Differential Characteristics and Subcellular Localization of Two Starch-branching Enzyme Isoforms Encoded by a Single Gene in Phaseolus vulgaris L.*

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Starch-branching enzymes (SBE) have a dominant role for amylopectin structure as they define chain length and frequency of branch points. We have previously shown that one of the SBE isoforms of kidney bean (Phaseolus vulgaris L.), designated PvSBE2, has a molecular mass (82 kDa) significantly smaller than those reported for isologous SBEs from pea (SBEI), maize (BEIIb), and rice (RBE3). Additionally, in contrast to the dual location of the pea SBEI in both the soluble and starch granule fractions, PvSBE2 was found only in the soluble fraction during seed development. Analysis of a pvsbe2 cDNA suggested that PvSBE2 is generated from a larger precursor with a putative plastid targeting sequence of 156 residues. Here we describe the occurrence of a larger 100-kDa form (LF-PvSBE2) of PvSBE2 found both in the soluble and starch granule fractions of the developing seeds. The determined N-terminal sequence, VKSSHDSD, of LF-PvSBE2 corresponded to a peptide sequence located 111 amino acids upstream from the N terminus of purified PfSBE2, suggesting that LF-PvSBE2 and PfSBE2 are products of the same gene. Analysis of the products by 5'-RACE (rapid amplification of cDNA ends) and reverse transcription PCR indicated that the two transcripts for pre-LF-PvSBE2 and pre-PvSBE2 are generated by alternative splicing. Recombinant LF-PvSBE2 (rLF-PvSBE2) was purified from Escherichia coli and the kinetic properties were compared with those of recombinant PfSBE2 (rPfSBE2). LF-PvSBE2 had much higher affinity for amylopectin (Kₐ = 4.4 mg/ml) than rPfSBE2 (18.4 mg/ml), whereas the Vₘₐₓ of rLF-PvSBE2 (135 units/mg) for this substrate was much lower than that of rPfSBE2 (561 units/mg). These results suggest that the N-terminal extension of LF-PvSBE2 plays a critical role for localization in starch granules by altering its enzymatic properties.

Starch, one of the most important carbon reserves in plants, is composed of two glucan polymers, amylose and amylopectin. Amylose is mainly a linear array of glucosyl residues connected by α-1,4-linkages, whereas amylopectin is a branched polymer of α-1,4-linked glucosyl residues with branched α-1,6-linkages. The biosynthesis of amylose and amylopectin are mediated by the enzymes ADP-glucose pyrophosphorylases, starch synthases, starch-branching enzymes, and starch-debranching enzymes (1, 2).

Starch-branching enzyme (SBE)1 (1,4-α-D-glucan: 1,4-α-D-glucan 6-α-β-(1,4-α-D-glucan)-transferase, EC 2.4.1.18) catalyzes the cleavage of α-1,4-linkages and the subsequent transfer of α-1,6 glucan to form an α-1,6 branch point in amylopectin (3). SBE is a member of the α-amylase family of enzymes, characterized by four highly conserved regions and a central (β/α)₈ barrel domain (4). Apart from the barrel domain, SBEs show considerable structural variation in the length and amino acid sequences at the N- and C-terminal regions. Multiple SBE isoforms have been found in individual plant species and are encoded by two gene families (families A and B) based on the primary sequences. Members of the two families display distinct enzymatic properties, presumably because of the differences in N- and C-terminal regions. Several studies have shown that the N-terminal region is important for specificity of transferred chain length and is required for maximum enzyme activity (5, 6), whereas the C-terminal region is involved in substrate specificity (5, 7).

Starch synthases (SS), except granule-bound starch synthase I (GBSSI), exist as both soluble and starch granule-bound forms (1, 8, 9). Likewise, SBEs from maize (10, 11), pea (8), and wheat (12–14) also occur in both soluble and granule fractions. However, the starch-bound SBEs and SSs are not considered active in starch biosynthesis. Their association with the starch granule has been suggested to be due simply to their being trapped within the starch granule during its growth and maturation (8, 11). Recently a novel granule-bound SBE has been isolated from wheat endosperm (15). This SBE is much larger (152 kDa) than previously characterized family A-type SBEs and contains not only the intact SBEI primary sequence but also an extra N-terminal domain formed by partial duplication of the SBEI sequence located near the N terminus. This N-terminal domain is likely responsible for binding of this SBE isoform to the starch granule.

