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Cloning, Characterization, and Expression of Mungbean (*Vigna radiata* L.) Starch Branching Enzyme II cDNA in *Escherichia coli*

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Full-length starch branching enzyme II (SBE, EC 2.4.1.18) cDNA from mungbean (*Vigna radiata* L. cv. Tainan no. 5), *VrsbeII*, was cloned, characterized, and expressed as an active enzyme in *Escherichia coli*. Gene-specific primers first amplified an internal cDNA by reverse transcriptase Polymerase Chain Reaction (RT-PCR), followed by obtaining 5′ and 3′ fragments by RT-PCR and rapid amplification of cDNA ends (RACE). *VrsbeII* possesses a complete open reading frame (ORF) of 2571 bp, and the deduced polypeptide includes the common catalytic $(\beta/\alpha)_8$-barrel domain and conserved regions of the α-amylase family. Phylogenetic analysis classified *VrsbeII* into SBE family A. Its partial 3D structure and functional features were predicted. *VrsbeII* has a shorter N-terminal among SBEs; however, two 6 bp (CCAGTT) direct repeat sequences (DRS) were found. A 24 bp shortened *VrsbeII* at the 3′ end, skipping one DRS, was ligated into pET21b vector and expressed as His_6-rVrSBEII in *E. coli* BL21 (DE3) cells. The optimal expression condition for rVrSBEII was evaluated and detected by Western blot with a molecular size of 108 kDa and activity of 6.4 U/mg.

KEYWORDS: ***Vigna radiata*; mungbean; starch branching enzyme; RACE; cDNA expression; DRS**

INTRODUCTION

Starch is a glucose homopolysaccharide consisting of two distinct types of polymers, the almost linear α-1,4 glucan (amylose; normally 15−30%) and α-1,4 glucan with α-1,6 branches (amylopectin; normally 70−80%). Its biosynthesis occurred in plastids, that is, the chloroplast in leaves and the amyloplast in nonphotosynthetic tissues of higher plants (*1*). The stroma of the amyloplast in the sink tissues (e.g., seeds, tuber, and endosperm) comprises a group of enzymes including ADP glucose pyrophosphorylase, starch synthase (SS), starch phosphorylase (SP), starch branching enzyme (SBE), debranching enzyme, and disproportionating enzyme, which contribute to starch biosynthesis and granule biogenesis. The α-1,6 branch formation in starch polymer was known to be catalyzed both by SBE and by SS in higher plants (*2*).

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) is a bifunctional enzyme possessing α-amylolytic hydrolase and glucosyl-transferase 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. Multiple isoforms of SBE were found and designated different names among plant species. According to amino acid sequence comparison, SBEs were classified into A and B families (*3*). The two families differ in enzymic properties such as the preferred length of chain transfer and substrate specificity, the two determinant factors of starch structure (*4*). This implied the potential use of SBE as a functional enzyme in biotechnology applications. Family A SBE genes or cDNAs have been cloned and analyzed in various plant sources such as rice SBE (*5*), pea (*3*), *Arabidopsis* (*6*), maize (*7*), potato (*8*), wheat (*9*), barley (*10*), kidney bean (*11, 12*), cassava (*13*), and sorghum (*14*).

Mungbean (*Vigna radiata* L.) is an essential ingredient used in making oriental foods such as greenbean cake and bean noodle. It contains as high as 45% dry weight of branched amylose content, which makes the retrogradation characteristics of mungbean starch more severe than those of legume starch and makes the mungbean noodle appear translucent and become more resistant to heating during cooking (*15*). Such unique starch structure is expected to be synthesized by a set of unusual biological machinery. Among the enzymes in the starch biosynthesis pathway, the major enzyme to produce branched structure within amylose/amylopectin chains in mungbean would point toward SBE. Our previous studies found two proteins possessing SBE activities in the mungbean starch granule (*16*), detected three SBE activity populations in the developing seed (*17*), and identified a family A SBE from the immature seed of

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the mid-developmental stage (*18*), indicating that SBE is an actively expressed enzyme in mungbean.

To understand mungbean SBE at the molecular level and to evaluate its application potential in biotechnology, this study reports the cDNA cloning of the family A SBE from mungbean seeds based on the conserved motif information of registered SBEs. Gene-specific primers were first designed to obtain partial cDNAs by reverse transcriptase Polymerase Chain Reaction (RT-PCR), and subset primers were designed from their internal partial sequences to amplify their 5′ and 3′ ends by rapid amplification of cDNA ends (RACE) and nested PCR. The full-length sequence was obtained, characterized, and used to predict its three-dimensional (3D) protein structure in silico. To further correlate its catalytic activity in α-1,6 branched glucan formation, the cDNA has been successfully expressed in the *Escherichia coli* system as an active recombinant enzyme.

MATERIALS AND METHODS

Materials. Immature mungbean pods (cultivar Tainan no. 5, VC3890A) were collected at 14 days after flowering (DAF) in the field growth arranged by Chao-Hui Wu at the Division of Crop Improvement in Tainan District Agricultural Research and Extension Station (Tainan Taiwan) and stored at −80 °C before use. Bacterial growth medium, antibiotics, and salts were from Ameresco (Solon, OH). dNTPs (dATP, dCTP, dGTP, dTTP) were from ABgene (Epsom, U.K.). Smart RACE cDNA amplification kit was from Clontech (Palo Alto, CA). DNA standard (Gen-100 DNA ladder) was from M&J ScinTek Co. Ltd. (Taichung, Taiwan). SuperScript One-Step RT-PCR system was from Invitrogen (Carlsbad, CA). Vector pGEM-T Easy and PolyATtract mRNA isolation systems were from Promega Corp. (Madison, WI). NovaBlue, *E. coli* BL21(DE3) competent cells, and pET-21b vector were from Novagen (Darmstadt, Germany). FastStart Taq DNA polymerase was from Roche (Mannheim, Germany). Agarose was from Cambrex Corp. (Rockland, ME). Molecular chemicals, media, and reagents were from Sigma Chemical Co. (St. Louis, MO). Gel Extraction, PCR-M Clean Up system, and plasmid DNA Extraction system were from Viogene Biotek Corp. (Taipei, Taiwan). Ultrafree-DA and Amicon YM-10 membrane microfilter were from Millipore (Bedford, MA). Protein analysis chemicals were from Bio-Rad (Hercules, CA). Enhancer chemiluminescence (ECL) detection kit, Histrap column, His buffer kit, and Tween-20 were from Amersham Biosciences (Uppsala, Sweden). Anti6x His tag mouse monoclonal antibody was from LTK BioLaboratories (Taoyuan, Taiwan). Peroxidase-conjugated affinipure goat anti-mouse IgG (H +L) was purchased from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA). Broad range 10−200 kDa molecular marker was from Fermentas (PageRuler Unstained Protein Ladder, Vilnius, Lithuania).

