COAGULATION AND GELATION OF SOY PROTEIN ISOLATES INDUCED BY MICROBIAL TRANSGLUTAMINASE

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ABSTRACT

The reaction process and corresponding mechanism of coagulation and gelation of native soy protein isolates (SPIs) induced by microbial transglutaminase (MTGase) were investigated. The protein constituents of SPIs, including a majority of subunits of β-conglycinin and acidic subunits of glycinin, could be polymerized by MTGase to form high weight molecular (WM) biopolymers. Both the coagulation and gelation reactions of native SPI solutions induced by MTGase were dependent upon the initial protein substrate concentration ($[C]_0$). In the coagulating reactions, the turbidity of SPI solutions continually increased with increasing $[C]_0$ in the range from 0.25 to 3.0%. As for the gelation reactions, with the concentration increasing from 3 to 8% (w/v), the onset time of gelation of native SPIs induced by 0.8 units/mL of MTGase at 37C shortened by ~5-fold, and the storage modulus (G') of finally formed gels (after 4 h) increased from ~1 to 1300 Pa. Both the coagulation and gelation reactions of SPI solutions were promoted remarkably by increasing the enzyme concentration. Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis analysis showed that the protein constituents of MTGase-induced aggregates of SPI (2% w/v) were mainly composed of basic subunits of glycinin and some of newly cross-linked high MW biopolymers. The solubility analysis of protein constituents indicated that the covalent cross-linkage, hydrophobic and H bindings and disulfide bonds were mainly involved in the coagulation of SPI induced by MTGase.

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INTRODUCTION

Soy proteins are often applied in a wide range of food products because of their highly nutritive value and ability to improve the texture. The gelling ability of soy proteins or their individual components (such as glycinin and β-conglycinin) have been widely investigated in heat-induced cases (Nakamura *et al.* 1984, 1986a, b; van Kleef 1986; Kang *et al.* 1991; Nagano *et al.* 1992, 1994; Renkema *et al.* 2000, 2001; Renkema and van Vliet 2002; Renkema 2004; Renkema and van Vliet 2004). However, the thermally induced gelation of soy proteins is not easily controlled, and the formed gels are coarse and stiff with poor water-holding capacity. In order to overcome the limitation of heat-induced gels of proteins, some other treatments (such as acidification, cross-linking or hydrolyzing) have been attempted to induce the gelation instead of the heat treatment (Nio *et al.* 1985; Nonka *et al.* 1989; Kang *et al.* 1994; Chanyongvorakul *et al.* 1995; Alting *et al.* 2002; Tay *et al.* 2005). Of all these treatments, the gelation of proteins by means of transglutaminase (TGase) seems to be more attractive.

TGase (EC 2.3.2.13) catalyzes an acryl transfer reaction between γ-carboxyamide groups of peptide-bound glutamine residues (acyl donor) and the primary amino groups in a variety of amine compounds (acyl acceptor) including peptide-bound ε-amino groups of lysine residues (Motoki and Seguro 1998). As a result, cross-links or ε-(γ-glutamyl)lysine isopeptide bonds and high molecular weight (MW) polymers of proteins are formed. To date, a lot of studies have shown that this kind of enzyme, including those from animal and microbial sources, could effectively and directly induce casein or casein micelles, soybean proteins (including glycinin or β-conglycinin) and even gelatin to form gels (Nio *et al.* 1985; Chanyongvorakul *et al.* 1994, 1995; Kang *et al.* 1994; Schorsch *et al.* 2000; Babin and Dickinson 2001). However, few detailed information about this gelation of soy proteins-induced TGase has been known, except some on the gelation or gel properties of glycinin or other 11S globulins (Nio *et al.* 1985; Kang *et al.* 1994; Chanyongvorakul *et al.* 1995). Covalent cross-linking is generally recognized as the direct reason to induce the gelation of native soy proteins, but the underlying gelation mechanism seems to be more complicated. In some cases, the hydrophobic interactions of some protein constituents of glycinin or β-conglycinin, which are not prone to the cross-linking of TGase, should also be not neglected. Therefore, it is necessary to investigate the details of gelation of native soy protein isolates (SPIs) induced by TGase.

In the heat-induced gelation of soy proteins, the protein denaturation induced by heat treatment is often a prerequisite for gel formation of soy proteins (Utsumi and Kinsella 1985a, b; Nakamura *et al.* 1986a, b; van Kleef 1986; Puppo and Añón 1998; Renkema and Vliet 2002) because the exposed

hydrophobic areas of denatured proteins could interact and aggregate. This kind of gelation of soy proteins is generally related to the protein substrate concentration. If the protein concentration is not high enough, a coagulation reaction rather than the gelation will occur as a result of the thermal treatment. Thus, the underlying mechanism of the coagulation and gelation would be similar. In the TGase-induced cases, whether the underlying mechanisms of these reactions are similar is nearly not mentioned in any literature. In the present study, the first objective was to investigate in detail both the coagulation and gelation of various concentrations of SPIs induced by a different level of microbial transglutaminase (MTGase). In addition, we also investigated the protein constituents and interactive forces of aggregates formed during the cross-linking reactions, and tried to describe a possible reaction mechanism for the coagulation and gelation induced by MTGase.

MATERIALS AND METHODS

Materials

Throughout the experiment, 0.05 M Tris-HCl buffer (pH 7.5, 25C) containing 0.05% sodium azide was used as the standard buffer. The whole soybean seed powder was provided by Henan Hebi Co. (China). Commercial MTGase was obtained from Chanshou Biological Co. Ltd. (China). N_{α}-CBZ-GLN-GLY and L-glutamic acid γ-monohydroxamate were purchased from Sigma Chemical Company (St. Louis, MO). The purification of MTGase and its activity's determination were according to the method of Tang *et al.* (2005). All other chemicals were of analytical reagent or better grade. Silicone oil was a gift from the Guangzhou office of Pharmacia Co.

Preparation of SPIs

The SPIs were prepared according to the method of Iwabuchi and Yamauchi (1987) but with a minor modification. The following procedures were performed at room temperature. Defatted soybean meal was prepared from ground soybean seed powder by solvent extraction with *n*-hexane. Defatted soybean seed meal was then extracted with 20-fold 0.03 mol/L Tris-HCl buffer (pH 8.0) containing 10 mmol/L β-mecaptoethanol (2-ME) and centrifuged to remove the insoluble material. The pH of the extract was adjusted to pH 4.8 at 4C with 2 N HCl, and the precipitate or curd was collected by centrifugation. The obtained curd was dissolved in water at 4C and adjusted to pH 7.5 with 2 N NaOH and then centrifuged at 4C, yielding the SPI supernatant. The supernatant was dialyzed thrice at 4C against desalted water (1:100,

3 times) and then lyophilized to yield the SPI product. The protein content of this SPI was 97.0% (dry basis) (determined by Kjeldahl method, $N \times 6.25$).

