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Molecular Characterization of Mungbean (*Vigna radiata* L.) Starch Branching Enzyme I

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Mungbean (*Vigna radiata* L. cv. Tainan no. 5) starch branching enzyme I (SBE, EC 2.4.1.18) cDNA, *VrsbeI*, was cloned, and its expression was characterized. Conserved regions of the family B SBE were used to amplify a full length cDNA of 2208 bp. Phylogeny was analyzed, and the partial 3D structure and functional features were predicted. Catalytic residues were identified in the $(\alpha/\beta)_8$-fold, and a unique loop from F365 to F376 between β3/α3 was located. Gene expression of *VrsbeI* in seeds during growth showed that the transcript appeared from week 1 and increased substantially at week 3−4. It was cloned into the pET30 vector and expressed in *E. coli* BL21(DE3) pLysS cells as a soluble recombinant protein. The affinity-purified recombinant VrSBEI exhibited a specific activity of 314.6 U/mg as an active enzyme with 114-fold activity enrichment from the crude extract.

KEYWORDS: Mungbean; starch branching enzyme; gene expression; recombinant enzyme

INTRODUCTION

Starch is the main source of carbohydrate in food. Starch molecules are composed of two groups of glucose homopolymers, the almost linear α-1,4 glucan amylose, and α-1,4 glucan with α-1,6-branched amylopectin; they are formed and stored as granules in the plastids of photosynthetic tissues and storage organs in higher plants such as seed embryo, tuber, and cereal endosperm. From different botanical origins, starch granules differ in morphology (shape and size) but have similar detailed structure hierarchies. A single granule shows amorphous and semicrystalline growth rings. The semicrystalline growth ring region is composed of alternating crystallinity of 200−400 nm thick subrings. Each subring is composed of a repeating unit of the crystalline and amorphous lamellae. The 7−10 nm thick amylopectin cluster is the basic unit composing the crystalline lamellae (*1*).

Several models were proposed for the formation of the amylopectin cluster unit during the biogenesis of the specialized granule architecture, including the phytoglycogen intermediate, phytoglycogen branching enzyme, glucan trimming, or the water-soluble-polysaccharide clearing model. In these models, the same group of enzymes is responsible for amylopectin cluster formation (*2*). These enzymes mainly include starch synthase (SS), starch branching enzyme (SBE), debranching enzyme, and disproportionating enzyme. The activity of amylopectin synthesis was proposed to be regulated by phosphorylation-dependent protein complex formation among two SBE isoforms and starch phosphorylase (SP) (*3*). A recent report demonstrated the identification of BE, SP, and sucrose synthase that are physically associated together to synthesize amylopectin in vitro (*4*). Nevertheless, the above reports show that the α-1,6-glucosidic branch in the amylopectin molecules is introduced mainly by SBE.

SBE (1,4-α-D-glucan:1,4-α-D-glucan-6-α-D-[1,4-α-D-glucano]-transferase; E.C. 2.4.1.18) possesses both α-amylolysis and glucosyl-transfer activities. It catalyzes the cleavage of α-1,4 linkages within a chain and the transfer of the released reducing end to a C6 hydroxyl of the hydrolyzed chain or a new chain, creating a α-1,6 linkage. SBE has been shown with application potentials. The *be* gene was used by transgenic technology to generate genetically modified crops with altered starch structure in vivo (*5*, *6*); also, SBE was acting as a functional biocatalyst for in vitro modifications on glucan structure (*7*). Multiple SBE isoforms are involved in starch biosynthesis and are classified into A and B families according to the sequence identity among amino acid sequences deduced from their corresponding genes (*8*). The two families differ in catalytic activities such as the preferred length of chain transfer and substrate specificities. Family A SBEs prefer amylose as the substrate and transfer longer chains, compared with family B SBEs, which prefer amylopectin and transfer shorter chains. The above features lead to starch polymers with diverse branching structures (*3*, *9*), ultimately impacting on the starch granule size and morphology (*10*).

Mungbean or the trivial name green bean (*Vigna radiata* L.) has been a Chinese medical plant since ancient times. Mungbean starch is one of the resistant legume starches (*11*). It is used as an essential ingredient in making bean noodle, a popular material in oriental dishes. The high amount dry weight of branched amylose

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content in mungbean starch is responsible for the noodle with severe retrogradation characteristics and heat persistency during cooking. Since SBE is an essential enzyme for composing the basic amylopectin cluster unit in the starch structure, it is necessary to understand the two gene families in mungbean SBE (VrSBE) at the basic molecular level in order to investigate how each of the isoforms is involved, perhaps in combination with other enzymes, in synthesizing the unique mungbean starch structure and, furthermore, to pursue application potentials. The full-length cDNA of the family A VrSBE isoform (*VrsbeII*) was recently cloned (*12*). The objectives of this study were to clone and characterize the family B SBE cDNA (*VrsbeI*) from mungbean seed of the mid-developmental stage, analyze gene expression, and produce a recombinant enzyme. On the basis of the conserved motif information among cloned SBEs, gene-specific primers (GSPs) and primers for the 5′- and 3′-ends were designed to perform RT-PCR (reverse transcriptase-polymerase chain reaction). Subset primers were then designed to amplify the internal sequence by RT-PCR and nested PCR. The obtained full-length sequence was characterized and used to predict its three-dimensional (3D) protein structure, identify domain features in silico, and compare these features with those of the family A SBEII. The gene expression for *VrsbeI* at the transcriptional level during seed development was analyzed by RT-PCR. In addition, to correlate its catalytic activity in α-1,6-branched glucan formation, the cDNA was expressed in the *Escherichia coli* system and produced as an active recombinant VrSBEI (rVrSBEI) enzyme.

