JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

J. Agric. Food Chem. **2004,** *52,* 4921−4927 **4921**

Insights into the Reaction Mechanism of the Coagulation of Soy Protein Isolates Induced by Subtilisin Carlsberg

KOUHEI NAGAI AND KUNIYO INOUYE*

Division of Food Science and Biotechnology, Graduate School of Agriculture, Kyoto University, Sakyo-ku, Kyoto 606-8502, Japan

The reaction mechanism of the coagulation of soy protein isolates (SPIs) induced by subtilisin Carlsberg was investigated. Formation of the coagula was monitored by measuring the turbidity (OD_{660}) of the SPI solution, which decreased at the initial stage (phase 1 or digestion phase) of the reaction, and then increased (phase 2 or coagulation phase) and finally reached the plateau level. The velocity of the coagulation increased with increasing enzyme concentration. The coagulation was inhibited dramatically by adding a serine protease inhibitor (phenylmethanesulfonyl fluoride, PMSF) when the turbidity reached the minimum value. This indicates that the SPI digests participating in the coagulation are produced mainly in phase 2; in other words, production of the coagulating fragments and their coagulation occur simultaneously in phase 2. Structural changes of SPI during proteolysis were measured by observing fluorescence changes of aromatic amino acids of SPI and an externally added hydrophobic probe. It was suggested that the hydrophilic surface areas of SPIs might be cleaved preferentially in phase 1, and that the hydrophobic inner areas might be cleaved in phase 2 with extensive decomposition of the 3-D structure of SPI proteins. The fragments formed in phase 2 are considered to coagulate through hydrophobic interactions.

KEYWORDS: Aggregation; coagulation; food protein; protease; protease inhibitor; soy protein

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 meal remains after extraction of the oil. Although this defatted soybean meal contains proteins with high nutritional values and functional properties (*1*), it is mainly used as feed, and hardly utilized in food industries. Therefore, new methods for utilizing soy proteins are needed. Limited proteolysis is one of the most promising approaches for the utilization, because it improves nutritional and functional properties of food proteins (*2*−*8*). In addition, it has been reported that proteolytic digests of many proteins contain bioactive peptides (*9*−*12*), suggesting the potential use of the protein digests for functional foods. In protease digestion of soy proteins, some of the digests coagulate and remain insoluble, resulting in lower yields of the soluble digests. The mechanism of the protease-induced coagulation of soy proteins must be different from those of the Ca^{2+}- and/or Mg^{2+}-mediated coagulation (tofu) and heat-induced coagulation (yuba). There might be a possibility that the insoluble coagulum could be used as a foodstuff with a novel texture and functional properties. Methods for controlling the coagulation would enable us to improve the yields of the soluble digests and/or insoluble coagula, and also to expand the use of soy proteins.

There have been several reports on the coagulation of soy proteins induced by proteases. Proteins prepared from defatted soybean meal show coagulation by treatment with bromelain, and the coagulation develops with increasing amounts of the fragments formed from the basic subunit of an 11S globulin (*13*). Some microbial enzymes have a clotting activity of soybean milk (*14*, *15*). A serine protease of *Bacillus pumilus* shows the coagulating activity by degrading the acidic subunit of an 11S globulin and α′-, α-, and β-subunits of a 7S globulin but not the basic subunit of the 11S globulin (*15*). However, these reports have never proposed the mechanism of the coagulation in detail.

In our previous paper (*16*), we reported a convenient method to follow the protease-induced coagulation of soy protein isolates (SPIs) by measuring continuously the turbidity of the reaction solution. During the hydrolysis, the turbidity decreased in the first phase due to digestion of SPI to reach the minimum (phase 1 or digestion phase). Then, it increased due to coagulation of the digests to reach the maximum (phase 2 or coagulation phase). After it reached the maximum, it decreased again slowly due to digestion of the coagula (phase 3). On the basis of these observations, we have proposed a hypothesis that the fragments produced in phase 1 participate in the coagulation observed in phase 2, and that the growth of the coagula is rate-limiting in the coagulating reaction, although there still remained a possibility that secondary slower digestion, if any, in phase 2 affects the velocity of the coagulation (*16*). To prove the hypothesis,

* To whom correspondence should be addressed. Phone: +81-75-753-6266. Fax: +81-75-753-6265. E-mail: inouye@kais.kyoto-u.ac.jp.

10.1021/jf034845g CCC: $27.50
Published on Web 06/24/2004

we have aimed to investigate in detail the coagulation of SPIs induced by subtilisin Carlsberg, which has relatively strong coagulating activity. The first point is to demonstrate that the digestion products in phase 2 are responsible for the coagulation by examining the effect of the enzyme concentration on the turbidity progress curves and the effect of a serine protease inhibitor on the coagulation. The other point is to characterize what structural changes may occur in SPI proteins to cause the coagulation by examining the fluorescence changes during the proteolysis and coagulation. On the basis of the evidence obtained, we propose insights into the mechanism of the coagulation of SPIs induced by subtilisin Carlsberg.

MATERIALS AND METHODS

Materials. Throughout the experiment, 20 mM phosphate buffer (at pH 8.0 and 37 °C) containing 0.05% sodium azide was used as a standard buffer.

SPI (Fujipro-R, lot 97.06.14.018) was purchased from Fuji Oil Co. (Osaka, Japan), being prepared from defatted soy meals in the procedures as described previously (*16*). SPI is composed of mainly 7S and 11S proteins (*17*). 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 was dispersed in the standard buffer at a concentration of 40 mg/mL (w/v), and the resulting dispersion was stirred at room temperature for 3 h. The suspension was filtered with Whatman no. 41 filter paper, and the filtrate was used as the SPI solution (*16*). The concentration was determined by the Lowry method using bovine serum albumin (BSA) as the standard (*18*). Unless otherwise mentioned in this study, the SPI concentration in the reaction mixture was 10 mg/mL (w/v). Subtilisin Carlsberg (lot 120K115; hereinafter subtilisin) was purchased from Sigma (St. Louis, MO) and used without further purification. It was dissolved in the standard buffer, and its concentration was determined using the molar absorption coefficient at 280 nm, ϵ_{280}, of 2.3×10^4 M^{-1} cm^{-1} (*19*). The activity of the preparation given by the manufacturer was 12 units/mg of solid [1 unit is the activity producing 1.0 μmol of tyrosine/min at pH 7.5 and 37 °C in the casein digestion as measured by the Lowry method (*18*, *20*)]. ANS (lot YLG 9113) and PMSF (lot CAK 7606) were from Wako Pure Chemical Co. (Osaka, Japan), and were dissolved in the standard buffer and 2-propanol, respectively. All other chemicals were of reagent grade and purchased from Nacalai Tesque (Kyoto, Japan).