Primer Design. Primers were designed by the SeqWeb service linked with the National Health Research Institute of Taiwan (Taipei, Taiwan). For designing gene-specific primers (GSPs), registered SBE sequences were first retrieved by Lookup, followed by using MEME to find their conserved motif sequences, and then were generated by Prime. Nested primers were designed from the internal sequence of cDNA fragments during the amplification process. Primer specificity was examined by Blastn and synthesized by Unimed Incorp. (Taichung, Taiwan) and Tri-I Biotech Co. (Taipei, Taiwan). Primers (**Figure 1A**) used in this study were F1 (5′TGGATATTGTTCACAGTCATGC3′), F2 (5′AAGATGAGGACTGGAAAATGGGCG3′),F3(5′ATGGTTTACACCATCTCGGGAATTCGATTTCCG3′), R1 (5′CTTGGGAAATCTATCCATTCAGGATGCC3′), R2 (5′TCAAGGATCAACTGGCTCTGGTTCA3′), R3 (5′AAGACGAGAATCCCACATCC3′), and NUP (5′AAGCAGTGGTATCAACGCAGAGT3′). F1 and R1 were designed from the nucleotides of two conserved motif sequences of SBE GTPEDLKYLVDKAHSLGLRVLMDVVHSHASNN and NFMGNEFGHPEWIDFPR. Universal primer mix (UPM) was provided in SMART RACE kit as a mixture of 1 to 5 ratio of 5′CTAATACGACTCACTATAGGGCAAGCAGTGGTATCAACGCAGAGT3′ and 5′CTAATACGACTCACTATAGGGC3′. F2 and R3 were nested primers designed from *VrsbeII*-800; F3 was designed from the 5′-terminus of SBE of *PvsbeII* (AB029548); R2 was designed from the 3′-terminal of *VrsbeII*-1200. NUP was from the Smart-RACE reaction.

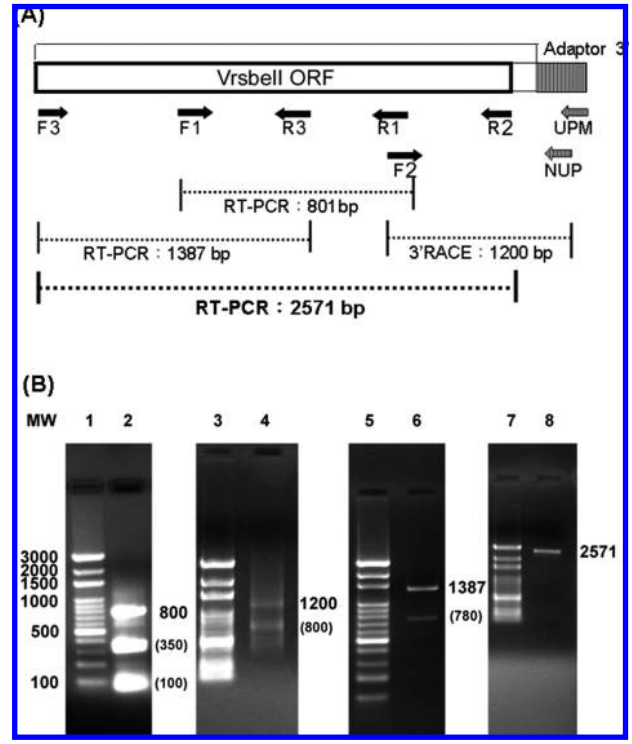

Figure 1. Cloning scheme for the full-length cDNA of *Vrsbell*. (**A**) Primers and amplicon length corresponding to the positions on ORF (open reading frame; the full-length cDNA from the starting codon ATG to poly A tail) of the cloned *Vrsbell*. Primer details are described under Materials and Methods section. (**B**) Agarose gels of the amplicons analyzed at different stages: (lanes 1, 3, 5, 7) 3 kb molecular marker; (lane 2) 800 bp (major band), 350 bp, and 100 bp RT-PCR products from F1 and R1; (lane 4) 1200 bp (major) and 800 bp 3′-RACE products from F2 and NUP; (lane 6) 1387 bp (major) and 780 bp RT-PCR products from F3 and R3; (lane 8) full-length cDNA of 2571 bp *Vrsbell* confirmed by RT-PCR and PCR using F3 and R2 primer pairs. Gels of 1% agarose were used.

Total RNA and mRNA Preparation. The hot phenol method (*19*) was used for total RNA preparation. All of the reagents were nuclease-free. The water used as solvent for handling and preparation for the reagent was 0.1% diethyl pyrocarbonate (DEPC)-treated and autoclaved. Extraction buffer [100 mM Tris-HCl, 100 mM lithium chloride, 100 mM ethylenediaminetetraacetate (EDTA), 1% sodium dodecyl sulfate (SDS), and 100 mM β-mercaptoethanol, pH 7.5] of 20 mL was preheated to 65 °C and mixed with 20 mL (1:1) of 65 °C hot phenol (acid phenol from Sigma) in a water bath. Mungbean seed of 4 g was ground into a fine powder under liquid nitrogen, mixed with 40 mL of the above mixed hot extraction solution, and incubated at 65 °C for 5 min with intermediate mixing. Then, 20 mL of chloroform/isoamyl alcohol (24:1, v/v) was added into the extraction mixture and extracted for 1 min, followed by centrifugation for 20 min. All of the centrifugation steps were performed at 15000 rpm at 4 °C. The aqueous layer was extracted with equal volumes of phenol/chloroform/isoamyl alcohol (25:24:1, v/v) for 1 min and centrifuged for 10 min. The aqueous layer was then extracted for 1 min with chloroform/isoamyl alcohol (24:1, v/v) and centrifuged for 5 min to remove residual phenol two times. RNA in the aqueous layer was precipitated by adding an equal volume of 4 M lithium chloride to precipitate under −70 °C for 2 h.

