Some Properties of a Selenium-Incorporating Sulfur-Rich Protein in Soybeans (*Glycine max* L.)[†]

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Sulfur-rich protein (SRP) incorporated selenium when soybeans were hydroponically grown and intrinsically radioactively labeled with ^{75}Se. SRP polypeptides with estimated molecular weights 17 000–18 000 contained twice the radioactivity compared to polypeptides with estimated molecular weights of 28 000–29 000, accounted for more (Se) than half of the radioactivity in the SRP, and had the highest in vitro resistance to trypsin hydrolysis. The amount of selenium in SRP was 14.86 μmol/mol of SRP. The chemical form of ^{75}Se in SRP was selenomethionine. Nonradioactive SRP had an absorption maximum at 280 nm, $A^{1\%}_{280nm}$ = 7.65 ± 0.95 (1 M NaCl), no free sulfhydryl group, no carbohydrates, a Stokes radius of 41.64 ± 0.96 Å, and a fluorescence maximum at 332–334 nm. Among the proteases tested, papain was most efficient in hydrolyzing SRP in vitro. Disulfide bonds conferred stability to SRP against proteolysis by trypsin and chymotrypsin in vitro.

INTRODUCTION

Soybeans (*Glycine max* L.) are high-protein (35–40% on a dry weight basis) seeds valued for their oil and protein content. The two major proteins in soybeans are glycinin (11S) and β-conglycinin (7S) which account for approximately 70% of the total seed proteins (Derbyshire et al., 1976). In recent years a high-sulfur-containing protein in soybeans (Hu and Esen, 1981, 1982; Yamauchi et al., 1984) has been reported. This protein is a salt-soluble globulin and has been purified to homogeneity by Yamauchi et al. (1984). This protein has been reported to be a basic (p*I* 9.05–9.26) 7S globulin that is distinctly different from the β-conglycinin (7S) and contains two kinds of polypeptides, with estimated molecular weight (MW) of the native tetrameric protein being 168 000. The heavy (MW 26 000) and light (MW 16 000) polypeptide chains are linked via disulfide linkage to form an intermediary subunit (MW 42 000), and four such intermediary subunits constitute the native molecule (Yamauchi et al., 1984). Sato et al. (1987) have shown that this 7S basic globulin is immunologically distinct from the 11S and 7S globulins and accounts for 3% of the total extractable proteins. They also reported that the heavy and light chains each consisted of two polypeptides with p*I* values of 7.7–7.9 and 6.5–7.0, respectively, and Val and Ser as the N-terminal amino acids, respectively. Hirano et al. (1987), using tryptic peptide mapping by high-pressure liquid chromatography (HPLC) and N-terminal amino acid sequencing, further confirmed that the basic 7S globulin and the β-conglycinin (7S) are different with respect to their primary structure. They also indicated that the heavy and light chains lacked homology with respect to their primary structures when evaluated for the tryptic peptide mapping by HPLC. These data indicate that the 7S basic globulin is a sulfur-rich protein (SRP) that is distinctly different from the major soybean proteins 11S and 7S.

In hydroponically grown, intrinsically labeled soybeans we have shown that the major portion of incorporated ^{75}Se is covalently bound to soybean proteins (Sathe et al., 1992). Anion-exchange (DEAE DE-52) column chromatography, preparative sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE), and solubility-based fractionation according to the method of Thanh and Shibasaki (1976) were used in those investigations to discern the radioactivity distribution of ^{75}Se in soybean proteins. When the proteins were analyzed by preparative SDS–PAGE, we typically recovered 65.6% (mean ± SEM = 65.5 ± 4.69%, n = 12) of the radioactivity loaded onto the gels and found that 7S (β-conglycinin) and 11S (glycinin), respectively, accounted for 9.22% and 47.22% of the recovered radioactivity. When the soluble soybean proteins were analyzed by ion-exchange column chromatography, we also noticed that the SRP, being a basic protein, eluted off the column before the salt gradient was developed and contained a significant amount (6.75%) of the total recovered radioactivity, indicating that the SRP incorporated the ^{75}Se.

Yasumoto et al. (1988) have shown that the major portion of selenium in soybeans is associated with methionine. Since sulfur amino acids are the first limiting amino acids in soybeans and because SRP is a high-sulfur protein which contains both methionine and cysteine, we were interested in finding the chemical form of selenium in SRP and the selenium distribution within the SRP polypeptides. Some of the molecular properties of SRP that have not yet been reported are also discussed in this paper.

