Mechanism of Physical Modification of Insoluble Soy Protein Concentrate

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ABSTRACT: The mechanism for solubilization of alcohol-leached soy protein concentrate (ALSPC) by physical modification was studied from the standpoint of molecular interactions, which are related to the differences in protein solubility under different conditions. The low solubility of ALSPC is caused by both noncovalent and covalent forces, but the noncovalent forces do not affect the solubility of modified soy protein concentrate (MSPC). Gel filtration shows that the major constituents of soluble protein from ALSPC and MSPC are protein molecules and protein aggregates, respectively. Physical modification promotes the formation of aggregates that are readily soluble in buffer. Fluorescence spectroscopy further proved that the hydrophobic groups are located in the interior of the aggregates. The reason for the formation of soluble protein aggregates during physical modification of ALSPC is discussed.

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KEY WORDS: Aggregating, denaturation, functional property, functionality, protein aggregate, protein modification, solubility, solubilization, soybean, soy protein.

Although soy protein concentrate (SPC), prepared by aqueous alcohol leaching, has desirable light color and bland flavor, its solubility is lost due to the denaturation effect of alcohol. Protein solubility is of paramount importance to the performance of functional properties in many food systems. Considerable efforts have been made to improve protein solubility by protein modification, including a physical method in which the material is subjected to heat and mechanical action (1,2). Generally, heat and mechanical treatments may cause protein to unfold and solubility to decrease; in fact, NSI (nitrogen solubility index) is used to determine the extent of denaturation of defatted soy meal. Nevertheless, Voustinas *et al.* (3) found that soy protein isolate (SPI) that was heated at alkaline pH had substantially improved solubility. Further studies proved that, on heating, soy protein molecules formed soluble aggregates and molecular interactions with diverse energy levels, such as van der Waals forces, hydrogen bonds, hydrophobic forces, and disulfide bonds, are suggested to be responsible for aggregate formation (4). According to Kinsella *et al.* (5), the solubility of a protein is the manifestation of the equilibrium between protein–solvent and protein–protein interactions. Thus, enzymatic or chemical modification will increase the protein–water interaction and weaken the protein–protein interaction through the introduction of electric charges and/or hydrophilic groups into the peptide chain. On the other hand, the effects of physical modification on molecular interaction have not been fully characterized. The present work was conducted to study the solubilization mechanism of SPC through changes of molecular interactions. The research was carried out by determining the solubility of protein in different solvents and by gel filtration analysis of soluble protein. This report concludes that the solubilization of the denatured soy protein is caused by transforming the strongly bound protein molecules to protein aggregates, which interact loosely with each other.

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MATERIALS AND METHODS

Defatted soy flakes, acid-washed SPC (AWSPC), and SPI were obtained from Sanjiang Food Co. (Jiamusi, China).

Alcohol-leached SPC was prepared in our laboratory by extracting defatted soy flakes with 60% (vol/vol) aqueous ethanol at 45°C for 40 min; after centrifuging, the extracted material was vacuum-dried at 50°C.

Modified soy protein concentrate (MSPC) was prepared by physically modifiying of ALSPC in a specially designed high-shear homogenizer. ALSPC suspended in water at a solids level of 14.5% (adjusted to pH 8.1) was treated in the device at 110°C for 5 min; after cooling to room temperature, the slurry was spray-dried with an inlet air temperature of 180°C and outlet temperature of 80°C.

Protein solubilities in 0.5 M phosphate buffer (pH 7), 0.025 M sodium dodecyl sulfate (SDS) solution, and 6 M urea solution were determined by suspending a 1.000-g sample in 50 mL of the appropriate solution, agitating at 25°C for 1 h. Then, the suspension was centrifuged, and protein content in the supernatant was determined: solubility = (protein in supernatant/total protein in sample) × 100.

Gel filtration of soluble protein was carried out in a 1 × 80 cm column (Sepharose 6B; Pharmacia, Uppsala, Sweden), eluted with 0.1 M phosphate buffer + 0.4 M NaCl (pH 7.6) at 7 mL/h. The void volume, determined by blue dextran 2000 (Sigma Chemical Co., St. Louis, MO), was 18 mL. A 0.5% SDS solution was used to elute 6 M urea solution-soluble protein samples according to Yamagishi *et al.* (6).

