Analysis of Protein Complexes in Wheat Amyloplasts Reveals Functional Interactions among Starch Biosynthetic Enzymes

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Protein-protein interactions among enzymes of amylopectin biosynthesis were investigated in developing wheat (Triticum aestivum) endosperm. Physical interactions between starch branching enzymes (SBEs) and starch synthases (SSs) were identified from endosperm amyloplasts during the active phase of starch deposition in the developing grain using immunoprecipitation and cross-linking strategies. Coimmunoprecipitation experiments using peptide-specific antibodies indicate that at least two distinct complexes exist containing SSI, SSIIa, and either of SBEIIa or SBEIIb. Chemical cross linking was used to identify protein complexes containing SBEs and SSs from amyloplast extracts. Separation of extracts by gel filtration chromatography demonstrated the presence of SBE and SS forms in protein complexes of around 260 kD and that SBEII forms may also exist as homodimers. Analysis of cross-linked 260-kD aggregation products from amyloplast lysates by mass spectrometry confirmed SSI, SSIIa, and SBEII forms as components of one or more protein complexes in amyloplasts. In vitro phosphorylation experiments with γ-32P-ATP indicated that SSII and both forms of SBEII are phosphorylated. Treatment of the partially purified 260-kD SS-SBE complexes with alkaline phosphatase caused dissociation of the assembly into the respective monomeric proteins, indicating that formation of SS-SBE complexes is phosphorylation dependent. The 260-kD SS-SBEII protein complexes are formed around 10 to 15 d after pollination and were shown to be catalytically active with respect to both SS and SBE activities. Prior to this developmental stage, SSI, SSII, and SBEII forms were detectable only in monomeric form. High molecular weight forms of SBEII demonstrated a higher affinity for in vitro glucan substrates than monomers. These results provide direct evidence for the existence of protein complexes involved in amylopectin biosynthesis.

Starch is produced by the majority of higher plant species inside plastids and represents a major storage product of many of the seeds and storage organs produced agriculturally and used for human consumption as well as many important industrial applications. The starch granule is a complex polymeric structure with a hierarchical order, allowing efficient packing of large amounts of Glc into a water-insoluble form, and is composed of two distinct types of Glc polymer: amylose and amylopectin.

Amylose comprises largely unbranched α-(1→4)-linked glucan chains and does not appear to participate in the formation of the ordered part of the matrix. Amylopectin is a branched glucan polymer typically comprising between 65% and 85% of the starch granule mass, produced by the formation of α-(1→6)-branch linkages between adjoining linear, i.e. α-(1→4)-linked glucan chains. The polymodal distribution of glucan chain lengths and branch point clustering within amyllopectin allows short- to intermediate-sized glucan chains to form double helices that can pack together in organized arrays, which are the basis of the semicrystalline nature of much of the matrix of the starch granule (Buléon et al., 1998; Thompson, 2000). Granule formation is driven by both the semicrystalline properties of amyllopectin, as determined by the length of...
the linear chains of amylopectin, and the clustering and frequency of α-(1→6)-branch linkages (French, 1984; Hizukuri, 1986; Myers et al., 2000). By contrast, the water-soluble glycogens of bacteria and animals have a more openly branched structure. As such, it is the amylopectin component of starch that essentially defines the highly conserved structure of the granule and is the major determinant of starch functionality (Davies et al., 2003; Tetlow, 2006).

The basic enzymatic steps required for the formation of amylopectin (the core pathway) are known and have been determined using a combination of biochemical techniques and forward and reverse genetics approaches. Amylopectin biosynthesis is initiated by the formation of α-(1→4)-linked linear glucan chains from the activated glucosyl donor ADP-Glc in a reaction catalyzed by starch synthases (SSs; EC 2.4.1.21). Branch linkages within glucan chains of varying length are introduced by starch branching enzymes (SBEs; EC 2.4.1.18), which cleave internal α-(1→4) linkages and transfer the released reducing end to a C6 hydroxyl to create a new α-(1→6) linkage. In addition to SSs and SBEs, the debranching enzymes (DBEs; EC 3.2.1.41 and EC 3.2.1.68) also appear to be an essential component in the formation of semicrystalline amylopectin, because DBE mutants often lack or are reduced in amylopectin content and in its place accumulate large amounts of a soluble, glycogen-like glucan polymer (Morris and Morris, 1939; James et al., 1995; Mouille et al., 1996; Zeeman et al., 1998; Wattebled et al., 2005). Plants possess multiple forms of SSs (SSs I–IV), SBEs (SBEs I and II), and DBEs (isoamylase-type DBEs 1–3 and pullulanase-type DBE), and the strong amino acid sequence conservation among these forms in plants suggests specific and unique roles for each during amylopectin biosynthesis (Jespersen et al., 1993; Myers et al., 2000; Ball and Morell, 2003). For example, recent studies with SSIII and SSIV in Arabidopsis (Arabidopsis thaliana) suggest SSIII can control the rate of starch synthesis in leaves (Zhang et al., 2005), and that SSIV may be involved in a granule initiation pathway (Roldán et al., 2007). Coordination of these activities, as well as other enzymatic steps, including starch-degrading activities, is likely to be required to produce the nonrandom clustered arrangement of glucan chains characteristic of amylopectin.

To date, information on how coordination between amylopectin-synthesizing enzymes is achieved is sparse. Recent work in wheat (Triticum aestivum) endosperm amyloplasts suggests protein phosphorylation is involved in modulating the catalytic activity of some key enzymes (the SBEII class) and their ability to form physical interactions with other starch-metabolizing enzymes (Tetlow et al., 2004b). Analysis of transgenic Arabidopsis and potato (Solanum tuberosum) plants also indicates a role for 14-3-3 proteins in the regulation of SS activity in the formation of assimilatory (transient) starch in leaves, presumably through protein-protein interactions (Sehnke et al., 2001; Zuk et al., 2005).

