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The Identification of Starch Phosphorylase in the Developing Mungbean (*Vigna radiata* L.)

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Starch phosphorylase (SP) in immature mungbean (*Vigna radiata* L. cv KPS1) seed soluble extract was detected by in situ activity staining and identified by MALDI-TOF mass analysis. After in situ SP assay on native-PAGE, a major starch−enzyme complex was located on the gel zymogram in a dose-dependent manner. This complex depicted two major SP-activity related proteins, 105 kDa and 55 kDa, by SDS−PAGE. The mass and predicted sequence of the tryptic fragments of the isolated 105 kDa protein, analyzed by MALDI-TOF spectroscopy and bioinformatic analysis, confirmed it to be mungbean SP as a result of high similarity to the L-SP of known plant. Polyclonal antibodies raised from the 55 kDa recognized both the 105 kDa and the 55 kDa proteins on the Western blot and neutralized partial SP activity, indicating that the two proteins were immunologically related. The 55 kDa protein possess high similarity to the N-terminal half of the 105 kDa SP was further confirmed. The SP activity and the activity stained protein density in mungbean soluble extract decreased as the seed size increased during early seed growth. These data indicate that mungbean 105 kDa SP and SP activity-related 55 kDa were identified in the developing mungbean.

KEYWORDS: Starch phosphorylase; SP; Pho; activity staining; 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. The structure, function, biosynthesis, and degradation of starch attract broad research interests in plant physiology for sugar and energy metabolism (*1*). Starch is synthesized in the chloroplast of photosynthetic tissues as transient starch and in the amyloplast of sink tissues for storage such as tuber, seed, and endosperm of cereals by several enzymes. The first enzyme found that uses glucose-1-phosphate (G-1-P) as a glycosyl donor to synthesize amylose-like starch in vitro is starch phosphorylase (SP or Pho; EC 2.4.1.1; 1,4-α-glucano:orthophosphate glucosyltransferase) since 1940 (*2*). Other starch synthetic enzymes studied include AGpase (ADP glucose pyrophosphorylase), SS (starch synthase), SBE (starch branching enzyme) and DBE (debranching enzyme) in the AGpase starch synthase pathway (*3*).

SP belongs to the α-glucan phosphorylase family in animals (i.e., glycogen phosphorylase), plants (i.e., SP), and microorganisms (i.e., 1,4-α-glucan phosphorylase) (*4*). In plants, it has been purified and studied from various botanical food sources such as maize (*5*−*8*), pea (*9*−*11*), banana (*12, 13*), spinach (*14*), potato (*15*), soybean (*16*), sweet potato (*17*−*20*), sorghum (*21*), fava bean (*22*), and rice (*23*). SP catalyzes a reversible reaction between synthesis and degradation of amylose depending on the relative concentration of substrates (*2*). In the synthetic direction, a glucosyl unit is transferred from G-1-P into a growing 1,4-α- amylose in vitro, either with or without a glucan primer, and inorganic phosphate is released. In the phosphorylytic direction, the addition of inorganic phosphate generates G-1-P from starch (*24*). Due to the bidirectional activity, SP has been considered to have a role mainly as a degradative enzyme in vivo where the long chain glucan molecules were utilized by SP through the phosphorylytic direction to generate G-1-P, which may be utilized by AGpase for regeneration of starch in the AGpase starch synthase pathway (*1*). The controversy of which reaction direction dominates for the roles of SP in starch formation in vivo remains unsettled. The studies with purified SP from maize (*7, 8*) and sweet potato (*17*−*20*) have observed the SP to synthesize starch in vitro. The study with pea (*9, 11*) showed that SP activity increased during the accumulation of starch in the developing seeds. In addition, by molecular approaches, the isolated plastidic SP cDNAs expressed in potato, spinach, and pea also correlated with starch accumulation (*8, 23*). All of the above evidence thus suggests that SP is involved in the starch biosynthesis direction.

Two isoforms were well identified on the basis of their affinities to glycogen, namely, the high-affinity form (H-SP, Pho 1) and low-affinity form (L-SP, Pho 2). The L-SP was found in the plastids (amyloplast and chloroplast), and the H-SP was found in the cytosol of plant cells (*14*). Although the molecular size of monomeric L-SP (approx 100−150 kDa) was larger than the H-SP (approx 90 kDa), over 60% homology throughout the

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two sequences was found (*25*). L-SP and H-SP were both purified as monomeric or dimeric forms (*6*, *13*, *19*), and the fragmented form was also purified (*12*, *19*). When the primary structures of L-SP and H-SP were compared, an insert sequence containing 78 amino acids (*15*, *20*, *25*) or 81 amino acids (*22*) was found uniquely in the L-SP (designated as L78). It was believed that this molecular insert located near the glucan binding site in L-SP produce a steric hindrance and lowered the affinity for the substrate (*25*). This extra sequence in L-SP was further proposed to be the proteolytic location where the nick occurs in the fragmented forms that might play a regulatory role in its catalytic behavior. It was proposed that if the L78 maintains its structure intact, L-SP in the plastids has a preference for the direction of starch synthesis. If the L78 is nicked or excised out as a H-SP in the cytosol, the enzyme binds to the starch readily and favors the direction of starch structure breakdown (*20*). This theory established that the differential roles of the two SPs may be under a switch control mechanism by proteases in starch biosynthesis.

