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Soybean Protein Agglomeration: Promotion by Ultrasonic Treatment

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Aggregate formation in soybean proteins after ultrasonic treatment was studied by gel filtration, disc gel electrophoresis, and ultracentrifugation. Ultrasonic-treated soybean proteins contained more aggregates than unsonicated samples. Gel filtration with Sephadex G-200 showed that the aggregates eluted near the void volume, ahead of 7 S and 11 S, and appeared to be stable above a critical protein concentration. Disc gel electrophoresis indicated that a large amount of protein from sonicated samples did not enter the stacking gel. Protein separation in the gel suggested transformational changes of 7 S proteins. Ultracentrifugal analyses also indicated conversion of 7 S into 40–50 S aggregates. Ultrasonic action seemed to promote protein aggregation rather than dissociation in water extracts prepared from defatted soybean flakes.

It has been shown previously that ultrasonic treatment increased soybean protein extractability in water from defatted flakes (Wang, 1975) and from commercial isolates, concentrates, and defatted flakes which were either heated or alcohol-washed before sonication (Wang, 1978). Sonication has been widely used in solubilizing animal and plant tissue components. However, its application to extracting soybean proteins is relatively new.

It is generally surmised that globular proteins in an ultrasonic field may be broken down or transformed into new molecules. Ultrasound induces cavitation which causes proteins to undergo either physical disruption and/or chemical transformations (El'Piner, 1964). The conditions under which proteins are transformed can be controlled in most cases. Changes in protein molecules may thus be studied. A recent example includes studies of the effect of ultrasonic irradiation on several proteins (O'Shea and Bradbury, 1973). Mayoglobin, apomyoglobin, and proteins from wool were sonicated and the modified proteins studied by gel permeation chromatography. Only myoglobin was aggregated, while apomyoglobin split into half-molecules and others showed little or no changes after sonication. It appears that the breakdown is a result of a physical rather than chemical process. The chemical process that is associated with cavitation may produce secondary effects such as formation of bilayers, micelles, and aggregates. Aggregation was also observed under various conditions in ultrasonic-treated samples including serum albumin (Searcy, 1966), apolipoprotein in a mixture of trioleins and lecithin (Forte et al., 1974), blood platelets (Miller et al., 1979), and goat immunoglobulin (Huang and Kennel, 1979).

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Reports on ultrasonic-treated soybean proteins are scanty. Thus, we have extended our earlier studies of ultrasonic effects on soybean proteins (Wang, 1975, 1978).

This paper reports a phenomenon of ultrasonic promoted agglomeration of soybean proteins as demonstrated by results obtained from gel filtration, disc gel electrophoresis, and ultracentrifugational analyses.

MATERIALS AND METHODS

Materials. Kanrich variety soybeans purchased locally were cracked, dehulled, flaked, and defatted. The defatted flakes contained 46.4% protein (dry basis) and had a nitrogen solubility index of 91 (Smith et al., 1966). The flakes were refrigerated until used. Fine-grade Sephadex G-200 was obtained from Pharmacia Fine Chemicals, Uppsala, Sweden. Acrylamide for electrophoresis was purchased from Eastman Kodak Co., Rochester, NY. A commercial preparation of soybean trypsin inhibitor (TI) for identification purposes in gel filtration was bought from Sigma Chemical Co., St. Louis, MO.

Protein Extractions. Protein extraction in a 1:10 ratio of flakes to water was used as described previously (Wang, 1975). Sonication was carried out for 8 min with a sonifier (Heat Systems-Ultrasonics Inc., Model J32A, Plainview, NY). The samples (10 g of meal; 100 mL of H_2O) in a 250-mL beaker were chilled in ice during sonication. After sonication, the mixture was centrifuged at 10000*g* for 15 min, and the supernatant was poured through a thin layer of glass wool to obtain a clear filtrate. The proteins were lyophilized and stored in a refrigerator for further use. Protein fractions (7 S, 11 S, and whey proteins) were prepared according to Thanh and Shibasaki (1976), dialyzed, lyophilized, and stored at 4 °C for future use in gel filtration and disc gel electrophoresis analysis.

