Purification and Structural Studies of the 11S Component of Soybean Proteins

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ABSTRACT

Soybean globulins were separated into at least 12 components by disc electrophoresis on polyacrylamide gel. The 11S component of the cold-precipitated globulins was purified by ammonium sulfate precipitation followed by DEAE-Sephadex column chromatography. The 11S protein was dissociated by 6*M* guanidine hydrochloride containing 0.2*M* mercaptoethanol into at least 12 subunits. Quantitative N-terminal amino acid analysis of the 11S component indicated that the protein contains at least twelve polypeptide chains, eight of which end in glycine, two in phenylalanine, and two in either leucine or isoleucine. Amino acid analysis showed that several of the essential amino acids exhibit lower values in the 11S protein than in the whole soybean globulin fraction.

Ultracentrifugal analysis of the globulin fraction of soybean protein revealed four components, namely, the 2S, 7S, 11S, and 15S (1,2). The two major components, 7S and 11S, which account for about 70% of the total soybean protein, have been purified and partially characterized (2–5). The 7S soybean globulin has a molecular weight of approximately 330,000, and the 11S, about 350,000. These components are capable of forming disulfide polymers and dissociating into subunits under various conditions (6,7). The purified 11S protein has been shown to contain three different N-terminal amino acids, namely glycine, phenylalanine, and leucine or isoleucine (5). The 7S fraction appears to be composed of at least four different components as shown by gel filtration with Sephadex G-200 (8) and by chromatography on calcium phosphate (9). A 2S component was isolated and shown to have a molecular weight of approximately 26,000 and to contain 1 mole of aspartic acid as the N-terminal amino acid (9).

Whole soybean protein was separated into at least 14 components by starch-gel electrophoresis in 7*M* urea (10). The cold-precipitated globulins showed one prominent band and four minor bands in the absence of urea. The main band was dissociated into several components in the presence of 7*M* urea and 0.02*M* mercaptoethanol. Three of these components were separated by DEAE-cellulose chromatography and were shown to be homogeneous by starch-gel electrophoresis (11). The components of soybean protein separated by electrophoresis appear to have no obvious correlation with ultracentrifugal components.

In this work, disc electrophoresis on polyacrylamide gel was used to separate the soybean globulin components, because of its resolving power and the sensitivity of this technique over paper and starch-gel electrophoresis. The 11S component of soybean proteins was purified on DEAE-Sephadex and partially characterized.

MATERIALS AND METHODS

Soybean Meal

The soybeans used in this investigation were Harosoy 63 variety grown

in 1964 and stored at 25°C. The seeds were cracked, dehulled, and flaked. The soybean flakes were defatted with pentane (b.p. 36°C.) in a Soxhlet apparatus for 4 to 5 hr. The extracted flakes were desolventized at room temperature.

Soybean Globulins

Soybean protein was obtained by extraction of the defatted flakes with water (flake:water ratio, 1:10) at 25°C. for 1 hr. and centrifugation at 10,000 r.c.f. for 15 min. to clarify the supernatant liquor (2). The globulins were separated from the whey by isoelectric precipitation at pH 4.5 with 10% HCl and washed free from whey constituents. The isoelectric globulins were then dialyzed against phosphate buffer, pH 7.6, made 0.4*M* in sodium chloride and 0.01*M* in mercaptoethanol, designated standard buffer (2), or treated as specified in each experiment.

Cold-Insoluble Fraction

For fractionation of the globulins by cooling (2), the defatted flakes were extracted with water (flake:water ratio 1:5) at 25°C. for 1 hr. at room temperature, and the extract was clarified by centrifugation at 10,000 r.c.f. for 15 min. Subsequently, the protein extract was cooled overnight and centrifuged at 4°C. The cold-insoluble fraction was then dialyzed against the standard buffer or treated as specified in each experiment.

Fraction X of the Cold-Precipitated Protein

The cold-insoluble fraction was subjected to ammonium sulfate fractionation, and the "fraction X" described by Wolf *et al.* (12) was isolated. Briefly, the procedure consists in precipitating the 11S protein twice with ammonium sulfate at pH 7.6 between 51 and 66% of saturation, followed by precipitation at pH 4.0 between 26 and 40% saturation. The ultracentrifugal composition of fraction X was reported to be 91% 11S and 9% 15S protein (12).

