Incorporation of Soy Milk Lipid into Protein Coagulum by Addition of Calcium Chloride

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Lipid holding in tofu (soybean curd) has such stability that it is not released even when it is cooked. The lipid in soy milk is incorporated into the tofu with coagulation of proteins on processing by the addition of a calcium salt. In this study, the incorporation of lipid into the coagulum was examined from the association with protein. The lipid in soy milk was obtained as the floating fraction by centrifugation. The floating fraction decreased by the addition of calcium chloride prior to forming a protein coagulum. When half of the protein coagulated, all of the floating fraction became inseparable. The protein in soy milk, then, was separated into particulate and soluble fractions by centrifugation. The decrease of the floating fraction with added calcium chloride was parallel to the coagulation of the particulate protein. The association of the floating fraction and the soluble protein occurred after the new particles formed from the soluble proteins. These results indicated that the lipid incorporation took place by the conjugation of the lipid and protein particles.

Keywords: *Soy milk; lipid; incorporation; coagulation*

INTRODUCTION

Soybean products, such as tofu (soybean curd) and douhua (soybean jelly), have been popular in some Asian countries since ancient times. These products contain a lot of lipid. Its content reaches >30% by dry weight (Resources Council, Japan, 1982). The lipid, however, is held in a stable state not to be released from these products even when they are cooked. The lipid influences the gelation of the soybean protein and plays an important role in the physical characteristics (such as texture and sensory quality) of the products (Catsimpoolas and Meyer, 1971; Shimada and Matsushita, 1981). However, the mechanism of lipid incorporation into soy milk gel with protein coagulation is not well understood. In this study, the incorporation of lipid into these products was examined from the association with protein. These conventional products are made by the addition of a coagulant such as bittern (mainly $MgCl_2$) to the water extracts of soybean (soy milk). In the unheated soy milk, most of the lipid is present in the protein particles and is liberated when heated above 90 °C (Ono et al., 1996; Guo et al., 1997). The lipid is separated as the floating fraction by centrifugation (Guo et al., 1997). The addition of the coagulant causes not only protein coagulation and gelation but also the incorporation of lipid into the protein gel (Yamano et al., 1981). Soy milk coagulation is known to depend on the concentration of coagulant (calcium chloride) and the pH (Ono et al., 1993). Temperature is an external factor and accelerates the soy milk coagulation (Saio and Watanabe, 1973). The coagulation at room temperature conveniently occurs slowly and shifts at high concentration of a coagulant. In this study, the incorporation of lipid into the coagulum was examined from the changes of the floating fraction and protein solubility by the addition of calcium chloride for coagulant at room temperature. On the basis of our results, we estimated a mechanism of lipid incorporation into soy milk gel.

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MATERIALS AND METHODS

Materials. Soybeans (species *Glycine max* var. Suzuyutaka) harvested at the Iwate University Experimental Farm located in Morioka, Iwate, Japan, were stored at 4 °C and used within one year. All chemicals were of the highest purity available and were used without further purification.

Preparation of Soy Milk. The soy milk used in this study was obtained as follows: 15 g of soybeans was soaked in deionized water for 18 h at 4 °C. The swollen beans were ground into a homogenate with 105 mL of water using an Oster blender (Oster Co.), and the homogenate was then filtered through a defatted cotton sheet. The filtrate was heated in a water bath for 5 min at 95 °C and then quickly cooled to 20 °C using ice water. The filtrate was designated soy milk.

Preparation of Particulate, Soluble, and Floating Fractions. The protein in soy milk was fractionated into a particulate fraction (a diameter of >40 nm) and a soluble fraction (a diameter of <40 nm), and the lipid was separated as the floating fraction by centrifugation at 156000*g* for 30 min at 20 °C (Ono et al., 1991, 1996). According to the method of Ono et al. (1991, 1996), 90 mL of soy milk was separated into precipitate, supernatant, and top layer by using an automatic preparative ultracentrifuge (Hitachi 85P-72, RP-65 rotor, Tokyo, Japan). The precipitate, supernatant, and top layer were designated the particulate, soluble, and floating fractions, respectively. Floating fraction dispersion was obtained by gently dispersing floating fraction in an ultrafiltrate (which will be described later) to 90 mL using a Wheaton Potter-Elvehjem tissue grinder. Using a similar method, the floating and soluble fraction dispersion was obtained by dispersing a floating fraction in the supernatant containing soluble protein. The floating and particulate fraction dispersion was obtained by mixing and dispersing the particulate and floating fractions in the ultrafiltrate to 90 mL. The protein

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content in the dispersion was adjusted equal to that of the floating and soluble fraction dispersion by controlling the quantity of the particulate fraction. To maintain constant pH throughout the experiments, a 750 mM Bis-tris buffer (pH 7.0) was added up to 50 mM into the above dispersions.

