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Effects of Thermal Treatment on the Coagulation of Soy Proteins Induced by Subtilisin Carlsberg

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The effects of thermal treatment on the subtilisin Carlsberg-induced coagulations of soy protein isolate (SPI) and soy proteins in 7S and 11S fractions, most of which are β-conglycinin and glycinin, respectively, were examined by measuring the turbidity (OD_{660}) of the reaction solutions. With the treatment at 37−60 °C, the turbidity did not increase at all by the proteolysis, while with the treatment at 70−96 °C, it drastically increased. The degree of the coagulation is the highest for the treatment at 80 °C and the most remarkable for 11S soy protein. Changes in the sodium dodecyl sulfate−polyacrylamide gel electrophoresis pattern of the digests during the proteolysis were in good agreement with those in the turbidities for SPI and 7S and 11S soy proteins. Circular dichroism analysis revealed that the amounts of nonstructured protein in SPI and 7S and 11S soy proteins were initially 40−50%, increased to 55−60% by the treatment at 80 °C, and further increased to 65−75% by the proteolysis. The maximum fluorescence intensity of SPI and 7S and 11S soy proteins increased with an increase in the incubation temperature up to 80 °C. These findings suggest that the thermal treatment at 80 °C most effectively changes the secondary structure of soy proteins and renders them coagulate when hydrolyzed by subtilisin Carlsberg.

KEYWORDS: Coagulation; soy protein; subtilisin Carlsberg; thermal treatment; turbidity

INTRODUCTION

Soybean is a major source of plant oil. About 150 million tons of soybeans are produced every year, and a large amount of defatted soybean meals remains after the extraction of oil. They are in part used as feed but are hardly utilized in food industries (*1*). Defatted soybean meals contain proteins with high nutritional values and functional properties. In addition, it has been reputed that a soy protein diet lowers blood cholesterol (*2*) and triglyceride (*3, 4*) levels. The proteolytic digests of soy protein also have bioactive effects such as insulin-mediated antilipolysis (*5*), insulin-mediated glucose transport and lipogenesis (*6*), and lowering of blood pressure (*7*), suggesting that soy protein and its digests could be useful for functional foods.

There have been some reports on the coagulation of soy proteins treated by protease (*8–10*). Aggregation and gelation of whey proteins induced by the proteases from *Bacillus licheniformis*, the main component of which is subtilisin Carlsberg, have also been reported (*11, 12*). In protease digestion of soy proteins, some of the digests coagulate and remain insoluble, resulting in lower yields of the soluble digests. The mechanism and characteristics of the protease-induced coagulation of soy protein isolate (SPI) must be different from those of Ca^{2+}- and/or Mg^{2+}-mediated coagulation (tofu) and heat-induced coagulation (yuba). We have found that subtilisin Carlsberg induced the coagulation more strongly than other proteases such as chymotrypsin, papain, trypsin, and subtilisin BPN' (Inouye, K., unpublished data), and we have examined the mechanism and characteristics (*13, 14*). If the mechanism of protease-induced coagulation is elucidated, the coagulation might be controlled, leading to the expansion of the use of soy proteins. We developed a convenient method to follow the protease-induced coagulation of SPI by continuously measuring the turbidity of the reaction solution (*13*). During the hydrolysis, the turbidity decreased in the first phase due to the digestion of SPI to reach the minimum (phase 1) and then increased due to the coagulation of the digests to reach the maximum (phase 2). After it reached the maximum, it decreased again slowly due to the digestion of the coagula (phase 3). On the basis of these observations, we demonstrated that the SPI digests participating in the coagulation are produced mainly in phase 2 but not in phase 1 (*14*). We showed that the fragments formed in phase 2 were involved in the coagulation through hydrophobic interactions (*14*) and reported that the proteolytic digestion of SPI promotes the removal of hexanal, one of the major compounds of the unfavorable soy bean odor, by the treatment with various absorbents (*15*).

SPI consists of two main protein components, β-conglycinin and glycinin. β-Conglycinin has α, α′, and β subunits and exists as a tetramer with a sedimentation coefficient of 7S. Glycinin is composed of an acidic polypeptide (38 kDa) and a basic one (20 kDa) linked by a single disulfide bridge and exists as a

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hexamer with a sedimentation coefficient of 11S. Generally, commercially available SPI has received thermal treatment with varying degrees depending on the procedures for its preparation. Indeed, the SPI that we used in our previous paper (*13, 14*) was prepared from defatted soybean flakes in the procedures including sterilization by heating (manufacturer's information). However, the effect of the thermal treatment on the protease-induced coagulation of soy proteins has not been known. Under these backgrounds, in this study, we prepared SPI and the proteins contained in the 7S and 11S fractions (hereinafter called 7S and 11S proteins, respectively) from defatted soybean flakes obtained in the procedures without any heating process (*16, 17*) and examined the effects of thermal treatment on their coagulations induced by subtilisin Carlsberg.

