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Purification and Molecular Genetic Characterization of ZPU1, a Pullulanase-Type Starch-Debranching Enzyme from Maize[1]

Mary K. Beatty[2,3], Afroza Rahman[2], Heping Cao, Wendy Woodman, Michael Lee, Alan M. Myers, and Martha G. James*

Department of Biochemistry, Biophysics, and Molecular Biology (M.K.B., A.R., H.C., A.M.M., M.G.J.), and Department of Agronomy (W.W., M.L.), Iowa State University, Ames, Iowa 50011

This study identified and purified specific isoamylase- and pullulanase-type starch-debranching enzymes (DBEs) present in developing maize (*Zea mays* L.) endosperm. The cDNA clone Zpu1 was isolated based on its homology with a rice (*Oryza sativa* L.) cDNA coding for a pullulanase-type DBE. Comparison of the protein product, ZPU1, with 18 other DBEs identified motifs common to both isoamylase- and pullulanase-type enzymes, as well as class-specific sequence blocks. Hybridization of Zpu1 to genomic DNA defined a single-copy gene, *zpu1*, located on chromosome 2. Zpu1 mRNA was abundant in endosperm throughout starch biosynthesis, but was not detected in the leaf or the root. Anti-ZPU1 antiserum specifically recognized the approximately 100-kD ZPU1 protein in developing endosperm, but not in leaves. Pullulanase- and isoamylase-type DBEs were purified from extracts of developing maize kernels. The pullulanase-type activity was identified as ZPU1 and the isoamylase-type activity as SU1. Mutations of the *sugary1* (*su1*) gene are known to cause deficiencies of SU1 isoamylase and a pullulanase-type DBE. ZPU1 activity, protein level, and electrophoretic mobility were altered in *su1*-mutant kernels, indicating that it is the affected pullulanase-type DBE. The Zpu1 transcript levels were equivalent in nonmutant and *su1*-mutant kernels, suggesting that coordinated regulation of ZPU1 and SU1 occurs posttranscriptionally.

Amylopectin is a branched Glc polymer that is a major constituent of plant starch granules and is the primary determinant of their structural and physical properties. The spatial positioning of $\alpha(1\rightarrow6)$ glycosidic bonds, i.e. branch linkages, is a critical aspect of the three-dimensional structure of amylopectin (Gallant et al., 1997). Branch linkages are introduced by the actions of starch branching enzymes and are hydrolyzed by the actions of DBEs (for recent reviews, see Preiss and Sivak, 1996; Smith et al., 1997). Mutations that result in DBE deficiencies, such as the *sugary1* (*su1*) mutations of maize (*Zea mays* L.) and rice (Pan and Nelson, 1984; James et al., 1995; Nakamura et al., 1996b; Rahman et al., 1998), alter the number and spatial distribution of branches in amylopectin. Therefore, DBEs are believed to be involved in branch-pattern determination, possibly providing an editing function (Ball et al., 1996).

Two classes of DBEs have been identified in plants that are distinguishable by their substrate specificities (Lee and Whelan, 1971; Doehlert and Knutson, 1991). "Isoamylase-type" DBEs cleave $\alpha(1\rightarrow6)$ branch linkages in amylopectin and glycogen, but do not hydrolyze the chemically identical bonds in pullulan, an $\alpha(1\rightarrow6)$-linked maltotriose polymer. In contrast, "pullulanase-type" DBEs, also referred to as R-enzymes or limit-dextrinases (Manners, 1997), readily hydrolyze $\alpha(1\rightarrow6)$ linkages of pullulan or amylopectin, but have little activity toward glycogen. Biochemical fractionation experiments identified both isoamylase- and pullulanase-type DBE activities in developing maize kernels during the starch biosynthetic period (Pan and Nelson, 1984; Doehlert and Knutson, 1991), but the genetic identities and specific functions of these two enzymes have not yet been established.

The primary sequences of a pullulanase-type DBE from rice endosperm and an isoamylase-type enzyme from maize endosperm were discovered from cloned cDNAs. Rice RE was purified and characterized as a pullulanase-type DBE, and the cDNA coding for RE was cloned (Toguri, 1991; Nakamura et al., 1996a). A maize cDNA identified from a cloned fragment of the *su1* gene codes for a protein similar to bacterial isoamylases (James et al., 1995). The *su1* gene product, SU1, functions as an isoamylase-type DBE and is present in amyloplasts of developing maize endosperm during the time that starch is synthesized (Rahman et al., 1998; Yu et al., 1998).

Expression of the isoamylase- and pullulanase-type DBEs of maize seemingly is coordinately controlled. Although the *su1* locus codes for an isoamylase-type enzyme (Rahman et al., 1998), previous studies have demonstrated

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[2] These authors contributed equally to this work.

[3] Present address: Pioneer HiBred International, Inc., Johnston, IA 50131.

* Corresponding author; e-mail mgjames@iastate.edu; fax 1–515–294–0453.

Abbreviations: DAP, days after pollination; DBE, starch-debranching enzyme; LOD, log of the odds; RE, R-enzyme; RI, recombinant inbred.

a reduction in the activity of a pullulanase-type DBE in *su1*-mutant endosperms (Pan and Nelson, 1984). Consistent with these data, a protein related immunologically to rice RE is present in nonmutant maize kernels at 20 DAP, but is deficient in *su1*-mutant kernels of the same age (Rahman et al., 1998). Thus, *su1* mutations apparently result in the deficiency of two distinct DBEs. A similar situation is likely to occur in rice, in which the *su1* mutation controlling RE expression maps to a chromosomal location that is distinct from the gene that codes for RE (Nakamura et al., 1996a).

To determine how DBEs affect starch structure, we are seeking to identify and characterize completely these enzymes in maize endosperm. Here we describe a full-length cDNA, designated Zpu1 (for *Zea mays* pullulanase-type DBE), which codes for a protein similar in sequence to known pullulanase-type DBEs. Zpu1 transcript accumulation was characterized, and the corresponding gene, *zpu1*, was mapped. The gene product ZPU1 was purified from developing endosperm and shown to be a pullulanase-type enzyme. Analyses of ZPU1 in a *su1-Ref* mutant indicated that its expression, electrophoretic mobility, and enzymatic activity are dependent on the presence of a functional *Su1* gene.

MATERIALS AND METHODS

Plant Materials and Nomenclature

Nonmutant plants in the maize (*Zea mays* L.) inbred lines W64A and Oh43 and plants homozygous for the reference mutation *su1-Ref* (Correns, 1901), introgressed in these same genetic backgrounds, were used for gel-blot and protein analyses. Standard genetic nomenclature for maize is used as described by Beavis et al. (1995). In addition, nonitalicized gene symbols are used to designate cDNAs and transcripts.

Characterization of Zpu1 cDNA

A random-primed maize endosperm cDNA library in λgt11 (K. Cone, University of Missouri, Columbia) was screened using a 1.2-kb *Hin*dIII fragment from the rice RE cDNA as a hybridization probe (Nakamura et al., 1996a). Standard procedures were followed for preparation of phage lifts, phage amplification, and single-plaque purification, plasmid construction, and growth of *Escherichia coli* cells (Ausubel et al., 1989; Sambrook et al., 1989). DNA was isolated from purified phage by the Wizard DNA Purification Kit (Promega). cDNA inserts were characterized with regard to their length by gel electrophoresis after digestion with *Eco*RI. The longest, in-clone λ14-1 was 2.3 kb in length and was subcloned into pBluescript KS+ to create plasmid pMB12. Subsequent screens of the endosperm cDNA library with the entire 2.3-kb insert from pMB12 (probe EE2.3) identified clones overlapping the 3′ end (λ6A, λ10A, λ16A, and λ17C), and screens using the 680-bp *Eco*RI/*Hin*dIII fragment as a probe (EH.68) identified clones overlapping the 5′ end (λ2A, λ3A, λ3C, and λ3E). Nucleotide sequences were determined by standard procedures (Ausubel et al., 1989) for both strands of the cDNA inserts in pMB12 and phage clones λ17C, λ3C, and λ3E. Universal and synthetic primers specific to portions of the cDNA inserts or to λ DNA were used for sequence analysis.

