Two starch-branching-enzyme isoforms occur in different fractions of developing seeds of kidney bean

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The nature and enzymic properties of starch-branching enzyme (SBE) are two of the dominant factors influencing the fine structure of starch. To understand the role of this enzyme's activity in the formation of starch in kidney bean (*Phaseolus vulgaris* L.), a study was undertaken to identify the major SBE sequences expressed during seed development and to characterize the enzymic properties of the coded recombinant enzymes. Two SBE cDNA species (designated *pvsbe2* and *pvsbe1*) that displayed significant similarity (more than 70%) to other family A and B SBEs respectively were isolated. Northern blot analysis revealed that *pvsbe1* and *pvsbe2* were differentially expressed during seed development. *pvsbe2* showed maximum steady-state transcript levels at the mid-stage of seed maturation, whereas *pvsbe1* reached peak levels at a later stage. Western blot analysis with antisera raised against both recombinant proteins (rPvSBE1 and rPvSBE2) showed that these two SBEs were located in different

amyloplast fractions of developing seeds of kidney bean. PvSBE2 was present in the soluble fraction, whereas PvSBE1 was associated with the starch granule fraction. The differences in location suggest that these two SBE isoenzymes have different roles in amylopectin synthesis in kidney bean seeds. rPvSBE1 and rPvSBE2 were purified from *Escherichia coli* and their kinetic properties were determined. The affinity of rPvSBE2 for amylose (K_m 1.27 mg/ml) was lower than that of rPvSBE1 (0.46 mg/ml). The activity of rPvSBE2 was stimulated more than 3-fold in the presence of 0.3 M citrate, whereas rPvSBE1 activity was not affected. The implications of the enzymic properties and the distribution of SBEs and amylopectin structure are discussed.

Key words: amylopectin chain length, *Phaseolus vulgaris*, stimulation with citrate, starch biosynthesis.

INTRODUCTION

Starch is a water-insoluble granule consisting of crystalline and amorphous regions. It is predominantly composed of two α -glucan types, amylose and amylopectin. Amylose is essentially a linear form composed of α -1,4-glucosidic linkages with rare (0.3–0.7%) α -1,6 branched points. Amylopectin has a much larger degree of polymerization (dp) in the range 50 000–500 000, is highly branched (4–5%) and has average chain lengths of 20–25 glucose residues [1]. Amylopectin is responsible for the crystalline structure of the starch granule [2].

Starches from different plants show different degrees of branching and distribution lengths. The enzyme responsible for the formation of the α -1,6 branch points is starch-branching enzyme [SBE; 1,4- α -D-glucan:1,4- α -D-glucan 6- α -D-(1,4- α -D-glucano)-transferase, EC 2.4.1.18]. SBE exists as multiple isoforms whose enzymic and physical properties are probably an important factor in determining amylopectin structure. However, the SBEs alone are not sufficient to dictate starch structure. Heterologous expression of the maize branching enzymes I and II (BEI and BEII) in branching-enzyme-deficient *Escherichia coli* resulted in the formation of a polymer with a structure greatly

different from that of amylopectin [3]. Similar observations were made when different combinations of maize starch synthases and SBEs were co-expressed in bacterial cells [4]. These observations in *E. coli* indicate that the function of starch synthase and SBE is essential for amylopectin biosynthesis but that other factors are also required for the formation of semicrystalline amylopectin [5–7].

A sequence comparison of SBEs from different plants indicates that they can be classified into two families, A and B. Family A SBEs show a lower affinity for amylose and preferentially transfer shorter chains than do SBEs of family B [8]. The SBE gene families also show different temporal patterns during endosperm or seed development. In most instances, family A and B SBE transcripts accumulate in the middle and late stages respectively of seed development [9–12]. Thus multiple forms of SBE with different temporal expression patterns might be an important factor in the synthesis of variegated amylopectin with chains of different lengths and branch points.

Mutants lacking an SBE isoform have been identified in maize [13–15] and rice [16]. These *ae* (amylose-extender) mutations correspond to the *rugosus* (*r*) gene, which confers a wrinkled phenotype in pea seeds (first described by Gregor Mendel) [17].

Abbreviations used: dp, degree of polymerization; BEI and BEII, maize branching enzymes I and II; DTT, dithiothreitol; HPAEC–PAD, high-performance anion-exchange chromatography–pulsed amperometric detector; PvSBE, kidney bean (*Phaseolus vulgaris* L.) SBE; rPvSBE, recombinant PvSBE; SBE, starch-branching enzyme.

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The nucleotide sequence data reported will appear in DDBJ, EMBL and GenBank® Nucleotide Sequence Databases under the accession numbers AB029548 (*pvsbe2*) and AB029549 (*pvsbe1*). The cDNA species *pvsbe2* and *pvsbe1* correspond to *kbe1* and *kbe3* on the databases.

The *ae* and *r* genes encode family A SBEs and their absence results in the accumulation of higher proportions of amylose and loosely branched polysaccharides. Similar results have also been observed in transgenic potato plants with decreased family A SBE activity [18]. No mutants of family B SBE have yet been reported. However, in transgenic potato expressing an anti-sense family B SBE, the transcripts and proteins were markedly decreased but no significant changes in amylose content and amylopectin structure were evident [19].

In our previous study [20] we reported the purification and properties of two SBE isoforms, PvSBE2 and PvSBE2-1, from the soluble fractions of kidney bean (*Phaseolus vulgaris* L.) seeds (in accordance with the nomenclature of SBEs [21], KBE1 and KBE2 in the original manuscript are here renamed PvSBE2 and PvSBE2-1 respectively). The two SBE isoforms, which had identical N-terminal amino acid sequences, displayed enzymic properties characteristic of family A SBEs. A comparison of N-terminal sequences of PvSBE2 with those of other plant SBEs revealed that PvSBE2 is shorter by approx. 100 amino acid residues. Although isoforms corresponding to family B SBEs have been purified from the soluble fractions of maize [22], rice [23], pea [24] and barley [25] seeds, a comparable form has not been detected in kidney bean seeds. Similarly, only one major SBE activity has been found in potato, although a second minor form has been detected [18,26]. The aim in the present study was to determine the basis for the shorter N-terminus of PvSBE2 and to elucidate the occurrence of family B isoform(s) in developing kidney bean seeds. Here we describe the isolation and expression of cDNA clones encoding A and B family SBEs. The two SBEs possess different catalytic properties and substrate preferences and are expressed at different stages of seed development. Unlike the SBEs from pea embryos, the two SBEs of kidney bean seeds are localized in different compartments of the amyloplast. Hence the unique branching properties of kidney bean starch are not only due to the unique catalytic properties of the SBE isoforms but are also a product of differences in gene expression at the RNA and in intracellular locations.