Fluorescence spectroscopy was carried out with a Hitachi-635 fluorometer (Hitachie Ltd., Tokyo, Japan) with a 1-cm

cell, at an excitation wavelength of 280 nm. The 8-anilino-1-naphathalenesulfonic acid (ANS) fluorescence probing method of Nakai and Li-Chan (7) was used to determine protein surface hydrophobicity.

RESULTS

Table 1 shows protein solubility in buffer, 2.5 mM SDS solution, and 6 M urea solution. The data show that solubility of all samples increases when the solvent shifts from buffer to SDS solution and finally to urea solution. From the viewpoint of molecular interaction, it can be concluded that urea is most prominent in breaking protein–protein interactions, SDS comes second, and buffer is the weakest. Determining the solubility in these media is a common practice in studying insoluble proteins, which is based on some generally accepted assumptions. In phosphate buffer, hydrophobic forces, hydrogen bonds interacting in the hydrophobic region, and disulfide bonds are assumed to be responsible for the insolubility of proteins. SDS is effective in breaking hydrophobic interactions, and urea breaks both hydrogen bonds and hydrophobic forces, but disulfide bonds remain intact in urea solution. The extent of insolubility caused by each type of interaction is well approximated by the difference of solubility (DS). Thus, DS1 is the solubility in SDS – solubility in buffer and represents the insolubility by hydrophobic force; DS2 is the solubility in urea – solubility in SDS, representing the insolubility by hydrophobic forces and stabilized by hydrogen bonds; and DS3 is 100% solubility – solubility in urea, representing the insolubility by disulfide bonds and the noncovalent forces that are stabilized by disulfide bonds.

DS1, DS2, and DS3 for each soy protein sample were calculated from data in Table 1, and results are shown in Figure 1. For ALSPC, which is alcohol-denatured and has the lowest solubility, the interaction pattern is unique among all samples in that both noncovalent interactions (DS1, DS2) and disulfide bonds (DS3) contribute to the decrease of solubility. After physical modification, the protein interaction pattern of the resulting MSPC changes substantially: noncovalent forces (DS1 and DS2) represent a minor effect on solubility, while disulfide forces emerge to be the major force. So, it appears that alcohol-denatured soy protein is solubilized by physical modification by selectively eliminating the low-energy noncovalent forces. The interaction patterns of AWSPC and SPI are also shown in Figure 1. They are characterized by rather high DS1 and DS2 values but a much lower DS3. Because the high-energy covalent forces are rare for AWSPC and SPI, physical modification will be useful in improving their solubility.

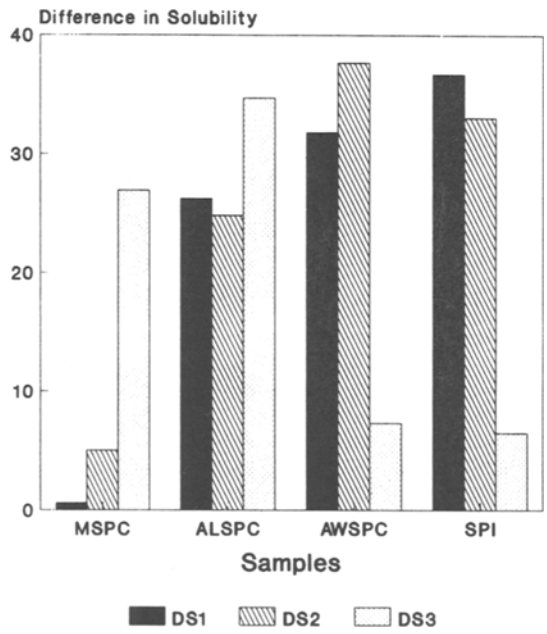

FIG. 1. Effect of the type of molecular interaction on the insolubility of soy proteins. DS1, protein solubility in sodium dodecyl sulfate (SDS) solution–protein solubility in buffer; DS2, protein solubility in urea solution–protein solubility in SDS solution; DS3, 100–protein solubility in urea solution. MSPC, modified soy protein concentrate; ALSPC, alcohol-leached soy protein concentrate; AWSPC, acid-washed soy protein concentrate; SPI, soy protein isolate.

