# Purification of the 11S Component of Soybean Protein[1]

**A. C. ELDRIDGE** and **W. J. WOLF,** Northern Regional Research Laboratory, Peoria, Illinois

**ABSTRACT**

Solubilities of the 2S, 7S, 11S, and 15S components in the cold-insoluble fraction of soybean protein were determined in sodium acetate-sodium chloride buffers at pH 4.6, 0°–2°C. Solubility of some components varied considerably with ionic strength. Extraction of the cold-insoluble fraction at 25°C. with pH 4.6, 0.5 ionic strength buffer, followed by cooling to 0°–2°C., yielded 3–4 mg. protein per ml. having a composition of 8, 21, and 70% for the respective 2S, 7S, and 11S components. When these solubles were diluted to pH 4.6, 0.3 ionic strength and cooled to 0°–2°C., a precipitate formed. The precipitate contained 5–8% 7S component and 92–95% 11S component; it was devoid of 2S and 15S material. Further fractionation of this precipitate on Sephadex G-200 resulted in an 11S fraction which appeared to be homogeneous.

Ultracentrifugal studies on the soluble proteins of soybeans revealed four prominent fractions having sedimentation constants of about 2, 7, 11, and 15S (1–3). The major fraction, the 11S component, represents 25–35% of the total protein in defatted soybean meal (4,5) and has been partially purified and characterized (3,6–10). This globulin has an isoelectric point of about pH 5, molecular weight of about 350,000, and a subunit structure. This paper describes purification of the 11S component by variation of ionic strength and temperatures at pH 4.6, followed by gel filtration on cross-linked dextran or chromatography on hydroxylapatite.

## MATERIALS AND METHODS

Harosoy soybeans stored at 5°C. for 5 months were cracked, dehulled, and flaked before being defatted with hexane at room temperature.

The cold-insoluble fraction (CIF) was prepared by extracting the defatted meal with water-to-meal ratio of 5:1 at 25°C. After centrifugation at 16,000 × *g* for 10 min., the clear supernatant was decanted and cooled at 0°–2°C. overnight. The precipitate (CIF) was then collected by centrifuging at 0°–2°C. at 16,000 × *g*. The CIF was dissolved in distilled water and dialyzed 1 week against distilled water at 0°–2°C. The contents of the dialysis casings were titrated to pH 7.8 with 1*N* NaOH, and a small amount of insolubles was removed by centrifugation before the solubles were freeze-dried.

Ultracentrifugal analyses of the protein solutions were performed at room temperature with a Spinco Model E ultracentrifuge with a 30-mm. cell, with a plastic double-sector center piece at 47,660 r.p.m. Areas were corrected for radial dilution, and compositions are expressed as percentages of the total area. Potassium phosphate-sodium chloride buffer, pH 7.6, 0.5 ionic strength, containing 0.01*M* 2-mercaptoethanol, was used for ultracentrifugal analyses (3).

In the fractionation of CIF, an acetic acid-sodium chloride buffer (11)

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was selected. The buffer was prepared by diluting 100 ml. *M* NaOH, plus 215 ml. *M* acetic acid, to 1 liter with deionized water. This procedure yielded a buffer of pH 4.6, 0.1 ionic strength. For higher ionic strengths, appropriate amounts of sodium chloride were added before dilution to volume. All buffers contained 0.01*M* 2-mercaptoethanol.

Column chromatography on hydroxylapatite was similar to the procedure of Wolf and Sly (5). A column $1 \times 25$ cm. was used. A gradient was produced by adding 1*M* potassium phosphate, pH 7.6, to a mixing chamber containing 320 ml. of the starting buffer, 0.03*M* potassium phosphate at pH 7.6. Before each chromatographic analysis, the column was washed with 1 liter of the starting buffer.

