Interactions among Protein Molecules in Freeze-Gel of Soymilk and Protein Structures in Heated Soymilk during Cooling

Makoto Shimoyamada,* Kayoko Tômatsu, Satomi Oku, and Kenji Watanabe

Laboratory of Food Material Engineering, Faculty of Agriculture, Gifu University, 1-1 Yanagido, Gifu 501-1193, Japan

To estimate the interactions forming in soymilk freeze-gel, lyophilized gel was extracted successively with various solvents. A mixture of urea, sodium dodecyl sulfate (SDS), and 2-mercaptoethanol (2-ME) dissolved the proteins effectively. The thiol−disulfide exchange reactions and hydrophobic interactions were shown to have a complex relation with a three-dimensional network. The addition of SDS or 2-ME resulted in an incomplete gel or no precipitation of soymilk. In our previous paper (Shimoyamada et al. *Food Sci. Technol. Res.* **1999**, *5*, 284−288), the significance of precooling to form small, homogeneously distributed ice crystals in soymilk was reported. In this study, precooling was shown to maintain the partially denatured structures of soybean proteins in soymilk that had unfolded due to heat treatment. These phenomena were considered to be other important functions of precooling in freeze-gelation.

Keywords: *Soymilk; heating and cooling; freeze-gel; surface SH content; hydrophobicity*

INTRODUCTION

Soybeans have been widely utilized as a foodstuff as well as a source of oil. They contain high levels of protein and oil, which have excellent qualities in terms of nutrition and functional properties. Soymilk, which is a kind of emulsion consisting of soybean proteins and lipids, is used not only as a beverage but also as the main ingredient in tofu (soybean curd) or yuba (sheets of dried soymilk skin) (Wu and Bates, 1972).

In our previous paper (Shimoyamada et al., 1999a), we reported that soymilk could be converted to a gel-like coagulate through successive processes of heating, precooling, and freezing. Soymilk freeze-gel is similar to soft tofu or yogurt. This freeze-gel is processed without the addition of coagulants such as calcium sulfate, magnesium chloride, glucono-δ-lactone, etc., thus differing from traditional tofu gel (Wang and Cavins, 1989; Sung and Breene, 1991; Kohyama et al., 1992; Evans et al., 1997). This process also differs from freeze coagulation (Watanabe et al., 1963; Hashizume et al., 1971; Hashizume, 1979), cold-gel (Soeda, 1994), and heat-gel (Kinsella, 1979; Mori et al., 1981; Utsumi et al., 1984), all processes in which gels are made from protein solution or paste containing no lipids.

Interactions among protein molecules in these aggregation processes have been widely investigated. Disulfide, hydrophobic, and electrostatic interactions are considered important for heat-gelation (German et al., 1982; Utsumi et al., 1984). In the freeze-insolubilization of soybean protein solutions, disulfide bond formation and exchange were reported to be important intermolecular interactions (Hashizume et al., 1971), while for cold gel formation, the hydrophobic interactions were more important (Soeda, 1994). We therefore decided to study the interactions among protein molecules during freeze-gelation, in which the precooling step is considered to be very important for uniform texture of the freeze-gel (Shimoyamada et al., 1999a,b). The precooling step allows the formation of spherical ice crystals, which were found to be distributed uniformly in frozen soymilk during freeze-storage (Shimoyamada et al., 1999b). We also attempted in the present study to clarify its significance in the protein structures of soymilk. For this, the free thiol content and the surface hydrophobicity of protein were estimated during precooling. The effect of precooling on the structure of heated soybean protein during cooling is discussed herein.

* To whom correspondence should be addressed (fax +81-58-293-2928; e-mail saponin@cc.gifu-u.ac.jp).

MATERIALS AND METHODS

Preparation of Soymilk. Soymilk was manually prepared as described in our previous paper (Shimoyamada et al., 1999a).

Processing of Soymilk. Raw soymilk preparations were heated in an autoclave at 110 °C for 3 min. The heated soymilk was put into individual plastic tubes (30 mL each) and stored in a temperature-controlled refrigerator. The soymilk samples were first cooled at −5 °C for 2 h and then frozen at −20 °C for 14 days as described earlier (Shimoyamada et al., 1999a). Freeze-stored soymilk was thawed in warm water at 30 °C.