MATERIALS AND METHODS

Materials. Mungbean pods (cultivar Tainan no. 5, VC3890A) were collected at 1−4 weeks after flowering in the field at Tainan Agricultural Research and Extension Station (Tainan, Taiwan) and stored at −80 °C. SuperScript One-Step RT-PCR, RNaseOUT, SuperScript III first-strand synthesis were from Invitrogen (Carlsbad, CA). *E. coli* NovaBlue and BL21 (DE3) pLysS cells, KOD HotStart DNA polymerase, and pET-30 EK/LIC (ligation independent cloning) vector were from Novagen (Darmstadt, Germany). Protein analysis chemicals were from Bio-Rad (Hercules, CA). The HisTrap column and Tween-20 were from Amersham Biosciences (Uppsala, Sweden). Chemicals, media, and reagents were from Sigma Chemical Co. (St. Louis, MO).

Primer Design. GSPs were designed as described previously (*12*). All primers listed are 5′ to 3′ sequences. For full-length cloning, primers were F1 (ATGTTTAACTGTCTGTGCCTTAATCCGTTC), F2 (GAGGGCTACCTTAAT TTCATGGGCAAT G), F3 (TTTGCAGCCCCATATGATGGT G), R1 (TTATGCGAGGTTCAG AGCTAC TCATC), R2 (TTAAATTTCCCTATCCAAAGAAGCTGCCAC), R3 (AGTGATCTG TATCCACCAGAT TCCACT G), and R4 (TTACCC CCAGAGATTAGGGCTCCTTACTCT). F1/R1 and F2/R2 pairs were from the common regions in the kidney bean *sbeI* (Genbank accession AB029549) and pea *sbeII* cDNA (X80010); F3 was from the internal amplicon of F1/R1; and R3 and R4 were from the internal amplicon and 3′-amplicon of F2/R2, respectively. For cloning into pET-30 EK/LIC with the LIC site, forward primer GACGACGACAAGATG and reverse primer GAGGAGAAGCCCGGTTA were used. For gene expression analysis, F2 and R4 were used to produce a 415 bp fragment. β-Actin gene (AF143208) was retrieved, and forward primer TTCGCAGCAACAAACAT and reverse primer TAAGCGGTGCCTCGGTAAGAAG were designed from DNASTAR software to produce an amplicon of 371 bp as the constitutive gene expression control.

cDNA Amplification and Analysis. Total RNA and mRNA were prepared (*12*). SuperScript One-Step RT-PCR was used. The reaction mix contained 25 μL of 2× the reaction mix (0.4 mM dNTP and 2.4 mM magnesium sulfate), 10 pg to 1 μg of mRNA, 10 μM sense and antisense primers, and 1 μL RT/Platinum Taq Mix in a final 50 μL volume. The amplification was programmed in an iCycler (Bio-Rad). The program was first strand cDNA synthesis (45 °C for 30 min), predenaturation (94 °C for 2 min), PCR amplification of 35 cycles (typically denaturing at 94 °C for 15 s, annealing at 60 °C for 30 s, and extension at 72 °C for 1−3 min), and final extension (72 °C for 10 min). DNA was sequenced in the Biotechnology Core Facility Center at National Chung-Hsin University (Taichung, Taiwan) or Tri-I Biotech, Inc. (Taipei, Taiwan).

Construction of Expression Vector. A construct encoding His-tag on the N-terminal of VrSBEI (His_6- VrSBEI) in the pET-30 EK/LIC expression vector was made. *VrsbeI* containing a LIC linker sequence was treated with T4 DNA polymerase in the presence of dATP to expose the LIC ends into a single strand sticky end. Ligation was performed at different molar ratios of insert and vector, then transformed into competent cells by 42 °C heat shock, and selected on selection medium. The cloning host was NovaBlue, and the expression host was BL21-(DE3)pLysS *E. coli* cells. NovaBlue is tetracycline resistant; the selection SOC medium contained 30 μg/mL kanamycin and 12.5 μg/mL tetracycline. The correct insert size in pET-30 EK/LIC-*VrsbeI* clone was confirmed by PCR using F1/R1, cutting with *Bam*HI/Not I, and sequencing. Then, the construct was transformed into BL21(DE3)pLysS competent cells and selected on an LB (Luria−Bertani)-agar plate. BL21(DE3)pLysS is chloramphenicol resistant; the selection LB medium contained 30 μg/mL kanamycin and 34 μg/mL chloramphenicol (LB-Kan/Chlor). To check the stability of the plasmid and correct insert size in the transformants, colony PCR was also routinely performed.

Protein Expression, Purification, and Analysis. A single transformant cell was inoculated into 50-mL LB-Kan/Chlor broth to grow at 37 °C, 250 rpm to $O.D._{600\ nm}$ of 0.6, and harvested by 5000*g* centrifugation at 4 °C for 10 min. The cell pellet was resuspended in 10 mL of fresh LB, and 1 mL was inoculated into another fresh 50-mL LB-Kan/Chlor broth in a 250 mL flask to grow at 37 °C, 250 rpm until $O.D._{600\ nm}$ of 0.5. The culture was induced with IPTG (isopropyl β-D-1-thiogalactopyranoside) under tested conditions. Cells were collected by 10000*g* centrifugation at 4 °C for 10 min, and the pellet was suspended in 2 mL of lysis buffer (50 mM sodium phosphate, 200 mM sodium chloride, 5 mM DTT, and 1 mM PMSF, pH 7.5), resting on ice for 10 min. Cells were then lysed by sonication for 10 short bursts of 10 s at 5 W followed by intervals of 30 s for cooling on ice. Soluble lysate was recovered by centrifugation 16000*g* for 30-min at 4 °C.