To investigate the relationships between structure and function of SBEs, as well as its mode of action in starch biosynthesis...
sis, we isolated and characterized a SBE isozyme (PvSBE2) from immature seeds of the kidney bean (*Phaseolus vulgaris* L.) (16). A comparison of the determined N-terminal sequence of purified PvSBE2 to the primary sequence derived from *pvsbe2* cDNA suggested that PvSBE2 was produced from a preprotein with a plastid signal leader sequence of 156 residues (16, 17). Additionally, comparison of the primary sequence of PvSBE2 to other plant SBEs such as pea SBEI (18, 19), maize BEIIb (20, 21), and rice RBE3 (22) showed that despite sharing considerable sequence homology with these other SBEs, PvSBE2 is shorter by about 50–100 amino acid residues (Fig. 1, A and B). Moreover, unlike the pea SBEI, which is observed in both the soluble and starch granule fractions (8), PvSBE2 protein is present only in the soluble fractions during seed development (17). Based on these observations, we suspected the existence of a larger form of PvSBE2 for which the molecular mass would be closer to pea SBEI. Here, we describe the occurrence of a larger form (LF-PvSBE2) of PvSBE2 containing an extended N terminus of 111 residues. This larger enzyme form is observed in both the soluble and starch granule fractions of kidney bean developing seeds. Immunoblot and molecular analyses suggest that there are two possible mechanisms for generating LF-PvSBE2 and PvSBE2: a post-translational modification and alternative splicing. In addition to their different subcellular locations, LF-PvSBE2 and PvSBE2 have distinct enzymatic properties. Based on the results obtained in this study, the relationship between the enzymatic properties and subcellular locations of both isoforms is discussed.

**EXPERIMENTAL PROCEDURES**

**Plant Materials**—Kidney bean (*P. vulgaris* L. cv. toramame) seeds were harvested from plants grown at an experimental field of Hokkaido University in Sapporo, Japan. Seeds were isolated at different stages of development and separated as small (4–8 mm), mid- (10–12 mm), and...
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Isolation of the Partial pvsbe2 Gene Fragment and Nucleotide Sequencing—Genomic DNA was isolated from the expanded leaves of kidney bean plants by the method of Murray and Thompson (28). The DNA was partially digested with Sau3AI and fragments (10–20 kb in size) were inserted into the BamHI site of pEMBL3 (Stratagene). The recombinant DNAs were propagated in E. coli (GIGA pack Gold-III; Stratagene) and grown on Escherichia coli LE392 (P2). The genomic library consisting of 3.6 × 10¹⁰ was screened with a digoxigenin-labeled probe using a digoxigenin DNA labeling kit (Roche Molecular Biochemicals). The probe (about 1.4 kb) was prepared by PCR using genomic DNA as a template and specific primers within the second and third exons of the pvsbe2 gene (5'-CTG TCT TGA AAT ATC TTG GTA CGA AAG G-3'). Based on the cDNA sequence, the methods used for hybridization and detection of the isolated was as described previously (17). The SalI/BglII genomic fragment containing the partial promoter region and the third intron was isolated and sequenced. DNA was sequenced by the dideoxy chain termination method (29) using a Thermosequenase Cycle Sequencing kit (United States Biochemical Corp.).

