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NMR-Based Screening Method for Transglutaminases: Rapid Analysis of Their Substrate Specificities and Reaction Rates

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Incorporation of inter- or intramolecular covalent cross-links into food proteins with microbial transglutaminase (MTG) improves the physical and textural properties of many food proteins such as tofu, boiled fish paste, and sausage. Other transglutaminases (TGases) are expected to be used in the same way, and also to extend the scope of industrial applications to materials, drugs, and so on. The TGases have great diversity, not only in amino acid sequence and size, but also in their substrate specificities and catalytic activities, and therefore, it is quite difficult to estimate their reactivity. We have developed an NMR-based method using the enzymatic labeling technique (ELT) for simultaneous analysis of the substrate specificities and reaction rates of TGases. It is quite useful for comparing the existing TGases and for screening new TGases or TGases variants. This method has shown that MTG is superior for industrial use because of its lower substrate specificity compared with those of guinea pig liver transglutaminase (GTG) and red sea bream liver transglutaminase (FTG). We have also found that an MTG variant lacking an N-terminal aspartic acid residue has higher activity than that of the native enzyme.

KEYWORDS: Transglutaminase; NMR; screening; enzymatic labeling

INTRODUCTION

Transglutaminases (TGase: protein-glutaminase γ-glutamyltransferase, EC 2.3.2.13) are a family of enzymes that catalyze the displacement of the amide ammonia at the γ-position in glutamine residues by replacing it with another amine, usually an ϵ-amino group from a suitable lysine residue (*1*−*4*). The formation of ϵ-(γ-glutamyl)lysine isopeptide bonds results in both intra- and intermolecular cross-linking of proteins, leading to polymerization. TGases are widely distributed in most tissues and body fluids, including liver, hair follicles, epidermis, prostate, and platelets, and are thought to be involved in diverse physiological functions, such as the maintenance of gross forms of structures and limited degrees of extensibility (*4*). They are also well-known for their improvement of food texture (*5*−*7*).

Guinea pig liver TGase (*8*), human epidermis keratinocyte TGase (*9*), and human blood coagulation factor XIII (*10*) are calcium ion-dependent enzymes. For instance, the enzymatic and structural properties of factor XIII have been well characterized for the cross-linking of fibrin, which stabilizes clots against redissolution by fibrinogen and plasmin (*11*−*14*). *Streptoverticillium* sp. S-8112, microbial transglutaminase (MTG), is a calcium ion-independent enzyme, and is the first TGase to be obtained from a nonmammal. Incorporation of inter- or intramolecular covalent cross-links into food proteins with MTG improves the physical and textural properties of many food products such as tofu, boiled fish paste, and sausage. In addition, TGases originating from marine organisms, including fish, crustaceans, and echinoderms, such as red sea bream liver (*15*, *16*), carp dorsal muscle (*17*), limulus hemocyte (*18*), lobster muscle (*19*), sea urchin eggs (*20*), and Japanese oyster gills and mantles (*21*), have been reported. However, these TGases are quite diverse, not only in amino acid sequence and size, but also in their substrate specificities and catalytic activities, although their enzymatic reactions generally involve an acyl transfer.

One of the methods for measuring TGase activity is the calorimetric hydroxamate procedure, using a small peptide including a glutamine residue, *N*-carbobenzoxy-L-glutaminylglycine (*22*). However, this is not adequate for an analysis of substrate specificity, because the reactivity of a glutamine residue depends on the type of TGase and the environment surrounding the glutamine residue in the substrate protein. If a substrate protein is modified with an ϵ-amino group of a lysine or any compound with an amino group, to investigate the substrate specificity for glutamine residues, its behavior would change when one of the glutamine residues in the protein is modified. Because it gives rise to several variants of the substrate protein, it is impossible to find the reactivity of a specific glutamine residue in the substrate protein. Moreover, the analysis of the modified protein requires significant time and labor. Therefore, it is worthwhile to develop a method for the rapid and easy analysis of substrate specificity and reaction rate.

