submitted to *Carbohydrate Polymers* for publication. The third paper, "Insertional Mutagenesis of Glycogen Synthase Genes in Cyanobacterium *Synechocystis* sp. PCC6803" and the fourth paper, "Insertional Mutagenesis of the Gene Encoding Glycogen Branching Enzyme in Cyanobacterium *Synechocystis* sp. PCC6803", will be submitted to *Plant Physiology* for publication. The four papers are preceded by a General Introduction and a Literature Review and followed by a General Conclusion and Acknowledgements.

LITERATURE REVIEW

General Overview of Structures and Properties of Starch and Glycogen

Starch and glycogen are the two largest biopolymers in nature. Starch is a major carbohydrate energy source in the plant kingdom that can be utilized by other living creatures, and glycogen is a primary energy storage form in both eukaryotes (animals, yeasts, etc) and prokaryotes (Candy, 1980). They consist of the same chemical building unit, i.e. glucose, same types of covalent bonds, i.e. α-(1,4)- and α-(1,6)-linkages, and have similar ranges of molecular mass (Manners, 1991). However, their morphology and physical properties are quite different. A semicrystalline granular structure developed in amyloplast is typical for starch, but soluble dispersion in the cytosol is found for glycogen in bacterial and eukaryotic organisms. Glycogen has an highly branched structure, similar to amylopectin, but exists in an amorphous form. The differences in the branch structure and molecule shape between starch and glycogen are mainly due to different functions of α-D-glucan synthesizing enzymes, e.g., synthase and branching enzyme.

Starch is one of the most important biopolymers in nature for both food and non-food applications. It is the predominant energy storing substance in plants and provides 70-80% of the calories consumed by humans worldwide (Whistler and Bemiller, 1997). Native and modified starches have an enormous number of applications such as thickener, gelling agent, water-holding substance, bulking agent, fat replacer, sizing agent and adhesive in food and non-food industries (Whistler and Bemiller, 1997).

Starch is primarily composed of glucose monomers and has two types of glycosidic bonds, α-(1,4)-linked backbones and about 5% α-(1,6)-linked branches. Even though it has simple formula of chemical structure, there are huge possibilities of different fine structures, depending on chain length, branching pattern and ratio, amylopectin/amylose ratio, and surrounding environment. Starch is unique among carbohydrates because it occurs naturally in a semi-crystalline granular shape. This granular form of starch leads to increase in the efficiency of storing conserved-energy and provides carbon skeletons and energy to geminating embryos (Preiss and Sivak, 1996).

Glycogen is another macromolecule with a chemical structure similar to amylopectin, but it has a greater proportion (10%) of α-D-(1,6)-glycosidic linkages and more random distribution of branch linkages (Manners, 1991). The regulatory and metabolic mechanism of glycogen is of interest in the field of nutritional and medical science. Glycogen is present in a much more dynamic state than is starch. Considering the metabolic time frame, glycogen should be catabolized within seconds for muscle movement and glucose level control in mammals (Robyt, 1998). In plants, it takes at least a 12 hr-time frame to mobilize starch synthesized in a leaf during daytime into sink tissue of seeds or roots (Preiss and Sivak, 1996). This form of energy deposited in sink tissue such as seeds may be stored for a long period until it regenerates the offspring.

Chemical and Physical Structure of Starch and Glycogen Molecules

Structure and Properties of Starch

Starch consists of two major glucose polymers, amylopectin and amylose. Amylopectin is a biopolymer consisting of D-glucose linear chains linked by α-(1,4)-glycosidic bonds and approximately 5% branches of α-(1,6)-glycosidic linkages. The average degree of polymerization (DP) reaches up to 10^4-10^6 glucose units/molecule, which corresponds to molecular mass of 10^6-10^8 g/mol (Hizukuri et al., 1983; Takeda et al., 1988; Aberle et al., 1994; Fishman and Hoagland, 1994; Millard et al., 1999). Depending on the source of starch, the *number*-average chain length (DP_n) of isoamylase-debranched amylopectin is in the range of 20-30 (Hizukuri et al., 1983; Hizukuri, 1985; Jane et al., 1999). Length of amylopectin branch-chains determines the crystalline structure of starch (Hizukuri et al., 1983; Hizukuri, 1985; Wild and Blanshard, 1986; Yasuko et al., 1990). The relationship between crystalline pattern and average branch-chain length of amylopectin showed that amylopectins of A-type starches had a shorter chain-length than did those of B-type starches (Hizukuri, 1985; Yasuko et al., 1990; Jane et al., 1999). The increase in chain-length of amylopectin in high-amylose maize resulted in the crystalline structure of maize starch changing from A- to B-type (Shi et al., 1998). Gidley and Bulpin (1986) report that the type of X-ray diffraction pattern, reflecting the crystalline structure, is determined by the chain length. Malto-oligosaccharides with DP10-12 and more than DP12 produced A- and B-types of crystalline structures, respectively (Gidley and Bulpin, 1986). The existence of extra-long chains of amylopectin has been reported from various normal starches (Takeda et al., 1987, Takeda et al.,

1988; Hizukuri et al., 1989; Shibanuma et al., 1994), but is not found in waxy varieties of rice, maize, and barley (Larson et al., 1953; MacGreger and Morgan, 1984). The ratio of A:B chains (1.0-2.0) and the β-amylolysis limit (55-60%) have been used to characterize the branch structure of amylopectin (Atwell et al., 1980; Manners, 1989a; Inouchi et al., 1987; Marshall and Whelan, 1974; Takeda et al., 1986, 1988; Takeda et al., 1993; Shibanuma et al., 1994).

Currently, the most convincing "cluster model" to describe the chemical structure of amylopectin was independently proposed by Nikuni (1969), French (1972), and Robin (1974), and further elaborated using more advanced analytical techniques (Hizukuri, 1986). Hizukuri (1986) described three types of chains that are involved in establishing the cluster model. A-chains are branch-free and are linked to B- or C-chains by α-(1,6)-glycosidic bonds. B-chains carry A- and/or other B-chains. B-chains that only carry A-chains and another B-chain were classified into Ba and Bb, respectively (Hizukuri and Maehara, 1990; Hizukuri and Maehara, 1991). B chains may extend two or more cluster units. Thus, it was subdivided into B1 to B4 chains depending on the number of clusters reached. C-chain is the only chain of molecule carrying a reducing end. Jane et al. (1997) propose a model showing that the branching patterns of amylopectin of A- and B-type starches are different from each other. Branch points in the A-type amylopectin are more scattered and distributed in both amorphous and crystalline regions, whereas branch points in the B-type amylopectin are preferentially localized in amorphous region. During acid hydrolysis of starch granules, branch points embedded in the crystalline region are preserved and are not readily hydrolyzed, and the residual α-(1,6)-branch linkages in

the crystalline region of a Naegeli dextrin can be determined by quantitative anion-exchange chromatography (Jane et al., 1997).

Amylose is an essentially linear polysaccharide of D-glucose units linked by α-(1,4)-glycosidic bonds, even though it contains a few α-(1,6)-branch linkages (Greenwood, 1964; French, 1973; Misaki and Smith, 1967; Hizukuri and Takagi, 1984; Hizukuri, 1986). The β-amylolysis limit of amylose is usually in the range of 70-85% (Banks and Greenwood, 1967; Takeda et al., 1986, 1988; Takeda et al., 1993). The *number*-average chain length of amylose molecules varies from DP990 for maize to DP2110 for potato starches (Takeda et al., 1988; Suzuki et al., 1994). Amylose is a unique glucan that is exclusively synthesized by granule-bound starch synthase I (GBSSI) during starch biosynthesis (Tsai, 1974; Shure et al., 1983; Hovenkamp-Hermelink et al., 1987; Nakamura et al., 1995) and is not found in bacteria or animals. The size and amount of amylose molecules in the starch granule could be related to the relative activity of GBSSI during granule development (Bogracheva et al., 1995; Van den Koomhuyse et al., 1996). The great tendency of crystallization by itself or with complexing agents makes amylose more distinct. V-type crystalline structure has been revealed as an amylose single helical complex with lipids present in the central cavity of the helix (Yamashita, 1965; Yamashita and Hirai, 1966; Yamashita and Monobe, 1971; Czuchajowska et al., 1991). Retrogradation (or recrystallization) of amylose in solution is rapid, producing imperfect, large crystallites (Flory, 1953; Greenwood, 1964; Colonna et al., 1992). It has been observed that different sizes of amylose affect the rate of retrogradation, and amylose of DP80-100 has fastest retrogradation (Pfannemuller et al., 1971;

Gidley et al., 1986; Eerlingen, 1993). Amylose has a great impact on pasting and gel properties of starch. Amylose interacts with iodine, lipids and aliphatic-chain alcohols to form helical complex, and this property is applicable to quantify the amylose content (Larson, 1953; Pfannemuller et al., 1978; Jane et al., 1999) and to separate amylose from amylopectin (Schoch, 1942b; Winter and Sarko, 1974; Billiaderis and Galloway, 1989).

It has been revealed that a few minor components of starch, such as lipids and phosphate mono-derivatives, affect physical properties of starches significantly despite their very minute quantity. Starch lipids are categorized into surface and internal lipids (Morrison, 1981). It is well known that normal cereal starches contain lipids proportional to their amylose content (Morrison et al, 1984; Soulaka and Morrison, 1985). The internal monoacyl lipids (free fatty acids and lysophospholipids) form a complex with amylose, resulting in changes of physical properties, including restricted starch swelling, increased gelatinization temperature, accelerated retrogradation, reduced gel viscosity, and decreased enzyme digestibility (Maningat and Juliano, 1983; Swinkels, 1985; Soulaka and Morrison, 1985; Biliaderis and Seneviratne, 1990; Morrison et al., 1993). Phosphorus in starch gives interesting features. Two major different types of phosphorus are found in starches, and they are phospholipids and phosphate monoester (Morrison, 1988; Lim et al., 1994; Kasemsuwan and Jane, 1996). The unique thermal properties of potato starches are partly attributable to the existence of substantial amounts of phosphate monoester (McPherson and Jane, 1999), whereas in cereal starches most of phosphorus is present as phospholipids that have completely different

effects on the properties of starches. In normal wheat starch, phospholipids account for most of the internal lipid and phosphorus contents (Meredith et al., 1978; Kasemsuwan and Jane, 1996), which can be detected by differential scanning calorimetry as an amylose-lipid complex (Kugimiya et al, 1980). The phosphate monoester on amylopectin of potato starch increases pasting viscosity, gel clarity and stability, but it decreases pasting and gelatinization temperature, and retrogradation rate as a result of the repulsion of negatively charged phosphate derivatives on starch molecules (Schoch, 1942ab; Galliard and Bowler, 1987; Jane et al., 1996). Efforts to find corresponding enzyme for starch phosphorylation has been made (Viskø-Nielsen et al., 1998; Lorberth et al., 1998), but the biochemical pathway of adding covalently bonded phosphate is still unknown. Aside from the minor components, most other differences in properties of starches are attributable to its granular structure, the content and structure of amylose, and the structure of amylopectin when compared with glycogen. Thus, starch shows distinct properties such as gelatinization and retrogradation (Atwell et al., 1988), which do not occur in glycogen.

The molecular mass and size of amylopectin are overwhelmingly larger than any other synthetic and natural polymers. Because of that, fine structure of starch is not well understood. Chromatographic techniques, such as gel permeation chromatography (Craig and Stark, 1984; Jane and Chen, 1992; Wang et al., 1993), high-performance size-exclusion chromatography (Hizukuri, 1985; Yuan et al., 1993; Ong et al., 1994), and high-performance anion-exchange (Koizumi et al., 1991; Wong and Jane, 1997; Jane et al., 1999) chromatography have been used to study

the branch structures of amylopectin molecules. However, lack of calibration standards, mainly due to the gigantic size of amylopectin, causes difficulties in determination of the molecular weight of amylopectin using size-exclusion chromatography. The molecular weights of amylose and amylopectin affect functional properties of starch. Larger molecular weight (DP_n) of amylose and amylopectin had a tendency to show greater pasting viscosity in wheat (Shibanuma et al., 1996) and sago starches (Takeda et al., 1989). It was reported that the long branch-chains of amylopectin and the intermediate size of amylose produced the greatest synergistic effect on the pasting viscosity of the reconstituted starch (Jane and Chen, 1992).

The multi-angle laser-light scattering (MALLS) technique combined with high-performance size-exclusion chromatography (HPSEC) is a powerful tool for determining the absolute molecular weights of the macromolecules such as starch (Aberle et al., 1994; Fishman and Hoagland, 1994). The good separation between amylopectin and amylose is prerequisite to conduct an accurate measurement of the molecular weight of amylopectin using this technique. Jackson (1991) reported that the solubility of starch increased with increasing proportion of amylose. Waxy-type starches, on the other hand, showed more susceptibility to shear-induced fragmentation than normal starches (Hanselmann et al, 1995; Millard et al., 1997; Bello-Pérez et al., 1998a). Thus, the methods used to obtain the completely-dispersed starch solution without breaking the covalent bonds are critical for the chromatographic analysis. A poorly dispersed starch solution could lead to the entangled amylose/amylopectin molecules and affect the resulting molecular weight

(M_w) and gyration radius (R_z) determined by the MALLS technique. Jackson (1991) studied the extent of solubility of maize starches in dimethyl sulfoxide (DMSO) using various conditions. The maximum dispersibility from this study was achieved using a solution of 90% DMSO/10% water solution (Jackson, 1991). Weight-average molecular weight (M_w) of waxy maize was reported to be 6.5×10^7 or 5.9×10^8 depending on dispersing conditions (Millard et al., 1997). The M_w range of 2.5-7.5 $\times$ 10^8 determined by Millard et al. (1997) was in agreement with that of other studies. Moreover, they indicated that the size and molecular shape of dissolved amylopectin was highly sensitive to the dispersing method. Bello-Pérez et al. (1998a) reported when microwave heating under high pressure for 35 s was used to disperse waxy maize starch, apparent M_w and R_z was $2.2 \pm 0.2 \times 10^8$ g/mol and 229 nm, respectively. Additionally, Bello-Pérez et al. (1998b) found molecular weight of amaranth starch decreased as the treatment time of microwaving increased from 35 to 90 s. However, there is still disagreement in M_w value of amylopectins reported between normal and waxy starches. Aberle et al. (1994) showed that M_w of normal maize amylopectin was higher than that of waxy maize, but the opposite result was achieved by Bello-Pérez et al (1996). These different results may be attributed to different methods of preparing starch dispersion and of isolating amylopectin from the starch. Bello-Pérez et al. (1998b) suggested that there would be difference in branching structure of amylopectin between waxy maize starch and amaranth starch. In their study, R_z value for amaranth starch (176 nm) was lower than waxy maize starch (234 nm), even though M_w of amaranth starch (6.9×10^8 g/mol) is larger than waxy maize starch (2.0×10^8 g/mol).

Organization of Starch Granule

The size (0.5-100 μm in diameter) and shape of starch granules vary depending on botanical source, maturity, and growing conditions (Banks et al., 1974; Greenwood, 1979; Morrison and Gadan, 1987; Jane et al., 1994). It is widely accepted that the polymodal chain-length distribution of amylopectin branch-chains (Hizukuri, 1986) is mainly responsible for double helix formation among branch-chains that eventually lead to form the semi-crystalline structure of starch granule (Kainuma and French, 1971; Oostergetel and Van Bruggen, 1993). The degree of crystallinity of native starch granule is related to the proportion of amylopectin. The type of crystallinity is classified into three different patterns based on X-ray diffraction patterns, i.e. A-, B-, and C-types (Sarko and Wu, 1978; Zobel, 1988). The unit lattices of A- and B-crystals are distinct from each other. The unit cell of the crystal in A-type starches is monoclinic whereas that in B-type starches is hexagonal (Imberty et al., 1991; Gallant et al., 1997). C-type is thought to be a combination of A- and B-type crystallinity (Sarko and Wu, 1978; Imberty et al., 1988). As the other category, crystalline structure of V-type (left-handed single helix) is not only observed in the retrograded amylose that makes a single-helical complex with lipids (Yamashita, 1965; Yamashita and Hirai, 1966; Yamashita and Monobe, 1971), but also found in non-waxy cereal starches (Morrison et al., 1993). The cluster model of semi-crystalline structure of the starch granule was proposed to explain the organization of amylopectin molecule in the starch granule (Robin et al, 1974; French, 1984). A repeat lamella of 9.0-9. nm was reported for wheat, potato, rice,

maize, barley, and tapioca starches, determined by small-angle X-ray diffraction (Jenkins et al., 1993; Jenkins and Donald, 1995). This repeated length of crystalline and amorphous domains corresponds to the size of amylopectin clusters, which is consistent with the cluster size estimated by analysis of the chain-length distribution of amylopectin (Hizukuri, 1986). Amlyose molecules are interspersed among the amylopectin molecules (Jane et al., 1992; Kasemsuwan and Jane, 1994). Since most starch granules display less than 50% crystallinity, the major portion of the granule is believed to be amorphous region contributed by amylose and branch linkages of amylopectin.

Characteristics of Glycogen

Glycogen, a homopolysaccharide that consists of glucose monomers linked by α-(1,4)-glycosidic linkages and is branched with about 10% α-(1,6)-glycosidic linkages. Glycogen is produced by many bacteria as well as other eukaryotes such as mammalian, shellfish, insect, and etc (Robyt, 1998). The structure of glycogen has been investigated enthusiastically over the last century since its discovery and isolation by Claude Bernard (Calder, 1991). Regardless of glycogen source, it seems that the degree of branching is fairly constant. Average branch chain-length of most glycogen examined falls in the range of DP10-14 (Calder and Geddes, 1985, 1986; Manners, 1991). Therefore, degree of branching is about twice of that in amylopectin. Amylopectin usually has an average chain-length ranging from DP20 to 30. Structures and properties of amylopectin and glycogen are summarized in Table I. It appears that branch points (i.e. α-(1,6)-glycosidic linkages) are randomly

Table 1. Characteristics of Amylopectin and Glycogen

	Amylopectin	Glycogen
Crystallinity	15-40%	amorphous
Degree of Branching	5-8%	10-12%
Average chain length	20-30	10-14
β-amylolysis limit (%)	55	45
Exterior chain length	13-16	6-8
Interior chain length	6-8	3-5
A:B chain ratio	1.0-1.5:1	0.7-1.0:1
Molecular weight (g/mol)	10^9-10^7	10^7

Source: Manners, D. J. 1991. Recent developments in our understanding of glycogen structure. Carbohydr. Polym. 16: 37-82.

located in the glycogen molecule (Marshall and Whelan, 1974; Goldsmith et al., 1982), although not evenly distributed throughout the whole molecule (Manners, 1991). Studies have shown that the branch chain-length of glycogen is up to about DP50 with unimodal distribution (Akai et al., 1971; Weber and Wöber, 1975; Craig et al., 1988), whereas amylopectin shows a polymodal distribution and with much longer chain-length (Hizukuri, 1986; Manners, 1989a). Most studies showed glycogen to have a lower % of β-amylolysis limit (45-50%) and A:B chain ratio (0.7-1.0:1) than amylopectin (Umeki and Yamamoto, 1977; Manners, 1989b).

Another interesting feature of glycogen is its amorphous nature comparing with the semi-crystalline amylopectin of starch granule. The mechanism of developing semi-crystalline granular structure of starch is not clear. It is known that the absence of amylose does not affect the granule formation. Therefore, the structural difference between glycogen and amylopectin is the reason to result in different conformations, i.e., semi-crystalline versus amorphous.

Most data reported on the structure of glycogen are for mammals; little information is available on branch structure of prokaryotic glycogen. A few studies indicate that the chemical structure of bacterial glycogen is quite similar to that of mammalian glycogen (Weber and Wöber, 1975; Fujimori et al., 1995), but whether glycogenin-like protein, an auto-glycosylating protein (Smythe et al., 1988; Campbell and Cohen, 1989), exists in bacterial glycogen is still questionable. Further studies are needed before any conclusion can be drawn.

Biochemical and Genetic Aspects of Starch and Glycogen

Biosynthesis of Starch and Bacterial Glycogen

Biosynthetic pathways of starch and sucrose are briefly illustrated in Fig. 1. Sucrose transported from leaves into sink tissue is hydrolyzed by alkaline invertase or sucrose synthase (SuSy) to glucose (or UDP-glucose) and fructose, which are then either further metabolized or transported in the form of hexose phosphates into amyloplast through translocators (Stitt and Sonnewald, 1995). Once ADP-glucose is formed, starch is synthesized by incorporation of ADP-glucose onto the non-reducing end of an existing α-D-glucan. The ADP-glucose is also shown as the glucosyl donor for bacterial glycogen synthesis (Greenberg and Preiss, 1964). The three main enzymes known to be involved in synthesizing both starch in plants and glycogen in bacteria are ADP-glucose pyrophosphorylase, glycogen/starch synthase, and branching enzyme (Smith et al., 1997; Preiss, 1999). The starch biosynthetic pathway consists of several isoforms of the aforementioned enzymes in plants, while bacteria show a relatively simple enzyme system for the biogenesis of glycogen (Preiss, 1999).

ADP-glucose pyrophosphorylase (ADPG-PP, EC 2.7.7.27) is a key enzyme to control carbon flux into starch and bacterial glycogen (Stark et al., 1992; Preiss and Sivak, 1996; Iglesias et al., 1991; Govons et al., 1973). ADPG-PP produces ADP-glucose from glucose-1-phosphate and ATP irreversibly. The change in the level of ADPG-PP may directly affect starch structure by altering the relative proportion of amylopectin and amylose (Bogracheva et al., 1995; Wang et al., 1997; Van den Koornhuyse et al., 1996: Lloyd et al., 1999). Drastic reductions of starch content in

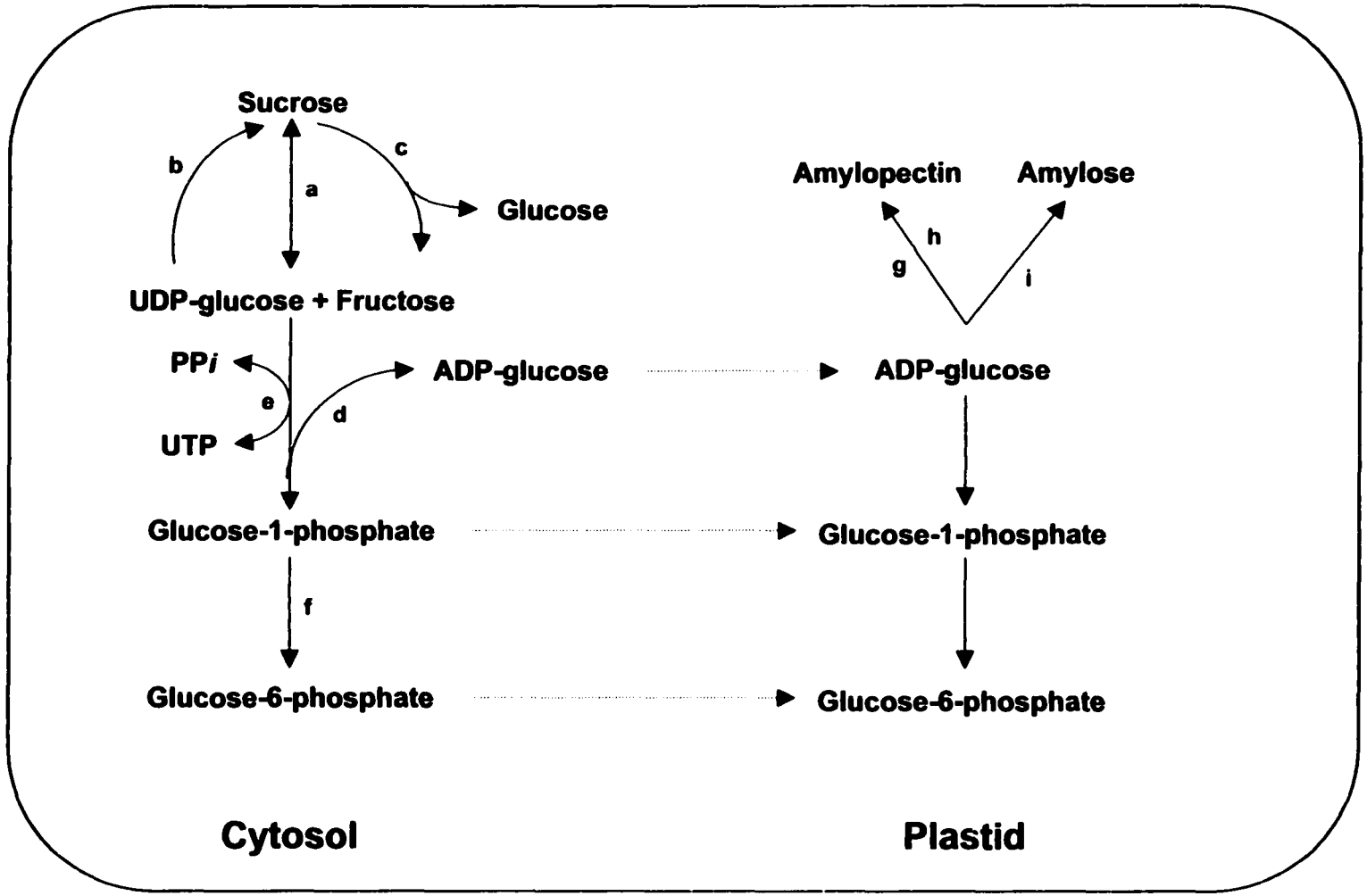

Fig. 1. The putative metabolic pathways of sucrose and starch in sink tissues of plants. a. Sucrose synthase (Susy), b. Sucrose-phosphate synthase (SPS), c. Invertase, d. ADPG pyrpphosphorylase, e. UGPase, f. Phosphoglucomutase, g. Soluble starch synthase (SSS), h. Branching enzyme (BE), I. Granule-bound starch synthase (GBSS) (Smith, Denyer, and Martin, 1997)

shrunken-2 and *brittle*-2 maize mutants were reported, which were caused by a significant decrease in the level of ADPG-PP (Tsai and Nelson, 1966; Dickinson and Preiss, 1969). Plants, such as tobacco, tomato, and potato tuber, were transformed with the mutated *E. coli* ADPG-PP that is insensitive to allosteric regulation (Stark et al., 1992) and the resulting transgenic plants accumulated larger amounts of starch than did untransformed controls. The content of glycogen in bacteria, such as *E. coli* and *S. typhimurium*, was also influenced by the mutation of ADPG-PP, but there is little information available about the glycogen structure of these bacterial mutants. The allosteric regulatory mechanism of ADPG-PP has been extensively studied in both plants and bacteria (Preiss, 1999).

Glycogen/starch synthase (GS/SS, EC 2.4.1.21) acts on ADP-Glc and then transfers a glucose moiety to an acceptor molecule such as maltooligosaccharide and/or preexisting branched glucan molecules. Several isoforms of starch synthases have been identified in plants (Kossman and Lloyd, 2000). Based on the amino-acid sequence, soluble SS are divided into at least three major classes, SSI, II, and III (Edward et al., 1995; Marshall et al., 1996; Abel et al., 1996; Kossmann et al., 1999; Knight et al., 1998; Harn et al., 1998; Gao et al., 1998). The roles of individual SS isoforms have been intensively studied using the mutant lines lacking specific isoforms (Gao et al., 1998; Craig et al., 1998) and transgenic plants (Edward et al., 1995; Marshall et al., 1996; Abel et al., 1996; Kossmann et al., 1999). It appears that each isoform plays a distinct role in the biosynthesis of starch, but their specific functions and metabolic regulations of starch synthesis have not been fully understood. A few studies hypothesize the interaction between certain isoforms of

SS and BE control the branching ratio of amylopectin (Boyer and Preiss, 1978, 1981; Gao et al., 1998). It is possible that the balance of the expressed proteins between SSs and BEs is an important factor determining the final structures of glycogens or starches (Preiss, 1991; Guan et al, 1995), but it is not clear whether the effect on the starch structure solely comes from the mutated gene or the disruption of the balance between SSs and BEs has a secondary effect. Some studies reported a pleiotropic effect resulting from antisensing or mutating the structural genes (Boyer and Preiss, 1981; Singletary et al., 1997; Craig et al., 1998). The bacterial GS exhibits no regulatory properties (Preiss, 1999), whereas in yeast and mammalian systems, glycogen synthase has been demonstrated to be the rate-limiting regulatory step (Roach and Larner, 1976, 1977; Cohen et al., 1982; Roach, 1990). It seems that *E. coli* GS can exist as dimers, trimers, and tetramers (Fox et al., 1976).

Granule-bound starch synthase I (GBSSI) is an isoform of starch synthase that is tightly bound to the starch granule. This enzyme plays an exclusive role in synthesizing amylose that is unique for the starch granule-forming organisms, such as various plants and algae, not for glycogen-producing organisms. The disruption of the gene encoding GBSSI by mutations essentially eliminates amylose production from the starch granule (Tsai, 1974; Shure et al., 1983; Nakamura et al., 1995; Hovenkamp-Hermelink et al., 1987). However, we could not exclude the possibility of GBSSI being involved in amylopectin biosynthesis. The branch structure of amylopectin from waxy-type rice, potato, and *Chlamydomonas* mutant differs from that of wild-type counterparts (Takeda and Hizukuri, 1987; Flipse et al., 1996; Delure

et al., 1992). GBSSI-deficient mutants appear to lack an extra-long chain fraction that is present in wild-type lines (Takeda and Hizukuri, 1987; Hizukuri et al., 1989; Takeda et al., 1988; Shibanuma et al., 1994; MacGreger and Morgan, 1984). It has been demonstrated that GBSSI elongates glucan-primer progressively without dissociation from the chain (Denyer et al., 1999), but SSII, one of the soluble SS, acts on primer in distributive manner by adding a single glucose unit at each encounter (Edwards et al., 1999).

Branching enzyme (BE, EC2.4.1.18) is also known to be involved in the starch/glycogen biosynthesis. This enzyme is responsible for creating α-(1,6)-linkages in starch and glycogen. The enzymatic function of each BE isoform is considerably better understood than the SS isoforms in plants. Bacteria encode only one BE gene, whereas plants contain at least two isofroms encoded by different genes (Guan et al., 1997; Preiss, 1999). In vitro biochemical studies shows that type I and type II BE in plants have different specificities and different branching manners. In maize, BEI is believed to transfer longer chains and have higher activity with amylose than BEIIa or BEIIb, which preferentially acts on amylopectin and transfer shorter chains, giving rise to the polymodal distribution of branch chain-length of amylopectin (Guan and Preiss, 1993; Preiss and Sivak, 1996). Guan and Preiss (1993) hypothesized that BEI is more involved in producing B-chains of amylopectin, whereas BEIIa and BEIIb are involved in creating A-chains.

Starch debranching enzyme (DBE) is an enzyme that specifically hydrolyzes α-(1,6)-linkages. Two types of different DBE have been found in plants. Isoamylase-type enzymes (glycogen 6-glucanohydrolase, EC 3.2.1.68) readily

hydrolyze α-(1,6)-linkages in both amylopectin and glycogen, but they have negligible or no activity toward branched oligosaccharides and pullulan (Kainuma et al., 1978; Evans et al, 1979; Rahman et al., 1998). Pullulanase-type enzymes (pullulan 6-glucanohydrolase, EC 3.2.1.41), which are also referred to as R-enzymes or limit-dextrinases in plants, have higher specific activity on pullulan and β-limit dextrin than amylopectin and glycogen (Ohba and Ueda, 1975; Yokobayashi et al., 1970; Doehlert and Knutson, 1991). Biochemical studies have revealed that *sugary-1* mutants of maize and rice, and *sta-7* mutant of *Chlamydomonas* are deficient in the activity of DBEs and accumulate phytoglycogen at the expense of starch (Rahman et al., 1998; Mouille et al., 1996; Nakamura et al., 1996). Two hypotheses have been proposed to explain the role of DBE in starch granule biosynthesis. One hypothesis, the so-called "glucan-trimming model," is that a glycogen-like random branching pattern, e.g., phytoglycogen, represents an intermediate or pre-amylopectin and that DBE removes and trims extra branch chains to form the clustered branching characteristic of amylopectin (Mouille et al., 1996; Ball et al., 1996). The other hypothesis is that phytoglycogen is a normal byproduct of starch synthesis formed through the action of SS and BE on soluble oligoglucans, and that the phytoglycogen normally is prevented from accumulation by action of amylases, phosphorylases, and DBEs (Zeeman et al., 1998). According to this second hypothesis, which is the "glucan recycling model," phytoglycogen synthesis competes with that of amylopectin for SSS, SBE, and ADP-glucose in the mutants deficient in DBEs, resulting in decreased amylopectin synthesis and accumulation of

phytoglycogen. At present, the question of whether DBEs are directly involved in amylopectin synthesis remains open.

