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Identification of the ε-(γ-Glutamyl)lysine Cross-Linking Sites in α-Lactalbumin Polymerized by Mammalian and Microbial Transglutaminases

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To investigate the site specificity of two transglutaminases (TGases), that is, the enzymes from guinea pig liver (GTGase) and *Streptoverticillium* (MTGase), the acyl acceptor and donor sites in α-lactalbumin were determined. α-Lactalbumin was cross-linked in the presence of dithiothreitol by GTGase and MTGase for 15 and 30 min, respectively. Cross-linked α-lactalbumins by GTGase and MTGase were digested with lysylendopeptidase followed by the separation of the resulting peptides using reverse-phase HPLC. By the sequence analysis of the peptide fragments containing two N termini, which indicates the presence of cross-linked peptide, the lysine residues targeted by TGases were identified as follows: for GTGase, Lys16, Lys93, and Lys122; for MTGase, Lys5. These peptide fragments were further digested by V8 protease. Separation and sequence analyses of the resultant peptides were performed to identify glutamine residue involved in cross-linking. It was found that Gln54 was cross-linked to lysine residues by GTGase and MTGase in common. It is suggested that the difference in the numbers of lysine residues targeted by GTGase and MTGase may be responsible for the difference in the polymerization process of α-lactalbumin between GTGase- and MTGase-catalyzed systems.

KEYWORDS: Mammalian transglutaminase; microbial transglutaminase; cross-linking site; α-lactalbumin; lysylendopeptidase; V8 protease

INTRODUCTION

Transglutaminase (protein-glutamine γ-glutamyltransferase, EC 2.3.2.13) (TGase) primarily catalyzes an acyl transfer reaction between the γ-carboxyamide group of peptide-bound glutaminyl residue and a variety of primary amines (*1*). When the ε-amino group of the peptide-bound lysyl residue acts as a substrate, peptide chains are covalently connected through ε-(γ-glutamyl) lysine bonds. Although the enzyme is widely distributed in various tissues, its biological functions have not been fully elucidated (*2*−*5*).

Recently, studies on the application of TGases have been carried out actively. TGase has been regarded as a potential means of improving the nutritional value and functional properties of food proteins by catalyzing the incorporation of biologically active amines or amino acids, the cross-linking of protein molecules, and the deamidation of the γ-carboxyamide group (*6*−*11*). At first, Ca^{2+}-dependent TGases were extracted from blood plasma or guinea pig liver to be used for the modification of food proteins (*6*−*11*). However, the use of such TGases of mammalian origin for food processing is impractical because of the high cost of extraction and purification. Therefore, the major source of TGases is shifting to microorganisms (*12*−*16*). Microbial TGases (MTGases) normally do not require Ca^{2+} for the expression of activity (*16*, *17*).

Considerable work has been undertaken to understand the sequence specificity around the glutamines in substrate proteins of mammalian-origin TGases (*18*−*21*). On the other hand, there have been a limited number of publications on the selectivity toward amine donor lysine residues in substrate proteins (*21*−*25*). Comparison of amino acid sequences around glutamine or lysine side chains known to be modified by TGases suggests that the chemical nature of the side chains adjacent to the target amino acids may not be the sole determinant of specificity. In other words, conformational factors as well as the primary structure of substrates may play important roles in TGase reaction. Because MTGases were recently found, there are scant data on the sequence specificity of substrate proteins. Matsumura et al. (*26*) demonstrated that Gln54 of α-lactalbumin was selectively modified by microbial TGase from *Streptoverticillium* when the protein was transformed from the native state to

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the molten globule state. This indicates the importance of conformational factors and sequence specificity for MTGase reaction, too.

It is believed that MTGase may have evolved along a different pathway from the Ca^{2+}-dependent TGases from mammals (*27*). MTGase consists of 331 amino acid residues with a molecular weight of 37863, whereas the molecular weights of mammalian TGases are normally 2-fold that. MTGase shows little homology with TGases of mammalian origin (*27*, *28*). A highly conserved amino acid sequence 374−381 (YGQCWVF) in the active site cysteine residue proposed for several mammalian TGases (*29*) is not present in the sequence of microbial TGase, but a similar sequence 62−66 (YGCVG) is present, which is predicted to be located in a β-turn (*27*). Kanaji et al. (*27*) have suggested that Cys64 is essential for its catalytic activity. The putative Ca^{2+}-binding, which is widely conserved in mammalian tissue TGases (*30*), is lacking in MTGase.

Little work has been done to compare the reactivity of mammalian and microbial TGases using the same substrate proteins under the same reaction conditions. Such comparable work is necessary for characterizing the properties of TGases and making full use of the activity of the enzymes in the application. We studied the reactivity of TGase from guinea pig liver (GTGase) and MTGase against several globular proteins such as α-lactalbumin, β-lactoglobulin, bovine serum albumin, and ovalbumin (unpublished data). Analyses by sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS-PAGE) showed a higher reactivity of GTGase, that is, a more rapid decrease of the monomeric form of substrate proteins. Moreover, analyses on the enzyme-catalyzed polymer formation of α-lactalbumin and bovine serum albumin using a technique of gel permeation chromatography combined with laser light scattering exhibited the more rapid growth of large polymers for the GTGase-catalyzed system (*31*). Such differences in cross-linking behavior may originate from the difference in the substrate specificity of the two enzymes. In the present study, we attempted to locate cross-linking sites in α-lactalbumin targeted by GTGase and MTGase, respectively, to assess the site specificity of the two enzymes.

