Major Proteins of Soybean Seeds. A Straightforward Fractionation and Their Characterization

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Adjusting the pH of the dilute tris(hydroxymethyl)aminomethane buffer extract of defatted soybean meal to 6.6 causes precipitation of the 11S globulin. Factors affecting the precipitation (namely, pH, Tris concentration, protein concentration, and ionic strength) were investigated in order to determine the optimum condition for simultaneous fractionation of the 7S and 11S globulins. It was found that the two globulins could be fractionated by their different solubilities in dilute Tris buffers. Protein concentration and NaCl (up to 0.04 M) had no noticeable effects on the separation of the two globulins. These results led to a simple, large-scale method for fractionation of the two globulins and whey proteins. Defatted soybean meal was extracted with 0.03 M Tris-HCl buffer (pH 8.0). The 11S globulin was precipitated at pH 6.4. The 7S globulin was separated from whey proteins by isoelectric precipitation at pH 4.8. Ultracentrifugal analyses, disc electrophoresis, and immunodiffusion indicated very little cross-contamination between the prepared fractions.

The two major reserve soybean proteins, 7S and 11S globulins, have been isolated by several methods (Wolf et al., 1962; Roberts and Briggs, 1965; Eldridge and Wolf, 1967; Koshiyama, 1965, 1972a,b). But none of these methods presented a straightforward simultaneous preparation of the two globulins; neither afforded a large-scale isolation of the two major proteins without cross-contamination. Recent studies revealed that the 7S soybean globulin capable of dimerization (conversion into the 9S sedimenting form) at 0.1 ionic strength is composed of at least three (Hill and Breidenbach, 1974) or five components (Thanh et al., 1975a,b). Thus, an effective isolation of the 7S globulin which ensures the presence of all of the individual 7S components would be of interest for further investigations and for the elucidation of conflicting data on the homogeneity of the major 7S globulin and the relative electrophoretic mobility of the 7S and 11S globulins by disc electrophoresis (Koshiyama, 1968; Hill and Breidenbach, 1974; Thanh et al., 1975a; Catsimpoolas et al., 1968a; Catsimpoolas and Ekenstam, 1969).

In a recent communication, a procedure for fractionation of the 7S and 11S globulins has been developed (Thanh et al., 1975b). This method was based on different solubilities of the two globulins in dilute tris(hydroxymethyl)aminomethane buffers at pH 6.6. The present paper deals with factors which account for the simultaneous fractionation of the two globulins, namely, pH, Tris concentration, and ionic strength. From these results, a simple, practical-scale isolation of the two globulins is described. Partial characterizations of the two globulins and whey proteins by ultracentrifuge, disc electrophoresis, and immunodiffusion are also presented.

MATERIALS AND METHODS

Protein Samples. The 7S and 11S globulins were isolated from soybean (*Glycine max* var. Raiden) by the method previously described (Thanh et al., 1975b). Ultracentrifugal analysis showed very little cross-contamination of the two fractions. The isolated globulins were dissolved in 0.30 M Tris-HCl buffer containing 0.01 M 2-mercaptoethanol (ME), pH 8.0. The protein solutions (0.2%) were centrifuged (10 000 rpm, 20 min). No significant precipitates were detected. All the protein solutions used in this investigation contained 0.01 M ME and experiments were carried out at room temperature (20 to 22 °C).

Precipitation of the 7S and 11S Globulins as a Function of pH. The protein solutions were diluted to 0.02%. Final Tris concentrations (0.03, 0.06, 0.10, and 0.20 M) were adjusted with concentrated Tris-HCl buffer (0.60 M Tris, pH 8.0). Turbidity of protein solutions at various pH values (pH adjusted with the appropriate HCl solution) was read at 600 nm.

Precipitation at Various Protein Concentrations. The 7S globulin was dissolved in 0.06 M Tris-HCl buffer, pH 8.0 (protein concentration varied from 0.02 to 4%). Turbidity of protein solutions at pH values between 5.7 and 6.6 was recorded. The 11S globulin solutions (0.02-3% protein in Tris-HCl buffer, pH 8.0) were adjusted to pH 6.4. After 30 min, the protein solutions were centrifuged (10 000 rpm, 20 min). Protein concentration in the supernatant was calculated using the value of $E_{280nm}^{1\%,1cm}$ = 8.04 (Koshiyama, 1972b).

