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Enzyme-Linked Immunosorbent Assay (ELISA) for Detection of Sulfur-Rich Protein (SRP) in Soybeans (*Glycine max* L.) and Certain Other Edible Plant Seeds

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As a result of methionine deficiency, legume proteins are considered to be incomplete, and therefore there is a need to explore ways to improve legume protein amino acid balance. Using rabbit anti-soybean sulfur-rich protein (SRP) polyclonal antibodies (pAb), sensitive immunoassays (nanogram sensitivity) were developed. The immunoassays detected SRP in all soybean seeds and soybean-based commercial samples examined. In addition, the presence of pAb cross-reactive proteins was detected in certain dry beans and oilseeds. The cross-reactive proteins were isolated using purified IgG-based immunoaffinity column chromatography. Biochemical analyses including N-terminal amino acid sequencing and amino acid composition indicated that the cross-reactive proteins were comparable to soybean SRP. The cross-reactive proteins contained methionine (1.6–2.4 residues/100 residues) and cysteine (2.4–3.6 residues/100 residues), which satisfies the FAO/WHO recommended pattern for sulfur amino acids in both adults and children (2–5 years old). The results suggest the presence of constitutive SRPs in several dry beans and oilseeds.

KEYWORDS: Sulfur-rich protein (SRP); legume; immunoassays; rabbit polyclonal antibody (pAb); amino acid composition

INTRODUCTION

Globally, legumes are valued as an inexpensive source of dietary proteins and lipids for human food and animal feed purposes (*1*). The term "legume" describes dry beans (e.g., common beans, lentils, and peas) and oilseeds (e.g., peanuts, soybeans, and lupine) (*2*). Among several thousand globally cultivated legumes, soybeans (*Glycine max* L.) are commercially the most important. In 2005, the United States (39%), Brazil (24%), Argentina (18%), and China (8%) accounted for ~89% of the global soybean production (*3*). Soybeans contain 35–40% protein, which is more than the typical protein content of most other legumes and oilseeds (20–30%) (*4, 5*). The majority of proteins in soybean are storage proteins, globulins being the most abundant (*6*). The two globulins, glycinin (11S) and β-conglycinin (7S), account for 70–80% of the total seed proteins (*7–10*) and thus determine the overall nutritional quality of the soybean proteins. Unlike "complete" animal proteins, legume proteins are "incomplete" as they are deficient in the sulfur amino acids methionine and cysteine. On average, most legumes contain ~50% of the FAO recommended sulfur amino acids. Methionine is an essential amino acid for humans and animals as it is required for protein synthesis both as a cysteine precursor and as a methyl group donor (*11–13*). The methionine content of soybean ranges from 10.7 to 12.6 mg/g of protein (*1*), which is below the sulfur amino acid requirement for adult humans (17–27 mg/g of protein) (*14–16*).

Attempts have been made to address the known deficiency of sulfur amino acids in legume proteins including genetic manipulations to produce transgenic seeds with improved amino acid balance (*17–20*). For example, the sulfur-rich 2S albumin gene from Brazil nut has been introduced into soybean (*21, 22*), rape seed (*Brassica napus*) (*23*), narbon bean (*Vicia narbonensis*) (*24–26*),

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Table 1. Comparative Immunoreactivity of Soybean Varieties*

no.	cultivar	origin	crop year	ELISA
1	Williams 82	USDA-ARS[a]	1992	60.6 ± 9.4
2	Century*	Purdue University[b]	1984	100.0 ± 0.0
3	Century 84	Purdue University[b]	1984	50.2 ± 15.8
4	Cumberland	Purdue University[b]	1984	68.7 ± 6.8
5	Cutler	Purdue University[b]	1984	65.0 ± 7.9
6	Hack	Purdue University[b]	1984	64.2 ± 13.1
7	Hardin	Purdue University[b]	1984	69.5 ± 9.0
8	Harper	Purdue University[b]	1984	66.6 ± 11.2
9	Hobbit	Purdue University[b]	1984	135.2 ± 16.3
10	Lawrence	Purdue University[b]	1984	97.8 ± 12.8
11	Miami	Purdue University[b]	1984	46.5 ± 5.9
12	Wells II	Purdue University[b]	1984	83.2 ± 20.7
13	Oakland	Purdue University[b]	1984	63.8 ± 11.9
14	Peller	Purdue University[b]	1984	70.5 ± 17.4
15	Pixie	Purdue University[b]	1984	41.4 ± 4.6
16	Regal	Purdue University[b]	1984	99.5 ± 39.7
17	Ripley	Purdue University[b]	1984	144.0 ± 21.5
18	Union	Purdue University[b]	1984	40.9 ± 6.2
19	Will	Purdue University[b]	1984	99.7 ± 12.9
20	Winchester	Purdue University[b]	1984	36.7 ± 1.7
21	Woodworth	Purdue University[b]	1984	84.6 ± 2.7
22	Raiden (PI 360.844)	University of Illinois[b] (from Japan)	1983	119.0 ± 13.2
23	Banesi	University of Illinois[b]	1984	63.2 ± 0.7
24	Cloud	University of Illinois[b]	1985	43.0 ± 5.4
25	Dunfield	University of Illinois[b]	1985	61.9 ± 5.8
26	Ebony	University of Illinois[b]	1985	94.4 ± 16.7
27	Kura	University of Illinois[b]	1984	51.2 ± 13.6
28	Giant Green	University of Illinois[b]	1984	109.2 ± 14.2
29	Kingwa	University of Illinois[b]	1983	54.8 ± 15.0
30	Mandarin	University of Illinois[b]	1983	70.1 ± 15.8
31	Jogan	University of Illinois[b]	1983	86.2 ± 6.1
32	R01-3235F	University of Arkansas[c]	2005	114.5 ± 8.6
33	R01-3597F	University of Arkansas[c]	2005	68.3 ± 12.3
34	R01-3525F	University of Arkansas[c]	2005	86.4 ± 11.3
35	R01-2623F	University of Arkansas[c]	2005	58.8 ± 15.3
36	R01-3606F	University of Arkansas[c]	2005	56.1 ± 10.9
37	R01-176F	University of Arkansas[c]	2005	95.2 ± 13.9
38	R01-3567F	University of Arkansas[c]	2005	*178.3 ± 27.4*
39	UA-4805	University of Arkansas[c]	2005	99.3 ± 1.9
40	R01-2655F	University of Arkansas[c]	2005	78.7 ± 17.3
41	R00-2059	University of Arkansas[c]	2005	69.8 ± 13.8
42	Ozark	University of Arkansas[c]	2005	110.1 ± 19.7
43	R01-3105F	University of Arkansas[c]	2005	91.3 ± 1.7
44	SS-516	University of Arkansas[c]	2005	85.9 ± 16.4
45	R00-2179	University of Arkansas[c]	2005	85.5 ± 6.0
46	R01-3464F	University of Arkansas[c]	2005	73.8 ± 9.7
47	R01-2753F	University of Arkansas[c]	2005	104.9 ± 6.5
48	R01-2111	University of Arkansas[c]	2005	*164.3 ± 24.0*
49	R03-410	University of Arkansas[c]	2005	*154.0 ± 22.9*
50	V99-2993	University of Arkansas[c]	2005	68.1 ± 10.7
51	R03-1134	University of Arkansas[c]	2005	137.5 ± 26.5
52	R01-4573	University of Arkansas[c]	2005	88.7 ± 14.4
53	R01-3615F	University of Arkansas[c]	2005	115.7 ± 7.2
54	R01-3309F	University of Arkansas[c]	2005	127.7 ± 29.4
55	V96-4181	University of Arkansas[c]	2005	93.3 ± 2.5
56	R01-2025	University of Arkansas[c]	2005	94.3 ± 14.8
57	R01-3651F	University of Arkansas[c]	2005	119.1 ± 4.6
58	R95-1705	University of Arkansas[c]	2005	105.8 ± 12.5
59	R02-1767	University of Arkansas[c]	2005	69.7 ± 12.6
60	1A 2032	North Dakota State University[d]	2005	110.8 ± 48.8
61	Tofueey	North Dakota State University[d]	2005	70.9 ± 12.4
62	Proto	North Dakota State University[d]	2005	*143.3 ± 13.3*
63	Vinton	North Dakota State University[d]	2005	*144.8 ± 11.8*
				LSD 43.2

* Data are expressed as mean + standard error (SEM), $n = 3$, $p = 0.05$. Sources: [a], Dr. W. J. Wolf, USDA-ARS, Peoria, IL; [b], previously described in Sathe and others (*4*); [c], Dr. Pengyin Chen, Department of Crop, Soil and Environmental Sciences, Fayetteville, AR; [d], Dr. Sam Chang, Department of Cereal and Food Science, North Dakota State University, Fargo, ND. *, arbitrarily designated = 100%; the SRP from this cultivar was used as the immunogen for rabbit pAb production. Statistically significantly lower (underlined) and higher (*italicized*) values are indicated.

and common bean (*Phaseolus vulgaris*) (*27, 28*). Additionally, the sulfur-rich 2S albumin gene from sunflower (*Helianthus annuus*) has been expressed in transgenic lupin seed (*Lupinus angustifolius*) (*18, 29*) and chickpea (*Cicer arietinum* L.) (*30*). However, sulfur amino acid content of the transgenic seeds remained at or below the FAO standard for nutritionally balanced food proteins (2.5 mg/g of crude protein) (*3, 29*). Among some of the concerns associated with the use of genetically modified organisms (GMOs) are low seed yield, decreased pest resistance, introduction of new allergens (*31*), and lack of consumer acceptance. One such example is the expression of the Brazil nut 2S albumin in soybeans with the former shown to be a major allergen (*31*). Similarly, sunflower seed (*32*) and sesame seed 2S albumins, two sulfur-rich proteins, have also been found to be major allergens (*33*). Other attempts to compensate for sulfur amino acid deficiencies include chemical attachment of methionine to legume storage proteins (*34, 35*), soil sulfur fortification to encourage increased accumulation of sulfur amino acids, and complementation of legumes with cereals, such as consuming beans and rice in the same meal (*36*).