α-Lactalbumin was chosen as a substrate for the following reasons. α-Lactalbumin consists of single polypeptide without any chemical modification such as glycosylation and phosphorylation. Its molecular weight is 14200, and it exists essentially as a monomeric form. Not only its amino acid sequence but also its three-dimensional structure have already been determined (*32*, *33*). These available data enable us to locate the target sites easily using peptide sequencing and to discuss the environment around the target sites. Moreover, we found that GTGase could act on α-lactalbumin even in the absence of Ca^{2+} (*31*), although this enzyme normally requires Ca^{2+} to exhibit activity as described above. We speculate that Ca^{2+} included in the α-lactalbumin molecule can be used by the enzyme. This situation enables us to compare the reactivity of GTGase and MTGase clearly without the influence of aggregates of substrate proteins caused by Ca^{2+} addition. Such aggregates were suggested to mask reactive glutamine and lysine residues during TGase reaction (*16*). Therefore, in the present study, TGase reaction was done in a Ca^{2+}-free system.

MATERIALS AND METHODS

Mammalian TGase (EC 2.3.2.13) (GTGase) was prepared from guinea pig liver by one-step purification using a monoclonal antibody immunoadsorbent according to the method of Ikura et al. (*34*). The specific activity was 6.4 units/mg, indicating almost homogeneous TGase (figure not shown) in a yield of 28.3%. Microbial TGase (specific activity = 1.5 units/mg) derived from *Streptoverticillium* sp. strain S-8112 (MTGase) was prepared according to the method of Ando et al. (*17*) and supplied by Ajinomoto Co., Inc. (Kawasaki, Japan). The specific activity was measured by using the method of Folk and Cole (*35*). Bovine α-lactalbumin (catalog no. L-5385, type I) was purchased from Sigma (St. Louis, MO). *Achromobacter* protease I and *Staphylococcus aureus* V8 protease were purchased from Wako Chemical (Osaka, Japan). Other reagents of analytical reagent grade were purchased from Wako Chemical and Nakarai Tesque (Kyoto, Japan).

Measurement of Circular Dichroism (CD). The far-UV CD spectra of α-lactalbumin solutions (0.2 mg/mL) were recorded at 37 °C with a spectropolarimeter (JASCO, J-720). α-Lactalbumin was solubilized in 200 mM Tris-HCl buffer (pH 7.5) with and without 15 mM dithiothreitol (DTT). The CD data were expressed as mean residue ellipticity (degrees·cm^2/dmol) by using 115.5 as the mean residue weight of α-lactalbumin.

Polymerization of α-Lactalbumin by Transglutaminase. TGase-catalyzed polymerization of α-lactalbumin was carried out in 200 mM Tris-HCl buffer (pH 7.5) containing α-lactalbumin (5 mg/mL), TGases (0.64 units/mL), and 15 mM DTT. The enzymatic reaction was carried out at 37 °C for 15 and 30 min for GTGase and MTGase, respectively. The reaction was stopped by adding 40 mM ethylenediaminetetraacetic acid (EDTA) and 10 mM *N*-ethylmaleimide (NEM) for GTGase and MTGase reaction, respectively, followed by heating at 100 °C for 5 min.

Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis. The reaction mixture was added with the equal volume of SDS solution (125 mM Tris-HCl, pH 6.8, containing 20% glycerol, 4% SDS, and 2% mercaptoethanol). SDS-PAGE was performed on gradient gel (5−20%) according to the method of Laemmli (*36*). The gel was fixed and stained in 50% methanol and 10% acetic acid with 0.1% Coomassie Brilliant Blue R-250

Reduction of Intact Disulfide Bonds of α-Lactalbumin. Disruption of disulfide bonds and carboxymethylation of generated sulfhydryl groups in nonmodified and modified α-lactalbumins were carried out by the following procedures, that is, incubation of α-lactalbumin (1 mg/mL) with 100 mM Tris-HCl buffer (pH 8.0) containing 8 M urea and 10 mM EDTA, followed by the addition of 100 mM iodoacetamide. After incubation at 37 °C for 2 h in the dark, the solution was dialyzed against 0.1 N acetic acid and lyophilized.

Digestion with Lysylendopeptidase. Lyophilized nonmodified and modified α-lactalbumins (10 mg) were dissolved in 0.9 mL of 8 M urea solution and added with 0.1 mL of 500 mM Tris-HCl buffer (pH 9.0), respectively. After incubation at 37 °C for 30 min, they were diluted with 1.25 mL of 50 mM Tris-HCl buffer (pH 9.0) to reduce the urea concentration to ~3 M and digested with lysylendopeptidase [*Achromobacter* protease I (2.8 AU/mg)] to give a 1:280 (w/w, protease/substrate) ratio for 6 h at 37 °C. Digestion was terminated by the addition 0.1% trifluoroacetic acid (TFA) for reducing the pH, and the reaction mixtures were loaded onto a reverse-phase high-performance liquid chromatography (RP-HPLC) column as described below.

