

# Cryoprecipitation of Soybean 11S Protein<sup>1</sup>

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## ABSTRACT

Cooling a concentrated aqueous extract of defatted soybean meal causes cryoprecipitation of protein consisting primarily of the 11S ultracentrifugal component. Factors affecting cryoprecipitation were studied to determine whether this component could be removed quantitatively from a water extract of meal by cooling, to increase the yield of 11S protein and simplify fractionation of 2S and 7S proteins. Factors studied were meal:water extraction ratio, extraction temperature, rate of cryoprecipitation, pH, and addition of mercaptoethanol, NEMI, sucrose, and salts. Extracts (meal:water ratio 1:5) prepared at 40°C. contained more 7S, 11S, and 15S than those prepared at 25°C. and yielded a nearly twofold increase in amount of 11S protein which precipitated when extracts were cooled. Cryoprecipitation was complete or nearly complete in 1-2 hr. Addition of 0.01M mercaptoethanol or NEMI had no effect on amount of cryoprecipitate obtained. Concentrations of NaCl greater than 0.3M and of sucrose greater than 0.6M prevented cryoprecipitation. Addition of CaCl<sub>2</sub> to water extract before cooling caused nearly quantitative precipitation of 11S and 15S, plus part of 2S and 7S proteins. Results were similar when water extract pH was lowered from initial 6.3 to 5.2 before cooling. Hydroxylapatite chromatography of 2S and 7S protein mixture remaining soluble at pH 5.4 and 0°-2°C. yielded 7S preparations of 83-90% purity. Such samples dimerized nearly completely at pH 7.6, 0.1 ionic strength.

In 1947, Lerner and Watson (1) proposed the name cryoglobulin for a group of pathological blood proteins that alternately precipitated and redissolved when serum was cooled and rewarmed to 37°C. In 1950, Briggs and Mann (2) reported that cooling concentrated aqueous extracts of defatted soybean meal precipitated protein which redissolved on warming. Ghetie and Buzila (3) called attention to the similarity between these studies and introduced the term "plant cryoproteins" to designate proteins that precipitate when aqueous extracts of seeds are cooled. Ghetie and Buzila confirmed cryoprecipitation for soybean meal extracts and demonstrated that cryoproteins also occur in other seeds (3-5). An interesting example of another animal cryoprotein system was reported recently (6,7); "cold cataract," a clouding of the lenses of young animals upon cooling, apparently is caused by cryoprecipitation of one of the lens proteins, gamma crystallin.

The cryoprotein of soybeans has been referred to as cold-precipitable protein or cold-insoluble fraction and identified as the 11S ultracentrifugal component, one of the major proteins in defatted meal (8-10). Although cryoprecipitation has been used to purify the 11S component (11,12), the phenomenon has not been studied in detail since the initial observations of Briggs and Mann (2). Here we describe studies to determine whether the 11S component can be removed selectively and quantitatively from an aqueous extract of meal by cryoprecipitation. Such a procedure would in-

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crease the yield of 11S protein and would simplify fractionation of the 2S and 7S fractions of soybean proteins.

#### MATERIALS AND METHODS

##### Soybean Meal

Soybeans were cracked, dehulled, and flaked, and the flakes then defatted with hexane at room temperature. Clark, Harosoy, and Hawkeye varieties of soybeans were used.

##### Ultracentrifugal Analysis

Sedimentation analyses were performed at room temperature with a 30-mm. double-sector cell in a Spinco Model E ultracentrifuge at 47,660 r.p.m. The buffer was potassium phosphate-sodium chloride (0.0325M  $K_2HPO_4$ , 0.0026M  $KH_2PO_4$ , 0.4M NaCl) pH 7.6, 0.5 ionic strength, containing 0.01M mercaptoethanol.

##### Protein Extraction and Cryoprecipitation

Aqueous extracts of defatted soybean meal were prepared by stirring the water-meal slurries for 30–45 min. at 25° or 40°C. The insoluble residue was removed by clarification in a Servall refrigerated centrifuge at the temperature of extraction for 10 min. at 12,000 r.p.m. ( $17,300 \times g$ ). Protein concentrations were determined by Kjeldahl analysis based on nitrogen-to-protein conversion factors of 6.06 for the water-extractable proteins and 6.20 for the cold-soluble fraction. These factors were calculated from average nitrogen contents for such protein fractions, as reported earlier (13). Cryoprecipitation was carried out at 0°–2°C. and cryoprecipitates were removed from meal extracts at the same temperature by centrifugation at 12,000 r.p.m. ( $17,300 \times g$ ) for 10 min.

##### Meal-to-Water Extraction Ratio

Meal samples ranging from 1 to 20 g. were extracted with 100 ml. of water at room temperature ( $\sim 25^\circ C.$ ). A portion of each extract was dialyzed against ultracentrifuge buffer, adjusted to a common weight-of-meal-to-water (3.3 g. meal/100 ml. water) basis by dilution or pervaporation, analyzed for protein (Kjeldahl nitrogen  $\times 6.06$ ), and then examined in the ultracentrifuge. Adjustment of extract volumes to a common extraction ratio permitted direct comparison of ultracentrifuge patterns to detect changes in extractability of protein components. A second portion of each extract was placed in a refrigerated centrifuge at 0°–2°C. for 16 hr. and then centrifuged. After removal of the cryoprecipitates, portions of the supernatants were analyzed as described for the original extracts.

