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Ultracentrifugation and Binding Studies of Acid-Sensitive Soybean Proteins[1]

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ABSTRACT

A fraction of the soybean protein that precipitates at pH 4.5 remains insoluble after neutralization. According to an assay developed, this acid-sensitive fraction (ASF) accounts for 25 to 30% of the water-extractable proteins. The ASF has been precipitated from an aqueous extract of soybean meal with $(NH_4)_2SO_4$ at pH 7.0 to yield preparations containing up to 67% ASF. Samples that contain ASF are tan or brown, and color intensity increases in proportion to the amount of ASF present. Difference spectroscopy of equilibrium dialysis samples indicates that ASF binds nonprotein material from 2 to 10 times more tightly than do the other soybean proteins. The 7S protein which lacks the ability to form a dimer at 0.1 ionic strength appears to function prominently in acid denaturation of ASF.

About 90% of the protein precipitates when an aqueous extract of defatted soybean meal is adjusted to pH 4.6 (1). Only 81 to 85% of this acid-precipitated protein curd is soluble in pH 7.6, 0.5 ionic strength, phosphate buffer containing 0.01M 2-mercaptoethanol (2), whereas virtually all the water-extractable soybean proteins are soluble in this buffer (3). This decreased solubility is attributable mainly to the acid-sensitivity of a portion of the 2 and 7S ultracentrifuge components (2,4).

Addition of 0.8M NaCl at pH 4.5 solubilizes at the most 78% of the acid-precipitable soybean protein because 41% of the 2S and 15% of the 7S components remain insoluble (5). Wolf et al. (2) reported 34 and 15% of the respective 2S and 7S components to be acid-sensitive.

The acid-sensitive fraction (ASF) is defined operationally in this paper as those proteins that are insoluble at pH 4.5 in the presence of 0.8M or greater concentrations of NaCl. The present study was undertaken to: a) evaluate an ASF assay; b) obtain a soluble form of the ASF; c) determine the ultracentrifugal composition of the ASF; and d) study the binding of diffusible constituents to the ASF.

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[2]Agricultural Research Service, U.S. Department of Agriculture. The mention of firm names or trade products does not imply that they are endorsed or recommended by the U.S. Department of Agriculture over other firms or similar products not mentioned.

MATERIALS AND METHODS

Protein Extraction Procedure

Hawkeye soybeans from the 1969 crop were cracked, dehulled, and flaked. The flakes were defatted with pentane-hexane (boiling range 33° to 57°C.) and then air-dried. Protein extracts were prepared with a ratio of water to flakes of 10 ml. per g. Extraction was carried out for 45 min. at ambient temperature. The insoluble residue was removed by centrifugation at 34,000 × g for 45 min. at 20° to 25°C.

Assay of the ASF

Three samples of equal volume are taken from a solution to be assayed for ASF content. To one is added sufficient NaCl to make the solution 1.0M. This solution and one of the remaining two are titrated to pH 4.5 with stirring. After 5 min. the volumes are corrected by addition of water equal to the volume of titrant consumed. The samples are then centrifuged at 34,800 × g for 15 min. at 18° to 22°C. Protein concentrations are determined on the supernatants by the biuret method noted below. No differences in the results of the protein determinations were noted when NaCl was added to bring all three samples to the same concentration, so this adjustment was not routinely made. Calculations are made according to the following:

$$\% \text{ ASF} = 100 (C - A)/C$$

$$\% \text{ APP}_i = 100 (A - B)/C$$

$$\% \text{ Whey}_i = 100 (B/C)$$

where C = the protein concentration of the nonacidified sample; A = the protein concentration of the solubles from the acidified portion containing 1.0M NaCl; APP_i = the nonASF acid-precipitable protein; B = the protein concentration of the solubles from the acidified sample devoid of NaCl; and $whey_i$ = the protein which is soluble at pH 4.5. Obviously this assay can only be used with soluble samples. Nor would this assay be suitable for a sample treated in such a manner so as to denature the ASF.

