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Detection of Proteins Related to Starch Synthase Activity in the Developing Mungbean (*Vigna radiata* L.)

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Proteins associated with starch synthase (SS) activities were identified in immature mungbeans (*Vigna radiata* L. cv KPS1). Seed soluble extract was separated by native-PAGE and subjected to in situ activity staining. The gel zymogram located starch−enzyme complex bands. The soluble extract was also partitioned by preparative-IEF and screened for SS activity using radioactive assay. IEF fractions eluted within pH 4−6 revealed enriched SS activity of 145-fold. Parallel comparison of the protein profiles among the activity stained enzyme complex and the active isoelectric focused fractions on SDS−PAGE depicted three SS-activity-related proteins with molecular size of 32, 53, and 85 kDa. The 85 kDa protein, however, was identified to be methionine synthase by MALDI-TOF analysis and should be a protein physically associated with the active SS. Polyclonal antibodies raised from eluted native enzyme complex neutralized up to 90% activity and antigenically recognize the other 53 and 32 kDa proteins on Western blot. Antibodies raised from the two individual denatured proteins were able to neutralize SS activities near 60% separately, indicating that the 53 kDa and 32 proteins associated with SS activity are potentially involved in starch biosynthesis during mungbean seed development.

KEYWORDS: Starch synthase; activity staining; SS; isoelectric focusing; mungbean; *Vigna radiata*; native-PAGE

INTRODUCTION

Starch is a glucose homopolysaccharide consisting of two distinct types of polymers, α-1,4-linked linear amylose and α-1,4-linked with α-1,6-branched amylopectin. Its structure, function, biosynthesis, and degradation attract broad research interests (*1*). It is characteristically synthesized in the chloroplast of photosynthetic tissues and in the amyloplast of sink tissues such as tuber, seed, and endosperm of cereals. Enzymes involved in starch biosynthesis play important roles in plant physiology for sugar metabolism and energy storage. During steps of starch formation, elongation, and branching in the amyloplast, the major starch biosynthetic enzymes include AGpase (ADP glucose pyrophosphorylase, EC 2.7.7.27), which activates G-1-P to produce a key intermediate ADP-glucose (ADPG), SS (starch synthase, EC 2.4.1.21), which elongates a α-1,4-linked glucose chain by the addition of the glucose moiety from ADPG to its nonreducing end, and SBE (starch branching enzyme, EC 2.4.1.18), which catalyzes the cleavage of an α-1,4-glucosidic linkage and subsequent transfer of α-1,4-glucan to α-1,6-branched point (*1*). Each of the three enzymes contains various types of isoforms that are differentially involved during the starch polymerization process that ultimately constitutes unique starch architecture in plants (*2*).

SS was found to exist in both soluble form (SSS) and starch-granule-bound form (GBSS). It was believed that GBSSs are essential to produce amylose in starch granules (*3*), while they contribute in a minor way to amylopectin synthesis (*4*), and SSSs participate in the synthesis of both amylose and amylopectin. The specific roles of the two SS forms remain unclear (*5*). SSS and GBSS were investigated in both monocot plants (*6*−*13*) and dicot plants (*14*−*24*) at biochemical and genetic levels during the past 30 years. In monocot plants, maize (*6*, *7*), rice (*8*, *9*), wheat (*10*, *11*), and barley (*12*, *13*) were studied. In dicot plants, cassava (14), potato (15, 16), sweet potato (*17*), pea (*18*−*22*), castor bean (*23*), and *Chlamydomoans reinhardtii* (*24*) were studied. There have been three major GBSS isoforms found, and only GBSS I (the waxy gene product, 58−60 kDa) was almost found as granule-bound form (*25*). In contrast, GBSS II (77−80 kDa) was also found in the soluble fraction (*20*) and GBSS III in wheat was a 100−105 kDa protein (*10*). SSS isoforms of different molecular size have been found in different sources such as the 75−77 kDa SSSI in pea (*20*) and wheat (*11*), and the 55 kDa and 57 kDa SSS isoforms in developing rice (*8*). A high molecular size of 140 kDa SSS III was expressed in potato (*26*). Their amino acid sequences, antigenic properties, and encoding genes were also investigated to derive their relationship and functionality in starch biosynthesis at a molecular level (*19*, *27*, *28*).

Mungbean (*Vigna radiata*, L. Wilczek) is an essential ingredient used in making traditional Chinese foods, such as greenbean cake and bean vermicelli. The average of 50% starch content in mungbean dry weight contributes to its specific applications. Particularly, mungbean starch possesses exclusive chemical compositions, such as the amylose content in total starch (amylose plus amylopectin) was higher (up to 35%) than

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its normal level among plants (15−30%). Besides, its long amylose chain and amylopectin structure are unique, which makes mungbean vermicelli appear translucent and become more persistent to heating without breaking or deforming during cooking (*29*). Mungbean starch is not replaceable by other starch raw materials in the manufacture (*30*). The earliest study with mungbean starch biosynthesis demonstrated that the activities of SSS and GBSS, after DAF13 (day after flowering), reached maxima and were responsible for starch accumulation in the late stage (*31*). As of yet, however, there is no further information pertaining to starch biosynthetic enzymes for mungbean until our laboratory recently continued this study. We found that there are high GBSS activities on the surface of isolated raw starch granules from mature beans, and active GBSS can be released into the lysate fraction by amylolytic treatment to the granule (*32*). It implied that mungbean GBSS should have been expressed and existed in a soluble state to perform its cellular functions before maturation. Since amylose was synthesized by either SSS or GBSS as mentioned earlier, the identification of these SS enzymes that contribute to the formation of the unique high-amylose mungbean starch is important in two phases. One is for our basic understanding of their roles in mungbean starch biosynthesis, and the other is their promising application as biological modifiers in native starch. We hypothesized that tracing the SS activities in the soluble extract for starch formation can be used for SS identification. Therefore, the aim of this study was to find mungbean SS isoforms during the developmental stage when the seeds contain less storage proteins and the SS enzymes are very active as the soluble form for starch formation.

