Differential Expression and Properties of Starch Branching Enzyme Isoforms in Developing Wheat Endosperm

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Three forms of starch branching enzyme (BE) from developing hexaploid wheat (Triticum aestivum) endosperm have been partially purified and characterized. Immunological cross-reactivities indicate that two forms (WBE-Iα, 88 kD, and WBE-Iβ, 87 kD) are related to the maize BE-I class and that WBE-II (88 kD) is related to maize BE-II. Comparison of the N-terminal sequences from WBE-Iα and WBE-II with maize and rice BEs confirms these relationships. Evidence is presented from the analysis of nullisomic-tetrasomic wheat lines demonstrating that WBE-Iα is located on chromosome 7B and that the WBE-Iα fraction contains polypeptides that are encoded on chromosomes 7A and 7D. The wheat endosperm BE classes are differentially expressed during endosperm development. WBE-II is expressed at a constant level throughout mid and late endosperm development. In contrast, WBE-Iα and WBE-Iβ are preferentially expressed in late endosperm development. Differences are also observed in the kinetic characteristics of the enzymes. The WBE-I isoforms have a 2- to 5-fold higher affinity for amylose than does WBE-II, and the WBE-I isoforms are activated up to 5-fold by phosphorylated intermediates and inorganic phosphate, whereas WBE-II is activated only 50%. The potential implications of this activation of BE I for starch biosynthesis are discussed.

Extensive studies of starch biosynthesis in plants provide compelling evidence from physiological, biochemical, genetic, and genetic manipulation studies supporting the view that starch is predominantly, if not entirely, synthesized within the plastid by a pathway involving the enzymes ADPglucose pyrophosphorylase (EC 2.7.7.27), starch synthase (EC 2.4.1.21), and BE (EC 2.4.1.18) (Preiss, 1991; Okita, 1992; Müller-Röber and Kossmann, 1994; Martin and Smith, 1995). Evidence from mutational studies supports the contention that starch debranching enzyme is also essential for shaping the final structure of amylopectin (Pan and Nelson, 1984; James et al., 1995; Mouille et al., 1996). Although these studies demonstrate the nature of the key steps in starch synthesis, there is complexity in the pathway deriving from the presence in plants of isoforms of each of the major activities. These isoforms may be expressed on a tissue-specific basis (e.g. leaf and endosperm ADPglucose pyrophosphorylase isoforms) or may be expressed at the same time in the same compartments (e.g. isoforms of both starch synthase and starch BEs in the cereal endosperm). Some isoforms are located only within the granule (e.g. granule-bound starch synthase), some are located only in the amyloplast stroma (e.g. starch BE I; Rahman et al., 1995), and some are partitioned between the granule and the amyloplast stroma (e.g. 77-kD starch synthase in pea; Denyer et al., 1993). The hexaploid nature of the bread wheat (Triticum aestivum) raises the possibility that not only are there likely to be isoforms from each of the classes of BE analogous to those found in maize and rice, but that there will also be forms of the enzyme from each class present in the developing endosperm encoded by genes on each of the wheat genomes. Different forms of each isoform deriving from different genomes are referred to as allozymes.

Starch BE catalyzes a transferase reaction in which a donor linear α-1,4-glucan chain (either amylose or a linear region of amyllopectin) is cleaved and attached to a recipient chain via an α-1,6 linkage. In maize and rice endosperm and in pea embryo two classes of starch BE activity are found. There is both biochemical and genetic evidence to suggest that these isoforms have different roles in starch synthesis.

Studies of purified maize BE I and II demonstrate that these isoforms differ in their specificity for a substrate with respect to both chain length and degree of branching. Maize BE I has a higher affinity for amylose than maize BE II and preferentially transfers longer chains than maize BE II (Takeda et al., 1993). Mutational studies in maize, rice, and pea demonstrate that high-amylose mutants in each case are deficient in the BE activity analogous to maize starch BE II (Martin and Smith, 1995; Morell et al., 1995). However, the linkage between the biochemical observations and the genetic evidence suggesting the differences in the roles remains unclear.

