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Effects of Ca^{2+} and Sulfhydryl Reductant on the Polymerization of Soybean Glycinin Catalyzed by Mammalian and Microbial Transglutaminases

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Two types of transglutaminases (TGases), Ca^{2+}-dependent TGase derived from guinea pig liver (GTGase) and Ca^{2+}-independent TGase derived from a variant of *Streptoverticillium mobaraense* (MTGase), were used to study the cross-linking of soybean 11S globulin (glycinin). The effects of sulfhydryl reductant (dithiothreitol, DTT) and Ca^{2+} on the conformation and TGase-catalyzed polymerization of glycinin were investigated. The conformational change of glycinin was probed by spectral methods. The degree of cross-linking and the polymer (aggregate) formation were analyzed by sodium dodecyl sulfate−polyacrylamide gel electrophoresis and dynamic light scattering, respectively. Addition of DTT stimulated the TGase-catalyzed cross-linking reactions without destroying the secondary and tertiary structure of glycinin but did not influence the polymer or aggregate formation. It was found that Ca^{2+} caused the formation of larger size polymers at lower concentrations, while it suppressed the polymerization at higher concentrations. In addition, the cross-linking behaviors of glycinin were shown to be different between MTGase- and GTGase-catalyzed systems.

KEYWORDS: Transglutaminase; polymerization; glycinin

INTRODUCTION

Transglutaminase (TGase; glutaminyl peptide γ-glutamyltransferase, EC 2.3.2.13) catalyzes the acyl-transfer reactions between γ-carboxyamide groups of peptide-bound glutamine residues and the primary amino groups in a variety of amine compounds, including peptide-bound ϵ-amino groups of lysine residues. TGase reactions result in modification of the charge of protein and a change in protein conformation by cross-linking within the protein itself or between different proteins, forming conjugates of higher mass up to supramolecular networks (*1*). The presence of TGases has been widely detected in mammals (from various organs, tissues, and body fluids), plants, and microorganisms (*2*, *3*). The activities of many TGases found in mammals are dependent on Ca^{2+}, such as the TGase derived from guinea pig liver (GTGase), while this is not an essential requirement for the TGases found in plants and microorganisms, such as the TGase derived from a variant of *Streptoverticillium mobaraense* (MTGase).

As substrates for TGase, caseins have been shown to be excellent, except for κ-casein, mainly because of its flexible structure with no disulfide bonds (*4*−*6*). In contrast, globular proteins such as α-lactalbumin, β-lactoglobulin, and bovine serum albumin cannot be completely attacked by TGase in the native state. A conformational change enhances the susceptibility of the proteins to TGase reaction (*7*, *8*). Reductions of intact disulfide bonds by reductants [dithiothreitol (DTT), glutathione, or cysteine] of these proteins are normally done to increase the susceptibility (*9*−*11*).

Soybean seeds are the main source of plant proteins worldwide, but the utilization of soybean proteins in food processing is still not efficient. To improve the functional properties, cross-linking of soybean proteins by TGases has been suggested as a good method. So far, several researchers have studied the cross-linking of glycinin and conglycinin, which are the major components of soybean proteins (*12*−*15*), and the cross-linking between soybean proteins and other globular proteins (*16*−*19*). Both glycinin and conglycinin were found to be available to form polymers or gels through covalent bonds with TGases. Ikura et al. (*12*) indicated that the cross-linking of glycinin proceeded faster than that of conglycinin. In MTGase-catalyzed systems, native glycinin was used mostly for the aforementioned studies, but heat-treated glycinin was also used by Kang et al. (*13*), who indicated that heat treatment could increase the reactive sites, lysine and glutamine residues, on the surface of glycinin. Although a reducing agent (DTT) was included in the cross-linking reaction of glycinin with GTGase, the effects of reducing agents on the conformational change of glycinin remain unclear. One of our main objectives is to determine whether DTT plays an important role in the enhanced susceptibility of glycinin to TGases, as in the case of globular proteins, or not.

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GTGase is a Ca^{2+}-dependent enzyme. Ca^{2+} is also an important factor in controlling the functionality of food proteins. Færgemand and Qvist (*20*) reported that Ca^{2+} induced noncovalent aggregation of β-lactoglobulin, which could retard the cross-linking by TGase. Glycinin is sensitive to Ca^{2+}. Therefore, our second objective is to determine the appropriate concentration of Ca^{2+} for the cross-linking of glycinin by TGases, especially GTGase.

Glycinin is a large globular protein with a complicated oligomeric structure. Five constituent subunits have been identified: $A_{1a}B_{1b}$, A_2B_{1a}, $A_{1b}B_2$, A_3B_4, and $A_5A_4B_3$. Each subunit is composed of an acidic polypeptide (acidic p*I*) and a basic polypeptide (basic p*I*) (*21*). It was reported that only the acidic polypeptides took part in the cross-linking reaction with TGases (*12*, *14*), whereas it is unclear what kind of molecular species in acidic polypeptides are attacked more easily by TGases. Therefore, our third objective is to compare the susceptibility by the enzymatic reaction of each acidic polypeptide, which should give some useful information for food processing and the genetic engineering of soybean proteins.

The conformational change and aggregate formation of glycinin induced by Ca^{2+} and DTT treatments should be closely related to the susceptibility of the protein to TGases. Therefore, CD spectra measurements and turbidometry were carried out to determine the effects of DTT and Ca^{2+} on the conformation and aggregation behavior of glycinin prior to the enzymatic reaction. The cross-linking reaction of glycinin catalyzed by TGases was analyzed by sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE), whereas dynamic light scattering (DLS) was used to monitor the polymerization of glycinin catalyzed by TGases under nondenaturing conditions.