##### Rate of Cryoprecipitation

Ten-milliliter portions of 1:5 meal:water extracts were cooled in an ice bath for various times and centrifuged at 0°–2°C. Cryoprecipitation was measured by Kjeldahl analysis of the extracts before cooling and after centrifuging in the cold.

##### Influence of Sucrose, Salts, and pH on Cryoprecipitation

The effect of sucrose on cryoprecipitation was determined by extracting meal with sucrose solutions of various concentrations (1:5 meal:sucrose) at room temperature. The sucrose extracts were cooled to 0°–2°C. for about 18

hr. and centrifuged in the cold. Inhibition of cryoprecipitation was measured by Kjeldahl analysis of the extracts before and after cooling.

Inhibition of cryoprecipitation by sodium chloride was shown by adding increments of solid sodium chloride to portions of 1:5 meal:water extracts, cooling to 0°-2° C., and centrifuging after 21 hr. Inhibition of cryoprecipitation was estimated by Kjeldahl analysis of the extracts before and after cooling.

The influence of calcium chloride on cryoprecipitation was measured by adding various amounts of 2*N* calcium chloride to a 1:5 extract of Clark meal prepared at 25°C. After the calcium chloride was added, all extracts were adjusted to pH 6.7 with *N* sodium hydroxide, held at 0°-2°C. for 4 hr., and then centrifuged in the cold. The resulting supernatants were dialyzed against ultracentrifuge buffer and then analyzed for Kjeldahl nitrogen and examined ultracentrifugally.

Cryoprecipitation at various pH values was studied with a 1:5 extract of Clark meal prepared at 25°C. Equal portions of the extract were adjusted from an initial pH of 6.25 to lower pH values with *N* hydrochloric acid and centrifuged after being held at 0°-2°C. for 16-18 hr. The resulting supernatants were dialyzed against ultracentrifuge buffer, adjusted to equal volumes, analyzed for protein, and examined ultracentrifugally.

#### Hydroxylapatite Chromatography

Commercially available hydroxylapatite, Hypatite C (Clarkson Chemical Co., Williamsport, Pa.) was used. Columns (2.2 × 37.5 cm.) were equilibrated with 0.03*M* potassium phosphate buffer (starting buffer). All buffers were prepared by titrating  $\text{KH}_2\text{PO}_4$  to pH 7.6 with KOH and diluting to appropriate volumes. Protein samples were dialyzed against ultracentrifuge buffer to remove nonprotein materials and to eliminate disulfide polymers of the 7S and 11S proteins, and then were equilibrated against starting buffer. Protein concentrations were estimated by absorbance measurements at 280  $m\mu$ , assuming  $E_{1\text{cm}}^{1\%} = 10.3$ , a value previously established for the water-extractable proteins (14). Although this value is probably in error for the proteins obtained by fractional precipitation in the cold at pH 5.4, it served as a relative measure of protein when sample size was varied. After the protein sample had been applied to the column, elution was begun with starting buffer, and buffer concentrations were increased stepwise as given later. Column effluents were collected in 2-ml. fractions and monitored at 280  $m\mu$ . Carbohydrate contents of the effluents were measured with phenol-sulfuric acid (15). Fractions were combined as indicated, pervaporated, equilibrated against ultracentrifuge buffer, and analyzed in the ultracentrifuge.

## RESULTS

#### Meal-to-Water Extraction Ratio

Briggs and Mann (2) found that cryoprecipitation occurred only when total protein concentration of the meal extracts was greater than 1%. They also found that about 30% of the total protein precipitated when extracts which contained 2-4% protein were cooled. We have re-examined the effect of meal:water extraction ratio on cryoprecipitation and determined

purity of the 11S fraction in the cryoprecipitates. Results obtained with Harosoy meal are shown in Fig. 1.

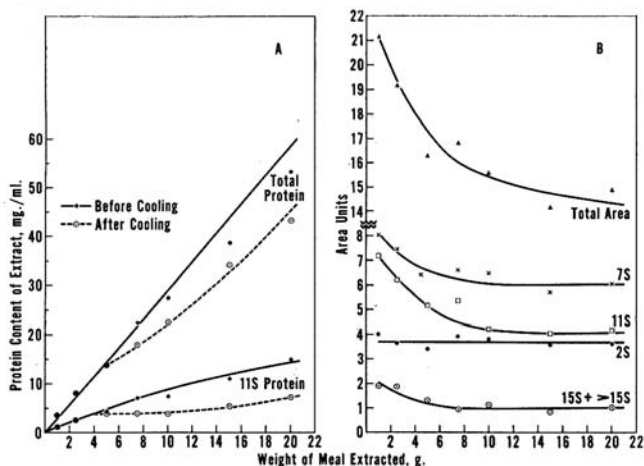


Fig. 1. Effect of meal:water extraction ratio on total and 11S protein concentrations of soybean extracts before and after cooling, A; and on ultracentrifuge composition of extracts, B. In B, the total area and areas for individual ultracentrifuge fractions are expressed in arbitrary units for each extract after adjustment to 3.30 g. of meal per 100 ml. of water.