There is no plausible hypothesis or theory with which the mechanism of the initiation of starch granule formation can be explained. The initiation step of glycogen biosynthesis in eukaryotes is known to require an auto-glycosylating protein, glycogenin (Smythe and Cohen, 1991; Lomako et al., 1991). There is currently no clear evidence that such a protein is necessary for the synthesis of starch and bacterial glycogen. As far as granule formation is concerned, it is a challenging task determining whether the combination of different isoforms could create a granule structure in vitro. Alternatively, a reconstituted system in bacteria could be utilized to test if starch granules can be created through the expression of plant starch-biosynthetic enzymes.

Genetic Modification of Starch Structure and Possible Usage in Food and Non-food Applications

ADPG-PP is a rate-limiting enzyme controlling the flux of carbon into starch. When the gene encoding ADPG-PP in *E. coli* was expressed in potato tubers, the starch content was significantly increased (Stark et al., 1992). This may be the first report of a positive result using a genetic engineering approaches. Overall carbon flux into starch synthesis may influence starch structure. Specific reduction of enzyme activities that precede ADP-glucose in the starch synthesis pathway result in a lower ratio of amylose to amylopectin in pea embryo (Bogracheva et al., 1995; Wang et al., 1997) and *Chlamydomonas* (Van den Koornhuyse et al., 1996). It

appears that amylopectin synthesis is favored over amylose synthesis when carbon flux decreases and concentration of ADP-glucose drops. Control of carbon flux into starch synthesis could make it possible to change the composition and structure of starch. Mutants, by nature or breeding, lacking GBSS accumulate waxy-type starch that is amylose-free. A specific down-regulation of GBSS was achieved by introducing an antisense RNA into transgenic potato (Visser et al., 1991; Kuipers et al., 1994), and rice (Shimada et al., 1993). Even GBSS was clearly shown to be primarily responsible for amylose synthesis by a study in which the waxy phenotype was complemented by expressing a genomic DNA coding for GBSS (Flipse et al., 1994). Thus, amylose-free starch can be relatively easily genetically engineered using current technology. On the other hand, high-amylose starch can be produced by suppressing branching enzyme. A few varieties of high-amylose maize starches are commercially available (Sidebottom et al., 1998). A significantly reduced activity of BEII isoform in maize resulted in a higher amount of amylose as well as longer average branch chain-length of amylopectin than normal maize (Shi et al., 1998). Overexpression of bacterial glycogen branching enzymes in potato resulted in the disappearance of amylose that is replaced by branched material (Kortstee et al., 1998). Schwall et al. (2000) reported that the simultaneous antisensing of BEI and II resulted in very-high-amylose potato starch. An altered amylopectin structure, e.g., a change in average branch-chain length, could also be obtained by regulating the total activity of SS or the activity of individual SS isoforms. The reduction of SSII activity in pea embryo altered the chain-length distribution of amylopectin (Craig et al., 1998). In this case, short and long chains increased at the expense of

intermediate size chains (DP15-50). This indicates SSII in pea embryo is responsible for the synthesis of intermediate size chains. In potato, however, mutation of either SSII or SSIII gave only minor change in chain-length distribution. The simultaneous mutation of SSII and SSIII significantly altered branch structure of amylopectin (Schwall et al., 2000). The proportion of chains larger than DP12 decreased, which was correlated with the reduction of total SS activity (Lloyd et al., 1999). The mechanism of starch phosphorylation has been recently studied and quite a few progresses have been achieved on the understanding of the enzymes involved (Lorberth et al., 1998; Kossmann et al., 1999). Complete understanding of the biochemical pathway is needed so that genetic engineering of starch can be specifically applied to modify the level of phosphate derivatives in starches. Because the physical property of starch is greatly affected by the negatively charged phosphate groups (Swinkels, 1985; Lim, 1990; McPherson and Jane, 1999), it is quite an interesting approach to genetically modify starch phosphate contents. So far, modification of starch structure by genetic engineering remains at the level of elucidation of the role of enzymes involved in starch biosynthesis. Productions of tailor-made structure of starch may be possible by using more advanced knowledge of the biological pathway and regulatory mechanism of starch biogenesis. The ultimate goal of this research is to find applications for genetically modified starches. More targeted and predictable direction of transgenic techniques could provide a big impact to food and industrial applications in the near future.

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CHAPTER 1. STRUCTURAL AND PHYSICAL CHARACTERISTICS OF WAXY AND OTHER WHEAT STARCHES[1]

A paper submitted to Carbohydrate Polymers

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ABSTRACT

Structures and properties of starches isolated from waxy wheat, amylose-reduced wheat (Kanto 107), and normal hard red winter wheat (Centura and a commercial product) grains were examined. Apparent amylose contents of the four starches were <0.2, 21.5, 26.2, and 26.6%, respectively. Waxy wheat amylopectin (AP) had the largest molecular weight (M_w) and no detectable extra-long branch-chains. The M_w of the AP displayed a negative correlation with the amylose (AM) content of the wheat starch, whereas the proportion of extra-long branch-chains of AP displayed a positive correlation with AM content. Relationships between the M_w and gyration radius (R_z) suggested that the structure of waxy AP was more compact than that of other wheat APs. Branch chain-length distributions of AP analyzed by high-performance anion-exchange chromatography (HPAEC) showed that the peak chain lengths of all the wheat starches were at DP12, and average chain-lengths varied between DP23.5 and 24.9. Centura AP had the highest proportion of long

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[1] Journal Paper No. J-19354 of the Iowa Agriculture and Home Economic Experiment Station, Ames, Iowa, Project No. 3756, and supported by Hatch Act and State of Iowa funds.

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branch-chains (DP≥25). Onset gelatinization temperatures of waxy wheat, Kanto107, Centura, and commercial wheat starches were 55.7, 57.5, 55.6, and 54.9°C, respectively, and enthalpy changes were 13.6, 11.8, 10.7, and 10.6 J/g, respectively. Differences in pasting temperature and peak viscosity between waxy and normal wheat starches were substantially greater than the differences between maize starch counterparts.

Keywords: Waxy wheat starch; Granule-bound starch synthase (GBSS); Amylopectin (AP); Extra-long chain (ELC); *Weight*-average molecular weight (M_w)

INTRODUCTION

Wheat is one of the oldest and most extensively cultivated crops. Commercial cultivars are classified into soft red winter (SRW), hard red winter (HRW), hard red spring (HRS), and durum wheat (Seib, 1994). Starch is the major component of wheat grain and wheat flour. Properties and structures of common and durum wheat starches of different varieties have been reported (Shibanuma, Takeda, & Hizukuri, 1996; Bhattacharya, Jafari-Shabestari, Qualset, & Corke, 1997; Vansteelandt & Delcour, 1999).

Many waxy mutants have been identified in cereals, such as maize, rice, barley, sorghum and amaranth. Natural mutation that leads to amylose-free varieties is considered unlikely in wheat because of its hexaploid genome. Waxy

hexaploid wheat has recently been produced by crossing the partial waxy mutants, Kanto 107 and Bai-Huo (Nakamura, Yamamori, Hirano, Hidaka, & Nagamine, 1995). Common wheat (*Triticum aestivum* L.) has three homologous waxy genes, WX-A1, WX-B1 and WX-D1 (Chao, Sharp, Worland, Warham, Koebner, & Gale, 1989). Kanto 107 is an amylose-reduced wheat carrying null alleles at two (WX-A1 and WX-B1) of the three WX loci and Bai-Huo is carrying one null allele at the D1 waxy locus. Waxy wheat mutants lack all three Wx proteins, also known as granule-bound starch synthases (GBSSI; EC 24.1.21). Genetic studies on mutants deficient in GBSSI have demonstrated its role in synthesis of amylose (Nakamura et al., 1995; Hylton, Denyer, Keeling, Chang, & Smith, 1996).

Potential use of wheat starch with reduced amylose content is a current topic of interest among wheat breeders and geneticists (Graybosch, 1998). One of the amylose-reduced wheat varieties is being used for producing Japanese Udon noodle because the starch contributes to overall textures of the cooked noodle. Several studies have reported structures and properties of starch derived from waxy wheat (Yasui, Matsuki, Sasaki, & Yamamori, 1996; Hayakawa, Tanaka, Nakamura, Endo, Hoshino, 1997; Fujita, Yamamoto, Sugimoto, Morita, & Yamamori, 1998).

In this study, we investigated the branch structure, molecular weight and gyration radius of AP, amylose content, and thermal and pasting properties of waxy wheat starch and compared them with that of Kanto 107 (amylose-reduced wheat), Centura (hard red winter wheat), and commercial wheat starch. We also attempted to unravel the relationship between molecular weight, gyration radius, and dispersed

molecular density of AP. Pasting properties of waxy and normal wheat starches were compared with those of maize starch counterparts.

MATERIALS AND METHODS

Materials

Waxy wheat, F4 seeds, descended from the cross of Kanto 107 and Bai-Huo, were grown in Southern California in 1998. Kanto 107 is an amylose-reduced wheat carrying null alleles at two (WX-A1 and WX-B1) of the three WX loci. Centura is a hard red winter wheat with three active WX alleles (wild type). A commercial hard red winter wheat starch was obtained from Midwest Grain Products, Inc. (Atchison, KS) and used for comparison. Waxy and normal maize starches were gifts of Cerestar, USA (Hammond, IN). Isoamylase (EC 3.2.1.68), from *Pseudomonas amyloderamosa*, was purchased from Hayashibara Biochemical Laboratories, Inc. (Okayama, Japan), and amyloglucosidase (EC 3.2.1.3), from *Rhizopus* mold [9032-80-0], was purchased from Sigma Chemical Co. (St. Louis, MO). Other chemicals (reagent grade) were used without further purification.

Starch Isolation

Starches were isolated from wheat grains using a method reported by Badenhuizen (1964) with slight modification (Kasemsuwan, Jane, Schnable, Stinar, & Robertson, 1995). Isolated starch was washed three times with distilled water,

rinsed twice with ethanol, and then recovered by filtration using Whatman No. 4 filter paper. Purified starch cake was dried in a convection oven at 35°C for 24 hr.

Starch Granule Morphology by Scanning Electron Microscopy

Starch granules spread on silver tape and mounted on a brass disk were coated with gold/palladium (60/40). Sample images were observed at 1500× magnification under a scanning electron microscope (JOEL model 1850, Tokyo, Japan) following the method of Jane, Kasemsuwan, Leas, Zobel, & Robyt (1994).

Crystalline Structure by X-ray Diffractometry

Crystallinity of starch granules was studied by using X-ray diffractometry. X-ray diffraction pattern was obtained with copper, nickel foil-filtered, Kα radiation using a Siemens D-500 diffractometer (Siemens, Madison, WI). The analysis was conducted by following the procedure of Song & Jane (2000). The degree of crystallinity was calculated based on the method of Hayakawa et al. (1997). The following equation was used to determine percent crystallinity:

$$\text{Crystallinity (\%)} = A_c/(A_c + A_a) \times 100$$

where A_c = crystalline area on the X-ray diffractogram and A_a = amorphous area on the X-ray diffractogram.

Molecular Weight Distributions of Amylopectin and Amylose by Gel Permeation Chromatography (GPC) and by High-Performance Size-Exclusion Chromatography (HPSEC)

Molecular weight distributions and amylose contents were determined by using GPC following the method of Jane & Chen (1992). An aliquot (5 mL) containing 15 mg starch and 0.75 mg glucose (as a marker) was loaded onto a Sepharose CL-2B gel (Pharmacia Inc., Piscataway, NJ) column (2.6 cm id × 90 cm). The column was run in ascending mode. The elution profiles were analyzed for total carbohydrate (anthrone-sulfuric acid method) and blue value (iodine staining) at 630 and 640 nm, respectively, by using an Autoanalyzer II (Technicon Instrument Corp., Elmsford, NY) (Jane & Chen, 1992). The AP fractions (Fraction No. 20 to 35) were collected for analyzing their branch structures.

The *weight*-average molecular weight (M_w) and z-average radius (R_z) of gyration of amylopectin were determined by using high-performance size exclusion chromatography equipped with multi-angle laser light scattering and refractive index detectors (HPSEC-MALLS-RI). Starch samples were prepared as described in Yoo & Jane (submitted, in Chapter 2). The HPSEC system consisted of an HP 1050 series isocratic pump (Hewlett Packard, Valley Forge, PA), a multiangle laser light scattering detector (Dawn DSP-F, Wyatt Tech. Co., Santa Babara, CA) and a HP 1047A refractive index detector (Hewlett Packard, Valley Forge, PA). To separate amylopectin from amylose, Shodex OH pak KB-G guard column and KB-806 and

KB-804 analytical columns were used. The operating conditions were described in Yoo & Jane (submitted, in Chapter 2) in details.

Apparent Amylose Contents by Potentiometric Autotitration

Apparent amylose content of starch was determined following the procedure of Lu, Jane, Keeling, & Singletary (1996). Analysis was based on iodine affinities of defatted starches using a potentiometric autotitrator (702 SM Titrino, Brinkmann Instrument, Westbury, NY). Starch samples were defatted using a DMSO (dimethyl sulfoxide, 90%) solution. Determination of amylose content was duplicated for each starch sample.

Amylopectin Branch Chain-Length Distribution by High-Performance Anion-Exchange Chromatography (HPAEC) and by HPSEC

The collected AP fractions from the GPC analysis were used for analyzing branch chain-length distribution. AP was debranched by using isoamylase following the method of Jane & Chen (1992). Branch chain-length distribution of AP was determined by using an HPAEC system (Dionex-300, Sunnyvale, CA) equipped with an amyloglucosidase post-column, on-line reactor and a pulsed amperometric detector (HPAEC-ENZ-PAD) (Wong & Jane, 1997). PA-100 anion exchange analytical column (250 × 4 mm) and a guard column were used for separating debranched starch samples. The gradient profile of eluents and the operating conditions were described previously (McPherson & Jane, 1999). Branch chain-

length distribution of AP was also analyzed to determine extra-long branch-chains by using an HPSEC equipped with an RI detector. The operating condition was the same as described earlier (McPherson & Jane, 1999), but the flow rate was 0.6 mL/min; the analytical column used for the analysis was Shodex OH pak SB-803HQ; the sample concentration was 1.0 mg/mL.

Thermal Properties by Differential Scanning Calorimetry (DSC)

Thermal properties of starch samples were determined by using a differential scanning calorimeter (DSC-7, Perkin-Elmer, Norwalk, CT) (Jane et al., 1999). Approximately 6 mg of starch was weighed in a stainless steel pan, mixed with 18 mg of deionized water and sealed. Sample was allowed to equilibrate for 2 hr and scanned at a rate of 10 °C/min over a temperature range of 25-140°C. An empty pan was used as the reference. The rate of starch retrogradation was determined by using the same gelatinized samples, stored at 4°C for 7 days, and analyzed using DSC and the same parameters as described above for starch gelatinization.

Pasting Properties by Rapid Visco Analyzer (RVA)

Starch pasting properties were analyzed by using an RVA (RVA-4, Newport Scientific, Sydney, Australia) (Jane et al., 1999). A starch suspension (8%, w/w) was prepared by weighing starch (2.4 g, dry starch basis (dsb)) into an RVA canister and making up the total weight to 30 g with distilled water. The sample suspension was equilibrated at 30°C for 1 min, heated at a rate of 6.0 °C/min to 95°C,

maintained at that temperature for 5.5 min, and then cooled to 50°C at a rate of 6.0 °C/min. A constant rotating speed of the paddle (160 rpm) was used throughout the analysis.

RESULTS AND DISCUSSION

Scanning electron micrographs of starch granules isolated from four wheat varieties, waxy, Kanto 107, Centura, and a commercial product, are shown in Fig. 1. Distinct bimodal size distributions were observed for all four starch samples. Large (A) granules showed a disk shape and had diameters of 18-33 μm, and small (B) granules, a spherical shape, had diameters of 2-5 μm. The results were in agreement with that reported by Jane et al. (1994). There were no detectable differences in the granule-size distribution and granule morphology between waxy and other wheat varieties. X-ray diffractograms of wheat starches displayed typical A-type patterns (Fig. 2). A minor peak at $2\theta = 20°$ was found in Kanto 107, Centura, and commercial wheat starches, reflecting the presence of amylose-lipid complex, which was not prominent in waxy wheat starch (Zobel, 1988). Percentage crystallinity of waxy, Kanto 107, Centura, and commercial wheat starches, calculated based on X-ray diffractograms, was 18.0, 14.5, 12.0, and 13.0%, respectively. Waxy wheat starch had significantly greater crystallinity than did others.

Apparent AM contents of defatted starches are shown in Table 1. Waxy wheat starch contained a negligible amount of amylose (<0.2%). Kanto 107 starch

had less amylose content (21.5%) than did other normal varieties (~26%). Total AM contents determined by GPC (Fig. 3) were greater than those determined by iodine affinity measurements. This was consistent with that reported by Fredriksson (1998). GPC profiles of starch molecular weight distribution showed no detectable amylose in waxy wheat starch (Fig. 3). Molecular weight distributions of amylose of other wheat starches were similar to each other.

Weight-average molecular weight (M_w) and *z*-average radius of gyration (R_z) of the APs, determined by using an HPSEC-MALLS-RI system, were in the range of 3.10-5.24 $\times$ 10^8 g/mol and 301-328 nm, respectively (Table 2). Among the wheat varieties, AP molecules of waxy wheat starch had the largest M_w and R_z. Molecular weights (M_w) of waxy maize AP (Bello-Perez, Roger, Baud, & Colonna, 1998; Yoo & Jane, submitted, in Chapter 2) and other cereal and tuber waxy APs (Yoo & Jane, submitted, in Chapter 2) have been found larger than that of normal starch counterparts. AP molecules of Kanto 107 (amylose-reduced starch) displayed the second largest M_w among the wheat starches (Table 2). However, there were no significant differences in R_z of APs among Kanto 107, Centura, and commercial wheat starches.

From the results, it appeared that the M_w of AP decreased as the AM content increased. Takeda, Takeda, & Hizukuri, 1993 reported that AP of amylomaize had a smaller M_w than did normal AP. This trend seems related to the absence of GBSSI protein in the waxy wheat mutant. It is plausible that the carbon flux of ADP-Glc (adenosine-5'-diphosphate glucose) exclusively goes into AP biosynthesis in

waxy wheat starch. In contrast, ADP-Glc partitions into the biosynthesis of AP and AM in the normal wheat starch. This could result in larger AP molecular weights of waxy wheat and Kanto 107 than those of normal wheat varieties.

Profiles of branch chain-length distributions of isoamylase-debranched APs of the starches analyzed by HPAEC-ENZ-PAD are shown in Fig. 4. The bimodal chain-length distribution showed peak chain-lengths at DP12 and DP47-51 (Table 3) and an obvious shoulder at DP18-21 for all four varieties of wheat starches. Song & Jane (2000) suggested that fewer branch-chains with DP18-21 (~6.8 nm average chain-length) to develop a crystalline array in the crystalline lamellae (6.6 nm) (Jenkins & Donald, 1995) of AP resulted in defective crystallites and lower gelatinization temperature. Branch chain-length distributions of the four varieties were similar to each other (Table 3).

It is worth noting differences in the blue values of AP fractions between different starches as shown on the GPC profiles (Fig. 3). APs of normal wheat starches displayed greater blue values than did that of waxy wheat starch. These results contradicted the similar branch chain-length distributions of the APs shown in Fig. 4. The greater blue values suggested there were longer branch-chains present in the AP of normal wheat starches, which were beyond the detectable chain-length range of HPAEC-ENZ-PAD. The HPSEC chromatograms of all the four debranched APs are shown in Fig. 5. The chromatograms showed two peaks at DP11 and ~32 and a shoulder at DP14 (Fig. 5). Profiles of normal wheat starches (Centura and commercial) displayed another peak (~DP770), known as extra-long chains (ELC),

which was not shown in waxy AP and was less prominent in Kanto 107. The profiles of normal wheat APs were in agreement with that reported by Shibanuma, Takeda, & Hizukuri (1994). Existence of ELC in AP of normal starches has been reported in various botanical sources (Takeda & Hizukuri, 1987; Hizukuri, Takeda, Maruta, & Juliano, 1989; Takeda, Shitaozono, & Hizukuri, 1988) including wheat (Shibanuma et al., 1994). The difference in the amount of ELC between waxy, amylose-reduced, and normal wheat starches suggested that GBSSI was responsible for the biosynthesis of ELC of AP. Denyer, Waite, Motawia, Lindberg-møller, & Smith (1999) reported that GBSSI synthesizes starch by a processive pattern. The dispersed molecular densities (ρ) of AP in a diluted solution showed that waxy wheat AP was more compact than normal wheat APs (Table 2). The ELCs, synthesized processively by GBSSI, are expected to have an amylose-like structure, carrying fewer branches, which could result in smaller M_w of normal AP with lower density (Yoo & Jane, submitted, in Chapter 2).

Thermal analysis of wheat starches showed that onset gelatinization temperatures (T_o) were similar (54.9-57.5°C) and enthalpy changes (ΔH) of the starches were 13.6, 11.8, 10.7, and 10.6 J/g, respectively, for waxy, Kanto 107, Centura, and commercial wheat starches (Table 4a), which were consistent with their degrees of crystallinity (18.0, 14.5, 12.0, and 13.0%, respectively). The DSC thermogram of waxy wheat starch showed no melting peak of AM-lipid complex, whereas other starches showed a peak (91-106°C), corresponding to an AM-lipid complex melting peak. The ΔH of melting AM-lipid complex proportionally increased

with the increase of the AM contents of the wheat starch. These results coincided with the fact that waxy wheat starch had a substantially lower lipid content (0.12 g/100g starch) than Kanto 107 (1.07 g/100g starch) reported by Yasui et al. (1996). Lim, Kasemsuwan, & Jane, (1994) reported there was undetectable or trace amount of phospholipids in waxy starches, whereas normal wheat starch contained a large concentration of phospholipids (0.058%, w/w) (Kasemsuwan & Jane, 1996). Phospholipids are known to form a strong complex with amylose (Morrison, Tester, Snape, Law, & Gidley, 1993). After storage at 4°C for a week, percentage retrogradation of waxy, Kanto 107, Centura, and commercial wheat starches were 33.7, 39.5, 45.1, and 35.9%, respectively (Table 4b). Centura wheat starch showed a greater degree of retrogradation than did other starches, which could be attributed to its larger proportion of branch chains of DP>25 (Yuan, Thomson, & Boyer, 1993) and smaller proportion of DP6-12 (Shi & Seib, 1992).

Pasting properties of the wheat starches, and normal and waxy maize starches are shown in Fig. 6. Pasting temperatures of waxy, Kanto107, Centura, and commercial wheat starches were 62.5, 88.4, 90.6, 85.0°C, respectively, and peak viscosities were 230.0, 164.1, 96.2, and 122.0 RVU, respectively. Like other waxy starches, such as waxy barley (Song & Jane, 2000), waxy potato (McPherson & Jane, 1999), and waxy cereals (Jane at al., 1999), waxy wheat starch had the lowest pasting temperature (62.5°C) and the largest peak viscosity among all wheat starch varieties, which was consistent with that reported by Kiribuchi-Otobe, Nagamine, Yanagisawa, Ohnishi, & Yamaguchi (1997). AM content had strong

negative correlation with the peak viscosity of the wheat starches analyzed (r = -0.94, P <0.05). This agreed with that reported for sweet potato starches (r = -0.89, P <0.001). (Collado, Mabesa, & Corke, 1999).

Comparing the pasting properties of wheat starches with that of maize counterparts, normal wheat varieties (Centura and commercial wheat) showed higher pasting temperatures (90.6°C and 85.0°C, respectively) and lower peak viscosity (96 RVU and 122 RVU, respectively) than did normal maize starch (81.5°C and 159.1 RVU, respectively, for pasting temperature and peak viscosity (Fig. 6). Waxy wheat, however, displayed a lower pasting temperature (62.5°C) but a higher peak viscosity (230 RVU) than did waxy maize starch (69.8°C and 200 RVU, respectively) (Fig. 6). The absence of amylose and lipids in waxy wheat starch had more profound effects on the pasting properties than did those of maize starch counterparts. The difference in pasting temperature between waxy-wheat starch and normal-wheat starches (Centura) (28.1°C) was much larger than the difference between maize starch counterparts (11.7°C), and the difference in peak viscosity between waxy and normal wheat starch (Centura) (134 RVU) was also much larger than that of maize starch counterparts (41 RVU). A lower gelatinization temperature (T_o) of waxy wheat starch (55.7°C) than waxy maize starch (64.1°C) (Jane et al., 1999) facilitated the lower pasting temperature of waxy wheat starch. The drastic differences in the pasting properties between waxy and normal wheat starches are attributed to the large phospholipid concentration in normal wheat starch (Lim et al., 1994; Kasamsuwan & Jane, 1996). Amylose-phospholipid complex (Table 4a)

restricted swelling of wheat starch granules (Tester & Morrison, 1990). Without the restriction on granule swelling caused by the amylose-lipid complex, waxy wheat starch could swell and develop viscosity at a much lower temperature.

CONCLUSION

The absence of amylose in waxy wheat starch did not affect crystalline polymorphism, granule size, morphology, and gelatinization temperature, but increased the degree of crystallinity. Waxy wheat AP had a significantly larger molecular weight and a greater dispersed molecular density than did APs of other wheat starches. Waxy wheat AP did not contain ELC that was synthesized progressively by GBSSI in normal wheat AP. Differences in pasting properties between waxy and normal wheat starches were greater than that between waxy and normal maize starch counterparts, which were attributed to phospholipid contents in normal wheat starches.

ACKNOWLEDGEMNETS

We thank Dr. Graybosch, USDA, Lincoln, Nebraska, for providing the waxy wheat, Centura, and Kanto 107 and his helpful suggestions.

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Table 1

Iodine affinities and apparent amylose contents of wheat starches

Sample	Iodine affinity (%)	Apparent amylose content (%)[a]	Amylose content by GPC
Waxy wheat	0.04 ± 0.02[b]	< 0.2 ± 0.1	0.0 ± 0.0
Kanto 107	4.29 ± 0.06	21.5 ± 0.3	24.0 ± 1.0
Centura	5.23 ± 0.09	26.2 ± 0.5	31.6 ± 0.6
Commercial wheat	5.32 ± 0.12	26.6 ± 0.6	33.4 ± 0.7

[a] Apparent amylose contents were averaged from at least two analyses.; Values were calculated from dividing iodine affinity by a factor of 0.2.

[b] ± Standard deviation.

Table 2

Average molecular weight and gyration radius of wheat starches[a]

Sample[b]	$M_w \times 10^{-8}$ (g/mol)[c]	R_z (nm)[d]	ρ (g/mol/nm^3)[e]	ρ_r[f]
Waxy wheat	5.24 ± 0.37[g]	328 ± 6	14.8	1
Kanto 107	3.98 ± 0.01	303 ± 2	14.3	0.95
Centura	3.50 ± 0.17	301 ± 7	12.8	0.87
Commercial wheat	3.10 ± 0.26	302 ± 3	11.3	0.77

[a] Data were obtained from at least three injections
[b] Starch samples were dissolved in 90% DMSO solution and precipitated with 5 vol. ethanol; Freshly prepared starch aqueous solution (100 μL; 0.4 mg/mL) was injected to HPSEC system.
[c] *weight*-average molecular weight.
[d] *z*-average radius of gyration.
[e] Density is equal to M_w/R_z^3.
[f] Relative density based on waxy wheat as 1.
[g] ± Standard deviation.

Table 3

Branch chain length distributions of wheat amylopectins

Sample	Peak DP		Average	Percent distribution				
	I	II	CL	DP6-9	DP6-12	DP13-24	DP25-36	DP≥37
Waxy wheat	12	51	24.4 ± 0.2[a]	4.6 ± 0.2	21.5 ± 0.2	45.0 ± 0.2	14.8 ± 0.0	18.7 ± 0.4
Kanto 107	12	47	24.2 ± 0.2	4.6 ± 0.1	21.3 ± 0.0	45.2 ± 0.1	15.2 ± 0.0	18.3 ± 0.3
Centura	12	48	24.9 ± 0.3	4.2 ± 0.2	20.2 ± 0.0	44.2 ± 0.5	15.6 ± 0.1	20.2 ± 0.7
Commercial wheat	12	47	23.5 ± 0.4	4.8 ± 0.0	22.4 ± 0.5	46.1 ± 0.0	14.4 ± 0.0	17.1 ± 0.7

[a] ± Standard deviation.

Table 4a

Thermal properties of native wheat starches

Sample[a]	Native starch				
	T_o (°C)	T_p (°C)	T_c (°C)	ΔH (J/g)	Amylose-lipid complex, T_p (°C)
Waxy wheat	55.7 ± 0.1[b]	61.4 ± 0.4	67.6 ± 0.3	13.6 ± 0.4	ND[c]
Kanto 107	57.5 ± 0.2	62.1 ± 0.4	67.0 ± 0.4	11.8 ± 0.2	97.9 ± 0.7
Centura	55.6 ± 0.1	59.1 ± 0.2	63.1 ± 0.2	10.7 ± 0.1	97.5 ± 0.9
Commercial wheat	54.9 ± 0.1	58.9 ± 0.1	63.8 ± 0.2	10.6 ± 0.3	97.9 ± 0.2

[a] Samples (~2.0 mg, dsb) and deionized water (~6.0 mg) were used for the analysis; T_o, T_p, T_c, and ΔH are onset, peak, conclusion temperature, and enthalpy change, respectively.
[b] Values were calculated from three analyses; ± Standard deviation.
[c] Not detectable.

Table 4b

Thermal properties of retrograded wheat starches

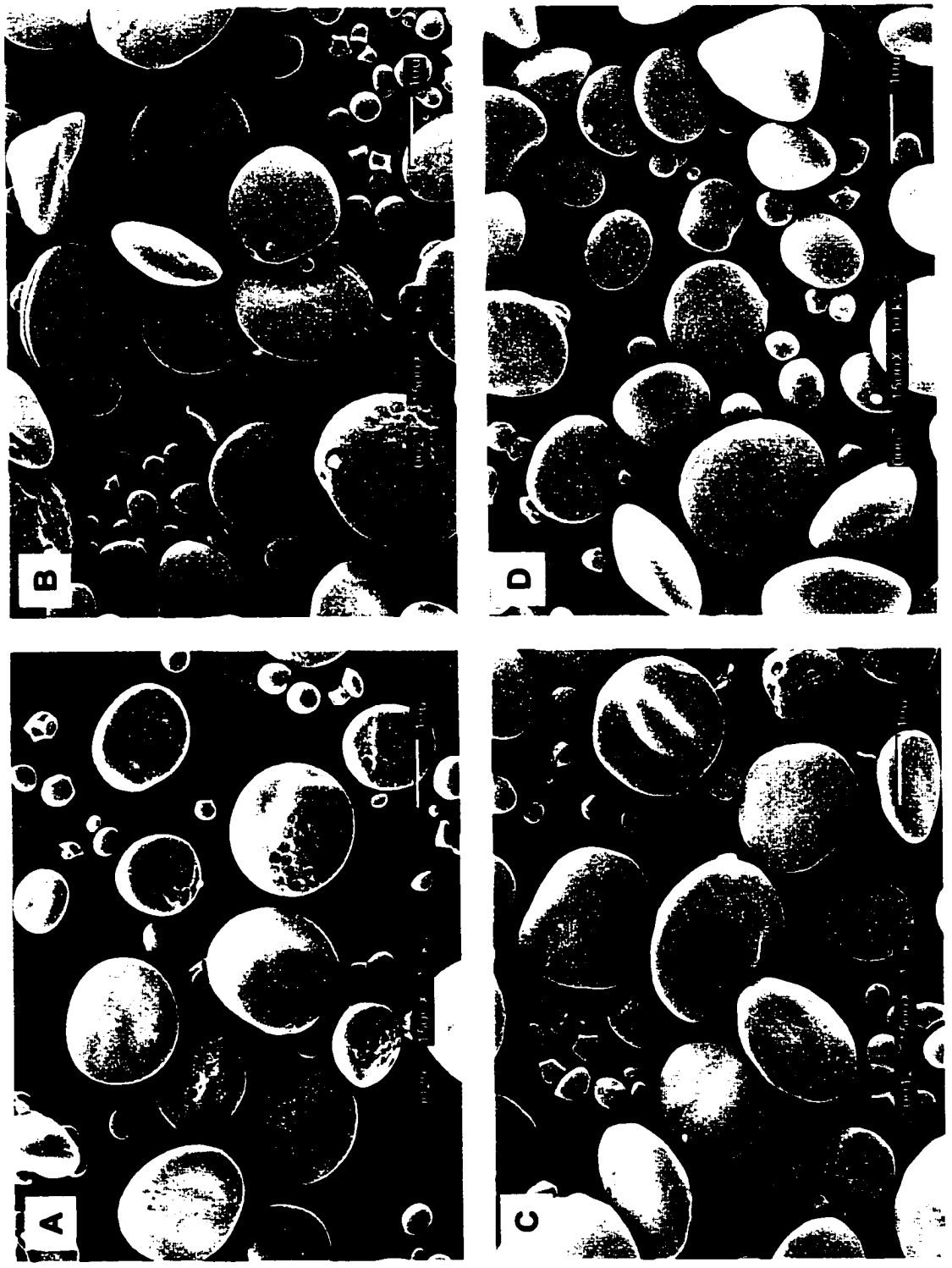
Sample	Retrograded starch[a]				Retrogradation (%)[b]
	T_o (°C)	T_p (°C)	T_c (°C)	ΔH (J/g)	
Waxy wheat	41.0 ± 0.2[c]	50.2 ± 0.5	56.5 ± 0.3	4.6 ± 0.2	33.7
Kanto 107	41.6 ± 0.5	50.4 ± 0.2	57.2 ± 0.2	4.7 ± 0.2	39.5
Centura	40.6 ± 0.5	49.4 ± 0.5	55.8 ± 0.4	4.8 ± 0.3	45.1
Commercial wheat	41.2 ± 0.3	49.4 ± 0.2	56.2 ± 0.5	3.8 ± 0.5	35.9

[a] After storage at 4°C for 7 days.

