Extraction of Soybean Proteins with Aqueous 2-Mercaptoethanol

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ABSTRACT

Water, 0.01M, and 0.1M 2-mercaptoethanol extracts of fresh and aged defatted soybean meals were equilibrated, either directly or after 10% sodium chloride solution-distilled water dialysis, with pH 7.6, ionic strength 0.5 buffer with and without 0.01M 2-mercaptoethanol. Kjeldahl and ultracentrifugal analyses of the extracts showed that reductant in the extraction medium increased protein extractability; mainly additional 7S and 11S proteins were solubilized. A portion of these proteins apparently exists in defatted meal as water-insoluble disulfide polymers that are depolymerized and solubilized by 2-mercaptoethanol. Ultracentrifugal analyses of the water extracts in buffer with and without 0.01M 2-mercaptoethanol also revealed significant amounts of water-soluble disulfide polymers of the 7S fraction. Similar analyses of the reductant extracts indicated that the 7S and 11S fractions repolymerized partially during dialysis against buffer to remove the reducing agent except when aged meals were extracted with 0.1M 2-mercaptoethanol. Exhaustive dialysis of the extracts against salt followed by distilled water failed to increase disulfide repolymerization over amounts obtained on shorter-term dialysis against buffer. Rather, losses of protein and changes in protein distribution occurred; generally, the 2S fraction increased and the 7S and 11S fractions decreased on exhaustive dialysis.

A variety of conditions have been devised to extract proteins from defatted soybean meal (1). Among the latest studied is the extractant aqueous 2-mercaptoethanol. Saio et al. (2) extracted defatted meal with 0.01M 2-mercaptoethanol to prepare crude 7S and 11S protein fractions. They also incorporated the reductant in pH 7.6 buffer to extract meals of several soybean varieties for a comparative study of the relative amounts of 7S and 11S fractions but did not assess the effect of the reducing agent on protein extractability. Shibasaki et al. (3) measured protein extractability of defatted meal heated for varying times by utilizing a series of solvents including water, buffer, and buffer containing 0.1M 2-mercaptoethanol. Protein extractability of heated and unheated meals increased with solvent in the order: water<buffer<buffer plus 2-mercaptoethanol.

It is well documented that the 7S and 11S proteins of soybeans are prone to form disulfide-linked polymers that are depolymerized by 2-mercaptoethanol (4–7). Aqueous extracts of defatted soybean meal contain disulfide polymers, and presumably the polymers pre-exist in the meal (7). Further polymerization of such soluble polymers to insoluble forms occurs during isolation of the proteins by precipitation (4,6). Increased extractability of meal proteins by buffer containing 2-mercaptoethanol as observed by Shibasaki et al. (3) indicates that water-insoluble, disulfide-linked polymers also occur in defatted meal.

We have, therefore, carried out a detailed study of the effect of 2-mercaptoethanol on protein extractability of undenatured defatted meal. We analyzed the protein extracts in an ultracentrifuge and also investigated such factors as removal of 2-mercaptoethanol from the protein extracts by dialysis and effect of the reductant in the buffer used for ultracentrifugal analysis of the soluble proteins.

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MATERIALS AND METHODS

Preparation of Soybean Meal

Freshly prepared hexane-defatted meals from Hawkeye-63, Adams, and Clark varieties were ground in a Wiley-type mill to pass a 40-mesh screen. Three lots of aged meal—Hawkeye (4 years old), Adams (7 years old), and Clark (9 years old)—were ground in a similar manner. The aged meals had been stored under ambient conditions in the laboratory.

Extraction of Soybean Meal

Extraction. Portions of the six lots of soybean meal were preweighed to give 8.0 g., dry basis. Eighty milliliters of water, 0.01 M 2-mercaptoethanol, or 0.1 M 2-mercaptoethanol was used to extract with stirring for 1 hr. at 25° C. The insoluble residue was removed by centrifuging (20,000 × g) for 10 min.