The upper series of points (solid circles) in Fig. 1, A, shows the relation between total protein concentrations of the extracts and weights of meal extracted. A regression curve (16) was fitted to the points, assuming an expression  $y = bx$  where  $y$  is the protein concentration,  $b$  is the regression coefficient, and  $x$  is the weight of meal. The value for  $b$  was 2.92. Experimental points fit the regression curve fairly well up to a meal weight of 10 g. and lie below the curve at higher extraction ratios. Ultracentrifugal analysis of the dialyzed extracts, however, revealed a more complex relation between extraction ratio and the proteins extracted than is suggested by Fig. 1, A. Since the extracts were adjusted to an equal weight of meal:water basis, changes in areas of the ultracentrifugal patterns were a measure of changes in extractability. Figure 1, B, shows total areas for the ultracentrifugal patterns and areas for the four major fractions of soybean proteins as a function of extraction ratio. Total area decreased rapidly from 1 to 10 g. of meal and then leveled off. The decrease in area was caused primarily by the 7S and 11S fractions. The 15S + > 15S fraction decreased by 50% but had little influence on the extraction curve because it represented only about 10% of the total protein. In contrast, the 2S fraction showed no significant change. Ultracentrifuge patterns are shown in Fig. 2 for four of the extracts.

In Fig. 1, A, the lower (broken) curves show the decrease in total and 11S protein content of the aqueous extracts as a result of cryoprecipitation during cooling. In agreement with the results of Briggs and Mann (2), cryoprecipitation did not occur until total protein concentration was greater

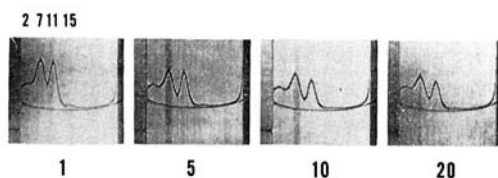


Fig. 2. Ultracentrifugal patterns showing the effect of extraction ratio on protein composition of the extracts. Numbers below each figure indicate g. of meal extracted with 100 ml. of water. Numbers across top of first figure are approximate sedimentation coefficients in Svedberg units.

than 1%. Although the extract from 2.5 g. of meal became turbid on cooling, nothing precipitated on centrifugation. Precipitation was detectable in the extract from 5 g. of meal. At the higher meal:water extraction ratios, from 16 to 20% of the total protein precipitated. Concentration of 11S protein rather than total protein is probably the most important factor controlling cryoprecipitation. Precipitation in the cold began when 11S protein concentration was about 4 mg./ml., and solubility of 11S at 0°-2°C. remained near this level up to 10 g. of meal (Fig. 1, A). At higher extraction ratios, solubilities in the cold were somewhat greater, perhaps because of increased concentrations of meal salts and sugars in the extracts.

Yields and ultracentrifugal analyses of the cryoprecipitates from the different extracts are shown in Table I. A small amount of 2S material

TABLE I

YIELDS AND ULTRACENTRIFUGAL COMPOSITIONS OF CRYOPRECIPITATES FROM SOYBEAN MEAL EXTRACTS PREPARED AT DIFFERENT EXTRACTION RATIOS

EXTRACTION RATIO MEAL TO WATER	YIELD		ULTRACENTRIFUGAL COMPOSITION			
	Extract <sup>a</sup> ml.	Protein <sup>b</sup> mg.	2S %	7S %	11S %	15S %
<i>g./100 ml.</i>						
5.0	81	88	3	16	74	7
7.5	76	244	3	15	76	6
10.0	74	388	0	12	82	6
15.0	74	605	0	9	82	9
20.0	68	636	0	5	88	7

<sup>a</sup>Extract, ml. per 100 ml. of extraction solvent.

<sup>b</sup>Cryoprecipitate, mg. per ml. × ml. of extract.

precipitated at the lower extraction ratios but became undetectable at higher extraction ratios. Likewise, the percentage of 7S fraction decreased progressively as the extraction ratio increased. Because yield and purity of the 11S protein were highest for the 1:5 extraction (Table I), this ratio was used in almost all subsequent studies.

A similar extraction study was also performed with a 32-month-old sample of Hawkeye meal. Although the effect of protein concentration on cryoprecipitation was similar to that in Fig. 1, A, the extraction curve (total protein) remained linear as the meal:water ratio was increased. Furthermore, ultracentrifugal patterns for the extracts did not show the changes in relative amounts of the various components with change in extraction ratio that were noted for the Harosoy meal (Fig. 1, B, and Fig. 2). Comparison of the

ultracentrifuge pattern for the 1:5 extract from the aged Hawkeye meal with a pattern for a 1:5 extract from freshly prepared Hawkeye meal indicated decreased extractability of the 11S component, apparently as a result of aging. The cryoprecipitates from the aged Hawkeye meal from 5 to 20 g. of meal per 100 ml. of water contained 69–74% 11S. In contrast to the Harosoy meal cryoprecipitates (Table I), none contained 2S fraction; the amount of 7S, however, also decreased as the meal:water ratio increased.

