Structural Characterization of High Molecular Weight Starch Granule-Bound Proteins in Wheat (*Triticum aestivum* L.)

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Starch granule-bound proteins in endosperms of common wheat (*Triticum aestivum* L.) included at least one major protein with a molecular weight of 61 000 (Wx protein: starch granule-bound starch synthase) and six minor proteins with molecular weights of 115 000, 108 000, 100 000, 92 000, 80 000, and 15 000 (SGP-A1, -D1, -B1, -2, -3, and friabilin, respectively). Peptide mapping of SGP-A1, -D1, -B1, -2, and -3 indicated that the primary structures of SGP-A1, -D1, and -B1 are highly homologous but differ considerably from that of SGP-2. The internal sequences of peptides obtained from SGP-D1, -2, and -3 showed that SGP-D1 and SGP-3 are structurally similar to rice soluble starch synthase, whereas SGP-2 has homology to rice starch branching enzyme 3. These results are in agreement with results of immunological and enzymatic studies suggesting that SGP-A1, -D1, -B1, and -3 are types of soluble starch synthases and that SGP-2 is a starch branching enzyme.

Keywords: *Starch granule-bound protein; amino acid sequence; wheat (Triticum aestivum L.)*

INTRODUCTION

Starch is a major constituent of seeds and tubers of the world's most important food crops such as cereals, legumes, and potatoes. Starch contains two structurally different components, amylose and amylopectin. Amylose is composed of large linear chains of α (1−4)-linked α-D-glucopyanosyl residues, whereas amylopectin is a branching form of α-glycan linked by α(1−6) linkages. In normal wheat, starch usually consists of 20−30% amylose and 70−80% amylopectin (Kuroda et al., 1989).

Waxy (amylose-free) mutants have been found in rice, maize, sorghum, barley, and potato (Hseih, 1988; Hovenkamp-Hermelink et al., 1987; Nelson and Rines, 1988; Sano, 1984). In these mutants, starch consists of almost 100% amylopectin and a starch granule-bound protein known as waxy (Wx) protein is missing, indicating that the production of amylose is linked to the amount of Wx proteins. Later, Wx protein was identified as starch synthase, which catalyzes amylose synthesis (Nelson and Rines, 1962). Comparisons of deduced amino acid sequences from waxy genes of wheat, rice, barley, and maize indicated that Wx proteins have a high degree of sequence similarity (about 85%) among these plants (Okagaki and Wessler, 1988; Rohde et al., 1988; Shure et al., 1983). A similar starch granule-bound protein found in potato tubers is also a granule-bound starch synthase (van der Leij et al., 1991).

In hexaploid common wheat, one of the endosperm starch granule-bound proteins was identified as Wx protein (Yamamori et al., 1992). Nakamura et al. (1995) showed that hexaploid common wheat has three Wx proteins encoded by waxy genes belonging to the A, B, and D genomes. Thus, the deletion of all three Wx proteins was required to produce waxy wheat. In nature, simultaneous deletion of all waxy genes belonging to three different genomes would rarely occur in common wheat. Nakamura et al. (1995) succeeded in breeding lines without Wx proteins by crossing partial waxy mutants with waxy genes located at one or two homoeologous chromosomes.

Since the ratio of amylose to amylopectin influences the texture and quality of flour, our understanding of the regulation of starch synthesis and deposition by starch-related enzymes is important for effective wheat breeding for seed quality.

However, with the exception of the Wx protein, none of the seven graunule-bound proteins identified in wheat (Schofield and Greenwell, 1987) have been characterized in detail. One of them is a 15 kDa protein, friabilin, the amino acid sequence of which is known (Rahman et al., 1994), but its function in vivo is not known. This protein has been proposed to be associated with grain quality (Greenwell and Schofield, 1986). The other five starch granule-bound proteins have higher molecular weights than Wx protein and are named high molecular weight (HMW) starch-granule bound proteins in this paper. The immunological reactions, enzyme activities, and N-terminal amino acid sequences of these proteins have been described (Denyer et al., 1995; Rahman et al., 1995). In this paper, we present the results of the structural characterization of these proteins based on peptide mapping and partial amino acid sequence determination.

