Thermal Denaturation of Soybean Protein at Low Water Contents

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Differential scanning calorimetry (DSC) thermograms of soybean protein isolate developed two peaks corresponded to 11S and 7S globulin, the denaturation temperatures of which were 93.3 and 76.5°C, respectively, with 94% water. These peaks shifted to higher temperatures with lower water contents of the sample. At 47% water, there were two peaks, at 149 and 118.7°C, and at 11% water, there was one peak at 180°C. The DSC thermogram measured during cooling and reheating gave no peak. The soybean protein isolate was heated with 24.5% water at 100°C and then mixed with more water to the water contents of 94%. This sample gave two peaks at temperatures close to those of the original soybean protein, indicating that the soybean protein was not denatured at temperatures even above 100°C when the water content was low.

The heat-denaturation temperature, T_D , of proteins in solution is usually below 100°C, and the T_D value for protein is generally independent of the concentration of protein in solution. However, the water content of a protein sample affects both the T_D value and the enthalpy of the protein when the water content is extremely low.¹⁾ That is, protein becomes stable toward heat when the water content is lower than 20%. In some proteins, the T_D value under such conditions is about 120°C.^{1–4)}

Heat treatment of food protein at temperatures higher than 100°C and a low water content is used in processes such as roasting, frying, retort treatment, and the relatively new method of extrusion. Extrusion cooking brings about texturization and sterilization with little loss of the available nutrients and improves bacterial control.⁵⁾ Changes in food constituents, including protein, when the water contents is low, have not been studied extensively.

To produce textured vegetable protein with extrusion-cooking, soybean protein is mainly used as a material. During extrusion-cooking, the soybean protein is heat-denatured under conditions of low moisture and high temperatures.⁶⁾ Soybean protein seems to be molten in

the extruder in these conditions. However, the state of soybean protein, what kind of changes in molecules occur, and whether the soybean protein, in fact, is molten inside the extruder are not known.

The purpose of this study was to identify the dependency of the thermal denaturation of soybean protein on the water content of the protein sample. A preliminary study was reported elsewhere.⁷⁾

Materials and Methods

Preparation of soybean protein isolate and soybean 11S, 7S globulins. Soybean protein isolate was prepared from defatted soybean flour (Fuji Oil Co., Ltd., Osaka, Japan) as follows. To the defatted soybean flour was added 15 volumes of distilled water, and the mixture was stirred for 30 min at room temperature. After centrifugation at 25,000g and 25°C, the pH of the supernatant obtained was adjusted to 4.5 by the addition of 6N HCl. The suspension was left for 15 min at room temperature, and centrifuged again at 8,500g. The precipitate was collected, suspended in distilled water, and solubilized by neutralization to pH 7.5 by the addition of 1 or 6N NaOH (or both). The protein was precipitated again by adjustment of the pH to 4.5. The suspension was centrifuged as above and the precipitate was collected. This procedure was repeated twice to remove whey protein from the isolate fraction. Finally, the soy protein isolate was neutralized to pH 7.5 with NaOH and lyophilized.

Each soybean globulin, 11S and 7S, was prepared according to the method of Thanh and Shibasaki.⁸⁾ The 11S and 7S globulin obtained were precipitated at pH 4.5 by the addition of 6N HCl. The precipitate was dissolved by neutralization to pH 7.5 by the addition of 6N NaOH. This treatment was repeated three times. Finally, the pH of the 11S and 7S globulins was adjusted to 7.5 by the addition of 6N NaOH, and the globulins were lyophilized.

The soybean protein powders obtained were dissolved in distilled water to prepare the protein samples for analysis. The protein concentration was assayed by the method of Bradford⁹⁾ with use of the Coomassie protein assay reagent (Pierce Chemical Co., Rockford, Ill.). Bovine serum albumin was used as the standard.

Differential scanning calorimetry. The thermal denaturation of soy proteins under various conditions was studied by differential scanning calorimetry (DSC) with the model DSC 100 calorimeter. Silver pans (No. 560-003; Seiko Instruments) were heated before use at 170°C for 1 hr in a special autoclave chamber saturated with water vapor.

