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Effect of Heat Treatment on Dispersion Stability of Soymilk and Heat Denaturation of Soymilk Protein

Makoto Shimoyamada[1*], Natsumi Tsushima[2], Kimiko Tsuzuki[3], Hiroaki Asao[3] and Ryo Yamauchi[2]

[1] *School of Food, Agricultural and Environmental Sciences, Miyagi University, 2-2-1 Hatatate, Taihaku, Sendai, Miyagi 982-0215, Japan*

[2] *Gifu University, 1-1 Yanagido, Gifu 501-1193, Japan*

[3] *Marusan ai Co., Ltd., 1, Arashita, Niki-cho, Okazaki, Aichi 444-2193, Japan*

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Soymilk was prepared by non-heated squeezing and then heated at various temperatures. For one-step heating, the precipitate produced by heating soymilk increased for heating at 70 and 80ºC and was much less at 90ºC or higher temperatures, showing that the dispersion stability of soymilk was dependent on the heating temperature. In the case of two-step heating (combinations of 115ºC and a lower temperature), soymilk heated at 115ºC in the first step and 70 or 80ºC in the second step resulted in increased precipitation. Changes in protein surface hydrophobicity were considered to be related to the precipitate formation of soymilk heated at the two different temperatures, indicating the significance of heat denaturation and aggregate formation of proteins on the dispersion stability of soymilk.

Keywords: soymilk, heating, heat denaturation, precipitation

Introduction

Soybeans are widely utilized as food materials, as well as feedstuffs. In particular, soymilk, the emulsion and dispersion consisting mainly of soybean proteins and lipids, is popular not only as a beverage, but also as the main ingredient in tofu (soybean curd) or yuba (sheets of dried soymilk skin). Recently, some soymilk gelation processes not using coagulants have been reported, and further possibilities have been shown for new food materials (Shimoyamada *et al.*, 1999a, 1999b, 2000, 2002; Kawase *et al.*, 2000; Kamata *et al.*, 2004). Soybeans contain high levels of proteins and oils, which have excellent nutritional and functional properties. Many kinds of physiological functions have also been reported for soybean (Lichtenstein, 1998; Messina, 1999). In Japan, consumption of soymilk is gradually increasing because of its healthy image.

An aseptic filling system has been used to prolong the shelf life of soymilk sanitarily; however, soymilk stored long-term occasionally shows some precipitation and this decreases the value of the product. Generally, heat treatment causes protein to denature, resulting in precipitation or gelation.

In this study, we investigated the effect of heat treatment on the precipitation of refrigerated soymilk and discussed the relationship between the dispersion stability of soymilk emulsion and heat denaturation of soymilk protein, namely surface sulfhydryl (SH) content and surface hydrophobicity. Further, considering that soymilk is heated using complicated temperature and time conditions in plant processing, we evaluated the effects of two-step heating as a first-model system, as well as one-step heating on the dispersion stability of heated and soymilk, by measuring precipitation of refrigerated soymilk and the surface SH content and the surface hydrophobicity of soymilk protein heated at two different temperatures.

Materials and Methods

Preparation of soymilk Dehulled soybean seeds (25 g), provided by Marusan ai Co., were soaked in water overnight at 4ºC. The soaked seeds, which imbibed about 30 ml of water, were then milled at 50–60ºC for 5 min with 170 ml hot water in a blender (10,000 rpm, Type R, Teraoka Co., Osaka, Japan) and filtrated by 5-ply gauze to provide raw soymilk. Namely, soymilk was prepared using one part dry soybean and 8 parts water.

*To whom correspondence should be addressed.
E-mail: shimoyam@myu.ac.jp

Heat treatment Raw soymilk samples were heated at 70, 80, and 90ºC in a water bath, and at 100ºC in boiling water. Actual temperatures of the samples were 70, 80, 88 and 98ºC, respectively. A pressure cooker was also used for heating at 115ºC, where the sample temperature was not measured. Heated soymilk was rapidly cooled in an ice bath. For rapid short-time heating, soymilk samples were aliquotted into polymerase chain reaction (PCR) tubes and heated in a thermal cycler (PCR PE2400, Perkin Elmer, Inc., USA) followed by rapid cooling to 4ºC after heat treatment.