MATERIALS AND METHODS

Plant Material. Mature grains of wheat (*Triticum aestivum* L.) cv. Norin 61, barley (*Hordeum vulgare* L. emend. Lam.) strain KI-29, and rice (*Oryza sativa* L.) cv. Nipponbare were used.

Preparation of Starch. Grains were ground by a coffee mill. Fifty grams of the crude flour was homogenized in 500

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mL of deionized water with a mortar and pestle and macerated at 4 °C for 4 h with occasional stirring. The flour solution was passed through a 75 μm mesh sieve and the filtrate centrifuged at 10000*g* at 4 °C for 10 min. Twenty grams of the pellet was resuspended in 400 μL of SDS sample buffer containing 60 mM Tris-HCl, pH 6.8, 10% (w/v) sodium dodecyl sulfate (SDS), 3% (v/v) β-mercaptoethanol, and 10% (v/v) glycerol, and the solution was centrifuged at 30000*g* at 4 °C for 10 min. This procedure was repeated three times. The pellet was washed with deionized water and centrifuged at 30000*g* at 4 °C for 10 min. This was repeated twice. Then, the pellet was washed with cold acetone and centrifuged at 30000*g* at 4 °C for 10 min. This washing procedure was repeated three times. Finally, about 8 g of the purified starch was obtained, dried at room temperature, and stored at −20 °C.

Extraction of Starch Granule-Bound Proteins. Sixteen grams of the dried starch was suspended in 10 volumes of the SDS sample buffer, placed in a water bath at 95 °C for 10 min, cooled on ice, and usually frozen at −80 °C for at least 1 h. After thawing at 50 °C, the gelatinized starch was centrifuged at 30000*g* at 4 °C for 10 min, the supernatant was transferred to a fresh tube, and the proteins were precipitated on ice for 1 h after the addition of 3 volumes of acetone. The samples were centrifuged at 30000*g* at 4 °C for 10 min. The pellet containing starch granule-bound proteins was dried at room temperature and resuspended in 1 mL of SDS sample buffer before being subjected to SDS−polyacrylamide gel electrophoresis (SDS−PAGE).

Electrophoresis. The starch granule-bound proteins were separated by SDS−PAGE (Kagawa et al., 1988). Gels contained 10% acrylamide [30:0.135 acrylamide/bis(acrylamide) (w/w)] and 0.1% (w/v) SDS. An aliquot (100 μL) of the sample solution was applied to gels, and electrophoresis was performed at 25 mA for 3.5 h. After electrophoresis, proteins were stained with Coomassie Brilliant Blue R or silver stain.

Peptide Mapping. Peptide mapping was carried out according to the method of Cleveland et al. (1977). After SDS−PAGE, protein bands were excised from gels and electroeluted at 2 W constant power. The eluted proteins were dialyzed against deionized water. The dialyzed solution was dried using a Speedvac concentrator and dissolved in 20 μL of SDS sample buffer. The protein solution was applied to an 18% SDS−polyacrylamide gel and 20 μL of SDS sample buffer diluted twice with deionized water, containing 2 μg of *Staphylococcus aureus* V8 protease, was overlaid on the protein solution. Electrophoresis was performed at 10 mA until the protein migrated to the center of the stacking gel and then interrupted for 1 h. Then, electrophoresis was continued to separate the digests.

Sequencing of Starch Granule-Bound Proteins. After peptide mapping, peptides in gels were transferred onto a poly(vinylidene difluoride) (PVDF) membrane by electroblotting (Hirano and Watanabe, 1990) and stained with Ponceau S; the bands were excised and applied to a gas-phase protein sequencer (Perkin-Elmer, Applied Biosystems, 491).

Homology Search. Peptide sequences were compared with proteins compiled in SwissProt and PIR databases using computer-aided analysis.

Extraction of a Soluble Protein Fraction. Flour was prepared from the mature grains of wheat and rice as described above for wheat. The soluble fractions were extracted in 100 mM MOPS, pH 7.2/1 mM DTT (Denyer et al., 1993). The sample was centrifuged at 10000*g* for 10 min, and the proteins in the supernatant were precipitated with 3 volumes of acetone on ice for 1 h. The sample was centrifuged at 30000*g* at 4 °C for 10 min, and the resulting pellet was diluted with the sample buffer, heated at 100 °C for 2 min, and applied to gels.