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 that amylose content was higher (up to 35%) than normal level (15−30%). In addition, its long amylose chain and amylopectin structure is unique, which makes mungbean vermicelli appear translucent and become more persistent to heating without breaking or deforming during cooking (*27*). Mungbean starch is not replaceable by other starch raw materials in the manufacture (*28*). The first report about mungbean starch biosynthesis was the analysis in 1983 of enzyme activities during seed development (*29*). Until recently, our laboratory continued this study (*30*−*32*). We utilized the in situ activity staining approach for the identification of enzyme activity-associated proteins for starch branching enzyme (*31*) and starch synthase (*32*). Because mungbean starch possesses high amylose content, it is expected to have unique machinery to elongate the amylose chain such as that performed by the synthetic activity of SP. In the current study, we hypothesized again that tracing the SP activities in the soluble extract for starch formation can be used for SP identification. Therefore, the aim of this study was to find mungbean SP during a developmental stage when the seeds contain less storage proteins. The identification of mungbean SP would not only advance our basic understanding of the members in mungbean starch biosynthesis but also act as a basis to further investigate the roles it would play and its possible biotechnology applications in the future.

We first separated mungbean soluble proteins through analytical approaches by native-PAGE. Together with in situ activity zymogram (iodine staining) and SDS−PAGE, we found SP activity-associated proteins. Then the suspected proteins were either identified by MALDI-TOF or used to raise polyclonal antibodies for immunological analysis. Results showed that we have identified mungbean SP in the developing mungbean seed.

MATERIALS AND METHODS

Materials. Mungbean cultivar KPS1 containing a high starch content of 53.5% was selected from the germplasm at Asia Vegetable Research and Development Center (AVRDC) in Shanhua, Taiwan. Immature mungbean pots were picked at three growth periods (approximately DAF 10, 12, and 14; DAF = days after flowering) during July and August and stored at −80 °C for further analysis. Bovine serum albumin (BSA), dithiothreitol (DTT), ethylenediaminetetraacetic acid (EDTA), G-1-P, glycerol, horseradish peroxidase (HRP) conjugated anti-mouse IgG, iodoacetic acid (IAA) 2-mercaptoethanol, phenylmethylsulfonylfluoride (PMSF), ponceau S, pristane, sodium citrate, and Tris-HCl were purchased from Sigma (St. Louis, MO). Reagent grade acid, base, salts and solvent were purchased from Sigma or Merck Chemical Co. (Darmstadt, Germany). Ammonium persulfate (APS), ECL detection kit, hyperfilm, radioactive ^{14}C-G-1-P, and scintillation cocktail were from Amersham Biosciences (Taipei, Taiwan, ROC.). Microcon, filter disk, PVDF membrane (Immobilon-P), and ZipTip$_{C18}$ were from Millipore Corp. (Bedford, MA). Acrylamide, *N*′,*N*′-methylenebisacrylamine, Bio-Safe Coomassie, glycine, molecular weight standard (broad range; No. 161-0317), protein assay dye reagent concentrate, sodium dodecyl sulfate (SDS), Silver Stain Plus, and *N*,*N*,*N*′,*N*′-tetramethylethylenediamide (TEMED) were purchased from Bio-Rad (Hercules, CA). Tris base was from USB (Cleveland, OH). Film developing agents were purchased from Kodak Corp. (Rochester, NY). Potassium iodine (KI) was from Wako Pure Chemical Industries (Japan), and iodine (I_2) was from Showa (Tokyo, Japan). Trypsin was from Promega (Madison, WI).

Soluble Fraction Preparation. Immature mungbeans were carefully collected from bean ovary on ice. The soluble fraction was prepared as previously described (*32*). Protein concentration was determined by the Bradford method (*33*) using BioRad protein assay reagent concentrate. BSA was used as standard to construct a calibration curve within a 10 μg/mL range.

PAGE (Polyacrylamide Gel Electrophoresis). Native-PAGE and SDS−PAGE were performed using Mini PROTEAN II apparatus (Bio-Rad), and SDS was omitted in all buffers of the native systems. The gel thickness of 0.75 mm was for routine analysis, and 1.5−3 mm was used for protein band preparation in electroelution. The lower separation gel contained final concentrations of either 7.5% or 12% acrylamide/bis and the upper stacking gel contained final concentrations of 4% acrylamide/bis with the same gel composition as previously described (*32*). Gel preparation was according to manufacture's instructions. The native-PAGE gels were used for SP activity staining, and SDS−PAGE gels were subjected to Coomassie blue or silver staining.

In Situ SP Activity Staining. In situ staining for SP activity was performed according toYu et al. (*8*) with minor modification. Samples were first separated by native PAGE in the minigel system. The harvested gel was then rinsed with 0.1 M sodium citrate (pH 7.0) several times followed by immersion in 10 mL of incubation solution (0.1 M sodium citrate, pH 6.0, and 20 mM G-1-P). The reaction was conducted for 2 h at room temperature with mild agitation. After incubation, the solution was decanted, and the gel was rinsed several times with deionized water. The gel was then incubated with 20 mL of Lugol's solution (10 mM I_2, 14 mM KI) at room temperature for 15 min followed by rinsing with deionized water to reduce background. The activity stained gel band strip was excised for protein profile by SDS−PAGE.

Protein Staining. Proteins were stained with either Coomassie staining or silver staining. Silver staining was conducted following the protocols provided in the Silver Stain Plus kit, and the aqueous system Bio-Safe was used for Coomassie staining. Gel photo documentation was performed with an Alpha Innotech ChemiImager IS4400 system loaded with software AlphaEase v. 2.3 (*32*).