MATERIALS AND METHODS

Plant materials

Kidney bean (*Phaseolus vulgaris* L., cv. Toramame) plants were grown in a field at Hokkaido University (Japan). The developing seeds were harvested at several stages of development and stored in liquid nitrogen until use.

Protein sequences

Native PvSBE2 protein was purified from immature seeds (10–14 mm) as described previously [20]. The digestion of enzyme with lysyl endopeptidase, separation of the peptides and sequencing of some peptides were performed as reported previously [27]. The N-terminal sequences of purified recombinant PvSBE isoforms (rPvSBE1 and rPvSBE2) and PvSBE1 protein identified in the starch granule fraction were determined with a protein sequencer after the polypeptides had been transferred to a PVDF membrane [28] from an SDS/PAGE gel.

Oligonucleotides

The oligonucleotides used in this study are listed in Table 1.

RNA isolation and reverse-transcriptase-mediated PCR

Total RNA was isolated from developing kidney bean seeds, leaves, stems and roots by the standard phenol/SDS method [29]. Poly(A)⁺ RNA was prepared from total RNA of the immature seeds (10–14 mm) with an oligo(dT)-cellulose column [30].

Reverse-transcriptase-mediated PCR for the preparation of each probe was performed with an RNA LA-PCR kit (Takara Shuzo) and oligonucleotide primers as described in the Results section. PCR products were cloned into pBluescript II SK(+) (Stratagene Cloning Systems, La Jolla, CA, U.S.A.) and sequenced on both strands with a Li-Cor 4000 automated DNA sequencer (Aloka, Tokyo, Japan).

Construction and screening of cDNA library

Double-stranded cDNA was synthesized by oligo(dT) priming from poly(A)⁺ RNA prepared from developing seeds (10–14 mm) with a cDNA synthesis kit (Amersham-Pharmacia Biotech, Tokyo, Japan). The synthesized cDNA was ligated to *EcoRI*–*NotI*–*BamHI* adaptors (5'-AATTCGGCGGCCGCGG-ATCC-3' and 5'-GGATCCGCGGCCGCGG-3') and then inserted into the *EcoRI* site of the λ gt10 phage vector (Stratagene). The recombinant DNA species were packaged into bacteriophage particles with a Gigapack Gold III packaging kit (Stratagene). Each probe for screening of the library was prepared

Table 1 Oligonucleotide primers used to isolate cDNA clones and to create truncated forms of the *pvsbe2* and *pvsbe1* primary sequences

The location of each primer other than the anchor primer is shown in Figure 2.

Primer	Sequence (5' → 3')	Comment
Anchor	GTTTCCAGTCACGAC	Supplied by an RNA LA-PCR kit (Takara Shuzo)
SBE2-N	GCIGGICARAARATITAYGARATIGAYCC	Based on the N-terminal amino acid sequence of native PvSBE2
SBE2-K23	TTYGGITAYCAYGTIACIAAYTTYTYGC	Based on the lysyl-ended peptide sequence of native PvSBE2
SBE2-K24	GGICCCRAAYTCRTTICCCATRAART	Based on the lysyl-ended peptide sequence of native PvSBE2
SBE2-sense	TTTGATGGITTYMGATTGATGGIGTIAC	Based on the consensus amino acid sequences of amylolytic enzymes
GSP-1	GGTTGATGCTTCTCCTATTGTTGAG	Based on the <i>pvsbe1</i> cDNA sequence
GPS-2	GATACACTTCATAACCATGGCATTG	Based on the <i>pvsbe1</i> cDNA sequence
Exp-2	GCTAAACCCATGGTCATACCCGAC	For expression of the <i>pvsbe2</i> cDNA
SBE2-A	GACTTAGGATCCTCTGGGGTTCC	For expression of the <i>pvsbe2</i> cDNA
Exp-1	GTTTCAAGGGTGCCATGGTTATGACAGATG	For expression of the <i>pvsbe1</i> cDNA
SBE1-A	GGGATGCGATCAACCAACTCCATC	For expression of the <i>pvsbe1</i> cDNA

Figure 1 Restriction-enzyme physical maps of *pvsbe2* and *pvsbe1* cDNA species and construction of expression vectors in *E. coli*

The solid bars indicate the 5' and 3' untranslated regions of each gene. The labelled arrows show the primers used for the amplification of fragments. The 2a and 2b fragments and the 1a and 1b fragments were used for constructing expression vectors (see also the Materials and methods section).

from cloned PCR fragments and labelled with a digoxigenin DNA-labelling kit (Boehringer Mannheim, Germany).

The phage plaques of the cDNA library were transferred to duplicate nylon membranes (Hybond N⁺; Amersham). The membranes were treated with the digoxigenin-labelled probe in the standard hybridization buffer (Boehringer) at 60 °C for 16 h. They were rinsed twice with 2 × SSC/0.1 % SDS at room temperature for 5 min each, then twice with 0.1 × SSC/0.1 % SDS at 68 °C for 15 min each. Detection of the signal was performed with a digoxigenin luminescent detection kit in accordance with the instructions of the supplier (Boehringer). The cDNA inserts of the positive clones were subcloned into pBluescript II SK(+) after digestion of the purified phage DNA species with *NotI*. The plasmids carrying the *pvsbe1* and *pvsbe2* cDNA species were named pBS-PvSbe1 and pBS-PvSbe2 respectively.

Northern blot analysis

Total RNA (15 µg) isolated from developing seeds at different stages was fractionated on a formaldehyde/1 % (w/v) agarose gel and transferred to a nylon membrane. The probes were prepared by PCR with a digoxigenin-dNTP labelling mixture (Boehringer), the SBE2-sense and SBE2-K24 primers and the cDNA clone as a template. The membrane was incubated with the digoxigenin-labelled probe in standard buffer containing 50 % (v/v) formamide (Boehringer) at 50 °C for 16 h. Membrane washing and detection of the signal were performed as described in the previous section.

