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Internal Structure of 7S and 11S Globulin Molecules in Soybean Proteins[1]

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ABSTRACT

The internal structure of soybean protein molecules (7S and 11S) was investigated by optical rotatory dispersion (ORD), UV difference spectra, infrared absorption spectra, and other techniques. The native soybean globulins of 7S and 11S possessed b_o values, near zero in the Moffitt-Yang equation, and their a_o values increased in the negative direction without accompanying changes in b_o values, upon urea denaturation. The far-UV ORD curve indicated a positive peak at 200–201 mμ and a negative trough at 233–235 mμ, but the shoulder near 210 mμ was not observed. Levorotation near the negative trough increased upon urea denaturation. In the infrared spectrum measurements, the amide I bands, were observed at 1,650–1,655 (shoulder), 1,638 (main peak), and 1,685 cm.$^{-1}$ (weak shoulder); the amide II bands, at 1,520–1,535 cm.$^{-1}$; and the amide V bands, at 698, 660, and 620 cm.$^{-1}$ (weak), in both proteins. UV difference spectra indicated that tyrosine (7S), or tyrosine and tryptophan (11S), are buried in the water-impenetrable hydrophobic regions. The denaturing abilities of various alcohols on these proteins completely depended upon the hydrophobicities of the alcohols used. The native protein molecules are quite compact, and were not hydrolyzed by proteinase without disruption of the internal structure.

Up to the present, a great number of investigations have been carried out on soybean proteins. In particular, much information on the molecular features of the major soybean proteins has been obtained recently; for example, the dissociation-association characteristics (1–4), intermolecular disulfide polymerization (5), molecular weights (4, 6), and N-terminal amino acids (4, 7, 8). However, this information is concerned mainly with the primary and the quaternary structures and not with the secondary and the tertiary. There is a report of optical rotatory measurements for soybean 11S globulins (2). Owing to the measurement at a single wave length, however, it is impossible to predict the details of the internal structures (i.e. the secondary and tertiary) from that report. Accordingly, knowledge of the internal structures of soybean protein molecules is meager.

At present the internal structures of biologically active proteins are under intensive study and are being determined gradually. However, little is known of the structures of biologically inactive proteins such as the proteins of soybeans in storage.

Soybeans or defatted soybean meals are used mainly for animal feeds.

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In the Orient they are also used very extensively for fermentation into *miso* or soypaste, *natto, shoyu* (soy sauce), *tempeh,* etc. Various protein materials made from soybeans are being added to other foods for functional effects. When used for feeds or fermentations, soybean proteins are proteolytically hydrolyzed, either by the digestive organs of animals or by microorganisms during the fermentation process. Hence, the susceptibilities of the soybean proteins to proteinase attack would depend to a great extent on their internal structures. The functional effects of soybean proteins also depend on their physical properties, which should be attributed fundamentally to the internal structure. Thus it is essential, not only for purely scientific research but also for practical or industrial purposes, to know the internal structure of the soybean protein molecules.

The present report deals with the secondary and tertiary structures of the 7S and 11S globulins, which comprise about 70% of soybean proteins. Results are based on measurements of optical rotatory dispersion, infrared spectra, ultraviolet (UV) difference spectra, and other techniques.

MATERIALS AND METHODS

Soybean Material

Soybeans, Var. *tsurunoko,* 1964 crop, in Hokkaido, Japan, were used throughout this study. The soybeans were dried, cracked, dehulled, and ground in a mill through a 40-mesh screen, and then defatted with n-hexane at room temperature. To 1 part of the resulting defatted soybean flour, 5 parts of water were added and the slurry was stirred for 1 hr. at 30°C. and centrifuged (12,000 r.p.m., 15 min.). The 7S and 11S preparations were separated from this supernatant by the method shown in Fig. 1.

7S or 11S Solutions

Two kinds of buffer solutions were used (except in special cases). One was 0.1*M* phosphate buffer, pH 7.6 (μ, 0.274), composed of disodium hydrogen phosphate and sodium dihydrogen phosphate. The other was 0.0365*M* phosphate buffer of pH 7.6, containing 0.4*M* sodium chloride (μ, 0.5). The 7S or 11S preparations were put into these buffers and, after 60 min. of standing, the pH was adjusted to 7.6 with sodium hydroxide. Centrifuging followed, at 40,000 r.p.m. for 60 min. The resulting clear solutions were used for the various measurements, after dilution to appropriate concentrations with the buffer solutions or with the urea solutions containing the buffers.

Electrophoretic Analysis

Samples were dissolved in 0.1*M* phosphate buffers at pH 7.6, adjusted to 3.0% concentration, equilibrated by dialysis against the buffers, and analyzed electrophoretically (Hitachi Model HTD-1 Tiselius apparatus) at 20°C. for 145 min.

Ultracentrifugal Analysis

Analysis was made with a Hitachi Model UCA-1A analytical ultracentrifuge equipped with schlieren optics, at 55,430 r.p.m., 20°C., in a single-sector 13-mm. cell. The samples were dissolved in the 0.5 μ phosphate buffers containing sodium chloride (pH 7.6); 0.1*M* phosphate buffer

was not used, because of partial dimerization of the 7S component in this buffer.

Amino Acid Analysis

Amino Acids Other Than Tryptophan and Cystine. The analysis was carried out on 22-, 40-, and 70-hr. hydrolysates in the Hitachi Model KLA-3 amino acid analyzer by the two-column system (Amberlite IR-120 columns of 0.9 × 150 and 0.9 × 15 cm.) of Moore *et al.* (9). Hydrolysis was done in evacuated, sealed tubes in 5.7*N* HCl at 110° ± 0.5°C. (5.0 ml. of 5.7*N* HCl with 10-mg. sample). The values reported here are 40-hr. hydrolysis values for those amino acids showing a maximum in 40-hr. hydrolysis, the extrapolated zero time values for those amino acids showing a decrease with time of hydrolysis, and the 70-hr. hydrolysis values for those amino acids which increased in amount with time of hydrolysis.

Tryptophan. The tryptophan content was determined by the dimethylaminobenzaldehyde method of Spies and Chambers (10,11). Forty-milligram samples were dissolved in 10 ml. of 0.1*N* NaOH solution. One milliliter of the resulting solution, 30 mg. of dimethylaminobenzaldehyde, and 9.0 ml. of 21.4*N* sulfuric acid were mixed and kept for 12 hr. at 20°C. in the dark; then 0.1 ml. of freshly prepared 0.04% sodium nitrate aqueous solution was added. After this had stood for 30 min. in the dark, the absorbance at 590 mμ was measured. Tryptophan content was determined with the standard curve after correction for blank value.

Cystine and Cysteine. These amino acids were measured as cysteic acid from an acid hydrolysate of performic acid-oxidized protein prepared as described by Hirs (12) on a column of Amberlite IR-120 (0.9 × 150 cm.) according to the procedure of Moore *et al.* (9).

Amide Ammonia. Ten milligrams of sample was added to 5 ml. of 1*N* HCl and kept for 3 hr. at 100°C. with a reflux condenser. The liberated ammonia was determined on a column of Amberlite IR-120 (0.9 × 15 cm.), with the automatic amino acid analyzer.

Carbohydrate Analysis

The carbohydrate contents were determined by the phenol-sulfuric acid method of Dubois *et al.* (13). Glucose was used to prepare a standard curve.

Optical Rotatory Dispersion (ORD) Analysis

ORD measurements were carried out with a JASCO Model ORD/UV-5 automatic recording spectropolarimeter (double prism, double monochromator, made by Japan Spectroscopic Co.), which has a xenon lamp as the light source.