A typical experiment was done as follows. First, 50 μ l of soybean protein solution (60 mg/ml) was put in a silver pan, and the pan was sealed. A sealed pan that contained 50 μ l of distilled water was used as a reference. The T_D value was defined as the temperature of the peak top of the thermogram measured with a programmed rate of increase of 5°C/min from 25 to 200°C. The sampling time was 0.5 sec.

The thermogram of soybean protein under low water conditions was measured as follows. To the silver pan, 50 μ l of soybean protein solution (70 mg/ml) was added, and then the pan was put into a bottle for lyophilization. After lyophilization, distilled water was added to the dried soybean protein to give a certain water content and then the pan was sealed. The closed pan was left for 24 hr at room temperature to temper the protein with water. The pans were heated to 180 or 200°C and then cooled to 25°C in the DSC instrument at a programmed rate of 5°C/min in the both cases.

The DSC instrument was calibrated with indium. The data were stored in a diskette and analyzed with software designed for DSC analysis. The onset temperature of heat absorption, T_m , the peak or denaturation temperature, T_D , and the heat of transition or enthalpy of denaturation ΔH , were computed from the thermograms.

Heating of soybean protein at low water contents with a metal reactor. Soybean protein with various water contents was heated to 100, 150 or 180°C in a metal reactor as follows. First, to the lyophilized soybean protein powder was added a given volume of distilled water, and then the mixture was kneaded in a Teflon mortar with a pestle. This kneading step was done in a water-saturated atmosphere at room temperature. The water content of the protein powder was measured with a Karl Fischer moisture meter (Model MCI KF-05, Mitsubishi-Kasei, Co., Ltd., Tokyo).

The sample with a given water content was put into a 10-ml glass bottle (V-10A, Nichiden Rika Glass Co., Ltd., Kobe, Japan), which was sealed with an aluminum cap. These bottles were placed in a metal reactor, which was a thermoregulated pressure vessel (type TMD-D 300M, Taiatsu Scientific Glass Co., Ltd., Tokyo) made of stainless steel. The vessel could be heated from the outside by a heat jacket. The temperature in the 10-ml glass bottle was checked with a thermo-indicator (Thermolable, Nichiyu Giken Co., Ltd., Tokyo). A small amount of distilled water was put inside the pressure vessel. Samples were heated to 100, 150 or 180°C, and then cooled to room temperature, with a programmed heating and cooling rate of 5°C/min. To find if the heated sample was denatured, it was hydrated and analyzed by DSC. A given amount of the heated sample was put into a silver pan, and distilled water was added to a water content of 94% before the DSC.

Results

Heating of soybean protein isolate solution

Figure 1 shows typical thermograms of 60 mg/ml soybean protein isolate solution, heated to 180°C (1), cooled from 180 to 60°C (2), and reheated to 180°C (3) with the programmed heating and cooling rate of 5°C/min. The soybean protein isolate gave two peaks below 100°C (at 93.3 and 76.5°C) and one more peak at 141.4°C. During the cooling and the second heating of the sample, no peaks appeared. This

Fig. 1. DSC Thermograms of Soybean Protein Isolate in the Temperature Range of 60–180°C.

The protein concentration was 60 mg/ml. Heating until 180°C (1), cooling from 180 to 60°C (2) and reheating until 180°C (3). The rate of temperature changes was 5°C/min for both heating and cooling.

indicated that the three peaks observed arose because of irreversible endothermic changes in the protein molecules.

The major proteins of soybean protein isolate are 11S and 7S globulins, and the two

Fig. 2. DSC Thermograms of the Fractions Rich in the 11S Globulin (A) or 7S Globulin (B) of Soybean Protein.

Fig. 3. Irreversibility of the Endothermic Denaturation of Soybean Proteins.