For purification, 250 mL of induced culture was prepared. The His_6-rVrSBEI was purified in a 1-mL HisTrap HP nickel ion affinity column, which was equilibrated with binding buffer (20 mM sodium phosphate, 0.5 M sodium chloride, and 40 mM imidazole, pH 7.4) in a AKTA prime system with a flow rate of 1 mL/min. The soluble lysate was filtered with a 0.45 μM membrane filter. Protein (78.2 mg) in 5 mL of lysis buffer was loaded onto the column, followed by the use of binding buffer to wash away unbound proteins. rVrSBEI was then eluted by a linear gradient of 40−500 mM imidazole in elution buffer (20 mM sodium phosphate and 0.5 M sodium chloride, pH 7.4) for 10 min. The lysed cell extract or the affinity-purified proteins were analyzed in 8% acrylamide-bis (37.5:1) SDS−PAGE. SBE activity was assayed by the amylose branching assay (*12*).

VrsbeI Gene Expression in Mungbean Seeds during Different Growth Stages. Mungbean pots of 4 different growth stages were used to extract total RNA. Reverse transcription was performed using the SuperScript III first-strand synthesis system, and RNaseOUT was added to 2 U/μL during first strand synthesis. The expression of the β-actin housekeeping gene of different growth stages was considered as a stable transcript and used to normalize the quantity of the total RNA among samples (*13*). At first, RNA normalization was achieved by amplification of the β-actin gene of different growth stages. Their ethidium bromide-stained intensities of the 371 bp β-actin gene fragment in 1% agarose gel were compared to adjust for the same amount of first stand cDNA that would be used in the subsequent PCR amplification of the target *VrsbeI* 415 bp fragment. PCR products were analyzed in an ethidium bromide-stained gel and quantified by AlphaImager (AlphaInnotech, San Leandro, CA). Then, the amount of first stand cDNA of different growth stages that were optimized from β-actin gene amplification was taken as template for the amplification of *Vrsbe I* and conducted in the same batch. The β-actin PCR reaction was conducted at 94 °C for 2 min, then the first 20 cycles (denaturing at 94 °C for 30 s, annealing at 55.7 °C for 30 s, and extension at 72 °C for 1 min), the second 20 cycles (denaturing at 94 °C for 30 s, annealing at 57.7 °C for 30 s, and extension at 72 °C for 1 min), and the final extension (72 °C for 5 min) were performed. PCR conditioning of the *VrsbeI* 415 bp fragment was conducted at 94 °C for 2 min, then 35 cycles

