A study of the impact of mazie SBE I on the [alpha]-polyglucan produced in Synechocystis sp. strain PCC 6803

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A study of the impact of mazie SBE I on the α-polyglucan produced in 
*Synechocystis* sp. strain PCC 6803

by

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A dissertation submitted to the graduate faculty

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This dissertation is dedicated to my Savior and Lord, Jesus Christ, who is the Creator of the starch granule. To Him be the glory for His awesome works. The order and grandeur of creation testifies to His great love and goodness!
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This is where I get the privilege of thanking all those who have helped me through the many years it has taken to complete this dissertation. I am very grateful to Professor Martin Spalding for giving me the opportunity to work in his lab and for tirelessly and patiently guiding me through to the finish line. Professor Spalding had a keen understanding of what was needed to complete the job and kept me focused on carrying out the necessary experiments and writing. Even with a busy schedule he has always been available to guide and direct my work and I am very grateful to him. Professor Jay-lin Jane has been a constant source of encouragement and a wealth of knowledge that I could refer to in working on this project and I owe her a debt of thanks. Professor David Oliver, Professor Paul Scott and Professor Madan Bhattacharyya my POS committee members and Professor Hanping Guan a former-POS committee member, have been very helpful in giving of their time and expertise.

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My husband, Shantha Pieris, and my parents have invested of themselves to help me graduate and I would not have completed my work if not for their prayers, love, and timely help.
Shantha has been my constant friend and helper throughout. My sister and my brother have encouraged me throughout. My two daughters, Anagi and Amila, have been a powerful motivation to help me complete my research. My extended family has also been a loving support to me.

The task of trying to understand, at least in part, a process that may impact granule formation has been challenging and rewarding. I am filled with thankfulness and gratitude to the Creator of the Starch granule, Jesus Christ, for sustaining me through the experiments and the many years of toil that have resulted in this dissertation. How can I completely and fully pen my gratitude to Him? My hope and strength is ultimately founded in Him alone. Everything else pales into insignificance when compared to Him. He holds the key to all knowledge and is graciously allowing us to discover the secrets of His creation. What a wonderful and merciful Creator God He is!
PREFACE

Since this dissertation is a scientific report of what was done and what results were obtained it does not portray, to any extent, my thoughts regarding this research and the processes that I have worked through in completing this research. Therefore, I wanted to take a few minutes to write down some of these things so that if some one, at some point in time, some where in the world does read this dissertation they will come to know more than science and be directed towards Jesus Christ, who is the Creator God of all that is good and who is the author of life and in whom we can have the gift of eternal life.

I am one who has accepted the redemption that Jesus alone can offer to us by His death and resurrection and am one who strives to allow Him to be the LORD of my life. The primary reason that propelled me to enroll in a Ph.D. program in Plant Physiology was to see how God through Jesus Christ has intricately and beautifully created all things with so much order and purpose. (It is interesting to me how the scientific community talks about the ‘structure function relationship’ and sometimes, if not always, misses the point that it is God who has created all things, including man, with a plan and a purpose! Even children have an instinct that says all things have a plan and purpose and do not exist because of random chance which is why they probably ask, at every opportunity, the question ‘Why?’ Children know there is an answer to that question and it is only as we grow older and, unfortunately, ‘seasoned into the lies of this world’ that we put aside thoughts of God being the Creator of all things and exchange it for ideas that give no purpose for the existence of man.) Also, I know that the scientific process of carrying out experiments to understand the world around us is a worthwhile process only because Jesus, the Creator God, is faithful to His creation. So, for instance, day follows night and night follows day and a cell will function under certain principles that will not change from day to day and our efforts at studying these principles will not be in vain. The Bible says in Isaiah 40: 26 ‘Lift up your eyes and look to the heavens: Who created all these? He who brings out the starry hosts one by one and calls them each by name. Because of His great power and mighty strength not one of them is missing.’

Given my desire to study His creation and my propensity to make many turns, I know that Jesus guided me to this research project that was designed to unravel, in the long term, the
mysteries behind the architecture of the starch granule. How amazing He is! To the undiscerning eye it would seem that I just ‘stumbled’ on this research since I changed labs and consequently the research project after almost a year and a half in my Ph.D. program. I had a wonderful Professor who graciously and without any negative feelings allowed me to change to a new lab and who continued to guide me by serving on my POS committee and my current Major Professor, Professor Spalding was willing to accept me. The inception of this project was a collaboration between Professor Spalding and Professor Jane and had funding for approximately a year or so and the fact that I joined Professor Spaldings’ lab so that I could be involved in this project is an act of God in my life since I have committed my life in to His hands.

While starting work on this project was a demonstration of Jesus’ hand of mercy on my life, staying the course and seeing it to an end was a demonstration of the sustaining power of Jesus in my life. There were times when I would pray over the experiments that I did and ask God to guide my hands as I mixed components together in a microcentrifuge tube and ask Him to guide my thinking and my planning. There were times when I had neither the strength nor the motivation to get up from bed and go to the lab and attempt to optimize another ‘failed experiment’! Jesus was my strength and His resurrection power worked in my life through the seemingly mundane duties of everyday life in the lab. Because of His great goodness I also had times when I was pleasantly surprised with good results from experiments. I had little notes posted on my lab bench that read ‘I lift up my eyes to the hills - where does my help come from? My help comes from the LORD, the Maker of heaven and earth’ which comes from the book of Psalms 121 : 1 & 2. The LORD has blessed my husband, Shantha, and myself with two beautiful little daughters, Anagi and Amila and has seen both Shantha and myself through the daunting Ph.D. process. We are so grateful to Jesus Christ for His mercies are new every morning in our lives.

At the end of this Ph.D. process I am more acutely and intensely aware of how science does not have the answers to all scientific quests let alone all of life’s questions. It blew my mind to know that a few decades ago starch and glycogen were regarded by the experts to have the same structure! Today it is readily accepted that they have very different structures. This
simple change in positions cautions me that science does not have all the right answers. Science is only a work-in-progress. As for me, a scientific quest can have great meaning and satisfaction only within the bounds of knowing the Creator God and giving Him praise for His wonderful works and for His graciousness in allowing us to discover how He created and set things in place. I am so grateful to Jesus Christ, my LORD and Savior for giving me the opportunity to understand and study a small portion of His creation, the starch granule, by studying the impact of a starch branching enzyme from maize on the polyglucan produced in *Synechocystis*.

I found an extremely interesting book written about the life of Dr. George Washington Carver, a great and well known scientist who introduced several uses for the peanut and other food crops. I shared the same feeling and anticipation about the starch granule while working in my lab bench as did Dr. George Washington Carver about the peanut. The Bible says in Jeremiah 33:2–3 “This is what the LORD says, He who made the earth, the LORD who formed it and established it—the LORD is His name: ‘Call to me and I will answer you and tell you great and unsearchable things you do not know.’” Dr. George Washington Carver is reported (The books you read, Harrisburg, PA: Executive Books, 1985, p. 132.) to have had the following exchange with the Committee Chairman on January 21, 1921 after an address before the House Ways and Means Committee (George Washington Carver – His Life & Faith in His Own Words by William J. Federer, Library of Congress, AmeriSearch, Inc., MO, 2002).

“Dr. Carver, how did you learn all of these things?”

Carver answered:

“What book?” asked the Chairman.

Carver replied, “The Bible”

The chairman inquired, “Does the Bible tell about peanuts?”

“No, Sir” Dr. Carver replied, “But it tells about the God who made the peanut. I asked Him to show me what to do with the peanut, and He did.”
Praise Jesus Christ, who is God the Son and God the Creator who has made all things good!
In Him alone do I find meaning in pursuing a career in the field of science.
ABSTRACT

Starch is a biopolymer synthesized primarily in plants and is made of $\alpha$-1,4 linked glucose chains with $\alpha$-1,6 glycosidic bonds at branch points. Amylose and amylopectin, the component molecules of starch are essentially linear and 4-5% branched respectively. Amylopectin, thought to be responsible for the semi-crystalline nature of starch, is chemically similar but physically distinct from water-soluble glycogen. Amylopectin is distinct from glycogen in having longer chains, fewer branch points and a clustered branching pattern. The hypothesis that the presence of a branching enzyme that can transfer long branches will facilitate the formation and perpetuation of a cluster branch pattern was tested using the cyanobacterium, *Synechocystis*, by introducing maize starch branching enzyme I (MSBE I) into a recipient strain, M3, that has only residual levels of branching enzyme activity to produce the double mutant SM3. In order to determine the effects of MSBE I on the nature of the glucan produced, the M3 mutant (Yoo et al., 2002) was also further characterized. Results indicate that the transfer of long, branch chains did lead to a molecule that has characteristics that are similar to amylopectin in size, branch chain length distribution, average chain length, solubility, and density characteristics. In addition, the glucan produced in SM3 had the appearance of stacked, plate-like structures when viewed under the transmission electron microscope. The further characterization of the M3 mutant revealed some similar characteristics when compared to SM3 although the data gave indications that the processes that led to these characteristics were different in the two mutants. The effort to produce a background free of glycogen branching enzyme in *Synechocystis* also led to the tentative conclusion that there is an apparent need for glycogen synthesis and that MSBE I cannot fully compensate for glycogen branching enzyme activity.
CHAPTER 1: INTRODUCTION/LITERATURE REVIEW

Biosynthesis

A unique function of photosynthetic organisms is their ability to capture and store light energy in the form of glucose. The light reactions of photosynthesis involve the capture of light energy by pigment molecules and the production of ATP and NADPH for use in the dark reactions of photosynthesis. The dark reactions of photosynthesis, also called the photosynthetic carbon reduction cycle or the calvin cycle, which also occur during the day just like the light reactions, utilize the ATP and NADPH produced in the light reactions along with CO₂ to produce triose phosphates that can be channeled toward the synthesis of transitory reserve starch in the chloroplast or can be transported out, as sucrose, into storage organs where they can be used to synthesize storage starch in amyloplasts.

In storage tissue, sucrose is converted to glucose and fructose in the apoplast by the action of apoplastic invertase or is transported via a sucrose transporter into the cytoplasm where it can be converted to the two hexose sugars by the cytosolic form of invertase. The hexoses produced in the apoplast can be moved into the cytoplasm by hexose transporters. Sucrose can also be broken down by the action of sucrose synthase into fructose and UDP-glucose in the cytoplasm. In the cytoplasm, the hexose sugar, glucose, can be phosphorylated by hexokinase to give glucose-6-phosphate, and fructose can be phosphorylated by fructokinase to give fructose-6-phosphate. Fructose-6-phosphate can be converted to glucose-6-phosphate by glucose phosphate isomerase. Glucose-6-phosphate can be converted to glucose-1-phosphate by the action of phosphoglucomutase. These hexose (glucose) phosphates can be transported, into the plastid by a hexose phosphate translocator or be converted through various reactions to give ADP-glucose which can be transported into the plastid by the action of the ADP-glucose translocator. Glucose-1-phosphate in the cytoplasm can be converted to ADP-glucose by the action of cytosolic ADP-glucose pyrophosphorylase in cereal endosperm tissue or glucose-1-phosphate is transported into the plastid where it is converted to ADP-glucose by a plastidic ADP-glucose pyrophosphorylase. The glucose donor ADP-
glucose, if produced in the cytoplasm can be transported into the plastid by an ADP-glucose translocator. Glucose-6-phosphate can be isomerized into glucose-1-phosphate and then converted to ADP-glucose in the plastid by the action of the plastidic isoforms of the relevant enzymes.

The formation of ADP-glucose is said to be the rate limiting step in starch synthesis. ADP-glucose is utilized by the soluble starch synthases (SSS) and the granule bound starch synthase (GBSS) to add glucose units onto the non-reducing ends of growing chains of glucose. While the action of GBSS in progressively adding glucose units results in the formation of amylose and extra long chains of mature amyllopectin, the action of the soluble starch synthases in distributively adding on glucose units is said to aid in the formation of the highly branched amyllopectin molecule. The starch synthases in general are involved in the formation of \( \alpha-1,4 \) bonds between glucose units. The action of branching enzymes results in the formation of \( \alpha-1,6 \) bonds at the branch points while the action of debranching enzyme results in the cleavage of \( \alpha-1,6 \) bonds. The combined action of the starch synthases, the branching enzymes and the debranching enzymes are said to result in the synthesis of amyllopectin molecules in the plastid.

**Starch and glycogen overview**

Starch

The amyllopectin molecule in starch is an \( \alpha-1,4 \) linked glucose polymer with 4-5 \% of \( \alpha-1,6 \) branch linkages arranged in clusters in amorphous regions that alternate with crystalline regions approximately 9 nm apart. The amyllose molecule in starch is an essentially linear molecule with \( \alpha-1,4 \) linked glucose units and only \( \sim 0.1 \% \) \( \alpha-1,6 \) branch points. Starch in photosynthetic organisms is formed as a transient polysaccharide or as a carbon reserve in storage organs in the organelles known as plastids. Transient starch is formed in chloroplasts whereas storage starch is formed in amylloplasts. Starch is also produced in the cytoplasm of rhodoplast-carrying organisms such as apicomplexa and is then known as floridean starch.
(Ball and Morell, 2003; Coppin et al., 2005). In molecular terms starch is made up of 70 – 80 % amylopectin and 20 – 30 % amylose (although amylose may be absent in floridean starch) (Ball and Morell, 2003). The amylopectin molecule can have a molecular weight of $10^7$ to $10^9$ Da whereas the amylose molecule can have a molecular weight of ~ $10^5$ Da (Yoo and Jane, 2002). Due to its higher order architecture, starch forms insoluble granules. Starch granules can vary in size from 100 nm (Ral et al., 2004) in Ostreococcus tauri to over 100 µM in diameter (Chanzy et al., 2006) and can take on various shapes often thought to reflect the botanical source from which it is obtained.

Glycogen

Glycogen is also a polysaccharide similar in chemical composition to starch in that it also is a polymer of glucose linked by $\alpha$-1,4 glucosidic bonds in the linear chains and $\alpha$-1,6 glucosidic bonds at the branch points. However, glycogen differs from starch by having as much as 7 – 12 % branch points that are randomly arranged, resulting in a molecule that is water soluble. Glycogen does not contain any amylose. Glycogen particles are about 50 nm in diameter (Ball and Morell, 2003). These glycogen particles are found as β particles (20 – 30 nm) or as α particles which are aggregated β particles (60 – 200 nm) (Manners, 1989). The maximum attainable size of glycogen particles is limited because of crowding at the periphery by the glycogen synthesizing enzymes (Melendez et al., 1998). According to mathematical modeling backed by experimentally derived information, glycogen is said to have an optimal size where the maximum possible amount of glucose available to phosphorylase is packed into the molecule to give it the highest density (Melendez et al, 1998). The structure of this optimized glycogen molecule is said to have chains of $\alpha$-1,4 linked glucose units with an average length of 13 glucose units with each chain carrying two branches and chains arranged in 12 concentric tiers (Melendez et al., 1998; Melendez et al., 1997).
Starch structure

Starch can be described at the micron level in terms of sizes of starch granules and in the nanometer level, in terms of semi-crystalline and amorphous growth rings, blocklets, and crystalline and amorphous regions (Vandeputte and Delcour, 2004). At the micron level starch granules can be described in terms of size, shape, distribution and whether simple or compound.

Starch granules can range in size from about 100 nm in *Ostreococcus* (Ral et al., 2004) to about 200 μm in *Phajus grandifolius* (Chanzy et al., 2006) and can take various shapes depending on its botanical origin (Jane et al., 1994; Vandeputte and Delcour, 2004; Jane, 2007). Starch granules have been documented to have spherical, disc, oval, polygonal, dome and elongated rod shapes (Jane, 2006). These shapes can vary depending on botanical origin. Maize is said to have a polyhedral starch granules whereas wheat can have disc shaped A granules and spherical B granules (Jane et al., 1994; Ao and Jane, 2007). The structure of amylopectin molecules in the A and B starch granules of wheat have been shown to differ such that A granules have more B2 chains and B granules have more A and B1 chains, suggesting that the biosynthesis of the granules may also be different (Ao and Jane, 2007). The sizes and shapes of starch granules are determinants of their end use. For instance, the large, disc shaped A granules are used in carbonless copy papers and the small, spherical B granules are used as plastic film filler (Ao and Jane, 2007). Starch granules can also have a unimodal, bimodal or trimodal distribution and can be simple or compound. In rice starch for instance, many polyhedral granules are produced and form a compound granule. In maize, starch granules are simple where only one granule is formed per amyloplast (Vandeputte and Delcour, 2004). In wheat, three size populations, less than 5 μM, 5 to 15 μM and greater than 15 μM, of starch granules are found using image analysis studies (Wilson et al., 2006) although traditionally wheat is thought to have the large, disc shaped A granules and the small, spherical B granules.
The next level of organization in starch structure is alternating semi crystalline and amorphous growth rings. The semi crystalline growth ring can vary in size from 120 nm to 500 nm depending on the botanical source and the amount of carbohydrate available at the time of biosynthesis (French, 1984; Vandeputte and Delcour, 2004). Studies by Cameron and Donald (1992) have shown that the amorphous growth ring could be as thick as the semi crystalline one. The amorphous growth rings are also suggested to contain amylose and less ordered amylopectin (Morrison, 1995). The organization of the granule into growth rings is also proposed to be the difference between starch produced during the day and starch produced at night since these growth rings are lost when wheat and barley are grown under continuous light and temperature. However, in potato tuber, these growth rings are present even when grown under conditions of continuous light (Smith, 2001). Therefore, day/night cycles may not totally account for growth rings.

Microscopic studies by Gallant et al. (1997) led them to propose that the growth rings are composed of blocklets as drawn in Figure 1 below. Blocklets are oval shaped structures with alternating crystalline and amorphous regions and the concept of blocklets was defined to support information derived from images taken by the transmission electron microscope (Gallant et al., 1997). The concept of blocklets is used to bridge the gap between the growth ring levels of organization in the starch granule and the alternating crystalline and amorphous regions of amylopectin. The semi crystalline growth rings generally have larger blocklets (20 – 500 nm) and the amorphous growth rings have the smaller blocklets (25 nm). The presence of blocklets with varying sizes has been shown in starches from various botanical sources (Szymonska and Krok, 2003; Ridout et al., 2003) by use of high resolution microscopic techniques such as the TEM, scanning electron microscope (SEM) and the atomic force microscope (AFM). For instance in potato starch (B type crystallinity), blocklets are larger in diameter (400 – 500 nm) than in wheat starch (A type crystallinity) where blocklets are smaller in diameter (25 – 100 nm).
Figure 1: Diagrammatic representation of a blocklet with alternating amorphous and crystalline regions, first proposed and drawn in Gallant et al., 1997 and redrawn in Myers et al., 2000.

The double helices of amylopectin are grouped into side chain clusters and these side chain clusters are grouped to form blocklets. In the report made by Gallant et al., (1997) the researchers have determined that on average an amylopectin side chain cluster is 10 nm wide and 9 – 10 nm long. Therefore, the diameter of the blocklet is proposed to serve as an indication of the number of side chain clusters present, where a diameter of 20 nm and 50 nm may have 2 and 5 side chain clusters, respectively. It follows that each blocklet is composed of crystalline and amorphous lamellae where the crystalline lamellae are made up of double helical structures present in the side chain clusters of amylopectin. The amorphous lamellae are proposed to be the regions where the branch points are clustered in a non-random arrangement. The amorphous and crystalline lamellae together span a 9 nm distance which is universal irrespective of the botanical source of starch (Jenkins et al., 1993).

It has also been proposed that the crystalline lamellae comprised of double helical structures are twisted into super-helical structures in potato starch (Oostergetel and van Bruggen 1993). These super-helical structures are said to have a width of 4 – 5 double helices with 18 – 40 double helices in a pitch of 9 nm (Waigh et al, 1999) and may be limited laterally into blocklets (Gallant et al., 1997).
A more recent model describes starch to be a side-chain-liquid-crystalline-polymer (SCLCP) with a flexible backbone, rigid units (also known as mesogens) and flexible spacers (Waigh et al., 2000). This model shows that in the presence of the plasticizer water, starch is in the smectic state where the mesogens are very well aligned together and on dehydration starch goes into a glassy nematic state.

The length of the branch chains in the amylopectin molecule gives starch granules a characteristic A-type (in cereal starches such as rice and maize), B-type (in tuber and root starches such as potato and tapioca respectively) or C-type (in leguminosae starches such as pea) X-ray diffraction pattern (Hizukuri, 1985). The packing of double helices in A- and B-type starches gives a monoclinic and hexagonal unit cell respectively where as the C-type starch is a mixture of the A- and B-type unit cells (Jane, 2006). The A-type starch is also easily digestible where as the B- and C-type starches are more resistant to enzyme hydrolysis (Jane, 2006).

Amylopectin structure

The amylopectin molecule has been subjected to investigation for many decades. This molecule was shown to have A chains that were linked only by their reducing ends to the C6 position of other chains, B chains that carried one or more other chains and were also linked by their reducing end to other chains and C chains that had a free reducing group (Peat et al., 1952). The development of methods to determine the ratio of A to B chains helped to decide between some of the early models proposed, individually, by Haworth, Staudinger and Meyer for amylopectin (Manners, 1989). An illustration of these early models is given in Figure 2 and show that the Haworth model would have an A:B ratio of 0, the Staudinger model would have an A:B ratio of infinity and the Meyer model would have an A:B ratio of 1. The experimental determination of the A:B ratio in amylopectin being greater than 1 was used to accept the Meyer model over the others proposed at that time. The multiply branched tree-like arrangement of chains seen in the Meyer model was deduced from the stepwise degradation of exterior chains by phosphorylase and amylol-1,6-glucosidase (Larner et al.,
1952). Subsequently the cluster model was developed to explain amylopectin structure (Nikuni, 1969; French, 1972). The polymodal chain length distribution of amylopectin chain lengths demonstrated by Hizukuri (1986) further clarified this model. The cluster model proposed for amylopectin is able to account for its semi crystalline nature, high viscosity and resistance to enzyme degradation (Manners, 1989).

Figure 2: Early models used to explain amylopectin structure. Haworth, Staudinger and Meyer models are as depicted in Manners (1989).
Enzymes involved in glycogen metabolism in *Synechocystis*

Synthesis/Anabolism

The core enzymes necessary for the synthesis of the glycogen polymer are ADP-glucose pyrophosphorylase, glycogen synthase, glycogen branching enzyme and glycogen debranching enzyme (Ball and Morell, 2003). Based on a search of the genome sequence site for *Synechocystis* ([http://www.kazusa.or.jp/cyanobase/Synechocystis/](http://www.kazusa.or.jp/cyanobase/Synechocystis/)) there are two glycogen synthases, one branching enzyme, one ADP-glucose pyrophosphorylase and two debranching enzymes. The gene product of *sll0158* (1,4-alpha-glucan branching enzyme) is involved in producing -1,6 linkages by the cleavage of an –1,4 glucosidic linkage in a pre-existing chain and the transfer of the reducing end of the cleaved chain to the C-6 position on the remaining segment of the chain. This enzyme is solely or in part responsible for the branch chain length distribution seen in glycogen with a peak DP of 6 (Yoo *et al*., 2002).