Electroelution for Antiserum Preparation. The 55 kDa protein in the SP-activity stained protein bands was excised and recovered by electroelution (BioRad model 422) after being separated by SDS−PAGE and visualized by Coomassie blue staining as described previously (*32*). Protein eluent was further concentrated in a Microcon for use as antigen to immunize mice. Polyclonal antiserum was prepared in the Core Facility Laboratories at the Department of Biochemistry and Technology in National Taiwan University under generous arrangement by Professor Rong-Huay Juang (*20*).

Mass Spectrometry. The purity of the105 kDa protein was verified in SDS−PAGE with silver staining before its Coomassie-stained gel slice was sent to the Mass Spectrometry Laboratory of DigitalGene Biosciences Co., LTD (Taipei, Taiwan) for in-gel trypsin digestion (*34*) and MALDI-TOF (matrix-assisted laser desorption/ionization-time-of-flight) MS analysis (*35*, *36*). The gel slice was first incubated with an adequate amount of 50% acetonitrile/0.2 M ammonium carbonate (pH 8) for 30 min at room temperature. The procedure was repeated two

more times to remove SDS and stain. Then, the gel was reduced by DTT (10 mg/mL) and alkylated with IAA (25 mg/mL) in 1 M NaOH solution. The gel was then minced and dehydrated by first incubating with an adequate amount of 100% acetonitrile, just enough to cover all gel pieces, for 10 min, followed by drying on a SpeedVac over 45−60 min. The dried gel pieces were then rehydrated with a concentrated trypsin solution (20 mg/mL, 0.1 M ammonium bicarbonate) using a 1:1 volume ratio to the original gel slice volume for 20−30 min. Then, 50 mM ammonium bicarbonate (pH 8) buffer was added to completely cover the gel pieces, and the mixture continued to incubate for 12−20 h at 37 °C. The resulting digested supernatant was saved. Ten percent formic acid was then added to cover the gel sample, and the mixture was incubated at 60 °C for 10 min, followed by sonication for 1 h. This formic acid supernatant combined with the digested supernatant was purified by ZipTips where the peptides were eluted with α-cyano-4-cinamic acid in 50% acetonitrile/0.2% TFA as sample for the MALDI-TOF.

The mass spectrometric peptide map analysis was performed on a Voyager-DE STR MALDI-TOF workstation (ABI, Applied Biosystems, Inc., Framington, MA) 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 the Sequazyme Peptide Mass Standard kit (PE Biosystems). Spectra were analyzed using software program Voyager, version 5 with Data Explorer software. The mass identity of the tryptic fragments of the 55 kDa protein was analyzed the similar manner in the Proteomic Center at China Medical University in Taichung, Taiwan.

SP Activity Radioactive Assay. SP activities in the crude soluble extract of different sized mungbean or activity neutralization was assayed without glucan primer in 200 μL volume containing final concentrations of 20 mM sodium citrate (pH 7.0) and 20 mM hot/cold ^{14}C-G-1-P (0.014 μCi). The reaction proceeded for 30 min at 30 °C in a water bath with 200 rpm agitation and was terminated by spotting the mixture onto a Whatman GF/A 2.4 cm filter disk on a heating block, followed by washing steps and radioactivity counting as described elsewhere (*30*−*32*). SP specific activity (SA) was calculated as SA = (disk-trapped cpm − blank-disk cpm)/(total cpm) × substrate (mole)/(sample protein (mg) × time (min)).

Western Blotting. Protein samples were first separated by 0.75 mm thickness SDS−PAGE using the minigel system. Gels were then electroblotted onto a PVDF membrane by BioRad Mini-transblot in transfer buffer, followed by successive hybridization with a 1:10 ratio of primary and a 1:1000 ratio of secondary antibodies and ECL detection as described previously (*32*).

RESULTS AND DISCUSSION

Mungbean SP Was Detected by Activity Staining. To detect SP activity during developing stage, DAF 10 mungbeans were used to prepare crude soluble extract. The extracts of various protein concentrations were separated by native-PAGE, and the gel was cut in two halves. One half was subjected to in situ activity staining, and the other half was subjected to protein staining with Coomassie blue. The two gels were aligned as shown in **Figure 1**. It showed that a major migrating protein population on the iodine-stained gel zymogram revealed SP activities as a starch−enzyme bluish-purple complex (**Figure 1B**). The parallel position corresponded to a single protein band population of the same mobility on the protein profile in **Figure 1A** by Coomassie staining. The density of this major activity-stained SP population was visualized in a dose-dependent manner.

There were also at least two minor slow migrating SP activity-stained proteins detected on the upper zymogram in the 90 μg sample. Many L-SPs were purified as both monomeric and dimeric forms, such as L-SP of 223 and 112 kDa in maize (*6*), 200 and 108 kDa in pea (*9*), 220 and 112 kDa in sweet potato (*19*), and 290 and 112 kDa in banana (*13*). Many H-SPs were also purified as different native size and subunit size, such as H-SP of 155 and 90 kDa in banana (*13*) and of 145 and 85 kDa in rice (*23*). This observation, thus, implied the possible presence of multimeric forms or isoforms of SP in the crude soluble extract. They were clustered with themselves, each other, or other protein species at native state, resulting in different mobility in the native-PAGE. These minor SP activity-associated bands, however, were not further investigated.

