Aging of Soybean Globulins: Effect on Their Solubility in Buffer at pH 7.6

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ABSTRACT

Isoelectric forms of freeze-dried soybean protein isolates were stored in the dark at room temperature for more than five years. During this time, protein solubilities decreased 1–4% per year in 0.5 ionic strength buffer at pH 7.6 when the buffer contained 0.01 *M* 2-mercaptoethanol and 4–6% when the reducing agent was absent. When measurements were made in buffer only, ultracentrifugal analyses of the soluble proteins indicated decreases in solubility of all fractions with time, with the exception of the >15S fraction. In buffer plus 2-mercaptoethanol, aging resulted in decreases in the solubilities of 2S, 7S, and 11S fractions, whereas the 15S and >15S fractions increased in concentration. Disulfide-crosslinked polymers were observed throughout the study, but loss in protein solubility appeared to be caused by formation of other kinds of polymers involving mainly the 7S and 11S proteins.

Solubility is physicochemical property of soybean proteins that is important in their preparation and ultimate use in both food and nonfood applications. To correlate previously reported protein solubility methods, Shen (1976a) used a range of conditions including protein concentration, blending speed, time, temperature, and centrifugal separations for measuring solubilities of several isolates at pH 7.0. He subsequently studied the effects of pH, ionic strength, and disulfide reductants on protein solubility (Shen 1976b). Although solubilities of isolates after acid precipitation and alcohol treatment have been studied (Wolf et al 1963, 1964; Nash et al 1971), solubilities as a function of isolate age have not been reported. Reduced solubility of aged isolates (Nash and Wolf 1967) and decreased protein extractability of defatted soybean meal with age (Nash et al 1971) prompted us to examine protein samples, when freshly prepared in the laboratory and after aging, to determine changes in solubility resulting from storage at ambient temperatures. Solubilities and ultracentrifugal component distributions were measured following dispersion and equilibration in phosphate buffer with and without 0.01 *M* 2-mercaptoethanol (2-ME) to determine the role of disulfide polymer formation of the major proteins during aging.

MATERIALS AND METHODS

Isoelectric soybean globulin samples were prepared from two lots of dehulled, undenatured, hexane-defatted meal approximately one year old, using water extraction and hydrochloric acid precipitation at pH 4.5. The resulting curds were separated by centrifuging and then freeze-dried. Protein preparations I (from Ottawa-Mandarin meal) and II (from Hawkeye meal, sample D in an earlier study [Nash and Wolf 1967]) were stored in sealed containers in the dark at laboratory temperatures (25 ± 5°C). Following initial values of 2.5%, moistures of isolates ranged from 6.5 to 7.5% throughout the aging period.

Periodically, 500-mg protein samples (dry basis as determined by drying at 105°C for 2 hr in vacuo) were dispersed in about 20 ml of potassium phosphate-sodium chloride buffer (0.0325 *M* K_2HPO_4, 0.0026 *M* KH_2PO_4, 0.4 *M* NaCl), pH 7.6, ionic strength 0.5, with or without 0.01 *M* 2-ME. The dispersions were then equilibrated by dialysis (regenerated cellulose casings) at 4°C against buffer for 24 hr or longer, adjusted to 25 ml, and clarified by centrifuging (20,000 × *g*) for 10 min at 25°C. Nitrogen concentrations of the solutions were determined by Kjeldahl analysis.

Solutions from the solubility experiments were analyzed at room temperature in a Spinco Model E ultracentrifuge (30-mm double sector cell) at 47,660 rpm. Sedimentation patterns were calculated according to Pickels (1952). Component areas were measured in arbitrary units obtained by a tenfold magnification of the cell at a 70° phase-plate angle.

Solubility comparisons and trends with age were tested at the 95% confidence level on ultracentrifugal component area and nitrogen concentration data fitted by linear regression analyses.

[1]Mention of firm names or trade products does not imply endorsement or recommendation by the USDA over other firms or similar products not mentioned.