5′ Rapid Amplification of cDNA Ends (5′-RACE) and Reverse Transcription Polymerase Chain Reaction (RT-PCR)—Total RNA was isolated from small-, mid-, and large-size developing seeds as well as mature seeds of the kidney bean as described previously (17). 5′-RACE was done using total RNA from mid-size developing seeds with a 5′-RACE System for Rapid Amplification of cDNA Ends kit (Version 2.0; Invitrogen) according to the manufacturer’s protocol. The first strand synthesis was primed with the specific primer to pvsbe2 cDNA (945ANT1, 5′-AAT TGG TGG TGA ACC ATC AAT GGT TTG TCT-3′). Terminal deoxynucleotidyl transferase was used to add homopolymeric (C) tails to the 3′-end of the first strand cDNAs. For amplification of target cDNA, the first round PCR was performed with the dCtailed cDNAs as templates using the Abridged Anchor Primer (5′-GGC CAC GCC TGG ACT ACT AGT ACG GGI IGG GII GIG-3′, where I is inosine) and the 945ANT1 primer. These amplified products were then subjected to a second round of PCR with the Abridged Universal Amplification Primer (5′-GGC CAC GCC TGG ACT AGT ACG GGI IGG GII GIG-3′) and the nested specific primer (745ANT1, 5′-ATG CAT CCA GCC GAC CCT CTT GCT G-3′). The amplified DNA was separated on a 2% agarose gel, purified, subcloned into pBluescript II vector (Novagen), and then sequenced.

RT-PCR was performed using an RNA LA-PCR kit (Takara Shuzo, Kyoto, Japan) according to the manufacturer’s protocol. The RT products were prepared from total RNA of small-, mid-, and large-size developing seeds and mature seeds using an oligo-dT adaptor primer and avian myeloblast virus reverse transcriptase. The RT products for pvsbe2 transcripts were amplified using the specific primers (231 SEN, 5′-TCT CAG AAA GAA CAA CTT CTC TC-3′ and 231 Nco, 5′-GCC GAC GCC TGG ACT ACT AGT ACG GGI IGG GII GIG-3′), the amplified DNA was separated on a 2% agarose gel, purified, subcloned into pBluescript II vector (Novagen), and then sequenced.

Production of Recombinant Large Form of PfSBE2 (rLF-PfSBE2) in E. coli—Plasmids for the expression of LF-PfSBE2 protein in E. coli were constructed as follows. A 1,092-bp cDNA fragment was amplified by PCR with LF-SEN (5′-AAT TGG TGG TGA ACC ATC AAT GGT TTG TCT-3′) and LF-Nco (5′-GCC GAC GCC TGG ACT ACT AGT ACG GGI IGG GII GIG-3′) primers, using the pBluescript II SK (pBS-PfSbe2; 17) as a template. The amplified fragment was digested with Nco I, and then cloned into the pBluescript II SK (pBS-PfSbe2; 17) as a template. The amplified fragment was digested with Ncol/KpnI and then cloned together with the KpnI/Sacl fragment from pB8-PvSBE2 into the Ncol/Sacl sites of PET23d (+) (Novagen, Inc.). The resulting plasmid, designated pET-LFPfSBE2, was introduced into E. coli BL21(DE3) strain (Novagen). Crude extract was obtained according to the previously described method (17) from cell cultures grown at 25 °C.

Purification of rLF-PfSBE2—After dialysis against buffer A (20 mM Tris-HCl buffer (pH 7.5) containing 1 mM dithiothreitol and 1 mM EDTA), the crude extract was applied onto a DEAE-Sepharose CL-6B column (2.0 × 11.5 cm). The column was washed with buffer A, and then the enzyme was eluted using a 0–0.4 M NaCl gradient. Active fractions were dialyzed against buffer B (20 mM Tris-HCl buffer (pH 6.8) containing 1 mM dithiothreitol and 1 mM EDTA) and then chromatographed on a DEAE-Sepharose CL-6B column (1.3 × 10 cm) using a 0–0.3 M NaCl gradient. Active fractions were pooled, concentrated, and fractionated on a Mono Q (Pharmacia, Uppsala, Sweden) column (1 × 10 cm) equilibrated with buffer B containing 0.1 mM NaCl. The active fractions were dialyzed against 50 mM sodium phosphate buffer (pH 8.0) containing 1 mM dithiothreitol and 1 mM EDTA and applied to a hydroxyapatite (Seikagaku Corp., Tokyo) column (1.3 × 8.0 cm). The enzyme solution was eluted with a 50–200 mM linear gradient in sodium phosphate buffer.