TABLE 1
Protein Solubility in Phosphate Buffer, Sodium Dodecyl Sulfate (SDS), and Urea Solution[a]

	MSPC[b]	ALSPC[c]	AWSPC[d]	SPI[e]
Phosphate Buffer (pH 7.0)	67.5	14.3	23.2	23.8
2.5 mM SDS solution	68.1	40.5	55.0	60.5
6 M Urea solution	73.1	65.3	92.7	93.5

[a]Protein solubility is expressed as [protein in supernatant (g)/protein in sample (g)] × 100.
[b]Modified soy protein concentrate.
[c]Alcohol-leached soy protein concentrate.
[d]Acid-washed soy protein concentrate.
[e]Soy protein isolate.

The buffer-soluble substances of ALSPC and MSPC were fractionated by gel filtration, and three fractions (F1, F2, and F3) were obtained in which F1 eluted at void volume and F2 at a volume corresponding to that of soy protein molecules. Of the three fractions shown in Figure 2, only F1 and F2 give characteristic ultraviolet absorption of protein around 220 and 280 nm (Fig. 3). Therefore, F1, F2, and F3 may be identified as protein molecular aggregates, soy protein molecules, and a nonprotein fraction, respectively. The concentration of buffer-soluble protein of ALSPC is low and predominantly composed of the molecular fraction, while MSPC gives much larger peaks than ALSPC; especially protein aggregates (F1) make a major contribution to the buffer-soluble protein of MSPC. Gel filtration of urea solution-soluble substances of ALSPC and MSPC also gives three fractions with elution volumes close to those of F1, F2, and F3 in Figure 2 (Fig. 4). For ALSPC, the molecular fraction increased significantly in urea solution, but the change of the aggregate fraction was relatively small. It is important to observe that the F1 content of MSPC remains almost unchanged, even in 6 M urea, which suggests that disulfide bonds are involved in intraaggregate forces.

Heating soy protein solution often causes aggregating if the concentration is adequate, but it is energetically unfavorable for the dissolved protein molecules to form soluble aggregates at

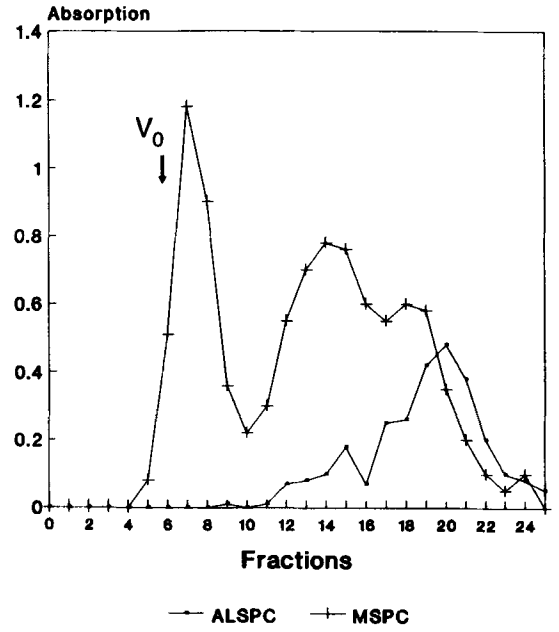

FIG. 2. Gel filtration of buffer-soluble substances from ALSPC and MSPC. A column of Sepharose 6B (1 × 80 cm; Pharmacia, Uppsala, Sweden) was eluted with the phosphate buffer (pH 7.6) in 3-mL fractions. V_0 indicates the position of void volume. See Figure 1 for abbreviations.