Heating from 60 to 110°C (1), cooling from 120 to 60°C (2), heating from 60 to 180°C (3), cooling from 180 to 60°C (4), and reheating from 60 to 180°C (5). The rate of temperature changes was 5°C/min.

peaks below 100°C seemed to correspond to these proteins. To check this, purified 11S and 7S globulins were analysed by DSC. Figure 2 shows that the 11S globulin fraction prepared gave a major peak at 90.2°C and the 7S globulin rich fraction had a peak at 77.4°C with a shoulder at 90.1°C. The 7S globulin preparation was contaminated with a small amount of 11S globulin to judge from SDS electrophoresis; therefore, the shoulder at 90.1°C was from the contaminating 11S globulin. These results showed that the two peaks in Fig. 1 below 100°C were of the 11S and 7S globulins, which was consistent with earlier reports.¹⁰⁻¹²⁾

The peak at 141.4°C was not symmetrical, and it had a shoulder at the side of lower temperatures, but this result was reproducible. To find whether this peak was related to the peaks of denaturation of the 11S and 7S globulin below 100°C, the soybean protein isolate was first heated to 100°C to denature both globulins (Fig. 3-1), and then after cooling, the sample was heated again to 200°C (Fig. 3-2 and -3). Below 100°C, no peaks appeared, but a similar peak to that in Fig. 2-A at above

Fig. 4. DSC Thermograms for Samples Containing 3.5mg of Soybean Protein Isolate at Various Water Contents.

100°C was observed (at 138.1°C). When the sample was cooled and heated again (Fig. 3-4 and -5), no peak appeared. This endothermic peak in the vicinity of 140°C seemed to be due to other changes in the molecular structure of the 11S and 7S globulin of soybean protein rather than the denaturation occurred at below 100°C.

In the experiments described above, soybean proteins solubilized with water (94%) were analysed with DSC. It is of interest to know the results of DSC when the protein had a lower water content.

Effects of the water content on the denaturation temperature

Figure 4 shows thermograms of soybean protein isolate with various water contents. The two peaks that appeared at below 100°C in 94% water shifted to higher temperatures as the water content decreased. That is, the decrease in the water level led to the shape of the peak of 7S globulin changing with changes in the water content. At 10.95% water, which was the water content of the original powder after lyophilization, 11S globulin gave only one peak.

To assess the change in the peaks with decreases in the water content, the following properties of 11S and 7S globulin were calculated from thermograms of the soybean protein and plotted as a function of the water contents: T_D (Fig. 5), T_m (Fig. 6), ΔH (Fig. 7),

Fig. 5. Effects of the Water Content on the Denaturation Temperature of the 11S Globulin (●) and the 7S Globulin (○) of Soybean Protein.

and the half-width of the denaturation peak ($\Delta T_{1/2}$; Fig. 8).

As the water content was decreased, T_D and T_m gradually increased from the high water content region. This effect is different from the results described for other proteins.¹⁻⁴⁾ The inset in Fig. 6 shows that both the 11S and 7S globulins gave straight lines when the horizontal axis was the % water content.

Calorimetric enthalpy can be calculated from the DSC thermograms. Figure 7 showed that there was no dependency of enthalpy on the

Fig. 6. Effects of the Water Content on the Onset Temperature (T_m) of the Endothermic Peak of the 11S Globulin (●) and the 7S Globulin (○) of Soybean Protein.

Fig. 7. Effects of the Water Content on the Heat of Denaturation (ΔH) of the 11S Globulin (●) and 7S Globulin (○) of Soybean Protein.

Fig. 8. Effects of the Water Content on the Half-width of the Denaturation Peak, $\Delta T_{1/2}$, of the 11S Globulin (●) and the 7S Globulin (○) of Soybean Protein

water content for either globulin. Decreasing water content raised the T_D of soybean protein, but the enthalpy values of the peaks were not changed. This result is different from those obtained for other proteins, where the enthalpy for denaturation decreases with decreasing water content.¹⁻⁴⁾

The $\Delta T_{1/2}$ value reflects changes in the mechanism of denaturation and in the overall co-operativity of the denaturation process.¹³⁾ Figure 8 shows the change in $\Delta T_{1/2}$ of the 11S and 7S globulins as the water contents changed. The values for both proteins increased with the decrease in the water content, and the change in the 7S globulin was particularly great.

