CHARACTERISTICS OF THE VARIOUS SOYBEAN GLOBULIN COMPONENTS WITH RESPECT TO DENATURATION BY ETHANOL[1]

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ABSTRACT

Of the four components of soybean globulins that are resolvable in the analytical ultracentrifuge, the 7S component is very rapidly denatured (rendered insoluble in phosphate buffer at pH 7.6, ionic strength 0.5) when the wet curd is brought into contact with ethanol-water mixtures of 20% or greater concentrations of the alcohol. By contrast, the rate of denaturation of the 11S and 15S components is slow, and the 2S component is not denatured at all. Variations in the extents of denaturation of the components in relation to the times of contact and concentrations of ethanol are reported.

The globulin fraction of soybean protein is the fraction of primary interest in the preparation of a protein for food uses from defatted soybean meal (11). The globulin fraction may be prepared most readily by acid precipitation in the pH range 4–5 of a water extract of soybean meal. This fraction is variously referred to as the acid-precipitable fraction, glycinin, or soybean casein.

A useful method for characterizing this heterogeneous globulin fraction is by ultracentrifugal analysis. By this method the globulin is found to contain four resolvable components (4,9). These components have been identified in the literature by their approximate $S_{20,w}$ values, as the 2S, 7S, 11S, and 15S components. Although the relative

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proportions of these components depend upon the variety of soybean and the method of extraction and precipitation, the 11S fraction is usually present in the highest percentage, followed by the 7S, the 2S, and finally the 15S. The composition of the globulin preparation used in the present experiments was 48% 11S, 33% 7S, 11% 2S, and 7% 15S.

It has been observed that aqueous solutions of ethanol and other water-soluble alcohols will reduce the solubility of part of the globulin fraction through denaturation (3,5). The purpose of the present study is to examine the denaturing action of aqueous ethanol on each of the four ultracentrifugally resolvable components of the soybean globulin.

The preparation of a bland, edible soybean protein can be achieved through the use of an alcohol extraction. The results of the experiments reported here can be of aid in assessing the changes in solubility of the product that may occur when the wet protein is subjected to ethanol treatments and in choosing conditions required to avoid extensive denaturation.

Materials and Methods

Preparation of Soybean Globulins. Soybean protein was extracted from hexane-extracted soybean flakes with water in the ratio of 1 g. of flakes to 8 ml. of water. The pH was maintained at 7.6 during the extraction period by addition of the necessary amount of 1*N* sodium hydroxide.

The globulin fraction was precipitated from the water extract by lowering the pH to 5.0 with 1*M* acetic acid, and was separated from the whey by centrifugation. The precipitate was redissolved at pH 7.6 and treated with two batches of Dowex 1 × 8 in the acetate form to remove phytic acid (6). The protein solution was clarified by centrifugation at 20,000 × *g* (Servall Angle Centrifuge) for 25 min., and freeze-dried.

The starting protein solution, in a typical denaturation experiment, was prepared by dissolving 2.00 g. of freeze-dried material per 100 ml. of water. Ten-milliliter portions of this stock solution were placed in 50-ml. centrifuge tubes and the protein in each was precipitated by lowering the pH to 5.0 by dropwise addition of 1*M* acetic acid. A Beckman probe combination-electrode was used to follow the pH change. The protein-free supernatants obtained upon centrifugation were discarded and the ethanol treatments were carried out on the remaining precipitates, each of which contained 200 mg. of protein, approximately an equal weight of water, and a negligible amount of salt.

Ethanol Treatment. All the experiments on ethanol denaturation were carried out at room temperature, in the range of 24° to 26°C. Absolute ethanol was mixed with water on a volume-to-volume basis, to prepare the ethanol solutions to be added to the protein precipitates. These solutions were brought to room temperature before addition to the protein.

Forty-milliliter aliquots of the various ethanol-water solutions were added to individual tubes containing the buttons of the precipitated protein to give the desired final ethanol concentration series. The tubes were shaken vigorously, then left undisturbed for the desired length of time. No significant difference in the amount of denaturation was observed between continuously stirred samples and those mixed only at the start.

Removal of the Ethanol. At the end of the ethanol treatment the tubes were centrifuged at approximately 10,000 × *g* for 10 min. The supernatants, which contained no material precipitable in 10% trichloroacetic acid, were discarded. The precipitates were washed once with 40 ml. of sodium acetate buffer of pH 5.0 and ionic strength 0.05 to remove residual ethanol. The total time for denaturation was measured from the moment the ethanol was added until the moment the wash buffer was added. None of the precipitated protein was dissolved by the wash buffer. After removal of the wash buffer by centrifugation and decantation, 10 ml. of phosphate buffer were added. This buffer has been adopted as the standard buffer best suited to observe the resolution of the four components of soybean globulins in the ultracentrifuge (7). The composition of this standard buffer was 0.0325*M* dipotassium monohydrogen phosphate, 0.0026*M* monopotassium dihydrogen phosphate, 0.40*M* sodium chloride, and 0.01*M* 2-mercaptoethanol (all A.R. reagents), giving a pH of 7.6 and an ionic strength of 0.5. The criterion for denaturation adopted in this work was that of insolubility of the protein in the standard buffer.