Assay of SBE Activity—SBE activity was determined by the following two methods. The iodine staining assay (Assay I) was performed ac-
according to the method of Boyer and Preiss (20), which monitors the decrease in absorbance at 660 nm for amylose (potato amylose type III, Sigma) or at 540 nm for amylopectin (potato amylopectin, Sigma) as substrates. One unit of enzyme activity was defined as the amount that decreased the absorbance of 0.1 at 660 or 540 nm/min at 30 °C.

The branching linkage assay (Assay BL) was done by the method of Takeda et al. (25) using reduced amylose as a substrate. One unit of the enzyme activity was defined as 1 H9262 mol of branch linkages formed/min at 30 °C.

**FIG. 3.** Analysis of the 5'-RACE and RT-PCR products and possible alternative splicing for the expression of the pvsbe2 gene. A, the 5'-RACE products (lane P) were subjected to 2% agarose gel electrophoresis. Lane Std, DNA marker containing the 1-kb and 100-bp ladders (Invitrogen). The arrows with “I” and “II” indicate the amplified bands. B, comparison of the nucleotide and deduced amino acid sequences between the 5'-RACE products I and II. The deduced amino acids from the I and II fragments are shown above and below, respectively, each nucleotide sequences. The white letters in black circles correspond to the N termini of mature LP-PvSBE2 (Val) and PvSBE2 (Ile). The lines between sequences I and II represent the specific primers used for RT-PCR (see also legend for panel D). C, schematic illustration of the generation of two mRNAs from the pvsbe2 gene. Each exon is shown by boxes. The open boxes indicate 5'-untranslated regions, and black and gray boxes represent regions translated into putative transit peptides and mature proteins, respectively. Two transcripts with different 5'-ends could be produced by alternative splicing, thereby generating two preproteins with different N-terminal structure. D, analysis of RT-PCR products for pvsbe2 and actin fragments during seed development. The RT products from each developing stage (S, M, L, or Ma) were used as templates. The products were separated on a 1.5% agarose gel. An actin fragment was amplified and used as a control (lower panel). The arrows preceded by “I” and “II” in the upper panel correspond to the amplified DNAs with the reasonable sizes predicted from sequences I and II in panel B, respectively. The middle panel represents the comparative levels estimated from each band strength by NIH Image software as the ratios to the estimated level of amplified fragment (I) in small seeds. E, the relative levels of total transcripts and total protein accumulation estimated by NIH Image software (Figs. 2B and 3D). The closed and open circles indicate total RNA expression by RT-PCR and total protein accumulation profiles by immunoblot, respectively. The gray triangles show the RNA expression profile by Northern analysis (17) and subsequent estimation from the band-strength by NIH Image software. In each profiles, the maximum level is defined as 1.

The branching linkage assay (Assay BL) was done by the method of Takeda et al. (25) using reduced amylose as a substrate. One unit of the enzyme activity was defined as 1 μmol of branch linkages formed/min at 30 °C.

**Analysis of Enzymatic Properties**—pH stability, optimum tempera-
characterization and localization of two SBE isoforms

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**TABLE I**

| Procedure                  | Total protein<sup>a</sup> | Total activity<sup>b</sup> | Specific activity | Purification | Yield % |
|----------------------------|---------------------------|-----------------------------|-------------------|--------------|---------|
| Crude extract              | 7,200                      | 7,040                       | 0.98              | 1            | 100     |
| DEAE-Sepharose CL-6B (1st) | 233                       | 5,140                       | 22                | 22           | 73      |
| DEAE-Sepharose CL-6B (2nd) | 64                        | 3,230                       | 50                | 51           | 46      |
| Bio-Gel F-200               | 19.6                      | 2,840                       | 145               | 148          | 40      |
| Hydroxyapatite             | 9.0                       | 1,530                       | 170               | 173          | 22      |

<sup>a</sup> Protein concentrations were calculated under the assumption that E<sub>1% 280</sub> is 10.

<sup>b</sup> These activities were measured by Assay I method using amylose as a substrate.