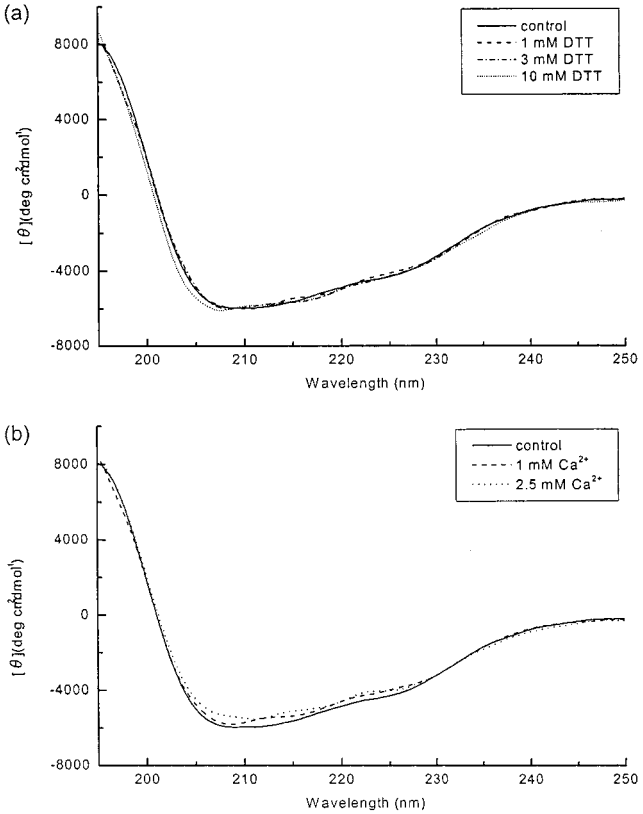

Figure 1. CD spectra (far-UV) of glycinin in 0.1 M Tris-HCl buffer (pH 7.5) in the presence of (a) 1, 3, and 10 mM DTT and (b) 1 and 2.5 mM Ca^{2+}. Control represents the native glycinin.

MATERIALS AND METHODS

Materials. Mammalian transglutaminase was prepared from guinea pig liver according to the method of Ikura et al. (*22*). The specific activity of the enzyme (4.08 units/mg) was measured by the method of Ando et al. (*23*). Microbial transglutaminase derived from *Streptoverticillium* (sp. no. 8112) with a specific activity of 1.0 unit/mg was kindly supplied by Ajinomoto Co., Ltd. (Kawasaki, Japan). Soybean (*Glycine max*, var. Tsurunoko) was kindly supplied by the Faculty of Horticulture of Chiba University in Japan. All chemicals used were reagent grade and were purchased from Wako Chemicals Co., Ltd. (Osaka, Japan).

Preparation of Glycinin. Glycinin was prepared from acetone powder according to the method of Mori et al. (*24*) and Zheng et al. (*25*) with a slight modification. Purified glycinin was dialyzed against 35 mM potassium phosphate buffer (pH 7.6) containing 0.4 M NaCl at 5 °C before use.

Enzymatic Polymerizations of Glycinin. All the enzymatic reactions were carried out at 37 °C in 0.1 M Tris-HCl (pH 7.5) buffer containing 1 mg/mL glycinin, an enzyme level of 0.1 unit/mg of protein substrate, and different concentrations of DTT (0, 1, 3, and 10 mM for both MTGase- and GTGase-catalyzed reaction systems) and/or Ca^{2+} (0, 1, 2.5, 5, and 10 mM for both MTGase- and GTGase-catalyzed reaction systems, and one more concentration of 20 mM for the GTGase reaction system). The reaction mixtures were incubated for various time intervals, and the reactions were terminated by adding 20 mM NEM and 40 mM EDTA at final concentrations for the MTGase- and GTGase-catalyzed systems, respectively. The zero-time samples were prepared by mixing the reaction mixtures with NEM and EDTA solutions before addition of enzymes. The reaction mixtures were analyzed immediately by SDS−PAGE and DLS.

SDS−PAGE. The reaction solution was mixed with the same volume of SDS solution [0.125 M Tris-HCl buffer, pH 6.8, containing 4% SDS, 20% glycerol, 5 M urea, and 5% 2-mercaptoethanol (2-ME)], followed by heating at 100 °C for 5 min before electrophoresis. Slab gel (4.5% stacking gel and 12.5% separating gel) was used according to the method of Laemmli (*26*). The gels were stained with Coomassie Brilliant Blue R-250.

Dynamic Light Scattering. The particle size distribution patterns of TGase-catalyzed glycinin polymers were characterized by the technique of DLS using the NICOMP model 370 submicron particle sizer (Particle Sizing Systems, Inc., Santa Barbara, CA). The various glycinin solutions treated by TGases were directly measured under the size range (diameters) from 10 nm to 10 μm.

Circular Dichroism (CD) Spectra. CD spectra measurements were obtained with a JASCO model J-720 spectropolarimeter in cells of path length 0.1 mm over the range of 190−250 nm (far-UV) or path length 10 mm over the range of 260−350 nm (near-UV). The sample was measured at 25 °C at 1 mg/mL protein concentration.

Self-Aggregation Measurements. The experiments of self-aggregation of glycinin were performed in 0.1 M Tris-HCl buffer (pH 7.5) containing 1−10 mM Ca^{2+} at 37 °C for various time intervals as in enzymatic polymerization experiments. The turbidity of the reaction mixtures was determined immediately at 420 nm by using a UV-2400 (PC) spectrophotometer (Shimadzu Co., Kyoto, Japan) at room temperature.

