Starch-Branching Enzymes Preferentially Associated with A-Type Starch Granules in Wheat Endosperm[1]

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Two starch granule-bound proteins (SGP), SGP-140 and SGP-145, were preferentially associated with A-type starch granules (>10 μm) in developing and mature wheat (*Triticum aestivum*) kernels. Immunoblotting and N-terminal sequencing suggested that the two proteins were different variants of SBEIc, a 152-kD isoform of wheat starch-branching enzyme. Both SGP-140 and SGP-145 were localized to the endosperm starch granules but were not found in the endosperm soluble fraction or pericarp starch granules younger than 15 d post anthesis (DPA). Small-size starch granules (<10 μm) initiated before 15 DPA incorporated SGP-140 and SGP-145 throughout endosperm development and grew into full-size A-type starch granules (>10 μm). In contrast, small-size starch granules harvested after 15 DPA contained only low amounts of SGP-140 and SGP-145 and developed mainly into B-type starch granules (<10 μm). Polypeptides of similar mass and immunologically related to SGP-140 and/or SGP-145 were also preferentially incorporated into A-type starch granules of barley (*Hordeum vulgare*), rye (*Secale cereale*), and triticale (× *Triticosecale* Wittmack) endosperm, which like wheat endosperm have a bimodal starch granule size distribution.

Wheat (*Triticum aestivum*), barley (*Hordeum vulgare*), rye (*Secale cereale*), and triticale (× *Triticosecale* Wittmack) mature endosperm contain large A- and small B-type starch granules, thus showing a bimodal granule size distribution (French, 1984). In wheat, the large A-type starch granules are more than 10 μm in diameter and lenticular in shape, whereas B-type starch granules are less than 10 μm in diameter and roughly spherical (Evers, 1973). Wheat A- and B-type starch granules have significantly different chemical compositions and functional properties (Seib, 1994), and therefore, the development of wheat cultivars with predominantly A- or B-type starch granules would be of value to the food and non-food industries. To produce such wheat cultivars, it is necessary to understand the ontogeny of A- and B-type starch granules during wheat endosperm development.

Anatomical studies have revealed that A-type starch granules are initiated at approximately 4 to 14 DPA, during which the endosperm cells are actively dividing (Briarty et al., 1979; Parker, 1985). On the other hand, B-type starch granules are initiated during the endosperm cell enlargement stage, which starts about 14 DPA and lasts until the wheat grain is mature (Briarty et al., 1979; Parker, 1985). This differential production of the two types of granules suggests that the biosynthesis of A- and B-type starch granules in wheat endosperm is developmentally regulated.

Starch synthases (SS), starch-branching enzymes (SBE), and starch-debranching enzymes participate in the biogenesis of plant starch granules (Ball et al., 1996; Preiss and Sivak, 1998). Each of these starch biosynthetic enzymes exists in multiple isoforms, some of which are soluble and others are localized to the starch granules. Mutations inactivating any of these enzymes result in modification of starch structure and sometimes also causes an altered starch granule morphology (Bhattacharyya et al., 1990; Mouille et al., 1996; Craig et al., 1998; Edwards et al., 1999). One enzyme that was suggested to have a role in determination of granule size in barley is the soluble SS. A mutation at the barley *shx* locus results in lower SSI activity and a concomitant reduction in the size of A-type starch granules thus giving the appearance of a unimodal granule size distribution (Schulman and Ahokas, 1990; Tyynelä and Schulman, 1993; Tyynelä et al., 1995). No mutant with altered starch granule size distribution, like *shx* endosperm in barley, has been reported in wheat.

Among the starch granule-bound proteins (SGP) in wheat, several are likely to be actively involved in the production of amylose or amylopectin. The 60-kD SGP, a granule-bound starch synthase (GBSSI), is required for synthesis of amylose (Shure et al., 1983), but GBSSI absence does not significantly affect granule size or structure (Fujita et al., 1998). However, absence of granule-bound SSII has been reported to cause deformation of large granules and production of starch with increased capacity to bind iodine (Yamamori, 1998). The major SGP in wheat starch