The objective of the research described in this communication was to investigate interactions between SSs and SBEs in starch-synthesizing plastids. This article describes the isolation and characterization of protein complexes comprising SSI, SSIII, and SBEII from amyloplast extracts of developing wheat endosperm. The data indicate that formation of the SS-SBE protein complexes and SBEII homodimers in the developing endosperm occurs from around 10 to 15 d after pollination (DAP), the major grain-filling period, and is phosphorylation dependent. This article presents the first direct evidence of catalytically active protein complexes involved in amylopectin biosynthesis and indicates that the kinetic properties of branching enzymes are altered in higher ordered states, most likely dimers.

RESULTS

Fractionation and Measurement of SS and SBE Activities in Developing Wheat Endosperm

Soluble proteins extracted from developing wheat endosperm at early stages of grain filling were separated by gel filtration chromatography to examine possible changes in aggregation state of specific enzyme activities involved in amylopectin biosynthesis. Measurements of the extractable catalytic activities of SS and SBE at early stages of endosperm development (6–9 DAP) following separation of protein extracts by gel filtration chromatography showed single, broad peaks of SS and SBE activities (Fig. 1, A and B). Both activities eluted from the gel filtration column in fractions with apparent molecular mass within the size range expected of monomeric forms of SBEII (approximately 88 kD) and of the SS isoforms SSI and SSIII, which are approximately 75 and 85 kD, respectively. At later stages of endosperm development (10–15 DAP and later), there was a notable shift in the elution patterns of both SS and SBE activities, indicating an apparent increase in molecular mass or aggregation state of the enzymes contributing to these measured activities. At later stages of endosperm development, the total eluted SS and SBE activity was divided almost equally between an apparently high M, peak of around 200 to 300 kD (termed HMW), as well as the peak originally observed at the earlier stages of endosperm development (6–9 DAP) with an apparently lower molecular mass, corresponding to the size of the monomeric proteins (termed LMW). Analysis of SS and SBE activities by gel filtration chromatography at later stages of endosperm development (beyond 15 DAP) showed essentially the same separation of peaks of SS and SBE activities as observed in Figure 1 at 10 to 15 DAP (data not shown).

The results described above were obtained with whole cell homogenates. Similar results were also obtained when amyloplast lysates prepared from endosperm were separated by gel filtration chromatography and fractions assayed for SBE and SS activities (data not shown).
Analysis of the fractions (following gel filtration chromatography of whole cell extracts) by immunoblotting with anti-SBE antibodies (Fig. 1C) showed that both forms of SBEII (SBEIIa and SBEIIb) were responsible for the measured SBE activity in both LMW and HMW fractions and that SBEI was not expressed at the stages of endosperm development used in these experiments, consistent with previous findings (Morell et al., 1997). Immunoblots of the gel filtration column fractions developed with anti-SSI and anti-SSII antibodies indicated the presence of both these forms at early stages of development (6–9 DPA), which is consistent with the known patterns of SS gene expression in wheat (Li et al., 1999a) and in both the LMW and HMW peaks of activity at later stages of endosperm development (Fig. 1D).

In Vitro Phosphorylation of SS and SBE Forms in Plastids

Previous work has shown that many stromal proteins rapidly become phosphorylated when amyloplasts and chloroplasts of wheat are incubated with \( \gamma^{32}\text{P} \)ATP (Tetlow et al. 2004b). Immunoprecipitation and autoradiography of stromal proteins following in vitro phosphorylation reactions showed that some isoforms of SS and SBE are phosphorylated. In particular, both isoforms of SBEII became phosphorylated in amyloplasts following incubations with \( \gamma^{32}\text{P} \)ATP, as well as SBEIa in chloroplasts (the only form of SBEII expressed in these organelles), in agreement with previous findings with amyloplasts isolated at later stages of endosperm development (Tetlow et al., 2004b; data not shown). Autoradiography of SDS-PAGE gels of stromal proteins previously immunoprecipitated with wheat anti-SSI and anti-SSII antibodies showed that plastidial forms of SSI are phosphorylated (Supplemental Fig. S1), in agreement with previous work (Tetlow et al., 2004b). However, we found no evidence to suggest that the soluble form of SSI is phosphorylated in amyloplasts. We observed no apparent alterations in the electrophoretic mobility of the different proteins as a result of phosphorylation using the one-dimensional (1D)-gradient gel systems.

Coimmunoprecipitation of SS and SBE Forms in Plastids

Amyloplast lysates were used as a source of material for coimmunoprecipitation experiments to examine protein-protein interactions and the possibility that phosphorylation has a role in facilitating such interactions (Fig. 2). Plastid lysates were employed to reduce
the cross-reactivity of antibodies with nonspecific proteins associated with the use of whole cell extracts.

Figure 2 shows the results of experiments using anti-SBEII antibodies as the immunoprecipitation agent. The data in Figure 2A show that each of the peptide-specific anti-SBEII antibodies (and the anti-SS antibodies; Supplemental Fig. S1) precipitates only the respective form of SBE and SS from plastid lysates.

Figure 2. Coimmunoprecipitation of SBE and SS isomers in amyloplasts. Amyloplast lysates (1.1–1.8 mg protein cm\(^{-3}\)) prepared from endosperm at early (6–9 DAP; seed weight approximately 18–25 mg; A) and late (10–15 DAP; seed weight approximately 32–45 mg; B and C) stages of development were preincubated with either 1 mM ATP or 10 units of APase for 20 min at 25°C. Lysates were then immunoprecipitated (IP) with peptide-specific anti-SBEII or anti-SS antibodies, separated by 1D-SDS-PAGE, electroblotted onto nitrocellulose, and developed with various anti-SBE antisera, and antisera against SSI and SSII as shown. Arrows indicate cross-reactions with the various antisera used: SBEIIa and SBEIIb at 88 kD, SSI at 74 to 75 kD, and SSII at 85 kD. The large band observed at approximately 50 kD in all lanes is due to autorecognition of the IgG heavy chain. [See online article for color version of this figure.]
that phosphorylation (preincubation with ATP) or dephosphorylation (preincubation with APase) does not affect the ability of any of the antibodies to bind to the respective forms of SBE and SS.