Digestion with V8 Protease. HPLC fractions containing peptides cross-linked via isopeptide bonds from the lysylendopeptidase digests were redigested with *S. aureus* V8 protease for identifying glutamine site targeted by TGases as follows. The HPLC fractions selected for redigestion were dried under nitrogen gas. Part of each dried fraction was hydrolyzed in a vacuum for 24 h at 110 °C with 6 N HCl containing 0.1% phenol followed by analysis with a Hitachi model 835 amino acid analyzer to determine the amount of peptide. Other parts of dried fractions were solubilized in 0.15 M sodium phosphate buffer (pH 7.8) containing 3 M urea and incubated with *S. aureus* V8 protease (enzyme/substrate = 1:50 w/w) at 37 °C for 24 h. For inactivation of enzyme, reaction mixtures were heated at 100 °C for 5 min followed by addition of TFA to 0.1%. Then the digests were loaded onto an RP-HPLC column.

Separation of Digested Peptides. The peptide mixtures obtained by lysylendopeptidase or V8 protease were separated by RP-HPLC on a YMC-Pack ODS-AP column of dimensions 250 × 4.6 mm i.d. and particle size 5 μm. Elution of peptides from the column was carried out by applying a two-step linear gradient of acetonitrile in 0.1% TFA.

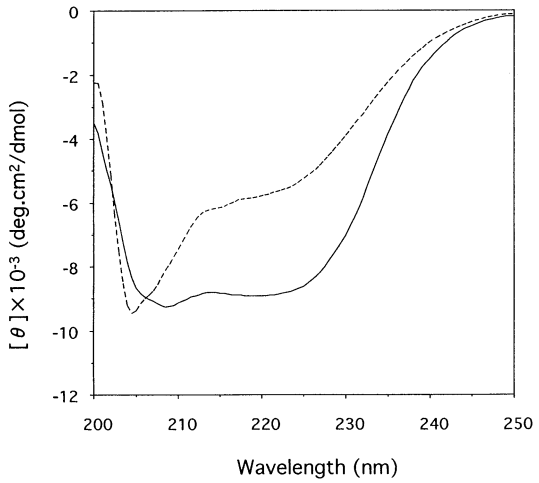

Figure 1. CD spectra of α-lactalbumin in the presence (- - -) and absence (—) of DTT. α-Lactalbumin was solubilized in a 0.2 M Tris-HCl buffer (pH 7.5) with and without 15 mM DTT. The far-UV CD spectra were recorded at 37 °C with a spectropolarimeter (JASCO, J-720).

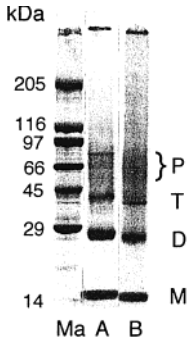

Figure 2. SDS-PAGE of α-lactalbumin cross-linked by TGases. TGase-catalyzed polymerization of α-lactalbumin was carried out in a 0.2 M Tris-HCl buffer (pH 7.5) containing 15 mM DTT. α-Lactalbumin was incubated with GTGase and MTGase at 37 °C for 15 and 30 min, respectively. The products were separated by electrophoresis using a gradient gel (5–20%). Ma, molecular weight marker; A, product from GTGase-catalyzed reaction; B, product from MTGase-catalyzed reaction. M, D, T, and P indicate monomer, dimer, trimer, and polymers (including oligomers larger than tetramer) of α-lactalbumin, respectively.

First, a linear gradient of between 100% solvent A (0.1% TFA, v/v) and 75% solvent A/25% solvent B (0.1% TFA in 70% acetonitrile, v/v) was applied over a period of 5 min. Then the second linear gradient between 75% solvent A/25% solvent B and 15% solvent A/85% solvent B was applied from 5 to 90 min. Elution of peptides was monitored by measuring UV absorption (215 nm). For the further purification of separated peptides, rechromatography was carried out using the same elution gradients but a different column, that is, a YMC Pack Protein-RP column.

Determination of Amino Acid Sequence. The fractions collected by RP-HPLC were applied to a protein sequencer, model 492 Procise (Applied Biosystems), which employs highly sensitive detection for phenylthiohydantoin (PTH) amino acids. PTH derivatives produced at each cycle were identified by using an Applied Biosystems model 785A programmable absorbance detector by reference to standard derivatives of amino acid.

RESULTS

Changes in CD Spectra of α-Lactalbumin Induced by Dithiothreitol. In the present study, the TGase reaction was carried out in the presence of 15 mM DTT. It is likely that DTT could alter the conformation of α-lactalbumin by disruption of intact disulfide bonds, thereby influencing the susceptibility to TGase reaction of α-lactalbumin. Therefore, the structural change of α-lactalbumin induced by DTT was investigated by measuring far-UV CD spectra.