Determination of precipitation The soymilk samples heated for 15 min at various temperatures were put into screw-capped test tubes and stored at 4ºC for one week. The stored samples were centrifuged at 1,500 × *g* for 30 min, and the supernatant was removed by placing the test tubes upside down for half a minute. The resulting precipitate was weighed to calculate precipitation as a fresh weight percentage. Measurements were made in triplicate.

Determination of protein Protein concentration was measured according to Lowry *et al.* (1951).

Sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) SDS-PAGE was performed using 12.5% gels according to the method of Laemmli (1970). The gels were stained with Coomassie Brilliant Blue R-250.

Protein surface SH content and surface hydrophobicity Protein surface SH content was estimated by using 2,2'-dithiobis-(5-nitropyridine) (DTNP) (Grassetti and Muray, 1969; Obata *et al.*, 1989). Thus, 2 ml samples, diluted with 0.1 M phosphate buffer (pH 7.6), were mixed with 0.5 ml of 5.0×10^{-4} M DTNP ethanol solution and incubated at 25ºC for 20 min. Then, 2.5 ml of 10% perchloric acid solution was added and samples were centrifuged at 1,500 × *g* for 10 min to remove protein. The resulting supernatant was passed through a 0.45-μm membrane filter, and the filtrate was measured at 386 nm.

Surface hydrophobicity of soymilk protein was measured using 8-anilino-1-naphtalene sulfonic acid (ANS) (Hayakawa and Nakai, 1985). Subsamples (100 μl) were diluted with 0.01 M phosphate buffer (pH 7.0) and mixed with 20 μl of 8×10^{-3} M ANS solution and 4 ml of the same buffer. The resulting mixtures were subjected to fluorescence spectrometry (F-2000 Spectrophotometer, Hitachi Co., Tokyo, Japan) and fluorescence was measured (excitation, 390 nm; emission, 470 nm). Measurements were made at least in duplicate and repeated twice, showing reproducible results.

Results and Discussion

Precipitation from heated soymilk during refrigerated storage and denaturation behavior of soymilk protein Protein is generally known to undergo changes in solubility or dispersion stability by heat denaturation. However, no data on the dispersion stability of raw and heated soymilk samples were available, so we prepared non-heated or raw soymilk and compared the precipitation of soymilk heated at various temperatures as an index of dispersion stability. For this purpose, raw soymilk was prepared by grinding and squeezing soaked soybean seeds at about 60ºC in order to increase the extraction rate of protein (Okubo, 1987) and to minimize heat denaturation of the main proteins in soybean (Guo *et al.*, 1997).

The raw soymilk obtained was therefore heated at various temperatures, rapidly cooled in an ice bath and then refrigerated for one week. As a result (Fig. 1), heat treatment at 70 and 80ºC increased precipitation of soymilk (raw soymilk, 4.6% and heated, 5.3% as fresh weight of precipitation to whole soymilk). However, the precipitation of soymilk heated at 90, 100 and 115ºC was lower than the raw soymilk (3.9 to 3.3%). These data clearly showed that heat treatment of soymilk at temperatures higher than 90ºC increased the dispersion stability of soymilk protein or soymilk emulsion consisting of proteins and lipids. For soybean protein dispersion (Hermansson, 1978) and soymilk (Zhang *et al.*, 2004), β-conglycinin and glycinin were reported to be thermally denatured at approximately 70 and 90ºC, respectively, by differential scanning calorimetry (DSC). From these reports, heat treatment of soymilk at 70 or 80ºC was thought to denature only β-conglycinin, but not glycinin. The precipitation results show that the coexistence of native glycinin and heat-denatured β-conglycinin decreased the dispersion stability of soymilk but heat denaturation of both proteins improved the stability. In the present study, the amount of precipitation appears to be larger than that of commercial soymilks. It is thought that because the soymilk used in this study was fil-