Alternative approaches to address sulfur amino acid deficiency in legumes may include detecting and increasing synthesis of sulfur-rich protein(s) in targeted legumes to help improve the amino acid balance. Soybeans contain a sulfur-rich protein (SRP) also referred to as a 7S basic globulin, that accounted for ~5% of the total extractable 'Century' soybean proteins (*37*). SRP contains significantly more methionine (1.30–2.64 residues/100 residues) than glycinin (1.1) or β-conglycinin (0.3) (*38–41*). Immunological and N-terminal amino acid sequencing studies have determined SRP to be different from glycinin and β-conglycinin (*41, 42*). The molecular mass of the native, tetrameric SRP is estimated to be ~148–168 kDa (*40, 43*). Under reducing conditions, SDS-PAGE of SRP reveals five polypeptides, a 42–45 kDa intermediate polypeptide, and one pair of heavy (26–29 kDa) and one pair of light (16–18 kDa) chain polypeptides. The heavy and light chains are linked by disulfide bond(s) (*41–44*).

The aim of the current investigation was to develop sensitive immunobased methods for the detection and quantification of soybean SRP and SRP-like proteins in select edible seeds. Protein G-purified anti-soybean SRP rabbit IgG was used to detect and isolate cross-reactive proteins from select edible seeds. Cross-reactive proteins were characterized by N-terminal amino acid sequencing and amino acid composition to determine homology with soybean SRP.

MATERIALS AND METHODS

Materials. Sources of soybean seeds are summarized in **Table 1**. With the exception of 2 lupine seed and 5 sunflower seed samples that were kindly provided by Dr. Sam Chang, Department of Cereal and Food Science, North Dakota State University, Fargo, ND, 39 unprocessed plant seeds (**Table 2**) and 5 commercial soy product samples were obtained from local grocery stores. Sources of electrophoresis, molecular mass markers, and staining and destaining chemicals were the same as reported earlier (*38*). Bovine serum albumin (minimum purity of 98% by electrophoresis, suitable for ELISA applications, catalog no. A 7030), cyanogen bromide-activated Sepharose 4B, Freund's complete and incomplete adjuvants, alkaline phosphatase labeled goat anti-rabbit IgG, Ponceau S, and phosphatase substrate [*p*-nitrophenyl phosphate, disodium (PNPP)] were from Sigma Chemical Co., St. Louis, MO. Microtiter ELISA plates (96 well, polyvinyl), Tween 20, salts, and other chemical reagents were from Fisher Scientific Co., Pittsburgh, PA. Protein G Sepharose 4 Fast Flow beads were from Pharmacia Inc., Piscataway, NJ. Nitrocellulose membrane (0.2 μm) was from Schleicher & Schuell Bioscience, Inc., Keene, NH. Disposable (5 mL) polypropylene columns were from Pierce Inc., Rockford, IL.

Table 2. Immunoassay Assessment of Seed Protein Extract Cross-Reactivities[a]

	ELISA			WB			DB		
protein extract	H	L	N	H	L	N	H	L	N
tree nuts									
almond			•			•			•
Brazil nut			•			•			•
cashew			•			•			•
hazelnut			•			•			•
macadamia nut			•			•			•
pecan			•			•			•
pinenut			•			•			•
pistachio			•			•			•
walnut			•			•			•
cereals									
barley			•			•			•
basmati rice			•			•			•
millet			•			•			•
sorghum			•			•			•
wheat berries		•			•			•	
wheat bran			•			•			•
whole oat			•			•			•
dry beans									
black bean	•			•			•		
black-eyed pea	•			•			•		
black gram	•			•			•		
chickpea	•			•			•		
Great Northern bean	•			•			•		
horse bean	•			•			•		
lentil			•			•			•
lima bean	•			•			•		
lupine 202 F2		•			•			•	
lupine 104 F2		•		•				•	
moth bean		•				•	•		
mung bean	•			•			•		
navy bean	•			•			•		
pinto bean	•			•			•		
small red bean	•			•			•		
split pea			•			•			•
tepary bean			•		•			•	
oil seed									
Inca peanut			•			•			•
pumpkin seed			•			•			•
sesame seed			•			•			•
Spanish peanut			•			•			•
soybean	•			•			•		
sunflower			•			•			•
sunflower, Cropland 30801 DMR		•		•				•	
sunflower, APF 30249			•			•			•
sunflower, Mycogen 8C488			•			•			•
sunflower, Panthers			•			•			•
sunflower, P63M02			•			•			•
Virginia peanut			•			•			•
winged bean	•			•			•		

[a] (H) high, (L) low, and (N) no cross-reactivity.

Methods. *Protein (11S, 7S, and SRP) Purification and SRP Heat Denaturation.* Soybean 11S and 7S proteins were prepared using the procedure described by Nagano et al. (*45*). SRP purification was according to the previously described procedure (*38*). SRP solution (1 mg/mL in BSB) was heated in a boiling water bath (100 °C) for the desired time, and cooled to room temperature (RT, ~25 °C), centrifuged (13600*g*, 20 min); aliquots of the supernatants were mixed with an equal volume of SDS-PAGE sample buffer (0.05 M Tris-HCl, 1% w/v SDS, 0.01% w/v bromophenol blue, 30% v/v glycerol, 2% v/v β-ME), heated for 10 min in a boiling water bath, and cooled to RT, and 8 μL of the sample was used for electrophoresis.

Preparation and Defatting of Flours. Plant seeds and commercial soy products were ground in an Osterizer blender (speed setting "grind", Galaxy model 869-18R, Jaden Consumer Solutions, Boca Raton, FL) and defatted for 6–8 h in a Soxhlet apparatus using petroleum ether (boiling point range of 38.2–54.3 °C, Fisher Scientific, Fair Lawn, NJ) as the extraction solvent. Soy milk was freeze-dried prior to defatting. The defatted samples were spread in a thin layer on aluminum foil and dried overnight under a fume hood at RT. Dried defatted samples were ground in an Osterizer blender to a fine flour able to pass through 40 mesh. The flour samples were stored in tightly capped plastic containers at −20 °C until further use.

Protein Extraction. Defatted seed flours and commercial soy products were extracted (flour to solvent ratio of 1:10 w/v typically 100 mg/1 mL) with borate saline buffer (0.1 M BSB, pH 8.45). The flour slurries were vortexed (Vortex Genie 2, setting 8, American Scientific Products, McGaw, IL) continuously for 1 h at RT, followed by centrifugation (13600*g*, 20 min., RT). Supernatants were collected and residues discarded. Routine protein extractions were performed using BSB as the solvent except where noted otherwise.

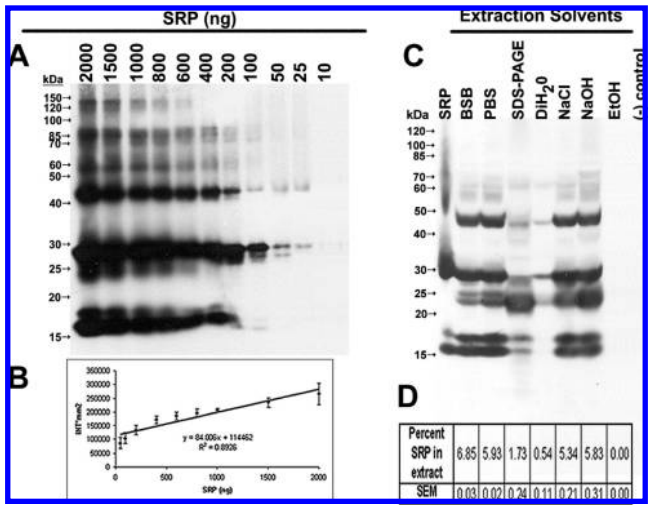

Figure 1. (**A**) Western blot of SRP used to construct standard curve (**B**) from densitometric scans. (**C**) Effect of extraction buffer on SRP ('Century' soybean) solubilization assessed by WB. Protein loads were 0.5 μg of SRP and 15 μg each for the rest. (−) control = preimmune rabbit sera. (**D**) Percent SRP in the protein extract as determined by densitometry.