Gel filtration was conducted on a $3 \times 100$-cm. column at room temperature with recycling chromatography (12) on an LKB 4900 assembly (LKB-Produkter AB, Stockholm, Sweden). The ultracentrifuge buffer was used in these studies.

After gel filtration or hydroxylapatite chromatography, the samples were pooled and concentrated by pervaporation. After analysis, the samples were dialyzed against distilled water until salt-free, and lyophilized.

## RESULTS

Since CIF is a mixture of globulins with isoelectric points of about pH 5, its solubility in the isoelectric range depends on ionic strength and temperature. Solubility of the CIF was determined at pH 4.6 at various ionic strengths, by dispersing 400–700 mg. CIF in 10 ml. of buffer of the desired ionic strength. The sample was then dialyzed against buffer at 1°C. for

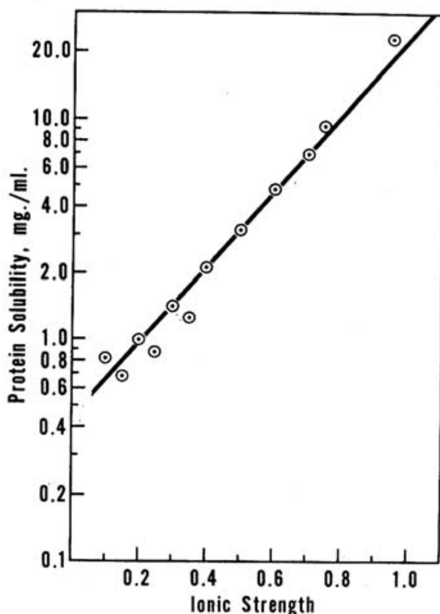


Fig. 1. Effect of ionic strength on solubility of cold-insoluble fraction (CIF) from soybean protein at pH 4.6 at 0°–2°C.

18–24 hr. After centrifugation at 0°–2°C. in a refrigerated centrifuge at 14,500 × *g* for 15 min., the solubles were collected. The protein concentration of the soluble fraction was determined in a Beckman Model DU spectrophotometer at 280 m$\mu$, assuming $E_{1\,cm.}^{1\%} = 9.2$ (3). Results are plotted in Fig. 1.

After solubilities of the total protein were determined (Fig. 1), the soluble portions were dialyzed against ultracentrifuge buffer prior to ultracentrifugal analyses. Total solubility and the ultracentrifugal distribution of components were then used to construct curves showing the effect of ionic strength at pH 4.6 on solubility of each component in the system at 0°–2°C. (Fig. 2).

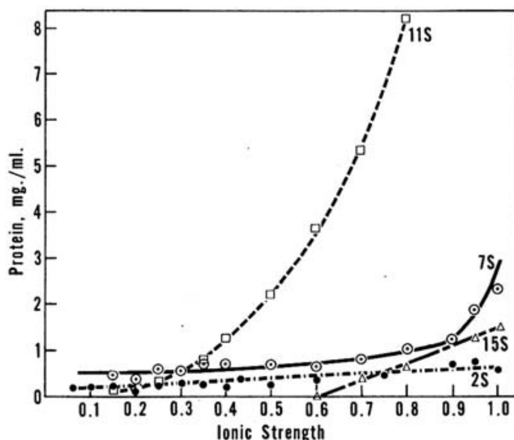


Fig. 2. Effect of ionic strength on solubility of the 2, 7, 11, and 15S components of CIF from soybean protein at 0°–2°C.

At 1.0 ionic strength, 1.5 mg./ml. of 15S is soluble. However, its solubility drops off sharply as ionic strength is decreased, and at 0.6 ionic strength it is completely insoluble.

The 2S and 7S fractions show a smaller dependence of solubility on ionic strength than the 15S fraction in the range of 0.9 to 0.1 ionic strength. The near-constant solubility of the 2S and 7S may suggest an insufficient amount of the globulins to saturate the extract. Thus the curves may not be true solubilities but merely the combined solubilities of several 2S and 7S species in the CIF.