Preparation of Acid-Precipitated Protein Fraction from Soybean Seeds. Soybean acid-precipitated protein fraction (APP) was prepared as described in our previous paper (Shimoyamada et al., 1998). Soybean seeds were milled and extracted with *n*-hexane. Defatted soybean seed flour was mixed with 20 vol of 0.03 M Tris-HCl buffer (pH 8) and stirred for 2 h at room temperature. The supernatant was collected by centrifugation (9000*g*, 30 min) and adjusted to pH 4.5. The resulting precipitate was dispersed in water, dialyzed, and lyophilized to afford APP.

Extraction of Lyophilized Soymilk Sample with Various Solvents. Soymilk, which had been heated, precooled, and then frozen for 2 weeks, was lyophilized without going through a thawing process. The lyophilized sample was milled and extracted with 10 vol of 0.1 M phosphate buffer (pH 7.6), 1.5 M urea in the same buffer, 0.1 M SDS in the buffer, and 1.5 M urea, 0.1 M SDS, and 0.01 M 2-ME in the buffer, successively. Each extract was subjected to SDS−PAGE for analysis of the protein constituents.

10.1021/jf0001132 CCC: $19.00
Published on Web 06/29/2000

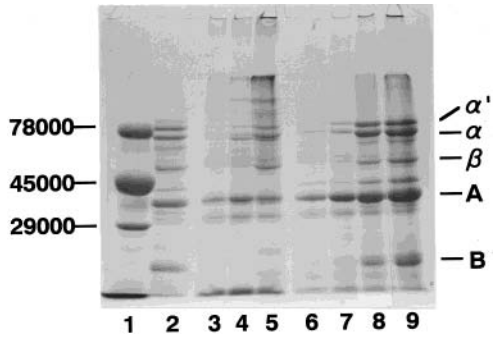

Figure 1. SDS−PAGE patterns of extracts from soymilk freeze-gel with various solvents. Each sample was extracted from lyophilized soymilk freeze-gel in which soymilk was heated, pre-cooled at −5 °C, and frozen for 2 weeks at −20 °C. Lane 1, Molecular weight markers; lane 2, APP; Lanes 3 and 6, 0.1 M phosphate buffer; lanes 4 and 7, phosphate buffer with 1.5 M urea; lanes 5 and 8, with 0.1 M SDS; lane 9, with 1.5 M urea, 0.1 M SDS, and 0.01 M 2-ME. Lanes 3, 4, and 5, without 2-ME; lanes 1, 2, 6, 7, 8, and 9, with 2-ME. α′, α′-subunit of β-conglycinin; α, α-subunit of β-conglycinin; β, β-subunit of β-conglycinin; A, acidic polypeptides of glycinin; and B, basic polypeptides of glycinin.

Sodium Dodecyl Sulfate−Polyacrylamide Gel Electrophoresis (SDS−PAGE). SDS−PAGE of proteins in soymilk was carried out by the method of Laemmli (1970), using 12.5% acrylamide gels. Electrophoresis gels were stained by Coomassie brilliant blue R-250.

Estimation of Surface Free Thiol Content in APP Solution and Soymilk. Free thiol content in APP solution was estimated by the method of Ellman (1959), using 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB). A sample solution was diluted with 0.1 M phosphate buffer (pH 7.6). The aliquot (5 mL) was mixed with 0.01 M DTNB solution (0.03 mL) and incubated for 20 min at room temperature. Absorbance of the resulting solution was measured at 412 nm.

The free thiol residue in soymilk was estimated using 2,2′-dithiobis(5-nitropyridine) (DTNP) instead of DTNB (Grasseti and Murray, 1969; Obata et al., 1989). Soymilk was similarly diluted with the phosphate buffer. An aliquot (2 mL) was mixed with 5×10^{-4} M DTNP in ethanol (0.5 mL) and incubated for 20 min at room temperature. The resulting solution was mixed with 10% perchloric acid solution (2.5 mL) and centrifuged for 10 min at 3000 rpm. The supernatant was filtered, and its absorbance was measured at 386 nm. Triplicate measurements were carried out for each sample.

