Denaturation of Soybean Proteins by Isoelectric Precipitation[1]

A. M. NASH, W. F. KWOLEK[2], and W. J. WOLF, Northern Regional
Research Laboratory, Peoria, Illinois 61604

ABSTRACT

Water extracts of soybean meal were acidified with hydrochloric acid; neutralized; equilibrated with a buffer of pH 7.6, ionic strength 0.5, containing 0.01M mercaptoethanol; and analyzed in an ultracentrifuge. Loss of solubility in the buffer, as compared to a nonacidified control, served as a criterion of denaturation. Factors causing denaturation were time of acid treatment and extremes of acidity. Two-hour acidification of water extracts to pH 4.5 decreased solubility and total ultracentrifuge area of the globulin fraction about 12%, with decreases in 2S, 7S, 15S, and >15S fractions. Alkylation of sulfhydryl groups did not prevent these losses. When whey was removed before neutralizing, protein solubility was reduced and all ultracentrifugal fractions decreased in area. Even though no loss of protein solubility occurred on pH 4.5 treatment of water extracts that were dialyzed to remove phytates, total ultracentrifuge areas decreased. The 7S and 11S fractions accounted for most of the area losses. Although stable on titration of a water extract to pH 4.5, the 11S protein showed marked sensitivity to lower pH values. The 7S and 11S fractions decreased in water extractability with aging of the meal.

Earlier studies showed that soybean globulins isolated by acid precipitation at pH 4.5 are no longer completely soluble in pH 7.6, 0.5 ionic strength potassium phosphate-sodium chloride buffer. Part of this insoluble protein dissolved when the buffer contained 0.01M mercaptoethanol, apparently because disulfide-linked polymers of the 7S and 11S ultracentrifugal fractions were reduced (1,2,3). Exploratory work indicated that the fraction remaining insoluble in buffer containing a reducing agent is derived primarily from the 2S and 7S fractions present in the initial aqueous extract of the meal (4).

Here we describe further research on insolubilization of soybean globulins by acid precipitation. Emphasis is on conditions of acid treatment which lead to decreased solubility of the globulins in buffer containing a reducing agent. Factors studied include time of acid treatment, alkylation of sulfhydryl groups, dialysis, removal of whey, and exposure to pH values below 4.5.

MATERIALS AND METHODS

Protein Extraction and Treatment of Extracts

Extraction. Unheated, hexane-defatted soybean meal (Hawkeye, 1963 crop) was extracted with 10 vol. of water for 1 hr. The insoluble residue was removed by

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centrifuging (20,000 X g) for 10 min. The supernatant is referred to as water extract.

Dialyzed Water Extracts. Water extracts were dialyzed at 4°C. against daily changes first of 10% sodium chloride solution for 1 week and then of distilled water for 1 week. These samples are referred to as dialyzed water extracts.

Alkylation of Protein Sulfhydryl Groups. Protein sulfhydryl groups were alkylated with 0.01M sodium iodoacetate for 30 min. at pH 6.6 by adding the solid reagent to water extracts while stirring.

Acidification. Untreated, alkylated, or dialyzed water extracts at 25°C. were acidified with 1N hydrochloric acid. The treatment was at pH 4.5 except when effects of pH were studied and for 2 hr. except when effects of time were studied. The acidified extracts were then neutralized to pH 7.6 with dilute sodium hydroxide.

Whey Removal. Near the end of treatment at a selected pH, the extracts, if at a pH other than 4.5, were adjusted to this pH and then centrifuged (20,000 X g) for 10 min. before the supernatant (whey) was removed.[3] At specified times after initial acidification, the centrifuged protein curd was dispersed in water and titrated to pH 7.6 with dilute sodium hydroxide.

Protein Solubility and Ultracentrifugal Analyses

To determine changes in protein solubility and in ultracentrifugal composition, water extracts, acidified-neutralized extracts, neutralized protein curds (from whey removal samples), and wheys were equilibrated against pH 7.6, ionic strength 0.5 buffer (0.0325M K_2HPO_4, 0.0026M KH_2PO_4, 0.4M NaCl, 0.01M 2-mercaptoethanol). The resulting solutions were then adjusted to twice their original water extract volumes and centrifuged (20,000 X g) for 10 min. at 25°C. After Kjeldahl analysis, the supernatants were also analyzed in a Spinco Model E ultracentrifuge at 47,660 r.p.m. and ∿25°C. with a 30-mm. double-sector cell. Pattern areas of sedimentation velocity runs were calculated by the method of Pickels (6). Data are expressed as square centimeters, which are arbitrary area units resulting from a tenfold magnification of the cell at 70° phase plate angle.

