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Coagulation of Soy Protein Isolates Induced by Subtilisin Carlsberg

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The coagulation of soy protein isolates (SPI) induced by subtilisin Carlsberg was studied. The proteins were digested to fragments of 16 kDa or less in the early stage of the reaction, followed by coagulation. The time−course of the coagulation measured by turbidity was separated into three phases. The turbidity decreased from the initial level observed at time zero to the minimum level (OD1) at time T1 (15−20 min) in the first phase. Then, it increased drastically to reach the maximum (OD2) at time T2 (60−70 min) in the second phase, which was followed by a slight decrease in the third phase. The coagulation was terminated at T2, where 30−35% of the weight of the SPI proteins was in coagula. Proteins in the coagula were degraded slowly in the prolonged incubation, and the protein content in the coagula was finally 15−20% of the weight. The time−course of the turbidity agreed well with that of the weight of the precipitates formed, indicating that the turbidity reflects the progress of the coagulation. The turbidity change (OD1 to OD2) from the start to the end of the coagulation increased proportionally to the SPI concentration (4.9−11 mg/mL), although the time (T1 to T2) needed for the coagulation was independent of the concentration. The growth of the coagula is promoted by increasing the SPI concentration and is rate-limiting in the coagulation.

KEYWORDS: Aggregation; coagulation; clotting; proteolysis; serine protease; soy protein; subtilisin Carlsberg

INTRODUCTION

Proteins in defatted soy meals possess a higher nutritional value and better functional properties, e.g., the ability of gel formation, emulsification, and foam expansion (*1*). However, they are mainly consumed as feed and hardly utilized in the food industry. Considering the enormous amount of defatted soy meals, better utilization of the soy protein is significantly important, and its development is desired. One of the most promising approaches to the utilization of the soy protein might be limited proteolysis of the protein, because it enhances the solubility of the soy protein. In addition, the bioactive effect of the soy protein digests seems to have attracted interest recently. The effect on insulin-mediated antilipolysis (*2*) and insulin-mediated glucose transport and lipogenesis (*3*) and a lowering activity of blood cholesterol (*4*) were reported, suggesting the potential application of the soy protein digests to functional food. On the other hand, a troublesome problem in the preparation of the soy protein digests has been known. That is, soy protein coagulates in many cases during the treatment with proteases. This coagulum is insoluble and lowers the yield of the soluble digests. The coagulation is different from that mediated by Mg^{2+} and Ca^{2+} in preparing tofu, and there might be a possibility that the coagulum will be used as a foodstuff with a novel texture and functional property. Thus, methods for controlling the coagulation would make it possible to improve the yields of the digests and coagulum desired for various uses. For this purpose, we intended to elucidate the molecular mechanism of the coagulation of soy protein isolates (SPI) in this study. The defatted soybean extract is prepared by adding soy flour defatted with *n*-hexane into water (1:15, w/w) and by removing the insoluble materials (okara) at pH 7.5. When the defatted soybean extract is added into water (1:4, w/w) at pH 4.5, SPI are prepared as precipitates and the whey proteins are removed in the supernatant and are composed of mainly 7S and 11S proteins (*5*). We have examined the digestion of SPI by proteases such as chymotrypsin, papain, trypsin, and subtilisins BPN′ and Carlsberg and showed that the coagulation process is different depending on the protease examined; subtilisin Carlsberg possessed the strongest activity among them (K. Nagai, Master's Thesis of Kyoto University, 2001). On the other hand, the reaction mechanism has been studied extensively in the case of the coagulation of milk casein induced by chymosin or pepsin (production of cheese) (*6*−*8*) and that of fibrin induced by thrombin (blood clotting) (*9*−*11*). However, there are only a few reports on the protease-induced coagulation of soy protein (*12*−*14*). It was reported that heat-induced water-soluble soybean proteins prepared from defatted soybean meal showed aggregation and gelation by treatment with stem bromelain (*12*). Bromelain attacked the basic subunit of 11S globulin and

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converted them to low molecular weight fragments (LMWF), and aggregation developed with an increase of the fragments. Some microorganisms were reported to secrete an enzyme with a clotting activity of soybean milk (*13*). Recently, it was reported that a novel serine proteinase of *Bacillus pumilus* showed the coagulating activity of soybean milk and that it degraded the acidic subunit in glycinin and α′-, α-, and β-subunits in β-conglycinin but not the basic subunit of glycinin (*14*). It was speculated that the coagulation was due to a hydrophobic interaction. In the present study, we intend to investigate precisely the SPI coagulation induced by subtilisin Carlsberg and describe a possible reaction mechanism for the coagulation.