RESULTS

Change of Protein Solubility with Age

Solubilities of two soybean protein isolates in phosphate buffer with and without 0.01 *M* 2-ME were measured periodically over several years. Nitrogen concentrations for solutions of Protein I in phosphate buffer are plotted in Fig. 1 (lower curve) from the time of isolate preparation through a storage period of five years. Despite some scattering of the points, a downward trend in solubility was

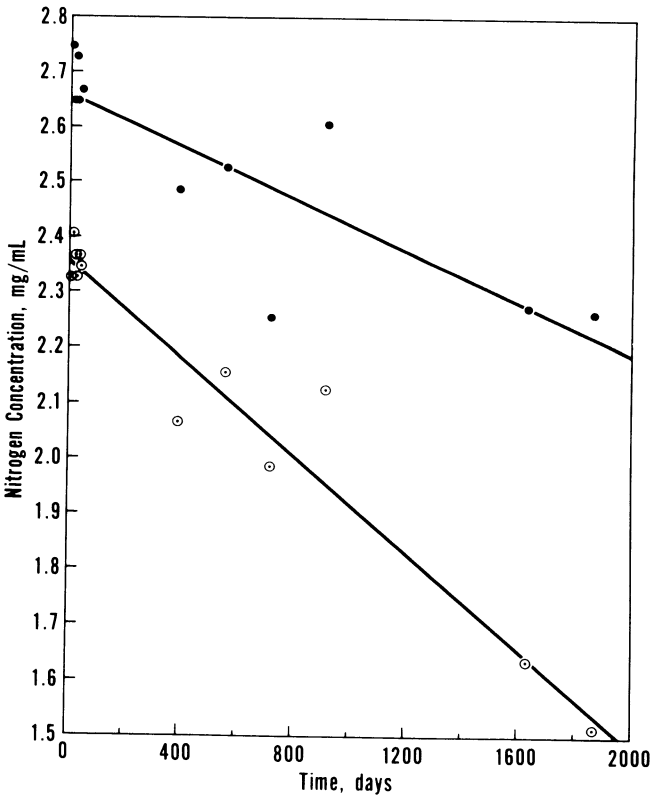

Fig. 1. Changes in solubilities of Protein I as a function of time as measured by Kjeldahl nitrogen determinations. **Lower** curve measured in buffer only; **upper** curve measured in buffer containing 0.01 *M* 2-mercaptoethanol. Curves were fitted by linear regression analyses.

obvious as the isolate aged. When the buffer contained 2-ME, protein solubilities were always higher (upper curve, Fig. 1) than in the absence of the reducing agent, but solubilities likewise decreased with time of aging. At any given age, the difference between the upper and lower curves in Fig. 1 represents the portion of the proteins that was present as buffer-insoluble, disulfide-linked polymers. These polymers were depolymerized by 2-ME, and the resulting monomers were soluble in buffer.

Figure 1 shows that disulfide polymers existed in the protein samples throughout the study. The divergence of the two curves with time of aging suggests that the amount of disulfide polymers may have increased with storage time. However, statistical analysis of the differences in the slopes of the two curves indicated a significant difference ($P < 0.05$) for Protein I but not for Protein II (data not shown). Hence, evidence for formation of additional disulfide polymers during aging is not clear-cut.

Ultracentrifugal analysis of the phosphate buffer solubles of Protein I (Fig. 2a) shows a downward trend with age of isolate for solubilities of all proteins except the > 15S fraction, which remained constant. Of the five ultracentrifugal fractions, the 11S fraction decreased at the fastest rate. The 7S fraction showed the next most rapid loss of solubility with time of aging, followed by the 15S and 2S fractions. By contrast, with reducing agent present (Fig. 2b), the 2S, 7S, and 11S fractions decreased in concentration, whereas the 15S and > 15S fractions increased in concentration with age. In this buffer system, the 7S fraction dropped more rapidly than did the 11S fraction; the latter decreased in solubility more slowly than in buffer without 2-ME (Fig. 2a).