Corrections were calculated for component distribution anomalies due to the Johnston-Ogston effect (7) for each of the components. Since the 7S and 11S were the only peaks showing this effect (a concentration dependence) and since the relative distributions of control samples and pH 4.5 treated samples were grossly similar, corrections were not applied. The observed distributions were Y (in percent of total pattern) = 27.19 + 1.35X (Standard Error = ± 0.57) for a 7S peak, and Y = 40.06 – 1.62X (Standard Error = ± 1.65) for an 11S peak. The value X is the Kjeldahl nitrogen concentration in milligrams per milliliter. In a typical concentration range for analysis, the observed 7S area was too large by about 4% of the total area, whereas the 11S area was too small by about 5% of the total area.

Statistical Methods

A least-squares linear regression program was used to fit component areas and

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[3]When "whey" was removed from dialyzed water extracts, the acidified slurries were adjusted to pH 5.0 before centrifuging, as a correction for the shift in isoelectric points of the acid-precipitated globulins resulting from removal of phytates (5).

nitrogen concentrations to the following independent variables: time of standing in acid slurry, pH of acid treatment, and age of the source soybean meal (age data of both control and acid treated).

Data from a designed experiment were examined by analyses of variance of component areas, total areas, and nitrogen concentrations. Significance of differences between means or other parameters was determined by Duncan's method (8), least significant differences, or confidence limits. All reported effects were significant at least at the 5% level unless otherwise stated.

RESULTS AND DISCUSSION

Meal Age and Globulin Extractability

In each series of experiments a portion of the water extract was buffer-equilibrated, adjusted to a standard volume, and analyzed as a control. In the time needed to complete this study, a decrease in extractability of protein from the defatted meal (stored at $\sim 25°C$.) was noted from Kjeldahl and ultracentrifugal analyses. Reduced extractability of protein with aging of the meal resulted in a loss of globulin components (Fig. 1). Data in Fig. 1 were obtained by subtracting nitrogen concentrations and ultracentrifuge pattern areas for whey proteins from the corresponding values for the control water extracts. This procedure yielded the calculated values for the globulin fraction in the absence of protein insolubility resulting from acid precipitation (4).

Regression lines were fitted to the experimental data in Fig. 1. Total area, 7S area, and Kjeldahl nitrogen had regression lines with slopes differing significantly from zero. The slope of the 11S line differed from zero at the 10% level of
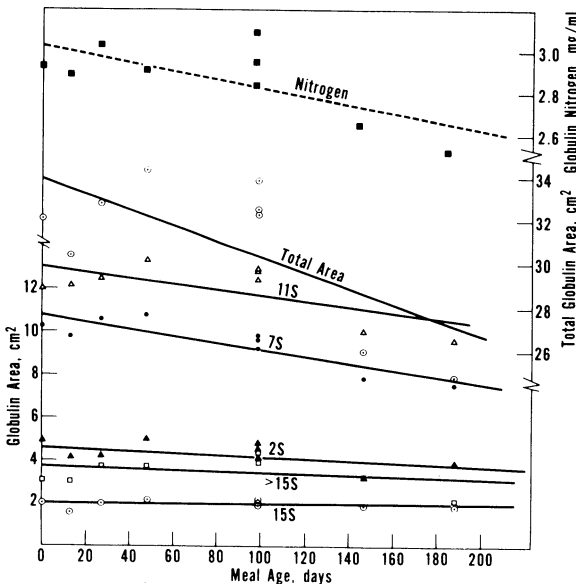

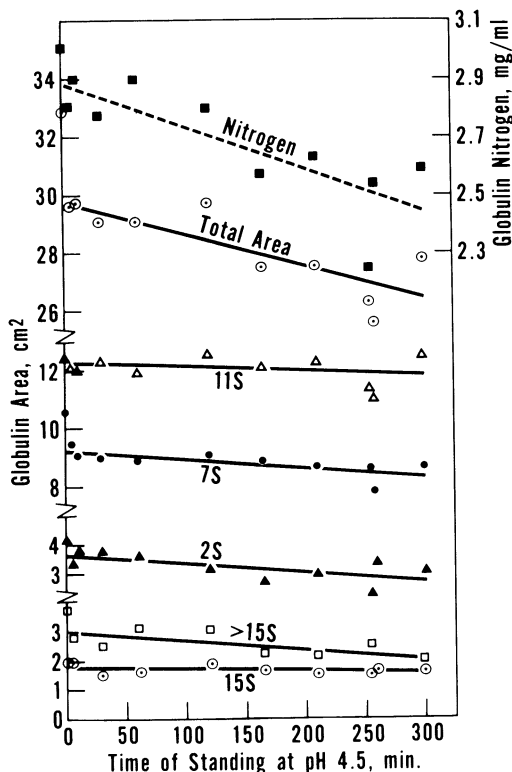Fig. 1. Change of globulin extractability with soybean meal age.