Previously, we have shown that there is a polypeptide that cross-reacts with antibodies to starch BE II in the wheat starch granule (Rahman et al., 1995), and Denyer et

Abbreviations: BE, branching enzyme; 3-PGA, D-glycerate 3-phosphate; TEA, triethanolamine; WBE, wheat BE.

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al., (1995) have presented evidence for the solubilization of starch BE from wheat starch granules. In this study we report on the separation and characterization of different classes of isoforms of starch BE in the soluble fraction of developing wheat endosperm. Differences between isoform classes have been found with respect to patterns of expression, to substrate kinetics, and in their activation by inorganic phosphate and a range of phosphorylated compounds. The possible significance of these results to the regulation of starch synthesis in wheat is discussed.

MATERIALS AND METHODS

Wheat (Triticum aestivum cv Rosella) was grown in Canberra and harvested in the field. Wheat (T. aestivum cv Chinese Spring) and a set of nullisomic-tetrasomic Chinese Spring lines were provided by Dr. Bob MacIntosh (University of Sydney, Australia) and were grown in greenhouse conditions and harvested 20 d after anthesis. Ears were snap-frozen in liquid nitrogen and stored at −90°C. Material for the partial purification of wheat starch BEs was collected 24 d after anthesis and prepared by excising the caryopsis from the head and removing the embryo. Developmental studies were carried out using endosperm that was dissected away from all of the maternal tissues.

Antibodies

Polyclonal antibodies raised against maize starch BE I and II were kindly provided by Dr. J. Preiss and Dr. H.P. Guan (Michigan State University, East Lansing). Polyclonal antibodies raised against potato starch BE were kindly provided by Professor Lars Rask (Swedish University of Agricultural Sciences, Uppsala). A polyclonal antibody against a peptide synthesized with the N-terminal sequence of WBE-IAD from wheat was raised in rabbits at the Commonwealth Scientific and Industrial Research Organization Division of Plant Industry by Dr. J.H. Skerritt and A. Hill. The antigen was prepared by coupling the synthesized peptide to keyhole limpet hemocyanin using m-maleimidobenzoyl-N-hydroxysuccinimide ester. This antibody was designated rabbit anti-WBE-I (N-terminal peptide).

Measurement of BE Activity

A phosphorylase a stimulation assay (Hawker et al., 1974) was used to monitor the enzyme purification. The assay measures the stimulation of phosphorylase a activity by BE and 1 unit of BE activity is defined as 1 µmole of Glc incorporated into α-D-glucan min−1 at 25°C.

Kinetic parameters were determined using the amylase/iodine assay essentially as described by Boyer and Preiss (1978). All reaction mixtures contained 20 mM Tris-acetate buffer (pH 7.5) containing 0.4 mM EDTA, 1 mM DTT, and 0.5 mg/mL BSA. Additions of different compounds to these mixtures were made as described in “Results.” Reactions were initiated by the addition of enzyme, performed at 25°C, and terminated by the addition of iodine solution. Absorbance of the iodine/glucan complex was determined at 680 nm. One ΔA unit is defined as a decrease in A0.1 of 1.0 per minute at 25°C.

Partial Purification of Wheat Endosperm BEs

All steps were carried out at 4°C. Wheat caryopsis (25 g) was homogenized with a mortar and pestle in 75 mL of 50 mM TAE-acetate buffer, pH 8.0, containing 10 mM EDTA, 5 mM DTT, 1 mM Pefabloc (Boehringer Mannheim), and 2 µg/mL leupeptin. The extract was filtered through Miracloth (Calbiochem) and centrifuged at 13,000g for 20 min. Solid (NH4)2SO4 was added to 15% saturation and centrifuged for 20 min at 13,000g after standing for 30 min. The supernatant was decanted and brought to 50% (NH4)2SO4 saturation. After standing for 30 min the precipitate was collected by centrifugation at 13,000g for 20 min. The supernatant was discarded and the pellet was resuspended in 6 mL of buffer A (50 mM TAE-acetate buffer, pH 8.0, containing 1 mM EDTA and 2 mM DTT). The sample was clarified by centrifugation at 13,000g for 20 min and the supernatant was applied to a 1.6 × 70 cm Sephadex G-25 column (pre-equilibrated in buffer A) and eluted with buffer A at a flow rate of 1 mL/min. Five-milliliter fractions were collected; fractions containing protein were pooled (32 mL) and water was added to give a final volume of 50 mL.