Expression in *E. coli* cells

Plasmids for the expression of *pvsbe2* and *pvsbe1* cDNA species in *E. coli* cells were constructed as follows (Figure 1).

pET-PvSbe2

The *NcoI/KpnI*-1a fragment was prepared from a PCR-amplified DNA by using the Exp-2 and SBE2-A primers and pBS-PvSbe2

as template. The fragment was cloned with the *KpnI/SacI*-2b fragment from pBS-PvSbe2 plasmid into the *NcoI/SacI* sites of pET23d(+) (Novagen, Madison, WI, U.S.A.); the resulting plasmid was termed pET-PvSbe2.

pET-PvSbe1

The *NcoI/HindIII*-1a fragment was made from PCR products by using the Exp-1 and SBE1-A primers and pBS-PvSbe1 as a template. This fragment, together with the *HindIII/EcoRI*-1b fragment from pBS-PvSbe1, was inserted into the *NcoI/EcoRI* sites of pET-23d(+) to construct plasmid pET-PvSbe1. *E. coli* BL21 (DE3) strain (Novagen) was transformed with plasmid pET-PvSbe2 or pET-PvSbe1 and the transformants were designated *E. coli*-PvSBE2 and *E. coli*-PvSBE1 respectively.

Assay of SBE activity

SBE activity was determined by the following three methods. The iodine-staining assay (assay I) was performed as described by Boyer and Preiss [31], which monitors the decrease in A_{660} for amylose (potato amylose type III; Sigma) as a substrate or in A_{540} for amylopectin (potato amylopectin; Sigma) in the presence of iodine solution. One unit of enzyme activity was defined as the amount of enzyme yielding a decrease in A_{660} or A_{540} of 0.1 per minute at 30 °C.

The phosphorylase *a* stimulation assay (assay P) was also performed by the method of Boyer and Preiss [31]. One unit of enzyme activity was defined as the amount of enzyme incorporating 1 µmol of glucose into the methanol precipitate per minute at 30 °C.

To determine the number of branching points introduced by SBE, the branching linkage assay (assay BL) was performed by the methods of Takeda et al. [8], with reduced amylose as a substrate. One unit of enzyme activity was defined as the amount of enzyme forming 1 µmol of branch linkages per minute at 30 °C.

(A) 1 catcaatgtacgcttctctcggacccctgaacacagcacactcgaacttttggatcgtttttctgctctcggaggaacatcttcaatcttccctcga
 101 atctcaagaactaaacctatccgcagcgaagatgggtttacacactcctgggaattcgaatttcggcgtgtttcttgcgtgcacacactcgaactcctcga
 1 M V Y T I S G I R F P A V L S L H N S T L R G
 201 CGATGCGAGGCGAGCCTCTCTCCAGTTTCTCAGAAAGACAACTTCTCTGTAATACTCTGCTGTAATACTCTCTCATGATTCTGATTCCCGCTCT
 24 D R R A A S L P V F L R K N N F S R K I L A V K S S H D S D S P S
 301 TCTGCAATCGCTGAATCTGATAAGTGCTTATCTCTCAAGATCATGATAACTCTGCACTTTTGACGAGTCAACTTGAACCTCTGTTATAACATCACTGG
 57 S A I A E S D K V L I P Q D H D N S A S L T D Q L E T P V I T S V
 401 ATGCACATACTAGAGGATCTAACCATGGAAGATGAGGATAAATACACATAGGTGAAGCAGATAGCAGTTACAGACAGATTGAAGATGGCGTAGGTTC
 90 D A H N L E D L T M E D E D K Y N I G E A D S S Y R Q I E D G L G S
 501 TGTTCATCATCAACCATAGATGTTGATATCCAGCCAAGACATCGGTATCTGTTGCAAGGAAGTAAAGATACCAAGTGTGAAGCTAAACCTAAG
 124 V A S S P V D V D I P A K K T S V S V G K E V K I P S V E A K P K
 SBE2-N
 601 ATCATACCCGACCTGGAGCTGGGCGAGAAATATATGAGATTGATCCATCTTTGCTAGCCTATCTGTAACCTTGTATTCGCTTTTGGCAATACAA
 157 S I P R P G A G Q K I Y E I D P S L L A Y R D H L D F R F G Q Y K
 701 GATTACATGATAAATTAACAGCATGAAGTGGCTGGATGATTTTCTCGGGCTATGAACAAATTTGGCTTCTACGAGTGTACAGCATTAATTA
 170 N L H D E I N K H E G G L D A F S R G Y E Q F G F L R S A T G I T T
 801 TAGAGATGGGACCTTGGAGCTAAGTCAGCAGCATTAATGGAGCTTCAACAAATGGAATCCAAATGCGAGTGTAAATGACTCGGAATGAGTTGGTGT
 224 R E W A P G A K S A A L I G D F N N N N P N A D V M T R N E F G V
 901 TGGGAGATTTTCTGCAAAATGATGGTTCACCAACCAATTCCTCATGGTCTCGGGTCAAGATCCGATGGGATCTCCCTCTGGAATCAAGAT
 257 W E I F L P N N V D G S P P I P H G S R V K I R M D T P S G I K D
 1001 CCATTCCTCATGATGATAAATTTCTGTACAGGCTCCTGGTGAATTCATATAGTGAATATATCTATGATCCCGCAGAGGAAATATGTTCTCA
 290 S I P A W I K F S V Q A P G E I P Y S G I Y Y D P P E E E K Y V F K
 1101 ACATCCACAGCCAAAGAAACAAATCACTTAGAATATACAGTCAATGTTGGAATGAGTATCCGAGCCAAATCAATCATATGCAATTTTGA
 324 H P Q P K K P K S P R I Y E S H Y G M S S P F P K I N T Y A N F R
 SBE2-K3
 1201 GATGATGATACCTCGCATTAAGAGCTTGGCTATATAGTCTGTCAGATATGGCTATCCAGAACATCTTATACCGACGCTTGGTGGTACCATGTA
 357 D D V L P R I K K L G Y N A V Q I M A I Q E H S Y Y A S F G Y H V
 SBE2-A
 1301 CAAATTTCTTGGACCTAGTAGTGTGATTTGAACCCAGAGGATCTTAAGTCTATGATAGACAAAGCCATGAACATGAGTCTGCTTCTCTGATGAT
 390 T N F F A P S S R F G T P E D L K S M I D K A H E L G L L V L M D I
 1401 TGTACAGATCATCATCAATTAATACATGGATGGGCTAAACATGTTTGTATGGAATGATGGTCACTTCCATCTCCGCTGAGGATGCAAGGATATCATTTG
 424 V H E N S S N N T L D G L N M F D G T D G H Y F H P G S R G Y H
 1501 ATGTGGGATCTCGCTTTTCAATATGGAAGCTGGGAAGTATTAAGTATCTACTTCAATCCGAGATGCTGGCTGGATGAATACAAATTTACGAT
 457 M W D S R L F N Y G S W E V L R Y L L S N A R W W L D E Y K F D S
 SBE2-long
 1601 TCGATTGATGTTGTTATCAATCATGAT
 490 F R P D G V T S M M Y T H G L Q V A F T G N Y S E Y F G L A T D V
 1701 TGATGCTGTGGTTTACCTGATGCTGGCTAATGATCTCATTCAGGATCTTCCCTGAGGCACTTACCATTGGTGAAGATGTTGATGGAATGCAACATTC
 524 D A V V Y L M L A N D L I H G L F P E A V T I G E D V S G M P T F
 1801 TGCCTTCTACCAAGATGCTGGGCTGGTTTGGATTATGCTGCTGCAATGGCCATTCAGACAAAGTGGATTGAGATCTCAAGAAAGCATGAGGACT
 557 C L P T Q D G G V G F D Y R L Q M A I A D K W I E I L K K Q D E D
 1901 GGAATATGGGTGATATTGTGCAACACTAACAAAGAGTGGCTGGAAATATGTTAGCTTATGCTGAGGATCATGACCGGCTTGGTGGTGACAA
 590 W K N G D I V H T L T N R R W L E K C V A Y A R P N D Q A L V G D K
 2001 GACAATTCATTTTGGTGTATGGAAGATATGAT
 624 T I A F W L M D K D M Y D F M S L D R P A T P R I D R G I A L H K
 SBE2-K24
 2101 ATGATTAGGCTTATACCATGGGACTTGGTGGTGAAGGATATTAATTTTATGGGAATGAGTTGGTCACTCTGAGTGGATTGATTTCCCAAGGGGTG
 657 M I R L I F M S L G S E S Y L N F M G N F F G R E F E F F R G
 2201 AACCAACACTCTTAATGGCTCTGTAATTCAGGGAACACTACAGTATGATAAATGAGGCTAGATTGACTTGGGAGATGCAAGATTTAAGATA
 690 E Q Q L P N G S V I P G N N Y S Y D K C R R R F D L G D A D Y L R Y
 2301 TCGAGGATGCAAGAAATGATGAGGATGAGGATGATGAGGATGATGAGGATGATGAGGATGATGAGGATGATGAGGATGATGAGGATGATGAGGAT
 724 R G M Q E F D R A N Q H L E E K F G F M T T E H Q Y I S R K N E G
 2401 GACAAAGTTAATCTTCGAAAGGGGAACTCGCTCTTGTCTTCAATTTTCTGGAACACAGCTATTGAGATTACAGAGTTGGCTGGCGAACCCCTG
 757 D K V I I F E R G N L V F V F N F E W N N S Y S D Y R V G C A T P
 2501 GGAATATAAGATTTCTTGGAT
 790 G K Y K I V L D S D D A L F G G F N R L N H S A E Y F T S E G W Y D
 2601 TGACCGAGCTAGATCTTTCTTATCTATGACCTCTGACAGCAGCAGTGGTTTATGCTCTGACATGATCTTGAACAGGCTTTTATGATGAAGTTGAA
 824 D R P R S F L I Y A P S R T A V V Y A L A D L E P A F L D E V E
 2701 CCAGCCCTTCAGATGAAGTTGAACAGAGCCGTTGATCTTGTGagaagctctgggaagatgatacaagtcacaaatggcagcagtcacatctcta
 857 P A L A D E V E P E P V D P *