Production of Anti-ZPU1 Polyclonal Antisera

To express part of ZPU1 as a fusion protein, the 2.3-kb *Eco*RI insert from pMB12 (see Fig. 1) was subcloned into plasmid pGEX-4T-2 (Pharmacia), creating pML1. *E. coli* DH5α cells containing pML1 were grown in 50 mL of LBA medium (Luria broth supplemented with 40 μg/mL ampicillin) at 37°C for 7 h, and the entire culture was then transferred to 1 L of LBA medium supplemented with 2.5 mM betaine and 1 M sorbitol and grown at 30°C for 24 h. Fusion protein expression was induced by the addition of isopropyl-β-D-thiogalactopyranoside to 0.1 mM, and incubation was continued at 37°C for 3 h. Cell lysis and affinity purification of glutathione *S*-transferase-ZPU1 using glutathione-agarose beads was performed as described previously (Rahman et al., 1998). The fusion protein was eluted in 100 mM Tris-HCl, pH 8.0, 120 mM NaCl, and 20 mM glutathione.

To produce polyclonal anti-ZPU1 serum, 0.5 mL (approximately 300 μg) of purified glutathione *S*-transferase-ZPU1 in 1× PBS was mixed with 0.5 mL of Freund's complete adjuvant (Sigma) and injected into each of two New Zealand White rabbits, according to standard procedures (Harlow and Lane, 1988). Inoculations were repeated four times at 3-week intervals using approximately 200 μg of fusion protein emulsified in Freund's incomplete adjuvant (Sigma). Immune serum was collected 6 weeks after the final inoculation, assayed for antibody titer, and stored at −80°C in the presence of 0.02% sodium azide.

DNA and RNA Gel-Blot Analyses

DNA Gel Blots

Genomic DNAs were isolated from line W64A seedling leaves according to the procedure of Dellaporta et al. (1983), digested with restriction enzymes, and electrophoresed and transferred to nylon membrane, as described previously (James et al., 1995). DNA gel blots were hybridized with probe EE2.3 or EH.68 labeled with ^{32}P by the random-primer method (Ausubel et al., 1989).

RNA Gel Blots

Total RNAs were isolated from maize tissues and subjected to gel-blot analysis, as described previously (Gao et al., 1998). RNA gel blots were hybridized with probe EE2.3.

Mapping of zpu1

The *zpu1* gene locus was mapped to a specific maize chromosome by analysis of restriction fragment-length polymorphisms in the T232×CM37 and CO159×Tx303 RI populations, consisting of 48 and 41 individuals, respectively (Burr et al., 1988). Segregation data produced from both populations were used (Burr et al., 1993; Matz et al.,

1994). Genomic DNAs were isolated from immature leaves of parental inbreds and RI plants according to the method of Saghai-Maroof et al. (1984), digested with *Eco*RI, and subjected to gel electrophoresis. Gel blots were hybridized with Zpu1 probe EE2.3. Maximum-likelihood estimates of linkage and map distances were determined using the Mapmaker program (Lander et al., 1987). Genetic linkage was determined with a recombination value of 50 and a LOD threshold of 4.0.

Fractionation, Enzymatic Assay, and Immunoblot Analysis of DBEs

Cell Extract Preparation and Ammonium Sulfate Precipitation

Kernels were harvested 20 DAP, quickly frozen in liquid nitrogen, and stored at −80°C. Approximately 15 g of frozen kernels or endosperm tissue was pulverized in liquid nitrogen, then stirred overnight at 4°C in 40 mL of extraction buffer (50 mM Hepes-NaOH, pH 7.5, 10 mM EDTA, 5 mM DTT, 1 mM PMSF, and 0.5 mL of protease inhibitor cocktail per gram of tissue [no. P2714, Sigma]). The suspension was centrifuged at 39,000*g* for 20 min. The supernatant was filtered through four layers of Miracloth (Calbiochem) and centrifuged again under the same conditions. The supernatant was then passed through a 0.45-μm syringe filter to yield the crude kernel extract. This solution was made up to 40% ammonium sulfate and stirred for 1 h at 4°C. Precipitated proteins were collected by centrifugation at 16,000*g* for 20 min, suspended in 20 mL of buffer A (50 mM Hepes-NaOH, pH 7.5, 10 mM EDTA, 5 mM DTT, and 5% glycerol), and dialyzed overnight at 4°C in 1 L of the same buffer.

Anion-Exchange Chromatography

The dialyzed protein solution was centrifuged at 10,000*g* for 15 min, and the supernatant was passed through a 0.45-μm syringe filter. The solution was then applied to a preequilibrated Q-Sepharose Fast-Flow column (1.5 cm × 46 cm column, 80 mL bed volume; approximately 1.3 mg of protein loaded per milliliter of bed volume; Pharmacia). After washing the column with 850 mL of buffer A, bound proteins were eluted with a linear, 600-mL gradient of 0 to 1 M NaCl in buffer A. Fractions (8 mL) assayed for DBE activity were concentrated approximately 80-fold using an Ultrafree-4 centrifugal filter-unit concentrator (model NMWL-10K, Millipore), pooled, made up to 50% in glycerol, quickly frozen in liquid nitrogen, and stored at −80°C.

Gel-Permeation Chromatography

Proteins from the 40% ammonium sulfate precipitate or, for purification A (see "Results"), pooled fractions from the Q-Sepharose column that exhibited pullulanase-type DBE activity, were applied to a Sephacryl S-200 superfine gel-permeation column (2.5 cm × 90 cm column, 440 mL bed volume; Pharmacia) and eluted with the equilibration buffer (10 mM Hepes-NaOH, pH 7.5, 5 mM DTT, and 5 mM $MgCl_2$) at a flow rate of 0.5 mL min^{-1}. Aliquots from 7.5-mL fractions were checked for pullulanase-type DBE activity. Fractions containing the enzyme were pooled and concentrated as described.

Fast-Protein Liquid Chromatography

Pullulanase-type DBE fractions from the Sephacryl S-200 column containing 3 mg of protein were diluted to 10 mL in buffer A, then loaded onto a Mono-Q column (1 mL bed volume; Pharmacia) equilibrated with buffer A. The column was washed with 10 mL of buffer A, then eluted with a linear, 50-mL gradient of 0 to 0.5 M NaCl in the buffer. Fractions were assayed again for pullulanase-type DBE activity, concentrated, and stored as described above.

Affinity Chromatography

Pullulanase-type DBE fractions pooled after anion-exchange chromatography with Q-Sepharose were dialyzed in citrate buffer (50 mM sodium citrate, pH 5.5, and 5 mM DTT) and concentrated as described. Approximately 0.4 mg of protein was applied to a column containing epoxy-activated Sepharose (Sigma) conjugated with cyclohexa-amylose (Sigma) (Vretblad, 1974) and equilibrated with the citrate buffer (0.7- × 8-cm column, 3 mL bed volume). Bound pullulanase-type DBE was eluted with 1 mg/mL β-cyclodextrin (cyclohepta-amylose) (Sigma) in citrate buffer. The fraction exhibiting pullulanase-type DBE activity was desalted and concentrated approximately 200-fold.