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**TABLE II**

The abilities of rLF-PvSBE2, rPvSBE2, and glucoamylase for adsorption to a raw starch

| Raw starch      | Remaining activity |
|-----------------|--------------------|
| None            | 100                |
| 100 mg/ml       | 98.8 ± 1.5         |
| 100 mg/ml       | 98.4 ± 1.5         |
| 100 mg/ml       | 41.2 ± 1.5         |

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**FIG. 4**

SDS-PAGE of purified rLF-PvSBE2 (A) and immunoblot analysis of granule-bound proteins of developing seeds (B). A, Coomassie Brilliant Blue-stained 8% SDS-PAGE gel. Lane Std was loaded with standard proteins; phosphorylase b (97.4 kDa), BSA (66.2 kDa), and ovalbumin (45.0 kDa). Lanes 1 and 2 are purified rPvSBE2 and rLF-PvSBE2, respectively. B, proteins from granule fractions of developing seeds were separated by SDS-PAGE on 8% gel and then transferred to a nitrocellulose membrane. The membrane was incubated with anti-rPvSBE2. Lane rLF, purified rLF-PvSBE2 (0.35 μg); lane M, proteins from 5 mg of starch granule of mid-size seeds.

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**RESULTS**

**Occurrence of an Alternative Form for PvSBE2**—Immunoblot analysis of the soluble and starch granule fractions prepared from developing kidney bean seeds (mid-size) showed that anti-rPvSBE2 reacted with the 82-kDa PvSBE2 located in the soluble fraction (Fig. 1C, lane SF). PvSBE2 was not detected in the starch granule fraction, although a 100-kDa protein associated with the starch granule fraction was readily evident (Fig. 1C, lane GF). To determine the nature of the 100-kDa starch granule-associated protein, its N-terminal sequence was determined by Edman degradation (Fig. 1D). The generated amino acid sequence, VKSSHDSD, aligns with the primary sequence deduced from the *pvsbe2* gene.
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Distribution and Amounts of LF-PvSBE2 and PvSBE2 during Seed Development—The spatial localization of LF-PvSBE2 and PvSBE2 during the development of kidney bean seeds was examined by immunoblot analysis (Fig. 2A). PvSBE2 was detected only in soluble fractions from small-, mid-, and large-size developing seeds but not in mature seeds. In contrast, LF-PvSBE2 was found mostly in the starch granule and soluble fractions at the latter stages of seed development when the rates of starch biosynthesis are at their maximal levels and then begin to slow down. The relative amounts of each SBE2 isoform were estimated from the intensities of known amounts of loaded rPvSBE2 protein (Fig. 2B). The levels of PvSBE2 protein peak at the mid-stage of seed development and then drop off at later stages when starch levels begin to increase rapidly. In contrast, the levels of LF-PvSBE2 protein parallel the increase in starch levels (17). They are relatively low in mid-developing seeds and then increase dramatically at the later stage when starch levels also increase. More than 75% of the total SBE2 protein is contributed by LF-PvSBE2 protein, whereas PvSBE2 comprises less than 25%. About half of the amount of LF-PvSBE2 was located in the starch granule fractions at the later stages of seed development. These results indicate that kidney bean seeds contain two SBE2 forms: a smaller PvSBE2 isoform, which attains maximum levels when starch levels begin to increase, and a larger LF-PvSBE2 isoform in which the temporal accumulation pattern coincides with starch synthesis.

Generation of Two Transcripts by Alternative Splicing—To determine whether LF-PvSBE2 and PvSBE2 were differently spliced products of the same gene, 5′-RACE was performed using total RNA from mid-size developing seeds of kidney bean (Fig. 3A). Two distinct bands of about 0.75 and 0.55 kb (denoted as I and II, respectively, in Fig. 3A) were detected in the 5′-RACE products, which were sequenced together with a partial psbe2 gene fragment corresponding to the 5′ coding sequences. The nucleotide sequence alignment between the two amplified cDNAs and the partial gene fragment demonstrated that both amplified cDNAs were derived from the same psbe2 gene and that the two transcripts were generated by alternative splicing (Fig. 3, B and C). The sequence of the larger RNA transcript I was identical to the previously determined psbe2 cDNA. In contrast, the sequence of the smaller transcript II was composed of truncated exons 1 (exon 1′) and 2 (exon 2′) as well as intact exon 3 at the 5′-end, suggesting that the translation commences with the Met residue at position 99 of the derived psbe2 primary sequence. Overall, pre-LF-PvSBE2 and Pvsbe2 are coded by two distinct RNA transcripts formed by alternative splicing of introns during transcription.