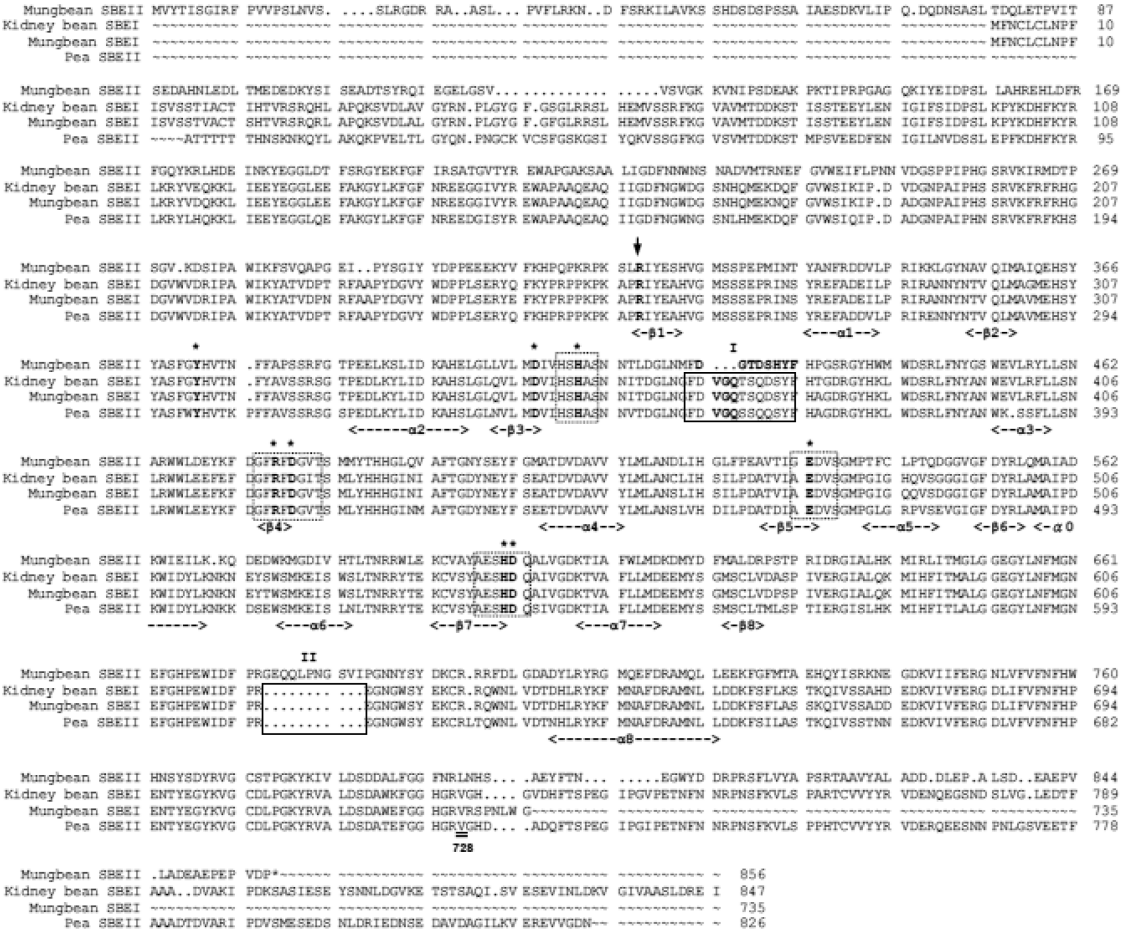


Figure 1. Alignment of the deduced amino acid sequence of mungbean VrSBEI with kidney bean SBEI (BAA82349), pea SBEII (CAA56320), and mungbean VrSBEII (AAT76444.1) by Pretty. The dot boxed amino acids indicate four conserved regions of the α-amylase family. The eight α-helix and eight β-sheet regions are labeled with dashed line arrows. Solid box I frames the unique loop sequence at 365−376. Solid box II frames the conserved loop, which is found in VrSBEII (675−685) and most family A isoforms ($^{P}/_{E}$QXLP$^{S}/_{N}$GK$^{F}/_{I}{}^{I}/_{V}$P) but is absent in most family B isoforms. Residues labeled with stars on top and in bold letters, Y313, D348, H353, R420, D422, E477, H545, and D546, are predicted to be important for catalytic activities.

(denaturing at 94 °C for 30 s, annealing at 58 °C for 30 s, and extension at 72 °C for 1.5 min) and the final extension (72 °C for 10 min) were performed.

Sequence Analysis and 3D Structure Prediction. DNA sequence was analyzed as previously described (*12*). The 3D structures of the deduced amino acid sequence were simulated in the structure homology-modeling server SWISS-MODEL. The simulated 3D images were then retrieved into Deep View-Swiss-PdbViewer to view proteins, to deduce structural alignments, and to compare active sites or relevant parts. R_EMUS (Reinforced Merging for Unique Segment) (*14*) was used to identify the location and compositions of unique peptide segments in SBE.

RESULTS

Cloning of *VrsbeI* cDNA. Three RT-PCR reactions were used to obtain the full length cDNA of *VrsbeI*. The 5′-terminal portion of 826 bp from F1/R1 and the 3′-terminal portion of 725 bp from F2/R2 were obtained first, and then F3/R3 primers were designed from the two fragment sequences and the middle 1234 bp amplified. The complete ORF (open reading frame) sequence of *VrsbeI* cDNA was derived and further amplified using the terminal primers (F1/R2) followed by a nested PCR using F1/R4 primers to obtain a full length of 2208 bp (GenBank accession no. AY667492). *VrsbeI* cDNA contains the start ATG to the stop TAA codon and encodes a predicted VrSBEI protein of 736 amino acid (**Figure 1**) with a predicted molecular mass of 84 kDa and a pI of 6.35.

Sequence Features and Characterization. The deduced amino acid sequence of *VrsbeI* was aligned with those of family B kidney bean SBEI, pea SBEII, and family A mungbean VrSBEII to compare sequence features and variation (**Figure 1**). The VrSBEI protein includes the common catalytic $(\beta/\alpha)_8$-barrel domain (regions are labeled with dashed arrows) and four conserved catalytic regions (labeled in dashed box), HSH$^{S}/_{A}$ S (351−355), GF RFDG VT (418−423), $^{G}/_{A}$EDVS (476−480), and AESHDQ (542−547), of the α-amylase family (*15*). The conserved catalytic residues including the Y313, D348, H353, R420, D422, E477, H545, and D546 (**Figure 1**, labeled with stars on top) were found. A high portion of the amino acid composition up to valine 728 (underline labeled) between mungbean and kidney bean SBEI was similar, but differences were apparent at the N- and C-termini. The essential R253 found in maize SBEI near the substrate binding site was observed in VrSBEI (R260) (labeled with an arrow) (*16*). Between β8 and α8, it is evident that the three family B SBEs do not have a loop structure (**Figure 1**, the solid box II, residue 618/619) of 11-amino acid residues that are found in the family A SBE isoforms (*8*, *12*).

Phylogenetic Analysis of VrSBEI among SBE Isoforms. The amino acid sequences of SBE isoforms were retrieved to construct a phylogenetic tree for VrSBEI by GrowTree (**Figure 2**). Mungbean VrSBEI has the highest evolutionary relatedness to kidney bean sbe1 (95%), followed by pea sbe2 (83%), cassava sbe (77%), sorghum seed sbe (65%), rice rbe1 (64%), maize sbe1 (64%), and