MATERIALS AND METHODS

Materials. Throughout the experiment, 20 mM phosphate buffer (pH 8.0) containing 0.05% sodium azide was used as a standard buffer. Subtilisin Carlsberg (lot 112k1327) was purchased from Sigma (St. Louis, MO) and dissolved in the standard buffer. Its concentration was determined using the molar absorption coefficient at 280 nm, ε_{280}, of 23.0 mM^{-1} cm^{-1} (*18*). All other chemicals were of reagent grade and purchased form Nacalai Tesque (Kyoto, Japan).

Preparation of SPI and 7S and 11S Proteins. Hexane-defatted soybean flakes (lot 05.10.27) were obtained from Fuji Oil Co. (Osaka, Japan). SPI was prepared according to the method of Tsumura et al. (*16*). Briefly, the defatted soybean flakes were dispersed with water (1:10, v/v), the pH was adjusted to 7.0 with 2 M NaOH, and the mixture was stirred for 1 h, followed by filtration through gauze. The filtrate was centrifuged at 5000*g* for 30 min to collect the supernatant. The pH of the supernatant was adjusted to 4.5 with 2 M HCl, and it was stirred for 1 h and centrifuged at 6500*g* for 20 min to collect the acid-precipitated soy protein. The precipitate was dispersed in distilled water and stored at 4 °C before use as the SPI solution. The proteins contained in the 7S and 11S fractions (hereinafter called 7S and 11S proteins, respectively) were prepared according to the method of Nagano et al. (*17*). Briefly, the defatted soybean flakes were dispersed with water (1:15, v/v), the pH was adjusted to 7.5 with 2 M NaOH, and the mixture was stirred for 1 h, followed by filtration through gauze and then by centrifugation at 9000*g* for 30 min. $NaHSO_3$ (solid) was added to the resulting supernatant, and it was adjusted to 0.98 g/L. After the pH of the supernatant was adjusted to 6.4 with 2 M HCl, it was left at 4 °C overnight and then was centrifuged at 6500*g* for 20 min to collect the precipitate and the supernatant. The precipitate was glycinin-rich and dispersed in distilled water. It was stored at 4 °C until use as an 11S protein. NaCl (solid) was added to the supernatant and was adjusted to 0.25 M. After the pH of the supernatant was adjusted to 5.0 with 2 M HCl at 4 °C, it was stirred for 1 h at 4 °C and centrifuged at 9000*g* for 30 min. The supernatant was mixed with ice-chilled water (1:1, v/v), and its pH was adjusted to 4.8 with 2 M HCl at 4 °C. After overnight incubation at 4 °C, the precipitate, which was β-conglycinin-rich, was collected by centrifugation at 6500*g* for 20 min, dispersed in distilled water, and stored at 4 °C before use as a 7S protein. The obtained solutions of SPI and the 7S and 11S proteins were dialyzed at 4 °C against the standard buffer with a ratio of 1:15 (v/v) three times to adjust their pH values to 8.0. After dialysis, the protein concentration was determined by the Lowry method using bovine serum albumin (BSA) as the standard (*19*). These solutions were prepared daily and kept at 4 °C before use. The solutions were slightly turbid and, thus, were not solutions but suspensions in a strict meaning, although we called these solutions in this study for convenience.

Thermal Treatment. SPI and 7S and 11S proteins were diluted with the standard buffer. After the incubation on ice for 10 min, 5 mL of each of the solutions (concentrations: >10 mg/mL) was incubated at 37, 50, 60, 70, 80, 90, and 96 °C for 30 min. They were used for turbidity, fluorescence, and circular dichroism (CD) measurements and sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS-PAGE).

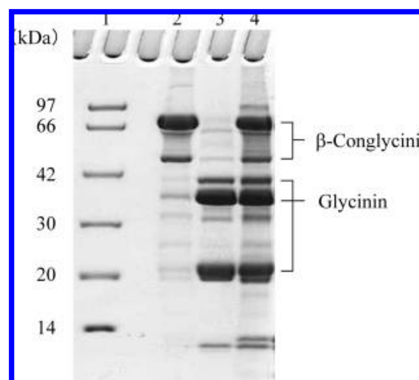

Figure 1. SDS-PAGE of soy proteins. Coomassie brilliant blue-stained 12.5% SDS-polyacrylamide gel is shown. Lane 1, molecular mass markers; lane 2, 7S protein; lane 3, 11S protein; and lane 4, SPI.

Turbidity Measurement. The turbidity of the soy protein solution was evaluated by measuring the optical density at 660 nm (OD_{660}) with a spectrophotometer DU800 (Beckman Coulter, Tokyo, Japan). After the thermal treatments of SPI and 7S and 11S proteins, they were immediately incubated at 37 °C for 10 min. The solution (2.4 mL) was transferred to an optical cuvette thermostatted at 37 °C and further incubated for 5 min. The turbidity measurement was performed for 2 min by adding 0.1 mL of the standard buffer to determine OD_{660} at the reaction time of 0 min. Then, another measurement was performed after adding 0.1 mL of 25 μM subtilisin Carlsberg. The concentrations of SPI and 7S and 11S proteins in the reaction solutions were 10 mg/mL, and that of subtilisin Carlsberg was 1.0 μM, at pH 8.0 and 37 °C.