The ultrafiltrate of soy milk was prepared by ultrafiltration with a UK-10 membrane filter (molecular weight cutoff = 10000; Advantec Toyo, Ltd., Tokyo, Japan).

Measurements of Floating Fraction Ratio and Protein Solubility in Various Concentrations of Calcium Chloride. The soy milk and the dispersions containing various concentrations of calcium chloride were prepared as follows: 0, 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, and 0.9 mL of 200 mM calcium chloride were added to 9 mL of soy milk or of the dispersions and stirred, and these mixtures were made up to 10 mL by distilled water. The mixtures were performed to a series of 0−18 mM concentration and kept at room temperature for 30 min. The separable floating fraction ratio in these mixtures was measured. Nine milliliters of the samples was centrifuged at 156000*g* for 30 min (Hitachi 85P-72, RP-65 rotor), the floating fractions obtained were lyophilized, and their dry weights were measured. The ratio of dry weights with calcium chloride against without calcium chloride was determined as the separable floating fraction ratio.

The protein concentration in the samples with and without calcium chloride was determined according to the method of Smith et al. (1985) using bicinchoninic acid (BCA) protein assay reagent (Pierce Chemical Co., Rockford, IL). The samples were centrifuged at 2000*g* for 10 min. One milliliter of the supernatant was lyophilized, defatted by *n*-hexane, and then dissolved into 50 mL of distilled water to adjust the protein concentration to 100−1200 μg/mL. Two milliliters of BCA protein assay reagent was added to 0.1 mL of each sample and subjected to mixing for 30 s. The mixtures were then incubated at 37 °C for 30 min, and the absorbance was measured at 562 nm (model 124 spectrophotometer, Hitachi Ltd., Tokyo, Japan.). The standard protein for this determination was a soybean protein that was prepared in our laboratory (Guo et al., 1997). The concentration of protein was converted to percentage against that from the sample without calcium chloride. The percentage was designated protein solubility.

pH Measurement. pH measurement was done by a pH meter model HM-30V (TOA Electronics Ltd., Tokyo, Japan)

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, whereas the buffers 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 0.2% protein, 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 sample of 10 μL was then put into a sample slot in the stacking gel and electrophoresed.

Coomassie brilliant blue G-250 was used to stain proteins in the gel according to 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 assignment of bands in the stained gels was determined with reference to those reported by Guo et al. (1997). The protein-staining bands on the gel were scanned with an HP Desk Scan II instrument (Hewlett-Packard Co.) connected to a computer (Win 95), and then the stain intensities were analyzed with the Scion image PC software (Scion Co.). A linear relationship between the stain intensity and the protein concentration was observed in each band, and the relative ratio among the protein bands of a sample was calculated from these stain intensity values.

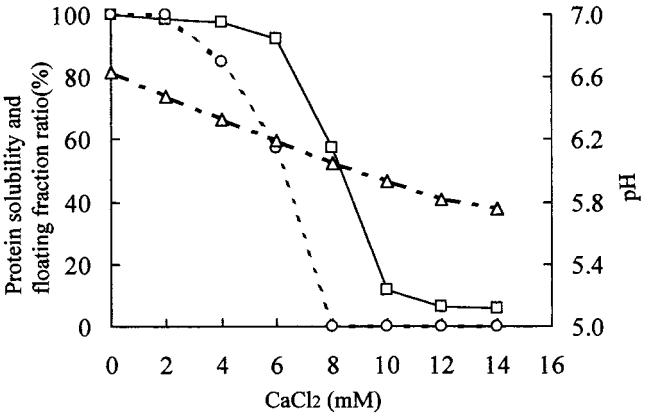

Figure 1. Changes in protein solubility and separable floating fraction ratio in soy milk by addition of calcium chloride: (□) protein solubility; (○) floating fraction ratio; (△) pH.