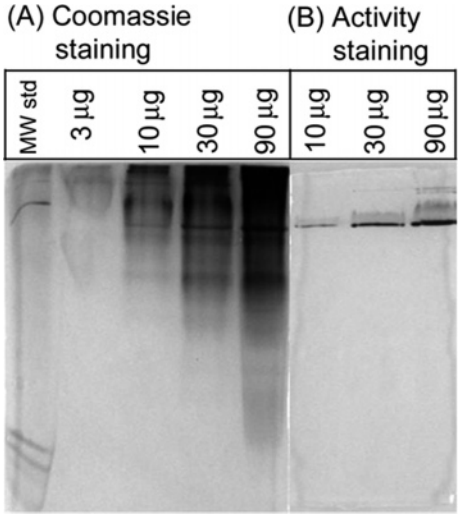


Figure 1. Dose-dependent detection of mungbean SP by activity staining: (**A**) native protein profiles of the soluble extract; (**B**) a major SP population detected in the gel zymogram. Various amounts (3, 10, 30, and 90 μg) of mungbean crude extract in two sets were loaded in a 0.75 mm thickness of 8% native-PAGE gel. Half of the harvested gel was stained with Coomassie blue (**panel A**); the other half excluding the 3 μg sample lane was subjected to in situ SP activity staining (**panel B**). SP visualized in **panel B** was stained bluish-purple with Lugol's solution, although the color does not show in the black and white print.

Another point made here is the ability of the major SP activity-stained native protein to directly use G-1-P as substrate without the addition of exogenous primers to synthesize starch in situ. This observation reinforced the role of SP in starch biosynthesis in mungbean like those observed previously (*5*, *7*, *8*, *17*−*20*). This major SP activity-stained native protein band was excised to analyze its protein components by SDS−PAGE.

SDS−PAGE Analysis of the SP Activity-Stained Band. The major SP activity-stained native protein band consisted of two main SP-activity-related proteins, 105 and 55 kDa as shown by SDS−PAGE in **Figure 2**, lane 1. These two proteins appeared to be the major protein species in the crude soluble extract as compared with **Figure 2**, lanes 2 and 3. A few minor proteins revealed by silver staining with approximate molecular size between 66 and 55 kDa and close to 31 kDa were also detected. Since the sample was excised from the major band of the native-PAGE in **Figure 1B**, lane 3, we cannot rule out the possibility that these minor protein species may be other physically associated proteins or protein cofactors of the SP activity-stained complex. The question of whether these minor proteins may also contribute to the in vitro formation of starch is interesting for further investigation. Because the density and appearance of the 105 kDa protein was a reproducible event compared to the 55 kDa protein, it was further submitted for mass identification.

Identification of the 105 kDa Protein To Be a SP by Mass Spectrometry. The 105 kDa protein band on one-dimensional SDS−PAGE appeared sharp enough to be judged as a homo-

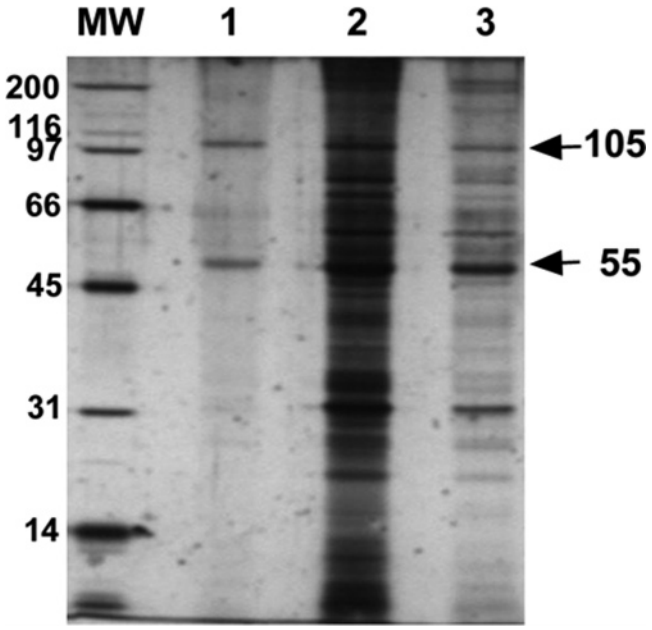


Figure 2. SDS–PAGE analysis of the SP activity-stained band. In the left lane, the molecular weight standard (0.5 μg) contained 200 kDa myosin, 116 kDa β-galactosidase, 97 kDa phosphorylase b, 66 kDa serum albumin, 45 kDa ovalbumin, 31 kDa carbonic anhydrase, and 14 kDa lysozyme. Sample lane 1 was prepared from the major activity-stained complex of **Figure 1B,** lane 3 (the 90 μg sample). The band was minced well with equal volume of the SDS–PAGE 2× sample buffer and sonicated for 1 min. After the sample was boiled at 95 °C for 1 min and immediately put on ice, half of the sample was loaded. Sample lanes 2 and 3 were protein profiles of 10 and 3 μg of the crude soluble extract. Samples were analyzed in a 12% SDS–PAGE gel and visualized by silver staining.