SDS-PAGE. SDS-PAGE was performed under reducing conditions according to the method of Laemmli (*20*). In the analysis of undigested SPI and 7S and 11S proteins, a 12.5% polyacrylamide gel (Daiichi Pure Chemicals, Tokyo, Japan) was used. The sample solution was mixed with 4 volumes of the SDS-PAGE sample buffer and was immediately boiled for 5 min. The solution (10 μL) was applied to SDS-PAGE. A constant current of 40 mA was applied for 60 min. In the analysis of digested SPI and 7S and 11S soy proteins, a 15%/25% gradient gel system (Multigel 15/25, Daiichi Pure Chemicals) was used. Five milliliters of each of SPI and 7S and 11S proteins (concentrations: >10 mg/mL) was treated at 37, 80, and 96 °C for 30 min. Then, the solution was incubated at 37 °C for 10 min and treated by subtilisin Carlsberg at pH 8.0 and 37 °C. At 5, 10, 20, 30, 60, 90, and 120 min from the start of the reaction, a part of the reaction solution was taken out and mixed with 4 volumes of the SDS-PAGE sample buffer. Separately, 1.0 mL of the reaction solution at 120 min was taken out and centrifuged (10000*g*, 1 min). The supernatant was collected and mixed with 4 volumes of the SDS-PAGE sample buffer. The solution was immediately boiled for 5 min, 10 μL of which was applied to SDS-PAGE. A constant current of 40 mA was applied for 60 min. After electrophoresis, proteins were stained with Coomassie Brilliant Blue R-250. A molecular mass marker kit consisting of phosphorylase b (97 kDa), BSA (66 kDa), rabbit muscle aldolase (42 kDa), bovine erythrocyte carbonic anhydrase (30.0 kDa), soybean trypsin inhibitor (20 kDa), and hen egg white lysozyme (HEL) (14 kDa) was from Daiichi Pure Chemicals, and heart muscle myoglobin (Mb) (17 kDa), bovine milk α-lactalbumin (14 kDa), Mb I + III (10.6 kDa), Mb I (8.2 kDa), and Mb II (6.2 kDa) were from Sigma.

CD Measurement. The far-UV CD spectra of SPI and 7S and 11S proteins were measured with a spectropolarimeter J-820 (JASCO, Tokyo, Japan). In the measurements of the undigested soy proteins, the solutions were immediately incubated at 37 °C for 10 min after the thermal treatments. The solution (10 μL) was mixed with the standard buffer (990 μL) in an optical cuvette thermostatted at 25 °C, and CD was measured for 5 min. In the measurements of the digested proteins, 5 mL of each solution (concentrations: >10 mg/mL) was treated at 37, 80, and 96 °C for 30 min. Then, the solution was incubated at 37 °C for 15 min and treated by subtilisin Carlsberg at pH 8.0 and 37 °C. At 5, 10, 20, 30, 60, 90, and 120 min from start of the reaction, a part of the reaction solution (10 μL) was taken out, and the reaction was

immediately stopped by adding 10 μL of 200 μM phenylmethanesulfonyl fluoride (PMSF). The standard buffer (980 μL) was placed in a cell at 25 °C, to which the above mixture (20 μL) was added, and CD was measured 5 min after. The CD spectrum was recorded as an average of three scans. The compositions of α-helix, β-structure, and random coil of the proteins were calculated based on the ellipticities in the range of 190−243 nm according to the method of Chen et al. (*21*).

Fluorescence Measurement. The changes in the fluorescence emission spectra of SPI and 7S and 11S proteins treated at specified temperatures were recorded at 37 °C using a JASCO FP-777 fluorescence spectrophotometer with an excitation wavelength of 295 nm. The solutions were diluted with the standard buffer to give a protein concentration of 0.1 mg/mL. The relative fluorescence intensity was expressed as the ratio to the fluorescence intensity of 2.0 mM L-tryptophan-amide (Sigma, lot T0629) at 356.5 nm. The spectrum was recorded as an average of three scans.

RESULTS

Preparation of SPI and 7S and 11S Proteins from Defatted Soybean Flakes. We prepared SPI and 7S and 11S proteins that had not received any thermal treatment. SPI was extracted from defatted soybean flake by alkaline and acidic solutions, and 7S and 11S proteins were extracted from the same material by alkaline and acidic solutions followed by salt precipitation based on the difference in the solubility of β-conglycinin and glycinin with sodium hydrogen sulfite, which are described in the Materials and Methods. Upon SDS-PAGE under reducing conditions, 7S protein yielded two bands with molecular masses of 70 and 50 kDa corresponding to α and β subunits of β-conglycinin, respectively, and 11S protein yielded three bands with molecular masses of 40, 35, and 22 kDa corresponding to two acidic subunits and one basic subunit of glycinin, respectively (**Figure 1**). It is considered that 7S and 11S proteins prepared in our method are exclusively composed of β-conglycinin and glycinin, respectively.