Protein Determination. Protein was determined by the methods of Bradford (*27*).

RESULTS AND DISCUSSION

Substrate Characterization. The far-UV CD spectral properties of glycinin are presented in **Figure 1**. These spectra were recorded in 0.1 M Tris-HCl buffer (pH 7.5), and because of the high absorbance, it was not possible to obtain data below 195 nm. The spectra in **Figure 1a** were recorded in the presence of 1, 3, and 10 mM DTT. No significant difference was found between the spectra obtained in the native state of glycinin (control) and those in the presence of DTT. The spectra in **Figure 1b** were recorded in the presence of 1 and 2.5 mM Ca^{2+}. There was only a slight difference between the spectra obtained

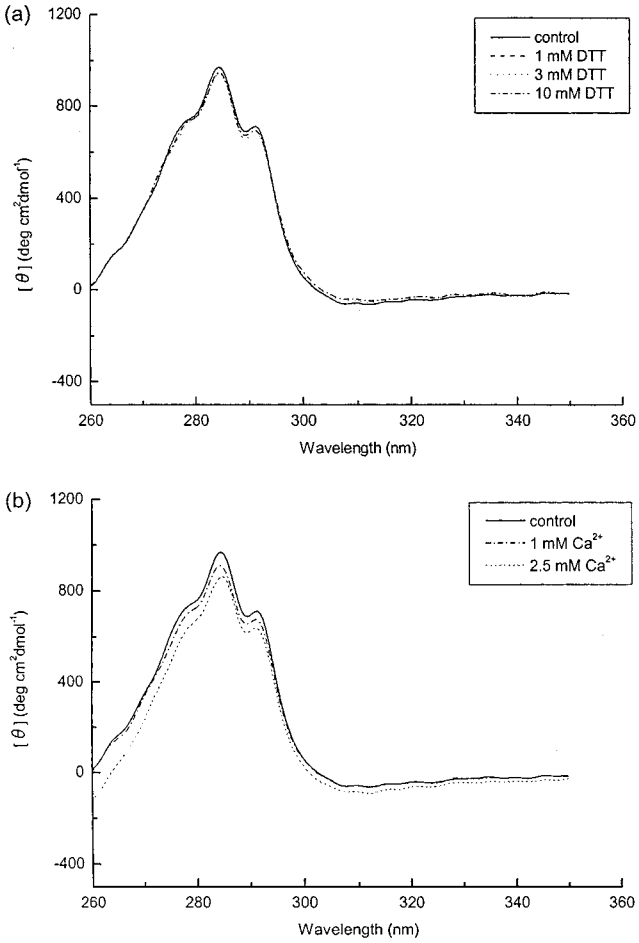

Figure 2. CD spectra (near-UV) of glycinin in 0.1 M Tris-HCl buffer (pH 7.5) in the presence of (a) 1, 3, and 10 mM DTT and (b) 1 and 2.5 mM Ca^{2+}. Control represents the native glycinin.

in the native state of glycinin (control) and those in the presence of Ca^{2+}, which could be neglected. An increase of Ca^{2+} concentration to 5 mM increased the protein solution turbidity, which will be described in the next section. This situation made it impossible to collect the CD data under high concentrations of Ca^{2+}.

The near-UV CD spectra of glycinin were recorded under the same conditions concerning Ca^{2+} and DTT concentrations as in the far-UV CD spectra experiments (**Figure 2**). The signals in the region of 280−300 nm characterized the environments of Tyr and Tyr side chains. Similar to the results of far-UV spectra, DTT (1−10 mM) did not induce any changes in the tertiary structure of glycinin as well. Also, 1 and 2.5 mM Ca^{2+} did not induce significant changes. All the CD spectra collected here indicated that the addition of the reducing agent (DTT) as well as the low concentrations of Ca^{2+} (1 and 2.5 mM) did not affect the secondary and tertiary structure of glycinin significantly.

Self-Aggregation of Glycinin. To study the self-aggregation behavior of glycinin in the presence of various concentrations of Ca^{2+} without TGase, turbidity at various incubation times was determined (**Figure 3**). It was clear that a low concentration of Ca^{2+} (1 or 2.5 mM) did not make any difference in turbidity in comparison with control (0 mM Ca^{2+}). However, visible aggregates were found when the level of Ca^{2+} was elevated to 5 or 10 mM, as shown by the high values of turbidity. When chelating agent (EDTA) was used to terminate the reaction, there was no significant difference in the turbidity among various concentrations of Ca^{2+} (results not shown). Apparently, high levels of Ca^{2+} can initiate the self-aggregation of glycinin.

Cross-Link Formation. The reactivity of cross-link formation catalyzed by MTGase and GTGase was investigated by analyzing the reaction products using SDS−PAGE. The extent of cross-linking was shown by the disappearance of polypeptide bands and the appearance of polymer products with higher molecular weights. As SDS−PAGE was carried out in the presence of the reductant 2-ME, the polymer products were not formed by disulfide bonds.

Effects of Calcium Ions. **Figure 4** (1 and 2) shows the electrophoretic patterns of glycinin cross-linked by MTGase and GTGase, respectively, in the presence of different concentrations of Ca^{2+} for various time intervals. Basic polypeptides and acidic polypeptides of A_3, A_4, and $A_{1,2}$ were found in the control sample (zero-time products). The bands of basic polypeptides did not show any significant change. This indicates the possibility that almost only the acidic polypeptides took part in the enzymatic cross-linking reactions, which is in agreement with the studies conducted by Ikura et al. (*12*) on a GTGase-catalyzed system and Chanyongvorakul et al. (*14*) on an MTGase-catalyzed system. This would be due to the oligomeric structure of glycinin, in which the acidic polypeptides are located mainly at the periphery of the protein molecule, making the reactive residues of the polypeptides more easily exposed to the TGases (*14*).