Fig. 2. Change in buffer solubility of the soybean globulin fraction of acidified-neutralized extracts with time at pH 4.5. Acidification was with hydrochloric acid.

significance. Since not all component lines were parallel, changes in component distribution of the soluble proteins are indicated. Effects of meal age were also observed when water extracts were given various treatments, as described later.

Treatment at pH 4.5

Effect of Time. Portions of a water extract (meal age 27 days) were titrated to pH 4.5 with hydrochloric acid, held at 25°C. for varying lengths of time, neutralized, and then dialyzed against standard buffer for analysis. Changes in solubility for the globulin fraction as a function of time of pH 4.5 treatment are shown in Fig. 2. Changes in globulin composition with time are illustrated by comparing component areas in one vertical segment with those areas in another vertical segment of the graph.

Although the whey proteins were not removed during the pH 4.5 precipitation step, data for both control samples (points at zero treatment times) and acid-treated samples are corrected for contributions by whey proteins as measured in a separate experiment.

Regression lines were fitted to the data (zero time values were not included). The lines for Kjeldahl nitrogen, total areas, and areas for 2S, 7S, and >15S had

negative slopes differing significantly from zero. In addition, the intercepts of the regression lines for total area and area of the 7S fraction were significantly below the corresponding control (zero time) values. This difference suggests that a portion of the 7S fraction is rapidly insolubilized on adjustment to pH 4.5, followed by the slower rate of insolubilization shown by the regression line. The rapid decrease in total area on adjustment to pH 4.5 (9.6% at the intercept) is not accompanied by a corresponding loss in solubility as measured by Kjeldahl nitrogen (4.9% at the intercept). A possible explanation of this discrepancy is that acid treatment converted some of the protein into aggregates which are not removed during the preliminary centrifugation (20,000 × g for 10 min.), and consequently are measured by Kjeldahl analysis. Such aggregates may, however, sediment out of solution early during ultracentrifugal analysis and thus go undetected in the normal ultracentrifuge pattern.

Alkylation, Dialysis, and Removal of Whey. A replicated experiment was run on extracts (meal age 99 days) to test the effect of the following factors on protein insolubilization during acid precipitation: a) alkylation of protein sulfhydryl groups with sodium iodoacetate; b) dialysis of the proteins to remove phytates and other low-molecular-weight compounds; and c) removal of whey by centrifugation.

A water extract was divided into nine portions: three portions were untreated, three were alkylated with sodium iodoacetate, and the remainder were dialyzed against 10% sodium chloride and then against water.[4] Each portion from each group of extracts then received one of the following treatments: a) dialysis against standard buffer (control samples); b) acidification to pH 4.5, neutralization, and dialysis against standard buffer; or c) acidification to pH 4.5, removal of whey by centrifugation, neutralization of the curd in a water slurry, and dialysis against standard buffer. This scheme was followed on three separate extractions. Acid treatment was for 2 hr. at 25°C. The results of this experiment are given in Table I. Values for whey protein removed from the centrifuged samples were added to the Kjeldahl nitrogen, to the areas of the 2S and 7S fractions, and to total areas, to permit direct comparison with the control and with the acidified samples still containing whey.

Since modification of protein from untreated and alkylated extracts led to essentially duplicate analyses, data presented are for the former and for the dialyzed extracts, although statistical calculations included alkylated extract data. The dialyzed control water extracts were lower in nitrogen concentration than untreated controls (P<0.10). The line of best fit through the concentrations of these dialyzed controls and six others plotted as in Fig. 1 was parallel to the line shown; thus the loss on dialysis was confirmed in other experiments.

After acidification the untreated samples were lower in protein concentration than their controls, irrespective of meal age. Protein insolubilization based on globulin nitrogen (both control and acidified-neutralized extracts corrected for whey nitrogen) was 10.6% at a 27-day meal age, 12.5% at 99 days, and 17.8% at 147 days. That meal age affected only the extractability is indicated since these losses are equivalent, i.e., the age regression of nitrogen concentrations in

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[4]In this group the control was dialyzed directly from 10% salt against standard buffer, omitting dialysis against water.