Gel Filtration of Proteins. Lyophilized protein samples (125 mg or less) were dissolved in 5 mL of sodium phosphate buffer (pH 7.6; $\mu = 0.1$) and applied to the top

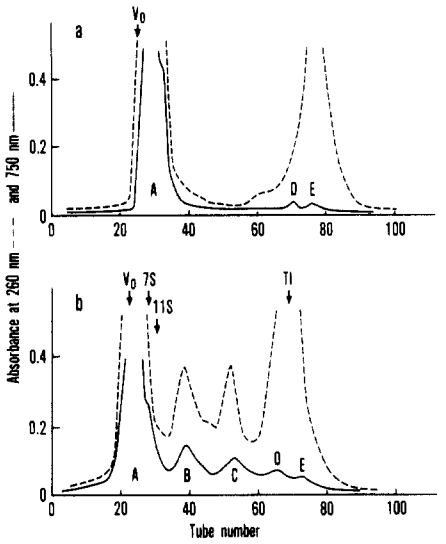

Figure 1. Sephadex G-200 gel filtration of soybean proteins (100 mg) in water extract obtained with (a) ultrasonic extraction and (b) stirring extraction. For elution conditions see the text. (---) 260 nm; (—) 750 nm.

of a Sephadex G-200 column (3 × 96 cm). The column was packed according to the specifications given by Pharmacia and was equilibrated with the same buffer. Effluent fractions (7 mL) were collected at room temperature with a fraction collector. The effluent was analyzed at 280 and 260 nm, and a portion was also analyzed by the Lowry phenol at 750 nm. Proteins in the desired peaks were pooled, dialyzed for 72 h against water, and freeze-dried for ultracentrifugal analysis.

Disc Gel Electrophoresis. Electrophoresis was performed with 5% gel concentration with 7% cross-linkage according to Davis (1964) and Ornstein (1964). A Bio-Rad electrophoresis cell, Model 300 B, was used. After electrophoresis the proteins were dyed with amido black, and the colored bands were either photographed or scanned at 600 nm with a Gilford spectrophotometer, Model 240. Identification of specific proteins (7 S, 11 S, and whey proteins) was made by coelectrophoresis of isolated protein fractions.

Ultracentrifugation. Protein samples were equilibrated with 0.03 M potassium phosphate buffer containing 0.4 M NaCl and 0.01 M 2-mercaptoethanol (pH 7.6; μ = 0.5). Sedimentation analyses were made with a spinco ultracentrifuge, Model E, operated at room temperature. A series of Schlieren patterns was taken at different time intervals for each sample after the centrifuging speed reached 48 000 rpm.

RESULTS

Gel Filtration of Ultrasonic-Treated Proteins. Sephadex G-200 separated ultrasonically extracted proteins in sodium phosphate buffer at pH 7.6, ionic strength 0.1 μ, into one major peak plus two minor ones (Figure 1a). About 80% of the total water-extractable proetins appeared in the first peak that eluted with the void volume. The low molecular weight proteins that were observed in a water extract prepared by mechanical stirring (peaks B–E, noted in Figure 1b) were either reduced in peak height or no longer seen. The virtual absence of peaks B and C in the sonicated proteins suggested that protein agglomeration occurred.

Figure 1b presents results from samples extracted by mechanical stirring. The water-extractable protein was separated into five peaks (A, B, C, D, and E). The protein solution from pooled peak A in Figure 1b was turbid,

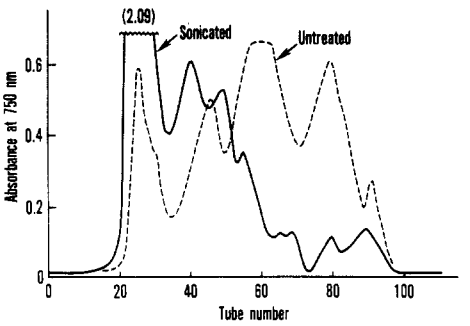

Figure 2. Sephadex G-200 gel filtration of a sonicated and untreated mixture of isolated whey proteins (50 mg) and proteins (50 mg) from water extract obtained by stirring extraction.

