Characterization of SU1 Isoamylase, a Determinant of Storage Starch Structure in Maize[1]

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Function of the maize (*Zea mays*) gene *sugary1* (*su1*) is required for normal starch biosynthesis in endosperm. Homozygous *su1*-mutant endosperms accumulate a highly branched polysaccharide, phytoglycogen, at the expense of the normal branched component of starch, amylopectin. These data suggest that both branched polysaccharides share a common precursor, and that the product of the *su1* gene, designated SU1, participates in kernel starch biosynthesis. SU1 is similar in sequence to α-(1→6) glucan hydrolases (starch-debranching enzymes [DBEs]). Specific antibodies were produced and used to demonstrate that SU1 is a 79-kD protein that accumulates in endosperm coincident with the time of starch biosynthesis. Nearly full-length SU1 was expressed in *Escherichia coli* and purified to apparent homogeneity. Two biochemical assays confirmed that SU1 hydrolyzes α-(1→6) linkages in branched polysaccharides. Determination of the specific activity of SU1 toward various substrates enabled its classification as an isoamylase. Previous studies had shown, however, that *su1*- mutant endosperms are deficient in a different type of DBE, a pullulanase (or R enzyme). Immunoblot analyses revealed that both SU1 and a protein detected by antibodies specific for the rice (*Oryza sativa*) R enzyme are missing from *su1*- mutant kernels. These data support the hypothesis that DBEs are directly involved in starch biosynthesis.

Starch is a reserve carbohydrate that accumulates in the storage organs of many higher plants. This storage compound consists of a mixture of two Glc homopolymers, amylopectin and amylose, in which linear chains are formed via α-(1→4) glucosyl linkages and branches are introduced by α-(1→6) glucosyl linkages. Starch synthesis in maize (*Zea mays*) occurs within the amyloplasts of endosperm cells during kernel development via the concerted actions of ADP-Glc pyrophosphorylase, starch synthases, and starch-branching enzymes (for reviews, see Preiss, 1991; Hannah et al., 1993; Martin and Smith, 1995; Nelson and Pan, 1995; Preiss and Sivak, 1996; Smith et al., 1996). In addition, selective removal of branch linkages by DBEs is proposed to play an essential role in the final determination of amylopectin structure (Ball et al., 1996).

Physical and chemical analyses of granular starch have led to a widely accepted model for amylopectin structure called the "cluster model," in which amorphous and crystalline regions alternate with a defined periodicity (for reviews, see French, 1984; Manners, 1989; Jenkins et al., 1993). Within amylopectin the crystalline component is composed of parallel arrays of linear chains packed tightly in double helices. Branch linkages, which account for approximately 5% of the glucosyl linkages in amylopectin, are located at the root of each cluster in the amorphous region. This periodic clustering of branches allows for the alignment of the intervening linear chains and their dense packing into crystalline regions, thus providing an efficient mechanism for nutrient storage. Undoubtedly, the enzymatic processes required to achieve this ordered spatial positioning of branch linkages and extension of linear glucans must be highly regulated and coordinated.

Mutations that alter or eliminate the cluster organization within amylopectin can provide clues to the molecular mechanisms that give rise to its structure. Such is the case with mutations of the maize *su1* gene. Phytoglycogen, which accumulates in *su1*- mutant kernels, has twice the frequency of branch linkages as amylopectin, a shorter average chain length (average degree of polymerization is approximately 10 versus an average of 20–25 for amylopectin), and a significantly different chain-length distribution (Yun and Matheson, 1993). Thus, phytoglycogen is multiply branched and lacks the packed crystalline helices of amylopectin (Gunja-Smith et al., 1970; Alonso et al., 1995). These structural alterations cause the molecule to be water soluble, whereas amylopectin in endosperm cells is insoluble. Biochemical analysis has revealed that *su1*- mutants are deficient in the activity of a specific DBE (Pan and Nelson, 1984). This fact, correlated with the accumulation of phytoglycogen in *su1*- mutant kernels, suggests that the DBE participates in the organization of regularly spaced clusters within amylopectin. Similar evidence is available from *sugary* mutants of rice (*Oryza sativa*) and the *STA-7*

Abbreviations: DAP, days after pollination; DBE, starch-debranching enzyme; ENZ, amyloglucosidase enzyme reactor; Glc-cGlc7, glucosyl-cycloheptaamylose; Glc2-cGlc7, maltosyl-cycloheptaamylose; GST, glutathione-*S*-transferase; HPAEC, high-performance anion-exchange chromatography; λ_{max}, wavelength at which the complex exhibited maximal absorbance; PAD, pulsed-amperometric detection; *Ps.* isoamylase, isoamylase from *Pseudomonas amyloderamosa*.

and *STA-8* mutants of *Chlamydomonas reinhardtii*, all of which accumulate phytoglycogen and also are deficient in the activity of a DBE (Mouille et al., 1996a, 1996b; Nakamura et al., 1996a, 1996b).

The two types of DBEs that have been identified in plants are classified as pullulanases (also referred to as R enzymes or limit dextrinases) and isoamylases (Lee and Whelan, 1971; Manners, 1971; Doehlert and Knutson, 1991). Both types of enzyme directly hydrolyze α-(1→6) branch linkages, but differ in their activities toward specific polysaccharides. Plant pullulanases hydrolyze both pullulan, a polymer of α-(1→6)-linked maltotriosyl units, and α-limit dextrins at much higher rates than amylopectin, but they have little or no hydrolytic activity toward glycogen. In contrast, isoamylases readily hydrolyze the α-(1→6) branch linkages of amylopectin and glycogen, but do not act on pullulan. The DBE shown to be missing in *su1-* mutants of maize and rice is of the pullulanase class (Nakamura et al., 1996b; Pan and Nelson, 1984).

Both isoamylases and pullulanases are present in developing maize endosperm during the time of starch biosynthesis (Doehlert and Knutson, 1991), consistent with the genetic evidence indicating DBE involvement in the determination of amylopectin structure. The participation of a specific pullulanase or isoamylase in the biogenesis of kernel starch, however, has yet to be demonstrated directly. In addition to having potential biosynthetic functions, both types of DBE are believed to be involved in the degradation of endosperm starch after seed germination (Manners and Rowe, 1969; Toguri, 1991).