RESULTS AND DISCUSSION

Lipid in soy milk can be separated as the floating fraction by centrifugation. The floating fraction contained 14 mg/mL soy milk on a dry weight basis. When calcium chloride was added to soy milk, the floating fraction decreased, with formation of aggregates. The decrease of floating fraction ratio and protein solubility in soy milk at various concentrations of calcium chloride was measured to analyze the incorporation of the lipid into the protein coagulum and is shown in Figure 1. The floating fraction decreased after the addition of more than 2 mM calcium chloride and became inseparable at 8 mM. The protein content was 34 mg/mL soy milk and decreased to 19 mg/mL by the addition of 8 mM calcium chloride. The protein solubility is 56%, calculating from this decrease. Almost all of the protein became insoluble at 10 mM calcium chloride. The decrease of floating fraction occurred before the formation of protein aggregates. When half of the protein coagulated with calcium chloride, almost all of the lipid in soy milk became inseparable.

It is known that soy milk is coagulated by the addition of calcium ion or pH decrease (Ono et al., 1993). The pH in soy milk decreased when calcium chloride was added (Figure 1). To examine the mixture at constant pH, Bis-tris (good buffer) was added to the soy milk up to 50 mM. The protein solubility and the floating fraction ratio of the soy milk containing 50 mM Bis-tris (pH 7.0) were analyzed again at various concentrations of calcium chloride and are shown in Figure 2. The protein solubility decreases from 10 to 18 mM calcium chloride. The calcium chloride concentration required for decreasing the protein solubility in the presence of Bis-tris buffer is 2 times higher than that without this buffer (Figure 1). The floating fraction ratio decreases from 8 to 14 mM calcium chloride. A similar difference at calcium concentration is obtained between both decreases in floating fraction ratio and protein solubility with and without buffer (Figures 1 and 2). The decrease in the floating fraction occurred at a lower concentration of calcium chloride than that in protein solubility. These results indicate that the floating fraction in soy milk became inseparable before all of the protein had coagulated with calcium chloride and that all of the lipids are trapped when half of the soy milk protein had coagulated with calcium chloride.

Soy milk shows a colloidal dispersion consisting of lipid, protein, and so on. When the soy milk coagulated

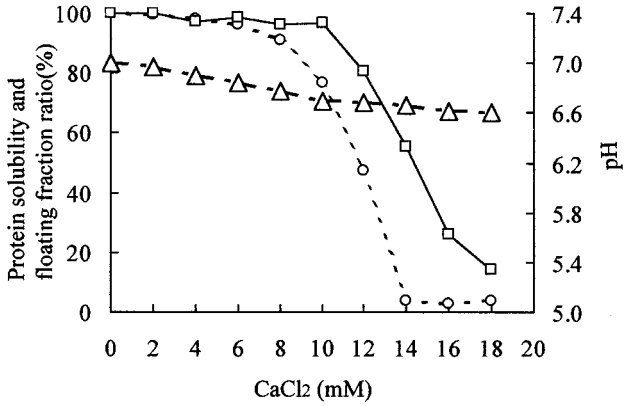

Figure 2. Changes in protein solubility and separable floating fraction ratio in soy milks containing 50 mM Bis-tris buffer (pH 7) by addition of calcium chloride: (□) protein solubility; (○) floating fraction ratio; (△) pH.

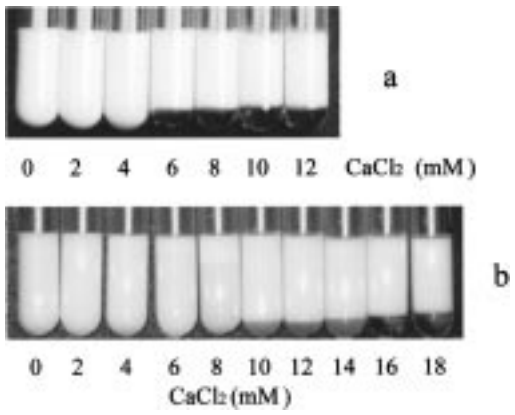

Figure 3. Phase changes in the floating fraction dispersion in ultrafiltrate by addition of calcium chloride. The floating fraction of soy milk was dispersed into ultrafiltrate of soy milk with and without 50 mM Bis-tris (pH 7). After calcium chloride was added to the dispersion from 0 to 18 mM, a series of the dispersions were kept at room temperature for 30 min and then stored in a refrigerator (4° C). Photographs were taken after 12 h of standing: (a) dispersion without buffer; (b) dispersion with 50 mM Bis-tris buffer (pH 7).