TABLE I. EFFECTS OF EXHAUSTIVE DIALYSIS AND WHEY REMOVAL
ON SOYBEAN PROTEIN MODIFICATION BY ACID PRECIPITATION[a]

Concentration or Ultracentrifuge Area	Control Water Extracts		Acidified Water Extracts		Acidified Water Extracts with Whey Removal[b]	
	Untreated	Dialyzed	Untreated	Dialyzed	Untreated	Dialyzed
Nitrogen (mg./ml.)	3.21 a	3.07 ab	2.84 cd	3.03 b	2.74 d	2.99 bc
Total area	35.33 a	33.89 a	31.61 b	29.25 cd	26.69 bc	27.60 d
2S	5.81 abc	5.43 cd	4.99 d	6.32 a	4.32 e	5.54 bcd
7S	10.54 a	10.42 a	9.52 b	7.90 c	9.21 b	7.78 c
11S	12.68 a	12.44 a	12.41 a	9.78 c	12.02 b	9.44 c
15S	2.03 a	1.95 ab	1.76 bc	1.60 cd	1.68 c	1.42 d
>15S	4.26 a	3.65 ab	2.93 bc	3.65 ab	2.46 c	3.42 ab

[a]For each line, means followed by similar letters are not significantly different at the 5% level.

[b]Whey was removed experimentally, but its contribution is included in the data below to permit direct comparison with other water extracts.

acid-treated samples showed the same slope (P<0.10) as the plot in Fig. 1. Little or no insolubilization occurred when the dialyzed extract was acidified (Table I). Protein solubilities of extracts after acidification plus whey removal were similar to extracts that were merely acidified.

Total areas of the ultracentrifuge patterns for the untreated extracts decreased after acidification with and without whey removal, in agreement with losses in solubility as measured by Kjeldahl analyses.[5] However, total areas for the dialyzed extracts after acid treatment also decreased. These area analyses were in contrast to the corresponding Kjeldahl analyses, which indicated no significant decreases in solubility when dialyzed extracts were acidified. Some of the dialyzed, acidified protein apparently was converted to a form (presumably aggregates) not estimated by the ultracentrifuge pattern. A similar discrepancy between Kjeldahl analysis and total ultracentrifuge area was noted earlier for an acidified-neutralized extract (see discussion of Fig. 2).

Also listed in Table I are the component areas that make up the total area. The 2S fraction in the untreated samples decreased on acidification with or without removal of whey. In the dialyzed extracts, the 2S fraction increased with acidification but remained the same with whey removal.

The 7S fraction in the untreated extracts decreased on acid treatment. Moreover, the losses of 7S area by acid treatment of dialyzed extracts (average 28% loss) were significantly greater than the losses from untreated extracts (average 12% loss).

The 11S fraction showed no change on acidification of an untreated extract; however, whey removal caused a loss of 11S. In contrast to this highly significant decrease of 11S area on whey removal from untreated extracts, no such loss occurred in the alkylated extract. This is the only exception to the observation that

[5]Area losses based on total globulin area (water extract area minus whey protein area) were 11.3% without and 17.1% with whey removal as compared to respective globulin nitrogen losses of 12.5 and 15.5%.
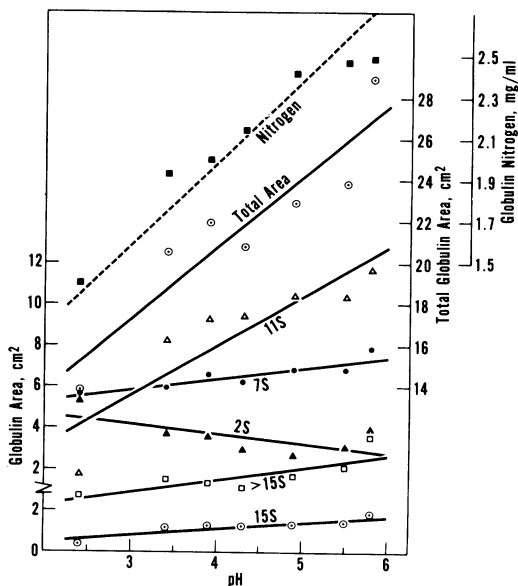
Fig. 3. Change in buffer solubility of the soybean globulin fraction of acidified-neutralized extracts with pH of exposure. Acidification was for 2 hr. with hydrochloric acid.

the alkylated samples are replicates of the untreated samples throughout Table I. The 11S area loss on acid treatment with whey removal was significantly greater from dialyzed (24.1% loss) than from untreated extracts (5.2% loss).