MATERIALS AND METHODS

Materials. Soybeans (*G. max* L. cv. Century) were from Indiana Crop Improvement Association, W. Lafayette, IN. Sources of electrophoresis chemicals, molecular weight markers, and staining and destaining chemicals are the same as reported earlier (Sathe et al., 1989). DEAE DE-52 cellulose was from Whatman Lab Sales, Inc., Hillsboro, OR. Sephacryl S200 and gel filtration calibration standards were from Pharmacia, Inc., Piscataway, NJ. Dowex AG 1 × 8 was from Bio-Rad Laboratories, Richmond, CA. Bovine TPCK–trypsin and TLCK–chymotrypsin, papain, and selenocystine and selenomethionine standards were from Sigma Chemical Co., St. Louis, MO. Fluoro-

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Hance was from Research Products International Corp., Mount Prospect, IL. The cellulose thin-layer chromatography (TLC) plates (20 × 20 cm, 250-μm thickness) were from Analtech Inc., Newark, DE. All other chemicals were of reagent grade.

Methods. *Preparation of Flour.* The soybean seeds were hydroponically grown and intrinsically labeled with ^{75}Se according to the method of Mason and Weaver (1986). Mature seeds were ground in a Wiley-type mill to obtain a 40-mesh flour. The flour was defatted with cold (4 °C) acetone with constant magnetic stirring provided. The defatted flour was air-dried in a fume hood and homogenized in a Waring blender for 1 min at the highest speed setting to obtain a homogeneous sample. The dried defatted soy flour was stored in airtight containers at −20 °C until further use.

Purification of SRP. The sulfur-rich protein (SRP) was purified from both ^{75}Se-labeled and unlabeled defatted soy flours as described earlier (Sathe et al., 1989) except the initial extraction was done using 50 mM Tris-HCl, pH 8.5, containing 0.1% β-ME and 0.02% NaN_3.

Analytical Methods. Protein concentration of appropriate samples was determined according to the Lowry procedure (Lowry et al., 1951) after the samples were dialyzed against 1 mM $NaHCO_3$ (dialysis tubing MW cutoff of 6000–8000) for 24 h.

SDS–PAGE was done according to the procedure of Fling and Gregerson (1986) as described earlier (Sathe et al., 1989). Nondenaturing nondissociating gel electrophoresis was done using 3–30% linear acrylamide gradient gels using 90 mM Tris, 80 mM boric acid, and 2.5 mM EDTA, pH 8.4, buffer system. Gels were typically run for 16 h at a constant current of 10 mA/gel. Cooling (approximately 15 °C) was provided with running tap water during the gel run. The cathode (+) was at the gel top, and anode (−) was at the gel bottom because SRP is positively charged at pH 8.4. All gels were stained in 50% methanol containing 10% acetic acid and 0.25% Coomassie Brilliant Blue R for 16 h. Destaining was with 50% methanol containing 10% acetic acid for 2 h followed by 5% methanol containing 7.5% acetic acid.

Radioactivity was measured using a γ counter (TM Analytic Inc., Elk Grove Village, IL) with a calculated efficiency of 27%. All radioactivity values were corrected for background and nucleotide decay (120.7-day half-life). Selenium content was measured using the gas chromatographic method of McCarthy et al. (1981). Autoradiography was performed as follows. Proteins were electrophoresed on a 12% acrylamide gel (SDS–PAGE) in the presence and abence of β-ME. The gels were treated with Fluor-Hance solution for 30 min and dried under vacuum on a filter paper. The dried gels were exposed to Kodak X-Omatic film for 3 weeks at −80 °C.

To determine the chemical form of selenium, radiolabeled protein was reduced and carboxymethylated according to the procedure of Crestfield et al. (1963), dialyzed against distilled water (4 °C, 24 h, three changes), and lyophilized. The reduced and carboxymethylated protein was then hydrolyzed in vacuo in 6 N HCl (110 °C, 24 h) and dried under vacuum. The dry hydrolysate was reconstituted in distilled water and filtered through a 0.45-μm filter (Whatman nylon filter), and the filtrate was loaded onto a Dowex AG 1X8 ion-exchange resin column (0.7 × 14 cm). The column was sequentially eluted with 12 mL each of distilled water, 1.5 M HCl, and 4.0 M HCl. Three-milliliter fractions were collected and counted for radioactivity to locate radioactive fractions. Fractions containing the radioactivity were pooled and lyophilized. The lyophilized fractions were reconstituted in distilled water, and aliquots were subjected to ascending analytical TLC. Acetone/water/pyridine/acetic acid (75:20:5:1 v/v/v/v) was the developing solvent. Selenomethionine and carboxymethylated selenocysteine were chromatographed simultaneously. Amino acids were visualized using ninhydrin spray and subsequent heating of plates at 80 °C for 20 min to develop the color.