Genes *sll0945* and *sll1393* code for glycogen synthases whose functions are the addition of single glucose units onto the non-reducing ends of glucose chains by the formation of an –1,4 linkage. Bacteria generally have only one form of glycogen synthase, although cyanobacteria such as *Synechocystis* have two isoforms of glycogen synthases. The glucose donor in *Synechocystis* is ADP-glucose. The gene product of *slr1176* (glucose-1-phosphate adenylyltransferase, also known as ADP-glucose pyrophosphorylase or AGPase) is the enzyme involved in the rate limiting step producing ADP-glucose from glucose-1-phosphate and ATP. Like all bacteria, *Synechocystis* also uses ADP-glucose as the activated form of glucose in the synthesis of glycogen, although this is in variance to animals and fungi where UDP-glucose is used as the glucose donor in glycogen synthesis (Guan and Keeling, 1998).

In *Synechocystis*, AGPase is a homotetrameric enzyme, unlike in higher plants and green algae where AGPase is a heterotetramer with large and small subunits. *Synechocystis* and higher plant AGPase enzymes also are regulated differently. AGPase is allosterically activated by PGA and inhibited by Pi in higher plants, green algae and cyanobacteria such as *Synechocystis* whereas in bacteria, the homotetrameric AGPase is activated by fructose-1,6-
bisphosphate, fructose-6-phosphate and pyruvate and inhibited by AMP and ADP (Miao et al., 2003; Iglesias et al., 1991).

Breakdown/catabolism

Breakdown or catabolism of glycogen generally requires the concerted action of glycogen phosphorylase, debranching enzyme, $\alpha$-1,4 glucanotransferase and malto-dextrin phosphorylase (Ball and Morell, 2003). In *Synechocystis*, two isoforms of glycogen phosphorylase, three types of debranching enzymes and one 1,4-$\alpha$-glucanotransferase are present, and, interestingly, *Synechocystis* seems to lack glucosidases and amylases (Ball and Morell, 2003 and [http://www.kazusa.or.jp/cyanobase/Synechocystis/](http://www.kazusa.or.jp/cyanobase/Synechocystis/)). Glycogen phosphorylases release glucose units as glucose-1-phosphate from the non-reducing end of the exterior chains of glycogen. They have been shown to act only on exterior chains with lengths greater than four glucose units. Three types of debranching enzymes are present in *Synechocystis*, including the gene products of *sll0842* (neopullulanase), *slr0237* (glycogen operon protein GlgX homolog) and *slr1857* (isoamylase). Of these three enzymes the glgX type of debranching enzyme is able to hydrolyze, very specifically, only those branch points that link an exterior chain with four glucose units (Ball and Morell, 2003).

**Enzymes involved in starch/glycogen biosynthesis**

1) Branching enzymes

Branching enzymes ($\alpha$-1,4 glucan: $\alpha$-1,4 glucan 6 glucosyltransferase, EC 2.4.1.18) are responsible for cleaving an $\alpha$-1,4 glucosidic bond and forming an $\alpha$-1,6 bond in starch and glycogen synthesis. Glycogen branching enzyme (GBE) is involved in the formation of glycogen in cyanobacteria, bacteria, yeast and mammals and starch branching enzyme (SBE) is involved in the formation of starch in algae and higher plants. Branching enzyme is important in that it is thought to be involved in determining amylopectin structure by the
different isoforms having different substrate and chain transfer specificities, as revealed in the study of mutants in various species that have no or reduced branching enzyme activity.

Branching enzymes belong to the structurally related superfamily of amylolytic proteins, along with cyclodextrin glucanotransferases, α-amylases and debranching enzymes (Jespersen et al., 1993), and contain the signature (beta/alpha)$_8$ barrel domain in their catalytic regions (Burton et al., 1995). Housed within these conserved sequences in all branching enzymes are, according to the consensus sequence numbering, Asp 474, Glu 536 and Asp 605 which are the residues participating in the catalysis of the α-1,4 bond and His 404 and His 604 which are the residues that bind to the glucosyl residue on the non-reducing side of the glucan bond to be cleaved in the substrate (Burton et al., 1995).

Many isoforms of branching enzymes have been characterized in different species. Biochemical studies on these isoforms have revealed the presence of two classes of branching enzymes, for instance, in maize (Boyer and Preiss, 1978). A more recent study by Burton et al., (1995) has placed branching enzymes in one of two classes according to sequence similarities, and these are class A and class B. Those that fall into class A are pea SBE I, maize SBE II, rice SBE II and potato SBE II and those that belong to class B are pea SBE II, maize SBE I, rice SBE I and potato SBE I. There are two isoforms of branching enzymes in Class A known as SBE IIa and SBE IIb in cereals such as maize and rice whereas only a single form has been identified in dicotyledonous plant tissue such as pea embryo (Tanaka et al., 2004). The presence of extra amino acids in the N terminal region characterizes those that are in class A, and the presence of a C terminal extension characterizes class B branching enzymes. Also, the comparison of sequences of branching enzymes from various sources showed that BEs that belonged to the same class and across species showed greater homology among each other than those that were across classes and in the same species (Burton et al., 1995). Branching enzymes that belong to class B are known to transfer longer chains than those that belong to class A (Guan et al., 1997; Rydberg et al., 2001). The expression of branching enzymes have been shown to change with developmental stage, such that pea SBE II, maize SBE I and potato SBE I belonging to class
B are more highly expressed in the later stages of development than their counterparts belonging to class A (Burton et al., 1995; Gao et al., 1996; Jobling et al., 1999). The expression of branching enzymes of these classes also shows tissue specificity, in that SBE IIa in maize is more highly expressed in leaves than in maize endosperms, and potato SBE II also is expressed more highly in leaves than in potato tubers. SBE IIb is more specifically expressed in the endosperm of cereals such as maize and rice (Takanka et al., 2004). In rice, of these two classes of branching enzymes, BE I is present in greater amounts (~60%) than the BE II isoforms (~20% of each) when measured by the phosphorylase a stimulation assay (Yamanouchi and Nakamura, 1992). Branching enzymes also are present in excess. Therefore, the many isoforms of branching enzymes that fall into two classes according to their biochemical characteristics, as well as their sequence similarities, also seem to show specificities both in the type of tissues where they are expressed and in the timing of their expression, and some of these specificities in the different isoforms have been related to their contribution in determining amylopectin structure (Burton et al., 1995; Guan et al., 1995; Jobling et al., 1999).

Since the presence of isoform specificities are thought to play an important role in determining amylopectin structure, we have used maize SBE I in our effort to use *Synechocystis* as a model system to study starch granule development. Before the study of mutants, maize SBEs from endosperm had been purified and biochemically characterized (Boyer and Preiss, 1978). These enzymes were further characterized with respect to their substrate preferences and the lengths of chains transferred using both native enzyme purified from maize kernels (Guan & Preiss, 1993) and recombinant enzymes (Guan et al., 1994). The glucan produced by the heterologous *E. coli* system expressing maize SBEs (Guan et al., 1995) and the minimum chain lengths required in substrates for maize SBEs and *E. coli* GBE action (Guan et al., 1997) were also studied. The glucans produced by expression of maize SBEs in a heterologous yeast system has also been studied (Seo et al., 2002).

Maize SBE I and II were separated using DEAE-cellulose chromatography, had a molecular weight of 89 kDa and 80 kDa respectively and were found to be monomeric (Boyer and
Preiss, 1978). Branching enzyme I represented 24% of the total activity based on phosphorylase a stimulation assay, 74% of the total activity based on the branching of amylose and had a lower $K_m$ for amylose. Maize SBE IIs were separated into two fractions using a 4-aminobutyl-Sepharose column and were very similar to each other, except in branching amylopectin, where SBE IIb had greater activity than SBE IIa (Boyer and Preiss, 1978). The optimum pH of all three isoforms is 7.5 but the temperature optima are 33, 25 and 15-20°C for SBE I, IIa and IIb, respectively (Takeda et al., 1993). The specific activity according to the branch linkages formed with reduced amylose as the substrate was highest for maize SBE I, even though it was the lowest according to the phosphorylase a stimulation assay. Maize SBE I has a lower $K_m$ for reduced amylose, with average chain length, 405, and has a higher $K_m$ for reduced amyloses of shorter average chain lengths compared to maize SBE IIa and IIb. Differentiation of maize SBEs purified from maize kernels revealed that maize SBE I is more active with amylose than with amylopectin and transfers longer chains in comparison to maize SBE II (Guan and Preiss, 1993). In other words, SBE I showed a greater affinity for amylose and its rate of branching amylopectin was less than 5% of its rate of branching amylose (Guan and Preiss, 1993). Also, the rate of SBE II branching amylose was only 9 – 12% of that of SBE I, and SBE II had a 6-fold greater activity in branching amylopectin than SBE I (Guan & Preiss, 1993).

The same characteristics were seen with recombinant enzymes expressed in *E. coli* (Guan et al., 1994). The study of the glucans produced by *E. coli* mutants expressing maize SBEs in a background lacking its native GBE showed that SBE II may be involved in production of short A chains of DP 6-9 and that SBE I may be involved in production of B chains of DP>14 (Guan et al., 1995). Further characterization of maize SBEs showed that maize SBE I, SBE II and *E. coli* GBE could branch chains with a minimum length of DP 16, 12 and 12 respectively (Guan et al., 1997). This paper also showed that maize SBE I produced chains of DP 4 – 15 with very few short chains of DP 3 – 5 and most of the chains of DP > 10, maize SBE II produced chains of DP 3 – 11 with a greater proportion of short chains of DP 3 – 5 and the most abundant being DP 6 – 7 and *E.coli* GBE produced chains of DP 3 – 11 with a greater proportion of short chains of DP 3 – 5 according to the plots obtained between 4
and 6 hours of incubation with reduced amylose (Guan et al., 1997). Maize SBEs expressed in an yeast heterologous system showed that SBE I was active, according to the phosphorylase a stimulation assay, only when expressed with maize SBE IIa or IIb and that expression of SBE I in the presence of SBE IIa and SBE IIb resulted in a change in the structure of the glucan produced (Seo et al., 2002). The requirement for the presence of SBE IIa or IIb to allow action of SBE I was not seen when these enzymes were expressed singly in E. coli (Guan et al., 1995).

SBE isoforms from potato have also been expressed in E.coli, isolated and studied. The substrates used were limit dextrins with chains lengths ranging from DP 8 to DP 200. When SBE I was incubated for 16 hours with this substrate and the resultant branched glucan debranched and analyzed, the new population of chains showed sharp peaks around DP 11 – 12 and DP 29 – 30. When SBE II was incubated the resulting population of chains had a broader peak around DP 13 – 14. In both cases there was an increase in chains of DP 6 – 9 and they were present in decreasing concentrations relative to each other such that DP 6 was present in greater concentration than DP 7 and so on (Rydberg et al., 2001). These results were in agreement with the finding that branching enzymes belonging to class B (potato branching enzyme I and maize branching enzyme I) transfer longer chains than branching enzymes belonging to class A (potato branching enzyme II and maize branching enzyme II).

GBE from E. coli showed a range of chains transferred from DP 5 – 16 with a peak at DP 10 and very few chains with DP > 20 (Guan et al., 1997). The GBE enzyme in Synechocystic sp. Strain PCC 6803 in the presence of the whole complement of starch synthesizing and degrading enzymes, gives a branch chain length distribution of DP 4 – 50 with a peak at DP 6 (Yoo et al., 2002).

Mutants affecting SBE activity are known for pea at the rugosus (r) locus which codes for SBE I (class A) and in maize and rice at the amylose extender (ae) locus which codes for SBE IIb (class A) (Bhattacharyya et al., 1990; Stinard et al., 1993; Nishi et al., 2001), and in these mutants amylose content is increased and amyllopectin has an increased average chain
length (Baba and Arai, 1984; Nishi et al., 2001). The ae mutant, which lacks SBE IIb, in rice, shows a decrease in chains less than DP 17 with the greatest decrease in chains of DP 8 – 12 that has led the researchers to propose that SBE IIb is involved in the production of A chains that are present within a single cluster (Nishi et al., 2001). Gene dosage experiments showed that when SBE IIb was present in greater amounts, the proportion of chains less than DP 17 increased while the activity of the other forms of SBEs, SBE I and SBE IIa, remained unchanged. This strongly suggests that SBE IIb is involved in the production of A chains with DP less than 17 and that this function cannot be complemented by either SBE I or SBE IIa (Nishi et al., 2001; Nakamura 2002). The effect of the lack of SBE IIb on chain length distributions reveals that SBE IIb function is involved in the determination of the fine structure of amylopectin. The introduction of SBE IIb into the ae mutant in rice gave transformants that showed varying degree of SBE IIb activity, which directly correlated with chain length distributions and gelatinization temperatures. When SBE IIb activity was high there were more of short chains of DP less than 13 and a lower gelatinization temperature compared to the case when SBE IIb activity was low and led to a decrease in short chains of DP less than 13 and a higher gelatinization temperature (Tanaka et al., 2004). When SBE IIb levels exceeded wild type levels, a water soluble polysaccharide was accumulated. These results further confirmed that SBE IIb was a determinant in the fine structure of the amylopectin molecule.

In a maize mutant lacking SBE I (Blauth et al., 2002), there were no changes in chain length distribution compared to WT suggesting that there may be no effect of SBE I in determining the fine structure of amylopectin. Subsequently it was hypothesized that SBEIIb is predominant to SBE I (referred to as SBE Ia in this paper) and therefore functional effects of SBE I can be determined only in the absence of SBE IIb (Yao et al., 2004.) This was tested by looking at the difference between the mutant pairs sbe1a wx and wx and sbe1a ae wx and ae wx (Yao et al., 2004). The authors reported that changes in chain length profiles were distinguishable only between sbe1a ae wx and ae wx where the difference is the absence or presence of SBE I in a background lacking SBE IIb. The data regarding chain lengths tabulated and described in this paper shows that when SBE I is absent in a background
lacking SBE IIb, short chains increase and long chains decrease, the ratio of intercluster B (ie. B2 + B3) chains to intracluster B (ie., B1a + B1b) chains decreases and the proportion of B3 chains decreases. The reasoning laid out by the authors focused, however, on the increase in branching density between *sbe1a ae wx* and *ae wx* and suggested that SBE I may be inhibiting the action of SBE IIa *in vivo* (Yao et al., 2004). In essence, the data described in this paper may support studies showing the transfer of long chains by maize SBE I which is an enzyme belonging to class B of branching enzymes.

In antisense mutants of potato SBE I, changes of the physical properties of the tuber were observed when expression levels were reduced below a threshold, although no changes were detected in branch chain length distributions (Stafford et al., 1998). In addition, a rice mutant, *sbe1*, lacking SBE I did show changes in chain length distributions (Satoh et al., 2003). The lack of SBE I in this mutant caused a reduction in long chains of DP greater than 37 and a reduction in chains with DP 12 – 21. This result is taken as support for the hypothesis that SBE I is involved in the production of long B1 chains and B2-B3 inter-cluster chains (Satoh et al., 2003). These reports of *ae* and *sbe1* mutants in rice have been used to propose that SBE IIb is involved in producing A chains while SBE I is involved in producing B1 chains and cluster-connecting B2 and B3 chains. The reports by Satoh et al., (2003) and Tanaka et al., (2004) also showed that even the total reduction in SBE I caused only a slight change in chain length distribution, while even a slight change in SBE IIb caused a greater change in chain length distribution. The *sbe1* mutant and the *flo2* mutant, which has ~ 10 % levels of SBE I activity, in rice and the antisense mutant in potato considered together, suggest that SBE I has to be reduced almost completely in order to see an impact in chain profile (Kawasaki et al., 1996; Yamanouchi and Nakamura, 1992; Stafford et al., 1998).

**Alpha-amylase superfamily**

About 25,000 different enzymes are known in nature although they fit into only about 1,000 topological motifs. Therefore, there are examples of enzymes that are similar in structure and yet unrelated in function (Kuriki and Imanaka, 1999). Structural similarities have been noted
among α-amylase (Matsuura et al., 1984), cyclodextrin glucanotransferase (CGTase; Klein and Schulz, 1991) and isoamylase (Katsuya et al., 1998) and glycogen branching enzyme (Abad et al., 2002) by X-ray crystallographic analysis. Sequences of these four types of proteins, the α-amylase, cyclodextrin glucanotransferase, isoamylase and branching enzyme have four conserved sequences (Kuriki and Imanaka, 1989). Within these conserved sequences, amino acid residues involved in catalysis are conserved, which led researchers to propose that the catalytic process of these different enzymes followed a common mechanism (Kuriki and Imanaka, 1989). The discovery of neopullulanase, which could carry out all of the four reactions, α-1,4 and α-1,6 hydrolysis and α-1,4 and α-1,6 tranglycosylation, combined with the finding that the replacement of the conserved residues corresponding to Asp-206, Glu-230 and Asp-297 of Taka-amylase A (the α amylase from Aspergillus oryzae) led to the loss of activity at both α-1,4 and α-1,6 sites, served as evidence that just one active center is present in this enzyme. This also showed that, since there was only one active site, the mechanism of catalysis of all the four different reactions should be the same (Takata et al., 1992). Therefore, the α-amylases, CGTases, isoamlyases and BEs share similarity in structure as well as similarity in catalytic mechanism. Hence, enzymes are placed in this α-amylase family based on similarity in structure and catalytic mechanism. In order to classify an enzyme to this group the following four conditions must be satisfied: 1) it acts on α-glucosidic linkages 2) it either hydrolyzes or transglycosylates α–glucosidic linkages 3) it should have four highly conserved regions it its protein sequence 4) it should have Asp, Glu and Asp residues as catalytic sites corresponding to the specific ones in Taka-amylase A (Kuriki and Imanaka, 1999).

Possible Mechanism of branching and effect on degree of branching

Studies have been conducted to investigate the mechanism of branching by using potato BE, also known as Q enzyme (Borovsky et al., 1975; Borovsky et al., 1976). It was shown by these researchers that Q enzyme breaks an α-1,4 bond in a donor chain and forms an α-1,6 bond in an acceptor chain by an inter-chain mechanism, although the possibility of intra-chain transfer was not excluded, and that the breaking of the α-1,4 bond occurs in an endo
acting random fashion (Borovsky et al., 1976). Since the minimum length of chain necessary to act as substrate for Q enzyme is 40 glucose units, it was suggested that some type of secondary or tertiary structure of the glucose chains may be a prerequisite to facilitate enzyme action. A substrate for Q enzyme of two chains forming a double helix was proposed and was confirmed by showing that the minimum length of chain required for branching action was not an absolute number but rather decreased with a decrease in temperature (Borovsky et al., 1975; Borovsky et al., 1979). In contrast to the early work done by potato BE, a recent report shows that branching must have occurred by a intra-chain transfer since the molecular weight of the products did not change, when different substrates were used (Rydberg et al., 2001). The authors suggest that external factors such as the phosphorylation of substrates may have an impact on whether inter or intra chain transfer takes place in the formation of branch linkages.

Reports comparing the polysaccharide synthesized \textit{in vitro} by SBEs (from potato and maize) and GBEs from (rat and rabbit) with their native polysaccharides showed that the BE alone, and not a ratio of BE to the synthase was responsible for the degree of branching (Tolmasky and Krisman, 1987). In this report it was shown that the SBEs introduced a lower degree of branching than the glycogen branching enzymes.

2) Starch synthases

Starch synthases can fall into one of five categories, which are granule bound starch synthase (GBSS), soluble starch synthase (SSS) I, IIa, IIb and III (James et al., 2003; Delvalle, 2005), depending on sequence similarities. The highest degree of sequence similarity is said to be in a 60 kDa region on the C-terminus which comprises the catalytic region, while the N-terminus remains variable (Kossmann and Lloyd, 2000; Commuri and Keeling 2001). These isozymes vary with respect to molecular weight, dependence on the presence of primer, Km values for different acceptors and antigenic properties (Kossmann and Lloyd, 2000). Mutations in the GBSS in, e.g., maize, rice, \textit{Chlamydomonas} etc., result in the synthesis of amylose-free or amylose-reduced starch. In cereals such as maize the amylose free mutants
are known as waxy mutants whereas in potato and pea they are known as \textit{amf} (amylose free) and \textit{lam} (low in amylose) mutants respectively (Kossmann and Lloyd, 2000). GBSS also has been reported to add glucose units processively without dissociation from the chain (Denyer \textit{et al.}, 1999). Analysis of starches from mutants lacking GBSS have shown the absence of extra long chains in amylopectin (Yoo and Jane, 2002). These results taken together indicate a role for GBSS in the synthesis of amylose and also the synthesis of extra long chains in amylopectin.

The SSSs are found entirely or primarily in the soluble phase, and different combinations of these are found in different plant species (Myers \textit{et al.}, 2000). For example, the dominant SSS in maize is SS I, in pea embryo is SS II and in potato is SS III (Myers \textit{et al.}, 2000). SSSs are known to add glucose units distributively, and studies with mutants lacking one or more starch synthases show that there are isoform specificities that are not simply additive in nature (Smith, 2001). The carboxy terminus of SS I from maize was shown to be important in starch binding (Commuri and Keeling, 2001). This study showed that SS I is primarily responsible for the synthesis of the shortest chains (DP <10). The affinity for glucan chains increased as chain length increased and catalytic activity decreased as chain length increased. These results have been used to propose that SS I in maize may be involved in the synthesis of shorter A and B1 chains (Commuri and Keeling 2001). The study of a mutant line of SS I in \textit{Arabidopsis thaliana} also suggests that this isoform is involved in the synthesis of short A and B1 chains that comprise the single cluster in amylopectin in leaves (Delvalle et al., 2005). The \textit{indica} and \textit{japonica} varieties of rice differ in having the L and S type starches, respectively. This difference has been attributed to the absence of SS IIa, leading to the increase of short chains (DP ≤10) and a decrease in intermediate-size chains (DP 13 -22) in \textit{japonica} rice varieties (Umemoto et al., 2002).

3) Starch debranching enzymes

Two types of DEs are known. These are the isoamylases and pullulanases. Isoamylases and pullulanases both cleave α-1,6 bonds. Substrate specificities allow isoamylase to be more
active with denatured amylopectin and glycogen molecules, and with molecules derived from their partial hydrolysis (limit dextrans). Pullulanases are active with pullulan, which is a polymer with \( \alpha-1,6 \) linked maltotriose units, and limit dextrans, and show little or no activity with denatured amylopectin and glycogen (Myers et al., 2000).

Mutations in the isoamylase type DEs in maize (James et al., 1995), in *Chlamydomonas* (Moille et al., 1996) and also in *Arabidopsis* (Zeeman et al., 1998), prevent starch accumulation to normal levels and cause, in addition to the accumulation of starch, the accumulation of phytoglycogen, which is a highly branched (twice as much as amyllopectin) glycogen-like molecule with more short chains and no long B chains. This serves as evidence that DE may be involved in amyllopectin biosynthesis (Myers et al., 2000). Two models have been proposed to account for isoamylase function in amyllopectin biosynthesis: the glucan trimming model, where improperly placed branches are cleaved from a preamyllopectin molecule (Myers et al., 2000), and, secondly, removal of water soluble polysaccharide that can accumulate at the expense of amyllopectin (Zeeman et al., 1998).