Exhaustively dialyzed extracts. Extracts were dialyzed in regenerated cellulose tubing (Visking Co., Chicago, Ill.) at 4°C. against changes of 10% sodium chloride solution (four times daily) for 1 week. The extracts were then dialyzed against changes of distilled water for 1 week.

Protein Concentrations and Ultracentrifugal Analyses

Before protein concentration and ultracentrifugal composition were determined, freshly prepared extracts and exhaustively dialyzed extracts were equilibrated against pH 7.6, ionic strength 0.5 buffer (0.0325M K_2HPO_4, 0.0026M KH_2PO_4, 0.4M NaCl). Like portions were equilibrated against this buffer containing 0.01M 2-mercaptoethanol. After a minimum of 48 hr. equilibration, the solutions were adjusted to twice their original volumes and centrifuged (20,000 × g) for 10 min. at 25°C.[2]. After Kjeldahl analysis, each supernatant was also analyzed in a Spinco Model E ultracentrifuge at 47,660 r.p.m. at 25° C. with 30 mm. double sector cells. Pattern areas were calculated by the method of Pickels (9) and were expressed in arbitrary area units, which resulted from a tenfold magnification of the cell at 70° phase plate angle. No corrections were made for the Johnston-Ogston effect (10).

Statistical Methods

Analyses of variance were run on data of each total protein measurement and component area. Variations associated with extraction media, reducing agent in the analytical buffer, and exhaustive dialysis were examined for significance. Trends common to the three varieties, which provided replication in the experiment, are reflected in the results. All effects reported are based upon statistical significance calculated at the 1% level.

RESULTS

Effect of Reducing Agent on Protein Extractability

Protein extractabilities with water were similar for the three fresh meals. Aged meals of the same varieties (but different lots) likewise yielded aqueous extracts

[2]One of the reviewers correctly pointed out that we did not measure extractable protein but rather the portion of the extractable protein remaining soluble after dialysis against the two buffer systems. Previous work (8) showed that when water extracts of defatted meal were dialyzed against buffer only 0.5 to 0.9% of the extractable protein precipitated. Consequently, this does not seem to be a serious source of error, and we have neglected it.

TABLE I. EXTRACTION OF FRESH, DEFATTED SOYBEAN MEALS[a]

Extraction Medium	Treatment: RSH[b] in buffer	Treatment: Exhaustive dialysis	Extract No.	Nitrogen Conc. mg./ml.	Meal Nitrogen Extracted %	Ultracentrifugal Areas[c] 2S	7S	11S	15S	>15S
Water	–	–	1A	2.62	55.3	7.78	7.03	7.90	2.28	4.06
	+	–	1B	2.66	56.3	7.96	10.12	8.88	1.17	2.41
	–	+	1C	2.51	53.2	7.50	7.03	5.92	2.30	4.93
	+	+	1D	2.44	51.5	8.05	7.81	6.49	1.05	3.00
0.01M RSH	–	–	2A	3.68	77.7	7.20	11.08	12.70	4.66	6.61
	+	–	2B	3.71	78.4	7.53	14.91	14.09	2.30	4.82
	–	+	2C	3.66	77.2	7.90	9.36	10.12	4.52	6.80
	+	+	2D	3.55	75.0	8.55	11.76	11.93	1.98	4.68
0.1M RSH	–	–	3A	3.86	81.5	6.50	10.72	14.98	5.07	7.17
	+	–	3B	3.90	82.5	7.04	14.69	16.18	2.31	4.73
	–	+	3C	3.72	78.5	7.97	10.48	11.67	4.06	7.37
	+	+	3D	3.67	77.4	8.51	11.59	13.42	2.70	5.58
Least significant differences (same extraction medium):										
5% level				0.10	2.1	0.35	1.32	1.03	0.82	1.45
1% level				0.13	2.8	0.47	1.81	1.41	1.12	1.99

[a]Values are means of single analyses for meals of three soybean varieties.

