Interaction between Protein and Lipid in Soybean Milk at Elevated Temperature

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Soymilk is prepared by heating swelled and ground soybeans, of which major components are lipid and protein. The stability of lipid in aqueous solution is important in soymilk. The interaction between lipid and protein in soymilk was studied from the viewpoint of changes in the lipid and protein distribution by heating. Soymilk was fractionated to particulate, soluble, and floating fractions by centrifugation. Most of the lipid in unheated soymilk existed in the particulate fraction. When heating from 65 to 75 °C, a part of lipid and almost α and α′ subunits of β-conglycinin in the particulate fraction liberated and moved to soluble fraction. When heating at 75 °C, the lipids in the soluble and particulate fractions began to liberate and to shift to the floating fraction. Almost all lipid (neutral lipid) shifted to the floating fraction at 90 °C. The major proteins in the floating fraction were two unknown proteins (named BX1 and BX2). BX1 and BX2 are minor proteins in soymilk and had relative molecular weights of 22000 and 16000, respectively.

Keywords: *Soymilk; lipid; heating*

INTRODUCTION

Soybean milk and its products have been popular in China and some Asia countries since ancient time. It is a colloidal solution that is obtained as a water extract from swelled and ground soybeans; therefore, almost all the components (protein, lipid, and saccharides) of the soybean seeds are present in it. The interaction between protein and lipid are important in achieving acceptable food characteristics. The lipid plays an important role in the physical characteristics (such as texture, sensory quality, etc.) of the products made from soybean milk (Catsimpoolas and Meyer, 1971; Yamano *et al*., 1981). It was reported that phosphatidylcholine was associated with soybean protein to produce multiform complexes by sonication and was nonspecifically bound to either 7S or 11S globulin (Kanamoto *et al*., 1977). The association of phosphatidylcholine and soybean protein occurs through hydrophobic interaction between phosphatidylcholine and the hydrophobic regions of the protein by the binding of phosphatidylcholine lamellae to the protein surface (Ohtsuru *et al*., 1976). Moreover, β-structure content of the protein decreased with this association (Ohtsuru *et al*., 1979). The lipid (neutral) and protein could form a complex of characteristic buoyant densities by the dissociation of native protein, providing an increased surface area and greater proportion of hydrophobic residues (Kamat *et al*., 1978). It is known that the denaturation of protein by heating increases the surface hydrophobicity (Sorgentini *et al*., 1995). While a lot of neutral lipid existed in the particles of unheated soybean milk and moved to the soluble phase by heating, however, half of the phospholipids was retained in the particles (Ono *et al*., 1996). The proteins in the particles were rearranged by heating (Ono *et al*., 1991). Almost all of the lipids in heated soybean milk could be separated as a floating layer containing a few proteins (Shibasaki *et al*., 1972; Ono *et al*., 1996). These results are insufficient to explain the stability of lipids in soybean milk. Therefore, it is important that the formation of the lipid complex in soybean milk is studied in view of the rearrangement of particles by heating. In this study, the change in lipid distribution of the particulate, soluble, and floating fractions from soybean milk was examined at elevated temperature.

MATERIALS AND METHODS

Materials. Soybeans (species *Glycine max* var. Suzuyutaka or Tatikogane) harvested at the Iwate University Experimental Farm located in Morioka, Iwate, Japan, were stored at 5 °C and used within one year. All the chemicals were of the highest purity available and were used without further purification.

Preparation of Soybean Milk (Soymilk). The beans were soaked in deionized water for 20 h at 4 °C. The swollen beans were ground to a homogenate containing 10% solids with water using an Oster blender, and the homogenate was next filtered through a defatted cotton sheet, the filtrate being designated unheated soymilk. The unheated soymilks were heated in a water bath for 5 min at 50, 55, 60, 65, 70, 75, 80, 85, 90, and 95 °C and then quickly cooled to 20 °C. They were designated heated soymilk.