#### Extraction Temperature

We have frequently observed that 1:5 meal:water extracts of defatted meal prepared at room temperature are a clear amber color immediately after centrifugation but become turbid on standing for 30–60 min. Because such extracts become clear again when warmed above room temperature, the extracts are evidently saturated with some protein component(s). The slight rise in temperature during centrifugation apparently solubilizes an additional amount of protein, which precipitates when the extract cools to room temperature after centrifugation. This interpretation was verified with 1:5 extracts prepared at 25° and 40°C. Figure 3 shows the ultracentrifugal patterns for the extracts, the cryoprecipitates, and the corresponding supernatants from freshly prepared Hawkeye meal. All solutions were adjusted in

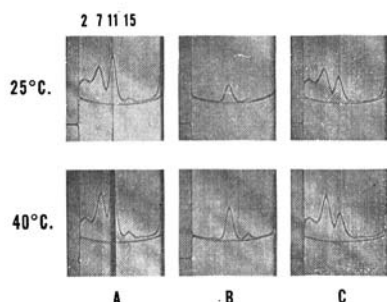


Fig. 3. Ultracentrifuge patterns for: A, 1:5 water extracts; B, cryoprecipitates from the extracts; and C, supernatants after removal of the cryoprecipitates. The extracts were prepared at 25°C. (upper row) and at 40°C. (lower row). Fractions are identified in Svedberg units across top of A.

volume to make the patterns directly comparable. Patterns for the two extracts (Fig. 3, A) show that more of the 7S, 11S, and 15S fractions were extracted at the higher temperature. On subsequent cooling ( $\sim 16$  hr.), the amount of 11S protein obtained from the 40°C. extract was 86% greater than from the 25°C. extract (Fig. 3, B). The amount of 11S component remaining soluble in both extracts upon cooling was nearly constant (Fig. 3, C). In another experiment, Harosoy meal was stirred with water (1:5 meal:water ratio) for 30 min. at 40°C., cooled to 25°C., stirred for 30 min. at the lower temperature, and then centrifuged. The resulting extract was similar to one prepared by stirring for 1 hr. at 25°C. Both extracts contained less 11S protein than a 30-min. extract prepared at 40°C. These results indicate that the additional 11S protein extracted at 40°C. precipitates when the extract is cooled to 25°C.

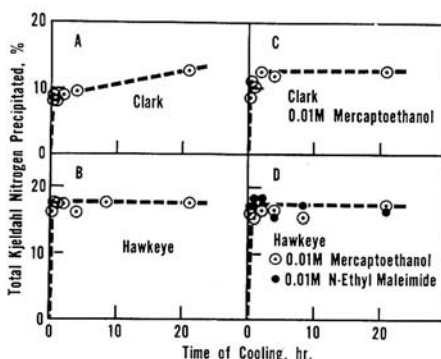


Fig. 4. Effect of time of cooling at 0°C. on percentage of total Kjeldahl nitrogen (uncorrected for nonprotein nitrogen) precipitated from 1:5 meal:water extract of: A, Clark meal; B, Hawkeye meal; C, Clark meal in which extract was made 0.01M with mercaptoethanol; and D, Hawkeye meal in which extracts were made 0.01M with mercaptoethanol and N-ethylmaleimide.

#### Time of Cooling and Cryoprecipitation

The rate of cryoprecipitation of the 11S component from aqueous extracts of soybean meal is shown in Fig. 4. A and B in Fig. 4 show results obtained for Clark and Hawkeye meal extracts prepared at 25°C. For the Clark extract, precipitation of protein was rapid in the first 30–60 min.; the rate was slower during 20 hr. of additional cooling. Cryoprecipitation from Hawkeye extract likewise was rapid but appeared complete after 1 hr. Also, precipitation was less from the Clark extract than from the Hawkeye extract. Rate of cryoprecipitation was also determined on a Hawkeye extract prepared at 40°C. A curve similar to that in A of Fig. 4 resulted, although more protein precipitated as expected because of the higher extraction temperature (Fig. 3). With the 40°C. extract, about 24% of the Kjeldahl nitrogen precipitated in 1 hr. and the amount increased to 28% in the next 20 hr.

#### Addition of Mercaptoethanol and N-Ethylmaleimide

Existence in isolated soybean proteins of disulfide polymers of the 7S and 11S components is well documented (9,17,18). However, experiments indicating that disulfide polymers of these proteins also occur in aqueous extracts of meal were reported only once (19). We therefore repeated and confirmed these experiments. Two portions of a 1:100 water extract of Harosoy meal were dialyzed against ultracentrifuge buffer with and without 0.01M mercaptoethanol. Figure 5 shows that 0.01M mercaptoethanol increased the amounts of 7S and 11S components with concomitant decreases in the amount of fast-sedimenting protein. The increases in area indicated that about 30% of the 7S fraction and about 12% of the 11S fraction were present in the original extract as disulfide polymers, which were depolymerized into their monomeric forms by mercaptoethanol. Similar analysis of a 1:5 water extract yielded 35% 7S and 10% 11S as disulfide polymers.

Elimination of disulfide polymers of the 7S and 11S proteins in the extracts before cooling was studied. Portions of the extracts used previously (Fig. 4, A and B) were made 0.01M with mercaptoethanol and the rate of

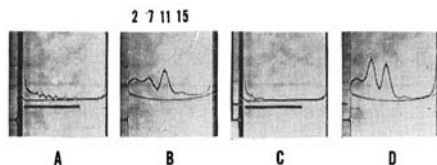


Fig. 5. Ultracentrifuge patterns for 1:100 extract of Harosoy meal in buffer without mercaptoethanol, A and B; and in buffer with 0.10M mercaptoethanol, C and D. Patterns A and C were taken after 8 min. of centrifugation, B and D after 48 min. Sedimentation of the disulfide polymers is indicated in A; C shows their absence. The extract was concentrated by pervaporation before analysis.

