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Changes in Characters of Soybean Glycinin Groups I, IIa, and IIb Caused by Heating

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Soybean glycinin groups I, IIa, and IIb were purified from soybeans composed of only glycinin groups I, IIa, and IIb, respectively. When these protein solutions were heated, the amount of the particulate protein formed in these solutions was greatest in the order of groups IIa, IIb, and I. The protein solubilities decreased upon the addition of magnesium chloride in the order of groups IIa, IIb, and I. It was determined by differential scanning calorimetry analysis that the denaturation temperatures of groups I, IIa, and IIb were 92.8, 96.0, and 97.9 °C, and that the enthalpies of their transitions were 24.2, 27.4, and 28.1 J g^{-1}, respectively. The α-helix rates of groups I, IIa, and IIb in aqueous solution were analyzed by circular dichroism and were 19, 16, and 15%, respectively. The β-sheet rates of groups I, IIa, and IIb were 44, 38, and 39%, respectively. In all group proteins, the α-helix rates were decreased by heating and the β-sheet rates were increased. The surface hydrophobicity of these group proteins increased as a result of heating, and those of groups IIa and IIb were larger than that of group I. The surface hydrophobicity of these protein groups increased by heating, and those of groups IIa and IIb were larger than that of group I and β-conglycinin. Breaking stress of curds prepared from these group proteins containing more than 1 of β-conglycinin ration showed similar values, but the order of those containing less than 1 in strength was groups I, IIb and IIa. These results suggest that the increase of particulate contents and the curd formation are related to the increase of surface hydrophobicity by heating.

KEYWORDS: Soybean; glycinin; surface hydrophobicity; sulfhydryl (SH) group; breaking stress; differential scanning calorimetry; circular dichroism

INTRODUCTION

When soybean is used as a food, it is usually heated, like in the case of tofu, soymilk, aburaage, and yuba. The acceptability and texture of soybean products are influenced by the denaturation of soybean protein on heating. Glycinin and β-conglycinin are two major protein components of soybean, accounting for about 70% of the total protein in soybean seed (*1*). There are many reports in which the proteins were gelled by heating (*2*−*6*). It is known that the gelation of glycinin proceeds with increasing number of disulfide bonds (*7*), and that β-conglycinin molecules also contributed to the gel texture by interacting with each other (*8*).

Glycinin consists of acidic (A) and basic (B) peptides, linked by a disulfide bond (*9*), and β-conglycinin has α, α′, and β subunits. Glycinin, before protein processing, is a single peptide in which A and B subunits are linked to each other (*10*). The peptide has five genetic variants, named A1aB1b, A2B1a, A1bB2, A5A4B3, and A3B4 (*11*). They are classified into two groups, group I (A1aB1b, A2B1a, A1bB2) and group II (A5A4B3, A3B4), on the basis of their homology in subunit sequence (*12*). Recently, group II has been further classified into two subgroups, named group IIa (A5A4B3) and group IIb (A3B4), on the basis of genetic analysis (*13*). Three group proteins (groups I, IIa, and IIb) differ especially at the carboxyl-terminal end of the acidic subunit. In amino acid sequences of groups I and IIb, about 60 and 30 amino acid residues are deleted from group IIa, respectively (*12*). The properties of these group proteins have not been studied yet, because purification of these proteins had been difficult. We have now succeeded in the purification of these three group proteins. In this paper, we tried to elucidate the changes in conformational and curd-forming characters of each group protein caused by heating.

MATERIALS AND METHODS

Materials. Soybeans (*Glycine max* (L.) Merr.) from three lines were used in this study, differing in the glycinin group proteins. Each of these three lines has only glycinin group I, IIa, or IIb. These soybeans were bred by backcrossing between Tamahomare, a recurrent parent

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lacking a group IIa, and a line having only the group IIa (*13*). Backcrossing was done for two generations. All seeds were stored at 5 °C and used for this experiment within one year.

Purification of Each Group Protein and β-Conglycinin. The crude group proteins and β-conglycinin were prepared by the method of Thanh et al. (*14*). They were then purified by a slight modification of the method of Kitamura et al. (*15*). Soybean having only group I, IIa, or IIb was crushed and defatted with hexane. Defatted soybean meal was stirred with 0.03 M Tris-HCl buffer (pH 8.0) containing 0.01 M 2-mercaptoethanol for 1 h and centrifuged at 3000*g* for 30 min. The extract was adjusted to pH 6.4 with 2 N HCl and left to stand overnight at 5 °C. The suspension was centrifuged at 18000*g* for 20 min to obtain the precipitate, which was washed by 0.03 M Tris-HCl buffer (pH 6.4). These supernatants (Sup 1) were used for preparation of β-conglycinin. The precipitate was dissolved in 0.03 M Tris-HCl buffer (pH 7.8) containing 0.01 M 2-mercaptoethanol and centrifuged at 18000*g* for 20 min to obtain the supernatant. The supernatant was dialyzed against deionized water and lyophilized. The material thus obtained was termed the crude group protein. The crude group protein was used for the measurement of curd hardness. The crude group protein was dialyzed against 0.1 M phosphate buffer (pH 7.1) containing 1.0 M NaCl and 0.01 M 2-mercaptoethanol and mixed with Con A-Sepharose 4B (Pharmacia, Uppsala. Sweden), equilibrated with 0.1 M phosphate buffer (pH 7.1) containing 1.0 M NaCl and 0.01 M 2-mercaptoethanol. The mixture was stirred for 30 min and then filtrated. The filtrate was dialyzed against deionized water and lyophilized. The material thus obtained was termed the group protein. The group protein was used for various experiments, except for the measurement of curd hardness.