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

[c] Values were calculated from three analyses; ± Standard deviation.

Figure 1. Scanning electron micrographs of waxy (A), Kanto 107 (B), Centura (C), and commercial (D) wheat starch granules (Scale bar = 10 μm).


A
B
C
D

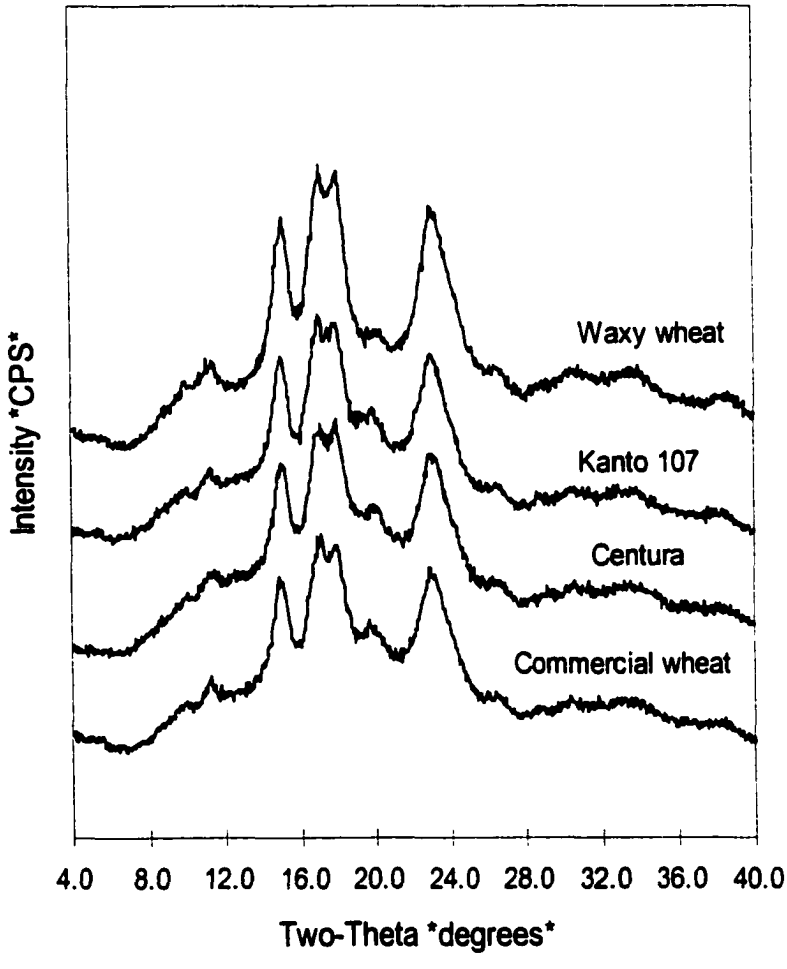


Fig. 2. X-ray diffraction patterns of waxy, Kanto 107, Centura, and commercial wheat starches.

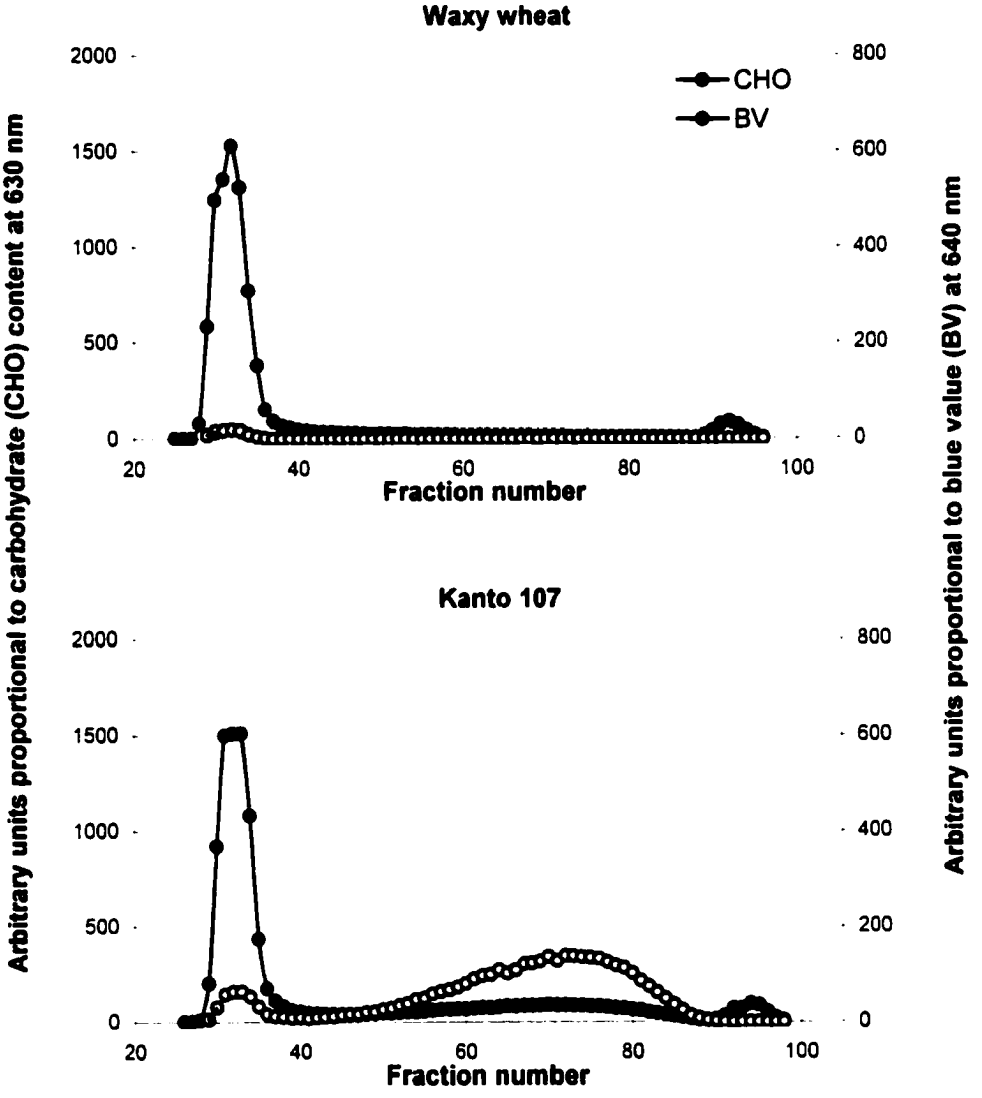


Fig. 3a. Sepharose CL-2B GPC profiles of waxy and Kanto 107 wheat starches.

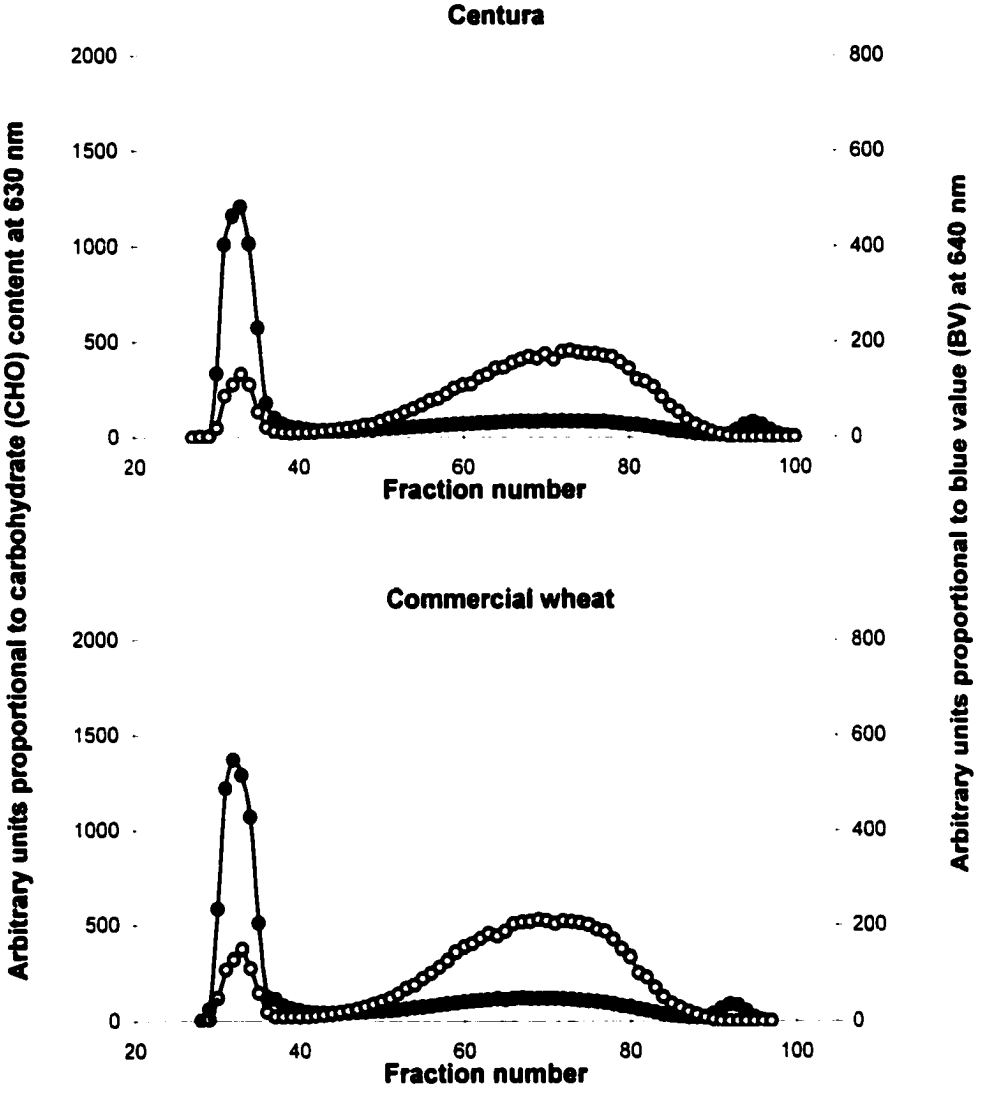


Fig. 3b. Sepharose CL-2B GPC profiles of Centura and commercial wheat starches.

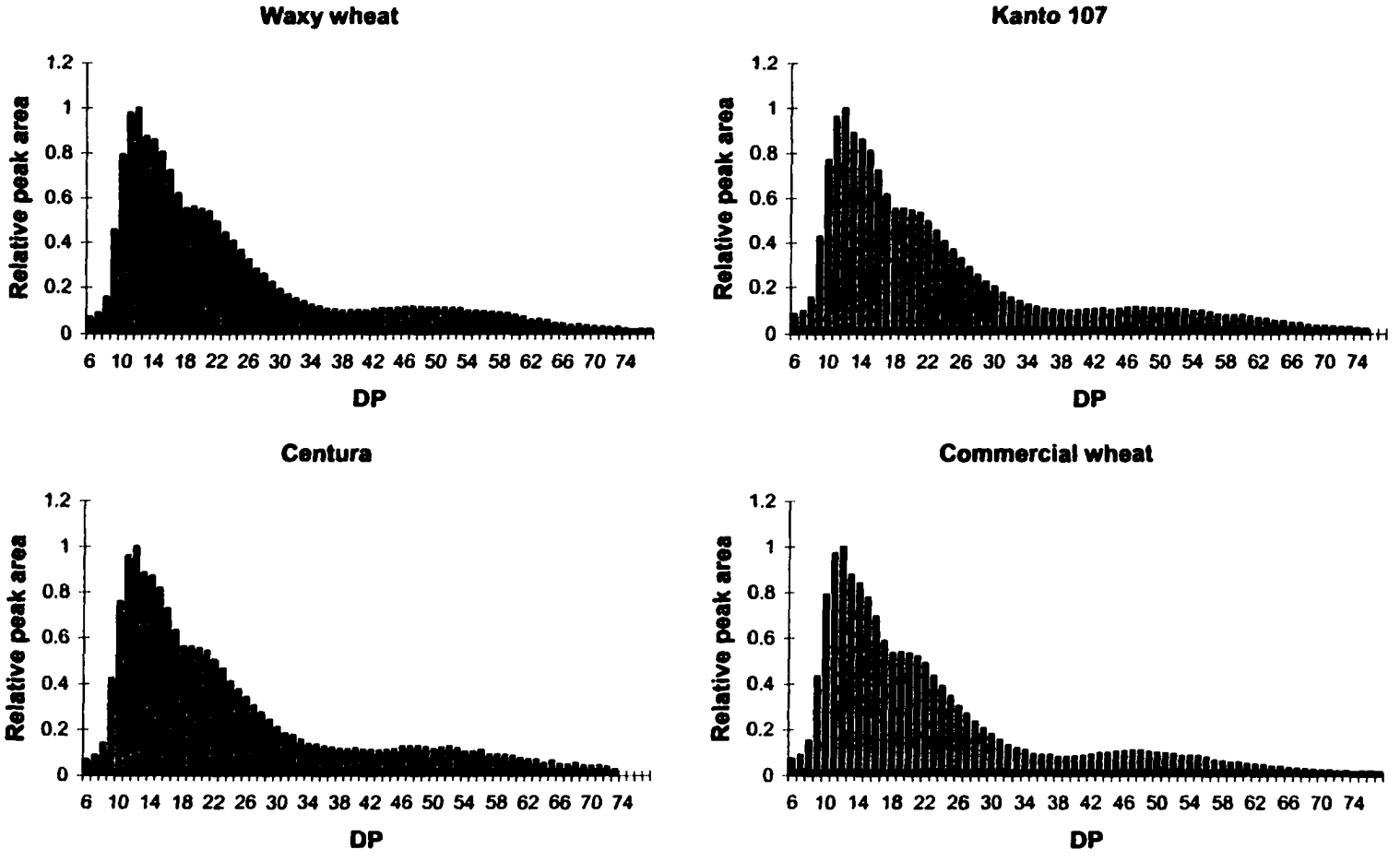


Fig. 4. Relative peak area distributions of wheat starches analyzed by using an HPAEC-ENZ-PAD. DP = Degree of polymerization.

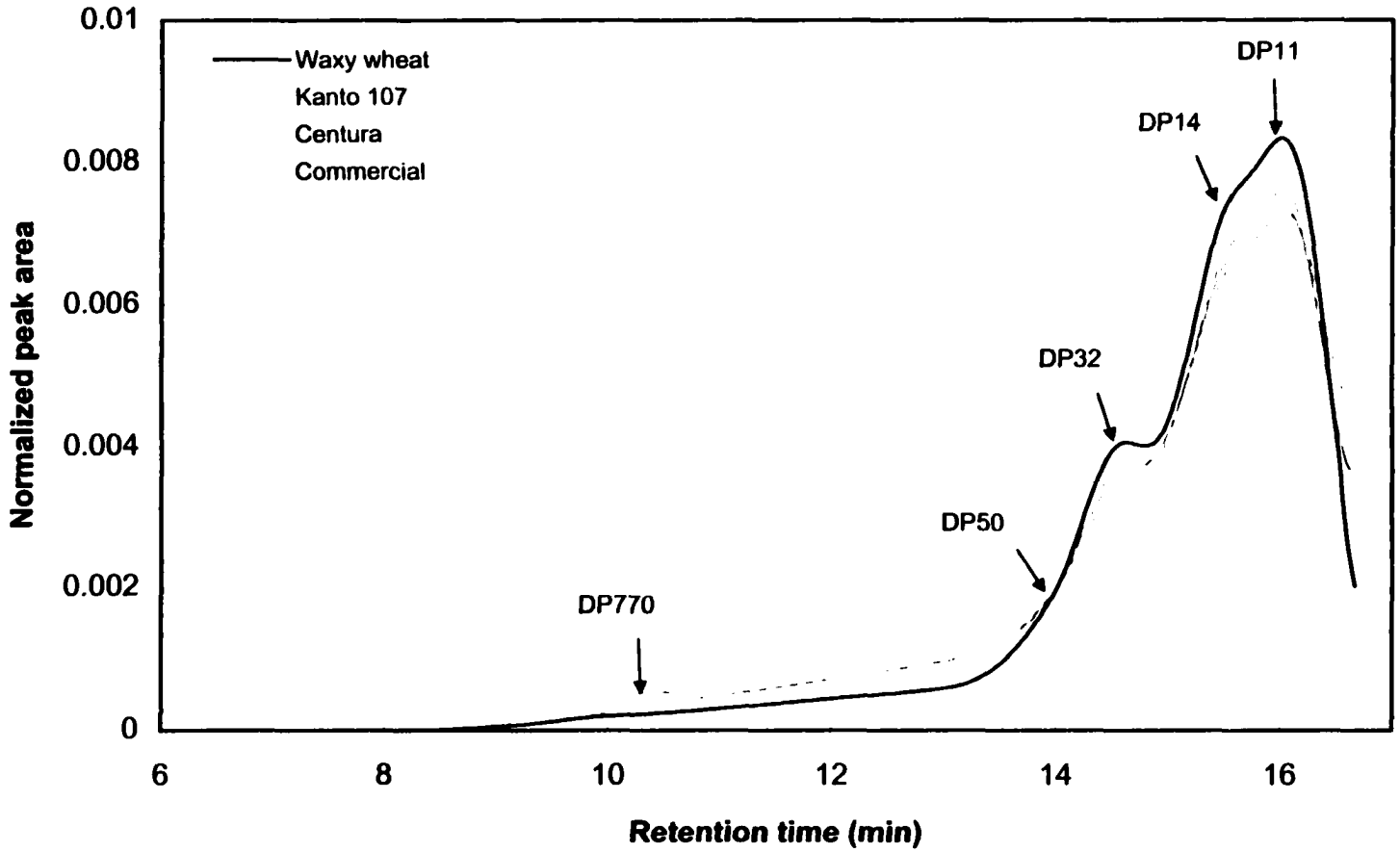


Fig. 5. Normalized HPSEC chromatogram of isoamylase-debranched amylopectins.

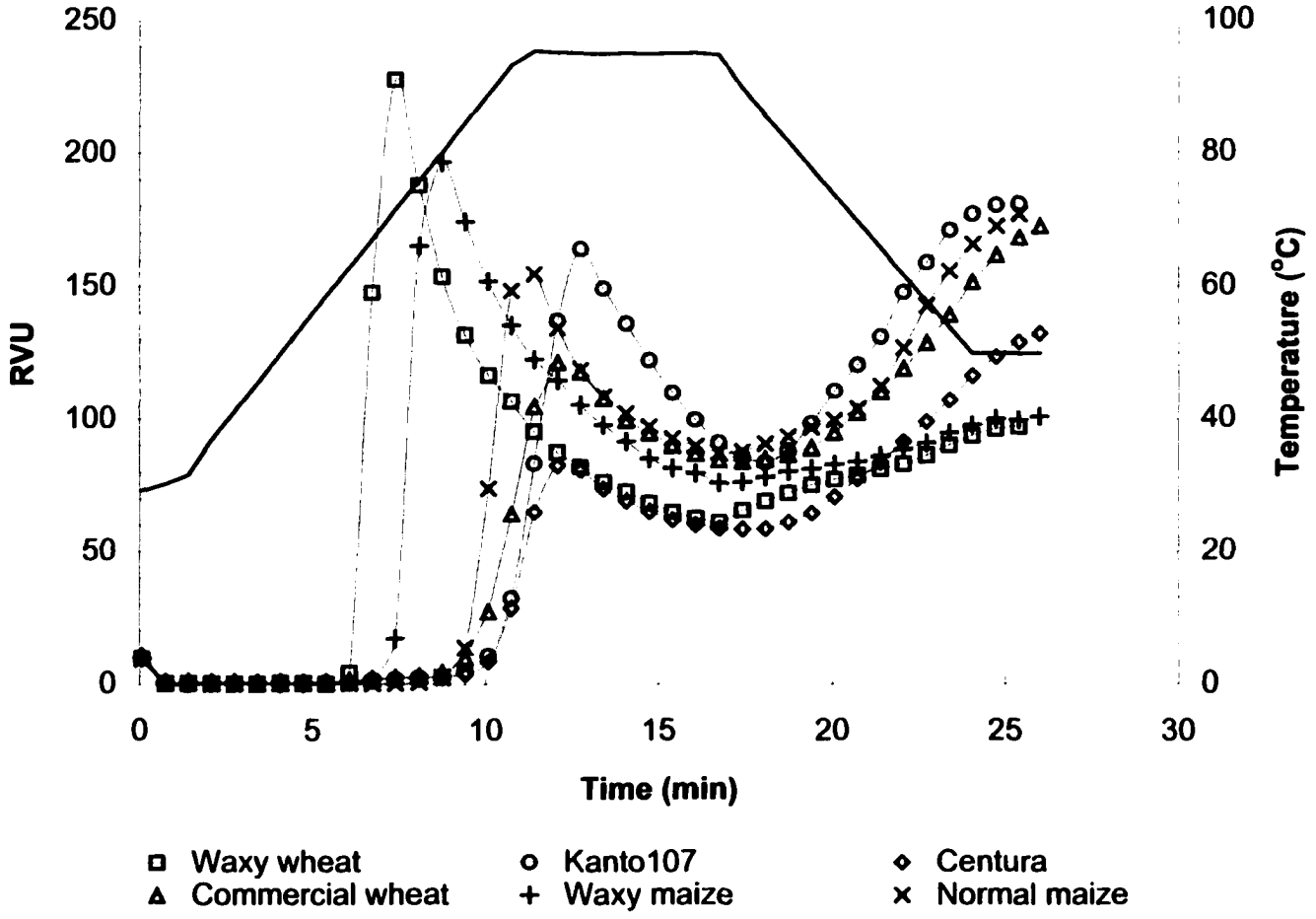


Fig. 6. Rapid ViscoAnalyzer pasting profiles of wheat starch varieties compared with that of normal and waxy maize starches (8.0% dsb, w/w).

CHAPTER 2. MOLECULAR WEIGHTS AND GYRATION RADII OF AMYLOPECTINS DETERMINED BY HIGH-PERFORMANCE SIZE-EXCLUSION CHROMATOGRAPHY EQUIPPED WITH MULTI-ANGLE LASER-LIGHT SCATTERING AND REFRACTIVE INDEX DETECTORS[1]

A paper submitted to Carbohydrate Polymers

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ABSTRACT

High-performance size-exclusion chromatography (HPSEC) equipped with multi-angle laser-light scattering (MALLS) and refractive index (RI) detectors was used to determine *weight*-average molecular weight (M_w) and *z*-average radius of gyration (R_z) of amylopectin of selected starches. Ranges of M_w and R_z values of amylopectin were 7.0×10^7 - 5.7×10^9 g/mol and 191 - 782 nm, respectively. Amylopectins of waxy starches had substantially larger M_w than did those of normal starch counterparts. On the basis of relationships between M_w and R_z, waxy amylopectins displayed, in general, larger dispersed molecular density than did normal amylopectin counterparts, and amylopectins of the A-type starches had larger dispersed molecular density than did those of the B-type starches. These results suggested that amylopectins of waxy starches had more branch-chains and

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[1] Journal Paper No. J-19335 of the Iowa Agriculture and Home Economics Experiment Station, Ames, Iowa, Project No. 3756, and supported by Hatch Act and State of Iowa funds.

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no extra long chains, which resulted in more densely packed molecules than did those of normal starch counterparts. The amylopectin of B-type starch had longer but fewer branch-chains, which resulted in smaller dispersed density than did that of the A-type starch. M_w and R_z values of amylose isolated from amylomaize VII starch also were determined to be 2.8×10^5 and 43 nm, respectively.

Keywords: Amylopectin; *Weight*-average molecular weight (M_w); *z*-average radius of gyration (R_z); Multi-angle laser-light scattering (MALLS)

INTRODUCTION

Starch is one of the most important biopolymers. There are two major components of starch: amylopectin that is a highly branched gigantic molecule and amylose, a primarily linear molecule. Functional properties of starch are affected by molecular weight of amylose and amylopectin. Larger molecular weight (DP_n) of amylose and amylopectin resulted in higher pasting peak viscosity in wheat (Shibanuma, Takeda, & Hizukuri, 1996) and sago starches (Takeda, Takeda, Suzuki, & Hizukuri, 1989). Jane & Chen (1992) reported that the long branch chain-length of amylopectin and the intermediate size of amylose produced the greatest synergistic effect on pasting viscosity of reconstituted starch.

Branch structures of amylopectin molecules have been studied using various chromatographic techniques such as gel permeation chromatography (Craig & Stark, 1984; Jane & Chen, 1992; Wang, White, Pollak, & Jane, 1993), high-

performance size-exclusion chromatography (Hizukuri, 1985; Yuan, Thompson, & Boyer, 1993; Ong, Jumel, Tokarczuk, Blanshard, & Harding, 1994), and high-performance anion-exchange chromatography (Koizumi, Fukuda, & Hizukuri, 1991; Wong & Jane, 1997; Jane et al., 1999). Determination of amylopectin molecular weight is challenging because of its gigantic molecules, which are larger than any other synthetic and natural polymers. Lack of calibration standards causes difficulties in determination of the molecular weight of amylopectin using size-exclusion chromatography.

High-performance size-exclusion chromatography (HPSEC) equipped with multi-angle laser-light scattering (MALLS) and refractive index (RI) detectors has been applied to determine absolute molecular weight of starches (Aberle, Burchard, Vorwerg, & Radosta, 1994; Fishman, Rodriguez, & Chau, 1996). The MALLS detection technique combined with HPSEC is a powerful tool for determining the absolute molecular weights of such macromolecules. To obtain an accurate molecular weight of amylopectin using this technique, complete separation of amylopectin from amylose is required.

Methods used to disperse starch molecules for chromatography are critical because entangled amylose/amylopectin molecules cannot be separated by SEC and will affect the molecular weight and gyration radius determined by the MALLS technique. It has been reported that total starch solubility increased with increasing proportion of amylose (Jackson, 1991). Waxy-type starches, on the other hand, are more susceptible to shear-induced fragmentation than are normal starches

(Hanselmann, Ehrat, &Widmer, 1995; Millard, Dintzis, Willett, & Klavons, 1997; Bello-Pérez, Roger, Baud, & Colonna, 1998). M_w of waxy maize was reported to be 6.5×10^7 or 5.9×10^8 depending on dispersing conditions (Millard et al., 1997). Jackson (1991) studied the extent of solubility of maize starches in dimethyl sulfoxide (DMSO) using various conditions. He confirmed that maximum dispersibility was achieved using a solution of 90% DMSO/10% water solution. In this study, we used a gentle procedure to prepare starch dispersion in an aqueous solution containing 90% DMSO and separated amylopectin from amylose using an HPSEC system. Molecular weights and gyration radii of amylopectins were determined by using on-line MALLS and RI detection. Branch structures of amylopectin molecules were proposed to explain the relationship between the molecular weights and gyration radii obtained for starches of A- and B-type polymorphisms.

MATERIALS AND METHODS

Materials

Chinese taro, mung bean, waxy rice, sweet rice, green leaf canna, lotus root, water chestnut, cattail millet, normal and waxy barley (from Dr. C. W. Newman, Montana State University), and waxy wheat (from Dr. R. A. Graybosch, USDA, NE) starches were isolated in our laboratory. Other starches were gifts from Dr. A. R. Bonilla, University of Costa Rica (green banana), Cerestar, USA (waxy, *ae*, and *du* waxy maize), National Starch and Chemical Co. (tapioca, normal maize, and

amylomaize V and VII), and Lyckeby Starkelsen Food and Fibre AB, Kristianstad, Sweden (waxy potato). Normal rice (from Matheson Coleman & Bel, Inc.), normal potato and wheat (from Sigma Chemical Co.) starches were purchased. Pullulan standards (Shodex STANDARD P-82) were purchased from Showa Denko K.K. (Tokyo, Japan). Isoamylase (EC 3.2.1.68) from *Pseudomonas amyloderamosa* was purchased from Hayashibara Biochem. Lab., Inc. (Okayama, Japan). Deionized water (18.2 MΩ cm) used as an eluent and for sample preparation was obtained from a Milli-Q Reagent Water System (Millipore, Bedford, MA). Other chemicals were reagent grade and used without further treatment.

Sample Preparation for High-Performance Size-Exclusion Chromatography (HPSEC)

Starch (120 mg) was evenly wetted with 1.2 mL of water and then dispersed in 10.8 mL of dimethyl sulfoxide (DMSO). The suspension was mechanically stirred while heating in a boiling water bath for 1 h and then stirred for 24 h at 25°C. An aliquot (0.4 mL) of starch dispersion (1.0%, w/v) was mixed with 5 volume of ethanol (2 mL) to precipitate starch. Ethanol-precipitated starch was separated by centrifugation at 6,750 × *g* for 20 min. The starch pellet was then redissolved in boiling water (10 mL) and stirred for 30 min in a boiling water bath. The hot sample solution was filtered through a nylon membrane filter (5.0 μm) and then injected into an HPSEC system. The final concentration of the starch solution filtrate injected (100 μL) was 0.4 mg/mL.

Molecular Weight Distribution of Starches Determined by HPSEC-MALLS-RI System

An HPSEC system consisted of an HP 1050 isocratic pump (Hewlett Packard, Valley Forge, PA) equipped with an injection valve (100μl sample loop, Model 7125, Rheodyne), a multiangle-laser-light-scattering detector (Dawn DSP-F, Wyatt Tech. Corp., Santa Barbara, CA) with a He-Ne laser source (λ = 632.8 nm) and a K-5 flow cell, and an HP 1047A refractive index detector (Hewlett Packard, Valley Forge, PA). To separate amylopectin from amylose, Shodex OH pak KB-G guard column and KB-806 and KB-804 analytical columns (Showa Denko K.K., Tokyo, Japan) were used. The temperature of the injector and columns was maintained at 55.0°C using a CH-460 column heater and a TC-50 controller (Eppendorf, Madison, WI). Temperature of RI detector was set at 30.0°C. The mobile phase was distilled-deionized water (18.2 MΩ cm) passed through in-line membrane filters (0.2 and 0.1 μm, Millipore, Bedford, MA) at a flow rate of 0.7 mL/min.

Data Analysis

A pullulan standard, P-20 (M_w = 2.28 × 10^4, 5 mg/mL), was used for normalization of multiangle photodiode detectors and for determination of delay volume (0.222 mL) between MALLS and RI detections. Data obtained from MALLS and RI detectors were analyzed using Astra software (Version 4.7.07, Wyatt Technology, Santa Barbara, CA). M_w was calculated using the following equation;

$$K^*c/R_\theta = 1/[M_wP(\theta)] - 2A_2c \quad (1)$$

where R_θ is the excess intensity of scattered light at angle θ; c is the sample concentration; M_w is the *weight*-average molecular weight; A_2 is a second viral coefficient; K* is an optical parameter equal to $4\pi^2n_o^2 (dn/dc)^2 / (\lambda_o^4N_A)$ where n_o is the solvent refractive index and dn/dc is the refractive index increment; N_A is Avogadro's number; λ_o is the wavelength of the scattered light in vacuum. Function $P(\theta)$ describes the angular dependence of scattered light. Expansion of $1/ P(\theta)$ to first order gives:

$$1/ P(\theta) = 1 + (16\pi^2/3\lambda^2)\langle r_g^2\rangle \sin^2(\theta/2) + f_4 \sin^4(\theta/2) +... \quad (2)$$

Curve fitting method in this study was based on a second order Berry method ($\sqrt{(K^*c/R_\theta)}$ vs. $\sin^2(\theta/2)$), and a second virial coefficient, A_2 was set at zero (Wen, Arakawa, & Philo, 1996; Yokoyama, Renner-Nantz, & Shoemaker, 1998). M_w and R_z (equal to r_g in equation (2)) values were calculated from intercept and slope of the equation (1) by extrapolating multiangle signals to zero angle, respectively. The dn/dc value of 0.146 mL/g was used in this calculation (Roger & Colonna, 1993; Fishman et al., 1996; Bello-Perez et al., 1998).