MATERIALS AND METHODS

Materials. Throughout the experiment, 20 mM phosphate buffer (pH 8.0, 37 °C) containing 0.05% sodium azide was used as the standard buffer. The SPI (Fujipro-R, lot 97.06.14.018) were purchased from Fuji Oil Co., Osaka, Japan. The protein and ash contents (dry) in SPI were 90.0 and 4.3% (weight), respectively, and the water content was 4.9% (weight). The SPI were dispersed in the standard buffer at 4% (w/v) and stirred at 25 °C for 3 h. The suspension was filtered with Whatman No. 41 filter paper, and the filtrate was used as the standard SPI solution. The filtrate cannot be regarded as solution in the strict sense of the word, because it was slightly turbid and a small amount of precipitate was formed by thorough centrifugation (see below). However, it looked transparent and clear. Thus, we called the filtrate the standard solution for the sake of convenience. The concentration was determined by the Lowry method using bovine serum albumin as the standard (*15*). This solution was prepared daily and was kept at 4 °C before use. Subtilisin Carlsberg (lot 128H1464) was purchased from Sigma (St. Louis, MO) and used without further purification. The activity of the preparation given by the manufacturer was 12 units per mg solid (1 unit was defined as the activity that produces color equivalent to 1.0 μmol of tyrosine per min at pH 7.5, 37 °C in the casein digestion as measured by the Folin−Ciocalteu method). It was dissolved in the standard buffer, and its concentration was determined spectrophotometrically using the molar absorption coefficient at 280 nm, ϵ_{280}, of 2.3×10^4 M^{-1} cm^{-1} (*16*). The active-site concentration of the enzyme preparation used was determined to be 85% by the active-site titration as described previously (*17*). For the titration, the burst of the liberation of the product, *p*-nitrophenol, in the subtilisin Carlsberg-catalyzed hydrolysis of *p*-nitrophenyl acetate was measured by using a stopped-flow method. The enzyme concentration in the present study was expressed as the active-site concentration. Trichloroacetic acid (TCA) was purchased from Wako Pure Chemicals Co. (Osaka, Japan). All other chemicals were of reagent grade and purchased from Nacalai Tesque (Kyoto, Japan).

Coagulation of SPI Induced by Subtilisin Carlsberg. The standard SPI solution was diluted with the standard buffer and incubated at 37 °C. The coagulating reaction was started on the addition of subtilisin Carlsberg into the SPI solution. The reaction was analyzed by the following measurements.

Measuring the Weight of the Precipitates. One milliliter of the reaction solution was taken in a centrifugal tube (1.5 mL) at a reaction time (*t*) and immediately centrifuged at 10000*g* for 1 min. After the supernatant was removed, the precipitates were dried in vacuo at 37 °C for 60 min with an EC-57CS centrifugal evaporator (Sakuma Seisakusho, Tokyo, Japan). The weight of the precipitates was measured using a chemical balance.

Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS−PAGE). SDS−PAGE was performed in a gradient gel system (Multigel 15/25, lot 247RJV, Daiichi Pure Chemicals, Tokyo, Japan) under reducing conditions (*18*). Proteins were reduced by treatment with 2.5% 2-mercaptoethanol at 100 °C for 3 min. A constant current of 40 mA was applied for 90 min. Proteins were stained with Coomassie brilliant blue R-250. A molecular mass marker kit was the product of Daiichi Pure Chemicals.

Measuring Turbidity. The turbidity of the SPI solution was evaluated by measuring optical density at 660 nm (OD_{660}) with a Shimadzu UV-2200 spectrophotometer (Kyoto, Japan). The SPI solution was placed in a cell and kept at 37 °C for 5 min. Thirty seconds after the enzyme was added into the cell, a change in OD_{660} was measured as a function of the reaction time. The total volume of the reaction solution was 2.5 mL.

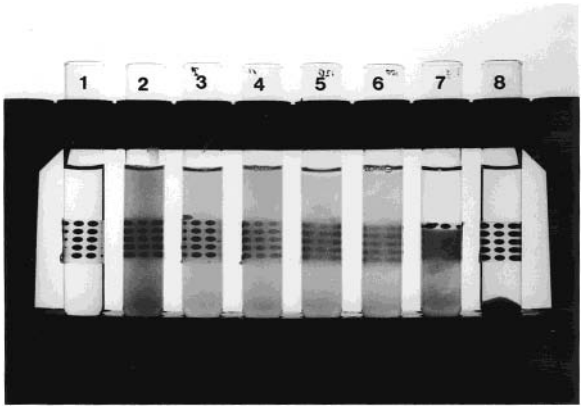

Figure 1. Visible change of the SPI solution observed on the addition of subtilisin Carlsberg. Subtilisin Carlsberg (0.56 μM) was added to the SPI solution (11 mg/mL) at pH 8.0 and 37 °C in the standard buffer. Lane 1: water; lanes 2–8: the reaction solution at the time of 0 and 20 min and 1, 2, 3, 5, and 24 h, respectively.