Defatted 'Century' soybean flour was extracted (1:10 w/v) in each of the following solvents: BSB (pH 8.45), PBS (pH 7.2), SDS-PAGE sample buffer with 2% β-ME, distilled–deionized water (diH_20), 1 M NaCl, 0.1 M NaOH, and 70% aqueous ethanol (v/v). The extracts of equal protein load (15 μg) were subjected to SDS-PAGE followed by Western blot analysis.

Protein Determination. Soluble protein was determined according to the method of Lowry and others (*46*) and Bradford (*47*). Bovine serum albumin (BSA fraction V) was used as the standard protein. Standard curves for BSA (prepared in appropriate buffer) and appropriate blanks were used in all assays.

Sodium Dodecyl Sulfate−Polyacrylamide Gel Electrophoresis (SDS-PAGE). SDS-PAGE was done as described previously by Acosta and others (*48*).

Polyclonal Antibodies (pAb). Purified SRP from 'Century' soybean (1 mg) in 1 mL of Freund's complete adjuvant was administered intradermally (id) to a female New Zealand white rabbit (*Oryctulagus cuniculus*). Approximately 4 weeks after the initial immunization, one booster dose (1 mg of SRP in 1 mL of incomplete Freund's adjuvant, id) was administered. Pre- and postimmunization blood was collected from the marginal ear vein. Blood was allowed to clot for 30 min, followed by centrifugation (5000*g*, 20 min, RT) and removal of supernatant (serum). Serum aliquots were kept at 4 °C for immediate use and at –20 °C for long-term storage.

Immunoassays. Seed protein extracts of interest were normalized to 1 mg/mL prior to immunoassays.

(1) Enzyme-Linked Immunosorbent Assays (ELISA). (a) Noncompetitive ELISA. Polyvinyl microtiter ELISA plates (96 wells/plate) were coated with SRP in citrate−phosphate buffer (pH 5.0) at concentrations starting at 500 ng with six successive 2-fold dilutions. The coated plates were incubated for 1 h at 37 °C. After the incubation, wells were washed three times with BSB and blocked with 100 μL of blocking buffer [0.5% BSA (w/v) in PBS, pH 7.2] for 1 h at 37 °C followed by three washings with BSB. The pAb was diluted in 0.1% (w/v) BSA in BSB, and the dilutions (ranging between 10^2 and 10^6) were tested to determine the lowest concentration for SRP detection. The pAb solution was then incubated for 1 h at 37 °C followed by three washings with BSB. Alkaline phosphatase-labeled goat anti-rabbit IgG (secondary antibody) was diluted 1:5000 v/v in 0.1% (w/v) BSA in BSB and added to each well (50 μL/well). The plates were incubated for 1 h at 37 °C followed by three washings with BSB. Phosphatase substrate [1 *p*-nitrophenyl phosphate tablet dissolved in 5 mL of alkaline phosphatase buffer (48 mL of diethanolamine and 24.5 mg of $MgCl_2$ to final volume of 500 mL, pH adjusted to 9.8 with 5 M HCl)] was added to each well (50 μL/well), and color development was allowed to proceed for 8 min at RT. Color development was stopped by adding 50 μL/well of 3.0 M NaOH. The optical density (OD) of each well was read at 405 nm using an ELISA reader (model EL 307, Bio-Tek Instruments Inc., Riverton, NJ).

(b) Competitive Inhibition ELISA. The ELISAs were performed similarly as described under noncompetitive ELISA. Normalized (1 mg/mL) test proteins were combined with rabbit anti-SRP pAb (antigen/antibody ratio of 1−10 v/v) and serially diluted 10-fold in successive wells. Appropriate reagent blanks (pAb, 10^{-4} dilution, with no test protein) and SRP (as control) were included on every plate assayed. The assay plates were incubated for 1 h at 37 °C, and then 45 μL from each well was transferred to their respective wells on previously prepared coated plate (250 ng/well SRP) and incubated for 1 h at 37 °C. After incubation, the assay plates were washed three times with BSB, and secondary antibody was added followed by color development as described above.

Cross-reactivity was assessed by comparing sample IC_{50} values to the IC_{50} value for SRP. A cross-reactive protein contains an IC_{50} value similar to that of SRP. IC_{50} is a term that describes the concentration of inhibiting protein that effected a 50% inhibition in ELISA response.

(2) Western Blotting (WB). WB analysis was done as described by Acosta et al. (*48*). Proteins from the gels were transferred onto 0.2 μm nitrocellulose paper (NC) using a Hoefer TE22 transverse electrophoresis unit as described by Towbin et al. (*49*). The NC membrane containing the transferred proteins was stained with 0.1% (v/v) Ponceau S in diH_2O to visualize the proteins. The membrane was then blocked with Tris-buffered saline containing Tween 20 [10 mM Tris, 0.9% (w/v) NaCl, and 0.05% (v/v) Tween 20 (TBS-T, pH 7.6)] containing 5% (w/v) nonfat dried milk (NFDM) for 1 h at RT. The membrane was washed with two changes of fresh TBS-T for 2 min each. The NC membrane was then incubated with diluted pAb ($1:10^4$; rabbit anti-SRP) solution overnight at 4 °C on a rocker (60 Hz, Rocker II model 260350, Boekel Scientific, Feasterville, PA). The membrane was washed once with TBS-T for 15 min followed by three washings with fresh TBS-T for 5 min each. The membrane was incubated with diluted secondary antibody [5×10^3; horseradish peroxidase (HRP) labeled goat anti-rabbit] for 1 h at RT on a rocker. The membrane was washed again as described above. Bands reacting to the pAb were visualized by using the luminol/*p*-coumaric acid system. The luminol and *p*-coumaric solutions were mixed together and spread evenly to cover the entire area of the NC blot. This solution was left on the blot for approximately 5 min at RT and blotted dry, and the membrane was placed in a translucent plastic cover and exposed to X-ray film (Kodak X-OMAT AR Film, Eastman Kodak Co., Rochester, NY) for autoradiographic visualization.

(3) Immunolabeling with Patients' Sera. Proteins were separated by SDS-PAGE (Nu-Page MES Bis-TRIS, Invitrogen, Carlsbad, CA) following the protocol from the manufacturer. Ten micrograms of protein extract was loaded into each well. The resolved proteins were subsequently transferred to Immobilon-P membrane (Millipore, Bedford, MA) and then stained with 2.2% (w/v) Coomassie Blue for total protein analysis. For molecular mass determination MultiMark Multi-Colored Standard (Invitrogen, Carlsbad, CA) was used. For detection of IgE binding to the separated soy proteins, immunolabeling was performed with individual patient sera from 11 soy-allergic individuals. In addition, sera from 6 sensitized but clinically tolerant individuals were used. Serum from an atopic but not soy-sensitized individual was used as a negative control. Patient and control sera diluted from 1:5 to 1:10, depending on the level of soy-specific IgE, in PBS-T plus 1% (w/v) bovine serum albumin and 10% (v/v) normal goat serum were incubated with immunoblots with gentle agitation at RT. After 2 h of incubation, immunoblots were briefly rinsed with PBS followed by addition of ^{125}I-labeled goat anti-human IgE (DiaMed, Windham, ME) diluted as per the manufacturer's recommendation. After agitation for 1 h at RT and subsequent washing with PBS, immunoblots were mounted on filter paper and exposed to Kodak X-OMAT Imaging Film (Eastman Kodak Co.).