RESULTS AND DISCUSSION

Molecular weight distributions of amylopectins of normal and waxy maize starches, eluted by distilled-deionized water as the mobile phase and traced by an RI detector, are shown as examples in Figs. 1A and 1B, respectively. A good separation of amylopectin from amylose was achieved by using the HPSEC system

with the operating conditions reported in this study, which enabled us to conduct accurate measurements of amylopectin molecular weights (Fig. 1A). *Weight*-average molecular weights (M_w) of amylopectins of different starches, calculated using a second order Berry method, are shown in Table 1. Molecular weights and gyration radii of amylopectins obtained in this study were larger than those reported in other studies (Aberle et al., 1994; Fishman et al., 1996). Differences between these results and others can be attributed to the methods of preparing starch dispersions. Millard, Dinzis, Willett, & Klavons, (1997) reported that methods used to prepare starch dispersion significantly affected molecular weight determination. Using a mild condition, such as direct dispersion in 90% DMSO, they reported M_w of waxy maize starch to be 7.5×10^8 g/mol, which was in agreement with our result (8.3×10^8 g/mol). Millard, Wolf, Dinzis, & Willett (1999) dispersed starch in 90% DMSO and compared the M_w of waxy maize starch obtained from static light scattering with that obtained from analytical ultracentrifugation. In their study, the M_w calculated using the Svedberg equation (5.93×10^8 g/mol) was in good agreement with the light scattering result (5.60×10^8 g/mol). The same authors also reported R_z value (342 nm) for waxy maize starch, which was comparable to R_z (372 nm) calculated from our result.

Another important factor affecting molecular weight calculation is the data fitting method. Yokoyama et al. (1998) applied three different methods (Zimm, 1948; Debye, 1947; Berry, 1966) to analyze M_w and R_z. The authors found that the M_w calculated by the Zimm method was significantly larger than that calculated by

Berry and Debye methods. The Zimm method can yield unreasonable results (Aberle et al., 1994), but the Berry method is demonstrated to determine M_w of larger molecules with greater accuracy (Hanselmann et al., 1995). Thus, we chose the second order Berry method (Millard et al., 1997) for our study. The second order Berry method gave a good curve fitting with laser signals obtained at different angles as shown in amylopectin of sweet rice starch at the peak of the RI signal (Fig. 2).

Among the M_w of the amylopectins reported in Table 1, amylopectins of waxy starches consistently displayed larger M_w than did that of normal starch counterparts. Carbon flux at a form of ADP-Glc (adenosine-5'-diphosphate glucose) is partitioned between amylose and amylopectin in normal starch biosynthesis. Granule-bound starch synthase I (GBSSI) is primarily involved in amylose biosynthesis of starch (Smith, Denyer, & Martin, 1997; Buléon, Colonna, Planchot, & Ball, 1998). In waxy mutants, GBSSI is missing and no amylose is synthesized. It is plausible that ADP-Glc is exclusively incorporated into amylopectin molecules resulting in amylopectin molecules with larger M_w in waxy mutants. Carbon partitioning could also explain substantially smaller M_w of amylopectins of amylomaize V and VII than that of normal and *ae wx* maize. Amylomaize VII has been reported to have less amylopectin of large molecular weight than does normal maize amylopectin revealed by HPSEC (Takeda, Takeda, & Hizukuri, 1993). It can also be postulated that space limitation in the normal starch granule because of the

presence of ca. 25% by mass of amylose molecules results in smaller M_w of normal amylopectin.

The M_w of amylopectins isolated from A-type starches varied to a larger range ($0.7 - 56.8 \times 10^8$) than did that isolated from B-type starches ($1.7 - 3.4 \times 10^8$). Many amylopectins of A-type starches had larger M_w than did those of B-type starches. Amylopectins of B-type starches also had larger R_z than did that of A-type starches having comparable molecular weights (Fig. 3). Because R_z is related to the volume occupied by the molecule in a solution (Millard et al., 1997), the branch chain-length and branching pattern of the amylopectin molecule are expected to affect the R_z of amylopectin in the solution. Glycogen, being highly branched and compact molecules, displayed a substantially larger dispersed molecular density ($\rho = M_w/R_z^3$) than did amylopectin (Table 1).

When the Log R_z was plotted against Log M_w of amylopectin (Fig. 3), the plot of A-type starches showed a linear relationship with a correlation coefficient of $r = 0.98$ ($P<0.05$). The strong linear relationship between Log R_z and Log M_w was likely due to similar branching structures of amylopectins among A-type starches. Most amylopectins of waxy starches displayed larger dispersed molecular densities than that of normal starch counterparts (Table 1). The larger dispersed molecular density of waxy amylopectin could be attributed to more branch-chains carried by waxy amylopectin, which was reflected by larger M_w. In addition, extra long chains (ELC) present only in normal amylopectin (Takeda & Hizukuri, 1987; Takeda, Shitaozono, & Hizukuri, 1988; Yoo & Jane, submitted, in Chapter 1), synthesized by a

progressive reaction of GBSSI, were likely to decrease the dispersed molecular density of normal amylopectin. The ELCs that have structures resembling amylose and carry few branches (Yoo & Jane, submitted, in Chapter 1) could also contribute to the smaller M_w of normal amylopectin. Two A-type amylopectins that displayed smaller dispersed molecular densities were Chinese taro and tapioca; both are root starches.

Molecular densities of dispersed B-type amylopectins were all smaller than that of the A-type amylopectins except *ae wx* amylopectin (Fig. 3 and Table 1). Amylopectins of B-type starches comprise longer branch-chains and larger proportions of long B chains than does that of the A-type starches (Takeda et al., 1993; Hizukuri, 1996; Jane et al., 1999). Hizukuri (1985) reported a ratio of short chains to long chains of normal maize amylopectin to be ten, which was at least three times larger than the ratio of 3 for amylomaize amylopectin. Differences in branch-structures between the A-type and the B-type starches were proposed by Jane, Wong, & McPherson (1997). They suggest that branch linkages of the A-type starch are scattered, and substantial branch-linkages are located within the crystalline region, whereas most branch linkages in the B-type starch are in the amorphous region.

The differences in the molecular weight and gyration radius of the A- and B-type amylopectin can be attributed to the molecular structures of the respective molecules. Structural models constructed using parameters of repeating distance of 9.0 nm (Jenkins, Cameron, & Donald, 1993; Jenkins & Donald, 1995), A:B chain

ratio of 1.2:1 (Yun & Matheson, 1993), and the chain-length distribution and molar chain ratio (Jane et al., 1999) for both normal maize (A-type) and amylomaize VII (B-type) amylopectins are shown in Fig. 4. Amylomaize VII has much longer exterior chains (Cheetham & Tao, 1997; Takeda et al. 1993) and larger long-chain/short-chain ratios (Hizukuri, 1985; Jane et al, 1999) than does normal maize.

It was difficult to calculate M_w and R_z of amylose by using the same on-line HPSEC-MALLS-RI system. With the sample concentration of 0.4 mg/mL used in this study and amylose molecules being highly polydisperse, we could not obtain sufficient laser-light scattering signals to calculate molecular weight distributions of amylose of most starch samples. We could, however, determine M_w and R_z of amylose of amylomaize VII that contained about 70% of apparent amylose. The *weight*-average molecular weight (M_w) and *z*-average radius of gyration (R_z) of amylose of amylomaize VII were 2.8×10^5 and 43 nm, respectively, which was similar to the M_w (1.8×10^5) of amylose calculated by Fishman et al. (1996) using HPSEC-viscometry (Fishman & Hoagland, 1994). Suortti, Gorenstein, & Roger (1998) reported the M_w of amylose to be $4.0\text{-}5.0 \times 10^5$ using laser-light scattering technique. The molecular weight distribution of amylomaize VII is shown in Fig. 5. The *weight*-average molecular weight of amylose was calculated by using first order Zimm method with the detectors 7-15. Amyloses of amylomaize V and VII have been reported to have the smallest molecular weights among all starches studied (Jane & Chen, 1992; Hizukuri, 1996).

CONCLUSION

Molecular weights (M_w) of amylopectins varied from 7.0×10^7 to 5.7×10^9, depending on the botanical source. Amylopectins of waxy starches had larger molecular weights, and most had larger dispersed molecular densities than did those of normal amylopectin counterparts. The M_w of A-type amylopectins varied to a larger range (7.0×10^7 to 5.7×10^9) than did that of B-type amylopectins ($1.7 - 3.4 \times 10^8$). At the same molecular weight, amylopectins of B-type starches had larger R_z than did those of A-type starches. Different branch structures of amylopectins of A- and B-type starches resulted in different dispersed molecular densities in dilute solutions. The M_w and R_z of amylose from amylomaize VII were 2.8×10^5 and 43 nm, respectively.

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Table 1

Amylopectin Molecular Weights and Gyration Radii of Selected Starches[a]

	M_w ($\times 10^8$)[b]	R_z (nm)[c]	ρ (g/mol/nm^3)[d]
A-type starches			
normal maize	4.9 (0.8)[e]	312 (23)	16.1
waxy maize	8.3 (0.2)	372 (11)	16.1
du wx maize	4.9 (0.5)	312 (13)	16.1
normal rice	26.8 (2.9)	581 (41)	13.7
waxy rice	56.8 (9.3)	782 (36)	11.9
sweet rice	13.9 (1.0)	486 (5)	12.1
normal wheat	3.1 (0.3)	302 (3)	11.3
waxy wheat	5.2 (0.4)	328 (6)	14.7
barley	1.3 (0.1)	201 (8)	16.0
waxy barley	6.8 (0.1)	341 (3)	17.1
cattail millet	2.7 (0.2)	278 (6)	12.6
mung bean	3.8 (0.2)	312 (3)	12.5
chinese taro	12.6 (3.6)	560 (15)	7.2
tapioca	0.7 (0.1)	191 (25)	10.0
B-type starches			
ae wx maize	3.2 (0.2)	306 (8)	11.2
amylomaize V	2.4 (0.0)	357 (24)	5.3
amylomaize VII	1.7 (0.0)	389 (57)	2.9
potato	1.7 (0.2)	356 (36)	3.8
waxy potato	2.0 (0.2)	344 (37)	4.9
green leaf canna	3.4 (2.2)	436 (85)	4.1
C-type starches			
lotus root	1.5 (0.4)	280 (57)	6.8
water chestnut	7.1 (1.5)	230 (25)	58.4
green banana	1.9 (0.8)	286 (29)	8.1
Glycogen			
cyanobacterial glycogen[f]	0.2 (0.0)	55 (4)	99.2

[a] Data were averages of at least two injections.
[b] *weight*-average molecular weight.
[c] *z*-average radius of gyration.
[d] Density (ρ) = M_w/R_z^3
[e] Standard deviation.
[f] Glycogen was isolated from *Synechocystis* sp. PCC6803 in our laboratory.

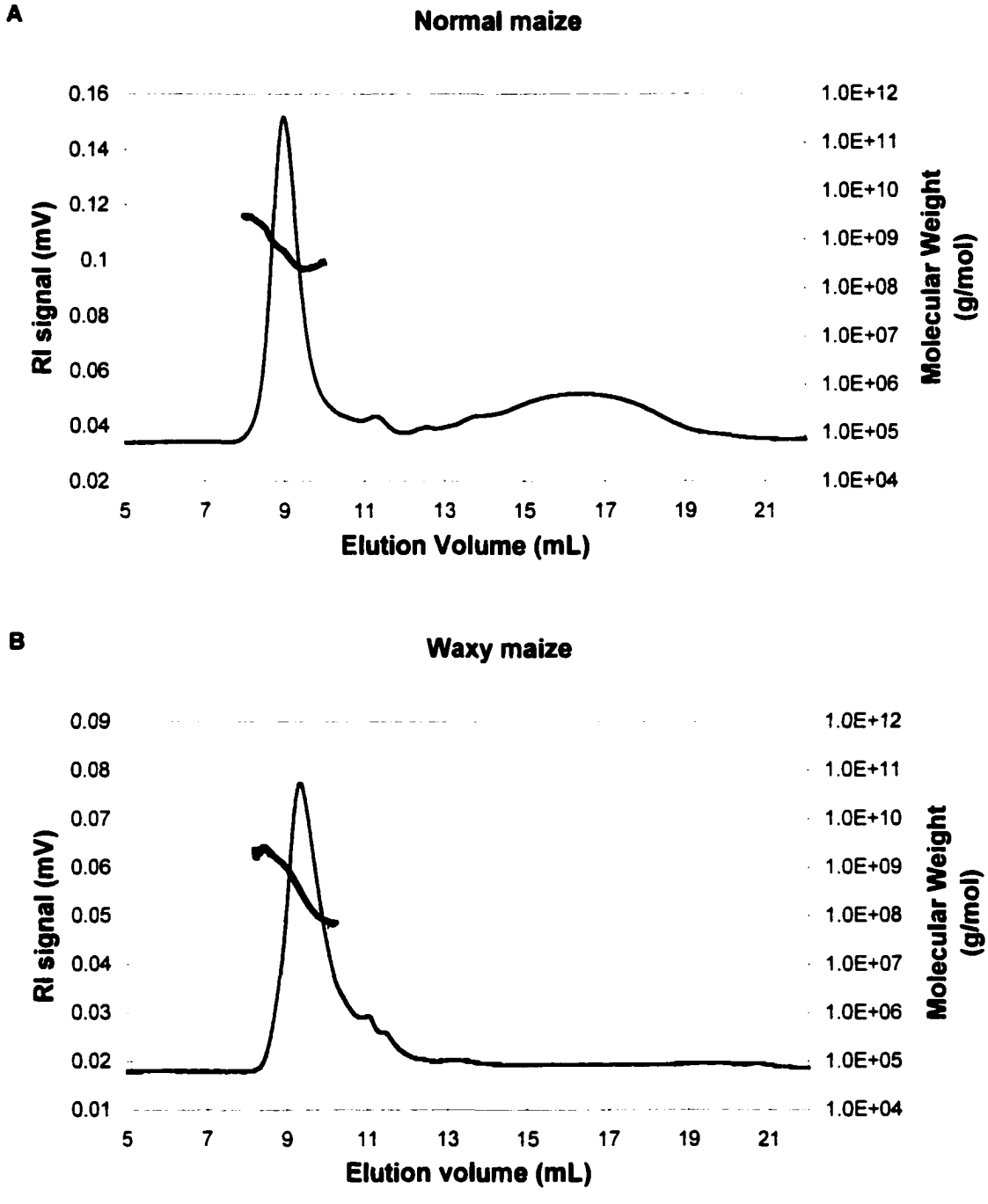

Fig. 1. Amylopectin molecular weight distributions (—) of maize (A) and waxy maize (B) determined by HPSEC-MALLS-RI system. The RI signal profile (—) is shown throughout the elution volume.

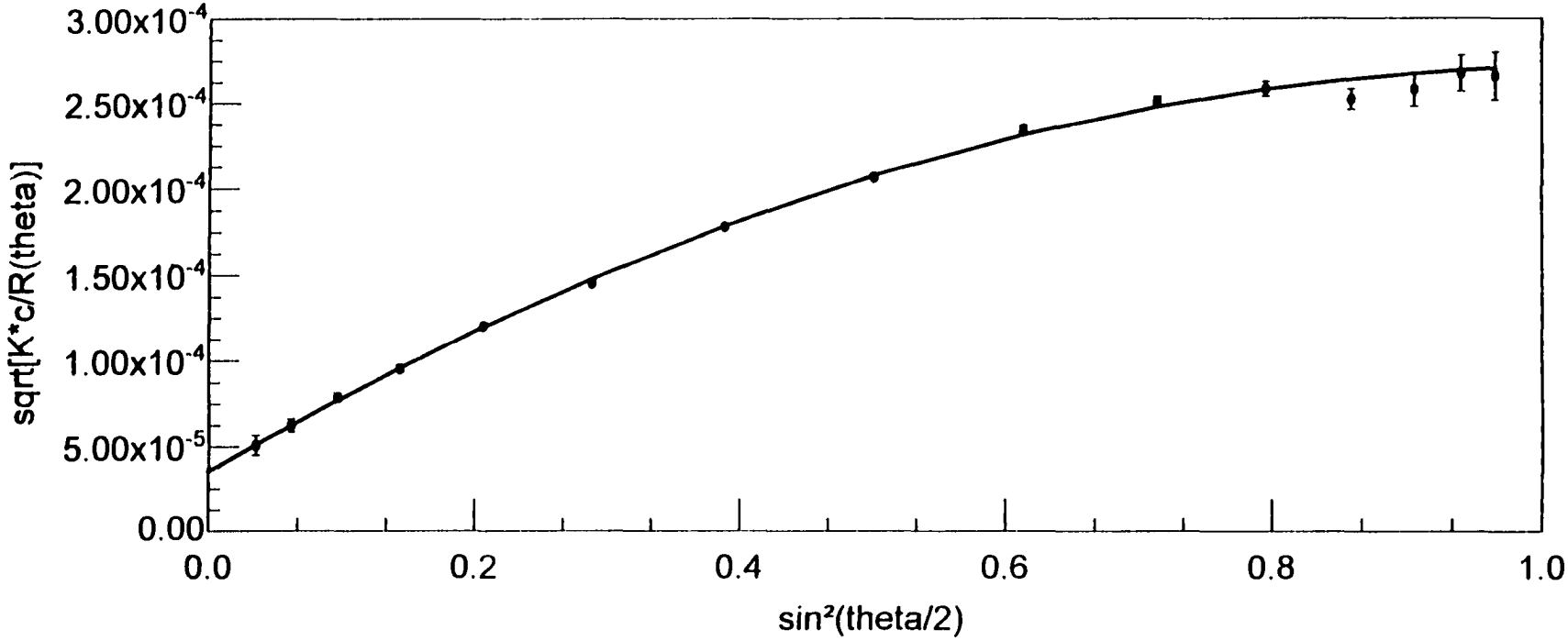

Fig. 2. Light scattering data plot ($\sqrt{(K^*c/R_0)}$ vs. $\sin^2(\theta/2)$) of sweet rice amylopectin at the peak concentration based on the second order Berry method. Three detectors at the lowest angle were not used for the curve fitting.

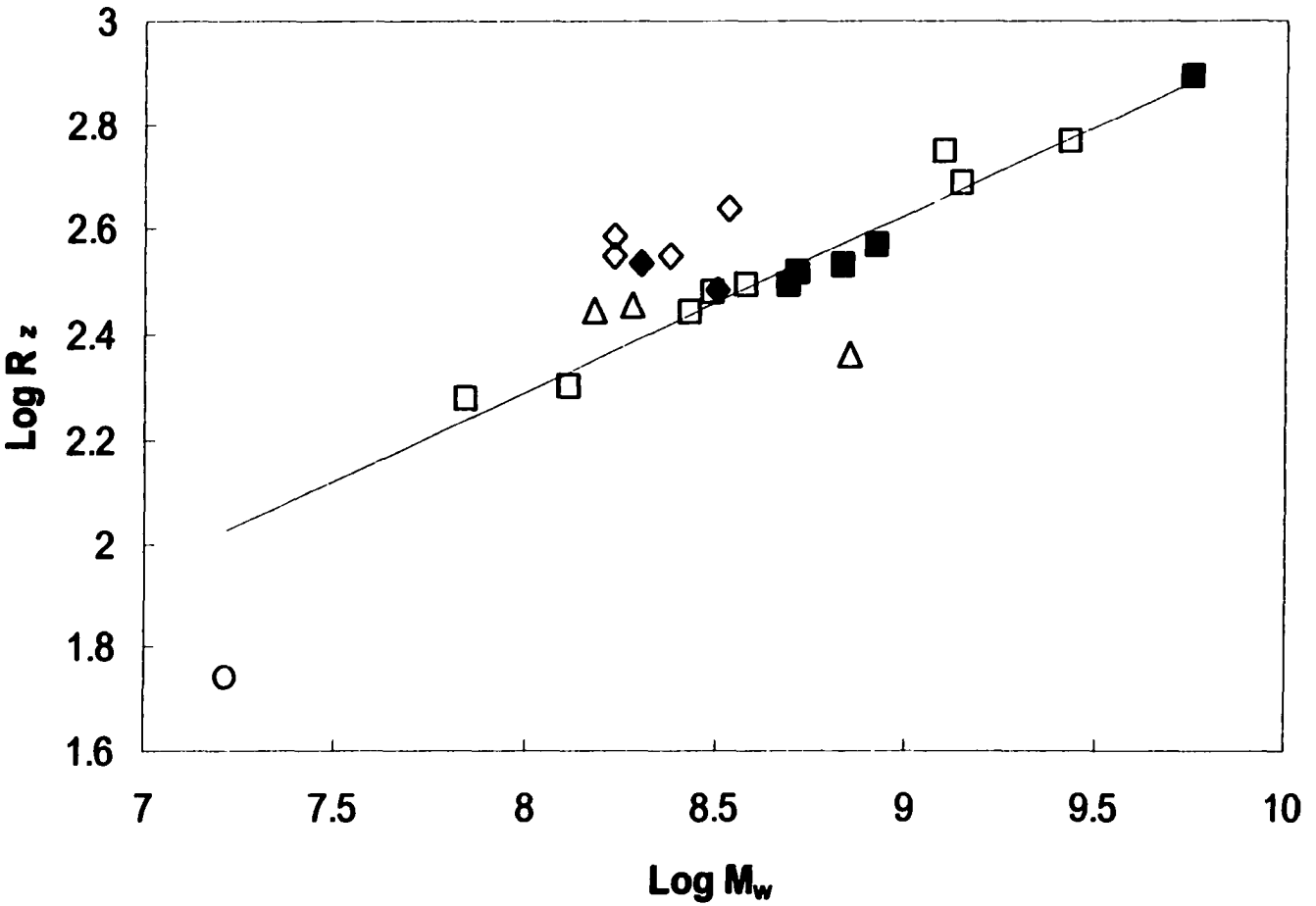

Fig. 3. Relationships of amylopectins between the *weight*-average molecular weight (M_w) and *z*-average radius of gyration (R_z). Data are plotted on Log-Log scale: A-type (□); B-type (◇); C-type (△); waxy A-type (■); waxy B-type (◆) amylopectins; glycogen (○). The linear regression line on the graph comprises data of A-type amylopectin.

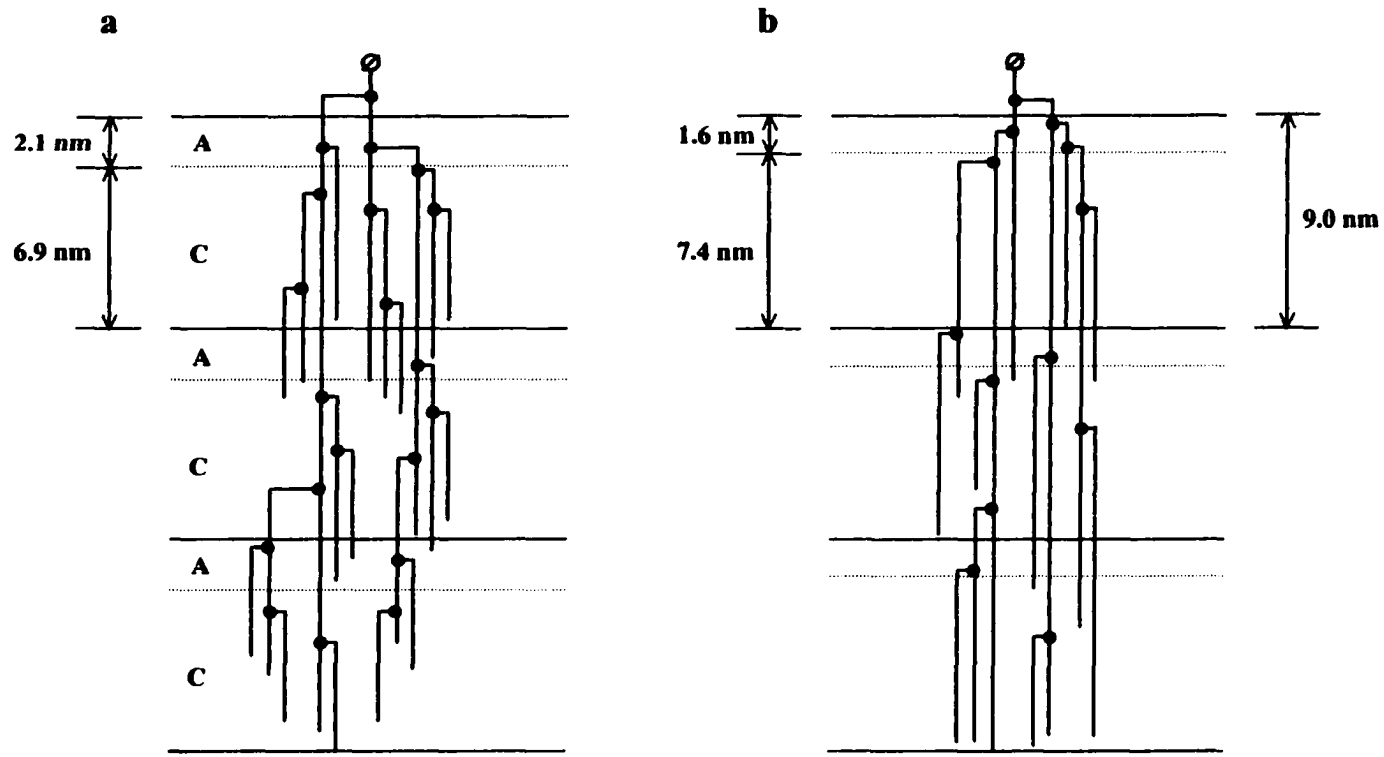

Fig. 4. Structure models of amylopectins of a, normal maize (A-type) and b, amylomaize VII (B-type) starches. **A** and **C** stand for the amorphous and crystalline regions, respectively. A repeating distance of 9.0 nm for the cluster (Jenkins et al., 1993; Jenkins & Donald, 1995) and A:B chain ratio of 1.2:1 (Yun & Matheson, 1993) for both starches are used for the models. Average branch chain- lengths of normal maize and amylomaize VII amylopectins are 24 and 31, respectively (Jane et al., 1999).

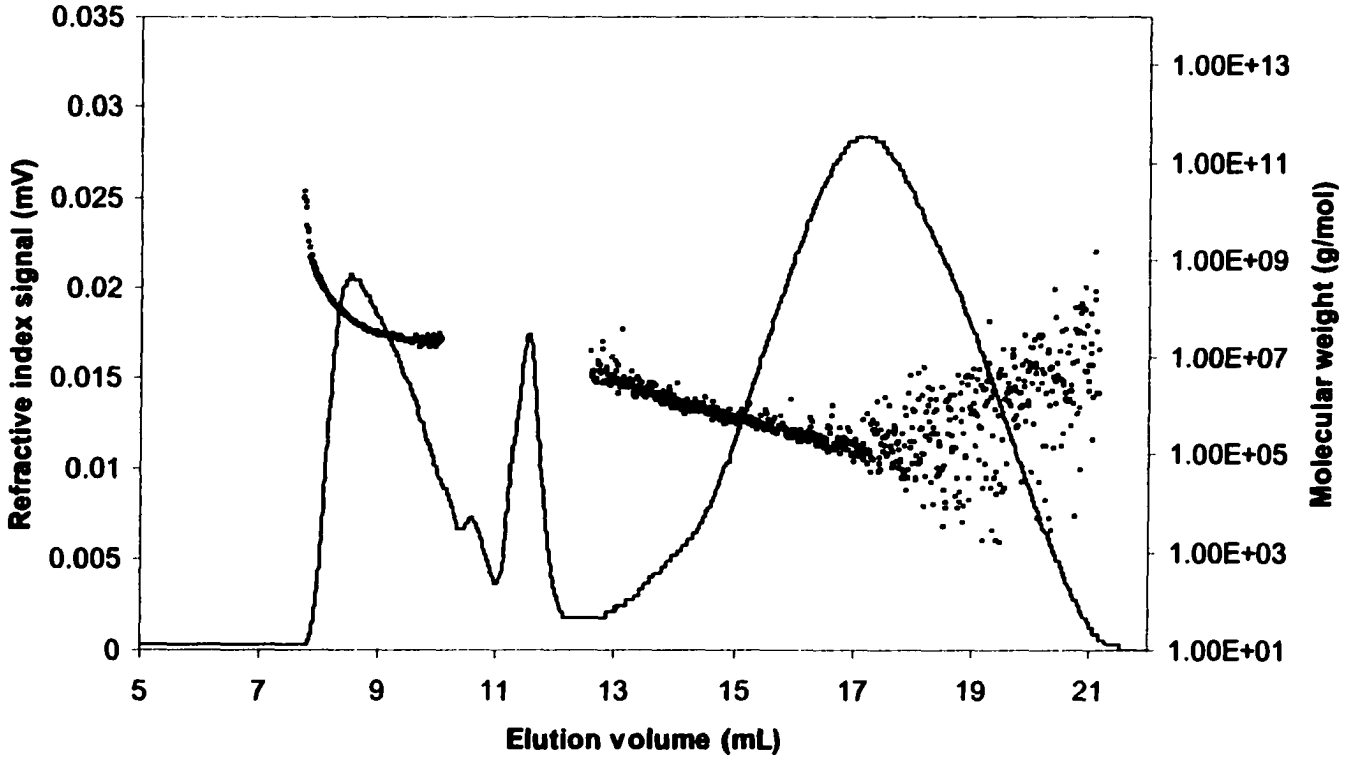

Fig. 5. Molecular weight distribution (▪) of amylomaize VII starch determined by an HPSEC-MALLS-RI system. The RI signal profile (—) is shown throughout the elution volume.

CHAPTER 3. INSERTIONAL MUTAGENESIS OF GLYCOGEN SYNTHASE GENES IN CYANOBACTERIUM *Synechocystis* sp. PCC6803[a]

A paper to be submitted to Plant Physiology

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ABSTRACT

As in animals, fungi and other bacteria, the primary carbohydrate storage form in cyanobacteria is glycogen. Many cyanobacterial genera produce substantial amounts of glycogen under nutrient-limiting conditions. Analysis of the genomic DNA sequence of *Synechocystis* sp. PCC6803 predicts this strain encodes two isoforms of glycogen synthase (GSI and II) and one glycogen branching enzyme. To examine the functions of the putative GS genes, each gene (*sll*1393 or *sll*0945) was disrupted by double cross-over homologous recombination. Zymogram analysis of the two GS disruption mutants and a double mutant allowed the identification of a protein band corresponding to each GS isoform. Results showed that two GS isoforms are present in *Synechocystis* sp. PCC6803, and both are involved in glycogen biosynthesis. Total GS activities in the mutant strains were not affected and compensated by the remaining isoform. Analysis of the branch-structure of

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Abbreviations: GS, glycogen synthase; BE, branching enzyme; HPAEC, high-performance anion-exchange chromatography; DP, degree of polymerization; *sll*1393, Genbank™ accession no. D90899; *sll*0945, Genbank™ accession no. D90915.

glycogen by high-performance anion-exchange chromatography revealed that the *sll*1393$^-$ mutant (GSI$^-$) produced glycogen containing more intermediate-length chains (DP8-18) at the expense of shorter and longer chains compared with the wild-type strain. The *sll*0945$^-$ mutant (GSII$^-$) produced glycogen similar to the wild-type, with only a slightly higher proportion of short chains (DP4-11). Results indicate that the GS isoforms in *Synechocystis* sp. PCC6803 have different reaction specificities in the biosynthesis of glycogen structure.

INTRODUCTION

Glycogen is the major carbon storage form in many prokaryotes. Biosynthesis of bacterial glycogen requires three enzymes, ADP-glucose pyrophosphorylase (ADPG-PP, EC 2.7.7.27), glycogen synthase (GS, EC 2.4.1.21), and branching enzyme (BE, EC 2.4.1.18). Many bacterial genes encoding glycogen synthase have been cloned, including those of *Eschericia coli* (Kumar et al., 1986), *Salmonella typhimurium* (Leung and Preiss, 1987), *Agrobacterium tumefaciens* (Uttaro and Ugalde, 1994), and *Bacillus subtilis* (Kiel et al., 1994). The structure of GS genes and the biochemical properties of many GS proteins have been extensively studied, but little information is available on GS of cyanobacteria and the structure of bacterial glycogen in general.