In the measurements of the 300- to 600-mμ region, the sample solutions of ionic strength 0.1 or 0.5 and a cell of 50-mm. path length were used, and the resulting data were treated by means of the Moffitt-Yang equation (14),

$$[m']_\lambda = 3/(n+2) \cdot M_o/100 \cdot [\alpha]_\lambda$$
$$a_o\lambda_o^2/(\lambda^2 - \lambda_o^2) + b_o\lambda_o^4/(\lambda^2 - \lambda_o^2)^2$$

and the one-term Drude equation,

$$[\alpha]_\lambda = A/(\lambda^2 - \lambda_c^2).$$

In the former equation, $[m']_\lambda$ is the reduced mean residue rotation at the

wave length of λ corrected for the refractive index (n) of the solvents, λ_0 is the absorption wave length associated with the rotation assumed to be 212 mμ, and M_0 is the mean molecular weight (MW) per residue. The refractive indices were measured with a Abbé refractometer. The dispersion of refractive index was neglected. The mean MW's of residues (M_0) were calculated on the basis of the present results of amino acid analysis (7S, 114.7; 11S, 113.0). The parameters a_0 and b_0 were calculated from the intercepts and slopes of $[m']$ *vs.* $(\lambda^2 - \lambda_0^2)^{-1}$ plots. The dispersion constant, λ_c, in the latter equation was similarly determined from the slopes of $[\alpha]_\lambda \cdot \lambda^2$ *vs.* $[\alpha]_\lambda$ plots.

In the ORD measurements of the region below 300 mμ, however, the buffer-free sample solutions were used, to make the measurable wave lengths as short as possible. The buffer-free sample solutions were prepared by removing the buffer salts from the sample solution with gel filtration of Sephadex G-100, lyophilizing, and thereafter dissolving in water (pH, 7.6). The protein concentrations were 0.02 to 0.15% and the thickness of the solution layer in the cells was 0.1 and 1.0 mm. In the region of this wave length, $[m']_\lambda$ was calculated on the basis of the data of Timmermans on dispersion of refractive index of water or urea-containing water, and Sellmeier's equation,

$$n_\lambda^2 = 1 + a\lambda^2/(\lambda^2 - \lambda_\nu^2),$$

where a and λ_ν are constant, respectively.

Infrared Spectrum Measurements

The measurements in solution were performed as follows. Samples were dissolved in 0.5*M* NaCl solution, centrifuged, and lyophilized. After the resulting samples were dried for 2 days in a desiccator over P_2O_5 at room temperature *in vacuo,* they were dissolved in D_2O to be 0.5*M* NaCl in the final concentration. The adjustment of pD value was made by throwing the metallic sodium into these solutions. These procedures were carried out at humidities below 10%. For the measurement, the calcium fluoride cells of 0.032-mm. light-path length were used.

The measurements in solid state were performed with a film cast to a AgCl plate about 1 mm. thick. The film was prepared by drying the buffer-free sample solution (see method of ORD measurements) on the plate in a desiccator over P_2O_5, first under atmospheric pressure and thereafter *in vacuo* for 2 days.

Measurements of infrared spectra were carried out with the infrared spectrophotometer Model IR-S (prism type, used in Figs. 9 and 11) and IR-G (grating type, used in Figs. 8 and 10), made by Japan Spectroscopic Co.

Ultraviolet Difference Spectrum Measurements

Difference spectra concerned with urea denaturation were recorded with a Hitachi Model EPS-2 automatic recording spectrophotometer in the wave length range of 230–300 mμ. The measurements were usually made on protein concentrations around 0.2, but at wave lengths below 250 mμ, con-

centrations about 0.04% were employed to eliminate effects such as those of stray light. The data were calculated as $\Delta A_{1\,cm.}^{1\%}$.

Native Protein Determination in Heat- and Alcohol-Treated Proteins

When soybean proteins are heated in water, particularly in the presence of alcohol, most of the denatured proteins precipitate, but some of them remain soluble. On the other hand, when the proteins in soybean preparations are denatured, some of the native proteins are not solubilized by water because they are trapped inside the denatured protein matrix (15,16). Moreover, such samples are heterogeneous systems containing both solid and liquid. Therefore, the usual methods are not suitable for measurement of the native protein contents. In these cases it is better to use the proteinases of *Aspergillus* spp. for determining native protein contents, as was shown (16). The method used herein is the same as described (16), except that the scale was reduced, and the alkaline proteinase of *A. sojae* refined by the method of Mizunuma and Iguchi (17) was used as protease solution. The procedure of the measurement is as follows: 35 mg. of the treated samples was extracted (or hydrolyzed) at 30°C. for 20 hr. by 3.5 ml. of the proteinase solution containing 0.1*M* phosphate buffer (pH, 7.6), with gentle shaking during the extraction. The total activity of the proteinase solution is 6.5×10^{-4} $[PU]_{m.\,eq.\,tyr.}^{Cas.30°FR}$, when it is measured by the modified Anson method (15). After 20 hr. of digestion, the digestion mixtures were centrifuged for 20 min. at 12,000 r.p.m., and 0.5 ml. of the supernatants was transferred into two test tubes (A and B). To tube A, 6.5 ml. of distilled water, 2 ml. of 2% gum arabic solution, and 1 ml. of 2.4*M* trichloroacetic acid (TCA) were added successively and mixed. To tube B, 9.5 ml. of 0.25*M* phosphate buffer was added and mixed. Tube A was incubated for 20 min. at 30°C. and then shaken vigorously. Three minutes later, the absorption of the solution in tube A was read against that in tube B (Hitachi Model EPO-B photometer with 660-mμ filter). The absorbances measured thus are proportional to the native protein content of the samples. The reason is as follows. Native soybean proteins are hydrolyzed with difficulty by the alkaline proteinase of *Aspergillus* spp., whereas the denatured ones are easily hydrolyzed, as was shown in both the previous (15,16) and the present reports. Therefore, when the proteinase solution is added to the samples, the native proteins in them are solubilized into the proteinase solution but not hydrolyzed. The denatured proteins are also solubilized but are completely hydrolyzed in 20-hr. digestion. Accordingly, the turbidities caused by TCA are ascribed to the native protein only. Further, the turbidities are proportional to the protein concentrations (16).

Determination of Degree of Proteolytic Hydrolysis with 2,4,6-Trinitrobenzene-1-Sulfonic Acid (TNBS)

The modified TNBS method of Satake *et al.* (18) was applied to determine the extent of hydrolysis of the 11S globulins partly denatured with alkali. Partly denatured 11S globulin (protein concentration 0.1%), 1 ml., and 1 ml. of alkaline proteinase of *A. sojae* ($[PU]_{m.\,eq.\,tyr.}^{Cas.\,30°FR}$: 2.2×10^{-4}), purified by the procedure of Mizunuma and Iguchi (17), were mixed and

hydrolyzed at 30°C. for various times. After the reactions were stopped with 2 ml. of 0.3*M* TCA solution, the precipitates were removed by filtration. To 1 ml. of the resulting filtrate, 1 ml. of the 0.5*M* Na_2HPO_4 solution containing NaOH equivalent to the TCA in the filtrate and 1 ml. of 0.1% TNBS solution were added and kept for 2 hr. at 40°C. in a dark place. To the resulting orange-colored solutions 4 ml. of acetic acid was added, and the absorbances were measured at 340 mμ (Hitachi Model EPU-2 spectrophotometer). Distilled water (1 ml.), instead of the filtrates, was read as a blank. Leucine was used as a standard.

Separately, the 11S globulin was hydrolyzed completely with an equal volume of 12*N* HCl at 110°C. for 22 hr., and the liberated alpha-amino residues were measured by the TNBS method described above. The data are shown as the ratio of the alpha-amino residues liberated by the proteinase to those liberated by HCl.

RESULTS AND DISCUSSION

Isolation of 7S and 11S Preparations

The 7S and 11S preparations were isolated as shown in Fig. 1.

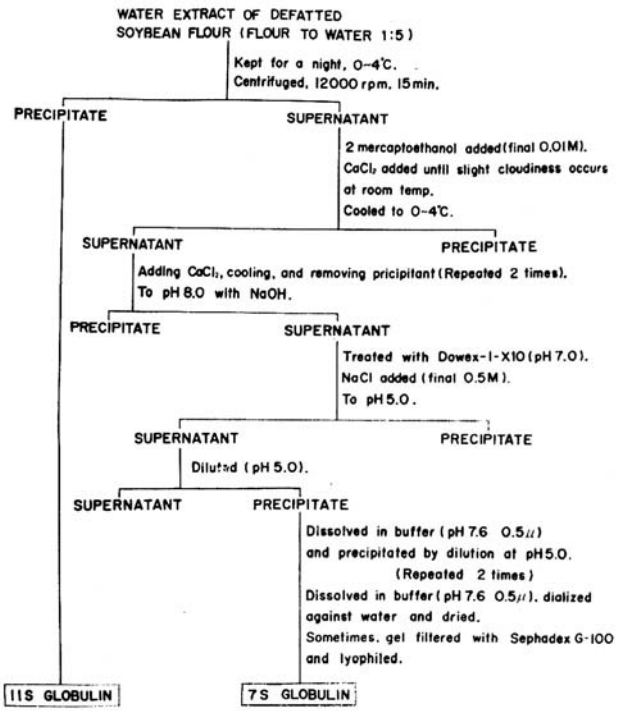


Fig. 1. Isolation of 7S and 11S globulins.