(4) Dot Blotting (DB). Stenciled circles (8 mm diameter) were drawn onto 0.2 μm NC paper using a no. 2 pencil. Aliquots of desired protein solutions (1 mg/mL) were pipetted (1–4 μL) carefully into the center

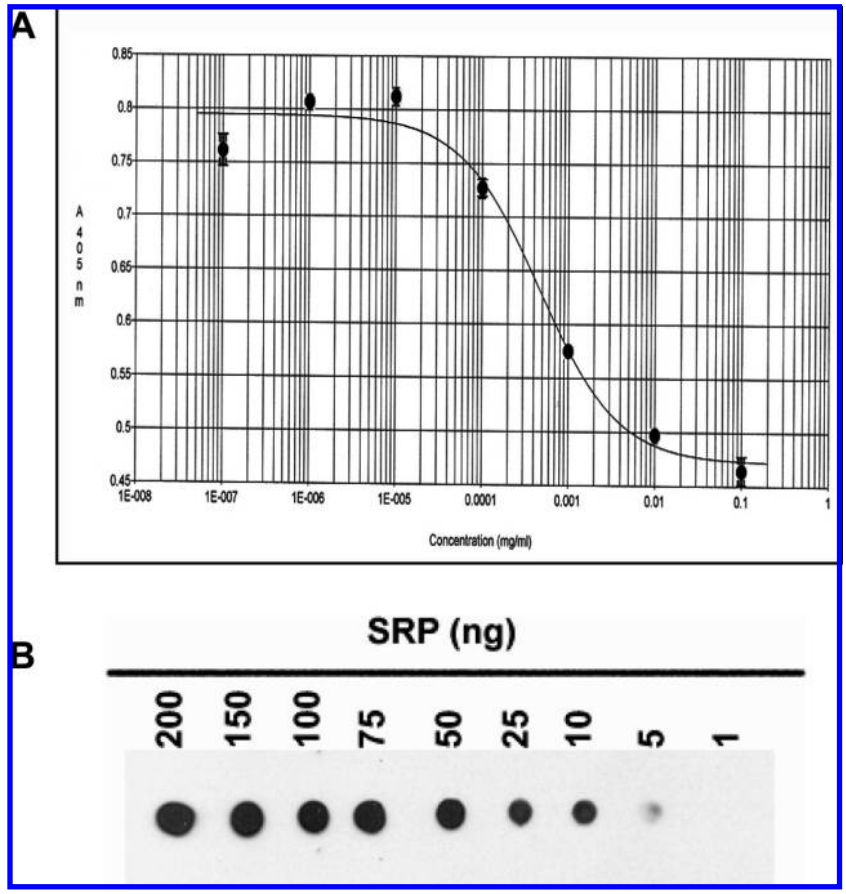

Figure 2. ELISA and dot blot detection limits for SRP: (**A**) representative competitive inhibition ELISA standard curve for SRP ($n = 18$, mean $IC_{50} \pm$ SEM 389.9 $\pm$ 31.1 ng/mL); (**B**) detection limit for SRP using DB analysis.

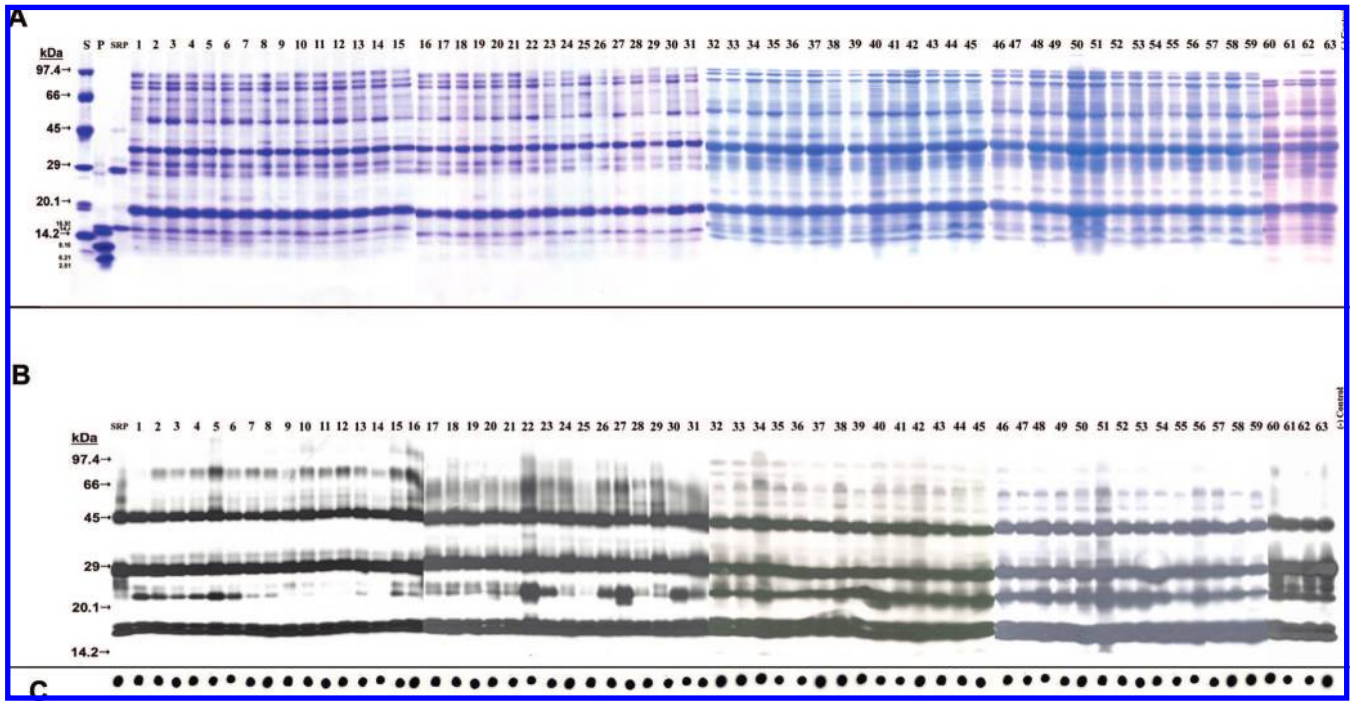

Figure 3. (**A**) SDS-PAGE (8–25% linear acrylamide gradient) electrophoretic profile of 63 soybean varieties. Panels **A** and **B** are a composite of five gels. Numbers on the top refer to the variety number identified in **Table 1**. S = low molecular mass standard markers. P = ultralow molecular mass peptide markers. (−) control = preimmune rabbit sera. Protein load was 30 μg each. (**B**) Corresponding Western blot. Protein loads for each variety and SRP were 30 and 1 μg, respectively. (**C**) Dot blot. Protein loads for each variety and SRP were 1 and 0.5 μg, respectively.

of each stenciled circle. The NC paper was dried at RT for 10–15 min and developed as described for Western blotting.

Densitometric Quantification. A densitometer [Molecular Imager ChemiDoc XRS System equipped with ChemiDoc software (version

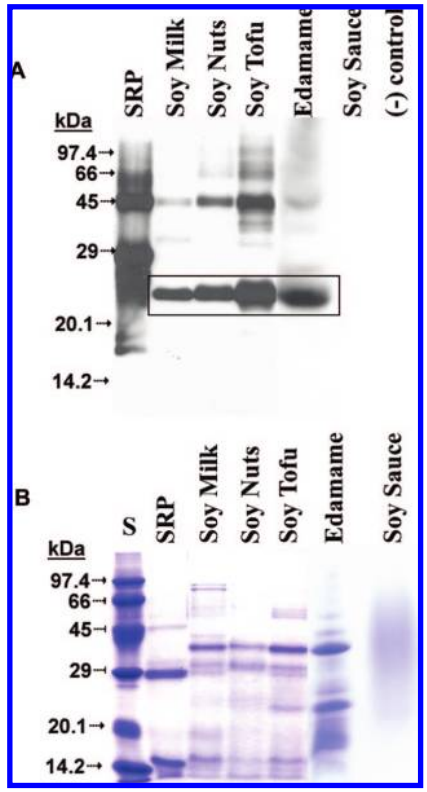

Figure 4. Western blot and SDS-PAGE analyses for commercial soy products: (**A**) Western blotting (protein loads for soy products and SRP were 10 and 2 μg, respectively). (**B**) SDS-PAGE (12% acrylamide) in the presence of 2% β-ME (protein loads for soy products and SRP were 18 μg). S = low molecular mass standards, and (−) control = preimmune rabbit sera.

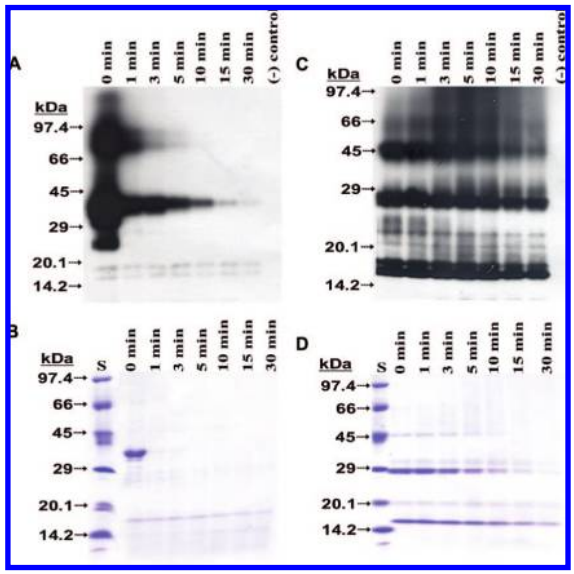

Figure 5. Western blots and SDS-PAGE analyses of cooked (100 °C) SRP at various time intervals in the presence and absence of 2% β-ME. Four microliters of sample for each time interval was used for SDS-PAGE. The protein load for low molecular mass standards was 58 μg (total proteins). (**A**) Western blotting for SDS-PAGE described in **B**; (**B**) SDS-PAGE (8–25% linear acrylamide gradient) in the absence of β-ME; (**C**) Western blotting for SDS-PAGE described in **D**; (**D**) SDS-PAGE (8−25% linear acrylamide gradient) in the presence of 2% β-ME. Note the SRP thermal stability indicated by the Western blots.