To access the relative levels of the two psbe2 transcripts during kidney bean seed development, RT-PCR was performed using RNA isolated from seeds at different stages of development (Fig. 3D). The amplified DNA products derived from actin RNA were constant during seed development and used as an internal control (Fig. 3D, lower panel). Two amplified bands corresponding to transcripts I and II were evident at all stages of seed development although their temporal accumulation patterns were distinct. Transcript I peaked during the midstage of seed development, whereas transcript II showed high levels at the mid- and late-stages of seed development (Fig. 3D, upper and middle panels). The temporal RNA accumulation patterns display by transcript I (pre-LF-PvSBE2) and II (pre-

PvSBE2) were different from those observed for the SBE2 isoforms (Fig. 3E), suggesting that the regulation of the two SBE2 isoforms occurs post-translationally.

Preparation of Recombinant LF-PvSBE2—rLF-PvSBE2 enzyme was produced in the E. coli cells and purified by four successive column chromatography steps (Table I) in order to investigate its enzymatic properties. The final enzyme preparation from 1.2 liters of cultured cells contained 9.0 mg of rLF-PvSBE2 with a specific activity of 170 units/mg using amylose as a substrate. The specific activity was about 80% of that (214 units/mg) measured for purified rPvSBE2. The specific activity of purified rPvSBE2 under the same assay conditions was nearly identical (223 units/mg) to that of the native PvSBE2 purified from immature seeds (16). Purified rLF-PvSBE2 migrated as a single polypeptide band with the same mobility as the immunoreacted protein from starch granule fractions on SDS-PAGE (Fig. 4, A and B).

The estimated molecular mass (100 kDa) of purified rLF-PvSBE2 obtained by SDS-PAGE was significantly larger than the 93.8 kDa predicted from the deduced primary sequence. Such electrophoretic behavior on SDS-PAGE has been also observed for the pea SBEs and for another kidney bean SBE.
protein (17, 19). Unlike rLF-PvSBE2, the estimated molecular mass (82 kDa) of rPvSBE2 agrees well with that predicted from the primary sequence. The anomalous electrophoretic behavior of the pea SBEs has been suggested to result from the high content of negatively charge residues. Indeed, about 21% of the extra 111 amino acids of rLF-PvSBE2 consist of negative charged residues (14 Asp and 9 Glu). These observations suggest that the additional N-terminal domain of rLF-PvSBE2 is responsible for its higher than expected mobility on SDS-polyacrylamide gels than if based on molecular size alone.

**Starch Binding Ability of rLF-PvSBE2**—Because the subcellular distribution profiles of LF-PvSBE2 and PvSBE2 suggested that the N-terminal region of LF-PvSBE2 participates in the interaction with starch granules, we examined whether this protein had the capacity to bind to raw starch (Table II). This was accomplished by incubating rLF-PvSBE2 (as well as rPvSBE2 to serve as a control) with raw starch and then assaying for the amount of SBE activity remaining in solution after removal of the starch granules by centrifugation. Such assays showed no significant amount of binding by either SBE form. In contrast, glucomylase, which is capable of binding directly to raw starch (30, 31), showed a 59% decrease in enzyme activity in the soluble fraction under the same conditions. These results suggest that rLF-PvSBE2 is unable to directly bind to raw starch in vitro.