The biosynthetic pathway of glycogen in bacteria is very similar to that of starch biosynthesis in plants, but different from that in non-photosynthetic eukaryotes, which use UDP-Glc as a major substrate for glycogen biosynthesis (Manners, 1991). There is a prominent difference between bacterial and plant

systems regarding whether isoforms of glycogen/starch-synthesizing enzymes are present. Isoforms of GS/starch synthase (SS) and BE have not been reported in bacteria, whereas multiple isoforms of SS/GS and BE have been identified in plants (Kossmann and Lloyd, 2000). The degree of branching on glycogen (10%) is about two times higher than that of amylopectin (5%). Another major difference is that amylopectin is found as a semi-crystalline granule and glycogen is amorphous. It is widely accepted that different specificities of BE and SS isoforms play important roles in determining starch structure (Preiss, 1991; Guan et al., 1995). Transposon-tagging and antisense down-regulation of specific genes involved in starch biosynthesis have helped to elucidate different functions for starch synthesizing enzymes (Gao et al., 1998; Edwards et al., 1999a), but the specific function and metabolic regulation of each isoform in starch biosynthesis is not fully understood. One of the major difficulties in revealing the starch biosynthetic mechanism by mutation or down-regulation of specific enzyme isoforms is the compensating effect of the remaining isoforms and/or the pleiotropic effect of the mutations on other starch biosynthetic enzymes in plants (Boyer and Preiss, 1981; Craig et al., 1998). Disruption of one SS isoform in potato has been shown not to affect the overall starch yield (Edwards et al., 1995).

Cyanobacteria, previously known as blue-green algae, are photosynthetic prokaryotes. They contain chlorophyll a, the same photosynthetic pigment that plants use. From an evolutionary point of view, they represent a link between bacteria and green plants, because cyanobacteria are proposed to represent the endosymbiotic progenitors of plastids in plants (McFadden, 2001). Cyanobacteria

have highly organized internal thylakoid membranes similar to those in plant chloroplasts. As in plants, cyanobacteria and other bacteria also use ADP-Glc as a precursor for the biosynthesis of glycogen (Greenburg and Preiss, 1964; Shen and Preiss, 1964). Therefore, the process of storing carbohydrates through CO_2 fixation in cyanobacteria may bear a closer resemblance to that in plants than do other bacteria or non-photosynthetic eukaryotes. A cyanobacterial system may therefore be ideal for examining the unique functionality of each plant SS isoform. On the basis of DNA sequence comparisons with other organisms, the cyanobacterium *Synechocystis* sp. PCC6803 appears to have two GS isoforms (Kaneko et al., 1996). The enzyme functions of individual GS isoforms have not been studied in this strain. Thus, it is worthwhile investigating whether the two putative GS genes encode functional GS proteins, and, if so, whether the two isoforms play different roles in glycogen biosynthesis.

Our goal is to establish a model system for starch/glycogen biosynthesis in cyanobacteria and to use the model to study the role of individual enzymes involved in starch/glycogen biosynthesis. We intend to modify the structure of native glycogen by replacing endogenous bacterial genes with plant genes and to investigate the development of starch granules by reconstituting a plant starch synthesis system in a cyanobacterium. As an initial step, we demonstrate the existence of two endogenous GS isoforms and characterize their functions in the process of glycogen biosynthesis in *Synechocystis* sp. PCC6803.

RESULTS

Mutagenesis of Putative Glycogen Synthase Genes *sll*1393 and *sll*0945 in *Synechocystis* PCC6803

To determine whether both *sll*1393 and *sll*0945 encoded functional GS proteins, we inactivated the genes by double cross-over homologous recombination. Two different mutants, M1 and M2, were generated by natural transformation of wild-type *Synechocystis* sp. PCC6803 with plasmids pSHK1393 and pSHK0945, respectively, both conferring kanamycin resistance (Fig. 1). The double mutant M12 was generated by a sequential transformation using plasmids pSHK0945 and pSHS1393, conferring kanamycin and spectinomycin resistance, respectively.

Since *Synechocystis* sp. PCC6803 contains multiple genome copies, complete segregation is necessary prior to functional analysis (Ermakova-Gerdes and Vermass, 1999). PCR analysis demonstrated complete segregation of the inserted Km^R or Sp^R genes and complete elimination of wild-type genomic copies of the targeted sequences. Structures of *sll*1393 and *sll*0945 are shown in Fig. 2A and 2B, respectively. As shown in Fig. 2C, a size difference of PCR products between WT and M1 was detected by ~0.8 kb. Insertion of the Km^R gene into *sll*1393 led to an increase in amplified DNA size as expected from the deleted size (0.4 kb) of the *sll*1393 gene. There was no detectable PCR product for M1 corresponding to the size of intact gene. From PCR analysis of the M2 strain, size difference (1.1 kb) in DNA bands between WT and M2 could be explained, considering the sizes of the inserted Km^R gene (1.3 kb) and the replaced *sll*0945 gene (0.2 kb). Insertion of the Sp^R gene in M12 caused a 1.5-kb increase in the PCR-amplified product size

relative to WT. Southern hybridization analysis, using the Km^R gene as a probe, revealed that the sizes of mutagenized DNA corresponded exactly to those predicted taking into account the size of the inserted Km^R gene from both M1 and M2 (Fig. 2D). The results showed that all mutant strains were completely segregated and free of wild-type copies of the targeted genes. Growth of the mutants was examined to determine whether the mutations have any gross effects on cell physiology.

Growth rates of M1, M2, and M12 were not significantly affected during exponential phase, but their final cell densities appeared to be slightly depressed (Fig. 3). Defective structural genes encoding glycogen biosynthetic enzymes also had no effect on bacterial (Steiner and Preiss, 1977) or yeast growth (Farkas et al., 1991).

Zymogram Analysis Confirmed Two Detectable Protein Bands with GS Activity

Two different protein bands with GS activity were identified using a native gel zymogram analysis. A strong purple-colored major band (GSI) stained with iodine was detected in WT and was the only detectable band in M2. A minor band (GSII) also was identified in WT and was the only detectable band in M1 (Fig. 4). Along with the lack of any detectable iodine-stained minor bands in the double mutant M12, these results indicate there are at least two distinct GS proteins in *Synechocystis* sp. PCC6803 and that the *sll*1393 and *sll*0945 genes encode GSI and GSII, respectively. The GSI band consistently stained darker than GSII, suggesting either that the apparent activity of GSI was higher than that of GSII or

that the pattern of glycogen chain-elongation by each isoform differed, leading to different iodine-staining characteristics (Buléon et al., 1997). For example, the darker iodine-stained band of GSI would be consistent with synthesis of longer chains by GSI. The elimination of GSI by mutation consistently appeared to result in decreased GSII activity, as judged by the GSII band intensity (Fig. 4). The apparent lack of any GS activity in the double mutant (M12) provides direct evidence that there is no detectable third enzyme synthesizing glycogen under our assay conditions.

Total GS Activities in the Mutant Strains Were not Affected and Were Compensated by the Remaining Isoform

To examine the total GS activity in mutant and wild-type strains, we determined GS activity using the rate of [^{14}C]glucose incorporation from ADP[U-^{14}C]glucose onto rabbit-liver glycogen. The incorporation rate was fairly linear for all strains over the initial 40 min of the assay (Fig. 5). The rates of [^{14}C]glucose incorporation for WT, M1, M2, and M12 were 0.080, 0.082, 0.10, and 0.018 μmol glucose min^{-1} mg^{-1} protein, respectively (Table II). Thus the disruption of either GS isoform did not significantly affect the total GS activity, suggesting a compensating increase in the activity of the remaining isoform in each case. Either GS activity might be regulated to control the level of glycogen production in the cells, or excess GS activity might be constitutively present during glycogen biosynthesis. Even though one of the isoforms was not expressed properly in each *Synechocystis* mutant strain, the expression level of the remaining GS apparently could be up-

regulated. The equivalent or even slightly higher GS activity in M2 relative to that of WT could explain why the GSI band on zymograms consistently showed more intense color for M2 than for WT. Surprisingly, the double mutant also showed around 23% of WT GS activity, even though no stainable activity was detected in zymograms of M12. Further study is required to understand the source of this apparent GS activity in M12.

Branch-Structure of Glycogen from M1 Was Significantly Different from M2 and WT

The amounts of glycogen produced by mutants and WT were determined to further quantify the GS activity of the corresponding isoforms. Glycogen yields of WT, M1, and M2 were 25.3, 23.2, and 22.5 mg g^{-1} wet cell mass, respectively (Table III), demonstrating that the quantities of glycogen accumulated in both mutants were not substantially reduced. This result is consistent with the lack of a decrease in the total GS activity in the individual mutants (Table II).

To identify any unique effects of each GS isoform on glycogen structure, glycogen produced by the mutants was isolated and analyzed. The branch chain-length distribution of glycogen from M1, M2, and WT was obtained using HPAEC (Fig. 6A). Regardless of strain types, a unimodal distribution of branch chain-length was displayed from isolated glycogen, whereas a bimodal distribution is observed for amylopectin from plant starch (Hizukuri, 1986; Jane et al., 1999). The chain-length distribution of M1-glycogen was quite different from WT-glycogen (Fig. 6B). M1-glycogen had fewer short chains (DP4-DP7) and long chains (>DP19), but had more

intermediate chains (DP8-DP18) than did WT. Peak chain-lengths of WT, M1, and M2 were at DP6, 8, and 6, respectively. The chain-length distribution of both M1 and M2 indicated a rather short average chain-length compared with WT (Table III), but, unlike for M1, the proportion of each chain in M2 was similar to WT (Fig. 6B), with only a slightly higher proportion of short chains (DP4-11) than WT. Thus the mutation in *sll*1393 had a more prominent impact on branch chain-length distribution of glycogen than that in *sll*0945. These two putative GS genes encode active GS proteins, and each GS isoform alone seemed to synthesize glycogen with different structure.

The average chain-length of each sample was calculated based on the peak area on HPAEC chromatograms. The *number*-average DPs (DP_n) of the branch chain-lengths were in the range of 8.4-9.5, as shown in Table III, and were not significantly different among the strains. DP_n, however, did not reflect the chain-length distribution. Longer exterior chains present in the glycogen of WT and M2 relative to M1 were demonstrated by λ_{max} of the iodine-glycogen complex (Table III), which increases with increasing chain length. These results agreed with the chain-length distribution analyzed by HPAEC (Fig. 6) and a darker purple-color stain shown on the zymogram (Fig. 4).

The DP_n of cyanobacterial glycogen samples (~DP9) were similar to that of rabbit-liver glycogen (DP11), but substantially shorter than that of waxy maize starch (DP19). Fujimori et al. (1995) also reported similar results in their study using a reducing-end determination method. The average chain-lengths of cyanobacterial glycogen reported from *Lyngbya-Phormidium-Plectonema* (DP11), *Nostoc*

muscorum (DP13), and *Anacystis nidulans* (DP9) (Fujimori et al., 1995; Chao and Bowen, 1971; Weber and Wöber, 1975) were similar to the chain-lengths determined here for *Synechocystis* sp. PCC6803.

DISCUSSION

The biosynthetic pathways of branched glucans in bacteria and plants share similar enzyme systems, but the structures of their products are different. The plants produce starch in granular form, but bacteria produce water-soluble glycogen. The difference in physical shape between starch and glycogen can mainly be attributed to different chemical structures, such as differences in branch chain-length distribution and in branching pattern, which, in turn, depend on the regulation and specificity of the glycogen/starch biosynthetic enzymes.

Two GS isoforms in *Synechocystis* sp. PCC6803 were identified at the expressed protein-level, and their functions during glycogen biosynthesis were found to be different. No significant change in total GS activity was observed in either M1 or M2, which was consistent with the mutant and wild-type strains accumulating comparable amounts of glycogen. It is not possible to compare the unique functions of each GS isoform in this cyanobacterium with most other bacteria, because other bacteria contain only one GS. Isoforms of GS and soluble starch synthase (SSS) were reported from a couple of unicellular eukaryotes, *Saccharomyces cerevisiae* (Farkas et al., 1991) and *Chlamydomonas reinhardtii* (Fontaine et al., 1993), respectively. The functions of each isoform of SSS also have been studied in plants

(Edwards et al., 1999a; Wang et al., 1993; Abel et al., 1996) with clear evidence that they play distinct roles in amylopectin synthesis.

The zymogram results (Fig. 4) showed the color intensity of the iodine-stained band of GSI was greater than that of GSII in WT. This staining pattern was similar to that in each mutant, i.e., GSI in M2 displayed a darker band than did GSII in M1. GS activity assays, however, demonstrated that M1 had a similar level of GS activity, contributed by GSII, as did M2, contributed by GSI (Fig. 5). These results suggested the apparently different intensity of iodine-staining did not reflect a quantitative activity difference between the isoforms, but rather a difference in the structure of the glycogen-primed product by each isoform.

Elimination of GSI in M1significantly altered the distribution of branch chain-lengths in the glycogen formed, whereas the pattern of branch chain-length distribution in M2 glycogen was not dramatically affected by the loss of GSII. The relatively minor change in overall branch-chain length distribution in the absence of GSII suggests that GSI played a predominant role in determining the final structure of glycogen in wild-type *Synechocystis* sp. PCC6803. M1 with the absence of GSI displayed the unique function of GSII, which is responsible for synthesizing excessive branch-chains of intermediate size (DP8-18). GSI apparently is involved in producing very short (DP≤6) and longer chains (DP≥20) compared with GSII, as can be seen from the comparison plot of the glycogen chain-length distribution (Fig. 6A and B). The preferential biosynthesis of longer chains (DP≥20) in M2 ($GSII^-$) than in M1 (GSI^-) confirmed that GSI is capable of extending the glycogen primer, possibly in a more progressive way to produce longer chains that developed darker

purple color bands with iodine as shown in the zymogram (Fig. 4). GSI may preferentially extend a chain by adding further glucose units to the same chain, whereas GSII may add single glucose units distributively to many chains (Fig. 7). Different manners of elongation have also been discussed for GBSSI and SSII from potato (Edwards et al., 1999b).

Disruption of the gene encoding a main starch synthase (SSII) in *Chlamydomonas reinhardtii* altered amylopectin structure and showed a significant decrease in the proportion of chains with DP8-50 and a slight increase in short chains (DP2-7) of amylopectin (Fontaine et al., 1993). In potato tubers, a substantial reduction of SSIII activity did not considerably change the overall branch-chain distribution (Abel et al., 1996), even though this isoform accounts for 80% of all soluble SS activity (Marshsall et al., 1996). A reduction of a minor SS, SSII, corresponding to 10-15% of SS activity, had a more severe impact on amylopectin structure (Edwards et al., 1999a). The starch contents of these mutant lines, however, were unaffected. This could be explained by the fact that either the total SS activity is present in excess in potato tubers or the remaining isoforms are capable of compensating for the absence of the other isoform (Abel et al., 1996). The alteration of amylopectin structure by an SSII mutation and the compensating effect by other starch synthase isoforms also was observed in pea embryos (Craig et al., 1998). Mutations in the gene encoding pea SSII resulted in an enrichment of short branch-chains (DP7-10) at the expense of longer branches. Farkas et al. (1991), however, showed that total GS activity in a wild-type yeast strain was contributed by two isoforms, and that the total amount of glycogen produced in

individual GS-deficient mutants was proportional to the remaining GS activity. In cyanobacteria, the remaining GS activity in each mutant clearly compensated for the lack of the other isoform to produce similar amounts of glycogen.

MATERIALS AND METHODS

Materials

ADP[U-^{14}C]Glc was purchased from Amersham Pharmacia Biotech (Piscataway, NJ). Chemicals for BG-11 medium were purchased from Fisher Scientific (Pittsburgh, PA). Molecular biology reagents were purchased from Promega Biotech (Madison, WI), and other chemicals and antibiotics were from Sigma (St. Louis, MO).

Strains and Growth Conditions

Synechocystis sp. PCC6803 was grown at 25°C under continuous illumination with fluorescent lights (~40 μmoles photons m^{-2} s^{-1}) in a BG-11 medium (Rippka et al., 1979) supplemented with 5 mM glucose. For different mutants, media contained 50 μg mL^{-1} kanamycin and/or 20 μg mL^{-1} spectinomycin. For glycogen isolation, cells were grown in a BG-11/glucose medium for 7-8 days (A_{730} = 1.6-1.8) before being transferred to a nitrogen-limiting BG-11/glucose medium, and then grown for another 2-3 days. The nitrogen-limiting BG-11 medium contained 8.34 mg mL^{-1}, which was less than 10 % of the normal sodium nitrate concentration as the only nitrogen source. Cell pellets grown in normal BG-11 medium, harvested by centrifugation at 6,000 × *g* for 15 min, were washed with sterile deionized water prior

to transfer of cells to a nitrogen-limiting medium. Cultures were started with an initial cell density of 1.0×10^6 cells mL^{-1} for determination of the cell growth rate.

Genomic DNA Isolation

For isolation of genomic DNA from *Synechocystis* sp. PCC6803 cell cultures in early stationary phase, cell pellets collected by centrifugation of 5 ml samples were resuspended in 400 μL TE (50 mM Tris-HCl, pH 8.0, and 5 mM EDTA) buffer following general protocol reported by Ausubel et al. (1999). The same volume (400 μL) of glass beads (acid washed, baked) was added to the cell suspension, followed by the addition of SDS (10%, w/v, 8 μL), N-lauryl sarcosine (5%, w/v, 16 μL), and phenol saturated with TE (400 μL). The cell suspension was then vortexed five times, 15 s each.

Targeted Mutagenesis of *sll*1393 and *sll*0945 Genes

Mutants lacking putative GS genes, *sll*1393 or *sll*0945, were generated by transforming the wild-type strain of *Synechocystis* sp. PCC 6803 with pSHK1393 or pSHK0945 plasmid, respectively, in which *sll*1393 or *sll*0945 was replaced by a gene conferring kanamycin resistance. A brief scheme for constructing the knock-out plasmids, pSHK1393 or pSHK0945, is shown in Fig. 1. DNA sequences from both 5' and 3' regions of each targeted structural gene were used to amplify corresponding ~0.5 kb fragments. Primers were obtained from *sll*1393 and *sll*0945 genes to avoid possible mutagenesis in flanking regions during recombination events (Table I). Two sets of primers, prA1 (forward) /prA2 (reverse) and prA3/prA4

for *sll*1393 were used for PCR amplification. For *sll*0945, prB1 (forward) /prB2 (reverse) and prB3/prB4 were used. Primers were designed to contain convenient restriction sites at both ends of the amplified DNA fragments as shown in Table I. Endonuclease-restricted *Bam*HI-*Eco*RI and *Eco*RI-*Hind*III fragments from each gene were subcloned sequentially into pBluescript KS vector. A kanamycin resistance (Km^R) gene from pUC4K (Pharmacia) was inserted between subcloned DNA fragments using the *Eco*RI restriction site. Transformation was carried out by the procedure of Williams (1988). Generated mutants in which either *sll*1393 or *sll*0945 was disrupted were named M1 and M2, respectively. The knock-out plasmid pSHS1393 was constructed to target disruption of the *sll*1393 gene by ligating an *Eco*RI-fragment of the spectinomycin resistance (Sp^R) gene from pHP45Ω (Prentki and Krisch, 1984) into pSHK1393 instead of the Km^R gene. A double mutant (M12), in which both *sll*1393 and *sll*0945 genes were disrupted, was established by transforming a pSHK0945-transformed mutant (M2) with pSHS1393. After transformants resistant to kanamycin and/or spectinomycin were segregated by single colony selection for several rounds, genomic DNA was isolated. Insertion of the Km^R or Sp^R gene cassette into the targeted sequence was confirmed by PCR amplification analysis. For southern blot analysis, genomic DNA from wild-type, M1, and M2 strains was isolated, purified, digested with *Hind*III, and separated in 0.8% (w/v) agarose gels and blotted onto nitrocelluose membrane (Hybond-C, Amersham). The DNA probe (a 1282 bp *Eco*RI-fragment of Km^R gene) was labeled with [α-^{32}P]-dCTP. Southern hybridization was performed as described previously

(Sambrook et al., 1989). Growth of wild-type and mutant strains was monitored by measuring absorbance of cell cultures at 730 nm.

Zymogram Analysis for Detecting GS Activity

Cells were harvested during early stationary growth phase from *Synechocystis* sp. PCC6803 culture (100 mL), collected by centrifugation and resuspended in 1.5 mL of 10 mM Tris-HCl (pH 7.0) extraction buffer containing 1 mM ethylenediaminetetraacetic acid (EDTA), 1 mM dithiothreitol (DTT), 0.2 mM phenylmethyl sulfonylfluoride (PMSF), and 1 mM benzamidine. An equal volume of 100-150 μm glass beads was added, and cells were broken with a Mini-beadbeater (Biospec Products, Bartlesville, OK). The method used for zymogram analysis was modified from the procedure of Tyynelä and Schulman (1993). The cell-free extract, obtained as the supernatant of a 15 min, 10,000 × *g* centrifugation, was used for detecting activity of GS proteins on a native gel following electrophoresis. The cell-free extract (15-25 μl, containing 10 μg of total soluble protein) was mixed with the same volume of sample loading buffer that contains 50% (v/v) glycerol, 0.25% (w/v) bromophenol blue, and 5 mM DTT in 75 mM Tris-HCl buffer (pH6.8). Native gel electrophoresis was carried out at 70 V using 7.5% polyacrylamide gel. Electrophoresis finished within 2 h. The gel was removed, gently washed with distilled water, and soaked in 25 mL of 50 mM sodium citrate buffer solution (pH 7.0) containing 20 mg glycogen and 15 mg ADP-Glucose as substrates for *in vitro* synthesis of glucan. The reaction mixture was incubated at 25°C for 16 h, and then the gel was stained with iodine solution (I_2/KI = 0.01/0.1%, w/v) in 0.1 M sodium

acetate buffer (pH 5.0) to detect dark purple-colored protein bands corresponding to GS activity.

GS Activity Assay and Protein Determination

The assay for the quantitative determination of GS activity was modified from Thomas et al. (1968). A cell-free extract was prepared as described above. Enzyme activity was determined in 50 mM sodium citrate (pH 7.0) containing 10.0 mg glycogen mL^{-1} and 0.5 μmol ADP[U-^{14}C]Glc at 0.04 μCi/mol (3.2 GBq/mol) and 20 μL of the cell extract in a final volume of 220 μL. The reaction was initiated by adding cell-free extract at 25°C. Zero-time controls were prepared within 5 sec of the addition of cell extract to the assay mixture. An aliquot (30 μL) of sample was taken from the reaction mixture and spotted immediately on a square (1.5cm × 1.5cm) of Whatman No. 3 filter paper at each time interval. Each spotted filter paper was dropped into a beaker containing 50 mL of 75% ethanol (v/v) and was washed twice with an equal volume of 75% ethanol. After being air-dried, the ethanol-insoluble ^{14}C retained on the filter papers was determined by liquid scintillation spectroscopy (Model LS 6500, Beckman, Fullerton, CA). Protein concentrations were determined using a protein assay kit (Bio-Rad). Bovine serum albumin (BSA) was used as a standard.

Determination of Glycogen Yield and λ_{max} of Iodine-Glycogen Complex

Yields of glycogen were determined using an enzymatic assay and a phenol-sulfuric acid method. For the enzymatic assay, α-D-glucans were completely hydrolyzed with amyloglucosidase (Sigma) (5 units mg^{-1} total carbohydrate) at 55°C for 2 h, and the amount of released glucose was quantified using a glucose diagnosis kit (Sigma). Total carbohydrate content was estimated following the phenol-sulfuric acid method described previously (Dubois et al., 1956). A glycogen dispersion (0.5 mg mL^{-1}) was prepared in a solution containing 0.01% I_2 and 0.1% KI. Absorbance of complex was scanned from 700 to 400 nm to determine the wavelength of highest absorbance (λ_{max}).

Analysis of Glycogen Structure

To examine the branch structure of glycogen produced by *Synechocystis* sp. PCC6803, 10 mg of glycogen was dissolved in 5 mL of 10 mM acetate buffer (pH 3.5), and 5 μL (300 units) of isoamylase (EC 3.2.1.68) were added to hydrolyze all branch points. To prevent microbial growth during enzyme reaction, 10 μL of 10% (w/v) sodium azide solution was also added. The reaction mixture was incubated for 48 h in a shaking water bath at 40°C with 120 strokes/min. Upon completion of the enzyme reaction, the pH was adjusted to 6.0 and the solution heated for 15 min in a boiling water bath. The branch chain-length distribution of glycogen was determined by separation of the hydrolyzed branches using an HPAEC system (Dionex-300, Sunnyvale, CA) equipped with amyloglucosidase reactor and pulsed-amperometric

detector (PAD) (Wong and Jane, 1997). A PA-100 anion exchange analytical column (250 × 4 mm) was used for separating 25 μL samples of debranched glycogen. Eluent A and B consisted of 100 mM NaOH and 100 mM NaOH containing 300 mM $NaNO_3$, respectively. The gradient of eluent B at 0, 39, 50, 170, and 220 min was 1, 5, 8, 30, and 45%, respectively, with an operating flow rate at 0.5 mL min^{-1}.

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Table I. Oligonucleotide sequences used to construct pSHK1393 and pSHK0945 plasmids

Final constructs were used to replace endogenous *sll*1393 and *sll*0945 genes, respectively, by homologous recombination. The position of the oligonucleotides in the nucleotide sequences of the *sll*1393 and *sll*0945 structural DNA are indicated. The restriction sites introduced by PCR amplification are underlined.

	Oligonucleotides	Position	Site inserted
sll1393 (1476 bp)			
prA1	5'-GTCGAGCGGATCCTACCCATGT-3'	109- 130 (22 bp)	BamHI
prA2	5'-GATCGTAGCTGAATTCATAACTGTC-3'	559- 583 (25 bp)	EcoRI
prA3	5'-CCGCCACCGAATTCAATCTGAG-3'	1009-1031 (23 bp)	EcoRI
prA4	5'-TGTATTCGTAAGCTTCCACATATTG-3'	1441-1465 (25 bp)	HindIII
sll0945 (1434 bp)			
prB1	5'-TAAAGTTCTGGATCCGTTGGGC-3'	81- 102 (22 bp)	BamHI
prB2	5'-AATACCGACTGAATTCCCACTC-3'	651- 672 (22 bp)	EcoRI
prB3	5'-GTCCTACACCGAATTCCAGTTA-3'	894- 915 (22 bp)	EcoRI
prB4	5'-TTCCATGAAGCTTAATTCCTCCG-3'	1374-1396 (23 bp)	HindIII

Table II. Effects of insertional mutation of *sll*1393 and *sll*0945 genes on GS activity

Total GS activities were quantified by determining the incorporation rate of ^{14}C on rabbit-liver glycogen primers.

Type of strain	^{14}C incorporation rate	Relative activity
	μmol glucose min^{-1} mg^{-1} protein	% of wild type
WT	0.080 ± 0.001	100
M1	0.082 ± 0.014	103
M2	0.100 ± 0.001	125
M12	0.018 ± 0.006	23

Table III. *Effects of sll1393 and/or sll0945 deletion on glycogen accumulation and structure*

Average chain-length was calculated based on peak area of each chain on HPAEC chromatograms. Commercial rabbit-liver glycogen and waxy maize starch were used for the comparison.

Type of strain	Glycogen yield	Average chain-length (DP_n[a])	λmax
	mg glycogen g^{-1} wet cell mass		nm
Wild type	25.3 ± 1.1	9.6 ± 0.4	512
M1 (*sll1393*)	23.2 ± 1.8	8.7 ± 1.4	490
M2 (*sll0945*)	22.5 ± 0.0	8.4 ± 1.1	508
Rabbit-liver glycogen	N/A[b]	11.3 ± 0.0	494
Waxy maize starch	N/A	18.8 ± 0.0	576

[a] *number*-average degree of polymerization = Σ peak area / Σ (peak area / number of glucose of each chain).

[b] Not Applicable.

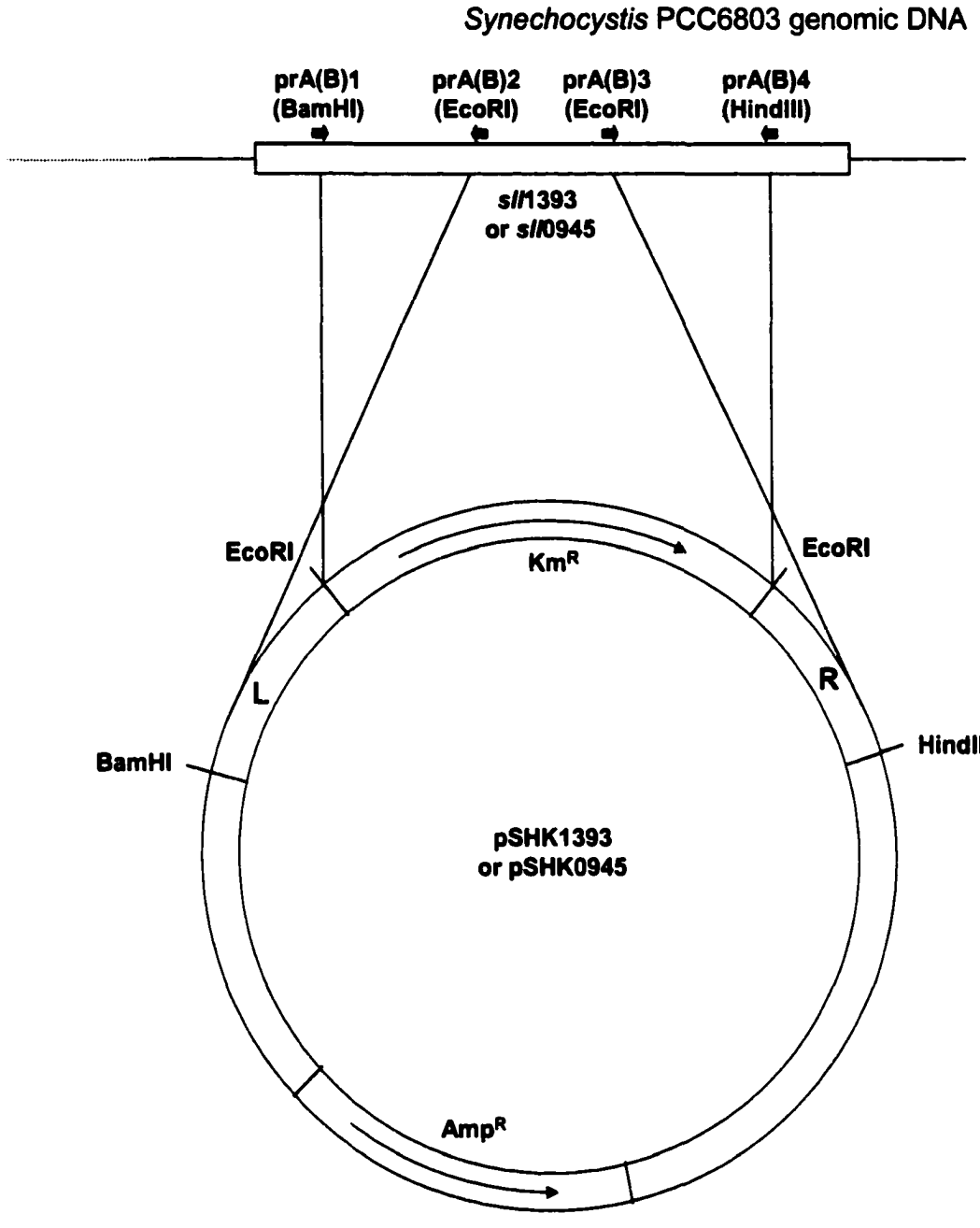

Fig. 1. Construction of vectors for insertional disruption of either the *sll*1393 or *sll*0945 gene. Two homologous DNA sequences were amplified using the primers, prA1/prA2 (for left fragment, L) and prA3/prA4 (for right fragment, R), to construct pSHK1393 by PCR. Introduced restriction sites of the primers are shown in Table I. The left homologous DNA fragment was restricted with *Bam*HI and *Eco*RI and the right fragment with *Eco*RI and *Hind*III. The restricted fragments and the Km^R gene were sequentially inserted into pBluescript KS. Using prB1/prB2 and prB3/prB4, two homologous DNA sequences (L and R) were amplified to construct pSHK0945. The pSHK0945 was constructed following the same procedure as for pSHK1393.