Experiments with stroma prepared from amyloplasts isolated from endosperm at 6 to 9 DAP showed no coimmunoprecipitation of other starch-synthesizing enzymes as judged by immunoblotting, regardless of the pretreatment conditions (Fig. 2A). However, at 10 to 15 DAP, when higher molecular mass/aggregation states of SSs and SBEs were observed in the gel filtration experiments (Fig. 1), both SBEII antibodies were able to coimmunoprecipitate SSI and SSII, indicating potential protein-protein interactions between each of the SBEII forms and SSI and SSII. Preincucation of plastid lysates with glucan-degrading enzymes (amyloglucosidase and α-amylase) did not prevent coimmunoprecipitation of SS and SBE isoforms, which indicates that their association is due to specific protein-protein interactions and not a result of SSs and SBEs binding to a common glucan chain.

Significantly, the SS-SBE interactions observed at 10 to 15 DAP of endosperm development in Figure 2B were not observed when plastid lysates were preincubated with APase (which causes nonspecific dephosphorylation), suggesting that the SS-SBE interactions observed in wheat endosperm are developmentally regulated and also phosphorylation dependent. Lysates of late-stage endosperm amyloplasts that were not incubated with ATP showed the same coimmunoprecipitation phenomena as those incubated with ATP (data not shown), suggesting that the phosphorylation state of the enzymes is not markedly altered by exogenous ATP (given at 1 mM). Reciprocal experiments demonstrate that both forms of SBEII are co-precipitated by antibodies to SSI and SSII. Figure 2C illustrates the coimmunoprecipitation of SBEIIb in a phosphorylation-dependent manner. Immunoblots of proteins immunoprecipitated with anti-SBEII antisera were also developed with other available wheat-specific antibodies: anti-plastidial starch phosphorylase, anti-plastidial 1,4-α-β-glucan:1,4-α-β-glucan, 4-α-β-glucanotransferase (disproportionating enzyme, d-enzyme), and anti-isoamylase. None showed coimmunoprecipitation with anti-SBEII and anti-SS antibodies at the early tissue developmental stages investigated or as a function of the phosphorylation status of the stromal preparations (data not shown).

Identification of Protein Complex Components in Amyloplasts by Chemical Cross Linking

Stromal proteins from amyloplasts isolated from endosperm at 10 to 15 DAP were separated by gel filtration chromatography. Eluted column fractions were then immediately incubated with the homobifunctional cross-linking reagent bis (sulfosuccinimidyl) suberate (BS²), and the proteins were separated by SDS-PAGE, electroblotted and developed with various anti-SBE and anti-SS antisera. Figure 3A shows the electroblotted, cross-linked proteins from column fractions containing the two major peaks of SS and SBE activities, corresponding to the HMW fractions (containing putative protein complexes) and the LMW fractions (containing monomeric forms of SS and SBE; Fig. 1). Cross linking of proteins in the HMW fraction facilitated detection of a product of approximately 260 kD that displays cross-reactivity with anti-SBEII, anti-SSI, and anti-SSII antisera (Fig. 3A). Another cross-linked product of approximately 180 kD was observed in the HMW fraction, which showed cross-reactivity only with anti-SBEIIa or anti-SBEIb antisera (Fig. 3A). Cross-linking experiments with the LMW fractions demonstrated the presence of only monomeric forms of SBEII (88 kD), SSI (75 kD), and SSII (85 kD), as expected. Similar HMW, cross-linked products were obtained when amyloplast lysates were incubated with BS² prior to separation of proteins by gel filtration chromatography (data not shown). Figure 3A shows that when proteins separated by gel filtration chromatography are incubated with APase prior to cross linking with BS² (eluted column fractions were pre-treated with APase prior to the addition of BS²), the protein complexes in the HMW fraction dissociate into monomers, and no aggregated SS or SBE products could be detected.

The cross-linked polypeptides that cross reacted with the various antisera described above and shown in Figure 3A were in-gel digested with trypsin (from corresponding silver-stained SDS-gels), and some of the resulting peptides were sequenced using quadrupole-orthogonal-acceleration-time of flight mass spectrometry (Q-TOF-MS). The MS survey acquisition data obtained from single representative analyses are shown in Figure 3B. The sequence data in Figure 3B shows that the amyloplast proteins present in the 260-kD cross-linked complex(es) were SSI, SSIIa, and SBEII (the close sequence homology between SBEIIa and SBEIIb means that the two forms cannot be distinguished on the basis of the peptide sequences acquired by the mass spectrometer), whereas only peptides from SBEII forms could be detected in the cross-linked complexes of approximately 180 kD (Fig. 3B). Figure 3C demonstrates that SSI protein could be detected in association with SSII following immunoprecipitation with a monospecific SSI antibody (Supplemental Fig. S1), confirming that SSI and SSII can coexist in the same complex. The proteins from the LMW fraction that cross-reacted with the various antisera shown in Figure 3A were also in-gel digested with trypsin, and the peptides were analyzed by Q-TOF-MS; these analyses confirmed that each of the antibodies recognized the respective monomeric protein (data not shown).

Immunoblotting of column fractions also revealed other cross-linked aggregation products containing SBEII, SSI, and SSII, with molecular masses greater than 260 kD, but these were of low abundance, and no measurable MS spectra could be obtained from them. Washed starch granules were also incubated with BS² to determine whether any of the granule-associated proteins formed aggregates/complexes. BS² is a low-Mr...
water-soluble, cross-linking reagent (mass of 368.4) that would be predicted to penetrate the starch granule structure. Nevertheless, we found no evidence to suggest that any of the granule-associated proteins were present as aggregation products (determined by immunoblotting and silver-stained SDS-gels), even when starch granules were partially digested by α-amylase prior to treatment with BS3 to increase the accessibility of the cross linker to the granule-associated proteins (data not shown).