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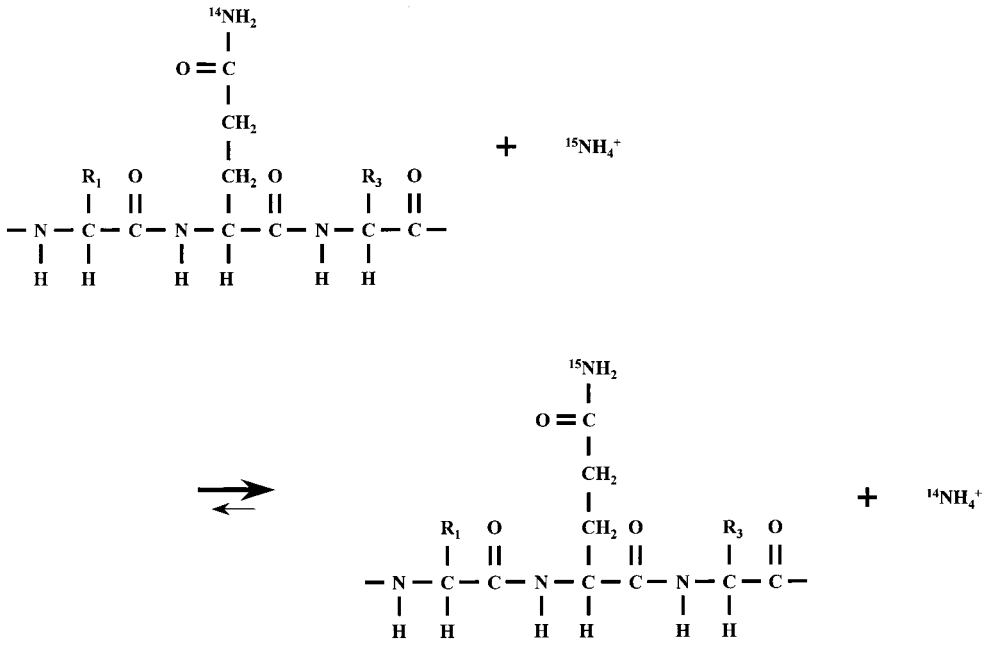

Figure 1. Schematic drawing of ELT by using TGases. The labeled ammonium ion reacts with the γ-carboxyamide groups of glutamine residues, and exchanges with their amide groups.

Chemical modifications of proteins with a fluorescent reagent or a chelating agent are useful for analyzing enzymatic activities, because the absorbance or fluorescence of a substrate or a product is easy to measure, and their sensitivities are quite high. However, this method is not appropriate for analyzing multiple reaction positions in one molecule simultaneously; for example, several glutamine residues in a substrate protein that have reacted with TGases. In such a case, nuclear magnetic resonance (NMR) is suitable for analyzing the reaction sites in a molecule individually, because NMR signals can be observed for individual glutamine residues.

The proposed TGase reaction mechanism is based on the crystal structure of factor XIII (*23*, *24*). At first, the γ-carboxyamide groups interact with TGases, and subsequently the primary amino groups of a variety of amines or the ϵ-amino groups of lysine residues are involved in the formation of new covalent bonds. On the basis of the proposed reaction mechanism, we have already developed an enzymatic labeling technique (ELT), in which ^{15}N nuclei are incorporated into the γ-carboxyamide groups of the glutamine residues in arbitrary proteins (*25*). In the present paper, we describe a novel method using ELT and the techniques for detection with NMR in order to determine the substrate specificities for glutamine residues and the reaction rates of TGases simultaneously. The method is quite useful for comparing existing TGases and for screening new TGases or TGase variants. By using this method, we have characterized the enzymatic properties of various TGases, and found one that would be preferred for improving the physical and textural properties of many food products, including tofu, boiled fish paste, and sausage.

MATERIALS AND METHODS

Materials. Expression and purification of MTG and TGase from red sea bream liver (fish-derived transglutaminase, FTG) were performed as described previously (*15*, *16*, *26*). TGase from guinea pig liver (GTG) was purchased from Sigma Chemical Co. The MTG variant lacking the N-terminal aspartic acid residue has a serine residue as the N-terminus, and thus will hereafter be referred to as "Ser-MTG". Recombinant Ser-MTG was expressed and purified as described previously (*27*).

Sample Preparation. Ovalbumin (albumin from chicken egg), which is a glycoprotein (43 kDa) including 14 glutamine residues, was used as the substrate protein. It is suitable for the establishment of our method because it is inexpensive and easy to use.

A solution of 2.3 mM ovalbumin was prepared by adding 5 mM $CaCl_2$, 200 mM $^{15}NH_4Cl$, and 4 μM MTG in 20 mM phosphate buffer, pH 6.0, in 95% H_2O/5% D_2O. After incubation at 310 K for 3 h, 500 μM of the protein solution was immediately placed into a 5 mm NMR tube for the NMR measurements. Replicate ovalbumin solutions were reacted under the same conditions, except for the addition of GTG or FTG instead of MTG. $CaCl_2$ was added for the enzymatic activities of GTG and FTG.