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range in size from 60 to 115 kD (Rahman et al., 1995; Båga et al., 1999), but no significant difference in polypeptide profiles for these proteins extracted from A- and B-type starch granules has been found (Sulaiman and Morrison, 1990; Rahman et al., 1995). Recently, we isolated and characterized a cDNA encoding a novel SBEI, SBEIc, with predicted molecular mass of 152 kD (Båga et al., 2000). SBEIc was found to be preferentially associated with starch granules of the wheat endosperm and corresponded to the 149-kD SGP identified by Schofield and Greenwell (1987). Here, we show that most hexaploid wheat starches contain two large SGP, SGP-140 (corresponding in mass to SBEIc) and SGP-145, that are preferentially incorporated into A-type starch granules. Polypeptides with masses similar to SGP-140 and SGP-145 were also present in other cereals showing a bimodal starch granule size distribution. The possible involvement of SGP-140 and SGP-145 in the development of A-type starch granules is discussed.

RESULTS

Identification of Granule-Bound Proteins Preferentially Associated with A-Type Starch Granules in Wheat Endosperm

To compare SGP localized in A- and B-type starch granules, we purified the two granule fractions from wheat endosperm of six wheat cultivars using a method previously reported (Peng et al., 1999). The extracted SGP were resolved by SDS-PAGE and visualized by silver staining. To quantitatively compare the different polypeptides in A- and B-type starch granules, the 60-kD GBSSI was used as an internal standard for equal loading of proteins. The major SGP of 60, 80, 92, 100, 108, and 115 kD were present in similar concentrations in A- and B-type starch granules from all the cultivars tested (Fig. 1), and no difference was observed among polypeptides with molecular masses lower than 60 kD (data not shown). These results were consistent with previous studies that reported almost identical polypeptide profiles for wheat A- and B-type starch granules (Sulaiman and Morrison, 1990; Rahman et al., 1995).

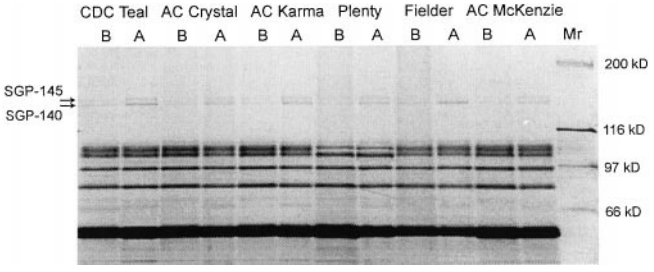

Figure 1. SDS-PAGE analysis of SGP extracted from wheat A- and B-type starch granules. Each lane was loaded with protein extract from 5-mg A- and B-type starch granules of five hexaploid and one tetraploid (cv Plenty) cultivars. Separated proteins were visualized by silver staining, and migration of protein molecular mass marker is indicated to the right.

In addition to the major SGP, it was recently found that a novel SBEI isoform, SBEIc, migrating as a 140-kD polypeptide on SDS-PAGE gels, was associated with starch granules of the cv Fielder (Båga et al., 2000). In this study, we observed that A-type starch granules of all wheat cultivars tested contained a polypeptide comigrating with SBEIc (Fig. 1). A slightly larger polypeptide with an apparent molecular mass of 145 kD was also present in A-type starch granules of all cultivars except cv Fielder (Fig. 1). Analysis of B-type starch granules from the six wheat cultivars showed a much lower abundance of the 140- and 145-kD polypeptides as compared with the A-type granules (Fig. 1). In the B-type granules of the cv Fielder, only the 140-kD band was observed, as was found for the A-type granules of this cultivar. The lower abundance of the 140- and 145-kD polypeptides in B-type starch granules suggested that SGP-140 and SGP-145 are incorporated into B-type granules, albeit with much lower efficiency than into A-type granules. Alternatively, the B-type granules do not contain SGP-140 and SGP-145, and the weak bands we observed resulted from contamination of B-type starch granules with some small-size A-type starch granules. Nevertheless, the conclusion from our data was that the SGP-140 and SGP-145 were preferentially associated with A-type starch granules.