In the MTGase-catalyzed system (**Figure 4**(1)), enzymatic cross-linking was determined at 1, 2.5, 5, and 10 mM Ca^{2+}, and the control reaction was done in the absence of Ca^{2+}. At 1 and 2.5 mM Ca^{2+}, the cross-linking behaviors of glycinin were nearly the same as that of the control sample. Upon increasing the concentrations of Ca^{2+} to 5 and 10 mM, however, the cross-linking reactions became slower, as shown by the increase of nonmodified acidic polypeptides of glycinin, even after a long incubation time. This indicated that the presence of a high concentration of Ca^{2+} retarded the enzymatic reactivity, while a low concentration of Ca^{2+} gave no notable effect.

In the GTGase-catalyzed system (**Figure 4**(2)), enzymatic cross-linking was performed at 1, 2.5, 5, 10, and 20 mM Ca^{2+}. Because GTGase is a Ca^{2+}-dependent transglutaminase, zero-time reaction with Ca^{2+} was used as a control sample, as described in Materials and Methods, in which chelating agent EDTA was added to the sample solution containing Ca^{2+} before addition of TGases. The results showed that 2.5 mM Ca^{2+} was sufficient to activate GTGase to the maximum level, as most of acidic polypeptides of glycinin disappeared after a reaction time of 120 min. This is somewhat different from the results of Ando et al. (*23*). When the short peptides were used as substrates, they found that the relative reactivity was nearly 100% when 5 mM Ca^{2+} was added to the GTGase reaction system. This difference could be due to the different substrates (short peptides and soybean globular proteins) used. Upon increasing the concentration of Ca^{2+} from 5 to 20 mM, the cross-linking reactions were gradually inhibited. Even after 120 min of reaction time, the bands of the constituent polypeptides of glycinin remained similar to that of the control sample.

The poor reactivity in both MTGase- and GTGase-catalyzed systems with high levels of Ca^{2+} (more than 5 mM) may be due to noncovalent self-aggregate formation, as mentioned above (**Figure 3**). Before starting and at the beginning of the enzymatic reaction, glycinin was easily aggregated in the buffer solution containing high concentrations of Ca^{2+}, which could have masked some glutamine and lysine reactive residues. The higher the concentration of Ca^{2+}, the more reactive sites were masked.

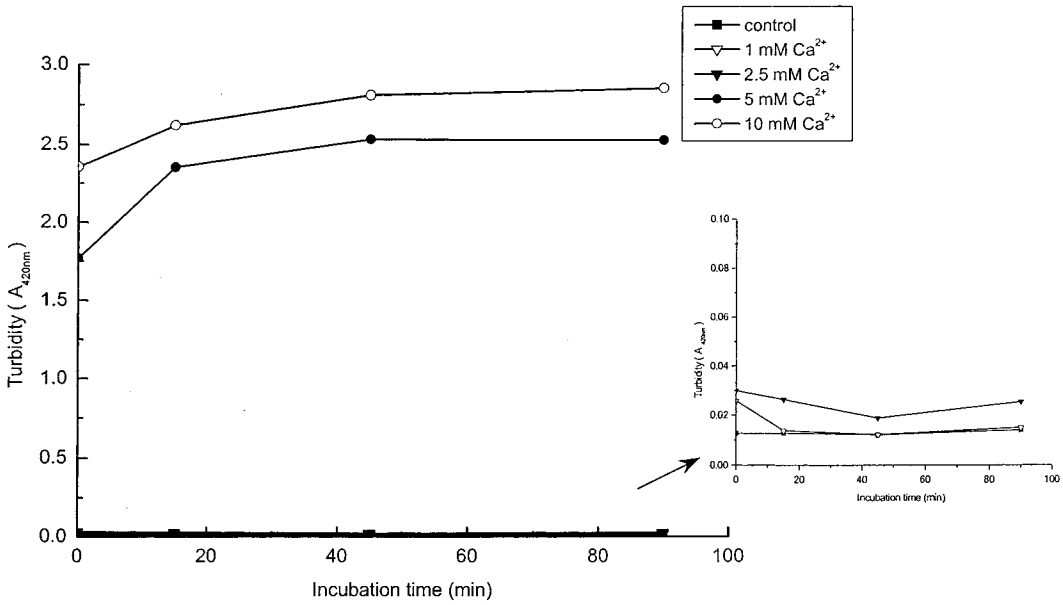

Figure 3. Effect of Ca^{2+} on self-aggregation of glycinin incubated at 37 °C for various reaction times.