For estimation of the reaction rate, solutions of 2.3 mM ovalbumin were prepared by adding 5 mM $CaCl_2$, 200 mM $^{15}NH_4Cl$, and 4 μM MTG or Ser-MTG in 20 mM phosphate buffer, pH 6.0, in 95% H_2O/5% D_2O. After incubations at 310 K for 1 and 3 h, the protein solutions were immediately placed into an NMR tube for $^1H-^{15}N$ HSQC measurements. For continuous measurements, concentrations of MTG and Ser-MTG were decreased to 0.4 μM.

NMR Measurements. NMR experiments were performed on a Bruker DMX600 spectrometer equipped with a triple resonance probe head with XYZ triple-axis gradient coils. All spectra were recorded at 310 K. The ^1H and ^{15}N chemical shifts were relative to the solvent H_2O as 4.66 ppm (*28*). For data processing and analysis, XWINNMR (Bruker Co., *29*), nmrPipe (*30*), and PIPP (*31*) were used.

The HSQC method is convenient for detection of ^{15}N labeled glutamine residues in a substrate protein; i.e., a two-dimensional NMR spectrum like an HSQC spectrum of the ^{15}N labeled sample reduces the overlap of the cross-peaks, and facilitates the observation of each signal separately, as shown in **Figure 2**. In addition, the HSQC measurement can be done in a short time, compared with the time required for one-dimensional ^{15}N NMR.

The HSQC spectra were recorded with spectral widths of 8400 Hz for ^1H and 1400 Hz for ^{15}N. The pulse sequence for the HSQC spectra was as described by Bodenhausen and Ruben (*32*). The WATERGATE water suppression scheme with the 3-9-19 refocusing pulse was incorporated into the reverse INEPT step (*33*). A total of 2048 data points were used in the t_2 dimension, and 200 transients were acquired for the t_1 points. Prior to 2D Fourier transformation, the acquired data were multiplied by Gaussian functions in t_2 and t_1, and were zero-filled to yield a 1024 (F_2) $\times$ 512 (F_1) matrix of the real data points.

$^1H-^{15}N$ HSQC spectra in the presence of MTG and Ser-MTG were measured after incubations for 1 or 3 h, and continuously 20 times, for

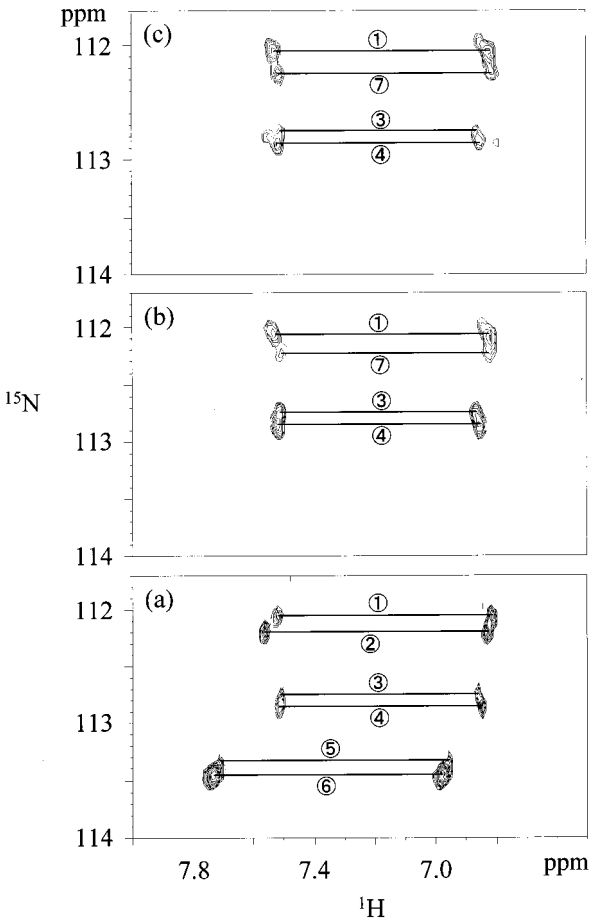

Figure 2. ^{1}H-^{15}N HSQC spectra of ovalbumin labeled with ^{15}N in the presence of (a) MTG, (b) GTG, and (c) FTG. Observed cross-peaks labeled with MTG are numbered 1–6. Cross-peaks observed in the presence of GTG or FTG are marked with the corresponding signal numbers, and newly observed cross-peaks are numbered 7.