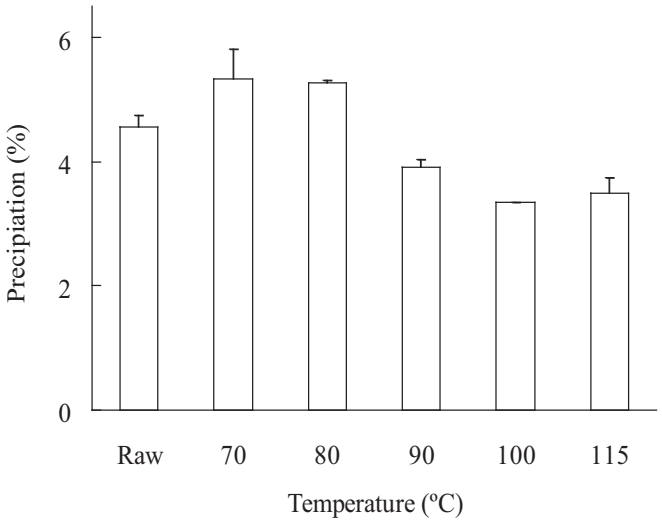

Fig. 1. Precipitation of soymilk heated at various temperatures. Mean±SD in triplicate experiments.

tered by gauze and not homogenized, it contained larger unstable fractions such as okara, although the soymilk showed had no visible precipitate.

From SDS-PAGE (Fig. 2), soymilk samples, which were unheated or heated at 70 and 80ºC, showed bands corresponding to glycinin subunits (Petrucceli and Añón 1995a). However, the glycinin subunits disappeared, and the aggregates formed through disulfide (S-S) binding were detected in soymilk heated to 90ºC or higher. These aggregates were considered to be mainly formed from basic polypeptides and β-subunits of β-conglycinin, according to a previous report (Petrucceli and Añón, 1995b). Ono *et al.* (1991) also reported that medium-sized particles (diameter, 40–100 nm), which were derived from soymilk proteins by heating in boiling water, contained more β-subunits and the basic polypeptides than raw soymilk. Xu *et al.* (1998) reported that soluble aggregate induced by dry-heating of egg white powder suppressed heat-insolubilization of egg white, so the aggregate formed in heated soymilk is considered to be related to the high dispersion stability of soymilk.

Precipitation of soymilk was decreased by heating at 90ºC or higher due to aggregate formation. As the precipitation of heated soymilk is thought to relate to the heat denaturation of soymilk protein, the denaturation of soymilk protein was therefore evaluated after heat treatment. Protein surface SH content of soymilk, which is changed by SH-SS exchange and conformation destruction during heating, was decreased by heat treatment (Fig. 3B), depending on the heating temperature. SH content of soymilk samples heated at 70 and 80ºC was slightly decreased by 5 min of heating and at relatively higher levels. SH content of soymilk heated at 90 and 100ºC was intermediate, and the SH content at 115ºC was the lowest of the samples in this study. However, SH content was maintained or slightly increased by heating from 5 to 15 min. As it was learned that SH changes plateaued at less than 5 min of heating, shorter heating was carried out using a thermal cycler. As a result, 1 min heating of soymilk showed the same decrease in surface SH content and maintained the same value as at 3 min of heating (Fig. 3A). According to these data, the SH-SS exchange reaction in soymilk was rapid and dependent mainly on the heating temperature. Further, SH content is likely to be related to the dispersion stability of refrigerated soymilk, and lower SH content was considered to be preferable to low precipitation. These results support the hypothesis that soluble aggregate formation through S-S binding may contribute to the increased dispersion stability of soymilk, based on SDS-PAGE results and a previous report (Xu *et al.*, 1998).