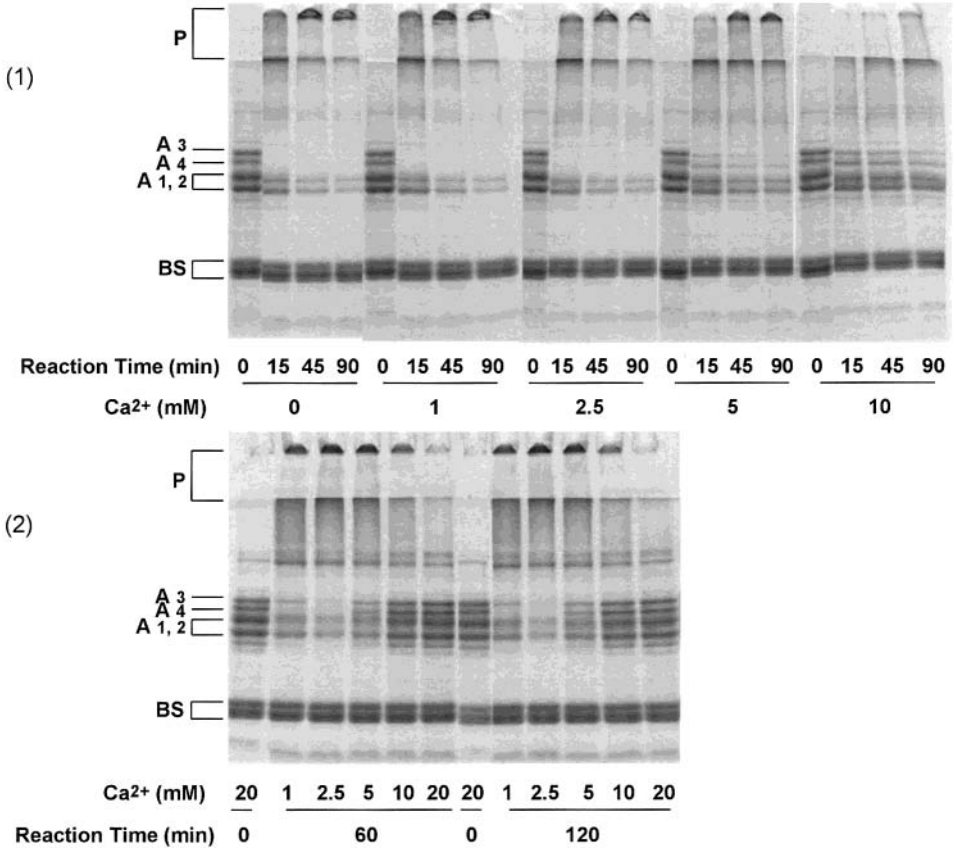

Figure 4. SDS–PAGE of glycinin incubated with (1) MTGase and (2) GTGase in the presence of Ca^{2+} at various reaction times. P, polymer products; $A_{1,2}$, A_3, and A_4, acidic polypeptides; BS, basic polypeptides.

On the other hand, the action of a low concentration of Ca^{2+} on the cross-linking with MTGase was different from that with GTGase. The presence of 2.5 mM Ca^{2+} significantly increased the activity of GTGase, but it showed no effect on MTGase. This result is reasonable, because of the nature of the two TGases, i.e., Ca^{2+} dependence of GTGase and Ca^{2+} independence of MTGase. For MTGase, Ca^{2+} can be omitted from the reaction mixture. For GTGase, however, 2.5 mM Ca^{2+} should be the best concentration to induce the cross-linking of glycinin to the maximum extent. Previous work had shown that gelation of β-lactoglobulin using Ca^{2+}-dependent TGase in the presence of DTT was caused mainly by noncovalent interactions (*10*, *28*). Also, using Ca^{2+}-independent TGase (MTGase), Færgemand and Qvist (*20*) reported the noncovalent aggregates of β-lactoglobulin in the presence of Ca^{2+}. In all of the above cases, cross-linking was performed in the presence of 5 mM Ca^{2+}, which inhibited the formation of covalent cross-links catalyzed by TGases. These results were consistent with our case in the presence of high concentrations of Ca^{2+} (more than 5 mM) in both MTGase- and GTGase-catalyzed systems.