MTG	R	E	W	L	S	Y	G	C	V	G	V	T	W	V	N
GTG	Q	R	V	K	Y	G	Q	C	W	V	F	A	A	V	A
FTG	R	P	V	K	Y	G	Q	C	W	V	F	A	A	V	A

Figure 3. Amino acid sequences neighboring the active cysteine residue in MTG, GTG, and FTG. The active residues, Cys64, Cys276, and Cys272, are boxed in MTG, GTG, and FTG, respectively.

the estimation of reaction rate. Continuous measurements were started within 20 min after the addition of the enzyme, and each measuring time was about 135 min. A series of peak intensities was extracted in a set of 2D data with the utility in nmrPipe, and are plotted in **Figure 5**.

RESULTS AND DISCUSSION

Figure 1 depicts the reaction mechanism for ELT using TGases. The labeled ammonium ion reacts with the γ-carboxyamide groups of glutamine residues and exchanges with their amide groups. A large excess of ^{15}N labeled ammonium ion increases the incorporation of the ^{15}N nucleus. The ^{15}N labeled glutamine residues, corresponding to the TGase substrate, are easily and rapidly observed in the^{15}N edited spectra using NMR.

Six pairs of cross-peaks were observed in the presence of MTG, showing that the corresponding γ-carboxyamide groups of the glutamine residues in ovalbumin were labeled with ^{15}N

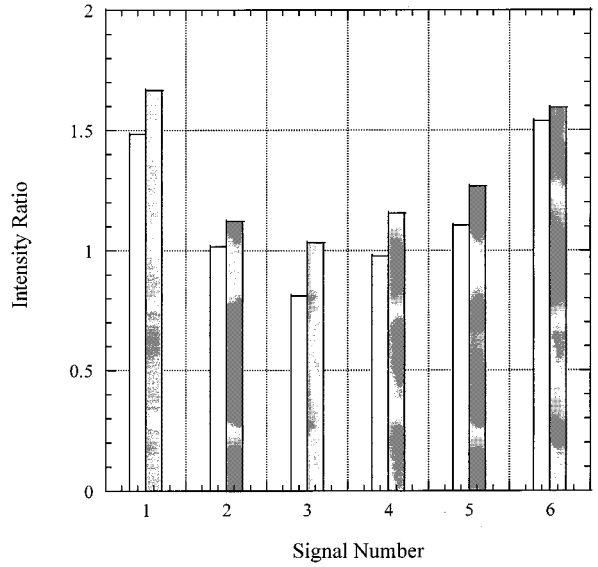

Figure 4. ^{15}N labeling ratios calculated from the signal intensities in the presence of Ser-MTG to those in the presence of MTG, were plotted for the signals indicated by 1–6 in **Figure 2a**. The open and gray bars correspond to the intensity ratios after incubations for 1 and 3 h, respectively. The value over 1 indicates that the ^{15}N labeling activity of Ser-MTG is higher than that of MTG.

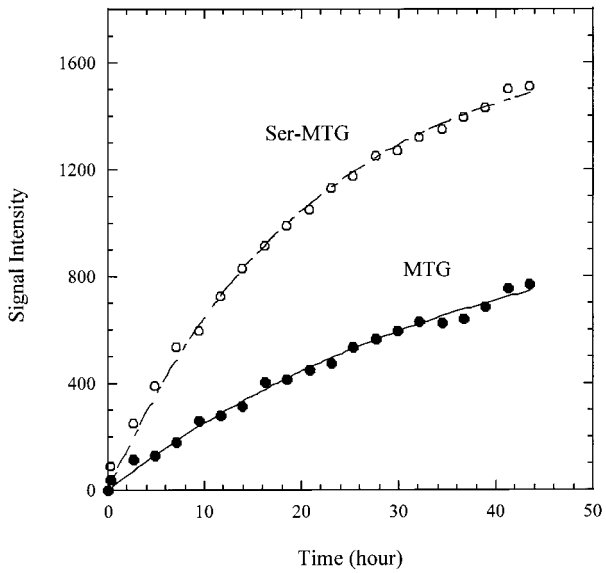

Figure 5. Sums of the intensities for the pair of signals indicated by 6 in **Figure 2a**, and those for the corresponding signals in ovalbumin reacted with Ser-MTG, were plotted against the reaction time. Signal intensities were fitted for the estimation of the relative reaction rates, using a single-exponential model function.