Carbohydrate analysis was done according to the method of Dubois et al. (1956). Stokes radius was measured using a 2.6 × 80.0 cm Sephacryl S200 column calibrated with standard proteins (known Stokes radii) according to the method of Siegel and Monty (1966).

The number of free sulfhydryl groups in the protein was determined according to the method of Ellman (1959) and the number of disulfide bonds was determined using the NTSB (nitro-2,5-thiosulfobenzoate) method as described by Thannhauser et al. (1984) with 13 600 as the molar absorbance at NTSB at 412 nm.

Amino acid analyses of unlabeled protein were done by hydrolyzing the protein at 105 °C in constant-boiling 6 N HCl (Pierce Chemical Co.) for specified times (24, 48, and 72 h) in vacuo. The hydrolysates were then lyophilized, reconstituted in dilute citric acid, and filtered through a 0.22-μm Millipore GS filter. Norleucine was added to all samples as an internal standard. A Beckman 7300 amino acid analyzer was used according to the manufacturer's instructions. Detection was by ninhydrin. Fifty microliters (injected volume) contained 10 μg of protein. Cysteine was determined after the samples were oxidized by performic acid. Tryptophan was determined according to the chemical method of Spies and Chambers (1948). Threonine and serine were calculated from zero time hydrolysis. Three separate preparations were analyzed for all of the amino acid analyses.

Ultraviolet spectrum was recorded using a Gilford Response UV–vis spectrophotometer equipped with a six-position automatic cell positioner, video display, and printer (Gilford Instrument Laboratories, Oberlin, OH) using 1 cm path length cuvettes. Fluorescence spectra were recorded using a Perkin-Elmer 650-10S fluorescence spectrometer equipped with a strip chart recorder (Cole Parmer Instrument Co., Chicago, IL) at 25 °C. The excitation wavelength was 280 nm, and emission was recorded at 300–480 nm. The excitation slit and emission slit were, respectively, 10 and 2 nm. The scan speed was 60 nm/min, and the chart speed was 2 cm/min. The spectrometer was checked for stability and the Raman band for water according to the manufacturer-recommended procedures prior to recording of the sample spectra. Appropriate solvent spectra were subtracted from sample spectra. Protein concentration was 0.05 mg/mL for all samples.

In vitro protein digestibility was determined using TPCK–trypsin (bovine pancreas, type III S, batch T-2395), TLCK–chymotrypsin (bovine pancreas, type II, batch C-4129), and papain (papaya latex, type IV, batch P-4762). All enzymes were from Sigma. For trypsin and chymotrypsin, Tris-HCl buffer, pH 8.1, and for papain, sodium phosphate, pH 6.2, buffer were used. Final digestion conditions were as follows: substrate protein concentration, 0.5 mg/mL; protein to enzyme ratio, 20:1 w/w; 37 °C (constant-temperature water bath); buffer concentration, 50 mM; digestion period, 30 min. When present, β-ME concentration was 20 mM. To discern the influence of heat on in vitro digestibility, appropriate samples were heated in a boiling water bath (100 °C) for 30 min in loosely stoppered (wooden stoppers) test tubes and cooled rapidly prior to enzyme treatment. Appropriate protein and enzyme controls were run simultaneously. At the end of the digestions, an equal volume of SDS–PAGE sample buffer (50 mM Tris-HCl, pH 6.8, 1% SDS, 0.01% bromophenol blue, 30% glycerol, and 2% β-ME) was added; the samples were heated (100 °C, 10 min) to inactivate the enzymes and immediately electrophoresed using SDS–PAGE. For kinetic analysis of protein digestibility, heat denatured (100 °C, 10 min) SRP was digested with TPCK–trypsin with the following final digestion conditions: substrate protein concentration, 0.5 mg/mL; 50 mM Tris-HCl, pH 8.1; 37 °C; protein to enzyme ratio, 50:1 w/w. Digestion times were 5, 10, 20, 30, 45, 60, 120, and 240 min. At the end of the digestion, the enzyme was inactivated as described above. Samples were electrophoresed (SDS–PAGE), and the gels were scanned at 595 nm (after staining and destaining) using a Beckman DU-8 gel scanner equipped with an integrator. Four separate gels (representing two separate digestions) using protein loads of 10, 15, 20, and 40 μg were scanned in this manner. The standard error of the mean was typically less than 10%. All experiments were done in duplicate.