Turbidity Measurement. The turbidity of the SPI solution was evaluated by measuring the optical density at 660 nm (OD_{660}) with a Shimadzu UV-2200 spectrophotometer (Kyoto). The SPI solution (2.4 mL) was placed in a cell and incubated at 37 °C for 5 min. Thirty seconds later, 0.1 mL of the protease solution was added, and a change in OD_{660} was measured as a function of the reaction time. The effect of PMSF on the coagulation was evaluated by adding 60 μL of the PMSF solution or 60 μL of 2-propanol (blank) to the reaction solution. All experiments for turbidity measurements were done four times at the same conditions, and the values for the parameters were evaluated by the method of least squares.

SDS−PAGE. SDS−PAGE was performed in a 15%/25% gradient gel system (Multigel 15/25, lot 247RJV, Daiichi Pure Chemicals, Tokyo) under reducing conditions (*21*). SPI (10 mg/mL) was treated with 0.13, 1.0, or 6.3 μM subtilisin at pH 8.0 and 37 °C. At a given time, the proteolysis was stopped by adding 2 mM PMSF, and 6 μL of the reaction solution was loaded onto each lane after SDS treatment in the presence of 2.5% 2-mercaptoethanol (2-ME) at 100 °C for 3 min. When the coagulum was analyzed, 1.0 mL of the reaction solution was centrifuged at 10000*g* for 1 min and the coagulum was collected as a precipitate. The coagulum was dispersed again with 1.0 mL of the standard buffer, and 6 μL of the dispersed solution was loaded onto each lane after SDS treatment in the presence of 2.5% 2-ME at 100 °C for 3 min. A constant current of 40 mA was applied for 90 min. Proteins in the gel were stained with Coomassie Brilliant Blue R-250. The molecular mass marker proteins, rabbit muscle phosphorylase b (97 kDa), BSA (64 kDa), rabbit muscle aldolase (42 kDa), bovine erythrocyte carbonic anhydrase (30 kDa), soybean trypsin inhibitor (20 kDa), horse heart muscle myoglobin (Mb) (17 kDa), bovine milk α-lactalbumin (14 kDa), Mb I+III (11 kDa), Mb II (8.2 kDa), and bovine lung aprotinin (6.2 kDa), were from Sigma.

Estimation of the Amount of TCA-Soluble Peptides. SPI (10 mg/mL) was treated with 1.0 μM subtilisin in the standard buffer. A 1 mL sample of the reaction solution was taken at a given time (*t*), followed by adding 1.0 mL of 20% TCA to stop the reaction, and the mixture was kept for 30 min with gentle stirring at 25 °C. Precipitates were removed by centrifugations at 10000*g* for 5 min and then for 10 min. The colorless and transparent supernatant was diluted five times with the standard buffer, and the absorbance at 280 nm (A_{280}) was measured as an index of the concentration of 10% TCA-soluble peptides. $A_{TCA}(0)$ and $A_{TCA}(t)$ designate the A_{280} values of the supernatant at the reaction times of zero and *t* min, respectively, and A_{SPI} designates the A_{280} value of the undigested SPI solution. Accordingly, $A_{SPI} - A_{TCA}(0)$ shows the total concentration of the SPI fraction which is precipitated with 10% TCA and $A_{TCA}(t) - A_{TCA}(0)$ the protein concentration which is converted to TCA-soluble fragments in the *t* min digestion from the TCA-precipitating SPI fraction. Under the conditions examined, A_{SPI} and $A_{TCA}(0)$ are 2.47 and 0.334, respectively. Thus, the degree of hydrolysis of SPI was estimated by the following equation:

$$\text{degree of hydrolysis (\%)} = \frac{A_{TCA}(t) - A_{TCA}(0)}{A_{SPI} - A_{TCA}(0)} \times 100 \qquad (1)$$

Fluorescence of SPI. Fluorescence spectra of proteins reflect well the microenvironments of tryptophyl and tyrosyl residues in the proteins, and could be a good probe for examining the change in their states (*22*). SPI (10 mg/mL) was treated with 1.0 μM subtilisin in the standard buffer. The fluorescence spectra of SPI were recorded at 37 °C using a JASCO FP-777 fluorescence spectrophotometer (Tokyo) with an excitation wavelength of 280 nm. For the measurement, SPI digests were diluted 100 times with the standard buffer to give a protein concentration of 0.1 mg/mL, at which the turbidity of the solutions was zero and no inner filter effect was observed. The relative fluorescence intensity (FI) was expressed as the ratio to the maximal FI of the reaction solution at 0 min.

ANS Fluorescence. ANS hardly radiates fluorescence in aqueous solution, but when it is bound to a protein surface, it radiates strong fluorescence depending on the hydrophobicity of its binding site. ANS could be a probe for exploring hydrophobic regions of proteins. SPI (10 mg/mL) was treated with 1.0 μM subtilisin, and was diluted 100 times with the standard buffer, to which 10 μM ANS was added. The fluorescence spectra were recorded at 37 °C with an excitation wavelength of 350 nm. No inner filter effect of ANS was observed. The fluorescence spectra of the SPI−ANS solution were not changed even after the solution was incubated at 37 °C for 30 min.