(**Figure 2a**). Labeling according to the enzymatic reaction of MTG was confirmed by measuring a spectrum of the same sample in the absence of MTG (data not shown). On the other hand, only four pairs of cross-peaks, in which the three pairs shown as 1, 3, and 4 correspond to the glutamine residues labeled with MTG, were detected in the presence of GTG and FTG (**Figure 2b**,**c**). As a result, among the glutamine residues 1 to 6 that were capable of being isotopically labeled using MTG, only glutamine residues 1, 3, and 4 were labeled. The glutamine residue 7 was newly labeled, in the presence of GTG and FTG. The results proved that when ovalbumin is the substrate protein, many glutamine residues may react with MTG, compared with GTG and FTG. In other words, MTG has a lower

substrate specificity for ovalbumin, compared with that of GTG and FTG. In addition, the resonances 2, 5, and 6 in **Figure 2a**, and 7 in **Figure 2b**,**c**, were observed only in the presence of MTG and GTG/FTG, respectively, thus revealing that this method is superior for identifying TGases with different substrate specificities.

It is difficult to assign the ^{15}NH signals, i.e., specific glutamine residues in the substrates for TGases. However, each ^{15}NH signal corresponding to a specific glutamine residue in a substrate protein is always observed at the same position in the HSQC spectrum, even if any TGases are used for labeling. Therefore, we can easily and rapidly classify the substrate specificities for TGases based on the resonances of the ^{15}NH signals. In reality, we could elucidate the difference in the substrate specificities among MTG, GTG, and FTG, and classify them based on the resonances of the ^{15}NH signals. This is the first example of an easy and rapid analysis of the substrate specificities for TGases using the protein substrate.

GTG and FTG share high sequence homology, especially in the vicinity of their putative active cysteine residue, suggesting their similar substrate specificities (**Figure 3**). On the other hand, the amino acid sequence of MTG is quite different from those of GTG and FTG (*34*). It has been reported that the thiol group of Cys64 in MTG is also essential for enzymatic activity, but the amino acid sequence around Cys64 is considerably different from those surrounding the active cysteine residue in GTG and FTG. This suggests that MTG evolved by a pathway different from that of GTG and FTG, and thus their substrate specificities are quite different, although they acquired acyl transfer activity during evolution.

ELT is useful not only for analyses of the substrate specificity, but also for the estimation of the reaction rate, because the ^{15}N labeling ratio is calculated from the signal intensity in the NMR spectrum. In this example, the reaction rates were compared with those of MTG and its variant, Ser-MTG, which lacks the N-terminal aspartic acid residue. Six pairs of cross-peaks were also observed in both ^{1}H-^{15}N HSQC spectra in the presence of MTG and Ser-MTG, respectively, and the intensity ratios are shown in **Figure 4**. The sums of the intensities of the signals, indicated by 6 in **Figure 2a**, are plotted in **Figure 5**. We found that the reaction rate for Ser-MTG is higher than that observed for MTG, at least at the site corresponding to the glutamine residue representing the signals indicated as 1 and 6 in **Figure 2a**. Thus, the method based on ELT permits monitoring of the reaction rate of any glutamine residue present in a substrate protein.

As a practical matter, the polymerization of several proteins with MTG progressed rapidly in comparison with the rate for GTG (*35*). It is quite easy to explain the lower substrate specificity of MTG, as compared with those of the GTG and FTG, as shown using ELT, because the polymerization proceeds according to the amount of cross-linking and the reaction rates. Our results show that in comparison with MTG, GTG or FTG would be restricted with regard to such industrial use, because of their high substrate specificities. However, GTG and FTG could be useful in a different field, as diverse glutamine residues reacted with GTG and FTG. On the other hand, Ser-MTG is expected to catalyze the protein polymerization rapidly, because the reaction rate for Ser-MTG was higher than that observed for MTG at the site described above, and their substrate specificities are identical to each other.

In conclusion, we have developed a novel method using ELT for analysis of the substrate specificities and reaction rates of TGases simultaneously. It is quite useful for comparing existing TGases and for screening new TGases or TGase variants. As a practical matter, we have characterized and classified the enzymatic properties of various TGases, such as MTG, GTG, and FTG, and have identified a preferable TGase variant, Ser-MTG, which is expected to improve the physical and textural properties of many food products, compared with the native TGase.

ABBREVIATIONS USED

TGase, transglutaminase; MTG, microbial transglutaminase; ELT, enzymatic labeling technique; GTG, guinea pig liver transglutaminase; FTG, red sea bream liver transglutaminase; HSQC, heteronuclear single quantum coherence.

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