$(NH_4)_2SO_4$ Fractionation

The pH of a solution to be fractionated was first adjusted to 7.0. During fractionation the solution was maintained between pH 6.9 and 7.0 with 1N NaOH. Solid $(NH_4)_2SO_4$ was added in small amounts with care being taken to withhold further addition until the preceding quantity was in solution. The mixture was stirred for 15 min. after the final portion of $(NH_4)_2SO_4$ had dissolved. The precipitates were collected by centrifugation at 10,800 × g for 45 min., dispersed in distilled water, and dialyzed against distilled water. To prevent the pH from decreasing below 6.0 during dialysis, the retentates were periodically removed from the dialysis bags and readjusted to pH 7.0. Retentates were dialyzed until the diffusates were free of NH_4^+ when tested with Nessler's reagent, which was prepared and used as described by Johnson (6).

Protein Determination

The biuret procedure of Layne (7) was followed for the determination of protein concentrations. Crystalline bovine albumin and an $E^{1\%}_{1\ cm.}$ of 6.6 (8) were used to prepare a standard curve.

Ultracentrifuge Analysis

Samples for ultracentrifugation were dialyzed against one of two buffers for 24 hr. at 2° to 4°C. These buffers were a) 0.035M phosphate, pH 7.6, 0.5 ionic strength containing 0.4M NaCl and 0.01M 2-mercaptoethanol; and b) 0.037M phosphate, pH 7.6, 0.1 ionic strength containing 0.01M 2-mercaptoethanol. Wolf and Briggs (9) found the 0.5 ionic strength buffer to be excellent for ultracentrifuge analysis of soybean proteins, whereas the 0.1 ionic strength buffer permits dimerization of some of the 7S ultracentrifuge component of soybean protein (10).

Sedimentation velocity runs were made with a Model E Spinco ultracentrifuge operated at 47,660 r.p.m. With the RTIC unit the temperature was maintained

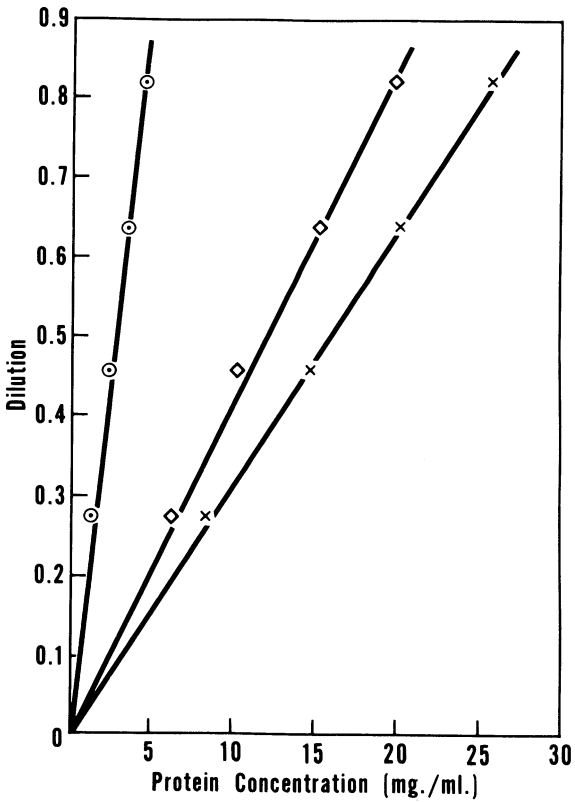

Fig. 1. Effect of protein concentration on an assay for acid-sensitive fraction (ASF). At each dilution three protein concentrations are plotted; crosses indicate nonacidified sample, squares = solubles from the acidified sample that contains 1.0M NaCl, and dotted circles = solubles from the acidified sample that contains no NaCl.

between 21° and 23°C. during each run. A 30-mm. double sector cell was used, and the schlieren patterns were recorded on photographic film.