Figure 2 For legend see facing page.

Purification of recombinant proteins

The recombinant proteins in the purification steps were monitored with assay I. Activity staining on the native electrophoresis gel [32] was also employed to distinguish the recombinant SBEs from endogenous glycogen-branching enzyme (*glbB* gene product) and amylose-degrading enzymes in *E. coli*. Cells were grown in 1.2 litres of Luria–Bertani medium containing 100 mg/l ampicillin at 25 °C for *E. coli*-PvSBE2 and at 16 °C for *E. coli*-PvSBE1. Cells were then induced with 0.5 mM isopropyl β -D-thiogalactoside (final concentration) at 25 °C for 27 h for PvSBE2 or 16 °C for 65 h for PvSBE1 when the D_{600} of the culture had reached 1.0. The cells were harvested by centrifugation and ruptured by passing the cell suspension through a French press (Ohtake Works, Tokyo, Japan) in 20 mM Tris/HCl buffer, pH 7.5, containing 50 mM NaCl, 1 mM EDTA and 1 mM dithiothreitol (DTT). After centrifugation, the supernatant (crude extract) was dialysed against buffer A [20 mM Tris/HCl

(pH 7.5)/1 mM EDTA/1 mM DTT] and then subjected to enzyme purification as follows.

Purification of rPvSBE2

The dialysed crude extract was passed through a DEAE-Sepharose CL-6B column (2.0 cm $\times$ 11.5 cm) equilibrated with buffer A. The column was washed with buffer A followed by elution with a gradient of 0–0.4 M NaCl. The active fractions were pooled, concentrated and fractionated on a Bio-Gel P-200 column (2.9 cm $\times$ 71 cm) equilibrated with buffer A containing 0.1 M NaCl. The active fractions were dialysed against 5 mM sodium phosphate buffer, pH 8.0, containing 1 mM EDTA and 1 mM DTT, then resolved on a column (1.3 cm $\times$ 8.0 cm) of Gigapite equilibrated with the same buffer. The enzyme solution was eluted with a 5–200 mM linear gradient of sodium phosphate buffer.

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Proteins of soluble or granule fraction were separated by SDS/PAGE and then transferred to nitrocellulose membranes. Membranes were blocked for 1 h in blocking solution {TBST [20 mM Tris/HCl (pH 7.5)/150 mM NaCl/0.1 % (v/v) Tween 20] containing 5 % (w/v) dried skimmed milk}, then incubated at room temperature for 1 h in blocking solution containing primary antibodies against rPvSBE1 or rPvSBE2. After four washings in TBST, the membranes were incubated with alkaline-phosphatase-conjugated goat anti-rabbit secondary antibodies at 1:500 dilution in blocking solution. After removal of the primary antibodies, signals were detected by reaction with 5-bromo-4-chloroindol-3-yl phosphate and 4-Nitro Blue Tetrazolium.