4.2), Bio-Rad Laboratories, Hercules, CA] was used to quantify SRP polypeptides. SRP standard curves were constructed using 1–10 μg (SDS-PAGE gels) and 10–2000 ng (Western blotting) SRP loads.

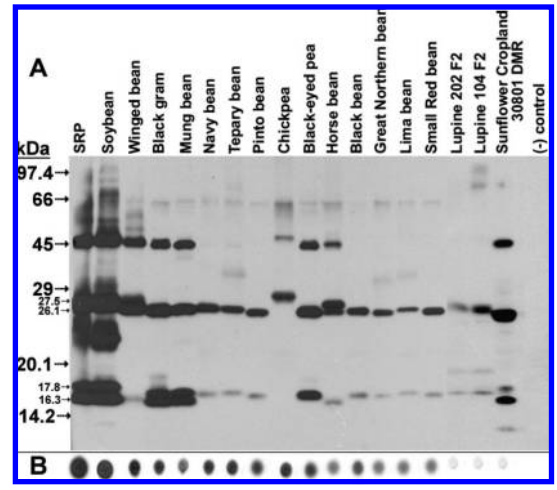

Figure 6. (**A**) Western blot for cross-reactive proteins. Protein loads were 30 μg each (seed protein extract) and 1 μg (SRP). (**B**) Dot blots for cross-reactive proteins. Protein load for each dot was 2 μg (seed protein extract) and 0.5 μg (SRP).

Using the standard curves, SRP concentrations in the desired samples were determined.

Immunoadsorption of Rabbit Anti-SRP Sera. A modified protocol previously standardized for purification of rabbit and goat anti-cashew sera was followed (*50*). Briefly, IgG was bound to and eluted from a protein G affinity column using 0.2 M glycine sulfate (pH 2.3) as the elution buffer. The eluate was immediately neutralized with 1.0 M TRIS (pH 9.0). The purified IgG was dialyzed against coupling buffer (0.1 M $NaHCO_3$ and 0.5 M NaCl, pH 8.5) to remove any amines contained in the buffers.

Purification of Cross-Reactive Proteins from Select Plant Seeds. Affinity chromatography protein purification was according to the manufacturer's recommended protocol (Sigma C9142). Cyanogen bromide (CNBr) activated Sepharose (4% agarose) resin was washed and allowed to swell in 1 mM cold HCl for 30 min (0.067 g swelled to ∼0.25 mL), followed by washing with coupling buffer. Protein G-purified rabbit anti-soybean SRP IgG (6.8 mg in 2 mL of coupling buffer) was covalently immobilized on CNBr activated resin (0.23 mL resin in 2 mL of coupling buffer) overnight at 4 °C. Slurry was gently centrifuged and supernatant discarded. Resin was washed with 5–10 resin volumes of coupling buffer followed by the addition of 2 mL of 0.2 M glycine (blocking buffer) for 2 h at RT. Slurry was gently centrifuged and supernatant discarded. Resin was extensively washed to remove the blocking buffer, first with coupling buffer followed by acetate buffer (0.1 M, pH 4.0) containing 0.5 M NaCl. The wash cycle was repeated five times followed by transfer into disposable (5 mL) plastic column. Using separate disposable columns to inhibit cross-contamination, BSB extracts from black gram (*Phaseolus mungo*), black-eyed pea (*Vigna unguiculata*), chickpea (*Cicer arietinum*), Great Northern bean (*Phaseolus vulgaris*), mung bean (*Phaseolus aureus*), pinto bean (*Phaseolus vulgaris*), and winged bean [*Psophocarpus tetragonolobus* (L.) DC] were passed through immunoaffinity columns to isolate cross-reactive proteins. The bound proteins were eluted from the immunoaffinity column using 0.2 M glycine sulfate (pH 2.3) as the elution buffer and immediately neutralized with 1.0 M TRIS (pH 9.0). The protein fractions (original extract, flow-through fraction, and eluate) were subjected to SDS-PAGE and were subsequently stained with Coomassie Brilliant Blue R-250 or transferred to NC for WB using rabbit anti-SRP pAb. The eluates were dialyzed against diH_2O, and the molecular mass cutoff of the dialysis tubing was 1 kDa (Spectrum, Spectrum Laboratories, Ranch Dominguez, CA). Dialysis was done at 4 °C with five buffer changes over 60 h. Sodium azide (0.02% w/v) was added to each buffer change to inhibit microbial growth. Dialysates were stored in sealed screw-top containers at −20 °C until further use.

N-Terminal Sequencing. Immunoaffinity column-purified cross-reactive proteins were electrophoresed on SDS-PAGE (8–25% linear monomer acrylamide gradient gels). Separated proteins were transferred

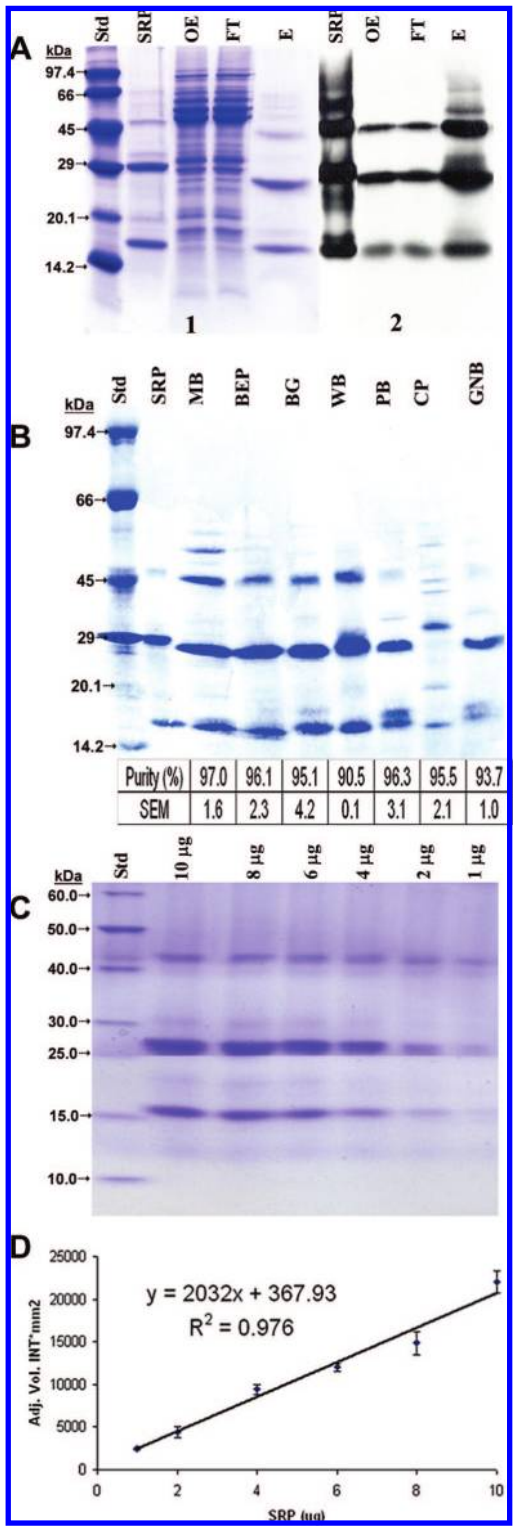

Figure 7. (**A**) Immunoaffinity column chromatography purification of cross-reactive protein from black-eyed pea [(**A1**) Coomassie Brilliant Blue R-250 stained SDS-PAGE (12% acrylamide) gel; (**A2**) corresponding Western blot]. Std = low molecular mass standards (25 μg total protein); soybean SRP (10 μg, **A1**; 0.5 μg, A2); OE = original seed protein extract (15 μg); FT = flow-through fraction from immunoaffinity column (15 μg); E = immunoaffinity column eluate (8 μg). (**B**) Coomassie-stained PVDF membrane of cross-reactive proteins (5 μg each) from MB (mung bean), BEP (black-eyed pea), BG (black gram), WB (winged bean), PB (pinto bean), CP (chickpea), and GNB (Great Northern bean) with corresponding percent purity below. SDS-PAGE for SRP (**C**) was used to construct standard curve (**D**) from densitometric scans.

onto a PVDF membrane (0.45 μm, previously soaked in 100% methanol), as described by Towbin and others (*49*). The protein bands were visualized by briefly staining (5 min) with 0.25% w/v Coomassie Blue R-250 in 10% v/v acetic acid and 50% v/v methanol and destained with 50% v/v methanol containing 10% v/v acetic acid for 30 min followed by 5% v/v methanol and 1% v/v acetic acid until the blue background was cleared. Bands representing SRP-like protein were excised from the PVDF membrane, and the N-terminal sequence was determined on a Procise cLC sequencer (Applied Biosystems, Foster City, CA). Sequence data were collected utilizing ABI Procise software (Applied Biosystems, Inc., model 610A) and analyzed with BLAST programming (National Center for Biotechnology Information, National Institutes of Health, Bethesda, MD; http://www.ncbi.nlm.nih.gov/BLAST/) (*51*).