Molecular cloning of the maize gene *su1* and the Su1 cDNA revealed that its polypeptide product, SU1, is similar in amino acid sequence to members of the α-amylase family of starch-hydrolytic enzymes (Jesperson et al., 1993; James et al., 1995; Beatty et al., 1997). SU1 is significantly similar to *Ps.* isoamylase, with 32% identical residues among 695 aligned amino acids, although its relation to known plant or bacterial pullulanases is significantly less (James et al., 1995). This result is an apparent discrepancy with the finding that the particular DBE deficient in *su1-* mutant endosperm is of the pullulanase type (Pan and Nelson, 1984).

To resolve this discrepancy and gain a better understanding of the role *Su1* plays in starch biogenesis, this study made use of two recombinant forms of the SU1 protein. Antibodies specific for SU1 were produced and used to characterize its native size, aspects of its subcellular location, and its expression pattern in developing endosperm. In addition, a nearly native-size recombinant form of SU1 was expressed in *Escherichia coli*, purified, and characterized in terms of its enzymatic properties. The results clearly demonstrate that SU1 is an enzyme of the isoamylase class and indicate that at least two distinct DBEs are deficient in *su1-* mutants as a result of a primary deficiency of SU1 isoamylase. Furthermore, SU1 is expressed in maize kernels during the period of starch production, consistent with the proposed biosynthetic role for this DBE.

## MATERIALS AND METHODS

Vector Construction

Plasmid pAR2 was constructed to express a portion of SU1 in *Escherichia coli* for the purpose of generating a polyclonal antibody. pAR2 contains a 577-bp region of the Su1 cDNA comprising nucleotides 202 to 778 (the A of the first ATG codon in the Su1 cDNA is designated as nucleotide 1) joined as an in-frame fusion with the 3′ end of the GST gene from *Schistosoma japonicum* in the expression vector pGEX-4T-3 (Pharmacia) (Fig. 1B). pAR2 was constructed by subcloning the 1.8-kb *Eco*RI fragment of the Su1 cDNA from pMJ67 (James et al., 1995) in pGEX-4T-3 to create pAR1, followed by removal of the 3′ end of the cDNA by digestion with *Hin*dIII and *Sal*I, subsequent repair of the 5′ overhangs with a DNA polymerase I Klenow fragment, and blunt-end ligation to recircularize.

Plasmid pAR4 was constructed to express biochemically active SU1 in *E. coli*. pAR4 contains the nearly full-length Su1 cDNA (nucleotides 202-2529) as an in-frame fusion with the 3′ end of the S-tag sequence (a 15-amino acid peptide derived from RNase A) in the expression vector pET-29b(+) (Novagen, Madison, WI) (Fig. 1C). pAR4 was constructed by first replacing the 1.3-kb *Kpn*I-*Xho*I fragment of the Su1 cDNA in pAR1 with the longer, 1.8-kb *Kpn*I-*Xho*I cDNA fragment from pMJ99 (James et al., 1995).

The resultant plasmid was linearized with *Xho*I and partially digested with *Eco*RI to excise a 2327-bp fragment comprising the nearly full-length Su1 cDNA. This fragment was cloned into pET-29b(+) to create pAR4.

Protein Expression and Purification

GST-SU1 expression from pAR2 was induced in *E. coli* strain TG-1 by the addition of 10 mM isopropylthio-β-
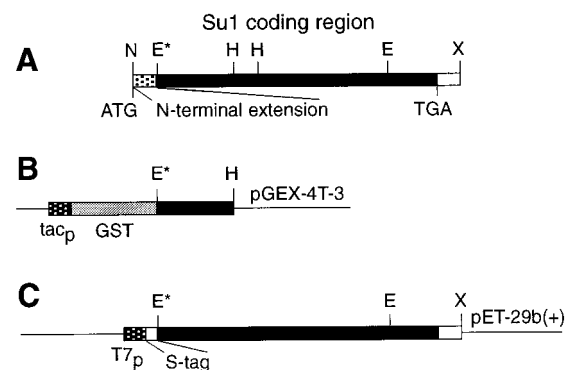
Figure 1. Su1 cDNA expression constructs. A, Restriction map of the coding region of the Su1 cDNA. The region denoted "N-terminal extension" indicates the first 67 amino acids of the polypeptide, which is not represented in *Ps.* isoamylase in its alignment with SU1. B, Plasmid pAR2, comprising the 580-bp *Eco*RI-*Hin*dIII fragment of the Su1 cDNA cloned downstream of the GST coding sequence. The fusion gene is under the direction of the *tac* promoter. C, Plasmid pAR4, comprising the 2327-bp *Eco*RI-*Xho*I fragment of the Su1 cDNA fused to the S-tag sequence in pET-29(b)+. The fusion gene is under the direction of the T7 promoter. Restriction sites are indicated as follows: N, *Nco*I; H, *Hin*dIII; X, *Xho*I; E, native *Eco*RI; and E*, *Eco*RI introduced during cDNA construction.

galactoside and incubation for 2 h at 30°C. Cells were lysed and separated into soluble and insoluble fractions, as described previously (Koerner et al., 1990). Soluble GST-SU1 fusion protein was purified using glutathione-agarose beads (Sigma) and eluted with 10 mM glutathione according to the manufacturer's protocol (Pharmacia).

The recombinant protein SU1r (for SU1 recombinant protein) was expressed from pAR4 in *E. coli* strain BL21(DE3)pLysS (Novagen) and purified as follows. A pure clone was grown overnight at 30°C in Luria-Bertani medium containing 30 μg/mL kanamycin and 34 μg/mL chloramphenicol, diluted 1:20 with fresh medium, and grown at 30°C until the A_{600} reached 0.8 to 1.2. Cultures were cooled to room temperature, and expression was induced by the addition of 1 mM isopropylthio-β-galactoside and growth at room temperature for 16 to 20 h. Cells from 100 mL of induced culture were harvested by centrifugation, suspended in 10 mL of 20 mM Tris-hydrochloride, pH 7.5, 150 mM sodium chloride, 5 mM DTT, 0.1% Triton X-100, 1 mM PMSF (GIBCO-BRL), and 0.01 mM E64 (Sigma), and lysed by treatment with lysozyme (100 μg/mL) and sonication. Lysates were centrifuged at 39,000g for 20 min. SU1r was affinity purified according to the manufacturer's protocol (Novagen) by incubating the total soluble extracts with S-protein agarose beads, followed by cleavage between the S-tag sequence and SU1r with biotinylated thrombin. Residual biotinylated thrombin was removed from the purified protein by treatment with streptavidin-agarose.