4) Disproportionating enzyme

D enzymes (disproportionating enzyme) catalyze the transfer of a segment of one linear chain to another by the breaking and forming of one \( \alpha-1,4 \) bond. Mutations in this gene (*STA11*) in *Chlamydomonas* cause a loss of D enzyme, leading to a decrease in amyllopectin, where the amyllopectin structure also is altered to have a higher frequency of short chains (Colleoni et al., 1999). However, a potato mutant expressing an antisense gene of D enzyme showed no obvious changes in starch biosynthesis (Takahara et al., 1998). A T-DNA insertion in the D enzyme in *Arabidopsis* showed no alteration in the structure of amyllopectin (Critchley et al., 2001). Therefore, D enzymes may have a role in amyllopectin biosynthesis, though this has to be verified with evidence from mutants of D enzyme in other species.
Current models explaining granule formation and our hypothesis

Granule formation considered in a simplistic manner may involve granule initiation, synthesis of the two polymer molecules amylose and amylopectin and their orderly arrangement into granules. The glycogenin protein can self glucosylate and add up to seven glucose units onto its Tyr (194) residue to produce a primer needed to initiate glycogen synthesis in animals and fungi (Guan & Keeling, 1998). A protein similar in function to glycogenin in glycogen synthesis has not been identified for starch synthesis. However, bacteria do not require a protein such as glycogenin to initiate glycogen synthesis so that it may be possible that such a protein is not necessary for starch synthesis (Smith, 2001). Amylose is known to complex with fatty acids and phospholipids (Jane & Robyt, 1984) and these, instead of a glycogenin-like-protein may serve as nucleation centers in starch synthesis. More evidence supporting complex formation between amylose and lipid molecules lie in the finding that sufficient amounts of lipids, that could serve as potential nucleating agents, are found in chloroplasts and that starch granules are found in close proximity to membranes. The amylopectin molecule is shown to have repeating crystalline and amorphous regions that are 9 nm apart. Therefore, the orderly arrangement of amylopectin into higher order structures is what gives starch granules their semi-crystalline characteristic. Therefore, in order to explain the semi crystalline nature of starch, many models have been proposed to explain the synthesis of amylopectin. Some of the current models used to describe amylopectin synthesis are 1) a physical process 2) the glucan trimming model 3) protein-protein interaction and 4) the water soluble polysaccharide clearing model 5) two-step branching and improper branch clearing model (proposed by Nakamura Y, 2002).

1) A physical process

Once granule initiation is completed, the formation of crystalline and amorphous regions in amylopectin giving rise to the semicrystalline nature of starch is suggested to be more a physical rather than a biological process (Smith, 2001). The argument that this may be a physical process is supported by the fact that all mutations identified to date that cause an
effect on the structure of starch lie in genes encoding starch synthase, SBE, isoamylase or D enzyme and none in proteins that serve a specific function in organizing carbon chains (Smith, 2001). Coordination may be required between the physical process at the granule surface allowing self assembly of chains into ordered lamellae and the biological process involving the starch synthesizing enzymes in determining the semicrystalline nature of amylopectin in granule formation (Smith, 2001; Thompson, 2000).

2) The glucan trimming model

Currently one of the well accepted models put forward to explain the biological processes surrounding amylopectin biosynthesis is the glucan trimming model, which highlights the role of DBEs. This model suggests that selected branches are removed by DBEs from a precursor called preamylopectin, converting it to amylopectin (Myers et al., 2000). Evidence for this model comes from the fact that mutants lacking an isoamylase type DBE produce phytoglycogen, which has a greater degree of branching (Myers et al., 2000).

3) Protein-protein interaction

Phosphorylation was shown to increase starch synthesis when amyloplasts and chloroplasts were incubated in the presence of ATP (Tetlow et al., 2004). This paper also reported the formation of a protein complex made up of SBE I, SBE IIb and starch phosphorylase when the proteins were phosphorylated, suggesting some impact on starch synthesis due to protein-complex formation.

4) Water soluble polysaccharide clearing model

This model (Zeeman et al., 1998; Smith, 2001) suggests that starch synthases and BEs are sufficient to produce amylopectin. In addition, these two enzymes also produce a water soluble polysaccharide that is cleared by the action of DBEs. Therefore, the synthesis of amylopectin and the water soluble polysaccharide are competing processes, and the action of
DBEs on the water soluble polysaccharide tilts the balance towards the net synthesis of only amylopectin. In the absence of DBEs however, both amylopectin and the water soluble polysaccharide (such as phytoglycogen) may be produced.

5) Two-step branching and improper branch clearing model

According to this model, (proposed by Nakamura, 2002 for amylopectin synthesis in rice) SBE I cleaves long chains from the preceding cluster and transfers to long B2 chains, extending from that cluster. The branch points formed are in the amorphous region and DBE cleaves off ill placed branches. This is followed by starch synthases elongating chains from the new cluster to approximately DP 12 at which point the SBE IIb can act on the newly forming cluster to produce branch points in the crystalline region, and DBE can act on the cluster to remove ill placed branches in the crystalline region. This is followed by SSIIa and SSI elongating the short and very short chains to complete both the preceding and newly forming clusters.

Therefore, starch granule development needs to be understood at many levels. It is also possible that some elements of some or all of the models described above work together, in reality, to produce the higher order structure of starch. We expect that the presence of a nucleating environment, i.e., an environment rich in lipids, and the transfer of longer branch chains facilitating formation of a bimodal chain length distribution in amylopectin will be sufficient to facilitate granule development.

**Our Hypothesis**

Our hypothesis is that cluster-branch structures and associated bimodal branch-chain length distribution of amylopectin, along with a membrane association providing an environment rich in lipids, are requisites for starch granule nucleation and development.

Therefore, the transfer of long, branch chains, resulting in double helix formation and a bimodal chain length distribution, along with an environment rich in lipids to serve as nucleation centers is sufficient for the formation of the alternating crystalline and amorphous
regions seen in amylopectin molecules. We expect that the transfer of longer branch chains resulting in double helical formation during amylopectin synthesis will lead to a steric hindrance that prevents further branching (Lineback, 1984), so that the branching degree will be similar to that in amylopectin rather than in glycogen.

Hypothesis tested in this dissertation

Assuming the presence of an environment rich in lipids in Synechocystis, due to the presence of thylakoid membranes, the transfer of long, branch chains due to the expression and activity of maize SBE I can lead to the formation of arrays of double helices forming a cluster branching pattern and a bimodal chain length distribution resulting in an insoluble glucose polymer that may resemble amylopectin.

The cyanobacterial system

Cyanobacteria are a group of prokaryotic organisms in the Kingdom Monera. As prokaryotes they lack a nuclear envelope, compartmentation of organelles within the cells and have peptidoglycan as part of their cell wall structures (Purves and Orians, 1983). Cyanobacteria are among the living forms that are important in carbon (C) and nitrogen (N) fixation. C fixation takes place through photosynthesis in the thylakoid membranes and N fixation takes place in heterocysts. These characteristics of cyanobacteria are taken advantage of to increase N levels in paddy fields and for use as a source of fixed nitrogen in some parts of the world. Cyanobacteria are also able to survive under extreme conditions such as high temperature and high salinity. Cyanobacteria perform oxygenic photosynthesis and therefore have been used as a model system in the study of photosynthesis. Synechocystis sp. Strain PCC 6803 is a unicellular, non N-fixing, fresh water living cyanobacterium (http://www.kazusa.or.jp/cyano/).

Some cyanobacteria such as Cyanobacterium sp. MBIC10216, Myxosacrina burmensis and Synechococcus sp. BG043511 have alpha polyglucans that have longer branch chain length distributions than those found in glycogen from most other cyanobacterial species
(Nakamura et al., 2005). The branch chain length distribution of these three species showed a greater proportion of chains with a DP greater than 37, such that the proportion, compared to the total number of chains, was 3.26 – 4.62% compared with a proportion of 6.71% for rice amylopectin and of less than 1% for most cyanobacteria. Gel permeation chromatography of these polyglucans showed a size and iodine complex lambda-maximum ($\lambda_{\text{max}}$) values similar to those of rice amylopectin. These alpha polyglucans have been designated semi-amylopectin by the authors (Nakamura et al., 2005). Synechocystis however was shown to have a branch chain length distribution similar to glycogen from most cyanobacteria. In addition, the $\lambda_{\text{max}}$ values of the molecules designated ‘semi-amylopectin’ are similar to that reported for Oscillatoria princeps (Fredrick, 1951), suggesting that Oscillatoria princeps also may produce semi-amylopectin.

Advantages and disadvantages in using the cyanobacterial system

One of the reasons for using Synechocystis sp. Strain PCC 6803 as a model system to study starch granule development is that they have an environment very similar to that in chloroplasts, where starch granules naturally form. The photosynthetic apparatus in Synechocystis is simply photosynthetic membranes which have the necessary photosynthetic pigments but are not organized as grana typically found in chloroplasts. The photosynthetic pigments found in Synechocystis are chlorophyll a, carotenoids and phycobilins (Buchanan et al., 2000). The presence of photosynthetic membranes within the cell also provides a lipid-rich environment which may favor formation of nucleation centers.

As a photosynthesizing organism, Synechocystis has a sufficient supply of ADP-glucose, the glucose donor used in glycogen synthesis. Generally UDP-Glucose is used as the glucose donor for glycogen synthesis in yeast and mammals, although ADP-glucose is used as the glucose donor in bacteria and cyanobacteria (Guan & Keeling, 1998; Buchanan et al., 2000). Starch synthesizing organisms also use ADP-glucose as the glucose donor. Therefore, Synechocystis sp. Strain PCC 6803, in addition to having a sufficient C flow through the system, uses the same glucose donor used by starch synthesizing enzymes. Therefore, when
starch synthesizing enzymes are expressed in *Synechocystis* they will not be deterred from action by a lack of ADP-glucose.

*Synechocystis* produces the osmoprotectant glucosylglycerol using ADP-glucose and is able to tolerate moderate salt concentrations up to 1.2M (Miao et al., 2003) and may not depend on the presence of glycogen to maintain cellular integrity. Since *Synechocystis* in nature produces glycogen, factors that promote formation of a semi-crystalline structure can be detected and assigned to the genetic manipulations we carry out in terms of introducing isoforms of plant SBEs and SSSs alone or in various combinations.

Although bacterial mutants lacking glycogen biosynthesis are known to survive as well as their parent strains, there seems to be a minimum requirement for starch synthesis in higher plants and glycogen synthesis in *Synechocystis* (Miao et al., 2003), which may be a drawback when using *Synechocystis* as a model system to study starch granule formation. ADP-glucose pyrophosphorylase is a rate regulating enzyme in the synthesis of starch in higher plants and in the synthesis of glycogen in cyanobacteria. Although in structure cyanobacterial AGPase is more similar to bacterial AGPase than the higher-plant AGPase, in terms of regulation it is more similar to higher-plants than to bacteria (Miao et al., 2003). Therefore, the AGPase in *Synechocystis* is a homotetramer and is up regulated by 3-PGA and down regulated by Pi. Replacement of a portion of this gene by an antibiotic resistance cassette resulted in a reduced growth rate in the mutant compared to wild type, leading to the conclusion that a minimal amount of glycogen synthesis is required for normal growth and development of cyanobacteria (Miao et al., 2003).

In addition to all the advantages mentioned above, the genome of *Synechocystis* is also fully sequenced (Kaneko & Tabata, 1997) and is available for use by the public at [http://www.kazusa.or.jp/cyano/](http://www.kazusa.or.jp/cyano/) along with the genome sequences of other cyanobacteria. The circular genome of *Synechocystis* sp. Strain PCC 6803 is 3,573,470 bp in size (Kaneko et al., 1996). Along with the circular chromosome, two extra chromosomal units which were 110 kb and 125 kb in size were also identified (Kotani et al., 1994). Only two glycogen
synthase enzymes, one GBE and two DBEs are listed for *Synechocystis*. Therefore, the relatively low number of genes possibly involved in glycogen synthesis and the characteristics detailed above make *Synechocystis* a very suitable model system in which to study the development of any crystalline characteristic in the glucose polymers produced.

**Photosynthesis in *Synechocystis* sp. Strain PCC 6803**

Like all oxygenic photosynthetic organisms, *Synechocystis* has two photosystems, PSI and PSII. The photosynthetic pigments present are chlorophyll a, carotenoids and phycobilins. A chlorophyll a dimer serves as the reaction center chlorophyll and both carotenoids and phycobilins serve as antenna pigments. In *Synechocystis*, the light harvesting antennae pigments, phycobilins, are water soluble and are found in association with proteins in structures known as phycobilisomes, which are found on the surface of the photosynthetic membranes.

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CHAPTER 2: NEED FOR GLYCOGEN BIOSYNTHESIS IN *Synechocystis* sp. PCC 6803 AND FURTHER CHARACTERIZATION OF THE M3 MUTANT LACKING GLYCOGEN BRANCHING ENZYME

Abstract

Glycogen is the water soluble glucose polymer accumulated in prokaryotic cells, including the cyanobacterium *Synechocystis* sp. PCC 6803. In order to use this cyanobacterium as a system in which to study the effects of starch synthesizing enzymes on the type of glucose polymer produced, efforts have been made to first study the native complement of glycogen synthesizing enzymes. Towards this end, the transgenic M3 mutant was constructed to replace the native glycogen branching enzyme with a Kan$^R$ cassette (Yoo et al., 2002). The present study further characterizes the M3 mutant to show the presence of residual glycogen branching enzyme activity and reports the production of additional mutants, each expressing maize SBE I (MSBE I), under the control of different promoters. PCR analysis has revealed an inability to completely replace GBE suggesting a need for glycogen synthesis in *Synechocystis*, at least under the conditions used in this work. This study also further characterizes the ability of the M3 mutant to produce, in addition to the small, linear molecules reported by Yoo et al. (2002), large branched molecules similar to wild type (WT) in size and in the distribution of branch chain lengths. An inverse relationship between the $\lambda_{\text{max}}$ of iodine complexes and size of the molecules in M3 indicated a limitation in branching enzyme activity levels had an effect on growth of the glucan molecule. The different populations of large, branched molecules produced in the presence of only low, residual levels of glycogen branching enzyme activity in M3 resulted in an increase in both peak and average DP (degree of polymerization). Results indicate that the limitation of branching enzyme activity cause a proportion of molecules to become sufficiently branched to allow the production of large molecules and that these large molecules may have a limited number of branch points because of limited branching enzyme activity. The limitation in number of branch points may have resulted in longer branch chains than WT in the large M3 glucans.
Introduction

Glycogen is a water soluble glucose polymer linked by α-1,4 glucosidic bonds within linear chains and 7 – 10 % α-1,6 glucosidic bonds at branch points. It is produced in animal cells, yeast, bacteria and cyanobacteria. In this group, cyanobacteria occupy a special niche as they are able to photosynthesize and, unlike photosynthetic organisms that produce starch granules, also synthesize glycogen.

Starch, composed of two types of molecules, amylose and amylopectin, is chemically similar to glycogen but is produced as a water-insoluble semi-crystalline granule, primarily, in higher plants and algae. As the storage carbon reserve in plants, starch serves an important role in the food industry. It also has many non-food uses such as in the production of carbon-less copy paper. Despite its usefulness and decades devoted to its study, all aspects of its synthesis and arrangement into higher-order structures are not fully understood. The plant systems that are generally used to study the biosynthetic aspects of starch present a great challenge to researchers due to the presence of several isoforms of starch biosynthetic enzymes. *Synechocystis* sp. PCC 6803 offers a simple cyanobacterial system in which the effects of each isoform of starch synthesizing enzymes could potentially be isolated and studied individually.

The presence of thylakoid membranes, the ability to photosynthesize and the use of ADP-glucose pyrophosphorylase as the enzyme involved in producing the glucose donor in glycogen synthesis are additional reasons why *Synechocystis* may be a good system in which to study the effect of the individual isoforms of starch synthesizing enzymes. However, in order to effectively use this as a model system it is necessary to characterize and study the functions of its own suite of glycogen synthesizing enzymes. This has led to the production of the putative knock-out mutant, M3, in which the endogenous glycogen branching enzyme (GBE) involved in glycogen synthesis was replaced by a Kan\(^R\) cassette (Yoo et al., 2002).
Although bacterial and yeast mutants that are deficient in glycogen synthesis have been produced, efforts to completely block glycogen synthesis in cyanobacteria have been unsuccessful. Therefore, of the two glycogen synthase genes available in *Synechocystis* sp. PCC 6803, both apparently can not be simultaneously replaced by antibiotic resistance markers, although they have been replaced completely in the presence of an introduced starch synthase gene (Li et al., manuscript in preparation). Reports (Miao et al., 2003) also have shown that when AGPase activity is down-regulated, growth rates of the resulting mutants were reduced compared to the wild type strain. The results reported here also demonstrate the inability to completely replace GBE in *Synechocystis* sp. PCC 6803 confirming the need for glycogen biosynthesis and display a possible inverse relationship between growth rate and the ability to synthesize glycogen. In addition, this report also presents data further characterizing the M3 mutant of *Synechocystis* sp. PCC 6803 and shows that ~10% of wild type levels of glycogen branching enzyme activity allows for the synthesis of more branched large glucan molecules with chains that are longer than the chains in the large glycogen molecules of wild type.

**Materials & Methods**

Mutant construction

**M3.** Construction of the M3 mutant was described in Yoo et al., (2002).

**SM3.** A plasmid (pBSSM3) was constructed to include the coding sequence of MSBE I, minus the transit peptide, and a Spec<sup>R</sup> cassette, flanked by 500 bp regions upstream and downstream of the *Synechocystis* slr1311 (*psbAII*) gene, as shown in Figure 1, and was used to replace the psbAII coding sequence region with MSBE I and the Spec<sup>R</sup> cassette in the M3 background of *Synechocystis* by double cross over homologous recombination, producing the SM3 double mutant. Forward (5’-GAG AGA GAG ACA TAT GGC TAC TGT GCA AGA AG-3’) and reverse (5’-ATT ATT ATT CTA GAA AAG CTA TAC-3’) primers with NdeI and XbaI restriction sites respectively, were used to amplify the MSBE I gene from a cDNA
clone (kindly provided by Dr. Jack Preiss; Baba et al., 1991). The Spec\textsuperscript{R} cassette was excised from the pH45\textsuperscript{Δ} plasmid (Prentki and Krisch, 1984) with BamHI. The upstream and downstream regions of slr1311 were amplified using primers as described in Lagarde et al. (2000) to give the upper flanking region (UFR) and the lower flanking region (LFR). Transformant colonies selected on BG-11 plates infused with 5 mM glucose, 50 µg/mL of kanamycin and 20 µg/mL of spectinomycin were carried forward by single colony descent for several plate-generations to facilitate complete segregation at the slr1311 locus, after which PCR analysis was performed on genomic DNA to confirm replacement of the \textit{psbAII} coding sequence with MSBE I. This gene replacement leaves the MSBE I gene under regulation of the \textit{psbAII} promoter.

**SM3G.** A plasmid (pBSSM3G) was constructed to include the coding sequence of MSBE I, minus the transit peptide, and a Spec\textsuperscript{R} cassette, flanked by 500 bp regions upstream and downstream of the slr0158 (GBE) gene. This was used to introduce MSBE I into the M3 recipient strain by double cross over homologous recombination (see SM3 construction) to completely replace the GBE coding sequence and also to express MSBE I under the control of the GBE promoter, resulting in the SM3G transgenic line. The MSBE I and Spec\textsuperscript{R} regions were prepared as in (b) above. The 500 bp flanking sequence of GBE to provide the upstream promoter region was amplified using the forward primer 5’-GBEUFR (5’-AAG ACT TGG GGC CCA TTG ATG-3’) with an \textit{Apa} I restriction site and the reverse primer 3’-GBELFR (5’-TGT AGG TCA TAT GGC TAT GGT G-3’) with an \textit{Nde} I site, and the downstream region was amplified by the forward primer 5’-GBELFR (5’-CTT AGA TAA CTG AAT TCCG CGA T-3’) with an \textit{EcoR} I restriction site and the reverse primer 3’-GBELFR (5’-CGA AAA TCC CGG AGC TCT TGT TG-3’) with a \textit{SacI} restriction site. Transformants were selected and tested as described in (b) above with the exception that only spectinomycin was used to exert selection pressure.

**SM3\textsubscript{b}.** For construction of the SM3\textsubscript{b} mutant, the MSBE I gene along with Spec\textsuperscript{R} was introduced into the \textit{psbAII} coding region of the WT strain first and then the GBE region was targeted for replacement with a Kan\textsuperscript{R} cassette to determine whether the presence of MSBE I
impacts the ability to replace GBE. The 1.3 kb KanR cassette was prepared from the pUC4K vector as in Yoo et al. (2002) and the flanking sequences were prepared as above for the construction of SM3G, and transformants were selected as described above for construction of SM3.

![Diagram](image)

Figure 1: Construct made to introduce MSBE I into *Synechocystis*, including a diagrammatic representation of intended double cross over homologous recombination. UFR and LFR refer to upper flanking region and lower flanking region respectively. (Not drawn to scale).

PCR analysis

A phenol/chloroform method (Ausubel et al., 1999) was used to extract genomic DNA from different *Synechocystis* strains following cell disruption in a Mini-Beadbeater™ (BiospecProducts Model). The 5’SBE-2F forward primer (5’-TTA CAA GAA AGT AAA ACT TAT G-3’) and the 3’SBE I reverse primer (5’-ATT ATT ATT CTA GAA AAG CTA TAC-3’) were used to detect the presence of the MSBE I gene coding sequence in SM3, the 5’SBE-6F forward primer (5’-CTT TGA ACG TGG AGA TTT AG-3’) and the 3’SBE I primer combination was used to detect the presence of MSBE I gene coding region in SM3G, and the 5’int psbA2 forward primer (5’-AAC TTC AAC CAG TCC ATC -3’) and the 3’psbA2 down reverse primer (5’-GAG AGA GAG AAA GCT TCG ATC GCC TTG GCA AAA CAA C-3’) were used to detect the presence of the slr1311 (psbAII) gene in SM3. The prC1 forward primer (5’-AAT CTT CAC CAG TAC GCC TTG GA-3’), prC4 reverse primer (5’-AAA GAC CAA GCT TCT GTC CAT T-3’), prK1 reverse primer (5’-ATA AAG TCT TCG GGA CTG CCA A-3’), and prK2 forward primer (5’-TTG GCA GTC CCG AAG ACT
TTA T-3’), as described in Yoo et al. (2002), and the prC7 forward primer (5’-TGG GAA GGG CAC ATC TAC G-3’) and the prC8 reverse primer (5’-CGG AAC CAG GGG TCC ATG C-3’) all were used to determine if the mutants were completely segregated at the sll0158 locus (which codes for GBE). The prK1, prK2, prC7 and prC8 primers were designed in the region of the sll0158 locus that was targeted for replacement by the KanR cassette in M3, SM3 and SM3G while in SM3b the prC7 and prC8 primers recognize regions that were targeted for replacement by the KanR cassette.

Growth conditions and growth curves

*Synechocystis* strains were grown in BG-11 liquid medium (Rippka et al., 1979) supplemented with 5 mM glucose at 30°C and approximately 13 µE m² s⁻¹ constant light with shaking at 150 rpm. Cultures used for the experiment where glucan was digested with amylglucosidase were grown at a slightly lower and variable temperature (~27.5°C+/-2.5°C). Cultures were transferred to nitrogen-limiting (N-limiting) BG-11 media for induction of glucan production and kept under the same conditions until harvested 2 days later either for analysis of glucan content and structure or for analysis of total protein extract. Cultures were always grown under selection pressure with the appropriate antibiotics incorporated into the media.