Amino Acid Analysis. Total amino acid composition was determined using a Pico-Tag Column Amino Acid Analyzer (Waters Chromatography Division, Milford, MA). One to two micrograms of lyophilized immunoaffinity column eluates was mixed with 10 μL of performic acid [19 volumes of 97% (v/v) formic acid to 1 volume of 30% (v/v) hydrogen peroxide as per the Pico-Tag method adapted from Tarr (*52*)] for 30 min at RT. Samples were lyophilized followed by microwave hydrolysis with 30 μL of 6 N HCl in the presence of nitrogen (18 min, 110 °C) and dried overnight at RT under vacuum. Dried protein hydrolysates were treated with a 2:2:1 v/v/v ethanol/triethylamine/water solution and dried under vacuum. Dried samples were derivatized with a 7:1:1:1 v/v/v/v ethanol/triethylamine/water/phenyl isothiocyanate (PITC) (99.9%) solution for 20 min at RT in a nitrogen atmosphere and dried overnight under vacuum. Eighty microliters of 5 mM sodium phosphate buffer (pH 7.6) containing 6% (v/v) acetonitrile was added to dried sample and vortexed to thoroughly mix the contents, followed by centrifugation (16100*g*, 20 min, RT). Supernatants were passed through a YM-3 Microcon centrifugal filtration device to remove insolubles. A known volume (20 μL) of filtered supernatant was used for analysis by HPLC. Norleucine was added to each sample prior to acid hydrolysis as an internal standard to calculate percent recovery of amino acids. Amino acid composition was reported as residues per 100 residues. Amino acid standards, Pierce H, methionine sulfone, and cysteic acid (250 pmol for all amino acids except cystine, which was 125 pmol), were run simultaneously.

Statistics. All experiments were done at least in duplicate. Data are reported as mean ± standard error of the mean (SEM). Data were analyzed for statistical significance ($p = 0.05$) using one-way ANOVA procedures provided by SPSS statistical software (SPSS for Windows 2003, Microsoft Corp., version 13.0, Chicago, IL).

RESULTS AND DISCUSSION

SRP Extraction Efficiency. Western blotting of extracted proteins indicated all solvents, with the exception of aqueous ethanol, extracted SRP (**Figure 1A−D**). Densitometric quantification indicated BSB to be the optimum solubilizer of SRP. Among the salt-containing buffers, BSB extracted the least amount of immunoreactive 23 and 25 kDa contaminant polypeptides (**Figure 1C**). BSB was therefore used for subsequent SRP extractions.

SRP Detection and Quantification. The optimal assay conditions for noncompetitive ELISA SRP detection and quantification were (1) rabbit anti-soybean SRP pAb (56 mg of Bradford protein/mL; calculated from the average of two aliquots at 53.8 and 58.2 mg/mL) diluted at 1×10^4 (v/v), (2) coating concentration of SRP at 250 ng/well, and (3) goat anti-rabbit secondary pAb concentration at a dilution of 5×10^3 (v/v). These optimized conditions were subsequently used to develop competitive inhibition ELISA. The detection sensitivities of the immunoassays were 389.9.1 ± 31.1 ng/mL (mean ± SEM, **Figure 2A**, ELISA), 200 ng (**Figure 1A**, WB), and 5 ng (**Figure 2B**, DB).

Immunoassays were able to detect SRP in all tested soybean varieties (**Table 1**; **Figure 3**). Competitive inhibition ELISA

Table 3. Amino Acid Composition of Sulfur-Rich Proteins[a]

amino acid	SRP	black gram	black-eyed pea	chickpea	Great Nothern bean	mung bean	pinto bean	winged bean	LSD
Asx	15.0	10.0	10.5	10.9	7.3	8.7	7.4	11.6	1.8
	0.0	*0.4*	*0.0*	*0.6*	*0.9*	*0.9*	*0.2*	*0.5*	
Glx	20.9	17.2	16.4	17.3	16.8	17.5	16.6	17.7	1.3
	0.3	*0.5*	*0.0*	*0.8*	*0.3*	*0.4*	*0.2*	*0.1*	
Ser	5.7	7.0	8.0	7.7	10.4	8.0	7.5	7.5	0.6
	0.1	*0.1*	*0.1*	*0.4*	*0.3*	*0.1*	*0.1*	*0.1*	
Gly	5.3	6.1	5.9	8.6	16.8	5.9	9.7	5.6	1.7
	0.1	*0.1*	*0.1*	*0.8*	*1.2*	*0.1*	*0.1*	*0.0*	
His (1.9/1.6)	1.9	3.3	3.4	2.9	2.5	3.4	3.8	2.6	0.2
	0.1	*0.0*	*0.0*	*0.0*	*0.1*	*0.0*	*0.0*	*0.1*	
Arg	6.4	5.3	4.8	7.4	7.3	4.9	6.1	6.5	0.4
	0.1	*0.0*	*0.0*	*0.3*	*0.1*	*0.1*	*0.0*	*0.1*	
Thr (3.4/0.9)	5.1	6.0	5.6	4.8	4.8	5.7	5.7	5.7	0.4
	0.0	*0.1*	*0.2*	*0.1*	*0.2*	*0.1*	*0.0*	*0.1*	
Ala	4.6	4.0	4.0	3.4	3.1	3.7	3.9	4.1	0.4
	0.1	*0.1*	*0.0*	*0.1*	*0.3*	*0.1*	*0.0*	*0.0*	
Pro	5.7	6.8	7.2	6.5	4.1	7.7	6.4	6.7	0.9
	0.1	*0.2*	*0.1*	*0.2*	*0.6*	*0.4*	*0.1*	*0.1*	
Tyr	0.3	0.5	0.4	0.7	0.6	0.5	0.7	0.4	0.2
	0.0	*0.0*	*0.0*	*0.1*	*0.1*	*0.1*	*0.0*	*0.1*	
Val (3.5/1.3)	5.2	6.3	6.3	4.7	3.5	6.7	5.6	5.3	0.4
	0.0	*0.2*	*0.1*	*0.2*	*0.2*	*0.2*	*0.0*	*0.1*	
Ile (2.8/1.3)	3.5	3.1	3.3	3.7	3.6	3.0	3.5	3.5	0.4
	0.0	*0.1*	*0.0*	*0.3*	*0.0*	*0.1*	*0.0*	*0.0*	
Leu (6.6/1.9)	8.6	10.1	10.6	8.9	7.9	10.8	10.2	10.2	2.0
	0.1	*0.5*	*0.1*	*1.3*	*0.9*	*0.6*	*0.1*	*0.0*	
Phe (6.3/1.9)	4.2	5.3	5.3	4.7	4.1	5.1	5.0	4.5	1.1
	0.0	*0.4*	*0.1*	*0.8*	*0.3*	*0.4*	*0.1*	*0.0*	
Lys (5.8/1.6)	3.4	3.4	3.2	3.5	2.6	2.9	3.0	3.4	0.6
	0.2	*0.0*	*0.2*	*0.2*	*0.3*	*0.0*	*0.1*	*0.1*	
Cys/2	2.4	3.3	2.9	2.7	2.3	3.6	2.6	3.0	0.6
	0.1	*0.1*	*0.1*	*0.2*	*0.5*	*0.0*	*0.1*	*0.1*	
Met (2.5/1.7)	1.8	2.3	2.3	1.8	2.4	2.1	2.3	1.6	0.6
	0.4	*0.2*	*0.1*	*0.1*	*0.2*	*0.1*	*0.0*	*0.1*	
Trp (1.1/0.5)	ND	ND	ND	ND	ND	ND	ND	ND	ND
				AAD[b] %					
hydrophobic	41.3	47.3	47.8	45.0	47.8	48.6	49.2	44.5	
hydrophilic	11.1	13.5	14.0	13.2	15.8	14.2	13.9	13.6	
basic	11.7	12.0	11.4	13.8	12.4	11.2	12.9	12.5	
acidic	35.9	27.2	26.9	28.2	24.1	26.2	24.0	29.3	
				LEAA (Pre-School Child)					
first	Lys	Lys	Lys	Lys	Lys	Lys	Lys	Lys	
second	Phe	Phe	Phe	Phe	Phe	Phe	Phe	Phe	
third									
				LEAA (Adult)					
first									
second									
third									
E/T[c] (%)	33.7	39.8	40.0	35.0	31.4	39.7	39.1	36.8	

[a] The data are expressed as mean residues/100 residues; standard errors of mean (SEM) are given in *italics*. ND, not determined. Values for amino acids are from two independent preparations. Numbers in parentheses represent essential amino acid scores compared to the FAO/WHO recommended pattern for pre-school child (2–5 years) and adult, respectively, and the LEAA value represents corresponding limiting essential amino acid (*70*). [b] Amino acid distribution (% of total). [c] E/T (%) represents essential to total amino acid ratio.