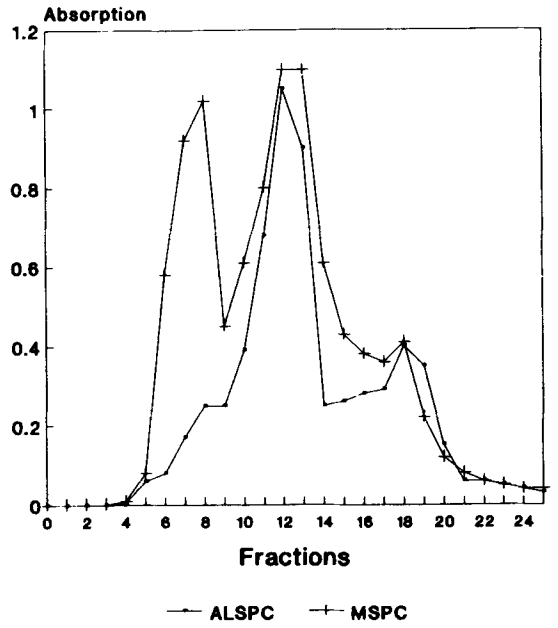

FIG. 4. Gel filtration of 6 M urea solution-soluble substances from ALSPC and MSPC. Conditions were the same as those used in Figure 2, except that the column was eluted with 0.5% sodium dodecyl sulfate solution. See Figures 1 and 2 for abbreviations.

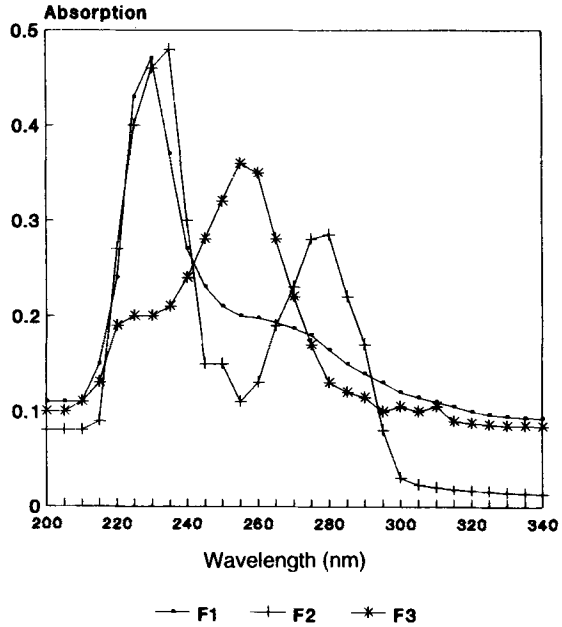

FIG. 3. Ultraviolet absorption curves of buffer-soluble substances fractionated on Sepharose 6B. F1, F2, and F3 were collected from gel filtration of buffer-soluble MSPC according to Figure 2. See Figure 1 for abbreviation and Figure 2 for company source.

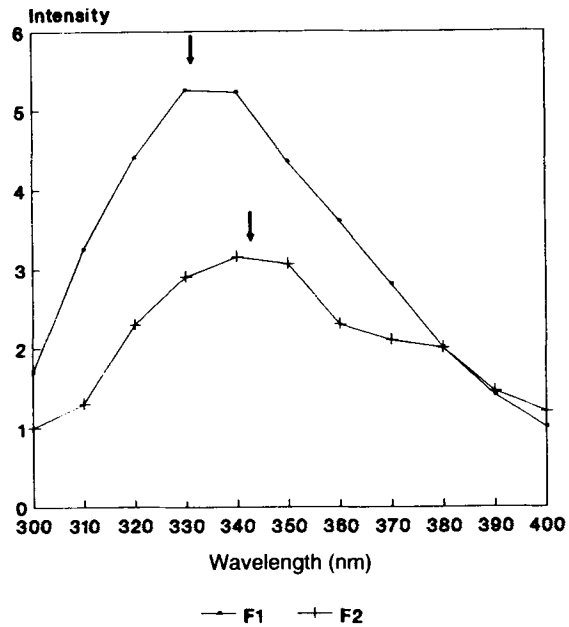

FIG. 5. Fluorescence spectrogram of two fractions from buffer-soluble protein of MSPC. F1 and F2 were collected according to Figure 2. The small arrows indicate the fluorescence peaks. See Figure 1 for abbreviation.