**Enzymatic Properties of rLF-PvSBE2**—To determine the effect of the N-terminal extension in rLF-PvSBE2 on enzyme function, the physical properties of purified rLF-PvSBE2 were measured and compared with those of rPvSBE2 (Figs. 5 and 6). rLF-PvSBE2 was fairly stable between pH 6.5 and 9.5, as more measured and compared with those of rPvSBE2 (Figs. 5 and 6). rLF-PvSBE2 was fairly stable between pH 6.5 and 9.5, as more measured and compared with those of rPvSBE2 (Figs. 5 and 6). rLF-PvSBE2 and rPvSBE2 were most active at around 30 °C. Nevertheless, almost no inactivation occurred when the enzymes were kept at pH 8.0 for 15 min at up to 45 °C for rLF-PvSBE2 as compared with a 50 °C level for rPvSBE2. The inconsistent data between the optimum temperature and thermal stability have been interpreted as the temperature-dependent stability of a secondary and tertiary structure of amylose as a substrate and compared with those obtained for rPvSBE2 (Table III). Lineweaver-Burk plots of the reaction catalyzed by rLF-PvSBE2 and rPvSBE2 showed Michaelis constants (Km) for amylose to be 4.80 and 1.27 mg/ml, respectively, and the maximum velocities (Vmax) to be 396 and 242 units/mg protein, respectively. In contrast, the Km and Vmax values for amylopectin were calculated to be 4.4 mg/ml and 135 units/mg protein for rPvSBE2 and 18.4 mg/ml and 561 units/mg protein for rPvSBE2. The Km and Vmax values for amylopectin by rLF-PvSBE2 are about one-quarter of those of rPvSBE2. This indicates that the Lineweaver-Burk plots of the reactions catalyzed by rLF-PvSBE2 and rPvSBE2 are parallel. An alternative way of looking at these enzymes is that the reactions catalyzed by rLF-PvSBE2 and rPvSBE2 are uncompetitive or competitive type of that catalyzed by rPvSBE2. Hence, the nonlinear kinetics exhibited by rLF-PvSBE2 reaction is due to competitive product inhibition. The Vmax/Km value for amylopectin is almost identical for rLF-PvSBE2 and rPvSBE2, indicating that both enzymes can act comparably on amylopectin or amylopectin-like glucans at a low concentration of the substrate. However, since it is reasonable to consider that the concentration of amylopectin in developing seeds is far higher than that in our assay, both isoforms with different kinetic parameters contribute to in vivo amylopectin biosynthesis.

**DISCUSSION**

SBE isoforms have a dominant role in determining the structure of amylopectin. Our previous data have shown that PvSBE2, one of the SBE isoforms detected in kidney bean seeds, is classified as a family A type on the basis of enzymatic properties and the predicted primary sequence (16, 17). This PvSBE2 protein was observed only in the soluble fraction during seed development (17). The data presented in this study show that a larger form of PvSBE2, LF-PvSBE2, exists that contains an extended N-terminal region. Unlike the soluble location of PvSBE2, LF-PvSBE2 is observed in both the soluble and starch granule fractions. Our studies also show that the extended N-terminal region in LF-PvSBE2 alters not only its subcellular location but also its kinetic properties as well. The two isoforms are encoded by the same gene, which produces two distinct transcripts generated by alternative splicing of the first two exons.

Although the pvsbe2 cDNA encodes a open reading frame similar in length to the pea sbcl cDNA, and the deduced amino acid sequences share a high homology each other, purified PvSBE2 from immature seeds of the kidney bean was significantly shorter than pea SBEI (8, 16). Additionally, PvSBE2
A hypothetical model for generation and localization of LF-PvSBE2 and PvSBE2 during amylopectin synthesis. Two precursors translated from alternative spliced RNAs would be localized to the plastid stroma. PvSBE2 could be also converted from LF-PvSBE2 by an unidentified processing activity (broken arrow). Because of the catalytic properties due to the extensive N-terminal region, LF-PvSBE2 distributes to the starch granule and soluble fractions. In contrast, PvSBE2 without the extensive N-terminal region is localized only in the soluble fraction.