RESULTS AND DISCUSSION

SRP Purification and Chemical Form of Selenium. Typical yields from ^{75}Se-labeled soybeans for SRP purification are shown in Table I. These yields are comparable to those from unlabeled soybean preparations reported earlier (Sathe et al., 1989). The SRP obtained using this

Table I. Summary of Soybean Sulfur-Rich Protein Purification[a]

purification step	total volume, mL	total protein, mg	total radioactivity, net cpm × 10^{-3}	net cpm/mg of protein
50 mM Tris-HCl, pH 8.5, containing 0.1% β-ME, 0.02% NaN_3 extract	162	3892.63	23234.729	5969
20–40% ammonium sulfate precipitate				
(a) before dialysis	50	1077.74	7573.2	7027
(b) after dialysis, centrifugation, and redissolution	30	81.37	828.33	10180
sulfur-rich protein off DEAE DE-52 column after dialysis against distilled water	42	26.79	699.731	26119

[a] Data are for a typical purification starting with 20 g of defatted soy flour containing 2.45 μg of ^{75}Se/g of flour (approximately 5 × 10^6 cpm/g).

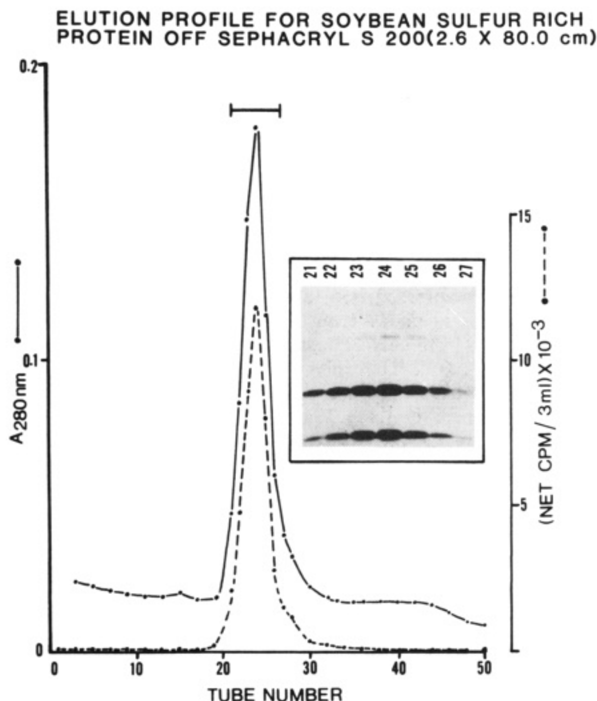

Figure 1. Elution profile for the purified sulfur-rich protein (SRP) off a 2.6 × 80.0 cm Sephacryl S200 column. Equilibrium and elution buffer for the column was 20 mM sodium phosphate buffer, pH 7.5, containing 100 mM NaCl and 0.02% NaN_3. Fractions were collected every 20 min. The column flow rate was 20 mL/h. (Inset) SDS–PAGE (8–25% linear acrylamide gradient) of the fractions (fraction number indicated on top). Fractions (75 μL each) were mixed with an equal volume of SDS–PAGE sample buffer and heat denatured (100 °C, 5 min) prior to electrophoresis. Horizontal bar indicates fractions pooled.

purification procedure was of 90+% purity based on SDS–PAGE as well as gel filtration on a Sephacryl S200 column (single peak) as shown in Figure 1. The nondenaturing nondissociating polyacrylamide gel electrophoresis of purified SRP (Figure 2) indicated that within the native molecules there were at least three different charge species (arrows) which were composed of both the heavy (MW 28 000–29 000) and light (MW 17 000–18 000) chains when analyzed by two-dimensional gel electrophoresis (Figure 3). The polypeptide with estimated MW 45 000 seemed to arise primarily from the highest positively charged species (highest mobility band in Figure 2). Selenium analysis of the purified SRP indicated that 14.86 μmol of selenium was incorporated per mole of purified protein, assuming the MW of native protein to be 148 600. Gel electrophoretic analysis in the absence and presence of 2% β-ME confirmed the selenium incorporation by SRP (Figure 4). Subsequent counting of gel slices (Figure 5) from two separate gel runs indicated that the radioactivity was associated with the protein bands. On average, 87–88% of the loaded radioactivity was recovered from gels. Of the recovered radioactivity, 86.06% radioactivity was associated with protein (in the absence of β-ME). In the

Figure 2. Nondenaturing nondissociating polyacrylamide gel electrophoresis (NDND–PAGE) of the purified SRP. This is a 3–30% linear acrylamide gradient gel. The gel and running buffer was 90 mM Tris, 80 mM boric acid, and 2.5 mM EDTA pH 8.4. The cathode (+) was at the gel top. Approximately 200 μg of SRP was electrophoresed. Arrows indicate the electrical charge variants.