room temperature either in buffer or urea solution; the aggregate must be dissociated directly from the solid protein. The fact that MSPC protein dissolves in the form of aggregates implies that the aggregates are condensed as an entity, together with protein molecules or subunits, so that the finished MSPC has a heterogeneous texture, and the interaggregate forces are weak enough to be ruptured in buffer. On the other hand, ALSPC dissolves in the form of molecules, which indicates that ALSPC has a homogeneous texture in which protein molecules are associated with each other by noncovalent forces. So, we propose that the protein interactions smoothly distributed among molecules are concentrated into aggregates during physical modification of ALSPC.

The physicochemical properties of the aggregates were studied further. Figure 5 shows fluorescence spectroscopy of F1 and F2. The emitting wavelength for F1 is 334 nm, close to that of native soy protein (333 nm), suggesting that the environment around the hydrophobic aromatic groups of the aggregates is the same as that of native soy protein. F2 has an emitting wavelength of 340 nm, indicating a different structure of this fraction. The surface hydrophobicity of several samples respectively—two buffer-soluble protein fractions and soy protein with different treatments—show that the surface of aggregates has a stronger hydrophobic character: 512.58 (F1), 157.30 (F2), 76.88 (ALSPC), 162.83 (AWSPC), 222.00 (SPI). The results also may indicate that the formation of aggregates follows the increase in hydrophobic regions on the surface.

DISCUSSION

Physical modification increases the solubility of ALSPC by promoting the formation of soluble protein aggregates with minimum interaggregate interaction. The aggregating behavior of heat-denatured protein is changed appreciably, which is determined by physicochemical properties of the protein and the feasibility of environmental conditions (8). A protein is of the coagulating type if it forms insoluble coagulum by random aggregation; if the denatured protein forms a reversible gel, then the protein is of the gelating type. Soy protein belongs to the latter category and forms gels by a stepwise gelling procedure: protein molecule → soluble aggregate → macro-aggregate → gel (9), in which protein molecules form aggregates readily, but soluble aggregates form gels only by approaching each other in the right direction. Moreover, disulfide bonds make important contributions to the gelation process because soy proteins will stay at the soluble aggregate stage if soy protein is heated in the presence of NEM (an -SH blocking reagent) (10). Therefore, after the protein molecules are forced to dissolve by action of elevated temperature and high shear stress, they are heat-denatured and form soluble aggregates, but further conjugation of aggregates is prevented because the aggregates cannot approach each other correctly so that disulfide bonds will not form in a turbulent system induced by high-speed homogenizing. On the other hand, the relative positions of amino acid residue groups in aggregates are somewhat fixed, which facilitates the formation of disulfide bonds within aggregates; the -SH group content is thus reduced, and the possibility of forming disulfide bonds between aggregates is decreased.

Because protein molecules are most likely associated with each other by hydrophobic interaction at high temperature, we can expect that the soluble protein aggregates possess the structure of a hydrophobic core surrounded by a polar shell. This structure is confirmed by fluorescence spectroscopy (Fig. 5), in which the emitting wavelength of F1 is 334 nm, indicating that hydrophobic groups are not in contact with water, while that for F2 is 340 nm, an indicator that hydrophobic groups are in contact with water (11). The hydrophobicity of F1 is much larger than that of other proteins, suggesting that aggregates contribute certain functional properties to MSPC. This is inconsistent with the conclusion obtained from Figure 5, because the surface hydrophobicity will not be high if the nonpolar groups are located in the interior of aggregates. This inconsistency may be caused by the vague interaction pattern of ANS with protein molecules (12). According to Damodaran (13), the data obtained from fluorescence probing do not represent the hydrophobicity of the molecule surface but that of "nonpolar cavities" into which the fluorescence probes can enter while water molecules are not allowed to enter. Therefore, the hydrophobicity data, shown above for F1, imply that many hydrophobic "holes" or "cracks" exist on the polar shell of soluble aggregates.

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