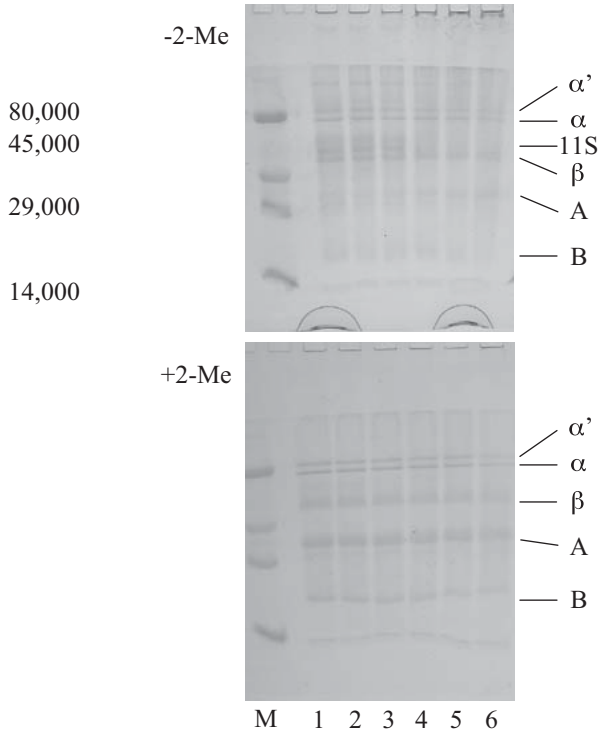

Fig. 2. SDS-PAGE patterns of soymilk protein. Soymilk samples were heated at various temperatures for 15 min and then cooled in an ice bath. -2-Me, without 2-mercaptoethanol; +2-Me, with 2-mercaptoethanol. M, molecular weight marker; 1, raw soymilk; 2, heated at 70ºC; 3, 80ºC; 4, 90ºC; 5, 100ºC; 6, 115ºC. Marker; lactoferrin (MW, 80,000), ovalbumin (45,000), carbonic anhydrase (29,000), lysozyme (14,000).

Next, surface hydrophobicity of soymilk protein, which is related to the exposure of the inner hydrophobic region of protein, was evaluated by measuring fluorescence derived from the interaction between ANS and protein (Fig. 4B). Surface hydrophobicity of proteins in soymilk heated at 70ºC gradually increased with an increase in heating time. However, soymilk heated at higher temperatures showed a rapid increase in hydrophobicity for 5 min. Heating of soymilk at higher temperatures caused the fluorescence intensity to increase faster and level off after 5 min of heating. Surface hydrophobicity of soymilk heated from 5 to 15 min at 90ºC was slightly higher than that heated at 70, 80, 100, 115ºC, and gradually increased with heating. Surface hydrophobicity of soymilk heated at 80, 100 and 115ºC showed almost identical values, and the hydrophobicity at 70ºC increased and leveled off at 15 min of heating. From the short-time heating trials using the thermal cycler (Fig. 4A), surface hydrophobicity was clearly shown to increase with heating time, as well as heating temperature.

These data showed that surface SH content decreased depending on heating temperature, but that the surface hydrophobicity increased depending on heating time rather than heating temperature. The precipitation of soymilk, which was heated for a relatively long time, was decreased by heating at 90ºC or higher temperatures. These dispersion stabilities

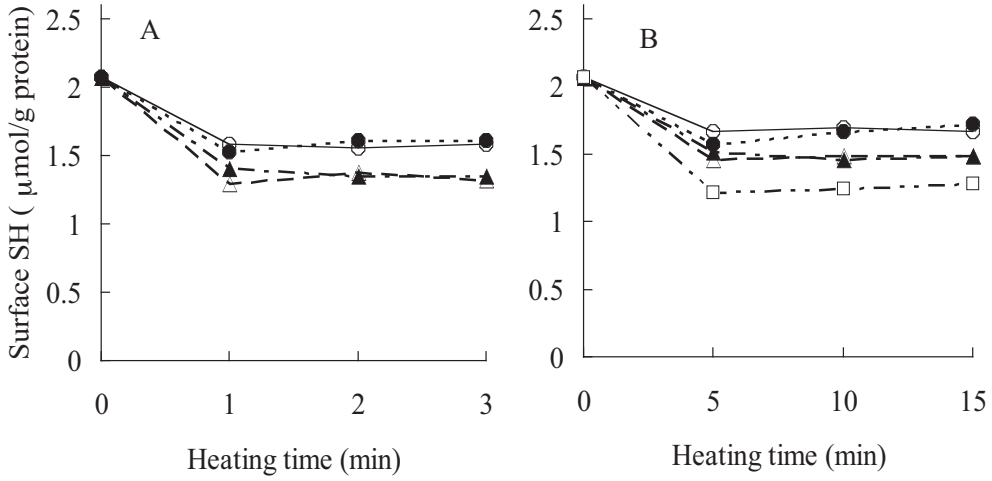

Fig. 3. Protein surface SH content of heated soymilk. A, Heated by a thermal cycler; B, Heated by a water bath. Closed circle, heated at 70ºC; Open circle, 80ºC; Closed triangle, 90ºC; Open triangle, 100ºC, Open square, 115ºC.

