PHYSICAL PROPERTIES OF ALCOHOL-EXTRACTED SOYBEAN PROTEINS[1]

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ABSTRACT

Isolated soybean proteins contain phospholipid-like materials which are extractable with aqueous alcohols. Since alcohols are protein denaturants, their effects on the physical properties of soybean proteins were investigated. Solubility of freeze-dried, isoelectric-precipitated soybean proteins in pH 7.6, 0.5 ionic strength, phosphate buffer was 59%. In the presence of 0.01*M* mercaptoethanol, solubility in buffer increased to 80% as a result of depolymerization of disulfide polymers of the 7S and 11S ultracentrifugal components. Extraction of the proteins for 2 hr. at 25°C. with 94% methanol, 83% ethanol, or 77% isopropanol decreased solubility (in the presence of mercaptoethanol) to 66–71%. Water-saturated butanol decreased solubility to 25%. The 7S and 11S components accounted for most of the protein insolubility. Loss of protein solubility apparently is caused by denaturation rather than by extraction of phospholipids by the alcohols. Optical rotation, viscosity, and ultracentrifugal measurements indicate that the proteins which retain their solubility are undenatured. Purified 11S component, in which the sulfhydryl groups are blocked with N-ethylmaleimide, forms soluble aggregates upon extraction with 83% ethanol. Extent of aggregation was reduced at low extraction temperatures.

Denaturation of soybean proteins by alcohols has been studied by several workers. For example, Smith *et al.* (9) studied the water dispersibility of the proteins of defatted soybean meal treated with methanol, ethanol, isopropanol, or acetone. They investigated the effects of alcohol concentration and temperature. Alcohol concentration proved to be important, and concentrations causing most

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extensive denaturation were 50–70% for methanol, 30–60% for ethanol, and 30–50% for isopropanol. Mann and Briggs (5) studied the effects of methanol and ethanol on solubility and electrophoretic properties of proteins in defatted soybean meal. They found that the globulin fraction was most sensitive to alcohol.

Recent studies in this laboratory have shown that isolated soybean proteins contain phospholipid-like materials which are extractable with aqueous alcohols (3). Removal of the phospholipids has a marked effect on the foaming properties of soybean proteins. Stable, low-density foams can be prepared after alcoholic extraction. Maximum foam stability is obtained when the proteins are extracted with 94% methanol, 83% ethanol, or 77% isopropanol by weight (3).[2] In this paper, we report the effects of these aqueous alcohols and water-saturated butanol on the solubility, optical rotation, ultracentrifugal properties, and viscosity of soybean globulins isolated by isoelectric precipitation at pH 4.5. Also presented are some studies on the effect of 83% ethanol on the 11S component, which represents more than one-third of soybean globulins.

Materials and Methods

Adams (1960 crop), Clark (1957 and 1958 crops), and Hawkeye (1957 crop) soybeans stored at 5°C. were used in this study. The samples were cracked, dehulled, and flaked prior to defatting with hexane at room temperature.

Acid-precipitated soybean proteins were isolated at pH 4.5 and freeze-dried as described by Eldridge *et al.* (3). The purified 11S component was obtained by ammonium sulfate fractionation (11). Sulfhydryl groups of the 11S component were blocked by treatment with N-ethylmaleimide at pH 7.6, 0.5 ionic strength (1).

Alcohol extraction of acid-precipitated soybean proteins at pH 4.5 was performed in 50-ml. screw-capped test tubes using 750 mg. of protein and 25 ml. of alcohol. Extraction was performed by mechanical shaking for 15 min., centrifuging at low speed, and decanting the solvent. The extraction procedure was repeated three times, and the last extract was removed after the protein was in contact with the alcohol for a total of 2 hr. To facilitate rapid removal of residual alcohol, the protein was slurried in diethyl ether, transferred to a sintered-glass funnel, and washed thoroughly with ether. The protein samples were then dried in a vacuum oven for 2 hr. at 37°C.

Solids extracted from acid-precipitated proteins were determined by

[2] Alcohol concentrations in reference 3 were expressed on a volume basis (v/v). In this paper concentrations are expressed on a weight basis to facilitate comparison with earlier studies on alcohol denaturation of soybean proteins.

evaporating aliquots of the alcoholic extracts and drying *in vacuo* for 2 hr. at 50°C.