We first separated and isolated mungbean soluble proteins through analytical and semipreparative approaches by native-PAGE, preparative-IEF, and electroelution. At the same time, we use in situ activity zymogram, iodine staining, and radioactivity assay to screen for SS activities. SS-activity-associated proteins were then revealed on SDS−PAGE, and both their native and denatured forms were prepared to raise their polyclonal antibodies. The activity neutralization and immunoblot results suggested that we have identified two proteins related to SS activity to be potentially involved in starch biosynthesis in the developing mungbean seed.

MATERIALS AND METHODS

Materials. Mungbean cultivar KPS1, which contained a high starch content of 53.5%, was selected from the germplasm at Asia Vegetable Research and Development Center (AVRDC) in Shanhua, Taiwan. It was grown in the field from July to August. Immature mungbean pods were picked at DAF13 and stored at −80 °C. ADP-glucose, amylopectin, Bicine (*N*,*N*,-bis[2-hydroxyethyl]glycine), bromophenol blue, bovine serum albumin (BSA), dithiothreitol (DTT), ethylenediaminetetraacetic acid (EDTA), glycerol, glutathione (GSH), horseradish peroxidase (HRP) conjugated anti-mouse IgG, 2-mercaptoethanol, phenylmethylsulfonyl fluoride (PMSF), ponceau S, pristane, and Tris-HCl were purchased from Sigma (St. Louis, MO). Reagent-grade acid, base, salts, or solvent were purchased from Sigma or Merck Chemical Co. (Darmstadt, Germany). Freund's complete adjuvant and Freund's incomplete adjuvant were from Gibco Invitrogen Corp. (Carlsbad, CA). Ammonium persulfate (APS), ECL detection kit, hyperfilm, radioactive [^{14}C]ADP-glucose, and scintillation cocktail were from Amersham Biosciences (Taipei, Taiwan, ROC). Microcon, filter disk, and PVDF membrane (Immobilon-P) were from Millipore (Bedford, MA). Acrylamide, ampholyte 3/10, N′,N′-methylenebisacrylamine, ampholyte, coomassie G250 stain, glycine, molecular weight standard (broad range; No.161-0317), protein assay standard II dye reagent concentrate, Rotofor standard kit, Rotofor buffer solution, sodium dodecyl sulfate (SDS), Silver Stain Plus, and TEMED were purchased from Bio-Rad (Hercules, CA). Tris base was from USB (Cleveland, OH). Potassium iodine (KI) was from Wako Pure Chemical Industries and iodine (I_2) was from Showa (Tokyo, Japan). Mouse SP2/0 myeloma cells, kindly provided by Prof. C.-Y. Hsiang of the Department of Microbiology, China Medical University, were from American Type Culture Collection (ATCC) (Rockville, MD). Cells were maintained in Dulbecco modified Eagle medium (DMEM) (Life Technologies, Gaithersburg, MD) supplemented with 10% fetal bovine serum (FBS) at 37 °C.

Soluble Fraction Preparation. Immature mungbeans were carefully collected from bean ovaries on ice. Beans were added with equal weight-to-volume ratio of ice-cold homogenizing buffer (5 mM PMSF, 1.25 mM DTT, 10 mM EDTA, 10% glycerol, and 50 mM Tris-HCl, pH 7.5) to a −20 °C precooled mortar and minced into consistency. The homogenate was centrifuged at 10 000*g* for 10 min at 4 °C. The resulting supernatant was the crude soluble extract.

Protein Determination. Protein concentration was determined by Bradford's method (*33*). Sample was properly diluted to a final 800 μL solution, followed by adding 200 μL of dye reagent to make 1 mL total. The mixture was vortexed, incubated at ambient temperature for 5 min, and read at 595 nm (Hitachi U-2000 spectrophotometer). Triplicate samples were used to obtain an average absorbance. BSA concentrations within the 10 μg/mL range were used to construct the standard curve.

PAGE (Polyacrylamide Gel Electrophoresis). Native-PAGE, preparative-PAGE, and SDS−PAGE were conducted. SDS−PAGE was conducted in a Mini PROTEAN II apparatus (Bio-Rad). The lower, degassed 12% separation gel was prepared by gently mixing 1.73 mL of H_2O, 1 mL of 1.5 M Tris-HCl (pH 8.8), 1.2 mL of 40% acrylamide/bis (37.5:1), 40 μL of 10% SDS, and 2 μL of TEMED. Fresh 10% APS (20 μL) was then mixed in just before loading into the 0.75 mm thickness gel space of the glass assembly. A few drops of 2-propanol were added on the gel surface to form a horizonal layer, and the gel polymerized for 45 min. The 2-propanol was then discarded and the gel surface was washed several times with deionized water. The upper, degassed 4% stacking gel was prepared by mixing 1.27 mL of H_2O, 0.5 mL of 0.5 M Tris-HCl (pH 6.8), 0.2 mL of 40% acrylamide/bis, 20 μL of 10% SDS, and 2 μL of TEMED. Fresh 10% APS (10 μL) was again mixed in just before loading. A comb was inserted into the stacking gel before polymerization for another 45 min. As for preparative-PAGE, a comb was not used and the gel thickness was 3 mm. SDS-containing running buffer (3 g/L Tris-base, 14.4 g/L glycine, 1 g/L SDS) was used as upper and lower tank buffer. Broad-range SDS−PAGE molecular weight standard was diluted 200-fold with deionized water, and 5 μL (0.09 μg/mL) was used to mix with equal volume of 2× sample buffer (0.0625 M Tris-HCl, pH 6.8, 5% glycerol, 1% SDS, 2.5% mercaptoethanol, and 0.025% bromophenol blue). After sample application, gel apparatus was run at 4 °C under constant 100 V for around 2 h until the tracing dye reached the gel bottom.