Figure 1 shows the CD spectra of α-lactalbumin in the presence or absence of DTT at 37 °C. The CD curve in the absence of DTT resembled those reported in the previous studies (*37*, *38*). There are two negative peaks around 208 and 222 nm in the CD spectrum, indicating the presence of α-helical structure. The estimated α-helix content was 26.3%, using the curve-fitting method of Yang et al. (*39*). DTT addition altered the shape of the CD spectra substantially. The intensity around 222 nm decreased, and the peak at 208 nm shifted to the direction of lower wavelength. The estimated α-helix content by Yang et al. (*39*) decreased to 15.2%. The simple calculation using the $[\theta]_{222}$ value based on the method of Chen et al. (*40*) also confirmed the decrease of α-helix content from 27.3 to 14.9%. The decrease of α-helix content was accompanied with the increment of β-sheet content from 13 to 22% by the addition of DTT. The increase of β-sheet can be partially attributed to the formation of intermolecular β-structure. However, we cannot avoid the inaccuracy for the estimation of contents of β-sheet, turn, and random coil using CD data. Therefore, in this case, it is appropriate to emphasize only the unfolding of α-helical structure in α-lactalbumin being induced by the addition of DTT and disrupting the intact disulfide bonds.

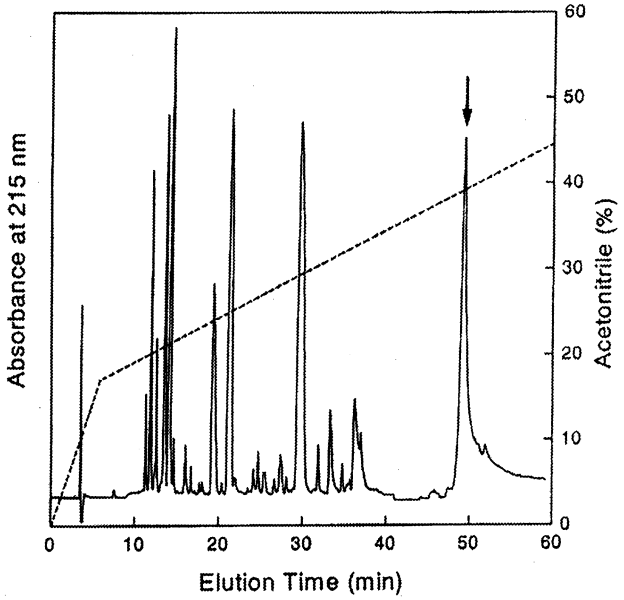

Figure 3. Separation by RP-HPLC of peptides produced by lysylendopeptidase digestion of nonmodified α-lactalbumin. Digestion of α-lactalbumin by lysylendopeptidase was performed as described under Materials and Methods. Peptides were eluted with a two-step gradient of acetonitrile (0–45%) in 0.1% TFA (- - -) on a YMC-Pack ODS-AP column and detected by recording the absorbance at 215 nm.

Reactivity of α-Lactalbumin as Substrate of GTGase and MTGase. The reactivity of α-lactalbumin as substrate of GTGase and MTGase was evaluated by SDS-PAGE (**Figure 2**). The patterns show that dimer, trimer, and polymers including oligomers larger than tetramer were formed via intermolecular cross-linkings by the TGases. The oligomers and polymers were not observed when α-lactalbumin was incubated with the TGases in the absence of DTT (data not shown), indicating the conformational change by disruption of intact disulfide bond of α-lactalbumin is essential for the induction of TGase-catalyzed polymerization. It should be noted that the longer incubation time for MTGase (30 min) was necessary to form

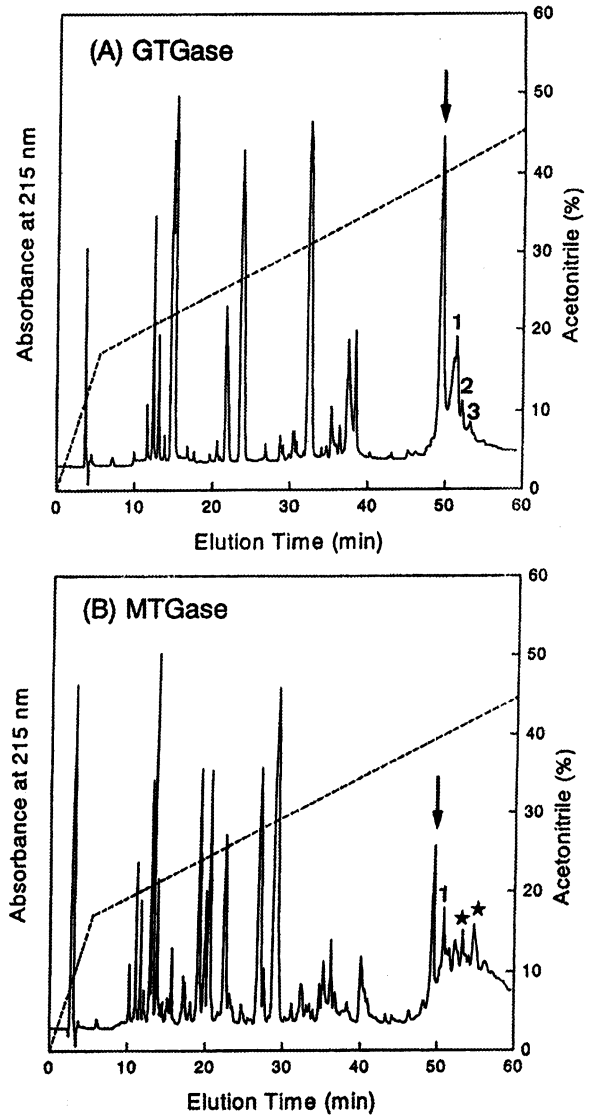

Figure 4. Separation by RP-HPLC of peptides produced by lysylendopeptidase digestion of TGase-modified α-lactalbumin. (A, B) Separations of peptides for cross-linked α-lactalbumin by GTGase and MTGase, respectively. Digestion of α-lactalbumin and elution conditions were as described in **Figure 3**. Numbers in (A) and (B) indicate the peaks containing cross-linked peptides (see text).