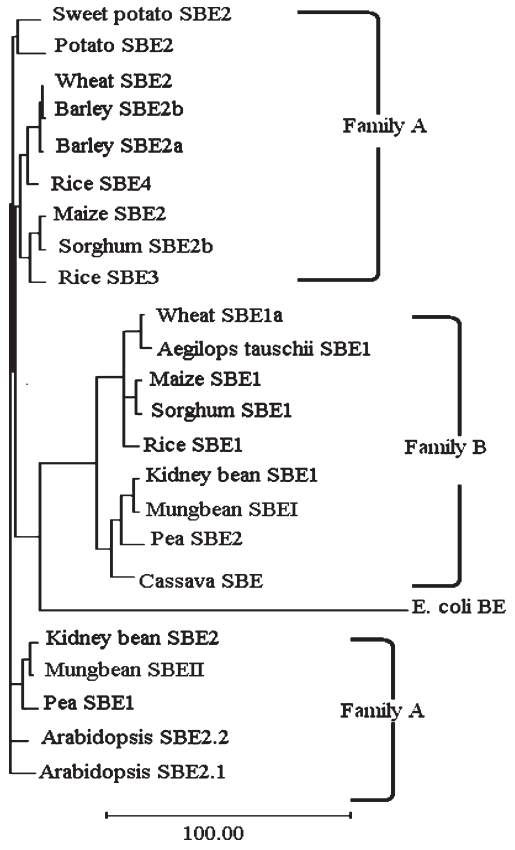


Figure 2. Phylogenetic analysis of mungbean SBEI among registered SBE isoforms by Grow Tree. The retrieved gi accession numbers were sweet potato SBE2 (15553090), potato SBE2 (2764395), wheat SBE2 (58618128), barley SBE2b (3822021), barley SBE2a (3822019), rice SBE4 (5689137), maize SBE2 (168482), sorghum SBE2b (32186929), rice SBE3 (436051), wheat SBE 1a (11037531), *Aegilops tauschii* SBE1 (32401224), maize SBE1 (600871), sorghum SBE1 (7547155), rice SBE1(218148), kidney bean SBE1 (5441247), mungbean SBEI (50400195), pea SBE2 (510546), cassava SBE (1771260), *E. coli* (146141), kidney bean SBE2 (42794061), mungbean SBEII (50400193), pea SBE1 (510545), and *Arabidopsis* SBE2.2 (30680139) and SBE2.1 (30686770).

wheat sbe 1a (62%). It showed clearly that mungbean SBEII and SBEI are classified into the families A and B, respectively.

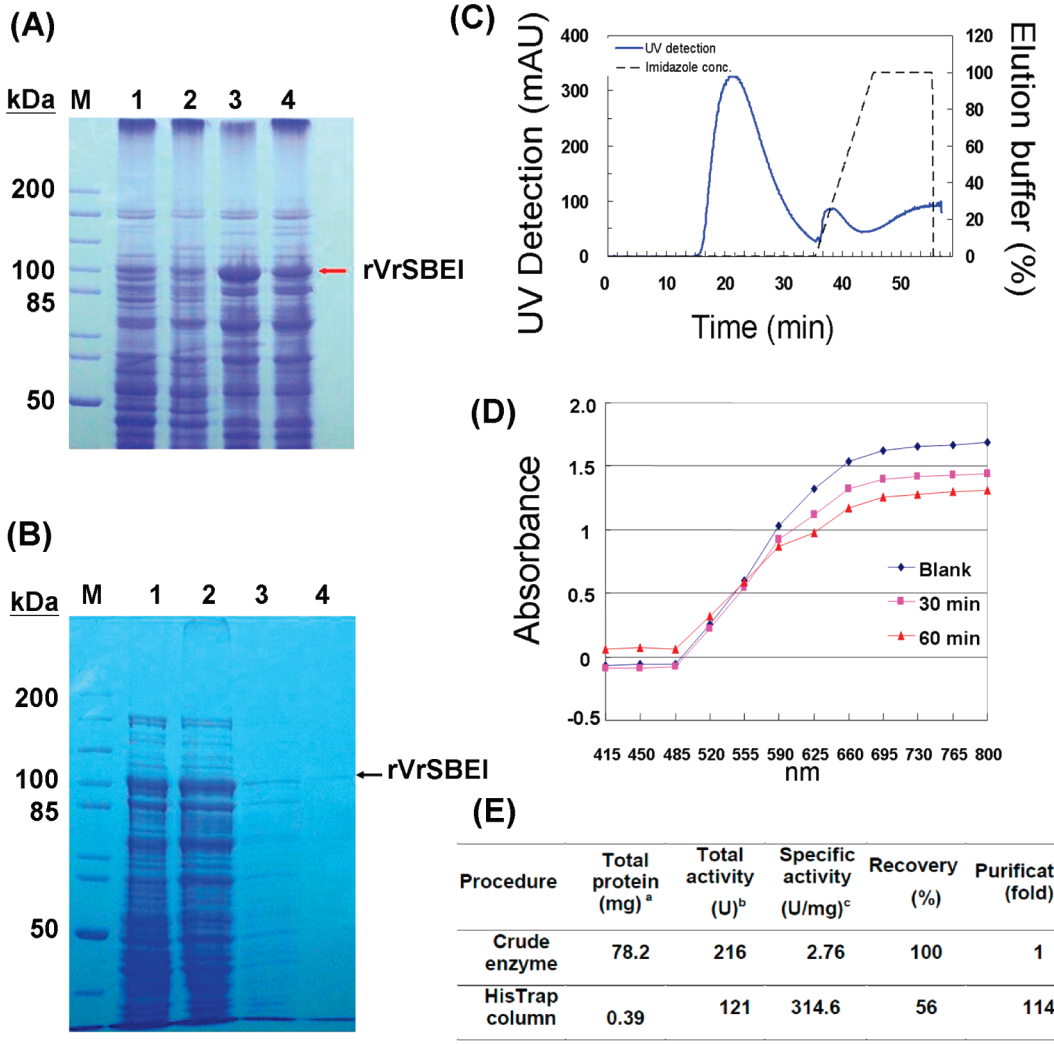
rSBEI Expression Optimization, Purification, and Activity. Optimization of the induction condition for rVrSBE1 expression showed that if it was induced only by 1 mM IPTG, the appearance of an approximately 100 kDa protein band was minor (**Figure 3A**, lane 1 vs 2). In order to increase the amount of target protein, the culture was supplemented with 1% glucose together with 1 mM IPTG and 1 mM PMSF; more abundant 100-kDa rVrSBEI was induced (**Figure 3A**, lane 3). PMSF was shown essentially to maintain the stability of the 100 kDa rVrSBEI protein band (**Figure 3A**, lane 3 vs 4). Therefore, rSBEI was induced by 1 mM IPTG in the presence of 1% glucose and 1 mM PMSF for 3 h, and 1 mM PMSF was also added in the cell suspension before lysis. The rVrSBEI was purified by affinity chromatography (**Figure 3B**). The peak fraction appeared at approximately 100−150 mM imidazole concentration (**Figure 3B**). When the crude extract and affinity-purified fractions (**Figure 3C**, lanes 3 vs 4) were examined, the 100-kDa protein was the major His-tagged rVrSBEI protein. Activity of the partial purified enzyme was analyzed by the amylose branching assay and showed a time-dependent decrease of absorbance (**Figure 4D**). The specific activity of rVrSBEI in the crude extract was 2.76 U/mg and in the purified fraction was 314.6 U/mg, indicating a 114-fold activity enrichment (**Figure 4E**).