Immunoblot Analysis

Proteins in concentrated fractions were separated by SDS-PAGE in 6% gels and transferred to nitrocellulose membranes according to standard procedures (Ausubel et al., 1989; Sambrook et al., 1989). Immunodetection was modified from the ECL protocol (Amersham) as described previously (Rahman et al., 1998) using affinity-purified anti-SU1 diluted 1:200 or crude anti-ZPU1 antiserum diluted 1:25,000. Alternatively, the chromatogenic 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium reagent system (Bio-Rad) was employed using crude anti-ZPU1 diluted 1:2,000. For comparative analysis of protein fractions from nonmutant and *su1-Ref* mutant kernels, equivalent amounts of protein were analyzed identically.

DBE Assays

For isoamylase assays, 100 μL of each column fraction was incubated in a total volume of 0.2 mL containing 5 mg of amylopectin (Sigma) and 50 mM Hepes-NaOH, pH 7.0, for 2 h at 30°C. A 50-μL aliquot of each reaction was mixed with 700 μL of water and 250 μL of 0.01 M I_2/0.5 M KI solution. The change in A_{550} was measured relative to a blank amylopectin reaction lacking protein as the reference. To measure reducing equivalent formation, the reactions were inactivated by mixing a 50-μL aliquot of each reaction with 25 μL of 1 M Na_2CO_3. Reducing equivalents

were determined as described by Fox and Robyt (1991) using maltose as the standard. For pullulanase-type DBE assays, 50 μL of each column fraction was incubated in a total volume of 0.1 mL containing 5 mg of pullulan (Sigma) and 50 mM citrate, pH 5.5, for 2 h at 37°C. Reducing equivalents were determined as described above using maltotriose as the standard. Determinations of specific activities were made on pooled fractions from each purification step. Aliquots were assayed after reaction times of 30 min, 1 h, and 2 h to demonstrate a linear increase in activity.

Computational Analyses

For sequence analyses we used the Genetics Computer Group Sequence Analysis Software Package (Madison, WI), and for multiple sequence alignment we used the program PILEUP. Conserved sequence motifs were assigned based on the presence of at least one invariant residue in the 19 polypeptides analyzed, as well as conservative substitutions of several nearby residues in aligned positions. Consensus sequences were determined by votes, according to the method of Posfai et al. (1989).

RESULTS

Characterization of a Maize cDNA Coding for a Predicted Pullulanase-Type DBE

The near-full-length Zpu1 cDNA was cloned from a random-primed maize endosperm cDNA library using a fragment of the rice RE cDNA as a hybridization probe. The complete Zpu1 cDNA nucleotide sequence of 3261 bp was determined from three overlapping bacteriophage λ clones (designated λ3C, λ14-1, and λ17C) (Fig. 1A) (accession no. AF080567). Zpu1 contains an ATG-initiated, continuous open reading frame of 2886 bp that predicts a 962-amino acid polypeptide, ZPU1, of approximately 106 kD. The ZPU1 protein is highly similar in sequence to rice RE, exhibiting 78% identity among 880 aligned residues with three gaps in the alignment (Fig. 1B). The mature rice RE begins with the Ala-Val sequence located at predicted residues 75 to 76 (Nakamura et al., 1996a). This sequence is conserved in ZPU1 at predicted residues 75 to 76 as well (Fig. 1B), suggesting that the preceding 74 residues constitute a transit peptide for protein targeting. ZPU1 also shows extensive similarity to a characterized pullulanase-type DBE from spinach leaves (accession no. X83969); these two proteins are 59% identical among 882 aligned residues with no gaps (data not shown). There are genomic and cDNA sequences from barley that predict another pullulanase-type DBE (accession no. AF022725); ZPU1 is identical to this deduced amino acid sequence at 79% of 832 aligned residues, with one gap of a single amino acid.

Sequence Motifs Conserved in Pullulanase- and Isoamylase-Type Enzymes

Further sequence comparisons among plant and bacterial α-(1→6) glucan hydrolases indicate that the pullulanase- and isoamylase-type DBEs have been conserved separately in evolution. The high degree of conservation in plants among pullulanase-type DBEs also occurs

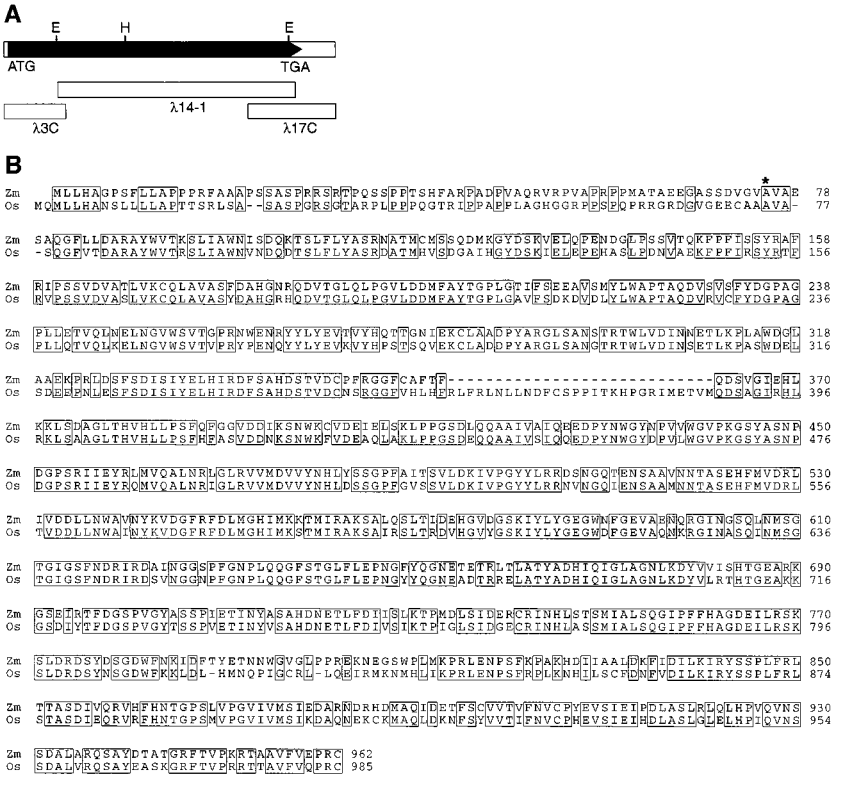

Figure 1. A, Overlapping bacteriophage λ clones provide the full-length Zpu1 cDNA. The 3261-bp nucleotide sequence of the Zpu1 cDNA was determined from overlapping sequences of three λ clones. The Zpu1 cDNA sequence is available as accession no. AF080567. B, Primary sequence alignment of ZPU1 with rice RE. Amino acid residues deduced from the Zpu1 cDNA (Zm) and rice RE cDNA (Os; accession no. D50602) are shown in alignment. Boxed residues are the same in both polypeptides. The asterisk designates the transit peptide cleavage site known in RE.

among the plant isoamylases: the maize isoamylase-type DBE SU1 is 71% identical over 690 aligned residues to an Arabidopsis protein predicted from genomic sequence data (accession no. AF002109; data not shown). However, each plant DBE is more similar to the bacterial enzymes of the same class than to the plant enzyme of the other class. For example, in the 200-residue span of ZPU1 and SU1 that is most similar, 32% of the amino acids are identical. Within the same 200 aligned residues, however, ZPU1 is 46% identical to pullulanase from *Klebsiella aerogenes* and SU1 is 47% identical to isoamylase from *Pseudomonas amyloderamosa* (data not shown). These observations suggest that isoamylase- and pullulanase-type DBEs diverged before establishment of the plant kingdom, and that the function of each type of DBE has been selected independently during the evolution of plants.