Effects of the Thermal Treatment on the Coagulation of Soy Proteins Induced by Subtilisin Carlsberg. As we reported previously (*13, 14*), SPI gets drastically coagulated by the proteolysis with subtilisin Carlsberg. The time course of the coagulation can be monitored by measuring the turbidity (OD_{660}) of the reaction solution (**Figure 2A**), in which OD_1 and OD_2 are the minimum and maximum values of OD_{660}, and T_1 and T_2 are the times at which OD_{660} reaches OD_1 and OD_2, respectively. We first examined the time courses of OD_{660} of the reaction solution containing soy proteins treated at various temperatures for 30 min (**Figure 2B−D**). OD_{660} of the SPI species, namely, SPI and 7S and 11S proteins, decreased in the initial phase of the reaction to reach OD_1 at time T_1 (phase 1). After that, OD_{660} did not increase at all for the SPI species treated at a temperature from 37 to 60 °C, while it increased drastically to reach OD_2 (phase 2) for the ones treated at a temperature from 70 to 96 °C. This indicates that the thermal treatment at 70−96 °C is required for the subtilisin Carlsberg-induced coagulation of soy proteins and that the subtilisin Carlsberg-induced coagulation of 11S protein was much more rapid and massive than that of 7S protein. **Figure 2E** represents time courses of the subtilisin Carlsberg-induced turbidity of the soy protein solutions treated at 80 °C for 30 min [SPI, 7S protein, 11S protein, and a mixture of 7S and 11S proteins (1:1, v/v)]. The order in the magnitude of T_2 is 11S protein < a mixture of 7S and 11S proteins < SPI < 7S protein (**Figure 2E**). Dependencies of the increase in the turbidity on the temperature at the thermal treatment and SPI species are summarized in **Figure 2F,G**. OD_1, OD_2, T_1, and T_2 for SPI and 7S and 11S proteins are markedly different: OD_1 = 0.1−0.3, 0−0.2, and 0−0.1, respectively; OD_2 = 0.4−0.7, 0.3−0.6, and 0.9−1.8, respectively; T_1 = 10−20, 10−30, and <5 min, respectively; and T_2 = 60−100, 120, and <5 min, respectively. OD_2 increased with increasing the temperature up to 80 °C and then decreased. This indicates that thermal treatment at 80 °C is the most effective for prompt and massive formation of the coagula. It was also observed that OD_2 of 11S protein treated at a temperature ranging from 70 to 96 °C was considerably higher than those of SPI and 7S protein treated at the same temperatures and that T_1 and T_2 of 11S protein were lower than those of SPI and 7S protein. Considering that glycinin is highly