geneous protein species; its gel slice was sent for in-gel trypsin digestion, followed by MALDI-TOF analysis and database searching. The mass spectrum shown in **Figure 3** displayed a unique fingerprint of the 105 kDa protein. The de-isotoped mass values of these tryptic fragments were subjected to MS−FIT analysis (http://www.ncbi.nlm.nih.gov). The lower table summarizes the searching result of the matched proteins in Swissprot database. The higher the MOWSE score, the higher the homology between the query protein and known protein is. Proteins of the top three highest MOWSE scores were the same SPs that were from *Ipomoea batatas* (sweet potato), *Zea mays* (corn), and *Oryza sativa* (rice). The detailed description in these accessible numbers showed it to be very similar to the known L-form of SP. Thus, the 105 kDa protein identified by activity staining was ascertained to be a form of mungbean SP. However, the question of whether this 105 kDa is indeed the L-form SP in mungbean is not known at this moment because the pure 105 kDa SP has not yet been isolated in native form to conduct the affinity experiment with starch, glycogen, or G-1-P, such as the studies with L-SP of maize (*7*), sweet potato (*20*), and rice (*23*).

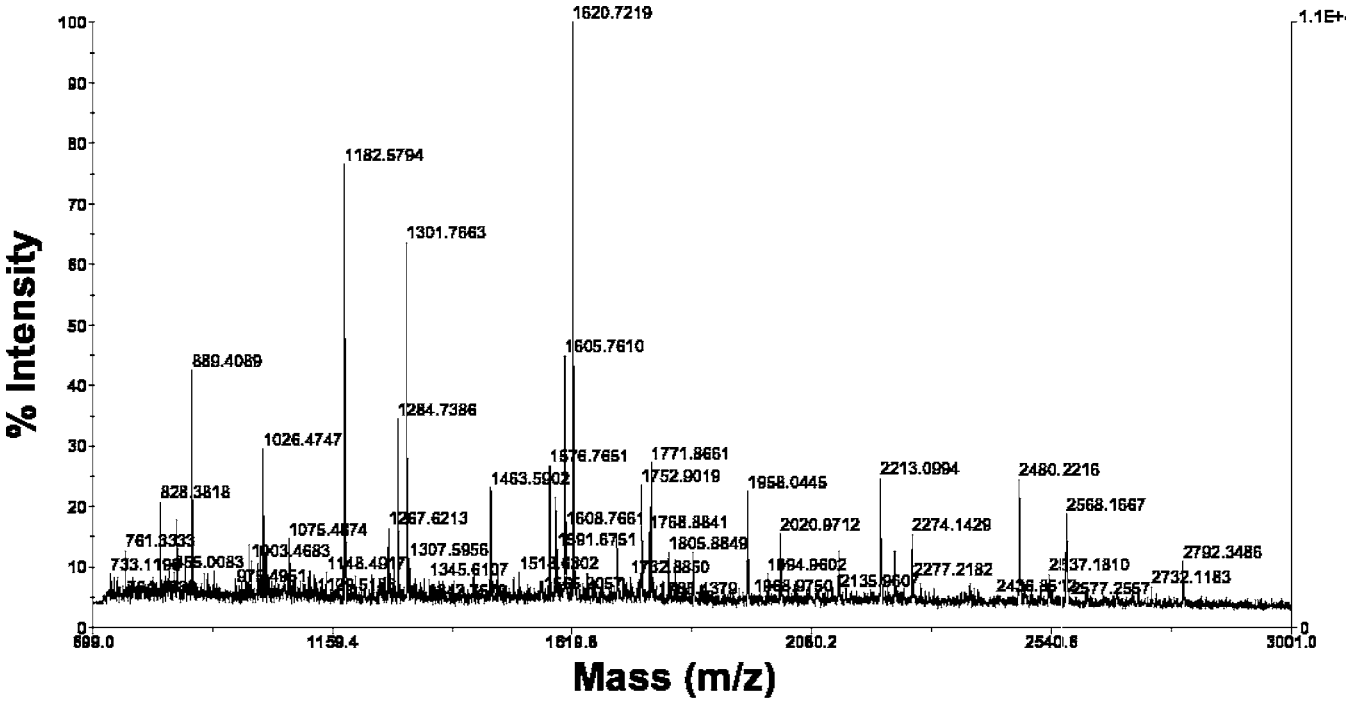
Through data analysis, three amino acid sequences from the mass of the tryptic fragments of mungbean 105 kDa protein were predicted and designated A (33 amino acids), B (23 amino acids), and C (20 amino acids) as listed in **Table 1**. Their sequences were then searched within the polypeptide of the retrieved L-SP and H-SP from GenBank to locate their corresponding positions in the published SP species by the BLAST function. Among the retrieved L-SP, it contained 955−1003 amino acids, and H-SP contained 809−841 amino acids. Fragment A was found near the N-terminus, whereas fragments B and C were found close to the C-terminus. In addition, fragments A and C were found in both L-SP and H-SP, whereas a similar fragment B was not found in H-SP except for that from potato. The homologies of these internal tryptic fragments of the 105 kDa protein again verified its identity to be a form of mungbean SP. Furthermore, fragments A and C were highly conserved in both L-SP and H-SP, implying the possible presence of H-SP in mungbean. This possibility was also raised by Western analysis as described below.