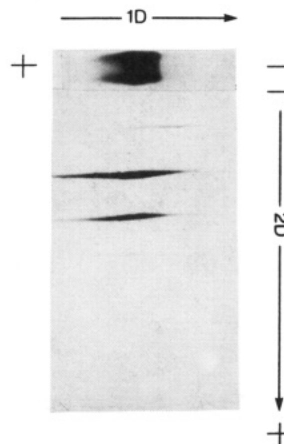

Figure 3. Two-dimensional electrophoretic analysis of the SRP, 1D, SRP electrophoresed in first dimension using 0.75 mm thick gels; 2D, SDS–PAGE (8–25% linear acrylamide gradient, 1.5 mm thick gel) in the presence of β-ME. Direction of migration is indicated by arrow.

presence of β-ME the radioactivity distributions with polypeptides of MW 45 000, 28 000–29 000, and 17 000–18 000 were, respectively, 6.17, 25.82, and 52.87% of the total recovered radioactivity. The combined recovered radioactivity from these three kinds of polypeptides represented 84.86% of the recovered radioactivity. It was also clear that the light chains had twice the amount of radioactivity compared with the radioactivity of the heavy chains. These data also indicate that the selenium incorporation by SRP is covalent in nature.

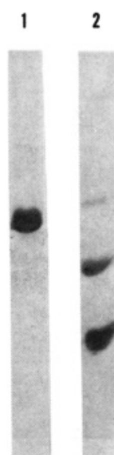

Figure 4. Autoradiographs of ^{75}Se-labeled SRP. SRP (200 μg each) was electrophoresed using 12% acrylamide gels (SDS–PAGE) in the absence (1) and presence (2) of β-ME. The gels were dried under vacuum on a filter paper (after treatment with Fluor-Hance for 30 min) and exposed to Kodak X-Omatic film for 3 weeks at −80 °C.

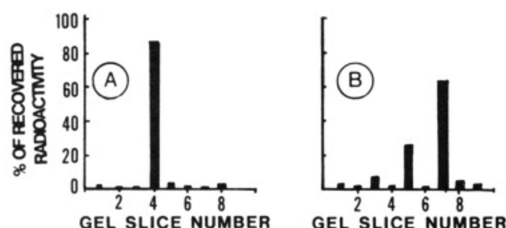

Figure 5. Radioactivity distribution in SRP polypeptides. The gels were run as in Figure 4, stained for 30 min to visualize protein bands, cut into appropriate slices (gel slice numbers 1–10 refer to gel top to bottom), and counted for radioactivity in a γ counter. (A) In the absence of and (B) in the presence of β-ME. Note the radioactivity associated with polypeptides of MW 17 000–18 000 was almost twice that associated with those with MW 28 000–29 000 (B).

Figure 6. TLC analysis of the radioactivity collected from Dowex AG 1X8 resin column. (Lane 1) Authentic selenecysteine; (lane 2) authentic selenomethionine; (lane 3) radioactive pool from Dowex AG 1X8 column when the purified SRP 6 N HCl hydrolyzate was fractionated. Direction of migration is from bottom to top (ascending TLC).

To discern the chemical form of selenium, we analyzed the radioactive fractions (using TLC) collected from Dowex AG 1X8 resin when the reduced, carboxymethylated SRP hydrolyzed in 6 N HCl was chromatographed. A typical TLC analysis is shown in Figure 6. On the basis of the identical mobility of the sample when compared with the authentic selenomethionine, the chemical form of the selenium in SRP is selenomethionine. On the basis of the amino acid composition of SRP from this study (Table II), the number of moles of methionine and Cys/2 in the SRP is 18 each, on a per mole of protein basis. Therefore, it appears that selenium is preferentially incorporated into methionine rather than cysteine. This observation is consistent with earlier studies indicating that the chemical form of selenium in soybean storage proteins (Yasumoto et al., 1988) and soybean lectin is also selenomethionine (Sathe et al., 1992). Selenium analysis of SRP also made it clear that selenium incorporation in SRP is not on a mole per mole of methionine basis because only 14.86 μmol of selenium was incorporated (per mole of SRP). The radioactivity distribution within the polypeptides, however, correlated well with their methionine content. This is because, on the basis of published data (Sato et al., 1987), the number of methionine residues in the light chains (4.695) is 2.86 times those in the heavy chains (1.64) and, although not in proportion, the light chains had twice the amount of radioactivity compared to the heavy chains.