Protein solubility was determined in potassium phosphate-sodium chloride buffer, pH 7.6, ionic strength 0.5, 0.01*M* mercaptoethanol (12), except for omission of mercaptoethanol in the first experiments shown in Table I. Protein solubilities were determined by suspending the

TABLE I

EFFECT OF MERCAPTOETHANOL ON SOLUBILITY, SPECIFIC ROTATION, AND ULTRACENTRIFUGAL COMPOSITION OF ACID-PRECIPITATED SOYBEAN PROTEIN[a] BEFORE AND AFTER EXTRACTION WITH 83% ETHANOL

ALCOHOL TREATMENT	MOLARITY OF MERCAPTOETHANOL	PROTEIN SOLUBILITY	$-[\alpha]_D^{25}$	ULTRACENTRIFUGAL COMPOSITION				
				2S	7S	11S	15S	>15S
		%		%	%	%	%	%
None	0.00	58.5	45.5	11.4	9.6	26.9	7.2	3.5
None	0.01	80.4	46.4	12.5	25.2	35.2	4.8	2.6
Extracted[b]	0.00	45.8	47.6	8.8	7.2	20.9	5.3	3.6
Extracted[b]	0.01	71.2	47.8	12.7	19.7	31.6	5.5	1.8

[a] Prepared from Adams soybeans, 1960 crop.
[b] Extracted for 2 hr. at 25°C.

protein in buffer at a ratio of 15 mg. protein per ml. of buffer and dialyzing against buffer at 4°C. for 48 hr. or longer. The insoluble protein was removed by centrifuging, and aliquots of the supernatant were analyzed for nitrogen by the Kjeldahl method. Results are expressed as percentages of the total Kjeldahl nitrogen remaining soluble after dialysis.

Optical rotations were measured on the protein solutions obtained in the solubility experiments. Measurements were made with a Bates-Fric saccharimeter at 25°C. with a sodium vapor lamp as the light source.

Viscosity measurements were made at 25°C. with a Cannon-Fenske modified Ostwald viscometer, ASTM No. 100. Viscosities were measured on the protein solutions from the solubility experiments. Densities of the buffer and protein solutions were measured with a 5-ml. pycnometer. Intrinsic viscosities were evaluated by plotting $(\eta_{rel}-1)/c$ versus c and extrapolating to zero concentration, where c is the concentration of protein in g./100 ml. of solution. Low solubility of the water-saturated butanol-treated protein resulted in a dilute solution in the solubility determination; therefore only the reduced viscosity at one concentration is given for this sample.

Ultracentrifugal analyses of the protein solutions obtained in the solubility experiments were performed at room temperature with a

Spinco,[3] Model E, ultracentrifuge at 47,660 r.p.m. A 30-mm. cell with a plastic double-sector centerpiece was used. Ultracentrifuge data are apparent compositions, since no correction has been made for the Johnston-Ogston effect (4). Ultracentrifugal compositions are expressed as percentages of the total protein.

Results

Studies on the Acid-Precipitated Proteins. Freeze-dried, acid-precipitated proteins were only about 59% soluble in pH 7.6, 0.5 ionic strength buffer (Table I). However, if the buffer contained 0.01*M* mercaptoethanol, an additional 22% of the proteins dissolved. Also noted in the presence of mercaptoethanol were increases in the areas under the 7S and 11S peaks of the ultracentrifuge patterns (compare Fig. 1a and 1b).[4] The proteins which are solubilized in the presence

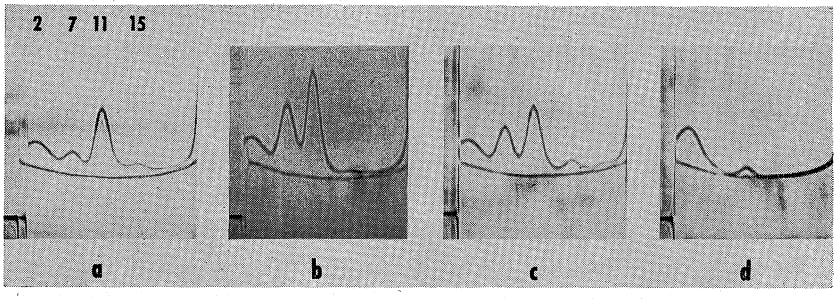

Fig. 1. Ultracentrifuge patterns for acid-precipitated proteins: (a) soluble in buffer; (b) soluble in buffer containing 0.01*M* mercaptoethanol; (c) soluble in buffer containing 0.01*M* mercaptoethanol after extraction with 94% methanol; and (d) soluble in buffer containing 0.01*M* mercaptoethanol after extraction with water-saturated butanol. Differences in areas under the patterns reflect differences in solubility, since the initial ratio of protein to buffer was 15 mg./ml. for all four samples. The major peaks are identified in Svedberg units across the top of Fig. a.