Preparation of Particulate, Soluble, and Floating Fractions. The soymilk was separated into the precipitate, supernatant, and top layer by centrifugation at 156000*g* for 30 min at 20 °C according to the method of Ono *et al*. (1991) using an automatic preparative ultracentrifuge (HITACHI85P-72, RP-65 rotor). The time required for a particle to move from position r_1 to position r_2, Ts, is described as follows (Ono *et al*., 1990):

$$T_s = \frac{1}{S}\frac{\ln r_2 - \ln r_1}{\omega^2} \quad (1)$$

According to Stokes's law, diameter of particle, d, is described below:

$$d^2 = \frac{18\mu S}{(\sigma - \rho)} \quad (2)$$

S is sedimentation coefficient, ω is revolutions per minute of rotor (rpm), μ is viscosity of solvent (0.1 poise), and the respective density of particle and solvent (water) were $\sigma = 1.2$ g/cm^3 and $\rho = 1.0$ g/cm^3. Combination of eqs 1 and 2 leads to the following equation:

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$$T_s = \frac{18\mu}{(\sigma - \rho)d^2} \frac{(\ln r_2 - \ln r_1)}{\omega^2} \quad (3)$$

The time and gravitation for the particles of size d were calculated by eq 3. The precipitate was designated as a particulate fraction with a diameter of more than 40 nm. The supernatant was designated as a soluble fraction which contains soluble proteins and smaller particles than 40 nm in diameter. The obtained top layer was designated as a floating fraction. In order to remove the adherent soluble component, the top layer was dispersed in water of the same volume as that of the unheated soymilk used and centrifuged again at 156000*g* for 30 min. This process was repeated three times until the protein was free in the supernatant. The particulate, soluble, and floating fractions were lyophilized and stored in the refrigerator at 4 °C.

Preparation of Soybean Protein. The soybean seeds were dehulled, ground, and defatted with *n*-hexane. Residual solvent in the defatted flour was removed by vacuum evaporation at room temperature. The defatted flour was suspended in 0.03 M Tris-HCl buffer (pH 8.0) containing 10 mM 2-mercaptoethanol (flour:buffer was 1:20) for extracting glycinin and β-conglycinin and stirred for 1 h at room temperature (Thanh *et al.*, 1976). The supernatant was obtained by centrifugation at 10000 rpm for 20 min, filtered through a 0.45 μm membrane filter, and adjusted to pH 8.0 with 2 N NaOH. The filtrate was dialyzed against deionized water at 4 °C to remove the low molecular components as salt, saccharides, etc., and lyophilized. This preparation was used for an extracted soybean protein.

Lipid Content. The total lipid contents of samples were determined using Folch's method (Folch *et al.*, 1957).

The Determination of Protein. After the lipids had been removed by *n*-hexane, the protein content of the samples was determined by the method of Smith *et al.* (1985) using bicinchoninic acid (BCA) protein assay reagent (Pierce Chemical Co., Rockford, IL).

Electrophoresis. The sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS-PAGE) was performed in a 1 mm thick vertical slab gel using an alkaline discontinuous buffer (Laemmli, 1970). The concentrations of the stacking and running gels containing 0.1% SDS, were 5% and 12.5%, respectively. The buffer in the reservoirs contained 0.025 M Tris, 0.192 M glycine, and 0.1% SDS, while the buffer in the stacking and running gels were 0.125 M Tris-HCl (pH 6.8) and 0.38 M Tris-HCl (pH 8.8), respectively. The samples containing 0.25 M Tris-HCl (pH 6.8), 1% SDS, and 2% 2-mercaptoethanol were allowed to stand overnight and were then mixed with the same volume of glycerol containing bromophenol blue. Each was then put into a sample slot in the stacking gel and electrophoresed. A relative molecular weight of the electrophoresed protein was estimated from followed standard proteins; bovine serum albumin (66000), ovalbumin (45000), β-casein (24000), and lysozyme (13000).

Coomassie brilliant blue G-250 was used for staining protein in the gel by the method of Blakesley and Boezi (1977). After electrophoresis, the gels were immersed in a staining solution containing 12% trichloroacetic acid for 12 h, and then the background stain of the gel was removed with water. The gels containing the protein-staining bands were densitometrically scanned at 600 nm with a Fujiriken FD-AIV instrument. A linear relationship between the stain intensity and the protein concentration was observed with each band, and the relative ratio among the protein bands of a sample was calculated from these stain intensity values.

RESULTS AND DISCUSSION

When the unheated and heated soymilks were fractionated to particulate, soluble, and floating fractions by centrifugation, it was found that lipid in the particles of unheated soymilk was liberated by heating at 95 °C (Ono *et al.*, 1996). The lipid content in the particles changed drastically from 35.6% to 4.2%. However it is not clear what temperature the lipid liberation began at and what change occurred on particulate proteins in the lipid liberation process.