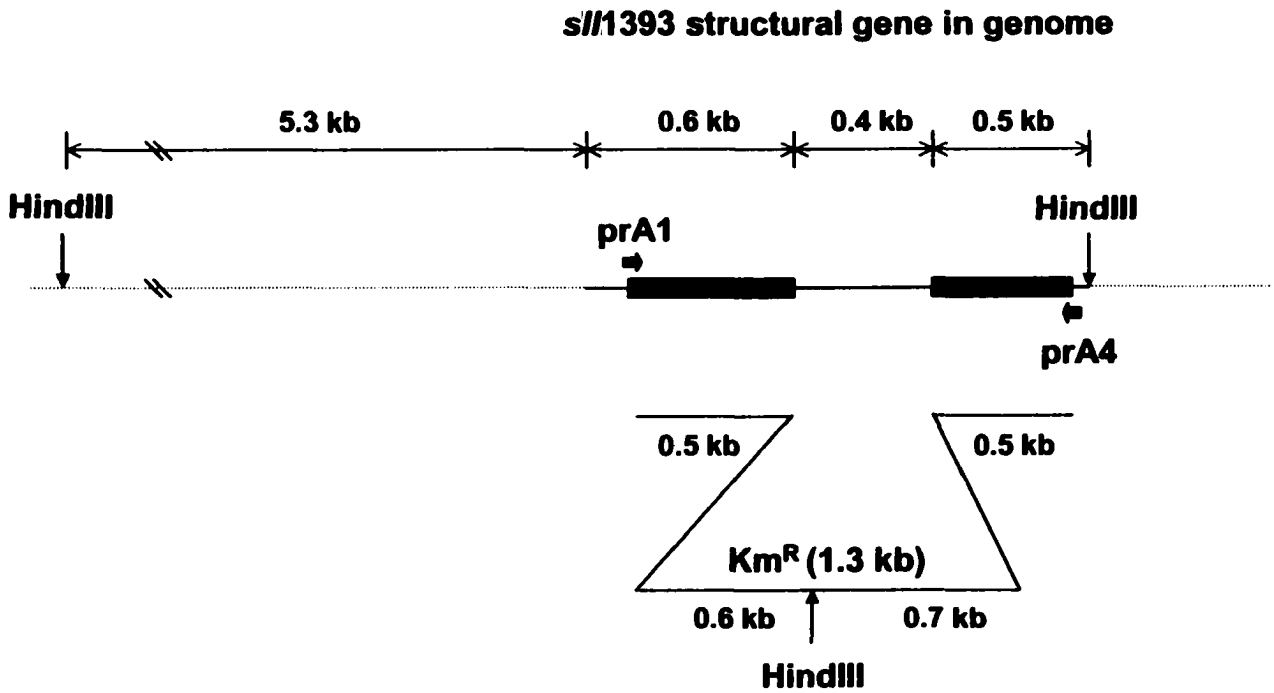

Fig. 2A. Gene structure of *sll*1393 on *Synechocystis* sp. PCC 6803 genomic DNA. *Hind*III restriction sites are indicated, along with the Km^R gene for targeted replacement of GS gene by homologous recombination.

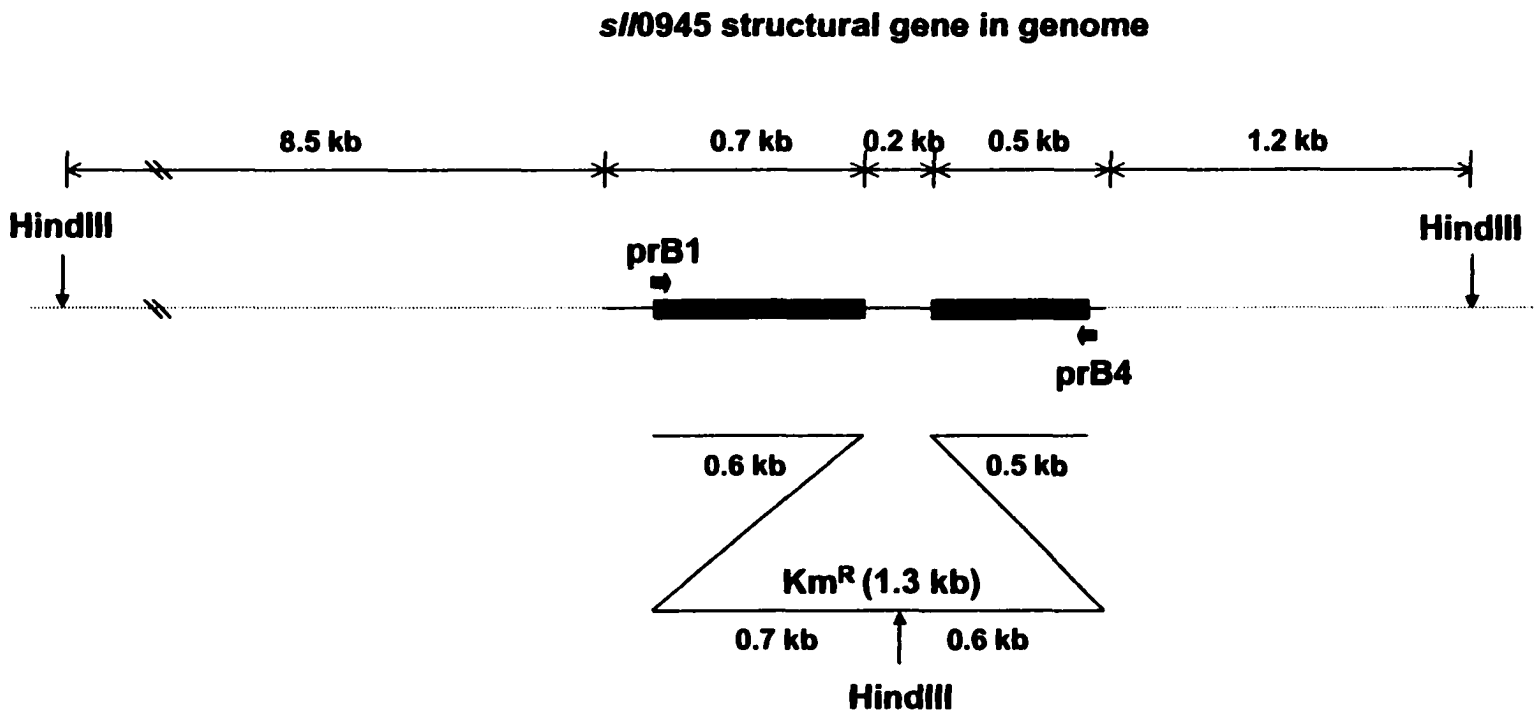

Fig. 2B. Gene structure of *sll*0945 on *Synechocystis* sp. PCC 6803 genomic DNA. *Hind*III restriction sites are indicated, along with the Km^R genefor targeted replacement of GS gene by homologous recombination.

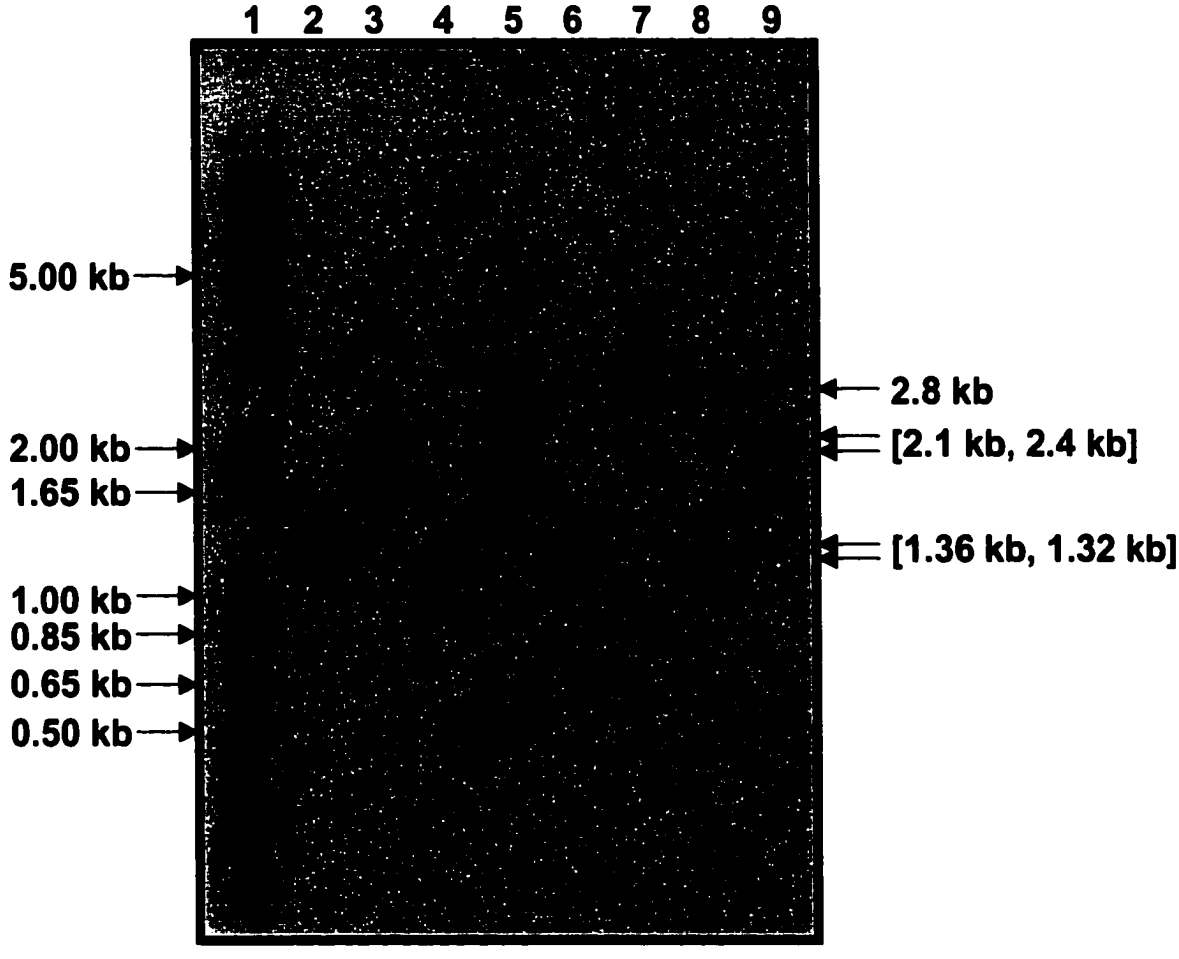

Fig. 2C. PCR analysis of *sll*1393 and *sll*0945 genes using genomic DNA from wild-type, *sll*1393^{-}, *sll*0945^{-}, and *sll*1393^{-}/*sll*0945^{-} strains as templates. PCR was performed using EX-Taq DNA polymerase (Takara) and an annealing temperature of 55°C. Lane1 is the size marker (1kb plus DNA ladder, Life Technologies). DNA bands on lane 2, 6 (WT), 3 (M1), and 7 (M12) were amplified using prA1 and prA4; Lane 4, 8 (WT), 5 (M2), and 9 (M12) were amplified using prB1 and prB4.

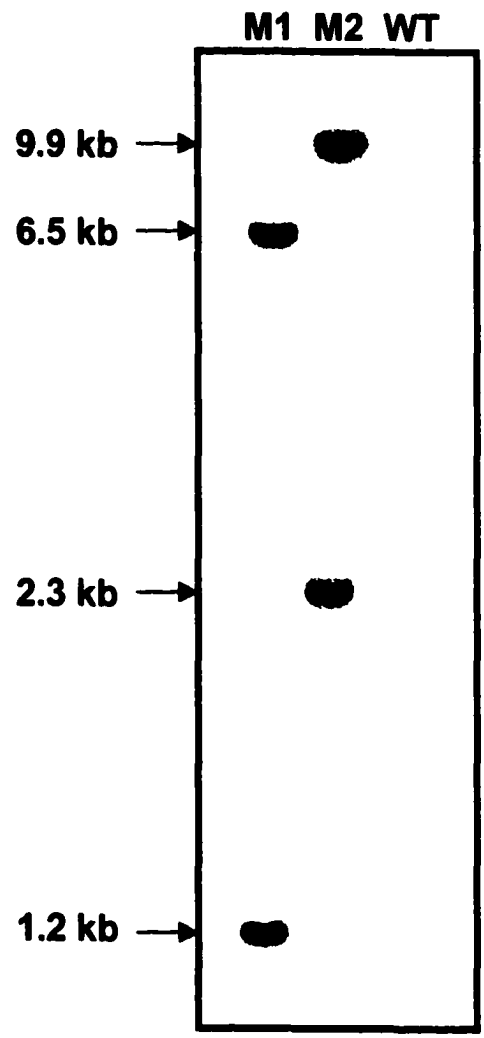

Fig. 2D. DNA hybridization analysis of the mutants and wild type. Isolated genomic DNA (5 μg) was digested with *Hind*III and probed with the *Eco*RI DNA fragment of the Km^R gene.

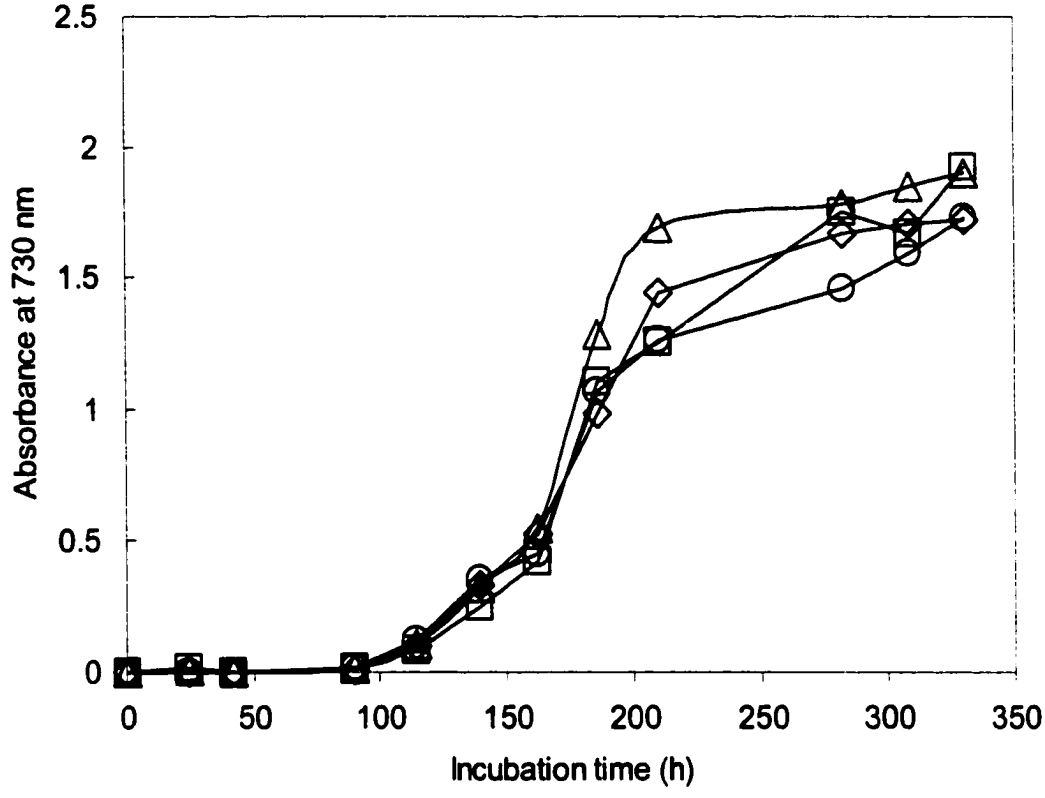

Fig. 3. Photoheterotrophic growth of WT (△), M1 (□), M2 (○), and M12 (◇) strains of *Synechocystis* sp. PCC6803. Cells were grown in a BG-11 medium containing 5 mM glucose and the number of cells were counted in the exponential phase. The same amount of the cells were inoculated in the new media. Each culture was inoculated with an equal number of exponential-phase cells, and growth of the cultures was monitored by measuring absorbance at 730 nm at various time intervals.

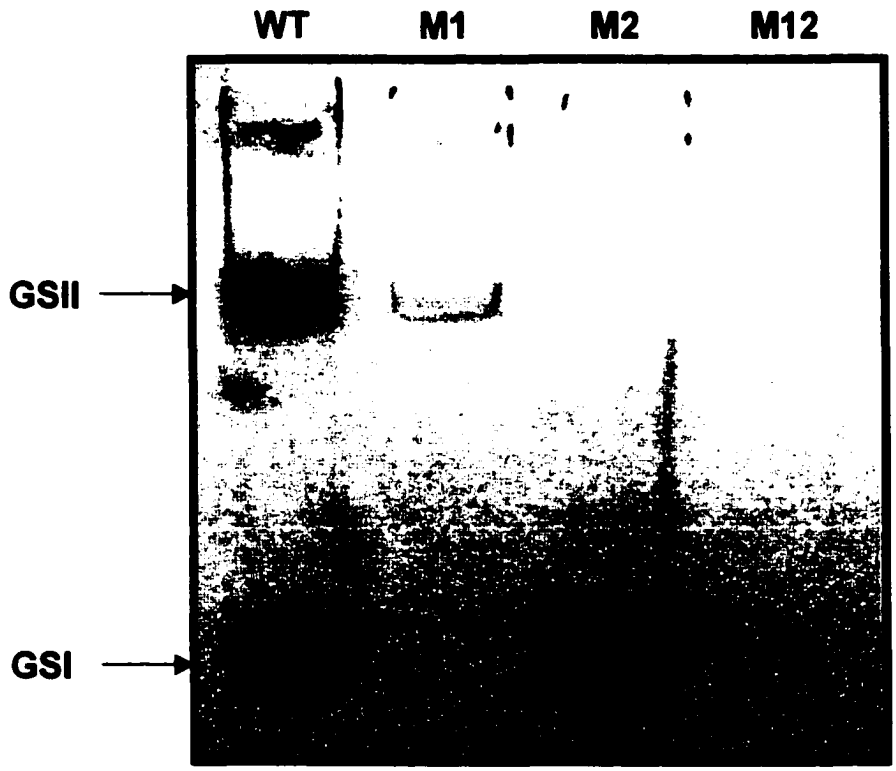

Fig. 4. Zymogram analysis of GS activity from wild-type and mutant cells. Ten μg of each soluble extract was subjected to native PAGE. The bands showing GS activity were visible after overnight incubation in a buffer containing ADP-Glc and rabbit-liver glycogen and were stained with iodine solution. Lane 1. WT, Lane2. M1 (*sll*1393$^-$), Lane3. M2 (*sll*0945$^-$), and Lane 4. M12 (*sll*1393$^-$/*sll*0945$^-$).

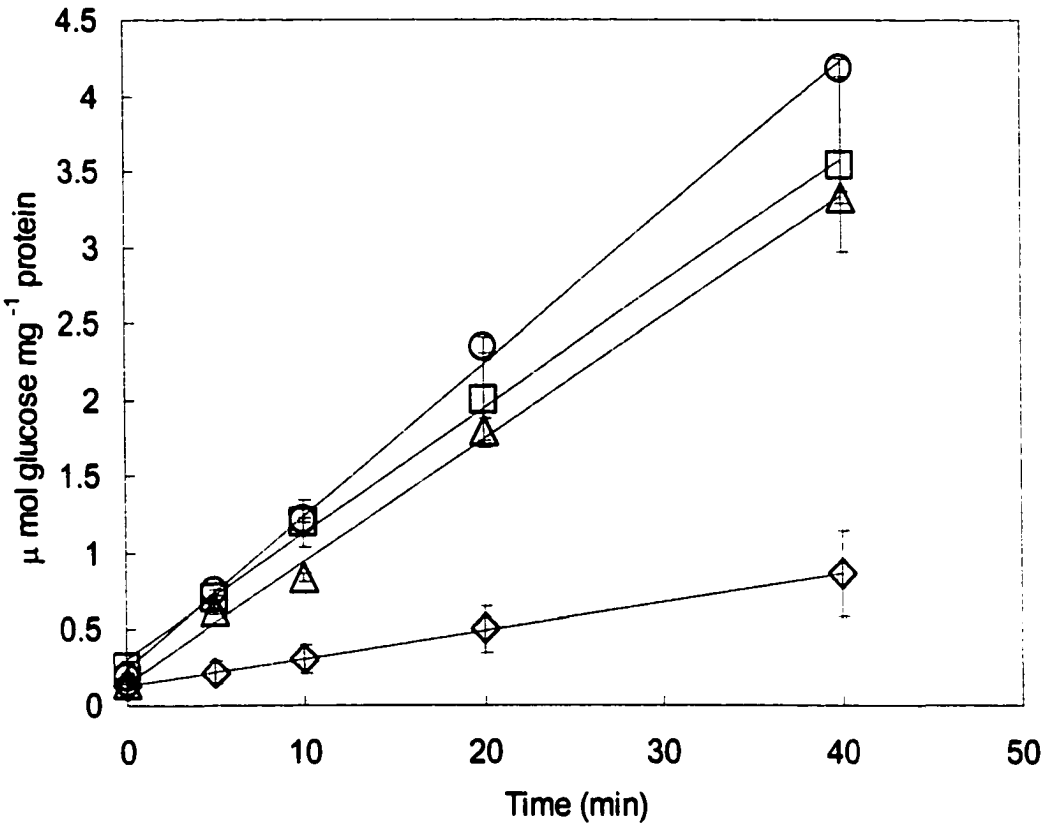

Fig. 5. Kinetics of glycogen-synthesizing activity from WT (△), M1 (□), M2 (○), and M12 (◇). The rate of [^{14}C]glucose incorporation onto rabbit-liver glycogen from ADP-[U-^{14}C]glucose was determined at various time intervals.

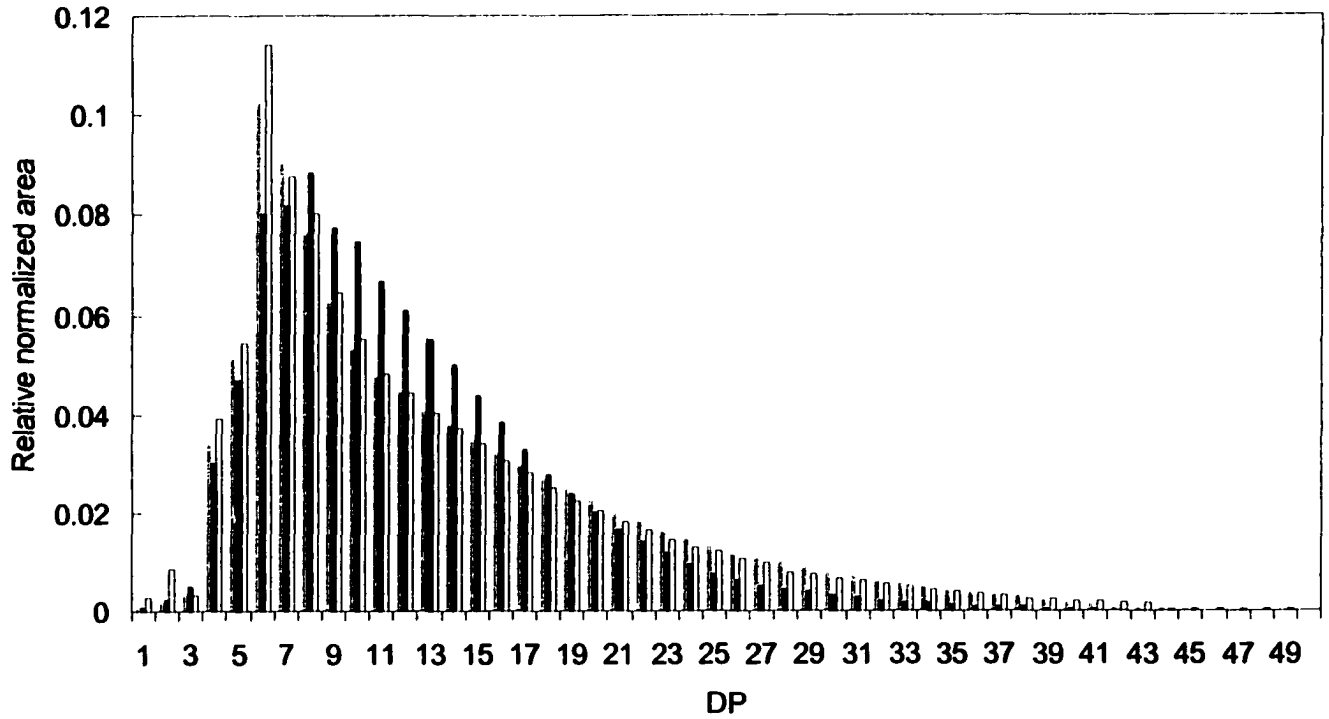

Fig. 6A. Branch chain-length distribution of glycogens from WT (■), M1 (■), and M2 (□). Glycogen samples were treated with a debranching enzyme, isoamylase, and the resulting debranched-samples were separated on an HPAEC system. The peak area was calculated and normalized from the chain-profile chromatogram.

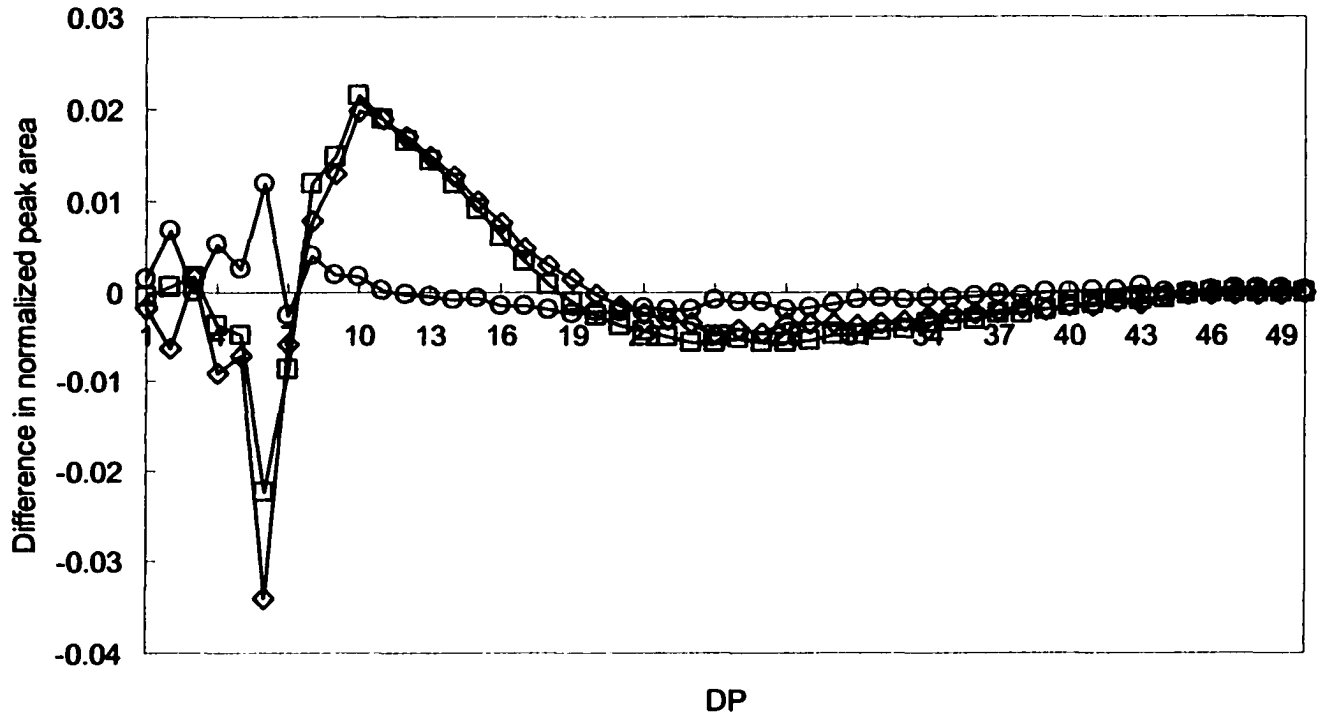

Fig. 6B. The comparison of difference in normalized peak area calculated from M1-WT (□), M2-WT (○), or M1-M2 (◇).

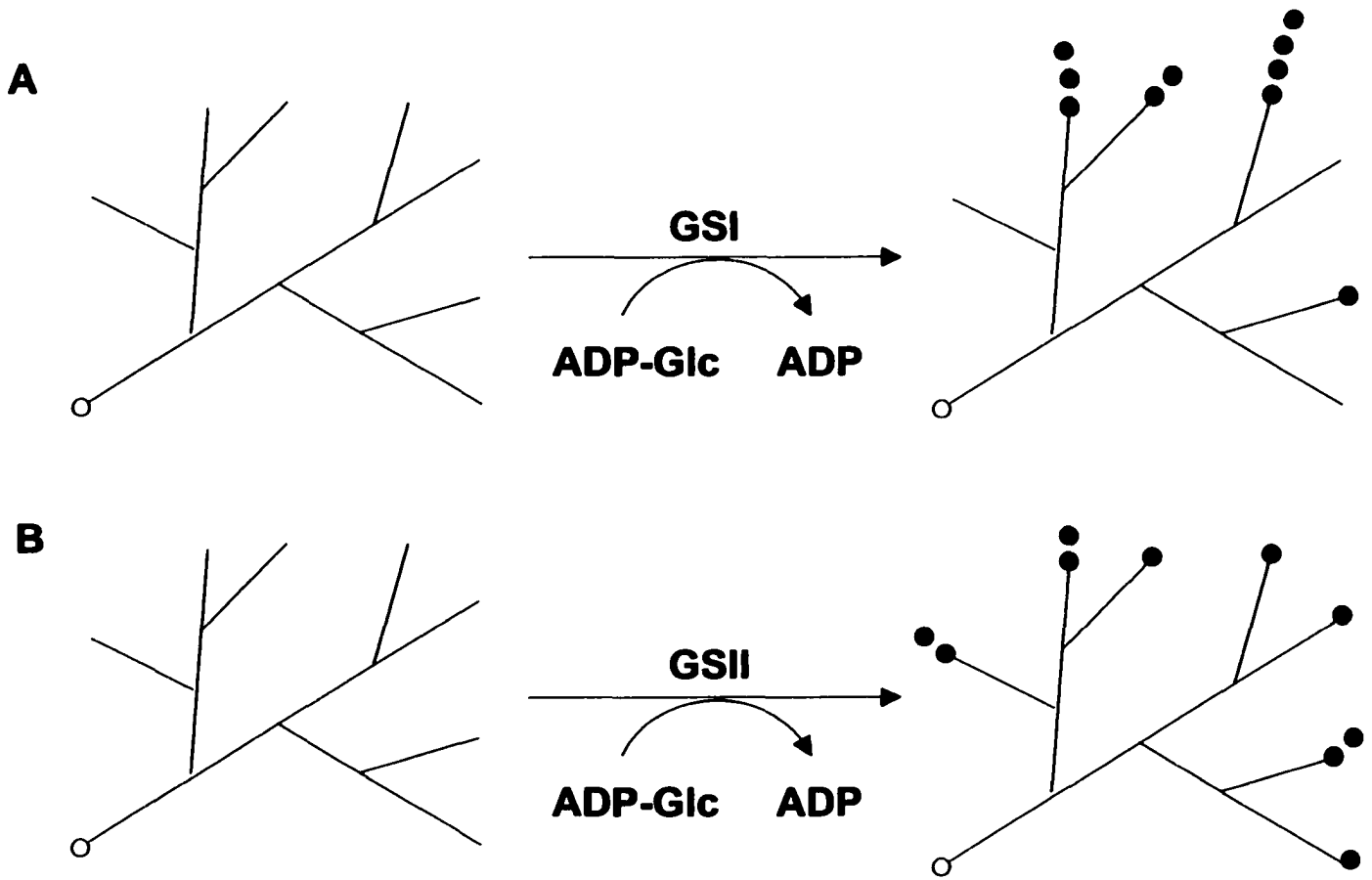

Fig. 7. Possible enzyme reactions of the GSI and GSII isoforms in elongation of rabbit-liver glycogen primers. Glucose units (●)from ADP-Glc are transferred by GS onto the non-reducing ends of the glycogen primer either progressively by GSI (A) or distributively (B) by GSII. The reducing-end of the glycogen primer is represented by the symbol (○).