For quantitative monitoring of growth, liquid cultures were started (from a previous liquid culture) in BG-11 liquid medium at an initial OD₇₃₀nm of 0.1 (cells at log phase) and OD₇₃₀nm was monitored at approximately 12 hour intervals. Cultures were grown in a shaker (150 rpm) at 30°C and approximately 13 µE m² s⁻¹ constant light. For a visual estimation of growth characteristics, a serial dilution of cultures to give an OD₇₃₀nm of 1, 0.1 and 0.01 were prepared and 3 µL of each dilution was spotted onto a BG-11 plate supplemented with 5 mM glucose. After 5 days of growth the plate was photographed to show differences in growth rates.
Glucan extraction, amylglucosidase digestion and HPSEC analysis

Cells were harvested by centrifugation at 6000 g for 15 minutes from cultures 2 days after induction in N limiting media for glucan extraction according to Yoo et al. (2002). Harvested cells from 1 L of culture were resuspended in 10 mL of deionized water, 3-4 mL of 0.1 – 0.15 mm glass beads were added and the cells disrupted in a Mini-Beadbeater™ 6 times, keeping the suspension on ice between disruption periods. The resulting extract was centrifuged at 10,000 g for 15 minutes at room temperature to separate glass beads, cell debris and insoluble glucan from soluble glycogen.

**Soluble glycogen separation:** The supernatant containing soluble glycogen was incubated in a boiling water bath, with stirring of the supernatant using a magnetic stir bar, for 15 minutes to denature proteins. The boiled extract was centrifuged at 8,000 g for 15 minutes at room temperature to pellet denatured proteins, and 5 times the volume of 100 % ethanol was added to the resultant supernatant, which was then centrifuged at 8,000 g for 15 minutes to precipitate soluble glycogen. The boiling water and centrifugation steps were repeated to remove color from the precipitated glycogen, after which the precipitated glycogen was dispersed in 10 mL of 90% DMSO for quantification and further analysis. Dispersion in 90% DMSO included addition of a magnetic stir bar and stirring in a boiling water bath for 15 minutes. Yield of soluble glycogen was measured as described in Yoo et al. (2002) using a glucose diagnostic kit from Megazyme.

**Insoluble glucan separation:** Insoluble glucan was separated from cell debris and glass bead residues by dispersion in DMSO as described by Yoo et al. (2002). The insoluble glucan was finally dispersed in 10 mL of 90 % DMSO for quantification and further analysis, including yield of insoluble glucan, as described above.

Molecular distribution and size information was obtained by separating 0.4 mg/mL of the glucan dispersion on an HPSEC-RI-MALLS (High Performance Size Exclusion column-
Refractive Index-Multi Angle Laser Light Scattering) system along with pullulan standards P-82 (Shodex, Japan) according to Yoo and Jane (2002).

The nature of the glucan was determined by digestion with amyloglucosidase (5 units per mg of total carbohydrate) at 55°C for 2 hours (Yoo et al., 2002) and separation on the HPSEC. The cultures used for this experiment were grown at 27.5°C +/- 2.5°C.

Branch chain length distribution of total glucan from the different strains following debranching with isoamylase was determined on an HPAEC-ENZ-PAD system (method described under Gel Permeation Chromatography and HPAEC analysis).

Soluble protein extraction and enzyme assay

Cell pellets obtained by centrifugation (6000 g; 15 minutes) from 100 mL of culture were resuspended in 1 mL of the extraction buffer described below with approximately 300 µL of glass beads. These cells harvested 2 days after induction in N-limiting BG-11 were disrupted in 10 mM Tris-HCl buffer (pH 7.0), 1 mM EDTA, 1 mM dithiothreitol (DTT), 0.2 mM phenylmethyl sulfonylfluoride (PMSF) and 1 mM benzamidine using a Mini-Beadbeater™ 5 times (at 5000 rpm for 20 seconds each time and kept on ice between disruptions) and centrifuged at 13,000 rpm in a bench-top centrifuge for 15 minutes to remove cell debris and glass beads. The resulting supernatant was subjected to a second centrifugation step for 1 hour at 4°C and 50,000 g to remove glycogen from the total soluble protein component. The total soluble protein concentration in the resultant supernatant was determined using the Bradford method (Bio-Rad Protein Assay; according to manufacturer’s specifications) with bovine serum albumin as standard prior to its use to assay branching enzyme (BE) activity.

Branching enzyme assay. To a reaction mix with 1 mM AMP, 0.1 mg/mL of phosphorylase a and 100 mM sodium citrate (pH 7.0) the enzyme extract was added, while vortexing, and was followed immediately by glucose-1-phosphate to give a 45 mM final concentration. The assay was conducted at 30°C in a final volume of 200 µL, and aliquots were removed at 5
min, 10 min, 30 min, 60 min and 90 min time points and boiled for 10 minutes to stop the reaction (Yoo et al., 2002). The phosphate released due to stimulation of phosphorylase a activity by BE was measured by the malachite-green assay as detailed in Baykov et al. (1988). Boiled enzyme extract was used as control. Centrifugation of the enzyme extract at 50,000g for 1 hour at 4°C was crucial to remove glycogen, which otherwise could overwhelm the stimulating power of the BE present.

Gel permeation chromatography (GPC) and high-performance anion exchange chromatography (HPAEC) analysis

Molecular size distribution of the glucans produced in each strain was analyzed by gravity flow gel permeation chromatography at room temperature using a 1 cm (internal diameter) x 78 cm column packed with Sepharose CL-2B (Amersham Pharmacia Biotech AB). Glucan (4 mg) dispersed in DMSO was precipitated using 5 volumes of 100 % ethanol, centrifuged and the resulting pellet dispersed in 2 mL of hot water with stirring in a boiling water bath. The 2 mL glucan dispersion was filtered using Whatman # 1 filter paper discs and the filtrate loaded onto the column while still hot. After the sample entered the column by gravity flow, an eluant (1 mM NaOH, 25 mM NaCl) was used to aid separation. Collected fractions (20 drops each) were analyzed for carbohydrate content using the phenol-sulfuric acid method (Dubois et al., 1956) by adding 200 µL of 5% aqueous phenol and 1 mL of concentration sulfuric acid (with mixing after each addition) to 200 µL of the collected fraction and measuring absorbance at 490 nm after 20 – 30 min. The blue value for each fraction was measured by determining the absorbance at 630 nm after adding 100 µL of a 0.01 %/0.1 % I₂/KI (pH 4.5) to 100 µL of each collected fraction. (Juliano BO, 1971). Absorbance measurements were made using a plate reader (Ultra Microplate Reader, ELx808, BIO-TEK Instruments, Inc.).

Fraction numbers 25 to 48 and fraction numbers 53 to 70 were combined to represent the large and small molecules observed in the mutant strains. The glucans from the combined fractions were precipitated with 5 volumes of 100 % ethanol and the resulting precipitate
dispersed in 0.9 mL of hot water with stirring in a boiling water bath. The glucan dispersion was prepared for debranching by addition of 100 µL of 0.1 N sodium acetate buffer (pH 3.5), 2 µL of 10% sodium azide and 15-30 units of isoamylase, and incubated in a 40°C shaking water bath at 120 rpm for 48 hours. Debranching was terminated by boiling for 15 minutes and raising the pH to neutral by addition of NaOH. The chain length distribution of the large and small molecules was analyzed by HPAEC (Dionex-300, Sunnyvale, CA) equipped with a post column amyloglucosidase reactor and pulsed amperometric detector (PAD) according to Wong and Jane (1997).

Results

Transgenic mutant M3 was produced by replacing a 1.2 kb portion of the *Synechocystis* GBE sequence with a KanR cassette (Yoo et al., 2002) by double cross-over homologous recombination. Similarly, transgenic mutant SM3 was produced by introducing MSBE I into the *psbA II* site (slr1311; Fig. 1) in the M3 mutant background.

As illustrated in Figure 2, several PCR primer pairs were used to determine whether the KanR insertion disrupting GBE in M3 and SM3 was fully segregated and present in all genomes. Again, as illustrated in Figure 2, no PCR products should be generated from M3 or SM3 when the primer combinations of prC1/prK1, prC4/prK2 and prC7/prC8 are used, if only an interrupted form of GBE, with a disruption caused by the KanR insert, is present. Figure 3A illustrates that the primer combinations of prC1/prK1 and prC4/prK2, used by Yoo et al. (2002), were not consistently reliable in detecting the presence of the 1.2 kb GBE region targeted for replacement by the KanR gene in M3, and Figure 3C shows that the prC7/prC8 primer combination yields products clearly demonstrating the 1.2 kb region of GBE is still present in both M3 and SM3. Figure 3B illustrates that the prC7/prC8 primer combination amplified the correct size product from the GBE region in the WT genome targeted for replacement by the KanR gene, but did not amplify a product of the correct size from negative controls, such as the KanR plasmid, *Chlamydomonas* genomic DNA, the MSBE I plasmid or in the absence of template. Possibly because of primers preferentially annealing to
more abundant Kan\textsuperscript{R}-containing genome copies rather than the less abundant GBE-retaining genome copies, only faint bands were produced using the prC\textsubscript{1}/prK\textsubscript{1} or prC\textsubscript{4}/prK\textsubscript{2} combinations. However, since the prC\textsubscript{7} & prC\textsubscript{8} primers are able to anneal only to the GBE region, they proved more reliable in detecting the presence of the small number of genome copies retaining the 1.2 kb GBE sequence. Therefore, even under antibiotic selection, detailed analysis revealed that, of the many genome copies per cell, most copies in M3 and SM3 have the Kan\textsuperscript{R} gene replacing the GBE, while at least some copies retain the native GBE (Figure 3A).

**Figure 2:** Primers used and size of expected PCR products to determine complete segregation at the GBE locus for M3 and SM3
Introduction of MSBE I into the psbA II site in a M3 background to produce SM3 gave complete segregation (Fig. 4A). In SM3, a 2 kb portion was amplified using primers specific to MSBE I and no product was amplified when primers specific to the psbA II site were used.
(Fig. 4B and Fig 4C). This result shows complete segregation at the psbA II site in the SM3 mutant.

Figure 4: PCR data showing presence/absence of MSBE I and psbA II coding sequence in WT, M3 and SM3. (A) A 2 kb product was amplified using the 5’SBE-2F and 3’SBE I primer in SM3 alone (B) A 0.65 kb product was amplified using the 5’int psbA2 and 3’psbA2down primer in WT and M3 (C) A 2 kb product was amplified using the 5’psbA2up and 3’psbA2down primers in WT and M3.

Figure 5: PCR data showing presence of both MSBE I and GBE in SM3G and SM3. A 0.6 kb portion of MSBE I was amplified using the 5’SBE-6F and 3’SBE I primers (shown in lanes 1 – 5) and a 1 kb portion of GBE was amplified using the prC7 and prC8 primers (shown in lanes 6 – 10). Lanes 1 – 4 and 6 – 9 are for verifying SM3G mutants and lanes 5 and 10 are for verifying SM3.
In SM3G, introduction of MSBE I into the GBE site of M3 to replace the original Kan\(^R\) insert and/or the residual GBE gene and to express MSBE I under the control of the GBE promoter also failed to give complete replacement of GBE (Figure 5). However, according to phenotypic evidence, all or most copies with the Kan\(^R\) gene were completely replaced resulting in complete loss of resistance to full strength kanamycin (no data shown).

The attempt to replace the entire GBE coding sequence with the Kan\(^R\) cassette, after introduction of MSBE I into the psbA II site, to produce SM3\(_b\) also was unsuccessful. PCR evidence showed a very low abundance of the conserved region for BE activity that was detected by the prC7 and prC8 primer combinations, showing that even in the presence of MSBE I, GBE coding sequence, or at least portions of it, cannot be completely replaced (data not shown).

Figure 6: Growth characteristics of WT and M3 represented by (A) absorbance data from liquid cultures WT M3 (B) serial dilution spot tests on agar plates.
Investigation of relative growth of WT and M3 in both liquid and solid phase indicated a decreased growth rate in the absence of WT levels of GBE (in M3) (Fig. 6). Analysis of BE activity, determined using the phosphorylase a stimulation assay, (Table 1) showed very little BE activity in M3, as reported previously (Yoo et al., 2002). When cultures were harvested at stationary phase, ~9% of the WT level of BE activity was observed in M3, whereas after induction in N-limiting media, only ~2.2% of WT levels of BE activity was observed in M3. These observations are consistent with the presence of one or a few intact copies of GBE, as suggested by the PCR data.

Table 1: Branching enzyme activity for WT and M3.

<table>
<thead>
<tr>
<th>Strain</th>
<th>BE activity (% of WT in similar condition)*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Stationary phase</td>
</tr>
<tr>
<td>WT</td>
<td>65.50 (100 %)</td>
</tr>
<tr>
<td>M3</td>
<td>5.80 (9 %)</td>
</tr>
</tbody>
</table>

*As nmols glucose incorporated per min per mg total soluble protein in harvested cells, measured using the phosphorylase a stimulation assay.

Table 2: Proportion of soluble and insoluble glucan in WT and M3.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Soluble glucan</th>
<th>Insoluble glucan</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>µg % of total</td>
<td>µg % of total</td>
</tr>
<tr>
<td>WT</td>
<td>9548 69.1</td>
<td>4265 30.9</td>
</tr>
<tr>
<td>M3</td>
<td>82 1.3</td>
<td>6278 98.7</td>
</tr>
</tbody>
</table>

M3 produced much less glucan than WT (Table 2) and these glucans also were of a different structure, as indicated by their solubility characteristics. Our results also confirm that the majority of glucan in WT is soluble whereas the majority in M3 is insoluble (Yoo et al., 2002). Staining of glucan from WT and M3 with I₂/KI revealed that the glucan-iodine complex for M3 has a much longer wavelength lambda-maximum ($\lambda_{max}$) than that of WT (Yoo et al., 2002). The combination of long chains and an insoluble glucan in M3 suggest that the low residual amount of GBE activity in M3 is insufficient to produce many branch points, leading to the presence of long, relatively linear chains that aggregate and precipitate, as concluded by Yoo et al. (2002).
Figure 7: HPSEC elution profiles of total M3 glucan, M3 glucan digested with amyloglucosidase, total WT glucan and WT glucan digested with amyloglucosidase. In M3, peak area from 8.04 mL to 10.74 mL reduced by 69 % and in WT peak area from 8.02 mL to 10.20 mL reduced by 47 % to account for large molecules hydrolysed in M3 and WT respectively.

Separation of the glucans from WT and M3 by HPSEC demonstrated that WT glucan is composed mainly of large molecules, and that M3 glucan comprise a combination of large and small molecules but is dominated by the small molecules (Fig. 7). Following digestion of glucans from M3 and WT with amyloglucosidase before separation by HPSEC to determine whether the large molecules in each were indeed carbohydrate, the peaks for the large and small molecules in M3 decreased markedly by 69 %, from 8.04 mL to 10.74 mL, (Fig. 7), and a peak for glucose increased tremendously. Similarly, in WT also, peaks related to the large molecule decreased by 47 %, from 8.02 mL to 10.20 mL, and a peak for glucose increased after digestion with amyloglucosidase (Fig. 7). These results demonstrate that the large molecules in both M3 and WT were subject to digestion, confirming that they represent carbohydrate with α-1,4 and α-1,6 linkages.
Glucan extracted from cultures grown at 30°C was also separated by HPSEC (Fig. 8). These chromatograms show the presence of only large molecules in WT and the presence of large and small molecules (DP 39; eluting at ~ 18.8 mL to 21.5 mL in Fig. 8) in M3. Table 3 shows that the average molecular weights for the large molecules in WT and M3 are similar. While there are many populations of molecules larger than the ‘small molecules’, molecular weights were calculated only for the populations with the highest molecular weights.

Figure 8: HPSEC elution profiles for WT and M3 (insoluble) glucan.
Table 3: Molecular weight of large glucan molecules in WT and M3.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Average MW (Da)*</th>
<th>% of total glucan</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>$1.05 \times 10^7$</td>
<td>~30</td>
</tr>
<tr>
<td>M3</td>
<td>$1.67 \times 10^7$, $9.37 \times 10^6$</td>
<td>~29</td>
</tr>
</tbody>
</table>

*Largest glucan population only: eluting at 9.3 mL to 11.8 mL for WT and 8 mL to 11.75 mL for M3. MW of large molecules calculated using the multi-angle-laser-light-scattering (MALLS) and refractive-index (RI) detector signals and the Astra software (Wyatt Technology, Santa Barbara, CA) (Yoo and Jane, 2002).

Figure 9: Gel permeation chromatography elution profiles for WT and M3 glucans. The molecular weight scale to the right indicates the molecular weights of pullulan standards ($85.3 \times 10^4$, $18.6 \times 10^4$ and $4.8 \times 10^4$ Da). ■ Normalized total carbohydrate ◇ Blue value (absorbance at 630 nm) ◆ Pullulan standards.

GPC analysis was also performed to confirm that the large and small molecules observed in HPSEC chromatograms of M3 samples are indeed carbohydrate molecules (Fig. 9). M3 does contain large molecules approximately the size of those in WT, and M3 also contains small molecules absent from WT. The different populations of large molecules seen in HPSEC analysis merge into one population for both WT and M3 due to the limited separating power of the column used. Previous HPSEC analysis of M3 samples determined the small molecules to have an average molecular weight of about $5.6 \times 10^3$ Da (DP 35) (Yoo et al., 2002). Analysis of GPC fractions for total carbohydrate content and blue value (OD$_{630\text{nm}}$ of
iodine complex) demonstrated that the large molecules ($\lambda_{\text{max}}$ of iodine complex $\sim 560$ nm) are carbohydrate in nature and suggested they are more branched than the small molecules ($\lambda_{\text{max}}$ of iodine complex $\sim 570$ nm). Characteristics of the small molecules in M3 are consistent with linear molecules, as indicated by their very high blue value relative to carbohydrate content. The high $\lambda_{\text{max}}$ values for fraction number 57 in M3 (Table 4) confirm that the small molecules in M3 may be primarily linear in nature. Comparison of the iodine complexes of peak fractions of WT and M3 large confirm that the large molecules in M3 have longer chains than those of WT and shorter chains than the small molecules in M3. Therefore, the large glucans in M3 with a higher iodine complex $\lambda_{\text{max}}$ than the WT samples, may have resulted from a slightly lower number of branches being produced than WT due to the very low level of BE activity.

Table 4: Iodine complex $\lambda_{\text{max}}$ values for GPC fractions from WT and M3.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Fraction Number</th>
<th>$\lambda_{\text{max}}$ (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>44</td>
<td>520</td>
</tr>
<tr>
<td></td>
<td>45</td>
<td>525</td>
</tr>
<tr>
<td>M3</td>
<td>44</td>
<td>560</td>
</tr>
<tr>
<td></td>
<td>45</td>
<td>560</td>
</tr>
<tr>
<td></td>
<td>57</td>
<td>570</td>
</tr>
<tr>
<td></td>
<td>58</td>
<td>570</td>
</tr>
</tbody>
</table>

Since the $\lambda_{\text{max}}$ values can serve as a good indicator of branching, in addition to comparing the $\lambda_{\text{max}}$ values for the peak fractions, $\lambda_{\text{max}}$ values were obtained for all other fractions (Fig. 10), and showed that the populations of large glucan molecules in WT may have different molecular weights but have the same degree of branching and as a result the $\lambda_{\text{max}}$ values remain fairly constant over all fractions. In contrast, among the populations of molecules present in M3 the $\lambda_{\text{max}}$ value increased as molecular weight decreased, suggesting an indirect relationship between molecular weight and degree of branching. Therefore, in M3, $\lambda_{\text{max}}$ increased steadily from 535 nm to 570 nm in the GPC elution profile, suggesting that as molecules are subjected to increased branching, they are able to increase in size. In essence, those molecules with fewer branches may remain smaller in size because of the small number of non-reducing ends generated, but have longer branches because of continued chain
elongation, resulting in a high $\lambda_{\text{max}}$ value. This suggests that the very small proportion of the largest of the large molecules in M3 might be similar to WT and that the smaller molecules probably are vastly different. A major proportion of the large molecules in M3, however, may have longer branches than the large molecules in WT (Fig. 10).

![Graph](image)

Figure 10: Change in $\lambda_{\text{max}}$ value of the glucan-iodine complex for each fraction collected during GPC analysis.

Preparative GPC also was used to separate the large and small glucans of M3 to facilitate independent analyses of their structure and characteristics. After separation of the large and small glucans, they were individually debranched and analyzed using HPAEC-ENZ-PAD. Figure 11 illustrates the branch chain length distributions of total glucans, as well as from the large and small (where present) glucans, for both WT and M3, and Figure 12 contains a difference plot of the branch chain length distributions between WT and M3 large glucans. A summary of the benchmarks from these data is provided in Table 5.
(A) Normalized peak area $\times 10^{-2}$ vs. Degree of polymerization for WT-total and WT-large.
Figure 11: Branch chain length distribution analyzed using HPAEC-ENZ-PAD for (A) total WT glucans and large WT glucans from pooled GPC fractions, and (B) total M3 glucans and large and small glucan populations from pooled GPC fractions.
Table 6: Summary of branch chain length distributions of WT and M3 glucans.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Peak DP</th>
<th>Average DP</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT total</td>
<td>6</td>
<td>16</td>
</tr>
<tr>
<td>WT large</td>
<td>6</td>
<td>16</td>
</tr>
<tr>
<td>M3 total</td>
<td>19</td>
<td>27</td>
</tr>
<tr>
<td>M3 large</td>
<td>7, 13</td>
<td>19</td>
</tr>
<tr>
<td>M3 small</td>
<td>7, 14</td>
<td>39</td>
</tr>
</tbody>
</table>

As would be expected, the WT total glucans and large glucans have essentially the same characteristics (Fig. 11; Table 6). The large glucans in M3 also have a unimodal branch chain length distribution similar to that of WT (total or large glucans), although the peak DP increased from 6 to 7 and 13, and the average DP increased from 16 to 19. However, when the total M3 glucan sample was considered, both the peak DP and the average DP increased dramatically to 19 and 27, respectively. Comparison of the large glucans of M3 with those of WT (Fig. 12) indicates that the M3 large glucans have more long chains of DP >10, consistent with a limitation in BE activity. Although small M3 glucans have peak DP values similar to the large M3 glucans, the shape of the branch chain length distribution is very different, in that the M3 small glucans have a distribution that is more symmetrical (increased abundance of longer chains) and the M3 large glucans have a distribution that is
skewed to the left (greater abundance of shorter chains). As shown in Table 6, this difference is reflected in the average DP of the small M3 molecules (39) being greater than that of the large M3 molecules (19). The higher average DP of the small M3 molecules is consistent with those molecules being mostly linear as reported in Yoo et al. (2002). Therefore, considering the large glucans of M3 separately provides a more realistic analysis of that sample and illustrates the presence of residual BE observed as assayable activity or inferred from PCR data.

**Discussion**

The lack of complete segregation of mutant lines M3, SM3 and SM3G at the GBE site despite complete segregation at the psbA II site in SM3 (as reported in Lagarde et al., 2000 regarding the psbA II site also) shows that GBE probably is essential for survival and may not be completely eliminated in *Synechocystis*. The characteristics of transgenic mutant M3 indicate that at least a low level of GBE (based on PCR and enzyme activity data) is required and sufficient for *Synechocystis* survival. Further, the lack of complete segregation of mutant SM3 shows that, when expressed under the control of the *psbA II* promoter, MSBE I activity does not compensate for the lack of GBE. The *psbA II* gene should exhibit high expression in log phase, where photosynthesis is unrestricted, but a decreased expression under N-limiting conditions, where photosynthesis is severely limited. In contrast, maximum expression of the GBE gene may occur under N-limiting conditions, where glycogen synthesis, or at least accumulation, is maximal. Therefore, under control of the *psbA II* promoter, MSBE I expression may be depressed in N-limiting conditions, when glycogen synthesis is induced. Consistent with the possibility of sub-maximal MSBE I expression in SM3 under N-limiting conditions, it also was observed that SM3 produced only ~30 % of WT levels of BE activity (Pieris et al. manuscript in preparation).