The crude β-conglycinin was prepared from above Sup 1, described above. Sup 1 was adjusted to pH 4.8 with 2 N HCl, stirred for 30 min, and centrifuged at 18000*g* for 20 min. The precipitate was washed by 0.03 M Tris-HCl buffer (pH 4.8), dissolved in 0.03 M Tris-HCl buffer (pH 8.0) containing 0.01 M 2-mercaptoethanol, and centrifuged at 18000*g* for 20 min. The supernatant was dialyzed against deionized water and lyophilized. The material thus obtained was termed the crude β-conglycinin. The crude β-conglycinin was used for the measurement of curd hardness. The crude β-conglycinin was dialyzed against standard buffer (0.1 M phosphate buffer (pH 7.1) containing 1.0 M NaCl and 0.01 M 2-mercaptoethanol) and mixed with Con A-Sepharose 4B (Pharmacia, Uppsala. Sweden), equilibrated with the standard buffer. The mixture was stirred for 30 min and filtrated. The Con A-Sepharose 4B was washed with standard buffer and then mixed with the same amount of standard buffer containing 1.0 M α-methyl-D-mannoside. The mixture was stirred for 30 min and filtrated. The filtrate was dialyzed against deionized water and lyophilized. The material thus obtained was termed β-conglycinin. The β-conglycinin was used for various experiments, except for the measurement of curd hardness.

Preparation of Protein Solution. Groups I, IIa, and IIb and β-conglycinin were dissolved in the deionized water and were termed groups I, IIa, and IIb and β-conglycinin solution, respectively. Unless otherwise noted, the heated protein solutions were prepared from these by heating at 97 °C for 5 min.

Preparation of Protein Particles. Protein fractions, which have different protein particles sizes, were prepared from the group proteins and β-conglycinin solutions before and after heating by the method of differential centrifugation (*16*). Protein particles of more than 100 nm in diameter were obtained as pellets by centrifugation at 32500*g* for 30 min from the group proteins and β-conglycinin solutions. Protein particles of 40−100 nm and less than 40 nm in diameter were obtained as pellets and supernatant, respectively, by centrifugation at 156500*g* for 30 min from the preceding supernatant. The particles of more than 100 nm, 40−100 nm, and less than 40 nm in diameter were termed large protein particles, medium protein particles, and supernatant protein, respectively. The contents of protein particles and supernatant protein were determined from the average of five runs.

Measurement of Protein Solubility. Measurement of protein solubility in the presence of magnesium chloride was performed following the method of Ono et al. (*17*). Each protein solubility of the heated group proteins and β-conglycinin solutions in various concentrations of magnesium chloride was determined by measuring the protein content of the supernatants after centrifugation at 1800*g* for 5 min.

Determination of Protein. Protein contents were measured by the method of Bradford (*18*).

Electrophoresis. SDS−polyacrylamide gel electrophoresis (SDS−PAGE) was carried out in a vertical slab gel of 1 mm thickness, using an alkaline discontinuous buffer system (*19*). Coomassie brilliant blue G-250 was used for protein staining following the method of Blakesley and Boezi (*20*). After electrophoresis, the gels were immersed in a staining solution containing 12% trichloroacetic acid for 12 h and then destained with water.

Densitometric analysis of the stained gels was carried out with a Bio-Rad Multi-Analyst instrument (Bio-Rad, Hercules, CA).

Differential Scanning Calorimetry (DSC). The calorimetry was carried out on a Pyris 1 differential scanning calorimeter (Perkin-Elmer, Wellesley, MA) within a temperature range of 30−110 °C, at scanning rate of 10 °C/min. Sixty microliters of 10% groups I, IIa, and IIb in the 10 mM glycine buffer (pH 8.0) containing 0.3 M NaCl were sealed in a hermetic pan. The reference pan was filled with 60 μL of 10 mM glycine buffer (pH 8.0) containing 0.3M NaCl and was sealed. Thermal stability was expressed as T_m (peak temperature), which is closely related to the temperature of denaturation. Enthalpies were calculated by using the Perkin-Elmer software, based on integration of the area of the transition. The calorimetric results were determined from the average of five runs.

Measurement of Circular Dichroism (CD). Circular dichroism spectra between 200 and 250 nm were obtained by using a Jasco J-720 spectropolarimeter (Jasco Co., Tokyo, Japan) equipped with a water bath to control the temperature at 25 °C. A solution of 5 mg/mL of group I, IIa, or IIb or β-conglycinin in 10 mM phosphate buffer (pH 8.0) containing 30 mM NaCl was placed in a cell of 1.0 mm path length. Ten scans were averaged to obtain each spectrum. To calculate θ, molecular weights of 54 800, 63 700, 58 000, and 201 000 and amino acid residue numbers of 468, 537, 493, and 1683 were used for groups I, IIa, and IIb (*12*) and β-conglycinin associate (*21*−*23*), respectively. To allow analysis of the features of the structural changes, secondary structure compositions of the proteins were calculated from the CD spectra by the method of Provencher (*24*).

Determination of Surface Hydrophobicity. Changes in the surface hydrophobicity of protein during heating were investigated by using 8-anilino-1-naphthalenesulfonic acid (ANS). Solutions of 230 μL of 2.5% of group I, IIa, or IIb or β-conglycinin in 10 mM glycine buffer (pH 8.0) were heated at elevated temperature for 5 min. A 35-μL portion of each of these solutions was filled up to 1.0 mL with deionized water, and 2.0 mL of various concentrations of ANS was added to the samples. These solutions were left at room temperature for 2 h. The fluorescence intensity of the solutions was measured at an excitation wavelength of 375 nm and an emission wavelength of 475 nm with an RF-5300PC fluorometer (Shimadzu Co., Tokyo, Japan).