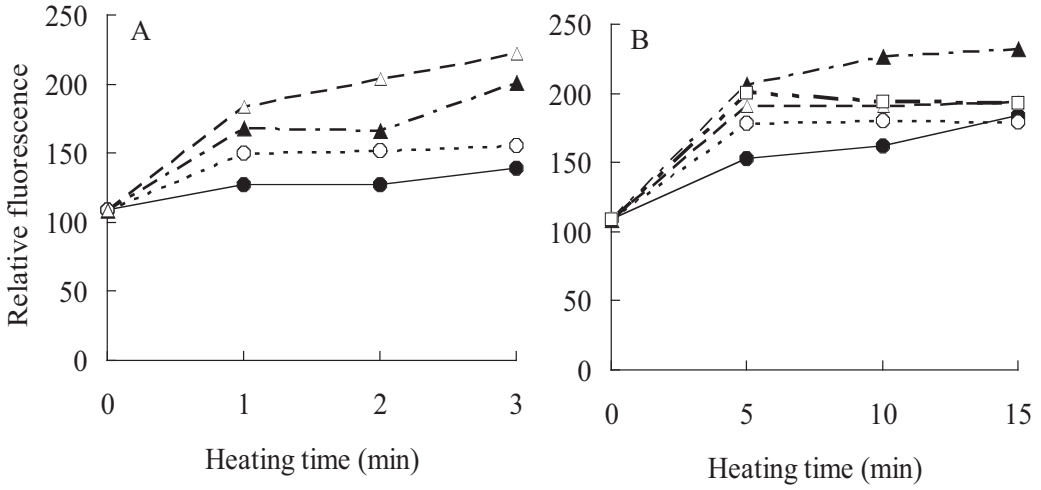

Fig. 4. Protein surface hydrophobicity of heated soymilk. A, Heated by a thermal cycler; B, Heated by a water bath. Closed circle, heated at 70ºC; Open circle, 80ºC; Closed triangle, 90ºC; Open triangle, 100ºC, Open square, 115ºC.

of the heated soymilk were likely to be related to the protein surface SH content of the heated soymilk because the surface hydrophobicities of proteins in heated soymilk were almost identical, except for soymilk heated at 90ºC.

Effect of two-step heat treatment on dispersion stability of soymilk Soymilk heated at higher than 90ºC showed decreased precipitation or increased dispersion stability. Heat treatment by a combination of two or more different temperatures is usually carried out in soymilk processing. In this study, we next estimated the effect of two-step heating on precipitation and protein denaturation of soymilk. For a first model, two-step heating was carried out using a combination of a higher temperature (115ºC) and lower (70–100ºC) temperatures. In Fig. 5, precipitate formations from soymilk heated at two different temperatures are summarized. The precipitation of soymilk, heated first at 70 to 100ºC and then successively heated at 115ºC, was at almost equal levels (3.1

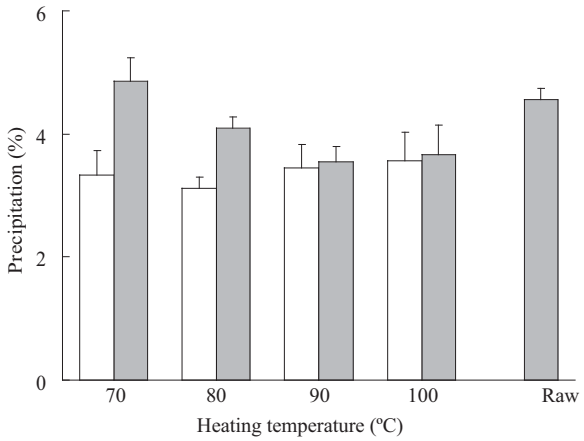

Fig. 5. Precipitation of soymilk heated at two different temperatures. Open square, Samples were heated first at lower temperature (70, 80, 90 or 100ºC) and then successively heated at higher temperature (115ºC); Hatched square, Samples were heated first at higher and then at lower temperatures. Heating time was 15 min at each step. Mean±SD in triplicate experiments.

to 3.6%) to the data from the single-heat treatment of soymilk at 90 to 115ºC. However, the precipitation of soymilk heated at 115ºC for the first step and then successively at 70 and 80ºC for the second heat treatment increased to 4.9 and 4.1%, respectively, resulting in levels almost equal to those of raw soymilk. These results indicated that the temperature in the second heating step had a greater effect on the dispersion stability of soymilk in two-step heating and that heating at a lower temperature than the soy protein denaturation temperature probably accelerates precipitate formation.