The soluble (i.e., undenatured) protein remaining in the ethanol-treated sample was extracted from any insolubles present with the standard buffer for a period of at least 30 min. with continuous stirring. The solubles were separated from the denatured material by centrifugation as supernatants which were saved for analysis in the ultracentrifuge. The total amount of denatured protein was measured by collecting the insolubles on tared moisture-free filter papers, washing with water to remove salts, drying *in vacuo* at 80°C. for 24 hr., and weighing.

Ultracentrifugal Analysis. The soluble fractions were examined in

a Spinco Model E Analytical Ultracentrifuge. In all cases a 12-mm. single-sector cell was used at a speed of 56,100 r.p.m. Photographs were taken, at constant bar angle for any given series, at 48 min. after the machine was started. The areas under the various peaks were determined from enlargements of the sedimentation patterns and base lines traced on graph paper. The base-line pattern was obtained by running solvent alone in the ultracentrifuge under the same conditions. In a series of samples where the areas under corresponding peaks were compared, the areas were considered to be directly proportional to the concentrations of the corresponding components in the samples under comparison. No corrections were applied for radial dilution or the Johnston-Ogston effect (1). (Corrections for radial dilution would be negligible, since differences in areas for corresponding components were being compared and such corrections would be constants that would not affect the ratios of the differences. Corrections for the Johnston-Ogston effects would be negligible because of the low total protein concentrations used.) The accuracy of the values obtained by this method is estimated to be ±10%.

Variations in Experimental Procedure. Experiments were made in which the procedure was altered in order to test for any influence that the physical state of the protein might have on its susceptibility to denaturation. In one case, 0.01*M* 2-mercaptoethanol was added to the protein solution before precipitation at pH 5.0, in order to depolymerize any disulfide polymers of the protein that might be present in the native globulins. In a second case, the ethanol denaturation was carried out on the stock globulin solution at pH 7.6. The ethanol concentration used was 50% and the protein was left in contact with the ethanol solution for 1 hr. Under these conditions of very low ionic strength, no precipitate formed but an increase was noted in the turbidity of the solution during the ethanol treatment. After 1 hr., the denaturation reaction was stopped by diluting with water to a low alcohol concentration and precipitating the protein at pH 5.0 by addition of 1*M* acetic acid. The precipitated protein was washed and extracted with standard buffer in the manner described above.

Results and Discussion

Dependence of Denaturation on Ethanol Concentration. Samples of the wet-precipitated soybean globulins, at pH 5.0, were mixed with 40 ml. of aqueous ethanol solutions ranging from 10 to 100% ethanol and left in contact for a period of 8 hr. The dependence of the total amount of denaturation, measured as the proportion of buffer-insolu-

ble proteins formed in that time period, on the ethanol concentration of the resulting slurries (uncorrected for the initial water contained in the wet protein) is shown in Fig. 1.

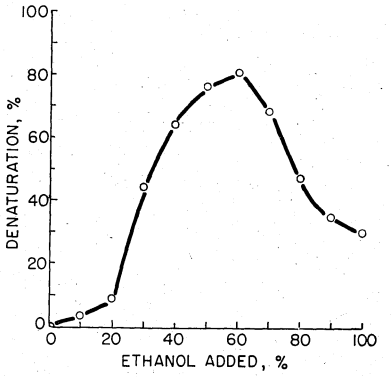

Fig. 1. Dependence of the total denaturation of soybean globulins on the concentration of ethanol, after 8 hours of contact with the ethanol solution. Percent denaturation is determined from the weight of insolubles.

Maximum denaturation occurred at about 60% ethanol, and the amount of denatured protein increased most rapidly with increase in ethanol concentration between 20 and 30% ethanol. At concentrations higher than 60% ethanol, the amount of denatured protein decreased rapidly with increase in ethanol concentration.

In the 100% ethanol sample, 30% of the protein was denatured as compared with 80% at 60% ethanol. When starting with the wet precipitate, the extent of protein denaturation at high ethanol content of the solutions is greater than that reported by Smith *et al.* (5) when aqueous ethanol solutions were added to dry protein. This difference can be explained by the presence of the initial small amount of water in the wet precipitate. During the initial mixing of the alcohol solutions with the wet precipitate, the ethanol concentration in the region immediately surrounding the precipitate would rise from zero to the final concentration of the added solution. This very short period of contact with intermediate concentrations of ethanol is apparently sufficient to allow for the observed increased degree of denaturation.