Native-PAGE was operated similarly to SDS−PAGE, except that SDS was omitted from all the gels and buffers, and the volume was compensated by deionized water. The native-PAGE gels were used for SS-activity staining and SDS−PAGE gels were subjected to coomassie blue or silver staining. Gel photodocumentation and protein molecular size calculations were operated with an Alpha Innotech ChemiImager IS4400 system loaded with software AlphaEase v. 2.3.

In Situ Activity Staining. In situ activity staining for SS activity was performed according to Edwards et al. (*12*) with some modification. Samples were first separated by native-PAGE in the minigel system, and the harvested gel was immersed in 10 mL of incubation solution (100 mM Bicine-NaOH, pH 8.0, 5 mM EDTA, 10 mM GSH, 25 mM potassium acetate, 5 mg/mL amylopectin, and 2 mM ADP-glucose). The reaction was conducted for 3 days at room temperature with mild agitation. After incubation, the solution was decanted and the gel was rinsed several times with deionized water. Gel was then incubated with 20 mL of staining solution (10 mM I_2, 14 mM KI) at room temperature for 15 min followed by rinsing with deionized water to reduce the background. The activity stained gel band strip was excised for electroelution and SDS−PAGE analysis.

Silver Staining. The gel was stained with a Bio-Rad silver staining kit. Briefly, the gel was first fixed with 10 mL of fixation solution (50% methanol, 10% acetic acid, 10% fixative enhancer concentrate)

for 20 min and then rinsed twice with deionized water for 10 min between of each step to remove residual chemicals. The gel image was then developed by soaking the gel in 10 mL of developing solution (5% silver complex solution, 5% reduction moderation solution, 5% image development reagent and development acceleration solution) to react for 10−20 min. When the image density appeared as the desired contrast, reaction was stopped by adding 10 mL of 5% acetic acid to neutralize for 15 min. Gel was then rinsed with deionized water and documented.

Electroelution. Desired proteins visualized either by coomassie blue staining or in situ activity staining on SDS−PAGE or native-PAGE gels were excised from the stained band strips or unstained gels of relative position and recovered by electroelution (Bio-Rad Model 422). Briefly, the band strip was sliced and put into individual glass tubes with a frit between the membrane cap. The whole electroelution unit was assembled in a vertical tube stand with electrophoresis running buffer filling the gel chamber. Unused tube holes were plugged with stoppers. The tube stand was put in a vertical electrophoresis chamber where upper and lower chambers were filled with 200 and 600 mL elution buffer (25 mM Tris-HCl, 192 mM glycine, pH 8.8), respectively. Elution was operated at 200 V for 4 h. Protein that eluted into the lower glass chamber solution was collected for protein concentration determination and analyzed by SDS−PAGE to verify its purity. It was further concentrated in Microcon for the use as antigen to immune mice.

Mass Spectrometry. After protein purity was verified in SDS−PAGE, the protein band was cut out of the gel and sent to the Mass Spectrometry Laboratory of DigitalGene Biosciences Co., LTD (Taipei, Taiwan) for in-gel trypsin digestion (*34*) and MALDI-TOF (assisted laser desoption ionization-time-of-flight) MS analysis (*35*). The mass spectrometric peptide map analysis was performed on a Voyager-DE STR MALDI-TOF workstation (ABI, Applied Biosystems) equipped with a linear detection system, a reflecting ion mirror, and a collision-induced disassociation (CID) cell. Spectra were acquired in reflect mode at an accelerating voltage of 25 000 V with an average of 100 laser shots. External or internal calibration used a SequazymeTM Peptide Mass Standard Kit (PE Biosystems). Spectra were analyzed using the software program Voyager Version 5 with Data Explorer Software.

Preparative Isoelectric Focusing. A standard Rotofor system (Bio-Rad) was used to fractionate proteins of the crude soluble extract to screen for SS activity. System operation followed the instruction provided by the manufacturer. Ampholyte 3/10 was used as sample medium in the focusing chamber. Ultrafiltered (0.22 μm filter disk) crude extract (40.6 mg/mL, 3 mL) was withdrawn to add with 2.5 mL of 40% ampholyte 3/10 and deionized water to give a final 2% ampholyte concentration in 50 mL sample volume. The sample mixture was injected into the sample chamber to run for 4 h at 13 W until a constant voltage was reached and run for an extra 30 min before harvesting. Twenty fractions were then aspirated from the sample chamber into individual collecting tubes. Isolated fractions were analyzed for pH distribution with Mettler MP220 (Mettler-Toledo, GmbH, Germany) and protein concentration. Each fraction was buffer exchanged by dialysis against 100 mM Bicine-NaOH (pH 8.5) before SS-activity radioactive assay.