Estimation of the Surface Hydrophobicity of Proteins in the Soybean Protein Solution and Soymilk. The surface hydrophobicity of the proteins was measured using 1-(anilino)naphthalene-8-sulfonate (ANS) (Hayakawa and Nakai, 1985). A properly diluted sample (0.1 mL) was mixed with 0.01 M phosphate buffer (pH 7.0, 4 mL) and 8×10^{-3} M ANS solution (0.02 mL). The mixture was excited at 390 nm, and the relative fluorescence intensity was measured at 470 nm in a fluorescence spectrophotometer (F-2000, Hitachi, Ltd., Japan). Triplicate measurements were carried out for each sample.

RESULTS AND DISCUSSION

Successive Extraction of Lyophilized Soymilk Freeze-Gel with Various Solvents. Soymilk freeze-gel was formed during freeze-storage, where the three-dimensional gel matrix consisted mainly of soybean protein molecules. To analyze the interactions among proteins, extracts with various solvents were subjected to SDS−PAGE (Figure 1). Only a fraction of the glycinin acidic polypeptides could be extracted from freeze-gel with phosphate buffer (lanes 3 and 6) and 1.5 M urea in the same buffer (lanes 4 and 7). Each subunit from soybean proteins, except glycinin basic polypeptides, could be extracted to a certain extent with 0.1 M SDS in the buffer (lane 5). In this sample, high molecular weight aggregates which could not enter the gel were also detected. With the addition of 2-ME, this fraction showed a decrease in aggregates and an increase in soybean protein subunits, especially glycinin basic polypeptides (lane 8). From the above data, it is thought that the aggregates detected in heat-frozen soymilk were formed by disulfide interactions and became insoluble due to hydrophobic interactions between aggregates. By using a buffer containing urea, SDS, and 2-ME, larger amounts of protein could be extracted (lane 9), and a small amount of precipitate remained. Even in this case, however, there was some aggregate detected at the top of the gel. Proteins in soymilk are thought to interact through disulfide bonds and hydrophobic interactions to form an insoluble network comparable to those in previous reports (Hashizume, 1979; Soeda, 1994). These aggregates were soluble in neither SDS-containing buffer nor 2-ME-containing buffer.

Effect of Sodium Sulfite and Sodium Dodecyl Sulfate (SDS) on Formation of Freeze-Gel of Soymilk. The above results revealed complex hydrophobic and disulfide interactions. To estimate the effect of the reduction of disulfide bonds, soymilk samples containing sodium sulfite were heated, successively cooled at −5 °C, and frozen at −20 °C. After 2 weeks, the frozen samples were thawed in water at 30 °C (Figure 2, panels B and C). In comparison with the control sample (Figure 2A), the addition of 0.1% sodium sulfite partially suppressed gelation and led to a heterogeneous precipitation (Figure 2B). Increased sodium sulfite (0.5%) completely inhibited the coagulation of soymilk (Figure 2C). Hashizume (1979) reported that the formation of intermolecular disulfide bonds was important for the insolubilization of soybean protein during freeze-storage.

Hydrophobic interactions were also considered to be important among protein molecules. The addition of SDS led to a partially collapsed surface on the freeze-gel at 0.1% (Figure 2D) and to heterogeneous precipitation at 0.5% (Figure 2E). Apparently, a greater concentration of SDS than sodium sulfite was needed to inhibit the formation of freeze-gel. From the above results overall, it is considered that glycinin basic polypeptides, which were dissociated from glycinin subunits during the heating step, were associated with other basic polypeptides or other proteins through intermolecular disulfide bonds. The resulting aggregates then interacted with other aggregates by disulfide and/or hydrophobic interactions to form insoluble coagulates.