[b]RSH = 2-mercaptoethanol.

[c]Expressed in arbitrary area units.

TABLE II. EXTRACTION OF AGED, DEFATTED SOYBEAN MEALS[a]

Extraction Medium	Treatment: RSH[b] in buffer	Treatment: Exhaustive dialysis	Extract No.	Nitrogen Conc. mg./ml.	Meal Nitrogen Extracted %	Ultracentrifugal Areas[c] 2S	7S	11S	15S	>15S
Water	–	–	1A	2.47	54.7	6.92	5.67	7.26	2.72	5.54
	+	–	1B	2.44	53.9	6.25	7.73	8.12	1.56	2.71
	–	+	1C	2.31	51.0	7.23	5.56	5.42	2.24	4.66
	+	+	1D	2.23	49.3	7.76	6.83	6.36	1.42	3.31
0.01M RSH	–	–	2A	3.28	72.6	6.64	8.95	11.35	4.19	5.99
	+	–	2B	3.29	72.8	7.25	11.66	12.34	2.52	4.33
	–	+	2C	3.04	67.3	7.76	7.47	8.74	3.53	5.67
	+	+	2D	3.08	68.1	8.40	9.32	9.89	2.21	4.38
0.1M RSH	–	–	3A	3.46	76.5	5.98	11.69	13.29	3.09	5.15
	+	–	3B	3.49	77.1	6.35	12.07	13.95	2.79	4.81
	–	+	3C	3.34	73.8	7.07	10.14	10.44	2.31	4.30
	+	+	3D	3.32	73.5	7.56	10.49	11.03	2.34	4.20
Least significant differences (same extraction medium):										
5% level				0.06	1.4	0.45	0.70	0.68	0.37	0.55
1% level				0.08	1.9	0.60	0.93	0.91	0.50	0.74

[a]Values are means of duplicate extractions of meals of three soybean varieties.

[b]RSH = 2-mercaptoethanol.

[c]Expressed in arbitrary area units.

alike in nitrogen concentration. On this basis results were averaged for the three varieties in each group. Extractabilities with water (Tables I and II) ranged from 54 to 56% for the two meal groups and these values agree with previous work where similar extraction conditions were used (8). When aqueous solutions of 2-mercaptoethanol rather than water were used, protein extractability of fresh and aged meals increased as measured by Kjeldahl analysis (compare extracts 1A, 2A, and 3A, Tables I and II). The most marked effect on protein extractability occurred with 0.01M reductant; further gain in protein extractability with 0.1M reducing agent was small but significant for both meal groups.

Ultracentrifugal analyses confirmed that aqueous 2-mercaptoethanol was a better extractant than water. Total ultracentrifuge pattern areas obtained by summing areas for individual fractions were always greater for the reductant extracts than for the water extracts. Each fraction resolved by ultracentrifugal analysis, except the 2S, increased in area with added reducing agent in at least one instance.

The 7S and 11S fractions made the largest contributions to higher protein solubility in reductant extracts as illustrated in Fig. 1a and 1b. In contrast, the amount of 2S fraction in the extracts declined when the extraction media contained 2-mercaptoethanol. For example, decreases in 2S fraction are noted in Tables I and II on comparing the aqueous and 0.1M 2-mercaptoethanol extracts. The >15S fraction showed enhanced extractability only from fresh meal at the 0.1M reductant level. The 15S fraction increased in extractability except from aged meal with 0.1M reducing agent. The higher protein extractabilities when 0.1M rather than 0.01M 2-mercaptoethanol was applied are net changes representing: a) gains in 11S but losses in 2S fraction from fresh meals and b) increases in 7S and 11S fractions but decreases in 15S fraction from aged meals.