For preparation of the 11S globulin, the cold-insoluble fraction was used, prepared by the method of Briggs and Mann (19). The 7S preparation

was prepared by removing: the cold-insoluble fraction by cooling under addition of $CaCl_2$ (19); the phytin-protein complex by the anion exchanger (20); and the whey proteins by acidification, from the water extract of the defatted soybean flour. When the 7S preparation was not dialyzed thoroughly it did not give a typical UV spectrum, perhaps because of contamination by low-MW nucleic acid compounds or genistin. In this case contaminants were removed by gel filtration (Sephadex G-100); dialyzing and lyophilizing followed.

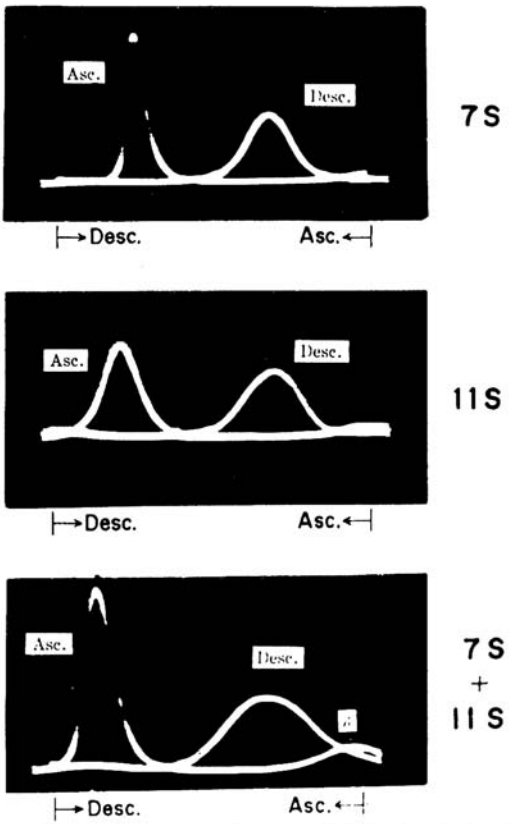


Fig. 2. Electrophoretic patterns. Solvent, 0.1*M* phosphate buffer (pH, 7.6; μ, 0.274); concentration of 7S or 11S, 3.0%; time, 145 min.; current, 6 mA; and temperature, 20°C.

Figure 2 shows electrophoretic patterns of the 7S and 11S preparations, both of which were electrophoretically uniform and revealed comparable electrophoretic behavior. The ultracentrifugal patterns of the 7S and 11S preparations contained a little of each other's component (Fig. 3). However, this contamination did not become an obstacle for the principal purpose of the present investigations, because both 7S and 11S globulins had almost the same internal structure, as will be shown later.

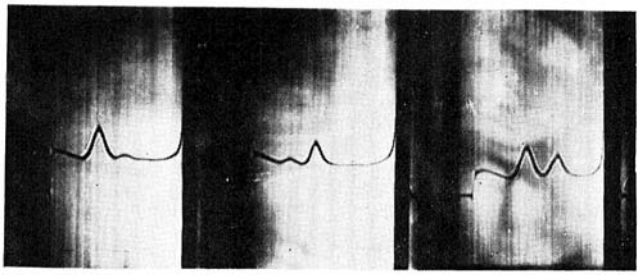


Fig. 3. Sedimentation patterns. Solvent, 0.0365*M* phosphate buffer containing 0.4*M* NaCl (pH, 7.6). Protein concentration; 7S, 0.755%; 11S, 0.582%. Patterns obtained at 20°C., 53 min., bar angle of 60°, and speed of 55,430 r.p.m.

Amino Acid and Carbohydrate Analysis

To the author's knowledge, complete analysis of the amino acid compositions in the 7S and 11S globulins has not been made so far, although reports have been published on a whole soybean globulin (21,22) and on partial analysis of 7S globulin (4). Complete analysis of amino acid compositions is necessary for the mean MW of amino acid residue of each protein molecule, which is required to calculate the reduced mean molecular rotation or the parameters a_o and b_o in the Moffitt-Yang equation in the optical rotatory dispersion.

Table I shows the amino acid and carbohydrate analysis of 7S and 11S globulins. The fact that the totals of a residue weight and its nitrogen in each amino acid residue and carbohydrate are near 100 show that these data are highly reliable. The difference in amino acid composition between 7S and 11S globulins is not marked; however, there were significant differences with regard to several amino acids and carbohydrate. The 7S globulin is somewhat lower than the 11S in content of ½-cystine, methionine, threonine, tryptophan, and glycine; whereas 7S is higher than 11S in content of arginine, phenylalanine, amido ammonia, and carbohydrate. The ½-cystine and methionine contents of 7S globulin were 43 and 57% of those of 11S globulin, respectively. The carbohydrate content of 7S globulin was four times that of 11S. Since these carbohydrates of both proteins were not removed by gel filtration, it is evident that they are not contaminants but are integral with the proteins. Mean MW's of the 7S and 11S globulin residues were 114.7 and 113.0, respectively.

Optical Rotatory Dispersion Analysis

To determine the secondary structure of the 7S and 11S molecules, ORD measurements from the visible to the far-UV region were made. In some preliminary experiments, the effects of protein concentrations, ionic strength, and the addition of the disulfide bond-splitting reagent on the optical rotation in the visible or near visible region were examined. According to these results (Fig. 4), the dependence of protein concentration on ORD was not observed in practice. Further, Fig. 4 shows that no differences of ORD exist, either between the solutions of 0.274 and 0.5 μ in ionic

TABLE I

AMINO ACID COMPOSITION OF 7S AND 11S GLOBULINS OF SOYBEAN

AMINO ACID	AMINO ACID PER 100 G. DRY PROTEIN	AMINO ACID RESIDUES PER 100 G. DRY PROTEIN	NITROGEN CONTENT[a]	RESIDUES IN ONE MOLECULE[b]
	g.	g.	%	
Aspartic acid[c]	13.42	11.60	8.59	181.4
Threonine[d]	2.77	2.34	1.98	41.8
Serine[d]	5.77	4.78	4.68	98.8
Glutamic acid[c]	24.17	21.21	14.00	295.7
Proline[e]	5.38	4.54	3.99	84.2
Glycine[c]	3.55	2.75	4.03	85.1
Alanine[c]	3.84	3.07	3.68	77.8
½-Cystine[f]	0.52	0.44	0.37	7.8
Valine[c]	5.29	4.48	3.86	81.4
Methionine[e]	0.87	0.76	0.49	10.5
Isoleucine[c]	5.81	5.00	3.77	79.5
Leucine[c]	8.87	7.64	5.76	121.6
Tyrosine[d]	3.73	3.36	1.75	37.1
Phenylalanine[c]	6.69	5.97	3.45	72.9
Lysine[c]	7.30	6.40	8.50	89.8
Histidine[e]	2.69	2.38	4.44	31.1
Arginine[e]	9.07	8.13	17.74	93.8
Tryptophan[g]	0.90	0.82	0.74	8.0
Amide NH_3[h]	2.01	–0.12[i]	10.04	212.4[j]
Carbohydrate[k]	4.26	3.38[l]	.....	42.6[j]
Total		99.28	101.86	1,498.3
Mean MW of residues				114.7[m] (116.0[n])