Preparation of soluble and granule fractions from kidney bean embryos

Starch granules were extracted from developing seeds of kidney bean as described by Smith [34], except that starch granules were left to sediment under gravity at room temperature for 1 h. The starch granule fraction was prepared as described by Båga et al. [35]. Soluble fraction was obtained by centrifugation of the homogenate. Developing seeds sized 4–8, 10–12 and 14–16 mm were selected as small, medium-sized and large seeds respectively.

Enzymic properties

The optimum pH, pH stability and temperature stability of the purified rPvSBE1 and rPvSBE2 were examined as described previously [20]. The effect of citrate on both enzymes was also measured as described previously [20].

Analysis of reaction products and amylopectin structure of developing seeds

The preparation of debranched α -glucan from amylopectin and the analysis of chain-length distribution were performed by the method of Hanashiro et al. [36]. The chain transfer patterns of SBEs were determined as follows. Reduced amylose was incubated in 500 μ l of 25 mM Mops (pH 7.5) at 30 °C with 1 m-unit (determined by assay BL) of rPvSBE1 or rPvSBE2. After reaction for 0.5 and 4 h, the samples were boiled for 1.5 min to inactivate the enzyme, then debranched by incubation with isoamylase (0.3 unit) in 0.1 M sodium acetate buffer, pH 3.5, for 2.5 h at 45 °C. The debranching reaction was terminated by boiling the reaction mixture for 3 min. Distribution of transferred chain length was analysed with a high-performance anion-exchange chromatography-pulsed amperometric detector (HPAEC-PAD) by the method of Tomlinson et al. [37].

RESULTS

Partial amino acid sequences of native PvSBE2

We had previously described the purification and characterization of family A SBE (PvSBE2) from developing seeds of kidney bean [20]. The purified enzyme was digested with lysyl endopeptidase and resulting peptides were separated by reverse-phase HPLC. Nine peptides were recovered and subjected to amino acid sequencing. The primary sequences generated (dashed underlined sequences in Figure 2A) of these peptides were used as a basis for the design of primers for molecular cloning of the corresponding gene sequences.

Isolation of cDNA clones and protein characteristics

To isolate a cDNA clone for PvSBE2, poly(A)⁺ RNA was obtained from mid-stage developing kidney beans and reverse

transcribed. The single-stranded cDNA mixture was then used as a template for PCR with a 5' primer covering the mature N-terminal amino acid sequences (SBE2-N) and a downstream 3' anchor primer. A 3 kb DNA product was generated that was subjected to a series of second (nested) amplification reactions with the primer combinations of SBE2-N and SBE2-K24, SBE2-sense and SBE2-K24, and SBE2-K23 and anchor (see Figure 2). The DNA sequences of the amplified PCR products, with the apparent exception of one product, showed significant similarity to other reported plant SBEs of the family A type. The cDNA amplified with SBE2-sense and SBE2-K24 primers had DNA sequences that differed significantly from family A SBEs. The clone containing this unique DNA fragment was named *pvsbe1*; two unique primers, GSP1 and GSP2, specific for *pvsbe1* were synthesized and employed with a downstream anti-sense primer to amplify the 3' cDNA sequences. Amplified cDNA products were then used to screen a cDNA library containing mRNA sequences from developing kidney bean seeds.

Multiple rounds of screening of the cDNA library resulted in the isolation of six *pvsbe2* clones and five *pvsbe1* clones. Restriction enzyme analysis indicated that all of the *pvsbe2* or *pvsbe1* phage DNA species had almost identical physical restriction maps (Figure 1). Plasmids containing the longest cDNA insert were named pBS-PvSbe2 and pBS-PvSbe1 and were subjected to further analysis. The isolated *pvsbe2* clone was 3360 bp in length and the *pvsbe1* clone was 2732 bp, with coding regions of 2613 and 2544 bp respectively. The coding regions corresponded to 870 and 847 amino acid residues with predicted molecular masses of 98.8 kDa (PvSBE2) and 96.3 kDa (PvSBE1). The N-terminus of PvSBE2 identified by protein sequencing was present at residue 157, suggesting that PvSBE2 is synthesized as a preprotein with a plastid signal leader sequence of 156 residues. Because native PvSBE1 has never been identified in kidney bean seeds, the mature N-terminus of PvSBE1 could not be determined. It was predicted, on the basis of homology with other SBEs (see below), that PvSBE1 is synthesized as a proprotein with a signal sequence of 70 residues (see also the section Distribution of PvSBE2 and PvSBE1). mRNA species corresponding to both clones have the typical polyadenylation signals of eukaryotic mRNA, namely AAUAAA [38]. The sequences were located at positions 3263 and 3293 in *pvsbe2* mRNA and 2688 in *pvsbe1* mRNA (Figure 2).

Both primary sequences had significant similarity to other plant SBEs (Figure 3). The PvSBE2 protein sequence showed significant sequence identity (70–80 %) with those of the family A members, and much lower similarity (40–50 %) to PvSBE1 and members of family B SBEs. PvSBE1 protein showed substantial similarity to family B members and much lower similarity to family A members. On the basis of sequence comparisons alone, PvSBE2 is a family A SBE and PvSBE1 is of family B type.

Differential expression of *pvsbe2* and *pvsbe1*

The expression profiles of *pvsbe2* and *pvsbe1* were investigated by Northern blot analysis using total RNA species from immature seeds at eight developmental stages and also from leaves, stems and roots (Figure 4). The temporal steady-state levels for these SBE RNA species were different during seed development. The accumulation of *pvsbe2* mRNA was highest at the mid-stage of seed development, whereas *pvsbe1* transcripts attained their maximum levels at the middle to late stages. Both transcripts were also detected in leaves and stems, indicating that both *pvsbe1* and *pvsbe2* gene products or closely related species

Figure 3 Phylogenetic tree of plant SBEs

Alignment of the sequences was created with the CLUSTAL W program and used to construct a phylogenetic tree. The sequences of both family members were obtained from the GenBank®/EMBL/DBJ databases: pea BEI (X80009), *Arabidopsis* SBE2.1 (AJ000497), *Arabidopsis* SBE2.2 (AL162506), barley SBEIIa (AF064560), barley BEIIb (AF064561), maize BEIIa (U65948), maize BEIIb (AF072725), potato SBE-A (AJ011888), rice RBE3 (D16201), rice RBE4 (AB023498), wheat SBEIIa (Y11282), maize BEI (U17897), pea BEII (X80010), potato SBE-B (Y08786), rice RBE1 (D11082) and wheat BEI (AJ237897).