Occurrence and Reformation of Disulfide Polymers in Extracts

The added protein extractability obtained with aqueous solutions of 2-mercaptoethanol can be ascribed to water-insoluble disulfide-linked polymers of several proteins in defatted meal. These polymers are depolymerized and solubilized by the reducing agent. Previous studies (7) suggested that meal also contains water-soluble disulfide polymers of these proteins. To measure the amounts of the water-soluble polymers in the aqueous extracts, we analyzed them after dialysis against buffer with and without 0.01M 2-mercaptoethanol. Similar treatment of the 0.01 and 0.1M 2-mercaptoethanol extracts enabled us to estimate the extent of reformation of the soluble disulfide polymers during dialysis against buffer (without 0.01M reducing agent) when the bulk of the reductant was removed.

As noted earlier (7) analyses of the aqueous extracts with 0.01M 2-mercaptoethanol in the buffer increased the ultracentrifugal areas of the 7S fraction (extracts 1A and 1B, Tables I and II). In the extracts from aged meal, the gains in 7S were accompanied by significant losses in 15S and >15S fractions as illustrated in Fig. 1a and 1e and Table II. Area changes in 11S protein caused by reducing agent in the buffer paralleled those in the 7S fraction, but such changes were smaller (significant only at the 5% level). Hence a sizable portion of the 7S fraction and a smaller part of the 11S protein were polymerized by disulfide linkages and these polymers sedimented in the regions of the 15S and >15S fractions. The 2S fraction of aged meal diminished in concentration with