Equilibrium Dialysis

All dialysis casing[3] was washed according to McPhie (11) before use. Sample solutions for equilibrium dialysis were prepared with 1M NaCl as solvent. Each solution was divided into four aliquots. One of these aliquots (retentate 4) was dialyzed against fresh changes of 1M NaCl until the ratio of the absorbance at 335 nm. to that at 280 nm. did not change for the retentate between two successive dialysis periods. This retentate served as the reference sample during generation of difference spectra. The remaining aliquots were dialyzed separately against individual portions of 1M NaCl equal to the retentate volumes to yield one aliquot (retentate 1) for which the diffusate was not changed, a second aliquot (retentate 2) for which the diffusate was changed once after 24 hr., and a third aliquot (retentate 3) for which the diffusate was changed once after 24 hr. and again after 48 hr. Thus a series of retentate-diffusate pairs from which increasing amounts of diffusibles had been removed was obtained from the same original sample. Retentates were removed from the dialysis casings 48 hr. after the last solvent change and the retentate and diffusate volumes were noted. The diffusates yielded no color with biuret reagent. Retentate volumes were corrected for changes during dialysis by diluting each retentate with its diffusate before difference spectroscopy so that all protein concentrations were again equal. Biuret reagent was used to verify the protein concentrations.

Difference Spectroscopy

Spectra of the retentates and diffusates from equilibrium dialysis were obtained with a Beckman DK-2A recording spectrophotometer. Difference spectra were also generated for each of the samples described in the equilibrium dialysis section. For the reference solution that aliquot was used that had been dialyzed to a minimum in the ratio A(335 nm.):A(280 nm.).

Diffusible chromophoric material bound to retentate protein was estimated by applying the following expressions at appropriate wavelengths:

$$\Delta A(r) = Ab(r) + Au(r)$$
$$Au(d) = Au(r)$$
$$\Delta A(r) - Au(d) = \Delta A(r) - Au(r) = Ab(r)$$
$$F.B. = Ab(r) \cdot v(r)/[Au(d) \cdot v(d) + \Delta A(r) \cdot v(r)]$$

where $\Delta A(r)$ = the magnitude of the retentate difference spectrum at a given wavelength; $Ab(r)$ = the absorbance, at the given wavelength, ascribable to diffusible material bound to the retentate; $Au(r)$ = the absorbance, at the given wavelength, ascribable to unbound diffusible material within the dialysis bag; $Au(d)$ = the absorbance, at the given wavelength, of unbound diffusible material in the diffusate; $v(r)$ = ml. of retentate; $v(d)$ = ml. of diffusate; and F.B. (Fraction Bound) = the fraction of diffusible material bound to retentate protein.

[3]The dialysis casing from the Visking Company, Chicago, Illinois, had a flat width of 0.984 in.

TABLE I. PURITY AND YIELD OF ACID-SENSITIVE FRACTION (ASF) IN $(NH_4)_2SO_4$ FRACTIONS OF WATER-EXTRACTABLE SOYBEAN PROTEINS

Fraction	Total Protein mg.	ASF			App_i[1]			$Whey_i$[1]		
		Protein mg.	Percent of Total ASF	Percent of Fraction	Protein mg.	Percent of Total APP_i	Percent of Fraction	Protein mg.	Percent of Total $Whey_i$	Percent of Fraction
Aqueous extract	2,348	674	100	28.7	1,340	100	57.1	334	100	14.2
$(NH_4)_2SO_4$ precipitate										
28%	326	219	32.5	67.2	77	5.7	23.6	30	9.0	9.2
28 to 41%	359	185	27.4	51.5	87	6.5	24.2	87	26.0	24.2
41 to 60%	327	134	19.9	41.0	123	9.2	37.6	70	21.0	21.4
60 to 100%	1,166	97	14.4	8.3	1,013	75.6	86.9	56	16.8	4.8

[1]APP_i = the nonASF acid-precipitable protein; $whey_i$ = the protein which is soluble at pH 4.5.

TABLE II. ULTRACENTRIFUGAL COMPOSITION OF $(NH_4)_2SO_4$ FRACTIONS OF WATER-EXTRACTABLE SOYBEAN PROTEINS

$(NH_4)_2SO_4$ Fraction	Buffer Ionic Strength	Percent				
		2S	7S	11S	>11S	11S + >11S
35% precipitate[1]	0.5	60	29	...	...	12
	0.1	57	23	...	...	20
35% precipitate after acid treatment[2]	0.5	64	23	....	...	13
	0.1	60	12	...	...	29
60 to 100% precipitate[3]	0.5	9	49	34	9	43
	0.1	10	11	29	51	80

[1]Assayed 74% ASF.
[2]Solubles remaining after removal of ASF at pH 4.5, 1M NaCl.
[3]Assayed 9% ASF.