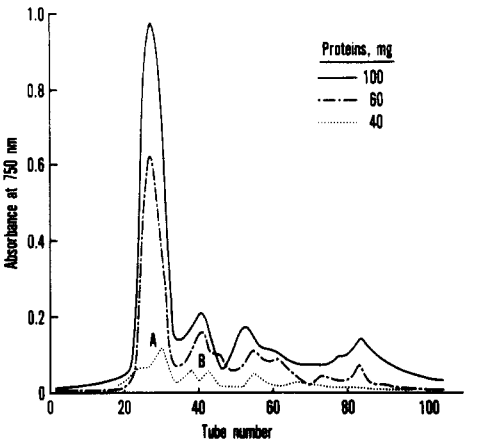

Figure 3. Gel filtration of varied amounts of proteins in water extract obtained by mechanical stirring.

Table I. Relative Protein Concentration Ratios in Peaks A and B As Chromatographed in Figure 1 with Varied Amounts of Proteins on a Sephadex G-200 Column

chromatographed mg of protein	rel protein concn, mg/mL		ratio of peak A to peak B
	peak A	peak B	
125	1.13	0.24	4.70
100	1.00	0.21	4.76
80	0.80	0.17	4.70
60	0.62	0.17	3.65
40	0.11	0.06	1.83

probably contained RNA (Obara and Kimura, 1967), and represented ~40% of the total protein in the extract. The 7 S and 11 S globulins were eluted at the back side of peak A; but under the conditions used (0.1 μ), the 7 S protein was in the 9 S or dimer form (Koshiyama, 1968). Little protein was found in peak E, but it contained high-UV-absorbing materials based on absorbance at 260 nm. The UV-absorbing materials found in the last peak appeared to emerge coincidentally with the small amount of protein found in this fraction. The water extract was not dialyzed to shorten the handling time of the sample after extraction. In a control experiment, dialysis removed nearly all of the UV-absorbing materials from the last peak but otherwise did not change the elution pattern.

Ultrasonic treatment of various protein samples produced the same effect as in Figure 1a. These samples included a water extract from defatted flakes obtained by mechanical stirring extraction and water-extractable proteins from alcohol-washed flakes or autoclaved flakes. For example, when a mixture of whey proteins and water-extracted proteins was sonicated, a much higher first peak was obtained (Figure 2). Again, sonication promoted agglomeration by increasing the height of the peak that eluted near the exclusion limit and altered the elution

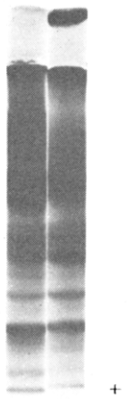

Figure 4. Disc gel electrophoresis of soybean proteins: (A) unsonicated flakes; (B) sonicated flakes.

pattern of those smaller sized proteins coming after the first peak by decreasing their peak height.

In the absence of ultrasonic treatment, a concentration-dependent disaggregation was observed by chromatographing different amounts of proteins (40, 60, and 100 mg of proetins) from water extracts prepared by stirring extraction. Figure 3 presents a noticeable chromatographic difference in the height of peak A relative to peak B. Table I gives the variation of the ratio of relative protein concentration in peak A to peak B. The ratio remained constant above a critical protein concentration of ~0.8 mg/mL for peak A, and at these higher concentrations the proteins apparently maintained their agglomerated form. At 0.6 mg/mL protein or lower concentration for peak A, the ratio decreased.

Other gel filtration materials such as Sepharose 4B, glass beads, and polystyrene, although they have a greater exclusion limit than Sephadex G-200, did not improve the resolution of the agglomerated proteins under chromatographic conditions such as varied ionic strength, amount of sample, and temperature.