The crude RNA was recovered by centrifugation for 30 min. The pellet was first resuspended with 10 mL of 2 M lithium chloride, recovered by centrifugation for 10 min, and then dissolved in 5 mL of 0.1% Lauro-sarcrosyl at room temperature. RNA was added with 5 mL of 100% ethanol and 200 μL of 3 M sodium acetate (pH 4.3) to precipitate again under −70 °C for 16 h and recovered by centrifugation

for 15 min. The resulting total RNA pellet was washed with 80% cold ethanol and recovered by centrifugation for 15 min, and the residual ethanol was removed by speed-vacuum. The final RNA pellet was dissolved in 200 μL of 0.1% Lauro-sarcrosyl to measure quantity and purity at A_{260}/A_{280}. The mRNA was prepared from total RNA by PolyATtract (Promega) to obtain mRNA quality of $A_{260}/A_{280} \geq 2.0$. The quality of RNA was analyzed by 1.2% formaldehyde agarose gel electrophoresis (1.2% agaroses, 1× MOPS buffer, 2.2 M formaldehyde) in a Sub-Cell GT system (Bio-Rad) to examine the intactness of the 28S and 18S rRNAs. 3-(*N*-morpholino)propanesulfonic acid (1×; MOPS) buffer contained 20 mM MOPS, 8 mM sodium acetate, and 1 mM EDTA, pH 7.0.

RT-PCR for Amplification and Cloning. The SuperScript One-Step RT-PCR (Invitrogen) was used. The reaction mix contained 25 μL of 2×reaction mix (0.4 mM dNTP, 2.4 mM magnesium sulfate), 10 pg−1 μg of mRNA, 10 μM sense and antisense primers, and 1 μL of 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 (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). A small product volume (2−5 μL) was withdrawn for 1% agarose gel electrophoresis analysis. The amplicons were cloned into either pGEM-T or pET21b vectors for characterization.

Rapid Aplification of cDNA Ends. 3′RACE was performed according to the manufacturer's instruction (Clontech). In short, template mRNA of 50 ng−1 μg was denatured with 10 nmol of lock-docking oligo (dT) primer and 10 nmol of SMART II A oligo in 5 μL at 70 °C for 2 min and cooled on ice. Then the template−primer mix was incubated with 50 mM Tris-HCl (pH 8.3), 75 mM potassium chloride, 10 mM dithiothreitol, 6 mM magnesium chloride, 200 U of PowerScript reverse transcriptase, and 1 mM dNTP in 10 μL at 42 °C for 1.5 h. The obtained first-strand cDNA was diluted with 250 μL of buffer (10 mM Tricine-potassium hydroxide, 1 mM EDTA, pH 8.5) to act as RACE-ready cDNA.

RACE was performed in a 50 μL reaction containing 2.5 μL of RACE-ready cDNA, 0.2 μM of either reverse GSP (for 5′ RACE) or forward GSP (for 3′ RACE), 1× universal primer mix (40 μM 5′CTAATACGACTCACTATAGGGCAAGCAGTGGTATCAACGC-AGAGT3′ and 0.2 μM 5′CTAATACGACTCACTATAGGGC3′), 0.2 mM dNTP, 1× Advantage 2 PCR buffer, and 250 U of DNA polymerase to react at 94 °C for 5 s, 68 °C for 10 s, and 72 °C for 3 min for 25−35 cycles. PCR product of 2−5 μL was withdrawn for gel analysis.

DNA Preparation, Purification, Analysis, and Sequencing. DNA was analyzed by 1% agarose gel electrophoresis using TAE buffer (50× TAE contained 40 mM Tris-base, 20 mM acetic acid, 1 mM sodium-EDTA, pH 8.0) in a Sub-Cell GT system (Bio-Rad). Homogeneous DNA fragment amplified from PCR was purified by PCR-M Clean Up system (Viogene) before sequencing. When multiple DNA products were obtained by PCR, the interested DNA size was excised from agarose gel and purified by Ultrafree-DA (Millipore) or Gel Extraction (Viogene). Purified DNA was sequenced by an ABI PRISM 3100 genetic analyzer and an ABI PRISM 377-XL using dideoxy-mediated termination reaction in the Biotechnology Core Facility Center at the National Chung-Hsin University (Taichung, Taiwan).

Sequence Aalysis and 3D Structural Prediction. DNA sequence was analyzed in NCBI (National Center for Biotechnology Information, http://www.ncbi.nlm.nih.gov) or in GCG (Genetics Computer Group) SeqWeb database services or by Vector NTI advance 10 software (Invitrogen). Programs Blast, Pileup, Pretty, Frame, Align, GrowTree, Map, Translate, and Reverse were used. Amino acid sequence homology of the deduced VrSBEII among registered SBE species used the Kimura protein-distance algorithm method. Homology percent was derived from 100 minus distance. Their 3D structures of the deduced amino acid sequences were simulated in the structure homology-modeling server, SWISS-MODEL (http://swissmodel.expasy.org). The registered structure found with sequence homology >25% was used as a template for the defined portion of the SBE to generate a superimposed image. 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 any relevant parts. R_EMUS (REinforced Merging for Unique Segment, http://140.121.196.30/remus) (*20*) was used to identify the location and compositions of unique peptide segments in SBE from a set of protein family sequences.

Construction of Expression Vector. A construct encoding 6× His-tag on the C-terminal of VrSBEII (His_6- VrSBEII) under the control of T7 promoter in pET 21b expression vector was made by the following. PCR forward primer F4 5′GCGGATCCAATGGTTTACA-CAATCTCGG and reverse primer R4 5′AATGCGGCCGCTATCTG-CAAGGACTG designed with *Bam*HI and *Not*I sites (underlined) that flank the *VrsbeII* were used to amplify in the high-fidelity Fast Start Taq DNA polymerase system (Roche). It amplified a 24-nucleotide shortened *VrsbeII* fragment at the 3′-end to avoid the one direct repeat CCAGTT sequence at 2557−2562. After the same *Bam*HI and *Not*I restriction enzyme cutting on the amplified insert and vector, the *VrsbeII* was ligated into pET21 vector at molar ratios of 3 to 1 at 16 °C overnight. The pET21-*VrsbeII* ligate was then transformed into *E. coli* NovaBlue cells by 42 °C heat shock approach and selected on SOC selection medium (contained 50 μg/mL ampicillin and 12.5 μg/mL tetracycline) for clone maintenance. The correct insert size in the pET21-*VrsbeII* clone was confirmed by PCR with F4/R4, cutting with *Bam*HI/*Not*I restriction enzymes, and sequencing of the plasmid preparation. The confirmed pET21-*VrsbeII* construct was transformed into *E. coli* BL21 (DE3) competent cells and selected on a Luria−Bertani (LB)−agar ampicillin (50 μg/mL) plate. To check the stability of the plasmid and correct insert size in the transformants, colony PCR was routinely performed during the study.