RESULTS AND DISCUSSION

To ascertain the influence of protein concentration on the outcome of the ASF assay, serial dilutions of an aqueous extract of soybean flakes were prepared and assayed for ASF content. The ASF assay yields valid results (Fig. 1) with sample concentrations at least as high as 26 mg. per ml. The lower limit is dictated by the sensitivity of the biuret method for determination of protein concentration. This

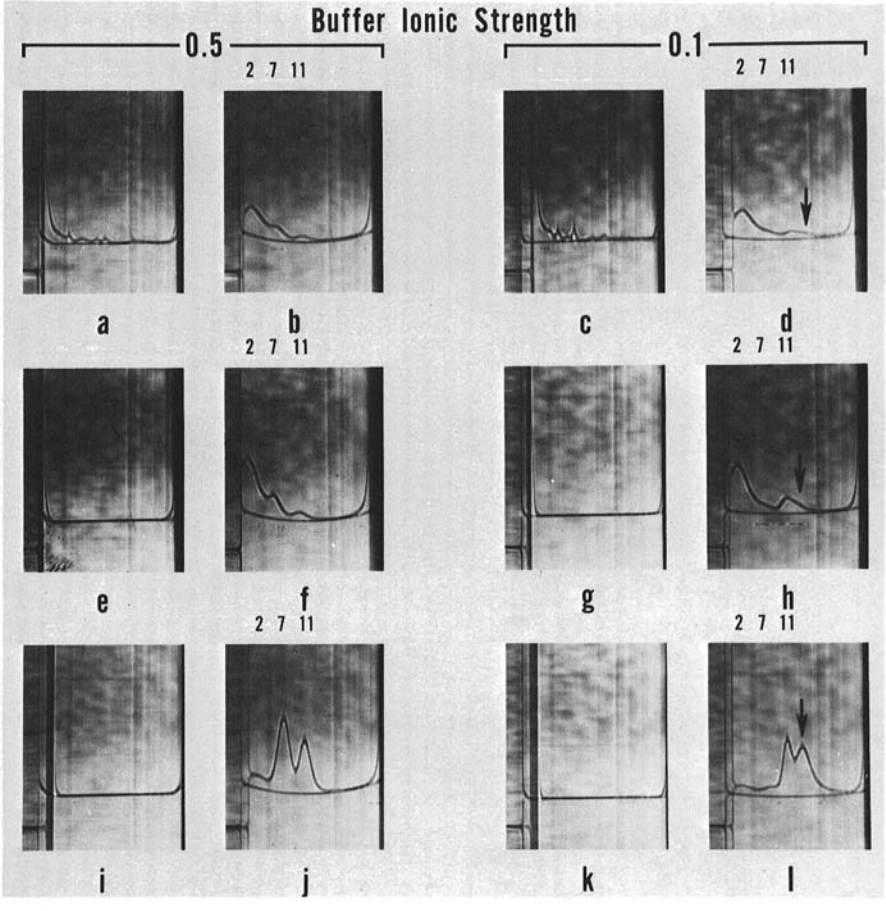

Fig. 2. Ultracentrifuge patterns of water-extractable soybean proteins precipitated with $(NH_4)_2SO_4$. a, b, c, d: 35% $(NH_4)_2SO_4$ precipitate containing 74% ASF. e, f, g, h: a, b, c, and d, respectively, after titration to pH 4.5 in the presence of 1M NaCl. i, j, k, l: 60 to 100% $(NH_4)_2SO_4$ precipitate containing 9% ASF. a, b, e, f, i, and j in 0.035M phosphate buffer, pH 7.6, 0.5 ionic strength, containing 0.4M NaCl and 0.01M 2-mercaptoethanol; c, d, g, h, k, and l in 0.037M phosphate buffer, pH 7.6, 0.1 ionic strength, containing 0.01M 2-mercaptoethanol. a, e, i, c, g, and k were taken 8 min. after the start of each run. The remaining exposures were made 40 min. later. Photographs taken with a 70° bar angle. Approximate sedimentation coefficients in Svedberg units (S) are given above the components in the 48 min. exposures. Arrows indicate the position of the 9S dimer.

limit is 1 to 2 mg. per ml. On the basis of data from Fig. 1, percentages of 25.5, 58.2, and 16.4 for the ASF, APP_i, and $whey_i$, respectively, can be calculated for these water-extractable soybean proteins.