**Immunoprecipitation of SS Activity with Anti-SBEII Antibodies**

In parallel to the previously described analysis of protein pellets after immunoprecipitation, the supernatants were also assayed for residual SS activity. The data presented in Figure 4A show that soluble SS activity is immunoprecipitated by both of the SBEII antisera only in the HMW fractions. In total, both of the anti-SBEII antibodies precipitated approximately 90% of the measurable SS activity in the HMW fraction, each accounting for approximately equal amounts of the soluble SS activity. Analysis of the pellets following immunoprecipitation of the HMW protein fractions by anti-SBEII antisera indicated the presence of SSI and SSIIa (Fig. 4B). However, addition of anti-SBEIIa or anti-SBEIIb antisera to LMW fractions caused no reduction in soluble SS activity in the supernatant, and only the respective forms of SBEII could be detected in the pellets following immunoblot analysis, indicating no interaction with SS forms (Fig. 4B). Similar results were obtained in an experiment in which both SBEII antibodies were added to the HMW and LMW fractions; approximately 90% of the

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**Figure 3.** Cross-linking protein complexes in amyloplasts. A, Amyloplasts isolated from wheat endosperm at 10 to 15 DAP were lysed and separated by 1D-SDS-PAGE and electroblotted onto nitrocellulose and developed with anti-SBEII, anti-SSI, and anti-SSII antisera. The blots shown are from fractions containing high-Mr peak SS and SBE activities (HMW), the same HMW fractions pretreated with 20 units APase for 30 min prior to cross linking, and the lower Mr (LMW) peak of SS and SBE activity containing monomeric forms of SS and SBE. The positions of the major cross-linked and monomeric proteins that react with the various antisera are marked with arrows and estimated molecular masses based on their migration in relation to molecular mass standards (M). B, Silver-stained cross-linked polypeptides corresponding to 260-kD and 180-kD bands observed on the immunoblots shown in A were excised from polyacrylamide gels and digested with trypsin, and the recovered peptides were sequenced using Q-TOF-MS to identify the cross-linked proteins. The amino acid sequences from the MS survey acquisition data for the cross-linked products of 260 kD and 180 kD are shown in B. The data presented are for single representative analyses, and in each case show the peptide sequences obtained from the respective cross-linked products and the identity of the constituent proteins assigned on the basis of these peptide sequences (shown in parentheses). C, Coimmunoprecipitation of SSI and SSII. Amyloplast lysates (1 mg protein cm⁻²) prepared from endosperm were incubated with anti-SSI antibodies, separated by 1D-SDS-PAGE, electroblotted onto nitrocellulose, and developed with antisera against SSI and SSII as shown. The large band observed at approximately 50 kD is due to autorecognition of the IgG heavy chain. [See online article for color version of this figure.]
measurable SS activity in the HMW fraction was precipitated (data not shown).

Analysis of the Glucan-Binding Properties of LMW and HMW SBE Activities

Affinity gel electrophoresis was employed to quantify the dissociation constant ($K_d$) of branching enzyme from the HMW fractions compared with the corresponding monomeric proteins by measuring relative migration ($R_m$) in the presence of different concentrations of $\alpha$-(1→4)-linked glucans. Given the relative sensitivities of the various antibodies, we chose to use anti-SBEIIa, anti-SBEIIb to locate, reliably, the SBE proteins associated with the HMW fraction on immunoblots of the affinity gels and compare them with the migration of the respective monomeric forms in the LMW fraction (Fig. 5). Reciprocal values of $R_m$ ($1/R_m$) of SBEII forms from LMW and HMW fractions were linearly related to the concentration of glucan substrate in the gels (Fig. 5, B and C). The SBEII forms present in the HMW fraction exhibited smaller $K_d$ values (equating to a higher affinity) with both starch and amylpectin in the gels than the corresponding monomeric forms in the LMW fraction (Table I). In contrast to the behavior of the SBEII forms in the gels, control proteins (bovine serum albumin and molecular mass standards) showed no change in migration, irrespective of the glucan concentration in the gel (data not shown). The behavior of the control proteins indicates that the reduced mobility observed with the different SBEII forms in the affinity gels is a result of their specific affinity for the glucan provided rather than being caused by a dense polyacrylamide/glucan matrix in the gel. Surprisingly, in gels containing no glucan substrate, the mobility of the different proteins tested was the same in LMW and HMW fractions. However, the $K_d$ values derived for both isoforms of SBEII in HMW fractions, run on native gels containing increasing concentrations of corn starch, were significantly lower ($P < 0.001$) than the respective SBEII isoforms from LMW fractions. Table I shows that the HMW forms of SBEII have a 2-fold higher affinity (as measured by the $1/K_d$ values) for the glucan substrates tested than their respective monomeric forms. The kinetic parameters determined for the monomeric forms of SBEII in wheat endosperm are similar to those determined for other branching enzymes (Matsumoto et al., 1990). The results indicate only small differences between monomeric forms of SBEIIa and SBEIIb in their respective affinities to $\alpha$-(1→4)-linked glucans.

DISCUSSION

This article presents evidence for the existence of functional interactions between the SBEII class and the SSI and SSII classes of amylpectin-synthesizing
enzymes in amyloplasts from developing wheat endosperm. Changes in elution profile following gel permeation chromatography, demonstration of coimmunoprecipitation of SSs with branching enzymes, chemical cross linking, and the loss of protein complexes in the presence of alkaline phosphatase provide direct evidence for their physical interaction. The observation that these complexes are seen at specific stages of development adds weight to the argument that they represent functional biochemical activities in vivo. In the endosperms of Festucoideae, such as wheat and barley (*Hordeum vulgare*), large A-type starch granules are formed first, with the synthesis of small B-type granules occurring later at around 15 DAP, depending on environmental conditions. While it is tempting to speculate that the formation of protein complexes is associated with B-granule synthesis, this seems unlikely, because of the presence of complexes

Table 1. Comparison of $K_d$ values and affinity constants of wheat endosperm SBEIIa and SBEIIb from HMW and LMW gel filtration fractions

Summary of kinetic data derived from affinity chromatography experiments with HMW and LMW gel filtration fractions following separation of whole cell extracts from developing wheat endosperm (10–15 DAP). Results presented for starch as the glucan substrate are the mean and SDs of three to five independent experiments. Data obtained with amylopectin as substrate are from single experiments.