Table II. Amino Acid Composition of Soybean Sulfur-Rich Protein: Comparison with Glycinin and β-Conglycinin[a]

amino acid	sulfur-rich protein *b*	*c*	*d*	*e*	glycinin	β-conglycinin
Asx	8.97	9.76	9.9	9.45	12.0	14.1
Thr	5.78	6.92	6.95	7.50	5.0	2.8
Ser	11.01	6.77	9.25	8.27	8.5	6.8
Glx	15.18	14.18	12.90	12.47	19.2	20.5
Gly	8.09	4.89	9.90	8.42	7.2	2.9
Ala	4.48	4.70	7.05	6.81	5.4	3.7
Cys/2	1.46	1.48	1.85	1.38	1.5	0.3
Val	6.10	7.12	6.45	7.87	4.9	5.1
Met	1.83	2.64	1.30	2.29	1.1	0.3
Ile	3.61	3.85	2.90	3.80	5.1	6.4
Leu	8.71	10.59	8.95	10.43	6.3	10.3
Tyr	2.62	2.09	1.40	1.50	2.7	3.6
Phe	5.33	5.67	4.20	4.43	3.8	7.4
Lys	3.35	3.35	3.45	2.96	3.8	7.0
His	4.06	4.82	3.50	3.60	1.9	1.7
Arg	3.89	4.42	3.00	3.28	5.1	8.8
Pro	4.90	6.75	7.05	7.59	5.7	4.3
Trp	1.37					

[a] The data are expressed as residues/100 residues. [b] Current study. Values for amino acids are from three independent preparations. Cys/2 values are from performic acid oxidation. Val, Ile, Leu, and Phe values are from 72-h hydrolysis of samples. Ser and Thr values are zero time hydrolysis values. Trp was determined by chemical method (Spies and Chambers, 1948). All amino acid values (except for Trp) are corrected for 100% recovery of norleucine used as an internal standard. [c] Yamauchi et al. (1984). [d] Hu and Esen (1982), calculated from the sum of their data for large and small chains. [e] Sato et al. (1987).

Molecular Properties of SRP. Molecular properties of SRP that have not previously been reported were investigated using SRP purified from nonradioactive beans. SRP tested negative for carbohydrates and therefore is not a glycoprotein. The protein had an absorption maximum of 280 nm (Figure 7). This spectrum also had shoulders at 260, 266, 269, and 291 nm. The shoulders at 260 and 266 nm are due to phenylalanine and at 291 nm reflect the tryptophan environment. $A^{1\%}_{280\text{nm}}$ values for SRP, using 1 M NaCl and 20 mM sodium phosphate, pH 7.5, containing 0.02% NaN_3 and 0.1 M NaCl as solvents, were, respectively, 7.65 ± 0.95 and 7.43 ± 0.32 (mean ± SEM, using three protein concentrations and making three measurements for each concentration). The protein concentrations used were in the range 0.5–1.0 mg/mL. The path length of the cuvettes was 1.0 cm. These values for SRP are greater than 5.47 for β-conglycinin (Deshpande and Damodaran, 1990) and less than that of 11S (8.04) reported by Koshiyama (1972). The molecular size of SRP is smaller compared to the size of 11S and 7S. The Stokes

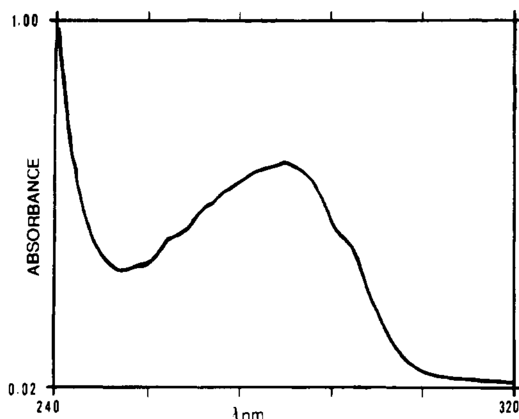

Figure 7. Ultraviolet absorption spectrum of the SRP. Protein was dissolved in 100 mM sodium phosphate buffer, pH 7.5 (1 mg/mL). Spectrum was recorded against solvent blank.

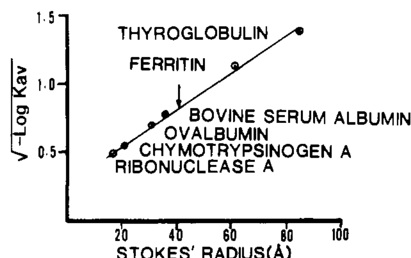

Figure 8. Calibration curve for measuring the Stokes radius of SRP using 2.6 × 80.0 cm Sephacryl S200 column. The standard curve was prepared using proteins of known Stokes radii (average of two runs for each protein). The column equilibrium and elution buffer was 20 mM sodium phosphate, pH 7.5, containing 100 mM NaCl and 0.02% NaN_3. Average column flow rate was 18 mL/h. Arrow indicates elution position of purified SRP.

radius of SRP was 41.65 ± 0.96 Å (n = 8, from one radioactive and four nonradioactive preparations). The calibration curve for the Sephacryl S200 column (2.6 × 80.0 cm) used for these determinations is shown in Figure 8. The typical elution volume for SRP off this column was 215.51 ± 2.57 mL (n = 6, mean ± SEM). The reported Stokes radii of 11S and 7S are 59 Å (Koshiyama, 1972).