The sample was applied at a flow rate of 2 mL/min to a 6-mL Resource-Q column (Pharmacia) pre-equilibrated with buffer A. The column was eluted with buffer A until the A280 of the effluent was <0.1. Ten-milliliter fractions were collected during the application and washing steps. The column was then eluted with a 120-mL linear gradient prepared by introducing buffer B (buffer A containing 0.6 M KCl) into buffer A. Four-milliliter fractions were collected during the gradient elution.

Individual pools were further purified prior to kinetic studies and N-terminal sequencing by chromatography over a 1 cm × 90 cm Sephacryl S-200 column that had previously been equilibrated with 0.1 M potassium phosphate buffer (pH 7.5) containing 1 mM DTT. The sample was applied in a volume of 1 mL and the column eluted at 0.25 mL/min. Fractions containing BE activity but no amylase activity were pooled. Amylase activity was determined by a modified Park Johnson reducing sugar assay following incubation of aliquots of each fraction with amylase under the incubation conditions of the amylase/iodine assay (Takeda et al., 1993).

Proteinase Inhibitors

Pefabloc and leupeptin were added to crude extracts at final concentrations of 1 mM and 2 µg/mL, respectively. Experiments that were used to investigate the effect of a broad spectrum of proteinase inhibitors involved the following inhibitors (final concentrations given in parentheses): Pefabloc (1 mM), leupeptin (2 µg/mL), antipain dihydrochloride (74 µM), apronitin (0.5 µM), bestatin (130 µM), chymostatin (100 µM), E-64 (2.8 µM), EDTA (1.3 mM), pep-
statin (1 μM), and phosphoramide (600 nM). All proteinase inhibitors were obtained from Boehringer Mannheim.

Gel Electrophoresis and Immunoblotting

SDS-PAGE and non-denaturing PAGE were carried out using 10% polyacrylamide gels according to Laemmli (1970). Western blotting from SDS-PAGE gels was carried out according to the method of Burnette (1981). The primary rabbit antibodies, rabbit anti-maize BE I, rabbit anti-maize BE II, and rabbit anti-potato BE, were diluted 1:1000 (v/v) and the immunoreactive bands revealed as described previously (Rahman et al., 1995). Nondenaturing gels were blotted following incubation in 0.25% SDS solution for 30 min at room temperature. The rabbit anti-WBE-I (N-terminal peptide) antibody was used at a 1:10,000 dilution. Immunoreactive bands were revealed using an Amersham ECL detection system according to the manufacturer’s instructions.

N-Terminal Sequencing

N-terminal sequences were determined after separation of proteins by SDS-PAGE and western blotting as described previously (Rahman et al., 1995). BE polypeptides were identified for N-terminal sequence analysis by identifying immunoreactive bands on sections of the blot and aligning these bands with specific protein bands revealed by amido black staining.

Nomenclature

In this study the properties of wheat starch BEs are related principally to the properties of maize BEs; therefore, the nomenclature of Boyer and Preiss (1978) was adopted to describe the wheat enzymes.

RESULTS

Separation of Soluble BE Isoforms

Starch BEs were extracted from developing wheat endosperm and partially purified by (NH₄)₂SO₄ fractionation. The fraction containing proteins precipitating between 15 and 50% (NH₄)₂SO₄ saturation contained greater than 95% of the BE activity. This fraction was desalted and chromatographed on a Resource-Q anion-exchange column. The results are shown in Figure 1. Three activity peaks were obtained and described as WBE-Iₐ, WBE-Iₐ, and WBE-II (see below).