AMINO ACID	AMINO ACID PER 100 G. DRY PROTEIN	AMINO ACID RESIDUES PER 100 G. DRY PROTEIN	NITROGEN CONTENT[a]	RESIDUES IN ONE MOLECULE[b]
	g.	g.	%	
Aspartic acid[c]	13.72	11.86	8.47	360.8
Threonine[d]	4.15	3.52	2.86	121.9
Serine[d]	6.17	5.11	4.82	205.5
Glutamic acid[c]	25.11	22.03	14.02	597.3
Proline[c]	6.21	5.24	4.43	188.8
Glycine[c]	4.96	3.77	5.43	231.2
Alanine[c]	4.27	3.41	3.94	167.8
½-Cystine[f]	1.22	1.04	0.83	35.4
Valine[c]	5.85	4.95	4.11	174.8
Methionine[c]	1.51	1.33	0.83	35.4
Isoleucine[c]	5.53	4.77	3.47	147.6
Leucine[c]	8.40	7.25	5.26	224.1
Tyrosine[d]	4.13	3.72	1.87	79.8
Phenylalanine[c]	6.13	5.46	3.05	129.9
Lysine[c]	6.97	6.11	7.84	166.9
Histidine[d]	2.53	2.24	4.02	57.1
Arginine[c]	8.75	7.85	16.50	175.8
Tryptophan[g]	1.36	1.24	1.10	23.3
Amide NH_3[h]	1.61	–0.09[i]	7.77	331.6[j]
Carbohydrate[k]	0.88	0.79[l]	.....	17.1[j]
Total		101.60	100.62	3,123.4
Mean MW of residues				113.0[m] (113.2[n])

[a] Nitrogen content as percentage, calculated on basis of: 7S, 16.44% and 11S, 17.05% nitrogen in the anhydrous protein.

[b] Molecular weight used for 7S: 180,000 *g*, the value obtained by Koshiyama (private communication). The value obtained by Roberts and Briggs (4), that is, 300,000 *g*, was not used, since it seems abnormal for the sedimentation coefficient of 7S. Molecular weight taken for 11S: 350,000 *g* (see ref. 6).

[c] Value obtained for the 70-hr. hydrolysates.

[d] Extrapolated to zero time from the value obtained for the 22-, 40-, and 70-hr. hydrolysates.

[e] Value obtained for 40-hr. hydrolysates.

[f] Determined as cysteic acid from an acid hydrolysate of performic acid-oxidized protein.

[g] Determined by the dimethylaminobenzaldehyde method.

[h] Value obtained for 3-hr. hydrolysates with 1*N* HCl at 100°C.

[i] Correcting value due to difference of molecular weight between $-CONH_2$ and $-COOH$.

[j] Not counted in adding number of residues.

[k] Determined by the phenol-H_2SO_4 method.

[l] Counted in the total.

[m] Value calculated without counting carbohydrate.

[n] Value calculated by regarding carbohydrate as one of residues.

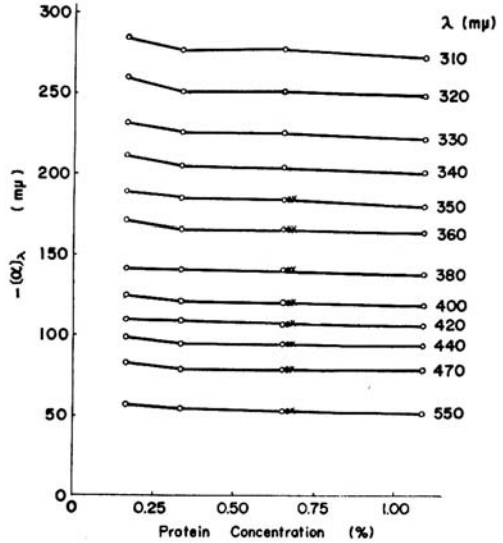


Fig. 4. Effect of protein concentration, ionic strength, and 2-mercaptoethanol on optical rotation in 7S globulin (pH, 7.6). ○, 0.5 μ; ●, 0.274 μ; and x, 0.5 μ buffer containing 0.01*M* 2-mercaptoethanol.

strength, or before and after addition of 2-mercaptoethanol (0.01*M* in final concentration) to the 0.5 μ buffer solution of 7S globulin. This indicates that optical rotation was not influenced in practice by the 7S $\rightleftarrows$ 9S dimerization reaction or by the –SS– polymerization reaction.

Next, the ORD measurements between 300 and 600 mμ were carried out in the presence of various concentrations of urea, followed by calcula-

TABLE II

VALUES OF a_o, b_o, AND λ_c OF 7S AND 11S GLOBULINS IN OPTICAL ROTATORY DISPERSION

	7S GLOBULIN		11S GLOBULIN	
	Native[a]	Denatured[b]	Native[a]	Denatured[b]
λ_c (mμ)	215	212	220	219
$-a_o$ (degree)	280	530	290	240
$-b_o$ (degree)	5	0	30	20

[a] Measured at the protein concentration of 0.6% in 0.1*M* phosphate buffer (pH 7.6).

[b] Measured after denaturation for 20 hr. with 9.0*M* urea solution containing 0.1*M* phosphate buffer (pH 7.6) at the protein concentration of 0.6%.

tion of a_o and b_o values in the Moffitt-Yang equation (Table II, Fig. 5, and Fig. 6). The $-b_o$ values of both the native 7S and 11S globulins are very low; and further, the large change of the a_o values to the negative direction upon urea denaturation occurs without any accompanying substantial change of the b_o values. It is evident from the large changes in $-a_o$ values upon denaturation that the $-b_o$ values near zero cannot be ascribed to compensating effects between right- and left-handed alpha-helices. Such behavior in these 7S and 11S globulins for a_o and b_o values bears a striking resemblance to that of poly-O-acetyl-L-serine having a cross-beta structure (23). This leads

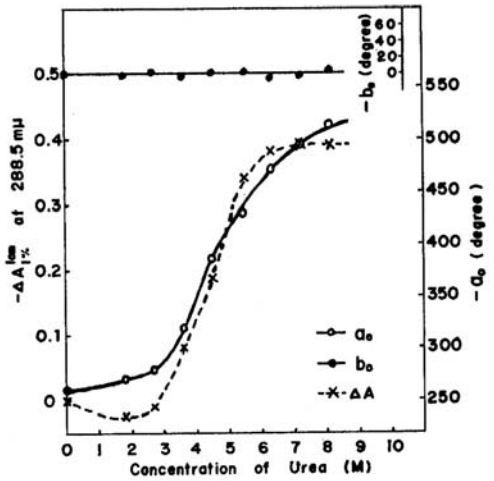


Fig. 5. Effect of urea on change of a_o, b_o, and difference spectra of 7S globulin. Buffer, 0.1*M* $Na_2HPO_4 \cdot NaH_2PO_4$ (pH, 7.6); temperature, 27°C.; time, 60 min.; protein concentration, 0.205%.

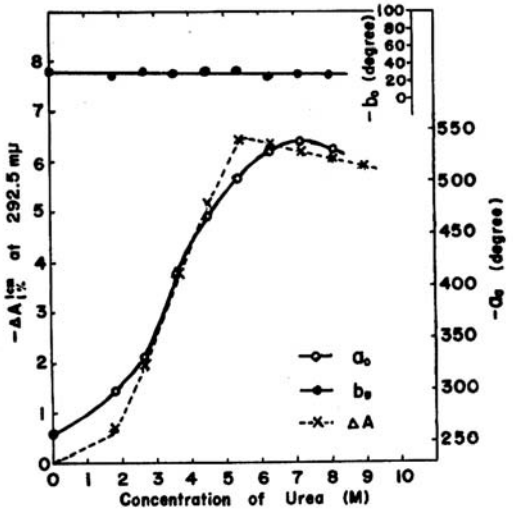
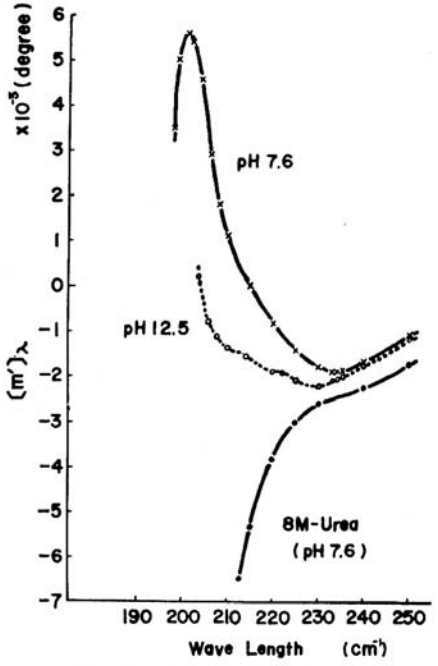


Fig. 6. Effect of urea on change of a_o, b_o, and difference spectra of 11S globulin. Buffer, 0.1*M* $Na_2HPO_4 \cdot NaH_2PO_4$ (pH, 7.6); temperature, 20°C.; time, 60 min.; protein concentration, 0.223%.

us to the idea that there may be an element of cross-beta structure in these proteins. Most recently, the Cotton effects characteristic of the beta-forms have been defined by several workers (24–26). Hence, the ORD in the far-UV region was measured on the 7S globulin. The results are shown in Fig. 7.