Visual Observation of the Coagulation. Coagulation of SPI induced by subtilisin Carlsberg was observed in a glass test tube (inner diameter, 15 mm) at 37 °C. The total volume of the reaction solution was set to 5.0 mL. A 40 W incandescent bulb was placed behind the test tube, the observer held up the test tube to the light, and the time required for the particles of the coagulum to be visible was defined as a visually observed coagulation time.

Estimation of the Amount of TCA-Soluble Peptides. One milliliter of the reaction solution was taken at a reaction time, followed by adding 1.0 mL of 20% TCA to stop the reaction. Precipitates were removed by centrifugation at 10000*g* for 5 min and then by centrifugation at 10000*g* for 10 min. The colorless and transparent supernatant was diluted 5 times with the standard buffer, and the absorbance at 280 nm (A_{280}) was measured as an index of the amount of 10% TCA-soluble peptides.

RESULTS AND DISCUSSION

Coagulation of SPI Induced by Subtilisin Carlsberg. Figure 1 shows the visual change of the SPI solution (11 mg/mL) induced by adding a catalytic amount of subtilisin Carlsberg (0.56 μM). As judged by the distinctness of the black circle pattern pasted on the back of the test tube, the turbidity decreased in the first 20 min and then increased by visual observation of the coagula (20 min to 3 h). The coagula fell down slowly after a while and finally accumulated at the bottom of the tube.

Weight of the Precipitate Obtained by Centrifugation. The SPI (11 mg/mL) were mixed with 1.0 μM subtilisin Carlsberg, and the precipitate was obtained by centrifugation at the reaction time indicated. The ratio of the weight of the precipitate to that of the total SPI proteins was plotted against the reaction time (**Figure 2**). Six percent (w/w) of the total proteins precipitated at time zero. The ratio decreased slightly to 4−5% in the first 15−20 min. Then, it increased and reached the maximum (35%) at around 1 h. After that, it decreased to 20% at 4 h and then decreased slowly afterward. About 15% of the total proteins precipitated even at 24 h.

SDS−PAGE. The SPI were mixed with subtilisin Carlsberg under the same conditions as those used in **Figure 2**, and the solution was analyzed by SDS−PAGE (**Figures 3** and **4**). The reaction solution (400 μL) was taken out at a reaction time,

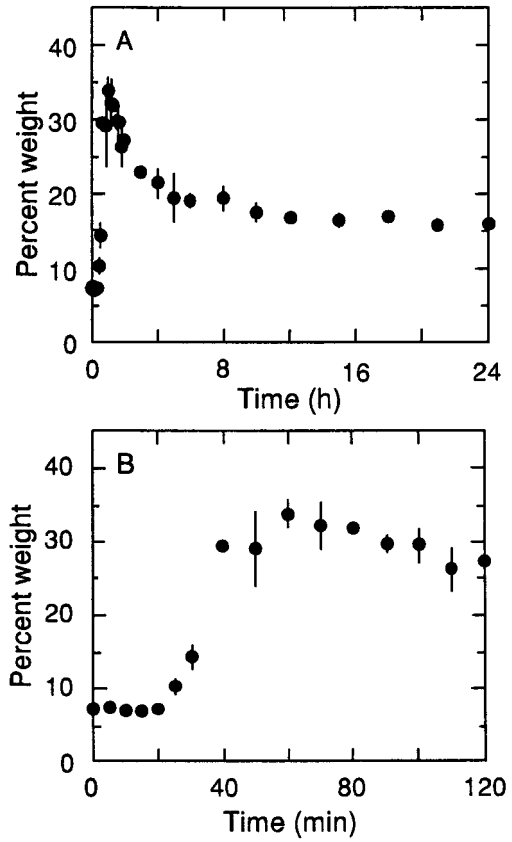

Figure 2. Change of the amount of precipitates formed in the SPI solution in the reaction with subtilisin Carlsberg. The reaction conditions are the same as those described in **Figure 1**. The precipitates were prepared as described in the Materials and Methods. The amount of the precipitates was represented as the percent ratio (w/w) to the total proteins. The average and deviation of the precipitated amounts for the triplicated measurements were shown. Time range of the reaction: A, 0–24 h; B, 0–20 min.