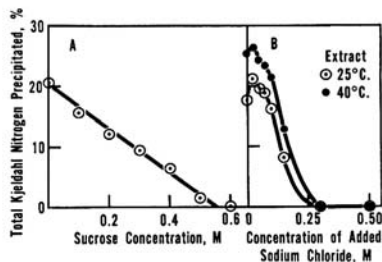


Fig. 6. Inhibition of cryoprecipitation from 1:5 Hawkeye meal extracts by sucrose, A; and by sodium chloride, B. In A, extracts were prepared with sucrose solutions at 25°C.

cryoprecipitation was determined (Fig. 4, C and D). Mercaptoethanol increased the rate of cryoprecipitation in the Clark extract, but the total amount of precipitate did not exceed the amount formed without mercaptoethanol (Fig. 4, A). Presence or absence of mercaptoethanol in the extract before cooling had no effect on ultracentrifugal composition of the cryoprecipitates. Neither did mercaptoethanol have any detectable effect on cryoprecipitation from the Hawkeye meal extract (Fig. 4, B and D). Likewise, addition of 0.01M N-ethylmaleimide (NEMI) to block protein sulfhydryl groups had no significant effect on the rate or extent of cryoprecipitation (Fig. 4, D). The Kjeldahl values in Fig. 4, D, are corrected for the nitrogen content of the added NEMI.

#### Sucrose Inhibition of Cryoprecipitation

Ghetie and Buzila (5) reported that sugars in concentrations of 0.1–0.55M prevented cryoprecipitation from seed extracts, but they gave no data for soybean meal extracts. In preliminary experiments 1:5 extracts of Hawkeye meal were adjusted to various sucrose concentrations by adding the solid sugar. Cryoprecipitation was inhibited, but volume changes at the higher sucrose concentrations were large enough to suggest that inhibition may be caused by dilution of the proteins. Meal extracts were then prepared with sucrose solutions (1:5 meal:sucrose) as the extraction solvent. Kjeldahl and ultracentrifugal analyses of the extracts indicated that sucrose had no significant effect on the amounts or kinds of proteins extracted. Increasing sucrose concentrations progressively inhibited cryoprecipitation; inhibition was complete at concentrations of 0.6M or higher (Fig. 6, A).

#### Effect of Salts on Cryoprecipitation

Briggs and Mann (2) described the influence of sodium chloride on



cryoprecipitation of the 11S protein, but did not carry out a systematic study of this factor. Accordingly, 1:5 meal:water extracts prepared from Hawkeye meal at 25° and 40°C. were used to reinvestigate the effect of added salt on cryoprecipitation. At low levels of added salt there appeared to be a small increase in the amount of cryoprecipitate formed, followed by a rapid decrease in cryoprecipitation as more sodium chloride was added (Fig. 6, B). No precipitation occurred at salt concentrations greater than 0.3M.

Calcium chloride has been used to precipitate the 11S component from aqueous meal extracts (2,11,20), but varying its concentration has not been studied. Precipitation of total protein and of the individual components as a function of added calcium chloride concentration is shown in Fig. 7 (lower). Results are expressed as percentages of the values for the water extract before cooling. The values at zero concentration of calcium chloride represent changes resulting from cooling the water extract. Thus 15% of the protein precipitated on cooling in the absence of calcium chloride. The total protein precipitating increased as calcium chloride was added until 60% of the protein precipitated at 0.1N calcium chloride. Of the protein components, the 11S and 15S + > 15S appeared most sensitive to calcium chloride and were precipitated almost quantitatively at 0.1N calcium chloride. It is apparent, however, that precipitation was not selective; the 2S and 7S fractions were also removed with increasing amounts of calcium but to a much smaller extent than the 11S and larger components. Representative ultracentrifuge patterns are shown in Fig. 7 (upper).

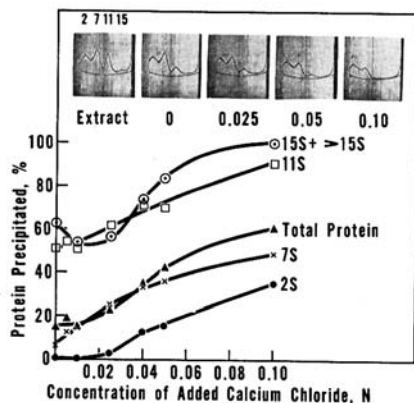


Fig. 7. Influence of added calcium chloride on proteins precipitated (lower) and on ultracentrifugal composition of proteins remaining soluble (upper), at 0°–2°C. Values in lower figure are expressed as percentages of total protein and protein components in original extracts before cooling. In upper figure the first pattern is for the water extract before cooling, and normalities of calcium chloride are given under other patterns.

#### Influence of pH on Cryoprecipitation

Cryoprecipitation of 11S protein from a meal extract is normally performed at the pH of the extract (6.2–6.5). Cryoprecipitation at lower pH values has not been reported. We therefore examined the effect of lowering the pH of a meal extract toward the isoelectric points of the 11S protein and

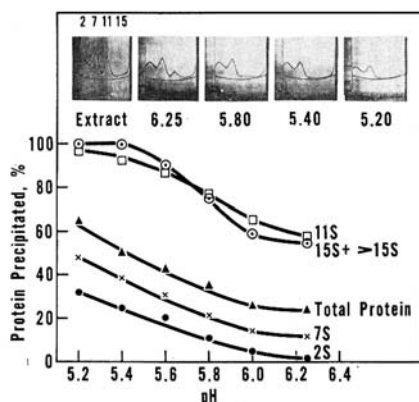


Fig. 8. Relation between pH of 1:5 extract and proteins precipitated (lower), and ultracentrifugal composition of proteins remaining soluble (upper), at  $0^{\circ}$ – $2^{\circ}$ C. Results (lower) are expressed as percentages of total protein and protein components in original extract at pH 6.25 and before cooling. First pattern in upper figure is for original extract before pH adjustment and cooling. Remaining patterns are for supernatants obtained at pH values indicated.