The dispersion curve of the native 7S globulin displayed a trough at 233–235 mμ ($[m']_{234\ m\mu}$: −1,900°) and a peak at 200–201 mμ ($[m']_{201\ m\mu}$: +5,500°), but it did not display the shoulder around 210 mμ. The position

and the magnitude of the positive maximum, and the absence of the shoulder around 210 mμ, suggest that these globulin molecules contain little alpha-helix or very small amounts, and that their major structures are beta- and disordered forms. Moreover, it should be noted that the optical rotation at 230–240 mμ increases in the negative direction upon urea denaturation (Fig. 7), in spite of the general phenomenon that the destruction of alpha-helix (if present) or beta-structure by urea, etc., is accompanied by the positive contribution around that wave length. Recently, it has been found in poly-L-glutamic acid (27) and silk fibroin[2] that the contraction of the polypeptide chains in random conformation was accompanied by a decrease in the magnitude of levorotations in both the visible and 230- to 240-mμ regions. Therefore, the disordered structure contained in the 7S globulin molecules may be presumed to be fairly compact. This was ascertained by infrared spectroscopy.



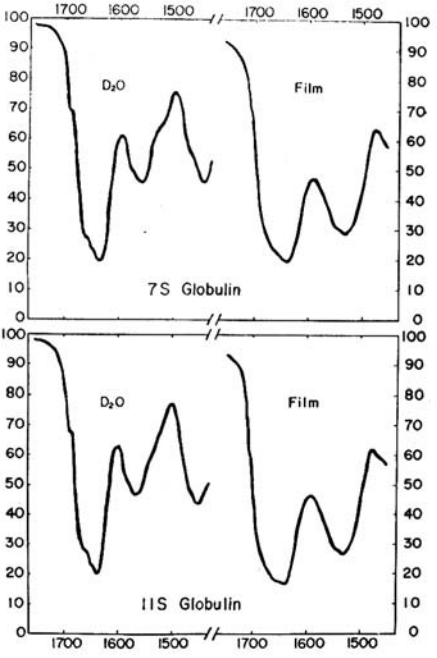


Fig. 7 (left). Reduced mean residue rotation of 7S globulin in 190- to 260-mμ region of wave length. Buffer-free solution of protein concentration of 0.02 to 0.15% was used.

Fig. 8. (right) Amide I and II bands in infrared spectra of 7S and 11S globulins. Protein concentration, 6.0%; pD, 7.0; path length, 0.032 mm.; and μ, 0.5; in D_2O solution. Thickness of film, cast on AgCl plate from buffer-free water solution was about 5 μ. Vertical axis, transmission; horizontal axis, wave number.

Infrared Spectrum Measurements

Infrared spectrum measurements of the 7S and 11S globulins were performed on the amide I, II, and V bands. A shown in Fig. 8, the main

[2] Iizuka, E. Private communication.

peak of the amide I bands was located at 1,638 cm.$^{-1}$; the subpeak was observed at 1,650–1,655 cm.$^{-1}$; and the shoulder was found at 1,685 cm.$^{-1}$, in both proteins. The substantial difference between the liquid and the solid was not observed in the amide I bands, but the difference was marked in the amide II bands (Fig. 8).

To obtain this pD value of the solution, the carboxyl groups in the side chain of the polypeptide are ionized; the ionized carboxyl groups have the absorption band around 1,575 cm.$^{-1}$ (28). This difference between the liquid and the solid in the amide II bands is due mainly to overlapping of ionized carboxyl groups in the solution. Therefore it is necessary, for obtaining the true amide II bands of soybean globulins in solution, to know their background absorption and to subtract its absorbances from those of the apparent amide II bands. It is known generally that the amide II bands between 1,500 and 1,600 cm.$^{-1}$, due to the peptide hydrogens, disappear when the peptide hydrogens are deutrated. This is caused by the shift of the deutrated amide II bands to the wave lengths around 1,450 cm.$^{-1}$ (29,30). In Fig. 9, the absorbances around 1,525 cm.$^{-1}$ decreased when the pD value of the 7S solution was changed to 12.5 from 7.6. This indicates that a part or all of the hard-to-exchange amide hydrogens in the solution at pD 7.6 was deutrated in the solution at pD 12.5. The absorbances of 1,500–1,600 cm.$^{-1}$ at pD 12.5 were regarded as the background absorbances of the amide II bands at pD 7.6, and the former was subtracted from the latter. The difference spectrum between the two showed the curve having one peak around 1,525 cm.$^{-1}$, which is very close to the curve from the solid film (one peak around 1,535 cm.$^{-1}$) (Figs. 8 and 9).

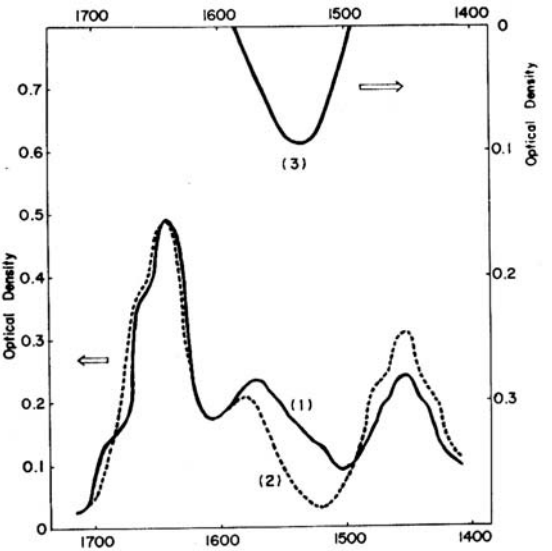


Fig. 9. Difference spectrum of 7S globulin between pD 7.6 and 12.5 solutions on amide II bands. Curve 1, pD 7.6; curve 2, pD 12.5; curve 3, difference spectrum between curve 1 and 2. Protein concentration, 6% and μ, 0.5 (NaCl).

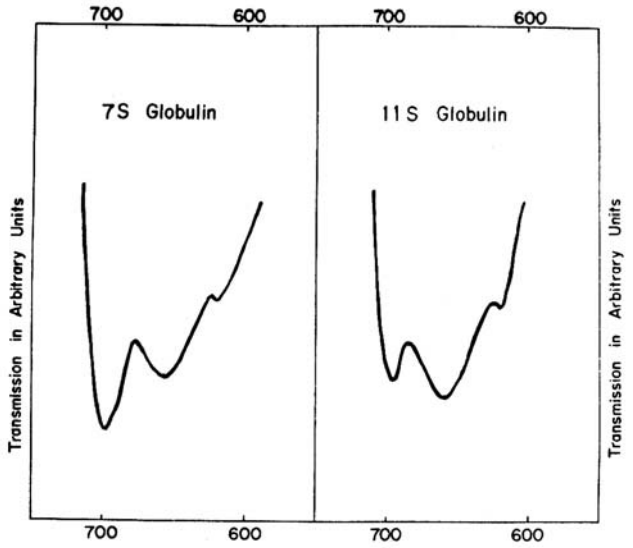


Fig. 10. Amide V bands in infrared spectra of 7S and 11S globulins. Measured with cast film of 15 μ thickness. Horizontal axis, wave number.

On the spectrum of the 400- to 700-cm.$^{-1}$ region in the cast film, two main peaks (698 and 660 cm.$^{-1}$) and one small peak (620 cm.$^{-1}$) appeared (Fig. 10). The intensities of these absorption bands decreased when the film was cast from D_2O solution. Therefore, these bands should be ascribed mainly to the amide V (31). In the 7S globulin, the peak at 698 cm.$^{-1}$ was higher than that at 660 cm.$^{-1}$, but in the 11S globulin, the reverse was true (Fig. 10).