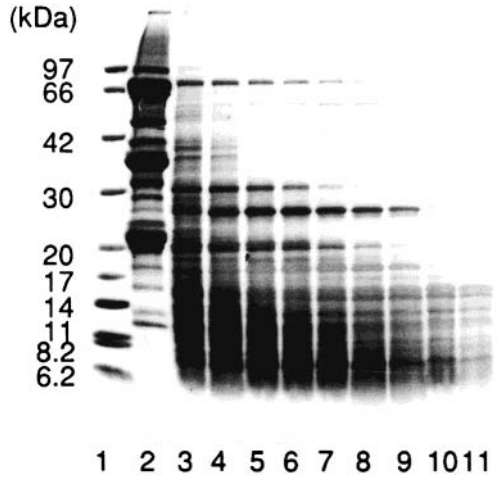

Figure 3. SDS–PAGE of the SPI proteins treated by subtilisin Carlsberg. The reaction conditions are the same as those described in **Figure 1**. SDS–PAGE was performed under reducing conditions. Lane 1: molecular mass markers; lanes 2–12: the SPI proteins digested for 0, 5, 10, 20, 30, and 60 min and 1.5, 2, 4, 6, and 24 h, respectively.

and the reaction was stopped by adding 200 μL of 1.3 M TCA. After 200 μL of 1.3 M NaOH was added for neutralization, 7.5 μL of the solution was loaded on each lane (**Figure 3**). It was shown that the SPI proteins were digested promptly, and most of the bands completely disappeared at 5 min. Only the 76 kDa protein remained until 90 min, suggesting that the protein, which might be assigned to α- or α'-subunits of β-conglycinin, was fairly resistant to the digestion. The digestion process appears to consist of two phases. One is the digestion in the first 30 min (the primary digestion), in which the digestion mostly progressed, while some bands of 58, 32, 27, 26, and 21 kDa were observed at 30 min in addition to the 76 kDa one. The second phase was observed subsequently after 30 min (the secondary digestion), in which these bands disappeared more slowly. It was clearly shown that the coagulation was accompanied with their extensive digestion.

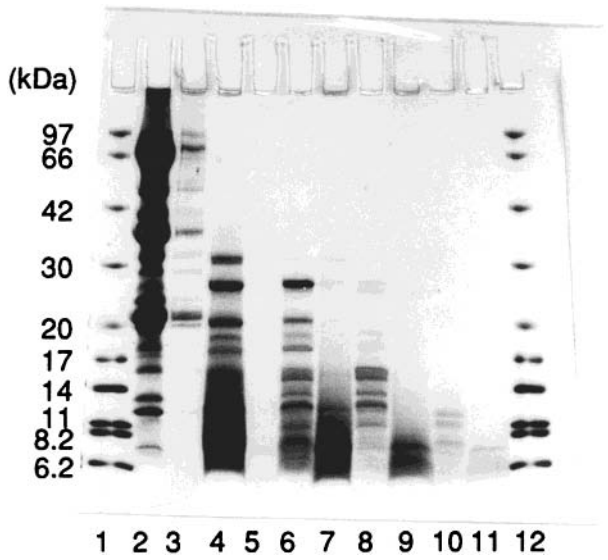

Figure 4. SDS–PAGE of the supernatant and the precipitates of the SPI proteins digested by subtilisin Carlsberg. The reaction conditions are the same as those described in **Figure 1**. The solution was taken out at the reaction time indicated and was centrifuged to prepare the supernatant and the precipitate (Materials and Methods). SDS–PAGE was performed under reducing conditions. Lanes 1 and 12: molecular mass marker; lanes 2, 4, 6, 8, and 10: the supernatants at 0 and 20 min and 1, 4, and 24 h, respectively; lanes 3, 5, 7, 9, and 11: the precipitates at 0 and 20 min and 1, 4, and 24 h, respectively.

The supernatant and the precipitate of the SPI solution treated by subtilisin Carlsberg were subjected to SDS−PAGE separately (**Figure 4**). The reaction solution (1.0 mL) was taken out at a reaction time indicated and centrifuged immediately. The precipitates were dispersed with 2.0 mL of the standard buffer. Five microliters each of the supernatant and the precipitate-dispersed solution was applied to SDS−PAGE. The precipitates obtained at time zero contained proteins of relatively large sizes (20−70 kDa). The protein content in the precipitates was fairly small in the first 20 min but increased drastically in the process from 20 min to 1 h, indicating that the coagulation occurred in this period. It is noted that the precipitates collected in this period contained proteins of relatively small sizes (<16 kDa). Most of the SPI proteins were of 20 kDa or more (**Figures 3** and **4**). Thus, it is obvious that the small proteins contained in the precipitates were formed in the digestion of the SPI proteins. In other words, it is the small fragments generated in the digestion that participate in the coagulation of the SPI solution. In the reaction of 1−24 h, the proteins in the precipitates as well as the fragments in the supernatant were digested, and those in the precipitates almost disappeared at 24 h (**Figure 4**).