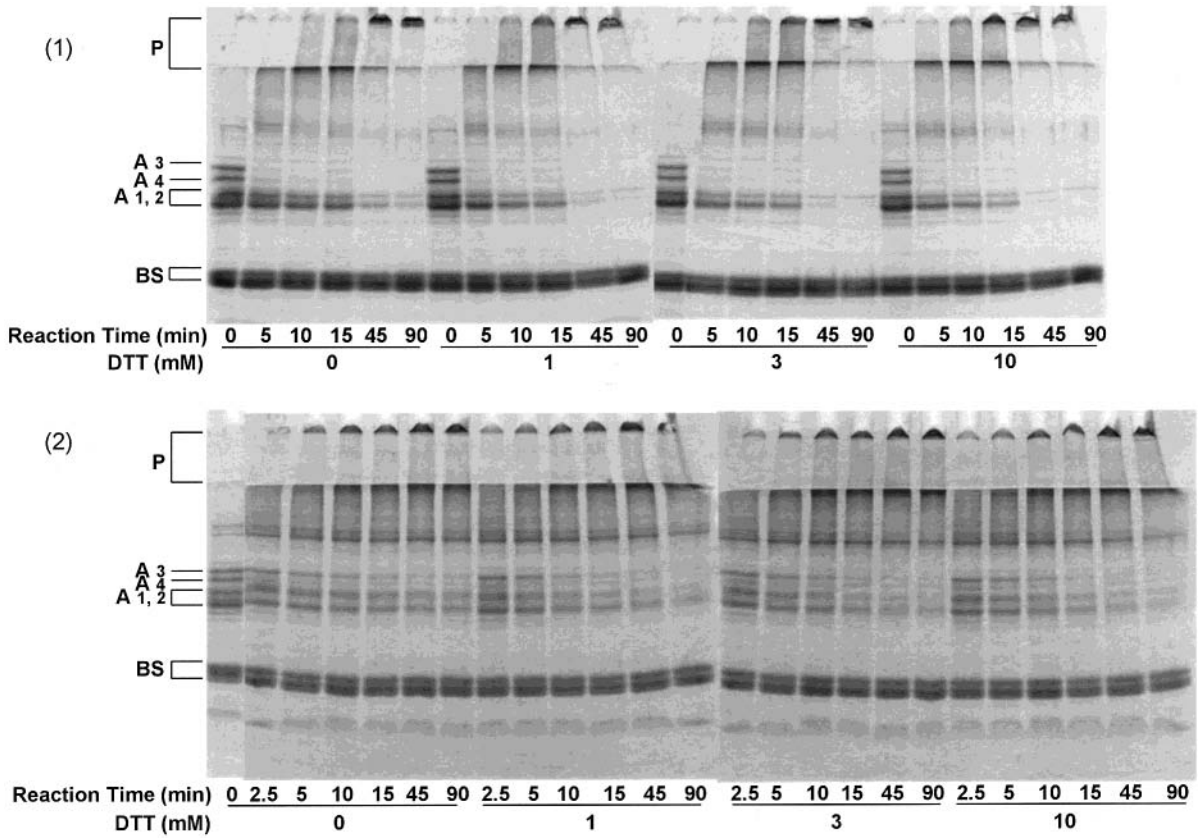

Figure 5. SDS–PAGE of glycinin incubated with (1) MTGase in the presence of DTT and (2) GTGase in the presence of 2.5 mM Ca^{2+} and DTT at various reaction times. P, polymer products; $A_{1,2}$, A_3, and A_4, acidic polypeptides; BS, basic polypeptides.

However, in our case, cross-linking was also performed in the presence of low concentrations of Ca^{2+}, which did not retard the covalent cross-linking reaction. Determination of an appropriate concentration of Ca^{2+} for the TGase-induced cross-linking is, therefore, suggested to be very important for each protein substrates. Upon determination and evaluation of an appropriate concentration of Ca^{2+}, it is important to consider the possible influence of phytate, which is known to have strong calcium chelating activity and is often copurified together with glycinin (*29*, *30*). However, we concluded that the influence of phytate is little or, at most, negligible in our study. We used anion-exchange chromatography (DEAE-Toyopearl) (*25*) in the preparation of glycinin, and it has been reported that most phytate can be removed by using anion-exchange resins (*29*, *30*). In addition, we observed that GTGase was activated by low concentrations (1 mM) of Ca^{2+} (**Figure 4**(2)), indicating that the possible chelating effect of phytate was very little, even if phytate was present in our glycinin preparation.

Effects of DTT. The electrophoretic patterns in **Figure 5** show the effects of DTT on the cross-linking of glycinin catalyzed by MTGase and GTGase at various time intervals. Without addition of DTT, it was observed that acidic polypeptides of glycinin were gradually cross-linked in both MTGase- and GTGase-catalyzed systems, which indicated the DTT-independent properties of TGases. In the case of the MTGase-catalyzed system (**Figure 5**(1)), with addition of 1 mM DTT, acidic polypeptides were extensively cross-linked within 90 min, much faster than the case with the control samples (0 mM DTT). However, increasing the DTT concentration to 3 or 10 mM promoted the cross-linking reactions only slightly. This means that a low concentration of DTT (1 mM) was sufficient to enhance the degree of cross-linking to the maximum. In the case of the GTGase-catalyzed system (**Figure 5**(2)), a concentration of 2.5 mM Ca^{2+} was used for activating GTGase highly, as mentioned above, with various concentrations of DTT. As was the case in the MTGase-catalyzed system, 1 mM DTT accelerated the cross-linking reaction highly.

To date, several researchers have studied the cross-linking of globular proteins catalyzed by TGase (*4*, *9*−*11*) and suggested that the reducing agent DTT was necessary to cause the conformational change (unfolding) of β-lactoglobulin in order to improve the reactivity toward TGase. Coussons et al. (*31*) also reported that significant incorporation of amine into β-lactoglobulin occurred only in the presence of reducing agent. They concluded that the effect of adding reductant did not arise from an effect of TGase itself. However, Dickinson and Yamamoto (*32*) reported that a concentrated solution of β-lactoglobulin at pH 7 could be used to make a gel slowly with TGase without any treatment or addition of a reducing agent. In this study, we have found that glycinin gradually polymerized with TGases, even without reductant DTT. However, addition of DTT significantly increased the cross-linking reaction catalyzed by TGases; particularly, the significant effect was observed with 1 mM DTT, as shown in **Figure 5**, while DTT did not induce any changes in the secondary and tertiary structures of glycinin (**Figures 1** and **2**). Further, the prolonged incubation of glycinin solution for 24 h at 37 °C with up to 10 mM DTT at pH 7.5 in 0.1 M Tris-HCl buffer caused no structural changes (the CD spectra data are not shown). This indicates that there is no contribution of DTT to the unfolding of glycinin molecule. Recent studies (*33*) on the primary and secondary structures of MTGase indicated that this enzyme had one cysteine (Cys) residue with a free thiol group, which is also the same in the case of GTGase. This Cys played an important role in the catalysis of an acyl-transfer reaction and is therefore essential for TGase enzymatic activity. It was known that there was a