It has been recognized generally that the beta-structure has the characteristic absorption bands at 1,625–1,635 (amide I), 1,520–1,530 (amide II), and 690–700 cm.$^{-1}$ (amide V); the alpha-helix, at 1,650–1,660 (amide I), 1,545–1,550 (amide II), and 610–620 cm.$^{-1}$ (amide V); and the random coil, at about 1,655 (amide I), about 1,535 (amide II), and about 650 cm.$^{-1}$ (amide V); and that the weak band at 1,685–1,705 cm.$^{-1}$ (amide I) should be observed only when the extended polypeptide chains in the beta-structure are antiparallel (31,32). Therefore, it can be strongly concluded from the data of the infrared spectra described above that the major internal structures of 7S and 11S globulins are both an antiparallel beta-structure and a disordered structure. This conclusion is quite compatible with that from the ORD measurements described above. Moreover, it should be mentioned from the results of the infrared and ORD measurements, that the alpha-helix is not a major structure in the internal structure of these protein molecules; the content is very small.

The amide I bands in solution are almost the same as those in film, whereas in the amide II bands there was a slight difference in the position of the main peak between the solution (difference spectrum) and the film (Figs. 8 and 9); this is not the substantial difference, since the background

absorbances in the amide II bands are fairly complicated. These facts indicate that no substantial changes in the internal structure of molecules occurred during the process from liquid to solid.

Next, to ascertain the rigidity of the molecules, the velocities of disappearance of the amide II bands in D_2O solution were investigated. Figure 11 shows the ratio of the amide II to the amide I as a function of time for D_2O solutions of 7S and 11S globulins. Generally, the ratio of the absorbances of the amide II to those of the amide I shows the value of 0.40–0.50 when the peptide groups are not deutrated at all (29). Therefore, it is clear that this ratio decreased rapidly in the first few minutes to 0.18–0.20 from 0.40–0.50 and reached equilibrium after 30 min. The rate of decrease of this ratio is very slow after 30 min. and the extrapolated value for zero time is about 0.17, indicating that about 40% of the peptide hydrogen remained unexchangeable for deuterium in 7S and 11S globulins.

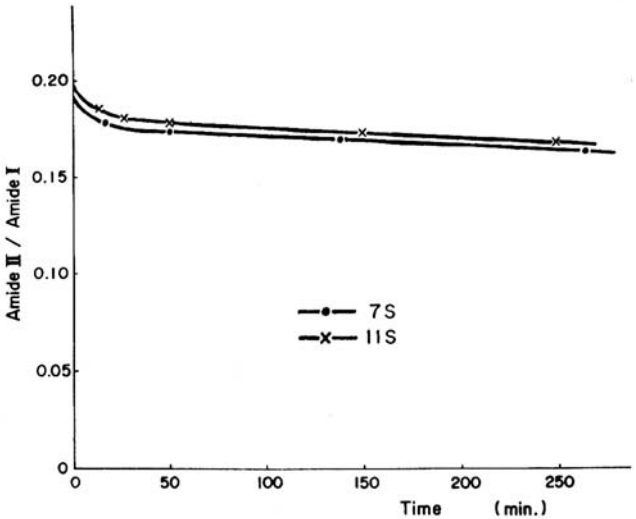


Fig. 11. Rate of D-H exchange of 7S and 11S globulins. Protein concentration, 6%; pD, 7.6; and μ, 0.5 (NaCl). The solution of pD 12.5 was used for determination of the background absorption of the amide II.

In Fig. 11, the absorbances of the solution at pD 12.5 were regarded as the background absorbances of the amide II bands of these protein solutions at pD 7.6, but their values may be overestimated as the background absorbances, because of imperfect denaturation of proteins at pD 12.4 as shown in Fig. 7. Hence, the true value of the ratio at the equilibrium state may be a little higher than that shown in Fig. 11. On the other hand, it is known that the D–H exchange reaction is strongly pH-dependent, and above neutral pH the exchange occurs even for the peptide hydrogen fixed in the secondary or tertiary structure (30,33). This suggests the possibility that much more peptide hydrogens than those presumed from the data at pD in Fig. 11 are trapped in the secondary or the tertiary structure. Thus, the native protein molecules in both 7S and 11S seem

to be quite compact and even in their randomly folded parts, which comprise a very large part of the internal structure of a molecule. Their randomly folded peptide chains are probably buried in the hydrophobic region, which is not penetrated by heavy water. This presumption, that the randomly folded structure in those globulin molecules is compact, is compatible with that from the ORD data described above.

Ultraviolet Difference Spectrum Measurements

The tertiary structure of these protein molecules was investigated through measurement of the UV difference spectra on urea solution. As shown in Fig. 12, both proteins indicated the typical denaturation blue shift on urea

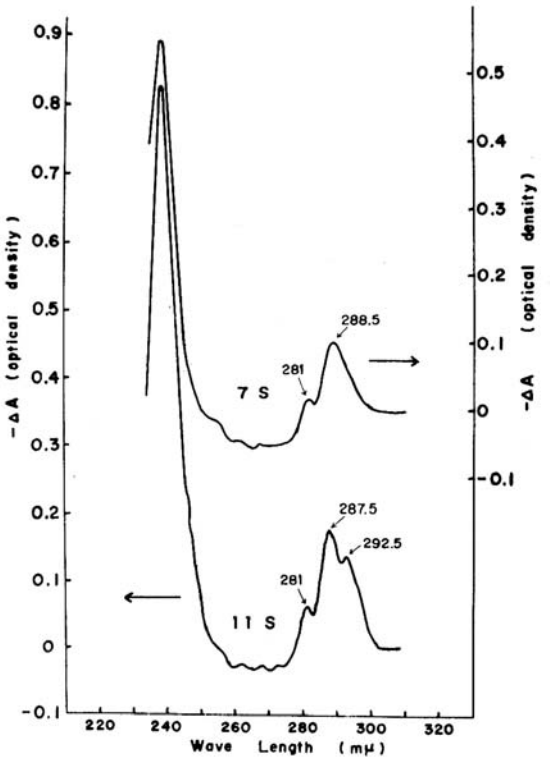


Fig. 12. Ultraviolet difference spectra of 7S and 11S globulins in 6.4 mol urea containing buffer *vs.* in buffer. Buffer, 0.1*M* $Na_2HPO_4 \cdot NaH_2PO_4$ (pH 7.6); protein concentration, 0.2%.

denaturation. In 7S globulin the negative peaks existed at 238, 281, and 288.5 mμ; in the 11S globulin they existed at 238, 281, 287.5, and 292.5 mμ. Recently, it has been recognized universally that the negative peaks at 285–288 and 287–280 mμ should be ascribed to changes in environment of tyrosine and those at 291–294 and 284–285 mμ to the tryptophan, although the strong negative peak at 230–236 mμ has not yet been assigned (34,35). Therefore, it is concluded that in the 7S globulin the tyrosine residue is buried in the hydrophobic region of the molecule, although the

tryptophan residue would be located on the surface of the molecule; whereas, in the case of the 11S globulin, both the tyrosine and tryptophan residues are buried in the interior of the molecule. This suggests that the antiparallel beta- or randomly folded chains are further folded and, as a whole, a fairly compact tertiary structure is formed, which is a hydrophobic domain impenetrable by water.

Next, difference spectra were measured in the presence of various urea concentrations. Figures 5 and 6 compare the a_0 values and the absorbances at the peaks of the difference spectra (7S, 288.5 mμ; 11S, 292.5 mμ) as a function of urea concentration. Results for the other peaks are not shown, because they showed almost the same tendency. According to these figures, the changes of a_0 values of the Moffitt-Yang equation in the ORD occur in parallel with changes of the absorbances in the difference spectra in both the 7S globulin and 11S globulin, indicating that, if the tertiary structure is broken, the secondary structure is also broken at the same time.