Changes of Free Surface Thiol Content of Soybean Protein During Heating and Cooling Processes. The above results showed that disulfide and hydrophobic interactions are important in forming the gel texture. The freeze-gelation was also shown to require the precooling step in which heated soymilk was cooled at −5 °C for 2 h before freeze-storage. In our previous paper (Shimoyamada et al., 1999a), uniform fine ice crystals were formed when soymilk was frozen after precooling by supercooling, and this was considered to be the significant aspect of precooling. However, heated soymilk kept at room temperature and then slowly cooled formed the freeze-gel with difficulty even if it was precooled at −5 °C (data not shown). The effect

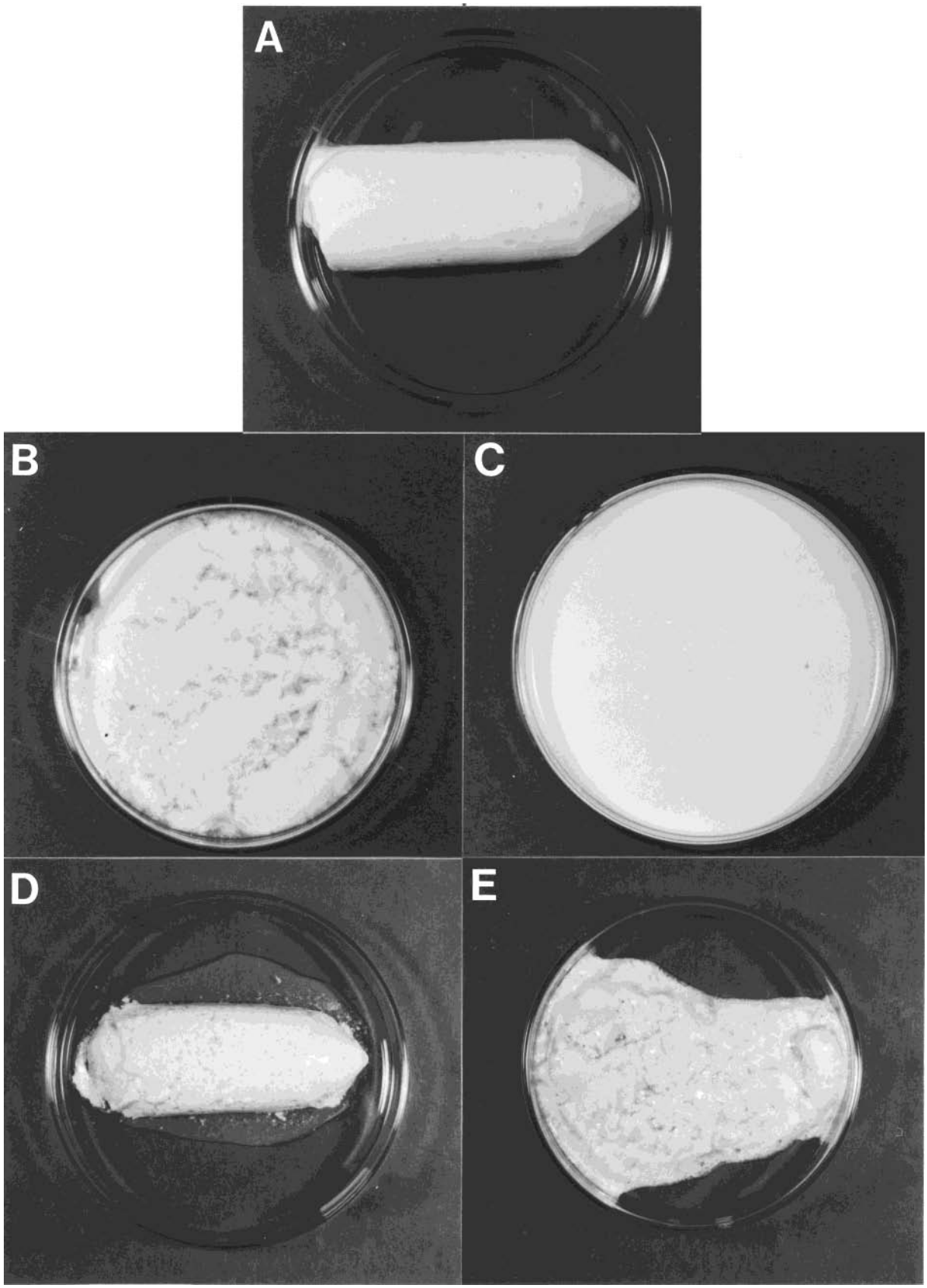

Figure 2. Effects of sodium sulfite and SDS on freeze-gelation of soymilk. (A) control (soymilk with no additives); (B) addition of 0.1% sodium sulfite; (C) 0.5% sodium sulfite; (D) 0.1% SDS; and (E) 0.5% SDS.

of precooling on the disulfide bonds among proteins in soymilk was therefore investigated.