Influence of Ionic Strength on the Precipitation of the 11S Globulin. The 11S globulin solution (0.02%) in dilute Tris buffer (0.03 and 0.06 M, pH 8.0) containing NaCl in various concentrations (up to 0.25 M) was adjusted to pH 6.4. Turbidity of the protein solutions was recorded.

Ultracentrifugal Analysis. Ultracentrifugal analysis was carried out at room temperature with a Hitachi UCA-1 ultracentrifuge at 55 430 rpm in the standard phosphate buffer (2.6 mM KH_2PO_4, 32.5 mM K_2HPO_4, 0.4 M NaCl, 0.01 M mercaptoethanol, pH 7.6, μ = 0.5) and the phosphate buffer (the same buffer without NaCl, μ = 0.1).

Disc Electrophoresis. The Ornstein–Davis disc electrophoresis (Ornstein, 1964; Davis, 1964) with 5% separating gel containing 0.02 M mercaptoethanol and 3.5% spacer gel (15% bisacrylamide) was carried out using 5 × 100 mm glass tubes. The samples were prepared as follows: the supernatant fractions were diluted with the phosphate buffer made 20% in sucrose to a concentration of 0.4% protein (total soybean proteins) or 0.2% (7S and 11S globulins). Whey proteins (freeze-dried sample) were dissolved in the buffer (4 mg/ml) containing sucrose. About 20 μl of the prepared samples was placed between two layers of spacer gel. Electrophoresis was conducted for 1.5 h with a current of 3 mA per gel column. Polyacrylamide gels were stained with Amido Black 10 B and destained by diffusion in MeOH–CH$_3$COOH–water (20:7:73, v/v). The destained gels were scanned at 550 nm

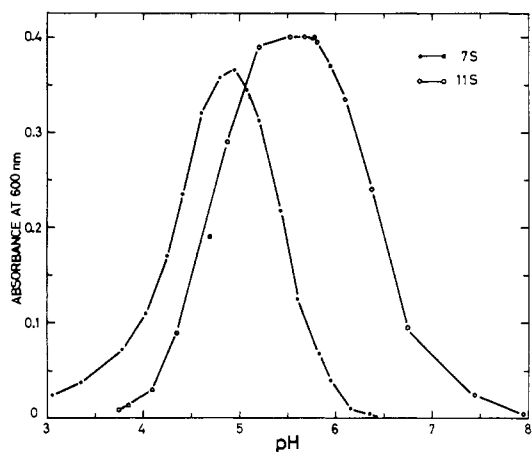Department of Food Chemistry, Faculty of Agriculture, Tohoku University, Sendai, Japan.

Figure 1. pH-dependent precipitation curves of the 7S and 11S globulins in 0.06 M Tris-HCl buffer. Protein concentration was 0.02%.
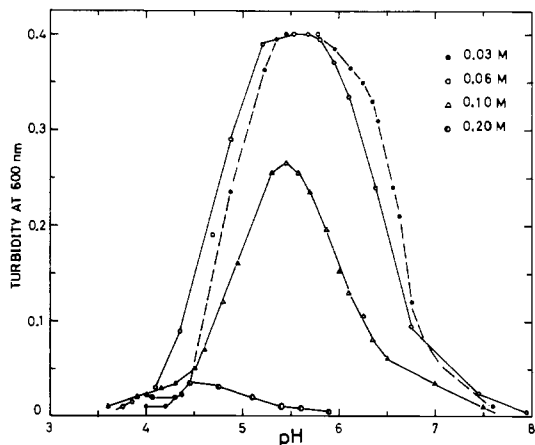

Figure 2. pH-dependent precipitation curves of the 11S globulin in various concentrations of Tris buffers. Protein concentration was 0.02%.
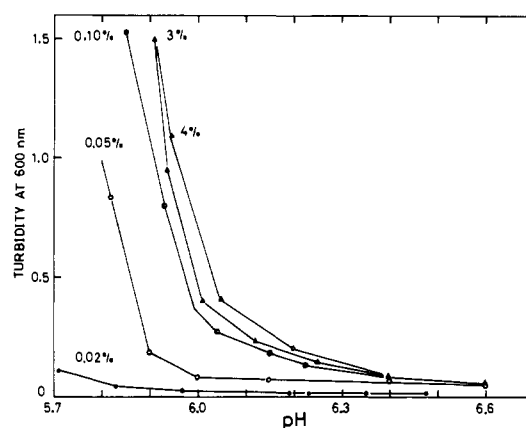