Coagulation of SPIs Induced by MTGase

The SPIs were dispersed in the standard buffer at 10% (w/v) and stirred at room temperature for at least 2 h. The dispersion was centrifuged at $8000 \times g$ for 15 min, and the supernatant was used as the standard SPI solution. This solution was prepared daily and was kept at 4C before use.

The standard SPI solution was diluted with the standard buffer to a certain concentration (0.25–3% w/v). The final SPI solution was incubated at 37C and mixed with a certain amount of MTGase to start the coagulation reaction. The reaction was analyzed by the turbidity, and the latter was evaluated by measuring optical density at 660 nm (A_{660}) (Inouye *et al.* 2002; Nagai and Inouye 2004). The corresponding SPI solution without the addition of MTGase was used as the blank.

Gelation of SPIs Induced by MTGase

The standard SPI solution was diluted with the standard buffer to a certain concentration (3–8% w/v). After the SPI solution was mixed well with a certain amount of MTGase, the mixture was immediately transferred to the lift plate of rheometer whose experiment temperature was preset at 37C. The gelation process of various SPI solutions was evaluated by the following dynamic oscillatory measurements:

Visual Observation of the Coagulation and Gelation. Coagulation and gelation of SPIs induced by MTGase were observed in 15-mm-diameter glass test tubes at 37C. The total volume of the reaction solution was set to 3.0 mL.

Determination of the Protein Solubility. The protein solubility of the SPI aggregates was determined according to Lupano *et al.* (1996). Samples (0.1% protein w/v) were dispersed by magnetic stirring at room temperature either in distilled water (DW), in a pH 8.0 buffer (0.086 M Tris, 0.09 M Glycine, 4 mM Na_2EDTA) (B), or in the same buffer containing 0.5% sodium dodecyl sulfate (SDS) and 8 M urea (BSU) or in the BSU plus 1% (v/v) 2-ME (BSUM) and then centrifuged at 15,000 g for 20 min. Protein solubility was determined from the supernatants and expressed as 100′ protein content in the supernatant/total protein content. Three independent extractions were carried out with each solvent. Average values (± standard deviations) were given. Protein concentration in various solvents was determined spectrophotometrically at 280 nm, and the corresponding solvent was used as the blank. The absorbency of 0.1% (w/v) native SPI in various solvents was defined as 100% protein solubility.

Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE). SDS-PAGE was performed on a discontinuous buffered system according to the method of Laemmli (1970) by using 12 and 4% stacking gels. The samples (the enzyme mixture directly mixed with sample buffer [×2], 1:1 [v/v]) or (the moderate samples dissolved with sample buffer [×1]) were heated for 5 min in boiling water before electrophoresis. Ten microliters of sample was applied to each lane. Before the sample entered the separating gel, electrophoresis had been performed at 10 mA, and the other was at 20 mA. The gel was stained with 0.25% Coomassie Brilliant Blue (R-250) in 50% trichloroacetic acid and destained in 7% acetic acid (methanol:acetic:water, 227:37:236 [v/v/v]).

Low Amplitude Dynamic Oscillatory Measurements. Rheological measurements using parallel plates (d = 27.83 mm) were carried out in a RS600 Rheometer (Thermo Haake, Dieselstr., Karlsruhe, Germany). The sample dispersions were placed between parallel plates, and the gap between two plates was set to 1.0 mm. Excess sample was trimmed off, and a thin layer of silicone oil was applied to the exposed free edges of the sample to prevent moisture loss. The lower plate was held at 37C. The equipment was driven through the RheoWin 3 Data Manager (Thermo Haake, Germany). Storage modulus (G'), loss modulus (G'') and phase angle (σ) or tan (σ), were recorded as a function of time. In order to ensure all measurements were carried out within the linear viscoelastic range, first stress amplitude sweep was performed at a shear oscillation of 0.1 Hz or other values.

The changes of rheological behaviors (G', G'' and σ or tan [σ]) of the mixture of SPI and enzyme at 37C were recorded and analyzed immediately after a SPI solution (preheated to this temperature) was mixed with MTGase and loaded onto the lift of the rheometer. The time of last crossover point of the G' and G'' during the incubation was defined as the onset time of gelation (T_{gel}).

Statistics

An analysis of variance of the data was performed, and a least significant difference test with a confidence interval of 95% was used to compare the means.

RESULTS AND DISCUSSION

Coagulation and Gelation of SPIs Induced by MTGase

The turbidity of native SPI solution (2% w/v) increased with the incubation time with MTGase and by visual observation of the coagulation phenom-

enon (Fig. 1A). The visual changes of 6% (w/v) native SPI solution incubated with MTGase (10 units/g of protein) was also observed. Not only did the turbidity of SPI solution observably increase with the incubation time, but also a gel was formed after incubated with MTGase for more than 30 min (Fig. 1B). Similar phenomena were observed in TGase-induced gelation of soy 7S globulins or β-conglycinin, glycinin, ovalbumin and other proteins (Nio *et al.* 1985; Schorsch *et al.* 2000; Alting *et al.* 2004). Figure 2 shows the SDS-PAGE patterns of native SPI (2% w/v) incubated with MTGase at 37C for various times (from 0 to 360 min). Once incubated with MTGase, the protein constituents (including α, α′ and β-subunits) of β-conglycinin and acidic subunits of glycinin were polymerized to form some new biopolymers, which could not enter either the separating gel or the stacking gel, while the basic subunits of glycinin were nearly unaffected after 360 min (Fig. 2). This result was in agreement with many previous reports (Nonka *et al.* 1989; Kang *et al.* 1994; Tang *et al.* 2002). Therefore, the occurrence of coagulation and gelation of native SPI solutions after addition of MTGase could be considered as the result of the covalent cross-linking reactions.