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<tr>
<th>Substrate</th>
<th>Monomer (LMW) Probed With</th>
<th>Complex (HMW) Probed With</th>
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<tbody>
<tr>
<td></td>
<td>Anti-SBEIIa</td>
<td>Anti-SBEIIb</td>
</tr>
<tr>
<td>Starch</td>
<td></td>
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<tr>
<td>$K_d$</td>
<td>0.42 ± 0.03</td>
<td>0.78 ± 0.06</td>
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<tr>
<td>Affinity ($1/K_d$)</td>
<td>2.44 ± 0.09</td>
<td>1.28 ± 0.11</td>
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<td>Amylopectin</td>
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<tr>
<td>$K_d$</td>
<td>0.44, 0.38</td>
<td>0.86</td>
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<tr>
<td>Affinity ($1/K_d$)</td>
<td>2.27, 2.63</td>
<td>1.16</td>
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in maize (*Zea mays*) endosperm (Hennen-Bierwagen et al., 2008 [accompanying article]).

Coimmunoprecipitation experiments in wheat and maize, as well as yeast (*Saccharomyces cerevisiae*) two-hybrid experiments (Hennen-Bierwagen et al., 2008) indicate that SBEIIa and SBEIIb form discrete protein complexes with SSI and/or SSIIa and are not found in the same heterocomplex. The fact that either SBEIIa or SBEIIb is present in the 260-kD complexes with SS isoforms suggests that assembly of these complexes is determined by conserved regions. Although Q-TOF-MS analysis of cross-linked products and coimmunoprecipitation experiments indicate the presence of heterotrimeric complexes involving SSI, SSII, and either form of SBEII, it is possible that other trimeric complexes also exist. Given the molecular masses of the SS and SBE isoforms in the protein complexes we have described, the 260-kD aggregation products could, in addition, include combinations of either SS isoform with SBEII homodimers (Fig. 6).

Other forms of SS and SBE are known to be expressed in the endosperm: SSIII, SSIV, and SBEI. These isoforms were not detected when the 260-kD cross-linked aggregates were analyzed by Q-TOF-MS. However, we cannot rule out that SSIII and SSIV may also be complexed, because we do not have specific probes for these proteins in wheat. Evidence suggests that SSIII also interacts with other starch-synthesizing enzymes in maize (Hennen-Bierwagen et al., 2008), although in gel permeation chromatography experiments, similar to those described here, SSIII was associated only with fractions of much higher molecular size (approximately 600 kD). In the case of SSIV, it is probable that the protein is not expressed at the stage of endosperm used in these experiments, since work with rice indicates that SSIV, like SBEI, is not expressed until late in endosperm development (Dian et al., 2005). SBEI could not be detected within the earliest stages of endosperm development (10–15 DAP) used in this study (up to 15 DAP; Fig. 1), which is in accordance with previous findings (Morell et al., 1997) and explains why the previously observed complex involving SBEI, SBEIIa, and starch phosphorylase was not observed here (Tetlow et al., 2004b).

The results show that as much as 40% to 50% of the measurable SS catalytic activity in the endosperm is in a high-Mₐ form at around 10 to 15 DAP (Fig. 1), although it should be noted that activities of SSs (Mu et al., 1994) and branching enzymes (Smith, 1990) may be underestimated in cell extracts, the latter because of interference by amylolytic activities. In the case of SS activity, the amount recovered from the gel permeation column exceeded the measurable activity applied by as much as 3-fold, suggesting that some inhibitory factor(s) may have been removed during fractionation. While this observation complicates interpretation, it at least suggests that loss of activity is unlikely. The coimmunoprecipitation experiments described in Figure 4 show that the SS-SBE protein complexes of around 260 kD are made up of catalytically active SSs, because each anti-SBEII antibody was able to immunoprecipitate approximately one-half of the SS activity in the HMW fractions. This observation therefore implies that most of the SS activity in the HMW fractions is associated with SBEII forms.

In addition to demonstrating their presence in heterocomplexes, evidence has been provided from chemical cross-linking studies that SBEII isoforms also form homodimers. To our knowledge, these experiments in wheat and maize are the first published evidence for homodimer formation among SBEII forms. SBEIIa and SBEIIb show a high degree of sequence identity, so it must be assumed that the less homologous regions at the amino- and carboxy-terminal ends of the proteins drive dimerization. Analysis of the mobility of HMW and LMW forms of SBEII in gels

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**Figure 6.** Summary of protein-protein interactions between enzymes of amylopectin biosynthesis. Potential phosphorylation-dependent protein-protein interactions within amyloplasts of wheat endosperm are shown, as deduced from the coimmunoprecipitation experiments and cross-linking studies presented in this article. All the proteins shown in Figure 6 are present at the earliest stages of endosperm development used in this study (6–9 DAP), but the various interactions shown are only detected later in development (10–15 DAP). It is assumed that all potential components of the complexes are present within the same cell-type. Broadly, three groups of potential interactions may be deduced from the experimental data: (1) homodimers of SBEII isoforms; (2) complexes formed between SS isoforms and SBEII homodimers; and (3) heterotrimeric complexes consisting of SSI, SSIIa, and one of the SBEI isoforms. In this study, there was no evidence for homo- or heterodimers of SSI/SSII forms, but we cannot rule out the possibility of complexes comprising SS dimers and an isozyme of SBEII being present.
containing presumptive glucan substrates (Fig. 5) suggests that the former may produce a catalytically functional unit with increased affinity for glucan substrates compared with the monomeric SBEII forms. This difference in affinity could represent the activity due to formation of homodimers of either SBEII isoform, or it could be the result of physical interaction with SSs in heteromeric complexes. The HMW and LMW forms of SBEII showed identical mobilities in native gels containing no glucan substrate (Fig. 5), which is more likely to be consistent with the activity of dimers. This is reinforced by the observation that immunodetection of SSI on the same blots did not show the same relative mobility of either form of SBEII (data not shown). The possibility that the differing affinity for starch may have arisen from the formation of a heteromeric complex cannot be ruled out completely, because it is possible that the complexes containing SBEII and SS might disassemble during electrophoresis. What is clear is that SBE isoforms in the HMW fraction show markedly different kinetic properties to their monomeric counterparts. Further work is needed to define the biochemical characteristics of SSs and branching enzymes when part of larger M, complexes.