First, for simplification, 5% APP solutions were used instead of soymilk. The APP solutions were heated and cooled at various temperatures. The cooled samples were diluted and mixed with DTNB solution to estimate surface thiol content. Relative thiol contents in which the value measured immediately after heating was 1

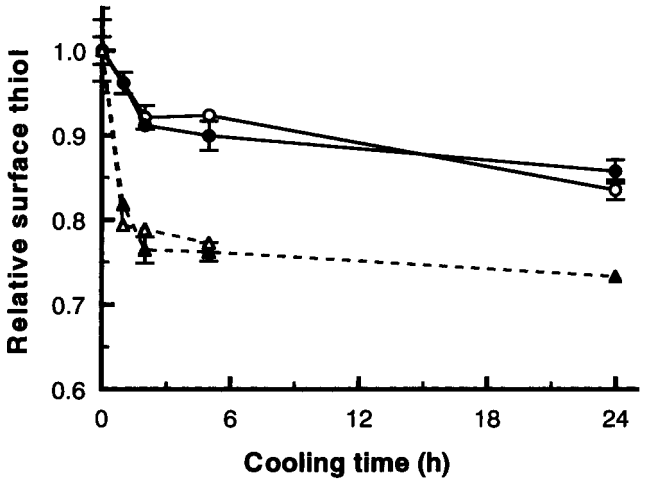

Figure 3. Surface thiol content of soybean APP solution after heating and cooling. Closed circles, heated soymilk sample was cooled at −5 °C; open circles, −2 °C; closed triangles, 4 °C; open triangles, room temperature.

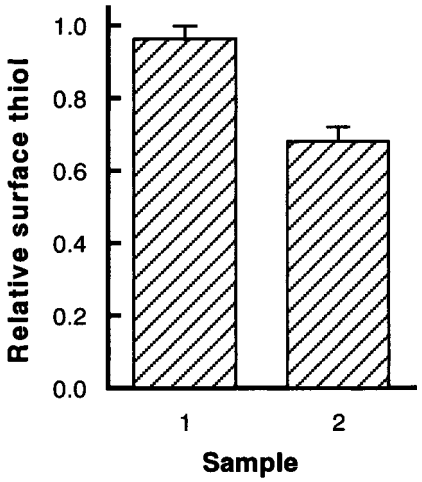

Figure 4. Surface thiol content of soymilk after heating and cooling. (1) Heated soymilk sample was cooled at −5 °C for 2 h; (2) heated soymilk sample was cooled at room temperature for 2 h.

are plotted in Figure 3. The surface thiol content in each sample decreased for the first 2 h, then remained nearly constant. However, the samples cooled at −2 or −5 °C showed significantly higher content than the sample cooled at 4 °C or room temperature. These results appear to indicate that cooling below about 0 °C inhibited the disulfide re-formation.

The surface thiol contents of soymilk were then measured. Soymilk, which was heated and cooled at each temperature, was diluted with phosphate buffer and mixed with DTNP solution (Obata et al., 1989). The results (Figure 4) coincide with the data on APP solution. The surface thiol content of the sample cooled at −5 °C was significantly higher than that of the sample cooled at room temperature and almost equal to the value measured just after heating. The precooling step inhibited disulfide re-formation during cooling, where free sulfhydryl residues were formed by heating, and allowed the protein molecules to become more reactive in disulfide interactions.

Changes in Surface Hydrophobicity of Soybean Protein during Heating and Cooling Processes. Following the test of free thiol residues, the hydrophobicity of the proteins was estimated. APP solution was heated and cooled at room temperature or at −5 °C. The fluorescence of ANS reagent was increased by heating the APP solution (Figure 5). The fluorescence intensity was a little but significantly higher in the sample cooled at −5 °C than that cooled at room temperature. The hydrophobicity of protein, which had increased due to heating, decreased during cooling. However, the cooling at −5 °C suppressed this decrease. The cooling at −5 °C led to more active hydrophobic interactions among proteins. This result supports the findings for free thiol contents. From the above data, it is thought that the renaturation of protein molecules that had been denatured by heating was suppressed by cooling at −5 °C.