Since the ASF is not readily resolubilized once it has been precipitated by acid, it is necessary to devise an alternative that will yield soluble ASF preparations for further study. Ammonium sulfate fractionation of an aqueous extract of soybean meal effectively fractionates ASF, APP_i, and $whey_i$ (Table I). The first line in the table presents data for an aqueous extract and each succeeding line, data on a fraction of the aqueous extract. The precipitate, which is collected at 28% saturation in $(NH_4)_2SO_4$, contains the largest quantity of ASF, and its ASF concentration is increased more than twofold over the ASF concentration in a water extract of soybean meal. However, only one-third of the total ASF occurs in this preparation. The ASF makes up a lesser percentage of each successive fraction. At 41% saturated $(NH_4)_2SO_4$, 60% of the total ASF is precipitated, and such a precipitate is 59% ASF; comparable figures for a precipitate with 60% saturated $(NH_4)_2SO_4$ are 80 and 53%, respectively.

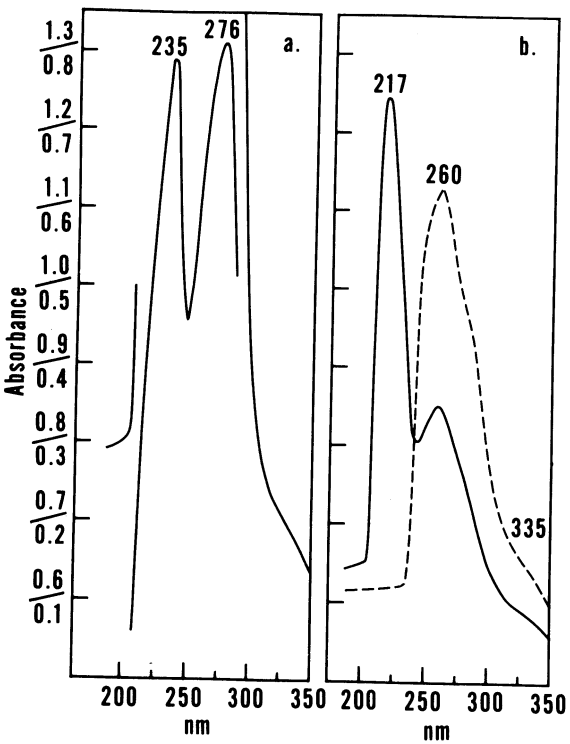

Fig. 3. Spectra of equilibrium dialysis samples. a. Spectra of retentate resulting from dialysis of a 44 to 100% $(NH_4)_2SO_4$ precipitate of an aqueous extract of soy meal against 1M NaCl. b._____, spectra of diffusate obtained in a; - - - - -, difference spectra between retentate sample in a and, as the reference sample, a portion of the same sample which was exhaustively dialyzed against 1M NaCl as described in Materials and Methods. Protein concentration 1.28 mg. per ml.

On occasion, when ASF samples were precipitated with $(NH_4)_2SO_4$ from a water extract of soybean meal, redissolved in distilled water, dialyzed to remove NH_4^+, and lyophilized, both solids and 2% aqueous solutions of them were tan or brown, and the color intensity increased in proportion to the percentage of ASF present in each sample.

The $(NH_4)_2SO_4$ fractions in Table I account for 93% of the protein in an aqueous extract. Apparently only 73% of the whey$_i$ is recovered, whereas recoveries of ASF and APP$_i$ are 94 and 97%, respectively.