the other globulins before cooling. Figure 8 (lower) plots total protein and ultracentrifuge components precipitated at different pH values. The results are expressed as percentages of the values for the original water extract. The amount of protein precipitating in the cold increased from about 25% at pH 6.25 to 65% at pH 5.2. As noted with calcium chloride, the 11S and 15S + > 15S proteins were most sensitive, even though precipitation was not completely selective for these two fractions. At pH 5.2, where precipitation of 11S and 15S + > 15S components was nearly complete, about 50% of the 7S and 30% of the 2S fractions precipitated. Ultracentrifuge patterns showing the effect of pH on cryoprecipitation are reproduced in Fig. 8 (upper). Results were essentially the same for Hawkeye meal; however, the pH of the initial extract was 6.4, the extract contained more protein, and more of the total protein precipitated at all pH values than shown in Fig. 8. For example, 30% of the protein precipitated at pH 6.4, and 75% at pH 5.2.

#### Preparation of 7S Protein by Hydroxylapatite Chromatography

One purpose of our studies was to remove the 11S and 15S fractions from an aqueous extract as a preliminary step in the separation of the 2S and 7S fractions. Of particular interest was isolation of the 7S component which undergoes dimerization at 0.1 ionic strength (21). This protein is obtainable in about 80% purity by hydroxylapatite chromatography of the supernatant from cryoprecipitation of the 11S protein in an aqueous meal extract (14). The major impurities remaining in such hydroxylapatite-purified 7S preparations are 11S and faster-sedimenting components. We therefore examined the hydroxylapatite-chromatographic behavior of 2S and 7S preparations similar to those in Figs. 7 and 8.

A stepwise elution procedure was developed on the basis of previous gradient elution chromatography of soybean proteins on hydroxylapatite (14). Figure 9 is an elution diagram of the stepwise procedure for a 1:10

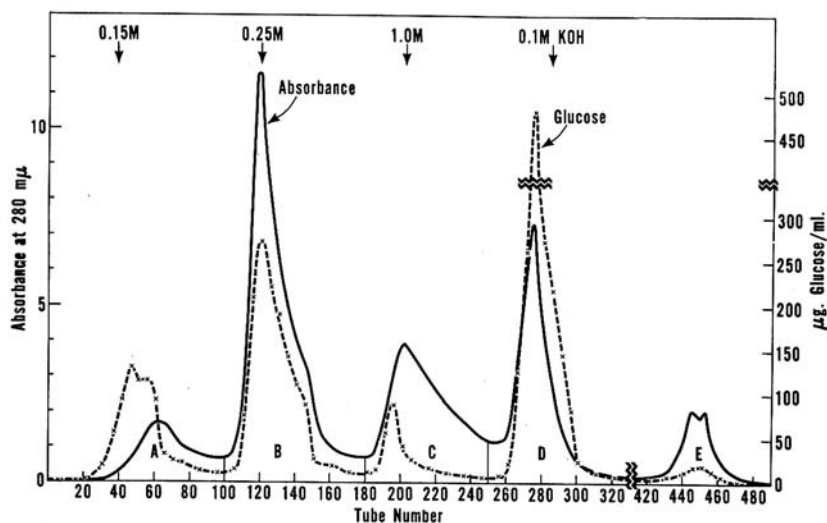


Fig. 9. Diagram of stepwise elution of water-extractable proteins from hydroxylapatite. Molarities of potassium phosphate and points of addition are indicated at top of figure. Solid curve is absorbance and broken curve is concentration of carbohydrates. Fractions contained 2 ml. per tube. Column load was 1,180 mg. of protein.

meal:water extract. Fractions A to D correspond to similarly designated fractions from gradient elution (14) and have the compositions given in Table II. Also apparent in Fig. 9 are the large differences between the ratio of carbohydrate to protein for the various fractions. Fraction E (Fig. 9) was eluted with dilute potassium hydroxide during column regeneration but was

TABLE II  
ULTRACENTRIFUGAL COMPOSITIONS OF FRACTIONS FROM HYDROXYLAPATITE CHROMATOGRAPHY

PREPARATION	CHROMA- TOGRAPHIC FRACTION	CHROMA- TOGRAPHIC COMPO- SITION <sup>a</sup>	ULTRACENTRIFUGAL COMPOSITION			
			2S	7S	11S	15S + >15S
Water-extractable proteins <sup>b</sup>	....	%	%	%	%	%
	A	100	21	31	34	14
	B	8	100 <sup>c</sup>	....	....	....
	C	40	44	40	8	8
	D	21	8	11	70	11
	E	20	2	44	37	17
Soluble proteins, pH 5.4	....	11	....	....	....	....
	....	100	45	50	5	....
	A	20	100 <sup>c</sup>	....	....	....
	B	50	52	48	....	....
	C	8	74	20	6	....
	D	16	3	90	7	....
E	4	....	....	....	....	

<sup>a</sup>Estimated from areas under peaks of elution diagrams.

<sup>b</sup>Values from a separate experiment by A. M. Nash of this Laboratory.

<sup>c</sup>About one-fourth to one-third of the protein sedimented as an unresolved shoulder in the 4S to 5S range.

not examined because of the high pH treatment received. From the ratio of carbohydrate to absorbance, it appears unlikely that fraction E is fraction D, which was not eluted by 1M phosphate buffer.