Protein Expression and Purification. A freshly transformed pET21-*VrsbeII*/BL21(DE3) colony was inoculated into 2 mL of LB−ampicillin (50 μg/mL) broth to grow at 37 °C overnight. Then 200 μL of the overnight culture was inoculated in 20 mL of LB−ampicillin (50 μg/mL) broth to grow at 37 °C until OD_{600nm} of 0.6, followed by induction with 0.2 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) for 5 h and harvest by 10000*g* centrifugation. Cell pellet was suspended in 20 mM Tris-HCl (pH 7.5) of 1 g of wet wt/5 mL. Cells were lysed by sonication at 5 W of 2 s with 5 s intervals for 10 min. Soluble cytosolic lysate fraction was recovered by 16000*g* centrifugation for 20 min at 4 °C.

For purification, the scaled-up 250 mL induced culture was prepared. The His_6-rVrSBEII was purified by immobilized metal-ion affinity chromatography (IMAC). A 1 mL HisTrap HP nickel ion affinity column (Amersham) was equilibrated with binding buffer (20 mM Tris-HCl, 0.5 M sodium chloride, 20 mM imidazole, pH 7.5) in AKTA prime system (Amersham). The soluble lysate was filtered with a 0.45 μM membrane filter and concentrated by Amicon YM-10 membrane filter, and 25.7 mg of protein in 0.5 mL of binding buffer was loaded onto the column, followed by washing with 10 mL of binding buffer. rSBEII was eluted by a linear gradient of 50−600 mM imidazole in elution buffer (20 mM Tris, 0.5 M sodium chloride, pH 7.5) with a flow rate of 1 mL/min. The pooled active fraction contained protein 0.124 mg/mL of 3 mL was collected for SDS-PAGE and Western analysis. For activity assay, the active fraction was desalted by passing through Sephadex G-25 (Sigma) and/or concentrated by Amicon YM-10 membrane filter (Millipore).

SDS-PAGE and Western Blotting. The crude lysate or the affinity-purified proteins were analyzed in 7.5% acrylamide/bisacrylamide (37.5:1) SDS-PAGE and detected by Western blotting according to similar procedures described previously (*21*). An ECL system (Amersham) was used for immunodetecting His_6- VrSBEII on the transferred PVDF membrane. The hybridization buffer was PBS−Tween (0.14 M sodium chloride, 3 mM potassium chloride, 8 mM disodium phosphate, 1.5 mM dipotassium phosphate, 0.1% Tween-20), blocking solution was 5% nonfat milk powder in PBS–Tween, first antibody was 1:1000 dilution of anti-6xHis-tag mouse monoclonal, and second antibody was 1:20000 dilution of peroxidase-conjugated affinipure goat anti-mouse IgG (H + L).

Amylose-Branching Assay. The amylose-branching assay (*17*, *22*) was used. Enzyme sample of 10 μL was mixed with 1 mg/mL amylose (type III, Sigma) in 1 M sodium citrate (pH 7.0) buffer of a final 100 μL volume to incubate at 30 °C for 90 min in a shaking incubator or

for 0−180 min in a time course assay. Then 0.9 mL of iodine solution (10 mM iodine, 14 mM potassium iodide) was added to measure the decrease of OD_{660nm}. One unit of SBE activity was defined as the decrease of 1.0 unit of absorbance per minute (Δ0.1/min). Specific activity (SA) was defined as units per milligram of protein.

RESULTS

Cloning of *VrsbeII* cDNA. Three amplification stages including two RT-PCR and 3′-RACE reactions were used to successfully obtain a full-length cDNA sequence of *VrsbeII* (**Figure 1A**) from mRNA. The major or longest amplicons obtained in each stage were sequenced and pursued further in the next stage. GSP pairs (F1/R1) amplified the first major fragment of 801 bp (**Figure 1B**, lane 2). Then, F2, designed from its 3′ terminus sequence, was used with UPM in the 3′-RACE reaction followed by using with NUP in a nested PCR to magnify the target product of 1200 bp (**Figure 1B**, lane 4). The sequences of the 801 bp and 3′ 1200 bp fragment were combined into a ∼1700 bp sequence. The 1700 bp sequence information was found with high similarity to that of the *sbeII* of kidney bean (GenBank AB029548) in the database. Therefore, the 5′ terminus sequence of kidney bean *sbeII* was used to design F3 and to amplify with the R3 designed from the first 801 bp fragment by RT-PCR, which eventually obtained the 5′-1387 bp fragment (**Figure 1B**, lane 6). As a result, the complete open reading frame (ORF) sequence of *VrsbeII* cDNA of 2571 bp was derived from overlapping the three fragments. The full-length size and nucleotide sequence of the *VrsbeII* cDNA was further amplified and confirmed by RT-PCR using F3 and the R2 designed from the 3′ terminus sequence (**Figure 1B**, lane 8) and cloned.

VrsbeII cDNA contains the complete ORF from the start ATG to the stop TGA codon and encodes a putative VrSBEII protein of 856 amino acids with a relative molecular mass of 97 kDa and a p*I* value of 5.47. The size of this putative VrSBEII matches closely with our previously identified family A type 96 kDa SBE isoform from partially purified mungbean SBE fractions (*18*). *VrsbeII* was registered in GenBank (accession no. AY622199).

Sequence Features and Characterization. Nucleotide and deduced amino acid sequences of the cloned *VrsbeII* were aligned (**Figure 2**). The known SBEII-specific N-terminal sequence features containing several serines and two or three proline residues (PP or PPP) (*3, 10*) were also observed. There were 22 serines among the N-terminal 135 residues, and two prolines were found as PRP at 144−146 residues (underlined). VrSBEII protein includes the common catalytic $(\beta/\alpha)_8$-barrel domain (regions are labeled with dashed arrows) and four conserved catalytic regions (labeled in dotted boxes), $HSH^{S}/_{A}S$ (410−414), GFRFDGVT (474−481), $^{G}/_{A}$EDVS (532−536), and AESHDQ (597−602), of the α-amylase family (*23*). In addition, between β-sheet-8 and α-helix-8, VrSBEII contains the 11 amino acid EQQLPNGSVIP (675−685) loop region, which was reported to be the conserved eQXLPnGsviP (capital letters represent the same amino acid, lower case letters represent not 100% identical, X represents any amino acid) sequence in family A but is lacking in the family B SBE isoforms (*3*).

A phylogenetic relationship among VrSBEII and 10 registered family A SBE species was constructed by GrowTree (**Figure 3A**). The percent of distance among the 11 SBE species was also compared (**Figure 3B**). This reflected that VrSBEII shares 94.6, 84, and 68.3% homologies to kidney bean SBEII (AB029548), pea SBEI (X80009), and potato SBEII (AJ011888). Both analyses showed that VrSBEII has the highest evolutionary intimacy to the SBE of kidney bean, followed by pea and potato.