and formed a gel, the lipid droplets were located in the networks of the protein gel (Saio and Watanabe, 1968). To examine what happens on the lipid droplets with the addition of calcium chloride, the phase change of floating dispersions was observed after 12 h of standing at concentrations of 0−18 mM calcium chloride with and without 50 mM Bis-tris (pH 7.0) in a refrigerator (4 °C). The dispersion without calcium chloride was stable even after 12 h, as shown in Figure 3. In the series of dispersions without Bis-tris (Figure 3a), the phase separation occurred above 4 mM calcium chloride (the change is not clear at 4 mM in this photograph). The lipid droplets were completely flocculated and floated at 8 mM calcium chloride. In the series of dispersions containing Bis-tris, the phase separation occurred at twice the concentration of calcium chloride without Bis-tris, being between 8 and 16 mM (Figure 3b). These results indicate that the lipid droplets conjugated by themselves in the presence of calcium chloride. It is reported that the floating fraction contains 3% phospholipids (Ono et al., 1996) and 14% protein (Guo et al., 1997). The phospholipids have both a hydrophobic hydrocarbon chain and a hydrophilic polar group in a molecule and express an amphiphilic property in an aqueous solution. Therefore, phospholipids can enhance

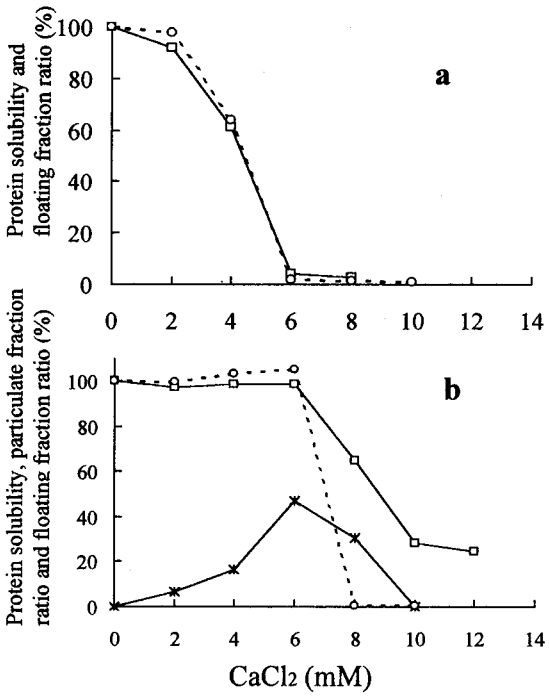

Figure 4. Changes in protein solubility and floating fraction ratio in dispersions of floating and particulate fractions (a) and floating and soluble fractions (b) at various concentrations of calcium chloride: (□) protein solubility; (○) floating fraction ratio; (*) newly formed particulate fraction ratio. The newly formed particulate fraction was obtained by centrifugation at 156000*g* for 30 min from the supernatant obtained by centrifugation of the dispersion of floating and soluble fractions at 2000*g* for 10 min in the presence of calcium chloride. The newly formed particulate fraction ratio is protein quantity of the newly formed particulate fraction against that of the dispersion of floating and soluble fractions.

the stability of the dispersion of protein or lipid (Kamat et al., 1978; Chen et al., 1985). The floating fraction was dispersed into the ultrafiltrate and formed a stable emulsion. This stability may be due to the fact that lipid droplets are covered by adsorbing phospholipids and proteins, which protect them from flocculation and coalescence. The hydrophobic surface of the oil droplets, therefore, changes to a hydrophilic one in aqueous solution. When calcium chloride is added, the ions on the surface will be suppressed and the droplets will come closer one another and flocculate. The concentration of calcium chloride at which the flocculation began (Figure 3) is in agreement with that at which the decrease of the floating fraction began in soy milk (Figures 1 and 2). These results suggest that the lipid droplets in soy milk combined with themselves and proteins by the addition of calcium chloride.