A new mutant, SM3G, was constructed with MSBE I expression under control of the GBE promoter with the expectation that it might provide greater expression of MSBE I under the N-limiting conditions used for glucan analysis. PCR analysis of SM3G showed that the GBE
replacement in this mutant also was incompletely segregated, indicating that MSBE I activity, even if expressed under control of the GBE promoter, could not compensate for the absence of GBE. The apparent inability to completely eliminate GBE coding sequences even when MSBE I is already present, as illustrated with the SM3b mutant, further demonstrates the inability of MSBE I to compensate for the absence of GBE.

These mutants as a whole suggest that loss of the ability to synthesize the water soluble glucose polymer, glycogen, by the loss of GBE apparently is lethal to *Synechocystis*. More puzzling, however, is the observation that the introduction of MSBE I, which is involved in the synthesis of amylopectin in maize and does contribute BE activity to the SM3 mutant (according to results in chapter 3), cannot compensate for the lack of GBE in *Synechocystis*. It will be informative to see if, for instance, GBE from *E. coli*, will allow for the complete replacement of the native GBE.

The growth rate of M3 was decreased compared to WT (Fig. 6), and this growth rate was similar in the presence or absence of kanamycin (data not shown), demonstrating that the decreased growth rate was not due to a growth effect of the antibiotic itself. Similar decreased growth rates have been observed in other mutants perturbed in glycogen biosynthesis, such as a mutant lacking glycogen synthase II (M2) (Li *et al.* manuscript in preparation) and a mutant lacking AGPase (Miao *et al.*, 2003). These common observations indicate that perturbations in the ability to produce glycogen may have some effect on the growth capacity of *Synechocystis*, which, of course, is consistent with the apparent inability to completely eliminate enzymes involved in glycogen biosynthesis.

M3, when initially reported (Yoo *et al.*, 2002) was not known to be incompletely segregated. In fact, complete segregation of the insertion in GBE was indicated by PCR analysis – an analysis demonstrated here to be flawed. In addition to the PCR evidence indicating complete segregation, the BE activity assays reported by Yoo *et al.* (2002) were low enough to be disregarded. As shown in this work, when M3 cells were harvested after induction in N-limiting media, very little BE activity (Table 1) was recorded, but the BE activity was
about 5-fold higher and clearly detectable in stationary phase cells prior to exposure to N-limiting medium. The soluble protein extract used for BE activity determination was not treated to remove glycogen in Yoo et al. (2002) and this compounded the difference between WT which has plenty of soluble glycogen and M3 which has only negligible amounts of soluble glucan. This factor in addition to the stage in which cells were analyzed made it difficult for Yoo et al. (2002) to detect the presence of BE activity in M3. *Synechocystis* is reported to have ~10 copies of the genome (Eaton-Rye, 2004). The presence of one copy of the unaltered version of the genome carrying GBE may result in ~10% of BE expression and activity, which is consistent with both the PCR data and enzyme activity data reported here. Although there may be only one functional GBE present in M3 the reason this does not result, in M3, in only a tenth of the growth rate seen in WT may be because GBE is present in excess under WT conditions.

Yoo et al. (2002) reported only the presence of small, mostly linear glucans in the transgenic M3 mutant, so the presence of large, branched glucan molecules represents another new observation in this mutant. HPSEC-RI elution data, before and after amyloglucosidase digestion, confirmed the presence of glucans in M3 as large as those in WT, and preparative GPC confirmed the presence of large glucans in M3 similar in size to those in WT. Yoo et al. (2002) did not detect a significant amount of large glucans, possibly because of different growth conditions (e.g., 25°C vs. 30°C), which we have observed to shift the proportion of large and small glucans (data not shown). The iodine-complex $\lambda_{\text{max}}$ values from peak fractions (Table 4) indicate that the large M3 glucans have longer chains than the WT glucans, and that the small, linear glucans in M3 have even longer chains. These observations are supported by the ratio of blue value to total carbohydrate for the WT and M3 samples (Fig. 9). Branch chain length distribution of the large glucans of M3 shows a distribution similar in shape to the unimodal distribution of the WT glucans (Fig. 11), although the distribution for the large M3 glucans exhibit a minor peak at DP 13, in addition to the prominent peak at DP 7. The minor peak at DP 13 in the branch chain distribution of the M3 large glucans may reflect an incomplete separation of the large and small glucan populations in the preparative GPC, resulting in the fractions used as M3 large glucans comprising a
mixture of molecules with varying degrees of branching, as reflected in the change in the iodine complex $\lambda_{\text{max}}$ through the GPC elution profile (Fig. 10).

A decreased branch number per molecule (degree of branching) could result in the formation of longer chains. It is readily apparent that the large glucans in M3 have a branch chain length distribution similar to the WT glucans, but, in spite of this similarity, the difference plot (WT-M3) of large glucans also demonstrates a greater abundance of branch chains of DP $>10$ in the large glucan of M3 (Fig. 12). The summary of branch chain length distribution in Table 5 shows also that the analysis of the total glucan from M3 is more a reflection of the small molecules in M3 and that the large molecules in M3 are similar to the glucans in WT.

The expression of BE IIb from *ae* rice (a mutant lacking BE IIb) resulted in the formation of lines showing varying levels of BE IIb activity (Tanaka et al., 2004). This report showed a positive relationship between the level of BE IIb activity and the proportion of short chains of DP less than 13 such that when BE IIb was present in greater amounts than WT, more of a water soluble polysaccharide was also produced. A slight change in BE IIb activity had a considerable impact on the chain length distribution and changed other physical characteristics of the glucan produced. Rice BE IIb is a branching enzyme that belongs to class B similar to GBE in *Synechocystis*. The observation that different populations of molecules with varying degree of branching may be produced in M3 may be a similarity shared with the mutant lines of rice expressing different levels of BE IIb.

Only a portion of the glucan produced in M3 are large glucans (Table 3). The presence of large glucans might be expected due to the presence of a low level of BE activity in M3. Because of the very low BE activity in M3, it is probable that only a fraction of the initially linear glucan molecules become branched prior to their removal as BE substrates by aggregation and precipitation. Therefore, only a percentage of glucan molecules may contain enough branches to keep their non-reducing ends accessible, and provide a platform from which the molecules can grow to a large size by addition of glucose units to their non-reducing ends.
The results in this study support the conclusion that *Synechocystis* requires glycogen synthesis for normal growth and that the introduction of MSBE I, which is involved in the synthesis of amylopectin in plants, is not sufficient to compensate for the loss of glycogen BE activity, even though addition of MSBE I increases the yield of large, branched glucan (Pieris *et al.* manuscript in preparation). This paper further reports the presence of residual BE activity in the M3 mutant, which leads to the production in M3 of some large branched glucans. Although the branch chain length distribution of this large glucan in M3 is similar to that of WT glucan, it has an increased abundance of long, branch chains (DP >10), probably because of the limitation of BE activity.

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CHAPTER 3: EFFECT OF MAIZE SBE I ON THE NATURE OF THE ALPHA POLYGLUCAN PRODUCED IN Synechocystis sp. PCC 6803

Abstract

Glycogen and starch, the α-1,4 and α-1,6 linked glucose polymers are chemically similar but differ in glycogen being water soluble and starch, due to the presence of amylopectin, being water insoluble/semi-crystalline (Jane, 2006). A factor thought to contribute towards the unique fine structure and semi-crystalline nature of amylopectin, is the type of branching enzyme (BE) present. We tested the hypothesis that the transfer of long chains may facilitate the formation and perpetuation of a cluster branching pattern by introducing maize starch branching enzyme I (MSBE I) into Synechocystis sp. PCC 6803 with a background of only residual glycogen branching enzyme (GBE) activity (mutant M3), to produce the double mutant SM3, and characterized the glucan produced. PCR, western and zymogram analyses showed that MSBE I was expressed and active in SM3, and characterization of the glucans produced by SM3 showed the formation of polyglucan molecules 10 times larger in size than those produced in WT. The introduced MSBE I resulted in synthesis of polyglucans with longer branch chains and a bimodal branch chain length distribution. Negative staining of glucan extracted with little or no change to its structure by gradient centrifugation showed unique structures and behaved similar to normal maize and amaranth starch granules and very differently from rabbit liver glycogen and WT Synechocystis glycogen when subjected to centrifugation in a discontinuous sucrose density gradient thereby suggesting that the glucan is synthesized, in vivo, with a high density. The density-gradient prepared glucans were similar in size and shape to structures seen in vivo when Theyr stained and viewed under transmission electron microscopy (TEM). These combined results indicate that MSBE I expression led to the formation of unique glucans and suggest that transfer of long chains does impact the nature of the glucan produced. Comparison of the glucans produced in the recipient strain, M3, revealed similar structures, suggesting that limiting BE activity had somewhat of a similar effect, although introduction of a BE that could transfer long chains resulted in end products that were larger, more abundant and more uniform than those of M3.
Introduction

Starch is produced from glucose units and serves as the stable, long term or transient carbohydrate reserve in plants and algae where as glycogen, the chemically comporable polymer is synthesized in an amorphous form (Jane, 2006). The high molecular weights of the constituent molecules of starch, amylopectin and amylose, and their arrangement into higher order granular structures give starch characteristics that make it economically valuable in the food and non-food industries (Jane, 2007). Amylopectin, which comprises about 70 – 80 % of the starch granule, is thought to determine the semi crystalline nature of starch. Researchers have struggled with elucidating the fine structure of amylopectin and with the change in techniques available to study characteristics, such as branch chain length distributions, the model that describes this molecule has been refined over the years.

The widely accepted model that describes this biopolymer is the cluster model (Thompson, 2000). Amylopectin is a glucose polymer with α-1,4 linkages within the chains and α-1,6 linkages at branch points. Amylopectin is known to have 4 – 5 % of residues at branch points with the branch points present in a cluster. Debranching of this molecule and separation according to size of the resultant chains shows the presence of a polymodal chain length distribution (Hizukuri, 1986; Hanashiro et al., 1996). This experimentally determined feature of amylopectin has led to the understanding that the amylopectin molecule has a tandem cluster structure, and that there may be chains that span one cluster, known as A and B1 chains, and there may be chains that span more than one cluster, known as B2 and B3 chains. The peak and average branch chain lengths of amylopectin molecules of various botanical origins also are high enough for chains to produce double helices with neighboring chains and ultimately form crystallites. The radial arrangement of these crystallites is shown by the presence of a Maltese cross when viewed under polarized light. Starch has been also shown to have 15 – 45 % crystallinity (Zobel, 1988), and this crystallinity is afforded by the presence of double helical crystallites.
The suite of biosynthetic enzymes that are responsible for the synthesis of starch are highly conserved among starch synthesizing organisms. The three main groups of enzymes that may be involved in starch synthesis are the synthases, branching enzymes and the debranching enzymes (Ball et al., 2003). Within each major group many different isoforms are present, although these are not functionally redundant. For instance there are five isoforms of starch synthases, including four soluble starch synthases (SSS), SSI, SSIIa and SSIIb, and SSIII, and one granule bound starch synthase (GBSS), and three isoforms of starch branching enzyme (SBE), SBE I, SBE IIa and SBE IIb in maize (Boyer and Preiss, 1978; Burton et al., 1995; Myers et al., 2000; James et al., 2003). Because of the highly conserved nature of these enzymes and the similarity in structural organization of the starch granules from different sources, it has been proposed that the biosynthetic enzymes play specific roles that contribute to the structural order of the amylopectin molecule.

We hypothesize that the transfer of longer branch chains in an environment rich in lipids may be sufficient to facilitate crystallite formation, because of the formation of double helices between adjacent chains, which then limit branching degree and position to give a cluster arrangement of branch points in relation to each other. To test this hypothesis we introduced maize SBE I (MSBE I), which is known to transfer long branch chains (Guan et al., 1997), into a *Synechocystis* mutant, M3, that has only residual branching enzyme activity (Yoo et al. 2002). This cyanobacterial model system was chosen to study the effect of MSBE I on the glucans produced because of its ability to photosynthesize, the presence of thylakoid membranes to provide an environment rich in lipids, the use of ADP-glucose as the glucose donor in glucan synthesis and the synthesis of glycogen as the carbon reserve.

Although MSBE I has previously been introduced into *E. coli*, yielding a glycogen-like soluble glucan (Guan et al. 1995), and into yeast, where no glucan accumulation was observed, possibly because of little or no expression of MSBE I (Seo et al. 2002) our *Synechocystis* model system yielded sufficient glucan that was insoluble in nature in order to study the branch chain length characteristics introduced by the action of MSBE I and that had a unique structure *in vivo*. 
Materials & Methods

Mutant construction

SM3. A plasmid (pBSSM3) was constructed to include the coding sequence of MSBE I, minus the transit peptide, and a Spec\(^R\) cassette, flanked by 500 bp regions upstream and downstream of the Synechocystis slr1311 (psbAII) gene, as shown in Figure 1, and was used to replace the psbAII coding sequence region with MSBE I and the Spec\(^R\) cassette in the M3 background of Synechocystis by double cross over homologous recombination, producing the SM3 double mutant. Forward (5’-GAG AGA GAG ACA TAT GGC TAC TGT GCA AGA AG-3’) and reverse (5’-ATT ATT ATT CTA GAA AAG CTA TAC-3’) primers with NdeI and XbaI restriction sites respectively, were used to amplify the MSBE I gene from a cDNA clone (kindly provided by Dr. Jack Preiss; Baba et al., 1991). The Spec\(^R\) cassette was excised from the pH45ω plasmid (Prentki and Krisch, 1984) with BamHI. The upstream and downstream regions of slr1311 were amplified using primers as described in Lagarde et al. (2000). The sequencing of the vector (pBSSM3) used for MSBE I introduction to produce SM3 revealed a single mutation at base pair number 447 from the first base pair of the coding sequence which is in the third position of a codon for Arginine. This change from CGA to CGT resulted in no change to the amino acid sequence. It has been reported that the CGA and CGT codons are used to equal extents in Synechocystis (Kanaya et al., 1999). Transformant colonies selected on BG-11 plates infused with 5 mM glucose, 50 µg/mL of kanamycin and 20 µg/mL of spectinomycin were carried forward by single colony descent for several plate-generations to facilitate complete segregation at the slr1311 locus, after which PCR analysis was performed on genomic DNA to confirm replacement of the psbAII coding sequence with MSBE I. This gene replacement leaves the MSBE I gene under regulation by the psbAII promoter.
PCR analysis

A phenol/chloroform method (Ausubel et al., 1999) was used to extract genomic DNA from different *Synechocystis* strains following cell disruption in a Mini-Beadbeater™ (BiospecProducts Model). The 5’SBE-2F forward primer (5’-TTA CAA GAA AGT AAA ACT TAT G-3’) and the 3’SBE I reverse primer (5’-ATT ATT ATT CTA GAA AAG CTA TAC-3’) were used to detect the presence of the MSBE I gene coding sequence in SM3, and the 5’int psbA2 forward primer (5’-AAC TTC AAC CAG TCC ATC TAC-3’) and the 3’psbA2<sub>down</sub> reverse primer (5’-GAG AGA GAG AAA GCT TCG ATC GCC TTG GCA AAA CAA C-3’) were used to detect the presence of the slr1311 (psbAII) gene in SM3. The prC1 forward primer (5’-AAT CTT CAC CAG GAT CCC TTT GA-3’), prC4 reverse primer (5’-AAA GAC CAA GCT TCT GTC CAT T-3’), prK1 reverse primer (5’-ATA AAG TCT TCG GGA CTG CCA A-3’), and prK2 forward primer (5’-TTG GCA GTC CCG AAG ACT TTA T-3’), as described in Yoo et al. (2002), and the prC7 forward primer (5’-TGG GAA GGG CAC ATC TAC G-3’) and the prC8 reverse primer (5’-CGG AAC CAG GGG TCC ATG C-3’) all were used to determine if the mutants were completely segregated at the sll0158 locus (which codes for GBE). The prK1, prK2, prC7 and prC8 primers were designed to hybridize to the region of the sll0158 locus that was targeted for replacement by the Kan<sup>R</sup> cassette.

Growth conditions and growth curves

*Synechocystis* strains were grown in BG-11 liquid medium (Rippka et al., 1979) supplemented with 5 mM glucose at 30°C and approximately 13 µE m<sup>-2</sup> s<sup>-1</sup> constant light with shaking at 150 rpm. Cultures were transferred to nitrogen-limiting (N-limiting) BG-11 media for induction of glucan production and kept under the same conditions until harvested 2 days later either for analysis of glucan content and structure or for analysis of total protein extract. Cultures were always grown under selective pressure with the appropriate antibiotics incorporated into the media.
Soluble protein extraction and enzyme assay

Cell pellets obtained by centrifugation (6000 g; 15 minutes) from 100 mL of culture were resuspended in 1 mL of the extraction buffer described below with approximately 300 µL of glass beads. These cells harvested 2 days after induction in N-limiting BG-11 were disrupted in 10 mM Tris-HCl buffer (pH 7.0), 1 mM EDTA, 1 mM dithiothreitol (DTT), 0.2 mM phenylmethyl sulfonylfluoride (PMSF) and 1 mM benzamidine using a Mini-Beadbeater™ 5 times (at 5000 rpm for 20 seconds each time and kept on ice between disruptions) and centrifuged at 13,000 rpm in a bench-top centrifuge for 15 minutes to remove cell debris and glass beads. The resulting supernatant was subjected to a second centrifugation step for 1 hour at 4°C and 50,000 g to remove glycogen from the total soluble protein component. The total soluble protein concentration in the resultant supernatant was determined using the Bradford method (Bio-Rad Protein Assay; according to manufacturer’s specifications) with bovine serum albumin as standard prior to its use to assay branching enzyme (BE) activity.

Branching enzyme assay

To a reaction mix with 1 mM AMP, 0.1 mg/mL of phosphorylase a and 100 mM sodium citrate (pH 7.0) the enzyme extract was added, while vortexing, and was followed immediately by glucose-1-phosphate to give a 45 mM final concentration. The assay was conducted at 30°C in a final volume of 200 µL, and aliquots were removed at 5 min, 10 min, 30 min, 60 min and 90 min time points and boiled for 10 minutes to stop the reaction (Yoo et al., 2002). The phosphate released due to stimulation of phosphorylase a activity by BE was measured by the malachite-green assay as detailed in Baykov et al. (1988). Boiled enzyme extract was used as control. Centrifugation of the enzyme extract at 50,000g for 1 hour at 4°C was crucial to remove glycogen, which otherwise could overwhelm the stimulating power of the BE present.
Western blot analysis

Protein was extracted as described in Chapter 2. A 7.5 % SDS-PAGE resolving gel (15 cm x 10 cm x 0.15 cm) was used to electrophoretically separate 25 µg of total soluble proteins from WT, M3 and SM3, and 1 µg of total soluble proteins from maize kernels. Protein extracts were centrifuged at 50,000 g to remove glycogen from Synechocystis samples, and the maize protein extract was treated similarly. As size markers, 40 uL of BenchMark Prestained Protein ladder (Invitrogen) and 0.5 µL of MagicMark Protein Ladder (Invitrogen) were included on the same gel for determination of apparent protein size during electrophoresis and after western analysis, respectively. Electrophoresis was performed at 4°C for 7 hours at 20 mA constant current. The resolving gel and filter papers used for transfer were equilibrated in transfer buffer for 10 – 30 minutes. Proteins on resolving gels were electroblotted onto a nitrocellulose membrane (equilibrated in 100% methanol for 15 seconds and in transfer buffer for 30 minutes) for approximately 2 hours at 350 mA constant current at 4°C with constant stirring of the transfer buffer. Following transfer, the nitrocellulose membrane was rinsed in TTBS solution (20 mM Tris-HCl, pH 7.6, 150 mM NaCl, 0.1 % Tween 20) for 5 minutes and blocked overnight in a shaker with TTBS enriched with 0.4 % (v/v) cold water fish skin gelatin. Following the overnight incubation, the blocking solution was removed, and the membrane was washed three times with TTBS solution for 5 minutes each time. The primary antibody in TTBS (1: 3000 dilution) was added to the membrane for 1 hour, followed by detection with a secondary antibody (goat α-rabbit horseradish peroxidase) in TTBS (1:15,000 dilution) for 1 hour. The membrane was washed twice with TTBS for 10 minutes in between the primary antibody and secondary antibody steps. After treatment with secondary antibody, the membrane was washed thrice with TTBS for 10 minutes each time. Electrochemical detection was performed using 1 mL of 4 mg/mL of Luminol, 1 mL of 1 mg/mL of iodophenol, 50 µL of 3 % H₂O₂ and 500 µL of 1 M Tris (pH 8.0) in 10 mL of water.
Zymogram analysis

Protein was extracted from 100 mL of frozen cells into 50 mM Tris/acetate buffer (pH 7.5) and 10 mM DTT by breaking cells using glass beads in a Mini Beadbeater™ (Biospec Products). The protein extract was centrifuged for 15 minutes at 13,000 rpm in a benchtop centrifuge to remove debris and glass beads, and the resulting supernatant was further centrifuged at 50,000g for 1 hour at 4°C to remove glycogen. Synechocystis protein samples (25 µg and 50 µg total protein extract) and maize kernel extract (6.25 µg of total protein, prepared according to Dinges et al., 2001) were loaded onto a 5 cm, 7 % (w/v) polyacrylamide native gel (1.5 mm thickness) with 375 mM Tris-HCl (pH 8.8) buffer and electrophoresed at 4°C for 45 minutes at 125 V with a running buffer composed of 25 mM Tris, 192 mM glycine at pH 8.8, and 2 mM DTT. The electrophoresis apparatus used for protein separation was placed in a container of ice to prevent over-heating. The proteins on the native gel were transferred overnight at 20 V and room temperature onto a 5 cm, 7 % (w/v) polyacrylamide gel with 0.3 % (w/v) potato starch. The potato starch stock solution was made by wetting 0.1 g of potato starch with 1 mL of water, followed by dispersion into 90 % DMSO final concentration and incubation in a boiling water bath for 1 hour. Stirring was continued for 24 hours at room temperature, although the transfer apparatus also was kept in a container of ice to prevent over-heating during the overnight transfer. The transfer buffer used was similar in composition to the running buffer. The polyacrylamide – starch gel was stained with I₂/KI solution (1g/10g in 1L of water) and photographed immediately after transfer. This protocol was adapted from that described in Dinges et al. (2001).

Glucan extraction and HPSEC analysis

Cells were harvested by centrifugation at 6000 g for 15 minutes from cultures 2 days after induction in N limiting media for glucan extraction according to Yoo et al. (2002). Harvested cells from 1 L of culture were resuspended in 10 mL of deionized water, 3-4 mL of 0.1 – 0.15 mm glass beads were added and the cells disrupted in a Mini-Beadbeater™ 6 times, keeping the suspension on ice between disruption periods. The resulting extract was
centrifuged at 10,000 g for 15 minutes at room temperature to separate glass beads, cell debris and insoluble glucan from soluble glycogen.