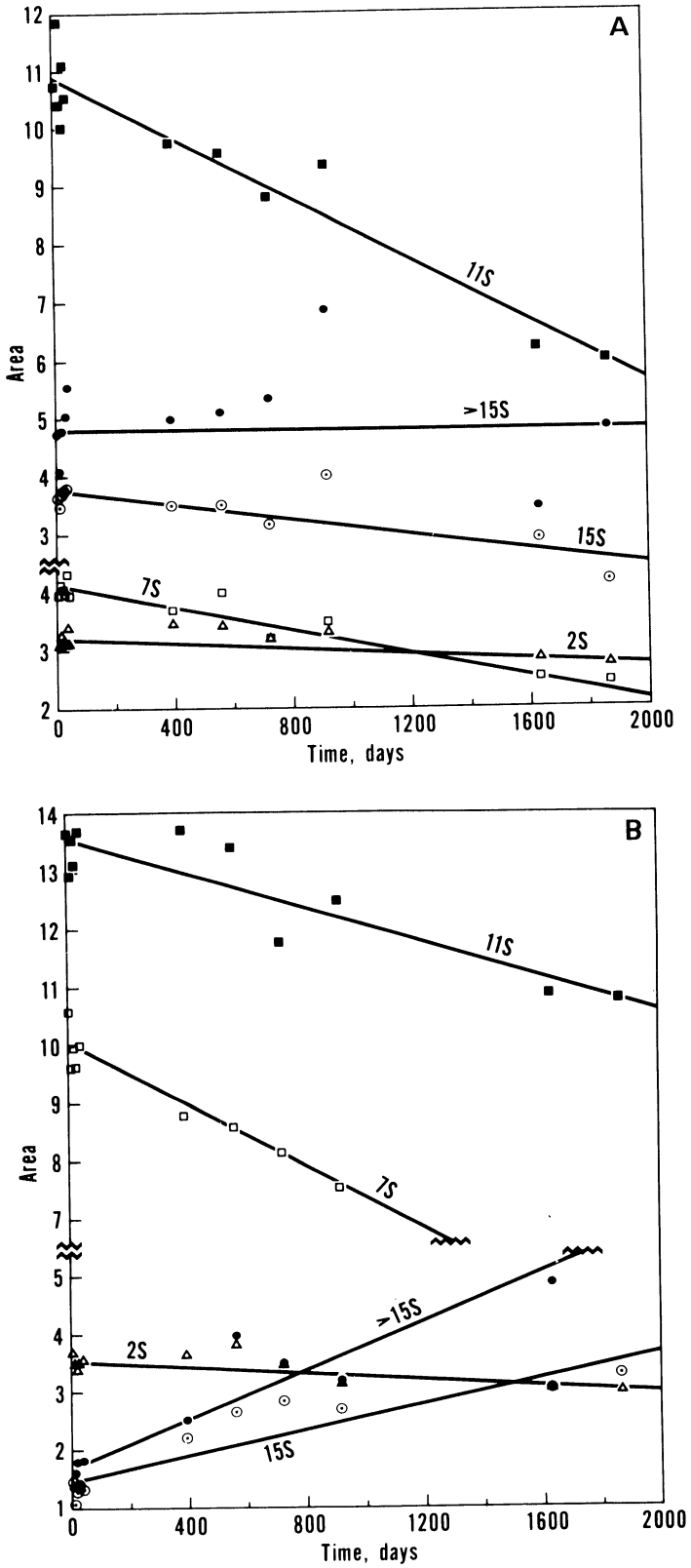

Fig. 2. Changes in ultracentrifugal areas of Protein I as a function of time (**A**) in buffer only and (**B**) in buffer containing 0.01 *M* 2-mercaptoethanol. Curves were fitted by linear regression analyses.