RESULTS

Effect of the Enzyme Concentration on the Coagulation of SPI. SPI (10 mg/mL) was treated with subtilisin ($[E]_o$ = 0.25−6.3 μM), and the turbidity (OD_{660}) of the reaction solutions was measured as a function of the reaction time (**Figure 1A**). At all enzyme concentrations, OD_{660} decreased in the initial phase of the reaction and reached the minimum (phase 1). Then, it increased and reached the maximum (phase 2). After reaching the maximum, it decreased again slowly (phase 3). With decreasing $[E]_o$, the progress of OD_{660} slowed without changing the maximal and minimal values of OD_{660}. To analyze the progress curves numerically, we introduced six parameters as shown in **Figure 1A**: OD_1 and OD_2, the minimum and maximum values of OD_{660}, respectively, T_1 and T_2, the times at which OD_{660} reaches OD_1 and OD_2, respectively, ΔOD, the difference between OD_2 and OD_1, or $OD_2 - OD_1$, which can be used as an index of the amount of the coagulum (*16*), and V_C, the maximum velocity of the OD_{660} increase, being the velocity of the OD_{660} increase at the inflection point in the progress curve. OD_1, OD_2, and ΔOD were hardly changed with

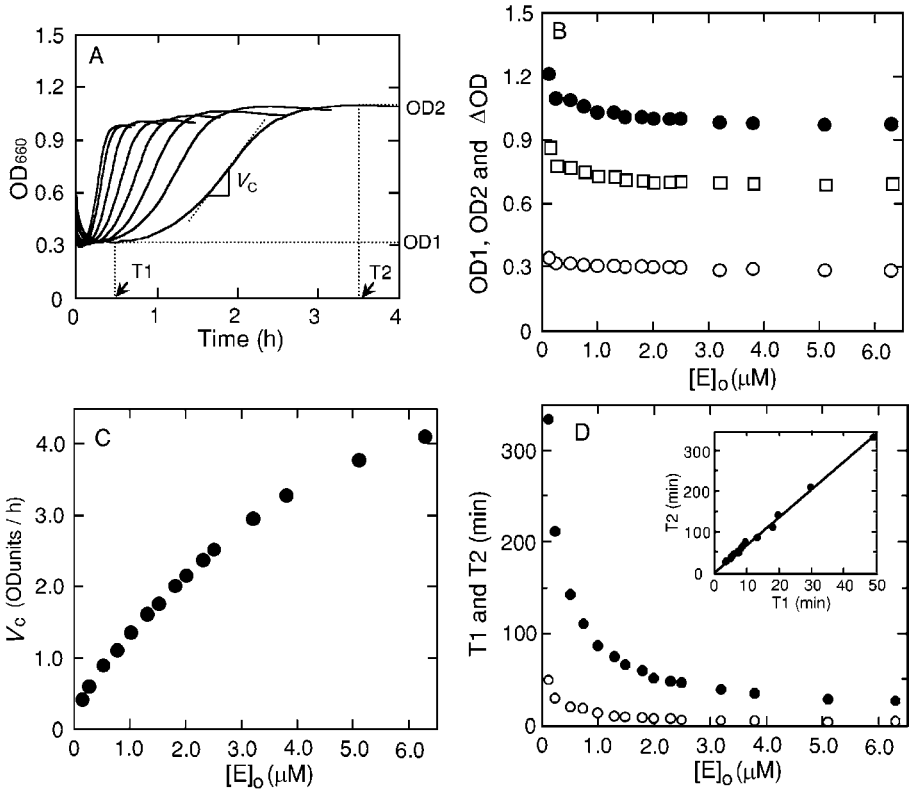

Figure 1. Dependence on the subtilisin Carlsberg concentration of the turbidity (OD_{660}) of the SPI solution treated by the enzyme. SPI (10 mg/mL) was treated with the enzyme ($[E]_o = 0.16–6.2\ \mu M$) at pH 8.0 and 37 °C. Panel **A**: Progress curves of the turbidity. $[E]_o = 5.1, 3.1, 2.0, 1.5, 1.0, 0.76, 0.51$, and $0.25\ \mu M$, respectively, for the eight progress curves from the leftmost to the rightmost. The curves changed most sharply at $5.1\ \mu M$ enzyme, and most slowly at $0.25\ \mu M$ enzyme. Panel **B**: Effect of $[E]_o$ on OD_1 (○), OD_2 (●), and ΔOD (□). Panel **C**: Effect of $[E]_o$ on V_C. Panel **D**: Effect of $[E]_o$ on T_1 (○) and T_2 (●). The inset shows the plot of T_2 against T_1. The equation for the regression is $T_2 = 6.87T_1$, with the correlation factor $R = 0.998$.

increasing $[E]_o$, although they slightly decreased in the $[E]_o$ range from 0.25 to 1.00 μM (**Figure 1B**). On the other hand, V_C increased dramatically from 0.4 ± 0.0 to 4.1 ± 0.0 (OD units/h) with increasing $[E]_o$ from 0.2 to 6.3 μM; the plots lay on a linear line at lower $[E]_o$ (<1.0 μM), and showed a saturated curve at higher $[E]_o$ (>1.0 μM), while they did not reach the saturated level even at $[E]_o = 6.3\ \mu M$ (**Figure 1C**). T_2 and T_1 decreased in an inverse-proportional manner with increasing $[E]_o$ (**Figure 1D**). The plot of T_2 against T_1 at the same $[E]_o$ lay on a straight line through the origin, indicating that a fixed ratio between T_1 and T_2 ($T_2/T_1 = 6.9 \pm 0.1$) was held at $[E]_o = 0.2–6.3\ \mu M$. Assuming that coagulating fragments are formed in phase 1 enzymatically and then coagulate in phase 2 nonenzymatically, the reaction of phase 1 should be dependent on $[E]_o$ but that of phase 2 should not. The results in **Figure 1** indicate that the velocities of the OD_{660} change in both phases 1 and 2 are dependent strongly on $[E]_o$, but the amount of the coagula formed (ΔOD) is not. It is noted that V_C increases with an increase in $[E]_o$ proportionally when it is relatively low (<1.0 μM). Under this condition, the coagulation observed in phase 2 should be an enzyme-catalyzed reaction and should be analyzed by the Michaelis–Menten treatment. On the basis of these results, we propose a possible scheme for the coagulation: SPI proteins are digested in phase 1, which is accompanied by increasing solubility of the products, and the products are further digested in phase 2 to form coagulating fragments. The enzyme-catalyzed formation of the coagulating fragments in phase 2 could be rate-limiting, and the fragments coagulate in a nonenzymatic fashion immediately after their formation.