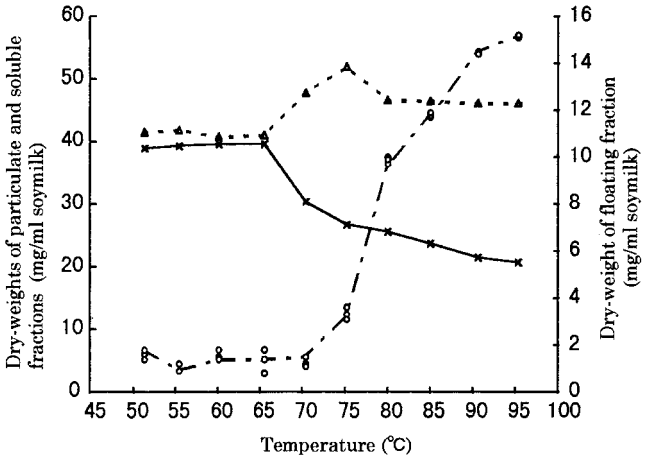

Figure 1. Dry-weight of each fraction obtained from unheated soymilk at elevated temperature. The soymilk was heated at a specific temperature for 5 min, cooled to 20 °C, and fractionated to particulate (×), soluble (△), and floating (○) fractions by centrifugation at 156000*g* for 30 min.

In order to clarify the lipid liberation process by heating, unheated soymilk was heated at a specific temperature (from 50 to 95 °C) for 5 min and cooled to 20 °C, and then the soymilk was separated to particulate, soluble, and floating fractions. The dry-weights of these fractions were measured and shown in Figure 1. The dry-weight of the particulate fraction sharply decreased as the temperature was increased from 65 to 75 °C, and declined gradually from 75 to 90 °C. On the other hand, the dry-weight of the soluble fraction increased between 65 and 75 °C, and then decreased from 75 to 80 °C. The dry-weight of the floating fraction increased with the increase of temperature from 70 to 90 °C. These results indicated that the dissociation of particles occurred above 65 °C, and that a part of the dissociated particles moved to the soluble fraction between 65 and 75 °C and then to the floating fraction above 70 °C.

The lipid quantity in the particulate and soluble fractions was then determined by Folch's method (Folch *et al.*, 1957). The lipid quantity in the particulate fraction decreased as the temperature was increased from 65 to 90 °C as shown in Figure 2. On the other hand, the lipid quantity in the soluble fraction increased between 65 and 75 °C. When heating above 75 °C, the lipid quantity in soluble fraction decreased with the increase of temperature and returned to the initial level at 90 °C. These results indicate that the lipid liberation occurred along with dissociation of the particles (Figure 1). The majority of liberated lipids shifted to soluble fraction between 65 and 75 °C, and the minority of lipid moved to the floating fraction. When heating above 75 °C, the floating fraction was increased remarkably with decreasing the lipid in particulate and soluble fractions. Therefore, it is considered that the lipid liberation process have two stages. In the first stage, the particles of unheated soymilk disrupted and a part of the lipids moved to the soluble fraction as the temperature was increased from 65 to 75 °C. In the second stage, lipid began to liberate from the particulate and soluble fractions at 75 °C, and almost all lipids were liberated and moved to the floating fraction above 90 °C.

It is reported that the particulate proteins in unheated soymilk were rearranged by heating at 95 °C

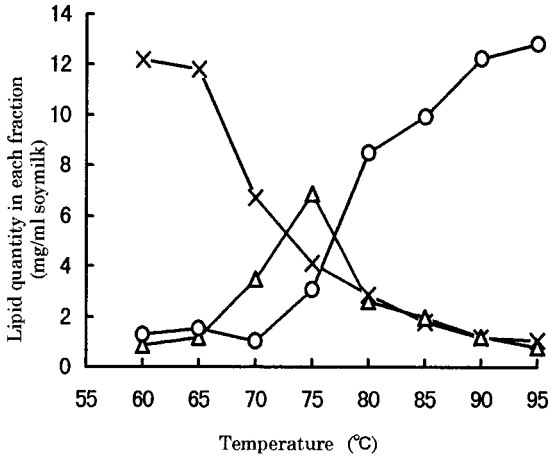

Figure 2. Lipid quantity in the particulate, soluble, and floating fractions of unheated soymilk at elevated temperature. The unheated soymilk was heated at a specific temperature for 5 min and cooled to 20 °C. Particulate (×), soluble (△), and floating (○) fractions were obtained by centrifugation at 156000*g* for 30 min.