Comparison of the entire ZPU1 sequence to 18 other known or predicted isoamylase- and pullulanase-type enzymes from plants and prokaryotes supported the preceding conclusion. As noted previously, both types of DBEs contain all four regions (motifs I–IV) conserved within the α-amylase superfamily of starch hydrolytic enzymes (Jesperson et al., 1993; James et al., 1995; Nakamura et al., 1996a, 1997). Two additional conserved sequence blocks, designated motifs V and VI, are identified here that occur in all of the DBEs that we examined, whether they fall within the isoamylase- or the pullulanase-type class (Fig. 2). Among these six common motifs, 20 residues are conserved in each of the 19 DBEs analyzed. Class-specific, conserved sequence blocks were also identified (Fig. 2). Enzymes grouped in the pullulanase-type class contain five conserved regions that are not found in the isoamylase-type enzymes. Similarly, eight motifs conserved among the isoamylase-type enzymes do not occur in the pullulanase-type enzymes.

Mapping of *zpu1* within the Maize Genome

DNA gel-blot analysis of genomic DNA from maize inbred line W64A revealed that the *zpu1* locus is unique

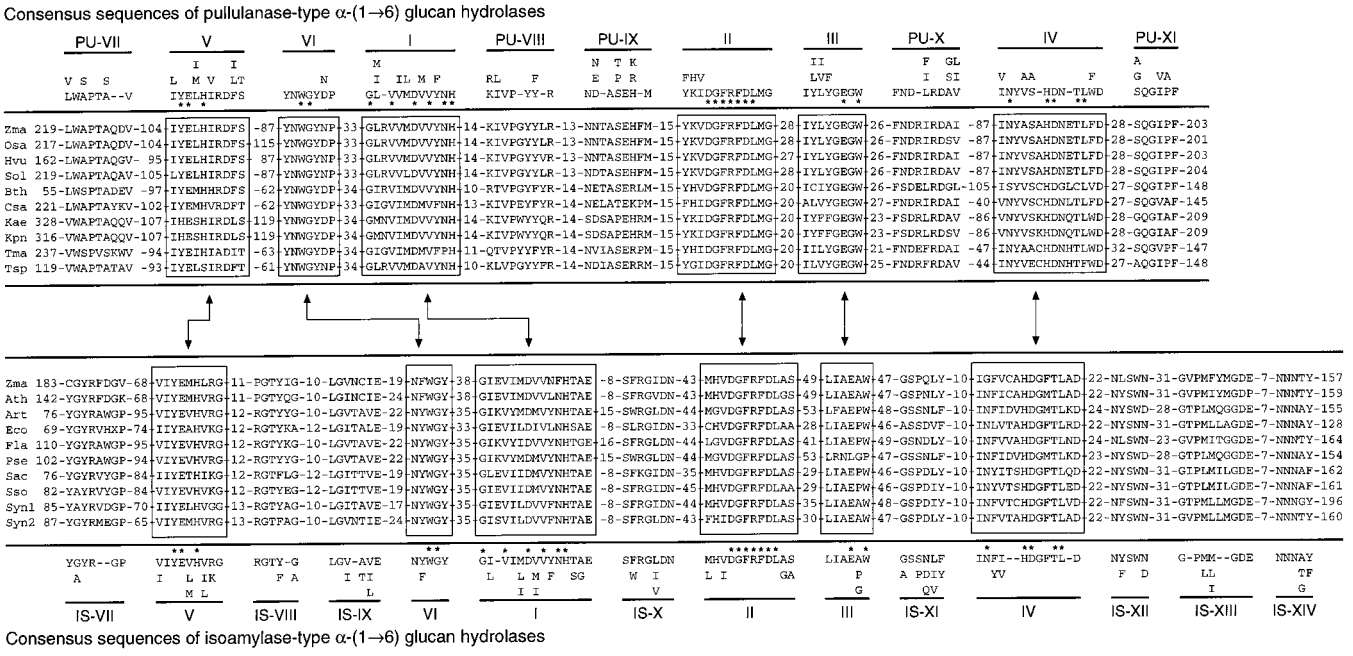

Figure 2. Multiple sequence alignment of pullulanase- and isoamylase-type DBEs from higher plants and prokaryotes. DBEs are grouped based on characterized enzymatic activity and/or sequence similarity; any polypeptide within a class is significantly more similar to others within that group than to those of the other class. Conservative substitutions in the consensus sequences are noted when they fall into the functional groups defined by Dayhoff and Orcutt (1979), which are AGPST, ILMV, HKR, DENQ, RWY, and C. Residues invariant in all 19 sequences are noted by asterisks. Rare exceptions to the consensus sequence are underlined. Numerals refer to amino acid position beginning at the first ATG codon of the open reading frame. The number of nonconserved amino acids adjacent to each conserved motif is indicated. Conserved motifs in boxes are present in both the pullulanase- and isoamylase-type classes, whereas conserved motifs without boxes are specific to one of the classes as indicated. Motifs I to IV are those defined previously that occur in all members of the α-amylase superfamily (Jesperson et al., 1993), and are numbered accordingly. Abbreviations and accession numbers are provided for the following DBES. Pullulanases and pullulanase-type DBEs: Bth (*Bacteroides thetaiotaomicron*, U67061); Csa (*Caldicellulosiruptor saccharolyticus*, L39876); Kae (*Klebsiella aerogenes*, M16187); Hvu (*Hordeum vulgare*, AF022725); Kpn (*Klebsiella pneumoniae*, X52181); Osa (*Oryza sativa*, D50602); Sol (*Spinacia oleracea*, X83969); Tma (*Thermotoga maritima*, AJ001087); Tsp (*Thermus* sp. IM6501, AF060205); Zma (*Z. mays*, AF080567, this study). Isoamylases and isoamylase-type DBEs: Art (artificial gene, A10906); Ath (Arabidopsis, AF002109); Eco (*E. coli*, U18997); Fla (*Flavobacterium* sp., U90120); Psu (*Pseudomonas* sp., A28109, A37035); Sac (*Sulfolobus acidocaldarius*, D83245); Sso (*Sulfolobus solfataricus*, Y08256); Syn1 (*Synechocystis* sp., U44761); Syn2 (*Synechocystis* sp., D90908); and Zma (*Z. mays*, U18908).

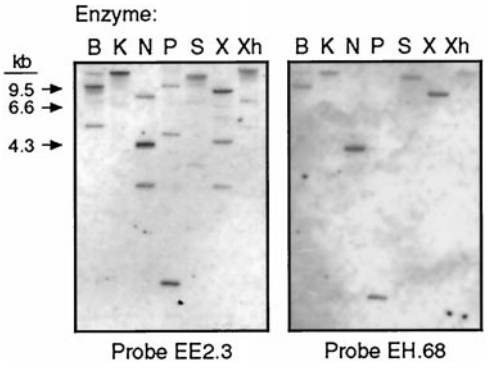

Figure 3. The copy number of the *zpu1* locus was determined by gel-blot analysis of genomic DNA. DNA from maize inbred W64A was digested with the indicated restriction enzymes (B, *Bam*HI; K, *Kpn*I; N, *Not*I; P, *Pst*I; S, *Sst*I; X, *Xba*I; and Xh, *Xho*I). The gel blot was hybridized at high stringency with probe EE2.3 (left panel), then stripped of probe and hybridized with probe EH.68 (right panel) of the Zpu1 cDNA.

within the maize genome. Restriction enzymes that do not cleave the Zpu1 cDNA were used, and the 2.3-kb *Eco*RI fragment of the cDNA containing codons 160 to 930 (Fig. 1) was used as a hybridization probe (designated probe EE2.3). The probe hybridized to a unique *Kpn*I genomic fragment (Fig. 3), indicating that *zpu1* is a single-copy gene. To support this conclusion, the blot was stripped of the probe and rehybridized with a smaller portion of the Zpu1 cDNA, the 680-bp *Eco*RI/*Hin*dIII fragment comprising codons 160 to 387 (designated probe EH.68; Fig. 1). In this analysis unique genomic fragments were identified using six different restriction enzymes (Fig. 3).