The protein surface SH contents of the soymilk samples heated at two different temperatures were lower than the results from the one-step heating experiment (Fig. 6). Some possible explanations include frequency of heating steps, prolonged heating time and number of heating temperatures, but the reason is unknown. The SH content was not dependent on the heating temperatures of the low temperature steps (namely 70, 80, 90 and 100ºC) nor the order of high and low temperature steps (namely, the combination of first the low and second the high or that of first the high and second the low temperature). The surface SH content was considered to be primarily affected by the higher temperature in the heating steps. From SDS-PAGE (data not shown), there were also no differences in aggregate formation through inter-subunit S-S bonding.

However, protein surface hydrophobicity depended on the combination of heating temperatures, in contrast to that of the SH content (Fig. 7). In the case of a combination of heating at 100ºC (low) and 115ºC (high), the surface hydrophobicity slightly increased after 5 min and then was almost maintained for 15 min of the second heating. There were no significant differences between treatments of different heating order, namely low to high or high to low.

These data showed that the denaturation behaviors of proteins heated at 100 and 115ºC were essentially identical. In

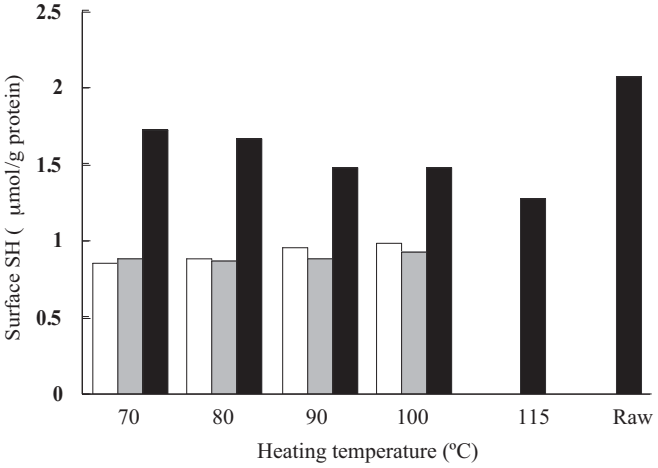

Fig. 6. Protein surface SH content of soymilk heated at two different temperatures. Closed square, one-step heating; Open square, heated first at lower temperature (70, 80, 90 or 100ºC) and then at higher temperature (115ºC); Hatched square, heated first at higher and then at lower temperatures. Heating time was 15 min at each step.