Table I shows the distribution of BE activity between the fractions obtained from the anion-exchange separation of wheat endosperm. The results show that the majority of the phosphorylase a stimulation activity is present in fraction WBE-Iₐ and approximately equal amounts of activity are present in the WBE-Iₐ and WBE-II pools. Although greater than 90% of the amylase activity was eliminated from the BE fractions by this procedure, residual α-amylase activity was removed from each pool by Sephacryl S-200 chromatography. No overall purification on the basis of total protein content was achieved by Sephacryl S-200 chromatography. Densitometry analysis of SDS-PAGE gels of the three BE pools indicated that BE constituted approximately 5 to 10% of the total protein in each fraction.

Immunological Characterization of Isoforms

Maize BE I, maize BE II, and potato BE I antibodies were available to assist in the characterization of the BE fractions. Amino acid sequence homology and immunological comparisons suggest that potato BE I is an isoform of the BE I class (Burton et al., 1995; Morell et al., 1995). SDS-PAGE western blotting experiments were conducted with each of the three antibodies. The cross-reactivities of the three partially purified wheat endosperm fractions with polyclonal potato BE and maize BE II antibodies are shown in Figure 2. Results with anti-maize BE I and anti-potato BE were identical in their reactions with proteins that consisted of 87 (WBE-Iₐ) and 88 kD (WBE-Iₐ). The anti-maize BE I antibodies also cross-reacted with polypeptides of 52, 60, and 64 kD (Fig. 3A) in wheat endosperm crude extracts. There is no evidence to suggest that these polypeptides are BEs or that they are related to BEs, since they do not cross-react with the anti-potato BE antibody and do not co-purify with BE activity, and the N-terminal sequence data from the 64-kD protein showed no homology to any known BE sequence (data not shown). These immunoreactions that were detected most likely derive from unrelated antibodies generated against wheat proteins by the rabbit inoculated with purified maize BE I antigen.

The SDS-PAGE western blotting analysis indicates that the proteins present in fractions WBE-Iₐ (88 kD) and WBE-Iₐ (87 kD) belong to the maize BE I class, whereas the 88-kD polypeptide in fraction WBE-II shows immunological similarity to the maize BE II class. Some cross-reactivity between anti-maize BE II antibodies and WBE-Iₐ and WBE-Iₐ was observed, although the cross-reactivity is approximately one-tenth as intense, as judged by western
Table 1. Partial purification of wheat endosperm starch BEs

| Fraction | Volume | Protein Content | Activity | Specific Activity | Recovery Relative to Previous Step % | Percent of Total Activity Eluted from Resource-Q
|----------|--------|----------------|----------|------------------|--------------------------------------|-------------------------------------------------
| Crude    | 74.5   | 137.5          | 14,118   | 76               | 100                                  | NA                                              |
| G25*     | 50     | 47.4           | 8,477    | 178              | 60.4                                 | NA                                              |
| WBE-I$_{AD}$ | 16  | 8.6            | 2,739    | 322              | 44.4                                 | 59                                              |
| WBE-I$_B$ | 16    | 10.7           | 1,293    | 121              | 15.3                                 | 20                                              |
| WBE-II   | 8      | 7.4            | 1,369    | 186              | 16.1                                 | 21                                              |

* Total recovery from Resource-Q column 76% relative to the activity applied. NA, Not applicable.

blotting experiments, as the reaction between anti-maize BE II antibodies and WBE-II.

Assignment of Wheat BE I Polypeptides to Chromosome 7

The chromosomal location of genes encoding the wheat BE I polypeptides was investigated by nondenaturing-PAGE analysis of nullisomic-tetrasomic lines of cv Chinese Spring (Fig. 4). Nondenaturing-PAGE of Chinese Spring endosperm extracts revealed the presence of four BE I polypeptides (Fig. 4, lane 7). Lines lacking chromosome 7A lack polypeptide A (Fig. 4, lanes 1 [N7AT7D] and 2 [N7AT7B]). Lines lacking chromosome 7D (Fig. 4, lanes 3 [N7DT7A] and 4 [N7DT7B]) lack the prominent polypeptide (D-i) and the weak immunoreactive band (D-ii). Although D-i and D-ii polypeptides are encoded on the same chromosome, this analysis does not resolve whether D-i and D-ii are the products of separate genes, or whether one polypeptide is derived from the other through posttranslational modification. The remaining two nullisomic-tetrasomic lines lack polypeptide B (Fig. 4, lanes 5 [N7BT7A] and 6 [N7BT7D]).