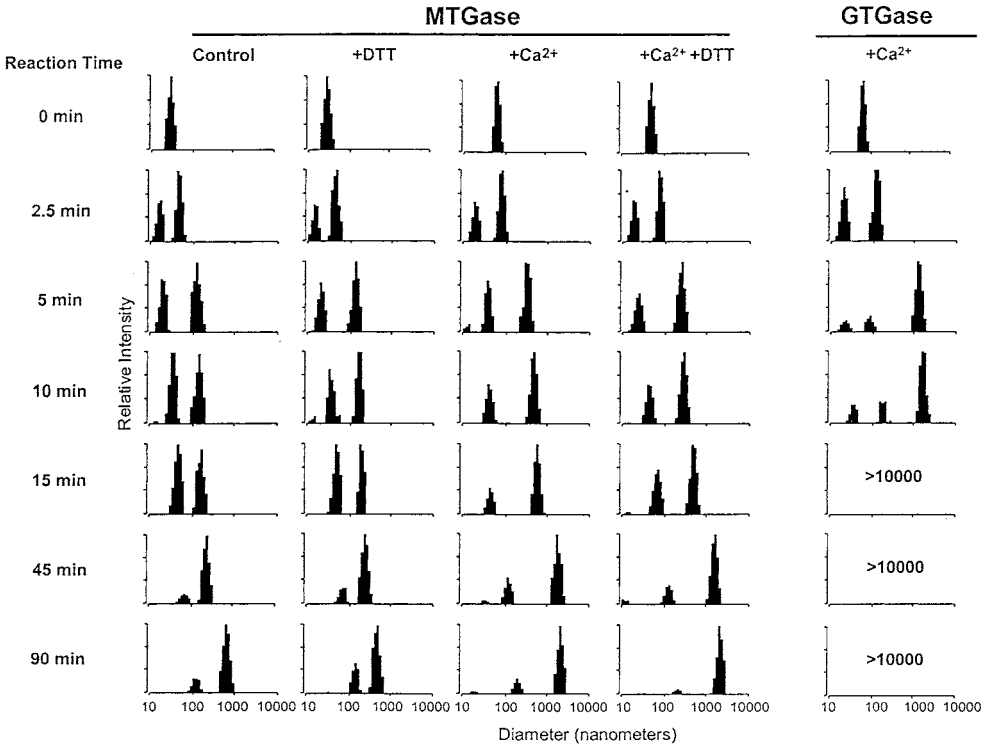

Figure 6. Behaviors of particle size distribution in glycinin polymerizations catalyzed by MTGase and GTGase at various times. Control, without Ca^{2+} and DTT; +DTT, with 1 mM DTT; +Ca^{2+}, with 2.5 mM Ca^{2+}; +Ca^{2+} + DTT, with 2.5 mM Ca^{2+} and 1 mM DTT.

loss in enzymatic activity to a certain extent after long-time storage. Therefore, it is possible that the reductant DTT played a role in activating that Cys residue of TGases to some extent when glycinin was used as the substrate. In the case of *β*-lactoglobulin, however, it was found that the breakage of intact disulfide bonds, induced by DTT, could lead to the exposure of a potential new site for the GTGase-catalyzed reactions (*31*). Further studies are needed to verify such a possibility in the case of glycinin as a substrate.

In the MTGase-catalyzed system, the cross-linking behavior of glycinin in the presence of both 1 mM Ca^{2+} and 1 mM DTT was also studied (results not shown). Compared with addition of 1 mM DTT alone, the enzymatic reactivity with 1 mM Ca^{2+} and 1 mM DTT presented no obvious difference, suggesting again that a low concentration of Ca^{2+} could not influence the cross-linking of glycinin catalyzed by MTGase.

Selectivity of Subunit Species by TGases. It is interesting to observe in **Figure 4** or in **Figure 5** that the cross-linking behavior of the polypeptides of glycinin was different between MTGase- and GTGase-catalyzed systems. As shown in **Figure 5**(1) in the case of the MTGase-catalyzed system, the bands of A_3 and A_4 polypeptides of glycinin almost disappeared after just 30 min of reaction time, whereas bands of $A_{1,2}$ polypeptides of glycinin were still observed clearly, suggesting the enzymatic reactivity for A_3 and A_4 polypeptides was much higher than that for $A_{1,2}$ polypeptides. Nevertheless, in the GTGase-catalyzed system, the cross-linking of A_4 polypeptides of glycinin was found to be more rapid than that of the other acidic polypeptides. The different preferences of MTGase and GTGase toward glycinin subunits as substrates for cross-linking reaction may be due to a difference in specificity of both enzymes toward glutamine and lysine residues.

The greater susceptibility of A_4 and/or A_3 polypeptides to TGases may be related to the primary structure of the polypeptides, e.g., the glutamine/lysine contents. The overall features concerning the number and position of glutamine/lysine residue are similar among acidic polypeptides (*34*, *35*). However, A_4 and A_3 polypeptides contain peptide sequences which are lacking in other acidic polypeptides (about 50 and 30 amino acid sequences, respectively). Of these peptides, the insertion sequence of A_4 contains glutamine and lysine residues, which are the potential reactive sites of the TGases. It remains unclear why A_3 polypeptide is also more susceptible to TGase than A_1- and A_2-type polypeptides. Probably, the A_3B_4 subunit is more exposed to the surface of the glycinin molecule and thus can be attacked by TGase more easily, although the arrangement of intermediary subunits in glycinin is not understood at present.

Polymer Formation. To further elucidate the effects of Ca^{2+} and DTT treatment on the polymerization of glycinin catalyzed by MTGase and GTGase, the formation of polymers was studied by monitoring time-dependent changes in the particle size distribution characterized by DLS (**Figure 6**). It should be noted that the buffer in DLS measurement contained no denaturants such as SDS and urea. Therefore, polymers or aggregates formed by noncovalent bonding as well as isopeptide bonds were analyzed. Low concentrations of Ca^{2+} (2.5 mM) and/or DTT (1 mM) were included. "Control" in the MTGase-catalyzed system means the reaction without Ca^{2+} and DTT.