almost the same amount of oligomers and polymers produced by GTGase for 15 min. This result is consistent with the previous finding that GTGase showed the higher efficiency in cross-linking reaction of α-lactalbumin than MTGase, that is, the more rapid decrease of monomeric form and the more rapid growth of large polymers (*31*). The reaction mixtures of **Figure 2** were used for the digestion by the proteases and determination of cross-linking sites in the following sections.

Fractionation of Peptides of α-Lactalbumin Digested by Lysylendopeptidase. The digestion by lysylendopeptidase of nonmodified α-lactalbumin was performed prior to the digestion of α-lactalbumin polymerized by TGases in order to compare the peptide maps between nonmodified α-lactalbumin and polymerized α-lactalbumin. **Figure 3** shows the RP-HPLC profile of peptide fragments produced by the digestion of nonmodified α-lactalbumin using an ODS type column. Most of peaks were eluted by 40 min, but one large peak appeared at ∼50 min, indicating the presence of a large and/or hydrophobic peptide.

^{1}EQLT**K**^{5} ^{6}CEVFREL**K**^{13} ^{14}DL**K**^{16}

^{17}GYGGVSLPEWVCTTFHTSGYD
TQAIVQNNDSTEYGLFQINN**K**^{58}

^{59}IWC**K**^{62} ^{63}DDQNPHSSNICNISCD**K**^{79}

^{80}FLDDDLTDDIMCV**K**^{93} ^{94}**K** ^{95}ILD**K**^{98}

^{99}VGINYWLAH**K**^{108} ^{109}ALCSE**K**^{114}

^{115}LDQWLCE**K**^{122} ^{123}L

Figure 5. Amino acid sequence and expected peptide fragments produced by lysylendopeptidase digestion of α-lactalbumin.

Next, α-lactalbumin polymerized by GTGase and MTGase as shown in **Figure 2** was digested by lysylendopeptidase, and the generated peptides were fractionated by RP-HPLC using the same chromatographic conditions. Parts A and B of **Figure 4** show the profiles of digests from α-lactalbumin polymerized by GTGase and MTGase, respectively. Comparing the patterns of nonmodified (**Figure 3**) and GTGase-polymerized α-lactalbumin (**Figure 4A**), we could not find any difference except that three peaks newly appeared after 50 min in the digests from the polymerized one. Comparing the patterns of nonmodified (**Figure 3**) and MTGase-polymerized α-lactalbumin (**Figure 4B**), the differences were observed in the elution profile at around 10−40 and 50 min. However, the newly appeared peaks at ∼10−40 min in **Figure 4B** were not recognized as cross-linked peptides in the subsequent analyses, so they were excluded from our targets. Consequently, only the new peaks appearing at ∼50 min were further analyzed. These peaks newly appeared in **Figure 4** were collected and purified to remove minor components by RP-HPLC using a different type of column (YMC Pack Protein-RP column). Identification of the resultant peptides was attempted using the amino acid sequencer as described in the next section.

Identification of Targeted Lysine Residues by GTGase and MTGase in α-Lactalbumin. It is expected that lysine residues involved in the isopeptide bonds are not acted on by lysylendopeptidase. Therefore, the fraction containing cross-linking isopeptide should be recognized by detection of two N termini. The peptide fractions found only in the digests of polymerized α-lactalbumin and separated as new peaks as described in the previous section were analyzed using the amino acid sequencer to determine whether they have two N termini or not and to identify amino acid sequences of cross-linked peptides. For each sample, at least a 15 amino acid sequence from the N terminus was analyzed. Peaks 1−3 in **Figure 4A** were shown to have two N termini. On the basis of the data of amino acid sequence (*32*) and the expected peptide fragments from the digestion of nonmodified α-lactalbumin shown in **Figure 5**, the peptides of the three new peaks 1−3 of **Figure 4A** were identified as follows:

Peak 1. Asp14-Leu-Xaa(Lys)- - -
Gly17-Tyr - - - -Lys58

Peak 2. Phe80-Leu- - - -Val92-Xaa(Lys)- - -
Gly17-Tyr- - - -Lys58

Peak 3. Leu115-Asp- - - -Glu121-Xaa(Lys)- - -
Gly17-Tyr- - - -Lys58

Xaa could not be identified, but it should be lysine from the sequence of the expected peptide fragments in **Figure 5**.