SGP-140 and SGP-145 Are Preferentially Incorporated into A-Type Starch Granules throughout Endosperm Development

In developing wheat endosperm, A-type starch granules are initiated at approximately 4 to 14 DPA, whereas B-type granules are formed after 14 DPA (Briarty et al., 1979; Parker, 1985). After initiation, both granule types continue to grow until maturity of the endosperm (Morrison and Gadan, 1987). An image analysis of purified large- and small-size starch granule fractions from developing endosperm of the cv CDC Teal showed that the growth of small starch granules formed before and after 15 DPA was significantly different (Fig. 2). Prior to 15 DPA, the newly formed small starch granules grew rapidly in size to become large-size (>10 μm) starch granules (Fig. 2A). During the time period 8 to 15 DPA, large-size starch granules accounted for more than 70% of total endosperm starch granules (Fig. 2B). Small-size starch granules formed after 15 DPA increased rapidly in number until maturity (25%–94%), but they grew very slowly and only reached diameters less than 10 μm (Fig. 2, A and B).

The preferential incorporation of SGP-140 and SGP-145 into A-type granules could be explained by synthesis of these polypeptides only during the first 15 DPA. To test this hypothesis, we analyzed the

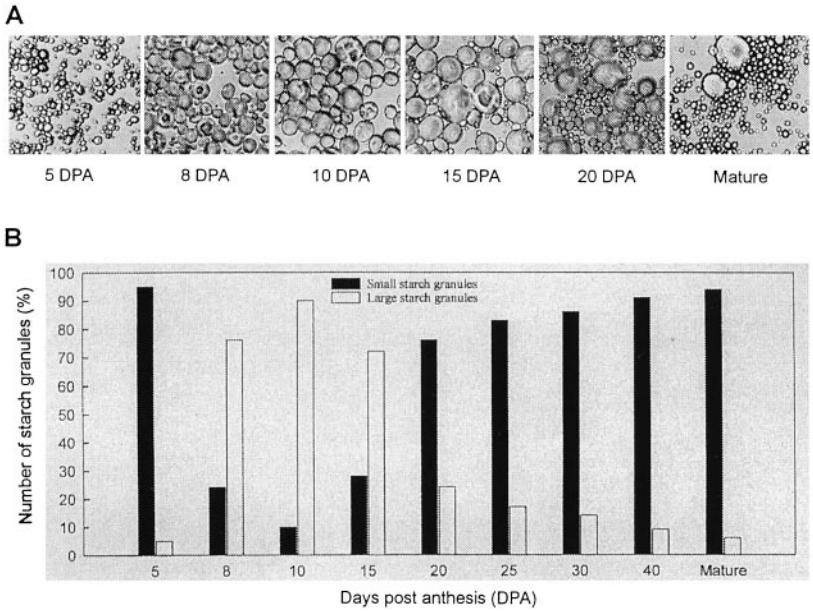

Figure 2. Analysis of starch granule size distribution in wheat endosperm. A, Light microscopic pictures (500×) of total starch granules harvested at different stages of endosperm development of the hexaploid wheat cv CDC Teal. B, Histogram of large-size (>10 μm) and small-size (<10 μm) granule size distribution during wheat endosperm development.

protein profiles of large- and small-size granules isolated at different DPA (Fig. 3). The large-size (>10 μm) A-type starch granules were found to show no variation in SGP-140 and SGP-145 concentration during development. Small-size starch granules (<10 μm in diameter) formed before 15 DPA, which were of the A-type, were also found to contain SGP-140 and SGP-145 at about the same concentration as in large-size granules. However, small-size starch granules harvested after 15 DPA, which are mainly of B-type, showed very low presence of SGP-140 and SGP-145. The analyses demonstrated no significant variation in concentration of the other major granule-bound polypeptides (60, 80, 92, 100, 108, and 115 kD) for both small- and large-size starch granules throughout endosperm development. In the cv CDC Teal, most of the A-type granule growth occurred after 15 DPA, when approximately 65% (w/w) of the starch in A-type granules was synthesized. Thus, the constant abundance of SGP-140 and SGP-145 in A-type granules strongly suggested that the two proteins were continuously incorporated into A-type granules throughout endosperm development.