Changes in the Turbidity or Mechanical Moduli of SPI Solutions

The turbidity (A_{660}) of a various concentration (from 0.25 to 3% w/v) of SPI solutions increases in a similar trend with the incubation time after the addition of MTGase (10 U/g protein substrate) (Fig. 3). This increase in the turbidity, induced by MTGase, is closely related to the initial SPI concentration ($[C]_0$), and at the same ratio of enzyme to substrate, the higher concentration of SPI solution is favorable for the coagulating reaction. At a low concentration (e.g., 0.25–1.0%) case, the turbidity increases fast at the initial stage of the reaction then slowly with further incubation with MTGase. It increases and reaches the maximum at the end of experimental time (near 600 min) (Fig. 3). However, in the case of 2% or higher SPI solutions, the increase in the turbidity undergoes a "turnabout," or a tangent point. Before the time of the tangent point, the turbidity increases in a similar manner as in the 1% or lower case. Whereas, after that, the turbidity almost increases exponentially to a value beyond the upper limit of the spectrophotometer.

Usually the turbidity of the SPI solution should be reflected by the colloidal properties of the SPI proteins and the coagulum formed (Inouye *et al.* 2002). Therefore, the increase in the turbidity of SPI solution induced by MTGase can be the formation of insoluble aggregates or coagula. Similar results have been obtained in the protease-induced coagulation cases of SPI (Inouye *et al.* 2002; Nagai and Inouye 2004). It is shown in Fig. 3 that the formation of the coagula was not only related to the $[C]_0$ but was also affected by the concentration of enzyme.

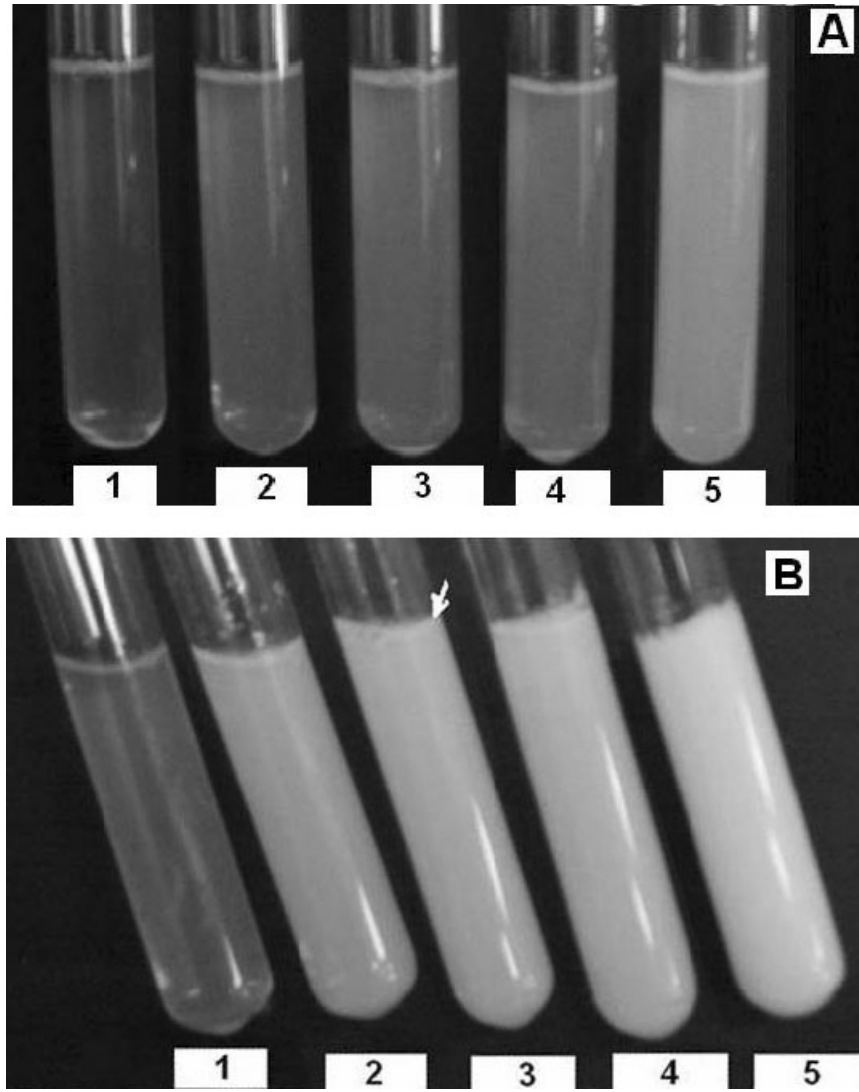

FIG. 1. VISIBLE CHANGES OF 2% (A) AND 6% (B) SPI SOLUTIONS OBSERVED AFTER THE ADDITION OF 10 UNITS PER GRAM OF PROTEIN SUBSTRATE OF MTGASE AT 37C Lane 1: the SPI solution without the addition of MTGase; Lanes 2–5: the reaction mixtures at the time of 15, 30, 60 and 240 min, respectively. The arrow indicates the formation of a gel or solution.