Fractionation on DEAE-Sephadex A-50

Chromatographic fractionation of the purified cold-insoluble fraction (from ammonium sulfate fractionation) was performed on a 4 × 40-cm. column of DEAE-Sephadex A-50 equilibrated with pH 7.6 phosphate buffer (0.0325*M* K_2HPO_4, 0.0026*M* KH_2PO_4), made 0.1*M* in sodium chloride and 0.01*M* in respect to mercaptoethanol. Ionic-strength gradient elution was achieved by adding 1*M* NaCl solution to a mixing chamber containing 700 ml. of the buffer at a rate equal to the rate of outflow from the mixing chamber. Detection and collection of protein fractions was made with the LKB Recychrom system.

Amino Acid Analysis

Amino acid analyses were carried out on 24-hr. hydrolysates in the Spinco Model MS amino acid analyzer by the two-column system of Moore *et al.* (13). Hydrolyses were done in 6*N* HCl at 110°C. under nitrogen. The values reported were corrected for the partial destruction of serine and threonine.

N-Terminal Analysis

Dinitrophenyl (DNP) protein derivatives were prepared by the method

of Sanger (14). The dinitrophenylation was done in 6*M* guanidine hydrochloride according to the method described by Phillips (15). The DNP protein was hydrolyzed with constant-boiling HCl for 12 hr. at 105°C. in an evacuated, sealed tube. Chromatographic analysis of the ether-soluble DNP amino acids was performed in the "toluene: 0.75*M* pH 6 phosphate buffer" two-dimensional system (16). For quantitative analysis the DNP amino acid spots were eluted from the paper with 1% $NaHCO_3$ at 60°C. and the absorbance was measured at 360 mμ. Correction for the extent of breakdown of the DNP amino acids during hydrolysis was determined by hydrolyzing the appropriate authentic DNP amino acids in the presence of the DNP soy protein. The protein content of the DNP soy protein was calculated from the amide nitrogen content of the soy protein and its DNP derivative. The amide nitrogen of the 11S component was found to be 1.87% and its DNP derivative 1.71% measured by the method of Lorand and Middlebrook (17).

Polyacrylamide Gel Electrophoresis

Polyacrylamide gel columns (7%) were prepared as described by Ornstein (18) and Davis (19). When applicable, the stacking and separating gels were made 8*M* in urea (20) and 0.02*M* in mercaptoethanol. Electrophoresis of a 0.2- to 0.4-mg. sample of protein was usually carried out for 30 min. in *tris*-glycine buffer (ionic strength 0.01; pH 8.3) with a current of 5 ma. per gel column. The separated protein components were detected by staining the columns for 1 hr. with Amido-Schwartz dye; this was followed by electrical destaining, as described by Davis (19).

RESULTS AND DISCUSSION

Figure 1 shows representative and reproducible results obtained by disc electrophoresis of soybean globulins. Column 1 shows the separation of the

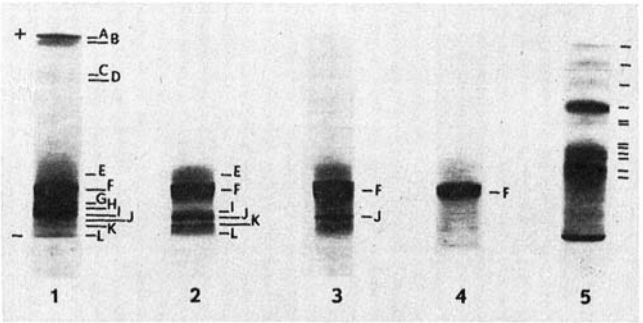

Fig. 1. Separation of components of soybean seed globulins by disc electrophoresis on polyacrylamide-gel. Column 1: unfractionated soybean globulins in standard buffer. Column 2: cold-insoluble fraction of soybean globulins in standard buffer. Column 3: fraction X obtained by ammonium sulfate fractionation of the cold-insoluble fraction. Column 4: purified 11S component obtained by DEAE-Sephadex chromatography of fraction X. Column 5: dissociation of 11S component into subunits by 6*M* guanidine hydrochloride containing 0.2*M* mercaptoethanol.

components present in unfractionated soybean seed globulins. A minimum of 12 components was obtained, which for comparison have been arbitrarily assigned the letters A through L.

A and B are the fastest-moving components and are located at the anodic end of the column. Other major components are those designated F, G, H, and I. The K and L components are very slow-moving and appear to be of minor intensity when the globulins are dissolved in standard buffer (pH 7.6 phosphate buffer 0.4*M* in NaCl and 0.01*M* in mercaptoethanol), and the resulting solution is used to prepare the sample gel. However, the staining density of the K and L bands increases when either mercaptoethanol or sodium chloride is omitted from the buffer system used to dissolve the proteins. This may be an indication that the K and L bands represent polymers of the soybean globulins.