**Soluble glycogen separation**: The supernatant containing soluble glycogen was incubated in a boiling water bath, with stirring of the supernatant using a magnetic stir bar, for 15 minutes to denature proteins. The boiled extract was centrifuged at 8,000 g for 15 minutes at room temperature to pellet denatured proteins, and 5 times the volume of 100 % ethanol was added to the resultant supernatant, which was then centrifuged at 8,000 g for 15 minutes to precipitate soluble glycogen. The boiling water and centrifugation steps were repeated to remove color from the precipitated glycogen, after which the precipitated glycogen was dispersed in 10 mL of 90% DMSO for quantification and further analysis. Dispersion in 90% DMSO included addition of a magnetic stir bar and stirring in a boiling water bath for 15 minutes. Yield of soluble glycogen was measured as described in Yoo et al. (2002) using a glucose diagnostic kit from Megazyme.

**Insoluble glucan separation**: Insoluble glucan was separated from cell debris and glass bead residues by dispersion in DMSO as described by Yoo et al. (2002). The insoluble glucan was finally dispersed in 10 mL of 90 % DMSO for quantification and further analysis, including yield of insoluble glucan, as described above.

Molecular distribution and size information was obtained by separating 0.4 mg/mL of the glucan dispersion on an HPSEC-RI-MALLS (High Performance Size Exclusion column-Refractive Index-Multi Angle Laser Light Scattering) system along with pullulan standards P-82 (Shodex, Japan) according to Yoo and Jane (2002).

Branch chain length distribution of total glucan from the different strains following debranching with isoamylase was determined on an HPAEC-ENZ-PAD system (method described under Gel Permeation Chromatography and HPAEC analysis).
Gel permeation chromatography (GPC) and high-performance anion exchange chromatography (HPAEC) analysis

Molecular size distribution of the glucans produced in each strain was analyzed by gravity flow gel permeation chromatography at room temperature using a 1 cm (internal diameter) x 78 cm column packed with Sepharose CL-2B (Amersham Pharmacia Biotech AB). Glucan (4 mg) dispersed in DMSO was precipitated using 5 volumes of 100 % ethanol, centrifuged and the resulting pellet dispersed in 2 mL of hot water with stirring in a boiling water bath. The 2 mL glucan dispersion was filtered using Whatman # 1 filter paper discs and the filtrate loaded onto the column while still hot. After the sample entered the column by gravity flow, an eluant (1 mM NaOH, 25 mM NaCl) was used to aid separation. Collected fractions (20 drops each) were analyzed for carbohydrate content using the phenol-sulfuric acid method (Dubois et al., 1956) by adding 200 μL of 5% aqueous phenol and 1 mL of concentration sulfuric acid (with mixing after each addition) to 200 μL of the collected fraction and measuring absorbance at 490 nm after 20 – 30 min. The blue value for each fraction was measured by determining the absorbance at 630 nm after adding 100 μL of a 0.01 %/0.1 % I₂/KI (pH 4.5) to 100 μL of each collected fraction. (Juliano BO, 1971). Absorbance measurements were made using a plate reader (Ultra Microplate Reader, ELx808, BIO-TEK Instruments, Inc.). Absorption spectra of fractions were determined with a VARIAN, CARY 50 UV-Visible spectrophotometer, using samples prepared by adding 6 μL of I₂/KI (0.01%/0.1%) and 54 μL of each fraction into a sub-micro cell cuvette (VARIAN). The spectrum was obtained for 5 nm increments in wavelength from 400 nm to 700 nm.

Fraction numbers 25 to 48 and fraction numbers 53 to 70 were combined to represent the large and small molecules observed in the mutant strains. The glucans from the combined fractions were precipitated with 5 volumes of 100 % ethanol and the resulting precipitate dispersed in 0.9 mL of hot water with stirring in a boiling water bath. The glucan dispersion was prepared for debranching by addition of 100 μL of 0.1 N sodium acetate buffer (pH 3.5), 2 μL of 10% sodium azide and 15-30 units of isoamylase, and incubated in a 40°C shaking water bath at 120 rpm for 48 hours. Debranching was terminated by boiling for 15 minutes
and raising the pH to neutral by addition of NaOH. The chain length distribution of the large and small molecules was analyzed by HPAEC (Dionex-300, Sunnyvale, CA) equipped with a post column amylloglucosidase reactor and pulsed amperometric detector (PAD) according to Wong and Jane (1997).

Note that glucan analysis was performed only on soluble WT glucans and insoluble M3 and SM3 glucans. Insoluble glucans from WT were used for measurement of $\lambda_{\text{max}}$ and analysis in sucrose gradient centrifugation experiments only.

Extraction of insoluble glucan in its native form

Following centrifugation at 6000g for 15 minutes, pelleted cells from 1 L of culture were resuspended in 10 mL of 10 mM Tris (pH 8) with approximately 3-4 mL of 0.15 mM glass beads and disrupted in a Mini-Beadbeater 6 times for 20 seconds at 5000 rpm. The mixture was kept on ice between each disruption and afterwards was centrifuged at 10,000 g for 20 minutes to pellet insoluble glucan. The glucan pellet was resuspended in 10 mL of 10 mM Tris (pH 8) buffer and filtered using a 30 µM mesh cloth to remove glass beads. The glass beads were resuspended in more buffer and filtered again to elute additional glucan. The combined filtrates were centrifuged at 10,000 g for 20 minutes, the supernatant removed and the tubes inverted to drain on paper towels. The drained pellets were resuspended in 2.5 mL of 90 % Percoll (Sigma) and centrifuged at room temperature for 30 minutes at 10,000 g using a swinging bucket rotor (Ral et al., 2004). Insoluble glucan with a density of 1.13 g/mL or greater that pelleted through the Percoll was resuspended again in 90 % Percoll and centrifuged at 10,000 g for 30 minutes at room temperature. The resultant pellet was washed with 1 mL of water 3 times (resuspended and centrifuged at 8,000 g for 10 minutes at room temperature). The resulting pellet was resuspended in 1 mL of 100 % ethanol and centrifuged at 8,000 g for 10 minutes, and the ethanol wash was repeated twice more. The final pellet was resuspended in 100 % ethanol and kept at room temperature for further analysis, including $I_2$/KI staining for observation with the light microscope, and, after negative staining with lead tungstate, for transmission electron microscopy (TEM). The glucan pellet
after fixation, embedding and sectioning (see below) also was used for Theiry staining of thin sections to identify carbohydrate.

Microscopy

For transmission electron microscopy (TEM), samples were collected and fixed with 2% (w/v) glutaraldehyde and 2% (w/v) paraformaldehyde in 0.1M cacodylate buffer, pH 7.2, for 48 hours at 4°C. Samples were rinsed 3 times in 0.1M cacodylate buffer and then post-fixed in 1% osmium tetroxide in 0.1 M cacodylate buffer for 1 hour at room temperature. The samples were rinsed in deionized, distilled water and dehydrated in a graded ethanol series, cleared with ultra-pure acetone, infiltrated and embedded using Spurr’s epoxy resin (Electron Microscopy Sciences, Ft. Washington, PA). Resin blocks were polymerized for 48 hours at 65°C. Thick and ultrathin sections were made using a Reichert Ultracut S ultramicrotome (Leeds Precision Instruments, Minneapolis, MN). For general morphology imaging, ultrathin sections were collected onto copper grids and counter-stained with 5% uranyl acetate in deionized distilled water for 15 minutes, followed by Sato’s lead stain (Sato, 1968) for 10 minutes. Images were captured using a JEOL 1200EX II scanning and transmission electron microscope (Japan Electron Optic Laboratories, Peabody, MA).

For Theiry staining, ultrathin sections were collected onto gold grids. Grids were exposed to 1% periodic acid for 45 min. (control grids were placed into 10% hydrogen peroxide for the same time). Grids were washed 4 times with distilled water and then placed in 0.2% thiocarbohydrazide overnight at room temperature. Grids were treated with descending acetic acid solutions 20%, 10%, 5% and distilled water for 5 minutes at each step. Sections were then placed in 1% silver proteinate (in the dark) for 130 minutes and then washed in distilled water 5 times, 10 minutes each step, before being allowed to dry. Images were captured using a JEOL 1200EX II scanning and transmission electron microscope (Japan Electron Optic Laboratories, Peabody, MA). Diameter and width measurements on structures were made using the Analysis Pro program (Olympus Soft Imaging Solutions).
Sucrose gradient centrifugation

In order to test the physical characteristics of solubility and density, samples were placed on a discontinuous sucrose gradient prepared in 400 µL microcentrifuge tubes (BIO PLAS, Inc.) and centrifuged for 30 seconds in a Beckman Microfuge B at room temperature. The sucrose solutions, supplemented with I$_2$/KI, were prepared at room temperature. The discontinuous sucrose gradient was prepared, on ice, by adding the solutions in decreasing order such that the most concentrated was added first and the least concentrated was added last. The solutions also were kept cold to reduce mixing at the interface of layers. The sucrose solutions were made in 5 mL batches, supplemented with 500 µL of I$_2$/KI (0.1%/1%) before addition of water to make up the final volume. Samples of glucan were extracted by treatment with DMSO or by Percoll gradient centrifugation, and 200 µg of each glucan sample was precipitated with 5 volumes of 100 % ethanol, and the resultant pellet was resuspended in 100 µL of 100 % ethanol. A pipette tip was used to break up the pellet, and 25 µL of the glucan suspension was applied to the discontinuous sucrose gradient, followed by the 30 second centrifugation and photography of the results.

Results

The double mutant SM3 was produced by introduction of MSBE I and a Spec$^R$ cassette into the psbA II coding region of *Synechocystis* in an M3 background containing residual amounts of GBE activity, as detailed in Chapter 2. Expression of MSBE I in the SM3 double mutant after 2 days of induction in nitrogen-limiting BG-11 was confirmed by western analysis (Figure 1A). The total BE activity in WT, M3 and SM3 was determined using the phosphorylase a stimulation assay (Table 1), which revealed that BE activity in SM3 had increased over its recipient strain, M3, suggesting that the increase in activity might be attributed to the expressed MSBE I protein. However, since the phosphorylase a stimulation assay only determines total BE activity and is unable to differentiate between GBE and MSBE I activity, zymogram analysis was carried out to give visual confirmation of the activity of MSBE I in SM3 (Figure 1B). A band in SM3 similar in color and position to the
BE band in maize and absent in WT and M3 confirmed that MSBE I was expressed and active in SM3.

Table 1 also shows that during stationary phase much more of the BE activity is detectable due to the lack of dead/inactive protein mass, which is present after glycogen induction and which may lead to a decrease in calculated activity that may not reflect the true state of the culture. In either case, the trend shows an increase in BE activity in SM3 relative to M3, the recipient/parent strain, and only ~ 35 – 50 % levels of WT activity.

Figure 1: (A) Western blot with proteins from maize kernels, WT, M3 and SM3 and molecular weight markers. The primary antibody was raised in rabbit against MSBE I. (B) Zymogram analysis of proteins from maize kernels, WT, M3 and SM3 Arrows in (B) point to MSBE I activity in Maize and SM3
Table 1: BE activity of WT, M3 and SM3.

<table>
<thead>
<tr>
<th>Strain</th>
<th>BE activity* (% of WT in similar condition)</th>
</tr>
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<tbody>
<tr>
<td></td>
<td><strong>Stationary phase</strong></td>
</tr>
<tr>
<td>WT</td>
<td>65.50 (100 %)</td>
</tr>
<tr>
<td>M3</td>
<td>5.50 (9 %)</td>
</tr>
<tr>
<td>SM3</td>
<td>35.20 (54 %)</td>
</tr>
</tbody>
</table>

*As nmols glucose incorporated per min per mg total soluble protein in harvested cells, measured using the phosphorylase a stimulation assay.

The expression of MSBE I in SM3 produced a glucan yield similar to that produced in M3. The glucans produced under these two conditions were not only similar in quantity but also similar in being primarily water-insoluble (Table 2). The iodine complex $\lambda_{\text{max}}$ values for water soluble and insoluble glucans for each strain is also provided in Table 2. The almost equal $\lambda_{\text{max}}$ values indicate that WT soluble and insoluble glucans may be very similar. The soluble glucan fractions of M3 and SM3 have lower $\lambda_{\text{max}}$ values compared to the respective insoluble fraction, which indicates more branching in the water soluble molecules compared to the insoluble molecules.

Table 2: Proportion of soluble and insoluble glucan in WT, M3 and SM3 and the respective $\lambda_{\text{max}}$ values for iodine complexes.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Soluble</th>
<th>Insoluble</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>µg</td>
<td>% of total</td>
</tr>
<tr>
<td>WT</td>
<td>9548</td>
<td>69.1</td>
</tr>
<tr>
<td>M3</td>
<td>82</td>
<td>1.3</td>
</tr>
<tr>
<td>SM3</td>
<td>171</td>
<td>3.8</td>
</tr>
</tbody>
</table>

Separation of glucan by high performance size exclusion chromatography (HPSEC) revealed the presence of many populations of molecules in WT, M3 and SM3 (Figure 2) such that there are only large molecules in WT and there are large and small molecules in M3 and SM3. The proportion of these molecules is provided in Table 3 and shows that in M3 a slightly greater proportion of molecules are present as large molecules. In contrast, SM3 has almost entirely large molecules.
Table 3: Proportion of glucan present as large and small molecules in WT, M3 and SM3.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Percentage of Large molecule</th>
<th>Percentage of Small molecule</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>100% (~9.1 mL to 17 mL)(^a)</td>
<td>absent</td>
</tr>
<tr>
<td>M3</td>
<td>56% (~8.1 mL to 17.5 mL)(^a)</td>
<td>44% (~18.8 mL to 21.5 mL)(^a)</td>
</tr>
<tr>
<td>SM3</td>
<td>97.8% (~8.1 mL to 16.9 mL)(^a)</td>
<td>2.2% (~19.7 mL to 21.3 mL)(^a)</td>
</tr>
</tbody>
</table>

\(^a\) As a proportion of the total glucan in HPSEC elution profiles.

The molecular weight of soluble WT glycogen was determined, using MALLS and RI detector signals from the HPSEC system using ASTRA software (Wyatt Technology, Santa Barbara, CA), to be 1.05 \times 10^7\text{ Da}, which is similar to values reported previously (2 \times 10^7\text{ Da in Yoo & Jane, 2002 and 6.6 \times 10^7\text{ Da in Yoo et al., 2002). The interesting difference seen in M3 compared to WT is the presence of a population of small molecules (see Chapter 2) with an average DP of 39 which is similar to DP 35 reported in Yoo et al., 2002, and the feature that separates M3 and WT from SM3 glucan is the presence of a population of very large molecules in SM3 with an average molecular weight of 2.60 \times 10^8\text{ Da (Table 4) which is close to the molecular weight of amylopectin reported in Yoo and Jane (2002). These very large molecules present in SM3 (eluting at ~ 8 mL to 9.0 mL) are 10 times larger than the molecules in WT and M3 (Table 4). The molecular size of the small molecules was calculated with respect to elution volumes of pullulan standards, which indicated the small molecules in M3 to have an average molecular size of DP 39 and those in SM3 to have an average molecular size of DP 26 (Table 4).}
Figure 2: HPSEC chromatograms of WT, M3 and SM3 and elution volumes of pullulan standards.

Table 4: Molecular weight of populations of large and small molecules in WT, M3 and SM3.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Molecular Weight (Da)(^a)</th>
<th>Large molecules</th>
<th>Large molecules</th>
<th>Small molecules</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td></td>
<td>1.05 x 10(^7)</td>
<td>(~ 9 – 11)(^b)</td>
<td>absent</td>
</tr>
<tr>
<td>M3</td>
<td>1.67 x 10(^7)</td>
<td>9.37 x 10(^6)</td>
<td>(~ 10.0 – 11)(^b)</td>
<td>6.32 x 10(^3)</td>
</tr>
<tr>
<td>SM3</td>
<td>2.60 x 10(^8)</td>
<td>7.70 x 10(^6)</td>
<td>(~ 9.0 – 11)(^b)</td>
<td>4.21 x 10(^3)</td>
</tr>
</tbody>
</table>

\(^a\) Determined for large and small molecule populations by MALLS-RI detectors and comparison with pullulan standards, respectively.

\(^b\) Elution volume in HPSEC.

\(^c\) Degree of polymerization given as DP.

In order to confirm that the large and small molecules are carbohydrate and to study the chain length characteristics of the large and small molecules independently, they were separated by gel permeation chromatography (GPC) and the total carbohydrate and blue value determined for each fraction (Fig. 3).
Figure 3: (A) GPC elution profiles for WT, M3 and SM3 glucan, including pullulan standards as molecular weight references. Fractions were analyzed for total carbohydrate and for blue value (OD$_{630nm}$ of iodine complex). (B) Total carbohydrate traces only—WT, □ M3 and ◊ SM3

GPC results (Fig. 3) confirmed the presence of large and small molecules in M3 and SM3 and showed that WT produces only large glucan molecules. Carbohydrate analyses of the eluted fractions showed only a single peak for the large molecule in all of the three strains, in contrast to the many peaks that were observed for the HPSEC elution profiles with the same samples. Because the GPC column is small, it is unable to yield complete separation of the molecules. Therefore, all the populations of large molecules observed with HPSEC (Fig. 2) apparently eluted together on the GPC. The population of small molecules observed in HPSEC chromatograms (Fig. 2) is observed in GPC chromatograms (Fig. 3A) also. The pattern of carbohydrate elution from GPC for each strain confirms that WT contains almost exclusively large molecules and that M3 and SM3 contain large and small molecules, although SM3 contains a greater proportion of large molecules relative to the small molecules (89 and 11 % respectively) compared to M3 (82 and 18 % respectively) (Fig. 3A).

Table 5 provides a summary of key data for each strain from the GPC analysis, including the peak fractions for the large and small molecules, values for the wavelength maximum ($\lambda_{\text{max}}$) of the glucan-iodine complexes for the peak fractions, and identification of the first carbohydrate-containing fraction. The carbohydrate trace shows that the WT and M3 glucans
may be eluting a few fractions after the SM3 glucans (Fig. 3B) indicating larger molecules in SM3 compared to WT and M3. However, it is difficult to use these elution profiles to determine the first fraction with carbohydrate and therefore, confirmation of the presence of larger carbohydrate molecules in SM3 compared to WT and M3 can be obtained from the HPSEC traces (Fig. 2). Consideration of the blue value (OD$_{630nm}$ of glucan-iodine complex) in relation to the total carbohydrate of each GPC fraction in Figure 3 reveals that WT fractions have a lower branch chain length distribution than M3 and SM3 (i.e., low blue value to carbohydrate ratios in WT compared to those in M3 and SM3). In M3, I$_2$/KI complexes of the large and small molecules give very different blue value to carbohydrate ratios, where iodine complexes with the large molecules yield a lower blue value to carbohydrate ratio than with the small molecules. In SM3, too, a similar but less pronounced trend is evident. This indicates that the large molecules in M3 and SM3 are more branched than the respective small molecules. As shown in Table 6, comparison of the $\lambda_{\text{max}}$ of glucan-iodine complexes indicates that the molecules in WT have shorter chains compared to those in M3 and SM3, and, based on similar evidence, large molecules in M3 apparently have shorter chains than those in SM3 in the first fractions. However, the relative $\lambda_{\text{max}}$ values for M3 and SM3 are reversed in the peak fractions. The small molecules seem to have equally long, branch chains in both M3 and SM3 when the $\lambda_{\text{max}}$ values of the peak fractions are considered.

Table 5: A summary of the first fraction with carbohydrate and the peak fraction with carbohydrate for WT, M3 and SM3 when analyzed through GPC.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Large Molecule</th>
<th>Small molecule</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>First fraction</td>
<td>Peaks fraction</td>
</tr>
<tr>
<td>WT</td>
<td>31 (525 nm)*</td>
<td>44,45 (525 nm)*</td>
</tr>
<tr>
<td>M3</td>
<td>22, 31 (535 nm)*</td>
<td>44 (560 nm)*</td>
</tr>
<tr>
<td>SM3</td>
<td>21, 26 (545 nm)*</td>
<td>40 (545 nm)*</td>
</tr>
</tbody>
</table>

$^*$ $\lambda_{\text{max}}$ value of glucan-iodine complex.

In an attempt to understand better why the $\lambda_{\text{max}}$ values for peak fractions of the large molecules in M3 were so high, the $\lambda_{\text{max}}$ values for all fractions for each of the three strains were obtained. Therefore, the $\lambda_{\text{max}}$ value of the glucan-iodine complex, which serves as a good indication of glucan chain lengths, is provided for all GPC fractions of WT, M3 and
SM3 in Figure 4. These data show that the molecules in WT, irrespective of size, have a similar branch chain length distribution and therefore, have a relatively constant $\lambda_{\text{max}}$ value (525 nm) for each fraction. The SM3 double mutant expressing MSBE I had a higher $\lambda_{\text{max}}$ value (545 nm) for all fractions up to fraction 53 and had higher $\lambda_{\text{max}}$ values (560 nm) for the following fractions. This suggests that molecules of varying sizes collected up to fraction 53 had a similar branch chain length distribution and that the smaller molecules eluting after fraction 54 had fewer branch points and therefore longer branches and a higher $\lambda_{\text{max}}$ value. The M3 mutant showed an interesting trend, where the fractions with larger molecules (lower fraction numbers) had lower $\lambda_{\text{max}}$ values and the $\lambda_{\text{max}}$ values increased as the molecules decreased in size (increasing fraction numbers). This trend suggests that the chain length from glucans in the M3 sample decreases as the molecules increase in size, i.e., shorter chains in the large molecules and longer chains in the small molecules. In M3, therefore, the branching pattern may reflect the limitation of BE activity, if there is non-uniform access of BE activity to all glucan molecules, i.e., that the larger and more branched glucans result from greater exposure to BE, and the smaller and mainly linear glucans result from little or no exposure to BE. In the WT and SM3 samples the $\lambda_{\text{max}}$ values remained fairly constant, suggesting that the branching of molecules in these strains is a reflection of the nature of the branching activity of the specific enzyme present. The small molecules in SM3 that do have higher $\lambda_{\text{max}}$ values (fractions $\geq 54$ in Fig. 4) might represent a population that has received more exposure to the limited BE activity. If so, this indicates that ~35 – 50 % of WT BE activity is sufficient for fairly uniform branching of most of the glucans, suggesting that BE activity may be present in excess in WT.
Figure 4: Glucan-iodine complex $\lambda_{\text{max}}$ values for all GPC fractions from WT, M3 and SM3.

- $\star$ WT
- $\blacksquare$ M3
- $\triangle$ SM3
Figure 5: Branch chain length distributions for glucans from preparative GPC for (A) total WT glucans (WT-total) and pooled large WT glucans, (WT-large), (B) total M3 glucans (M3-total), pooled large M3 glucans (M3-large) and pooled small M3 glucans (M3-small), and (C) total SM3 glucans, (SM3-total), pooled large SM3 glucans (SM3-large) and pooled small SM3 glucans (SM3-small).