Disulfide Polymers of Unaged Isolates

Table I summarizes the ultracentrifugal and solubility data for Protein I and its replicate, Protein II, in buffer without and with 2-ME at zero time and at an age of 1,000 days. Buffer containing 2-ME solubilized one-sixth more protein from freshly prepared Protein I (Fig. 1) and Protein II than did phosphate buffer alone. These solubility increases caused by 2-ME resulted in twice as much 7S area, one-third more 11S, but only about one-half as much 15S and > 15S fraction. For Protein I, these area shifts are also shown by comparison of the intercepts (zero days of aging) of Fig. 2a with those of Fig. 2b. One-half to two-thirds of the increase in 7S plus 11S as sulfhydryl-monomeric forms can be accounted for by the disappearance of phosphate buffer-soluble, disulfide-linked polymers in the 15S and > 15S size range when 2-ME is present in the buffer. The remaining increase in the amounts of 7S and 11S proteins noted in buffer plus 2-ME resulted from depolymerization of buffer-insoluble, disulfide-linked polymers of these proteins. Thus, fresh protein isolate samples contain buffer-soluble and buffer-insoluble disulfide polymers of the 7S and 11S fractions plus the proteins that are insoluble in buffer plus 2-ME as a result of being precipitated at pH 4.5 (Nash et al 1971).

Disulfide and Nondisulfide Polymers of Aged Isolates

As with the freshly prepared samples, treatment of aged isolates with 2-ME resulted in greater protein solubility in buffer than when 2-ME was absent (Fig. 1 and Table I). This increased solubility manifested itself primarily as more 7S and 11S proteins in solution. For example, the 1,000-day-old samples (Table I) show that twice as much 7S and one-half more 11S protein was solubilized with 2-ME. Consequently, the disulfide polymers seen in the aged isolates consist of polymeric forms of 7S and 11S proteins as observed in fresh isolates.

Table I also shows that the 15S and > 15S fractions behave differently in aged than in fresh isolates. At 1,000 days, 2-ME fails to depolymerize 15S and > 15S to the extent observed at 0 days, thus resulting in decreased amounts of 7S and 11S monomer forms. Moreover, 15S and > 15S fractions increase with further storage (Fig. 2b). Apparently, the 7S and 11S proteins undergo a slow polymerization, not reversed by 2-ME, resulting in polymers sedimenting in the 15S and > 15S range. Presumably, these nondisulfide polymers continue to aggregate until they are no longer dispersible in buffer containing 2-ME.

DISCUSSION

Earlier, we measured solubilities of commercial and laboratory-prepared soybean protein isolates (Nash and Wolf 1967). Our results suggested that aging caused insolubilization of protein, but we did not examine the effects of long-term storage at that time. In

TABLE I
Solubilities of Two Protein Isolates in Phosphate Buffer at 0 and 1,000 Days as Determined by Ultracentrifugation[a] and Kjeldahl Analyses

	Without 2-Mercaptoethanol		With 2-Mercaptoethanol	
	0 Days	1,000 Days	0 Days	1,000 Days
Protein I				
2S	3.18 ± 0.16	2.98 ± 0.17	3.55 ± 0.17	3.24 ± 0.17
7S	4.04 ± 0.25	3.13 ± 0.26	9.90 ± 0.26	7.33 ± 0.27
11S	10.82 ± 0.45	8.20 ± 0.47	13.48 ± 0.48	11.98 ± 0.48
15S	3.72 ± 0.25	3.07 ± 0.26	1.38 ± 0.27	2.48 ± 0.27
>15S	4.81 ± 0.61	4.80 ± 0.64	1.66 ± 0.65	3.75 ± 0.66
N,mg/ml	2.36 ± 0.084	1.92±0.090	2.66 ± 0.091	2.42±0.090
%N soluble	75.3 ± 2.7	61.4 ± 2.9	84.9 ± 2.9	77.4 ± 2.9
Protein II				
2S	3.01 ± 0.20	2.98 ± 0.18	3.34 ± 0.19	3.42 ± 0.16
7S	3.79 ± 0.31	3.01 ± 0.27	7.79 ± 0.30	6.04 ± 0.26
11S	8.49 ± 0.58	6.20 ± 0.49	11.92 ± 0.55	9.78 ± 0.47
15S	3.45 ± 0.32	2.45 ± 0.28	2.27 ± 0.30	2.70 ± 0.26
>15S	4.84 ± 0.77	3.86 ± 0.67	2.80 ± 0.74	4.23 ± 0.63
N,mg/ml	2.05 ± 0.108	1.74±0.090	2.43 ± 0.103	2.27±0.090
%N soluble	66.4 ± 3.5	56.4 ± 3.0	78.6 ± 3.3	73.6 ± 2.9