Nondenaturing-PAGE analysis showed that the first BE I peak to elute from Resource-Q anion-exchange chromatography, WBE-I$_{AD}$, contained 88-kD polypeptides encoded on chromosomes 7A and 7D. The WBE-I$_B$ fraction contains a single 87-kD polypeptide (Fig. 2) encoded on chromosome 7B.

The relative intensities of the bands suggests that BE I genes residing on different wheat genomes are expressed at different levels. Western blotting experiments using a range of polyclonal antibodies (rabbit anti-maize BE I, rabbit anti-potato BE I, and rabbit and BE I N-terminal peptide) yield similar ratios of expression for the BE I genes from the various genomes (data not shown), suggesting that differences in the intensities are not due to differences in the relative affinities of the antibodies for individual polypeptides. The expression levels observed are ranked in the following order: WBE-I$_{D(i)}$ > WBE-I$_B$ > WBE-I$_A$ > WBE-I$_{D(ii)}$.

A similar analysis of the chromosomal location of the BE II genes was not possible because the products of different genomes could not be resolved by either SDS-PAGE or nondenaturing-PAGE.

Figure 2. SDS-PAGE immunoblot of partially purified wheat endosperm BE fractions using anti-potato BE I and anti-maize BE II antibodies. Aliquots of each fraction containing equivalent amounts of activity (determined by the phosphorylase a assay) were electrophoresed in 10% SDS-PAGE and electroblotted to nitrocellulose. Immunoreactive proteins were revealed following reaction with primary antibodies raised against (A) maize BE II or (B) potato BE.

Figure 3. Western blot analysis of the soluble fraction of crude extracts of wheat endosperm extracted from endosperm harvested at a defined number of days after anthesis. Aliquots containing 10 μg of protein were separated by SDS-PAGE in a 10% gel and blotted and probed. A, Probed with antibodies raised against maize BE I; B, probed with antibodies raised against maize BE II. Labeled arrows indicate immunoreactive bands at 88 (a), 87 (b), 64 (c), 60 (d), and 52 kD.
88-kD proteins and the WBE-I<sub>B</sub> 87-kD proteins are not detectable until 18 d after anthesis and increase in relative expression late in endosperm development. The low-molecular-weight, non-BE polypeptides (Fig. 3, c–e) that react with the maize anti-BE I proteins are expressed at a constant level relative to total protein and provide a useful internal calibration of the relative expression of the BE I class of proteins.

**Kinetic Characteristics of Isoforms**

The K<sub>m</sub> values for the three wheat endosperm isoforms measured by the amylose/iodine assay are given in Table III. The K<sub>m</sub> values are similar to those reported for maize BE I and BE II (Takeda et al., 1993). The ratio of activity in the phosphorylase a stimulation and amylose/iodine assays has been shown to be diagnostic for the BE classes in pea and maize (Smith, 1988; Guan and Preiss, 1993). Table III gives values for the ratio of activity of each of the wheat BE fractions in these assay systems. WBE-II has a 7.8- and 5.5-fold higher phosphorylase a stimulation assay to amylose/iodine assay activity ratio than WBE-I<sub>AD</sub> or WBE-I<sub>B</sub>, respectively. These results are consistent with the results from maize (BE Ia has an 18-fold higher ratio than BE I; BE Ib has a 17-fold higher ratio than BE I [Guan and Preiss, 1993]) and pea (the BE II activity ratio is 11-fold higher than the BE I ratio [Smith, 1988]).