Figure 3. pH-dependent precipitation curves of the 7S globulin in 0.06 M Tris buffer. Protein concentration was: (●) 0.02%; (○) 0.05%; (◐) 0.10%; (▲) 3%; (△) 4%.
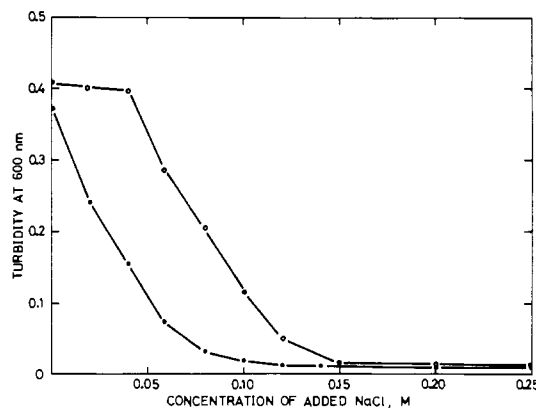

Figure 4. Effect of NaCl on the solubility of the 11S globulin in 0.03 M Tris buffer (○) and 0.06 M Tris buffer (●). The protein concentration was 0.02%.

on a Gilford linear transport attachment to the Beckman DU monochromator.

Double Gel Immunodiffusion. Antisera to the water-extractable proteins and the 7S and 11S globulins were prepared by the procedure of Catsimpoolas et al. (1968a,b). Double gel immunodiffusion in agar was carried out according to the method of Ouchterlony (1949). The gel medium consisted of 1% agar in the standard phosphate buffer containing 0.05% NaN$_3$, pH 7.6. Protein samples were dissolved in the standard buffer. The reactants were allowed to diffuse at room temperature for 24 h and results were recorded photographically.

RESULTS

Factors Affecting Differential Solubility of the 7S and 11S Globulins. Figure 1 shows the pH-dependent precipitation curves of the two globulins in 0.06 M Tris-HCl buffer. It is clear that the two globulins behaved differently: the 7S globulin precipitated at pH values between 4 and 5.6, and the 11S globulin, between 4.4 and 6.8. In the pH 6.1 to 6.6 region, the 7S globulin dissolved, while most of the 11S globulin precipitated. The reported isolation of the two globulins (Thanh et al., 1975b) was based on this result: they were separated by dialysis against 0.06 M Tris-HCl, pH 6.6.

As the Tris concentration decreased from 0.06 to 0.03 M, there were no remarkable changes in the pH-dependent precipitation behavior of the 11S globulin (Figure 2). But the 11S globulin became more soluble in the 0.1 and 0.2

M Tris buffers. This result indicated that the Tris concentration should not exceed 0.06 M for quantitative precipitation of the 11S globulin. The 7S globulin was quite soluble in 0.03 and 0.06 M Tris buffers at pH greater than 6.0.

The solubility of the 7S globulin at pH greater than 6.0 was independent of protein concentration (Figure 3). The recovery of 11S globulin by precipitation at pH 6.4 increased from 63 to 85% as the protein concentration increased from 0.05 to 1%. At protein concentrations greater than 1%, 85 to 90% of the 11S globulin was precipitated at pH 6.4. This indicates that high protein concentration (up to 4%) is favorable for the resolution of the two globulins.

Adding NaCl to the 11S globulin solution (0.02% protein in 0.06 M Tris, pH 6.1) increased its solubility drastically (Figure 6). At 0.1 M NaCl the 11S globulin was quite dissolved. In the case of the protein solution containing only 0.03 M Tris, NaCl up to 0.04 M (ionic strength = 0.07) did not affect the precipitation of the 11S globulin at pH 6.1 (Figure 4).