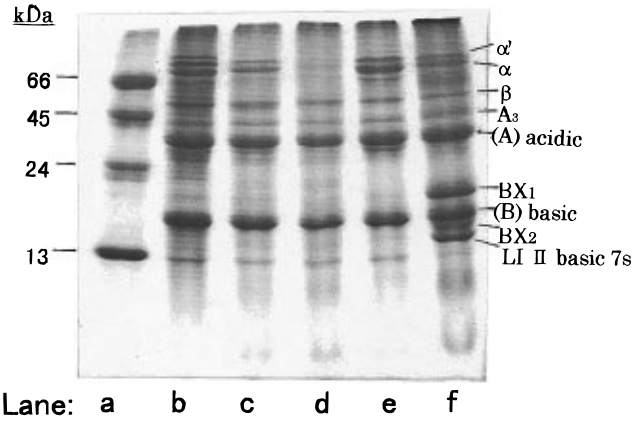

Figure 3. SDS-PAGE patterns of each fraction obtained from heated dispersion of unheated particulate fraction of soymilk. The dispersion was heated at 75 °C for 5 min and cooled to 20 °C, and then fractionated to particulate, d, soluble, e, and floating, f, fractions by centrifugation at 156000*g* for 30 min. a, molecular weight standard; b, soybean protein; c, unheated particulate fraction; α′, α, and *β*, subunits of *β*-conglycinin; A and B, acidic and basic subunits of glycinin.

(Ono *et al*., 1991). The compositional change of the particulate protein accompanying the liberation of lipid by heating was analyzed by SDS-PAGE. The unheated particulate fraction was prepared from unheated soymilk by centrifugation as the statement of materials and methods. This fraction was dispersed in water of the same volume as that of the unheated soymilk used for the sample and heated at 75 °C for 5 min. The heated dispersion was then fractionated into the particulate, soluble, and floating fractions by centrifugation (156000*g* for 30 min at 20 °C). The SDS-PAGE pattern of proteins in each fraction are shown in Figure 3 compared with that of a extracted soybean protein (Figure 3, lane b). The extracted protein with Tris-HCl buffer is saline soluble and consists of 7S (primarily *β*-conglycinin), 11S (glycinin), and 2S (whey protein) (Wolf *et al*., 1961). These proteins on SDS-PAGE as shown in Figure 3 are assigned by Hirano *et al*. (1987). The patterns were densitometrically scanned, and the relative ratio of bands was estimated. The protein in particulate fraction of unheated soymilk was comprised of 70% glycinin and 30% *β*-conglycinin. This result is in good agreement with that reported by Ono *et al*. (1991). When heating

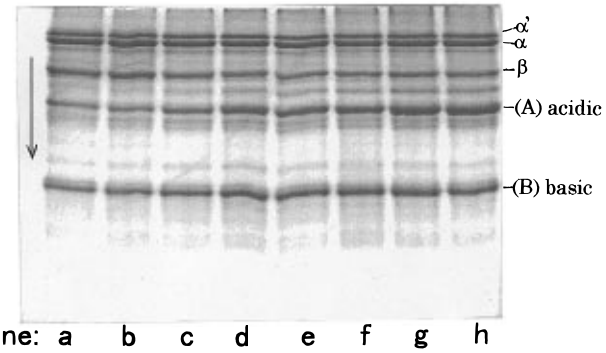

Figure 4. SDS-PAGE patterns of the proteins in soluble fraction of soymilk obtained at various temperatures. The sample was obtained from heated soymilk by centrifugation at 156000*g* for 30 min. a, 60 °C; b, 65 °C; c, 70 °C; d, 75 °C; e, 80 °C; f, 85 °C; g, 90 °C; h, 95 °C.