Change in the Turbidity during the Coagulating Reaction. **Figure 5** shows the change in the turbidity during the coagulation of SPI under the same conditions as those used in **Figures 2**−**4**. The SPI solution is slightly turbid, and the turbidity (OD_{660}

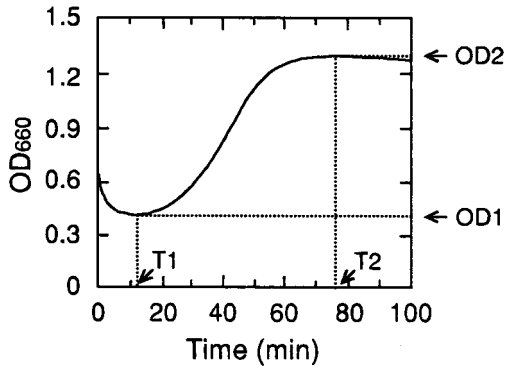

Figure 5. Change in the turbidity of the SPI solution in the reaction with subtilisin Carlsberg. The reaction conditions are the same as those described in **Figure 1**. The turbidity was evaluated by OD_{660}. Four parameters were introduced on the reaction curve of the turbidity: OD1 and OD2 are the minimum and maximum of OD_{660}, respectively. T1 and T2 are the times at which OD_{660} reached OD1 and OD2, respectively.

= 0.6) observed at time zero corresponds with that 6% of the total protein that was precipitated (**Figure 2**). The turbidity decreased at the initial stage of the reaction to reach the minimum (OD_{660} = 0.4) at 12.7 min. Then, it increased and reached the maximum (OD_{660} = 1.3) at 77.2 min. After that, it decreased again slightly. We introduced four parameters: OD1 and OD2 are the minimum and maximum values of OD_{660}, respectively, and T1 and T2 are the reaction times to be required so that OD_{660} reaches the minimum and maximum values, OD1 and OD2, respectively.

The turbidity of the SPI solution added with subtilisin Carlsberg must be reflected by the colloidal properties of the SPI proteins and the coagulum formed. In the progress of the coagulation, the turbidity due to the SPI proteins decreases, and that due to the coagulum increases. Because the precipitates were not formed in the period until T1, the decrease in OD_{660} observed in this period must be solely due to the digestion of the SPI proteins (**Figure 2**). The decrease in OD_{660} due to the digestion of the SPI proteins after T1 may be much smaller than that (ΔOD_{660} = 0.2) observed in the first 10 min, because the SDS−PAGE pattern changed drastically in the first 5−10 min whereas it did not change much from 10 min to 2 h (**Figure 3**). As the primary digestion progressed, the coagulation occurred and the turbidity increased from OD1 to OD2 (ΔOD_{660} = 0.9). The turbidity decreased slowly again after T2 because of the secondary digestion. Consequently, it is reasonable to use the increase in the turbidity (OD1 to OD2) as an indicator of the progress of the coagulation. Comparing the results of **Figures 2** and **5**, it was shown that the turbidity change (OD1 to OD2) of 0.9 was given by the precipitates formed in the primary digestion, the amount of which corresponds to 30−35% of the weight of the total SPI proteins (11 mg/mL).

Effect of the SPI Concentration on the Coagulation. Various concentrations of SPI (2.0−11 mg/mL) were digested by 1.0 μM subtilisin Carlsberg, and the coagulation was monitored by turbidity and by visual observation (**Figure 6**). As the initial SPI concentration, $[SPI]_0$, decreased, the change in the turbidity decreased, and the visually observed coagulation time became longer. When $[SPI]_0$ was less than 4.9 mg/mL, particles of the coagulum were not observed visually in the reaction up to 100 min, and an increase in the turbidity was not detected at $[SPI]_0$ = 2.0 mg/mL, indicating that coagulation did not occur at $[SPI]_0$ less than 2.0 mg/mL. OD1 increased linearly with increasing $[SPI]_0$ from 3.0 to 11 mg/mL (**Figure 7A**). OD2 was nearly constant at $[SPI]_0$ of 3.0−4.0 mg/mL but increased

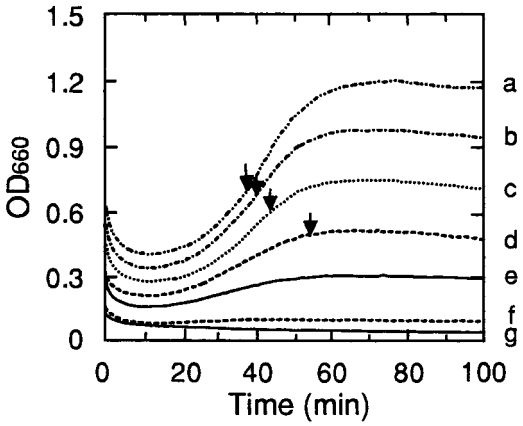

Figure 6. Effect of the SPI concentration on the turbidity of the SPI solution treated by subtilisin Carlsberg. Various concentrations of SPI (2.0–11 mg/mL) were digested by 1.0 μM subtilisin Carlsberg at pH 8.0 and 37 °C. The turbidity was evaluated by OD_{660}. SPI concentrations: a, 11; b, 9.7; c, 8.1; d, 6.5; e, 4.9; f, 3.0; and g, 2.0 mg/mL. Arrows indicate the visually observed coagulation times.