Simple, Practical-Scale Isolation of the 7S and 11S Globulins. The above results indicate that the two globulins can be simultaneously fractionated based on their different solubilities in dilute Tris buffers (0.03 or 0.06 M) in the pH 6.1 to 6.6 region (optimum pH for their resolution was 6.2–6.4). The protein concentration has no obvious interference and the ionic strength should be less than 0.07. A simple method is outlined in Figure 5. Defatted soybean meal is extracted with 0.03 M Tris buffer (pH 8.0) at room temperature for 1 h. The extract is

Table I. Yield and Ultracentrifugal Composition of the Isolated Fractions

Isolated fractions	Pattern figure	Fraction of total protein,[a] %	Ultracentrifugal composition, %						Yield,[b] %	
			2S	7S			11S	≥15S	7S	11S
				[7S]	7S	7S				
Buffer extract[c]		100	16	7	41	4	31	1	100	100
11S globulin	6A	36	9			10	78	3		92
	6A′	24					57	19		
7S and whey proteins	6B	64	20		76[e]		4		100	8
	6B′			11	65					
7S globulin	6C	44	14		86[e]				91	
	6C′				86					
Polymerized form[d]		6								
Whey proteins		14								

[a] Based on dry weight of the isolated fractions. [b] Calculated from fraction of total protein and ultracentrifugal composition. [c] Based on ultracentrifugal composition of the 11S globulin and the 7S–whey protein fractions. [d] About 33% of soybean proteins was irreversibly insolubilized by precipitation at pH 4.5 (Wolf and Sly, 1965). This acid-sensitive fraction was composed mainly of 2S and nondimerizing 7S (Anderson, 1974). In this investigation, precipitation of the 7S globulin at pH 4.8 resulted in the polymerization of only 6% of the total extractable proteins. [e] Ultracentrifugal composition of [7S] and 7S.
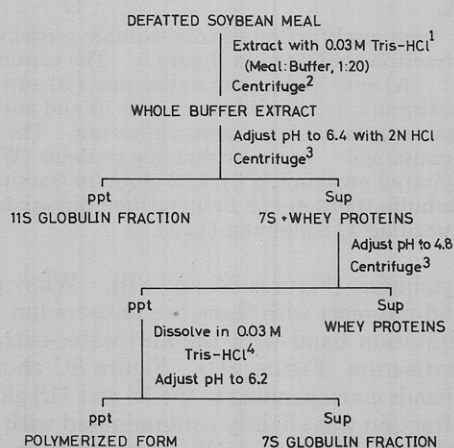

Figure 5. Simple, practical-scale method for the simultaneous isolation of the 7S and 11S globulins: superscript 1, extract with 0.03 M Tris buffer (pH 8.0) containing 0.01 M mercaptoethanol at room temperature (20 to 22 °C) for 1 h; superscript 2, centrifuge (10 000 rpm, 20 min, 20 °C); superscript 3, centrifuge (2–5 °C); superscript 4, add 2 N NaOH dropwise while stirring until the protein dissolves (pH 7.6), then adjust to pH 6.2 with 2 N HCl.
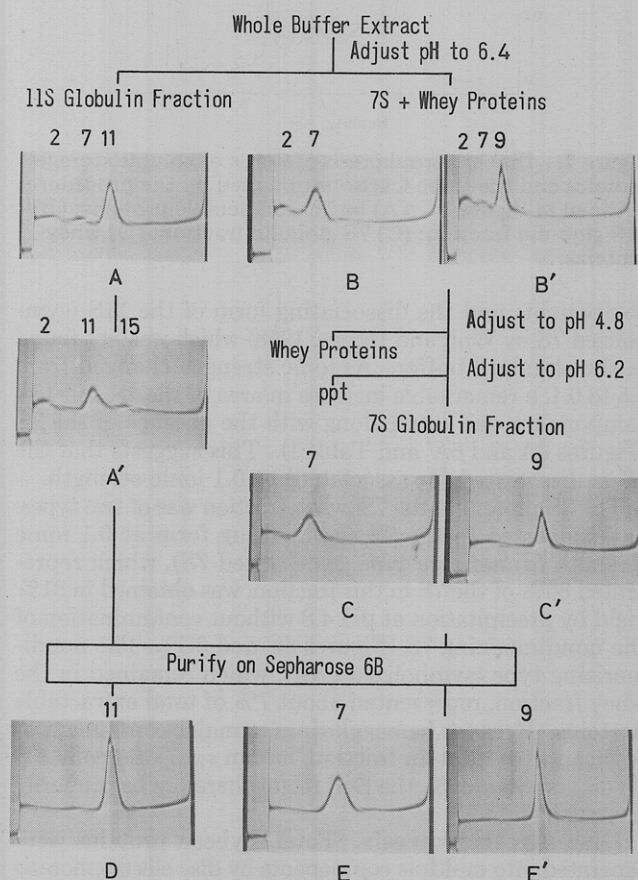