The *zpu1* locus was mapped to chromosome 2 (Burr et al., 1994). Probe EE2.3 (Fig. 1) was used to identify restriction fragment-length polymorphisms in two populations of RIs. In the two sets of parental inbreds polymorphisms were detected by digestion with *Eco*RI, which produced Zpu1-homologous fragments of 4.3 and 3.0 kb in line CM37, 5.2 kb in line T232, 5.4 and 4.3 kb in line Tx303, and 5.0 and 3.9 kb in line CO159 (data not shown). The detection of more than one band in three lines is most likely attributable to the presence of an internal *Eco*RI site in the *zpu1* locus, given that each pair of bands was inherited as a single allele. Identification of the parental allele in individual plants of the CM37×T232 and Tx303×CO159 RI populations allowed us to determine the genetic linkage to previously mapped physical markers using the program Mapmaker. These linkage data placed *zpu1* approximately 2.7 centimorgans from marker accA and 1.2 centimorgans from marker pps15 in the Tx303×CO159 RI population, with a LOD score of 9.4. Similar results were obtained with the CM37×T232 population, with *zpu1* mapping approximately 2.5 centimorgans from marker accA and 1.1 centimorgans from marker isu142, with a LOD score 11.4. Thus, *zpu1* was localized to the region of Bin 2.05 to 2.06 (Gardiner et al., 1993), although specific placement to either the short or the long arm of the chromosome could not be made.

Tissue and Developmental Expression of Zpu1 mRNA

The tissues in which Zpu1 mRNA accumulates were identified by RNA gel-blot analysis. Total RNAs isolated from maize embryos, developing endosperm, leaves, roots, and tassels were separated by gel electrophoresis and hybridized with probe EE2.3. A transcript of approximately 3.2 kb was abundant in endosperm from kernels harvested 20 DAP, and was weakly expressed in both the embryo and tassel tissues (Fig. 4A). Transcript was not detected in leaf or root tissue, indicating that *Zpu1* expression is specific to the reproductive tissues of the plant. The 3.2-kb size of the transcript matches the length of the cloned cDNA, providing further confirmation that the clone is nearly full length.

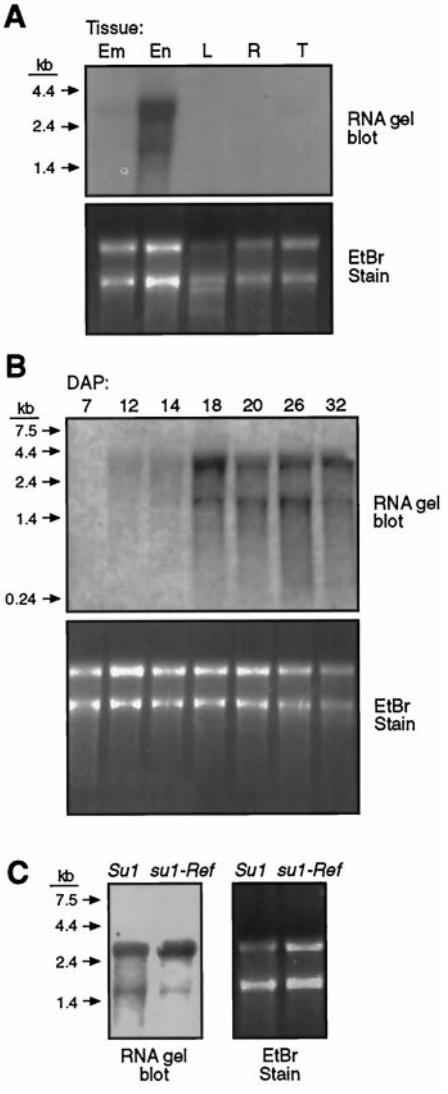

Figure 4. Total RNAs from various sources were hybridized with probe EE2.3. The RNAs as they appeared in the ethidium bromide (EtBr)-stained gel before transfer are shown to indicate RNA integrity and loading differences. A, RNAs from embryo (Em) and endosperm (En) harvested 20 DAP, seedling leaves (L), immature root (R), and immature tassel (T). B, RNAs from maize endosperm harvested at various times after pollination. C, RNAs from nonmutant (*Su1*) and mutant (*su1-Ref*) kernels harvested 20 DAP.

Probe EE2.3 also identified a smaller-sized transcript of approximately 1.4 kb that corresponded on all RNA blots with accumulation of the larger transcript. The identity of the 1.4-kb transcript is not known at this time. Although the RNAs detected by the *Zpu1* probe migrate at nearly the same rate as the rRNAs, the signal does not result from nonspecific binding because the rRNAs are equally abundant in all of the samples, whereas the Zpu1 transcript is tissue specific.

Zpu1 mRNA levels over the course of endosperm development were determined as well. Total RNAs isolated from wild-type kernels at 7, 12, 14, 18, 20, 26, and 30 DAP were analyzed. Zpu1 mRNA was shown to be weakly expressed at 12 and 14 DAP, but strongly and uniformly expressed from 18 to at least 32 DAP (Fig. 4B).

Immunological Detection and Purification of DBE Activities from Developing Maize Kernels

ZPU1 was detected in soluble kernel extracts by immunological methods. The polyclonal antiserum anti-ZPU1 was raised in rabbits against the 770 residues of ZPU1 derived from codons 160 to 930. Anti-ZPU1 detected a major polypeptide of approximately 100 kD among proteins from crude extracts of developing wild-type kernels (data not shown) and in specific fractions (Fig. 5A). The apparent size of this protein corresponds with that predicted by the Zpu1 cDNA. Anti-ZPU1 failed to detect ZPU1 in protein extracts from seedling leaves harvested during the light or dark cycle (data not shown). This observation, together with the transcript-accumulation data, demonstrated that *zpu1* is not expressed in leaves during either phase of the photosynthetic period.

The product of the Zpu1 cDNA cofractionated with a pullulanase-type DBE activity purified from extracts of developing maize kernels, thereby confirming the identity of ZPU1 as a pullulanase-type enzyme. The DBE activities present in the 40% ammonium sulfate precipitate from extracts of nonmutant kernels harvested 20 DAP were separated by anion-exchange chromatography on Q-Sepharose (Fig. 5A). Pullulanase-type DBE activity was assayed by measuring increases in reducing sugar concentrations after incubation of the substrate pullulan with protein fractions. DBE activity was also determined by increased reducing value measurements using amylopectin as the substrate, and by changes in the A_{550} value of the glucan-iodine complexes formed after incubating amylopectin with the protein fractions. Owing to substrate specificity, the assays using pullulan were expected to detect only pullulanases, whereas the amylopectin assays could identify either isoamylase- or pullulanase-type DBEs.