at 75 °C, α and α′ subunits of *β*-conglycinin almost disappeared in the particulate fraction (Figure 3, lane d), and the major protein bands were acidic, basic and A_3 subunits of glycinin, and the *β* subunit of *β*-conglycinin. This result is in agreement with the dissociation of α and α′ subunits in *β*-conglycinin solution by heating at 75 °C which was reported by Iwabuchi *et al*. (1991a). It is known that the conformational change in *β*-conglycinin occurs between 65 and 75 °C, and that *β*-conglycinin is thoroughly denatured and the denaturation of glycinin begins at 80 °C (Hermansson, 1986; Iwabuchi *et al*., 1991b; Sorgentini *et al*., 1995; Nagano *et al*., 1995). This suggests that glycinin of the unheated particulate fraction was not denatured at 75 °C. A part of the proteins in unheated particles dissociated to soluble and floating fractions at 75 °C (Figure 3, lanes e, f). The temperature for producing the soluble fraction from the unheated particulate fraction was between 65 and 75 °C (Figure 1) and agrees with that for the conformational change of *β*-conglycinin.

Additionally, two unknown proteins were found in the floating fraction by heating at 75 °C, moving near the position of basic subunit of glycinin on SDS-PAGE (Figure 3, lane f). The unknown proteins are named BX1 and BX2 in this paper. The BX1 and BX2 occupy 19% and 8% of the protein in the floating fraction, respectively. The protein in floating fraction contained not only *β*-conglycinin and glycinin but also other proteins as BX1 and BX2.

The changes in protein composition of the soluble fraction of soymilk after heat treatments (60−95 °C) were analyzed by SDS-PAGE. The major protein of the soluble fraction in heated soymilk between 60 and 65 °C was *β*-conglycinin (α, α′, and *β* subunits) as shown in Figure 4. The bands of *β*-conglycinin and glycinin (acidic and basic subunits) changed with elevating temperature. Glycinin bands, particularly, appeared strongly above 75 °C. Therefore, a relationship between each subunit concentration (of *β*-conglycinin and glycinin) and temperature is shown in Figure 5 by estimating from both of the relative ratios of each subunit and the protein concentration in the soluble fraction. α and α′ Subunits of *β*-conglycinin in the soluble fraction increased from 65 to 70 °C, and decreased at 75 °C. The *β* subunit was not changed between 60 and 70 °C and slightly decreased at 75 °C. Glycinin concentration increased markedly from 65 to 70 °C and slowly increased from 70 to 75 °C. These results indicate that the proteins (*β*-conglycinin and glycinin) containing lipids, except the *β* subunit, were liberated from the particles and moved to the soluble fraction between 65

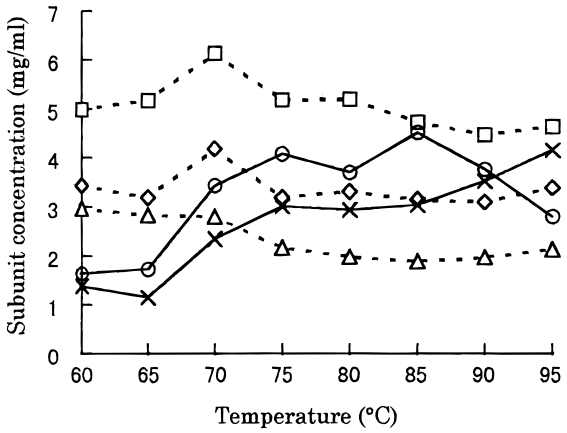

Figure 5. Changes in subunit concentration of glycinin and β-conglycinin in the soluble fraction of soymilk by heating from 60 to 95 °C. β-conglycinin: α' subunit (◇); α subunit (□); β subunit (△); glycinin: acidic subunit (×); basic subunit (○).

and 75 °C (Figure 2). Almost all the α and α' subunits of β-conglycinin in the particulate fraction dissociated at 75 °C (Figure 3, lane d), and then the lipid shifted to the floating fraction by heating above 75 °C (Figure 2). The decrease of β-conglycinin in the soluble fraction from 70 to 75 °C is considered to be due to forming the floating fraction containing β-conglycinin (Figures 2 and 3, lane f). The shift of lipids to the soluble fraction between 65 and 75 °C (Figure 2) may proceed with dissociation of β-conglycinin, and the shift to floating fraction above 70 °C may occur by further denaturation of β-conglycinin.