Role of Hydrophobic Forces

To ascertain the contribution of hydrophobic forces to these internal structures, the denaturation abilities of the lower alcohols to 7S or 11S globulin were examined systematically. Aliquots of the samples (35 mg.) were suspended in 9.45 ml. of phosphate buffer solutions containing various amounts of the lower alcohols up to 2.5 moles in the final alcohol concentrations (0.045*M* Na_2HPO_4-NaH_2PO_4, pH 7.6). They were heated and then dried by aeration at room temperature, and the amounts of the native protein remaining in the samples thus treated were measured. The results are shown in Figs. 13 and 14.

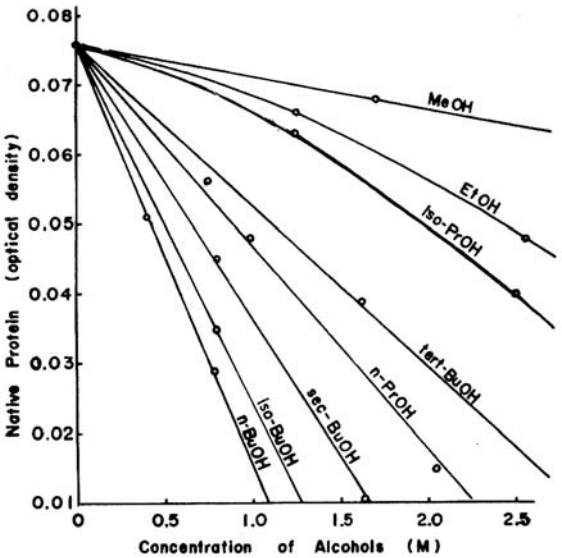


Fig. 13. Effect of alcohols on denaturation of 7S globulin. Denaturation condition: buffer, M/45 $Na_2HPO_4 \cdot NaH_2PO_4$; pH, 7.6; temperature, 60°C.; time, 60 min.

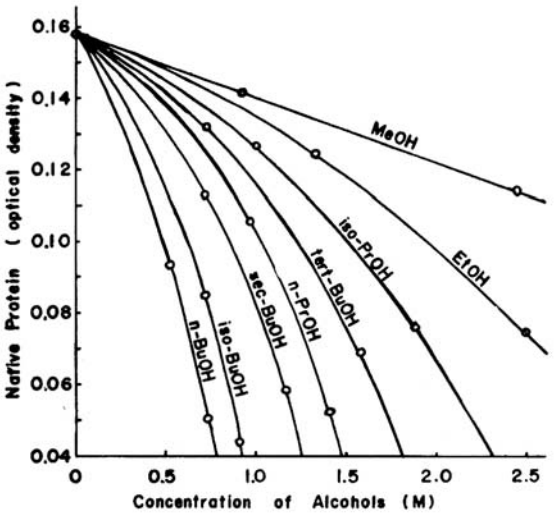


Fig. 14. Effect of alcohols on denaturation of 11S globulin. Denaturation condition: buffer, M/45 $Na_2HPO_4 \cdot NaH_2PO_4$; pH, 7.6; temperature, 70°C.; time, 60 min.

As shown in these graphs, the denaturation abilities depended completely upon the order of the hydrophobicity of the lower alcohols used, both in 7S and 11S. In other words, the ability to disrupt the internal structures of these molecules decreased with the number of the branches in the molecule of the alcohols used; whereas they increased with the length of the straight $-CH_2-$ chain, with the distance of the branching position for the alpha-carbon atom, and with the hydrocarbon contents, in the alcohol molecules. These facts strongly support the belief that the internal structures of the native molecules of both 7S and 11S globulin are stabilized by hydrophobic bonds.

Relation between Molecular Conformation and Proteinase Attack

When the protein molecules are folded compactly, they would be expected not to be hydrolyzed at all by proteinases, before the internal structure of the molecules is broken to a flexible form. Moreover, it is expected that there will exist a proportional relation between initial velocity for proteinase hydrolysis and degree of disruption of the internal structure. To ascertain this, the following experiments were carried out. Phosphate buffer (0.1*M*, 5 ml.), containing the various amounts of alkali, was added to 100 mg. of the 11S preparations and kept at various alkaline pH values for 90 min. at room temperature. Thereafter, the resulting mixtures were neutralized to pH 7.6 and centrifuged. The supernatant extracts containing the proteins which were partly denatured to various degrees were adjusted to a definite concentration and hydrolyzed by the purified alkaline proteinase of *A. sojae* (17), by the method described in "Material and Methods." At the same time, the ORD of these substrates was measured, and the a_0 values in the Moffitt-Yang equation were calculated. The degree of disruption of the internal structure (Δa_0) was expressed as the ratio (%)

of the change of a_o value to that of the complete disruption, that is, complete denaturation by 8*M* urea, as shown in the following equation:

$$\Delta a_o = (a_o^{\text{sample}} - a_o^{\text{native}}) / (a_o^{\text{urea}} - a_o^{\text{native}})$$

The results are shown in Figs. 15 and 16.

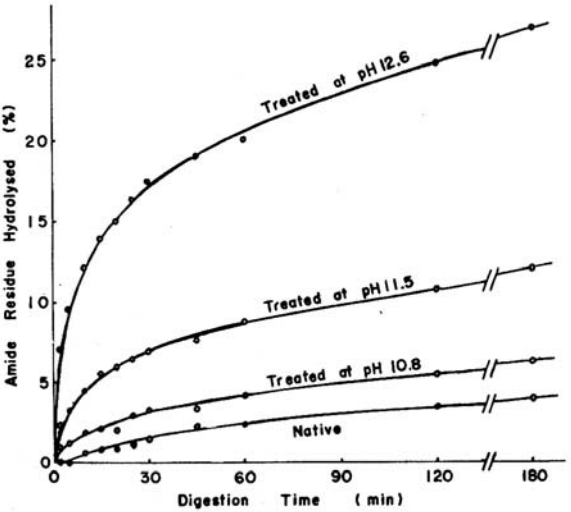


Fig. 15. Enzymatic hydrolysis of partially denatured 11S globulin. Substrates were neutralized to pH 7.6 after treatment at various pH values for 90 min. at 20°C. and hydrolyzed by alkaline proteinase of *Aspergillus sojae* at 30°C., pH 7.6. Details are given in text.

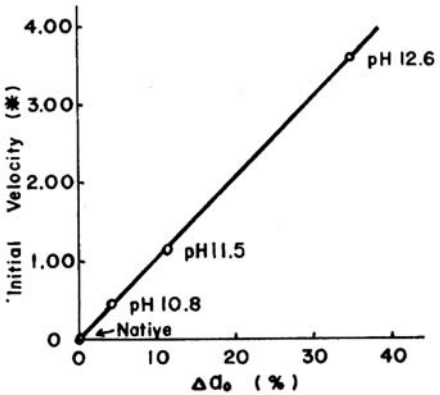


Fig. 16. Relation between Δa_o and proteinase susceptibility of soybean 11S globulin. (*): percent of peptide bonds hydrolyzed in the first minute by alkaline proteinase of *Aspergillus sojae*. The explanation of Δa_o is given in the text.

The native 11S globulin was not hydrolyzed at all during the initial several minutes of digestion time, whereas the partly denatured globulins were hydrolyzed easily; the higher the alkalinity of their treatment, the more they were hydrolyzed (Fig. 15). Moreover, there is a linearity between the initial velocities of the hydrolysis and the change of a_o value (Δa_o

indicates the degree of disruption of the secondary and tertiary structure) (Fig. 16). These results suggest that the native soybean proteins possess a fairly compact internal structure as a whole, in which most of the peptide bonds to be hydrolyzed are buried. When the closely packed peptide chains are unfolded upon denaturation, their atomic groups which serve as points of the combination with active centers of the proteinase become accessible to the proteinase molecules. As a result, the substrates are hydrolyzed in proportion to the degree of denaturation of the substrate protein molecules.

The hydrolysis seems to occur step by step, especially in the native protein, as is understood by detailed observation of the spots along the curves in Fig. 15. This could be explained as follows. When a peptide bond located at the surface of the tightly folded molecule is hydrolyzed, the molecule would be partly unfolded in the form close to a random coil once, and then it would be refolded in the new conformation after a certain time. This would lead the protein hydrolysis by proteinase into step-by-step degradation. Such a phenomenon is also found in ovalbumin (36).