Column 2 shows the constituent components of the cold-insoluble fraction (CIF) of the soybean globulins which is obtained by cooling a concentrated aqueous extract of defatted soybean meal (2). The most predominant component is the F, which appears to be contaminated with the E, I, J, K, and L components. Assignment of groups in different gel columns was based on the Rp values of the visible bands. Wolf and Briggs (2) observed that the cold-insoluble fraction of soybean globulins consists principally of a component having a sedimentation constant of 11S (82%). The other components are 2S (6%), 7S (7%), and 15S (5%). Disc-electrophoresis studies of the cold-insoluble fraction indicated the presence of at least six components (E, F, I, J, K, and L). Both the 11S ultracentrifugal component and the disc-electrophoresis component F are the major components of the cold-insoluble fraction.

Wolf *et al.* (12) reported that fraction X of the cold-insoluble fraction obtained by ammonium sulfate precipitation yields an 11S protein which is estimated to be 91–93% pure by ultracentrifugation. The only other contaminant present is the 15S protein. The results of disc electrophoresis of fraction X is shown in Fig. 1, column 3. The main component is band F, contaminated mainly by component J. Other components may be present in very small amounts, since the area between E and L bands contains diffused protein. However, ammonium sulfate precipitation improved the purity of component F in a fashion very similar to that described for the 11S protein. Furthermore, the existence of only two major components, as determined by both the reported ultracentrifugal (12) and the disc-electrophoresis analyses, suggests that the 11S protein is identical with component F, and that the 15S protein is identical with component J. Also, as discussed later, the 11S protein and the purified protein migrating as band F have identical N-terminal amino acids and immunochemical specificities.

The 11S component was further purified by ionic-strength gradient elution chromatography on a 4 × 40-cm. DEAE-Sephadex A-50 column. Elution was performed with pH 7.6 phosphate buffer containing 0.1*M* NaCl and 0.01*M* mercaptoethanol, and the ionic-strength gradient was achieved by adding 1*M* NaCl solution. The elution pattern is shown in Fig. 2. Four fractions, I, II, III, and IV, were obtained, of which fraction III contained the purified 11S protein as determined by disc electrophoresis (Fig. 1, column 4). The protein purified by DEAE-Sephadex and migrating as band F was found to be immunochemically homogeneous and identical with the major

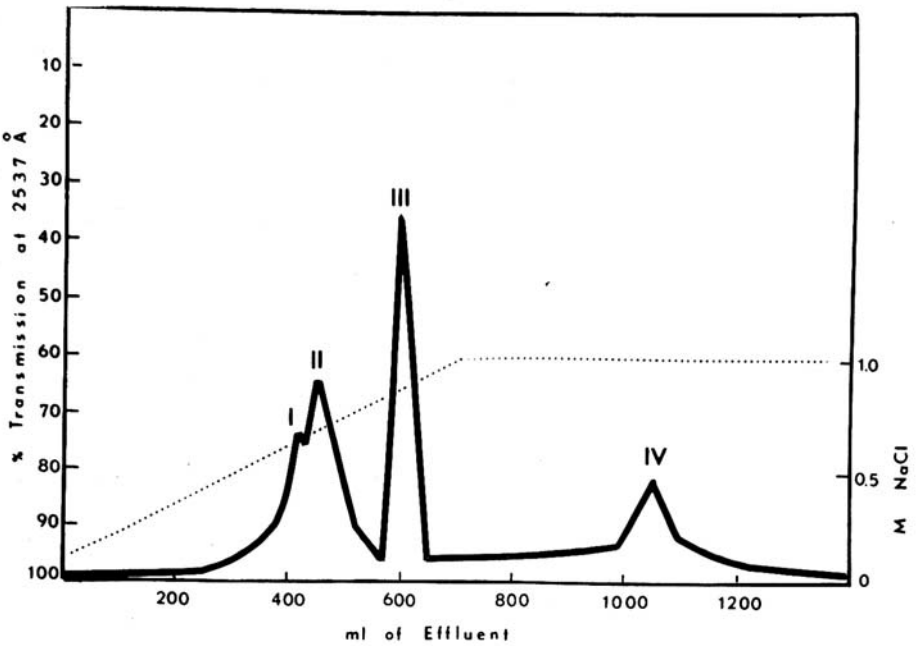

Fig. 2. Chromatography of fraction X with DEAE-Sephadex A-50. Column size: 4 × 40-cm. Sample: 200 mg. of fraction X in 10 ml. of pH 7.6 phosphate buffer containing 0.1*M* NaCl and 0.01*M* mercaptoethanol. Elution: same buffer containing NaCl in gradient concentration of 0 to 1.0*M*, flow rate 20 ml./hr. Full line: UV absorption at 2537 A, 1-mm. cell. Dashed line: gradient of NaCl concentration.

component (11S protein) of fraction X by double gel immunodiffusion and disc immunoelectrophoresis. Details of the immunochemical work will be published elsewhere.[1] Mercaptoethanol (0.02*M*) was incorporated into the polyacrylamide gel to avoid polymerization of the protein during electrophoresis. No precipitate was obtained from fractions I, II, and IV on saturation with ammonium sulfate. Failure to precipitate protein from chromatographic peaks of soybean proteins measured at 280 mμ has been reported by other investigators (5,8). The authors have no sound explanation for this phenomenon.