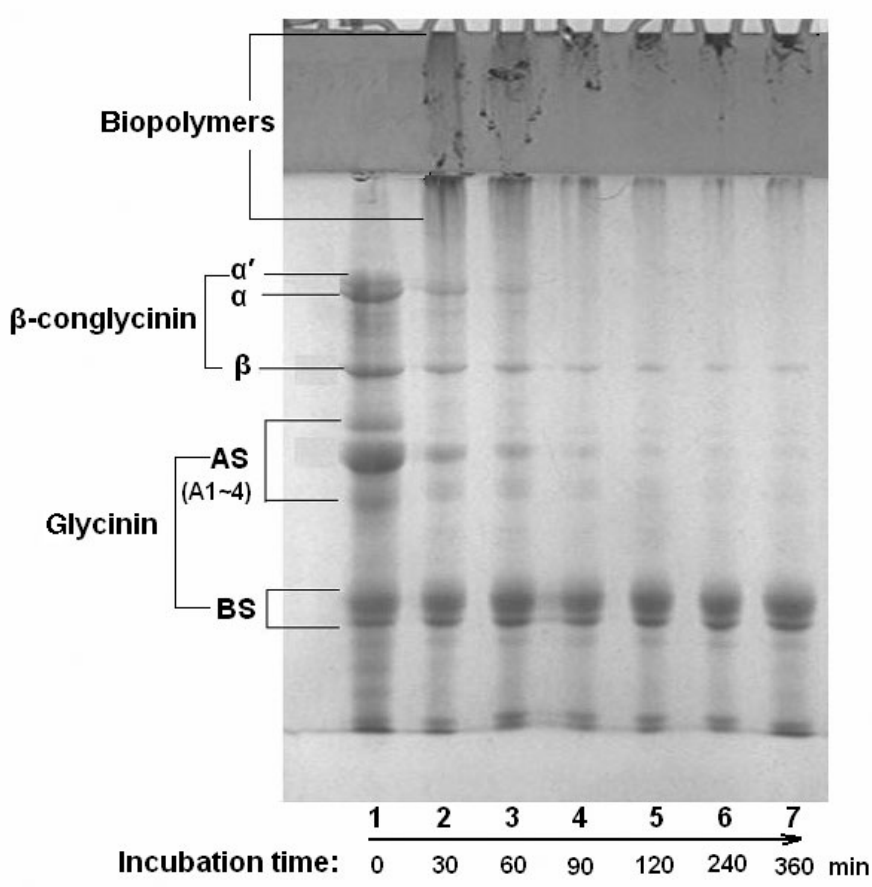

FIG. 2. SDS-PAGE PROFILES OF NATIVE AND MTGASE-TREATED SPI
Lanes 1–7 indicate the 2% (w/v) SPI incubated with 10 units per gram of protein substrate of MTGase at 37C for 0, 30, 60, 90, 120, 240 and 360 min, respectively.

The change patterns of mechanical moduli (including the storage modulus G′ and loss modulus G″) could reflect the gelation process and the development of formed gels (Tabilo-Munizaga and Barbosa-Cánovas 2005). Figure 4 shows the dynamic gelation process of high concentration (≥3%) of native SPI by a constant concentration of MTGase at 37C. A typical gelation curve of native SPI (8% w/v) incubated with MTGase is shown in Fig. 4A. In this case, the G′ and G″ did not change remarkably until after incubation with MTGase for about 15 min. After that, the G′ increased much faster than G″, indicating the onset of gelation or the initial formation of gel network with

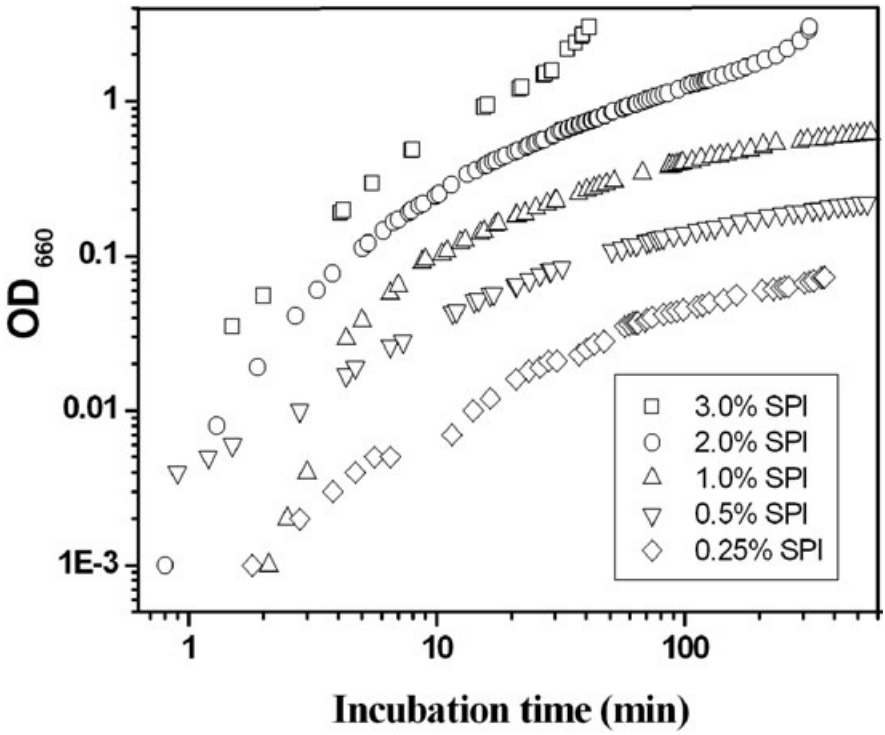

FIG. 3. CHANGES IN THE TURBIDITY OF A VARIABLE CONCENTRATION OF NATIVE SPI SOLUTIONS DURING THE INCUBATION WITH 10 UNITS PER GRAM OF PROTEIN SUBSTRATE OF MTGASE AT 37C

incubation with MTGase. The sudden increase in the G′ during the incubation with MTGase (Fig. 4A) also suggests that the gel-formation of SPI by MTGase be a dynamic process, and the initial formation of gel-network needs enough cross-links of native SPI induced by MTGase.

In the MTGase-induced gelation cases, the influence of protein concentration on the G′ and G″ of native SPI solutions (Fig. 4B) was like that in the MTGase-induced coagulation cases. Similarly, the gelation of a high concentration of native SPIs induced by MTGase is tightly related with the initial substrate concentration ($[C]_0$). While the $[C]_0$ increases in the range from 3 to 8%, the onset time (T_{gel}) shortens from near 80–15 min, and the G′ value increases from ~1 to −1300 Pa. In addition, the G″ value increases from a value less than 1 Pa to 35 Pa while the $[C]_0$ increases from 3 to 8% (data not shown). From Figs. 3 and 4, it is obvious that either in MTGase-induced gelation cases at a high concentration of protein (>3%) or in MTGase-induced coagulation