Figure 6. Ultracentrifugal analyses of the isolated fractions in 0.5 ionic strength standard buffer (A, B, C, D, and E) and 0.1 ionic strength phosphate buffer (A′, B′, C′, and E′). The protein concentration was 0.8%. Photographs were taken after 52 min of centrifugation (except for Figures 6A′ and 6B′, 35 min) at 55 430 rpm. Direction of sedimentation is from left to right. Fractions are identified in Svedberg units across the top of the figures.

adjusted to pH 6.4. The 11S globulin is collected by centrifugation. The 7S globulin is separated from the whey proteins by isoelectric precipitation at pH 4.8.

The prepared 11S globulin was washed with pH 6.4 Tris buffer, and then dispersed in the standard buffer (protein concentration, 2–3%). NaOH solution was added dropwise while stirring until the protein dissolved (pH 7.8). The protein solution was kept at 3–5 °C overnight. A trace of precipitate was removed by centrifugation. The 7S globulin solution was adjusted to pH 7.8 before dialysis. Whey fraction (supernatant) was kept overnight at 3–5 °C (pH 8.0) to precipitate phytate (Smith and Rackis, 1957). After clarification by centrifugation, whey proteins were concentrated by full saturation with ammonium sulfate.

Ultracentrifugal Study. Ultracentrifugal analyses are shown in Figure 6. The 11S fraction contained primarily 11S globulin (Figures 6A and 6A′). The 7S–whey fraction was nearly devoid of the 11S globulin (Figures 6B and 6B′). Concentration of the 7S globulin at pH 4.8 resulted in the significant decrease of the 2S fraction (Figures 6C and 6C′). The purified 7S and 11S globulins (Figures 6D, 6E, and 6E′) were obtained by gel filtration on Sepharose 6B.

The yields of the prepared fractions and their ultracentrifugal composition are given in Table I. The total amount of proteins extractable with 0.03 M Tris-HCl (pH

8.0) was 42.6 g per 100 g of defatted soybean meal. Thirty-six percent of these proteins was found in the 11S globulin fraction. This fraction contained about 10% of 7S. However, neither 7S nor the 9S sedimenting form was found when analysis was carried out at 0.1 ionic strength (Figure 8A′). Therefore, the 7S in this fraction, which was different from the 7S globulin which remained soluble in Tris buffer at pH 6.4, might be either a dissociating form of the 11S or another kind of the 7S globulin. It is symbolized as 7S in order to differentiate it from other 7S
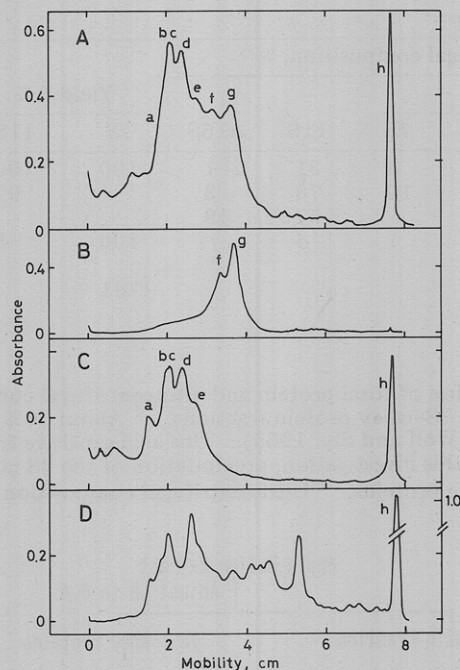
Figure 7. Disc electrophoresis patterns of soluble storage proteins and the three fractions obtained by the procedure outlined in Figure 5: (A) buffer-extractable proteins; (B) 11S globulin fraction; (C) 7S globulin fraction; (D) whey proteins.