Preparation of Polyclonal Antibodies

Antibodies reactive with SU1 were produced in a New Zealand White rabbit using a protocol approved by the Laboratory Animal Resource Facility of Iowa State University. Purified GST-SU1 fusion protein (300 μg in 1 mL of PBS) was emulsified in an equal volume of Freund's complete adjuvant (GIBCO-BRL) and administered by injection. Subsequent 200-μg booster inoculations of GST-SU1 emulsified in Freund's incomplete adjuvant were administered three times at 3-week intervals, and serum was harvested 3 weeks after the final injection. Preimmune serum was collected before the initial injection to serve as a negative control. Anti-SU1 antibody was purified from the crude antiserum by means of its affinity to SU1r immobilized on nitrocellulose filters (Harlow and Lane, 1988).

Nomenclature and Maize Stocks

Standard genetic nomenclature for maize (*Zea mays*) is used as described by Beavis et al. (1995). Alleles beginning with an uppercase letter indicate a functional, nonmutant form of the gene (e.g. *Su1*). Unspecified mutant alleles are indicated by dashes with no following designation (e.g. *su1-*). Gene products are indicated by nonitalicized uppercase letters (e.g. SU1). Transcripts and cDNAs are indicated by the nonitalicized gene symbol (e.g. Su1).

Wild-type plants were in the W64A or Oh43 genetic background, as were plants homozygous for the reference mutation of the *su1* locus *su1-Ref* (Correns, 1901). Plants homozygous for *su1- R4582::Mu1* were of a mixed genetic background (James et al., 1995).

Isolation of Proteins from Maize Kernels

Ears were harvested 18 to 22 DAP. Kernels stripped from the cob were quick frozen in liquid nitrogen and stored at −80°C. Total protein was extracted from frozen kernels according to the method of Ou-Lee and Setter (1985), with the following modifications: 10 g of maize kernels was homogenized in liquid nitrogen, stirred in extraction buffer (50 mM Hepes-sodium hydroxide, pH 7.5, 5 mM magnesium chloride, 5 mM DTT, 1 mM PMSF, and 0.01 mM E64) at 4°C for 3 to 4 h, then filtered through four layers of cheesecloth. Soluble extracts were separated from insoluble material in the filtrate by centrifugation at 39,000g for 20 min at 4°C. Granule-associated proteins and postgranule soluble extracts were isolated from frozen kernels as described by Mu-Forster et al. (1996).

SDS-PAGE and Immunoblot Analysis

SDS-PAGE in 10% polyacrylamide gels, silver staining, and blotting to nitrocellulose membranes were performed according to standard procedures (Sambrook et al., 1989). Immunodetection was modified from the enhanced chemiluminescence western blotting system (Amersham), modified as follows: blots were blocked in 3% BSA (fraction V, Sigma), 0.02% sodium azide in 1× PBS for 1 h, then incubated overnight at room temperature with affinity-purified anti-SU1 diluted 1:200 in blocking solution, or with a 1:10,000 dilution of the rice (*Oryza sativa*) anti-R-enzyme antibody (Nakamura et al., 1996a). Secondary antibody was goat anti-rabbit IgG conjugated with horseradish peroxidase (Santa Cruz Biotechnology, Santa Cruz, CA). For immunoblot analysis of soluble protein extracts, approximately 30 μg of protein was loaded into each lane. Granule-associated proteins extracted from 5 mg of purified starch granules were loaded into each lane for immunoblot analysis.

Enzyme Assays

DBE activity was measured by the increased blue value of glucan-iodine complexes according to the method of Doehlert and Knutson (1991), modified as follows. Reactions incubated at 30°C included 1 μg of purified SU1r and 1 mg of polysaccharide substrate in 50 mM citrate, 100 mM sodium phosphate, pH 6.0, and 0.02% sodium azide in a total volume of 1 mL. Immediately after the addition of SU1r and after 1 h of incubation, 100-μL aliquots were combined with 900 μL of iodine/potassium iodide stain. Substrates tested were: maize amylopectin (Sigma), β-limit dextrin of maize amylopectin prepared according to the method of Doehlert and Knutson (1991), oyster glycogen (Sigma), and phytoglycogen prepared according to the method of Sumner and Somers (1944).

Quantitative determination of reducing-end formation was performed according to the method of Fox and Robyt (1991). Assay conditions were the same as for blue-value

assays, except that all substrate concentrations were increased to 5 mg/mL. Pullulan (Sigma) was included as an additional potential substrate. SU1r was present at 0.5 μg/mL for assays of its activity toward amylopectin and β-limit dextrin, and at 1 μg/mL for other substrates. Products were assayed over a time course of 1 to 6 h; aliquots of 100 μL were removed after each hour, followed by inactivation of the enzyme with 50 μL of 1 M sodium carbonate. A baseline reducing value obtained for each substrate before the addition of the enzyme was subtracted from the reducing value measured at each time point. Maltose was used as the standard for reducing value. In preliminary experiments the rate of product formation obtained under these reaction conditions was in all instances shown to be approximately linearly proportional to the enzyme concentration (data not shown). Thus, the reactions were performed under conditions in which the enzyme was saturated with substrate.

Enzyme activity as a function of pH was determined from the reducing activity measured after incubating 5 mg of amylopectin with 0.5 μg of SU1r in 50 mM sodium citrate, 100 mM sodium phosphate buffers varying from pH 3.0 to 7.0; in 100 mM sodium phosphate, pH 8.0; or in 50 mM Gly-sodium hydroxide buffers at pH 9.0 or 10.0. Formation of reducing ends was assessed hourly during a 6-h time course, and specific activities were calculated. In a similar time course, the thermal optimum for SU1r activity toward amylopectin was measured at reaction temperatures varying from 15 to 60°C at pH 6.0. The thermal stability of SU1r was determined by preincubating the enzyme at temperatures from 30 to 60°C for 10 min, then assessing enzymatic activity at pH 6.0 at 30°C. The effect of divalent cations on SU1r activity toward amylopectin was determined by adding 10 mM calcium chloride, 10 mM magnesium chloride, or 10 mM EDTA to the pH 6.0 reaction buffer, and then measuring activity according to the reducing-sugar assay.

Analysis of SU1r Reaction Products

The chain lengths of the products formed after incubation of *waxy* maize amylopectin (Cerestar, Hammond, IN) with SU1r were analyzed by HPAEC-PAD (Dionex, Sunnyvale, CA) according to the quantitative methods of Wong and Jane (1995, 1997), with modification of the separation gradient. Eluent A was 100 mM sodium hydroxide and eluent B was 100 mM sodium hydroxide and 300 mM sodium nitrate for the following gradient: 0 to 5 min, 99% A, 1% B; 5 to 10 min, linear gradient to 5% B; 10 to 30 min, linear gradient to 8% B; 30 to 150 min, linear gradient to 30% B; 150 to 200 min, linear gradient to 45% B. Debranching reactions included 20 μg of SU1r and 5 mg of *waxy* amylopectin in 50 mM Mes, pH 6.0, in a total volume of 1 mL. After incubation at 30°C for 1 h an aliquot of 25 μL was removed for HPAEC-ENZ-PAD analysis. A similar analysis of *waxy* maize amylopectin debranched by *Ps.* isoamylase (300 units) (Hayashibara Biochemical Laboratories, Okayama, Japan) was carried out for comparison with SU1r, except that the reaction buffer was 50 mM sodium acetate, pH 3.5, and incubation was for 24 h.