***VrsbeI* Expression during Different Growth Stages.** The amount of a 415 bp amplified cDNA fragment from total RNA was used for monitoring *VrsbeI* gene expression at the transcriptional level in different growth stages. The specificity of the primer pairs was evaluated by BLAST, as well as to check the inability to amplify any fragments on the cDNA template of *VrsbeII*. The R4 primer was designed from the 3′-end of *Vrsbe1*, which is completely different from the corresponding segment in *VrsbeII*, and R4 did not anneal with endogenous *VrsbeII* in the first strand cDNA populations, only with *VrsbeI*. The relative invariant amplification of 371 bp β-actin was used as a control for the integrity and normalization of the total RNA template. RNA normalization was achieved by the amount of first strand cDNA used in PCR for β-actin to obtain a constant level. Then, parallel PCR of the *VrsbeI* and β-actin for different growth stage samples were performed in their own batch instead of coamplification. In this manner, the interference/competition among the primers of internal standard and target gene during reaction is avoidable (*13*). Results showed that as the seed size enlarged (**Figure 4A**), *VrsbeI* gene expression increased (**Figure 4B**) when β-actin gene expression remained at almost a constant level. The intensity of the 415 bp fragment increased from week 1 and increased substantially at weeks 3−4 during growth, indicating that there is more abundant *VrsbeI* gene expression in the late growth period than the early stage. This result conformed to the previous result that a family B 84-kDa SBE activity-related protein was found as starch granule-associated proteins, and it belongs to the late expressed and the trapped SBE form within the granule during mungbean maturation (*17*).

Prediction of 3D Structure and Function of VrSBEI. *E. coli* glycogen synthase (GS) (*18*) is the template in Swiss Model having 27.4% sequence identity (higher than the minimal value of 25%), which was matched with VrSBEI for 3D structural prediction (**Figure 5A**). GS resembles SBE in catalyzing the similar reaction except for its native substrate and product, glycogen, which is the α-1,4 glucan with short α-1,6 branches in animals and microorganisms. This GS enzyme also consists of three major domains, an N-terminal domain, a C-terminal domain, and a central $(\beta/\alpha)_8$ TIM barrel domain containing the enzyme active site. The eight conserved catalytic residues (**Figure 5A** bottom, color in red) within the central $(\beta/\alpha)_8$ domain were Y300, D 335, H340, R403, D405, E458, H525, and D526. Partial structures were simulated from this template where the 252 amino acids (34.3% of full length) of VrSBEI ranging from E272 to K523 (**Figure 5B**) were predicted. Six of the conserved catalytic residue counterparts (**Figure 5B** top, colored in blue; Y313, D348, H353, R420, D422, and E477) in VrSBEI were located in the structure. The sequence of VrSBEI was analyzed by R_EMUS to identify locations and compositions of unique peptide segments from a set of protein family sequences. These unique sequences were located and segmented from the predicted 3D structure by Swiss-Pdb Viewer. It is interesting that one region unique to the SBEI species (F365F to F376) was found in the loop motifs between β3/α3 (**Figure 1**, solid square labeled with I), which are extended toward the exterior of the molecules (**Figure 5B**, label in red). The unique sequence was retrieved and would be the potential epitope region to design VrSBE1 monoclonal antibodies (*14*).

DISCUSSION

In the family B SBE isoforms in plants, sequence variation of the deduced SBEs of different origin was reported mainly at the N- and C-termini and have different apparent molecular size (*9*). When comparing with either genes or cDNAs reported in



(E)

Procedure	Total protein (mg)[a]	Total activity (U)[b]	Specific activity (U/mg)[c]	Recovery (%)	Purification (fold)
Crude enzyme	78.2	216	2.76	100	1
HisTrap column	0.39	121	314.6	56	114

Figure 3. Induction and activity of VrSBEI expression in the *E. coli* BL21 (DE3) cell. (**A**) Induction optimization: lane M, molecular marker; lane 1, no-induction; lane 2, induction by PMSF and IPTG; lane 3, induction by PMSF, IPTG, and glucose; lane 4, induction by IPTG and glucose. Each of the final concentrations is 1 mM PMSF, 1 mM IPTG, and 1% glucose, respectively. The crude cell extract prepared from the 2-mL culture was analyzed. (**B**) Elution profile. A linear gradient was programmed for the elution buffer of 40 mM (0%) to 500 mM (100%) imidazole in 20 mM sodium phosphate containing 0.5 M sodium chloride (pH 7.4) in a low pressure liquid chromatography system; protein of 34.6 mg in 5 mL of lysis buffer was loaded onto the column; the peak fractions at 100−150 mM imidazole concentration were pooled, containing 306 μg of protein for gel and activity analysis; (**C**) SDS−PAGE analysis of partially purified rVrSBEI. Lane M, molecular marker; lane 1, noninduced crude cell extract; lane 2, induced crude cell extract (~28 μg); lane 3, unbound proteins from the column fraction (~2.5 μg); lane 4, eluted rVrSBEI in peak fraction (~0.6 μg). (**D**) Absorption spectra of the iodine-stained products of rVrSBEI action on amylose for 0, 30, and 60 min. Each data point is taken from the average of triplicate samples in which ~0.19 μg of protein was included in the assay mixture. The 0-min sample represents the unreacted sample; a blank scanning has been conducted to auto zero the baseline scan. (**E**) Summary of rVrSBEI purification. a, total protein content was from the crude extract of the 250-mL batch *E. coli* BL21 (DE3)/pET30-*VrSBEI* culture; b, one unit of activity was defined as the decrease in absorbance at 660 nm of 0.1 per min (Δ0.1/min); c, specific activity (S.A.) was defined as U/mg protein.