Figure 4 Differential accumulation of *pvsbe2* and *pvsbe1* mRNA species during seed development

Each lane was loaded with 15 µg of total RNA isolated from each tissue. The numbers along the top show the size of the developing seeds in millimetres. Ma, L, S and R indicate mature developing seeds, leaves, stems and roots respectively. The molecular sizes of the signals for *pvsbe2* and *pvsbe1* RNA species were approx. 3.5 and 3 kb respectively.

participated in the biosynthesis not only of storage starch but also of transitory starch.

Distribution of PvSBE2 and PvSBE1

For identifying native PvSBE2 and PvSBE1 proteins in developing seeds, Western blot analysis was performed with specific antisera against rPvSBE2 and rPvSBE1. Immunoblot analysis of developing seed extracts showed that anti-PvSBE2

Figure 5 SDS/PAGE of purified rPvSBE2 and rPvSBE1 (A) and Western blot analysis of each fraction of developing seeds (B, C)

(A) CBB-stained SDS/8% PAGE gel. Lane M was loaded with standard proteins: myosin (200 kDa), β -galactosidase (116 kDa), phosphorylase *b* (97 kDa), BSA (66 kDa) and ovalbumin (45 kDa). Lanes 1 and 2 contained purified rPvSBE2 and rPvSBE1 respectively. (B, C) Proteins from soluble and granule fractions of developing seeds were separated by SDS/PAGE [8% (w/v) gel] and then transferred to nitrocellulose membranes. The membranes were incubated with anti-rPvSBE2 (B) or anti-rPvSBE1 (C). Lanes rSBE2 and rSBE1, purified rPvSBE2 and rPvSBE1; lanes S, M, L and Ma, fractions from small (4–8 mm), medium (10–12 mm) and large (14–16 mm) sizes and from mature seeds respectively.

reacted with a polypeptide band of approx. 82 kDa, whereas anti-PvSBE1 recognized a band at approx. 100 kDa (Figure 5). The sizes of the reacting polypeptides are identical with that predicted for the mature PvSBE2 but not for the mature PvSBE1 polypeptide (results not shown). The molecular mass of rPvSBE1 estimated by SDS/PAGE (Figure 5A) was significantly larger than that predicted from the sequence. A similar situation has also been observed for the pea SBEII protein [9,24]. It has been suggested that the high concentration of negative charges of pea SBEII causes an abnormal electrophoretic behaviour on

Table 2 Purification of rPvSBE2 and rPvSBE1 from *E. coli*

rPvSBE2 and rPvSBE1 were purified from *E. coli*-PvSBE2 and *E. coli*-PvSBE1 cultured cells, respectively, as described in the Materials and methods section. Protein concentrations were calculated based on the assumption that its $A_{1\text{ cm}, 280}^{1\%}$ was 10.0. SBE activities were measured by the assay I method.

Enzyme	Procedure	Total protein (mg)	Total activity (units)	Specific activity (units/mg)	Purification (fold)	Yield (%)
rPvSBE2	Crude extract	2800	9400	3.4	1	100
	DEAE-Sepharose	87	8700	100	29	93
	Bio-gel P-200	21	3760	179	53	39
	Gigapite	16	3420	214	63	36
rPvSBE1	Crude extract	5600	5400	0.96	1	100
	DEAE-Sepharose (first)	109	3990	36.6	38	74
	Bio-gel P-200	16	2320	145	151	43
	DEAE-Sepharose (second)	8.2	1760	215	224	33
	DEAE-cellulose	0.45	114	254	265	2.2

SDS/PAGE. Considering the close primary sequence similarity between PvSBE1 and pea SBEII including the high net negative charge character of the mature polypeptide, the electrophoretic behaviour of PvSBE1 is likely to be inconsistent with its actual molecular mass.

To determine the intracellular location of these SBEs, soluble and starch granule fractions were prepared from developing kidney bean seeds. When these fractions were analysed by immunoblotting, PvSBE2 protein was detected mainly in the soluble fractions during all phases of development, whereas PvSBE1 protein was found in only the starch granule fraction in the middle to late stages of seed development. The temporal accumulation of these proteins during seed development paralleled the gene expression pattern (Figure 4). To verify that the cross-reacting polypeptide band in the granule fraction was PvSBE1, the protein band was transferred to a PVDF membrane from the SDS/polyacrylamide gel and then subjected to amino acid sequencing. The resulting N-terminal amino acid sequence of the granule protein was VMTDDK and was identical with those predicted for the mature N-terminus of the product encoded by *pvsbe1*.

Expression of rPvSBE2 and rPvSBE1 in *E. coli* and purification

To investigate the enzymic properties of PvSBE2 and PvSBE1, recombinant proteins were obtained by expression in *E. coli*. The optimum conditions of temperature and culture duration were examined in *E. coli*-PvSBE2 and *E. coli*-PvSBE1 cell cultures (results not shown). The maximum activities for the recombinant *E. coli*-PvSBE2 and *E. coli*-PvSBE1 cultures were 8.3 units/ml for 27 h at 25 °C and 2.8 units/ml for 65 h at 16 °C respectively. In contrast, under the same conditions, control cell cultures showed lower activities, approx. 0.35 unit/ml, corresponding to the endogenous branching-enzyme activity from the *glgB* gene product. SDS/PAGE analysis showed that most (more than 80%) of the recombinant branching enzyme was found in inclusion bodies.

Although the bulk of the expressed PvSBE2 and PvSBE1 was insoluble, sufficient activity was present in the soluble phase for protein purification (Table 2). The final enzyme preparations contained 16 mg of rPvSBE2 and 0.45 mg of rPvSBE1, with specific activities of 214 and 254 units/mg respectively, using amylose as a substrate. The specific activity of rPvSBE2 was close to that obtained for native PvSBE2 [20]. Purified rPvSBE2 and rPvSBE1 migrated as a single polypeptide band on SDS/polyacrylamide gels (Figure 5A). The N-terminal sequences of purified rPvSBE2 and rPvSBE1 were determined and the expected sequences were obtained.