Nothing indicates the number or variety of proteins making up the ASF. The only known property common to ASF in $(NH_4)_2SO_4$ fractions of Table I is that each is insoluble in 1.0M NaCl at pH 4.5.

Ultracentrifuge patterns were recorded for a $(NH_4)_2SO_4$-precipitated fraction (35% saturation) of soybean proteins containing 74% ASF (Fig. 2a, b, c, d); for the same preparation after titration to pH 4.5 in the presence of 1M NaCl (Fig. 2e, f, g, h); and for a $(NH_4)_2SO_4$-precipitated fraction containing 9% ASF (Fig. 2i, j, k, l). The percentage composition of each of these samples is given in Table II. The protein fraction, which contains a large amount of ASF, is comprised of 89% 2S and 7S components (Fig. 2b), whereas the sample with only 9% ASF is 83% 7S and 11S (Fig. 2j). Anderson et al. (5) inferred such results when they reported that incomplete solubility of soybean protein in salt solutions at pH 4.5 was primarily due to insolubility of the 2S and 7S components which were 59 and 85% soluble, respectively, in 0.8M NaCl at pH 4.5.

Comparison of the sedimentation patterns from an ASF-containing sample before and after acid treatment (Fig. 2b, f) suggests that ASF has the same

TABLE III. ABSORBANCE OF EQUILIBRIUM DIALYSIS RETENTATES

Retentate[1]	Sample 1[2]		Sample 2[2]	
	260 nm.	335 nm.	260 nm.	335 nm.
1	...	...	1.149	0.168
2	1.605	0.458	0.996	0.122
3	1.520	0.412	0.782	0.073
4[3]	1.178	0.303	0.521	0.031

[1]See **Materials and Methods** for preparation of retentates 1-4.
[2]Sample 1 contained 74% ASF. Sample 2 contained 9% ASF.
[3]Retentates dialyzed until the absorbances at 260 and 335 nm. remained constant between two successive changes of diffusate.

TABLE IV. FRACTION OF DIFFUSIBLE CHROMOPHORES BOUND TO EQUILIBRIUM DIALYSIS RETENTATES

Sample	260 nm.	335 nm.
1[1]	0.480 ± 0.030	0.836 ± 0.045
2[1]	0.254 ± 0.058	0.207 ± 0.013

[1]Same samples as in Table III.

proportion of each ultracentrifuge component found in a 35% $(NH_4)_2SO_4$ precipitate from a water extract of soybean meal. From data in Table II, the ultracentrifugal composition of ASF can be calculated: 58% 2S, 31% 7S, and 11% 11S plus >11S.

Rapidly sedimenting (>15S) material occurs in those samples which contain ASF (Fig. 2a, c), whereas such material is absent in samples containing little or no ASF (Fig. 2e, g, i, k). The same phenomenon is apparent in ultracentrifuge patterns for salt extracts made at pH 4.5 (5).

The percent of the 7S component which dimerizes when the buffer ionic strength is decreased from 0.5 to 0.1 can be calculated from data in Table II. The sedimentation coefficient of the 7S dimer is concentration dependent (10) and the dimer does not always resolve from the 11S component. For this reason the percentage of 11S plus >11S is included in Table II. The increase in the area of 11S plus >11S approximates the decrease in the area of the 7S when ionic strength is lowered to 0.1 with each of the three samples. Twenty-four percent of the 7S in the 74% ASF sample is dimerizable 7S. When this fraction is acidified to pH 4.5 in the presence of 1M NaCl, the 7S which remains soluble is composed of 59% dimerizable 7S.The 60 to 100% $(NH_4)_2SO_4$ precipitate has 77% dimerizable 7S. Therefore, the nondimerizable 7S must function more prominently in ASF precipitation than does the dimerizable species of 7S. The ultracentrifuge patterns in 0.1 ionic strength buffer are shown in Fig. 2d, h, and l.