Irreversibility of endothermic peaks appearing at low water contents

The soybean protein isolate of 94% water gave two endothermic peaks, with no peaks appearing during cooling or reheating. It was possible that such irreversibility would be absent at low water contents. Figure 9 shows the thermograms of heating, cooling, and reheating of the soybean protein with 11% (w/w) water. No peaks appeared during the cooling and reheating. The irreversibility of the thermal denaturation of soybean protein was independent of the water contents.

Resistance to thermal denaturation of protein at low water contents

The T_D of soybean protein at low water

Fig. 9. DSC Thermograms of the Soybean Protein Isolate at 11% (w/w) Water Content with Heating from 60 to 200°C (1), Cooling from 200 to 60°C (2), Reheating from 60 to 200°C (3), and Recooling from 200 to 60°C (4).

The rate of temperature changes was 5°C/min.

content could be estimated from Figs. 5 and 6. The temperatures were both higher than 100°C. To check the estimates, the following experiment was done. A powder of soybean protein isolate containing 24.5% (w/w) water, was heated to 100, 150 and 180°C. After the powder was cooled, water was added to each heated sample to give a water content of 94%. From Figs. 5 and 6, it can be predicted that 11S globulin would not be denatured by being heated at 100 or 150°C, but that it would be denatured by being heated at 180°C. The 7S globulin would probably not be denatured by being heated at 100°C, and would be denatured at either 150 or 180°C. Therefore, it seemed that in the DSC thermograms, two peaks could be expected with the sample heated at 100°C, one peak with the sample heated at 150°C, and no peaks with the sample heated at 180°C.

The experimental results were consistent with these predicted results (Fig. 10). When the water content was 24.5%, heating at 100°C did not denature either globulin, heating at 150°C denatured only the 7S globulin, and heating at 180°C denatured both globulins. These results suggest that soybean protein with a low water content was not denatured at temperatures higher than 100°C. Even 150°C did not cause

Fig. 10. DSC Thermograms of the Soybean Protein Isolate at 94% (w/v) Water Content Previously heated at 100°C (1), 150°C (1), 150°C (2), or 180°C (3) with the Water Content of 24.5%.

denaturation.

Discussion

Heating converts a protein from its native state to a denatured state, accompanied by the disruption of intramolecular bonding and the unfolding and aggregation of protein molecules. The unfolding step is usually highly cooperative, and requires heat, which is seen as an endothermic peak in DSC thermograms. The DSC used here was useful in the analysis of the characteristics of thermal transitions in proteins.

DSC is not done at above 100°C as often as below 100°C, and the change in protein molecules at low water contents have not often been studied at high temperatures. One reason may be a technical problem. When a silver pan is used without treatment beforehand, an endothermic peak of unidentified origin appears at above 120°C, which interferes with observations of the peak from the protein. However, this peak was eliminated when the silver pan was first heated at 170°C for 1 hr in water-saturated conditions. The pan could then be used for thermal analysis of protein denaturation at high temperatures.

For tropocollagen,²⁾ β -lactoglobulin,³⁾ myoglobin,⁴⁾ and carboxypeptidase A,⁵⁾ T_m values

are almost constant when the water content is above about 35% (1.6 g water/g protein); T_m increases and ΔH decreases below 35% water. These findings mean that a similar change in the molecules took place in solution and in the range from 70% (2.3 g water/g protein) to 35% water. That is, in these proteins, the unfolding of the protein molecule is little affected by intermolecular contacts in a paste or solid-like state. However, for the soybean proteins 11S and 7S globulin, the T_m depended on the water content even in the region of a high water content. This is different from proteins that have been reported before. The unfolding of soybean protein was influenced by the water level and seemed to be affected by intermolecular contacts. For the proteins mentioned above, differentiation between the primary and the secondary hydration phases has been done.⁴⁾ However, with soybean protein, the water molecules from the boundary layer of the bulk phase seem to be continuously interacting with the protein molecule. It might be difficult to decide whether these water molecules are involved in the primary or the secondary hydration phase. Soybean protein can contain much water,¹³⁾ which means that soybean protein interacts with strongly bulk water. This might be reflected on the T_m profiles of 11S and 7S globulins, that is, the removal of water from soybean protein sample increased in the T_m values even in the region of a higher water content.