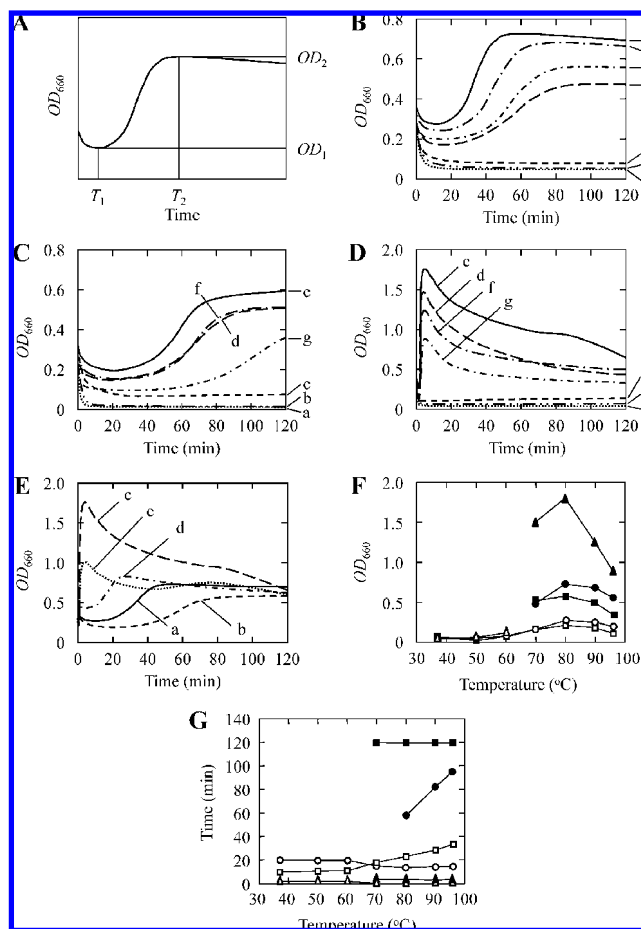

Figure 2. Effect of the thermal treatment on the coagulation of soy proteins treated by subtilisin Carlsberg. (**A**) Introduced four parameters, OD_1, OD_2, T_1, and T_2. The turbidity was evaluated by OD_{660}. OD_1 and OD_2 are the minimum and maximum of OD_{660}, and T_1 and T_2 are the times at which OD_{660} reached OD_1 and OD_2, respectively. (**B**−**D**) Time course of the subtilisin Carlsberg-induced turbidity of soy protein solutions treated at various temperatures. SPI (**B**) and 7S (**C**) and 11S proteins (**D**) were treated at 37 (a), 50 (b), 60 (c), 70 (d), 80 (e), 90 (f), and 96 °C (g) for 30 min and then treated by subtilisin Carlsberg (1.0 μM) at pH 8.0 and 37 °C. (**E**) Time course of the subtilisin Carlsberg-induced turbidity of soy protein solutions treated at 80 °C. SPI (a), 7S protein (b), 11S protein (c), and a mixture of 7S and 11S proteins (1:1 for w/v) (d) were treated at 80 °C for 30 min and then treated by subtilisin Carlsberg (1.0 μM) at pH 8.0 and 37 °C. A combined diagram of the time courses of 7S (b) and 11S (c) protein solutions is also shown (e). Zero minute means the start of enzyme reaction (**B**−**E**). (**F** and **G**) Effect of the thermal treatment on OD_1, OD_2, T_1, and T_2. OD_1 and OD_2 (**F**) and T_1 and T_2 (**G**) of SPI solution (open and closed circles, respectively) and 7S (open and closed squares, respectively) and 11S (open and closed triangles, respectively) protein solutions are plotted against the temperature at the treatment. One of the representative data of three separate experiments is shown.