Immunological Analysis between Mungbean 105 kDa-SP and the 55-kDa Protein. Mungbean 105 kDa-SP has been confirmed in the first place as above; the identity of the SP activity-associated 55 kDa protein was further investigated by an immunological approach. The 55 kDa protein was prepared by electroelution from preparative SDS−PAGE gels of the 55 kDa protein band as shown in **Figure 2**, lane 1, for mouse immunization. The anti-55 kDa antibodies were then used to probe the mungbean crude extract of two protein concentrations on a Western blot **(Figure 4**). The protein profile in the crude extract was revealed by silver staining (lane 1) and the performance of protein transferred onto the PVDF membrane was revealed by ponceau S staining (lanes 2 and 3). The band signals detected by antibodies (lanes 4 and 5) thus clearly showed that the anti-55 kDa antibodies recognize mainly the 105 kDa protein and the 55 kDa protein itself.

In addition, an approximately 90 kDa protein band with similar size to the known H-SP (*13*, *23*, *25*) was also detected in minor densities in both lane 4 and lane 5. Owing to the high similarities between the known L-SP and H-SP (*25*), if the 55 kDa protein possesses homology to either the L-SP or the H-SP, the detection of either SP by anti-55 kDa was possible. When the results are combined in **Figure 4** and **Table 1**, it demonstrated first that an approximately 90-kDa protein was detected by the SP activity-associated anti-55 kDa antibodies. Second, the tryptic fragments A and C in mungbean L-SP were also the conserved sequences in H-SP. Third, the crude soluble extract contained plastid stromal and cytosolic fractions where the L-SP and H-SP were located, respectively. It suggested the possible presence of an H-SP in developing mungbean.

The anti-55 kDa antibodies were also used in the activity neutralization experiment (**Figure 5**) to correlate the 55 kDa protein with SP activities. It showed that there was dose-dependent neutralization of SP activities up to 55%. The removal of 55% activity clearly suggested that the antibodies recognized proteins with SP activities in the crude extract. In contrast, the residual 45% activity implied the possibility that there may be SP isoforms other than the 55 kDa-related 105 kDa L-SP and possible 90 kDa H-SP or unknown enzymes that shared the same G-1-P substrate. Nevertheless, the above two immunological experiments demonstrated that the 55 kDa and the 105 kDa SP were immunologically related protein species.

Identification of the 55 kDa Protein by Mass Spectrometry. The possibility that the 55 kDa protein might be a fragment derived from the 105 kDa-SP was disclosed by a subsequent MALDI-TOF mass analysis. Five tryptic fragments were derived from the 55 kDa protein, and their mass values corresponded to predicted peptide sequences of QAYYLSMEFLQGR, AVAHDVPIPGYK, HTEASEALANAEK, DAWNITQR, and TIIAEYGTADSDLLDKK. After database searches, the five sequences were matched separately with the N-terminal half of the 1003 amino acid-containing L-SP of fava bean (*Vicia faba*) at positions 148−160, 291−302, 331−343, 427−434, and 477−493 with very high MOWSE scores. Previous reports showed that fragmented forms of plant SP were found during purification



MOWSE Score	Protein MW (Da)/pI	Accession No.	Species	Protein Name
1.119e+005	108521/5.3	168276	IPOMOEA BATATAS	(M64362) starch phosphorylase
6.613e+004	110434/5.6	6733896	ZEA MAYS	(A85269) starch phosphorylase
6.099e+004	104645/5.4	13195430	ORYZA SATIVA	(AF327055) alpha 1,4-glucan phosphorylase L isozyme
3526	110701/5.2	313349	SOLANUM TUBEROSUM	(X73684) starch phosphorylase

Figure 3. Mass spectral identification of the mungbean 105 kDa protein. The 105 kDa protein, after Coomassie blue staining on SDS–PAGE, was cut from the gel. The gel slice was subjected to in-gel trypsin digestion, MALDI-TOF analysis, and database searching as described in Materials and Methods. Percent of intensity was plotted from the normalized values using the highest peak signal as 100%. The table summarizes the searching result.

in pea (*9*), *Voandzeia subterranean* seed (*26*), and sweet potato (*19*), and the occurrence of similar sized products of 46−55 kDa from the nicked L-SP of sweet potato (*20*) were detected by proteolytic modification. Therefore, the mungbean 55 kDa protein may be a fragmented product from the N-terminal half of the 105 kDa-SP. As to what happened to the C-terminal half of the SP, it was possible degraded or fragmented into too many pieces for us to examine. However, whether the 55 kDa protein itself may be an independent SP like the purified 55 kDa banana SP (*12*) that performed SP activities in vitro was not clear at this moment. Therefore, the 55 kDa may be simply a proteolytic fragment from the 105 kDa as discussed above, or an independent active form that is expressed as a truncated form of the 105 kDa on the same gene or one of separated multiple SP genes under certain regulatory control at the molecular level.

Analysis of Mungbean SP Activities during Early Growth. SP has been reported to be active in the developing stage of pea seeds (*9*), banana (*13*), *Voandzeia subterranean* seed (*26*), young sweet potato (*18*), and rice (*23*). In this study, mungbeans were collected at approximately DAF10, DAF12, and DAF14 at three different early growth stages (the maturity is approximately DAF 28) to analyze their SP activities by both radioactivity assay and activity staining in the soluble extracts. It showed that as the seed grows 2-fold in size, the specific activities of SP decreased almost 6-fold, demonstrating an inverse correlation between seed size and SP activity during early stage seed growth **(Figure 6A,B)**. The crude extracts of the three samples were also subjected to native-PAGE followed by SP activity staining as shown in **Figure 6C**. Half of the gel was Coomassie-stained (**left** panel), and the other half was activity stained (**right** panel). It showed that although the intensities of this corresponding major protein population as visualized by Coomassie blue (**left** panel) were not obviously different among the three seed extracts, the intensities of their in situ iodine-stained starch−enzyme complexes were different. This result again was consistent with the result of the radioactivity assay that the stained SP activities decreased as the seed size enlarged during early growth.