RESULTS

Immunological Identification of Native SU1

Polyclonal antibodies were raised against a fusion protein containing GST at its N terminus and SU1 residues 69 to 262 at its C terminus (Fig. 1) (residue 1 is defined by the first ATG codon in the Su1 cDNA). The presence of high-titer antibodies in the crude antiserum that specifically recognized SU1 was demonstrated by immunoblot analysis of soluble extracts from *E. coli* cells expressing the GST-SU1 fusion protein (Fig. 2A). Antibodies specific for SU1 were purified from the crude serum by means of their affinity for nearly full-length recombinant SU1 immobilized on nitrocellulose filters. The resultant antibody fraction is called anti-SU1.

Anti-SU1 identified a protein of approximately 79 kD in immunoblot analysis of total soluble extracts from maize kernels collected 20 DAP (Fig. 2B). This protein was not detected in extracts of kernels homozygous for either of two independent *su1-* mutations, the reference allele *su1-Ref* (Correns, 1901) or the transposon insertion allele *su1-R4582::Mu1* (James et al., 1995). These data, together with previous characterizations of the cloned *su1* gene (James et al., 1995), indicate that the 79-kD protein is native SU1. The 79-kD size of the protein is approximately that predicted for native SU1 based on the length of the Su1 cDNA, considering the likely removal of an amyloplast-targeting peptide.

Investigation of the physical association of SU1 with starch granules revealed that it is exclusively a soluble endosperm protein. Proteins from maize endosperm cells
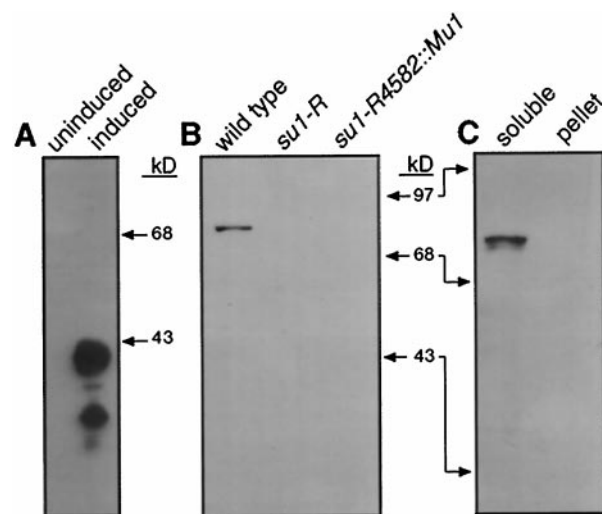

Figure 2. Immunoblot analyses with anti-SU1. A, *E. coli* extracts. Anti-SU1 detects GST-SU1 in soluble extracts of *E. coli* cells bearing pAR2 only when GST-SU1 expression is induced from the *tac* promoter. B, Total kernel extracts. Anti-SU1 identified a polypeptide of approximately 79 kD in wild-type kernels of inbred W64A collected 20 DAP. This polypeptide was not detected in the indicated homozygous *su1-* mutant kernels. C, Soluble and granule-associated proteins. Anti-SU1 detected the 79-kD polypeptide in the soluble fraction of proteins from wild-type maize kernels, but not in the fraction containing granule-associated proteins (pellet).

collected 20 DAP were separated into those bound to starch granules and those in the postgranule fraction. Immunoblot analysis indicated that SU1 is present solely in the postgranule fraction (Fig. 2C). These results suggest that SU1 acts at the surface of the granule or within the amyloplast stroma, rather than within the granule interior or tightly bound to a substrate that is integral to the granule.

The expression pattern of *Su1* during the course of endosperm development was determined by immunoblot analysis using anti-SU1 and also by RNA gel-blot analysis (Fig. 3). Anti-SU1 detected the 79-kD protein in extracts from kernels harvested 10 to 30 DAP; this protein was present in nearly constant abundance throughout this time period (Fig. 3, bottom). However, the 79-kD protein was not detected in extracts from 6-DAP kernels. These results were supported by an analysis of Su1 transcript accumulation in kernels harvested at various times after pollination. Gel blots of RNAs isolated from kernels 8 to 20 DAP showed that the Su1 transcript was present at a relatively constant level during this period (Fig. 3, top and data not shown). Thus, SU1 is present in the endosperm coincident with the initiation of starch biosynthesis, and persists at a high level throughout the time period of starch accumulation.