Measurement of Sulfhydryl (SH) Group Content. Changes in the sulfhydryl (SH) group content during heating were determined by a slight modification of the method of Obata et al. (*25*). Solutions of 230 μL of 2.5% of groups I, IIa, and IIb and β-conglycinin in 10 mM glycine buffer (pH 8.0) were heated at elevated temperature for 5 minutes. To a 200-μL portion of each solution were added 1.8 mL of deionized water, 1.0 mL of 0.2 M phosphate buffer (pH 6.8), and 500 μL of ethanol containing 5.0×10^{-4} M 2,2′-dithiobis(5-nitropyridine) (DTNP), and the resulting solutions were termed the P-samples. In a subsequent dilution, instead of protein solution, deionized water was used, and the resultion solutions were termed the S-blanks. Ethanol was added instead of ethanol containing DTNP, and the resulting solutions were termed the R-blanks. These were left for 30 min at room temperature and centrifuged at 3000*g* for 30 min. The absorbance of the supernatant was measured at 386 mm by using a UV-1200 UV−vis spectrophotometer (Shimadzu Co., Tokyo, Japan). Test values were determined by subtracting S-blank and R-blank from P-sample. SH group contents were determined from the test values using a molecular extinction coefficient of 14 700.

Preparation of Protein Curd. The mixtures of crude group proteins and/or β-conglyinin were dissolved to 5% solution. Three-milliliter portions of each of them were preincubated at 97 °C for 2 min and

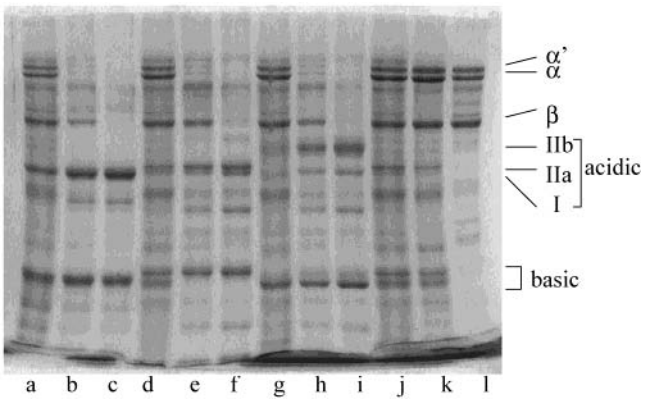

Figure 1. SDS–PAGE patterns of the group proteins and β-conglycinin. a, d, and g are proteins extracted from soybean having only groups I, IIa, and IIb, respectively. j is protein extracted from Tamahomare. b, e, and h are crude groups I, IIa, and IIb, respectively. k is crude β-conglycinin. c, f, and i are groups I, IIa, and IIb, respectively. l is β-conglycinin.

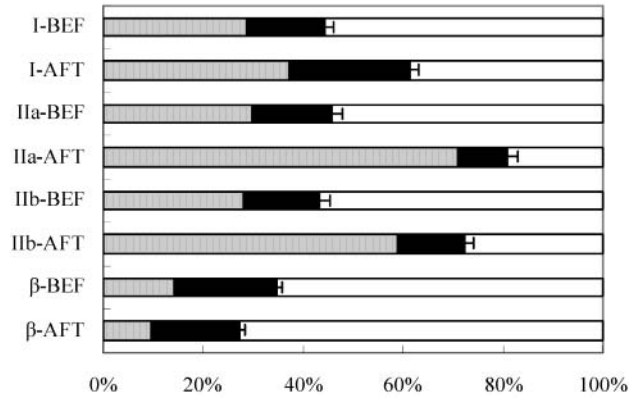

Figure 2. Distribution of the protein particles obtained by differential centrifugation from 2% groups I (I), IIa (IIa), IIb (IIb), and β-conglycinin (β) in aqueous solution, before (BEF) and after (AFT) heating at 97 °C for 5 min. The particles of more than 100, 40–100, and less than 40 nm in diameter were assigned as large protein particle (gray bars), medium protein particle (black bars), and supernatant protein (white bars), respectively. Values are means ± standard deviation (sum of large and medium protein particles); $n = 5$ per group.

then quickly cooled to 5 °C. Thirty-eight microliters of a magnesium chloride solution (50 g of $MgCl_2 \cdot 6H_2O$ + 5.0 g of NaCl/200 mL of H_2O) was added to the cooled crude protein solution, which was then heated for 30 min at 85 °C to prepare protein curd. Protein curd thus prepared was then kept at 10 °C

Measurement of Curd Hardness. A compression test was carried out at a compression rate of 6.0 cm/min using a Sun Rheo Meter model CR-200D (Sun Scientific Co., Ltd., Tokyo, Japan) with a cylindrical plunger of 1 cm diameter. The breaking stress value of protein curd was expressed as the mean of five measurements for each sample.