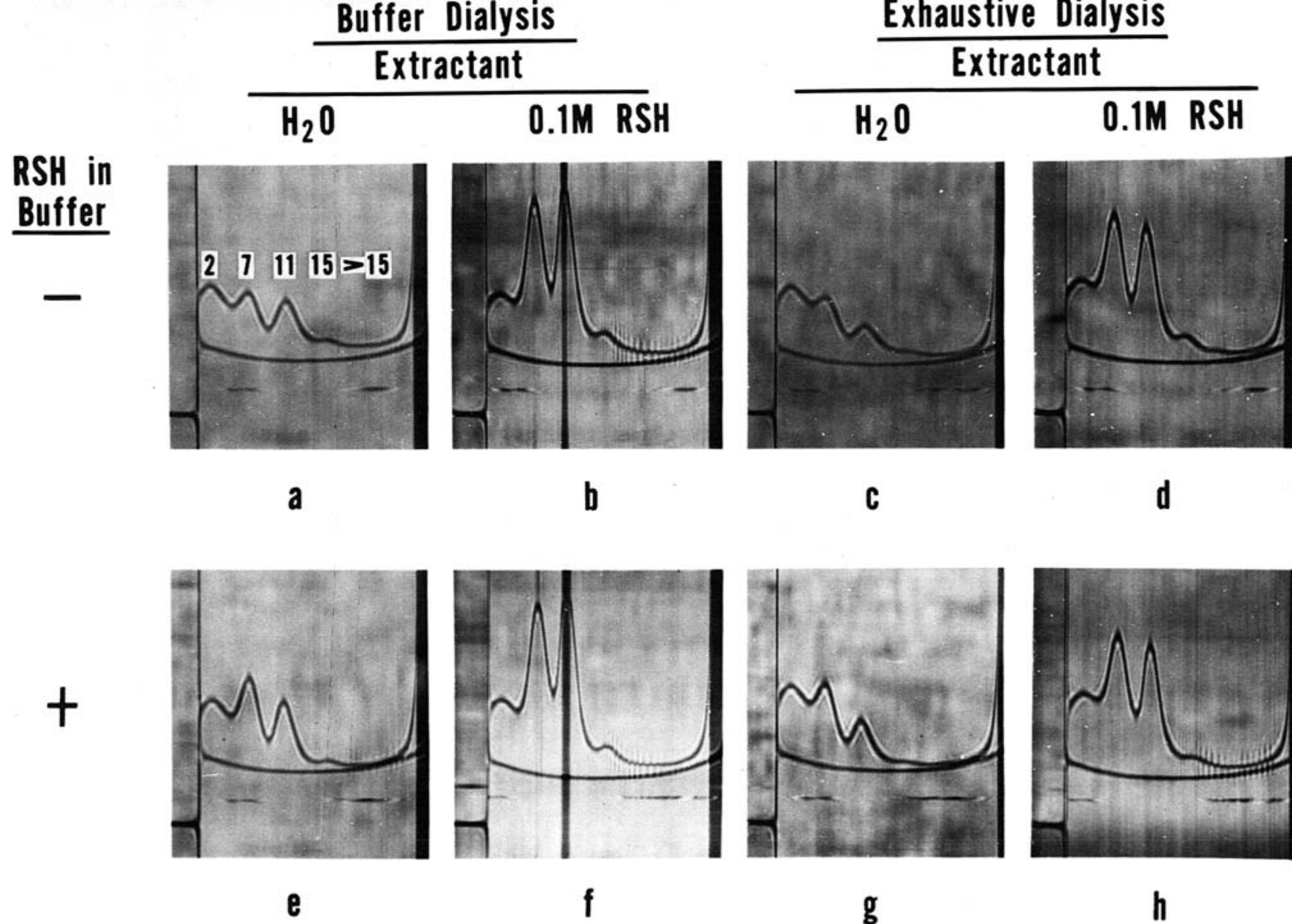

Fig. 1. Ultracentrifuge patterns for water and 0.1M 2-mercaptoethanol (RSH) extracts of aged, defatted Clark soybean meal. Extracts were dialyzed against buffer only or exhaustively dialyzed. Ultracentrifugal analysis was in absence or presence (0.01M) of 2-mercaptoethanol. Direction of sedimentation is from left to right and approximate sedimentation coefficients are given for 1a in Svedberg units. Letters are given under figures for identification in the text.

reducing agent present; this result suggests that some forms in this size range either depolymerized and diffused through the dialysis bag or aggregated and precipitated.[3]

When the 0.01M 2-mercaptoethanol extracts were analyzed under identical conditions, the 7S of aged and fresh meals and 11S of aged meals repolymerized during dialysis against buffer. The polymer forms were 15S in aged and fresh meal extracts and >15S in aged meal extracts. The amounts of polymers formed were equivalent to those quantities found on depolymerization of the aqueous extracts. Similar analysis of the 0.1M 2-mercaptoethanol extracts of fresh meals showed repolymerization of the 7S and also some 2S probably to the 15S and >15S fractions, which were the ones appearing to be polymer forms. However, no fractions in the 0.1M reductant extract of aged meal displayed significant amounts of repolymerization. Behavior of this extract (Fig. 1b and 1f) clearly was different from that of the aqueous and 0.01M 2-mercaptoethanol extracts.

Effects of Exhaustive Dialysis

Two portions of each extract were also dialyzed exhaustively against 10% sodium chloride followed by distilled water to remove reducing agent completely when present. Since partial precipitation of protein occurred during dialysis, greater polymerization was expected (4) than takes place during mere dialysis against buffer. Comparison of extracts B and D in each series discloses that one of the major effects of exhaustive dialysis was a loss of protein. Accompanying this loss of protein was a change in relative amounts of the various protein fractions. The water extracts showed no significant change in 2S fraction except for extract 1D (Table II) where an increase occurred. However, all extracts made with 2-mercaptoethanol and dialyzed exhaustively (Tables I and II) contained more 2S fraction than the corresponding portions of the extracts dialyzed directly against buffer. In contrast to the gains in 2S fraction on exhaustive dialysis, the 7S and 11S fractions decidedly decreased in concentration, except for the 7S of aqueous extracts of aged meal (Fig. 1e and 1g).

The exhaustively dialyzed extracts were analyzed in buffer with and without 0.01M 2-mercaptoethanol (extracts C and D, Tables I and II). In the water extracts of fresh meals, the only significant change noted was an apparent depolymerization of 15S fraction to 2S fraction. The water extracts of aged meals after equilibration against 0.01M reductant-buffer (Fig. 1c and 1g) exhibited 7S and 11S gains and 15S and >15S losses while the 2S fraction did not change significantly. This behavior of the 2S fraction is in contrast to the decrease that occurred when the extract was dialyzed directly against buffer (compare with extracts 1A and 1B, Table II).