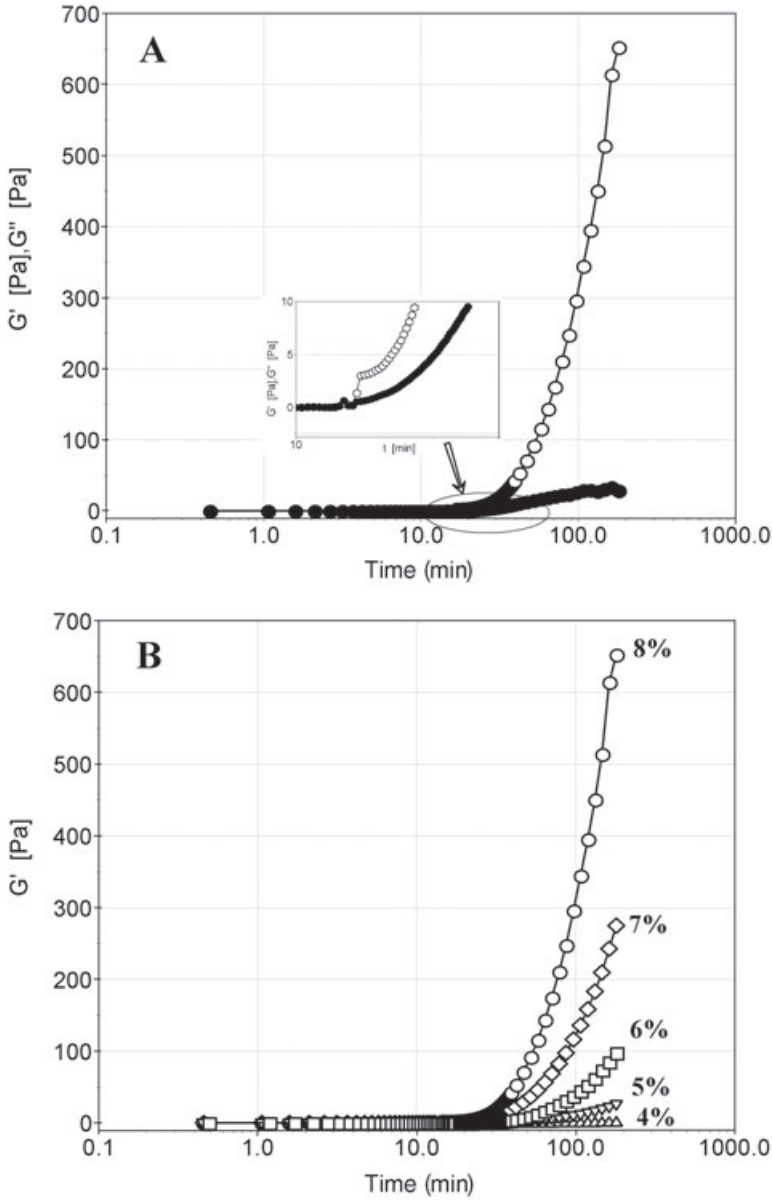

FIG. 4. EFFECT OF SUBSTRATE CONCENTRATE ON THE GELATION OF NATIVE SPI INDUCED BY MTGASE

Panel A: Typical gelation curve of native SPI induced by MTGase at 37C; Panel B: The dynamic gelation profiles of a various concentration (4, 5, 6, 7 and 8% w/v) of native SPI induced by 0.8 units per mL of MTGase at 37C

cases at a low concentration of protein (<3%) the underlying mechanism of gelation and coagulation may be similar.

Effect of the Initial Enzyme Concentration ($[E]_O$) on the Coagulation or Gelation

Native SPI solution (2% w/v) was treated with various concentrations of MTGase ($[E]_o$ = 0.1–0.5 U/mL), and the turbidity (A_{660}) of the reaction solutions was measured as a function of the incubation time (Fig. 5A). At all enzyme concentrations, the A_{660} values persistently increased with the incubation time. This coagulation process seems to be divided into two phases according to the inflectional tangent point (ITP) in the progress curve. Up to the ITP (phase 1), the slope of the A_{660} gradually declines, and after the ITP (phase 2), on the contrary, the slope of the OD_{660} increases. The length of phase 1 reflects the ease extent of coagulation reaction of SPI induced by MTGase. With increasing $[E]_o$ from 0.1 to 0.5 U/mL, phase 1 remarkably shortens, suggesting that the coagulation reactions occur much more easily. To analyze the progress curves numerically, we introduced two parameters as shown in Fig. 5A: *k* and A_0, the tangent slope at the ITP and the OD value of the tangent (at 0 min), respectively. The dependence of *k* on the enzyme concentration was plotted in Fig. 5B. At 2% (w/v) SPI, the k increased dramatically from 0.24 ± 0.04 to 1.25 ± 0.08 A units/min (×100), with increasing $[E]_o$ from 0.1 to 0.5 U/mL. At various enzyme concentrations, the change of A_0 value was negligible (data not shown), suggesting this value seems to only reflect the coagulation potential of a concentration of SPI, independent of the applied enzyme concentration.

We also investigated the effect of enzyme concentration on the gelation process of a high concentration (6% w/v) of native SPI (Fig. 6). Like the MTGase-induced coagulation cases, the gelation process of native SPI was also remarkably affected by the enzyme concentration. With increasing $[E]_o$ from 0.30 to 1.2 U/mL, the T_{gel} of gelation was markedly shortened from 47.0 to 10.8 min, and the G′ value of final gels increased by sevenfold (from 35.2 to 243.7 Pa). Similarly, the G″ increased by ~2-fold (from 3.5 to 7.2 Pa) (data not shown). These results suggest that at high enzyme concentration, the initial gel-network be more quickly formed, the viscoelasticities of finally formed gels be stronger. It is also obvious that the initial network formation require a certain amount of cross-links among various protein constituents of SPI, which can be accelerated by increasing the $[C]_o$ or $[E]_o$.

SDS-PAGE Analysis of Aggregates or Precipitates

The mixtures of native SPIs (2% w/v) and MTGase, incubated at 37C for various times, were centrifuged at 20,000 g for 2 min to obtain the