RESULTS AND DISCUSSION

Identification of HMW Starch Granule-Bound Proteins. Starch granule-bound proteins were separated by SDS−PAGE (10% gel) and detected by silver staining as one major band (MW 61 000) and five minor HMW bands (MW 115 000, 108 000, 100 000, 92 000, and 80 000). Another starch granule-bound protein with a low molecular weight, friabilin, was not separated in the 10% gel. The electrophoretic patterns of the MW 92 000, 80 000, and 61 000 proteins of wheat were similar to those of their rice and barley counterparts but the MW 115 000, 108 000, and 100 000 proteins of wheat were not found in rice and barley (Figure 1). The anti-100 000−105 000 MW wheat protein monoclonal antibodies do not react with the starch granule-bound proteins of rye, barley, rice, maize, potato, and pea (Rahman et al., 1995). This suggests that the MW 115 000, 108 000, and 100 000 proteins are specific to wheat. The relative molecular weights of wheat starch granule-bound proteins were deduced on the basis of their electrophoretic mobility, as shown in Figure 1. The values differ slightly from those deduced by Denyer et al. (1995) and Rahman et al. (1995). Comparison of relative molecular weights reported to date for the starch granule-bound proteins is shown in Table 1.

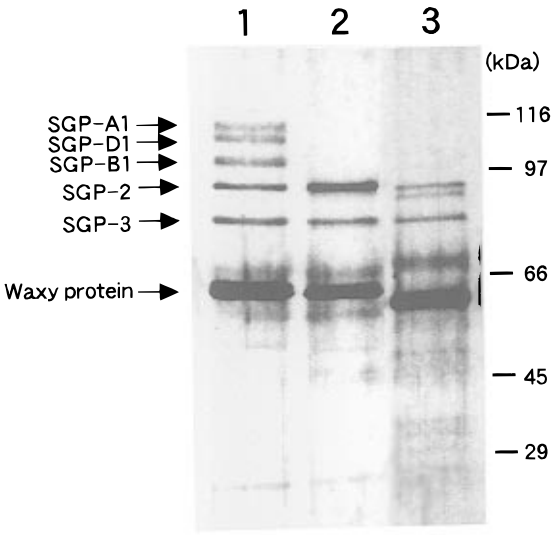

Figure 1. SDS−PAGE patterns of starch granule-bound proteins from wheat (lane 1), barley (lane 2), and rice (lane 3). Proteins were extracted from starch granules as described under Materials and Methods, separated by electrophoresis on 10% SDS−polyacrylamide gels, and visualized by silver staining. Positions of moleular mass marker proteins (Pharmacia) are shown on the right side of the gel.

The major MW 61 000 protein of wheat and barley showed slightly higher relative molecular weights than those of rice and maize. The determination of molecular weight and N-terminal sequence confirmed that the major protein is the Wx protein. The N-terminal sequence of the major protein of wheat was identical to that of Wx protein, which has been determined by actual protein sequencing (Nakamura and Hirano, unpublished data) and deduced from the nucleotide sequence of the cDNA encoding the Wx protein (Ainsworth et al., 1993).

Peptide Mapping of HMW Proteins. There were five minor proteins with higher relative molecular weights and weaker Coomassie Blue staining intensity than the Wx protein. According to nomenclature based on the genetic analysis (Yamamori and Endo, 1996), the MW 115 000, 108 000, 100 000, 92 000, and 80 000 proteins are designated SGP-A1, -D1, -B1, -2, and -3, respectively, in this paper. The electrophoretic patterns of peptides generated from SGP-A1, -D1, and -B1

Table 1. Molecular Weights of Starch Granule-Bound Proteins of Wheat[a]

	relative molecular weight			
	Denyer	Rahman	Yamamori	chromosome location
SGP-A1 (115 000)	100 000−105 000	100 000−105 000	115 000	7AS
SGP-D1 (108 000)			108 000	7DS
SGP-B1 (100 000)			100 000	7BS
SGP-2 (92 000)	90 000	85 000	92 000	
SGP-3 (80 000)	77 000	75 000	80 000	
waxy (61 000)	60 000	60 000	61 000	7A, 4B, 7D

[a] Results obtained in the present study are compared with previously reported values (Denyer et al., 1993; Rahman et al., 1995; Yamamori et al., 1996).