One peak of DBE activity was observed using pullulan as the substrate, and two peaks were observed with amylopectin as the substrate, one of which coincided with the pullulanase-type DBE peak (Fig. 5A). Immunoblot analysis using anti-ZPU1 or anti-SU1 (Rahman et al., 1998) was used to determine whether ZPU1 or SU1 could be correlated with either activity. ZPU1 was identified only in the fractions exhibiting pullulanase-type DBE activity, whereas SU1 was present only in those DBE fractions that constituted the second peak of activity toward amylopectin (Fig. 5A), i.e. each activity peak yielded a positive immunoblot signal with only one of the two antisera. This analysis provided a clear distinction between the pullulanase- and isoamylase-type activities in developing maize kernels, and identified the particular DBE responsible for each activity peak. Thus, the *su1* gene product was identified specifically as an isoamylase-type DBE active in developing kernels, and the *zpu1* gene product was identified specifically as an active pullulanase-type DBE in the same tissue. The increased A_{550} of the glucan-iodine complex ("blue value") obtained after amylopectin digestion (Fig. 5A) indicates that a DBE, as opposed to contaminating α-amylase activity, is responsible for the increased reducing value in the peak assigned as isoamylase (fractions 42–49). Contaminating α-amylase activity can also be excluded as the cause of the peak assigned as pullulanase-type DBE (fractions 25–32), because the former enzyme does not hydrolyze pullulan.

Pullulanase-type DBE activity was further purified by gel-filtration chromatography followed by another anion-exchange chromatography step. Q-Sepharose fractions that displayed pullulanase-type DBE activity were pooled and separated on the basis of size using Sephacryl S-200 chromatography. Assays of these fractions identified a pullulanase-type DBE activity, and immunoblot analysis revealed that ZPU1 again cofractionated with the activity peak (Fig. 5B). The enzyme was further purified by anion-exchange chromatography on a Mono-Q column; once again, ZPU1 cofractionated with the purified pullulanase-type DBE (Fig. 5C).

Measurements of the pullulanase-type DBE activity after the anion-exchange and gel-permeation chromatography steps (purification A) are presented in Table I. Specific activity increased with each round of purification, resulting in a 100-fold purification of the enzyme from the ammonium sulfate precipitate. This purification stage was used as the baseline because contaminating hydrolases have been shown by others to artificially elevate the apparent pullulanase-type DBE activity in crude extracts (Lee et al., 1971; Maeda et al., 1978).

A further purification of the pullulanase-type DBE was achieved by means of affinity chromatography (Fig. 5D). Q-Sepharose fractions that exhibited pullulanase-type DBE activity (Fig. 5A) were pooled and the proteins separated on the basis of their affinity for cyclohexa-amylose Sepharose (purification B). Pullulanase-type DBE activity was detected in only one of the four fractions eluted from the affinity column. SDS-PAGE and silver staining of the proteins in these fractions revealed one band of approximately 100 kD, which coeluted with pullulanase-type DBE activity. This protein was identified as ZPU1 by immunoblot analysis (Fig. 5D). The Q-Sepharose and affinity chromatography steps resulted in a 200-fold purification of ZPU1 (Table I), again using the ammonium sulfate precipitate as the baseline. The fact that ZPU1 was the only protein present in the most pure enzyme preparation provides definitive evidence that ZPU1 and the purified pullulanase-type DBE are one and the same.

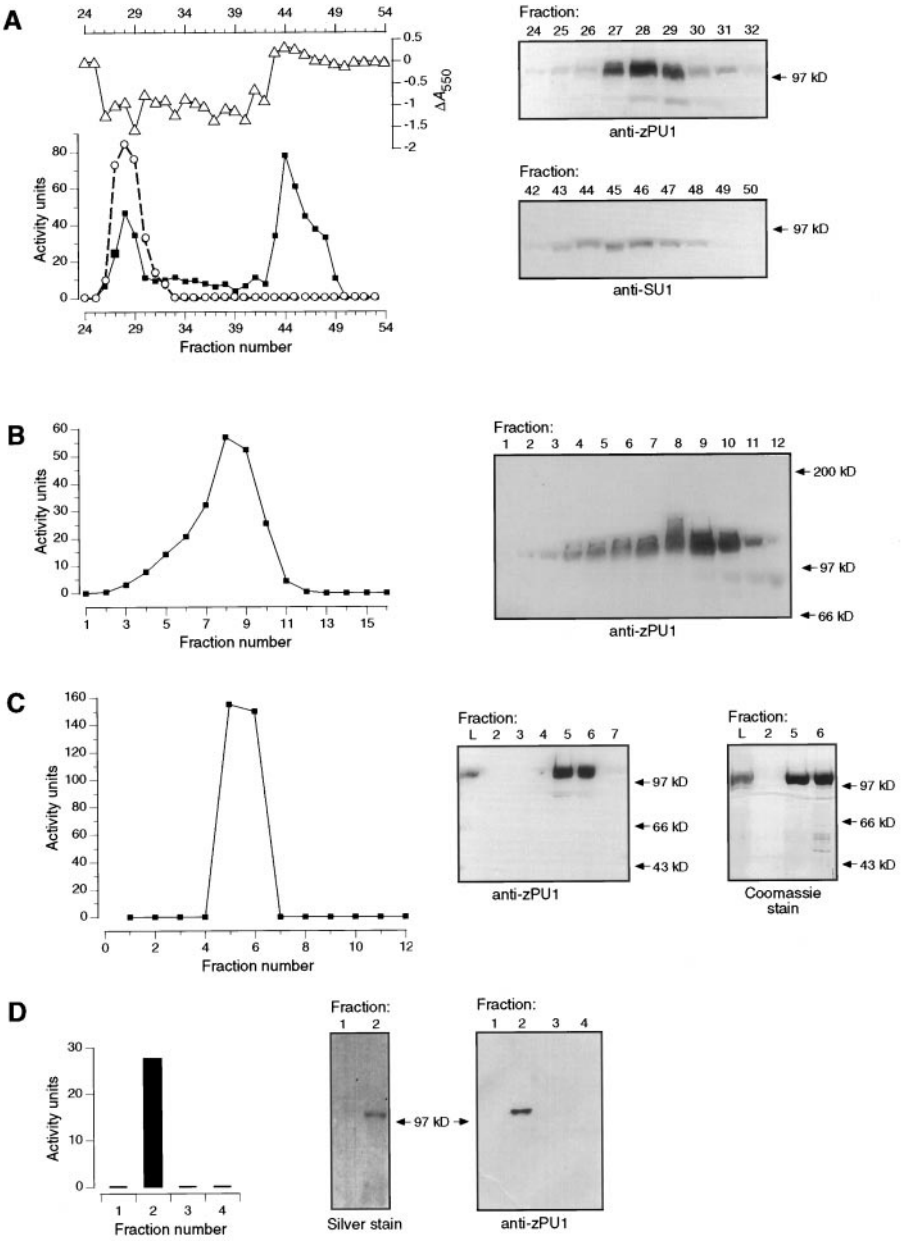

Figure 5. A, Q-Sepharose chromatography. Fractions eluted from the column were assayed for DBE activity using pullulan (○) or amylopectin (■) as a substrate. Products of the amylopectin reaction were complexed with iodine, and the change in A_{550} value relative to untreated substrate was plotted (△). Activity units for the amylopectin digestion are microgram maltose equivalents produced after a 2-h incubation of substrate with 100 μL of protein fraction. Activity units for the pullulan digestion are microgram maltotriose equivalents produced after a 2-h incubation of the substrate with 50 μL of the protein fraction. Fractions with DBE activity were subjected to immunoblot analysis with anti-ZPU1 or anti-SU1 antiserum, as indicated (right-hand panels). B, Gel-filtration chromatography. The peak fractions of pullulanase-type activity from Q-Sepharose columns were pooled, concentrated, and applied to a Sephacryl S-200 superfine gel-permeation column. DBE activity in the fractions eluted from this column was assayed using pullulan as the substrate; activity units are as described for A. Fractions were also assayed for the presence of ZPU1 by immunoblot analysis (right-hand panel). C, Mono-Q chromatography. The peak fractions (7–11) of pullulanase-type activity from the Sephacryl S-200 column were pooled, concentrated, and applied to a Pharmacia fast-protein liquid chromatography Mono-Q column. DBE activity in fractions eluted from this column was assayed using pullulan as the substrate; activity units are as described for A. Fractions were assayed for the presence of ZPU1 in immunoblots (right-hand panels). D, Affinity chromatography. The peak fractions of pullulanase-type activity from Q-Sepharose columns were pooled, concentrated, and applied to a column containing epoxy-activated Sepharose conjugated with cyclohexa-amylose. DBE activity in the four fractions eluted from this column was assayed using pullulan as the substrate; activity units are as described for A. Proteins from two of the fractions were separated by SDS-PAGE and the gel was silver stained; a duplicate gel was subjected to immunoblot analysis with anti-ZPU1 (right-hand panels).