Expression of Recombinant SU1 and Purification to Apparent Homogeneity

A nearly full-length version of SU1, SU1r, was produced in *E. coli* for the purpose of analyzing the enzymatic properties of this protein. SU1r was produced initially as a fusion protein consisting of SU1 residues 68 through the C terminus, plus a 33-residue N-terminal sequence that included an S-tag useful for affinity purification followed by
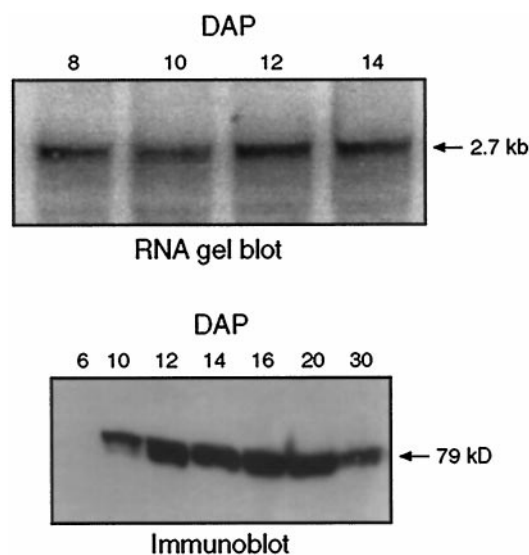
Figure 3. Temporal expression of Su1 mRNA and SU1 protein. Top, RNA gel-blot analysis using a Su1 cDNA probe. Su1 mRNA was detected in total RNA isolated from wild-type kernels of maize inbred W64A harvested 8, 10, 12, and 14 DAP. RNA loading was standardized based on ethidium-bromide staining of rRNAs. Bottom, Immunoblot analysis with anti-SU1. SU1 was detected in wild-type kernels of inbred Oh43 harvested 10 DAP and later.
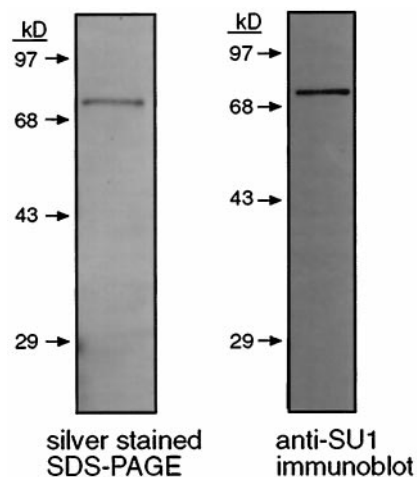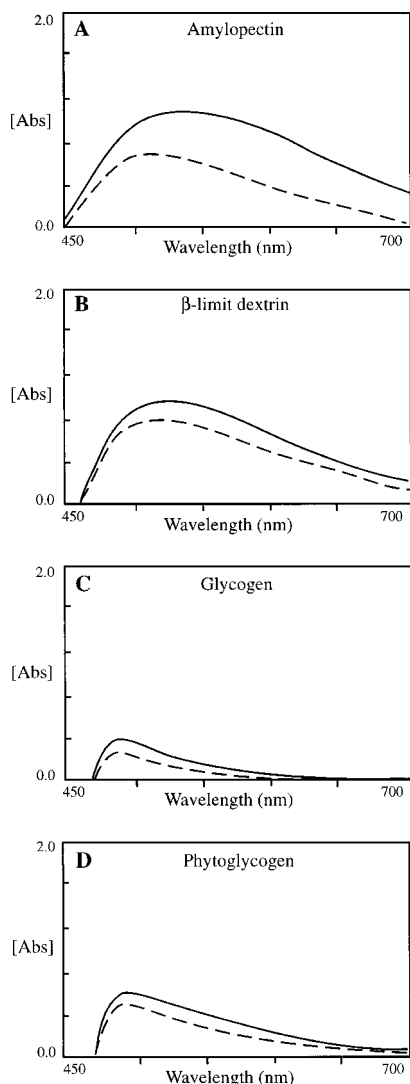

Figure 4. Purification of SU1r. A, Silver stain of an SDS-PAGE gel. The gel contains approximately 0.5 μg of affinity-purified protein collected from *E. coli* cells bearing pAR4 grown in inducing conditions, and shows a single polypeptide of approximately 75 kD. B, Immunoblot analysis with anti-SU1. The gel analyzed was a duplicate of that shown in A. The purified 75-kD protein is recognized by anti-SU1.

a thrombin proteolytic-cleavage site (Fig. 1C). The N-terminal 67 residues of SU1 missing in the fusion protein are not represented in *Ps.* isoamylase (James et al., 1995), and thus most likely are not required for enzymatic activity.

Inducible expression of a fusion protein of approximately 75 kD was detected in both the soluble and insoluble fractions of crude *E. coli* cell extracts by SDS-PAGE, and was identified specifically as SU1r by immunoblot analysis using anti-SU1 (data not shown). Equivalent protein fractions from uninduced *E. coli* cells and also from induced *E. coli* cells harboring the empty plasmid pET-29(b)+ served as negative controls. SU1r was purified from the total soluble extracts by means of the affinity of the fused S-tag region for S-protein derived from RNase A (Novagen), and was released from the affinity matrix by cleavage with thrombin. Analysis of the purified material by SDS-PAGE revealed a single polypeptide of approximately 75 kD on a silver-stained gel, and immunoblot analysis with anti-SU1 also identified a 75-kD protein (Fig. 4). The expressed protein is approximately the size predicted by the Su1 cDNA sequence, and at this sensitive level of detection appears to be free of contaminating proteins.

SU1r Cleaves α-(1→6) Branch Linkages

SU1r was found to possess DBE activity. Purified SU1r cleaves, presumably by hydrolysis, the α-(1→6) branch linkages of amylopectin, the β-limit dextrin of amylopectin, oyster glycogen, and maize phytoglycogen, as measured by an increase in the maximal absorbance (blue value) of the glucan-iodine complex after treatment with the enzyme (Fig. 5). In the case of amylopectin, a shift also occurred in

isoamylase, presumably because of the overall shorter and more uniform chain lengths in the glycogen molecule (Yokobayashi et al., 1970).

Substrates of SU1r were also identified by a quantitative colorimetric assay that measures increases in the reducing value of the reaction products relative to the substrate (Fig. 6). The formation of new reducing ends was assessed hourly during the course of a 6-h incubation period of various substrates with SU1r. Again, SU1r was shown to have significant activity toward amylopectin, with the number of reducing ends formed increasing linearly for the entire incubation period (Fig. 6). Hydrolysis of β-limit dextrin proceeded in a similar manner but at a slower rate under these reaction conditions (Fig. 6; Table I).

Phytoglycogen and glycogen are also substrates of SU1r, although the rate of formation of new reducing ends leveled off after 5 and 3 h, respectively (Fig. 6). Significantly, there were no new reducing ends formed during the incubation of pullulan with SU1r, indicating that SU1r is unable to hydrolyze α-(1→6) glycoside bonds in this substrate. These results were supported by TLC analysis of the products of the pullulan/SU1r reaction, which indicated that no maltotriose was released (data not shown). The specific activities of SU1r toward each of the substrates tested under these reaction conditions is provided in Table I, as is a comparison of the activities relative to that for amylopectin. The finding that SU1r has DBE activity toward each of the substrates tested except pullulan classifies it as an isoamylase rather than a pullulanase type of DBE. The *Su1* product, therefore, is referred to as SU1 isoamylase.

SU1r activity toward branched cyclodextrins containing either glucosyl or maltosyl side chains was also tested.