[a]Ultracentrifugal data expressed in arbitrary area units.

this study, which began with freshly prepared samples of isoelectric protein isolates, we found that protein solubility does decrease as the isolates age (Fig. 1). By measuring solubilities in buffer with and without 2-ME, we were able to differentiate between proteins presumably insolubilized as a result of disulfide polymer formation and those insolubilized by some other mechanism. Previous studies indicated that, under conditions similar to those used here, 0.01 *M* 2-ME caused no detectable conformation changes in the soybean globulin mixture as judged by ultracentrifugation, optical rotation, and viscosity measurements (Wolf et al 1963). Consequently, we are tentatively assuming that 2-ME causes an increase in protein solubility by cleaving intermolecular disulfide bonds that act as crosslinkages between the monomeric proteins.

Disulfide-Linked Polymers in Fresh and Aged Isolates

Incomplete solubility of isolates is attributable, in part, to disulfide-linked polymers that are insoluble in phosphate buffer. With freshly prepared isolates, as shown in Table I, 10–12% more protein dissolved in buffer with 2-ME. The proteins solubilized by 2-ME are derived from disulfide polymers of the major proteins. Our studies also confirm that about 80% of the protein in fresh isolates is soluble in buffer containing 2-ME (Wolf et al 1963). Figure 1 indicates that the disulfide polymers were present at an approximately constant level throughout the study.

Nondisulfide Insolubilization with Age

Our results also show that formation of disulfide polymers does not account for the decrease in protein solubility on aging, but rather that the decrease in solubility exhibited by the upper curve (Fig. 1) represents protein insolubilized by some other means. Additional evidence for reaction(s) other than disulfide polymerization is the increase in 15S and > 15S fractions as noted in the portion soluble in buffer with 2-ME (Fig. 2b). However, these increases in the 15S and > 15S fractions do not totally account for the losses in the 7S and 11S proteins. Presumably, the aggregates sedimenting in the 15S and > 15S range continue to grow in size by an unknown mechanism until they are no longer soluble.

Aggregation and insolubilization of proteins on aging have also been noted in dried blood (Sensabaugh et al 1971). Both disulfide and nondisulfide crosslinkages appeared to be responsible for the changes noted in the blood proteins. Evidence implicated the Maillard reaction between glucose (naturally occurring plus that present in the anticoagulant) and ε-amino groups of lysine. This reaction appears less likely in our samples because the content of reducing sugars in soybean meal is very low and is lowered further by preparation of the isolates by isoelectric precipitation. On the other hand, soy protein isolates contain small amounts of lipid (Nash et al 1967). A low rate of protein insolubilization could result from lipid peroxy radical addition reactions postulated as leading to protein insolubilization in studies by Roubal and Tappel (1966). Highly unsaturated lipids of fish, which were major components in Roubal and Tappel's model system, have long been implicated in a high relative rate of protein insolubilization during fish storage (Kuusi et al 1975). Studies by Shimada and Matsushita (1978) indicate that the 11S protein undergoes polymerization when it is treated with peroxidized linoleic acid. Autoxidation of residual lipids in soy protein isolates may, therefore, be involved in the slow insolubilization that occurs in protein isolates during storage.

Decreases in protein solubilities with isolate age, 1–4% per year in the reductant buffer or 4–6% per year in the phosphate buffer, are intermediate between losses occurring with commodity age, 1% per year as beans (Smith et al 1952) and 1% per month as defatted soybean meal (Nash et at 1971, Smith and Circle 1938).

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