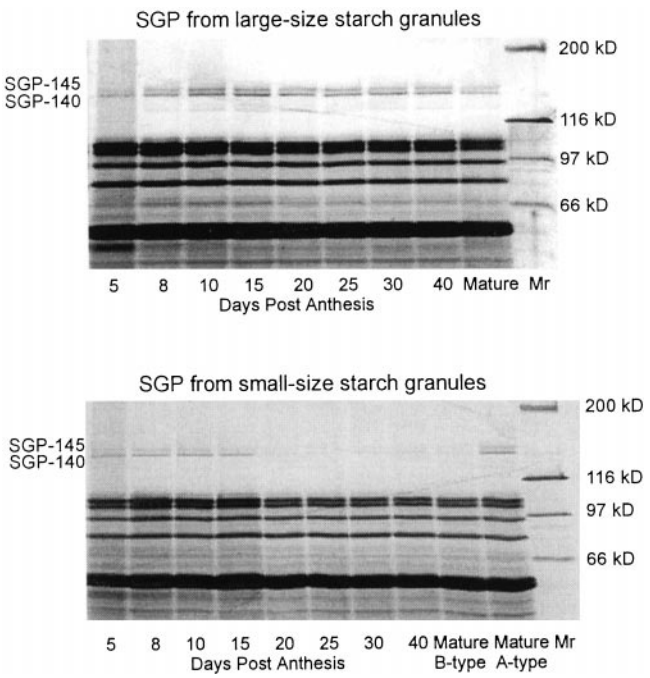

Figure 3. SDS-PAGE analysis of SGP extracted from large-size (>10 μm) and small-size (<10 μm) starch granules of the hexaploid wheat cv CDC Teal. Samples of SGP from 5-mg starch granules were from different stages of wheat endosperm development as indicated. Gel-separated proteins were visualized by silver staining, and migration of protein molecular mass marker is indicated to the right.

SGP-140 and SGP-145 Are Immunologically Related to SBEI

To confirm the identity of SGP-140 as a SBEI isoform in the cv CDC Teal and to possibly identify SGP-145, immunoblots of SGP from A- and B-type starch granules were reacted with polyclonal antibodies raised against wheat SBEI, SBEII, SSI, SSII, and GBSSI, respectively (Fig. 4). The major polypeptides of 60 kD (GBSSI), 80 kD (SSI), 92 kD (SBEII), and 100 to 115 kD (SSII) were recognized by their respective antibodies, as expected, with no difference in intensity between A- and B-type granules (Fig. 4). Among the five antibodies tested, only the wheat SBEI antibodies reacted with SGP-140 and were also found to recognize SGP-145. A weaker interaction between the SBEI antibodies and a protein comigrating with SBEII and proteins of approximately 63 kD were also seen. Similar to the analysis of SGP-140 and SGP-145 by SDS-PAGE, the immunoreactive bands

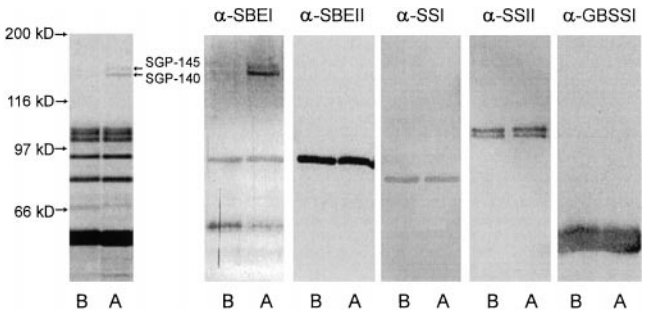

Figure 4. Immunoblot analysis of extracted SGP from wheat A- and B-type starch granules. Each lane was loaded with SGP extracted from 2-mg A- and B-type starch granules harvested from mature endosperm of the hexaploid wheat cv CDC Teal. To the left is shown SGP separated by SDS-PAGE and visualized by silver staining. To the right is shown immunoblot analyses of gel-separated SGP using polyclonal antisera prepared against different wheat starch biosynthetic enzymes as indicated.

were strong in A-type but weak in B-type starch granules (Fig. 4).

To compare SGP-140 and SGP-145, both protein bands were purified from SDS-PAGE gels and subjected to direct amino acid sequencing. The sequence information from this analysis suggested variation in amino acid sequence as indicated in Table I. This is likely due to presence of several polypeptides that differ slightly in sequence within the same protein band as suggested by reverse transcription PCR analysis (Båga et al., 2000). Nevertheless, alignment of the determined N-terminal sequences of the SGP-140 and SGP-145 with those predicted for SBEIc and wSBEI-D2 revealed striking similarities, thus suggesting that all four polypeptides were closely related (Table I). A lower level of similarity was noted to the predicted N-terminal sequence for the wheat 87-kD SBEIb isoform (Repellin et al., 1997). Since the molecular masses of SGP-140 and SGP-145 were reasonably close to that of SBEIc (152 kD) predicted from *Sbe1c* cDNA, the data suggested that SGP-140 and SGP-145 were isoforms of SBEIc.