RESULTS AND DISCUSSION

Isolation of Groups I, IIa, and IIb and β-Conglycinin. Crude group proteins of glycinin and crude β-conglycinin were prepared by the method of Thanh et al. (*14*), and group proteins and β-conglycinin were purified by using Con A-Sepharose 4B. **Figure 1** shows the SDS−PAGE patterns of crude and purified group proteins of glycinin and β-conglycinin. The purities of groups I, IIa, and IIb and β-conglycinin prepared by the method of Thanh et al. were 88.1, 78.2, 82.1, and 88.8%, respectively, and were increased to 94.2, 89.4, 88.9, and 91.1%, respectively, by using Con A-Sepharose 4B. The purified proteins were used for the experiments on group proteins of glycinin and β-conglycinin.

Particulate Protein in the Solution of Group Proteins and β-Conglycinin. It is known that half of soy milk protein exists in the form of particles which are more than 40 nm in diameter (*16*), and that the particulate content increases with increasing glycinin content (*26*, *27*). In the case of identical levels of glycinin content, the amount of particulates in soy milk was greatest in the order of groups IIa, IIb, and I, as described previously (*26*). The group IIa had the highest ability to form particles among these glycinin group proteins. It was reported that glycinin in solution formed gel and aggregates by heating (*2*). It is presumed that a part of glycinin forms particles in solution. The distribution of protein particles in 2% protein solution of groups I, IIa, and IIb and β-conglycinin was analyzed before and after heating. The results are shown in **Figure 2**. The proportion of protein particles in heated protein solution was higher in each group protein than that before heating. The proportion of protein particles in heated β-conglycinin solution was less than that before heating. The particulate contents after heating were greatest in the order of groups IIa, IIb, and I and β-conglycinin. Group IIa formed particles most easily among these proteins. The particulate content after heating was greatest in the order groups IIa, IIb, and I, in agreement with the results of soy milk containing groups I, IIa, and IIb of glycinin (*26*).

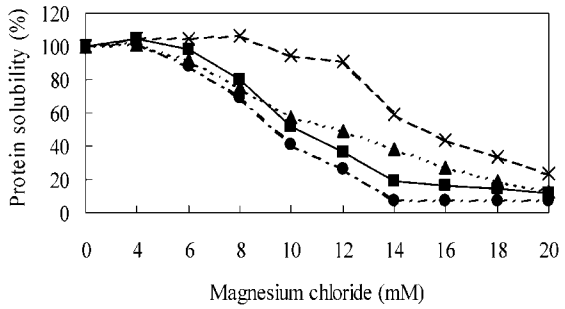

Figure 3. Changes in protein solubility in 2.5% groups I (■), IIa (●), and IIb (▲) and β-conglycinin (×) in solutions with the addition of magnesium chloride. The samples were evaluated after heating at 97 °C for 5 min.

Effects of Magnesium Ion on Solubility of Group Proteins. When magnesium chloride was added to soy milk, the ion concentration leading to a decrease in the protein solubility was different, based on the protein composition (*28*). The protein solubility of the group proteins and β-conglycinin was measured at various magnesium chloride concentrations. Each 2.5% solution of group proteins or β-conglycinin was prepared with deionized water, and the changes in the protein solubility caused by the addition of magnesium chloride were measured after heating. The results are shown in **Figure 3**. The lowest range that led to a decrease in the protein solubility in group IIa was between 6 and 14 mM magnesium chloride. Decreases in protein solubility for group IIb were observed between 6 and 20 mM, and between 8 and 16 mM for group I. β-Conglycinin solubility decreased at 10 mM or more of magnesium chloride as the highest range. The protein solubility after heating decreased in the order of groups IIa, IIb, and I and β-conglycinin, the same as that of the particulate fraction (**Figure 2**). It has been known that the protein particulate fraction in soy milk precipitates at lower calcium chloride concentration than the supernatant protein (*17*). It has also been known that soy milk contains more particulate protein precipitates at lower magnesium chloride concentrations (*29*). Thus, this experiment shows that, after heating, group IIa, containing the most particulate protein,

Table 1. Differential Scanning Calorimetry Denaturation Temperatures and Calorimetric Denaturation Enthalpies of the Group Proteins[a]

sample	high temperaturepeak T_m (°C)	enthalpy ΔH (J g^{-1})
group I	92.8 ± 0.5	24.2 ± 1.2
group IIa	96.0 ± 0.4	27.4 ± 1.0
group IIb	97.9 ± 0.2	28.1 ± 0.9

[a] Groups I, IIa, and IIb were dissolved in 10 mM glycine buffer (pH 8.0) containing 0.3 M NaCl. Values are means ± standard deviation; $n = 5$ per group.

precipitated at the lowest magnesium concentration, and β-conglycinin, containing the least particulate protein, precipitated at the highest magnesium concentration.

Denaturation Temperatures of Group Proteins. After heating, the order of particulate protein contents was groups IIa, IIb, and I and β-conglycinin (**Figure 2**), and the decreasing protein solubility resulting from addition of magnesium chloride followed the same order. The particulate fraction was formed by heating from the soybean proteins. To determine the denaturation temperatures (T_m) and the enthalpies of the transitions of the group proteins, DSC experiments were performed. The results are shown in **Table 1**. The T_m values of groups I, IIa, and IIb were 92.8, 96.0, and 97.9 °C, respectively. The T_m value of group IIb was the highest. The enthalpies of groups I, IIa, and IIb were 24.2, 27.4, and 28.1 J g^{-1}, respectively. Grinberg et al. (*30*) reported that the T_m value and enthalpy of glycinin were 92 °C and 25 J g^{-1} at pH 8.0, and the ion strength was 0.3. The T_m values and enthalpies we found were similar to Grinberg's data. These values agreed with those of group I. The values of groups IIa and IIb were larger than that of group I, indicating that the holding power of the molecule may be higher than that of group I. It means that the higher holding power is due to the higher hydrophobicity inside of the molecule. Therefore, when the hydrophobic regions were exposed by heat denaturation, the hydrophobic attraction of the surface must be enhanced more than that of group I. These results suggest that the particulate protein content from group I was less than those of groups IIa and IIb.