The activity-stained protein complexes of three DAF samples were also analyzed by SDS−PAGE and show the common

Table 1. Bioinformatic Analysis of the Tryptic Fragments of Mungbean 105 kDa SP[a]

matched enzyme	accession no.	species name	enzyme length (amino acids)	matched sequence range		
				fragment A	fragment B	fragment C
L-SP	130172	sweet potato (*Ipomoea batatas*)	955	106–138	740–762	802–821
	130173	potato (*Solanum tuberosum*)	966	113–145	751–773	813–832
	2506470	fava bean (*Vicia faba*)	1003	131–163	788–810	850–869
	7433830	spinach (*Spinacia oleracea*)	971	136–168	756–778	818–837
	13195430	rice (*Oryza sativa*)	928	65–97	713–735	775–794
	13236668	rice (cv japonica) (*Oryza sativa*)	951	115–147	736–758	798–817
	15228683	thale cress (*Arabidopsis thaliana*)	962	130–162	747–769	809–828
H-SP	417488	potato (*Solanum tuberosum*)	838	61–93	623–645	685–704
	15983803	thale cress (*Arabidopsis thaliana*)	841	64–96		688–707
	34908348	rice (cv japonica) (*Oryza sativa*)	841	63–95		688–707
	12025466	rice (*Oryza sataiva*)	809	31–63		656–675
	14916632	bread wheat (*Triticum aestivum*)	832	55–87		679–698

[a] The predicted amino acid sequences of tryptic fragment A, B and C were generated from MALDI-TOF analysis and Data Explorer software searching: fragment A, DALIVNWNATYDTTEKLNMKQAYYLSMEFLQGR; fragment B, LAKFITDVGATINHDPEIGDLLK; fragment C, FAMNGCILIGTLDGANVEIR. Each individual sequence was used to search within each polypeptide query sequence of the retrieved SP species from GenBank by the BLAST function. The matched amino acid sequence ranges of sequences A, B, and C in the known SP enzymes were organized.

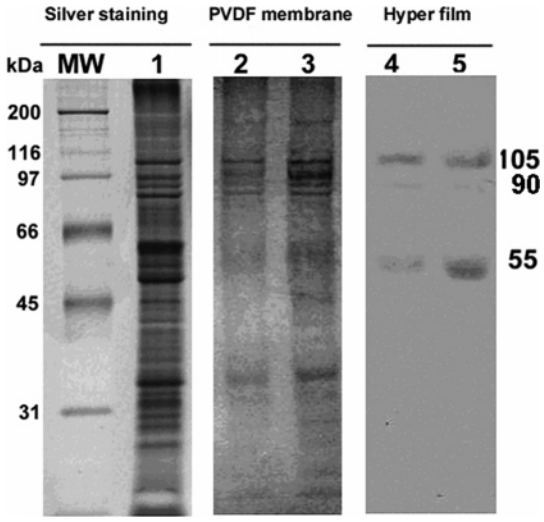


Figure 4. Western detection of the 55 and 105 kDa proteins by anti-55 kDa antibodies. Lane 1 contains the total protein profile of a SDS–PAGE gel of 3.6 μg of crude soluble extract as visualized by silver staining. Lanes 2 and 3 were 3.6 and 7.8 μg of crude soluble extract separated in 10% SDS–PAGE and blotted onto PVDF membrane. The blot was stained with ponceau S and photographed to inspect the transfer efficiency of the proteins. Lanes 4 and 5 were from the same PVDF membrane in lanes 2 and 3 probed with anti-55 kDa antibodies, and their hybridization signals were detected by chemiluminescent ECL reagent after exposure on a hyper film.

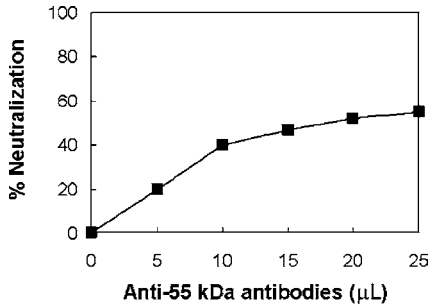


Figure 5. SP activity neutralization by anti-55 kDa antibodies in the crude soluble extract. The preimmune serum was performed as negative background control in the SP activity radioactive assay. Increasing volumes of preimmune serum were performed along with antiserum in duplicates. Both serums were incubated with SP reaction mixture for 10 min on ice before hot/cold ^{14}C-G-1-P was added to react at 30 °C for 30 min. The background radiation in the preimmune sample has been subtracted from the antiserum sample for plotting. The percent neutralization was calculated by using the total SP activities in the positive control as 100% to subtract the residual activities in the antiserum samples.

appearance of the major 105 kDa SP and 55 kDa despite other minor proteins present in the complex (data not shown). This study used early growth beans; therefore, it was not compatible with the study of pea (*9*, *11*), which showed that SP activity increased during the accumulation of starch. Particularly we used bean size instead of starch content, and we measured the specific activity rather than total activity. In addition, the growth stage prior to DAF 14 was not the starch accumulation period when the activity of most of the starch biosynthetic enzymes started to increase after DAF 14 as reported by Tsay et al. (*29*). When previous results with pea (*9*, *11*), mungbean (*29*), and this study were combined, it thus showed that high activity of SP starts in early seed growth and is involved throughout the starch accumulation and germination period (*9*), indicating that SP plays important roles not yet fully understood in the lifetime of seed.