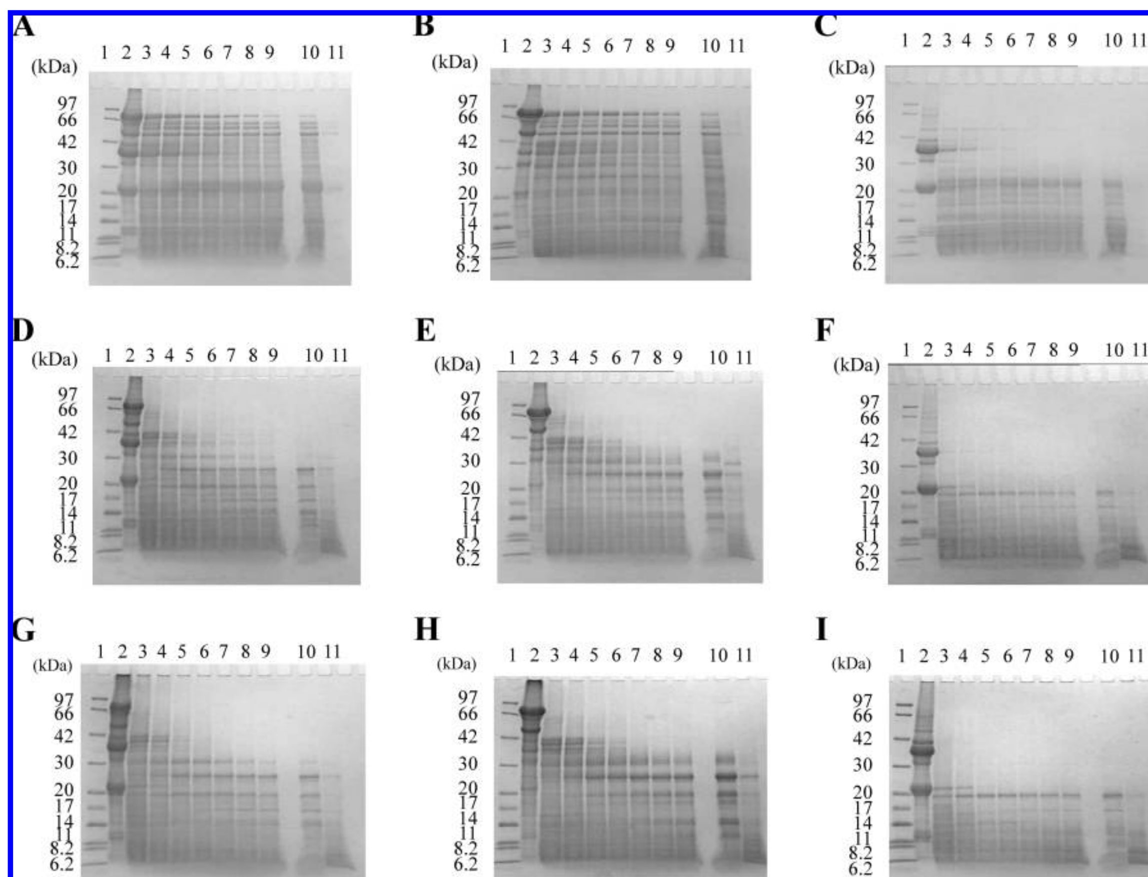

Figure 3. Effect of the thermal treatment on the SDS-PAGE pattern of soy proteins treated by subtilisin Carlsberg. SPI (**A**, **D**, **G**) and 7S (**B**, **E**, **H**) and 11S (**C**, **F**, **I**) proteins were treated at 37 (**A**−**C**), 80 (**D**−**F**), and 96 °C (**G**−**I**) for 30 min and then treated by subtilisin Carlsberg (1.0 μM) at pH 8.0 and 37 °C. SDS-PAGE was performed under reducing conditions. Lane 1, molecular mass markers; lanes 2−9, soy protein digests at 0, 5, 10, 20, 30, 60, 90, and 120 min, respectively; and lanes 10 and 11, the supernatants and the precipitates at 120 min, respectively. Zero minute means the start of the enzyme reaction.

concentrated in 11S fraction (**Figure 1**), the fragments resulted from glycinin seem to be easier to form the coagula than those resulted from β-conglycinin, which is concentrated in 7S fraction.

Effects of the Thermal Treatment on Proteolysis of Soy Proteins Induced by Subtilisin Carlsberg. SPI and 7S and 11S proteins were treated at 37, 80, and 96 °C for 30 min and then mixed with subtilisin Carlsberg (1 μM). The mixtures were incubated for specified times, followed by SDS-PAGE (**Figure 3**). With the treatment at 37 °C, the SDS-PAGE patterns of SPI and 7S protein did not change substantially in the progress of enzyme reaction, although proteins with molecular masses higher than 20 kDa gradually decreased (**Figure 3A**,**B**). On the other hand, for 11S protein treated at 37 °C, acidic subunits of glycinin (35 and 40 kDa) disappeared completely at 10 min (**Figure 3C**). With the treatment at 80 or 96 °C, proteins with the mass higher than 20 kDa in SPI and 7S and 11S proteins drastically decreased in the progress of the reaction as compared to the ones with the treatment at 37 °C (**Figure 3D−I**). There is no difference in the time course of the SDS-PAGE patterns between the digests derived from soy proteins treated at 80 and 96 °C.

Effects of the Thermal Treatment and the Digestion with Subtilisin Carlsberg on far-UV CD Spectra of Soy Proteins. The secondary structure of soy proteins was studied by far-UV CD (**Figure 4**). Each spectrum was characterized by negative (200−210 nm) and positive (around 190−195 nm) ellipticities. There is little difference in CD spectra of SPI and 7S and 11S proteins, which received the treatment at 37 °C. Their maximum

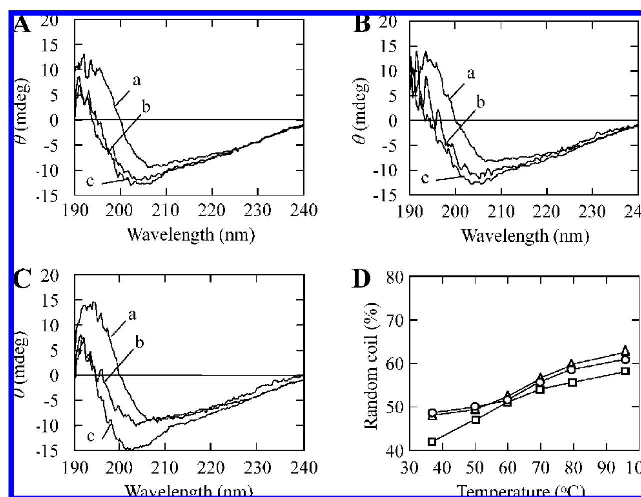

Figure 4. Effect of the thermal treatment on far-UV CD spectra of soy proteins. (**A**−**C**) CD spectra of SPI (**A**) and 7S (**B**) and 11S (**C**) proteins treated at 37 (a), 80 (b), and 96 °C (c) for 30 min. The protein concentration at CD measurement is 0.1 mg/mL. (**D**) Estimated amount of nonstructured protein in SPI (circle) and 7S (square) and 11S (triangle) proteins as a function of the temperature at the treatment. One of the representative data of three separate experiments is shown.

and minimum wavelengths were around 193 and 206 nm, respectively. On the other hand, soy proteins treated at 80 or 96 °C showed a negative change in the ellipticity at 190−220 nm as compared to the ones treated at 37 °C with the maximum