Changes in Secondary Structure of Group Proteins Caused by Heating. The secondary structures of the group proteins and β-conglycinin before and after heating (97 °C, 5 min) were analyzed by using circular dichroism spectrometry between 200 and 250 nm. The results are shown in **Figure 4**. The spectrum of group I after heating was almost unchanged from that before heating (**Figure 4A**). In groups IIa and IIb, the θ values between 205 and 230 nm were greatly increased by heating (**Figure 4B,C**). In β-conglycinin, the θ values between 200 and 220 nm were decreased a little by heating (**Figure 4D**). The ratios of α-helix, β-sheet, β-turn, and random structures were calculated from these CD spectra by the method of Provencher (*24*), and the results are shown in **Table 2**. In all group proteins, α-helix and β-turn contents were decreased by heating, and β-sheet was increased. The β-sheet contents of groups IIa and IIb were increased more than that of group I by heating. Ker et al. (*31*) reported that α-helix and β-sheet contents of glycinin were about 8 and 30%, respectively. In this experiment, the α-helix ratios of groups I, IIa, and IIb were 19, 16, and 15%, respectively, about 2 times larger than Ker's data. The β-sheet contents of groups I, IIa, and IIb were 44, 38, and 39%, respectively, a little larger than Ker's data. It has been reported that the α-helix rate of β-conglycinin is 15% and increases to 20% upon heating at 95 °C for 30 min, and that the β-sheet rate of β-conglycinin is 50% and decreases to 20% upon heating at the same conditions (*32*). The same protein pointed out that the α-helix and β-sheet contents changed upon heating and then returned to near native values after cooling.

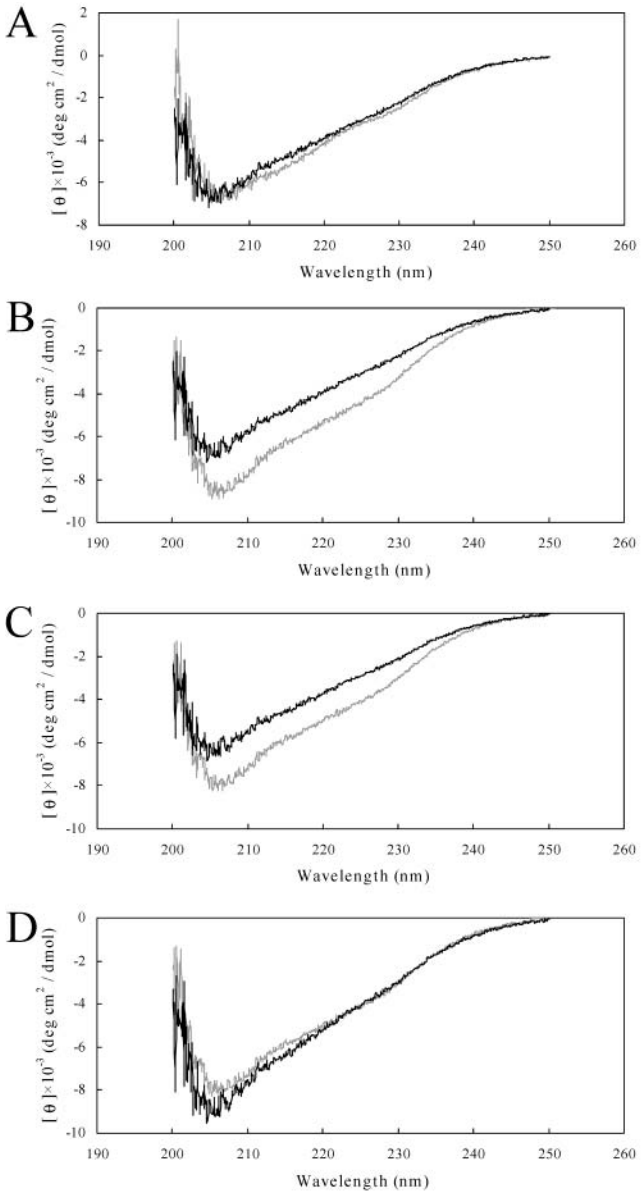

Figure 4. Circular dichroism spectrometry (CD) spectra of groups I (A), IIa (B), and IIb (C) and β-conglycinin (D) in 10 mM phosphate buffer (pH 8.0) containing 30 mM NaCl. The samples were evaluated before (gray line) and after (black line) heating at 97 °C for 5 min. The CD spectra were measured between 200 and 250 nm using a cell path length of 1.0 mm at 25 °C. To calculate θ, molecular weights of 54 800, 63 700, 58 000, and 201 000 and numbers of amino acids residues of 468, 537, 493, and 1683 were used for groups I, IIa, and IIb and β-conglyicinin, respectively. Ten scans were averaged to obtain each spectrum.

Table 2. Ratio of Secondary Structure of the Group Proteins and β-Conglycinin before and after Heating by Provencher's Method[a]

	group I		group IIa		group IIb		β-conglycinin	
	before	after	before	after	before	after	before	after
α-helix	19	10	16	10	15	10	15	15
β-sheet	44	50	38	50	39	50	39	50
β-turn	21	17	20	17	21	18	21	15
random	17	23	25	23	25	23	25	21

[a] Values are reported as %.