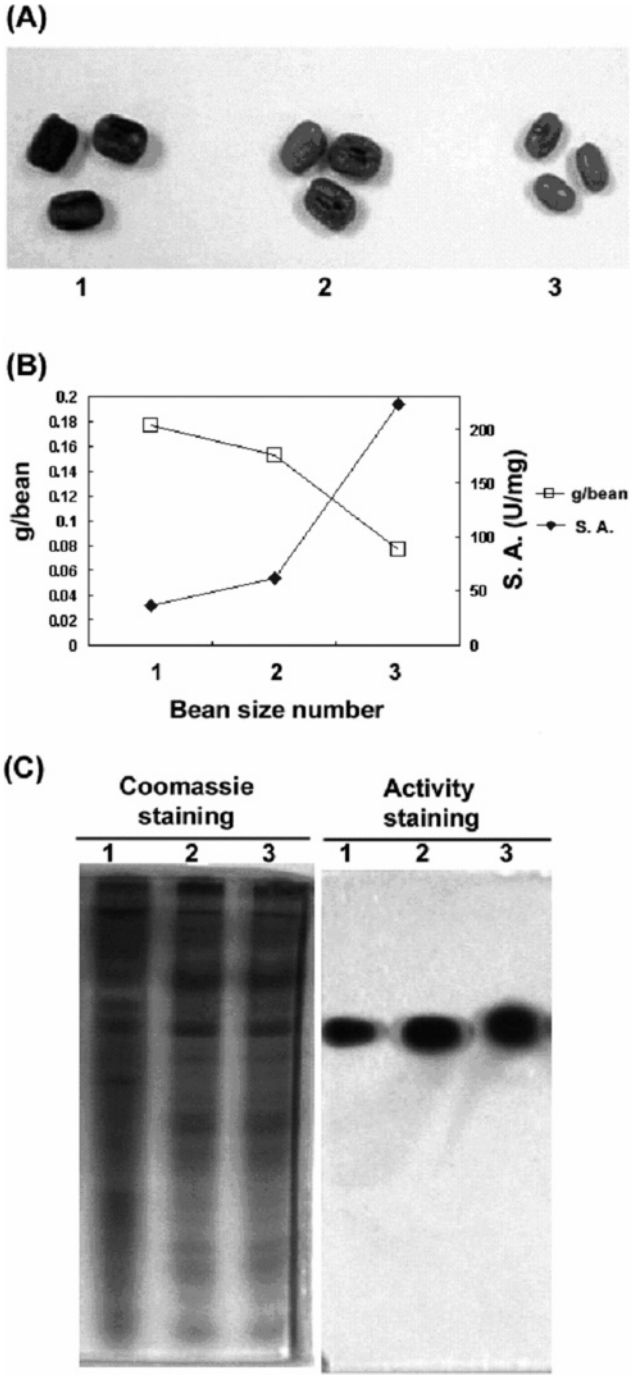


Figure 6. Mungbean SP activities of different seed size. The sample labeling is in the same order in **panels A, B,** and **C**. In **panel A**, three mungbean groups of different sizes were photographed in parallel. Size group 1 was DAF 14 seeds, size group 2 was DAF 12 seeds, and size group 3 was DAF 10 seeds. Each bean group was picked from the same mungbean pot. **Panel B** presents the specific activities of SP in three samples. The SP activities were assayed by the radioactive method. **Panel C** shows the native protein profile and SP zymogram in three crude soluble samples. Forty micrograms of each crude extract in two sets were separated in a 7.5% native-PAGE. Half of the gel was visualized by Coomassie staining to show proteins. The other half was visualized by SP activity staining to show the SP population.

In summary, this study successfully identified an L-form-like 105 kDa SP, an SP activity-related 55 kDa protein and a possible H-form-like 90 kDa SP in mungbean. The 105 and 55 kDa proteins appeared to be high-quantity species in the soluble proteins. The native form of mungbean SP should exist physically as a complex that includes the 105 kDa SP and the 55 kDa protein complexed with themselves, each other, or other protein species. In addition, mungbean SP was able to synthesize starch from G-1-P even in the absence of exogenous primer, reinforcing some vital roles of SP in the starch biosynthesis in mungbean.

Finally, the finding of mungbean SP with similar molecular size and sequence homology among known species was not a surprise; on the contrary, our results opened a door to further study of many unsolved questions at a basic level in the future, such as the following: What mechanism is involved for each of the 105 kDa SP and the 55 kDa proteins in such high quantities in early mungbean growth? How and where are other forms of SP expressed throughout the development? What is the size of the starch product that SP synthesizes? How does mungbean SP cooperate with other starch biosynthetic enzymes to produce unique mungbean starch architecture? Does the 105 kDa SP also regularly exist as a dimer form and, if so, what force is associated between the monomers? What is the benefit of SP dimerization for starch elongation? Does the same 105 kDa SP protein exist as both active and inactive forms during seed development? Is the activity of the 105 kDa SP under regulation control, such as by other cofactors or translational modification (phosporylation, acetylation, methylation etc.)? Is SP able to be promoted to act as a biological modifier for application in food biotechnology? All of the above questions are expected to resolve relying on our initial understanding of mungbean 105 kDa SP and SP activity-related 55 kDa as reported here.

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