Phosphorylated and nonphosphorylated metabolic intermediates and various anions were included in amylose/iodine assays at a 10 mM final concentration and were found to have different effects on the individual WBE activities (Fig. 5). Most of the phosphorylated compounds and inorganic anions (tested at 10 mM concentration) showed a 2- to 3-fold activation of WBE-I<sub>AD</sub> and WBE-I<sub>B</sub>. WBE-II was only about 50% stimulated by these compounds; the noncharged compounds maltose and Rib did not affect the activities. However, Pi, Fru-6-P, and Rib-5-P exhibited a 5-fold activation on the BE I-type enzymes. Half-maximal activation constants for Pi for the three enzyme forms were determined using the amylose/iodine assay with increasing amounts of potassium phosphate, pH 7.5 (Fig. 6; Table III). 3-PGA and Pi are allosteric modulators of ADPglucose pyrophosphorylase in the majority of the plants investigated; however, 3-PGA and Pi do not have mutually antagonistic effects on wheat en-

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**Table II. N-terminal sequences of cereal starch BEs**

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<th>Protein</th>
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<tbody>
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<td>A</td>
<td>T</td>
<td>A</td>
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<td>K</td>
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<sup>a</sup> N-terminal amino acid of the mature polypeptide.  
<sup>b</sup> Kawasaki et al. (1993).  
<sup>c</sup> Baba et al. (1991).  
<sup>d</sup> Mizuno et al. (1993).  
<sup>e</sup> Positions where a gap has been inserted into the sequence to maximize homology between the members of a BE class.  
<sup>f</sup> Fisher et al. (1993).
Table III. Kinetic parameters of wheat starch BEs

<table>
<thead>
<tr>
<th>Fraction</th>
<th>(K_m) (Amylose) (\mu)mol</th>
<th>Phosphorylase Stimulation: Amylose/Iodine Activity Ratio</th>
<th>(K_{M,Pi}) (Pi) (\mu)mol</th>
<th>Activation by 10 mM Pi</th>
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</thead>
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<tr>
<td>WBE-I(_{AD})</td>
<td>0.30 ± 0.06</td>
<td>1.91</td>
<td>6.5 ± 0.8</td>
<td>5.3</td>
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<tr>
<td>WBE-I(_B)</td>
<td>0.11 ± 0.02</td>
<td>2.7</td>
<td>12 ± 1.5</td>
<td>4.3</td>
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<tr>
<td>WBE-II</td>
<td>0.65 ± 0.11</td>
<td>14.9</td>
<td>22 ± 4.6</td>
<td>1.5</td>
</tr>
</tbody>
</table>

\(a\) Amylose/Iodine assay procedure. \(b\) Ratio of activity determined by the phosphorylase stimulation assay \((\mu\)mol min \(^{-1}\) mg \(^{-1}\)) to the activity determined using the amylose/Iodine assay \((A_{abs} \text{ min}^{-1} \text{ mg}^{-1})\). \(c\) Concentration required for one-half maximal activation. \(d\) \(n = 3\).

dosperm BE activity, since 10 mM 3-PGA stimulated both WBE-I\(_{AD}\) and WBE-I\(_B\) activities 3-fold and WBE-II activity 1.5-fold. A similar stimulation of activity by Pi has also been seen by Blennow (1992) for potato BE I. Potato BE I was 2-fold more active in 50 mM phosphate buffer (pH 7.5) than in 50 mM TEA-Cl buffer (pH 7.5). However, a 5-fold activation with 10 mM phosphate was also observed for the potato enzyme when using 20 mM TEA-acetate as a buffer.

**DISCUSSION**

The basic features of the starch biosynthetic pathway in plants have been established through both biochemical and genetic studies over the past two decades. However, deficiencies in our knowledge remain with respect to the roles of particular isoforms of the starch biosynthetic enzymes (Preiss, 1991; Martin and Smith, 1995). Studies on a number of plants, including maize, rice, and pea, suggest that a general feature of starch biosynthesis in sink tissues is the presence of two classes of starch BE. The recent description of a BE II-type activity in potato tubers maintains this pattern, despite earlier suggestions that there is a single BE activity in this tissue (Larsson et al., 1996). Therefore, it is not surprising that we found two classes of starch BE in wheat endosperm and that, on the basis of immunological comparisons, these isoforms are either a class-I or class-II type. Two forms of wheat BE class I activity were separated by anion-exchange chromatography from wheat endosperm. These forms differ in molecular weight and kinetic properties. Nondenaturing-PAGE of Chinese Spring nullisomic-tetrasomic lines demonstrate that there are BE I genes located on the wheat group 7 chromosomes A, B, and D. Chromosome 7D may encode two forms of BE I.