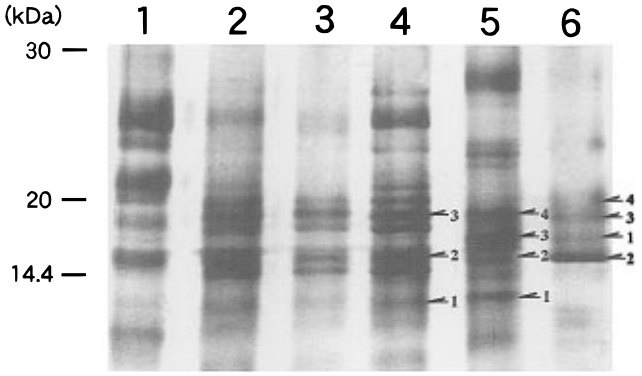

Figure 2. Peptide maps of *S. aureus* V8 protease digests of wheat Wx protein (lane 1), SGP-A1 (lane 2), SGP-D1 (lane 3), SGP-B1 (lane 4), SGP-2 (lane 5), and SGP-3 (lane 6). After digestion with *S. aureus* V8 protease in the gel, the digests were separated on a 20% SDS−polyacrylamide gel. The separated peptides were transferred onto PVDF membranes and detected with Ponceau S. The numerals show the peptides of which amino acid sequences were determined and shown in Table 2. Positions of molecular mass marker proteins are shown on the left side of the gel.

(Figure 2, lanes 2−4) were identical, indicating the structuralsimilarity of these proteins. These proteins are referred to as SGP-1 here. However, the patterns of peptides obtained by the digestion of SGP-1, -2, and, -3 differed from one another. This suggests that the HMW starch granule-bound proteins can be divided into three groups based on differences in primary structure.

Amino Acid Sequences of HMW Proteins. Attempts to sequence the five HMW starch granule-bound proteins using a gas-phase sequencer were not successful, possibly due to in vivo or in vitro N-terminal blockage. Therefore, to obtain sequence information, the internal sequences of the HMW starch granule-bound proteins were analyzed. After Cleveland peptide mapping on SDS−PAGE gels, the generated peptides were electroblotted onto PVDF membranes and subjected to gas-phase sequencing. The amino acid sequences of a total of 35, 31, and 60 residues for SGP-B1, -2, and -3, respectively, were determined as shown in Table 2.

SGP-1 Is Structurally Related to Soluble Starch Synthase. Denyer et al. (1995) reported that SGP-A1, -D1, and -B1 exhibit starch synthase activity and that antiserum raised against the pea soluble starch synthase cross-reacted with SGP-1, suggesting that SGP-1 is soluble starch synthase which is present in starch granules.

In Western blotting analysis, the antisera raised against rice starch branching enzyme (RBE1) 1 and RBE3 (Mizuno et al., 1992) did not cross-react with SGP-1 (Takaoka et al., unpublished data). SGP-A1, -D1, and -B1 had similar molecular weights and almost identical peptide compositions, suggesting that these proteins are structurally related and may be the products of homoeologous genes.

Table 2. Internal Amino Acid Sequences of Peptides from SGP-B1, -2 and -3

peptide	peptide no.	sequence
SGP-B1	1	LGGYIYGQNPAFV
	2	AGLYDAGGD/GE/A
	3	VNYFHAYGDGXDF
SGP-2	1	IFLPTADGSFXN
	2	T/DGVHSVI/E
	3	KYGFMLSDG
	4	IDPT
SGP-3	1	AAPYAKSGGLGDVSGSLPIAL
	2	LGRYIYGQNPMFVVNDAPAS
	3	GPMRSTESKYID
	4	GMAEN/STE

Yamamori and Endo (1996) demonstrated that individual SGP-1 proteins are produced by different homoeologous groups of chromosome 7. Developing grains of nullisomic−tetrasomic lines of cv. Chinese Spring lacking chromosomes 7A, 7B, and 7D did not have SGP-A1, -D1, and -B1, respectively (Table 1). This agrees with the results of Denyer et al. (1995).