When the N-terminal amino acid sequence among SBE species was aligned (data not shown), from residue 124 of VrSBEII, this protein is short of either the 15 amino acids found in kidney bean SBEII (GenBank ID BAA82828, 125−139, ASSPVDVDIPAKKTS) or the 18 amino acids found in pea SBEI (GenBank ID CAA56319, 127−144, TSSSLVDVNT-DTQAKKTS). When the C-terminal amino acid sequence among SBE species was aligned (data not shown), VrSBEII, kidney bean SBEII, and pea SBEI contain longer C termini than maize SBEII, wheat SBEI, rice RBE, and *Arabidopsis* by at least 25 amino acids after the conserved region PSRTAAVYAL (819−828 of VrSBEII).

Finding and Solving the Two Direct Repeat Sequences (DRS) in *VrsbeII*. One unexpected feature found in the *VrsbeII* cDNA sequence was the presence of two direct repeat sequences (DRS) CCAGTT in 5′-nucleotide 335−340 and 3′-terminal 2557−2562 (**Figure 2**, framed in box). Their presence resulted in homologous recombination in the flanking region of *VrsbeII* and excised away the main part of the cDNA during plasmid replication in the host cells. Therefore, mutation occurred, and only an approximate 350 bp PCR product was observed during our examination on the recombinant plasmid of the full-length clone by colony PCR. The presence of the DRS was confirmed by sequencing check on the shortened amplicon that only one copy of the direct 6 bp repeat CCAGTT sequence remained and connected with the 5′ 1−334 and 2563−2571 3′-end of *VrsbeII*.

The DRS indeed caused a dilemma for us to smoothly express the full-length VrSBEII in the *E. coli* expression system. To avoid the DRS problem during protein expression, a 24 bp shortened *VrsbeII* at the 3′-end, skipping the CCAGTT at 2557−2562 and designing with the *Not*I site, was ligated in-frame into the pET21b vector to express as His_6- rVrSBEII in *E. coli* BL21 (DE3) cells.

Prediction of 3D Structure and Function of VrSBEII. *E. coli* glycogen synthase (GS), 1m7x (*27*), was the only template retrieved by Swiss Model where 36.7% sequence homology (higher than the minimal value of 25%) was matched with VrSBEII for 3D structural prediction. GS resembles SBE in catalyzing the similar reaction except that its native substrate and product are glycogen, which is the α-1,4 glucan with α-1,6 branches in animals and microorganisms. This GS 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 β-sheets locate inside and the α-helixes locate outside the barrel domain. A partial structure of VrSBEII was simulated from this GS template, where 380 amino acids (44.4% of full length) ranging from E195 to D574 (**Figure 4A**, labeled Glu 195 and Asp 574) were predicted.

The sequence of VrSBEII was analyzed by R_EMUS (*20*) 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-PdbViewer. It is interesting that one region unique to VrSBEII (D425 to F432) (**Figure 4B**) was found in the loop motifs between β3 and α3, which is extending toward the exterior of the molecules. Another region found unique to VrSBEII (P299 to I320) is also an extended loop before the β1 region. These unique sequences were retrieved as the potential epitope regions to be designed for making monoclonal antibodies (*20*) and may work for VrSBEII.

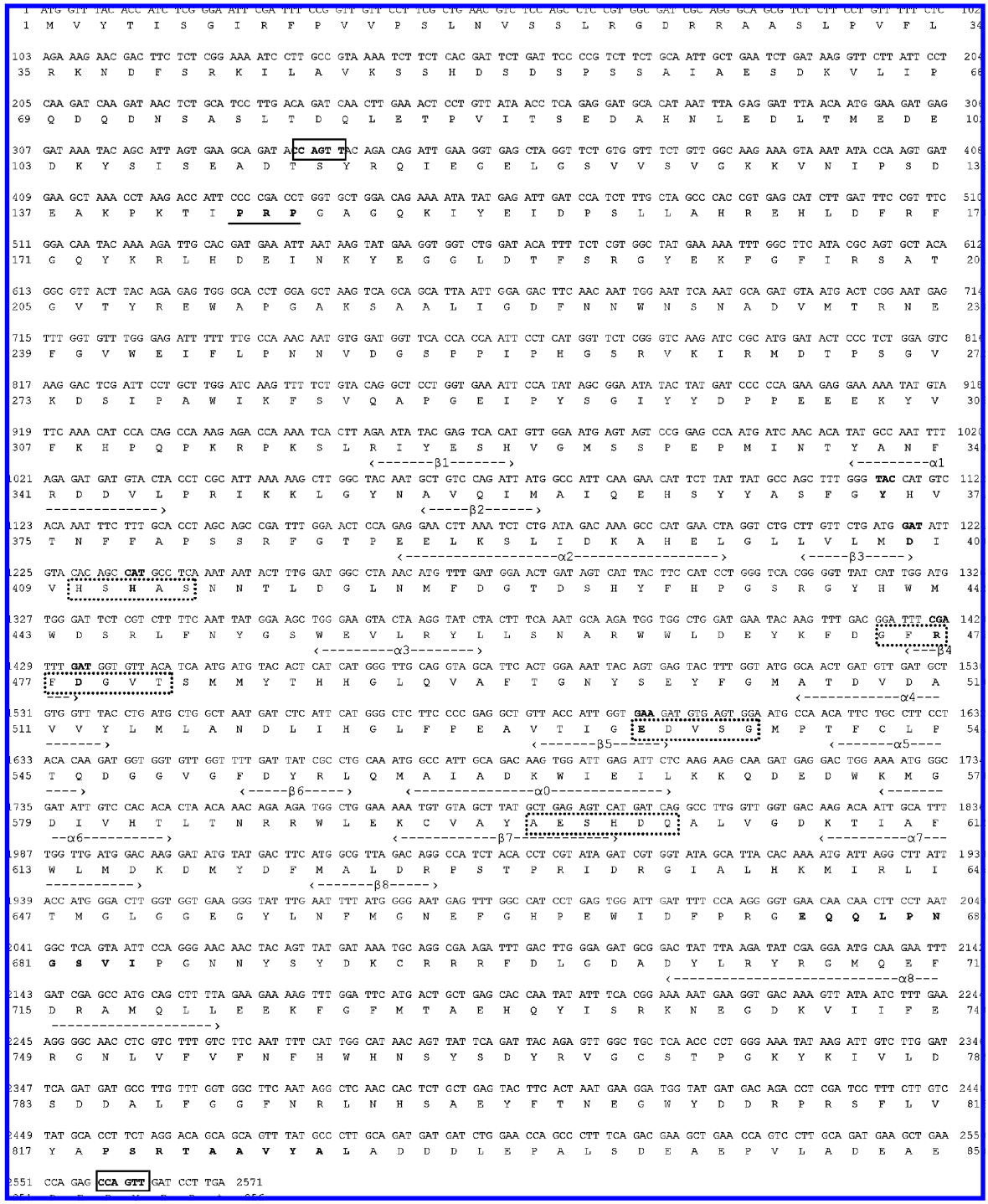

Figure 2. Nucleotide and deduced amino acid sequence of *Vrsbell* cDNA. The dot boxed amino acids indicate four conserved regions of the α-amylase family. The eight α-helixes and eight *β*-sheet regions are labeled with dash-line arrows. The two open boxes locate the direct repeat sequence, CCAGTT, found in *Vrsbell*. The underline labels the two conserved proline residues in the N-terminal. The residues given in bold letters, Y372, D407, H412, R476, D478, and E533, were predicted to be important for catalytic activities.