rice (*19*), cassava (*20*), maize (*21*), potato (*22*), pea (*8*), *Arabidopsis* (*23*), wheat (*24*), kidney bean (*25*), sorghum, and barley (*26*), the full length *VrsbeI* cDNA of a predicted protein of apparent molecular size 84-kDa in this study encodes a shorter C-terminal region. The full length of the VrSBEI protein is 97 amino acids shorter than rice RBEI (D 10752, from residue 724−820), 103 amino acids shorter than wheat SBEI (AF286318, from residue 731−833), 93 amino acids shorter than maize SBEI (U17897, from residue 730−823), 116 amino acids shorter than kidney bean SBEI (AB029549, from residue 732−847), and 104 amino acids shorter than pea SBEII (X80010, from residue 723−826). As a result, it shows that the conserved 39 amino acids in most family B SBEs starting from FTSP EGIPGIPETN FNNRPNSFKV LSPP-RTCVVY YRVDE to their C-termini including the insertion-like sequence PEGIPGVP (*27*) are not present in the expressed rVrSBEI (**Figure 1**). One would expect that the shorter C-terminal and total length in mungbean SBEI would have smaller molecular size and be determinative of enzyme activities. However, when the size of the native form of the known family B SBEs was compared, the molecular size of the predicted 84-kDa VrSBEI reported here was found to be even longer than these active SBEs. For instance, potato SBEI is an active 80-kDa protein in tubers and fresh leaves (*22*). Kidney bean SBEI is purified as an active 82-kDa protein (*25*), and active rice RBE1 is 82-kDa (*28*). Therefore, it is not surprising that the rVrSBEI in this study is catalytically active, let alone that the predicted length of VrSBEI is the shorter one among registered SBE species. Some of those predicted sequences in the N- and C-termini among the above registered SBE species of longer length would not relate to enzyme activities. The data may imply that the C-terminal ends of other SBEs may not be necessary

for catalytic activity. This is certainly the case in maize SBEs (*30*) but not in kidney bean (*27*).

When the two isoforms VrSBEI and VrSBEII are compared, there are only 59% in cDNA and only 56% in amino acid sequence homologies between them. The corresponding 60% and 57% homologies were found between *sbeII* and *sbeI* of the kidney bean. These results suggest that *VrsbeII* and *I* should be from distinct mRNA transcripts and coded by different genes (*25*). Between the β3 and α3 regions, both isozymes contain a unique loop motif as identified by R_EMUS. The protruding region was reported as the Domain B identified in the α-amylase family (*29*). However, three more residues VGQ (**Figure 1**, 367−369 labeled in bold in the solid square box I) in the unique loop (**Figure 1**, box I; **Figure 5B**, lower panel, F365−F376) were found in VrSBEI compared with that in VrSBEII (**Figure 1**, D425-F432, DGTDSHYF labeled in bold). It is reflective that VGQ are the extra three residues found exclusively in family B SBE but not in family A SBE (*8*). Although they both contain the common catalytic $(\beta/\alpha)_8$-barrel domain (labeled α1-α8 and β1-β8) and four conserved active site regions (**Figure 1**, the dashed-line squares) of the α-amylase family, it is noteworthy in the $^{G}/_{A}$EDVS region that G is found in SBEII, and A is found in SBEI, which is another sequence difference between the two families (*1*, *27*).

In addition, the predicted VrSBEI is shorter than VrSBEII in both the N-terminal (77 residues) and in the C-terminal (55 residues) (**Figure 1**) domains. The difference at their N-terminal domain between the two would indicate different flexibility and local structure, and ultimately affect their interactions with the starch substrate such as substrate chain length and the chain length of transferring from α-1,4 to the α-1,6 position (*1*). The difference at their C-terminus thus represents that the substrate preference and catalytic efficiency for amylose or amylopectin would be dissimilar as found in maize and kidney bean (*27*, *30*). Maize SBEI transfers longer chains of d.p. (degree of polymerization) 40−100 and has higher affinities for amylose, whereas SBEII transfers shorter chains of d.p. 6−14 and prefers amylopectin as a substrate. These substrate binding kinetics differentiate their distinct roles between the two families in starch biosynthesis (*21*). Therefore, sequence information obtained from the current report will allow us to further investigate the effects and roles of the shorter N- and the C-terminal domains in rVrSBEI compared to rVrSBEII in catalytic activities for α-1,6-branched starch formation, the structure utilization of amylose