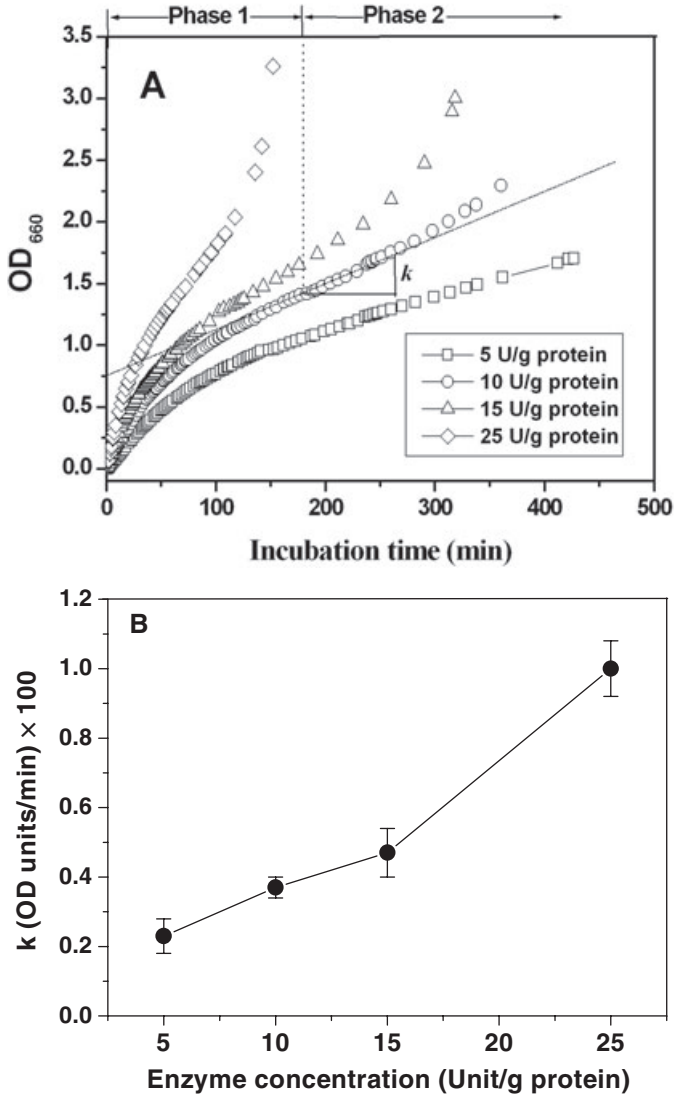

FIG. 5. EFFECT OF THE ENZYME CONCENTRATION ON THE TURBIDITY (A_{660}) OF NATIVE SPI SOLUTION (2%) W/V INCUBATED WITH MTGASE AT 37C

The turbidity was evaluated by A_{660}. Two parameters were introduced on the reaction curve of the turbidity: k and A_0 are the tangent slope of inflectional point and the A value of the tangent (at 0 min), respectively. Panel A: Progress curves of the turbidity. $[E]_o = 5$, 10, 15 and 25 units per gram of SPI, corresponding to 0.1, 0.2, 0.3 and 0.5 U/mL, respectively. Panel B: Effect of $[E]_o$ on the k. The bars show standard deviation ($n = 3$).

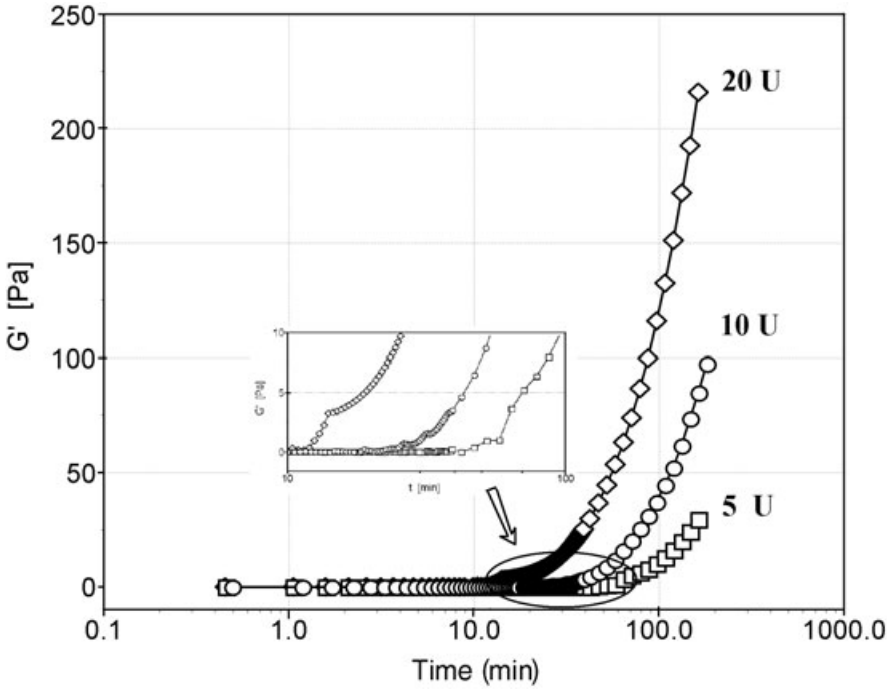

FIG. 6. EFFECT OF ENZYME CONCENTRATION ON THE MTGASE-INDUCED GELATION OF NATIVE SPI (6% W/V), AT 37C
$[E]_o$ = 5, 10 and 20 units per gram of SPI, corresponding to 0.3, 0.6 and 1.2 U/mL, respectively.

supernatants and corresponding aggregates. The SDS-PAGE patterns of the protein constituents of various supernatants and corresponding aggregates were dependent upon the incubation time with MTGase (Fig. 7). With the incubation time increasing from 15 to 240 min, the MW of biopolymers formed from the protein constituents of glycinin or β-conglycinin in the supernatants and corresponding aggregates gradually increased. After 15 min, only a small quantity of aggregates was obtained after centrifugation. In this case, the main protein constituents of native SPI (including glycinin and β-conglycinin) were practically unchanged in the supernatant while the aggregates were mainly composed of basic subunits (BS) of glycinin and a few biopolymers not entering the separating gel (Fig. 7, Lane 1 and 2). After 30 min, a majority of various constituents of β-conglycinin and acidic subunits (AS) of glycinin were absent from the supernatant and corresponding aggregates. Correspondingly, a lot of high MW biopolymers appeared on the top of separating gel and stacking gel (Fig. 7, Lanes 3 and 4). In this case, the BS of

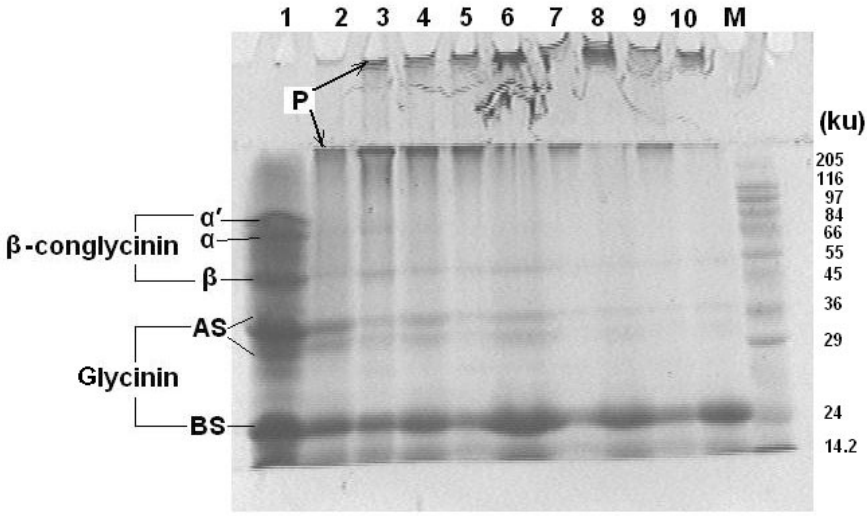

FIG. 7. SDS-PAGE PROFILES OF THE PROTEIN CONSTITUENTS OF SUPERNATANTS AND CORRESPONDING PRECIPITATES OF SPI SOLUTIONS (2% W/V) TREATED BY MTGASE AT 37C FOR VARIOUS TIMES