Disc Gel Electrophoresis. Disc gel electrophoresis was employed to separate the proteins according to their size, shape, and charge. Results in Figure 4 show that the sonicated sample contained large amounts of proteins that accumulated at the top of stacking gel (B). These proteins were either too large in size or too low in mobility to enter the gel. On the other hand, the unsonicated sample (A) with the same amount of protein revealed less protein on the top of the stacking gel. When tracings at 600 nm were used, differences in the separation gel were noticed between sonicated and untreated samples (Figure 5). Untreated samples of 7 S, 11 S, and 2 S (TI) were found to have different mobilities; 7 S had the lowest mobility, 2 S had the highest, and 11 S fell in between (Figure 5a). Sonication caused disappearance of 7 S bands in the sonicated sample (Figure 5b). Smaller changes in mobilities of 11 S and other proteins were noticed. Apparently sonication causes significant changes in 7 S proteins.

Ultracentrifugation. Figure 6 shows results of ultracentrifugal analysis of sonicated and unsonicated water extracts in potassium phosphate buffer with 2-mercaptoethanol (pH 7.6; ionic strength 0.5 μ). In the sonicated sample, a 40–50 S peak representing agglomerated proteins was clearly observed at 8 min, during acceleration. This peak sedimented to the bottom of the cell in ~24 min. Examination of the ultracentrifuge pattern after 64 min of centrifugation shows that 7 S proteins are missing in

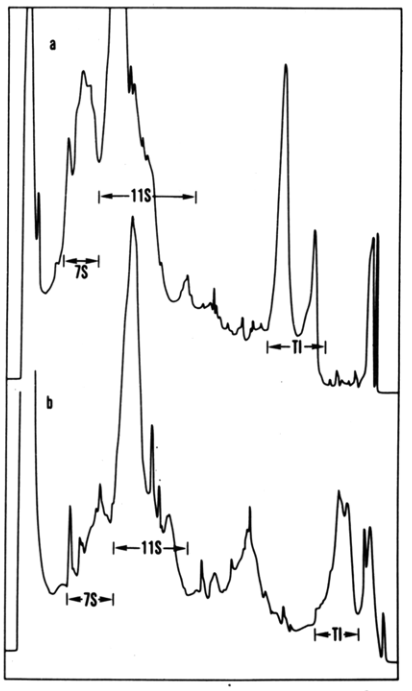

Figure 5. Densitometer tracings at 600 nm of disc gel electrophoretograms of (A) and (B) from Figure 4.

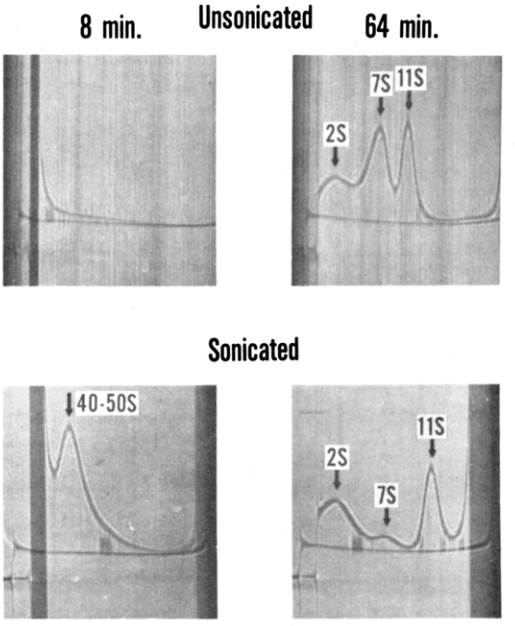

Figure 6. Ultracentrifugal analysis of sonicated and unsonicated samples, pictures taken at 8 and 64 min after the start of centrifugation.

the sonicated as compared with the unsonicated sample. Obviously, 7 S proteins are agglomerated by sonication.