Figure 10 (lower) contains the elution diagram for the proteins that re-

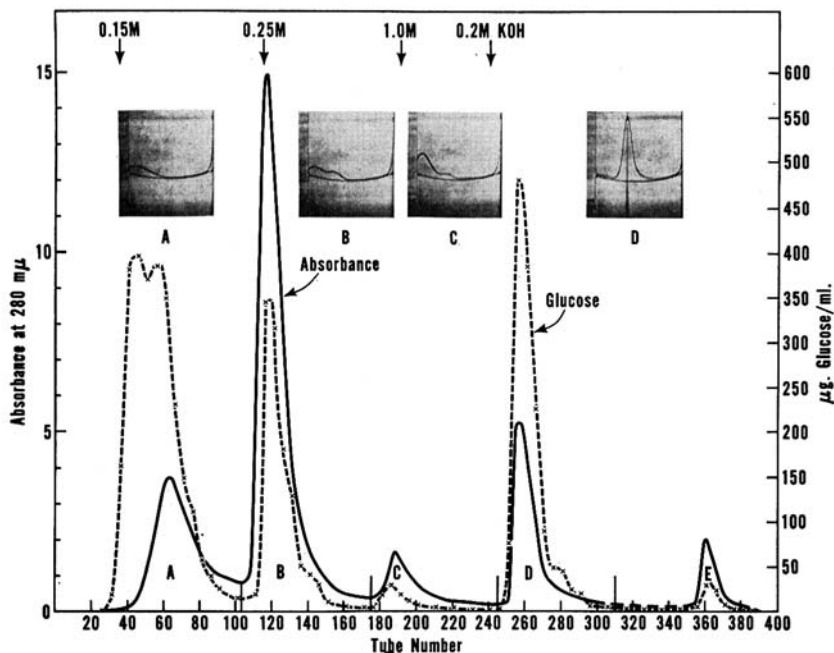


Fig. 10. Stepwise elution diagram of proteins soluble at pH 5.4 and 0°-2°C. in a 1:5 extract. Conditions were similar to those of Fig. 9. Column load was 980 mg. of protein. Ultracentrifuge patterns for fractions A to D are shown across top of figure.

remained soluble when a 1:5 meal:water extract was adjusted to pH 5.4 and cooled to 0°-2°C. (Fig. 8). The ultracentrifuge patterns for the four fractions appear in Fig. 10 (upper). Fraction C was noticeably decreased in size and changed in composition (Table II) as compared to the same fraction from the water-extractable proteins. Removal of 11S and 15S fractions by cryoprecipitation also markedly improved purity of the 7S protein in fraction D. Besides a trace of 2S material, marked skewing of the leading edge on the 7S peak (Fig. 10) indicated about 7% 11S component. In six other chromatographic runs similar to the one shown in Fig. 10, column loads were varied from 400 to 5,400 mg. with nearly identical results. One of these runs (column load 1,900 mg.) yielded fraction D with a 7S purity of only 83%, but all others yielded 7S preparations in the range of 87 to 91% purity. In several of the runs, fraction D was divided into two parts and analyzed separately; the front half of the peak always contained a higher percentage of 2S material than the back half. Evidently the 2S protein is not a reversible breakdown product of the 7S protein. The possibility of irrever-

sible breakdown of 7S protein at an earlier stage of preparation, however, is not ruled out.

A fraction D preparation was also examined in the ultracentrifuge at pH 7.6, 0.1 ionic strength. Under these conditions about 90% of the 7S material was converted into a faster-sedimenting form, presumably a dimer. Therefore the major protein of fraction D is similar to, if not identical with, the 7S globulin isolated by Roberts and Briggs (21).

#### DISCUSSION

Previous work (22) indicated that meal-to-water extraction ratio did not particularly affect the ultracentrifuge pattern for the proteins. Likewise in our study, the ultracentrifuge patterns showed no change in the number of components, but distinct quantitative differences occurred as the extraction ratio was varied (Fig. 1, B, and Fig. 2). Our studies, however, covered a wider range of extraction ratios and were carried out with the use of more sensitive analytical methods. The ultracentrifuge buffer contained mercaptoethanol, which eliminated disulfide polymers of the 7S and 11S fractions (Fig. 5), thereby giving a simpler sedimentation pattern; in addition, analyses were carried out with a double-sector cell, which eliminated errors in fitting the baseline to the sedimentation patterns.

Several factors must be considered in attempting to explain why extractability of the 7S, 11S, and 15S fractions decreased with increases in the meal:water extraction ratio (Fig. 1, B, and Fig. 2). One of these factors is solubility of the protein components in the extraction solvent. The influence of extraction temperature on extract composition (Fig. 3) indicates that the solubility limit of the 11S protein is reached at high extraction ratios. This explanation is unlikely, however, at low extraction ratios. For example, no 11S component precipitated when the 2.5 g. extract was cooled, although a decrease in extractability occurred. It is also doubtful that the solubility limit of the 7S fraction is reached during extraction, since the 7S fraction did not precipitate in appreciable amounts when any of the extracts were cooled.

Decreased protein extractability as a result of a decrease in cellular rupture is improbable for the following reason. Extractability of 2S fraction remained constant at all meal:water ratios (Fig. 1, B). This result indicates that disruption of cell walls is as complete at 20 g. of meal as at 1 g. of meal per 100 ml. of water.