Rahman et al. (1995) determined the N-terminal amino acid sequenes of SGP-A1, -D1, and -B1, but could not identify any proteins with structural homology to this region. We determined that the internal sequences of SGP-B1 are similar to those of rice soluble starch synthase (Mizuno et al., 1992) (Figure 3A), suggesting that SGP-1 may be soluble starch synthase.

Rahman et al. (1995) have recently reported that the anti-100 000−105 000 MW protein (SGP-A1, -D1, and -B1) monoclonal antibodies did not cross-react with proteins in the wheat soluble fraction. It was demonstrated that these proteins are localized in starch granules. In the present study, we could obtain no evidence that SGP-1 is localized only in the starch granules.

SGP-2 Is Structurally Related to Starch Branching Enzyme. The internal sequence of wheat SGP-2 showed high similarity to that of RBE3 (Mizuno et al., 1992) (Figure 3B) or maize starch branching enzyme IIb (BEIIb) (Mu-Forster et al., 1996). Western blot analysis using anti-RBE1 and -RBE3 antibodies indicated that the molecular weights of major immunoreactive proteins in the soluble fraction of rice were 80 000 and 90 000 on SDS−PAGE, respectively (Takaoka et al., unpublished data). The anti-RBE3 antibody reacted weakly with SGP-2 of wheat. In contrast, the anti-RBE1 antibody did not react with any of the wheat granule-bound proteins. It has been previously reported that antibody raised against BEIIb cross-reacted with SGP-2 and a soluble protein similar in size to SGP-2 from wheat (Rahman et al., 1995). As well, starch branching enzyme activity has been detected in the fraction including SGP-2 in wheat (Denyer et al., 1995).

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Rice starch          V T F F H E Y R D S V D      217
synthase             |   | |  |   |    |
SGP-B1(peptide3)     V N Y F H A Y G D G X D

Rice                 - G N L Y G D N F - G        238
synthase               |  | |  |      |
SGP-B1(peptide2)     A G - L Y - D A G - G

Rice                 L G G Y I Y G Q K C M F V    275
synthase             | | | | | | | |     | |
SGP-B1(peptide1)     L G G Y I Y G Q N P A F V
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(A)Sequence alignment of rice soluble starch synthase with SGP-B1.

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Rice branching       I F L P N N A D G S          248
enzyme               | | | |   | | | |
SGP-2(peptide1)      I F L P - T A D G S

Rice branching       K Y G F M T S D              726
enzyme               | | | | |  | |
SGP-2(peptide3)      K Y G F M L S D
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(B) Sequence alignment of rice starch branching enzyme 3 with SGP-2.

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Rice starch          A S P Y A K S G G L G D V C G S L P I A L     162
synthase             |   | | | | | | | | | | |  | | | | | | |
SGP-3(peptide1)      A A P Y A K S G G L G D V S G S L P I A L

Rice                 L G G Y I Y G Q K C M F V V N D W H A S       282
synthase             | |  | | | | |   | | | | | |   | |
SGP-3(peptide2)      L G R Y I Y G Q N P M F V V N D A P A S

Rice                 G W M R S T E S G Y R D                       503
synthase             |  | | | | | |  |  |
SGP-3(peptide3)      G P M R S T E S K Y I D
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(C) Sequence alignment of rice soluble starch synthase with SGP-3.
The enclosed sequence (K-S-G-G-L) has been implicated as the binding site for the substrate, ADP-glucose.

Figure 3. Comparison of amino acid sequences of wheat HMW starch granule-bound proteins, rice soluble starch synthase (Baba et al., 1993) (A, C), and rice starch branching enzyme 3 (Mizuno et al., 1993) (B). Identical residues are indicated by |. Amino acid positions are numbered from the N-terminal end of proteins.

Multiple forms of starch branching enzyme have been found in various plants. There are at least three forms of branching enzymes (BEI, BEIIA, and BEIIB) in maize (Boyer and Preiss, 1978; Fisher et al., 1995) and four forms (RBE1, RBE2, RBE3, and RBE4) in rice (Mizuno et al., 1992). RBE3 and BEIIB have high sequence similarity, although the N-terminal region is poorly conserved. Affinity-purified anti-RBE3 antibody did not react with either RBE1, REB2A, or RBE2B (Mizuno et al., 1992), indicating that RBE3 is distinguishable from these three starch branching enzymes. In our preliminary experiments, anti-RBE1 antibody did not react with the wheat SGP-2 (Takaoka et al., unpublished data), suggesting that multiple forms of starch branching enzyme exist in wheat and that SGP-2 is structurally divergent from RBE-1.