In the case of the MTGase-catalyzed system (**Figure 6**, MTGase), no significant change in the distribution patterns was caused by addition of DTT, which was demonstrated by comparison between "Control" and "+DTT " or "+Ca^{2+} " and "+Ca^{2+} + DTT ". The results of SDS−PAGE (**Figure 5**) showed the enhancement effects of 1 mM DTT on the cross-linking reactivities of the glycinin molecule, while the DLS results here indicated that the polymer sizes of glycinin molecules induced by MTGase were the same, irrespective of the presence or absence of 1 mM DTT.

In contrast, with regard to the effects of low concentrations of Ca^{2+} on the polymer formation in MTGase-catalyzed system, we observed a great difference in polymer size according to the presence or absence of Ca^{2+}, which was demonstrated by

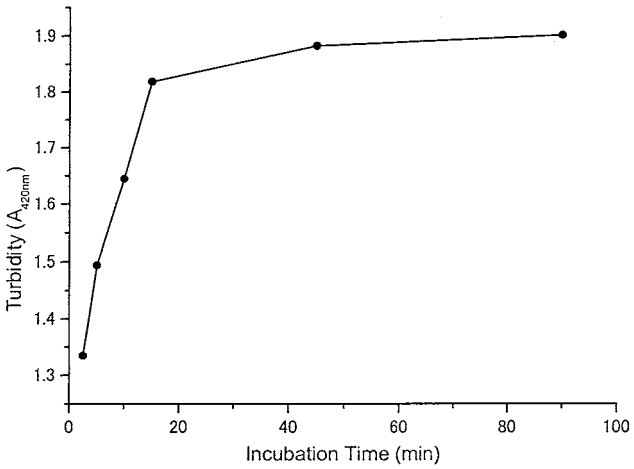

Figure 7. Turbidity behavior of the particle products of glycinin catalyzed by GTGase at various reaction times.

comparison between "Control" and "+Ca^{2+} " or "+DTT " and "+Ca^{2+} + DTT ". At the initial stage of the enzymatic reaction (0 min), the particle size of the reaction mixtures with Ca^{2+} was found to be somewhat larger than that of the mixtures without Ca^{2+}, and after 45 min of reaction time with MTGase, the formation of extremely larger polymers was detected. The reason for such effects may be the Ca^{2+}-induced aggregation of glycinin to a certain extent via electrostatic interactions from the beginning of reaction. However, this polymerization did not progress to a larger extent to hide the reactive residues for MTGase. As mentioned earlier, the low concentration of Ca^{2+} did not induce the visible self-aggregation of glycinin, and it also did not influence the enzymatic reactivity. So there was a possibility that polymers of glycinin proteins formed through the isopeptide cross-linking with MTGase became more sensitive to Ca^{2+}, leading to the formation of larger polymers (aggregates). Alternatively, the formation of an isopeptide bridge by MTGase may enable the close contact of the glycinin molecules. Therefore, the aggregation of glycinin proteins catalyzed by MTGase in the presence of a low concentration of Ca^{2+} is presumably caused by the combination of enzymatic covalent bonds and noncovalent bonds (probably electrostatic interactions).

In the case of a GTGase-catalyzed system (**Figure 6**, GTGase), the experiments were carried out with addition of 2.5 mM Ca^{2+}, because of the Ca^{2+}-dependent property of GTGase. The results showed that the polymer formation could be detected only at the early stage of the reaction, not more than 15 min. The gigantic polymers (aggregates) after 15 min were so large that is was difficult to detect them by the technique of DLS, as shown by the results of extremely high turbidity (**Figure 7**).

Comparing the MTGase- and GTGase-catalyzed systems (**Figure 6**), a great difference in polymerization behavior was observed. With MTGase treatment, large polymers gradually formed as the small polymers slowly decreased. In contrast, with GTGase treatment, the enormous polymers formed rather rapidly, and just after a reaction time of 5 min, the amount of smaller polymers (less than 1000 nm) significantly decreased. Apparently, GTGase caused more rapid generation of large polymers (aggregates) of glycinin than MTGase. As demonstrated already in this paper, the cross-linking sites for glycinin polypeptides were very different between MTGase- and GTGase-catalyzed systems. Probably the difference in the covalent cross-linking sites and the noncovalent bonds induced by Ca^{2+} subsequently induced the difference in formation of larger polymers. GTGase is supposed to be more versatile for modifying the lysine and glutamine residues, leading to the much larger polymers.

Conclusions. This work has demonstrated the effects of Ca^{2+} and DTT on the polymerization of glycinin catalyzed by TGase from mammals and microorganisms. Addition of a low concentration of DTT could activate the TGase reaction via reacting SH residues in the catalytic site without destroying the secondary and tertiary structure of glycinin, but could not influence the size of the polymer formed by the TGase-catalyzed reaction. It was found that up to 2.5 mM Ca^{2+} activated GTGase to a high level when glycinin protein was used as the substrate, but higher concentrations of Ca^{2+} (>5 mM) slowed the cross-linking reaction catalyzed by GTGase and MTGase. In the MTGase-catalyzed system, 2.5 mM Ca^{2+} did not affect the cross-linking reaction, whereas it did induce the formation of larger polymers. In addition, it was indicated that the sites that were reactive toward glycinin polypeptides and the polymer formation catalyzed by MTGase and GTGase were different, which may provide useful information on the structure of the glycinin molecule and the applications of TGase in food processing. Further studies are needed to clarify the difference effects of various TGases at reactive sites of glycinin as well as the different sol and gel properties of soybean proteins catalyzed by MTGase and GTGase.

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