rVrSBEII Expression Optimization and Detection. The induction condition for the expression of His_6- rVrSBEII in *E. coli* BL21 (DE3) cells was optimized by varying the IPTG concentration and induction time. IPTG was added to final concentrations of 0, 0.2, 0.4, 0.6, 0.8, and 1 mM during induction at 37 °C for 5 h, and soluble cytoplasmic proteins in the crude extract were analyzed by SDS-PAGE (**Figure 5A**) and detected by Western blot (**Figure 5B**,**C**). This showed minor protein profile change under different induction conditions in SDS-PAGE; however, there were approximately five His-tagged proteins detected by Western blotting using the anti-6xHis-tag mouse monoclonal antibodies (**Figure 5B**, lanes 3−7). These induced protein bands were detected when 0.2 mM IPTG was used, and their amount was not much different when 1 mM IPTG was used. It seemed to include basal levels of nonspecific interaction of one protein detected in host BL21 cell (**Figure 5B**, lane 1) and another protein detected in noninduced cells (**Figure 5B**, lane 2). There were three truly induced bands. The one with an approximate molecular size of 108 kDa protein matched closely with the size of rVrSBEII. These shorter bands might result from incomplete expression or minor proteolysis during expression

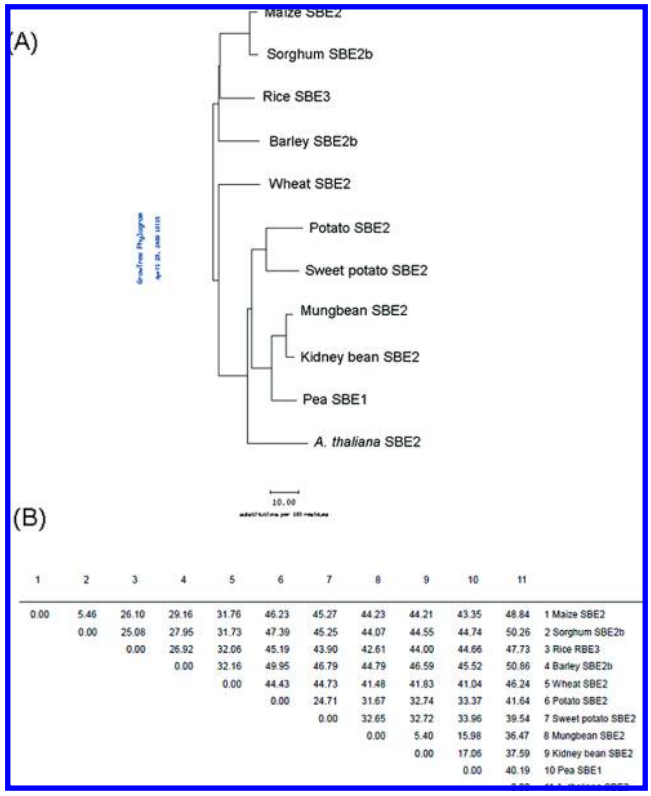

Figure 3. Phylogenetic analysis of mungbean SBEII among registered family-A BE isoforms: (**A**) analysis by GrowTree; (**B**) analysis by distance. Kimura protein-distance algorithm in the distance program of GCG was used. The retrieved sequences were maize SBE2 (L08065), sorghum SBE2b (AY304540), rice SBE3 (D16201), barley SBE2b (AF064561), wheat SBE2 (Y11282), potato SBE2 (AJ011888), sweet potato SBE2 (AB071286), mungbean SBE2 (AY622199), kidney bean SBE2 (AB029548), pea SBE1 (X80009), and *Arabidopsis thaliana* SBE2 (AK226896).

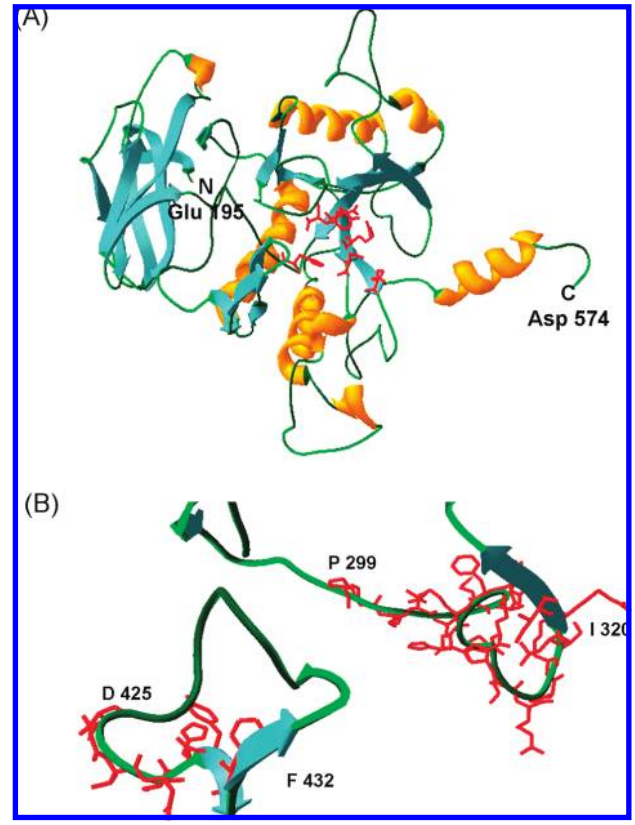

Figure 4. 3D homology modeling of VrSBEII. (**A**) simulated *VrSBEII* E195-D574 from *E. coli* glycogen synthase (GS) 1m7x template generated by SWISS MODEL (labeled side chains were the residues in the conserved active site); (**B**) unique D425-F432 and P299-I320 sequence regions of VrSBE identified by R_EMUS (the two locally enlarged loop regions correspond to the 3D view of graph **A** by Swiss-PdbViewer).

and operation. When different induction times were used from 5 to 20 h and detected by Western blot (**Figure 5C**), 5 h induced the highest density of the target 108 kDa. With the increase of incubation time, the induction of this target protein decreased. Therefore, the optimized induction condition for rVrSBEII expression was 0.2 mM IPTG for 5 h.