Between 75 and 90 °C, β-conglycinin concentration slightly decreased as shown in Figure 5. On the other hand, the basic subunit increased between 80 and 85 °C and decreased above 85 °C. The acidic subunit increased above 85 °C. It is reported that the denaturation of glycinin begins at 80 °C and ends at 91 °C (Hermansson, 1986; Sorgentini *et al*., 1995). Moreover, the particles of soymilk formed by heating at 95 °C are suggested to be mainly consisted of the β subunit of β-conglycinin and the basic subunit of glycinin (Yamagish *et al*., 1980; Utsumi *et al*., 1984; Ono *et al*., 1991). The increase of the acidic subunit and decrease of the basic subunit above 80 °C can be attributed to the denaturation and dissociation of glycinin and to the rearrangement of particles. The increase of the floating fraction above 80 °C could be due to the denaturation and dissociation of glycinin.

The liberated lipid could be separated as the floating fraction from the soymilk by centrifugation. The floating fraction was dispersed in water and centrifuged again to remove the adherent soluble components. The protein content of the floating fraction was 13.6%. The protein composition of the floating fraction was analyzed with SDS-PAGE and was compared with those of soybean and soymilk proteins as shown in Figure 6. The major protein bands of the floating fraction from unheated soymilk was glycinin (Figure 6, lanes d, f), while the major bands from heated soymilk (95 °C) were two unknown bands named BX1 and BX2 above (Figure 6, lanes e, g). The relative ratios of protein bands were estimated densitometrically and are shown in Table 1. BX1 and BX2 occupy 28% and 27% of the protein in the floating fraction (Figure 6, lane f, from Suzuyutaka variety), respectively, from heated soymilk at 95 °C. A similar result was obtained from that of a different variety of soybean such as tatikogane (Figure 6, lane e). BX1 and BX2 appeared as minor bands in soymilk

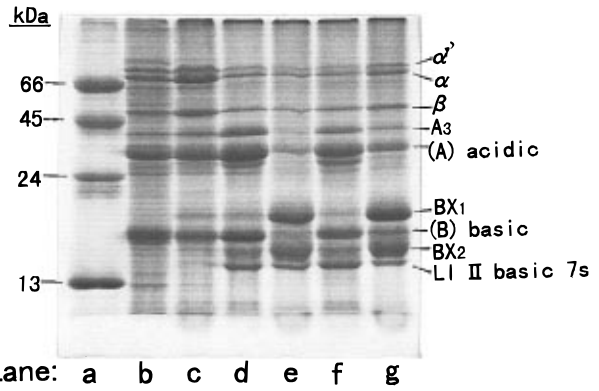

Figure 6. SDS-PAGE patterns of protein in the floating fraction obtained from soymilk (95 °C). a, molecular weight standards; b and c are proteins of soybean and soymilk, respectively; d and e are proteins in the floating fractions from unheated and heated soymilk for tatikogane, respectively; f and g are proteins in the floating fraction from unheated and heated soymilk for Suzuyutaka, respectively.

Table 1. Relative Ratio of Each Subunit in Protein of the Floating Fraction and in Soymilk Protein[a]

	relative ratio of each subunit (%)			
	from unheated soymilk		from heated soymilk (95 °C)	
subunit	in floating fraction	in soymilk	in floating fraction	in soymilk
α'	2.6	0.03	4.5	0.22
α	4.6	0.06	7.1	0.35
β	3.4	0.04	4.9	0.24
A_3	6.3	0.08	2.3	0.11
A	21.4	0.26	8.7	0.43
BX_1	2.8	0.03	27.7	1.53
B	18.2	0.22	10.6	0.52
BX_2	13.1	0.16	27.0	1.32
others	26.9	0.33	7.3	0.34
sum	100.0	1.21	100.0	4.88

[a] The samples were prepared from soybean Suzuyutaka variety.

protein (Figure 6, lane c) and were found little in the extracted soybean protein (Figure 6, lane b). It is considered that BX1 and BX2 could not be extracted with Tris-HCl buffer from defatted flour of soybean. The relative ratio of BX1 plus BX2 in the unheated floating fraction to the total proteins of soymilk is 0.2% (Table 1), and the relative ratio in the heated one (at 95 °C) is 2.7%. These results suggest that BX1 and BX2 are minor proteins in soymilk and may have come from the particles with the lipid liberated by heating. The respective molecular weights of BX1 and BX2 were estimated to be 21000 and 16000 from their relative mobility on SDS-PAGE in comparison with the molecular weights of the known proteins (bovine serum albumin, 66000; ovalbumin, 45000; β-casein, 24000; lysozyme, 13000).

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