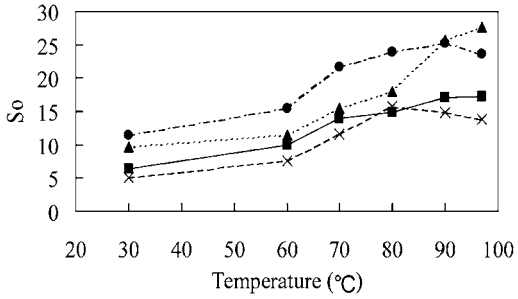

Figure 5. Changes in surface hydrophobicity of groups I (■), IIa (●), and IIb (▲) and β-conglycinin (×) in 10 mM glycine buffer (pH 8.0) after being heated for 5 min at elevated temperature. A slope (S_o) was calculated from a plot of fluorescence intensity against 8-anilino-1-naphthalenesulfonic acid (ANS) concentration from 0 to 2.0×10^{-5} M.

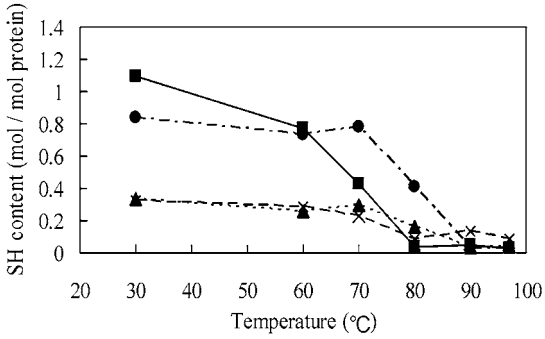

Figure 6. Change in sulfhydryl group contents of groups I (■), IIa (●), and IIb (▲) and β-conglycinin (×) in 10 mM glycine buffer after being heated for 5 min at elevated temperature.

In this experiment (**Table 2**), the α-helix rate did not change but the β-sheet rate increased from 39 to 50%. This increase might depend on the mutual association of these molecules caused by heating.

Changes in Surface Hydrophobicities of Group Proteins Caused by Heating. It has been proposed that the curd formation of soy milk is related to the increase of hydrophobicity due to the denaturation of protein by heating (*16*). It has also been known that surface hydrophobicity of glycinin was increased by heating (*33*). However, the change in surface hydrophobicity of each group protein caused by heating has not been clear yet. Therefore, the change in surface hydrophobicity of each group protein caused by heating was examined. The group proteins and β-conglycinin were heated for 5 min at 30, 60, 70, 80, 90, and 97 °C, respectively. The fluorescence intensities of ANS bound to protein surface were then measured. As an indication of surface hydrophobicity, a slope (S_o) was determined from the plot of the fluorescence intensity against ANS concentration from 0 to 2.0×10^{-5} M. The changes in the S_o of each group protein as a result of heating are shown in **Figure 5**. The S_o of groups I and IIa increased from 60 to 90 °C. The S_o of group IIb increased from 60 to 97 °C. The S_o of β-conglycinin increased from 60 to 80 °C. In DSC data (**Table 1**), the T_m of group IIb was 97.9 °C, the highest value in these proteins. Group IIb denatured at higher temperature than groups I and IIa, and the increase of hydrophobicity took place at higher temperature than those of the groups I and IIa. The values of S_o's of groups IIa and IIb were 24 and 28, and those of group I and β-conglycinin were 17 and 14 at 97 °C, respectively. Therefore, it was shown that the surface hydrophobicity of groups IIa and IIb was much larger than that of group I and β-conglycinin. In the CD data described above (**Figure 4**), the change in secondary structures of groups IIa and IIb caused by heating was greater than that of group I and β-conglycinin, and the β-sheet rates of groups IIa and IIb were increased more by heating than those of group I and β-conglycinin (**Table 2**). These results suggest that the greater change in secondary structures of groups IIa and IIb caused by heating introduces the more hydrophobic surface of these proteins. On the other hand, the particulate content in protein solutions was greatest in the order of groups IIa, IIb, and I (**Figure 2**). If the hydrophobic surface of the protein is larger, protein association is accelerated and protein particles should be formed. The surface hydrophobicities of groups IIa and IIb were larger than those of group I and β-conglycinin. Therefore, groups IIa and IIb must form the protein particles more easily. After heating, group IIa was precipitated at the lowest magnesium concentration among the group proteins and β-conglycinin, and β-conglycinin was precipitated at the highest magnesium concentration (**Figure 3**). It is known that protein particles are precipitated at lower calcium concentration than soluble proteins (*17*). It is suggested from these results that protein with higher surface hydrophobicity forms protein particles more easily and could aggregate at lower magnesium concentration.

Changes in SH Group Content of Group Proteins Caused by Heating. It is well known that protein gelation occurs as the result of making intermolecular disulfide (S−S) bonds from SH groups and hydrophobic bonds by intermolecular interaction. Participation of the SH group has been known in the gel formation of soy protein upon heating (*3*). Since the primary structure of each group protein had already been decided, the numbers (mol/mol) of cysteine residues in groups I, IIa, and IIb were 10 or 11, 8, and 8, respectively (*12*). Changes in SH group contents of the group proteins caused by heating were examined. The group proteins and β-conglycinin were heated for 5 min at elevated temperatures, and SH group contents were measured. The results are shown in **Figure 6**. The SH group contents of these proteins were decreased above 70 °C, expect for that of group I, which was decreased already at 60 °C. The SH group contents at 30 °C were greatest in the order of groups I, IIa, β-conglycinin, and group IIb. The SH group content of group I decreased extremely upon heating and approached 0 mol at 80 °C. The decrease of SH group content in group IIb and β-conglycinin was gentler than in the other proteins. The amount of SH group after heating decreased in the order of groups I, IIa, and IIb and β-conglycinin. It has been known that the SH group content in soy protein isolate was decreased by heating (*34*). Decreasing the SH group content brought about the formation of S−S bond. Group I, therefore, would form a S−S bond more easily by heating, and β-conglycinin and groups IIa and IIb would make less of this bond.