Table 3 Properties of rPvSBE2 and rPvSBE1

The molecular masses of rPvSBE2 and rPvSBE1 were predicted from the deduced amino acid sequences and estimated from SDS/PAGE. Optimum pH is the pH at which there was maximum activity under the conditions described in the Materials and methods section. pH stability is defined as the pH range over which more than 95% of the residual activity remained after being treated as described in the Materials and methods section. Thermal stability is the temperature limit within which more than 95% of the original activity remained after being treated as described in the Materials and methods section.

Property	rPvSBE2	rPvSBE1
Molecular mass (Da)		
Predicted	81 932	88 590
Estimated	82 000	100 000
Optimum pH	7.0	7.0
pH stability	7.0–9.0	7.0–8.5
Thermal stability (°C)	< 50	< 40
Specific activity (units/mg)		
(A) Assay I for amylose	214	254
(B) Assay I for amylopectin	91	20
(C) Assay P	823	292
(A)/(B)	2.35	12.7
(A)/(C)	0.26	1.0
K_m for amylose (mg/ml)	1.27	0.44
Effect of citrate	Activation	None

Enzymic properties of rPvSBE2 and rPvSBE1

The enzymic properties of purified rPvSBE2 and rPvSBE1 are summarized in Table 3. Both enzymes were active at approx. pH 7.0 at 30 °C. More than 90% of the original activity remained after incubation at 4 °C for 16 h between pH 7.0 and 9.0 for rPvSBE2 and between pH 7.0 and 8.5 for rPvSBE1. Almost no inactivation occurred when the enzymes were kept at pH 8.0 for 15 min at up to 50 °C for rPvSBE2 and up to 40 °C for rPvSBE1.

When amylose and amylopectin were used as substrates in assay I, the specific activities were 214 and 91 units/mg respectively for rPvSBE2, and 254 and 20 units/mg respectively for rPvSBE1. The ratios of activity towards amylose and amylopectin as substrates were 2.35:1 for rPvSBE2 and 12.7:1 for rPvSBE1, indicating that rPvSBE2 prefers amylopectin over amylose in comparison with rPvSBE1. The linear substrate in assay P was much shorter than that in assay I because α -glucans synthesized by phosphorylase α are relatively short, showing a chain-length distribution peak at dp 33 [39]. The ratios of specific activities obtained with assay I (amylose) to assay P were 0.26 for rPvSBE2 and 0.87 for rPvSBE1, suggesting that rPvSBE1 preferentially transfers longer chains than rPvSBE2. The Lineweaver–Burk plots of the reaction catalysed by rPvSBE2 and

Figure 6 Effects of citrate on rPvSBE2 and rPvSBE1

(A) Activities of rPvSBE2 (●) and rPvSBE1 (○) in the presence of citrate. Activity is shown relative to the original activity without citrate (100%). (B) Double-reciprocal plots of reactions for amylose by rPvSBE2 in the presence of 0.3 M citrate (□) and 0.1 M citrate (●) or in the absence of citrate (○). U, units.

Figure 7 Chain-length distributions of reaction products by rPvSBE2 (A–C) and rPvSBE1 (D–F)

All reaction products with amylose as a substrate were analysed by HPAEC–PAD after digestion with isomylase. (A, B) Chain-length distributions of the 0.5 h (A) and 4 h (B) reaction products of rPvSBE2. (D, E) Chain-length distributions of the 0.5 h (D) and 4 h (E) reaction products of rPvSBE1. The solid bars denote the percentage area of dp 6 glucose units. (C, F) Differences in percentage area between 0.5 h and 4 h products of rPvSBE2 (C) and rPvSBE1 (F).

rPvSBE1 yielded K_m values for amylose of 1.27 and 0.46 mg/ml respectively. These results indicate that the affinity of rPvSBE1 for amylose was approx. 3-fold that of rPvSBE2.

Citrate has been used for the activation and stabilization of plant SBEs. The activity of native PvSBE2 in the presence of 0.3 M citrate increased more than 3-fold. The dose dependence

of rPvSBE2 activity on citrate (Figure 6A) was the same as that of native PvSBE2 [20]. The activation by citrate was non-competitive (Figure 6B), suggesting that citrate had no influence on the binding between enzyme and substrate. Unlike rPvSBE2, a high concentration of citrate inhibited rPvSBE1 activity (Figure 6A).

Figure 8 Starch amounts and chain-length distributions of amylopectins isolated from developing seeds of kidney bean

(A) Starch types were isolated from approx. 20 g of small (4–8 mm), medium-sized (middle; 10–12 mm), large (14–16 mm) and mature seeds. (B–E) The chain-length distribution of each amylopectin was determined by a HPAEC–PAD after digestion with isoamylase. The solid bars denote the percentage area of dp 6 glucose units.

Analysis of reaction products and amylopectin structure

The chain-length distribution pattern of debranched products was analysed by HPAEC–PAD after incubation of rPvSBE2 or rPvSBE1 with reduced amylose (AS-320) followed by isoamylase treatment to generate branched chains (Figure 7). The general chain-length distribution pattern generated by rPvSBE2 was similar to that generated by maize BEII [40,41]. rPvSBE2 preferentially transferred short chains of dp 6 (Figures 7A and 7B). The chain-length distribution pattern produced by rPvSBE1 was also similar to that obtained by maize BEI [40,41] except that rPvSBE1 preferentially transferred chains of dp 6 in the early branching reaction (Figures 7D and 7E). Such preferential transfer of dp 6 has never been observed in the reaction products generated by maize BEI [40,41]. Overall, the transferred products generated *in vitro* by rPvSBE2 were predominantly short chains (Figure 7C; dp 6–12), whereas rPvSBE1 transferred a much broader distribution of chain lengths (Figure 7F; dp 6–30).

To investigate whether the characteristics of chains transferred by SBEs *in vitro* influenced starch structure, the distribution of branched chain lengths from starches of developing seeds was analysed (Figure 8). The amounts of starch isolated increased during seed development (Figure 8A) but there was almost no difference in the chain-length distribution patterns, indicating

that amylopectin synthesis was a uniform process at every stage of seed development (Figures 8B–8E). Although the chain-length distribution in amylopectin of kidney bean seeds was similar to those in other plants [36], it contained a relatively large quantity of dp 6 branch chains, a pattern not evident in starches from maize and rice endosperms.