SGP-140 and SGP-145 Are Endosperm Starch Granule-Bound SBEI

Subcellular localization of starch biosynthetic enzymes is of importance for understanding their function. To localize SGP-140 and SGP-145 in the developing kernels, SGP from pericarp and endosperm starch granules and the soluble endosperm fraction were prepared from developing wheat kernels and analyzed by SDS-PAGE and immunoblotting. The results of these analyses confirmed that SGP-140 and SGP-145 were present within the endosperm starch granules but could not be found in the endosperm soluble fraction (Fig. 5). Nor were SGP-140 and SGP-145 observed in pericarp starch granules harvested from 5 to 10 DPA but could be seen as two very faint bands in pericarp granules of 15 DPA (Fig. 5). Since pericarp from kernels older than 15 DPA was rather difficult to separate from the endosperm, it is possible that the two faint bands seen in 15 DPA pericarp sample originated from some endosperm starch granules mixed with the pericarp starch granules.

SGP-140 and/or SGP-145 Exist in Plant Species Known to Produce A- and B-Type Starch Granules

We extended the study of starch granule proteins to other plants than wheat to determine if any association could be found between the size-distribution of granules produced and the presence of SGP-140 and SGP-145 homologs. This study included starches from plants with bimodal (rye, barley, and triticale) and unimodal (rice, maize, potato, and canary seed) starch granule size distribution (French, 1984). SDS-PAGE analysis of extracted SGP from triticale, barley, and rye revealed one (barley and rye) or two protein bands (triticale) with similar relative mobility as SGP-140 and SGP-145 of wheat (Fig. 6A). These protein bands were also found to react with SBEI antibodies (Fig. 6B) and thus appeared to be SGP-140 and SGP-145 homologs. Analysis of canary seed, rice, maize, and potato SGP did not reveal presence of any polypeptides similar in size to SGP-140 and SGP-145 and reacting with SBEI antibodies (Fig. 6, A and B). Thus, it appeared that proteins similar to SGP-140 and SGP-145 were only present in cereal starches with bimodal granule size distribution.

To determine if the SGP-140 and SGP-145 counterparts in triticale, barley, and rye were, like in wheat, preferentially associated with A-type starch granules, the A- and B-type starch granules from these

Table I. *Alignment of SGP-140 and SGP-145 N-terminal sequences to those predicted for wheat endosperm SBEI and SBEI-like proteins*

Wheat wSBEI-D2 is a SBEI-like protein predicted to be produced in wheat endosperm (Rahman et al., 1997). SBEIc is deduced from a wheat endosperm transcript (Båga et al., 2000). SBEIb is deduced N-terminal sequence of 87-kD SBEI expressed in wheat endosperm (Repellin et al., 1997). Identical amino acids are in bold type.

Polypeptide	Sequence
SGP-140	**K/H/A I/V N G Y G** R **D** R **L P**/R **M Y D**/R
SGP-145	Q **T T G Y G S?D H L P M?Y D L**
SBEIc (152 kD)	A **N G Y G S D H L P M Y D L**
wSBEI-D2 (87 kD)	**T T G Y G S D H L P I Y D L**
SBEIb (87 kD)	V S A P R D Y T **A T** A E D **G** V G D **L P I Y D L**