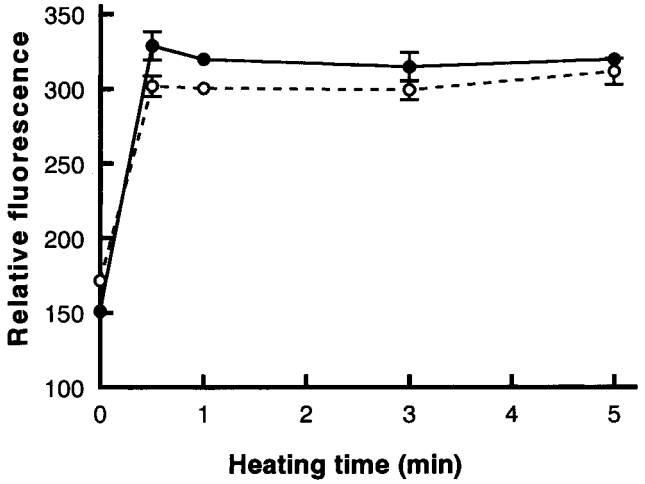

Figure 5. Surface hydrophobicity of soybean APP solution after heating and cooling. Closed circles, cooled at −5 °C for 2 h; open circles, cooled at room temperature for 2 h.

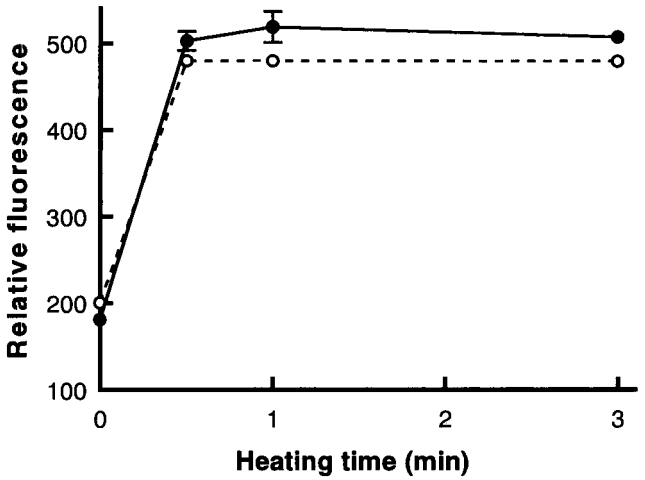

Figure 6. Surface hydrophobicity of soymilk after heating and cooling. Closed circles, cooled at −5 °C for 2 h; open circles, cooled at room temperature for 2 h.

On the basis of the results with APP solution, the surface hydrophobicity of soybean protein was measured in soymilk (Figure 6). There is higher fluorescence intensity in the sample cooled at −5 °C. Because of the lipids and other constituents, the measurements of hydrophobicity in soymilk may contain some inaccuracies. Even given these restrictions, the data coincided well with the result from surface hydrophobicity in APP solution. The surface hydrophobicity, which had increased with the heat treatment, might have been maintained at a relatively higher level by rapid cooling to the supercooling state.

The step of precooling heated soymilk at −5 °C suppressed the decrease in surface thiol content and surface hydrophobicity of soybean proteins, which had increased during the heating step. This result might also show that precooling suppressed the refolding of the protein molecules, which had been partially destroyed or unfolded by heat treatment. High levels of surface thiol and surface hydrophobic areas were required to form three-dimensional networks during freeze-storage. In our previous paper (Shimoyamada et al., 1999b), the significance of the precooling step was reported to be the formation of uniform, fine ice crystals. The high level retention of surface thiol and hydrophobicity is considered to be another significant aspect of precooling for freeze-gelation. Other interactions such as hydrogen bonds and electrostatic interactions should be considered in the future to characterize the gelation mechanism of soymilk freeze-gel.

ABBREVIATIONS USED

APP, acid-precipitated protein fraction; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); DTNP, 2,2′-dithiobis(5-nitropyridine); ANS, 1-(anilino)-naphthalene-8-sulfonate.

ACKNOWLEDGMENT

We thank Dr. T. Hara and Dr. H. Koyama for use of a fluorescence spectrophotometer.

LITERATURE CITED

Ellman, G. L. Tissue sulfhydryl groups. *Arch. Biochem. Biophys.* **1959**, *82*, 70−77.