The $\Delta T_{1/2}$ of 11S globulin was not dependent on the water level, but that of 7S globulin increased with decreasing water contents. Thus, the peak of 7S globulin broadened as the water content decreased; at 11% (0.13 g water/g protein), the peak would disappear. However, ΔH changed little as the water content changed. An endothermic peak reflects the cooperative uptake of heat by unfolding of a protein. With a decrease in the water level, 7S globulin seemed to lose this cooperativeness and the protein conformation became less stable and unhomogeneous.

At 94% water (16 g water/g protein), and endothermic peak other than the peaks from

11S and 7S globulin was observed at 140°C in the DSC thermogram. This peak shifted to the region of higher temperatures with a decrease in the water level, where it was difficult to see. A similar endothermic peak was observed with ovalbumin in 93% water (unpublished). An endothermic peak at 140°C when the water content was 94% meant that conformational change in the protein different from that observed at below 100°C occurred (unpublished). Therefore, the structure and aggregation behavior of 11S globulin heated at 160°C with a 94% water level was probably quite different from those of the 11S globulin heated at the same temperature with a 50% water level. DSC of the protein at various water contents suggested that the effect of being heated at 160°C and 50% water (1 g water/g protein) corresponded to that of being heated at 100°C with 94% water. It is not known if the protein structure after heating under these different conditions were the same or not.

Denaturation of protein has been explained by the two-state transition model. Denaturation of lysozyme or ribonuclease is by this process, and these proteins undergo reversible denaturation when heating conditions are controlled.^{20,21)} Their denaturation is not accompanied by aggregation of proteins; monomeric denatured molecules exist. However, aggregation or coagulation (or both) of many kinds of protein occurs after heat denaturation.¹⁴⁾ At high protein concentrations, the chance of protein molecules colliding with each other increases, and aggregation may occur. In this study, the protein concentration was so high that such interaction must be common, and so the heat denaturation was irreversible. Treatment at high temperature causes deamidation, hydrolysis, and other reactions of protein molecule with water molecules. These also lose the reversibility of heat denaturation.¹⁵⁾

Resistance of soybean protein to heat-denaturation at low water content is surprising in comparison with results obtained for other water-soluble proteins.¹⁶⁻¹⁹⁾ The physiological meaning of this phenomenon of

resistance is not known. We speculated as follows. After maturation in the early summer, it is advantageous for soybeans for survive dryness and heat until the autumn. Thermostability of soybean protein is therefore essential to seed viability. To end dormancy, certain moisture and temperature conditions are needed.²⁰⁾ Most soluble proteins in animal and bacterial cells do not need thermostability, because they exist in an aqueous environment. Natural selection may have operated in the evolution of thermostable soybean protein. If so, other storage proteins of beans and seeds are probably also stable.

Soybean protein, a popular edible protein, has been widely used traditionally as a protein resource, and also recently used in extrusion cooking. Textured soybean protein gives a meat-like texture after it is rehydrated. To produce such textured vegetable protein, soybean protein containing little water (15-40%) is cooked at a high temperature for a short time in an extruder. The heating temperature is about 140 to 150°C. This temperature was found empirically to be suitable for the texturization of soybean protein, and it has generally been believed that the material was molten in the extruder during cooking. However, the melting temperature of this protein has not been reported and even the concept of melting is rather vague.¹⁶⁾ The extrusion of the melting soybean protein makes it possible to form textured protein after cooling. The result reported here showed that the temperature needed for texturization in the extruder was that for the denaturation of soybean protein, which temperature depended on the water content. After denaturation of the protein, its viscosity decreased and the fluidity of the soybean protein paste might give rise to a certain order under some shearing conditions, which would result in texturized tissue after cooling.

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