Breaking Stresses of Curds Prepared from Group Proteins. We reported previously that, for tofu curds made from soybeans having groups I, IIa, and IIb, after adjustment to the same protein concentration, the order of hardness according to their breaking stress was IIa, IIb, and I (*26*). To make clear the relationship between the group proteins and the curd hardness, the breaking stress of curds prepared from these group proteins was measured. A curd was prepared from a mixed solution of crude group proteins and β-conglycinin after preincubation. **Figure 7** show the breaking stress values of protein curds at a preincubation time of 2 min at 97 °C. The breaking stress of curd prepared from group I increased with increasing glycinin content. The breaking stress of the curd from groups IIa and IIb had maximum values in the mixture ratio of 4:6 glycinin to β-conglycinin, and showed the lowest value without β-conglycinin. When the ratio of glycinin to β-conglycinin was

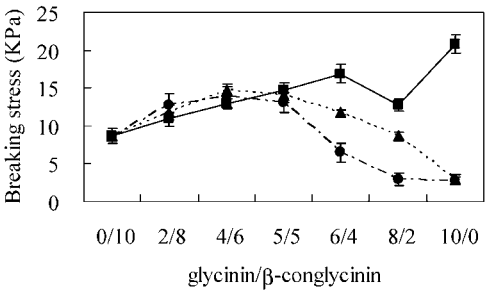

Figure 7. Effects of glycinin/β-conglycinin ratio on the breaking stress of groups I (■), IIa (●), and IIb (▲) curds made by the addition of magnesium chloride. Three-milliliters samples of 5% crude proteins were preincubated at 97 °C for 2 min and then quickly cooled at 5 °C, and then 38 μL of a magnesium chloride solution (50 g of $MgCl_2 \cdot 6H_2O$ + 5.0 g of NaCl/200 mL of H_2O) was added. The solution was heated at 85 °C for 30 min to make curd. Values are means ± standard deviation; $n = 5$ per group.

10:0, the curds from groups IIa and IIb did not form normally, and the breaking stress value of the curd from group I was higher than others. These results indicate that since the surface hydrophobicities of heated groups IIa and IIb were significantly higher than those of group I and β-conglycinin (**Figure 5**), the curd of holding water became hard by reducing β-conglycinin ratio. However, group I having low hydrophobic surface made a curd without β-conglycinin and showed high breaking stress. Therefore, it shows that a tofu made from normal soybeans containing group proteins and β-conglycinin is easy but that from soybeans not having β-conglycinin is hard, except from soybeans having only group I.

CONCLUSIONS

The solutions of groups IIa and IIb, after heating, had much more particulate proteins than the group I solution. The protein solubility, in response to addition of magnesium chloride, decreased faster in groups IIa and IIb than in group I. The increase of surface hydrophobicity in groups IIa and IIb upon heating was larger than that in group I. The SH group content of group I was decreased by heating more than those of groups IIa and IIb. The breaking stress value of group I curd was larger than those of groups IIa and IIb curds. From these results, it is suggested that the surface hydrophobicity of glycinin molecules is an important factor in determining the particulate content and the curd formation.