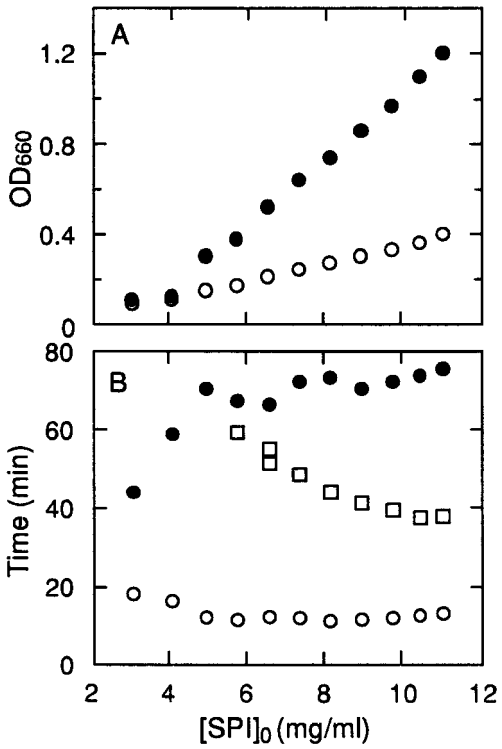

Figure 7. Effect of the SPI concentration on the parameters determined from the time–course of the turbidity of the SPI solution treated by subtilisin Carlsberg. Various concentrations of SPI (2.0–11 mg/mL) were digested by 1.0 μM subtilisin Carlsberg at pH 8.0 and 37 °C. Four parameters (OD1, OD2, T1, and T2) determined from the time–course of the turbidity as well as the visually observed coagulation time were plotted against the initial SPI concentration, $[SPI]_0$. (A) Effect of $[SPI]_0$ on OD1 (○) and OD2 (●). (B) Effect of $[SPI]_0$ on T1(○), T2 (●), and visually observed coagulation time (□).

drastically in a linear fashion with increasing $[SPI]_0$ from 4.9 to 11 mg/mL. Consequently, the turbidity change (OD1 to OD2) increased linearly with increasing $[SPI]_0$ in the range of 4.9−11 mg/mL. In contrast, the effect of $[SPI]_0$ on T1 and T2 was relatively small (**Figure 7B**). Although T1 increased and T2 decreased with increasing $[SPI]_0$ from 3.0 to 4.0 mg/mL, they were almost constant over 4.9 mg/mL. Despite the negligible change in T1 and T2 at $[SPI]_0$ of 4.9−11 mg/mL, the visually

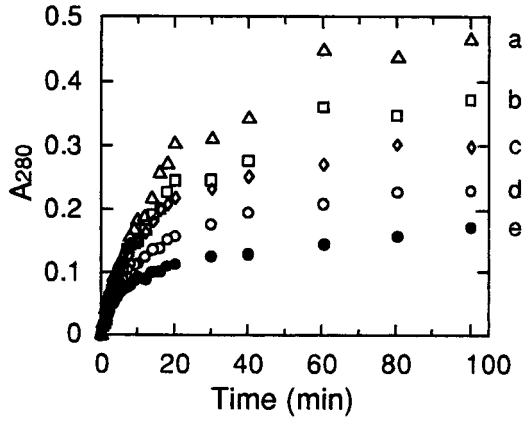

Figure 8. Formation of the 10% TCA-soluble peptides in the SPI solution treated by subtilisin Carlsberg. Various concentrations of SPI (2.0–11 mg/mL) were digested by 1.0 μM subtilisin Carlsberg at pH 8.0 and 37 °C. The 10% TCA-soluble peptides in the SPI solution were prepared by the method described under Materials and Methods. The difference in A_{280} of the TCA-soluble peptides obtained at the reaction time indicated and at time zero was plotted against the reaction time. The SPI concentrations are as follows: a (△), 11; b (□), 8.1; c (◇), 5.0; d (○), 3.0; and e (●), 2.0 mg/mL.

observed coagulation time decreased from 60 to 40 min with the increase in $[SPI]_0$.

The SPI (2.0−11 mg/mL) were digested by 1.0 μM subtilisin Carlsberg, and the increase in A_{280} due to the formation of the TCA-soluble peptides was plotted against the reaction time (**Figure 8**). The initial rate of the increase was determined to be 0.10−0.12 A_{280} /min and was independent of $[SPI]_0$. Here, we assume that the digestion of SPI by subtilisin Carlsberg obeys the Michelis−Menten equation

$$v = k_{cat}[E]_0[SPI]_0/(K_m + [SPI]_0) \quad (1)$$

where v is the initial reaction rate and k_{cat}, K_m, and $[E]_0$ are the molecular activity, the Michaelis constant, and the initial enzyme concentration, respectively. When $[SPI]_0$ is in large excess of K_m (pseudo-zero order approximation), the equation is transformed to

$$v = k_{cat}[E]_0 = V_{max} \quad (2)$$

The reaction rate gives the maximum value with this approximation. The result that the reaction rates were constant at $[SPI]_0$ of 2−11 mg/mL suggests that V_{max} of the hydrolysis of SPI by subtilisin Carlsberg is 0.10−0.12 A_{280} /min and K_m is much less than 2 mg/mL.