Some of the components of the SS-SBE complexes have been shown to be phosphorylated (SBEIIa, SBEIIb, and SSI, but not SSI; Tetlow et al., 2004b; Supplemental Fig. S1), indicating the presence of protein kinase(s) in amyloplasts. Phosphorylation of these proteins may be an important factor in their ability to form protein complexes, as demonstrated by their dissociation following treatment with APase prior to cross linking (Fig. 3). The recent observation reported by Alexander and Morris (2006), indicating that SSI, SSI, and SBEIIa of barley are able to bind with amyloplast 14-3-3 proteins in a phosphorylation-dependent manner, suggests a potential mechanism for assembly of the SS-SBEII complexes via protein phosphorylation and plastidial 14-3-3 proteins. Sehnke et al. (2001) demonstrated that altered expression of a plastidial, granule-associated 14-3-3 protein in Arabidopsis influenced starch deposition, further reinforcing the connection between posttranslational modification mechanisms and starch synthesis. Mass spectrometric analysis of the cross-linked protein complexes described here did not indicate the presence of 14-3-3 proteins but does not rule out their involvement, because that association may be transient. Recently, Kempa et al. (2007) have identified a glycogen-synthase-like protein kinase in Medicago sativa. Modification of the expression of the Arabiopsis homolog (AtK-1) of this gene led to altered starch synthesis, particularly under conditions of high salinity. The AtK-1 protein was shown to be plastid localized and, in particular, bound to the starch granule. It will be interesting to determine the relationship between this protein and the regulation of protein phosphorylation and protein complex formation as observed in this study of wheat endosperm.

Previous genetic and biochemical data are consistent with the existence of protein-protein interactions among amylopectin biosynthetic enzymes, in particular, between SBE and SS forms. For example, the dul1 maize mutant that conditions a loss of SSIII function (Gao et al., 1998; Cao et al., 1999) also causes a decrease in SBEIIa activity (Boyer and Preiss, 1981; Cao et al., 2000). In rice endosperm, the ae mutation (causing a loss of SBEIIa activity) also shows a significant (50%) reduction in the activity of soluble SSI (Nishi et al., 2001). Loss of SSIla in wheat, barley, and rice endosperms also causes a reduction in amylopectin synthesis and abolishes the presence of SSI, SBEIIa, and SBEIIb within the starch granules (Yamamori et al., 2000; Morell et al., 2003; Umemoto and Aoki, 2005). All of these genetic observations could be explained by interactions between specific enzymes within a complex.

Because amylopectin is made by the ordered elongation and branching of glucan chains, the SS and SBE classes of enzymes would be logical partners in any amylopectin-synthesizing protein complex. In addition to the genetic evidence for interactions between the SSs and SBEs (see above), additional in vitro evidence exists for functional interactions between these enzyme classes. In maize kernel extracts, the activity of SSI was greatly stimulated by the addition of purified SBEI or SBEII (Boyer and Preiss, 1979), and Seo et al. (2002) showed that functional interactions exist between heterologously expressed SBEs from maize and yeast glycogen synthases, which were proposed to work in a cyclically interdependent fashion, consistent with the idea that SSs and SBEs may operate within hetero-protein complexes. Functional assemblies of this kind would presumably improve the efficiency of polymer construction as the product of one reaction becomes a substrate for another within the complex (substrate channeling). At a higher level of organization, the formation of protein complexes during amylopectin biosynthesis may promote a favored, three-dimensional structure within the growing polymer necessary for crystallinity (clustered branch points, side chains of defined length, particular side chain packing). In this hypothetical context, such multiprotein complexes may act as a form of “carbohydrate chaperonin” (Tetlow et al., 2004a).

Previous models have attempted to explain how individual enzymes contribute to the distinct unit structure of the cluster in amylopectin, often making use of well-characterized mutants or using antisense or Mutator transposon approaches. However, recent genetic and biochemical evidence (Colleoni et al., 2003; Dinges et al., 2003; Morell et al., 2003; Tetlow et al., 2004b) and the results presented here indicate that the distinct structure of amylopectin is probably the product of many combinations of interacting enzymes, some of which are components of protein complexes that may be active or inactive at different times. Future studies will focus on determining the distinct glucan products produced by the action of the protein complexes described and identifying the regulatory proteins involved in assembly and disassembly of the protein complexes.
MATERIALS AND METHODS

Plants and Growth Conditions

Spring wheat (Triticum aestivum) 'Tahoe' was grown under glasshouse conditions in a soil medium containing local topsoil, Surface MVP (Profile Products), peat moss, lime, and Nutricote (14-14-14; Morton’s Horticulural Products) in a ratio of 3:1:1:0.01:0.01. All other plant growth conditions are as previously described (Tetlow et al., 1993). Developing ears were tagged at the onset of anthesis (the first appearance of anthers) to consistently monitor seed development. Endosperm tissue was obtained from developing grains taken from the mid-ear region of the head at various stages of endosperm development between 8 and 45 DAF. The different stages of endosperm development were determined by measuring the fresh weight of harvested seed from the tagged wheat plants.

Plastid Isolation

Amyloplasts were isolated from the endosperm obtained from developing grains taken from the mid-ear region of the head at various DAP (see above) using the methods described by Tetlow et al. (2003), typically using between 70 and 110 g fresh weight of seed for each preparation. Intact amyloplasts were lysed osmotically by the addition of ice-cold rupturing buffer containing 100 mM Tricine/KOH, pH 7.8, 1 mM Na₂-EDTA, 1 mM dithiothreitol (DTT), 5 mM MgCl₂, and a protease inhibitor cocktail (Sigma-Aldrich, catalog no. P 9599, used at 10 μL/cm³). Starch granules were removed by centrifugation at 14,000g for 5 min at 4°C and the supernatant centrifuged at 120,000g for 15 min in a Beckman Airfuge (at 25 psi) to remove plastid membranes and particulate material. The supernatant from the ultracentrifugation step, termed plastid stroma, was used for subsequent experiments. Starch granules were washed, and granule-associated proteins were extracted following the methods described by Denyer et al. (1995).

Protein Extraction from Developing Endosperm

Whole cell extracts were prepared by rapidly homogenizing approximately 0.5 to 0.8 g endosperm (of various individual seed weights corresponding to different stages of development) in 1 cm³ of ice-cold rupturing buffer followed by centrifugation at 13,500g for 2 min at 4°C. The resulting supernatant was subjected to ultracentrifugation as described above and immediately loaded onto the size exclusion column (below).