The 11S component of CIF has the greatest variation in solubility with changes in ionic strength. At pH 4.6, 0.25 ionic strength, at 0°–2°C., the 11S component is nearly insoluble, whereas at 0.8 ionic strength, this globulin component has a solubility of 8 mg./ml.

The differences in solubilities of the four components with changes in ionic strength provided a basis for a fractionation procedure outlined in Fig. 3. Usually 1% CIF was suspended in pH 4.6, 0.5 ionic strength buffer containing 0.01*M* 2-mercaptoethanol and then stirred gently for a few hours at room temperature. The solubles were separated by centrifugation. Ultracentrifugal analysis of the solubles in phosphate-sodium chloride buffer at pH 7.6 indicated

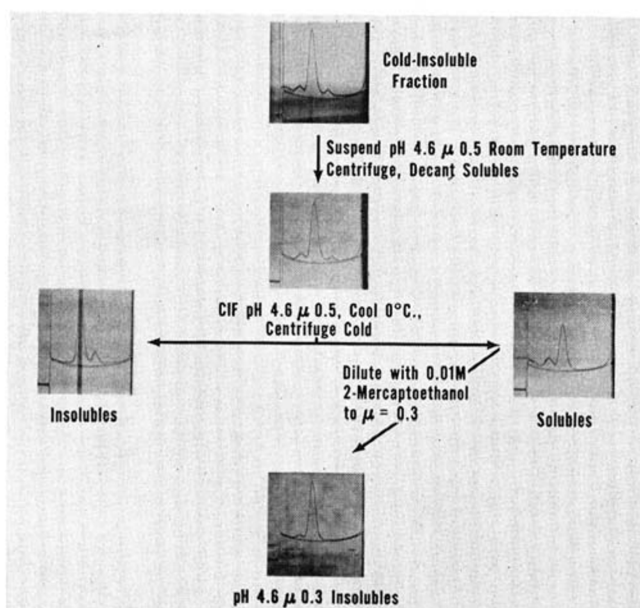


Fig. 3. Fractionation of CIF from soybean protein by variation of ionic strength and temperature at pH 4.6.

a loss of about 50% of the 2S material present and enrichment of the 11S material (Table I).

TABLE I
COMPOSITION OF SOYBEAN PROTEIN AFTER FRACTIONATIONAL PRECIPITATION

| FRACTION | ULTRACENTRIFUGE COMPOSITION | | | | | YIELD OF 11S |
|---|---|---|---|---|---|---|
| | 2S | 7S | 11S | 15S | >15S | |
| | % | % | % | % | % | % |
| Cold-insoluble fraction (CIF) | 7 | 10 | 65 | 11 | 7 | 100 |
| Solubles, pH 4.6 ($\mu$ = 0.5, 25°C.) | 3 | 12 | 74 | 11 | .... | 92 |
| Insolubles, pH 4.6 ($\mu$ = 0.5, 1°C.) | .... | 4 | 76 | 16 | 6 | 59 |
| Solubles, pH 4.6 ($\mu$ = 0.5, 1°C.) | 8 | 21 | 70 | .... | .... | 33 |
| Insolubles, pH 4.6 ($\mu$ = 0.3, 1°C.) | .... | 6 | 94 | .... | .... | 15 |

The room-temperature extract was cooled overnight at 0°–2°C., and then centrifuged in the cold. The insolubles from this centrifugation contained some of the 11S and all of the faster-sedimenting material, whereas the soluble fraction was composed of 11S and slower-sedimenting proteins. An analysis of these fractions is given in Table I.

The fraction from the pH 4.6, 0.5 ionic strength extract which remained soluble in the cold (Fig. 3 and Table I) was then diluted to 0.3 ionic strength by adding cold 0.01*M* 2-mercaptoethanol. The resulting precipitate was separated by centrifugation at 0°–2°C. to yield an oily, translucent pellet which dissolved readily in ultracentrifuge buffer; it contained approximately 94% 11S protein when analyzed in the ultracentrifuge. The usual impurity was 7S component, but occasionally a trace of material sedimenting faster than 11S was observed.