Figure 8. Immunodiffusion of total soluble proteins and the three fractions obtained in Figure 5. The center wells contained: (A) anti-7S globulin antiserum; (B) anti-11S globulin antiserum; (C) a mixture of anti-7S and anti-11S; (D) anti-water-extractable proteins antiserum. The peripheral wells contained: water-extractable proteins (WEP); acid precipitated protein (APP); 11S globulin fraction (11S Fr.); 7S globulin fraction (7S Fr.); purified dimerizing 7S (7S); and purified 11S globulin (11S).

components and the dissociating form of the 11S (symbolized $\overline{7S}$ by Wolf and Briggs, 1958) which was not found in the standard buffer. As ionic strength changed from 0.5 to 0.1, a remarkable increase in area of the 2S and 15S components was noted along with the absence of the 7S (Figures 6A and 6A' and Table I). This suggests that the 7S is dissociated (or associated) at 0.1 ionic strength.

The 7S protein in the 7S–whey fraction was of two types: one converted to the 9S sedimenting form at 0.1 ionic strength (dimerizing type, symbolized 7S), which represented 85% of the 7S in this fraction, was obtained in 91% yield by precipitation at pH 4.8 without contamination of the nondimerizing 7S (Figures 6C and 6C'). The nondimerizing type (symbolized [7S]), which remained in the whey fraction, represented about 7% of total extractable proteins. Because hemagglutinin, a major component of soybean whey protein fraction, has an $s_{20,w}$ value of 6.5 S (Wolf and Sly, 1965), the [7S] might therefore be primarily hemagglutinin.

Disc Electrophoresis. Total soybean proteins were separated into multiple components by disc electrophoresis (Figure 7A). The pattern was similar to that obtained by Hill and Breidenbach (1974). Band h with the highest relative mobility was demonstrated to correspond to the 2S fraction (Hill and Breidenbach, 1974). Bands f and g (Figure 7B) may refer to the 11S and its dissociating form (7S). The 7S globulin fraction exhibits five major bands (a to e, Figure 7C). These bands have been related to the multiple 7S globulins (Thanh et al., 1975a,b). The whey protein fraction composed mainly of 2S and 6S fractions by ultracentrifugation (Eldridge et al., 1966) shows a great number of bands (Figure 7D). Some of the major bands were observed in the region of the 7S globulin. The isolated 7S and 11S fractions showed very little cross-contamination upon electrophoresis.

Immunodiffusion. The results of immunodiffusion in agar indicated that the isolated fractions were antigenically different from each other. The 7S and 11S fractions exhibit one precipitation band against antisera to the 7S
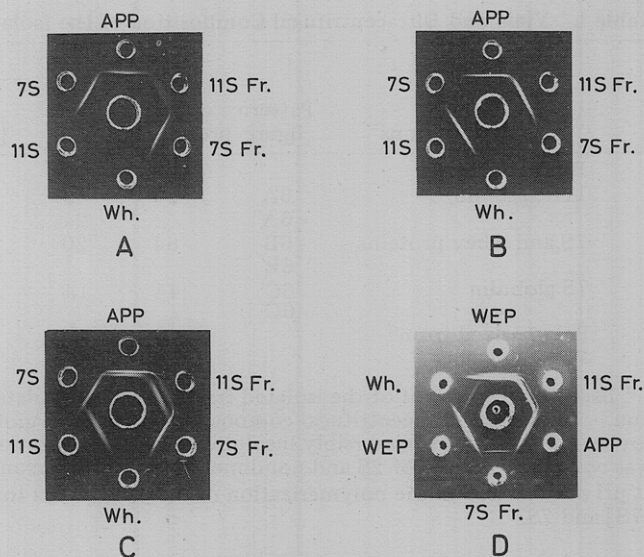
and 11S globulins (Figures 8A and 8B). Whey protein fraction did not react with these two antisera but formed one precipitation band with the anti-water-extractable protein antiserum (Figure 8D). Figure 8C shows two different bands corresponding to the 7S and 11S globulins. The 11S fraction was slightly contaminated with a component which was antigenically identical with the 7S globulin (Figures 8C and 8D). No detectable contamination was found in the 7S fraction.