Size Exclusion Chromatography

Amyloplast lysates and whole cell extracts were separated by size exclusion chromatography using a Superdex 200 10/300GL column using an AKTA FPLC (Amersham Biosciences) at 4°C. The column was pre-equilibrated and run in a buffer containing 10 mM HEPES-NaOH, pH 7.5, and 100 mM NaCl at a flow rate of 0.25 cm³/min. Protein extracts (between 1.2 and 2.2 mg/cm³ protein) were loaded onto the column in a final volume of 0.5 cm³ and fractions of 0.5 cm³ were collected using a Frac 950 fraction collector (Amersham Biosciences). The column was routinely calibrated using commercially available standards; those used for calibration were: thyroglobulin (669 kD), ferritin (440 kD), catalase (232 kD), aldolase (158 kD), albumin (67 kD), ovalbumin (43 kD), chymotrypsinogen (25 kD), and ribonuclease (13.7 kD) purchased from Amersham Biosciences.

Enzyme Assays

Subcellular marker enzyme assays were performed as previously described (Tetlow et al., 2003). SBE activity was assayed semiquantitatively using a modification of the phosphorylase a stimulation assay described by Smith (1988). Reaction mixtures contained 100 mM sodium citrate, pH 7, 1 mM Na₂-EDTA, 1 mM DTT, 2.5 mM AMP, and 0.2 unit rabbit muscle phosphorylase a (product no. P-1261, Sigma-Aldrich). Reactions were initiated by the addition of 50 μL [U-14C]Glc 1-P (3.7–7.4 kBq per assay; Amersham Biosciences) and incubated at 25°C for 5 min. Reactions were terminated by heating the mixture at 90°C for 5 min and the radio-labeled glucan washed in methanol-KCl as for SBE assays above. The assay for amylolytic activity involved incubating 0.1 cm³ of washed [14C]-labeled glucan with 0.1 cm³ protein fractions for 10 min at 25°C. The reaction was terminated by heating to 95°C for 5 min and the remaining [14C]-labeled glucan was precipitated using methanol-KCl; the [14C]-labeled products released into the supernatant were taken as a measure of amylolytic activity and counted using a liquid scintillation counter.

Phosphorylation of Amyloplast Proteins in Vitro

Phosphorylation reactions in which intact amyloplasts were incubated with γ-32P-ATP were performed as described previously (Tetlow et al., 2004b). Reactions were terminated by lysis in ice-cold rupturing buffer followed by immediate desalting on NAP-10 columns (Amersham Biosciences) that had been preequilibrated in rupturing buffer with no protease inhibitor cocktail present. The desalted stromal proteins were used in immunoprecipitation experiments (see below). The phosphorylation status of proteins was also determined using ProQ Diamond stain (Invitrogen Canada) in conjunction with APase as a control and following the manufacturer’s instructions.

Preparation of Peptides and Antisera

Polyclonal antibodies were raised in rabbits against the synthetic peptides derived from the N-terminal sequences of wheat SBEI (VSPADTYMALTSGA, Tetlow et al., 2004a), and wheat SBEIIb (AGGPGSEVM ICCG, Regina et al., 2005). The antigen was prepared by coupling the synthesized peptide to keyhole limpet hemocyanin using the heterobifunctional reagent m-maleimidobenzoyl-N-hydroxysuccinimide ester. Anti-wheat SSI and anti-wheat SSI antisera were prepared as described by Li et al. (1998b), and anti-wheat v-enzyme antisera was prepared as described by Bresolin et al. (2006).

Immunoprecipitation

Immunoprecipitation experiments were performed with samples of amyloplast stroma and size exclusion column fractions, using methods previously described (Tetlow et al., 2004b). SBE antibodies were added to plastid stroma at concentrations described previously (Tetlow et al., 2004b), and other antibodies were added at the following concentrations, unless stated otherwise: anti-SSI and anti-SSI, 5 μL cm⁻³, anti-isoamylase, 0.2 μL cm⁻³, anti-starch phosphorylase, 2 μL cm⁻³. Specificity of the SS antibodies used in immunoprecipitation experiments was confirmed (Supplemental Fig. S1). Proteins were immunoprecipitated by adding 40 μL of Protein A-Sepharose (Sigma-Aldrich) made up as a 50% (w/v) slurry with phosphate buffered saline (137 mM NaCl, 10 mM Na₂HPO₄, 2.7 mM KCl, 1.8 mM KH₂PO₄ to the samples and incubating for a further 30 min at 4°C. The Protein A-Sepharose/protein complex was washed three times, each with 1.2 cm³ phosphate buffered saline, followed by three similar washes with 10 mM HEPES/KOH, pH 7.5. To exclude the possibility that the immunoprecipitation of the proteins observed in the immunoprecipitation pellet was a result of SSs and SBEs binding to the same glucan chain, plastid lysates used for immunoprecipitation...
and cross-linking (see below) were preincubated with glucan-degrading enzymes as follows. Plastid lysates were incubated with five units each of amylolytic enzymes (EC 3.2.1.3; Sigma product no. A7255, from Rizopus and α-amylase (EC 3.2.1.1; Sigma product no. A2643, from porcine pancreas) for 20 min at 25°C. Control experiments indicated that 0.1 mg glucan (glycogen, starch, or amylopectin) could be completely digested under these conditions (data not shown).

**SDS-PAGE and Immunoblotting**

Prior to electrophoresis, proteins were mixed with SDS sample buffer (62.5 mM Tris-HCl, pH 6.8, 2% [w/v] SDS, 10% [w/v] glycerol, 5% [v/v] β-mercaptoethanol, 0.001% [w/v] bromophenol blue) and boiled for 2 min. Protein samples were separated on gels using precast NuPAGE Novex 4% to 12% Bis-Tris gradient gels (Invitrogen Canada) with MOPS running buffer and following the manufacturer’s instructions for sample preparation and electrophoresis. SDS-solubilized cross-linked proteins were either separated using four to 12 Bis-Tris gradient gels (see above) or with precast NuPAGE Novex 3% to 8% Tris-acetate gels following the manufacturer’s instructions for sample preparation and electrophoresis. Gels were either stained with a colloidal Coomassie C-250 kit (Simply Blue Safestain, Invitrogen) or silver-stained according to methods described by Shevchenko et al. (1996). Stained proteins that were to be analyzed by TOF-MS were excised from gels with a sterile razor blade and stored at −20°C until required.