In pea, two starch branching enzymes (SBEI and II) occur in both the soluble and the granule-bound fractions (Denyer et al., 1993), and in maize, BEII is also found in both soluble and granule-bound forms (Mu-Forster et al., 1996). Western blot analyses (Rahman et al., 1995) have indicated that SGP-2 exists in both soluble and granule bound forms. Thus, like SBEI, SEBII, and BEII, SGP-2 may be one of the starch branching enzymes which are found in both soluble and granule-bound fractions.

SGP-3 Is Structurally Related to Soluble Starch Synthase. The partial amino acid sequences obtained for SGP-3 showed high similarity to that of rice soluble starch synthase (Baba et al., 1993) (Figure 3C) but did not share significant homology with starch granule-bound starch synthase. In pea seeds, two major isoforms of starch synthase, GBSSI (MW 59 000) and GBSSII (MW 77 000), are associated with starch granules (Smith, 1990). GBSSI is similar in amino acid sequence to Wx proteins isolated from other species. However, although GBSSII is antigenically related to the starch synthase (SSSII) found in the soluble fraction of pea seeds, it bears no such relationship to GBSSI or Wx protein (Denyer et al., 1993). The amino acid sequence of GBSSII, as well, was completely consistent with the sequences obtained for SSSII (Denyer et al., 1993). These results suggest that GBSSII exists in both soluble and granule-bound forms in pea. Denyer et al. (1995) reported that both SGP-3 and the soluble starch synthase are antigenically related to the pea GBSS II

(MW 77 000) and SGP-3 has a starch synthase activity. This, combined with the sequence data reported here, leads us to conclude that SGP-3 is one of the starch synthases which is present in both soluble and granule-bound forms.

The ADP-glucose binding site consensus sequence motif (K-X-G-G-L) is highly conserved in soluble starch synthase from rice, Wx protein from rice, wheat, and barley, and BGSS from potato (Ainsworth et al., 1993; Baba et al., 1993). This domain has been reported to function as the substrate ADP-glucose-binding site of *Escherchia coli* glycogen synthase (Furukawa et al., 1990). In the present study, the motif (K-S-G-G-L) was identified in SGP-3 (Figure 3C). Sequence analysis confirmed the wheat SGP-3 to be a rice soluble starch synthase-like protein which may play a role as a starch synthase in soluble and granule-bound fractions.

Possible Function of HMW Starch Granule-Bound Proteins. Denyer et al. (1993) provided evidence that the soluble starch synthase (SSSII) of wild-type pea embryos is the same protein as the starch granule-bound starch synthase (GBSSII). This protein may be enzymatically active only in the soluble fraction but is finally trapped with the granule and becomes inactive, thus existing in both soluble and starch granule-bound fractions (Denyer et al., 1995). It is probable that SGP-3, as well, is active only in the soluble fraction, being involved with starch branching enzymes in elongation of amylopectin (Denyer et al., 1995). Peptide sequencing of SGP-3 suggested that SGP-3 might be the same protein as one of the starch synthases in the soluble fraction. In maize endosperm, it is evident from peptide-sequencing and -mapping studies that the starch branching enzyme (BEII) exists in both soluble and granule-bound forms (Mu-Forster et al., 1996). Antibodies raised against BEIIb reacted with both SGP-2 and a MW 85 000 soluble protein (Rahman et al., 1995). This suggests that SGP-2 might exist in both fractions and, like SBGP-3, might be concerned with synthesis of amylopectin.

Edwards et al. (1996) have shown that for maize, pea, and potato, the granule-bound activity is not important in the synthesis of amylose. Both SGP-2 and -3 might be inactive when bound to the granule, although it is not clear whether SGP-1 binds to the granule. It is likely that the role of SGP-1 differs from that of SGP-3, since SGP-1 is present only in the granule-bound form (Rahman et al., 1995).

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