CHAPTER 4. INSERTIONAL MUTAGENESIS OF THE GENE ENCODING GLYCOGEN BRANCHING ENZYME IN THE CYANOBACTERIUM *Synechocystis* sp. PCC6803[a]

A paper to be submitted to Plant Physiology

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ABSTRACT

Cyanobacteria, like animals, fungi, and other bacteria, produce glycogen as their primary form of carbohydrate storage. Analysis of the genomic DNA sequence of *Synechocystis* sp. PCC6803 indicates that this strain encodes two isoforms of glycogen synthase (GS) and one glycogen branching enzyme (GBE). To confirm the putative GBE and to demonstrate the presence of only one GBE gene, we generated a mutant lacking the putative GBE gene, *sll*0158, by replacing it with a kanamycin resistance gene by homologous recombination. GBE activity in *sll*0158$^-$ mutant was nearly eliminated; the mutant strain produced less glucan, 48% of that produced by the wild type. In contrast to that 74% of the glucan extracted from the wild-type strain was water-soluble, there was only 14% of the glucan extracted from the mutant in a water-soluble form. The molecular structures of glucans produced

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[a] Journal Paper No. J.-XXXXX of the Iowa Agriculture and Home Economics Experiment Station, Ames, IA. Project No. 3756.

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Abbreviations: GBE, glycogen branching enzyme; GS, glycogen synthase; HPAEC, high-performance anion-exchange chromatography; HPSEC, high-performance size-exclusion chromatography; DP, degree of polymerization; *sll*0158, Genbank™ accession no. D63999.

by the mutant and the wild type were characterized by using high-performance size-exclusion and anion-exchange chromatography. In contrast to glycogens of molecular weights of 6.6×10^7 daltons and10% branch-linkages produced by the wild type, α-D-glucans with negligible branching structure and molecular weights of 4.7-5.6×10^3 daltons (equivalent to DP29-35) were isolated from the mutant strain. The results confirmed that *sll*0158 was the only functional GBE gene in *Synechocystis* sp. PCC6803.

INTRODUCTION

Glycogen is a major reserve carbohydrate in both prokaryotes and eukaryotes. It is an α-D-(1,4)-glucan polymer containing about 10% α-(1,6)-branch linkages (Calder, 1991). Three major enzymes, ADP-glucose pyrophosphorylase, glycogen synthase, and branching enzyme, are involved in bacterial glycogen biosynthesis (Preiss, 1984; Preiss and Romeo, 1989; Preiss, 1999). Branching enzyme (GBE; 1,4-α-D-glucan: 1,4-α-D-glucan 6-α-D-[1,4-α-D-glucano]-transferase) plays a key role in determining glycogen structure by forming branch linkages. A similar type of BE also is responsible for the branch-structure of starch in plants.

Cyanobacteria and other bacteria are known to produce glycogen as their carbohydrate reserve (Chao and Bowen, 1971; Weber and Wöber, 1975; Fujimori et al., 1995). Genes encoding BE have been cloned from several bacterial species and their activities examined (Baecker et al., 1986; Kiel et al., 1989, 1991, 1992; Homerova and Kormanec, 1994). Bacteria generally possess one branching enzyme (Preiss, 1999), whereas plants possess at least two isoforms with differing

specificities (Smith, 1988; Guan and Preiss, 1993). Analysis of the genomic DNA of *Synechocystis* sp. PCC6803 on the basis of sequence similarity with other organisms revealed that this cyanobacterium had one gene encoding branching enzyme (Kaneko et al., 1996). However, this putative GBE gene has not been proven to encode a functional GBE, nor is it certain that no other GBE genes exist in the genome.

It is of additional interest to determine whether a GBE-deficient bacterial mutant could accumulate any linear, unbranched glucan material. Studies using iodine staining have shown that GBE-deficient mutants of *E. coli* and yeast accumulate amylose-like glucan (Romeo et al., 1988; Rowen et al., 1992). It is expected that in GBE-deficient mutants, glycogen synthase (GS) can elongate linear glucan to an unknown length. A reduced efficiency in glucan biosynthesis is expected because of limited non-reducing ends of the glucan, comparing with branched molecules, available for elongation by GS. A GBE-deficient mutant of cyanobacteria can be used to investigate what chain-lengths of linear glucans are synthesized by GS alone.

To determine whether *sll*0158 encodes a functional GBE, we generated a BE-deficient mutant in *Synechocystis* sp. PCC6803. The yield and structure of the glucan produced by the mutant were studied and compared with that by the wild type. Because most linear α-(1,4)-glucans are known not soluble in water, it is necessary to develop a method to extract insoluble glucan material from cell debris. Our goal is to establish a model system to study the mechanism of starch/glycogen biosynthesis in cyanobacteria. As an initial step of the study, we examined if *sll*0158

encodes a functional GBE and is the only GBE, and determined the structure of glucan produced by GBE$^-$ mutant.

RESULTS

*sll*0158 Gene Encoding Putative Glycogen Branching Enzyme Was Mutagenized in *Synechocystis* sp. PCC6803

A mutant (M3) lacking a putative GBE gene, *sll*0158, was generated by transforming a wild-type strain of *Synechocystis* sp. PCC6803 with a plasmid pSHK0158 (Fig. 1). The transformation resulted in a replacement of *sll*0158 with a gene conferring resistance to kanamycin (Km^R) via homologous recombination. PCR analysis was performed to detect insertion of the Km^R gene cassette into the targeted sequences and to verify complete elimination of wild-type copies of the targeted gene. The structure of *sll*0158 is shown in Fig. 2A. Primers, prC1 and prC4, were used to amplify both the mutagenized and intact *sll*0158 genes, generating the corresponding PCR products (2.3 and 2.2 kb for M3 and WT, respectively). Because the size difference between PCR products of the mutant and the wild type was less than 100 bp, it was too close to be identified with confidence. Thus, new primers (prK1 and prK2) were designed in the middle of the *sll*0158 gene and utilized to amplify the wild-type gene. The expected sizes of the PCR amplified fragments were identified in WT (Fig. 2B), but no detectable bands were found in M3, demonstrating that the wild-type *sll*0158 gene was no longer present in M3. Southern hybridization analysis confirmed that the restriction fragment sizes of the mutagenized *sll*0158 genome region corresponded exactly to those expected for the

inserted Km^R gene (Fig. 2C). The results confirmed that the targeted *sll*0158 gene was disrupted and completely segregated in the M3 strain.

This M3 strain was used for studying the function of the enzyme encoded by *sll*0158 gene. The growth rate of M3 appeared not to be significantly affected by the insertional mutation, comparing with that of the wild-type strain (Fig. 3). Defective structural genes encoding glycogen biosynthetic enzymes also had no effect on bacterial (Steiner and Preiss, 1977) or yeast growth (Farkas et al., 1991).

Insertional Mutation of *sll*0158 Gene Affects Branching Enzyme Activity

Glycogen branching enzyme (GBE) activity was assayed over a 1-h time course (Fig. 4) by measuring the amount of inorganic phosphate (P_i) released during a phosphorylase *a* stimulation assay (Boyer and Preiss, 1978; Sun et al., 1997). Following a lag of approximately 5 min, the amount of released P_i increased substantially over time for the WT strain. The rate of P_i release from 5 to 60 min was fairly linear for the WT strain (1.5 mM min^{-1} mg^{-1} protein), but no apparent branching activity was detected for M3. This analysis confirmed that the insertional inactivation of the *sll*0158 gene significantly reduced the total cellular GBE activity to an undetectable level.

Glucan Extracted from *sll*0158$^-$ Mutant (M3) Is Primarily Linear with Negligible Amount of Branching

To examine the effect of the disruption of *sll*0158 on glycogen biosynthesis, we quantified the α-D-glucan produced in M3 and analyzed its structure in

comparison with that of glycogen produced in WT. The yield of α-D-glucan produced by M3 was about 48% of that by WT (Table II), but the structure was very different. The soluble fraction extracted from M3 contained only a small amount of α-D-glucan (2.3 mg g^{-1} cell mass). Mostly 86% of the total α-D-glucan (14 mg g^{-1} cell mass) was found in a cold-water-insoluble form present with the cell debris. In contrast, 74% of the glycogen (25.3 mg g^{-1} cell mass) in WT was in a cold-water-soluble form (Table II). High-performance size-exclusion chromatograms (HPSEC) showed that the relative molecular weight (M_r) of the M3-glucan was much smaller than that of the WT-glucan (Fig. 5). The M_r of the insoluble glucan isolated from WT (6.6×10^7 daltons) was identical to that of the soluble glucan (Table III). The M_r of the soluble and insoluble glucans isolated from M3 were 4.7×10^3 (~DP29) and 5.6×10^3 daltons (~DP35), respectively.

The chain-length profiles analyzed by using high-performance anion-exchange chromatography (HPAEC) were similar between the soluble and insoluble glucan of M3 (Fig. 6A and B), which were agreeable with the range of the molecular weights of M3-glucans analyzed by using HPSEC. Peak chain-lengths of both soluble and insoluble M3-glucans were DP19. The HPAEC chromatograms of the insoluble M3-glucan with (Fig. 6C) and without isoamylase hydrolysis (Fig. 6B) were very similar, which suggested that M3-glucan consisted of negligible branches. The very high λ_{max} (632 nm) of iodine-complex of insoluble M3-glucans (Table III) also suggested that insoluble M3-glucan had an amylose-like structure.

The insoluble glucan isolated from WT (Fig. 7) showed a unimodal branch chain-length distribution, typical of cyanobacterial glycogen (Yoo et al., submitted, in Chapter 3). The insoluble glucan consisted of more longer chains than did the soluble glucan from WT (Fig. 7), which agreed with an increase of λ_{max} from 512 nm for soluble glucan to 518 nm for insoluble glucan (Table III).

DISCUSSION

Glycogen branching enzyme (GBE) has a dual-function of hydrolyzing an α-(1, 4)-linkage and synthesizing an α-(1, 6)-linkage. On the basis of the genomic sequence of *Synechocystis* sp. PCC6803, this strain is predicted to encode one GBE as is typical for bacteria. By generating a mutant strain lacking GBE, we demonstrated that *sll*0158 gene encoded a functional GBE that was responsible for the branch-structure formation of glycogen.

Results showed that after the putative GBE gene was disrupted, the cellular GBE activity was diminished. It was confirmed that M3, without GBE activity, produced a reduced amount of linear glucan, equivalent to 48% of that produced in the WT strain (Table II). By using iodine-staining of *E. coli* cells, Romeo et al. (1988) suggested that *E. coli* strain lacking GBE synthesized unbranched glucan. Guan et al. (1995) reported that glucans produced by an *E. coli* mutant (*glgB*$^-$), missing GBE, had an average molecular weight of DP42 and a glucan yield of more than 50% of that produced by cells transformed with maize BE. GBE-deficient mutants of *Saccharomyces cerevisiae*, a unicellular eukaryote, have been reported to

accumulate approximately 20-60% of the glycogen produced by the wild type (Rowen et al., 1992).

As a result of lacking GBE activity, once glucan chains are elongated by GS to a sufficient length, the linear chains form double helices, crystallize spontaneously, and precipitate out from the solution. The precipitation of the glucan chains stops them from being elongated by GS. As a result, glucans produced by the M3 strain were found with the cell debris and could not be extracted by cold water. The M3-glucans were linear and had average molecular weight of about DP35 that was 10^4 times smaller than the WT glycogen. Moreover, because of the absence of branches, the rate of glucan biosynthesis in M3 was reduced substantially.

From the analysis of the GBE activity using phosphorylase *a* stimulation assay, we found no detectable branching activity in M3 (Fig. 4). Through isoamylase debranching and HPAEC analysis, it was demonstrated that glucans produced in M3 were primarily linear (Fig. 6B and C). However, when we quantified the proportion of individual chains by calculating their peak area, some residual GBE activity was still detected. Isoamylase treatments of M3-glucan resulted in a slight change in the overall pattern of molecular weight (chain-length) distribution in comparison with that of the untreated M3-glucans. The distribution profile shifted towards smaller molecular weights as shown in Figure 8, suggesting that some branched molecules were hydrolyzed into smaller molecules. Currently, we do not expect another GBE to be present in *Synechocystis* sp. PCC6803. Because BE has highly conserved domains for substrate binding and active sites (Svensson, 1994),

the inability to detect another conserved sequence corresponding to a putative BE gene in the genomic sequence suggests it is unlikely another GBE isoform exists. Alternatively, a partially functional GBE from *sll*0158 gene may be expressed and show some branching activity. During disruption of *sll*0158 by homologous recombination, however, four conserved regions (Svensson, 1994) considered very important for BE activity (Kuriki et al., 1996) have been replaced by the antibiotic resistance marker, as indicated by the PCR and Southern analysis results. Additionally, no detectable PCR product was amplified by primers designed to detect the wild-type *sll*0158 gene, so residual GBE activity resulting from a meridiploid condition is also unlikely. Therefore, it is not possible to expect any residual activity originating from the *sll*0158 gene product. At this point, the source of residual BE activity is not clear.

In the HPAEC analysis of WT-glucan, there was a detectable difference in the branch chain-length distribution between the water-soluble and water-insoluble fractions (Fig. 7). The insoluble fraction of WT-glucan showed a larger proportion of longer chains (DP $\geq$ 14) than the soluble fraction, implying that water solubility of glucans is a result of the branch chain-length distribution. Thus, glucan having larger proportion of longer branch chains was trapped in the cell debris and was extracted during DMSO treatment.

Our results showed that the *sll*0158^{-} mutant, deficient in GBE, had a retarded glucan-synthesizing rate, and that most glucans accumulated as an insoluble form with a negligible amount of branch structure.

MATERIALS AND METHODS

Materials

Chemicals for the BG-11 medium were purchased from Fisher Scientific (Pittsburgh, PA). Molecular biology reagents were obtained from Promega Biotech (Madison, WI). Most other chemicals and antibiotics were from Sigma (St. Louis, MO).

Strains and Growth Conditions

Synechocystis PCC6803 strains were grown at 25°C under continuous fluorescent light (40 $\mu E\ m^2\ s^{-1}$) in BG-11 media (Rippka et al., 1979) supplemented with 5 mM glucose. For initial growth of the mutant strain, the medium contained 50 $\mu g\ mL^{-1}$ kanamycin. For glycogen isolation, cells were grown in a BG-11 medium for 7-8 days (A_{730} = 1.6-1.8) before being transferred to a nitrogen-limiting BG-11 medium for another 2-3 days of growth. The nitrogen-limiting BG-11 medium contains less than 10 % of the normal sodium nitrate (8.34 mg mL^{-1}) as the only nitrogen source. Cell pellets harvested by centrifugation at 6,000 × *g* for 15 min were washed with sterile deionized water prior to transfer of the cells to the nitrogen-limiting medium. Cultures were started at an initial cell density of 1.0×10^6 cells mL^{-1} for determination of the cell growth rate.

DNA Isolation and Mutagenesis of *sll*0158 Gene in Synechocystis sp. PCC6803

Genomic DNA of *Synechocystis* PCC 6803 was isolated following the procedure of Yoo et al. (submitted). A brief scheme for constructing the knock-out

plasmid, pSHK0158, is shown in Fig. 1. To replace the putative BE gene, *sll*0158, with a kanamycin resistance (Km^R) gene by a double cross-over homologous recombination, we used two PCR-amplified DNA fragments from the coding regions close to 5' and 3' ends of the *sll*0158 gene. Two sets of primers, prC1 (forward)/prC2 (reverse) and prC3/prC4 were used for PCR amplification (Table I). Primers were designed to contain *Bam*HI/*Eco*RI and *Eco*RI/*Hind*III at the 5'/3' ends of the amplified DNA fragments. The resulting two amplified DNA fragments were subcloned sequentially into a pBluescript KS vector, and the Km^R (~1.3 kb) gene from pUC4K (Pharmacia) was inserted between them using the *Eco*RI restriction site. Mutant strain (M3) was obtained by transforming *Synechocystis* sp. PCC6803 (Williams, 1988) with the final construct, pSHK0158, and selecting for kanamycin resistance. After several rounds of segregation by single colony selection, insertion of the Km^R gene cassette into the targeted sequence was confirmed in the transformants by PCR and Southern blot analyses. Growth of wild-type and mutant strains was monitored by measuring the absorbance of the cell cultures at 730 nm.

PCR and Southern Blot Analysis of *sll*0158$^-$ Mutant Strain

Insertion of the Km^R gene cassette into the targeted sequence was confirmed by PCR analysis. The oligonucleotides, prC1 and prC4, which were designed to amplify DNA fragments used for homologous recombination, were used to obtain full length intact (WT) and Km^R-disrupted (M3) structural genes. Oligonucleotides, prK1 (reverse, 5'-ATAAAGTCTTCGGGACTGCCAA -3') and prK2 (forward, 5'-TTGGCAGTCCCGAAGACTTTAT-3') from the middle part of *sll*0158 replaced with

the Km^R gene, were used to confirm the complete absence of the wild-type *sll*0158 gene in M3. Primer pairs, prC1/ prK1, and prK2/prC4, were used to amplify the left and right regions of *sll*0158, respectively, in wild-type and M3 strains. For Southern blot analysis, genomic DNA from wild-type and M3 was isolated, purified, digested with *Hind*III, separated in 0.8% agarose gels and blotted on to nitrocelluose membrane (Hybond-C, Amersham). The DNA probe (a 1282 bp *Eco*RI fragment of the Km^R gene) was labeled with [α-^{32}P]-dCTP and Southern hybridization performed by standard techniques (Sambrook et al., 1989).

Determination of Branching Enzyme Activity

GBE activity was quantified using the phosphorylase *a* stimulation assay (Boyer and Preiss, 1978; Sun et al., 1997). The reaction mixture contained 20 μL of 1 mg mL^{-1} rabbit-liver phosphorylase *a* (equivalent to 0.04 units), 1mM AMP, 100 mM sodium citrate (pH7.0), 45 mM α-D-glucose-1-phosphate, and 80 μL of cell-free extract (equivalent to 20 μg of total soluble protein) in total volume of 200 μL. The reaction was initiated at 30°C by adding α-D-glucose-1-phosphate. At different time intervals, 30 μL samples were quenched by boiling for 10 min. The resulting samples were diluted with deionized water and the P_i released from the reaction determined as described by Baykov et al. (1988), using a Microplate reader (Bio-Tek Instruments, Inc., Winooski, VT) to measure the absorbance at 630 nm in 200 μL of the mixture from each assay. The boiled cell extracts were used as controls at the same reaction condition.

Protein concentrations were determined using a Protein assay kit (Bio-Rad). Bovine serum albumin (BSA) was used as the standard.

Glucan Extraction from M3 Deficient in Branching Enzyme (BE)

Wild-type and mutant cell cultures (1 L; grown for 2 days in N-limited BG-11 medium as described above) were harvested at 6,000 × *g* for 15 min. The cell pellets frozen with liquid nitrogen were stored at -20°C until used. The cell pellets were ground in liquid nitrogen using pestle and mortar, and the soluble glucan was extracted by suspension of the broken cells in 10 mL deionized water and washed twice with an equal volume of water. After centrifugation (10,000 × *g* for 15 min), the supernatant was removed and boiled for 15 min. The cell debris (1.2 g, wet basis) was dissolved in 4 mL of dimethyl sulfoxide (DMSO) and boiled for an hour with stirring. The supernatant from a centrifugation (8,000 × *g*, 15 min) was precipitated at 4°C overnight with 3 vol. of ethanol. The pellet, recovered by another centrifugation (8,000 × *g* for 15 min), was again dissolved in DMSO and recovered as described above. Residual DMSO was removed with two ethanol washes (0.5 mL each) and precipitates dried in the air. The dried pellets were dissolved in 10 mL of boiling water for 30 min and the resulting solutions used for further study.

Glycogen Yield, λ_{max} of Iodine-Glycogen Complex and Analysis of Glucan Structure

Yield of glycogen and λ_{max} of iodine-glycogen complex were determined following the procedure of Yoo et al. (submitted, in Chapter 3). The relative molecular weight (M_r) of glucan/glycogen was determined using an HPSEC system. Pullulan standards P-82 (Shodex, Japan) were used to calibrate the analytical columns. The operating condition of the system was established previously (Yoo and Jane, submitted, in Chapter 3). The branch chain-length distribution of glucans was determined using an HPAEC system (Dionex-300, Sunnyvale, CA) equipped with an amyloglucosidase reactor and a pulsed amperometric detector (PAD) (Wong and Jane, 1997). The procedure for analysis of debranched-glucan samples by HPAEC has been described in detail previously (Yoo et al., submitted, in Chapter 3).

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Table I. Oligonucleotide sequences used to construct pSHK0158

The vector construct was used to replace endogenous *sll*0158 gene by homologous recombination. The position of the oligonucleotides in the nucleotide sequences of the *sll*0158 structural DNA are indicated. The restriction sites introduced by PCR amplification are underlined.

	Oligonucleotide sequence	Position	Site inserted
prC1	5'-AATCTTCACCAG/GATCCCTTTGA-3'	46-68 (23 bp)	BamHI
prC2	5'-GATAAAG/AATTCCCACACCATGTT-3'	523-546 (24 bp)	EcoRI
prC3	5'-GGCAAAAG/AATTCCAACGTCAG-3'	1715-1736 (22 bp)	EcoRI
prC4	5'-AAAGACCA/AGCTTCTGTCCATT-3'	2171-2192 (22 bp)	HindIII

Table II. Effect of *sll*0158 deletion on accumulation of glycogen

The isolated glucans were treated with amyloglucosidase and converted to glucose. Total content of α-D-glucan from each source was determined using a glucose diagnosis kit.

Source of Glucan	Glucan yield	
	M3	WT
	mg g^{-1} wet cell mass	
Water-soluble fraction (A)	2.3 ± 0.2 (14%)	25.3 ± 1.1 (74%)
Water-insoluble fraction (B)	14.0 ± 0.5 (86%)	8.8 ± 0.8 (26%)
Total amount (A)+(B)	16.3	34.1
Glucan yield	48%	100%

Table III. Effect of *sll*0158 deletion on glycogen structure

Source of Glucan	Molecular weight (M_r[a])		λ_{max}[b]	
	M3	WT	M3	WT
	Dalton (Da)		nm	
Water-soluble fraction	4.7×10^3 (DP29)	6.6×10^7	546	512
Water-insoluble fraction	5.6×10^3 (DP35)	6.6×10^7	632	518

[a] Relative molecular weight was calculated from a standard curve of pullulan standards, which was based on the elution time from the HPSEC system.
[b] Glucan-iodine complex was scanned from 400 to 700 nm. Maximum wavelength (λ_{max}) was determined from the absorption spectrum.

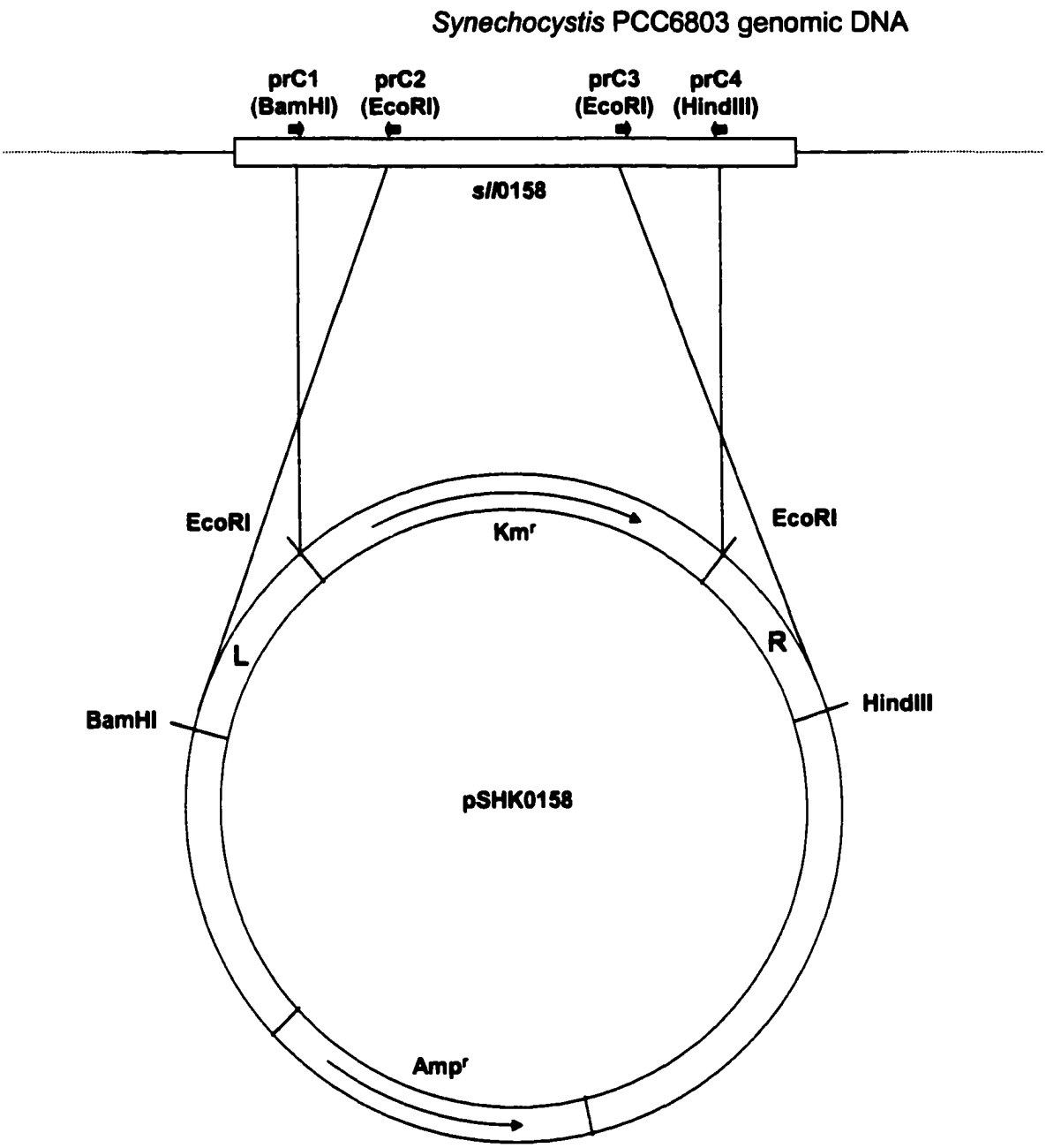

Fig. 1. Construction of a vector for insertional mutation of the *sll*0158 gene. Two homologous DNA sequences were amplified using prC1/prC2 (for left fragment, L) and prC3/prC4 (for right fragment, R), respectively, by PCR. Introduced restriction sites in the primers are shown in Table I. Left homologous DNA fragment was restricted with BamHI and EcoRI and right fragment with EcoRI and HindIII. Restricted fragments and Km^R gene were sequentially inserted into pBluescript KS. The resulting vector was used for transformation of wild type of *Synechocystis* sp. PCC6803.

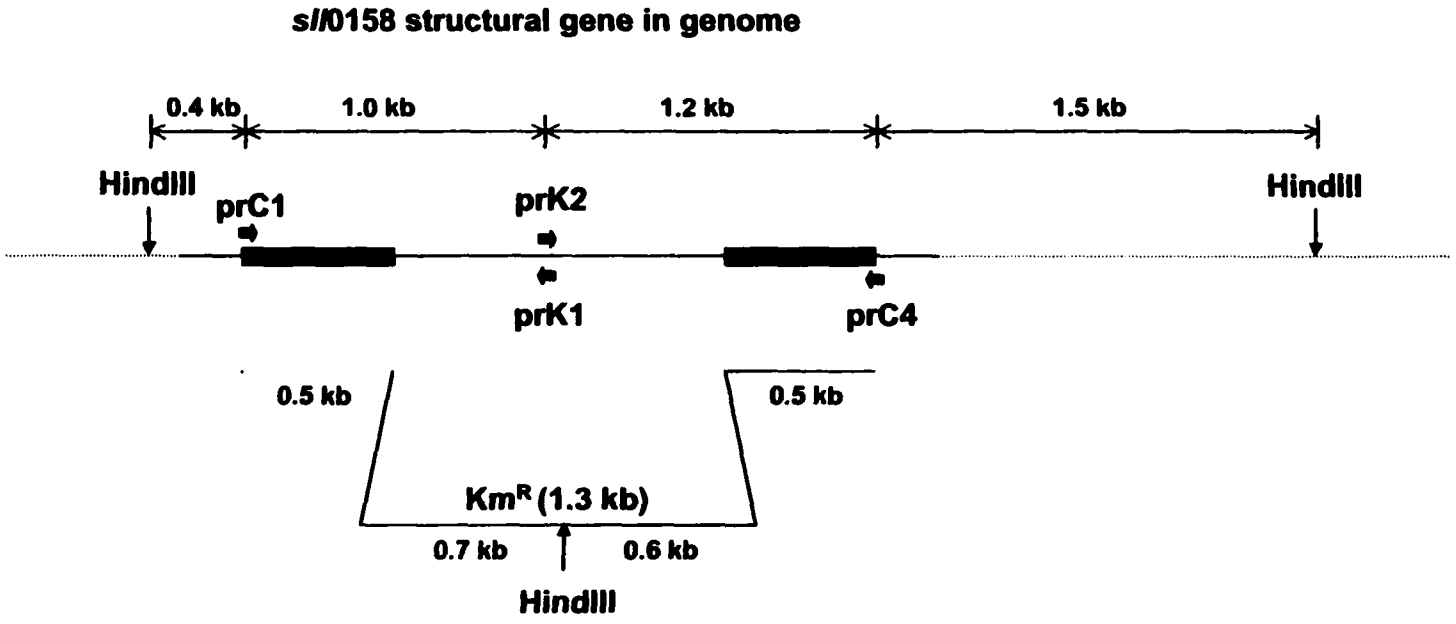

Fig. 2A. Gene structure of *sll*0158 in *Synechocystis* sp. PCC6803 genomic DNA. *Hind*III restriction sites are indicated, along with the Km^R gene used for targeted replacement of *sll*0158 by homologous recombination.

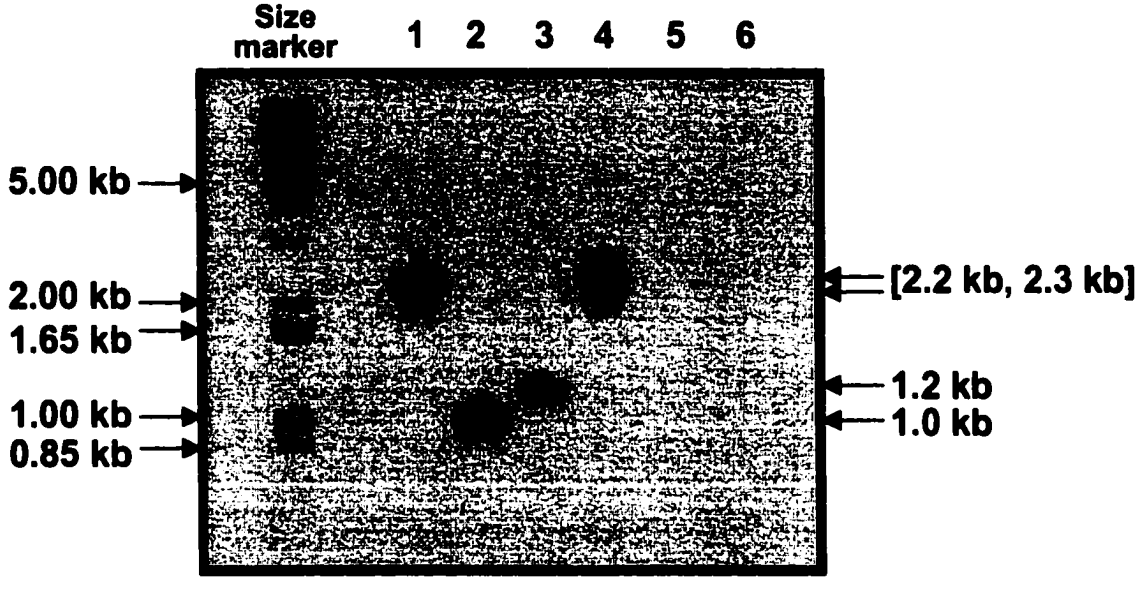

Fig. 2B. PCR analysis of the *sll*0158 gene using genomic DNA from wild type and *sll*0158^{-} strains as templates. The amplified bands in lanes 2 (WT) and 5 (M3) show similar sizes because, by using prC1 and prC4 primers (see Table I), the amplified bands for the intact *sll*0158 gene (2.2 kb) and for the inserted Km^R gene (2.3 kb) are almost the same size. When primers of PrC1/PrK1 and PrK2/PrC4 were used, 1.0 and 1.2 kb bands showed on lane 3 and 4 (WT), respectively, and there were no band on lane 6 and 7 (M3). These results showed that *sll*0158 gene has been replaced with the Km^R gene. Lane1 is the size marker (1 kb plus DNA ladder, Life Technologies).