Wolf *et al.* (5,6) have indicated in hydroxylapatite chromatographic experiments that the 7S material could be removed from preparations containing 7S, 11S, and 15S components, but the 15S material was not removed. Since the pH 4.6, 0.3 ionic strength precipitate contained little 15S fraction, attempts were made to separate the residual 7S by chromatography on hydroxylapatite (Fig. 4).

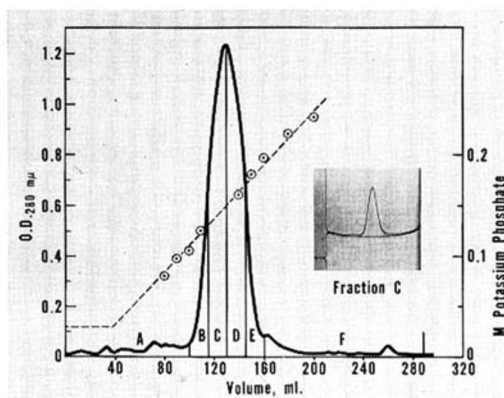


Fig. 4. Gradient elution of the pH 4.6, 0.3 ionic strength insolubles (Fig. 3) on a hydroxylapatite column. Solid curve is absorbance at 280 mμ in a 1-cm. cell after 1:4 dilution. Dashed line is gradient determined by conductivity. There was 221 mg. of protein applied to column. The eluted peak was divided into six samples and analyzed (Table II). Inset is ultracentrifuge pattern of fraction C. Sedimentation is from left to right after 42 min. at 47,660 r.p.m., 70° bar angle.

Although the elution peak from the precipitate appeared symmetrical, the peak was divided, pooled, pervaporated, and analyzed in the ultracentrifuge so that differences in composition could be detected (Table II). Composition varied through the elution peak, and the purest 11S preparation was fraction C (Table II, inset Fig. 4); it contained 96% 11S, the only impurity being a 7S component. Trace amounts of 2S and 15S materials apparently were present in the protein applied to the column, but these were detectable only after removal of 11S by chromatography and extensive concentration of fractions A, D, E, and F.

Hasegawa *et al.* (13) reported their inability to separate the 11S and 7S by gel filtration with Sephadex G-200 and a column 3 × 210 cm. We confirmed their report; however, we were able to fractionate the pH 4.6, 0.3 ionic strength insolubles by recycling chromatography with Sephadex G-200 and a column 3 × 100 cm. Upward flow with column loads of 250–300 mg. was

TABLE II

COMPOSITION OF ELUTED SOYBEAN PROTEIN FROM HYDROXYLAPATITE CHROMATOGRAPHY

| FRACTION | ULTRACENTRIFUGE COMPOSITION | | | | ELUTED PROTEIN[a] |
|---|---|---|---|---|---|
| | 2S | 7S | 11S | 15S | |
| | % | % | % | % | % |
| A | 100 | .... | .... | .... | 7 |
| B | 4 | 6 | 90 | .... | 9 |
| C | .... | 4 | 96 | .... | 33 |
| D | .... | 3 | 92 | 5 | 35 |
| E | .... | 6 | 85 | 9 | 8 |
| F | 9 | 36 | 55 | .... | 7 |

[a]Expressed as percent of area under elution diagram.