DISCUSSION

The present study shows that developing kidney bean seeds contain two different SBE isoforms, PvSBE2 and PvSBE1, with distinct enzymic properties. rPvSBE1 had a higher affinity for amylose and preferentially transferred longer glucan chains than rPvSBE2. Our results also showed that citrate stimulates rPvSBE2 activity 3.3-fold but has little influence on rPvSBE1 activity. These enzymic properties, together with the primary sequences and accumulation patterns of transcripts of PvSBE2 and PvSBE1, are typical of the properties displayed by members of families A and B respectively of SBEs (Figure 3). One unique finding from our studies is that the SBEs of developing kidney bean seeds are located differently within the amyloplast. PvSBE2, like other SBEs of other plants, was observed in the soluble fraction of amyloplast extracts, whereas PvSBE1 was located in the starch granule fraction.

PvSBE2 has 81 % identity and 94 % similarity to pea (*Pisum sativum* L.) SBEI [9] and shares approx. 70 % identity with other family A SBEs. Likewise, PvSBE1 shows 80 % identity and 94 % similarity to pea SBEII [9] and has 60–70 % identity with those of other family B members. The members of family A share approx. 50 % similarity to family B. SBEs belong to the α -amylase family whose members have the $(\beta/\alpha)_8$ barrel structure containing the active centre [42]. The high sequence similarity displayed by the SBEs is largely attributable to conservation within the $(\beta/\alpha)_8$ barrel regions. Therefore differences in the enzymic properties between individual SBEs would be determined by their N-terminal or C-terminal domain [40]. Indeed, analyses of chimaeric enzymes between maize BEI and BEII revealed that the N-terminal domain determines the size of oligosaccharide chain transferred and that the C-terminal domain has a role in substrate preference or maximum catalytic efficiency [40,43].

Despite the close sequence similarity between PvSBE2 and pea SBEI, the molecular masses of the mature proteins are quite different: that of PvSBE2 is 82 kDa and that of pea SBEI is 99.8 kDa [9]. When the sequence of PvSBE2 was analysed for a putative signal peptide with the TargetP [44,45] and ChloroP [46] network programs, the protein was predicted to contain a plastid transit peptide with a processing site between Lys-47 and Ser-48. The predicted site is very close to that in pea SBEI. The region between Ser-48 and Lys-156 in the derived PvSBE2 primary sequence (Figure 2A), which showed a high similarity (62 % identity and 82 % similarity) to the N-terminal region of pea SBEI, was not observed in the native PvSBE2. Because pea SBEI is found in both the soluble and granule fractions, whereas PvSBE2 was detected in only soluble fractions, these observations might account for the location of this enzyme within the amyloplast. PvSBE2 is found only in the soluble phase because it lacks the homologous pea SBEI N-terminal sequence that might be responsible for localization to the starch granule. Burton et al. [9] suggested that this N-terminal region of pea SBEI, which is predicted to be a flexible domain, might be important in interactions between enzymes or between enzymes and starch. More recently, a novel type of 152 kDa SBE has been isolated from wheat endosperm [35]. This unique SBE consisted of an N-terminal 74 kDa SBEI-like domain 1 lacking branching enzyme activity and a C-terminal 87 kDa catalytically active SBEI domain. In addition to its unusually large molecular size, it was associated with the granule. Because the wheat SBEI is located only in the soluble fraction of the endosperm [47], domain 1 of this unique SBE might function to bind this enzyme to the granule.

In the pea embryo, SBEI and SBEII are found in both the soluble and granule fractions [24]. Despite the localization of these SBEs to the starch granule, it has been suggested that the bound SBEs were not active in starch biosynthesis but were simply trapped during synthesis of the starch granule [24]. Such a view is unlikely to apply to PvSBE1 because it is found only in the granule fractions (Figure 5C). This restricted localization of PvSBE1 within the starch granule accounts for an inability to detect PvSBE1 enzyme activity. The high affinity of rPvSBE1 for amylose would be one of the reasons for its occurrence in the granule fractions. From the relationships between the enzymic properties of rPvSBE1 and amylopectin structure mentioned below, we believe that PvSBE1 is active in starch formation.

rPvSBE2 is non-competitively activated by citrate (Figure 6), indicating that citrate has a significant influence on the catalytic activity but has no effect on its substrate affinity for amylose [20]. rPvSBE1 showed no effects by citrate. Citrate often stimulates the activities of plant starch synthases [31,48,49]. Although the biological significance is unclear, it was shown that potato

granule-bound starch synthase I (GBSSI) and starch synthase II activities were stimulated approx. 10-fold and 2-fold respectively by 0.5 M citrate in the presence of a low concentration of amylopectin [48]. Edwards et al. speculated that the effect of citrate on GBSSI activity might be mimicking a high amylopectin concentration by altering the physical nature of amylopectin solutions [48]. Given that the stimulation by citrate resulted from the variation in phase of amylopectin, PvSBE2 could also prefer polyglucans with an altered physical nature, as does GBSSI or starch synthase. PvSBE2 occurring in soluble fractions might have a role in amylopectin synthesis synchronizing with soluble starch synthase, whereas PvSBE1 could serve in a different environment from PvSBE2. At present, we cannot precisely explain the physiological roles of PvSBE2 and PvSBE1 but both enzymes seem to have different roles for amylopectin synthesis because of differences in their enzymic properties, in gene expression patterns and in distribution in the amyloplast fractions.

Both rPvSBE2 and rPvSBE1 had a strong bias in transferring chains with lengths of dp 6 *in vitro* (Figure 7). Similarly to rPvSBE2, maize BEII [40,41] also preferred to transfer chain lengths of dp 6 and dp 7. In contrast, rPvSBE1 has a high capability for transferring chains of dp 6 in comparison with maize BEI [40,41]. Additionally, a chain length of dp 6 was also found in amylopectin from kidney bean seeds at each developmental stage (Figure 8) but not in amylopectin of maize and rice endosperms [36]. Moreover, during seed development the accumulation of *pvsbe1* transcript and PvSBE1 protein paralleled the amount of starch (Figures 4, 5 and 8). These results not only suggest that PvSBE1 is active in the granule but also could provide us with a hypothesis that a portion of the ultimate chain-length distribution of kidney bean amylopectin reflects the enzymic properties of family B SBE or starch-granule form (PvSBE1) rather than family A SBE or soluble form (PvSBE2). Analysis of amylopectin in the rugosus (*r*) mutant of pea suggested that the family A SBE might have no specific role in the basic chain-length distribution of amylopectin [37]. The generation of genetic mutants without PvSBE1 and/or transgenic plants with a decreased level of PvSBE1 will address the relationships between SBEs and the chain-length distribution of amylopectin.

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