Pooled total (pre-GPC) glucan fractions (WT-total, M3-total, SM3-total), as well as pooled preparative GPC fractions from WT, M3 and SM3 containing either large glucans (WT-large, M3-large, SM3-large) or small glucans (M3-small, SM3-small) were subjected to HPAEC-ENZ-PAD to determine the branch chain length distribution for each (Fig. 5). Inspection of the branch chain length distributions for the total glucan pools and the large and small glucan pools reveals that the distributions for WT-total and WT-large are very similar, as are the distributions for SM3-total and either SM3-large or SM3-small. However, very obvious differences can be seen when the M3-total branch chain length distribution is compared to those of M3-large and M3-small. These data support the observation, based on GPC fraction $\lambda_{max}$ values (Fig. 4), that the various populations of glucans in M3 exhibit branching, and that this differential branching might result from non-uniform access of the different populations to the very limited GBE activity. On the other hand, the chain length distributions for the various SM3 glucan pools seem fairly uniform whether the total pool or pooled fractions are considered, which supports the suggestion, based on the relatively uniform $\lambda_{max}$ values across the SM3 GPC fractions, that BE access to the glucan populations in SM3 is relatively uniform and that the branching characteristics and branch chain length distribution likely result from the specific characteristics of the MSBE I activity in this mutant. A summary of the peak DPs and the average DPs for the chromatograms given in Figure 5 are provided in Table 7.
Table 7: Summary of DP peaks and average DP for WT, M3 and SM3 pooled glucans, debranched and analyzed by HPAEC-ENZ-PAD. Average DP was calculated using the formula $\Sigma CiMi/\Sigma Ci$ where $Ci$ is peak area and $Mi$ is molecular weight at each DP.

<table>
<thead>
<tr>
<th></th>
<th>WT Total</th>
<th>WT Large</th>
<th>M3 Total</th>
<th>M3 Large</th>
<th>M3 Small *(GPC)</th>
<th>SM3 Total</th>
<th>SM3 Large</th>
<th>SM3 Small *(GPC)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DP of Peaks</td>
<td>6</td>
<td>6</td>
<td>19</td>
<td>7,13</td>
<td>7,14</td>
<td>6</td>
<td>7,15</td>
<td>7,17 and 49</td>
</tr>
<tr>
<td>Average DP</td>
<td>16</td>
<td>16</td>
<td>27</td>
<td>19</td>
<td>39</td>
<td>23</td>
<td>23</td>
<td>22</td>
</tr>
</tbody>
</table>

*Samples were pooled fractions collected after GPC separation

The data summarized (Table 7) show that the peak DP for the pooled large glucans in WT (DP 6) is lower than either of the two DP peaks for the pooled large glucans of M3 (DP 7, 13), which also is lower than either of the two DP peaks for the pooled large glucans of SM3 (DP 7, 15). However, because of the asymmetric distribution of branch chains in these distributions, a more valid comparison is obtained by considering average rather than peak DP values (Table 7). Among the pooled large glucans, the average DP for SM3-large (23) is the longest, followed by M3-large (19) and WT-large (16). As expected based on the other characteristics, WT and SM3 glucan samples seem fairly similar whether considered as pooled total glucans or as pooled large and small glucan fractions, and the average DP values for M3 glucan samples vary greatly depending on which pooled samples are considered.

In addition to the increased DP peak values and average DP values in SM3, a bimodal chain length distribution also was observed in SM3 (Figure 5 and Table 7), consistent with the expected transfer of longer chains by MSBE I relative to those transferred by GBE. Also supporting this expectation are the increased SM3 DP peak values and average DP values themselves compared to the same characteristics for the WT sample.
In order to compare branch chain length distributions between strains, we used difference plots. The difference plot of (SM3-large - WT) shows that SM3-large has more of the long chains with DP > 15 compared to WT and that of (SM3-large – M3-large) has more of the long chains with DP > 20 compared to M3-large (Fig. 6). This is consistent with peak and average DP values for SM3 being greater than those for WT. M3-large also has more of the long chains with DP > 11 compared to WT. This plot also shows that SM3-large is very different from WT and M3-large. The difference plot between M3-large and WT shows the least difference of the three pair-wise comparisons (0.50 for (SM3-large – WT), 0.32 for (SM3-large – M3-large) and 0.26 for (M3-large – WT) when the absolute difference at each DP value is taken and summed over the whole range of DP values), showing that, M3-large may be similar to WT, although M3-large does possess more of the long, branch chains of DP 11 to 28 to a greater extent than DP 29 – 56 compared to WT. By the same token, it can be seen that SM3-large is very different from WT and that SM3-large has more of the long, branch chains of DP 15 – 44 and DP 45 to 64 compared to WT. The comparison of SM3-large to M3-large quantifies, in terms of chain lengths, the action of MSBE I over and above the effect of residual GBE action. Therefore, SM3-large has more chains with DP > 20 compared to M3-large, which may the due to the long chains transferred by MSBE I.

The glucan structures in WT, M3 and SM3 were subjected to the Theiry stain and observed under TEM. Spherical glycogen particles that stained evenly throughout were observed in WT, a linear, mesh-like arrangement of glucan was observed in M3, and structures that were stained in the periphery and not in the centers as well as structures similar to those in M3, were observed in SM3 (Fig. 7). These observations provide visual confirmation that the introduction of MSBE I has resulted in synthesis of glucans that differ from those present in either WT or M3.
Normalized peak area x 10^-1

Degree of polymerization
Figure 6: Difference plots of branch chain length distributions for glucans from preparative GPC

Figure 7: *In vivo* sections of WT, M3 and SM3 cells. Theiry stained to reveal the shape and nature of accumulated glycogen/glucan. The full length of the scale bar denotes 200 nm.

Insoluble glucan with a density $\geq 1.1 \text{ g/mL}$ was extracted using Percoll (Sigma) gradient centrifugation to preserve its native structure/form, negative stained and examined by TEM to study its surface/shape/size characteristics (Fig. 8). The same Percoll-gradient prepared samples also were sectioned and Theiry stained to determine if the nature of the extracted structures is similar to those seen *in vivo*. Preliminary confirmation of the glucan nature of the insoluble material collected by Percoll gradient centrifugation was obtained by I$_2$/KI
staining of the material, which exhibited brown/purple coloration when viewed under the light microscope (data not shown).

Figure 8: Negative stain of structures extracted using Percoll gradient centrifugation for WT (A), M3 (C), SM3 (D) and a control with no cells (B). In (A) the scale bar denotes 200 nm with each division representing 40 nm, and in (B), (C) and (D) it denotes 500 nm with each division representing 100 nm. Arrows in M3 and SM3 point to stacked, plate-like structures forming single oval/spherical units and those in the no cells control point to silica particles.

As expected because of the almost complete absence of material isolated in the insoluble fraction, examination of the negative-stained, Percoll-purified samples from WT (Fig. 8A) revealed no material notably different from that observed in the control (no cells), which included silica particles (open circular shape, ~30 nm in diameter) from the Percoll (Fig 8B). In M3 and SM3, the oval/spherical structures which appear to be formed from stacked, plate-
like structures are marked with block arrows (Figs. 8 C and D). First impressions that these structures in M3 are smaller than those in SM3 are validated by measurement of diameters using the analySIS program (Olympus soft imaging systems). The plate-like structures also appear to be discontinuous (Fig. 8 C and D). Based on the average height of the plate-like structures in M3 (~3 nm; range of 2.1 to 4.1 nm) and SM3 (~5.8 nm; range of 3.6 to 8.3 nm), and assuming the width of a glucose moiety from C1 to C4 to be ~3.4 Å (Buleon et al., 1998), the expected number of glucose units in plate-like structures 3 nm and 5.8 nm long would be 9 and 17 glucose units, respectively.

In addition to negative staining and I$_2$/KI staining, the extracted, Percoll-purified insoluble material also was embedded, sectioned and Theiry stained (Fig. 9) to confirm that the structures under study are carbohydrate in nature and to determine whether they are similar to structures seen in vivo (Fig. 7). Since WT cells have mostly water soluble glycogen and nearly no material was extracted using the Percoll gradient centrifugation, the WT samples and control (no cells) samples were very similar whether negative stained or Theiry stained, so these samples are not included in Figure 9.
In SM3, Theiry stained sections of Percoll-purified material was similar in size (~43 nm vs ~30 nm in diameter; Table 8) and shape to the spherical, open structures seen in vivo. The Theiry stained sections of M3 cells showed mostly linear structures in vivo, whereas the extracted, Percoll-purified material from M3 was similar in shape to that from SM3 but smaller in size (~28 nm). This size was larger than that (~11 nm) of the low abundance of peripherally-stained, spherical structures observed in vivo in M3.
Table 8: Sizes of glycogen/glucan particles in vivo and in vitro

<table>
<thead>
<tr>
<th>Strain</th>
<th>Diameter(^a) of glycogen particle/insoluble glucan from in vivo Theiry stain</th>
<th>Dimensions(^a) of in vitro negative-stained oval/spherical structures</th>
<th>Diameter(^a) of in vitro oval/spherical structure Theiry stained</th>
<th>Width(^a) range of plate-like structures in vitro</th>
<th>Average width(^b) of plates in plate-like structures in vitro</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>22.6</td>
<td>Length 27.7 Width 27.7</td>
<td>Diameter 22.6</td>
<td>Width 2.11-4.14</td>
<td>Average width 3.0 (9)(^b)</td>
</tr>
<tr>
<td>M3</td>
<td>11.1</td>
<td>Length 27.7 Width 27.7</td>
<td>Diameter 22.6</td>
<td>Width 2.11-4.14</td>
<td>Average width 3.0 (9)(^b)</td>
</tr>
<tr>
<td>SM3</td>
<td>30.3</td>
<td>Length 39.4 Width 55.6</td>
<td>Diameter 43.2</td>
<td>Width 3.57-8.27</td>
<td>Average width 5.9 (17)(^b)</td>
</tr>
</tbody>
</table>

\(^a\) in nm and \(^b\) number of glucose units needed

In order to compare the glucan produced in M3 and SM3 with normal maize and amaranth starch granules, Percoll-gradient purified M3 and SM3 glucans and DMSO-extracted WT glucans were placed on a discontinuous sucrose gradient along with normal maize and amaranth starch granules and rabbit liver glycogen, subjected to a 30 second centrifugation and photographed immediately (Fig. 10).

Figure 10: DMSO-extracted WT soluble glycogen, M3 and SM3 insoluble glucan extracted by Percoll gradient centrifugation, maize and amaranth starch granules and rabbit liver
glycogen centrifuged on a discontinuous sucrose gradient supplemented with I₂/KI for 30 seconds.

These results show that the insoluble glucan extracted by Percoll gradient centrifugation from M3 and SM3 behave similar to the starch granules and different from the soluble, precipitated WT or rabbit liver glycogen under these sedimentation conditions. Figure 10 illustrates the conclusion that M3 and SM3 insoluble glucans have a density ≥1.376 g/ml, because, like starch granules, they sediment through 75% sucrose. On the other hand, DMSO-extracted M3 and SM3 insoluble glucans behave like the WT soluble glucan on a discontinuous sucrose gradient (Fig. 11). This may also suggest that after treatment with DMSO the structure of the insoluble glucans is altered, even after reprecipitation with ethanol.

Figure 11: WT insoluble and soluble glucan, and M3 and SM3 insoluble glucan, extracted by treatment with DMSO, placed on a discontinuous sucrose gradient supplemented with I₂/KI and centrifuged for 30 seconds.
The results portrayed in Figures 10 and 11 are not absolute indicators of the density of the sample but serve as an indication of the similarities and dissimilarities between samples. Comparison of Figures 10 and 11 also suggest that there is a structure to the glucan produced in M3 and SM3 that makes it behave similar to maize and amaranth starch granules when they are extracted using Percoll gradient centrifugation rather than when they are extracted using DMSO.

The results as a whole suggest that the expression of MSBE I has led to the formation of a molecule that is insoluble, has longer branch chain lengths with a bimodal chain length distribution and that this molecule has acquired solubility and density characteristics similar to that in maize and amaranth starch granules and has a structure that gives the impression of stacked plates when negatively stained and viewed under TEM.

**Discussion**

As discussed in chapter 2, the greatly reduced GBE activity of the M3 mutant results in decreased levels of glucan accumulation, of which ca. 50% appears to be linear glucan while the other ca. 50% is branched. The abundance of small, linear glucan illustrates the impact of limiting branching enzyme activity in M3, and the decreased glucan accumulation in M3 attests to the connection between formation of non reducing ends by the action of branching enzymes and molecular growth and ultimately the amount of glucan accumulated. In addition, as concluded in chapter 2, the linear small molecules and the inverse relationship between branch length and glucan molecular size in M3 resulted from the extremely limited BE activity. They reasoned that the limited activity of native GBE resulted in transfer of short chains to a limited number of molecules, and that the larger glucans in M3, which had the shortest branch chains (most similar to WT), resulted from the greatest exposure to GBE activity. Thus, as the glucan molecules of M3 were exposed to less GBE activity, they had fewer branch chains and longer branches, as the result of continued GS extension of the chains in the absence of branching. Therefore, the chain lengths seen in M3 may result from
uneven exposure to branching and elongation activities rather than the result of branching activity alone.

SM3, like M3, does not completely lack the native GBE activity, probably because complete elimination of GBE is lethal (as referred to in chapter 2). The presence of some residual GBE activity in SM3 somewhat confounds the interpretation of the impact of MSBE I expression, but the substantial differences between the glucans produced by SM3, M3 and WT provide insight into the effect of MSBE I activity on glucan structure in SM3. In contrast to the <10% of WT BE activity in M3, BE activity in SM3 was increased to 35 – 55 % of WT levels due to the expression of MSBE I. Nonetheless, this increased BE activity resulted in no net increase in amount of glucan accumulated in SM3, relative to M3, but rather, impacted the structure of the glucan produced and increased the proportion of branched glucans to ca. 98 % of the glucans produced.

It is generally expected that increased branching activity would form more non reducing ends, which would ultimately lead to stimulation/increase of glucan synthesis and accumulation. However, the lack of an increase in glucan synthesis concomitant with the increase in branching activity, already concluded to be greatly limiting in M3, suggests that the native glycogen synthases may not have utilized the non reducing ends produced by MSBE I as efficiently as those of their normal, soluble glucan substrates. This argues that the branches observed in the SM3 glucans result primarily from MSBE I action alone rather than from limited exposure to BE activity in combination with elongation by the native glycogen synthases, as concluded for M3, and that molecular growth of the SM3 glucans occurred primarily by increasing the number of branches rather than by the elongation of branches. The fairly constant glucan-iodine complex $\lambda_{\text{max}}$ values observed for SM3 branched glucans demonstrates that the branch chain lengths are constant, irrespective of the molecular weight/size of the glucan, which was confirmed by the observed branch chain length distributions of SM3 large glucan and total glucan pools. The constancy of the SM3 branch chain length regardless of glucan molecular size further argues that MSBE I was responsible for the transfer and formation of branch chains with little but uniform impact from the
glycogen synthases. The branch chain lengths observed in SM3 glucans are also in agreement with MSBE I action as recorded in literature (Guan et al., 1995; Guan et al., 1997), further supporting the conclusion that the branch chain length distribution of SM3 glucans largely reflects action of MSBE I activity alone. Taken together, the data leads us to the conclusion that the branches produced in branched glucan molecules in SM3 is a reflection of, primarily, MSBE I action alone.

Despite a substantial increase in total BE activity relative to M3, SM3, like M3, produces almost exclusively insoluble glucans. Thus, the action of MSBE I in transferring branch chains longer than DP 10 (Guan et al., 1997) possibly led to the formation of double helices, which requires a minimum length of DP 10 (Gidley MJ & Bulpin PV, 1987), resulting in synthesis of branched, insoluble glucans. The formation of such insoluble, branched glucans, therefore, is consistent with the transfer of long, branch chains that can form double helices without further elongation. This is in contrast to the rationale for the formation of insoluble glucan in M3 (as referred to in chapter 2), where long branch chains resulting from very limited BE activity in combination with elongation by GS also facilitated the formation of double helices and led to the synthesis of insoluble glucans.

The formation of insoluble, branched glucan by action of MSBE I in SM3 is different from what was reported when MSBE I was expressed in E. coli, where it was concluded that a glycogen-like polysaccharide was produced (Guan et al., 1995). However, it is not clear if the procedure used (according to the method reported in Preiss et al. (1975) was aggressive enough to extract a representative portion of the insoluble glucan for characterization. The similarity of the glucans produced in the AC71 strain expressing MSBE I to soluble glycogen from E. coil B, as reflected in the glucan-iodine complex $\lambda_{\text{max}}$ value may be recapitulated by our observation that the soluble glucans produced in the SM3 mutant had a glucan-iodine complex $\lambda_{\text{max}}$ value similar to WT Synechocystis soluble glycogen. Thus, some glucan molecules (a small proportion) may experience a net action of BE and native GS activity such that they are readily water soluble because of the formation of numerous short branches. Although the proportion of such soluble glucans produced in the E. coli AC71 strain may
have been fairly high, the proportion of soluble glucans produced in SM3 was minimal relative to the insoluble glucans produced. For this reason, and because the insoluble glucans may reflect the formation of double helices when long chains were transferred in SM3, we have focused only on the insoluble form of glucan produced in SM3.

The activity of MSBE I in SM3 led to a greater occurrence of chains with DP >14 compared to WT, which is similar to the trend observed when MSBE I was expressed in *E. coli* (Guan et al., 1995). This is also in agreement with the report that MSBE I is able to transfer longer chains than GBE from *E. coli* (Guan et al., 1997). MSBE I also has been expressed in a yeast heterologous system, where it was concluded that MSBE I required the prior action of maize SBE IIa (MSBE IIa) and maize SBE IIb (MSBE IIb) and where, in the presence of the MSBE IIb and MSBE IIa, MSBE I was associated with the production of only short chains of DP 4 – 11 (Seo et al., 2002). This association of MSBE I with short branch chain lengths differs markedly from the chain lengths of DP >10 reported to be preferentially transferred by MSBE I (Guan et al., 1997). When MSBE I was expressed in a yeast mutant lacking the native GBE activity, no net increase in BE activity was observed in the resultant strain, which did not support the accumulation of glucan (Seo et al., 2002). It is not clear whether insufficient levels of MSBE I activity may have confounded the ability of this approach to provide a more complete understanding of MSBE I action.

Visual comparison of HPSEC and GPC data show that SM3 produced molecules larger in size than those of M3 and WT. A similar conclusion can be reached by examining the negatively stained samples exhibiting stacked plate-like structures. Calculations using HPSEC data show that SM3 produced glucan molecules (ca. $10^8$ Da) approximately 10 times greater in size than those produced in WT or M3 (ca. $10^7$ Da). The action of MSBE I in transferring longer branches may have increased the capacity to carry more branches resulting in an ultimate increase in molecular weight. In glycogen, exterior chain lengths (ECL) are about 8 glucose units and interior chain lengths (ICL) are about 4 glucose units (Manners, 1989). Therefore, a chain of DP 28 may be expected to have four ICLs and to carry four branches whereas, a chain of DP 13 may have just one ICL and carry only one
branch. Because a minimum number of glucose units between branch points appears to be required, the number of branch chains carried should increase as the chain length increases, resulting in a higher molecular weight. Therefore, the increase in molecular weight seen in SM3 also is consistent with the expectation that MSBE I transfers longer chains than GBE.

Not only are the glucans produced by SM3 insoluble, the results from sucrose density gradient centrifugations indicate they also exhibit a high density, similar to that of starch granules from maize and amaranth. Their behavior in this respect is very different from that of soluble glycogen from WT *Synechocystis* and rabbit liver, and similar to those from M3. In addition, comparisons of the glucans extracted by Percoll gradient centrifugation and by treatment with DMSO confirm that the insoluble glucans of M3 and SM3 have a structure *in vivo* giving them a density similar to maize and amaranth starch granules, but that extraction with DMSO leads to the loss of the native structure and thus, to a loss of their high densities. DMSO treatment produced samples more like the soluble and insoluble forms of WT glycogen, although the difference due to the presence of long chains was still apparent in the color formed by the glucan-iodine complex. The water soluble characteristic of WT glycogen is explained by the presence of abundant, short branch chains in a non-clustered arrangement (Manners, 1989).

The solubility and density characteristics of M3 and SM3 glucan can be explained by the presence of long, branch chains that form double helical crystallites, which are not readily soluble in water and may also lead to higher density glucans relative to WT but similar to maize and amaranth starch granules (density of ca. 1.4 g/mL). It is interesting to note that the process by which the chains are synthesized gives the glucans a high density characteristic that can be lost by dispersion in DMSO, even after re-precipitation with ethanol. This suggests that long, branch chains in themselves do not confer a high density to the glucan molecules but that the mechanism of branch chain production *in vivo* must facilitate the formation of double helices, thus conferring high density characteristics.
The high density and insoluble nature of the SM3 glucans apparently results from transfer of long, branch chains by MSBE I leading to formation of double helices and crystalline arrays. This conclusion is consistent with the conclusion discussed above that the increased BE activity producing more branch points without increasing the glucan accumulation can be explained by the substrate being produced in an insoluble form. In M3, also, the glucans display high density. However, as concluded in chapter 2, this may result from a severe GBE activity limitation, where a limited number of short branches formed are elongated by GS, and, the absence of further branching eventually leads to the formation of double helices and crystallites. As a result, the presence of limited GBE activity in M3 also leads to the formation of high density glucan. This is somewhat similar to the result obtained in maize ae mutants where the lack of SBE IIb activity results in synthesis of high amylose starch with amylopectin molecules having more of the long chains (Klucinec & Thompson, 2002).

The large glucans of SM3 exhibit a slightly bimodal chain length distribution, with peaks at DP 15 and DP 49, reminiscent of the more prominent bimodal distribution of maize amylopectin, where peaks occur at DP 13 and 45 (Perera et al., 2001). The bimodal branch chain length distribution of SM3 is in contrast to the unimodal distribution of the WT sample classically observed with glycogen (Yoo et al., 2002). Although a bimodal branch chain distribution supports the tandem cluster arrangement of branch chains for amylopectin from various botanical sources, a single enzyme has not been identified as being responsible for this feature. In fact, mutants defective in specific enzymes involved in amylopectin biosynthesis, such as the ae (lacking SBE IIb) and sbe 1 (lacking SBE I) mutants in rice, still exhibit a bimodal chain length distribution (Nishi et al., 2001 and Satoh et al., 2003). Therefore, it is interesting to note that the expression of MSBE I in SM3, is able to establish, even to a limited extent, a similar bimodal chain length distribution.

The large glucans of M3 also exhibit a very slight second peak around DP 46 – 50 (as referred to in chapter 2). However, the shape of the branch chain length distribution for M3 is skewed to the shorter chains, unlike the distributions of amylopectin (Perera et al., 2001) and
is probably the result of glycogen synthases and a much reduced amount of GBE working together as described earlier.

A bimodal chain length distribution is associated with the presence of A and B1 chains that span one cluster and B2 and B3 chains that span more than one cluster (Hizukuri, 1986; Hanashiro et al., 1996), and an average chain length of 20 – 22 interconnects clusters in amylopectin (Buleon et al., 1998). Therefore, the observed bimodal branch chain length distribution and average branch chain length of 22 - 23 of SM3, increased from 16 in wild type, are consistent with but does not demonstrate a cluster arrangement of tandemly-linked chains. Furthermore, the chain lengths seen in SM3 suggest that some chains can serve as inter-cluster chains. In addition, the proportion of chains greater than DP 37 is ca. 15 % in SM3, compared to 21.4 % in maize (Perera et al., 2001) and only ca. 5 % in WT *Synechocystis*. Chains of DP ≥37 are classified as B3 chains based on the periodicity intervals of DP 12 (Hanashiro et al., 1996). Thus, SM3 also produces a significant proportion of branch chains sufficiently long to interconnect three or more clusters.