of disulfide-cleaving reagents therefore appear to be mainly disulfide polymers of the 7S and 11S fractions (1,13). Calculations from the data in Table I indicate that approximately 60% of the 7S fraction and nearly 25% of the 11S fraction are polymerized in this manner. The nature of the proteins which remain insoluble in the presence of mercaptoethanol is unknown, although freeze-drying causes aggregation of the 11S component, as shown below and by Wolf *et al.* (11).

If the acid-precipitated proteins are extracted with 83% ethanol, protein solubility (in the absence or presence of mercaptoethanol) drops to 46 and 71%, respectively. These solubilities are 9–13% lower

[3] Reference to specific equipment or organizations does not necessarily constitute endorsement by the U.S. Department of Agriculture.

[4] Substitution of 0.01*M* sodium sulfite for 0.01*M* mercaptoethanol gave similar increases in protein solubility and areas under the 7S and 11S peaks.

than the values obtained for the unextracted protein in the presence or absence of reducing agent indicating denaturation by alcohol. Increase in protein solubility in the presence of mercaptoethanol was 22 and 25%, respectively, for unextracted and extracted proteins. This relatively constant value suggests that the disulfide polymers are resistant to denaturation by 83% ethanol. Comparison of the ultracentrifugal compositions in the presence of mercaptoethanol shows that the 7S and 11S proteins are the major fractions involved in the loss of solubility upon alcohol extraction. The specific rotations (Table I) of the alcohol-extracted proteins are unchanged from those for the unextracted proteins.

Extraction of soybean proteins with 94% methanol, 83% ethanol, 77% isopropanol, or water-saturated butanol removes 7–8% of the weight of the proteins (Table II). About one-third to one-half of the alcohol-soluble material is also soluble in chloroform and appears to

TABLE II

EFFECT OF ALCOHOL EXTRACTION ON SOLUBILITY, SPECIFIC ROTATION, INTRINSIC VISCOSITY, AND ULTRACENTRIFUGAL COMPOSITION OF ACID-PRECIPITATED SOYBEAN PROTEIN[a]

EXTRACTION SOLVENT[b]	SOLIDS EXTRACTED	PROTEIN SOLUBILITY	$-[\alpha]_D^{25}$	INTRINSIC VISCOSITY	ULTRACENTRIFUGAL COMPOSITION				
					2S	7S	11S	15S	>15S
	%	%		*dl./g.*	%	%	%	%	%
None	..	80.4	46.4	0.052	12.5	25.2	35.2	4.8	2.6
83% ethanol	7.3	71.2	47.8	0.055	12.7	19.7	31.6	5.5	1.8
94% methanol	7.4	66.3	50.1	0.057	11.8	18.4	25.1	6.4	4.7
77% isopropanol	8.3	68.1	48.3	0.047	12.4	18.1	31.1	4.5	2.0
Water-saturated butanol	8.2	25.4	54.8	0.053[c]	20.3	3.0	2.2[d]	..	..

[a] Prepared from Adams soybeans, 1960 crop.
[b] Extracted for 2 hr. at 25°C.
[c] Value for reduced viscosity of solution containing 0.35 g. protein/100 ml.
[d] $s_{20,w}$ value 11.85.

be phospholipid; the remainder of the alcohol-soluble material probably consists of sugars, salts, and other nonprotein constituents present in the crude proteins.