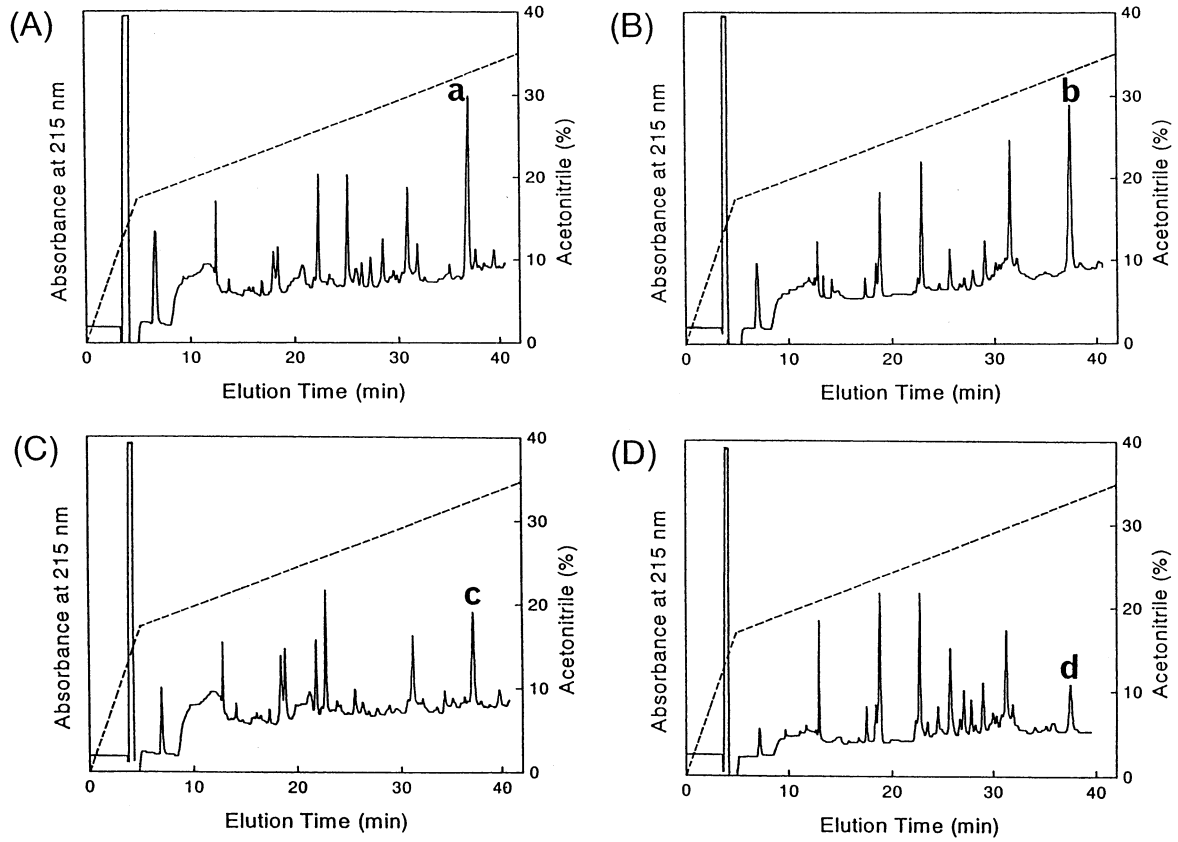

Figure 6. Separation by RP-HPLC of peptides produced by the V8 protease digestion of the cross-linked peptides. Cross-linked peptides separated and purified from lysylendopeptidase digests of α-lactalbumin polymerized by GTGase and MTGase were further cleaved by V8 protease as described under Materials and Methods. (A, B, C) Separations of peptides produced by V8 protease digestion of peak 1, peak 2, and peak 3 of **Figure 4A**, respectively, that is, cross-linked peptides originating from GTGase-induced α-lactalbumin polymer. (D) Separation of peptides produced by V8 protease digestion of peak 1 of **Figure 4B**. Elution conditions and column were the same as those of **Figure 3**. (a–d) Peaks containing cross-linked peptides.

The peak eluting just before these peaks and appearing also in the HPLC pattern of nonmodified α-lactalbumin as shown by the arrow (**Figures 3** and **4**) was sequenced for comparison. This peak was found to be a peptide of Gly17−Lys58, not cross-linked. On the bais of the results of identification of peaks 1−3 as shown above, we conclude that three different peptides, namely, Asp14-Leu-Xaa(Lys)- - -, Phe80-Leu- - -Val92-Xaa-(Lys)- - -, and Leu115-Asp- - -Glu121-Xaa(Lys)- - -, are linked to the same peptide Gly17−Lys58 to form the cross-linked peptides of peaks 1−3, respectively, via isopeptide bonds. Because one peptide involved in peak 3 (Leu115- - -Lys122) contains one glutamine residue, this glutamine residue could be a potential cross-linking site. However, Gln117 was detected by amino acid sequencer, and we could exclude the possibility of the cross-linking of this glutamine residue. Therefore, we have shown that Lys16, Lys93, and Lys122 are targeted sites of GTGase.

The new peaks appearing from the digest of α-lactalbumin polymerized by MTGase were also analyzed by the amino acid sequencer, and peak 1 (in **Figure 4B**) was found to have two N termini. This fraction contained the following two peptides: Glu1- - -Xaa (Lys5)- - - and Gly17- - -Lys58. Analysis of the two starred peaks in **Figure 4B** showed the same sequence data as that from peak 1, indicating the peptides in these two peaks are the derivatives of the peptides in peak 1. Therefore, it was demonstrated that MTGase could act on Lys5.