Lanes 1, 3, 5, 7 and 9 indicate the supernatants for 15, 30, 60, 120 and 240 min, respectively; Lanes 2, 4, 6, 8 and 10, the corresponding precipitates of SPI solution, respectively; M indicates the standard markers corresponding to 205, 116, 97, 84, 66, 55, 45, 36, 29, 24 and 14.2 kDa, respectively; P indicates the MTGase-induced biopolymers, which cannot enter the stacking and separating gels.

glycinin was still contained in both the supernatant and aggregates. After 60 min or more, almost all of high MW biopolymers in the aggregates were stacked on the top of stacking gel, accompanied by occurrence of BS of glycinin (Fig. 7, Lanes 6, 8 and 10) and, in the supernatants, the relative ratio of BS of glycinin evidently declined but a few high MW biopolymers still remained (Fig. 7, Lane 5, 7 and 9). These results suggested that the covalent cross-linking by MTGase remarkably decreased the solubility of native SPI, or resulted in the formation of some insoluble aggregates, composed of high MW biopolymers and BS of glycinin. At the same time, some soluble high MW biopolymers were formed, indicating the rupture of spatial structure of glycinin or β-conglycinin and the formation of new and stable spatial structure.

Protein Solubility Analysis of Aggregates or Precipitates

Figure 8 shows the respective solubility of the MTGase-treated SPI sample in various solvents including DW, B, BSU and BSUM. The low solubility (27%) of the protein constituents of the sample in DW confirmed the

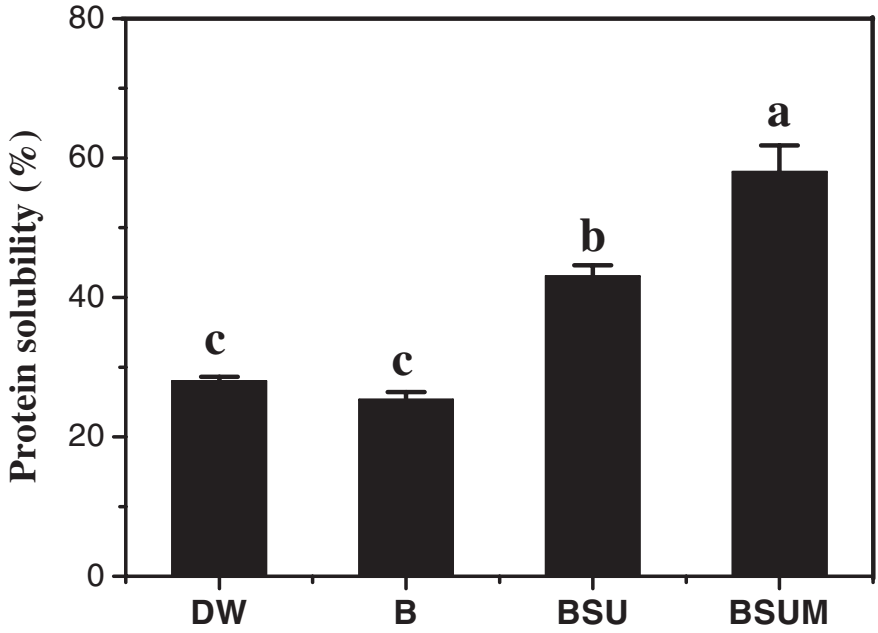

FIG. 8. SOLUBILITY OF THE PROTEIN CONSTITUENTS OF MTGASE-TREATED SPI IN VARIOUS SOLVENTS

This sample was obtained by incubating native SPI (2% w/v) in standard buffer with 20 units per gram of protein substrate of MTGase at 37C for 4 h. DW, distilled water; B, a pH 8.0 buffer (0.086 M Tris, 0.09 M Glycine, 4 mM Na_2EDTA); BSU, the same buffer containing 0.5% SDS and 8 M urea; BSUM, BSU plus 1% (v/v) 2-ME. Protein concentration of all solubilization assays: 0.1% w/v. The different alphabetic symbols on the top of column indicate the significant difference ($P < 0.05$).

effect of cross-linking by MTGase on the solubility of native SPI, and the solubility in DW was the same as that in B, indicating the decline in the solubility was not due to the electrostatic forces (Fig. 8). The solubility of the protein constituents was significantly improved by the presence of SDS (0.5%) and urea (8.0 M), which caused the disruption of hydrophobic and H-bondings. Thus, the hydrophobic and H-bondings would be the principal forces responsible for the insoluble aggregates of SPI after the cross-linking reactions by MTGase. This fact was expected because the structure of native glycinin or β-conglycinin was destroyed during the cross-linking reactions by MTGase, and the hydrophobic areas initially buried in the interior were exposed, particularly those BS of glycinin, which coagulated with other protein constituents or self-aggregated to result in the formation of insoluble aggregates. In addition, the solubility in BSU was further increased by the presence of 1%

(v/v) 2-ME (Fig. 8), indicating there would also be disulfide bonds involved in the formation of insoluble aggregates. This fact was also expected because the disulfide bonds linking the AS and BS were in a certain extent broken during the cross-linking reactions, which would favor the new contact between the free sulfhydryl groups in different protein constituents of glycinin. Therefore, besides the covalent cross-linking, hydrophobic and H- bondings and disulfide, bonds were involved in the formation of insoluble aggregates during the coagulating reactions of a low concentration of native SPI induced by MTGase.