Evans, D. E.; Tsukamoto, C.; Nielsen, N. C. A small scale method for the production of soymilk and silken tofu. *Crop Sci.* **1997**, *37*, 1463−1471.

German, B.; Damodaran, S.; Kinsella, J. E. Thermal dissociation and association behavior of soy proteins. *J. Agric. Food Chem.* **1982**, *30*, 807−811.

Grassetti, D. R.; Murray, J. F., Jr. The use of 2,2′-dithiobis-(5-nitropyridine) as a selective reagent for the detection of thiols. *J. Chromatogr.* **1969**, *41*, 121−123.

Hashizume, K. Studies on production of new textured protein by freeze denaturation of soybean protein. *Nippon Shokuhin Kogyo Gakkaishi* **1979**, *26*, 450−459 (in Japanese).

Hayakawa, S.; Nakai, S. Relationships of hydrophobicity and net charge to the solubility of milk and soy proteins. *J. Food Sci.* **1985**, *50*, 486−491.

Hashizume, K.; Kakiuchi, K.; Koyama, E.; Watanabe, T. Denaturation of soybean protein by freezing. Part I. *Agric. Biol. Chem.* **1971**, *35*, 449−459.

Kinsella, J. E. Functional properties of soy proteins. *J. Am. Oil Chem. Soc.* **1979**, *56*, 242−258.

Kohyama, K.; Yoshida, M.; Nishinari, K. Rheological study on gelation of soybean 11S protein by glucono-δ-lactone. *J. Agric. Food Chem.* **1992**, *40*, 740−744.

Laemmli, U. K. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **1970**, *227*, 680−685.

Mori, T.; Nakamura, T.; Utsumi, S. Gelation mechanism of soybean 11S globulin: Formation of soluble aggregates as transient intermediates. *J. Food Sci.* **1981**, *47*, 26−30.

Obata, A.; Matsuura, M.; Fukushima, D. Spectrophotometric determination of sulfhydryl groups in soymilk using 2,2′-dithiobis-(5-nitropyridine). *Nippon Shokuhin Kogyo Gakkaishi* **1989**, *36*, 707−711 (in Japanese).

Shimoyamada, M.; Ikedo, S.; Ootsubo, R.; Watanabe, K. Effects of soybean saponins on chymotryptic hydrolyses of soybean proteins. *J. Agric. Food Chem.* **1998**, *46*, 4793−4797.

Shimoyamada, M.; Tômatsu, K.; Watanabe, K. Insolubilisation and gelation of heat-frozen soymilk. *J. Sci. Food Agric.* **1999a**, *79*, 253−256.

Shimoyamada, M.; Tômatsu, K.; Watanabe, K. Effect of precooling step on formation of soymilk freeze-gel. *Food Sci. Technol. Res.* **1999b**, *5*, 284−288.

Soeda, T. Gelation of soy protein isolate during cold storage. *Nippon Shokuhin Kogyo Gakkaishi* **1994**, *41*, 670−675 (in Japanese).

Sung, N.; Breene, W. M. Calcium sulfate concentration influence on yield and quality of tofu from five soybean varieties. *J. Food Sci.* **1991**, *56*, 1604−1607, 1610.

Utsumi, S.; Damodaran, S.; Kinsella, J. E. Heat-induced interactions between soybean proteins: Preferential association of 11S basic subunits and β-subunits of 7S. *J. Agric. Food Chem.* **1984**, *32*, 1406−1412.

Wu, L. C.; Bates, R. P. Soy protein-lipid films. 1. Studies on the film formation phenomenon. *J. Food Sci.* **1972**, *37*, 36−39.

Wang, H. L.; Cavins, J. F. Yield and amino acid composition of fractions obtained during tofu production. *Cereal Chem.* **1989**, *66*, 359−361.

Watanabe, T.; Nakayama, O.; Iwasaki, N. Studies on the changes of the properties of soybean protein by freezing. Part I. Changes in water extract of soybean. *Nippon Shokuhin Kogyo Gakkaishi* **1963**, *10*, 163−166 (in Japanese).

Received for review January 26, 2000. Revised manuscript received April 24, 2000. Accepted April 25, 2000.

JF0001132