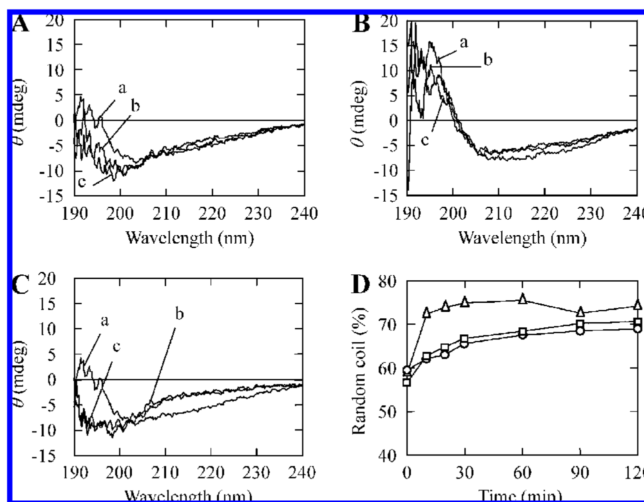

Figure 5. Effect of the digestion with subtilisin Carlsberg on far-UV CD spectra of soy proteins. (**A**−**C**) CD spectra of SPI (**A**) and 7S (**B**) and 11S (**C**) proteins treated by 1.0 M subtilisin at pH 8.0 and 37 °C for 0 (a), 30 (b), and 120 min (c). The protein concentration at CD measurement is 0.1 mg/mL. (**D**) Estimated amount of nonstructured protein in SPI (circle) and 7S (square) and 11S (triangle) proteins as a function of the reaction time. One of the representative data of three separate experiments is shown.

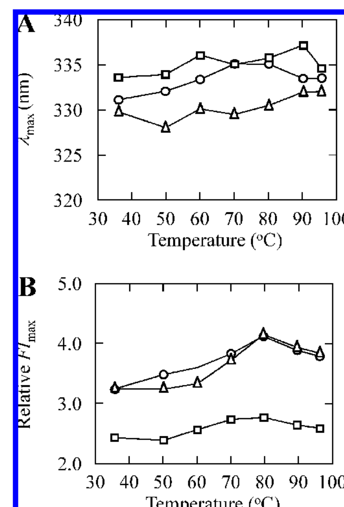

Figure 6. Effect of the thermal treatment on fluorescence of soy proteins. The wavelength for the maximum fluorescence intensity (λ_{max}) (**A**) and the maximum fluorescence intensity (FI_{max}) (**B**) of SPI (circle) and 7S (square) and 11S (triangle) proteins are plotted against the temperature at the treatment. The protein concentration at fluorescence measurement is 0.1 mg/mL. One of the representative data of three separate experiments is shown.

and minimum wavelengths of around 191 and 202 nm, respectively. Using reference spectra (*21*), the content of the secondary structure was estimated. **Figure 4D** presents the content of nonstructured protein. The amount of nonstructured protein for SPI treated at 37 °C was 48% but increased to 57% for SPI treated at 80 °C. Such a correlation was observed in 7S and 11S proteins.

We next examined far-UV CD spectra of soy proteins that were incubated at 80 °C and then digested by subtilisin Carlsberg (**Figure 5**). The ellipticity at 190−220 nm of SPI and 7S and 11S proteins decreased in the progress of the enzyme reaction (**Figure 5A**−**C**). The estimated amount of nonstructured protein increased in the progress of the enzyme reactions for SPI and 7S and 11S proteins (**Figure 5D**). The degree of this subtilisin Carlsberg-induced increase was the most remarkable in 11S protein (58% before the enzyme reaction and 72% at 10 min). Taken together, the amounts of nonstructured protein in SPI and 7S and 11S proteins were initially 40−50%, increased to 55−60% by the treatment at 80 °C, and further increased to 65−75% by the proteolysis.

Effects of the Thermal Treatment on Fluorescence Spectra of Soy Proteins. Fluorescence spectra of soy proteins (**Figure 6**) show that the temperature did not affect the wavelengths (λ_{max}) for the maximum fluorescence intensity of SPI and 7S and 11S proteins. However, their relative maximum fluorescence intensity (FI_{max}) increased with increasing the temperature up to 80 °C and then decreased. The degrees of the increase, which are expressed as the ratios of the difference in the relative FI_{max} of soy proteins treated at 80 and 37 °C to the relative FI_{max} at 37 °C of SPI and 7S and 11S proteins, are 28, 22, and 28%, respectively.

DISCUSSION

Role of the Thermal Treatment in the Coagulation of Soy Proteins Induced by Subtilisin Carlsberg. Our results in this study clearly showed that the role of the thermal treatment is crucial in the coagulation: With increasing the temperature of the treatment from 37 to 80 °C, all aspects reflecting the degrees of the coagulation increased as examined by the turbidity (**Figure 2**), the rate of proteolysis as examined by SDS-PAGE (**Figure 3**), the amount of nonstructured protein as estimated by CD (**Figure 4**), and the relative FI_{max} (**Figure 6**). However, with increasing the temperature from 80 to 96 °C, such a correlation was not observed; the degree of the coagulation and the relative FI_{max} decreased, and the rate of proteolysis did not change, while the amount of nonstructured protein increased.