SDS–PAGE of SPI Treated with Enzyme. Parts of the reaction solutions of the enzyme-treated SPI were taken out at the reaction times of T_1 and T_2 and the midpoint between them (T_m), and the reaction was stopped by adding 2 mM PMSF. The SPI digests and the coagula prepared by centrifugation were subjected to SDS–PAGE (**Figure 2**). SDS–PAGE patterns for the SPI digests obtained at the respective reaction times were almost the same notwithstanding the difference in the enzyme concentrations; all SPI components except the 76 kDa protein were digested promptly until T_1. After that, the fragments formed as well as the 76 kDa protein were degraded slowly. Smaller fragments (<10 kDa) produced in the SPI digests mainly participated in the coagulation at all enzyme concentrations examined.

Effect of PMSF on the SPI Coagulation. The inhibitory effect of PMSF against 1.0 μM subtilisin in the SPI solution (10 mg/mL) was examined at 20 μM to 2 mM PMSF (**Figure 3**). Addition of PMSF slowed considerably the decrease in OD_{660} in phase 1, and increased the T_1 and T_2 values. PMSF inhibited subtilisin, but the inhibition was not completed even with 2 mM PMSF. At 200 μM PMSF, a lag time of 5 min was needed for the inhibition, and the inhibition lasted for at least 3.5 h (**Figure 3C**). At every PMSF concentration, a prompt decrease in OD_{660} was observed with a lag time of a few minutes after the addition of enzyme to the SPI–PMSF solution. It is noted that 20 μM PMSF inhibited promptly the hydrolysis of *p*-nitrophenyl acetate catalyzed by 1.0 μM subtilisin (data not shown). It was considered that an excess amount of SPI proteins interfered with interactions between subtilisin and PMSF, and this interference might cause the lag time for the inhibition. It was examined whether PMSF affected the coagulation by either direct or indirect interactions with the enzyme (**Figure 4**). When PMSF was added to the SPI–subtilisin mixture at 15 min after their

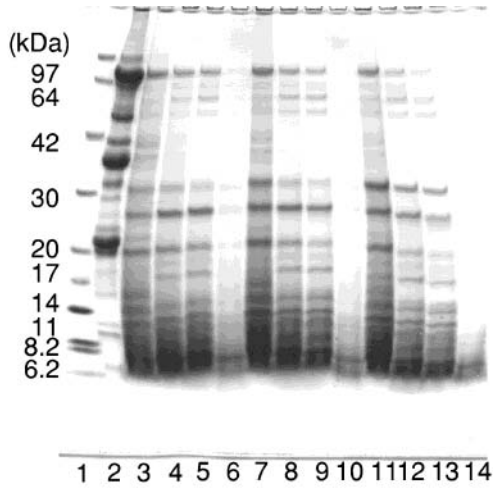

Figure 2. SDS–PAGE of the SPI proteins digested by subtilisin. SPI (10 mg/mL) was treated at $[E]_o = 0.13$, 1.0, and 6.3 μM. The digests were taken out at T_1, T_2, and T_m. The digests at T_2 were centrifuged to prepare the precipitates. Key: lane 1, molecular mass marker; lane 2, undigested SPI; lanes 3–5, digests produced by 0.13 μM enzyme at T_1, T_m, and T_2, respectively; lane 6, precipitates produced by 0.13 μM enzyme; lanes 7–9, digests produced by 1.0 μM enzyme at T_1, T_m, and T_2, respectively; lane 10, precipitates produced by 1.0 μM enzyme; lanes 11–13, digests produced by 6.3 μM enzyme at T_1, T_m, and T_2, respectively; lane 14, precipitates produced by 6.3 μM enzyme.

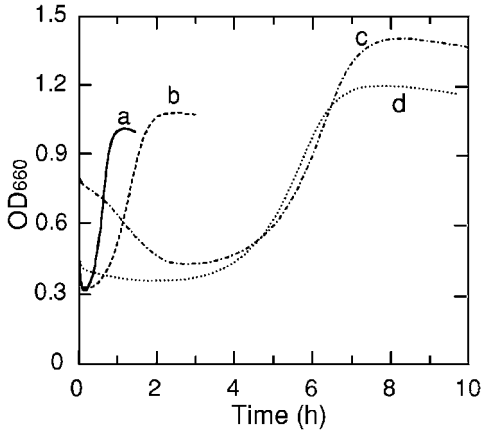

Figure 3. Inhibition of subtilisin by PMSF. PMSF was added to SPI (10 mg/mL) and incubated for 5 min before addition of 1.0 μM enzyme. The turbidity of the solution was measured. $[2\text{-propanol}]_o = 2.4\%$. $[PMSF]_0$ = a, 0 μM; b, 20 μM; c, 200 μM; and d, 2 mM.

mixing, the velocity (V_C) of the OD_{660} increase was $(7.9 \pm 0.1) \times 10^{-2}$ OD unit/h (**Figure 4A**), being much smaller than that for the blank (1.48 ± 0.10 OD units/h). The inhibition of the coagulation was attenuated when PMSF was added to the SPI–subtilisin mixture at the later positions (30, 40, or 50 min) of the progress curve (**Figure 4B**,**C**), and disappeared when PMSF was added at 50 and 55 min (**Figure 4D**,**E**). Addition of PMSF at any point always increased the OD_2 value in comparison with that of the blank curve. OD_{660} reached the maximum at 75 min in the blank, but it increased further with addition of PMSF at 75 min (**Figure 4F**).