SS-Activity Radioactive Assay. SS-activity assay was conducted according to Hara et al. (*36*). Basically, final concentrations of 100 mM Bicine-NaOH (pH 8.5), 5 mM EDTA, 10 mM GSH, 25 mM potassium acetate, and 5 mg/mL amylopectin were first prepared as a cofactor mix. A sample of defined volume was then gently mixed into the cofactor mix followed by adding a final concentration of 1 mM hot/cold [^{14}C]ADP-glucose (4.2 × 105 cpm/μmol) into a total volume of 100 μL to react at 30 °C in a water bath with 200 rpm agitation. Reaction continued for 90 min and terminated by spotting the mixture onto a Whatman GF/A, 2.4 cm filter disk on a heating block set at 120 °C to dry for 10 min. The filter disk was washed successively with 66% (v/v) ethanol containing 85 μM EDTA, 66% ethanol, and 70% ethanol. Each solution wash used 2 mL of nine repeats with suction in a filtration sampling manifold apparatus (model 1225, Millipore, Bedford, MA) (*37*). Each disk was then put into a 5 mL counting vial with addition of 4 mL of scintillation cocktail (ACS, NACS104) to measure [^{14}C]starch trapped on the filter by a scintillation counter (Beckman LS-6500, Beckman Coulter, Inc., Fullerton, CA). SS specific activity (SA) was calculated as SA = (sample cpm − blank cpm)/standard cpm × substrate (mol)/sample (mg)/time (min).

Antiserum Preparation. A total of three samples including the SS-activity-stained band (as native protein complex) and two individual activity-associated proteins were used as antigens for mice immunization. Crude extract (40.6 mg/mL, 750 μL) in SDS-free sample buffer was loaded in the sample slot well of 3 mm thickness to separate proteins on a first preparative 16 cm native-PAGE gel using a Bio-Rad PROTEAN II xi cell. The SS-activity-associated protein region in this nondenaturing gel was positioned by in situ SS-activity staining as mentioned above. The activity-stained gel band was excised to recover proteins by electroelution to be used directly for preparing anti-activity-stained-band antibodies or continued to be separated by SDS−PAGE. The electroeluted activity-associated band protein solution (1.17 mg/mL, 750 μL) in SDS sample buffer was then separated by a second preparative 16 cm SDS−PAGE, also performed in a PROTEAN II xi cell, except that protein bands were visualized with coomassie blue staining. The stained bands were excised separately and again electroeluted to recover proteins. All the recovered electroeluents were subjected to protein quantification.

For mice immunization, 4−6-week-old female Balb/c mice, with four or five mice per group for one antigen, were used. Each mouse was injected intraperitoneally with 0.2 mL of pristane with a 1 mL syringe. After 14 days, 5−10 μg of electroeluted antigenic proteins were diluted in 100 μL PBS buffer (phosphate buffered saline, pH 7.2) and mixed with an equal volume of complete Freund's adjuvant to form emulsion, and the mixture was injected into mice subcutaneously with a 1 mL syringe. After 14 days, mice were boosted intraperitoneally with another 5−10 μg of antigenic proteins in 100 μL of PBS buffer emulsified with an equal volume of incomplete Freund's adjuvant and boosted again after 14 days. PBS buffer was substituted for antigenic protein in a control group. Fourteen days after the last boost, each mouse was infected with 3×10^5 SP2/0 myeloma cells. These cells were grown to confluency in DMEM and washed with PBS to remove serum by centrifugation at 1500 rpm (5 min × 3), and 200 μL of cells was injected into mice intraperitoneally. After 1 week, the ascite fluid was collected daily for 5−8 days. Fluid was centrifuged at 2000 rpm at 4 °C for 5 min and the supernatant was used as polyclonal antiserum to conduct activity neutralization and immunoblot experiments (*38*).

Activity Neutralization. The above-mentioned three polyclonal antibodies were used in activity neutralization experiments. Samples were conducted in triplicate. Each polyclonal antibody fluid or control fluid of 0−10 μL was mixed with 37 μL of the crude extract to incubate at room temperature in SS cofactor mix (final 90 μL) for 1 h. The hot/cold [^{14}C]ADP-glucose substrate was then added, followed by the rest of the procedures described for the SS-activity radioactive assay (*39*).

Western Blotting. Protein samples were first separated by SDS−PAGE using the minigel system. Gels were then electroblotted onto PVDF membranes by a Bio-Rad Mini-transblot in transfer buffer (Tris base 3 g/L, glycine 14.4 g/L, and 10% methanol) according to the manufacturers instructions at 4 °C. Ponceau S staining was then performed to monitor the transferred proteins on the membrane before incubating for 1 h with blocking solution [5% nonfat milk in PBS-T (0.1% Tween-20 in PBS)]. The membrane blots were always rinsed several times with PBS-T between each incubation step. Membranes were then incubated with polyclonal antibody fluid diluted in 1:100 ratio in PBS-T for 1 h and then with HRP conjugated anti-mouse IgG diluted in 1:1000 ratio in PBS-T for 1 h. The HRP-linked second antibodies captured on membranes were detected by chemiluminescent substrate with ECL detection kit performed in the dark. Finally, air-dried membranes were exposed to Hyperfilm.

RESULT AND DISCUSION

In Situ Activity Staining of the Soluble Extract on Native-PAGE. Immature mungbean pods (45, 118 g) were used for soluble extract preparation. Mungbeans (67 g) were separated from 51 g of pod ovaries and prepared into 64 mL of crude soluble extract with a protein concentration of 121.8 mg/mL. Each gram of the immature mungbean was calculated to contain

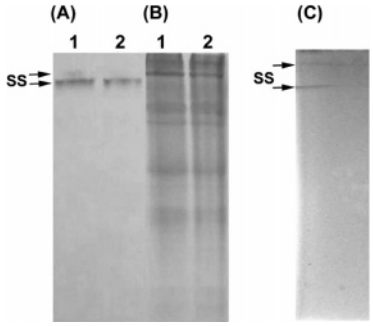

Figure 1. Native-PAGE and SS visualization by in situ activity staining with iodine. (A) SS zymogram of the crude soluble extract. (B) Native protein profile of the crude soluble extract. Crude extract (65 μg/lane) was loaded in a 0.75 mm thickness 12% native-PAGE gel. The harvested gel was cut in half; half (panel A, lanes 1, 2) was subjected to in situ activity staining, and the other half gel (panel B, lanes 1, 2) was stained with coomassie blue. (C) A partial portion of a preparative SS zymogram. The crude extract was run in a preparative 10% native-PAGE gel for the recovery of the major SS-activity-associated band strip. The arrow lines pointed out the two SS-activity-associated protein populations.