DISCUSSION

Present studies with gel filtration, electrophoresis, and ultracentrifugation show agglomeration of soybean proteins after sonication. Gel filtration was useful in demonstrating aggregates in sonicated water extract (Figure 1) and their formation on sonication of a mixture of isolated whey proteins and water extract (Figure 2). However, the utility of Sephadex G-200 columns was limited because of poor resolution of soybean proteins in the high molecular weight range. Electrophoresis and ultracentrifugation were helpful in confirming agglomeration and conformation changes of 7 S proteins (Figures 4–6). Thus far, studies have revealed that sonication not only improves solubility

of soybean proteins in water but also agglomerates 7 S proteins. These observations suggest that sonication may cause significant changes in physical properties of soybean proteins and their food products.

The major storage proteins in soybeans (7 S and 11 S) are globular in nature and water soluble. Mechanical stirring produces water extracts with ~80% of the total proteins from fresh defatted soybean meal. The yield is lower if the meal is toasted, alcohol-washed, or stored for several months. Ultrasonic extraction, however, improves protein solubility from many of these treated meals (Wang, 1978). Present findings indicate that sonication promotes aggregate formation and that one of the major storage proteins, the 7 S fraction, is involved in the aggregation process. Thus, 7 S proteins may have unique properties that, unlike other soybean proteins (2 S and 11 S), are sensitive to ultrasonic treatment. Perhaps under sonication 7 S transforms into an aggregate of bilayer, micelle structures and aggregates. At high protein concentration such as 0.8 mg/mL the aggregates are more stable (Figure 3 and Table I). The aggregation formation may have no relationship to increased protein solubility as observed previously (Wang, 1975, 1978).

Ultrasonic action promotes agglomeration rather than dissociation of soybean proteins. Agglomeration of the 7 S proteins may affect physical properties in such a way, as observed by Saio et al. (1971) in making tofu. Tofu coagulated from 7 S and 11 S protein fractions has distinct differences in hardness. Ultrasonic action may serve as an alternative means to achieve desired properties in soybean food products.

Further studies on the mechanism of aggregate formation by sonication may reveal several possibilities; ultrasonic action may promote hydrophobic interaction of globular proteins in water (Tanford, 1977, 1978), may induce formation of complex mixture as in apolipoproteins (Forte et al., 1974), or may alter the equilibrium condition of protein–protein or protein–lipid interactions to favor the formation of a cluster type of structure. More results along these lines of investigation should be helpful to broadening this basic understanding of soybean proteins.

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COMMUNICATIONS

Synthesis of Some Highly Brominated Naphthalenes

The chemical synthesis and characterization of 2,3,6,7-tetra-, 1,2,4,6,7-penta-, and 1,2,3,5,6,7- and 1,2,3,4,6,7-hexabromonaphthalene are reported. These compounds are representative of the brominated naphthalene contaminants of the fire-retardant chemicals polybrominated biphenyls.

Highly brominated naphthalenes (primarily penta and hexa) have been identified (Hass et al., 1978) as associated contaminants of the fire-retardant chemicals polybrominated biphenyls (PBB's). The PBB's were introduced to the Michigan environment through accidental contamination of animal feeds, thereby exposing a large part of the population of the state to these substances. While the PBB's themselves have not been found (Allen et al., 1978) to be particularly toxic to animals, there is a great deal of concern about the highly toxic potential of associated contaminants such as the brominated naphthalenes. Interest in carrying out toxicity studies on these potentially toxic environmental contaminants engendered a need for synthesis of selected isomers and homologues. In view of the demonstrated (McConnell et al., 1978; McKinney et al., 1980) dependence of toxicity on the number and position of halogens in related planar compounds, it was important to have regiospecific synthetic methodology. A search of the chemical literature revealed a scarcity of information on synthetic methodology for the highly brominated naphthalenes. Our specific interest was in synthesizing symmetrical tetra-, penta-, and hexa-substituted isomers. Especially important was the need to have methodology that would permit substitution in at least three of the lateral positions (2, 3, 6, 7) which is always a requirement for high toxicity in these compounds (McKinney et al., 1980). It was clear that the easiest route into these systems would be selective functionalization of