Changes in the Microenvironments around the Aromatic Amino Acids in SPI. SPI was treated with subtilisin, and the degree of hydrolysis of SPI was estimated in the course of the hydrolysis (**Figure 5**). Compared with the reaction curve shown in **Figure 1A**, the degree of hydrolysis increased rapidly in the initial phase of the reaction and reached 8% at T_1 (10 min) and 18% at T_2 (60 min). After T_2, it increased slowly, and finally reached 25% in the reaction for 5 h. This indicates that 18% of the SPI proteins which can be precipitated with 10% TCA were converted to TCA-soluble fragments in phases 1 and 2. The ratios of the weight of the precipitate to that of the total SPI protein are 6% at time zero, 4−5% at T_1, 35% at T_2, and 20% at the end of phase 3 ($t = 4-24$ h) (*16*). This suggests that 30% and 15% of the total SPI protein are in the coagula formed at T_2 and the end of phase 3, respectively. This is discrepant from that in **Figure 5.** It is suggested that the coagula are formed not only with TCA-soluble but also with TCA-insoluble fragments. **Figure 6A** shows the fluorescence spectra of SPI digests and SPI treated with 6 M Gdn·HCl. SPI showed a spectrum with a maximum at 332 nm, and the wavelength (λ_{max}) at which the emission maximum is given is considerably shorter than that (357 nm) of *N*-acetyltryptophan ethyl ester (data not shown). This suggests that most of the aromatic amino acid residues locate in the hydrophobic internal regions of SPI. Treatment with Gdn·HCl decreased the fluorescent intensity (FI) with a red shift of λ_{max} from 332 to 351 nm, suggesting that the aromatic residues are exposed extensively to the aqueous environment due to denaturation of SPI. Treatment with the enzyme also decreased FI with a red shift of λ_{max}, and the spectrum after 5 h of reaction was similar to that of the SPI treated with Gdn·HCl. The time courses of the maximum relative FI (RFI_{max}) and λ_{max} showed that the changes in the spectrum almost finished at 50 min and only slight changes were observed after that (**Figure 6B**). RFI_{max} decreased by 32% when the digestion progressed from 0 to 50 min compared with that of the untreated SPI, and λ_{max} red-shifted simultaneously by 13 nm. On the other hand, RFI_{max} decreased by 6% and λ_{max} shifted by 7 nm in the course from 50 min to 5 h.

ANS Fluorescence. Figure 7A shows the fluorescence spectra of ANS added to SPI or the SPI digests. ANS added to SPI showed intense fluorescence with a maximum around 470 nm, which was much shorter than the maximum (530 nm) of free ANS in aqueous solution, suggesting that the ANS-binding sites on the SPI proteins are highly hydrophobic. ANS added to the SPI digests showed decreased FI, and it decreased with increasing degree of digestion. However, the FI maximum remained at around 470 nm. This suggests strongly that the amount of ANS molecules bound to SPI was decreased because of the proteolysis of the ANS-binding sites. The relative FI at 470 nm decreased until 120 min, and then approached a constant level around 40% of that of the untreated SPI (**Figure 7B**).

DISCUSSION

Mechanism of SPI Coagulation by Subtilisin Carlsberg. The amount of the coagulum formed (ΔOD) is constant at the $[E]_o$ examined (0.16−6.2 μM) (**Figure 1B**). In addition, SDS–PAGE showed that the digestion patterns of SPI were essentially the same at $[E]_o = 0.13-6.3$ μM **(Figure 2)**. This suggests that the coagulating potential of the SPI digests is kept constant at $[E]_o = 0.16-6.2$ μM. If we assume that the SPI digests participating in the coagulation were produced mainly by the primary digestion until T_1 and that the following secondary digestion in phase 2 hardly affects the coagulation, an $[E]_o$ change might affect the reaction in phase 1 but not the reaction in phase 2. As long as the assumption stands, the values of V_C and $T_2 - T_1$ must be independent of $[E]_o$. However, V_C increases in a saturating fashion, and $T_2 - T_1$ decreases inverse-proportionally with increasing $[E]_o$ (**Figure 1**). This result indicates strongly that fragments produced in phase 2 might have a major influence on V_C. Therefore, the digestion in phase

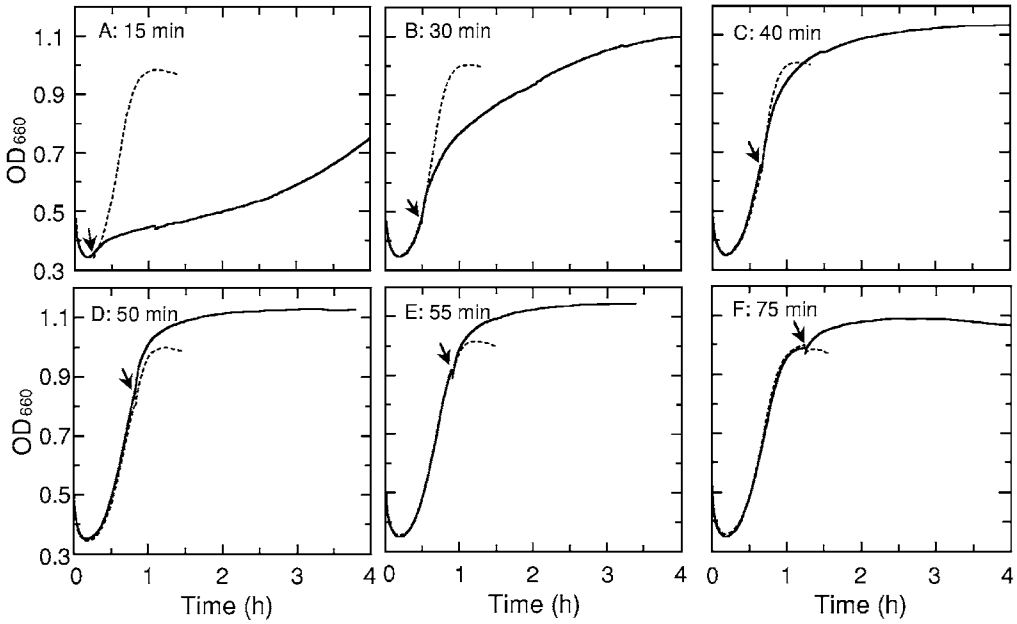

Figure 4. Effect of PMSF on the SPI coagulation by subtilisin. SPI (10 mg/mL) was treated with 1.0 μM enzyme, and the turbidity of the solution was measured. At a given time, PMSF (solid lines) or 2-propanol (blank, dashed lines) was added. $[PMSF]_0$ and $[2\text{-propanol}]_0$ were 200 μM and 2.4%, respectively. Time of the PMSF addition: **A**, 15 min; **B**, 30 min; **C**, 40 min; **D**, 50 min; **E**, 55 min; and **F**, 75 min.