The protein in soy milk consists of particulate (>40 nm in diameter) and soluble proteins (Ono et al., 1991). In this study, the incorporation of lipid into the coagulum was examined from the conjugation of the lipid droplets with the particulate and soluble proteins. Soy milk was fractionated into floating, particulate, and soluble fractions by centrifugation. The mixed dispersions of the floating and particulate fractions or of the floating and soluble fractions were prepared. The floating fraction ratio and protein solubility in the floating and particulate fraction dispersion were analyzed at various concentrations of calcium chloride and are shown in Figure 4a. The decrease of the floating fraction ratio and the protein solubility in this dispersion

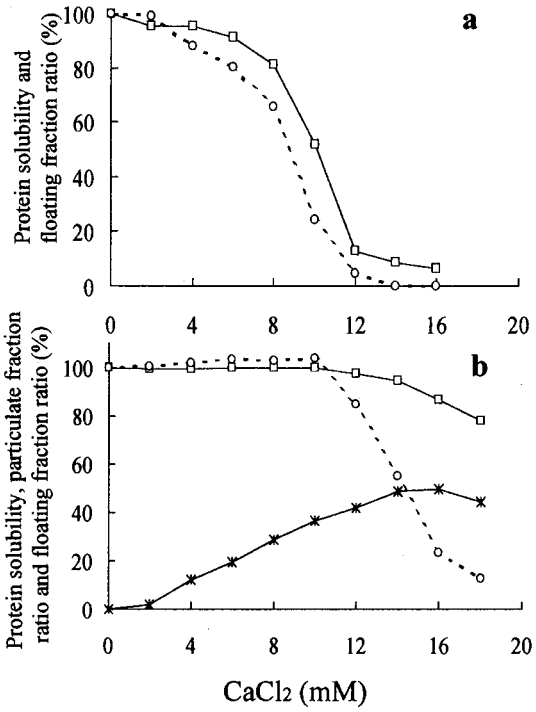

Figure 5. Changes in protein solubility and floating fraction ratio in both dispersions (presence of 50 mM Bis-tris buffer, pH 7) of floating fraction containing particulate protein (a) and soluble protein (b) by addition of calcium chloride: (□) protein solubility; (○) floating fraction ratio; (*) newly formed particulate fraction ratio.

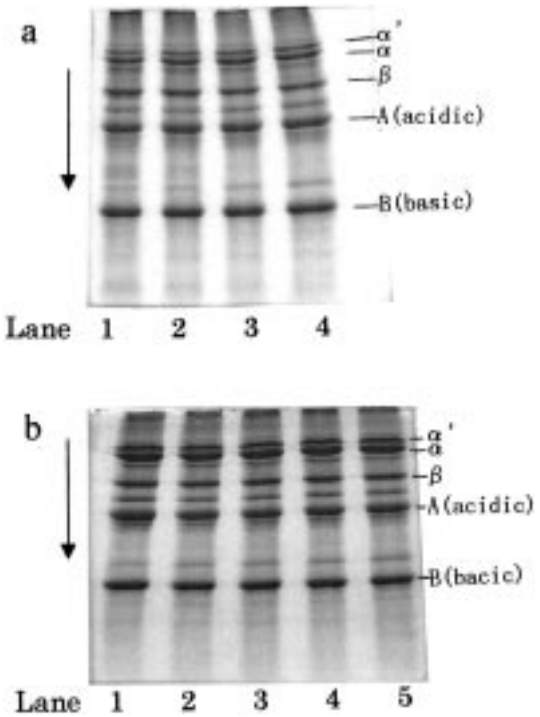

Figure 6. SDS−PAGE patterns of the protein in particulate fraction: (a) lanes 1, 2, 3, and 4 were obtained from soy milk at calcium chloride concentrations of 0, 4, 6, and 8 mM, respectively; (b) newly formed particulate fraction from the dispersion of the floating fraction containing soluble protein (lanes 1 and 2) and in the presence of 50 mM Bis-tris buffer (pH 7) (lanes 3−5) at various concentrations of calcium chloride. Lanes 1, 2, 3, 4, and 5 were obtained at calcium chloride concentrations of 6, 8, 10, 12, and 14 mM, respectively.