Figure 5. Absorption spectra of glucan-iodine complexes. The indicated substrates were incubated with SU1r, and aliquots of the reaction mixtures were combined with iodine/potassium iodide stain. Spectra were recorded before the addition of SU1r (dashed lines) and after 1 h of incubation (solid lines).
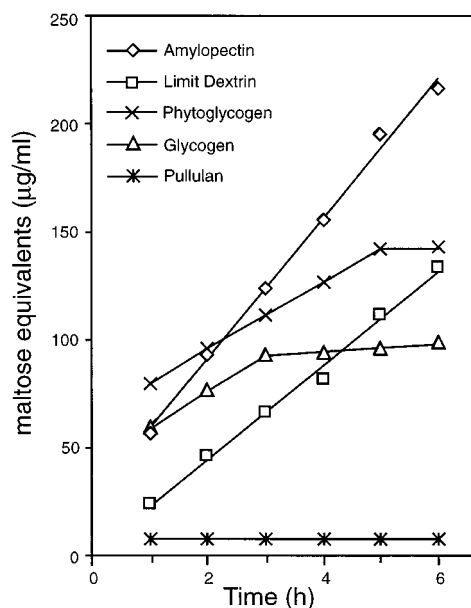
the λ_{max}. An increased blue value reflects greater linearity of the polysaccharide, and a shift in the λ_{max} normally indicates that the reaction product consists of longer, unbranched chains (Banks et al., 1971). Incubation of amylopectin with SU1r for 1 h resulted in an increase in the blue value of 0.5 absorbance unit at 550 nm, as well as a shift in the λ_{max} from 520 to 550 nm (Fig. 5). Thus, SU1r was shown to hydrolyze branch linkages within amylopectin, producing linear molecules with longer effective chain lengths, which complexed more readily with iodine. Smaller increases in blue value resulted from a similar incubation of SU1r with the β-limit dextrin of amylopectin (0.15 unit at 520 nm) and both oyster glycogen and maize phytoglycogen (0.15 unit at 470 nm), although in each instance there was little or no shift in λ_{max} (Fig. 5). A constant λ_{max} also was observed after debranching of glycogen with *Ps.*

Figure 6. Hydrolytic activity of SU1r. The indicated polysaccharides were incubated with SU1r, and the concentration of reducing ends present in the reaction mixtures was determined in terms of micrograms of maltose equivalents per milliliter. The amount of SU1r present in the reactions using glycogen and phytoglycogen was twice that present in the reactions using amylopectin and β-limit dextrin.

Table I. *Substrate specificity of SU1r*

Substrate	Specific Activity	Relative Activity
	$\mu mol\ ME^a$ $min^{-1}mg^{-1}protein$	%
Amylopectin	3.34	100[b]
β-Limit dextrin	2.04	61
Phytoglycogen	0.74	22
Oyster glycogen	0.74	22
Pullulan	0	0

[a] ME, Maltose equivalents. [b] Activity toward amylopectin is set at 100%.

Cycloheptaose (also called β-Schardinger dextrin) to which a glucosyl or a maltosyl branch was attached by an α-(1→6) linkage (called Glc-cGlc7 and Glc2-cGlc7, respectively), was incubated with SU1r, and the reducing value of the product was measured over time. No increase was seen in the number of reducing ends after the incubation of either Glc-cGlc7 or Glc2-cGlc7 with SU1r, indicating that SU1r is unable to hydrolyze the α-(1→6) linkage between the glucosyl or maltosyl side chain and the cyclodextrin (data not shown). These results suggest that SU1 requires a chain length of greater than two Glc units as a minimal substrate for its presumed hydrolytic activity. This interpretation is supported by TLC analysis of the products of the β-limit dextrin/SU1r reaction, in which no maltose was released (data not shown). Maltotriose was observed in this TLC analysis, however, indicating that a minimum chain length of three Glc residues is needed for debranching by SU1r.

Properties of SU1r Isoamylase

The debranching activity of SU1r toward amylopectin, as measured by the changes in reducing value, occurred only within the narrow range of relatively neutral pH, from 5.5 to 8.0. Maximal activity occurred at pH 6.0 (Fig. 7A). At this optimal pH value, SU1r hydrolyzed branch linkages in amylopectin at temperatures between 15 and 40°C, with maximal activity occurring at approximately 30°C (Fig. 7B). SU1r was completely inactive at 50°C and above. The thermal stability of SU1r was tested by a 10-min preincubation of the enzyme at temperatures of 30°C and higher, followed by assessment of its activity toward amylopectin at 30°C. The results mirrored those of the thermal-activity tests, indicating that enzyme stability declined until it was lost at 50°C (Fig. 7C).

Neither divalent cations nor sulfhydryl agents were required for the activity of SU1r. Although SU1r enzymatic reactions were conducted routinely in buffers devoid of divalent cations, the addition of 10 mM calcium or magnesium ions to the buffer did not alter SU1r activity (data not shown). SU1r activity was not dependent on residual calcium ions, because the addition of 10 mM EDTA to the reaction buffer did not alter the reaction rate. Maintenance of enzyme stability did require the addition of 5 mM DTT to the storage buffer.