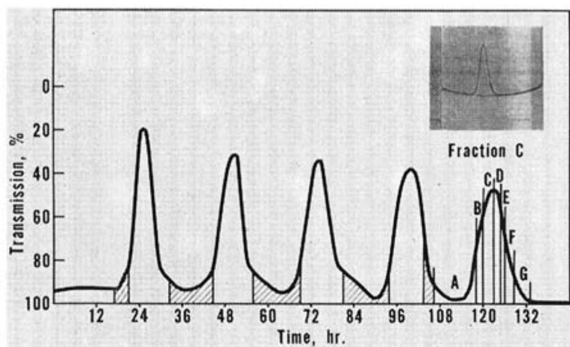


Fig. 5. Elution diagram of the pH 4.6, 0.3 ionic strength insolubles (Fig. 3) on Sephadex G-200. Solid curve is percent transmission. Shaded areas were withdrawn after each cycle and fractions A–G were collected after five cycles. Inset is ultracentrifuge pattern of fraction C. Sedimentation is from left to right after 42 min. at 47,660 r.p.m., 70° bar angle.

used. Slow flow rates were essential, and we generally used 13 ml./hr. for the entire analysis. Results from a typical run are shown in Fig. 5. After the major peak emerged, sample was withdrawn until the major peak re-emerged. Portions removed are indicated as shaded areas in Fig. 5.

As the peak emerged the fifth time (equivalent to a 500-cm. column) the sample was collected in several fractions. The fractions were concentrated and

TABLE III

COMPOSITION OF ELUTED FRACTIONS FROM SEPHADEX G-200 AFTER RECYCLING CHROMATOGRAPHY

| FRACTION | ULTRACENTRIFUGE COMPOSITION | | | | |
|---|---|---|---|---|---|
| | 2S | 7S | 11S | 15S | >15S |
| | % | % | % | % | % |
| A | -----42----- | | 47 | 7 | 5 |
| B | .... | 5 | 83 | 12 | .... |
| C | .... | .... | 100 | .... | .... |
| D | .... | .... | 100 | .... | .... |
| E | 4 | 6 | [illegible]0 | 4 | .... |
| F | 4 | 6 | 85 | 5 | .... |
| G | 19 | 12 | 68 | .... | .... |

analyzed in the ultracentrifuge. Their compositions are given in Table III.

Fractions C and D (Table III, inset Fig. 5) sedimented as a single component in the ultracentrifuge and accounted for 40% of the area under the elution peak. All other fractions were contaminated to various extents (Table III).

## DISCUSSION

The experimental results show clearly that a fractional precipitation has been devised which will yield a purified 11S protein containing only about 7% of a component sedimenting in the 7S region. Yield of 11S protein of this purity is only about 15%, based on the starting 11S material, but the ease of preparation compensates for the low yield.

Roberts and Briggs (14) separated a 7S fraction with a molecular weight of 330,000 as compared to 350,000–360,000 reported previously for the 11S protein (3). Resolution of the two proteins in the ultracentrifuge must thus depend on differences in molecular shape. Poor separation of these proteins by gel filtration which depends on molecular size substantiates these findings. The 7S component is but slowly separated from the 11S component by recycling gel filtration.

Although we have considered the 7S component as a contaminant, we cannot exclude the possibility of partial dissociation of 11S into half-molecules. This process has been observed previously on N-ethylmaleimide-treated 11S but only on prolonged storage in solution, on freezing and thawing, or on freeze-drying (6). In preliminary studies we observed reappearance of 8–10% 7S component in 11S preparations from gel filtration within 3 weeks at pH 7.6, 0.5 ionic strength, 0°–2°C. The 11S protein is readily dissociated into subunits at low ionic strengths and slightly alkaline pH values (15). We have found extensive dissociation of the purified 11S into 2S and 7S components in 0.03*M* *tris*-citrate buffer, pH 8.6. However, reassociation into 11S component was low when the sample was subsequently dialyzed against pH 7.6, 0.5 ionic strength phosphate-sodium chloride buffer. Apparently some irreversible conformational changes occur in the subunits when they are dissociated under these conditions.

Further studies on stability of the purified 11S are being made to determine whether the small amount of 7S material usually present is an impurity or merely a breakdown product of the 11S component.

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