Figure 2 shows the dependence of the denaturation of each component of the globulin fraction, after 8 hours' contact, on the ethanol concentration (as measured from ultracentrifuge patterns obtained with the buffer-soluble protein remaining after that time period). The percent denaturation of each component is calculated as equal to the decrease in area under that component peak compared with the area

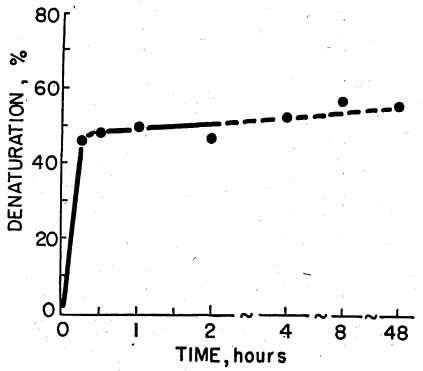

Fig. 3. The dependence of the denaturation of total soybean globulins by 50% ethanol solutions with time. The percent denaturation was determined from the weights of the insolubles.

from the ultracentrifuge patterns of the buffer-soluble protein, appears in Fig. 4. The percent denaturations of the various components, as shown in Fig. 4, were estimated as described for Fig. 2, and are subject to the same limitations.

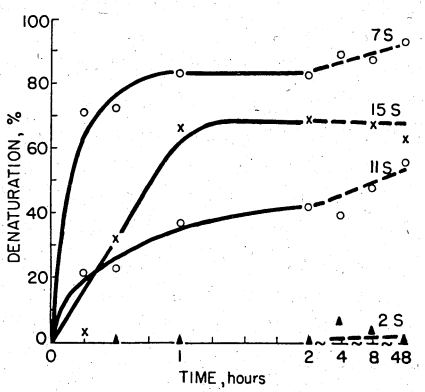

Fig. 4. A comparison of the rates of denaturation of the four components of soybean globulin by 50% ethanol solutions. The percent denaturation was calculated from area changes in the sedimentation patterns.

The rate of denaturation of the 7S component in 50% ethanol is considerably greater than that of the 11S or of the 15S component. The 7S component is 70% denatured in 15 min. and it is maximally denaturated in less than 2 hr. The 2S component does not appear to be denatured at all.

In Fig. 5 some examples of sedimentation patterns are presented: *a* shows a typical globulin fraction (no treatment with ethanol); *b, c,*

and *d* show the soluble fraction remaining after consecutively longer treatments with 50% ethanol. The patterns illustrate the large decrease in the amount of 7S that remains soluble after short treatments with aqueous ethanol.

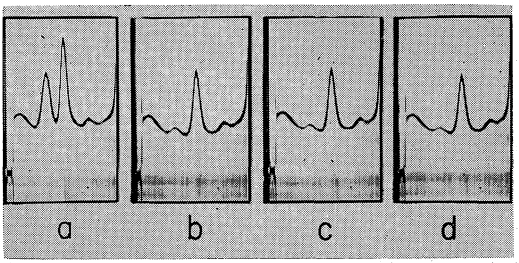

Fig. 5. Sedimentation patterns of soybean globulin: a, soybean globulin before treatment with 50% ethanol; b, after 15 minutes' treatment with 50% ethanol; c, after 30 minutes' treatment with 50% ethanol; d, after 1 hour's treatment with 50% ethanol. Sedimentation from left to right. From left to right, the peaks represent 2S, 7S, 11S, and 15S components, respectively.

Even after a period of 48 hr. in contact with 50% ethanol, none of the four components completely disappeared from the buffer-soluble fraction. It was particularly surprising that, even though 70% of the 7S was denatured in the first 15 min. of 50% ethanol treatment, 7–10% of the 7S still remained soluble after 48 hr. Conceivably this could have resulted from some kind of physical protection of a part of the protein against denaturation.

Some of the 7S and 11S components are present as disulfide polymers in the native globulin preparation (7). The possibility that the polymers of these components offered some degree of protection against the denaturing action of ethanol was tested. A comparison was made of the amounts of denaturation occurring in 1 hr. in 50% ethanol in a sample, pretreated with 0.01*M* 2-mercaptoethanol to reduce any disulfide polymers present, with a sample containing no reducing agent. No significant differences in the extents of denaturation of the 7S and 11S components were observed.

A comparison of the amount of denaturation occurring at 50% ethanol in a sample of protein dissolved in water at pH 7.6 with the amount occurring on a similar sample of protein in the precipitated state at pH 5.0 showed that, when in solution, all components except the 2S were denatured at a faster rate. However, the denaturation was not completed; 7–10% of the 7S, for example, still remained in the soluble fraction.

The reason why the 7S component is not completely denatured,

under any of the conditions tried, is not known. One possible explanation for this behavior could be that there exist two different native components having 7S sedimentation values, one of which, like the 2S component, is resistant to alcohol denaturation. The possible occurrence of two 7S components has been suggested by Wolf (8) from ammonium sulfate fractionation studies. Another explanation could be that the 11S component may dissociate to an intermediate, buffer-soluble, 7S component during the denaturation process, in a manner similar to its behavior at low pH's (10). In this case, the residual 7S (remaining undenatured) would not actually be a part of the natural 7S component. Both of these explanations are plausible, but the correct answer must await experiments using purified 7S and 11S components.

The use of the analytical ultracentrifuge has shown clearly that the various components of soybean globulins are denatured at different rates when brought into contact with aqueous ethanol solutions. This indicates that each component behaves as a discrete species with respect to ethanol denaturation. This conclusion does not support the proposal by Kretovich *et al.* (2) that all sedimenting fractions of soybean globulins are made up of the same subunit.

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