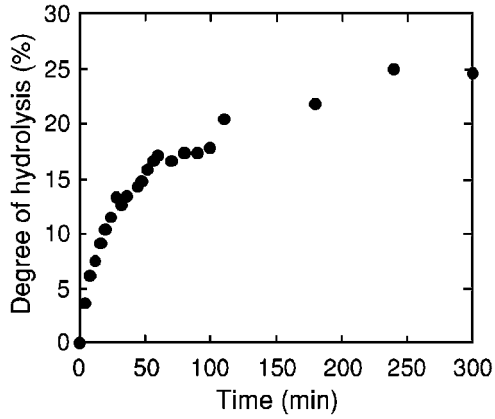

Figure 5. Changes in A_{280} of the 10% TCA-soluble fraction. The 10% TCA-soluble fraction was prepared from the reaction mixture of the enzyme-treated SPI as obtained in **Figure 4**. The degree of hydrolysis (eq 1) is plotted against the reaction time.

2 which produces coagulating fragments could be accelerated with increasing $[E]_0$, and this may result in an increase in V_C. The assumption is supported by the observation on the effect of PMSF on the coagulation (**Figure 5**). Addition of PMSF at T_1 (15 min) inhibits the coagulation, suggesting that the digestion occurring after T_1 dominates the coagulation more than that after T_1. It has been indicated that the SPI fragments produced in phase 2 participate in the coagulation, and both production of coagulating fragments and their coagulation occur simultaneously in this phase. As a consequence of these observations, the possibility of participation of the fragments produced in phase 1 in coagulation should be excluded. It is noted that molecular sizes of the fragments produced at the point of T_1 are similar to those of the coagulating fragments produced in phase 2 (**Figure 2**). Probably, the fragments produced in phase 1 are converted to coagulating fragments in phase 2 by secondary proteolysis with a minor change in molecular sizes. PMSF added after T_2 increased considerably the maximum value of the turbidity, namely, the amount of coagulum, without

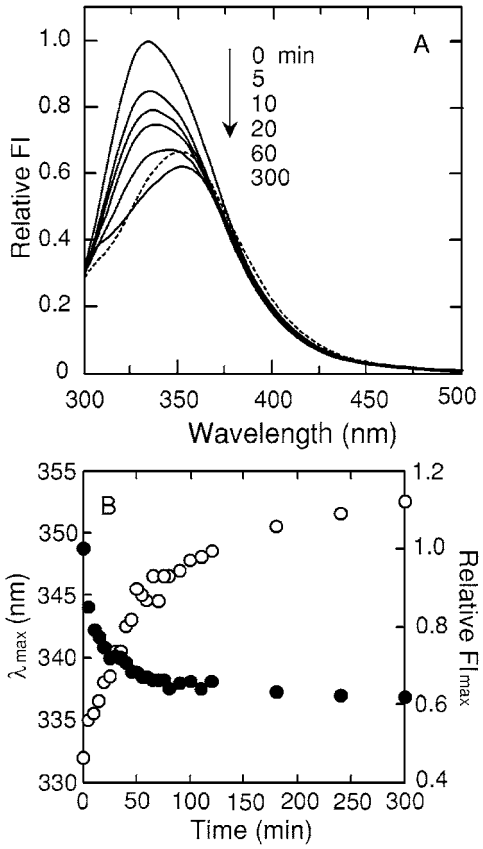

Figure 6. Fluorescence of SPI. Panel **A**: The solid lines show emission spectra, from the top to the bottom, at reaction times of 0, 5, 10, 20, 60, and 300 min, respectively. The dotted line is the spectrum of SPI (0.1 mg/mL) treated with 6 M Gdn·HCl. Panel **B**: progress curves of the maximal FI (FI_{max}, close circles) and the wavelength of FI_{max} (λ_{max}, open circles).

changing the velocity of coagulation (**Figure 4**). The coagulum is digested by the enzyme in phase 3, and the yield of the coagulum is decreased in a prolonged enzyme treatment (*16*).

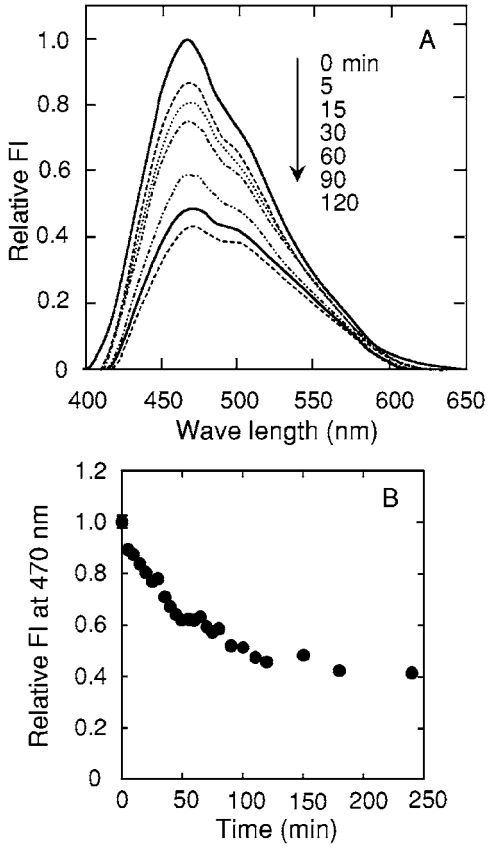

Figure 7. Fluorescence of ANS. Panel **A**: spectra obtained at reaction times of 0, 5, 15, 30, 60, 90, and 120 min, respectively, from the top to the bottom. Panel **B**: progress curve of the relative FI at 470 nm.