Mechanism of the Coagulation of SPI. The coagulation of SPI induced by subtilisin Carlsberg was measured by various methods. The SPI proteins were digested mostly in the first 20 min, and the digestion hardly progressed from 20 min to 1 h as compared to the first 20 min (**Figure 3**). On the basis of the results obtained in this study, we propose a mechanism of the coagulation (**Figure 9**). In the initial stage of the reaction, the protease digests the SPI proteins indiscriminately (the primary digestion). The fragments produced are classified into two groups. One is the high molecular weight fragment (HMWF) larger than 16 kDa, and the other is the LMWF smaller than 16 kDa. In the following stage, LMWF forms the coagulum, while HMWF remains soluble without involving in the coagulation. After the primary digestion, the protease digests both LMWF in the coagulum and HMLF in the solution (the secondary digestion). The half-life times for the primary and secondary

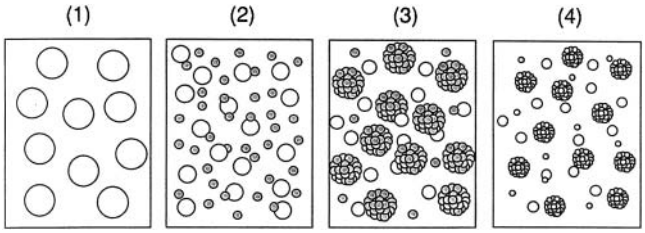

Figure 9. Postulated mechanism for the coagulation of the SPI proteins by subtilisin Carlsberg. Panel 1, intact SPI proteins; panel 2, digestion of SPI (primary digestion); panel 3, coagulation of LMWF; and panel 4, digestion of the coagulum (secondary digestion). Marks: large open circles, intact SPI proteins; small open circles, HMWF; and small closed circles, LMWF.

digestions were estimated to be 10 and 60 min, respectively (**Figure 2**). The secondary digestion, which progresses more slowly than the primary one, reduces the size of the fragments in the coagulum.

Effect of the SPI Concentration on the Turbidity. When $[SPI]_0$ was 4.9−11 mg/mL, the maximum velocity holds for at least 13 min (**Figure 8**). Assuming that HMWF does not interfere with the coagulation of LMWF, this suggests that the progress of the coagulation of LMWF was almost the same until 13 min instead of the variety in $[SPI]_0$. T1 is supposed to change when the turbidity changes, depending on the coagulation and the primary digestion. However, T1 was constant at $[SPI]_0$ of 4.9−11 mg/mL (**Figure 7**), suggesting that it is dominated by the coagulation and the effect of the primary digestion is negligible. Thus, T1 is considered to be the time when enough of LMWF is accumulated to start the coagulation. On the other hand, when $[SPI]_0$ is less than 4 mg/mL, the rate of the SPI digestion slowed before 13 min, because the SPI proteins were consumed out and the amount of LMWF generated was not enough to form coagula. With a large amount of SPI, LMWF was produced even after T1, and the maximum amount of LMWF given at 100 min increased with increasing $[SPI]_0$ (**Figure 8**). This might be the reason that the turbidity change (OD1 to OD2) increased with increasing $[SPI]_0$. T2 is expected to increase with an increasing amount of LMWF depending on $[SPI]_0$. It increased with increasing $[SPI]_0$ up to 4.9 mg/mL according to the expectation but was constant at $[SPI]_0$ of 4.9−11 mg/mL. This unexpected behavior of T2 might be explained by assuming that the coagulation is promoted cooperatively depending on an increase in $[SPI]_0$ from 4.9 to 11 mg/mL. Furthermore, it is suggested that the step where LMWF molecules meet together to form coagulum particles may be rate-limiting. Actually, at $[SPI]_0$ higher than 4.9 mg/mL, the coagulating rate increased remarkably (**Figure 6**). Because the amount of LMWF at T1 is considered similar at the $[SPI]_0$ examined, the LMWF produced after T1 must be related to the coagulation. On the other hand, the visually observed coagulation time decreased with increasing $[SPI]_0$. It is reasonable because it reflects the time when the coagulum grows large enough to be visible. Provided that the same size of coagulum is observed visually, the decrease in the time with increasing $[SPI]_0$ means faster growing of the coagulum. When $[SPI]_0$ was less than 4.9 mg/mL, the amount of LMWF generated was so small that the coagulum was not large enough to be visible. The lowest $[SPI]_0$ necessary for the coagulation was 4.0 mg/mL (**Figure 7**). The cleaved sites in the primary and secondary digestions of SPI by subtilisin Carlsberg, the amino acid sequence of LMWF, and its characterization are presently under investigation. The cleavage pattern might be dependent on the proteinases used and the conditions of soybean proteins in

soybean milk or in SPI. The effect of fatty acids and proteinase inhibitors included in the soybean milk on the action of proteinases and coagulum formation should be examined. The application of a particle analyzer instead of visual inspection would be favorable in further studies.