The amino acid composition of SRP (Table II) is comparable with the published amino acid composition. Hydrophobic amino acids (36.33%) dominate the amino acid composition of SRP. The acidic, uncharged polar, and basic residues accounted for 24.15%, 28.96%, and 11.3%, respectively, of the total amino acids. Because sulfur amino acids are the first limiting amino acids in soybean proteins and because the SRP contains more methionine than either 11S or 7S, increasing the SRP quantity in soybeans may prove nutritionally beneficial.

The fluorescence spectra of SRP in several solvents are shown in Figure 9. Regardless of the solvent, all spectra registered maximum fluorescence at 332–334 nm, indicating the hydrophobic environment around tryptophan residues. Since NaCl indirectly promotes the hydrophobic interactions, SRP registered the highest fluorescence when 1 M NaCl was used as a solvent. The presence of 0.1% SDS did not cause a shift in the peak, indicating that the tryptophan environment was stable. Because the peak intensity was not changed significantly and there was no red shift in the emission maximum, addition of 0.1% SDS did not expose the tryptophan residues to more polar environment. This suggests that the SRP structure is stable. As expected, addition of 0.5 N NaOH did cause a significant decrease (approximately 50%) in the peak height but not the half-peak width. However, it did not completely eliminate the fluorescence, indicating that the

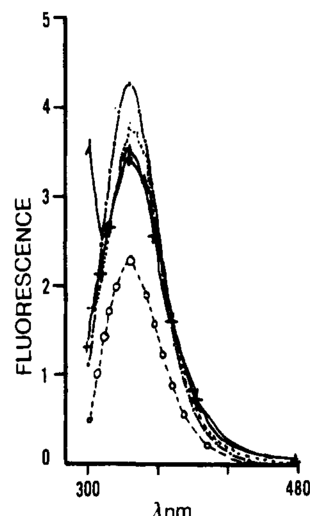

Figure 9. Fluorescence spectra of purified SRP (0.05 mg/mL) at 25 °C using excitation wavelength of 280 nm, scan spped of 60 nm/min, excitation slit of 10 nm, emission lits of 2 nm, and chart speed of 2 cm/min. All spectra are corrected for appropriate solvent blanks. Solvents: distilled water (—); 20 mM sodium phosphate buffer, pH 7.5 (- - -); 1.0 M NaCl (●); 0.5 M NaOH (O); 0.1% SDS (+). Fluorescence is expressed in arbitrary units.

protein structure is quite compact. Usually the denaturation of proteins by guanidine hydrochloride and urea causes a red shift (emission maximum at higher wavelength) and exposure of tryptophan residues to polar environment causes a blue shift (emission maximum at lower wavelength compared to that of the native protein) in protein fluorescence spectra (Lakowicz, 1983). Since exposure of SRP to SDS and NaOH did not cause any shifts in the fluorescence spectrum of the SRP, it is apparent that the tryptophan residues in SRP are buried in the interior of the protein structure, which is stable.

SRP in Vitro Digestibility. Since the bioavailability of selenium partly depends on the food source and the chemical form (Yoshida et al., 1981; Beilstein and Whanger, 1986; Mason and Weaver, 1986), susceptibility of SRP to proteolysis may be important from a nutritional viewpoint. Therefore, we evaluated the in vitro digestibility of SRP using different proteinases (Figure 10). In the native state SRP was resistant to both trypsin and chymotrypsin. Heating significantly improved the susceptibility of SRP to proteinases. This improvement was more pronounced for trypsin compared to chymotrypsin. The inability of chymotrypsin to effect significant proteolysis of SRP is consistent with earlier observations of Nielsen et al. (1988) on β-conglycinin. They found that, even after heating for 30 min (moist heat), chymotrypsin was unable to cause complete hydrolysis of β-conglycinin (7S). However, heated SRP was completely broken down to small polypeptides. The addition of 20 mM β-ME in the digestion buffer helped improve proteolysis by both trypsin and chymotrypsin. However, it did not allow complete proteolysis by either of the proteinases. This suggests that although the disulfide bonds contribute to the SRP's stability toward proteinases, they are not solely responsible for it. We failed to detect any free sulfhydryl groups in SRP using Ellman's reagent. This would suggest that all cysteine molecules in SRP are disulfide-linked. Using NTSB reagent, we determined the number of disulfide bonds in SRP to be 8.5, which accounts for 17 cysteine residues of 17.8 residues obtained from amino acid analysis. However, the SRP was completely degraded by the cystine proteinase papain. Papain digestion was done in the presence of β-ME regardless of heat treatment of SRP.