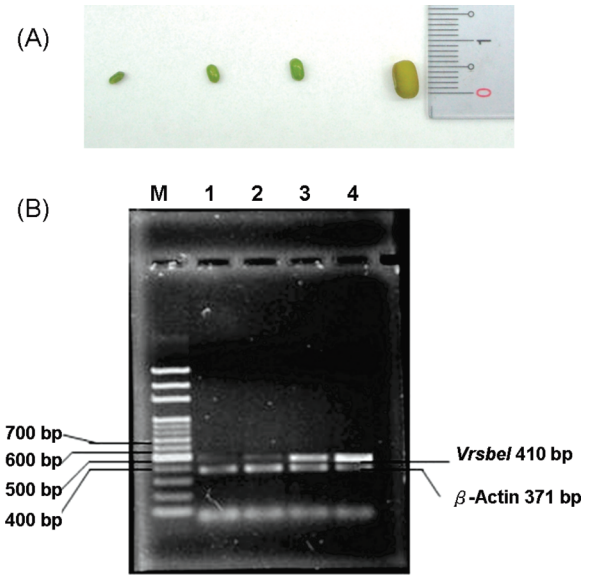


Figure 4. *Vrsbel* gene expression in mungbean during growth. (**A**) Size of developing mungbean. Beans from left to right were from the bean pod of weeks 1, 2, 3, and 4 after flowering; the small grid on the ruler is in the millimeter scale. (**B**) *Vrsbel* expression profile during growth; lane M, 3-kb marker. The 700 bp, 600 bp, 500 bp, and 400 bp contained 10 ng, 10 ng, 30 ng, and 10 ng of DNA, respectively. Lanes 1−4 are the RT-PCR products from mungbeans 1−4 weeks after flowering.

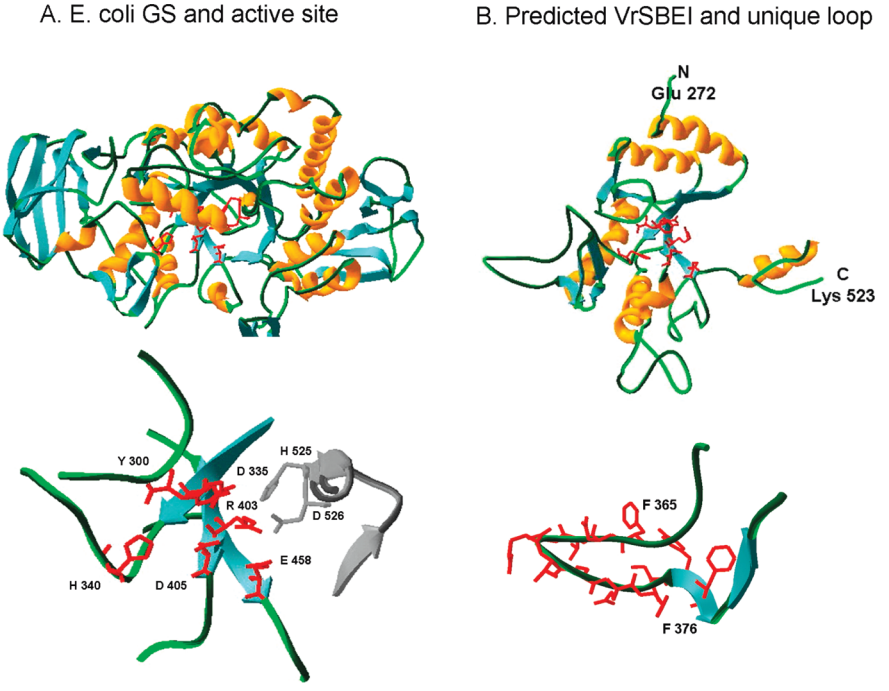


Figure 5. Homology modeling of the predicted VrSBEI with *E. coli* glycogen synthase (GS). (**A**) Template *E. coli* GS, 1m7x (top) and 8 conserved residues in the active site (bottom). (**B**) VrSBEI, E (Glu) 272-K (Lys) 523 (top) and its unique F365−F376 region (bottom). The belt portion colored in gray shows the H525 and D458 in the active site of *E. coli* GS (A panel bottom), where the corresponding region was not predicted in VrSBEI. The red-color side chains in *E. coli* GS (A panel top and bottom) and in VrSBE I (B panel top) are the residues located in the conserved active site.

and amylopectin as the substrate, and how these features would be different from those in other plant species resulting in the unique structure in mungbean starch.

rVrSBEI was expressed as a heterologous protein and partially purified with a specific activity of 314.6 U/mg by the amylase-branching assay (**Figure 3**E). Previously, the native SBE activity prepared from developing mungbean seed (*31*) had a crude extract enzyme activity of 0.002 U/mg; after sucrose gradient purification, the specific activity increased to 0.191 U/mg. Therefore, the recombinant VrSBEI was endowed with a much higher specific activity (314.6 vs 0.191 U/mg) than the partially purified native enzyme. When the activity of rVrSBEI in this study was compared with other *E. coli* expressed recombinant SBEs under the same assay method, the specific activity of maize rBEI was 574 U/mg (*21*), kidney bean rSBEI was 254 U/mg (*25*), and rice rSBEI was 20.8 U/mg (*32*). Wheat pABEI was able to branch the amylose-like molecules when expressed in the branching enzyme-deficient *E. coli* strain KV832 cells (*24*). These results clearly demonstrate that the polypeptide chain of the recombinant form of family B SBE is able to be properly folded in the prokaryotic host cells and expressed into active enzymes.

In conclusion, we have successfully cloned and expressed the full length cDNAs of family B mungbean SBEI in *E. coli* system. The deduced primary sequence established its phylogenetic relationship among SBEs. The features in their N-terminal, central and C-terminal regions and information from the predicted 3-D structures were described. Gene expression profile of *VrsbeI* in seeds showed that it accumulated at the late growth stage. The *VrsbeI* clone and the active rVrSBEI protein are useful for further molecular manipulation and to exploit application potential.

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