Samples for immunoblot analysis were translotted onto nitrocellulose membranes (Pall Life Sciences), blocked with 1.5% bovine serum albumen, and exposed to antibodies using the methods described by Harlow and Lane (1999). The various anti-wheat antisera were used in immunoblot analyses at the following dilutions: anti-SBEI and anti-SBEIIa, 1:5,000; anti-SBEIIb, 1:2,000; anti-α-1,000,000, anti-SS and anti-SSII, 1:2,000; anti-acylase, 1,500; and anti-barley (Hordeum vulgare) endosperm starch phosphorylase, 1,200. Bound antibodies were detected with alkaline phosphatase-conjugated goat anti-rabbit IgG using a 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetra-azolium liquid substrate system (Sigma-Aldrich, catalog no. B-8119).

**Zymogram Analysis and Affinity Electrophoresis**

For native zymogram analysis, protein samples were mixed with native gel sample buffer (50% [v/v] glycerol, 0.2% [w/v] bromophenol blue) in a ratio of 20:1 and separated on native 5% (w/v) polyacrylamide gels in 1.5 mM Tris-HCl, pH 8.8. For analysis of SB activity, gels contained 0.2% (w/v) maltotetraose, 1.4 units phosphorylase a (from rabbit muscle; Sigma-Aldrich, catalog no. P-1261) and 10 mg of the α-amylase inhibitor Acarbose ("Prandase," Bayer) for 2 h at 4°C. Gels were washed, incubated for 2 h at 30°C, and stained, as previously described (Nishi et al., 2001). Gels were photographed immediately after staining. The different SBE isoforms from wheat endosperm separated on the native gels were identified as described previously (Tellow et al., 2004b).

For affinity electrophoresis, various concentrations of glucan substrates (corn starch or amylopectin) were added to the native gel polymerization mixture. Protein samples (0.5–2 mg) from gel filtration experiments corresponding to either complexes or monomeric forms of SS and SBE were loaded on these gels, and electrophoresis was carried out at 100V constant at 25°C in running buffer (25 mM Tris, 192 mM Gly) containing 1 mM DTT. The migration distances of the proteins were measured after immunoblotting and probing for specific enzymes as described above.

**Calculation of Kd**

Affinity electrophoresis was used as a means of measuring protein-glucan interactions, and Kd are calculated from the retardation of the electrophoretic mobility of enzyme/protein by the substrate contained in the supporting medium. We followed the methods described by Commuri and Keeling (2001) and Matsumoto et al. (1990). Using native polyacrylamide gels containing various concentrations of amylopectin and starch (both from maize [Zea mays], Sigma), the relative mobilities of wheat SSs and SBEs in monomeric and aggregated forms were measured at room temperature (23°C–25°C). Bovine serum albumen was used in control experiments, and its mobility was detected in gels by staining with Coomassie G-250 (see above). Paired-sample t tests were used to compare the Kd values of SBEII isoforms from the HMW and LMW fractions following electrophoresis in native gels containing corn starch.

**Cross Linking**

Amyloplasts were prepared for cross-linking experiments using buffers free of EDTA and DTT and were lysed in gel filtration chromatography running buffer containing a protease inhibitor cocktail (see section above on plastid isolation).

Fractons of amyloplast lysates separated by size exclusion chromatography were immediately incubated with 1 mg of the homobifunctional cross-linking reagent BS3 (Pierce/BioLynx) on a rotating table at 25°C for 30 min. The cross-linking reaction was quenched by the addition of 10 μL of a 1 M solution of Tris, pH 8, to the 110-μL reaction mix, and the sample volume reduced to 40 to 50 μL using MicroCon concentrators (Millipore). The concentrated cross-linked samples were mixed with SDS sample buffer prior to electrophoresis (see above).

**MS**

In-gel digestion with trypsin and preparation of peptides for MS were as described previously (Tellow et al., 2004b). Tandem electrospray mass spectra were recorded using a hybrid Q-TOF spectrometer (Micromass) interfaced to a Micromass CapLC capillary chromatograph. Samples were dissolved in 0.1% aqueous formic acid, and 6 μL was injected onto a Pepmap C18 column (300 μm × 0.5 cm; LC Packings) and washed for 3 min with 0.1% aqueous formic acid (with the stream select valve diverting the column effluent to waste). The flow rate was then reduced to 1 μL min−1, the stream select valve was switched to the data acquisition position, and the peptides were eluted into the mass spectrometer with an acetonitrile/0.1% formic acid gradient (5%–70% acetonitrile over 20 min). The capillary voltage was set to 3,500 V, and data-dependent tandem mass spectra acquisitions were performed on precursors with charge states of 2, 3, or 4 over a survey mass range of 400 to 1,300. Known trypsin autolysis products and keratin-derived precursor ions were automatically excluded. The collision voltage was varied between 18 and 45 V depending on the charge and mass of the precursor. Product ion spectra were charge-state de-encrypted and de-isotoped with a maximum entropy algorithm (MaxEnt 3, Micromass). Peptides were identified by correlation of uninterpreted tandem mass spectra to entries in SwissProt/TREMBL, using ProteinLynx Global Server (Version 1, Micromass). One missed cleavage per peptide was allowed, and an initial mass tolerance of 50 ppm was used in all searches. Cysteines were assumed to be carboxymethylated, but other potential modifications were not considered in the first pass search. When this approach failed, amino acid sequences were deduced manually from the charge state de-encrypted spectra (Wait et al., 2002) and were used as queries for searches using BLAST (Altschul et al., 1997) and FASTS (Mackey et al., 2002).

**Protein Determination**

The protein content of wheat endosperm whole cell extracts and plastid preparations was determined using the Bio-Rad protein assay (Bio-Rad Laboratories Canada) according to the manufacturer’s instructions and using thyroglobulin as a standard.

**Supplemental Data**

The following materials are available in the online version of this article.

**Supplemental Figure S1.** Antibody specificity, phosphorylation, and immunoprecipitation of SS and SSI.

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