Mechanism of Coagulation or Gelation of SPI Induced by MTGase

The process of coagulation and gelation of native SPI induced by MTGase is dynamic, in which the MW and molecular structure of protein substrate and the pattern of interactive forces between different proteins are changing. This kind of enzyme (MTGase) can effectively bring different individual proteins together and covalently cross-link them to form higher MW biopolymers. As far as a protein consisting of several subunits (e.g., glycinin and β-conglycinin) is concerned, only those subunits located in the exterior of protein, which have relatively low hydrophobicity, can be catalyzed effectively (Fig. 2). On the basis of the results obtained in this study, we propose a mechanism of the coagulation or gelation induced by MTGase (Fig. 9). In order to express well the mechanism of this kind of coagulation or gelation reactions, we divide the reaction process into two stages, namely "primary stage" and "terminal stage." In the initial stage of the reaction, the initially stable spatial structure of native SPI is gradually destabilized because of the covalent linkage. The hydrophobic areas initially buried in the interior of protein molecular, such as basic subunits of glycinin and some β-subunits of β-conglycinin, are partially exposed. This partially exposed or cross-linked SPI proteins are intermediate and unstable (Fig. 9B). In the latter stage of the reaction, this kind of modified protein could be cross-linked further and aggregate itself or each other or gelate because of their hydrophobic or other interactions (Fig. 9C,D). The occurrence of coagulation or gelation of SPI solutions is dependent upon the initial protein concentration ($[C]_0$) (Figs. 3 and 4). In the coagulation cases of low concentration ($<3\%$ w/v) of SPI, it was obvious that the coagulation reaction was initially induced by covalent cross-linking (at initial stage) and further accelerated by hydrophobic and other interactions (at poststage of coagulation). This phenomenon was particularly evident in the 1 or 2% cases (Fig. 3). In the cases of high concentration ($>3\%$ w/v), the formed cross-links among various cross-linked SPI (particularly those of glycinin) increased remarkably, and the hydrophobic interactions also became outstanding at poststage of the reaction. As a result, the gel network was onset (Fig. 9D). After the initial formation of gel network,

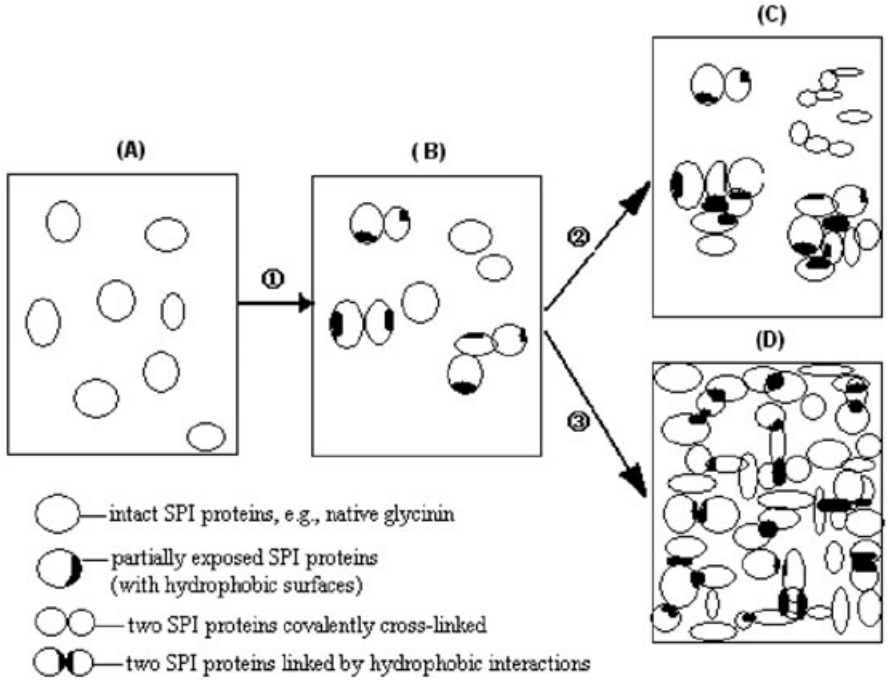

FIG. 9. POSTULATED MECHANISM FOR THE COAGULATION OR GELATION OF NATIVE SPI INDUCED BY MTGASE

Panel A, native SPI solutions; Panel B, polymerization of SPI by MTGase (primary stage); Panels C and D, coagulation and gelation of SPI induced by MTGase, respectively (terminal stage). Step ①, the addition of MTGase; step ②, the coagulation of low concentration (<3% w/v) of SPI solutions; step ③, the gelation of high concentration (>3% w/v) of SPI solutions.

the gel strength of formed gels increased markedly with further incubation with MTGase (Fig. 4).

The influence of cross-linking on the pattern of interactive forces among SPI proteins (Fig. 8) confirms that the coagulation of SPI is caused by the rupture of the structure; and covalent cross-links, hydrophobic and H- bondings and disulfide bonds were involved in the formation of the coagula or insoluble aggregates. Furthermore, the formation of insoluble aggregates (composed of basic subunits of glycinin and some of high MW biopolymers) usually accompanies the formation of some of soluble high MW biopolymers (Fig. 7). Thus, there are two possibilities in the coagulation of native SPI induced by MTGase. First, the covalent cross-linking induced by MTGase is not enough to completely destroy the interactive forces maintaining the intact structure. In this case, those new cross-linked proteins may have enough time

to rearrange themselves to form more stable molecules, which are still soluble in the solution. If the spatial bulk of a newly formed protein is too large, it will also separate out. Second, the covalent cross-linking by MTGase can completely destroy the structure of SPI components and pull apart the subunits of glycinin and β-conglycinin, especially for acidic subunits of glycinin. In this case, the formed biopolymers are stable and can be catalyzed further by MTGase while the remaining fragments are very unstable and aggregate together.

In the heat-induced gelation, the protein denaturation is a prerequisite to form a gel of soy proteins (Utsumi and Kinsella 1985a,b; Nakamura *et al.* 1986a,b; Puppo and Añón 1998; Renkema and Vliet 2002), because the exposed hydrophobic areas of denatured proteins can interact to form gel-network upon cooling. In the MTGase-induced gelation cases, both the covalent cross-linking and the following hydrophobic interactions among exposed hydrophobic areas of fragmentary components of SPI contribute to the formation of gel network. In addition, the relative importance of individual protein constituents (including glycinin and β-conglycinin) of SPI to the gel formation induced by MTGase may be different because of the differences in their molecular characteristics.

To elucidate the more detailed mechanism of coagulation and gelation of SPI induced by MTGase, the changes of molecular structure of SPI, dynamic changes of the size of formed aggregates, and how different kinds of interactions act upon one other are under investigation.

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