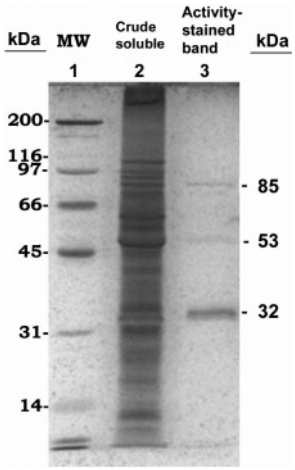

Figure 2. SDS–PAGE analysis of SS-activity-stained band. Samples [lane 1, molecular weight standard (0.45 μg); lane 2, crude soluble extract (15 μg); and lane 3, activity-stained band] were analyzed in a 10% SDS–PAGE gel and visualized by silver staining. Lane 3 was from the major iodine-stained band of Figure 1C after being excised and electroeluted (0.5 μg was loaded).

approximately 116.3 mg of soluble proteins, corresponding to 11.6% of mungbean wet weight.

SS in the crude soluble extract was detected by in situ activity staining of the starch synthesized on native-PAGE slab gels. This combined approach was designed as a crucial selection and purification experiment for SS to show up in situ on the gel by a unique property of its product, starch, which can be visualized by iodine. The native gel was first incubated in SS assay buffer to form starch, followed by iodine staining. Therefore, only proteins possessing SS activity can synthesize starch in situ and then be visualized as blue bands by iodine (zymogram). The zymograms in parts **A** and **C** of Figure 1showed two protein populations including a major strong band and a minor band on the top. This result strongly suggested that the two activity-stained protein bands are associated with SS activities. Besides, this detection approach was reproducible throughout our study such that the same two active SS populations seen in Figure 1A,C, were performed in different experimental batches (as described in the figure legend). When the two SS populations (panel **A**) and their corresponding band densities in total protein profile visualized by coomassie blue (panel **B**) were compared in parallel, it showed that the lower major activity-stained band belonged to minor protein species in the soluble extract.

The incubation time required for the in situ SS assay before visualization by iodine was found to be much longer than the 2 h needed for SBE (*40*). We monitored the reaction progress and it finally took 3 days to show appreciable iodine signal on protein bands. We however can actually see the white starch band on the gel before iodine staining. Therefore, it is possible that the amount of SS or the activity of SS in the gel was low, requiring longer time for starch synthesis, or the newly synthesized amylose needed to accumulate enough quantity before binding with iodine. Amylose has been shown to have much lower affinity than amylopectin to associate with iodine (*41*). It was postulated that starch conformation affects the iodine binding. Therefore, it is suggested here that the starch product formed on the gel should come from SS activity, which has been well-documented to participate in the synthesis of amylose (3).

Furthermore, the zymogram showed that the upper minor band sometimes was not seen, which might be caused by loading with high sample concentration on native-PAGE. The possibility that this minor band implied the presence of a second form of SS cannot be ruled out. Because the lower band strip was dominant, it was excised for electroelution to identify SS-activity-associated proteins.

Analysis of Activity-Stained Band by SDS−PAGE. The major activity-stained band was electroeluted and analyzed by SDS−PAGE. Three proteins were detected by silver-staining on SDS−PAGE with molecular masses of 85, 53, and 32 kDa (Figure 2, lane 3). The intensity of the 32 kDa protein band was stronger and seemed to be a doublet. When compared with the crude extract (Figure 2 lane 2), which containing hundreds of proteins, these three proteins (Figure 2, lane 3) can be clearly differentiated from other proteins, and their corresponding protein positions in the crude extract were evident. They were thus considered as potential SS candidates. Although the 53 kDa protein region in the crude showed a protein population with the same molecular mobility stacked together on SDS−PAGE, the SS-related 53 kDa was again observed as a minor protein species. Later, however, we found the occurrence and intensity of the 85 kDa protein was not stable, which led us to analyze its identity by MALDI-TOF separately.

Mass Spectrometry. Since the 85 kDa protein band on one-dimensional SDS−PAGE was sharp enough to be judged as a homogeneous protein species, it was selected to be analyzed by MALDI-TOF MS. The mass spectrum shown in Figure 3 displays the unique fingerprint of the 85 kDa protein. The deisotoped mass values of these tryptic fragments were subjected to MS−FIT analysis (http://www.ncbi.nlm.nih.gov). The matched proteins in Swissprot database with the top higher MOWSE scores were the same methionine synthases of *Arabidopsis thaliana* (NP_187028), *Solanum tuberosum* (AAF74983), *Mesembryanthemum crystallinum* (P93263), and *Zea mays* (AAL33589). Therefore, this 85 kDa protein was proven to be mungbean methionine synthase. The reason the 85 kDa methionine synthase appeared with SS activity could be rationalized as follows. It may be due to an artifact of physically associated proteins when cells broke. The breakage promoted the oxidation of the sulfur hydroxyl residues among the three polypeptides to form cross-links that resulted in their comigration as a complex form in native-PAGE. The disulfide bonds in the complex were then reduced by 2-mercaptoethanol in the gel sample buffer and separated by SDS in the SDS−PAGE to