Table 4. N-Terminal Amino Acid Sequence Homology of SRP-like Proteins with Soybean 7S Basic Globulin[a]

Protein	N-terminal amino acid
7S basic globulin (45 kDa)	<---------ND--------->
Black gram (43.8 kDa)	NYVMNPAYLLVL
Black-eyed pea (43.8 kDa)	NNVTNPAYMLVLPTQ
Chickpea (46.5 kDa)	<---------ND--------->
Great Northern bean (43.8 kDa)	<---------ND--------->
Mung bean (43.8 kDa)	<---------ND--------->
Pinto bean (43.8 kDa)	<---------ND--------->
Winged bean (43.8 kDa)	<---------ND--------->

Protein	N-terminal amino acid sequence	7S basic globulin isoform[a]	S	I	G	7S basic globulin[b]	S	I	G
7S basic globulin (28 kDa)	<---------ND--------->	NPTKPINLLVLPVQ	100	100	0	VTPTKPINLVVL	100	100	0
Black gram (26.8 kDa)	NYVMNPAYLLVL	--------LLVL	100	100	0	------------	NM	NM	-
Black-eyed pea (26.7 kDa)	NNVTNPAYMLVLPTQ	NVTNPAYMLVLPTQ	64	57	0	------------	NM	NM	-
Chickpea (28.7 kDa)	<---------ND--------->	----------------	NM	NM	-	------------	NM	NM	-
Great Northern bean (26.7 kDa)	NYVSNPAYLLVL	--------LLVL---	100	100	0	------------	NM	NM	-
Mung bean (27.0 kDa)	NYVMNPAYVLVLPTQK	--------LVLPTQ	83	83	0	------------	NM	NM	-
Pinto bean (26.5 kDa)	NYVSNPAYLLVL	--------LLVL---	100	100	0	------------	NM	NM	-
Winged bean (27.5 kDa)	VSPTTPSKLLVL	-PTTPSKLLVL---	70	70	0	VSPTTPSKLLVL	75	58	0

Protein	N-terminal amino acid sequence	7S basic globulin isoform[a]	S	I	G	7S basic globulin[b]	S	I	G	7S basic globulin LII subunit[c]	S	I	G
7S basic globulin (16 kDa)	<---------ND--------->	STIVGSSGGTMISTS	100	100	0	STIVGSTSGGTMISTS	100	100	0	STIVGSTSGGTMISTS	100	100	0
Black gram (16.3 kDa)	STTVGHYGGTMI	STTVGHYGGTMI	75	75	0	STTVGHY-GGTMI	69	69	1	STTVGHY-GGTMI	69	69	1
Black-eyed pea (16.5 kDa)	STTVGHSGGTMISTS	STTVGHSGGTMISTS	86	86	0	STTVGH-SGGTMISTS	81	81	1	STTVGH-SGGTMISTS	81	81	1
Chickpea (16.1 kDa)	GYPEGVIGGTLI	GYPEGVIGGTLI	NM	NM	-	----------------	NM	NM	-	----------------	NM	NM	-
Great Northern bean (16.3 kDa)	STIVGHSGGTMI	STIVGHSGGTMI	91	91	0	STIVGH-SGGTMI	84	84	1	STIVGH-SGGTMI	84	84	1
Mung bean (16.3 kDa)	STTVGHSGGTMIST	STTVGHSGGTMIST	85	85	0	STTVGH-SGGTMIST	80	80	1	STTVGH-SGGTMIST	80	80	1
Pinto bean (16.5 kDa)	STIVGHSGGTMI	STIVGHSGGTMI	91	91	0	STIVGH-SGGTMI	84	84	1	STIVGH-SGGTMI	84	84	1
Winged bean (16.1 kDa)	STVPGHSGGTMI	STVPGHSGGTMI	83	75	0	--------SGGTMI	100	100	0	--------SGGTMI	100	100	0

[a] Sequence analysis was subjected to BLAST analysis (*51*). [a], Ishizu, Sassa, and Hirano. Sequence of a cDNA encoding soybean basic 7S globulin isoform, accession AB084260.1, http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=Protein&list_uids=20302594&dopt=GenPept; [b], Kagawa and Hirano (*71*), Watanabe and Hirano (*72*); [c], Kagawa and others (*42*); S, % similarities; I, % identities; G, number of gaps introduced by BLAST for matching; NM, no match; ND, not determined.

and dot and Western blotting help to assess immunoreactivity of a protein in native, immobilized, and reduced and denatured forms, respectively. With the exceptions noted for ELISA data, all tested soybean samples registered comparable immunoreactivity in ELISA (**Table 1**), Western blots (**Figure 3B**), and dot blots (**Figure 3C**). Similarly, a pea (*Pisum*) seed sulfur-rich albumin mainly composed of two polypeptides (molecular masses of 8 and 22 kDa) has been identified in 45 lines of peas (*53, 54*). Interestingly, the author noted that 68% of the seed protein sulfur was accounted for by these two polypeptides. Sulfur-rich storage proteins have also been found in several additional seeds that include Brazil nut (*55*), chickpea (*56*), pea (*57*), sunflower (*58*), and canola, castor bean, cotton, lupine, and pumpkin (*59, 60*).

Commercial use of soybean proteins in food products often involves thermal processing. It was therefore important to investigate SRP processing stability. With the exception of fermentation (soy sauce), the immunoassays were able to detect SRP in the tested commercial soy products regardless of type of processing (**Figure 4A**,**B**). However certain immunoreactive polypeptides with mobility different from that of the parent SRP polypeptides, for example, a molecular mass of 23.8 kDa, were noted in several heat-processed samples. Sathe and others (*38*) had earlier reported formation of soluble (molecular masses of 14.2, 20, 30–37, and 50–60 kDa) and insoluble (molecular masses of 30–31 and 45–66 kDa) aggregates upon heating SRP at 100 °C in 0.4 M NaCl. In the current investigation, when SRP was heated at 100 °C in 0.1 M BSB, insoluble aggregates were formed with the concurrent disappearance of soluble polypeptides (**Figure 5A**−**D**). The differences in the molecular masses of aggregates noted in the current study and the one by Sathe and others (*38*) may arise from the differences in the experimental conditions used (particularly buffer type, pH, and the ionic strength). The observed MW heterogeneity in thermally processed soy products may arise from variable aggregate formation as a consequence of inherent differences in processing and the food matrices. However, SRP remained immunoreactive after thermal processing either alone or when present in soy products. Together, these results demonstrate that rabbit pAb-based immunoassays can be used for SRP detection in raw unprocessed soybeans as well as processed soybean/soy products.

Detection of Cross-Reactive Proteins. Of the 46 plant seed extracts tested, several exhibited differential cross-reactivity toward rabbit anti-soybean SRP pAb (**Table 2**; **Figure 6**). The observed cross-reactivity in many tested seeds suggests SRP-like proteins may be conserved across different genera. Kagawa and others (*42*) have similarly reported the presence of cross-reactive proteins using antiserum raised against the LII subunit (16.5 kDa peptide) of 7S basic globulin with protein extracts from azuki, mung, and winged beans. In the same study, a high degree of amino acid sequence homology was observed between the LII subunit of the 7S basic globulin and the lupin conglutin γ; however, no cross-reactivity was observed with the LII subunit antiserum (*42*). Two lupine seed varieties (202 F2 and 104 F2) in the current investigation exhibited cross-reactivity with the rabbit antisoybean SRP pAb, suggesting differences among lupine varieties. Seed protein extracts exhibiting strong cross-reactivity in all of the immunoassay formats were selected for immunoaffinity purification and characterization to evaluate their relatedness to SRP.

Purification and Characterization of Cross-Reactive Proteins. *Protein Purity.* Cross-reactive proteins were purified to apparent homogeneity using immunoaffinity column chromatography (**Figure 7A**,**B**). The purity of the cross-reactive proteins was determined using densitometric scanning of the Western blots with soybean SRP (1–10 μg) as the standard. The results suggested that the purity of cross-reactive proteins (**Figure 7C**,**D**) is in the range of 90.5–97.0%.

N-Terminal Amino Acid Sequence. With the exception of chickpea, N-terminal amino acid sequence results of cross-reactive proteins exhibited significant similarity and identity with the soybean 7S basic globulin (**Table 4**). The N-terminal residues found in the current investigation for mung bean and winged bean compare favorably with the previously published studies by Hirano and others (*61*) and Mendoza and others (*62*).