The purified 11S protein was dissolved in 6*M* guanidine hydrochloride containing 0.2*M* mercaptoethanol—a reagent capable of dissociating globular proteins into their constituent subunits (21)—and the sample was subjected to disc electrophoresis on polyacrylamide gel containing 8*M* urea and 0.02*M* mercaptoethanol. The results are shown in Fig. 1, column 5. The 11S component was dissociated into at least 12 subunits. These subunits do not necessarily represent single polypeptide chains with different electrophoretic mobilities. The formation of aggregation components is not excluded. However, work is under way in our laboratory to establish the N-terminal groups of these components by the method described by Catsimpoolas (22). Dinitro-

[1]N. Catsimpoolas and E. W. Meyer: Immunochemical study of soybean proteins (MS in preparation).

phenylation of the 11S protein followed by hydrolysis with HCl showed the presence of glycine, phenylalanine, and leucine (or isoleucine) as N-terminal amino acids. The data presented below, on the quantitative N-terminal amino

N-Terminal Amino Acid	*moles/350,000 g. protein*
Glycine	8.05
Leucine + isoleucine	2.14
Phenylalanine	1.85

acid analysis of the 11S protein (350,000 used as the average molecular weight of the cold-precipitated 11S protein (2)), indicate that the protein contains at least twelve polypeptide chains, eight of which end in glycine, two in phenylalanine, and two in either leucine or isoleucine. Mitsuda *et al.* (5) reported that the 11S protein purified by calcium phosphate-gel chromatography contained glycine, phenylalanine, and leucine or isoleucine as N-terminal amino acids. Okubo and Shibasaki (11) found that the cold-insoluble fraction was dissociated into several components in 8*M* urea containing 0.01*M* mercaptoethanol. These were partially separated by means of column chromatography on DEAE cellulose. Three of these components were homogeneous, as shown by starch-gel electrophoresis, and the fourth component, a mixture of subunits, migrated slowly. The results of these workers cannot be evaluated clearly; their preparation was a mixture of the total cold-precipitated globulins. However, their inability to obtain separation of a larger number of subunits could be explained either as a result of the low mercaptoethanol concentration (0.01*M*), leading to incomplete cleavage of disulfide bonds, or the low resolving power of the starch-gel electrophoresis system in comparison to disc electrophoresis on polyacrylamide gel.

A partial amino acid analysis of the 11S protein is compared in Table I

TABLE I
COMPARISON OF AMINO ACID ANALYSES OF THE 11S PROTEIN AND WHOLE GLOBULIN

AMINO ACID	11S PROTEIN	WHOLE GLOBULIN	AMINO ACID	11S PROTEIN	WHOLE GLOBULIN
	g. AA/16 g. N	*g. AA/16 g. N*		*g. AA/16 g. N*	*g. AA/16 g. N*
Lysine	4.3	6.0	Alanine	4.0	3.9
Histidine	2.0	2.4	Cystine[a]	1.4	1.2
Arginine	7.3	7.8	Valine	4.5	4.7
Aspartic acid	9.8	12.6	Methionine	1.1	1.2
Threonine	2.9	3.6	Isoleucine	4.2	4.8
Serine	3.8	5.7	Leucine	6.9	7.9
Glutamic acid	21.7	22.4	Tyrosine	3.2	3.8
Proline	5.8	5.4	Phenylalanine	4.8	5.5
Glycine	4.0	4.1			

[a] Determined as cysteic acid (23,24).

with that of the whole globulin fraction. In general, the amino acid composition of the 11S protein is similar to that of the whole globulin. However, several of the essential amino acids and especially lysine, threonine, and leucine have lower values in the 11S component than in the whole globulin fraction.

Although additional work will be necessary to establish the correlation of ultracentrifugal and electrophoretic components, the disc-electrophoresis

technique described above appears to be a useful tool for separating the soybean globulins and determining their purity. The purification of the 11S protein offers an opportunity for the study of the chemical structure of this protein and its physicochemical properties.

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