SPI and 7S and 11S proteins treated at 96 °C were not fully unfolded. They retained nonstructured and structured proteins with the ratio of 60 and 40%, respectively, according to the CD measurement at 25 °C (**Figure 4D**), and their λ_{max} values were 330−337 nm by the fluorescence measurement at 37 °C (**Figure 6A**). Considering that the SPI, which was fully denatured by guanidine hydrochloride or urea, had the λ_{max} at around 350 nm (*14, 22*), our results indicate that the irreversible denaturation of soy proteins at 96 °C is limited. On the other hand, according to the reports on the differential scanning calorimetry (DSC) measurement of soy proteins, the denaturation temperatures of β-conglycinin and glycinin isolates were 73 and 88 °C, respectively (*23*), and that of each glycinin subunit was in the range of 93−98 °C (*24*), indicating that the thermal treatment with such high temperatures must cause substantial denaturation to soy proteins. Interestingly, Mills et al. reported that proteins in 11S protein treated at 95 °C not only retained much of the intramolecular β-sheet structure but also formed newly the intermolecular β-sheet structure by Fourier transform infrared spectroscopy (FT-IR) measurement at the same temperature (*25*), although it is not known whether these changes are reversible or irreversible. These lines of evidence suggest that the thermal treatment at such high temperatures causes substantial soy protein structural changes, a part of which is irreversible.

The denatured structure of soy proteins, resulting from the thermal treatment, is crucial to the subtilisin Carlsberg-induced coagulation. The denaturation seems to have followed two effects on soy proteins; one is an increase in the amount of nonstructured proteins susceptible to enzyme digestion, and the other is maintenance, or appearance, of the secondary structures

involved in the coagulation. Upon proteolysis, intramoelcular hydrophobic interactions forming such secondary structures might be changed into the intermolecular ones between the digests. This speculation is supported by the observation that the λ_{max} of SPI did not change after the thermal treatment at 80 or 96 °C (**Figure 6A**) and increased by 20 nm by the subsequent proteolysis (*14*). The digests still retained structured proteins with the ratio of 30% (**Figure 5D**) despite complete disappearance of proteins with a molecular mass higher than 20 kDa by SDS-PAGE (**Figure 3**). The reason why the degree of the coagulation decreases with the temperature at 96 °C for 30 min might be explained by the decrease in the secondary structures of soy proteins involved in the subtilisin Carlsberg-induced coagulation.

Difference in the Degree of Subtilisin Carlsberg-Induced Coagulation between 7S and 11S Soy Proteins. The subtilisin Carlsberg-induced coagulation of 11S protein was much more rapid and massive than that of 7S protein (**Figure 2C**,**D**), suggesting that the fragments resulting from glycinin seem to be easier to form the coagula than those resulting from β-conglycinin. In addition, the combined diagram of the time courses of 7S and 11S protein solutions did not resemble those of SPI or the mixture of 7S and 11S proteins in terms of T_2, suggesting that the digests of 7S protein inhibit coagulation of those of 11S protein through forming soluble aggregates composed of the digests of both 7S and 11S proteins (**Figure 2E**). Clara Sze et al. reported that urea, guanidine hydrochloride, and SDS caused unfolding of the structure of 11S protein more potently than 7S protein as assessed by CD and fluorescence measurements in the presence of the denaturants (*22*). This seems contradictory to the report above-mentioned that the denaturation temperature of β-conglycinin (73 °C) was lower by that of glycinin (88 °C) (*23*). We speculate that hydrophobic amino acid residues of 11S protein are easily exposed at the molecular surface by the treatment with denaturants but the proteins still retained the secondary structure.

Recently, the peptide fragments released from soy proteins and whey proteins by various proteases and involved in coagulation have been characterized and summarized (*26–28*). The common mechanism for production of insoluble aggregates has also been provided (*26*): Unfolded peptides with exposed hydrophobic areas released from soy proteins or whey proteins during hydrolysis bind to hydrophobic areas of other molecules to form coagula. The findings observed in this study support this mechanism. However, we emphasize the importance of thermal treatment on the protease-induced aggregation of soy proteins. Special attention has to be paid for the preparation of the materials. With regard to the coagulation mechanism, we previously examined the effect of PMSF on the subtilisin Carlsberg-induced coagulation and showed that the coagulation occurred simultaneously with, rather than subsequently after, the production of the coagulating fragments (*14*). The effects of PMSF and other inhibitors such as *Streptomyces* subtilisin inhibitor (*29, 30*) on the coagulation of soy and whey proteins induced by subtilisins should be important subjects.

In conclusion, the role of the thermal treatment is crucial in the coagulation of soy proteins induced by subtilisin Carlsberg and probably other proteases. Further study is required to elucidate the mechanisms of the effects of thermal treatment on the coagulation to establish a novel method with which the coagulation is fully controlled. The study of coagulation of thermally treated soy protein by other proteases is currently underway.

ABBREVIATIONS USED

BSA, bovine serum albumin; CD, circular dichroism; HEL, hen egg white lysozyme; Mb, myoglobin; PMSF, phenylmethanesulfonyl fluoride; SPI, soy protein isolate.

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