DISCUSSION

Fractionation of seed proteins based on their different solubilities is a major method to prepare pure globulins for biochemical research and for their nutritional characterization. Although the method afforded complete separation of globulin fractions from some legume seeds (Sun and Hall, 1975), its application to soybean globulins was not so successful (Naismith, 1955; Wolf and Briggs, 1959; Koshiyama, 1965; Roberts and Briggs, 1965). An acidic extraction procedure developed by Koshiyama (1972a) which was effective in separating the 7S protein from soybean globulins seems unsuitable for the preparation of 11S globulin. A simultaneous fractionation of the two globulins in the neutral pH region will, therefore, be of interest and is the objective of this investigation.

In this study, the differential solubility of the 7S and 11S globulins in the pH 6.1 to 6.6 region is the basis for their fractionation. The only major factor which may interfere with their resolution is the ionic strength of the buffer used. Thus, increasing the ionic strength ($\mu > 0.07$) by increasing Tris concentration or by adding NaCl results in partial solubilization of the 11S globulin (Figures 2 and 4). The solubilized 11S fraction will remain in the 7S globulin. In order to eliminate the possible interference of ionic strength, defatted soybean meal was extracted with a relatively large volume of dilute Tris buffer (Figure 5). This allows the separation of the two globulins directly by adjusting to pH 6.4. The soluble fraction thus obtained contained no detectable 11S globulin (Figures 6B and 6B'). This finding is unexpected since a considerable part of the

11S globulin was reported to remain soluble at pH 6.25 after dialysis against water (Wolf and Briggs, 1959). The solubility characteristic of the two globulins might be altered by their interaction with Tris buffer ions and, therefore, accounts for their effective separation.

Ultracentrifugal analysis of the fractions obtained by the simultaneous fractionation suggests that the proteins which constitute the 7S sedimenting form of soybean protein at 0.5 ionic strength are composed of at least three types. Two of them, dimerizing and nondimerizing 7S, are completely separated by adjusting the pH to 4.8. The third type which is found in the 11S preparation is immunochemically identical with the dimerizing 7S globulin (Figure 8). Although ultracentrifugation at 0.1 ionic strength tends to prove it as a distinct type, this might not be conclusive evidence. Further investigations will be necessary to clarify this problem.

The dimerizing 7S globulin is composed of multiple components detected by disc electrophoresis (Figure 7), but immunodiffusion in agar fails to discriminate these multiple components (Figure 8). Investigation of these 7S components isolated by ion-exchange chromatography indicates that they are isomers containing varying proportions of common subunits (Thanh and Shibasaki, 1976) and, therefore, antigenically similar.

The complexity, presented in this study, of proteins which constitute the 7S sedimenting form leads to the necessity of an appropriate soybean protein nomenclature. Disc gel electrophoresis, because of its high resolution, is useful in characterizing and designating the multiple components of the dimerizing 7S globulin. But as all components of soybean proteins are concerned, this technique alone has only limited value for a nomenclature system because some whey proteins which are immunochemically different from globulin fractions (Figure 8) are not clearly separated from the 7S and 11S globulins on polyacrylamide gels (Figure 7). A combination of disc electrophoresis and ultracentrifugal terminologies would be a solution to the problem: soybean proteins would be called glycinin and would be distinguished from one another by their ultracentrifugal term, by their association–dissociation character, and by gel electrophoresis. This nomenclature proposal, a modification of proposal VI reported by Wolf (1969), is summarized as follows:

2S glycinin:	2S α-glycinin (2S α-glycinin I, . . .), . . .
7S glycinin:	7S α-glycinin (7S α-glycinin I, . . .)
	7S β-glycinin (. . .)
	7S γ-glycinin (. . .)
11S glycinin:	
15S glycinin:	15S α-glycinin (. . .), . . .

In this proposal 7S α- and 7S β-glycinins designate the dimerizing and nondimerizing types, respectively. The two types of 7S globulins have been identified with β- and γ-conglycinins (Catsimpoolas, 1969; Koshiyama and Fukushima, 1976a,b). The other possible type is assigned 7S γ-glycinin. The Roman numeral differentiates various proteins detected by gel electrophoresis.

The use of Greek letters and Roman numerals makes it possible for this nomenclature system to be extended in the future as new proteins are isolated from each ultracentrifugal fraction.

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