CONCLUSION

On the basis of the results of the ORD through the visible, near-visible, and far-UV regions of the infrared spectra in the amide I, II, and V bands, of the ultraviolet difference spectra on urea denaturation, etc., the author concluded that 1) the major internal structures of both 7S and 11S globulins of soybeans are not alpha-helix, but are both the antiparallel beta-structures and the disordered structures; 2) the molecules are fairly compact as a whole and even in their disordered parts; 3) the molecules are folded tertiarily and the water-impenetrable hydrophobic region is formed; 4) the hydrophobic bonds play an important role for stabilization of the internal structure; and 5) the molecules are not hydrolyzed by proteinase before the internal structure is disrupted, and the initial velocity of the hydrolysis by proteinases is proportional to the degree of its disruption.

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Literature Cited

1. NAISMITH, W. E. F. Ultracentrifuge studies on soya bean protein. Biochim. Biophys. Acta 16: 203–210 (1955).
2. WOLF, W. J., RACKIS, J. J., SMITH, A. K., SASAME, H. A., and BABCOCK, G. E. Behavior of 11S protein of soybean in acid solutions. I. Effects of pH, ionic strength and time on ultracentrifugal and optical rotatory properties. J. Am. Chem. Soc. 80: 5730–5734 (1958).
3. WOLF, W. J., and BRIGGS, D. R. Studies on the cold-insoluble fraction of the water-extractable soybean proteins. II. Factors influencing conformation changes in the 11S component. Arch. Biochem. Biophys. 76: 377–393 (1958).
4. ROBERTS, R. C., and BRIGGS, D. R. Isolation and characterization of the 7S component of soybean globulins. Cereal Chem. 42: 71–85 (1965).

5. BRIGGS, D. R., and WOLF, W. J. Studies on the cold-insoluble fraction of the water-extractable soybean proteins. I. Polymerization of the 11S component through reactions of sulfhydryl groups to form disulfide bonds. Arch. Biochem. Biophys. 72: 127–144 (1947).
6. WOLF, W. J., and BRIGGS, D. R. Purification and characterization of the 11S component of soybean proteins. Arch. Biochem. Biophys. 85: 186–199 (1959).
7. WEINTRAUB, I. A. N-terminal amino acids of soybean seed globulin. Trans. Chim. Natural Compounds, Kishinev State Univ. (Russia), No. 4 (1961).
8. MITSUDA, H., KUSANO, T., and HASEGAWA, K. Purification of the 11S component of soybean proteins. Agr. Biol. Chem. (Tokyo) 29: 7–12 (1964).
9. MOORE, S., SPACKMAN, D. H., and STEIN, W. H. Chromatography of amino acids on sulfonated polystyrene resins. Anal. Chem. 30: 1185–1195 (1958).
10. SPIES, J. R., and CHAMBERS, D. C. Chemical determination of tryptophan. Anal. Chem. 20: 30–39 (1948).
11. SPIES, J. R., and CHAMBERS, D. C. Chemical determination of tryptophan in proteins. Anal. Chem. 21: 1249–1266 (1949).
12. HIRS, C. H. W. The oxidation of ribonuclease with performic acid. J. Biol. Chem. 219: 611–621 (1956).
13. DUBOIS, M., GILLES, K. A., HAMILTON, J. K., REBERS, P. A., and SMITH, F. Colorimetric method for determination of sugars and related substances. Anal. Chem. 28: 350–356 (1956).
14. MOFFITT, W., and YANG, J. T. The optical rotatory dispersion of simple polypeptides. Proc. Nat. Acad. Sci. (U. S.) 42: 596–603 (1956).
15. FUKUSHIMA, D. Studies on soybean proteins. I. Water dispersibility of protein of defatted soybean flour as a criterion for degree of denaturation. Bull. Agr. Chem. Soc. Japan [*now* Agr. Biol. Chem., Tokyo] 23: 7–14 (1959).
16. FUKUSHIMA, D. Studies on soybean proteins. II. A new method for quantitative determination of the degree of denaturation of protein in soybean flour. Bull. Agr. Chem. Soc. Japan [*now* Agr. Biol. Chem., Tokyo] 23: 15–21 (1959).
17. MIZUNUMA, T., and IGUCHI, N. Purification of alkaline proteinase of *Aspergillus sojae* by ion exchange resin. Bull. Agr. Chem. Soc. Japan [*now* Agr. Biol. Chem., Tokyo] 22: 35–38 (1958).
18. SATAKE, K., OKUYAMA, T., OHASHI, M., and SHINODA, T. The spectrophotometric determination of amine, amino acid and peptide with 2,4,6-trinitrobenzene-1-sulfonic acid. J. Biochem. (Tokyo) 47: 654–660 (1960).
19. BRIGGS, D. R., and MANN, R. L. An electrophoretic analysis of soybean protein. Cereal Chem. 27: 243–260 (1950).
20. SMITH, A. K., and RACKIS, J. J. Phytin elimination in soybean protein isolation. J. Am. Chem. Soc. 79: 633–637 (1957).
21. VAN ETTEN, C. H., HUBBARD, J. E., MALLAN, J. M., SMITH, A. K., and BLESSIN, C. W. Amino acid composition of soybean protein fractions. J. Agr. Food Chem. 7: 129–131 (1959).
22. RACKIS, J. J., ANDERSON, R. L., SASAME, H. A., SMITH, A. K., and VAN ETTEN, C. H. Amino acid in soybean hulls and oil meal fractions. J. Agr. Food Chem. 9: 409–412 (1961).
23. IMAHORI, K., and YAHARA, I. Optical properties and conformations of poly-O-acetyl-L-serine and poly-L-serine. Biopolymers Symposia No. 1: 421–432 (1964).
24. IIZUKA, E., and YANG, J. T. Optical rotatory dispersion and circular dichroism of the β-form of silk fibroin in solution. Proc. Nat. Acad. Sci. 55: 1175–1182 (1966).
25. SARKAR, P. K., and DOTY, P. The optical rotatory properties of the β-configuration in polypeptides and proteins. Proc. Nat. Acad. Sci. 55: 981–989 (1966).
26. DAVIDSON, B., TOONEY, N., and FASMAN, G. D. The optical rotatory dispersion of the β structure of poly-L-lysine and poly-L-serine. Biophys. Res. Comm. 23: 156–162 (1966).
27. IIZUKA, E., and YANG, J. T. Effect of salts and dioxane on the coiled conformation of poly-L-glutamic acid in aqueous solution. Biochemistry 4: 1249–1257 (1965).
28. BELLAMY, L. J. The infrared spectra of complex molecules (2nd ed.), pp. 174–176. Wiley: New York (1958).
29. BLOUT, E. R., LOZÉ, C. D., and ASADOURIAN, A. The deuterium exchange of water-soluble polypeptide and proteins as measured by infrared spectroscopy. J. Am. Chem. Soc. 83: 1895 (1961).
30. HVIDT, A. The deuterium exchange of lysozyme as followed by an infrared spectrophotometric method. Compt. Rend. Trav. Lab. Carlsberg 33: 475–495 (1963).

31. MIYAZAWA, T., MASUDA, Y., and FUKUSHIMA, K. Chain conformation and amide V band of polypeptides. J. Polymer Sci. 62: S62–S64 (1962).
32. MIYAZAWA, T., and BLOUT, E. R. The infrared spectra of polypeptides in various conformational amide I and II bands. J. Am. Chem. Soc. 83: 712–719 (1961).
33. HVIDT, A., and KANAREK, L. The deuterium exchange of lysozyme as followed by the gradient method. Compt. Rend. Trav. Lab. Carlsberg 33: 463-474 (1963).
34. GLAZER, A. N., and SMITH, E. L. Studies on the ultraviolet difference spectra of proteins and polypeptides. J. Biol. Chem. 236: 2942–2947 (1961).
35. EISENBERG, D. S., and EDSALL, J. T. Spectrophotometric titrations of human serum albumin and reduced carboxymethylated albumin. Science 142: 50–51 (1963).
36. SATAKE, K., SAKAGAWA, S., and HONDA, M. Partial hydrolysis of ovalbumin. Abstr., Proc. Symp. on Protein Structures, Japan (1964), pp. 60–63.

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