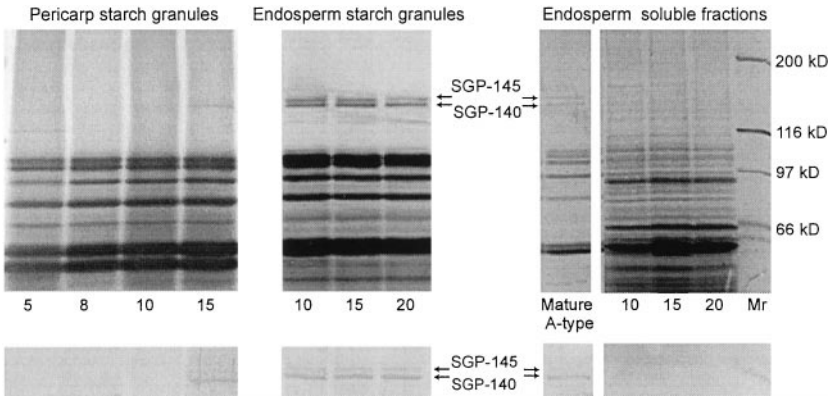

Figure 5. Subcellular localization of SGP-140 and SGP-145 in immature wheat kernels. SDS-PAGE analysis of SGP extracted from cv CDC Teal pericarp starch, endosperm starch, and soluble endosperm proteins were prepared from different DPA of endosperm development as indicated. Samples of soluble protein (280 [10 DPA], 250 [15 DPA], or 250 μg [20 DPA]) and starch granules (5 mg) analyzed were derived from the same amount of endosperm tissue. Gel-separated proteins were visualized by silver staining (pericarp and endosperm starch analysis) or Coomassie Blue staining (soluble endosperm analysis). Migration of molecular mass marker is shown to the right. Below is shown immunoreactive bands formed between gel-separated SGP-140 and SGP-145 and wheat SBEI antibodies.

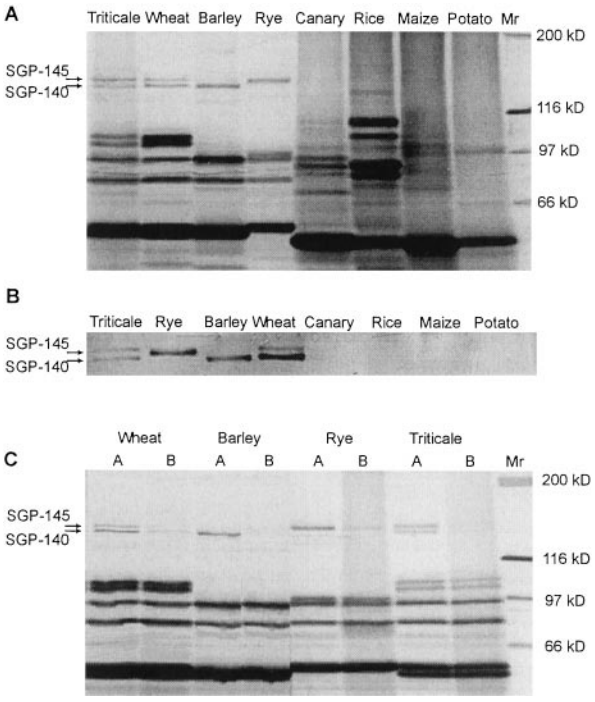

Figure 6. Analysis of SGP in starches from various plant sources. A, SDS-PAGE analysis of SGP extracted from 5-mg starch of: A-type starch granules from endosperm of triticale, wheat, barley, and rye; total starch from endosperm of canary seed, rice, and maize; and potato tubers. Proteins were visualized by silver staining. Migration of molecular mass marker is shown to the right. B, Immunoblot analysis of gel-separated proteins shown above. Immunoreactive bands obtained from interaction between wheat SBEI polyclonal antibodies and SGP-140 and SGP-145 are indicated. C, SDS-PAGE analysis of extracted SGP from 5-mg A- and B-type starches isolated from wheat, barley, rye, and triticale endosperm. Proteins were visualized by silver staining. Migration of molecular mass marker is shown to the right.

cereals were analyzed. Similar to wheat endosperm starch, the SGP-140 and SGP-145 homologs were abundant in A-type starch granules, but very scarce in B-type starch granules (Fig. 6C).

DISCUSSION

The biogenesis of starch granules in plant amyloplasts involves two successive steps: the formation of small starch granule nuclei, and the production of mature granules by apposition of starch molecules onto the nuclei (Badenhuizen, 1965; Shannon et al., 1970). Thus, the biosynthesis of A- and B-type starch granules in developing wheat endosperm could be regulated at two stages. The first stage would be during the formation of the starch granule nuclei. Previous reports and our data strongly suggest that one peak of granule nuclei formation occurs before 15 DPA and another occurs after 15 DPA. The second stage of regulation could be during the development of the nuclei into A- and B-type granules. During this stage, the A-type granules are able to grow larger than 10 μm in diameter, and B-type granules lack this ability.