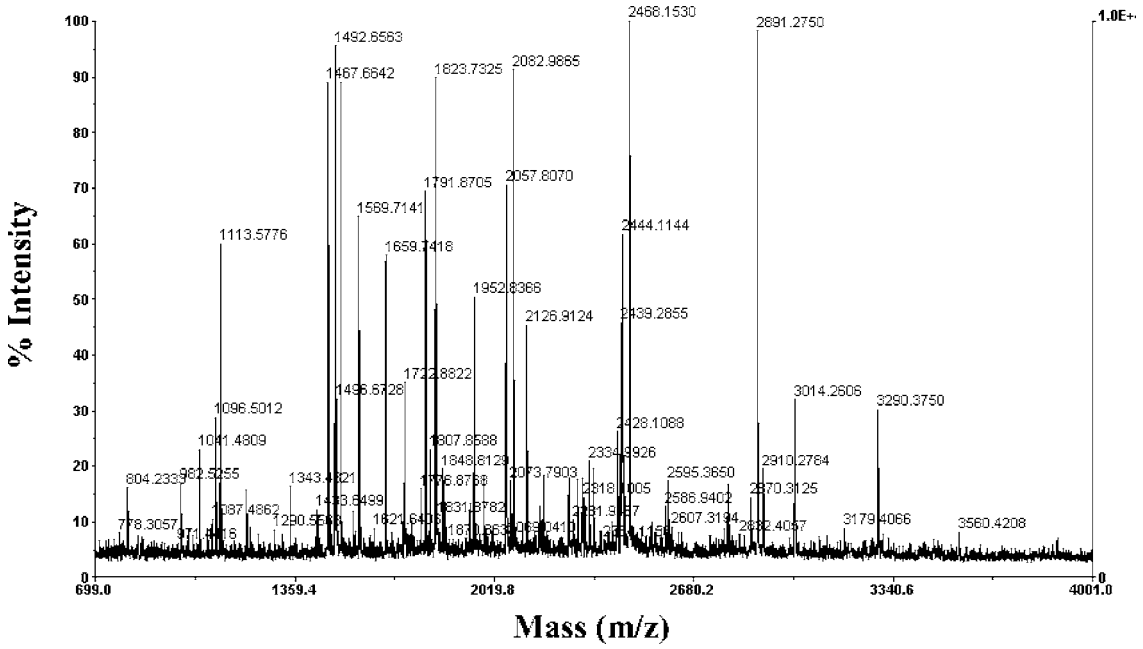

Figure 3. Mass spectral identification of the tryptic fragments of the activity-associated 85 kDa protein by MALDI-TOF analysis. The 85 kDa protein sample visualized by coomassie blue on SDS–PAGE was cut from the gel. The gel slice was sent for in-gel trypsin digestion. Percent of intensity was normalized values using the highest peak signal as 100%.

reveal individual components. The association may also be the methionine synthase located in very close proximity to SS under normal physiological condition. Particularly, we observed that the association is a reproducible event. Since methionine is an essential amino acid, in this case, the spatial association of methionine synthase with SS activities implied a close relationship between the synthesis of methionine and starch in mungbean cells at a molecular level. In addition to the 85 kDa protein, we have attempted to identify the isolated 53 and 32 kDa proteins by either N-terminal sequencing or MALDI-TOF, but the analysis failed. This phenomenon suggested possible molecular modification of the two polypeptides.

Fractionation of Soluble Extract by Preparative Isoelectric Focusing. We further used liquid-phase isoelectric focusing system, a Rotofor cell, to separate the soluble extract into 20 fractions and analyze their pH values, total protein percent, and SS specific activities (Figure 4). This system has been a useful tool as the first dimensional sample separation to isolate target proteins or enzymes, such as studies for peptide mapping in cerebrospinal fluid (*42*), purification of biotinidase (*43*) and fractionation of cytoplasmic proteins to characterize glucose-1-phosphate (*44*). Result showed the pH distribution ranged from 13 to 1.5 in a descending order to the fraction number and was broader than the ampholyte provided range of 3−10 (Figure 4A). The proteins were distributed into two peaks (Figure 4B). The main peak between fractions 10 and 14 corresponded to p*I* values of 4−8. There were more acidic proteins compared to alkaline proteins. Each Rotofor soluble fraction was sampled to assay SS activity by radioactive assay. The highest SS specific activity of 367.2 pmol/min/mg was found in fraction 14, followed by fraction 13 of 166.3 pmol/min/mg (Figure 4C). Therefore, 40.7% of total activity was concentrated in fraction 14 and the p*I* of SS proteins was focused within pH 4−6. When compared with the specific activity of the crude fraction (2.5 pmol/min/mg), SS was purified 145-fold in fraction 14 and 66-fold in fraction 13. The total activity in all 20 fractions was 1815 IU (pmol/min), which was higher than the total activity of 1530 IU loaded from the crude extract. The increased SS activity should probably be due to the removal of inhibitory material from SS proteins during Rotofor separation.