We could identify amine donor lysine sites by sequence analyses of cross-linked peptides liberated from α-lactalbumin polymerized by GTGase and MTGase. We also showed that amine acceptor glutamine site was positioned in the sequence from Gly17 to Lys58. Becasue this peptide contains three glutamines, Gln39, Gln43, and Gln54, we could not know which was the glutamine residue(s) targeted by TGases. Therefore, all of the cross-linked peptides were further digested to smaller peptides and separated using RP-HPLC to identify amine acceptor glutamine residue(s) as described in the next section.

Identification of Glutamine Residue Involved in Cross-Linking. Peptides of peaks 1−3 of **Figure 4A** and of peak 1 of **Figure 4B** were digested by V8 protease, and the resultant peptide fragments were separated using RP-HPLC as shown in panels A, B, C, and D of **Figure 6**, respectively. The conditions and column used were the same as those of **Figures 3** and **4**. Peaks a−d were found to have two N termini and thought to be cross-linked peptides. These fractions contained peptides (YGLF- - -) in common. This peptide can be identified as Tyr50- - -Lys58 on the basis of the expected peptide fragment (**Figure 5**). Therefore, it is thought that Gln54 should be the common site targeted by GTGase and MTGase.

DISCUSSION

The results reported here showed that, of 12 lysine residues in α-lactalbumin, 3 lysine residues (Lys16, 93, and 122) and one lysine residue (Lys5) could be selectively modified by GTGase and MTGase, respectively, when α-lactalbumin was treated by the addition of DTT. Only one glutamine residue was available to the enzymes, although six glutamine residues are present in α-lactalbumin. The low numbers of lysine and glutamine residues available to both GTGase and MTGase may restrict the rapid cross-linking of α-lactalbumin as compared

to the case of using caseins as substrates (*11*). A number of glutamine residues, at least three, are found to be TGase-reactive for each casein (*20*). Flexible conformation of caseins may enable lysine and glutamine residues to be modified by TGases, whereas globular proteins such as α-lactalbumin cannot be good substrates of TGases even if the proteins are partially denatured.

It is worthwhile to note the difference in the number and the site of reactive lysine residues between GTGase- and MTGase-catalyzed systems in relation to the efficiency of polymer formation. In our previous studies, polymer formation of α-lactalbumin caused by incubation with GTGase and MTGase was investigated using SDS-PAGE (unpublished data) and gel permeation chromatography combined with a multiangle laser light scattering detector system (*31*). GTGase showed the high cross-linking activity at the early stages of incubation within 60 min, namely, the more rapid decrease of monomeric α-lactalbumin and the more rapid growth of large polymers. Such a difference in the efficiency of polymer formation should originate from the difference in the number of TGase-reactive lysine residues in α-lactalbumin demonstrated in the present study. It is thought that GTGase can catalyze cross-linking of α-lactalbumin more efficiently acting on three lysine residues than MTGase, which can use only one lysine residue.

There are limited data about the sequence specificity around lysine residues targeted by TGases (*22*−*25*). Grootjans et al. (*25*) investigated the primary structure requirements around amine donor lysines in α-crystallin using a site-directed mutagenesis technique. On the basis of their results, they pointed out the trends on the influence of the residue preceding the amine donor lysine site. That is, glycine and aspartic acid before the amine donor lysine have strong adverse effects on substrate reactivity, and proline, histidine, and tryptophan are less favorable. In contrast, valine, arginine, and phenylalanine and, to a lesser extent, serine, alanine, leucine, tyrosine, and asparagine were shown to have an enhancing effect. This pattern of preference was shown to be largely in agreement with that previously observed for the limited number of characterized amine donor lysines in protein substrates for TGases. We discuss the applicability of this finding to our data as follows.

The amino acid residues preceding Lys93 and Lys16 were valine and leucine, satisfying the requirement suggested by Grootjans et al. (*25*). The residue preceding Lys5 is threonine. The preference of this amino acid residue was not studied by Grootjans et al. (*25*), but they confirmed the appearance of threonine as a preceding residue in the past data. Therefore, our data about substrate requirement around lysine residues in α-lactalbumin for GTGase and MTGase are consistent with the suggestion of Grootjans et al. (*25*), except Lys122.

The residue preceding Lys122 was glutamic acid. There is no example that glutamic acid precedes a lysine targeted by TGases. For instance, the sequence of - - -Glu-Lys- - - was found in rat seminal vesicle SV-IV protein, but this lysine residue was not attacked by TGase (*22*). As described above, aspartic acid before the amine donor lysine has strong adverse effects. It is possible that the negative charge of glutamic acid and aspartic acid discourages the reactivity of lysine against TGases. Actually, Lys79, Lys98, and Lys114 after aspartic or glutamic acids in α-lactalbumin (see **Figure 5**) were not modified by both of the TGases. Therefore, it seems to be unusual that GTGase attacks lysine residues after glutamic acid in substrate proteins.

Although the amino acid sequence before the reactive lysine satisfies the requirement of Grootjans et al. (*25*), some lysine residues of α-lactalbumin were not attacked by the TGases. For instance, Lys13 after leucine was not found to be cross-linked. This indicates that conformational factor as well as primary sequence may also affect the reactivity of lysine for TGases. Our result that α-lactalbumin was not cross-linked by the TGases in the absence of DTT also supports the importance of conformational factor with respect to the induction of the enzyme reaction. The data and discussion on the conformational environment of reactive lysine residues were scant, but it is suggested that TGase may prefer solvent-exposed lysine residues (*21*). As for the case of TGase-reactive glutamine residues, the secondary structure around the target lysine might be also important for determining the susceptibility of proteins to TGase reactions.