The 0.01M 2-mercaptoethanol extracts of both aged and fresh meals revealed increased 2S, 7S, and 11S fractions and decreased 15S and >15S fractions upon re-addition of the mercaptan. Although the 2S and 11S fractions of the 0.1M reductant extracts of fresh meal repolymerized somewhat, probably to the 15S range, the 7S unexpectedly failed to repolymerize significantly. Apparently exhaustive dialysis modifies that portion of the 7S which is capable of polymerizing. Extensive dialysis failed to promote repolymerization of any fractions in the comparable extracts from aged meals as illustrated in Fig. 1d and

[3]All extracts contained small amounts of insoluble material after equilibration against buffer.

1h. Similar behavior was noted earlier when the 0.1M reductant extracts were dialyzed directly against buffer (extracts 3A and 3B, Table II).

DISCUSSION

The enhanced protein extractability we obtained when extractions were made with 2-mercaptoethanol instead of water indicates that the meal contains insoluble forms of disulfide polymers, as well as water-soluble forms of these polymers (7). The 7S and 11S fractions accounted for most of this increase in protein solubility. In 0.01M and 0.1M 2-mercaptoethanol extracts of fresh meals, the 7S plus 11S proteins made up 76 and 82%, respectively, of the gains in total ultracentrifuge areas (calculated for extracts B, Table I, where disulfide polymers were absent). With aged meals (extracts B, Table II), the respective increases in total ultracentrifuge areas accounted for by the 7S plus 11S proteins were 70 and 75% for the reductant extracts. The remaining gain in protein extractability is attributed mainly to the 15S and >15S fractions. We do not know whether the insoluble polymers preexist in intact soybeans, although their presence is suggested in fresh, as well as in aged, meals. The possibility cannot be ruled out that polymers formed when beans were flaked and defatted.

Buffered solutions containing 0.01M 2-mercaptoethanol have been extensively studied in soy protein research, but higher concentrations have received little attention. Except for increased extractability, we found no gross changes in the properties of the proteins when they were extracted with 0.1M 2-mercaptoethanol. The 2S and 7S fractions retained their ability to repolymerize when the reducing agent was removed by dialysis against buffer except when the proteins were prepared from aged meals. The 7S and 11S fractions from aged meals behaved normally when extracted with 0.01M reducing agent but apparently were sensitive to treatment with the higher level of 2-mercaptoethanol.

Attempts were not successful to enhance the extent of repolymerization of reductant-treated proteins by exhaustive dialysis. We did, however, confirm that loss of protein and changes in protein distribution take place as observed earlier (11). We do not know whether the protein was lost by dialysis through the membrane or by aggregation to an insoluble form that was removed by preliminary centrifugation before Kjeldahl and ultracentrifugal analyses.[3]

The increase in 2S fraction generally found after exhaustive dialysis was probably caused by irreversible dissociation of either 7S or 11S protein, or both, since each fraction decreased in concentration. The 11S protein is known to dissociate to 7S and 2S entities under conditions of low ionic strength near neutrality (12) such as occurred during exhaustive dialysis.

Although proteins from fresh and aged meals were examined, differences between them cannot be attributed solely to aging since the aged meals were not of the same lots as the fresh meals. Environmental factors, such as location and year of growth, may also contribute to the variations observed. However, effects that appear attributable to meal age are: a) decreased effectiveness of 0.01M 2-mercaptoethanol as a protein extractant for aged as compared to fresh meals and b) failure of the proteins in 0.1M 2-mercaptoethanol to repolymerize on removal of the reductant.[4]

[4]Results were similar with 0.1M 2-mercaptoethanol extracts of the fresh meals after the meals were steamed mildly (4 min. at 100°C.).

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