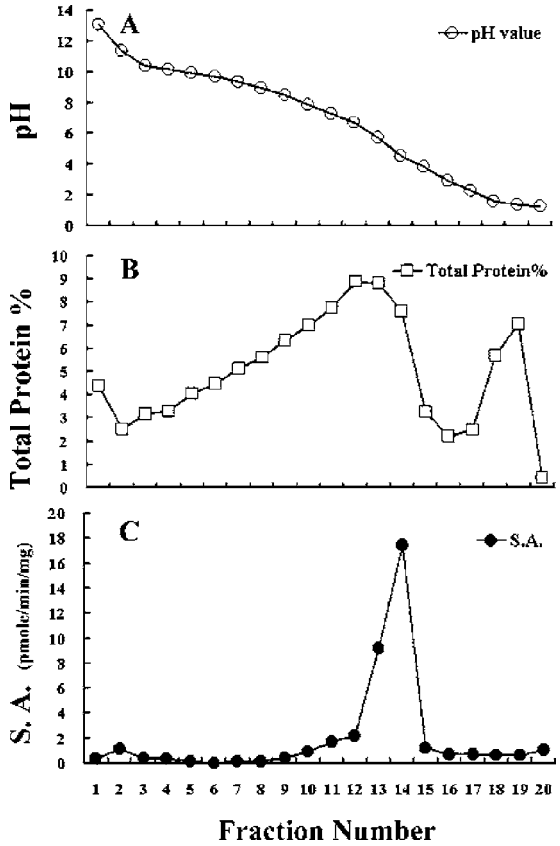

Figure 4. Analysis of the IEF fractions from a Rotofor cell. (A) pH distribution. (B) Total protein distribution. (C) Enriched SS-specific activity peak. Fractions were collected from vacuum suction of the isoelectric focused sample chamber into 20 individual tubes/fraction that obtained an average of 2 mL. Protein concentration in each fraction was measured using the Bradford assay. Percent of total protein calculation was based on the sum of soluble protein recovered in all the focused fractions.

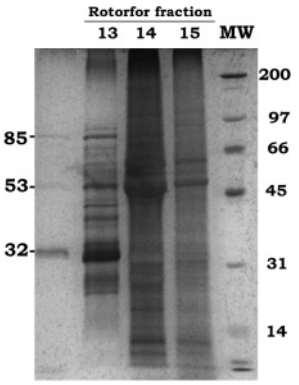

Figure 5. Comparison of protein profiles of the active IEF fractions with the SS-activity stained band on SDS–PAGE. Lane 1 was the same sample as lane 3 of Figure 2. Lanes 2–4 were the Rotofor fractions 13–15 of SS-activity peak, which were loaded with concentrated forms of the same volume (32 μL) in 10% SDS–PAGE gel and visualized by silver staining.

Identification of Potential SS Proteins. Protein profiles in Rotofor fractions from 13 to 15 spanning SS-activity peak were compared with that of the SS-activity-stained band (Figure 5) The most active fraction 14 contained mostly the 53 kDa protein and less 32 and 85 kDa proteins (lane 3). Whereas, the less active fraction 15 contained much less 53 and 32 kDa proteins (lane 4). The 85 kDa protein appeared as the major component in fraction 13 and a minor one in fraction 14, but was in negligible amounts in fraction 15. Interestingly, the three major 85, 53, and 32 kDa proteins found together in the SS-activity-stained band were also present in the highly enriched SS fractions. Although native-PAGE separates proteins by molecular size, Rotofor cell separates proteins by isoelectric point, and they are of two independent approaches; the association of the three proteins was evident. Therefore, the MALDI-TOF-identified 85 kDa methionine synthase should probably be with SS to form a protein complex in the native state, as proposed previously. The correlation of protein profiles between the active Rotofor-focused fractions and the activity-stained band urged us to strongly speculate that 53 and 32 kDa proteins are potentially related with SS in mungbean soluble extract.

SS-Activity Neutralization by Antiserum. To identify SS, three mouse ascite fluids immunized from the native SS-activity-stained protein complex band and denatured form of the two potential SS candidates were prepared. We conducted several runs of activity staining to collect and to cut out the iodine stained band strips, followed by electroelution to prepare the native SS-activity-stained protein complex band as antigens. The electroeluted activity-stained protein complex was subjected to preparative SDS−PAGE to excise the 53 and 32 kDa proteins, followed by electroelution to separately collect the two denatured proteins as antigens.

The three polyclonal antibodies were used in activity neutralization experiments (Figure 6) to correlate the proteins with enzyme activities and in Western blotting (Figure 7) to detect their antigenic proteins. The preimmune serum (see Material and Method, the PBS group) was used as a negative control in the neutralization assay, and the signal has been subtracted from the sample for plotting. It showed that there was dose-dependent neutralization of SS activities in the crude extract with the three antibodies. Both anti-53-kDa (Figure 6A) and anti-32-kDa (Figure 6B) antibodies neutralized SS activities only up to 60% compared to the anti-activity-stained-band (Figure 6C) antibodies, which neutralized up to 90% activities. The removal of 90% activity clearly suggested that the antibodies recognized proteins with SS activity in the crude extract. Besides, this result showed

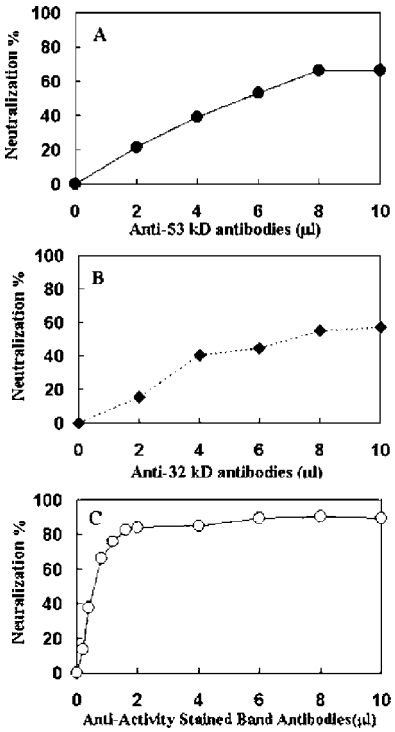

Figure 6. Neutralization of SS activities of the crude soluble extract with polyclonal antibodies raised from denatured 53 and 32 kDa protein and native SS-activity-stained band. (A) Neutralization with anti-53-kDa antibodies. (B) Neutralization with anti-32-kDa antibodies. Anti-53-kDa and anti-32-kDa antibodies were prepared separately from their electroeluted and denatured forms of the activity-stained band (Figure 2, lane 3) from preparative SDS–PAGE gels. (C) Neutralization with anti-activity-stained-band antibodies. The anti-activity-stained-band antibodies were raised from the electroeluted and native form of the activity-stained band from preparative native-PAGE (the lower band of Figure 1A,C).