Purification and Activity of rVrSBEII. The pET21b vector expressed rVrSBEII protein has a six histidine sequence tag on the C terminus. A HisTrap HP nickel ion affinity column was used to purify the protein from the crude cytoplasmic extract (**Figure 6A**) and assay the amylose-branching activity. It showed that only a few bands were purified into the affinity-eluted His-tagged protein fraction, and SDS-PAGE showed the 108 kDa rVrSBEII was the major species (**Figure 6A**, lane 4). After purification, these ECL-detected His-protein signals were more enhanced than they were in the crude fraction (**Figure 6B**, lanes 3 and 4). Therefore, the intensity and expected molecular size among the noninduced crude soluble extract, the induced crude soluble extract, and the purified fraction were compared on either SDS-PAGE or the expressed His-protein by Western blots, demonstrating that the 108 kDa is the expressed rVrSBEII.

The activity of the rVrSBEII was examined by amylose-branching ability (*17, 22*) in a time course assay. It showed a time-dependent decrease of OD_{660nm} reflected by branching on the amylose substrate up to 90 min, demonstrating the rVrSBEII was expressed as an active form. The specific activity of rVrSBEII was enhanced from 0.252 to 6.402 U/mg (25-fold) after purification (**Table 1**).

DISCUSSION

The deduced amino sequence of mungbean VrSBEII (**Figure 2**) was found to be shorter at the N terminus than its related family A SBE in kidney bean and pea (**Figure 3**). VrSBEII lacks either the 15 amino acids found in kidney bean SBEII (125−139, ASSPVDVDIPAKKTS) or the 18 amino acids found in pea SBEI (127−144, TSSSLVDVNTDTQAKKTS). The N-terminal domain is known to act as a "flexible arm", where its local structure would affect the interaction of SBE with the starch substrate and determine the chain length of transferring from α-1,4 to the α-1,6 position (*1, 4, 22, 24*). Therefore, the shorter N-terminal domain in VrSBEII implied its unique roles in catalytic activities for branched starch formation in mungbean and would be expected to be distinct from those in other plant species.

As for the C-terminal features, VrSBEII, kidney bean SBEII and pea SBEI contain longer C termini than maize SBEII, wheat SBEII, rice RBE 3, and *Arabidopsis* SBEII by at least 25 amino acids after the conserved region PSRTAAVYAL (819−828 of VrSBEII). The C terminus was reported to be involved in substrate preference and catalytic efficiency for amylose or amylopectin (*25, 26*). The C terminus of maize SBEII was reported to transfer short chains of dp 6−14 and prefers amylopectin as a substrate (*22*). The longer C terminus in mungbean VrSBEII than in maize SBEII implied that VrSBEII would possess different chain length transfer functionalities and need to be investigated further.

The 3D structure of VrSBEII was simulated from the only *E. coli* GS template 1m7x in the database (**Figure 4**). According

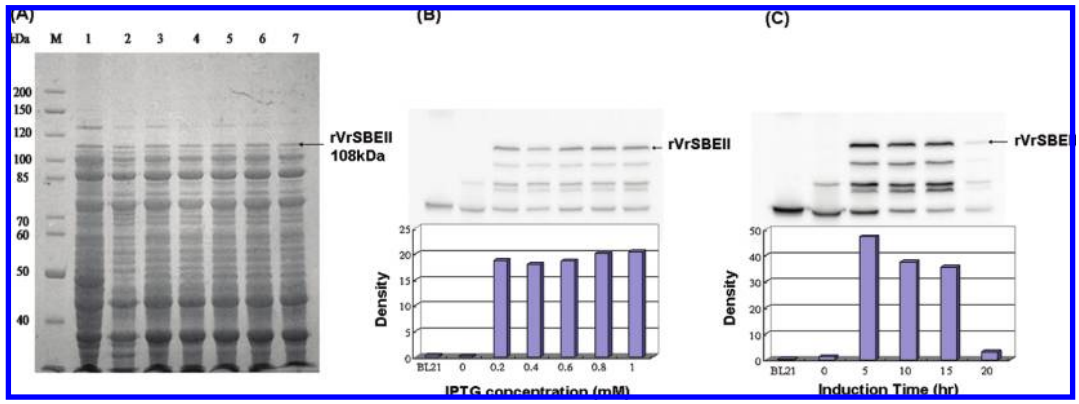

Figure 5. Induction optimization of rVrSBE expression in the *E. coli* BL21 (DE3)/pET21b-*VrSBE*II culture: (**A**) SDS-PAGE and (**B**)Western blotting analysis of the crude soluble fraction of the culture induced under different IPTG concentrations (lane M, broad range protein marker, 0.1−2.5 μg/band; lane 1, *E. coli* BL21 (DE3) control; lanes 2−7, culture induced with 0, 0.2, 0.4, 0.6, 0.8, and 1.0 mM IPTG for 5 h; (**C**) Western blotting of the *E. coli* culture induced with 0.2 mM IPTG for 5, 10, 15, and 20 h. *E. coli* BL21 (DE3) is the nontransformed culture that served as endogenous protein control. The signal of the expressed target His_6- rVrSBEII band is indicated. Sample load of each lane was 60 μg taken from 0.5 mL of the crude soluble cytosolic fraction prepared from 20 mL of induced culture (lysed by sonication of 1 g of cell wet wt/5 mL of 20 mM Tris-HCl, pH 7.5) buffer. SDS-PAGE gel of 4% stacking and 7.5% separation gel was used and stained with Coomassie blue.