The data on three-dimensional structure (*32*, *33*) show that all of the lysine residues in α-lactalbumin are exposed to solvent in the native state. GTGase-reactive Lys16 is involved in the sequence forming turn structure, and GTGase-reactive Lys122 is positioned in the flexible region of C termini, which are predicted to show no structural preferences. Such secondary structural environment probably encourages the susceptibility to the enzyme reaction. Furthermore, the disruption of superreactive Cys6−Cys120 disulfide bond, which is 140 times more reactive than normal disulfides in the fully accessible state and easily reduced by 1 mM DTT at pH 7.0 (*37*), should occur in the presence of 15 mM DTT, thereby enhancing the accessibility of TGases to Lys122.

On the other hand, Lys5 and Lys93 are positioned in the N-terminal end of α-helix (5−11, called the A-helix) and the center of the α-helix (85−98, called the C-helix), respectively. It has been suggested that glutamine residues in α-helices are difficult to be attacked by TGase. On the basis of our results, we can speculate that lysine residues in α-helices are available to TGases, in contrast to the case of glutamine residue. However, the results of CD spectra (**Figure 1**) suggest the unfolding of α-helices of α-lactalbumin in the reduced form to greater extent, and it is also likely that such unfolding enhances the reactivity of Lys5 and Lys93. Chyan et al. (*41*) demonstrated that the C-helix was not persistent in partially denatured states, that is, the molten globule state, using a hydrogen exchange technique combined with a NMR spectroscopy. Judging from the substantial change of far-UV CD spectra (**Figure 1**), the secondary structure of reduced α-lactalbumin seems to be more unfolded than the α-lactalbumin molecule in the molten globule state, which does not affect far-UV CD spectra (*41*). Therefore, Lys93 in the C-helix of α-lactalbumin should be susceptible to TGase reaction in the reduced condition because of unfolding of the helix. The A-helix of α-lactalbumin is found to be persistent in the partially denatured state (*41*). However, disruption of the superreactive disulfide bond (Cys6−Cys120) may weaken a native-like tertiary fold, thereby partially destabilizing the A-helix structure. It is unlikely, therefore, that lysine residues in persistent α-helices are susceptible to TGase reactions.

In the present study, only Gln54 was modified by GTGase and MTGase in the presence of DTT. This is consistent with the previous result, that is, the selective modification of Gln54 by MTGase when α-lactalbumin is in the molten globule state (*26*). These results imply that TGases are much more selective toward amino acceptor glutamine residues in proteins than they are toward the lysine substrates, irrespective of the origin of the enzyme. It is interesting that the specificity on reactive glutamine residues has been conserved, although MTGases have evolved along a different pathway from the Ca^{2+}-dependent TGases.

Coussons et al. (*19*) derived some rules to identify "discouraging" sequence features for the TGase reaction. For substrates with little ordered structure, they claimed that the positively charged amino acids within the five positions next to the C-terminal side of a glutamine residue are a discouraging feature. Gln54 does not follow this charge rule, because the fourth position after Gln54 is lysine. Several TGase-reactive glutamines in the caseins (*29*) and histones (*42*) were found to not follow the charge rule, too. Coussons et al. (*18*) also discussed conformational factors affecting reactivity of glutamine residues. According to their criteria, the glutamine must satisfy an accessibility either by being in a highly flexible region of polypeptide chain or by being clearly exposed to solvent in a more structured region. Furthermore, the target glutamine side chains should be in regions that are predicted to be reverse turns, at the end of β-sheet structure, or to show no structural preferences. Gln54 of α-lactalbumin is involved in a type-I turn structure and exposed to the solvent in the native state, satisfying the requirement on the secondary structure to be attacked by TGase (*32*, *33*). However, our present and previous results have shown that Gln54 cannot be modified by TGase when α-lactalbumin is in the native state. Tertiary and/or secondary structural change induced by disruption of intact disulfide bond(s) may be essential for the full exposure of Gln54 to be available to TGases. Possible reasons of no reaction of other glutamine residues in α-lactalbumin were already discussed in detail in the previous paper (*26*).

In conclusion, the present results showed that there were a limited number of potential GTGase- and MTGase-reactive lysine and glutamine residues in the bovine α-lactalbumin: in the case of 12 lysine residues, 3 residues and 1 residue were used for GTGase and MTGase, respectively; and in the case of 6 glutamine residues, only 1 was used for both of them. Although the same site was recognized commonly as a reactive glutamine residue by both of the TGases, the site specificities for reactive lysine residue were completely different. The reason for variable lysine substrates should be due to the difference in the amino acid sequence and secondary structural environment around active site pocket between GTGase and MTGase (*27*). In addition, it is possible that two regions outside the active site pocket that are important for substrate recognition play dominant roles in determining the specificity for amine donor lysine sites, because these regions are lacking in MTGase, whereas they are present in factor XIIIa and tissue TGases (*43*).

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