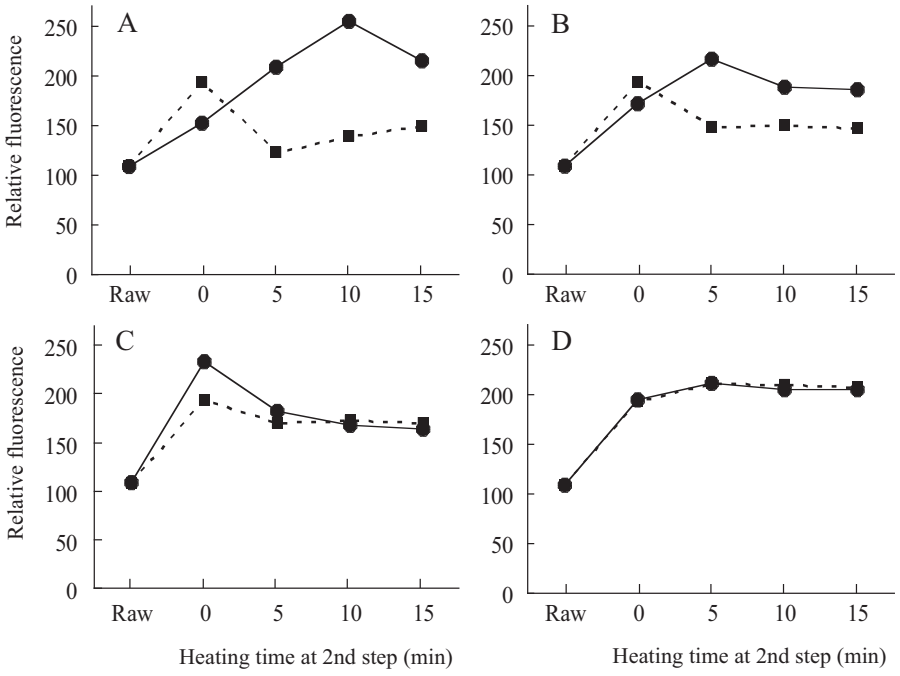

Fig. 7. Protein surface hydrophobicity of soymilk heated at two different temperatures. A, two-step heated at 70 and 115ºC; B, 80 and 115ºC; C, 90 and 115ºC; D, 100 and 115ºC. Solid line, heated first at lower temperature (70, 80, 90 or 100ºC) and then at higher temperature (115ºC); Dotted line, heated first at higher and then at lower temperatures. Heating time at first step was 15 min.

the case of a combination of 90 and 115ºC, heat treatment at 115 followed by 90ºC showed that the hydrophobicity level was maintained in the second-heating step, but heating at 90 and then 115ºC produced a different result. At the first heating step, extraordinarily higher hydrophobicity, similar to the results observed in one-step heating experiments, decreased in the second-heating step at 115ºC, suggesting that heating at 90ºC caused protein to denature more than did heating at 100 and 115ºC in terms of hydrophobicity. Further, in the case of the combination of 70 and 115ºC or 80 and 115ºC, heating at 70 or 80ºC as the first step caused insufficient thermal denaturation to soymilk protein, so that hydrophobicity further increased at the second-heating step (115ºC). However, two-step heating of soymilk at 115 and next at 70 or 80ºC first decreased the hydrophobicity for 5 min of heating and then increased it gradually at the second heating. Two possible mechanisms of the decrease in hydrophobicity for 5 min of heating time at the second-heating were considered, namely an association of dissociated subunits through the hydrophobic surfaces, or a kind of refolding of denatured peptide chains. Then, hydrophobicity increased with the increase in heating time at 70 or 80ºC, so it is likely that the protein molecules were denatured again in a manner different from that for heating at 115ºC. Considering that this heating sequence decreased the dispersion stability of soymilk, the structural changes in protein molecules during two-step heating at high- (115ºC) and successive low-temperatures (70 or 80ºC) were thought to be at least partially responsible for precipitate formation.

Based on the above data, even if soymilk is heated by either a one- or two-step process, the dispersion stability of soymilk decreased from heating at 70–80ºC. Between 70 and 80ºC, soybean β-conglycinin was thermally denatured, but glycinin was not, according to the result of DSC (Hermansson, 1978; Zhang *et al.*, 2004). For one-step heating, these data lead to the hypothesis that the combination of denatured β-conglycinin and native glycinin lower the dispersion stability of soymilk, whereas the combination of denatured β-conglycinin and denatured glycinin increase dispersion stability. Guo *et al.* (1997) reported that heat treatment at 75ºC dissociated α and α' subunits of β-conglycinin from particulate fraction, which was recovered as precipitate from soymilk by centrifugation at 156000×*g* for 30 min and that the resulting particulate fraction contained mainly acidic and basic subunits of glycinin and β-subunit of β-conglycinin. Heating soymilk in boiling water allowed both β-conglycinin and glycinin to denature and resulted in decrease in large particles (>100 nm diameter) and increase in medium-sized particles (40 - 100 nm) (Ono *et al.*, 1991). The dispersion stability of soymilk may be related to these protein particles. For two-step heating, the glycinin subunits, which were denatured by heating at 110ºC, were supposed to be associated or partially refolded during the second heating at 70 or 80ºC, considering the results from protein surface hydrophobicity (Fig. 7). These altered molecules, which were expected to differ from both native and heat-denatured forms, may also be related to decreases in the dispersion stability, similar to that observed for the native glycinin. The interactions between β-conglycinin and glycinin were changed by the denaturation behavior of individual molecules, and the formation of the soluble aggregate or particles was expected to be accelerated by the combination of denatured protein molecules.

In soymilk processing, a steam-injection system is also used for sterilization. Soymilk heated with high-pressure steam is transferred to a flash vessel where the temperature drops instantly under reduced pressure. In this process, soymilk heated at ~140ºC was instantly cooled to ~80ºC and maintained for a short time. These data show the possibilities that vessel temperature has important effects on the quality of the processed soymilk and should be optimized in terms of the dispersion stability of heated soymilk.

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