The spectra in Fig. 3 are typical of those obtained in the equilibrium dialysis experiments. Absorption maxima occur at 217, 235, 260, and 276 nm. with a shoulder at 335 nm. This shoulder tails off toward longer wavelengths with an extinction coefficient proportional to ASF content of the sample. The peak at 217 nm., probably attributable to tryptophan (12) or tryptophan-containing peptides, was prominent in the diffusate, overshadowed by the large 235 nm. peak in the retentate, and absent in the difference spectra. Such an absorption would not be evident in the difference spectra because equal protein concentrations of the same protein were used in the reference and sample solutions. The peptide bond is likely responsible for the 235 nm. peak (13). The calculations of diffusible material bound were made at 260 and 335 nm. The former absorption maximum might be at least partially attributable to genistin (14).

Table III contains the absorbance at 260 nm. and at 335 nm. for the equilibrium dialysis retentates of two samples. The samples are $(NH_4)_2SO_4$-precipitated soybean proteins which differ greatly in the amount of ASF each contains. The retentate numbers in Table III refer to the retentates obtained by equilibrium dialysis as described in **Materials and Methods**. Proceeding from retentate 1 to retentate 4 progressively more diffusible material has been removed. The number 4 retentates for the two samples in the table were dialyzed simultaneously, in separate dialysis casings, against the same portion of diffusate. Therefore, diffusible chromophores which passed through the semipermeable membrane enclosing one sample were free to dialyze into the other sample. The protein concentrations of all the retentates for the two samples were equal as determined by the biuret assay. The results in Table III demonstrate the greater ease with which dialysis removes diffusible material from a protein fraction containing only 9% ASF than from a fraction which is 74% ASF. Although retentate 2 of sample 1 initially had a higher absorbance at

both wavelengths than did the first retentate of sample 2, the minimum absorbance that can be achieved (retentate 4) by dialysis of sample 1 is more than twice as high at 260 nm. and nearly 10 times higher at 335 nm. than is the minimum absorbance of sample 2 at these wavelengths.

Yellow-brown copolymers are readily formed by the oxidation of unsaturated fats in the presence of proteins (15,16). Such copolymers exhibit absorption maxima near 278 nm. with trailing off to 600 nm. (17). Large amounts of protein can be incorporated into such copolymers, and Tappel (16) indicated that the interactions of proteins with the products of unsaturated fat oxidation readily account for alterations of the fundamental properties of the proteins. The higher absorbances shown in Table III for the exhaustively dialyzed sample 1 may be due to such a copolymerization phenomenon.

The fraction of diffusible material bound to retentate protein was calculated for each retentate of the two samples in Table III and averaged for each sample at either 260 or 335 nm. The averages are given in Table IV. The ranges between the extremes which make up each average are also included. Evidently ASF binds diffusible material more tenaciously than does the remainder of the protein. The binding at 260 nm. is nearly twice as great for sample 1 as for sample 2, while at 335 nm. the difference is increased to greater than fourfold more material bound by sample 1 than by sample 2.

The results presented here demonstrate that, at pH 4.5 in the presence of 1M NaCl, a group of soybean proteins with distinguishing characteristics is precipitated. These proteins are differentiated by their ultracentrifugal composition, their binding of diffusible materials, and their solubility behavior during $(NH_4)_2SO_4$ precipitation from those proteins which remain soluble under these conditions.

Soybean protein isolates commercially prepared contain from 19 to 94% insoluble protein (18). Since these isolates are prepared by isoelectric precipitation, a certain amount of denatured ASF is undoubtedly present even though the wide range of solubility reported by Nash and Wolf (18) cannot be ascribed solely to the ASF. Certain functional properties of soybean protein isolates may be altered by the presence of ASF. Undesirable solubility properties limit the use of these isolates in carbonated beverages, coffee whiteners, and protein beverages (19).

Wolf and Cowan (19) and also Eley (20) list flavor as another important factor limiting the expanded usage of soybean proteins. Kalbrener et al. (21) reported that six commercial isolates had flavor scores ranging from 5.9 to 6.4 on a scale of 1 to 10. A score of 10 was defined as bland. Since ASF binds diffusible material more tenaciously than does the remainder of the soybean protein, it seems reasonable to expect a relationship between flavor and ASF proteins. It has already been noted that preparations containing large amounts of ASF tend to be more intensely colored than preparations with less ASF. The relationship of ASF to flavor and color of soy protein isolates is currently under investigation.

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