Some of the physical properties of soybean proteins extracted with the different alcohols are given in Table II. All data were obtained with buffered solutions containing 0.01*M* mercaptoethanol. Solubility is reduced from 80 to 66–71% for the first three alcohols; water-saturated butanol reduces solubility to 25%. Specific rotations and intrinsic viscosities of the soluble portions of the proteins are essentially the same as for the unextracted proteins, indicating: (a) the soluble portion is undenatured, and (b) the buffer used separates the denatured and undenatured proteins by differences in solubility. The

ultracentrifugal patterns for the alcohol-extracted proteins also suggest that the buffer-soluble proteins are undenatured; the peaks are sharp and similar to those of patterns obtained with unextracted proteins (compare Fig. 1c with Fig. 1a and 1b). The ultracentrifuge data (Table II) show that the 7S and 11S fractions are sensitive to alcohol treatment. The 7S fraction appears to be more sensitive to 83% ethanol and 77% isopropanol than tthe 11S fraction. With 94% methanol, solubility of the 7S fraction is decreased to the same extent as with the other two alcohols, but there is also a marked decrease in solubility of the 11S fraction (compare Fig. 1b and 1c). All of the proteins (including the disulfide polymers of the 7S and 11S components) except the 2S fraction are extremely sensitive to water-saturated butanol (Fig. 1d). Only traces of the 7S and 11S fractions remain soluble after 2 hours' contact with this alcohol. The apparent increase in 2S fraction may be the result of breakdown of the faster sedimenting proteins into soluble 2–3S subunits. Upon longer contact (6–8 hr.) with butanol the 7S and 11S components are completely insolubilized and protein solubility decreases to about 15%.

Two factors may explain insolubilization of protein on extraction with alcohols: (a) removal of phospholipids which act as solubilizing agents and (b) alcohol denaturation of the proteins. Previous studies (3) showed that maximum extraction of phospholipids with 83% ethanol occurs at an alcohol-to-protein ratio of 20:1 (ml. alcohol per g. of protein) or greater. At an alcohol-to-protein ratio of 2:1 only about 40% of the phospholipids are extracted. Table III shows no

TABLE III

EFFECT OF VARYING SOLVENT:PROTEIN RATIO ON PROTEIN SOLUBILITY AND ULTRACENTRIFUGAL COMPOSITION OF ACID-PRECIPITATED SOYBEAN PROTEIN[a]

SOLVENT RATIO[b]	PROTEIN SOLUBILITY	ULTRACENTRIFUGAL COMPOSITION				
		2S	7S	11S	15S	>15S
	%	%	%	%	%	%
Control[c]	80.4	12.5	25.2	35.2	4.8	2.6
2:1	72.6	11.9	19.6	36.0	4.1	0.9
50:1	71.2	11.0	19.6	35.6	3.9	1.2

[a]Prepared from Adams soybeans, 1960 crop.
[b]Ratio is expressed as ml. of solvent per g. of protein. Solvent was 83% ethanol.
[c]No alcohol treatment.

appreciable differences in the protein solubility and ultracentrifugal composition for protein extracted with 83% ethanol at solvent:protein ratios of 2:1 and 50:1 for 2 hr. at room temperature. These results suggest that the difference in solubility between the control and the extracted samples is the result of denaturation rather than removal of phospholipids.

Phospholipid extraction and drying of wet soybean protein curd can be combined in one step by using alcohol and ether (3). Table IV compares results of freeze-drying and drying with absolute ethanol

TABLE IV

COMPARISON OF FREEZE-DRYING AND ALCOHOL-ETHER DRYING OF SOYBEAN CURD[a]

DRYING METHOD	PROTEIN SOLUBILITY	ULTRACENTRIFUGAL COMPOSITION			
		2S	7S	11S	15S
	%	%	%	%	%
Freeze-drying	72.3	8.2	22.9	35.8	5.3
Absolute ethanol-ether[b]	66.8	10.0	13.5	35.7	7.7

[a] Prepared from Clark soybeans, 1958 crop.
[b] Time of contact with alcohol was about 1 hr.

and diethyl ether. The ethanol-ether-dried sample has a solubility only about 5% lower than the freeze-dried preparation; the decrease in protein solubility occurs in the 7S fraction. The lower solubility for the freeze-dried sample in Table IV as compared to the values for the untreated samples in Tables I–III is probably the result of using protein samples from different defatted meals.