Products of SU1r Hydrolysis

The debranching activity of SU1r was characterized further by analysis of the linear chains released from amylopectin after digestion with the recombinant enzyme. The chain-length profile obtained after treatment of amylopectin from *waxy-* mutant maize with SU1r for 1 h, as determined by HPAEC-ENZ-PAD, is shown in Figure 8. This pattern is very similar to that obtained after an analogous treatment of the same substrate with *Ps.* isoamylase (Fig. 8) and to that reported by others for the complete debranching of amylopectin by *Ps.* isoamylase (Wong and Jane, 1995, 1997). These results lend further support to the identification of SU1 as an isoamylase.
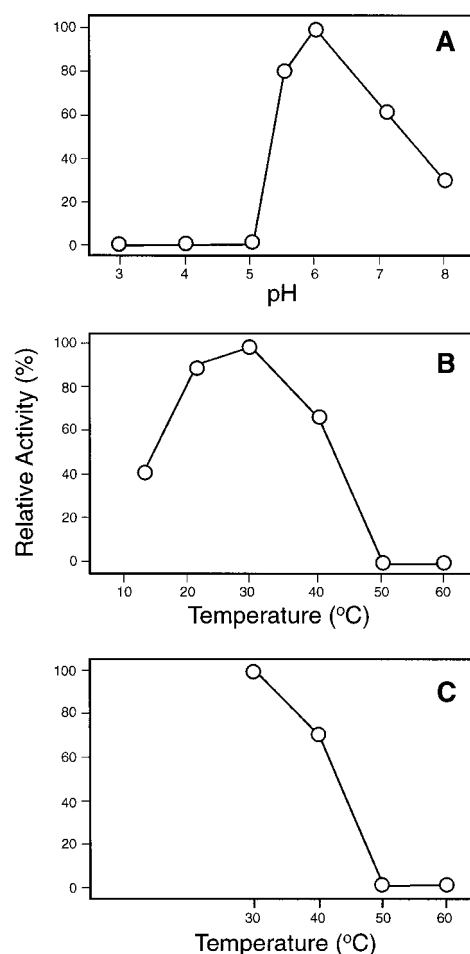
Figure 7. pH and temperature optima for SU1r activity and stability. A, Activity of SU1r relative to pH. SU1r was incubated with amylopectin in buffers adjusted to pH values from 3.0 to 10.0, and activity was determined at 30°C as illustrated in Figure 6. B, Activity of SU1r as a function of temperature. SU1r was incubated with amylopectin at pH 6.0 at temperatures ranging from 15 to 60°C. C, Thermal stability of SU1r. SU1r was preincubated for 10 min at temperatures ranging from 30 to 60°C before its incubation with amylopectin at 30°C and pH 6.0.
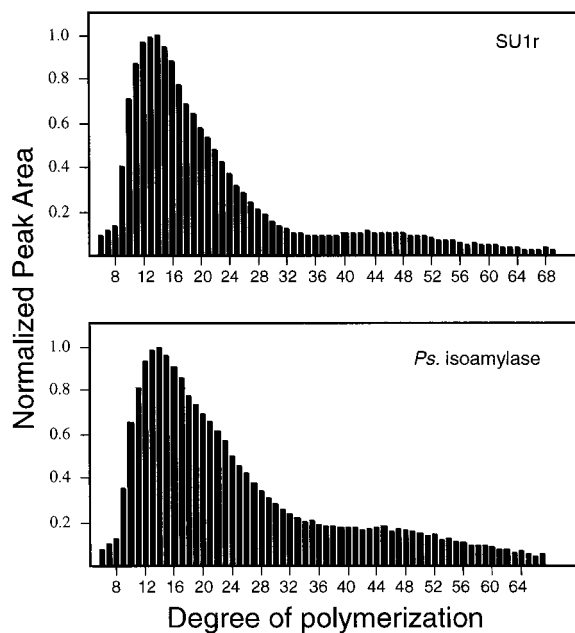
Figure 8. Chain-length distribution produced by SU1r and *Ps.* isoamylase. Amylopectin from *waxy* maize was debranched by SU1r or *Ps.* isoamylase for 1 or 24 h, respectively. Chain-length distribution was determined quantitatively by HPAEC-ENZ-PAD and normalized to the most abundant glucan chain.

Potential Regulation of a Second Maize DBE by *Su1*

The enzymatic properties of SU1r are distinct from those of the DBE known to be deficient in *su1-* mutant endosperms. Specifically, SU1r fails to hydrolyze pullulan, whereas the DBE activity previously found to be lacking in su1- mutants was defined using pullulan as the substrate (Pan and Nelson, 1984). To investigate this apparent discrepancy, endosperm extracts were examined for the presence of a pullulanase with expression dependent on SU1 function. An antiserum raised against purified R enzyme from rice endosperm (Nakamura et al., 1996a) was used to detect antigenically related proteins in extracts from wild-type maize endosperm harvested 20 DAP. This antiserum identified four polypeptides in immunoblot analysis, one of which was approximately 100 kD in size, and therefore coincided with the 105-kD molecular mass known for the native R enzyme in rice (Toguri, 1991; Nakamura et al., 1996a) (Fig. 9). The abundance of this 100-kD protein was greatly reduced in extracts from homozygous *su1-Ref* kernels (Fig. 9). These data suggest that a pullulanase structurally related to the rice R enzyme exists in maize, and that accumulation of this protein may be affected by *su1-* mutations. Immunoblot analysis using the rice R-enzyme antiserum was performed on starch-granule-bound proteins and postgranule fractions from maize endosperm. The results showed that, like SU1 isoamylase, the putative maize pullulanase is a soluble endosperm protein (data not shown).

Further support for the conclusion that a rice R-enzyme homolog is present in maize, provided by characterization of a nearly full-length cDNA sequence isolated from an endosperm library, will be described in detail in a paper in preparation (M. Beatty, A. Rahman, A.M. Myers, and M.G. James, unpublished data). This cDNA codes for a protein of approximately 100 kD that has high identity to the rice R enzyme (Nakamura et al., 1996a). Genetic mapping by restriction fragment-length polymorphism linkage analysis showed that the gene that codes for this pullulanase homolog is located on chromosome 2 (data not shown). This map location is distinct from that of *su1*, which is located on chromosome 4S (Neuffer et al., 1997).

DISCUSSION

This report demonstrates that the product of the maize gene *su1* is a polypeptide with an apparent molecular mass of 79 kD that is located exclusively in the soluble fraction of endosperm cells and is present during the time of starch biosynthesis. Furthermore, SU1 was specifically identified as an α-$(1\rightarrow6)$ glucan hydrolase of the isoamylase class. Together with previous characterizations of the cloned *su1* gene (James et al., 1995), the fact that the polypeptide identified by anti-SU1 antibodies is missing in various *su1-* mutant endosperms is confirmation that the cloned cDNA was correctly identified as a copy of the Su1 transcript. Thus, the phenotypic effects of *su1-* mutations must be explained as a result of the primary defect in the DBE characterized here as SU1 isoamylase.
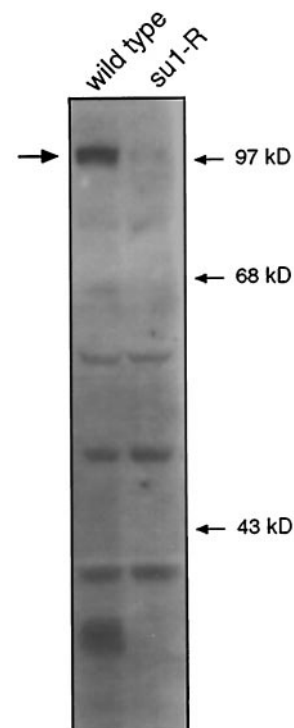
Figure 9. Immunoblot analysis with rice R-enzyme antibody. Total protein extracts from kernels in the Oh43 inbred background were analyzed. The polypeptide of approximately 100 kD present in wild-type kernels was deficient in the homozygous *su1-Ref* mutant kernels.

An increase in the absorbance of a glucan-iodine complex after incubation with an enzyme is the standard means of demonstrating debranching activity; this measurement, the blue value, is known to increase with the linearity of the polysaccharide (Banks et al., 1971). In addition, a shift in the λ_{max} is known to reflect an increase in the number of linear chains. Increases in both the blue value and the λ_{max} were observed after incubation of amylopectin with SU1r, defining SU1 as a DBE. This conclusion was confirmed by analysis of the glucan chains produced after a relatively brief digestion of amylopectin with SU1r. The resultant chain-length distribution indicated complete debranching of amylopectin, and closely matched the distribution obtained by digestion with *Ps.* isoamylase.