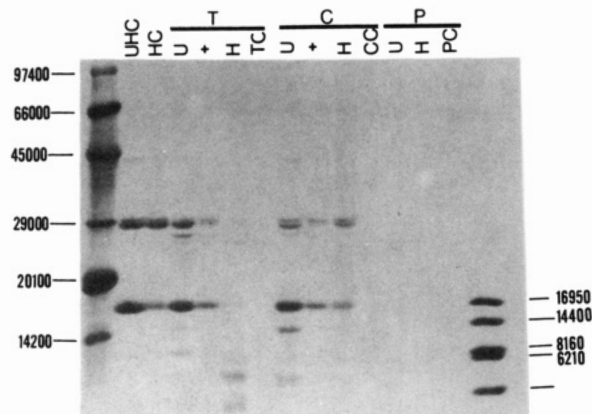

Figure 10. SDS-PAGE (8–25% linear acrylamide gradient) analysis for the in vitro digestion of purified SRP with TPCK–trypsin (T), TLCK–chymotrypsin (C), and papain (P). (Leftmost lane) Protein MW standards (MWs indicated in the left margin); (rightmost lane) peptide MW standards (MWs indicated in the right margin). UHC, SRP unheated control (20 μg); HC, heated (100 °C, 30 min) SRP control (20 μg); U, unheated SRP digested with enzyme; +, unheated SRP digested with enzyme in the presence of 20 mM β-ME; H, heated (100 °C, 30 min) SRP digested with enzyme. TC, CC, and PC are, respectively, trypsin, chymotrypsin, and papain enzyme controls (contained same amount of enzyme that would be present in the SRP digestion). Protein SRP load for enzyme-digested SRP was 20 μg each. Final digestion conditions: SRP concentration of 0.5 mg/mL; SRP to enzyme ratio 20:1 (w/w); 37 °C; 30 min; 50 mM appropriate buffer (Tris-HCl, pH 8.1, for trypsin and chymotrypsin digestions and sodium phosphate, pH 6.2, for papain digestion).

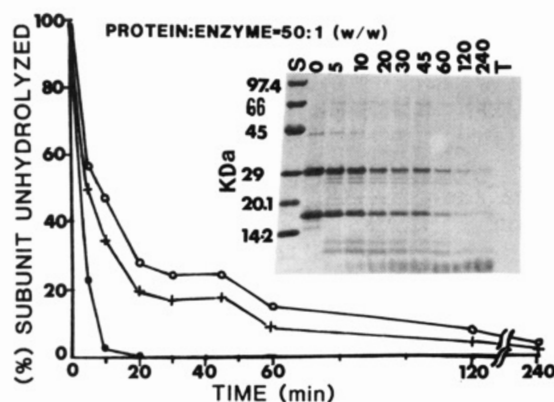

Figure 11. Rates of in vitro trypsin digestion of heat-denatured (100 °C, 30 min) SRP subunits. Final digestion conditions: 0.5 mg SRP/mL; 50 mM Tris-HCl, pH 8.1; SRP to TPCK–trypsin ratio 50:1 (w/w); 37 °C, for desired time length. (●) 45 000; (+) 28 000–29 000; (○) 17 000–18 000. SDS-PAGE (8–25% linear acrylamide gradient) analysis of SRP digested with TPCK–trypsin for the length of time (minutes) indicated in the left margin); T, trypsin control; O, no enzyme SRP control. Protein (SRP) load in each lane was 40 Mg, and that for trypsin was 0.8 μg. The rates were determined from four separate gel runs using SRP loads of 10, 15, 20, and 40 μg. The data points on graphs are therefore average values from four determinations.

To discern the rates of proteolysis of SRP subunits, heat-denatured SRP was subjected to trypsin treatment at a protein to enzyme ratio of 50:1 w/w (Figure 11) for up to 4 h. As can be seen from this figure, the polypeptide with MW 45 000 was degraded at the highest rate among all of the subunits, while those with MW 17 000–18 000 were hydrolyzed at the slowest rate. The slow rate of proteolysis of SRP is consistent with the observations of Nielsen et al. (1988) and Deshpande and Damodoran (1990) that the soybean proteins 7S and 11S are proteolyzed slowly to several intermediate polypeptides before they are broken down to very small (MW ≤ 5000) peptides even after heat treatment. Such a slow rate of proteolysis of soybean proteins taken together with deficiency of sulfur amino acids may at least partly account for the lower protein quality of soybean proteins when compared with the high-quality animal proteins.

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