Studies on the 11S Component. The 11S component is the major fraction of the acid-precipitated proteins (Fig. 1 and Tables I–IV) and can be obtained in better than 90% purity from the cold-insoluble fraction of soybean proteins. Purification consists of blocking sulfhydryl groups with N-ethylmaleimide and fractionating with ammonium sulfate (11). The purified 11S component contains only a trace (0.1%) of material extractable by 83% ethanol (3) and is stable to changes in molecular size in solution at pH 7.6, 0.5 ionic strength (11). However, it is sensitive to other conditions, including treatment with 83% ethanol (Table V and Fig. 2). Upon dialysis and freeze-drying,

TABLE V

EFFECTS OF DIALYSIS, FREEZE-DRYING, AND ALCOHOL EXTRACTION ON THE ULTRACENTRIFUGAL PROPERTIES OF THE 11S COMPONENT[a]

TREATMENT	ULTRACENTRIFUGAL COMPOSITION[b]				
	2S	7S	11S	15S	>15S
	%	%	%	%	%
None	..	..	94	6	...
Dialysis to remove buffer salts	..	2	89	9	...
Freeze-drying after dialysis	1	2	81	16	...
Extraction with 83% ethanol[c]	2	3	35	12	48[d]

[a] Prepared from Hawkeye soybeans, 1957 crop.
[b] Ultracentrifugal analysis in phosphate-sodium chloride buffer, pH 7.6, 0.5 ionic strength, 0.01*M* mercaptoethanol.
[c] Extraction for 2 hr. at 25°C. with 100 ml. alcohol per g. of protein.
[d] Two peaks faster than the 15S peak plus faster unresolved material were present.

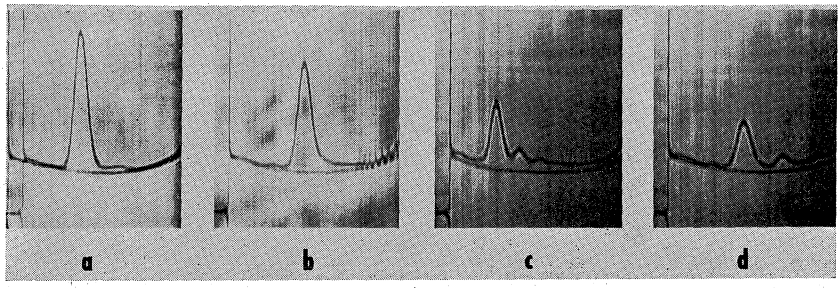

Fig. 2. Ultracentrifuge patterns for purified 11S component (a) before dialysis and freeze-drying (b) after dialysis and freeze-drying; (c) and (d) after extraction with 83% ethanol at 25°C. Solvent used was phosphate-sodium chloride buffer, pH 7.6, 0.5 ionic strength, containing 0.01*M* mercaptoethanol.

3% of the protein dissociates into 2S and 7S material and 10% is converted to material sedimenting as a broad boundary in the region of the 15S peak (compare Fig. 2a and 2b). On extraction of the freeze-dried material with 83% ethanol at room temperature, aggregation becomes extensive (Fig. 2c, 2d), although the protein is still completely soluble in the buffer.

Table VI shows the effect of temperature on extraction of the 11S

TABLE VI

EFFECT OF TEMPERATURE ON 83% ETHANOL EXTRACTION OF THE 11S COMPONENT[a]

TREATMENT	ULTRACENTRIFUGAL COMPOSITION			
	7S	11S	15S	>15S
	%	%	%	%
Control	2	89	9	..
Control after dialysis and freeze-drying	4	77	18	..
Extraction at 25°[b]	4	27	13	56
Extraction at 4°[b]	4	49	14	33

[a] Prepared from Clark soybeans, 1957 crop.
[b] Time of contact with alcohol was 2.5–3 hr.

component with 83% ethanol. On extraction at room temperature, the 11S component decreases from 77 to 27%, indicating that about 50% of the protein aggregated. The sample extracted at 4°C. was only 28% aggregated. The difference in degree of aggregation was also noted when the extracted proteins were dissolved at pH 7.6, 0.5 ionic strength, in 0.01*M* mercaptoethanol for ultracentrifugal analysis. The sample extracted at 4°C. gave a solution nearly as clear as the control. The sample extracted at 25°C., while completely soluble, was turbid. Insoluble aggregates would probably be obtained on extraction for longer times or at higher temperatures.

Discussion

The experimental results clearly show that the 7S and 11S ultracentrifugal fractions of acid-precipitated soybean protein are sensitive to treatment with aqueous alcohols. This sensitivity is manifest primarily as a loss in solubility. With 83% ethanol and 77% isopropanol the 7S fraction shows a greater lability than the 11S fraction, while the 11S fraction appears to be more sensitive to 94% methanol. Roberts and Briggs (8) have found a marked lability of the 7S fraction to 50% ethanol, indicating that alcohol concentration may be an important factor in determining relative stabilities of the 7S and 11S proteins.