Table I. *Purification of ZPU1*[a]

Step	Total Protein	Total Activity	Specific Activity	Purification	Recovery
	mg	*units*[b]	*units/mg*	*fold*	*%*
Purification A					
Crude extract	328	6.56	0.02	ND[c]	ND
Ammonium sulfate (40%)	146	1.46	0.01	1	100
Q-Sepharose	17	0.85	0.05	5	58.2
Sephacryl S-200	3	0.75	0.25	25	51.4
Mono-Q	0.01	0.01	1.00	100	0.68
Purification B					
Crude extract	288	20.16	0.07	ND	ND
Ammonium sulfate (40%)	104	2.09	0.02	1	100
Q-Sepharose	4	0.42	0.11	5.5	20.1
Cyclohexa-amylose	0.06	0.24	3.99	199.5	11.4

[a] Total and specific activities are apparent values, because contaminating hydrolytic enzymes may cleave the substrates or products of the assay reactions. [b] Units are micromole maltotriose equivalents per minute. [c] ND, Not determined because of potential inaccuracy resulting from contaminating hydrolases.

Accumulation of ZPU1 Protein and Zpu1 mRNA in Nonmutant and *su1*-Mutant Kernels

The previous finding that pullulanase-type DBE activity is greatly reduced in *su1* mutants (Pan and Nelson, 1984) prompted further characterization of the DBEs in *su1-Ref* kernels. Proteins from nonmutant and mutant kernels harvested 20 DAP were separated on the basis of size using Sephacryl S-200 chromatography. Pullulanase-type DBE activity was assayed by measuring hydrolysis of pullulan, and isoamylase-type DBE activity was assayed by blue-value determinations after hydrolysis of amylopectin. As expected from the Q-Sepharose fractionation (Fig. 5A), distinct peaks of activity were observed for each DBE in the nonmutant extracts (Fig. 6A). Immunoblot analyses again confirmed that the pullulanase-type DBE activity corresponded with ZPU1 and the isoamylase-type DBE activity corresponded with SU1. Both peaks of DBE activity were reduced in the *su1* mutant (Fig. 6B). Immunoblot analyses of the protein fractions from both genotypes were performed under identical conditions. The pullulanase- and isoamylase-type DBE activities affected by *su1-Ref* corresponded with reduced accumulation of the ZPU1 and SU1 proteins, respectively (Fig. 6B). However, a shift was also detected in the electrophoretic mobility of ZPU1 in certain *su1-Ref* fractions. Direct comparison of corresponding nonmutant and *su1-Ref* fractions revealed that the anti-ZPU1

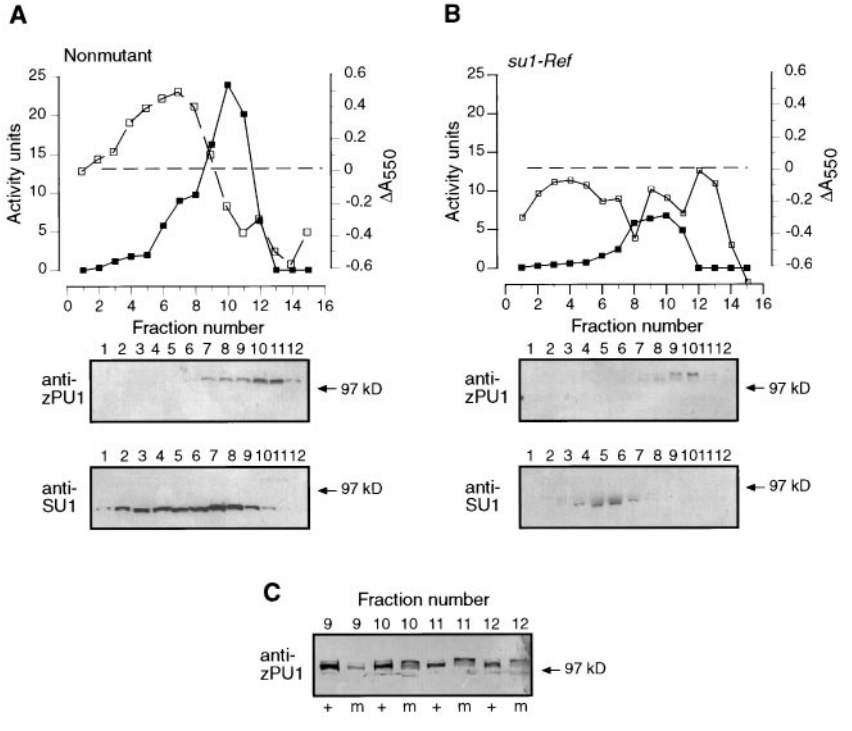

Figure 6. Fractionation of DBEs from nonmutant and *su1-Ref* kernels by gel-permeation chromatography. A, DBEs in nonmutant kernels. Proteins from nonmutant kernels harvested 20 DAP were applied to a Sephacryl S-200 superfine gel-permeation column. Fractions were assayed for pullulanase-type DBE activity by measuring formation of new reducing ends after incubation with pullulan (activity units, ■), and for isoamylase-type DBE activity by determination of iodine-complex absorbance maxima after incubation with amylopectin (A_{550}, □). Equivalent amounts of protein from fractions exhibiting DBE activity were analyzed for the presence of ZPU1 or SU1 on immunoblots with the indicated antisera. B, DBEs in *su1-Ref* kernels. Proteins from *su1-Ref* kernels harvested 20 DAP were fractionated and assayed for DBE activity, and immunoblot analysis was performed, as described for A. C, Comparative immunoblot analysis. Nonmutant proteins in fractions 9 to 12 from A (lanes +) and *su1-Ref* proteins in fractions 9 to 12 from B (lanes m) were subjected to immunoblot analysis with the anti-ZPU1 antibody. Equivalent amounts of protein were loaded, and each lane contained twice as much protein as the immunoblots shown in A and B.

antiserum identifies a polypeptide doublet of approximately 100 and 105 kD, respectively, and that the form with the apparent lower molecular mass predominates in the nonmutant kernels (Fig. 6C). This polypeptide was greatly reduced in the *su1-Ref* mutant, but the form with the apparent higher molecular mass was increased (Fig. 6C). Similar results were observed with the independent allele *su1-R4582::Mu1* (James et al., 1995; data not shown).

To determine whether the effect of *su1* mutations on ZPU1 expression occurs at the level of transcription, the steady-state level of Zpu1 mRNA was compared in nonmutant and *su1-Ref*-mutant kernels harvested 20 DAP. Full-length Zpu1 transcripts were approximately equal in both size and abundance in the nonmutant and mutant kernels (Fig. 4C). Thus, no obvious changes were detected in the transcription of Zpu1 in the *su1* mutants compared with nonmutant kernels.