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LITERATURE CITED

(1) Hill, J. E.; Beridenbach, R. W. Protein of soybean seeds. *Plant Physiol.* **1974**, *53*, 742−746.
(2) Mori, T.; Nakamura, T.; Utsumi, S. Gelaiton mechanism of soybean 11S globulin: formation of soluble aggregates as transient intermediates. *J. Food Sci.* **1981**, *47*, 26−30.
(3) Yamagishi, T.; Miyakawa, A.; Noda, N.; Yamauchi, F. Isolation and electrophoretic analysis of heat-induced products of mixed soybean 7S and 11S globulins. *Agric. Biol. Chem.* **1983**, *47*, 1229−1237.
(4) Nakamura, T.; Utsumi, S.; Mori, T. Network structure formation in thermally induced of glycinin. *J. Agric. Food Chem.* **1984**, *32*, 349−352.
(5) 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.
(6) Nagano, T.; Mori, H.; Nishinari, K. Effects of heating and cooling on the gelation kinetics of 7S globulin from soybean. *J. Agric. Food. Sci.* **1994**, *42*, 1415−1419.
(7) Yamauchi, F.; Yamagishi, T.; Iwabuchi, S. Molecular understanding of heat-induced phenomena of soybean protein. *Food Rev. Int.* **1991**, *7*, 283−322.
(8) Petruccelli, S.; Añón, M. C. pH-induced modifications in the thermal stability of soybean protein isolates. *J. Agric. Food Chem.* **1996**, *44*, 3005−3009.
(9) Staswick, P. E.; Hermodson, M. A.; Nielsen, N. C. Identification of the cystines which link the acidic and basic components of glycinin subunits. *J. Biol. Chem.* **1984**, *259*, 13431−13435.
(10) Barton, K. A.; Thompson, J. F.; Madison, J. T.; Rosenthal, R.; Jarvis, N. P.; Beachy, R. N. The biosyntehsis and processing of high molecular weight precursors of soybean glycinin subunits. *J. Biol. Chem.* **1982**, *257*, 6089−6095.
(11) Nielsen, N. C. The structure and complexity of the 11S polypeptides in soybeans. *J. Am. Oil Chem. Soc.* **1985**, *62*, 1680−1686.
(12) Nielsen, N. C.; Dickinson, C. D.; Cho, T.; Thanh, V. H.; Scallon, B. J.; Fisher, R. L.; Sims, T. L.; Drews, G. N.; Goldberg, R. B. Characterization of the glycinin gene family in soybean. *The Plant Cell* **1989**, *1*, 313−328.
(13) Yagasaki, K.; Kaizuma, N.; Kitamura, K. Inheritance of glycinin subunits and characterization of glycinin molecules lacking the subunits in soybean (*Glycine max* (L.) Merr.). *Breed. Sci.* **1996**, *46*, 11−15.
(14) Thanh, V. H.; Okubo, K.; Shibasaki, K. Isolation and characterization of the multiple 7S globulin of soybean proteins. *Plant Physiol.* **1975**, *56*, 19−22.
(15) Kitamura, K.; Okubo, K.; Shibasaki, K. The purification of soybean 11S globulin with Con A-sepharose 4B and Sepharose 6B. *Agric. Biol. Chem.* **1974**, *38*, 1083−1085.
(16) Ono, T.; Choi, M. R.; Ikeda, A.; Odagiri, S. Changes in the composition and size distribution of soymilk protein particles by heating. *Agric. Biol. Chem.* **1991**, *55*, 2291−2297.
(17) Ono, T.; Katho, S.; Mothizuki, K. Influence of calcium and pH on protein solubility in soybean. *Biosci., Biotechnol., Biochem.* **1993**, *57*, 24−28.
(18) Bradford, R. W. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **1976**, *72*, 248−254.
(19) Laemmli, U. K. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **1970**, *227*, 680−685.
(20) Blakesley, R. W.; Boezi, J. A. A new staining technique for protein in polyacrylamide gels using coomassie brilliant blue G250. *Anal. Biochem.* **1977**, *82*, 580−582.
(21) Coates, J. B.; Medeiros, J. S.; Thanh, V. H.; Nielsen, N. C. Characterization of the subunits of β-conglycinin. *Arch. Biochem. Biophys.* **1985**, *243*, 184−194.
(22) Doyle, J. J.; Schuler, M. A.; Godette, W. D.; Zenger, V.; Beachy, R. N.; Slightom, J. L. The glycosylates seed storage proteins of Glycine max and Phaseolus vulgaris. Structural homologies of genes and proteins. *J. Biol. Chem.* **1986**, *261*, 9228−9238.
(23) Sebastiani, F. L.; Farrell, L. B.; Schuler, M. A.; Beachy, R. N. Complete sequence of cDNA of a subunits of soybean β-conglycinin. *Plant Mol. Biol.* **1990**, *15*, 197−201.
(24) Provencher, S. W. A constrained regularization method for inverting date represented by linear algebraic or integral equations. *Comput. Phys. Commun.* **1982**, *27*, 213−227.
(25) 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).

(26) Tezuka, M.; Taira, H.; Igarashi, Y.; Yagasaki, K.; Ono, T. Properties of tofus and soy milk prepared from soybean having different subunits of glycinin. *J. Agric. Food Chem.* **2000**, *48*, 1111−1117.

(27) Guo, S.-T.; Tsukamoto, C.; Takahashi, K.; Yagasaki, K.; Nan, Q.-X.; Ono, T. Incorporation of soymilk lipid into soy-protein coagulum by the addition of calcium chloride. *J. Food Sci*. **2002**, *67*, 3215−3219.

(28) Lakemond, C. M. M.; Jongh, H. H. J.; Hessing, M.; Gruppen, H.; Voragen, A. G. J. Heat denaturation of soy glycinin: Influence of pH and ionic strength on molecular structure. *J. Agric. Food Chem.* **2000**, *48*, 1991−1995.

(29) Tezuka, M.; Ono, T.; Ito, T. Properties of soymilk prepared from soybeans of different varieties. *Nippon Shokuhin Kagaku Kogaku Kaishi* **1995**, *42*, 556−561 (in Japanese).

(30) Grinberg, V. Y.; Danilenko, A. N.; Burova, T. V.; Tolstoguzov, V. B. Conformation stability of 11S globulins from seed. *J. Sci. Food Agric.* **1989**, *49*, 235−248.

(31) Ker, Y.; Chen, R.; Wu, C. Relationship of secondary structure, microstructure, and mechanical properties of heat-induced gel of soy 11S globulin. *Biosci., Biotechnol., Biochem.* **1993**, *57*, 536−541.

(32) Nagano, T.; Akasaka, T.; Nishinari, K. Study on the heat-induced conformation change of β-conglycinin by FT-IR and CD analysis. *Food Hydrocolloids* **1995**, *9*, 83−89.

(33) Matsudomi, N.; Mori, H.; Kato, A.; Kobayashi, K. Emulsifying and foaming properties of heat-denaturated soybean 11S globulin in relation to their surface hydrophobicity. *Agric. Biol. Chem.* **1985**, *49*, 915−919.

(34) Shimada, K.; Cheftel, J. C. Determination of sulfhydyl groups and disulfide bonds in heat-induced gels of soy protein isolate. *J. Agric. Food Chem.* **1988**, *36*, 147−153.

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