Similarity of the extractability curves for the 7S, 11S, and 15S fractions (Fig. 1, B) suggests a common cause of decreased extractability with increase in extraction ratio. A common property for the three fractions may be their location within the cell in the protein bodies or aleurone grains (23,24). Lowered extractability could then be attributed to a decrease in the extent of rupture of the protein bodies as the extraction ratio is increased. Assuming that the meal salts and sugars are completely soluble at all extraction ratios, the salt and sugar content of the extracts should be lowest in the 1-g. extract and highest in the 20-g. extract. Thus osmotic shock should be greatest with the 1-g. meal sample and should become smaller as the extraction ratio is in-

creased. If this interpretation is correct, the 2S fraction must be located outside of the protein bodies and readily solubilized at all extraction ratios.<sup>2</sup>

Alternatively, raising the extraction ratio may cause an interaction of 7S, 11S, and 15S components to form an insoluble aggregate. This explanation for reduced extractability of the three fractions seems unlikely, however. A 1-g. meal extract was pervaporated to one-tenth of its original volume, which made it equivalent to an extract from 10 g. of meal. Since no protein precipitated, increasing the concentration of proteins and of the other soluble meal components does not cause aggregation of any of the proteins during extraction.

Extraction of meal at 40° instead of 25°C. resulted in an almost twofold yield of 11S protein from a 1:5 extract (Fig. 3); consequently, extraction temperature is important if maximum yield of 11S protein is desired. At this extraction ratio, the extract appears to be saturated with 11S component at 25°C. Raising the extraction temperature thus causes an increase in solubility of the 11S component. This interpretation, however, does not explain why more 7S protein is extracted at 40° than at 25°C., since the additional 7S fraction does not precipitate on cooling. Increased efficiency of protein body rupture at the higher temperature seems more plausible.

Yield of cryoprecipitate reached a maximum or near maximum in 1 to 2 hr. of cooling and was unaffected by mercaptoethanol or NEMI in the extracts (Fig. 4). Insensitivity of cryoprecipitation of the 11S protein to reducing agents is in marked contrast to the behavior of blood cryoglobulins, which no longer precipitate in the cold after treatment with 0.1M cysteine (26). Cryoprecipitation is therefore not limited to the disulfide polymers of the 11S protein, although the polymers will also precipitate (9). If formation of disulfide polymers were a prerequisite for cryoprecipitation, the 7S fraction would also be expected to precipitate on cooling, since part of it is also present in polymerized form in aqueous extracts (Fig. 5).

Our results confirm the report of Ghetie and Buzila (5) concerning the ability of sugars to inhibit cryoprecipitation from soybean extracts, although there is no obvious explanation for this behavior. Possibly sucrose complexes with 11S protein and prevents it from aggregating and precipitating in the cold. The ability of high concentrations of salt to prevent cryoprecipitation indicates that electrostatic interactions are involved in the aggregation occurring during cryoprecipitation. In this connection, Naismith (8) showed that both the 7S and 11S globulins undergo association reactions at low ionic strength which are reversed by increasing ionic strength. If association of the 7S globulin is limited to the dimer state, as suggested by Roberts and Briggs (21), cryoprecipitation may be unique for the 11S component, because it can associate until the aggregates become insoluble.

Inhibition of cryoprecipitation by salts is also a function of pH. In this study, addition of 0.3M sodium chloride to an extract with a pH of 6.4 inhibited cryoprecipitation. Other work (27) shows that the 11S and 15S

<sup>2</sup>Tombs (25) recently reported that about 21% of the total protein (corrected for 5% non-protein nitrogen) is located outside of the protein bodies of soybean meal. This value is in good agreement with a 2S content of 19-24% calculated from the data in Fig. 1, B.

fractions will precipitate at  $0^{\circ}$ – $2^{\circ}$ C. from 0.5 ionic strength buffer at pH 4.6. Since the 15S fraction has lower solubility at pH 4.6 than the 11S fraction, it was possible to separate the 15S fraction from 11S by selective precipitation.

Attempts to precipitate the 11S protein completely and selectively by modification of conditions during cooling were only partially successful. The 11S fraction from aqueous extracts was nearly completely removed either by adding calcium chloride or by lowering pH, followed by cooling. Both procedures, however, caused some loss of 2S and 7S fractions, plus complete removal of the 15S fraction (Figs. 7 and 8). By combining cryoprecipitation at pH 5.4 with hydroxylapatite chromatography, we obtained a 7S globulin of 80–90% purity that dimerized at 0.1 ionic strength. This preparation corresponded closely in composition and properties to the one Roberts and Briggs (21) secured in low yield by ammonium sulfate fractionation. Our yields were on the order of 9–12% of the total 7S fraction when calculated on the basis used by Roberts and Briggs; their yields were about 4% of the total 7S protein. Both of these yields are likely to be low, because they are calculated by assuming that all the 7S fraction in soybeans is capable of undergoing dimerization at low ionic strength. Hydroxylapatite chromatography showed that this assumption is incorrect; a part of the 7S fraction is eluted in fraction B (Table II) but does not dimerize at 0.1 ionic strength (14). In addition, we have analyzed the water-extractable proteins in the ultracentrifuge in 0.1 and 0.5 ionic strength buffers (pH 7.6 plus 0.01M mercaptoethanol). Only about 60% of the 7S fraction observed at 0.5 ionic strength or 20% of the total protein dimerized at 0.1 ionic strength.

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