Therefore, it is supposed that PMSF inhibits the digestion of the coagulum, resulting in an increase in its yield. This indicates that V_C is affected by the protease activity as well as the coagulating potential of the SPI digests. The progress curve of OD_{660} (**Figure 1A**) should be expressed by a rate equation. V_c is dependent on both $[E]_o$ (**Figure 1C**) and $[SPI]_o$ (*16*), while the velocity of the decrease in OD_{660} in phase 1 is independent of $[SPI]_o$ (*16*). From these lines of evidence, the reactions in phases 1 and 2 are supposed to be zeroth-order and first-order, respectively, with respect to the SPI concentration. However, the entities expressed by OD_{660} in phases 1 and 2 are substantially different. We need more kinetic information on the protease-catalyzed coagulation reaction of SPI to formulate the rate equation for the progress curve of OD_{660}.

Structural Change of SPI Occurring during Proteolysis. It has been speculated that the fragments participating in the coagulation are derived from the peptide segments buried in the internal hydrophobic regions of SPI, and they might coagulate through hydrophobic interactions (*13*, *15*). Glycinin and β-conglycinin are the main components of SPI. Glycinin proteins are observed as 20, 35, and 42 kDa bands in SDS−PAGE, and β-conglycinin proteins as 50, 70, and 76 kDa bands (**Figure 2**). There are 3−6 tryptophyl and 10−15 tyrosyl residues in every subunit of glycinin, and 0−2 tryptophyls and 12−13 tyrosyls in every subunit of β-conglycinin (*23*, *24*). It has been shown that the structural changes of SPI occur in a time range including phase 1 and phase 2 by observing the changes in the microenvironments around the aromatic residues in SPI and those of ANS externally added (**Figures 5**−**7**). On the other hand, the SPI proteins are digested into fragments (<10 kDa) promptly in phase 1, and further digestion in phase 2 is much slower, although the fragments produced in phase 2 are involved in the coagulation. The states of aromatic residues and ANS-binding regions are not much affected in phase 1 compared with those in phase 2, suggesting that the degree of decomposition of the 3-D structure of the SPI proteins in phase 1 seemed to be less than that observed in phase 2. In general, when a protease hydrolyzes a protein in its folded form, flexible regions such as loops or hinges are more likely to be attacked than structured regions such as α-helices or β-sheets (*25*, *26*). If the same is true for the hydrolysis of SPI by subtilisin, the results described above are explained as follows: In the primary digestion in phase 1, flexible regions are digested. It is relatively fast, but the structured regions are almost maintained. The structured regions are digested by the secondary digestion in phase 2. This digestion is slower than that in phase 1, but the 3-D structure of the proteins decomposes largely in phase 2.

The molecular sizes of the fragments in coagula are less than 10 kDa, and the fragments of 76, 32, 27, and 20 kDa, which are fairly resistant to digestion, are in the soluble fraction (*16*). We are currently examining the digestion of the respective components of conglycinin and β-conglycinin to characterize the coagulating fragments and identify their origin. Soy proteins contain protein trypsin inhibitors and allergenic proteins. The representative inhibitors are of Kunitz type and Bowman−Birk type, their molecular sizes being 21.5 and 8.0 kDa, respectively (*27*). A protein band corresponding to the Kunitz inhibitor is observed in SDS−PAGE (**Figure 2**). In our examination, however, the trypsin inhibitory activity of SPI was not detected, and the inhibitors had no inhibitory activity against subtilisin Carlsberg (K. Inouye, unpublished data). More precise study of the effect of the protease inhibitors contained in SPI on its coagulation is needed. We studied extensively the characterization and inhibitory mechanism of a protein subtilisin inhibitor, *Streptomyces* subtilisin inhibitor (SSI) (*28*−*30*). SSI inhibits subtilisins Carlsberg and BPN′ specifically and strongly with an inhibitor constant (K_i) of 1 pM or less, but inhibition against α-chymotrypsin and trypsin is much weaker (K_i > 1 mM). In the present study, we have demonstrated that PMSF is useful to control the coagulating reaction. However, PMSF inhibits strongly various serine proteases, and is toxic in the human body. Therefore, SSI is promising in preparing SPI coagula for food applications. A major soy protein allergen is a 30 kDa β-conglycinin protein named *Gly m* Bd 30K (*31*). It has been preliminarily demonstrated that the allergenic activity disappears in the coagula and soluble fractions obtained by subtilisin Carlsberg-catalyzed hydrolysis of SPI (K. Inouye, unpublished data), suggesting that the protease treatment of SPI may improve its immunological demerits. Generally, peptides produced by proteolysis have a bitter taste (*32*). A point of further study will be how to reduce the taste or mask it. To elucidate the more detailed molecular mechanism of coagulation of the SPI digests, microscopic and macroscopic examinations including identification of key components for the coagulation, characterization of peptides in the coagula, effects of medium conditions on the coagulation, and nutritional properties of the coagula are under way.

LITERATURE CITED

(1) Kinsella, J. E. Functional properties of soy proteins. *J. Am. Oil Chem. Soc.* **1979**, *56*, 242−258.

(2) Kunst, T. Protein modification to optimize functionality: Protein hydrolysates. In *Handbook of Food Enzymology*; Whitaker, J. R., Voragen, A. G. J., Wong, D. W. S., Eds.; Dekker: New York, 2003; pp 221−236.

(3) Chobert, J.-M.; Bertrand-Harb, C.; Nicolas, M.-G. Solubility and emulsifying properties of caseins and whey proteins modified enzymatically by trypsin. *J. Agric. Food Chem*. **1988**, *36*, 883−892.