LITERATURE CITED

(1) Lusas, E. W.; Riaz, M. N. Soy protein products: Processing and use. *J. Nutr*. **1995**, *125*, 573S−580S.

(2) Minami, K.; Ishikawa, Y.; Moriyama, R.; Makino, S. Stimulation of *in vitro* insulin action by glycinin acidic subunit A_{1a}. *Agric. Biol. Chem*. **1991**, *55*, 1033−1039.

(3) Ishikawa, Y.; Kumagai, C.; Moriyama, R.; Makino, S. Stimulation of insulin-dependent glucose transport and lipogenesis in isolated rat adipocytes by trypsinized soybean glycinin acidic subunit A_{1a}. *Biosci., Biotechnol., Biochem*. **1994**, *58*, 590−591.

(4) Wang, M. F.; Yamamoto, S.; Chung, H. M.; Chung, S. Y.; Miyatani, S.; Mori, M.; Okita, T.; Sugano, M. Antihypercholesterolemic effect of undigested fraction of soybean protein in young female volunteers. *J. Nutr. Sci. Vitaminol*. **1995**, *41*, 187−95.

(5) Samoto, M.; Miyazaki, C.; Kanamori, J.; Akasaka, T.; Kawamura, Y. Improvement of the off-flavor of soy protein isolate by oil-body associated proteins and polar lipids. *Biosci., Biotechnol., Biochem*. **1998**, *62*, 935−940.

(6) Hyslop, D. B.; Richardson, T.; Ryan, D. S. Kinetics of pepsin-initiated coagulation of κ-casein. *Biochim. Biophys. Acta* **1979**, *566*, 390−396.

(7) Payens, T. A. J. Casein micelles: the colloid-chemical approach. *J. Dairy Res*. **1979**, *46*, 291−306.

(8) Brinkhuis, J.; Payens, T. A. The rennet-induced clotting of para-ê-casein revisited: inhibition experiments with pepstatin A. *Biochim. Biophys. Acta* **1985**, *832*, 331−336.

(9) Blombäck, B.; Hessel, B.; Hogg, D.; Therkildsen, L. A two-step fibrinogen-fibrin transition in blood coagulation. *Nature* **1978**, *275*, 501−505.

(10) Smith, G. F. Fibrinogen-fibrin conversion. The mechanism of fibrin-polymer formation in solution. *Biochem. J*. **1980**, *185*, 1−11.

(11) Visser, A.; Payens, T. A. On the kinetics of the thrombin-controlled polymerization of fibrin. *FEBS Lett*. **1982**, *142*, 35−38.

(12) Fuke, Y.; Sekiguchi, M.; Matsuoka, H. Nature of stem bromelain treatments on the aggregation and gelation of soybean proteins. *J. Food Sci*. **1985**, *50*, 1283−1288.

(13) Park, Y. W.; Kusakabe, I.; Kobayashi, H.; Murakami, K. Production and properties of a soymilk-clotting enzyme system from a microorganism. *Agric. Biol. Chem*. **1985**, *49*, 3215−3219.

(14) Aoyama, M.; Yasuda, M.; Nakachi, K.; Kobamoto, N.; Oku, H.; Kato, F. Soybean-milk-coagulating activity of *Bacillus pumilus* derives from a serine proteinase. *Appl. Microbiol. Biotechnol*. **2000**, *53*, 390−395.

(15) Lowry, O. H.; Rosenbrough, N. J.; Farr, A. L.; Randall, R. J. Protein measurement with the Folin phenol reagent. *J. Biol. Chem*. **1951**, *193*, 265−275.

(16) Markland, F. S.; Smith, E. L. Subtilisins: Primary Structure, Chemical and Physical Properties. In *The Enzymes*, 3rd ed.; Boyer, P. D., Ed.; Academic Press: New York, 1971; Vol. 3, pp 561−608.

(17) Inouye, K.; Tonomura, B.; Hiromi, K.; Sato, S.; Murao, S. The stoichiometry of inhibition and binding of a protein proteinase inhibitor from *Streptomyces* (*Streptomyces* subtilisin inhibitor) against subtilisin BPN′. *J. Biochem*. **1977**, *82*, 961−967.

(18) Laemmli, U. K. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **1970**, *227*, 680−685.

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