Our results show that SGP-140 and SGP-145 are preferentially found on both small- and large-size A-type granules (Fig. 3). No reduction was noted in SGP-140 and SGP-145 concentrations in large granules harvested after 15 DPA (Fig. 1), a developmental stage when most of the A-type granule starch is being produced. This argued against SGP-140 and SGP-145 being incorporated at a specific stage of A-type granule development, but rather, being continuously targeted to A-type granules, even when B-type granules are produced. Since SGP-140 and SGP-145 did not accumulate in the soluble phase of the endosperm, these proteins must be actively produced both before and after 15 DPA. This was also indicated by RNA

analysis of SGP-140 gene expression during kernel development, which showed only a small reduction in transcript levels after 15 DPA as compared with before 15 DPA (Båga et al., 2000).

In developing wheat endosperm, only one A-type starch granule is produced in each amyloplast (A-type amyloplast) from 4 to 14 DPA, a stage when the endosperm cells are dividing (Briarty et al., 1979). During the cell expansion stage of endosperm development (approximately 15 DPA to maturity), the B-type starch granules appear in the protrusions extending from A-type amyloplast (Parker, 1985). The formation of A- and B-type starch granules at different locations could be the reason for preferential localization of SGP-140 and SGP-145 to A-type starch granules.

SGP-140 and SGP-145 were also found to be associated with A-type starch granules in the endosperm of barley, rye, and triticale, but no presence of similar polypeptides could be detected in the potato starch granules, which are also relatively large in size. These results suggest that SGP-140 and SGP-145 homologs are not generally associated with large starch granules in plants. Furthermore, we were unable to detect SGP-140 and SGP-145 in starch granules from endosperm of canary seed, rice, and maize, which like potato starch granules, have a unimodal size distribution. These data suggest that the presence of SGP-140 and SGP-145 was related to the presence of A- and B-type starch granules in wheat, rye, barley, and triticale.

The amino-terminal sequencing and immunoblotting of SGP-140 and SGP-145 produced in the wheat cv CDC Teal strongly suggested that these polypeptides are isoforms of SBEIc identified in the wheat cv Fielder (Båga et al., 2000). Both SGP-140 and SGP-145 were present in the endosperm starch granules and absent in the soluble fraction. Thus, SGP-140 and SGP-145 differ in subcellular localization from the main isoforms of SBEI (87–88 kD), which are primarily found in the soluble fraction of the endosperm (Morell et al., 1997). The different locations of the 87- to 88-kD SBEI and the much larger SGP-140 and SGP-145 may imply that the two classes of SBEI have different activities and functions in the wheat endosperm. It is possible that the 87- to 88-kD SBEI are functional in the synthesis of amylopectin in the endosperm soluble fraction, but become inactive when trapped within starch granules, like their counterparts in pea and maize (Denyer et al., 1993; Mu-Forster et al., 1996). In contrast, SGP-140 and SGP-145 may, like the exclusively granule-bound GBSSI, be primarily active only on polymers of the starch granule. Thus, it is conceivable that the amylopectin produced by the soluble 87- to 88-kD SBEI and SGP-140 and SGP-145 may differ in structure.

Since SS, SBE, and starch-debranching enzyme participate in the biogenesis of plant starch granules, we speculate that one or several isoforms of these enzymes are involved in the regulation of initiation and size growth of A- to B-type starch granules in the developing wheat endosperm. Identification of the SGP-140 (SBEIc) and SGP-145 and their occurrence coinciding with A-type starch granules suggest that these proteins may play some role in the growth of small-sized A-type into full-sized A-type starch granules. This role may be to regulate the amount and/or structure of amylopectin molecules formed in the small-size A-type starch granules, which allows the A-type granules to expand to a larger extent than the B-type granules. However, to test this hypothesis further, characterization of SGP-140 and SGP-145 isoforms and their action on glucan polymers is needed.