(4) Hevia, P.; Whitaker, J. R.; Olcott, H. S. Solubilization of a fish protein concentrate with proteolytic enzymes. *J. Agric. Food Chem.* **1976**, *24*, 383−385.

(5) Chen, S. X.; Swaisgood, H. E.; Foegeding, E. A. Gelation of β-lactoglobulin treated with limited proteolysis by immobilized trypsin. *J. Agric. Food Chem*. **1994**, *42*, 234−239.

(6) Turgeon, S. L.; Gauthier, S. F.; Paquin, P. Interfacial and emulsifying properties of whey peptide fractions obtained with a two-step ultrafiltration process. *J. Agric. Food Chem*. **1991**, *39*, 673−676.

(7) Caessens, P. W. J. R.; Gruppen, H.; Visser, S.; Van Aken, G. A.; Voragen, A. G. J. Plasmin hydrolysis of β-casein: foaming and emulsifying properties of the fractionated hydrolysate. *J. Agric. Food Chem.* **1997**, *45*, 2935−2941.

(8) Caessens P. W. J. R.; Visser, S.; Gruppen, H.; Voragen, A. G. J. β-lactoglobulin hydrolysis. 1. Peptide composition and functional properties of hydrolysates obtained by the action of plasmin, trypsin, and *staphylococcus aureus* V8 protease. *J. Agric. Food Chem.* **1999**, *47*, 2973−2979.

(9) Yamamoto, N. Antihypertensive peptides derived from food proteins. *Biopolymers* **1997**, *43*, 129−134.

(10) Fukudome, S.; Jinsmaa, Y.; Matsukawa, T.; Sasaki R.; Yoshikawa, M. Release of opioid peptides, gluten exorphins by the action of pancreatic elastase. *FEBS Lett.* **1997**, *412*, 475−479.

(11) Parker, F.; Migliore-Samour, D.; Floc'h, F.; Zerial, A.; Werner, G. H.; Jollès, J.; Casaretto, M.; Zahn, H.; Jollès, P. Immunostimulating hexapeptide from human casein: amino acid sequence, synthesis and biological properties. *Eur. J. Biochem*. **1984**, *145*, 677−682.

(12) 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.

(13) 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.

(14) Park, Y. W.; Kobayashi, H.; Kusakabe, I.; Murakami, K. Purification and characterization of soymilk-clotting enzymes from *Bacillus* sp. K-295G-7. *Agric. Biol. Chem.* **1987**, *51*, 2343−2349.

(15) 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.

(16) Inouye, K.; Nagai, K.; Takita T. Coagulation of soy protein isolates induced by subutilisin Carlsberg. *J. Agric. Food Chem*. **2002**, *50*, 1237−1242.

(17) Samoto, M.; Miyazaki, C.; Kanamori, J.; Akasaka, T.; Kawamura, Y. Improvement of the off-flavor of soy protein isolate by removing oil-body associated proteins and polar lipids. *Biosci. Biotechnol. Biochem.* **1988**, *52*, 935−940.

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

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

(20) Inouye, K. Thermolysin. In *Handbook of Food Enzymology*; Whitaker, J. R., Voragen, A. G. J., Wong, D. W. S., Eds.; Dekker: New York, 2003; pp 1019−1028.

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

(22) Inouye, K.; Tanaka, H.; Oneda, H. States of tryptophyl residues and stability of recombinant human matrix metalloproteinase 7 (matrilysin) as examined by fluorescence. *J. Biochem*. **2000**, *128*, 363−369.

(23) Wright, D. J. The seed globulins. In *Developments in Food Proteins-5*; Hudson, B. J. F., Ed.; Elsevier: London, 1987; pp 81−157.

(24) Wright, D. J. The seed globulins-Part II. In *Developments in Food Proteins-6*; Hudson, B. J. F., Ed.; Elsevier: London, 1988; pp 119−178.

(25) Fontana, A.; Fassina, G.; Vita, C.; Dalzoppo, D.; Zamai, M.; Zambonin, M. Correlation between sites of limited proteolysis and segmental mobility in thermolysin. *Biochemistry* **1986**, *25*, 1847−1851.

(26) Hubbard, S. J.; Eisenmenger, F.; Thornton, J. M. Modeling studies of the change in conformation required for cleavage of limited proteolytic sites. *Protein Sci*. **1994**, *3*, 757−768.

(27) Birk, Y. *Plant Protease Inhibitors: Significance in Nutrition, Plant Protection, Cancer Prevention and Genetic Engineering*; Springer-Verlag: Berlin, 2003.

(28) 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.

(29) Inouye, K.; Tonomura, B.; Hiromi, K. Interaction of α-chymotrypsin and a protein proteinase inhibitor, *Streptomyces* subtilisin inhibitor. The formation of ternary complex of *Streptomyces* subtilisin inhibitor, α-chymotrypsin, and proflavin. *J. Biochem*. **1979**, *85*, 601−607.

(30) Inouye, K.; Tonomura, B.; Hiromi, K. Interaction of a tyrosyl residue and carboxyl groups in the specific interaction between *Streptomyces* subtilisin inhibitor and subtilisin BPN′. A chemical modification study. *J. Biochem*. **1979**, *85*, 1115−1126.

(31) Ogawa, T.; Tsuji, H.; Bando, N.; Kitamura, K.; Zhu, Y.-L.; Hirano, H.; Nishikawa, K. Identification of the soybean allergic protein, Gly m Bd 30K, with the soybean seed 34 kDa oil-body-associated protein. *Biosci. Biotechnol. Biochem*. **1993**, *57*, 1030−1033.

(32) Ishibashi, N.; Kouge, K.; Shinoda, I.; Kanehisa, H.; Okai, H. A mechanism for bitter taste sensitivity in peptides. *Agric. Biol. Chem*. **1988**, *52*, 819−827.

Received for review July 28, 2003. Revised manuscript received May 5, 2004. Accepted May 20, 2004. This study was supported in part (K.I.) by Grants 0150 and 0345 from the Salt Science Foundation (Tokyo) and grants from the Fuji Foundation for Protein Research (Osaka).

JF034845G