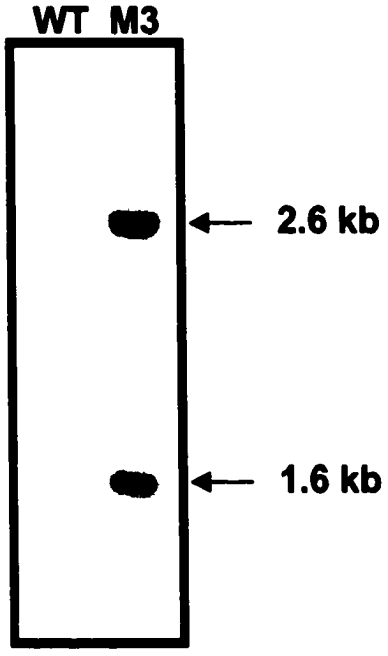

Fig. 2C. Southern blot analysis of the mutant and wild-type *Synechocystis* sp. PCC6803. Genomic DNA (5 μg) was digested with *Hind*III and probed with the *Eco*RI-fragment of Km^R gene.

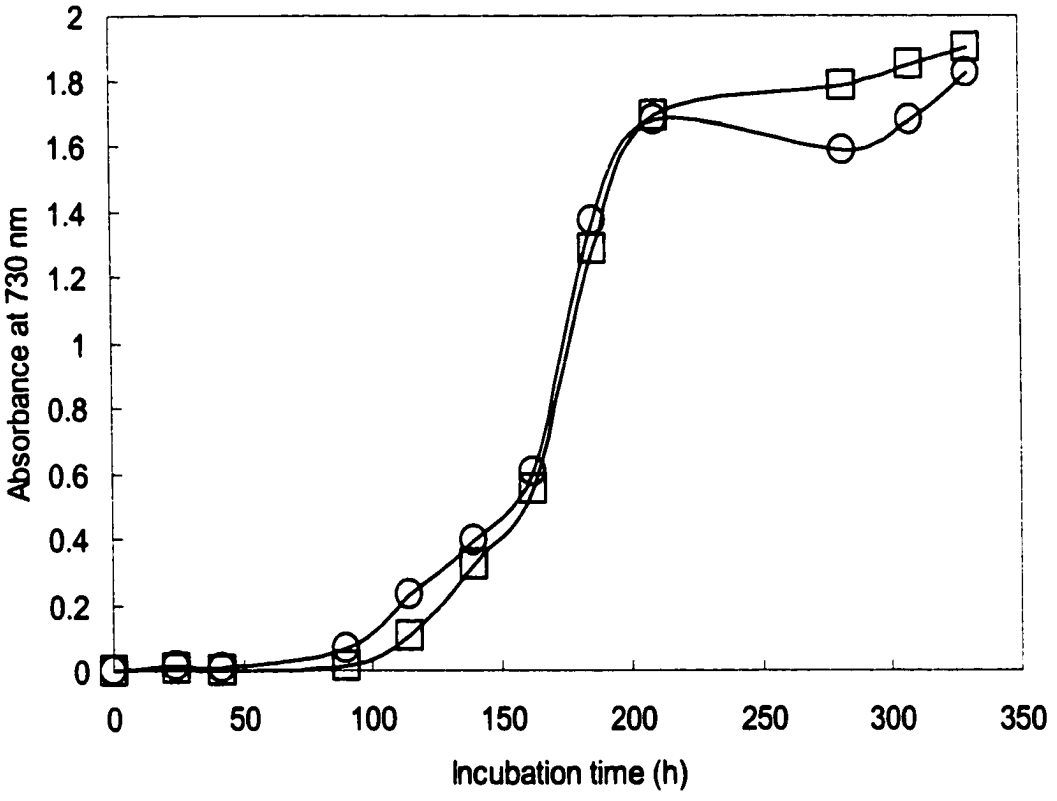

Fig. 3. Photoheterotrophic growth of WT (□) and M3 (○) strains of *Synechocystis* sp. PCC6803. Cells were grown in a BG-11 medium containing 5 mM glucose, and the numbers of cells were counted in the exponential phase. The same amount of cells was inoculated in the new media. Each culture was inoculated with an equal number of exponential-phase cells, and growth of the cultures was monitored by measuring absorbance at 730 nm at various time intervals.

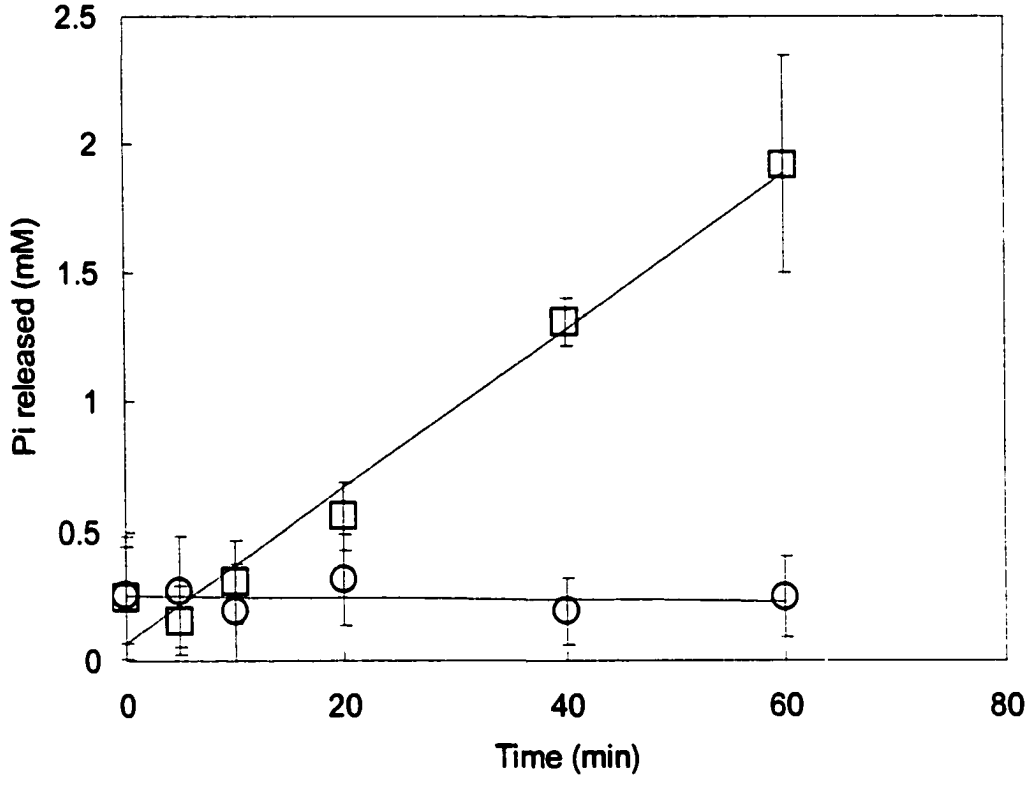

Fig. 4. Time course of glycogen branching enzyme activities from WT (□) and M3 (○), as measured by the phosphorylase *a* stimulation assay.

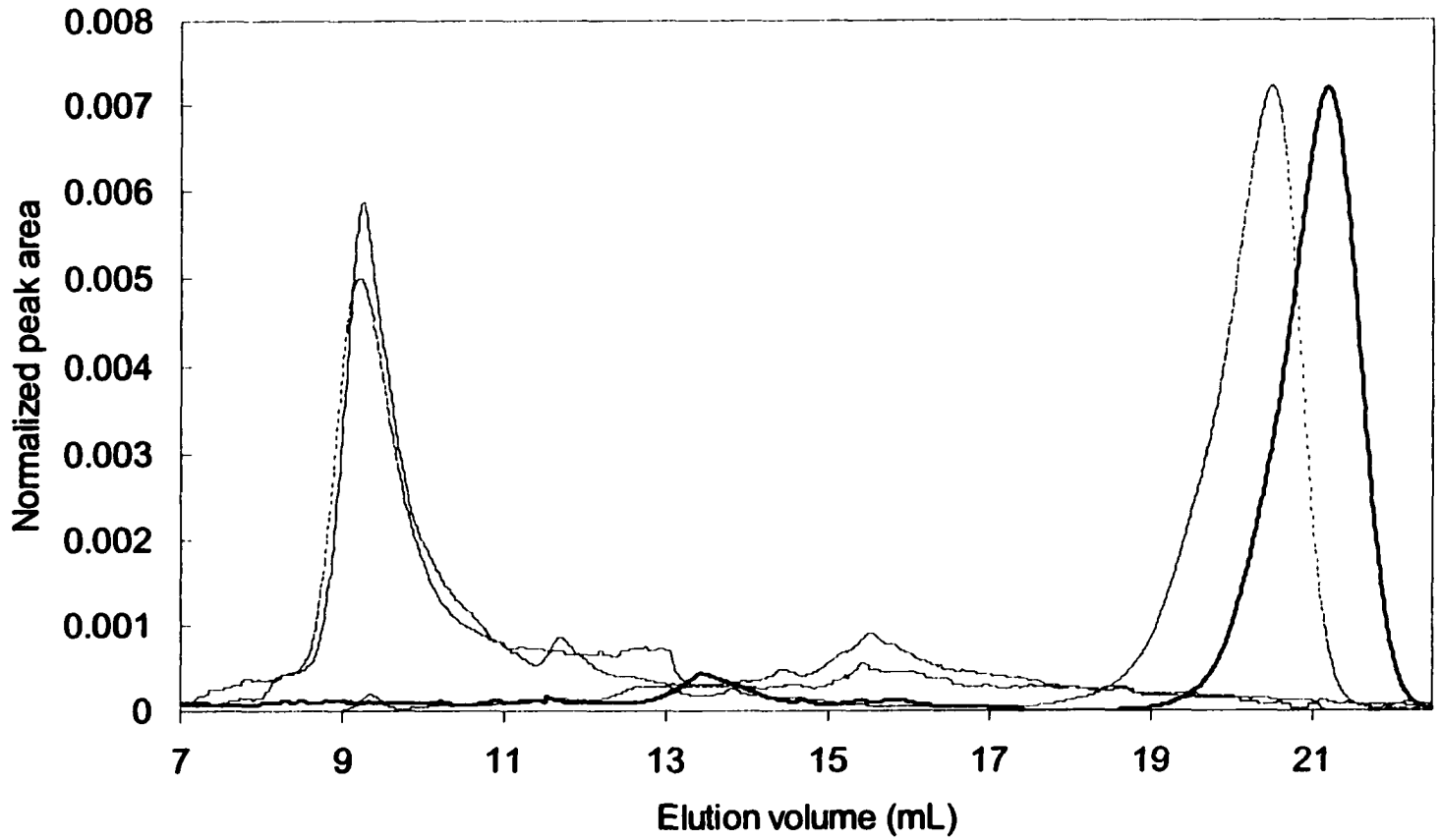

Fig. 5. High-performance size-exclusion chromatograms of the glucans isolated from WT and M3 strains. Peak areas of soluble (—) and insoluble (----) M3-, and soluble (—) and insoluble (-·-) WT-glucans were normalized.

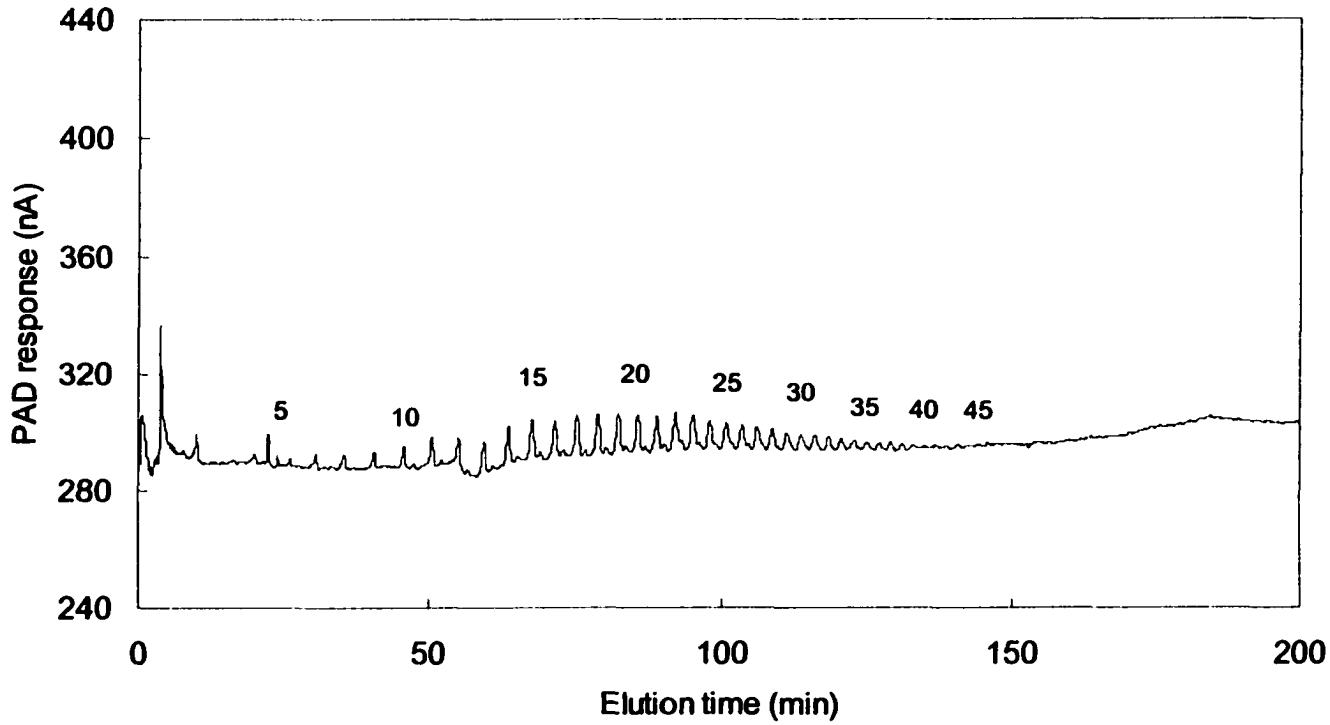

Fig. 6A. Chain-length distribution of the water-soluble glucan fractions from M3.

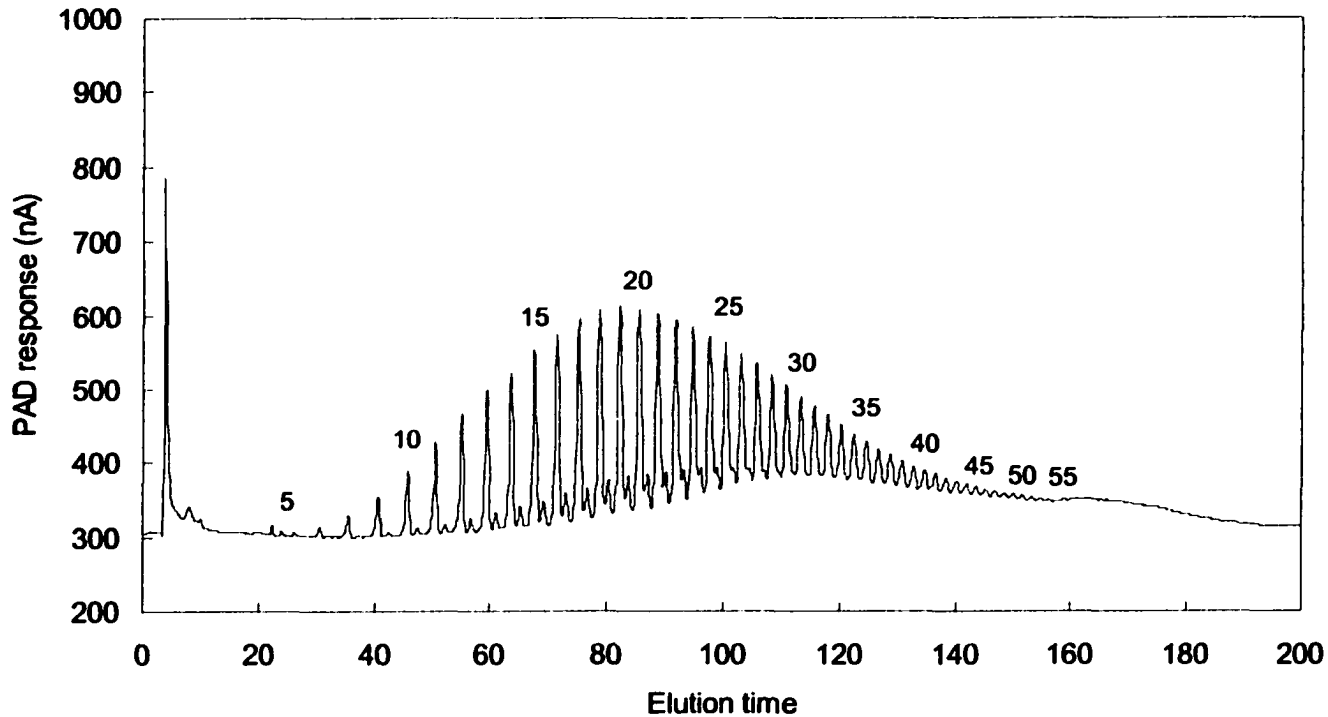

Fig. 6B. Chain-length distribution of the water--insoluble glucan fractions from M3.

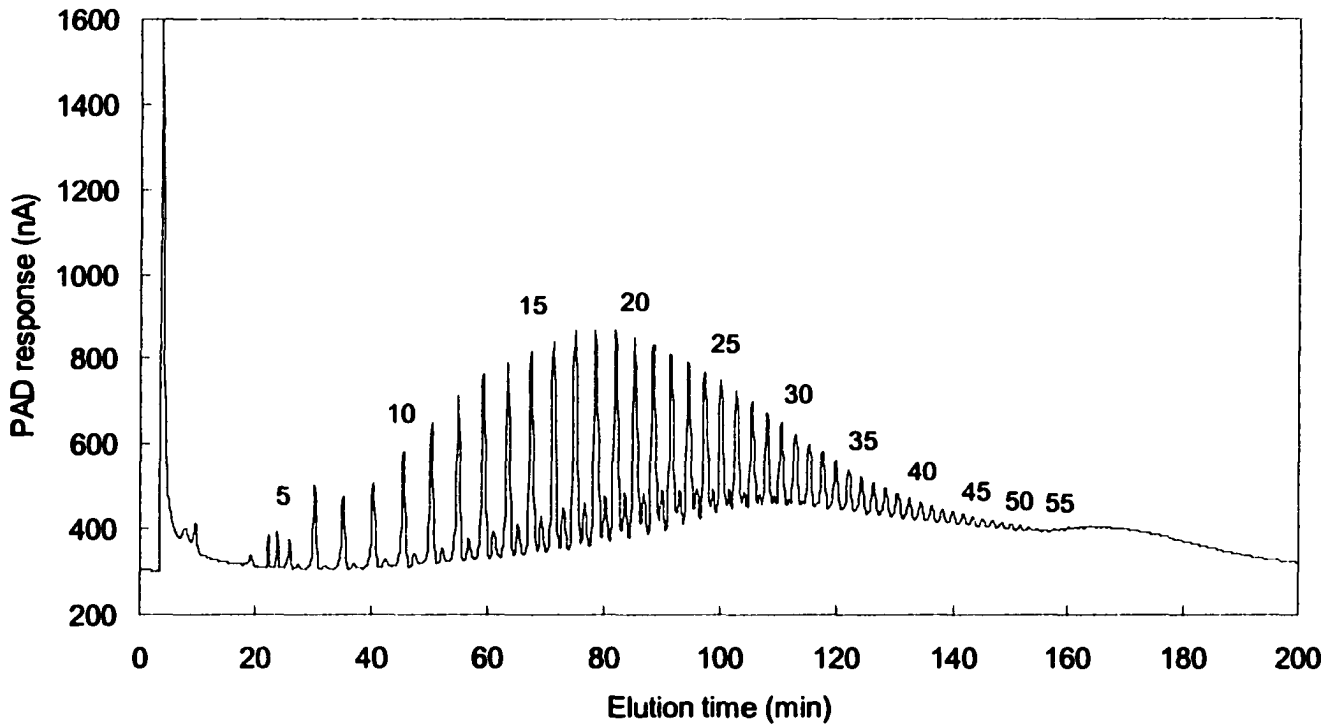

Fig. 6C. Chain-length distribution of the water-insoluble glucan from M3 with isoamylase hydrolysis (48 h, 40°C).

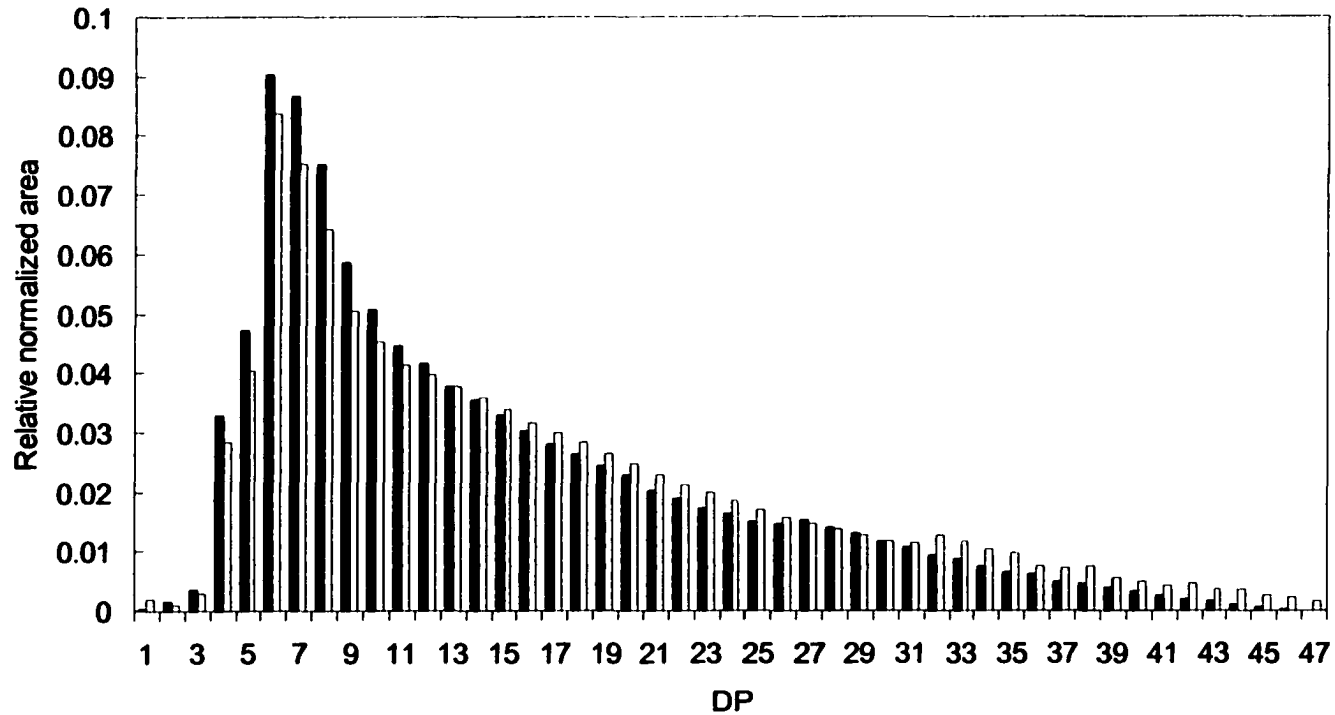

Fig. 7. Branch chain-length distributions of water-insoluble (□) and water-soluble WT-glucans (■). Glucan samples were treated with a debranching enzyme, isoamylase, and the resulting debranched-samples were separated on an HPAEC system. The peak area was calculated and normalized from the chain-profile chromatograms.

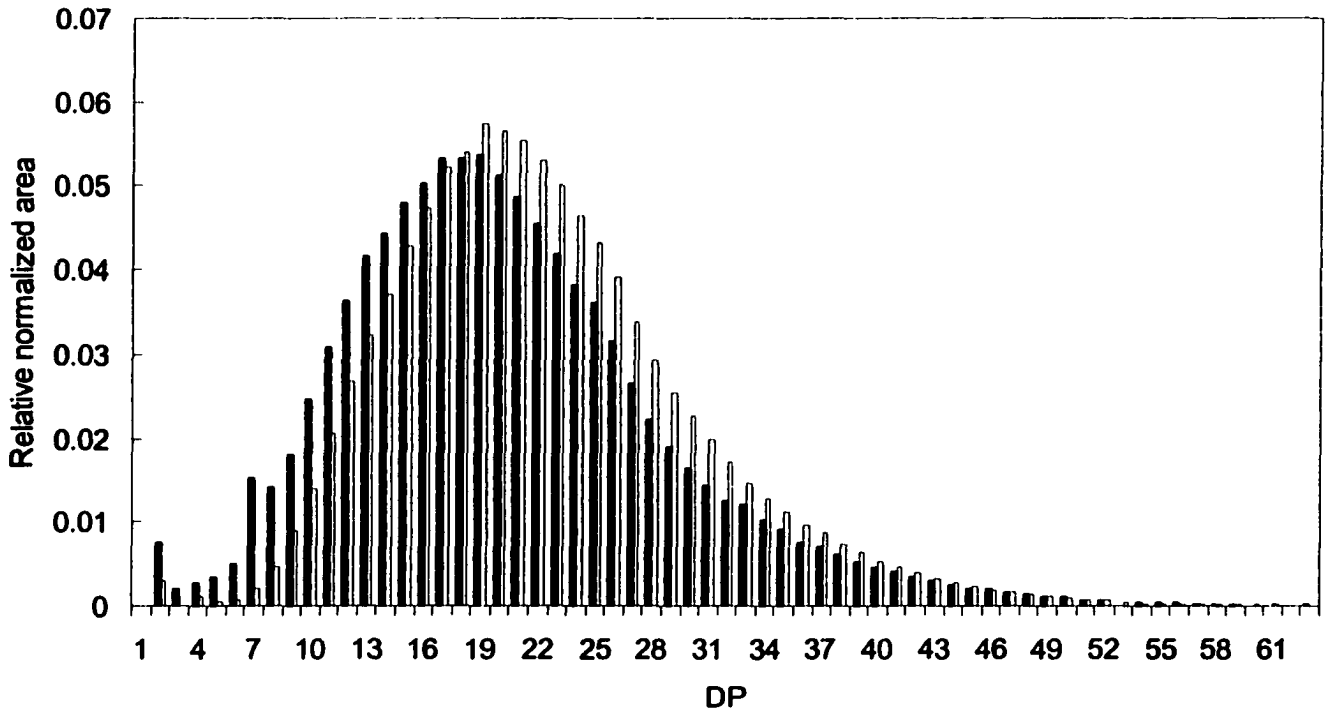

Fig. 8. Chain-length distributions between water-insoluble M3 glucans, either with (■) or without (□) isoamylase treatment. The peak area was calculated and normalized from the chain-profile chromatograms.

GENERAL CONCLUSIONS

Branch structures and physical properties of waxy wheat starch were characterized and compared with those of amylose-reduced (Kanto 107) and normal (Centura and commercial product) wheat starches. Apparent amylose contents were <0.2, 21.5, 26.2, and 26.6%, respectively. The absence of amylose in waxy wheat starch did not affect crystalline pattern (A-type), granule size and morphology, and gelatinization temperature, but increased the degree of crystallinity and changed pasting properties. Differences in pasting temperature and peak viscosity between waxy and normal wheat starches were substantially greater than maize starch counterparts.

The *weight*-average molecular weight (M_w) of the wheat amylopectin (AP) displayed a negative correlation with AM content, whereas the proportion of extra-long branch-chains of wheat AP were positively correlated with AM content. The M_w of APs varied from 7.0×10^7 to 5.7×10^9, depending on botanical source. The APs of waxy starches had larger M_w, and most had larger dispersed molecular densities than did that of normal AP counterparts. It was shown that waxy wheat AP did not contain extra-long chains (ELC) that was synthesized progressively by granule-bound starch synthase I (GBSSI) in normal wheat AP. These results indicated that the APs of waxy starches carried more branch-chains and no ELCs, which resulted in more densely packed molecules than did that of normal AP counterparts. Loss of Wx gene should not significantly affect other enzymes involved in starch biosynthesis. GBSSI is not a primary enzyme for the AP biosynthesis, but small

amount of ELC in normal AP appears to be attributed to GBSSI activity. The existence of ELC in AP of normal starches has been reported for various botanical sources, including wheat. Differences in the amount of ELC detected among waxy, partial-waxy, and normal wheat starches suggest that the GBSSI is directly involved in ELC synthesis of AP molecules. Fewer branches on ELC that is synthesized progressively by GBSSI affect branch-structures and make normal AP molecules less dense during starch biosynthesis. The results also suggest that carbon distribution between AP and AM during starch biosynthesis determines M_w of AP molecule. In waxy mutants, GBSSI is absent and no AM is synthesized. It is plausible that ADP-Glc is exclusively incorporated into AP, resulting in AP with larger M_w in waxy mutants.

Different branch structures of APs from A- and B-type starches resulted in different dispersed molecular densities in dilute solutions. Because more clustered and fewer branch linkages do not allow to organize AP effectively, the APs in B-type starches are less dense when dispersed. Having the same molecular mass, the dispersed B-type AP occupies more space than does the dispersed A-type AP. This is attributed to longer branch-chain length (in other words, fewer branch linkages), which is more extended. The M_w and R_z values of amylose isolated from amylomaize VII starch were also determined to be 2.8×10^5 and 43 nm, respectively.

Individual glycogen synthases (GSI and II) and glycogen branching enzyme (GBE) from *Synechocystis* sp. PCC 6803 strain were disrupted by homologous recombination conferring kanamycin resistance. Significantly different branch-

structures of glycogen were found between GSI$^-$ and GSII$^-$ mutant strains. Each isoform of GS seems to play a different role in glycogen structure. A prominent increase in intermediate size of chains (DP 8-18) was observed from the GSI$^-$ mutant compared to the wild-type and the GSII$^-$ mutant. Products of the two mutants suggest that GSI extends chains in a distributive manner whereas GSII in a more progressive manner. It is not possible to compare the unique functions of each GS isoform of this cyanobacterium with most other bacteria, because other bacteria contain only one GS. The isoforms of GS and soluble starch synthase (SS) has been reported in a couple of unicellular eukaryotes, *Saccharomyces cerevisiae* and *Chlamydomonas reinhardtii*, respectively. The functions of SS isoforms also have been studied in plants, with evidences that they play distinct roles in amylopectin synthesis. The different action patterns of cyanobacterial GS isoforms do not appear to be a result of changes in total GS activities, because no apparent decrease in total GS activity was observed in both GSI$^-$ and GSII$^-$ mutant cells. Thus, the different specificities of GS isoforms on glucan primers could be a more important factor in determining the structure of glycogen in the cyanobacteria. It is also possible that the interaction of GS isoforms among themselves or with other enzymes, such as GBE, affects the degree of elongation as postulated for unicellular eukaryote, *Chlamydomonas reinhardtii*. There is a possibility that GSII exists as a hetero-polymeric form with GSI in WT and the disappearance of GSI causes the change in GSII specificity. It was suggested that GS could exist as dimer, trimer, and tetramer in *E. coli*. In yeast, a hetero-tetramer consisting of two GS isoforms has been reported, with similar molar ratio of the isoforms in a tetramer form.

Our results showed *sll*0158^{-} mutant, deficient in GBE, had retarded glycogen-synthesizing rate, and most glucans accumulated as an insoluble form with negligible amount of branch linkages. The GBE played a role in increasing overall rate of glycogen biosynthesis in the cyanobacterium. We plan to continue controlling GBE activity by regulating the gene expression, and study the roles of GBE/SBE (starch branching enzyme) in glucan structure by expressing plant-derived SBE in cyanobacteria.

Long-term goal of this study is to establish a model system of starch/glycogen biosynthesis in cyanobacteria and to use the model to study individual enzymes involved in starch/glycogen biosynthesis. We intend to modify the structure of native glycogen by replacing endogenous bacterial genes with the plant genes and investigate the development of starch granules by reconstituting plant system of starch biosynthesis in bacteria. As an initial step, we demonstrated the existence of two endogenous GS isoforms and one GBE and characterized their function in the process of glycogen biosynthesis in *Synechocystis* sp. PCC 6803 strain.

ACKNOWLEDGEMENTS

I truly respect my major professor, Dr. Jay-lin Jane, for her enthusiastic attitude to research. Furthermore, I'd like to thank her for letting me pursuing this project. It was not easy to start a new field for both of us, but we trusted each other and accomplished some important results.

I also appreciate the members of my committee, Dr. John Robyt, Dr. Martha James, Dr. Larry Johnson, and Dr. Pam White for their interest in my research and their valuable advice.

Without help from my colleagues, I could not imagine how I would complete my Ph. D. degree: David, Cassie, Mou, Kit-Sum, Perminus, Milica, Andy, Youyoun, and Jingwen. I also thank faculty and staff members of Food Science and Human Nutrition Department for their support and assistance.

Finally, I wish to thank my wife, Ji-Won, my parents, and family for their unfailing love, patience, and support throughout my study.