A comparison of N-terminal primary sequences between family A and B SBEs has indicated that there is an extra flexible domain in some family A SBEs such as pea SBEI, maize BEIIb, and rice RBE3. Burton et al. (19) predicted that this extra domain might be involved in the interactions between SBE and starch or in determining the type of glucan chain the enzyme can utilize as a substrate. The N-terminal extension of LF-PvSBE2 corresponds precisely to this extra domain seen in these other SBEs. The difference in subcellular localization between LF-PvSBE2 and PvSBE2 (Fig. 2A) indicates that the N-terminal domain is responsible for the association with the starch granule. However, since rLF-PvSBE2 does not directly bind to raw starch in vitro (Table II) and lacks a starch-binding domain (29, 30, 36), it is unlikely that LF-PvSBE2 binds directly to the starch granule. Additionally, rLF-PvSBE2 does not bind stably to amylopectin-like glucans formed during the in vitro branching assay described in Fig. 6B (data not shown). Despite the apparent lack of direct binding of rLF-PvSBE2 to starch, we propose an explanation that can account for the association of LF-PvSBE2 with starch granules during seed development. One possibility is that LF-PvSBE2 becomes buried within the growing amylopectin structure as it becomes converted into a crystallized starch granule because of its high affinity for amylopectin but low catalytic turnover (Fig. 6B and Table III). This may provide a basis for the distribution of LF-PvSBE2 to soluble and starch granule fractions, particularly in the late-stage of seed development. This explanation is consistent with the hypothesis that entrapment of maize SSI within the starch granule is affected by its high affinity for a longer glucan (37).

It has been predicted from a comparison of primary sequences between SSs and granule-bound starch synthases or bacterial glycogen synthases that SSs also contain a flexible polypeptide...
N-terminal domain (38). Analyses of the recombinant truncated N-terminal forms of pea SSII (39) and maize SSII (40) revealed that the flexible domain does not participate in the catalysis but may be involved in regulating the association between enzymes and α-glucans, and/or in partitioning between soluble and granule-bound phases. In particular, the truncated form of maize SSII has reduced affinity for amylopectin, compared with the extended form (40). Additionally, in developing pea embryo, there are truncated and native forms of SSII in both soluble and granule fractions (39). These characteristics of native and truncated SSs are similar to those of rLF-PvSBE2 and rPvSBE2, suggesting the possibility that branching enzyme activity is coordinated with SS polymerizing activity. The possible interaction between SBEs and SSs has been suggested from the analysis of the amyllose-extender mutants of maize (41) and rice (42), where genetic disruption results not only in a loss or decrease of branching enzyme but also of starch synthase activity as well.

Comparison of the enzymatic properties between rLF-PvSBE2 and rPvSBE2 suggests that the N-terminal extension of LF-PvSBE2 has a significant effect on the kinetic parameters but is not essential for enzyme catalysis (Figs. 5 and 6 and Table III). A similar situation was also reported for the maize BEIIb (6); the truncated form lacking the N-terminal 39 residues of mature maize BEIIb not only possessed about 70% net catalytic activity but also showed a chain transfer pattern identical to that obtained for the mature enzyme. These observations indicate that the 39 residues of the N terminus of maize BEIIb are not required for catalysis. Interestingly, the N-terminal of the truncated form of maize BEIIb is very close to that of PvSBE2.

Results from this study lead to a hypothetical model for production and distribution of LF-PvSBE2 and PvSBE2 (Fig. 7). The psbse2 gene transcribes two different mRNAs by alternative splicing, and the protein products of this gene are targeted to plastids. Because of their enzymatic properties, part of LF-PvSBE2 is entrapped within starch granule, whereas PvSBE2 distributes only in soluble phase. In the mid-stage, when starch biosynthesis begins to be promoted, PvSBE2 isoform, in which the enzymatic properties are affected only slightly by changes in the concentration and structure of the substrate, is mainly responsible for formation of α-1,6 branch points in amylopectin biosynthesis. The conversion of LF-PvSBE2 to PvSBE2 by an unidentified processing enzyme activity is probably important for the effective increase in the number of nonreducing ends for starch syntheses. In the later stage, the level of PvSBE2 protein reduces in concert with the transcript level, and possibly the processing enzyme activity is also decreased. Instead, LF-PvSBE2 protein not only is entrapped within the starch granule but also becomes accumulated in the soluble phase.

This study demonstrates that two SBE isoforms encoded by a single gene have different subcellular localization and protein accumulation profiles as well as distinct enzymatic properties. Our results shed new insights on our understanding of the regulatory mechanism of amylopectin biosynthesis. A proteolytic processing enzyme will be particular significant because the enzyme could control the activities and localization of not only SBES but also SSs by the removal of their N-terminal domains.

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