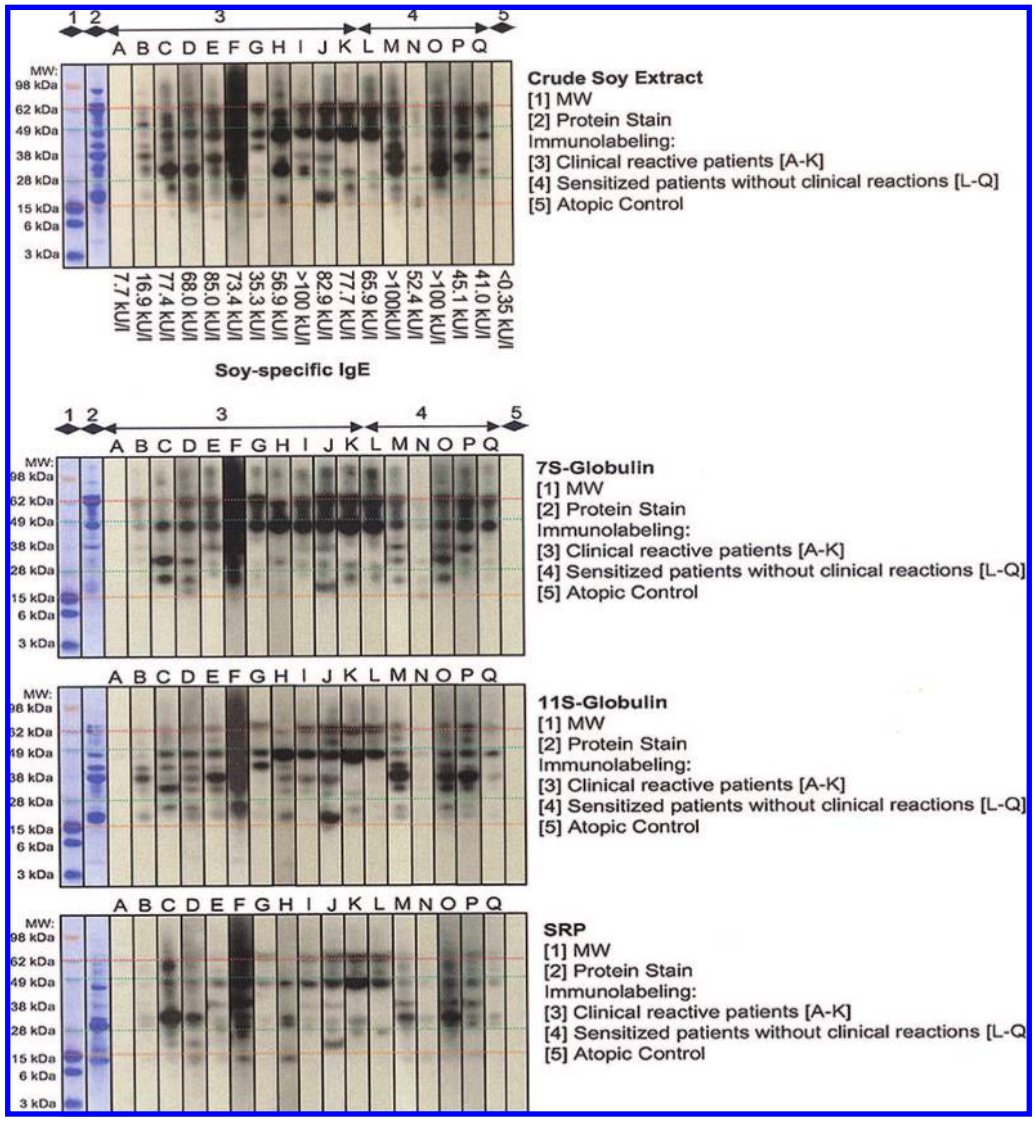

Figure 8. IgE immunoblot analyses of crude soy extract, purified 7S (β-conglycinin), 11S (glycinin), and SRP.

The N-terminal amino acid sequences of cross-reactive proteins from azuki bean and cowpea (*61*) were comparable to those of cross-reactive protein sequences noted in the current investigation.

Amino Acid Composition. The amino acid analyses of SRP and the cross-reactive proteins are summarized in **Table 3**. The amino acid composition of SRP from the current study is in agreement with earlier reports of Sathe and others (*43*), Hu and Esen (*39*), Yamauchi and others (*40*), and Sato and others (*41*). Soybean SRP was characterized by high methionine (1.8 g) and cysteine (2.4 g/100 g of protein basis). The methionine and cysteine contents of all of the cross-reactive proteins were also comparable to those of the soybean SRP, ranging from 1.6 to 2.4 methionine residues/100 residues and from 2.3 to 3.6 cysteine residues/100 residues. Hydrophobic amino acids (41.3–49.2%) dominate the amino composition of SRP as well as the cross-reactive proteins. The acidic, hydrophilic, and basic residues accounted for 24.0–35.9, 11.1–15.8, and 11.2–13.8%, respectively, of the total amino acids. On the basis of the FAO/WHO recommended essential amino acid pattern for 2–5-year olds, lysine and phenylalanine are the first and second limiting amino acids (**Table 3**). Compared to the recommended pattern for adults, all of the cross-reactive proteins are complete proteins. The essential-to-total amino acid ratio range for the cross-reactive proteins was from 31.4% for Great Northern bean to 40.0% for black-eyed pea (**Table 3**).

SRP. Soybeans are one of the "big eight" food allergens (*63, 64*), and therefore assessing the potential immunoreactivity of soybean SRP with soybean allergic patients' sera IgE was of interest. Magni et al. (*65*) have demonstrated cross-reactivity between soybean basic 7S globulin, anti-Ara h 3 basic subunit (a major peanut allergen), and lupin conglutin γ. Similarly, lupin-sensitive patient IgEs were found to cross-react with soybean 17 and 30 kDa polypeptides (*66*). These reports suggest that soybean SRP may potentially be an allergen. For these and several additional reasons, it was important to determine if soybean SRP is an allergen. Western blotting experiments (**Figure 8**) using crude soybean extract as well as biochemically pure 11S, 7S, and SRP were performed using serum from 11 soy-allergic patients and 6 negative controls. Lane 2 in each panel contains Coomassie Blue stained protein patterns. It can be seen that each of the purified proteins contains multiple bands, most of which are unique, but some appear to share common electrophoretic mobilities. The latter bands might represent either minor contaminants or unique proteins with similar mobilities.

Western blots are orders of magnitude more sensitive than Coomassie staining, and thus it is not surprising that strong signals appear on Western blots in positions with minimal or no corresponding protein staining signal. With these caveats in mind, the SRP Western blot signals of most significance are those at ~32 kDa (band just above the 28 kDa standard) and ~14 kDa (band just below the 15 kDa standard). SRP is known to be composed of two low molecular mass peptide chains (16–18 kDa), two high molecular mass peptide chains (27–29 kDa) and a minor peptide (45 kDa) (*38*). Consequently, patients C, D F, H, and likely K, are designated as being positive and patients M and O and possibly P and Q, who are not clinically sensitive to soybeans, are judged to be positive. The fact that not all positive patients recognize the same putative SRP bands is not surprising because different patients might target different epitopes. These data suggest that SRP may indirectly, through protein–protein interactions, contribute to soy allergenicity in some but not most patients and that the production of IgE anti-SRP may not be associated with allergic symptoms in some individuals. Thus, additional investigations into SRP allergenicity are warranted.

Conclusion. The findings of the current investigation suggest that in addition to soybeans, SRPs appear to be conserved in tested dry beans and oil seeds. The soybean 7S and 11S globulins are likely to be soy allergens with SRP being a less likely candidate. Recent papers (*64, 67–69*) suggest soybean β-conglycinin (7S) to be more immunogenic than glycinin (11S). Further research is warranted to find ways to increase SRP expression to improve seed protein nutritional quality without the use of GMOs.

ABBREVIATIONS USED

BSB, borate saline buffer (0.1 M H_3NO_3, 0.025 M $Na_2B_4O_7$, 0.075 M NaCl, pH 8.45); diH_2O, deionized, distilled water; ELISA, enzyme-linked immunosorbent assay; GMO, genetically modified organisms; HRP, horseradish peroxidase; kDa, kilodalton; MES, 2-(*N*-morpholino)ethanesulfonic acid; NC, nitrocellulose; NFDM, nonfat dried milk; pAb, polyclonal antibody; RT, room temperature (25 °C); SDS-PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; SRP, sulfur-rich protein; TBS-T, Tris-buffered saline, Tween 20 [10 mM Tris, 0.9% (w/v) NaCl, and 0.05% Tween 20 (v/v)].

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