The 15S fraction showed an overall tendency to decrease on acid treatment. The >15S fraction of the untreated sample was likewise sensitive to acid treatment either with or without whey removal.

In summary, the major acidification effects in Table I, relative to area changes, were contributed by 2S, 7S, and >15S of the untreated samples and by 7S and 11S of the dialyzed samples.

Treatment at pH Values Below 4.5

Since precipitation of soybean globulins at pH 4.5 insolubilizes or modifies part of the protein, the effect of lower pH values on the proteins was also examined. Portions of a water extract (meal age 147 days) were adjusted to pH values ranging from 5.8 to 2.4 with hydrochloric acid, allowed to stand for 2 hr. at 25°C., neutralized to pH 7.6 with sodium hydroxide, and then dialyzed against standard buffer. Changes in protein solubility as a function of pH are shown in Fig. 3. For depicting changes in composition, heights of the component regression lines relative to that height for the total area line can be compared at different pH values. Although the experimental points for the 2S and 11S components suggest curvilinear rather than linear regressions, the fitting was limited to a linear model, as illustrated in Fig. 3. All except the 2S line had a slope that deviated significantly from zero, although the slope for the 7S fraction was significant only at the 10% level.

Nitrogen solubility, and also total area, decreased progressively as the pH was

lowered to 2.4. Areas of all components decreased with decreasing pH except for the 2S fraction, which appeared to increase, especially at low pH. Loss of the 11S fraction appears moderate through the midrange of pH in Fig. 3, but appreciable in the region below pH 3. Previous studies indicate that the 11S is unstable and dissociates in the region of pH 2.2 to 3.8 (9). Reassociation to parent 11S form following acid exposure is low (10). Although experimental conditions are not the same, our results are in agreement with those earlier findings; the apparent increase in 2S area complements the decrease observed in 11S area in the low pH region.

Although nitrogen concentrations calculated at pH 4.5 from the regression line of Fig. 3 and at 2 hr. from the regression line of Fig. 2 confirmed the results of the replicated study, such calculations for the components were quite variable. The change in relative distribution of the extracted protein with meal age (Fig. 1) probably results from loss of acid-labile forms of some components and loss of acid-stable forms of others.

CONCLUSIONS

All protein solubility measurements were made in buffer containing 0.01M mercaptoethanol. As opposed to treatment with higher concentration of this reductant, which could cause cleavage to subunits or alcohol denaturation, or both, this concentration has been shown to reduce accessible disulfide bonds, yet maintain the protein system in its native state. Insolubilization and also distribution changes as measured in this study therefore indicate modification of protein. Acid modification of the protein is a function of pH and time (Figs. 2 and 3). Under the usual conditions for isolation of soybean globulins (adjustment of a water extract to pH 4.5), part of the 7S fraction rapidly insolubilizes. After initial acidification, this fraction, and also the 2S and >15S fractions, slowly but progressively lose solubility. Formation of the insoluble protein is not prevented by alkylation of accessible protein sulfhydryl groups. Therefore, if disulfide linkages are involved, sulfhydryl groups other than those accessible to the alkylating agent must have formed disulfide bonds during acidification, bonds which are inaccessible to the reducing agent.

Possibly soybean whey contains substances that aid in dispersing proteins in water (11). The nitrogen concentrations of samples with and without whey removal were equivalent although the 2S areas were greater in the latter samples; the 11S area of the untreated series was also greater in the sample without whey removal. Although no clear-cut evidence exists for a protein-solubilizing factor in whey, the 2S and 11S fractions appear more stable when whey is not removed.

When phytic acid complexes with the globulin fraction during isoelectric precipitation, an insoluble phytate protein complex is formed (5). When we dialyzed the water extracts to remove phytates (Table I), no significant losses of protein solubility occurred on acidification. Instead, appreciable amounts of 7S and 11S fractions were modified by the combined treatment. Changes were apparently by dissociation into 2S fraction and by aggregation. Such protein modification may have occurred, in part, during dialysis to remove the phytate.

The primary cause of protein modification (protein insolubilization as loss of total area with phytate present or loss of 7S and 11S with phytate absent) is suggested to be acid. From Table I these changes appear to be similar in quantity.

The effect of acid modification is small at pH 4.5 but increases as the pH is lowered.

Our results confirm the effect of meal age on extractability of the proteins observed earlier (12), and we have identified the 7S and 11S proteins as the major fractions affected by aging.

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