DISCUSSION

This study identified the specific genetic elements responsible for each of two distinct DBE activities in developing maize endosperm tissue, extending the analysis of DBE activities described previously by Doehlert and Knutson (1991). Activities of both isoamylase- and pullulanase-type DBEs were purified from developing maize kernels. The pullulanase-type enzyme activity corresponds with the product of the gene *zpu1* identified in this report, and the isoamylase-type enzyme activity corresponds to the product of the *su1* gene. In a previous study recombinant expression of *su1* produced an active isoamylase-type DBE (Rahman et al., 1998). Taken together, these data clarify the cast of DBEs present in maize endosperm cells: *zpu1* codes for a pullulanase-type DBE, *su1* codes for an isoamylase-type DBE, and both enzymes are present in amyloplasts of endosperm tissue during the time that starch granules are being produced.

Two lines of evidence support the conclusion that ZPU1 is a pullulanase-type DBE. First, the polypeptide predicted by the Zpu1 cDNA is highly similar in sequence to all known bacterial pullulanases and plant pullulanase-type DBEs (Figs. 1 and 2). Second, antibodies raised against the *Zpu1* product detected an endosperm protein (or protein doublet) that cofractionated with pullulanase-type DBE activity in four different chromatography purification steps (Figs. 5 and 6). After a nearly 200-fold purification of the pullulanase-type enzyme activity, the 100-kD protein that reacts with anti-ZPU1 appeared to be the only polypeptide present in the fraction.

The *zpu1* gene is expressed predominantly in endosperm. The small amount of transcript observed in the embryo could indicate a role for the pullulanase-type DBE in embryo starch metabolism or, alternatively, may result from endosperm contamination of the tissue sample. Small amounts of Zpu1 transcript were detected in the tassel, possibly indicating a role in pollen starch metabolism. The fact that Zpu1 transcript was not detected in leaves is significant because pullulanase-type DBEs have been characterized in photosynthetic tissue from several species (Okita and Preiss, 1980; Li et al., 1992; Ghiena et al., 1993). Presuming that one or more pullulanase-type DBEs are present in maize leaves, they must be coded for by genes other than *zpu1*.

The presence of both isoamylase and pullulanase types of DBEs may be a general feature of tissues that produce storage starch. Both enzymes have been reported in developing maize kernels (Doehlert and Knutson, 1991) and potato tubers (Drummond et al., 1970; Ishizaki et al., 1983). In a recent study pea embryos were found to possess two distinct pullulanase-type DBEs in addition to an isoamylase (Zhu et al., 1998). From the fact that both *zpu1* and *su1* are highly conserved within the plant kingdom, we speculate that most starch-producing sink tissues contain functional homologs of the DBEs coded for by these two maize genes.

Two possibilities can be envisioned for the function of ZPU1. The simplest explanation is that this DBE hydrolyzes storage starch during seed germination. Even though ZPU1 is expressed during starch biosynthesis, it may accumulate in an inactive form and be restricted from action until after germination. Such restriction, however, would have to occur even though the enzyme is catalytically active in endosperm cell extracts. Furthermore, ZPU1 was shown by immunoblot analysis to be enriched in the amyloplast stromal fraction of developing endosperm (H. Mu, B. Wasserman, personal communication). Thus, ZPU1 is present during the time that starch biosynthesis occurs and in the same subcellular compartment. For these reasons we favor the explanation that ZPU1 functions directly in starch biosynthesis as opposed to starch utilization. A starch biosynthetic function has been proposed for SU1 isoamylase (Ball et al., 1996), based on the fact that *su1* mutations result in the production of an overly branched polysaccharide (Sumner and Somers, 1944). Mutations of *zpu1* are not known; however, because the cDNA sequence is currently available, reverse genetic strategies can be used to identify a mutant allele. Such a mutation could be used to determine whether ZPU1 is needed for normal starch biosynthesis.

Previous studies showed that a pullulanase-type enzyme activity is deficient in maize endosperm homozygous for the *su1-Ref* mutation (Pan and Nelson, 1984). The current study, however, together with the characterization of recombinant SU1 (Rahman et al., 1998), demonstrates unequivocally that *Su1* does not code for a pullulanase-type enzyme but instead specifies an isoamylase-type DBE. Therefore, the reduction in pullulanase-type DBE activity in *su1* mutants must be explained by a pleiotropic effect. This report shows that some or all of the pullulanase-type DBE affected pleiotropically by *su1* mutations is ZPU1. Pan and Nelson (1984) identified three peaks of pullulanase-type DBE activity in hydroxyapatite columns, all of which were affected to some extent by the *su1-Ref* mutation. Further analysis is required to determine whether all three peaks are attributable to *zpu1* or, alternatively, if additional pullulanase-type DBEs exist in maize endosperm.

Previously, our laboratory reported that *su1-Ref* mutant kernels are deficient in an approximately 100-kD polypeptide identified by antisera to rice RE (Rahman et al., 1998). Anti-rice RE identified only a single polypeptide in non-

mutant kernels in the region of 100 kD. The current study shows that antiserum raised against the maize protein ZPU1 identifies a polypeptide doublet of approximately 100 and 105 kD. The lower band of this doublet is strongly reduced in *su1* mutants. This is consistent with the previous report, because anti-rice RE specifically identifies the protein of apparent lower molecular mass (data not shown). Identification of the larger form of ZPU1, which is increased in *su1* mutants, suggests that the overall decrease in ZPU1 protein resulting from *su1* mutations is less than previously thought. Rather, the data imply that the relative accumulation of the two ZPU1 polypeptide forms is dependent on SU1 isoamylase, and that the form with the apparent lower molecular mass is enzymatically active, whereas the larger form is functionally inactive.

The pleiotropic effect of *su1* mutations on *zpu1* gene expression could occur at either the transcriptional or the posttranscriptional level. Transcriptional mechanisms regulating starch biosynthetic gene expression have been demonstrated previously for sugar-accumulating mutants of maize (Giroux et al., 1994). Zpu1 transcription is normal in *su1*-mutant kernels as far as can be resolved by RNA gel-blot analysis, which suggests that changes in transcription initiation are not responsible for the effects of *su1* mutations on ZPU1.

Several potential posttranscriptional mechanisms could account for the changes in ZPU1 in *su1* mutants. The possibility that altered splicing of the Zpu1 pre-mRNA occurs as the result of *su1* mutations has not been ruled out. At the level of protein-protein interaction, the possibility exists that SU1 and ZPU1 participate in an enzyme complex. According to this model, loss of SU1 could lead to destabilization and/or altered modification of ZPU1. Observation of SU1 and ZPU1 in distinct chromatographic fractions makes this model less plausible, although the possibility remains that the complex dissociates upon cell lysis or during fractionation. An explanation that does not require direct SU1-ZPU1 interaction is that loss of the isoamylase-type DBE results in an altered concentration or form of the preferred substrate for the pullulanase-type DBE. Substrate binding in turn might affect the stability of ZPU1 and/or alter its ability to undergo further posttranslational modification. Finally, *Su1* activity may be directly required to achieve or maintain an active form of ZPU1. For example, SU1 might remove covalently linked glucan from ZPU1, analogous to the known ability of *Pseudomonas* sp. isoamylase to cleave the glucosyl-tyrosyl linkage in glycogenin (Lomako et al., 1992). In any event, coordinate regulation of the two types of DBE in maize endosperm cells suggests a cooperative function. Both the timing of gene expression and the effects of *su1* mutations on starch structure suggest that SU1 and ZPU1 cooperate to play a direct role in the biosynthesis of amylopectin.

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