Quantitation of hydrolysis rates revealed that the specific activity of SU1r toward amylopectin was significantly greater under these reaction conditions than that toward the β-limit dextrin of amylopectin, phytoglycogen, or oyster glycogen. For β-limit dextrin this effect may be attributable to the short lengths of the exterior branch stubs that remain in the dextrin after treatment of amylopectin with β-amylase. Maltosyl stubs were shown directly to be poor substrates by the lack of activity of SU1r toward Glc2-cGlc7. This is also true for *Ps.* isoamylase, which cleaves maltosyl branches at a lower rate than maltotriosyl branches, and in general is more active toward longer glucan chains (Kainuma et al., 1978). Reduced activity of SU1r toward phytoglycogen and glycogen compared with amylopectin is also likely to result from the substrate structure, given that these molecules have shorter average chain lengths than amylopectin and relatively uniform distributions of branch-linkage positions. Overall, these results suggest that SU1r does not require the ordered branching structure of amylopectin to recognize a substrate, and that chains with as few as three Glc residues can be released by the enzyme. There was no observable activity of SU1r toward pullulan. Taken together, the data clearly indicate that SU1r is a DBE of the isoamylase class.

The recombinant form of SU1 analyzed in vitro exhibits a slightly smaller apparent molecular mass than the native protein (75 as opposed to 79 kD). The amino acid sequence of the mature N terminus of native SU1 has not yet been determined, and it is likely that the native protein and SU1r have distinct N termini. The possibility exists, therefore, that the enzymatic activity of SU1 in cells could differ from the properties determined in vitro. That SU1 functions as an isoamylase in vivo is most likely, however, considering the in vitro characterization and the fact that SU1 is more closely related to isoamylases than to pullulanases (James et al., 1995).

SU1 is distinct from bacterial isoamylases with respect to substrate preference and optimal pH and temperature conditions. *Ps.* isoamylase, a monomeric enzyme of 81 kD, is optimally active in the acidic pH range of 3.0 to 4.0 and at a temperature of 52°C (Yokobayashi et al., 1970). This isoamylase completely hydrolyzes the branch linkages of amylopectin and glycogen, releasing maltotriose and larger maltosaccharides, but its activity is relatively equal toward both substrates (Yokobayashi et al., 1973; Kainuma et al., 1978). In contrast, SU1r debranches amylopectin more

readily than glycogen, and is optimally active within the neutral range of pH 6.0 to 7.0 and at temperatures between 25 and 37°C. A plausible explanation for the different conditional and substrate requirements for such similar-acting enzymes is that each has developed a functional adaptive response to its own unique environmental conditions while still using the same structural motifs for substrate binding and catalysis.

Based on its enzymatic characteristics, SU1 may be the same enzyme that was defined biochemically as isoamylase II (Doehlert and Knutson, 1991). Like SU1r, isoamylase II is present in extracts of developing maize kernels, is active at a neutral pH, hydrolyzes amylopectin more rapidly than phytoglycogen, and requires reducing agents in the buffer for maintenance of activity. Isoamylase II has an estimated molecular mass of 141 kD as determined by gel filtration, which is significantly larger than SU1. However, this observation by itself does not exclude the possibility that SU1 and isoamylase II are the same, because the size determination was made on relatively crude fractions and the possibility exists that SU1 is oligomeric in vivo. Another maize isoamylase was identified in extracts from mature *su1-* mutant sweet corn kernels (Manners and Rowe, 1969). The fact that SU1 is not present in *su1-* mutant kernels rules out identity with this sweet-corn isoamylase, which most likely participates in endosperm-starch degradation after seed germination.

The finding that *su1-* mutants are deficient in a DBE was first demonstrated by Pan and Nelson (1984). In that study, the assay used to detect hydrolysis of α-(1→6) linkages employed pullulan as a substrate, so the missing enzyme must be classified as a pullulanase. These results do not agree with the characterization of SU1 as an isoamylase that has no activity toward pullulan. Further analysis of *su1-* mutant endosperms in the present study, however, revealed deficiencies of two proteins, SU1 isoamylase and a protein immunologically related to a known R enzyme. These data suggest that the *su1* locus controls the accumulation of two different DBEs.

An alternative possibility, that a deficiency of the pullulanase occurs as the result of a second mutation present in the *su1-* stock, can be ruled out by a previous characterization of multiple, independent *su1-* mutations (Pan and Nelson, 1984). In that study, pullulanase activity was assayed in six distinct *su1-* mutant lines and found to be deficient in each. If a mutation other than *su1-* were responsible for the pullulanase deficiency, it would have had to occur simultaneously with the *su1-* mutation in each instance. The likelihood of this happening is remote. We conclude, therefore, that *su1* function is necessary for accumulation of a pullulanase type of DBE in addition to the isoamylase for which it codes. Such a pleiotropic effect would reconcile the results of the initial study of Pan and Nelson (1984) with the current characterization of SU1. A similar situation exists in rice, in which the R enzyme found to be deficient in *sugary-* mutants is not coded for by the *sugary* gene (Nakamura et al., 1996b).

Two hypotheses are offered that might explain why the primary deficiency in SU1 isoamylase secondarily results in a decrease of the putative pullulanase. First, the two

DBEs may associate in vivo in a multisubunit complex. Deficiency of SU1 could disrupt the complex, which could result in decreased accumulation of the pullulanase. The second suggestion is that *su1-* mutations indirectly result in an altered expression of the gene coding for the pullulanase, possibly as a result of the increased mono- and disaccharide concentrations in the mutant endosperm.

Mutations of *su1* have been studied for nearly a century because of their unique effects on kernel starch. Here we show that these mutations cause a primary deficiency in an isoamylase normally present in the endosperm at the time that starch is produced. These observations suggest that SU1 isoamylase participates in starch biosynthesis. Furthermore, the fact that the combined deficiencies of SU1 isoamylase and a pullulanase result in phytoglycogen production at the expense of amylopectin is most simply explained by direct participation of DBEs in amylopectin synthesis. A mechanism by which this might occur was recently proposed in a model for the synthesis of storage starch, which suggests that DBEs act to selectively trim newly introduced branches to restore spatial order to the growing amylopectin molecule (Ball et al., 1996). Further analysis of the nature of the conditions and substrates required by both SU1 isoamylase and pullulanase, as well as characterization of the regulatory mechanisms that govern these enzymes, is expected to elucidate the specific roles played by DBEs in starch biosynthesis.

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