that the major activity stained group of SS (Figure 1 A,C, the fast migrating band) contributed most of the enzyme activity, whereas there were minor SS isoforms other than the proteins in the major group that constitute 10% of the total SS activities.

Western Detection of SS. The specificity of the three antibodies was examined by probing against the crude extract on Western blots. The anti-53 kDa and anti-32 kDa antibodies recognized only their own corresponding molecular weight signals in the crude extract (Figure 7A,B). The anti-activity-stained-band antibodies recognized mainly the 53 and 32 kDa proteins in both the crude extract and the electroeluted active band (Figure 7C). The signals for the 32 kDa protein, however, were stronger than the 53 kDa protein, probably because the SS-associated 32 kDa protein was composed of a pair of similar sized proteins as shown in Figure 2 (lane 3).

The above results showed that we have successfully utilized analytical and biochemical methods as a novel approach to sort out the 53 and 32 kDa proteins as the components involved in SS activities from hundreds of mungbean soluble proteins. All the experiments in the current study were reproducible and successful events in our laboratory, duplicated at least twice by different investigators, except the N-terminal sequencing and MALDI-TOF mass identification of the two proteins, which remained unsolved. Therefore, the possible topology of the two molecules was implied from our current observations. For instance, the intensity of the 32 kDa protein band in the activity-stained band was stronger than that of the 53 kDa protein and seemed to be a doublet (Figure 2, lane 3). Therefore, it raised many possibilities, such as whether the similar-sized 32 and 53

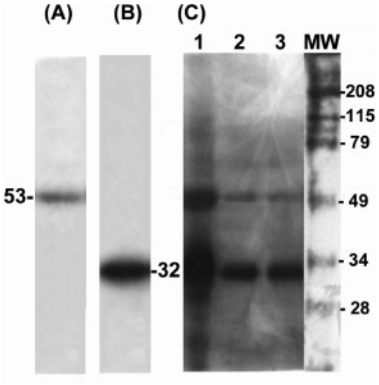

Figure 7. Western detection of the 53 and 32 kDa proteins. (A) Specificity of anti-53-kDa antibodies. (B) Specificity of anti-32-kDa antibodies. A membrane blot was transferred from a SDS–PAGE gel of the crude soluble proteins (31.8 μg/lane). The blot was cut into strips before probing with the two antibodies separately. (C) Detection of the 53 kDa and 32 kDa proteins by anti-SS-activity-stained-band antibodies. Crude soluble proteins (31.8 μg, lane 1), the electroeluted SS-activity-stained band (0.5 μg, lanes 2, 3), and prestained broad range molecular weight standard (BioRad 161–0318) (5 μL, lane 4) were separated on a SDS–PAGE gel and blotted onto PVDF membrane. The blot was stained with ponceau S and photographed, and then the molecular standard lane was cut out before Western detection to locate the sample position. The blot was probed using the anti-activity-stained antibodies. Hybridization signals were detected by chemiluminescent ECL regent.

kDa proteins are contributing to the activities of different SS isoforms or working together as part of a whole SS cluster to synthesize starch, or they are they simply proteins to hold the SS complex together near the active site without conferring activity, or are they cofactors of proteineous nature to regulate the activity. The exact molecular size of mungbean SS is also a puzzle, such as whether the 32 and 53 kDa proteins are subunits of a multimeric complex with different molecular ratios or either the 32 kDa protein or the 53 kDa one or both are fragmentation products from a large SS molecule in a complex. When we looked into the known starch synthases studied, the 60 kDa maize waxy protein, which has been shown a thermal labile protein, was prone to fragmentation into 47 and 32 kDa proteins. One possible mechanism is deamidation of glutaminyl and asparaginyl residues (45). Other evidence of protein fragmentation was demonstrated in potato SBE, where SBE-specific antibodies recognized a 63 kDa proteolyzed product beside its full size of 79 kDa (*39*). These proposed issues need to be further investigated. In addition, the 53 kDa protein may be real mungbean SS, since the two SS isoforms identified in developing rice (*8*) were 55 and 57 kDa, whose molecular size was similar to the mungbean 53 kDa found in this study. Furthermore, if the 32 kDa protein was the fragmented doublet form, the possible full size of 64 kDa will be similar to the known GBSSI (60 kDa) (25). Nevertheless, the results in the present study showed that the 53 and 32 kDa proteins were found together in two independent separation approaches (native-PAGE and preparative-IEF) and were found to associate with SS activities by two activity assays (radioactivity assay and activity staining), and their antibodies prepared from the activity-stained proteins were antigenic specific and able to neutralize SS activity, demonstrating that the 53 and 32 kDa proteins were directly related to SS activities and in very closely physical proximity. In conclusion, we have found SS-activity-related 53 and 32 kDa proteins that are potentially involved in starch biosynthesis in the soluble extract of developing mungbeans.

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