The expression of BEs during development was monitored by western blotting and showed that there are differences in expression patterns between isoforms, with the BE II class expressed from 13 to 32 d after anthesis, whereas the BE I isoforms were not detected until 18 d after anthesis. The BE I class of proteins appear to be subject to coordinated regulation of expression. The WBE-I\(_{AD}\) and WBE-I\(_B\) fractions are not expressed early in en-

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**Figure 5.** The effects of various metabolic intermediates and anions, each at a final concentration of 10 mM, on the activities of wheat endosperm BEs. Aliquots of each isoform containing equal amounts of activity (in the absence of effectors) were included in the assays. BE activity is expressed relative to control activity (absence of added effectors). Solid bars, WBE-I\(_{AD}\); gray bars, WBE-I\(_B\); and open bars, WBE-II. DHAP, Dihydroxyacetone phosphate.

**Figure 6.** Effect of Pi on isoforms of wheat starch BE. ■, WBE-I\(_{AD}\); ○, WBE-I\(_B\); and △, WBE-II. Starch BE activity was assayed using potato amylose as a substrate in the iodine assay in the presence of varied levels of Pi. α-Cyclodextrin was included at a concentration of 2 mM in assays to completely suppress α-amylase activity.
dosperm development but appear during mid-endosperm development and are increasingly strongly expressed in late endosperm development. These results mirror similar findings by Burton et al. (1995), who showed that pea BE I (a maize BE I protein) and BE II (a maize BE I protein) are differentially expressed in the developing pea embryo, and that the BE I protein is expressed later in development than the BE II protein.

Evidence from biochemical studies and from the characterization of mutants in maize, rice, and pea suggests that there are significant differences between class I and class II BEs with respect to their substrate specificities, modes of action, and physiological roles. In this study we present preliminary kinetic evidence of the affinities of starch BE isoforms with potato amylose as a substrate. These data show that the wheat BE I isoforms had higher affinity for amylose than the wheat BE II preparation. These results are consistent with the findings of Takeda et al. (1978), who found that the maize BEs had K_m values for long-chain (CL 405) amylose of 0.13 (BE I) and 0.66 mg/mL (BE II).

The activation of BE activity by anions, Pi, and phosphorylated intermediates differs between the wheat BE I and II classes. This difference in response emphasizes that in addition to the kinetic differences between the BE classes, there also may be important differences between the active sites of the enzymes that may be important to the regulation of both the rate and branching pattern during starch biosynthesis. Although the range of phosphorylated intermediates that activate BE I suggests that there is a broad response to phosphorylated compounds, the concentrations of phosphate required for half-maximal activation of the isoforms are physiologically reasonable. Differences in the action pattern of BE isoforms, arising through distinctions in substrate recognition, cleavage, and chain transfer, are very important to the fine structure of amyllopectin. The results presented in this report raise the possibility that Pi, phosphorylated intermediates, or other effectors can modulate the specificity of the chain-cleavage or chain-linkage reactions of BEs, influencing the fine structure of amyllopectin.

Sequence, mechanistic, and structural considerations have led to the view that starch BEs are members of the α-amylase superfamily of proteins (Jespersen et al., 1993; Burton et al., 1995). The effect of phosphate may be primarily to stabilize the enzyme in an active form in an manner analogous to the chloride activation of some α-amylases. The chloride activation of α-amylase alters maximum reaction rate and not substrate affinity (Levitzki and Steer, 1974), and preliminary investigations of the wheat BEs indicate that phosphate also does not alter substrate affinity.

The respective roles of BE I and BE II in starch biosynthesis have not yet been unambiguously defined. However, the data presented here show that the wheat BE I and BE II enzymes differ in their patterns of expression and kinetic properties. The constant expression level and insensitivity to activation by metabolites suggest that BE II is essentially constitutive, whereas BE I is likely to be important to starch biosynthesis late in endosperm development.

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