MATERIALS AND METHODS

Isolation of A- and B-Type Starch Granules

Starch granules were isolated from mature endosperm of five hexaploid wheat cultivars (*Triticum aestivum* L. cv CDC Teal, cv McKenzie, cv AC Karma, cv AC Crystal, and cv Fielder), one tetraploid wheat (*Triticum turgidum* L. cv Plenty) cultivar, barley (*Hordeum vulgare*), rye (*Secale cereale*), triticale (× *Triticosecale* Wittmack), rice (*Oryza sativa*), maize (*Zea mays*), canary seed (*Phalaris canariensis*), and potato (*Solanum tuberosum*) tubers as described (Peng et al., 1999). Pericarp and developing endosperm tissues were manually dissected from wheat cv CDC Teal kernels and immediately placed in extraction buffer B (50 mM Tris [tris(hydroxymethyl)aminomethane]-HCl, pH 7.5, 10 mM EDTA, 5 mM dithiothreitol, 10% [v/v] glycerol, 0.1% [w/v] polyvinyl pyrrolidone) held at 4°C. The pericarp fraction was washed three times with extraction buffer B to remove endosperm starch granules. Thereafter, the endosperm and pericarp fractions were homogenized with a mortar and pestle in 3 volumes of extraction buffer B and filtered through four layers of Miracloth (Calbiochem, San Diego) to remove cell debris. The crude starch granule fraction was pelleted by centrifugation at 15,000*g* for 30 min and further purified as described (Peng et al., 1999). The endosperm starch granules were separated into large-size (diameter >10 μm) and small-size (diameter <10 μm) fractions and studied by image analysis as described (Peng et al., 1999).

Preparation of Endosperm Soluble Fractions

The supernatant remaining from centrifugation of the homogenized endosperm (see above) constituted the endosperm soluble fraction. Protein concentration in the extract was determined using a dye-binding assay from Bio-Rad Laboratories (Hercules, CA). For each endosperm fraction, the total amount of extracted soluble protein was determined.

SDS-PAGE and Immunoblot Analysis

To extract SGP, 50-mg starch granules were suspended in 350 μL of extraction buffer A (62.5 mM Tris-HCl, pH 6.8,

10% [w/v] SDS, and 5% [v/v] β-mercaptoethanol), boiled for 15 min, cooled to room temperature, and centrifuged at 15,000*g* for 20 min. SDS-PAGE analysis of SGP was done on 10% (w/v) resolving gels (30:0.135) and proteins were visualized by Coomassie Blue staining and/or silver staining (Bio-Rad Laboratories). For immunoblot analysis, the gel-separated proteins were electrophoretically transferred at 4°C onto polyvinylidene fluoride membranes (Immobilon P, Millipore, Bedford, MA) using transfer buffer (25 mM Tris-HCl, pH 8.3, 192 mM Gly, and 20% [v/v] methanol). Membranes were incubated for 1 h in Tris-buffered saline (TBS) buffer (20 mM Tris-HCl, pH 7.5, and 150 mM NaCl) containing 3% (w/v) bovine serum albumin, to block non-specific binding sites. Antibodies, at a dilution of 1:4,000 in TBS buffer, were then added to the blot and incubated for 4 h at room temperature. Following three washes in TBS buffer containing 0.05% Tween 20 and one wash in TBS buffer, membranes were incubated with alkaline phosphatase-conjugated goat anti-rabbit IgG (Stratagene, La Jolla, CA) at a dilution of 1:5,000 for 1 h. Membranes were washed three times in TBS buffer containing 0.05% Tween 20, once in TBS buffer, and equilibrated in 20 mM Tris-HCl, pH 9.5, 100 mM NaCl, 5 mM $MgCl_2$. Immunoreactive bands were detected with 4-nitroblue tetrazolium chloride and 5-bromo-4-chloro-3-indolyl phosphate (Stratagene).

N-Terminal Sequencing of SGP-140 and SGP-145

SGP were extracted from 10 g of A-type starch granules of cv CDC Teal and resolved on preparative SDS-PAGE gels. The migration of SGP-140 and SGP-145 was determined by silver staining a slice of the gel. The proteins were eluted from the unstained part of the gel using an electro-eluter (model 422 Electro-Eluter, Bio-Rad Laboratories) and elution buffer (25 mM Tris, 192 mM Gly, and 0.1% [w/v] SDS). The eluate was dialyzed for 8 h against 2 L of dialysis buffer (50 mM Tris-acetate, pH 6.8, and 5 mM dithiothreitol) with one buffer change. The dialyzed solution was concentrated to 500 μL through an ultrafiltration unit (Amicon 100, Amicon, Beverly, MA), and 200 μL of the concentrate was loaded on a preparative SDS-PAGE gel. Gel-separated proteins were blotted on a polyvinylidene fluoride membrane, as described above. SGP-140 and SGP-145 were identified by amido black staining and subjected to N-terminal sequencing using a gas-phase protein sequencer (model 476A, Applied Biosystems, Foster City, CA).

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