The 7S and 11S fractions represent the major proteins of glycinin (12). Our results thus confirm the electrophoretic studies of Mann and Briggs (5) which indicated that the glycinin fraction of soybean protein is most sensitive to alcohol denaturation. However, ultracentrifugation shows more clearly which components of glycinin are involved. Electrophoretic analysis of 83% ethanol-extracted protein and unextracted protein in pH 7.6, 0.1 ionic strength, phosphate buffer failed to show significant differences.

With water-saturated butanol the 7S, 11S, and 15S fractions are nearly completely insolubilized in 2 hr. This result is surprising in view of Morton's studies (6) showing that a large number of tissues can be defatted with water-saturated butanol without loss of enzymatic activities. Sensitivity of the 7S, 11S, and 15S fractions to alcohols may be a reflection of their subunit structure (13). The subunits are held together by noncovalent bonds that may be broken by alcohols. Disruption of the noncovalent bonds probably results in irreversible conformational changes within the subunits, and on subsequent re-formation of noncovalent bonds the subunits recombine in a nonspecific manner resulting in insoluble aggregates.

Extent of extraction of the phospholipid impurities from soybean protein depends upon the ratio of alcohol to protein (3). However, protein solubility and ultracentrifugal composition were not affected by varying the ratio of alcohol to protein (Table III). Smith *et al.* (9) also found that the alcohol-to-protein ratio had no effect upon protein solubility in alcohol-extracted soybean meal. Maximum loss of protein solubility in soybean meal occurs at alcohol concentrations of 40–60% (9), while optimum removal of phospholipids from the isolated proteins occurs at higher alcohol concentrations (3). Loss of protein solubility thus appears to be the result of denaturation rather than removal of phospholipids. The studies of Nagel *et al.* (7) support

this conclusion, since addition of lecithin to alcohol-extracted meal failed to restore protein solubility.

The physical properties of the alcohol-extracted proteins, which are soluble in buffer at pH 7.6, 0.5 ionic strength containing 0.01*M* mercaptoethanol indicate that these proteins are undenatured. Solubility measurements in this buffer system therefore appear suitable for measuring extent of denaturation by various alcohol treatments.

The data of Tables I–III show that the 11S component in the impure state (acid-precipitated protein) is quite stable to 83% ethanol. However, when the 11S component is purified and modified by blocking its sulfhydryl groups with N-ethylmaleimide, it appears more susceptible to aggregation by 83% ethanol (Tables V and VI). Blocking of the sulfhydryl groups in the 11S component includes a pretreatment with 0.01*M* mercaptoethanol to eliminate disulfide polymers of the protein. Although mercaptoethanol causes no conformational changes detectable by optical rotation measurements (Table I) or ultracentrifugation (Fig. 1a and 1b), intramolecular disulfide linkages may be broken. Cleavage of such bonds may labilize the protein to subsequent treatment with alcohol. Roberts and Briggs (8) found that the presence of 0.01*M* mercaptoethanol in ethanol increased the susceptibility of the 11S component to denaturation by the alcohol.[5]

Behavior of the purified 11S protein on extraction with alcohol resembles that of serum albumin on extraction with chloroform-methanol (10). Albumin also forms polymers in the presence of sulfhydryl-blocking reagents, but, unlike those of the 11S protein, the albumin polymers are broken down by reducing agents.

Although the 7S and 11S components are sensitive to alcohol, the decrease in solubility of the entire acid-precipitated proteins upon 2-hr. extraction with high concentrations of methanol, ethanol, and isopropanol is relatively low. Further decrease of insolubility might be effected by minimizing extraction time. The use of a reducing agent in the solvent also appears necessary if maximum protein solubility is desired. However, intended use of the protein determines the importance of solubility. For the formation of foams with alcohol-extracted soybean protein, partial insolubility is not serious; turbid suspensions of the protein obtained in the absence of reducing agent foamed satisfactorily (2).

Acknowledgments

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[5] Since the present manuscript was submitted, the paper under discussion has been published: Roberts, R. C., and Briggs, D. R. Characteristics of the various soybean globulin components with respect to denaturation by ethanol. Cereal Chem. *40:* 450–458 (1963).

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