proceeded concurrently with the addition of calcium chloride from 2 to 6 mM, whereas in the floating and soluble fraction dispersion, the floating fraction ratio slightly increased with added calcium chloride up to 6 mM, as shown in Figure 4b. The increase may be due to conjugation of the lipid droplets with the soluble protein. This result indicates that the lipid droplets did not become inseparable even if these droplets associated with soluble protein. On the other hand, new particles formed and increased with added calcium chloride up to 6 mM. The ratio of floating fraction and protein solubility, then, decreased with the decrease of the newly formed particulate fraction at >6 mM calcium chloride. The decrease of floating fraction needed a higher concentration of calcium chloride than that in the floating and particulate fraction dispersion. These results suggest that the lipid droplets conjugated with particulate and soluble proteins in soy milk by the addition of calcium chloride and became inseparable by the association with particulate protein.

The floating and particulate or floating and soluble fraction dispersion containing 50 mM Bis-tris buffer (pH 7.0) was prepared for the measurements at constant pH. The separable floating fraction ratio and the protein solubility in various concentrations of calcium chloride are shown in Figure 5. The floating fraction ratio in the floating and particulate fraction dispersion decreased from 4 to 12 mM calcium chloride, and the decrease was parallel to that of the protein solubility as shown in Figure 5a. On the other hand, the floating fraction ratio in the floating and soluble fraction dispersion decreased at >10 mM as shown in Figure 5b. The newly formed particulate fraction increased with added calcium chloride up to 16 mM. The decrease of floating fraction ratio took place after the new particles formed. The lipid droplets conjugated with particulate and soluble proteins by the addition of calcium chloride and became inseparable by the association with particulate protein. The decreases of the floating fraction ratio and the protein solubility with Bis-tris buffer are similar to those without buffer (Figure 4) but took place at a higher concentration of calcium chloride than that without buffer. These results indicate that the protein particles are essential for the incorporation of lipids into aggregates (such as the tofu gel).

To clarify the difference in the composition between the original particles in soy milk and the newly formed particles from the soluble protein, the protein composition was analyzed by SDS−PAGE. The main subunits of the soy milk protein are developed successively into α′, α, and β subunits of β-conglycinin and acidic and basic subunits of glycinin (Guo et al., 1997), as shown in Figure 6. Lane 1 in Figure 6a shows protein bands from the particulate fraction in soy milk. The relative ratio among the bands was estimated from their stain intensity values, and it is estimated that the protein particle is composed of 40% β-conglycinin and 60% glycinin. Lanes 3, 4, and 5 and lanes 1 and 2 in Figure 6b show bands from the newly formed particulate fraction formed in the floating and soluble fraction dispersion with and without Bis-tris buffer, respectively. The ratio of these bands on each lane showed almost the same values and the newly formed particulate fraction consisted of 48% β-conglycinin and 52% glycinin at various calcium chloride concentrations. The newly formed particles contain more β-conglycinin than the original protein particles in soy milk. Glycinin consists of an acidic peptide (A) having an isoelectric point (p*I*) of 4.2−4.8 and a basic peptide (B) having a p*I* of 8.0−

8.5 (Badley et al., 1975; Utsumi et al., 1981). β-Conglycinin consists of α, α', and β subunits having p*I* values of 5.3, 5.2, and 5.8−6.2, respectively (Yamauchi et al., 1981; Sykes and Gayler, 1981). It is known that glycinin coagulates at a lower calcium chloride concentration than β-conglycinin (Saio et al., 1969, 1973). The newly formed particles containing more β-conglycinin coagulated at a higher calcium chloride concentration than the original particles in soy milk (Figures 4 and 5). These effects are due to the lower p*I* of β-conglycinin relative to that of glycinin. Lanes 2−4 in Figure 6a show bands from the particulate fractions in soy milk after calcium chloride had been added. These relative ratios of β-conglycinin slightly increased from 40 to 44% with increasing calcium chloride. This increase must be due to the joining of the newly formed particles from the soluble fraction to the original particles. The lipid droplets ultimately form into coagulates by conjugating with the original and the newly formed particles.

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