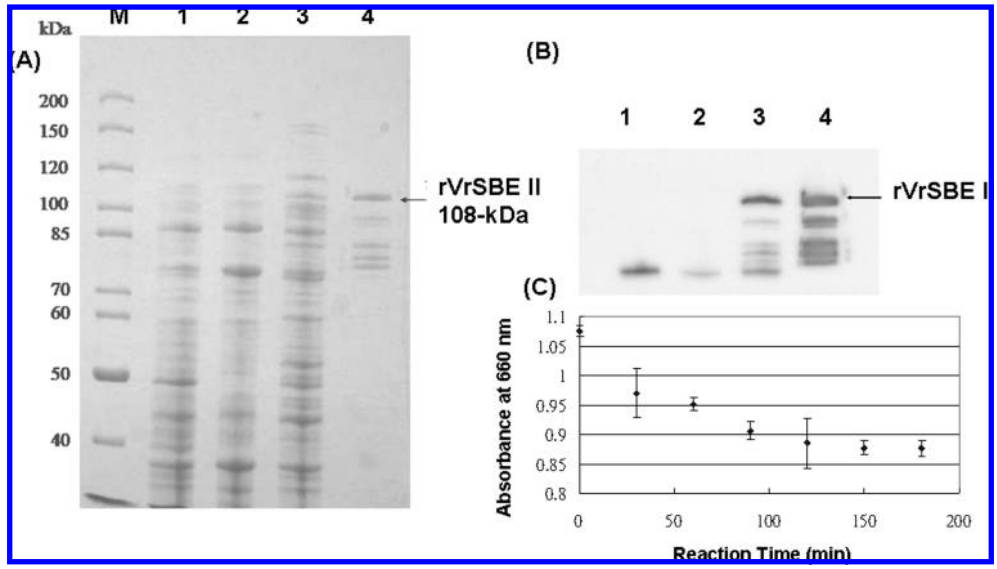

Figure 6. SDS-PAGE, Western blotting, and activity of partially purified rVrSBEII protein: (**A**) lane M, broad range protein marker, 0.1−2.5 μg/band; lane 1, *E. coli* BL21 (DE3); lane 2, *E. coli* BL21 (DE3)/pET21b-*Vrsbell* without induction; lane 3, *E. coli* BL21 (DE3) pET21b-*Vrsbell* with 0.2 mM IPTG induction for 5 h; lane 4, partially purified peak fraction after HisTrap affinity chromatography (sample loaded was 60 μg of the same described in **Figure 5**, and partially purified rVrSBEII was loaded with ~10 μg; gel was Coomassie blue stained); (**B**) Western blotting of the gel in (**A**); (**C**) time-dependent amylose branching activity of the rVrSBEII. The reaction contained 1 mg/mL amylose, 0.1 M sodium citrate (pH 7.0), and 250 μL of crude rVrSBEII (12.86 μg/μL) in 2.5 mL and was incubated at 30 °C. Aliquots of 100 μL were taken at every 30 min interval to add with 900 μL of iodine reagent. Triplicate samples were taken for each time point. Buffer was used as blank. Enzyme activity was measured by monitoring the decrease in absorbance of the amylose−iodine complex at 660 nm. SDS-PAGE gel of 4% stacking and 7.5% separation gel was used.

Table 1. Purification of Recombinant VrSBEII

procedure	total protein[a] (mg)	total activity (U)[b]	specific activity (U/mg)[c]	yield (%)	purification fold
crude enzyme	25.72	6.49	0.252	100	1
HisTrap column	0.214	1.37	6.402	21.11	25.4

[a] Total protein content was from the crude extract of a 200 mL batch of *E. coli* BL21 (DE3)/ pET21b-*VrSBEII* culture. [b] One unit of activity is defined as the decrease in absorbance at 660 nm of 0.1 per minute (Δ0.1/min). [c] Specific activity is defined as U/mg of protein.

to the studies with *E. coli* GS, there are eight conserved catalytic residues within this central $(\beta/\alpha)_8$ domain, Y300, D 335, H340, R403, D405, E458, H525, and D526. Y300 is known to interact with substrates and inhibitors in the enzyme/inhibitor complex structures. D335 plays a structural role by hydrogen bonding and orienting both R403 and Y300. E458, D405, and H340 have molecular motion in their side chains. H340 would be hydrogen bonded to D405. The four negatively charged residues, D 335, D405, E458, and D526, in the catalytic cavity are known to be involved in substrate binding and hydrolysis in all of the α-amylase family members. D405 is the nucleophile in the reaction, and E458 is responsible for protonations and deprotonations necessary on the leaving group and attacking oxygen (*27*). Six of the eight corresponding conserved catalytic residues for VrSBEII including the Y372, D407, H412, R476, D478, and E533 were located in the simulated structure (**Figure 4A**, shown by side chains; **Figure 2**, labeled in bold). It also includes the arginine residue (R476) in the active site, which is claimed to be essential for SBE catalytic activity of maize SBEs (*28*).

These equivalent residues for VrSBEII would possibly display catalytic properties in similar manners.

The rVrsBEII was expressed as a heterogeneous protein with His-tag at the C terminus and partially purified with amylase-branching activity (**Table 1**). When compared with the native SBE activity prepared from developing mungbean seed (*17*), the specific activity of the crude enzyme was 0.002 U/mg and after sucrose gradient purification, the specific activity increased to 0.191 U/mg. Therefore, the recombinant VrSBEII has been endowed with a much higher specific activity (6.402 vs 0.191 U/mg) than the partially purified native enzyme. When the activities of rVrSBEII in our study and the recombinant SBE of the most homologous kidney bean SBEII (rPvSBE2) (*11*) that was assayed by the same method were compared, the specific activity of rPvSBE2 in the crude extract was 3.4 U/mg, and it increased to 214 U/mg after purification. The purified rPvSBE2 activity was 36-fold the rVrSBEII′s in the study here. Why was rVrSBEII activity lower than the construct reported in rPvSBE2? One of the reasons may be the truncation of the eight amino acids (24 bp) at the C terminus of rVrSBEII that was designed for avoiding the DRS. Such an effect is supported by a similar situation studied with PvSBE2 (*26*), in which omitting the 52 bp at the C terminus and constructing into the pET23d expression/*E. coli* system, the activity of the expressed ΔC52-PvSBE2 protein dramatically decreased to <0.01 U/mg (vs the rPvSBE2 control, 3.4 U/mg). This result showed that the C terminus of rPvSBE2 plays an important role in catalytic efficiency. Such an effect may be also true for the rVrSBEII counterpart in mungbean. As a result, it is pertinent to further resolve the C-terminal DRS problem. This goal would be achievable by modifying the 6 bp DRS codon without changing the amino acid type and length and expressing rVrSBEII in the *E. coli* system with a removable tag sequence. Such a construct would reflect the catalytic efficiency of native VrSBEII for characterizing the products.

In conclusion, we have successfully cloned and expressed the full-length cDNA of family A mungbean SBEII in the *E. coli* system. The deduced primary sequences were used to establish its phylogenetic relationship. Features in their N-terminal, central, and C-terminal regions and information from the predicted 3D structures were obtained. The *VrsbeII* clone and rVrSBEII protein are useful for further molecular manipulation and pursuing biotechnology application potential.

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