The increased proportion of chains with DP ≥37 associated with the presence and activity of MSBE I is reminiscent of the decrease in chains of DP ≥37 in the *sbe 1* rice mutant lacking SBE 1, which belongs to class B of BEs, as does MSBE I (Satoh et al., 2003). Therefore, the activity of MSBE I expressed in SM3 is associated with production of long, branch chains characteristic to class B branching enzymes. Therefore, the chain length distribution of SM3 has attributes, such as a bimodal chain length distribution, an average chain length of 22 – 23, and a high proportion of chains with DP ≥37, similar to those of amylopectin that reflect a cluster arrangement of tandemly-linked chains. The increased abundance of chains with DP ≥37 in SM3 also is similar to that reported in molecules designated semi-amylopectin by Nakamura et al. (2005). In the study done by Nakamura et al., (2005) the proportion of chains with DP ≥37 was essentially less than 1 % in cyanobacterial glycogen and phytoglycogen and was 6.7 % in amylopectin from rice cv. Kimmaze and ranged from 3.3 to 4.6 % in what was defined as semi-amylopectin from *Cyanobacterium* sp. MBIC10216, *Myxocarcina burmensis* and *Synechococcus* sp. BG043511 when the branch chain length
distribution was analyzed using the fluorescence-labelled capillary electrophoresis method. When branch chain length distribution from our study is transformed to account for number of chains, those with DP ≥37 was determined to be 1.3 %, 2.4 % and 4.2 % for WT, M3 and SM3 respectively showing that the value obtained for SM3 lies within the range of values obtained for what was called semi-amylopectin. However, these authors do not disclose the solubility and density characteristics of these semi-amylopectin molecules, so we cannot make further comparisons with the molecules produced in SM3.

In contrast to the case in SM3, in WT, the average DP is 16, the proportion of chains that are DP ≥37 is only 5 % and the branch chain length distribution is unimodal, giving rise to soluble glycogen. In the large glucans of M3, the average DP is 19, the proportion of chains that are greater than DP 37 is 8 % and the branch chain length distribution is largely unimodal like WT and less bimodal in nature than SM3. Therefore, the M3 glucans exhibit characteristics that lie between those of WT and SM3. The glucans in M3 show a distribution similar to WT but have a greater occurrence of long chains, which has resulted in insoluble glucans. According to the information gained from the difference plots, it is evident that SM3-large has more long chains than M3-large which in turn has more long chains than WT.

The glucan produced in SM3 and M3 appeared roughly spherical or rather plate-like, respectively, when Theiry stained for carbohydrate in vivo and observed under TEM. Both appear quite different from the glycogen particles observed in WT cells, consistent with the substantial differences in glucan structure between Wt and either M3 or SM3. The glucan produced in M3 and SM3 extracted by Percoll gradient centrifugation has a shape and size similar to that seen in vivo and this served as confirmation that the material isolated by Percoll gradient centrifugation was carbohydrate material and that it had retained its in vivo structure during the extraction process. However, the sizes of negative stained structures were slightly larger than those of the corresponding Theiry stained structures, which may result from shrinkage during chemical treatments such as periodic acid oxidation included in the Theiry staining procedure (Oostergetel & Van Bruggen, 1989).
An interesting feature of the surface view of the negatively stained glucans was the presence of stacked, plate-like structures. These structures resembled blocklet structures reported in Gallant et al. (1997). The absence of amylose-like molecules in M3 and SM3 may have allowed better penetration of the negative stain to give a clearer representation of the stacked, plate-like structures, much as it was easier to see the rippled effect of amylopectin from waxy maize, barley and low amylose potato starches (Oostergetel and Van Bruggen, 1989). The plate-like structures in M3 and SM3 are also discontinuous, similar to the discontinuities reported in lamellae regions that make up blocklets in amylopectin (Gallant et al., 1997).

The negatively stained samples support the conclusion, based on the HPSEC and GPC data, that the glucans in SM3 are larger than those of M3. The sizes of the stacked plate-like structures in SM3 were ca. 40 – 50 nm, while these structures in M3 were ca. 30 nm. French (1972) estimated that an amylopectin molecule may be about 50 nm, indicating the size of the stacked plate-like structures to be similar in size to an amylopectin molecule. The height of each plate in the stacked, plate-like structures, was, on average, 5.9 nm in SM3 and 3.0 nm in M3. If the plate-like structures represent many side chain clusters lined up adjacent to each other, the height of the plate-like structure should be approximately the height afforded by a chain of glucose units present within the cluster. In SM3, the average height of 5.9 nm corresponds to 17 glucose units, assuming the distance of one glucose unit in a chain to be 3.4Å (Buleon et al., 1998). It has also been reported that 85 % of chains in amylopectin are A and B1 chains that span a single cluster (Manners, 1989). According to the branch chain length distribution for the large branched molecule of SM3, 85 % of the chains would be less than DP 37 and the average length of this population of chains is 18.6 glucose units. This suggests that the height of the plate-like structures in SM3 (5.9 nm) could be explained as a row of side chain clusters arranged adjacent to each other, assuming the side chain clusters to be made of glucose chains corresponding to DP 17, which agrees reasonably well with the peak DP of 15 (and an average DP of 18.6 for chains less than DP 37) for the large molecules in SM3.
The pool of GPC fractions collected as the ‘large’ molecules is probably a mixture of molecules with different degrees of branching and molecular weights, so the branch chain length distribution reflects an average that may not agree with plate-heights of negative stained M3 samples. Also, the height of plate-like structures in M3 did not show a normal distribution, unlike those in SM3 (data not shown), suggesting that variable plate heights may result from the combination of limiting GBE activity and GS activity. The interesting phenomenon that emerges from the presence of structures in M3 similar to those in SM3 is that, limiting BE activity also can give rise to branched molecules containing crystallites and produce insoluble glucans in *Synechocystis*.

The addition in SM3 of MSBE I expression and activity into a mutant background with very low GBE activity produced glucans that are larger than those in WT and M3, have bimodal branch chain length distribution consistent with the presence of tandem branch clusters, appear as stacked plate-like structures in TEM when negatively stained, and have dimensions explained by their branch chain length distribution. In addition, the glucans extracted to retain their *in vivo* structure also exhibited solubility and density characteristics similar to maize and amaranth starch granules. All these characteristics can be explained by the transfer of long branch chains by MSBE I, leading to the formation of double helices that can form crystalline arrays and limit further elongation and branching in those areas due to steric hindrance, as proposed by French (1972). These characteristics provide evidence that the glucan in SM3 may have a structure that resembles, to some extent, that of amylopectin. This leads us to propose that the transfer of long branch chains by MSBE I may facilitate formation of branch chains that form double helices with neighboring chains to give crystallites, leading to the formation of large branched molecules with tandem branch cluster arrangements that are insoluble has and have density characteristics markedly different from WT glycogen and similar to maize and amaranth starch granules. Therefore, SM3 may produce glucans that have made a transition to form molecules similar to amylopectin.

In addition to producing glucans with some similarities to amylopectin, the branch chain length distributions and the size of the plate-like structures in SM3 suggest that each plate-
like structure may represent a row of side chain clusters arranged with their long axis adjacent to each other, and that there may be inter-cluster chains connecting the side chain clusters present in adjacent, stacked, plate-like structures. Therefore, the chain length transferred by MSBE I may have been a determinant in deciding/setting the height of the crystalline region of the side-chain clusters and ultimately the height of the plate-like structures. This suggests that, the action of MSBE I may play a role in determining the physical, water-insoluble characteristics of amylopectin as hypothesized, and, also is a determinant of the height of crystalline regions that alternate with amorphous regions with clustered branch points seen in amylopectin.

Further characterization of the glucans produced in SM3 to determine the presence/absence of crystallinity by X-ray crystallographic analysis is difficult because of the small amounts of material extracted. However, DSC analysis of these glucans may provide conclusive evidence regarding the presence/absence of crystallinity. It would also be interesting to determine if the expression of potato SBE I, which is in the same class as MSBE I and can transfer long branch chains, also is able to elicit the same response from *Synechocystis* as has been found for MSBE I in the SM3 mutant. If so, this would allow us to draw wider conclusions about the function of branching enzymes transferring longer branch chains in determining the unique fine structure of amylopectin. This system can also be used to express the soluble starch synthases in combination with a plant BE to gain an understanding of the enzymatic processes that lead to the synthesis of the amylopectin molecule. This report has established the usefulness of the *Synechocystis* model system in studying starch granule development.

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CHAPTER 4: CONCLUSIONS AND RECOMMENDATIONS

Conclusions

Starch, made up of the two biopolymers amylose and amylopectin, is a widely used raw material in many food and non-food industries. It also plays a crucial role in the life of the plant, because it can be produced for transient storage in the chloroplast or for long term storage in sink tissue such as endosperm, tubers, etc. Amylose and amylopectin are polymers of glucose linked by $\alpha$-1,4-O-glucosidic linkages between glucose units and $\alpha$-1,6-O-glucosidic linkages at branch points. Amylose is primarily linear, with only ca. 0.1% branch points, and amylopectin is multiply branched, with 4 – 5% branch points. The scientific community has actively sought to unravel the factors that control the higher order arrangement of these molecules into starch granules and the factors that control the synthesis and breakdown of the two specific molecules, although insight into these biopolymers comes only in small increments.

The study of the starch granule has become an interdisciplinary science due to the great deal of order mixed with complexity that is present at many levels. As such, it is studied by microscopists to understand its size, shape and surface characteristics, it is studied by biologists in terms of the enzymes that are involved in its synthesis and degradation, it is studied by food scientists and chemists to determine the pasting and gelling properties afforded by its component molecules, and so on. While it is known that synthases and branching enzymes are involved in the synthesis of starch, recent studies have also implicated debranching enzymes and disproportionating enzymes as having some role in this process. However, the individual role of each of the isoforms of each category of enzyme is not fully known. Mutants have been used to study the roles of the branching enzymes, which are thought to control the lengths of the chains transferred and the frequency and pattern of branching, and the roles of synthases that are expected to elongate the transferred chains. However, the difficulty with interpreting information obtained from mutants has been that, often, pleiotropic effects are seen in other starch synthesizing enzymes. The branching enzymes have also been expressed in heterologous systems such as E. coli and yeast to
determine their specific functions without interference from the other starch synthesizing enzymes. However, the expression of branching enzymes in these systems did not lead to the production of amylopectin-like molecules, although in the *E. coli* system they did not characterize any insoluble glucan that may have been produced and in the yeast system MSBE I action was very low according to activity assays (Guan et al., 1995; Seo et al., 2002). This dissertation has focused on using a different system, the cyanobacterium *Synechocystis* sp. Strain PCC 6803, as one in which the effects of the maize branching enzyme I (MSBE I), a class B branching enzyme, can be studied. As such, the mutant M3, which was partly characterized and reported in Yoo et al. (2002), and SM3 have been studied in some detail, and the highlights of the results are stated here.

The conclusions gained from this dissertation, therefore, fall into two main categories, which are regarding the (1) M3 mutant and the (2) SM3 mutant. The M3 mutant has been defined according to our results as one with very limited glycogen branching enzyme activity, and the SM3 mutant has been defined as one with very limited glycogen branching enzyme activity inherited from the M3 host strain, as well as maize SBE I activity.

Regarding M3

In M3, PCR analysis and enzyme activity data support the presence of a copy of the native glycogen branching enzyme. However, the branching enzyme activity in M3 is significantly reduced compared to the wild type strain and the growth habit of M3 is reduced compared to the wild type strain. This result suggests a relationship between growth habit and the capacity to synthesize soluble glycogen, such that when glycogen synthesis is impaired due to reduced branching enzyme activity, the growth rate of the culture is reduced. This may be similar to the AGPase mutant of *Synechocystis*, where a down-regulation of photosynthesis was seen when glycogen synthesis was limited, suggesting a feed back inhibition on photosynthesis by the absence of glycogen synthesis (Miao et al., 2003).
The mutants SM3 and SM3G retained some native glycogen branching enzyme according to PCR analysis despite the presence of maize starch branching enzyme I. The recipient strain used in the production of both SM3 and SM3G was M3. Therefore, the inability to completely replace the native glycogen branching enzyme with maize starch branching enzyme I may be due to the need for *Synechocystis* to synthesize soluble glycogen or due to M3 being a merodiploid such that one copy of its genome has retained an uninterrupted form of the glycogen branching enzyme.

The further characterization of glucans produced by M3 showed the presence of large, insoluble, branched molecules, with some longer chains than those in wild type, in addition to the small mostly linear molecule reported in Yoo et al. (2002). This result suggests that either the presence of a reduced level of branching enzyme activity in combination with the glycogen synthesizing enzymes is able to produce large molecules with longer chains or that a truncated version of glycogen branching enzyme that could be present in a merodiploid is able to transfer longer branch chains than the native glycogen branching enzyme. In order to demonstrate that the glycogen branching enzyme cannot be completely replaced by the MSBE I, additional mutants need to be generated where the endogenous glycogen branching enzyme is targeted for replacement. The insoluble glucan produced in M3 showed characteristics similar to the insoluble glucan produced in SM3 when viewed under TEM. However, the stacked, plate-like structures produced in M3 were smaller in size than in SM3. Inferences regarding chain lengths relating to the height of the plate-like structures in M3 could not be made since the molecules in M3 seemed to show different degrees of branching, as represented by the change in iodine complex $\lambda_{\text{max}}$ values over different fractions collected by GPC.

Regarding SM3

The results obtained from the SM3 mutant fall into two categories, which are to do with (i) the nature of glucan accumulation and its relationship to branching enzyme activity and (ii)
the lengths of the branches transferred and their relationship to the nature of the structures synthesized in vivo, as viewed under TEM.

(i) Since a low level of branching enzyme activity compared to WT was sufficient to produce almost completely branched glucan in SM3, we surmise that branching enzyme activity is present in excess under WT conditions. The insoluble nature of the glucan produced in SM3 also suggests that the increased branching due to MSBE I action in transferring longer branch chains did not result in a more soluble glucan. This served as an initial confirmation that the transfer of long, branch chains may have led to the formation of double helices and ultimately crystalline arrays of double helices that prevented the glucan being synthesized being water soluble. The inability of SM3 (similar to M3) to accumulate glycogen to levels seen in WT suggest that there may have been some effect on the glycogen synthesizing enzymes or that the native glycogen synthesizing enzymes were unable to use, efficiently, an insoluble glucan (which is produced in SM3 and M3) as a suitable substrate. The insoluble glucan produced in SM3 is characterized by a high density, similar to densities of maize and amaranth starch granules, as demonstrated by discontinuous sucrose gradient centrifugation. The action of DMSO in dispersing this native structure was seen when DMSO-dispersed glucan from SM3 was subjected to the same centrifugation in a discontinuous sucrose gradient. This apparent high density in insoluble glucan from SM3 may have resulted from the formation of crystalline arrays of double helices produced by the transfer of long, branch chains by MSBE I action.

(ii) The transfer of long, branch chains by MSBE I action in SM3 resulted in the formation of molecules with very high molecular weight, similar to those of amylopectin molecules present in starch granules. This may be explained by the long, branch chains produced in SM3 having the capacity to hold more branches and thereby leading to greater molecular growth. A slight bimodal branch chain length distribution of the large molecules in SM3 suggest that MSBE I action in transferring long branch chains, coupled with the action of the native glycogen synthesizing enzymes in Synechocystis sp strain PCC 6803 was responsible for this. This argues, therefore, that the action of MSBE I is partly responsible for the setting
up of the bimodal chain length distribution that is very evident in amylopectin molecules. The insoluble glucan produced in SM3, when extracted by Percoll-gradient centrifugation to avoid the dispersing effects of DMSO and retain a more in vivo structure, showed a stacked plate-like arrangement when viewed under the TEM. This stacked plate-like arrangement of the glucan fits the description of the amylopectin blocklet in terms of appearance and size as reported in Gallant et al. (1997). The size of the stacked plate-like arrangement also is in the same order of magnitude as the predicted size of an amylopectin molecule (French, 1972), suggesting that the stacked plate-like objects observed in SM3 may represent molecules similar to amylopectin but produced by the combined action of MSBE I and the endogenous glycogen synthesizing enzymes. Assuming the height of the plates in this structure is one that is constrained by the lengths of the chains, the length of chains needed to form double helices to fit the height of the plates in this structure should be somewhat equal to the peak DP or average DP of 85 % of the chains (which is said to be the proportion of chains compared to the total that make up a cluster in the amylopectin molecule – Manners, 1989) of the branch chain length distribution seen in SM3. We have determined the plates to have an average height of 5.9 nm that corresponds to 17 glucose units and also determined the peak DP and average DP of 85 % of the chains to be 15 and 18.6, respectively. Since these numbers lie in the same range, we have concluded that the stacked plate-like structures may have been produced by clusters of chains arranged adjacent to each other within one plate and that inter-cluster chains may link the individual plates comprising of clusters of chains. The average DP, 22 – 23, of SM3, determined from its branch chain length distribution suggests that there are chains long enough to serve as inter-cluster chains.

The combined information gathered from the insoluble glucan produced in SM3 suggests that the activity of MSBE I in transferring long chains may have resulted in the formation of glucans with somewhat of a cluster arrangement of branches, a high density and a stacked, plate-like structure. The cluster arrangement, if present, in SM3 may not be as distinct or as organized as seen in amylopectin, since the whole suite of starch synthesizing enzymes is not present in SM3. However, the presence of such a cluster arrangement of branch chains giving rise to high molecular weight glucan with density characteristics similar to amylopectin, and
a bimodal branch chain length distribution similar to amylopectin, is consistent with it also exhibiting stacked plate-like structures similar to the blocklets proposed to represent branch chain clusters in the amylopectin of starch granules. Therefore, we are led to conclude that the expression of MSBE I in *Synechocystis* sp. Strain PCC 6803 led to the formation of a glucan that has characteristics similar to amylopectin, fitting our hypothesis that the transfer of long, branch chains, coupled to an environment rich in lipids may facilitate the production and the maintenance of a cluster branching pattern and the formation of crystalline and amorphous regions.

The results from the SM3 mutant also support the model that a physical process may be controlling the formation of double helices and crystallites at the granule surface, since all known starch mutants have a defect in one of the enzymes involved in starch metabolism rather than in proteins that may be involved in granule assembly. Our results do not contradict the water soluble polysaccharide clearing model, which suggests that the synthesis of amylopectin and a water soluble polysaccharide are competing processes. However, our approach specifically looked at the effect of MSBE I on the nature of the glucan produced and did not study the role of debranching enzymes and soluble starch synthases. Therefore, the results from this dissertation do not provide evidence to support or reject the glucan trimming model or the two-step branch clearing model that have also been proposed to explain the synthesis of amylopectin.

The starch granule is a higher order structure composed of amylose and amylopectin, although only amylopectin is said to be responsible for the semi-crystalline nature of starch. Our goal has been only to elucidate the role of MSBE I in the formation of a molecule such as amylopectin. The effect of amylose synthesis on the arrangement of amylopectin side chain clusters has not been addressed and much more characterization of the *Synechocystis* system will be required before efforts to answer questions related to amylose synthesis in connection with amylopectin synthesis can be carried out.
Additional observations

We also have observed that the temperature at which the culture is grown plays a role in the rate of growth of the culture. This in turn appears to have impacted the glucan produced. Therefore, we have observed that when the culture is grown at less controlled, lower temperatures, the glucan from M3 had different peak and average DP values, and the same was observed with glucan from SM3. GPC analysis also showed that there was a great reduction in the proportion of the small molecules produced in SM3 when the temperature of the cultures was variable and less than 30°C. This suggests that when temperature is set at less than 30°C, growth of *Synechocystis* is reduced, and the enzymes produced may be less active, leading to changes in the chain length distribution. This also corroborates the finding in Takeda et al. (1993) that MSBE I is more active at higher temperatures (for instance, 30°C) than at lower temperatures.

Branch chain length distributions from cultures grown at two different temperatures showed that in M3, the branch chain length distribution for 30°C cultures included more of the short chains relative to a lower and more variable temperature, suggesting greater GBE activity at 30°C, which is the optimum temperature for growth of *Synechocystis* cultures (Eaton-Rye, 2004; Rippka et al., 1979). The same distribution for SM3 grown at two different temperatures showed that at the higher temperature of 30°C, where MSBE I is expected to be more active (Takeda et al., 1993), increased branching enzyme activity led to the formation of more of the long, branch chains. This also fits with the explanation that at higher temperatures the potato branching enzyme was shown to transfer longer chains (Borovsky et al., 1975; Borovsky et al., 1976).
Recommendations for future experiments

(1) Further characterization of M3 and SM3 mutants

The genetics of the M3 and SM3 mutant can be more clearly characterized by carrying out extensive Southern analysis to determine whether they are merodiploids. In addition, the glucans produced, although insufficient in amounts for X-ray analysis, should be sufficient for analysis using DSC (diffraction scanning calorimetry) to gain insight into the presence/absence of crystallinity. The production of crystalline arrays of double helices by the transfer of long branch chains by MSBE I action or the combined action of limited GBE activity and glycogen synthase activity in SM3 and M3, respectively, as predicted from interpretations of negative stained material viewed in TEM, in combination with branch chain length distribution data could be more definitively determined by showing that there is a temperature at which these crystallites can absorb latent heat and undergo melting.

(2) Production of new mutants to determine need for glycogen synthesis in Synechocystis sp. Strain PCC 6803

It has been shown that a reduction in glycogen biosynthesis causes a feedback inhibition on photosynthesis (Miao et al., 2003) and causes a reduction in growth of the mutants produced (Miao et al., 2003; referred to in chapter 2). However, it has not been demonstrated that glycogen synthesis is essential for the survival of the strain. This can be determined by producing more M3-like and SM3-like mutants that are completely segregated. The selection for these mutants should be done under conditions of low CO₂ and in the absence of supplemented glucose. This approach may be complemented by introducing a GBE from a different source (for instance E. coli) to determine if complete replacement of the native GBE is possible. From lessons learned, it would be necessary to screen several possible transformants in order to obtain a mutant that has undergone double cross over homologous recombination and that is not a merodiploid.
(3) Characterization of native enzymes involved in glycogen metabolism in *Synechocystis* sp. Strain PCC 6803

Biochemical analysis of the function of the debranching enzymes in *Synechocystis* help relate the data gained from this dissertation with some of the models currently used to explain the synthesis of amylopectin. In addition, it would be interesting to determine the function of neopullulanase, which, from other sources, has been shown to be able to catalyze the formation and hydrolysis of α-1,4 and α-1,6 bonds between glucose units.

(4) *In vitro* experimentation with glycogen synthases

Since the action of MSBE I and limited GBE, in combination with at least the glycogen synthases, has given rise to a glucan with an interesting structure it would be useful to understand whether this can be replicated *in vitro*. This would give insight into the role of an environment rich in lipids proposed to be necessary to serve a nucleating function in granule development.

(5) Expression of soluble starch synthases in combination with MSBE I

In higher plants a whole suite of enzymes have been shown to be involved in the synthesis of amylopectin, although the specific function of each is not well understood. Since *Synechocystis* has proven to be a useful system to unravel, at least in part, the function of MSBE I and has resulted in the formation of a glucan that has a branch chain length distribution with similarities to that of amylopectin, it would be even more instructive to add soluble starch synthases that have a role in elongating the branch chains of amylopectin and to determine if the bimodal nature of the branch chain length distribution is accentuated to resemble amylopectin even more. The mutants produced could be characterized by experiments similar to those described in this dissertation. Additional experiments to determine A:B chain ratios and external and interior chain lengths would assist in classifying the glucan produced as similar to or different from glycogen. Further, TEM analysis of
negative-stained samples to obtain a tilt series to enable 3-D reconstruction of the structures and/or atomic force microscopy to study the surface characteristics of the structures produced would provide a more complete understanding of the role of these enzymes involved in the production of amylopectin, which appears to determine the semi-crystalline nature of starch.

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