Substrate Specificities of Microbial Transglutaminase for Primary Amines

T. Ohtsuka,* A. Sawa, R. Kawabata, N. Nio, and M. Motoki

Food Research and Development Laboratories, Ajinomoto Company, Inc., 1-1 Suzuki-cho, Kawasaki-ku, Kawasaki-shi, 210-8681 Japan

Transglutaminase (ϵ-glutaminyl-peptide:amine γ-glutaminyl-transferase, EC 2.3.2.13) (TGase) is an enzyme that catalyzes acyl transfer reactions between primary amines and Gln residues in proteins and peptides. The substrate specificity of TGase for primary amines was investigated to incorporate various functional groups into proteins and peptides. In this study, microbial transglutaminase and guinea pig liver transglutaminase were used. For the primary amines to be incorporated into benzyloxycarbonyl-L-Gln-Gly (Z-Gln-Gly), they were required to have more than four carbon chains without side chains between the functional groups. These results suggest that with appropriate primary amines as spacers, various functional groups, carboxyl groups, phosphate groups, saccharides, and so on, can be incorporated into proteins by using TGase.

Keywords: *Transglutaminase; substrate supecificity; acyl acceptor; primary amine; ϵ-(γ-glutamyl)-lysine bond*

INTRODUCTION

Transglutaminase (TGase) catalyzes the acyl transfer reaction between the carboxyamide groups of peptide-bound glutamine residues and a variety of primary amines. This reaction leads to the post-translational modification of proteins through either the formation of intra- and intermolecular isopeptide bonds or the covalent attachment of amines such as polyamine and putrescine (Ikura et al., 1980; Motoki et al., 1983). Recently, TGase from *Streptoverticillium mobaraense* var. (MTGase) has become commercially available, and on the basis of the cross-linking of protein, there are now numerous applications for improving food protein functionality (Kuraishi et al., 1996; Seguro et al., 1995; Soeda et al., 1995). In addition to cross-linking, several studies to incorporate primary amines into proteins have been conducted (Yan et al., 1984, 1987; Ikura et al., 1992; Colas et al., 1993; Nonaka et al., 1996).

Folk et al. (1983) reported that guinea pig liver transglutaminase (GTGase) recognized a wide variety of primary amines. This property has been well exploited for fortifying amino acids. Nonaka et al. (1996) and Ikura et al. (1992) suggested that various amino acids were incorporated into proteins using Lys as a spacer. Yan et al. (1984, 1987) showed that oligosaccharide extended chemically with an alkylamine could act as an amine donor for TGase. Colas et al. (1993) also reported that galactose was incorporated into legmine and β-gliadin using *n*-hexylamine as a spacer by GTGase. The ability to arbitrarily incorporate various functional groups into the Gln residues of proteins by using TGase would therefore be very useful.

In this study, the structure of primary amines available as spacers for the incorporation of functional groups into proteins has been investigated using MTGase as a Ca^{2+}-independent type TGase. For comparison, GTGase, a Ca^{2+}-dependent type TGase, was also used.

* Author to whom correspondence should be addressed (telephone +81-44-244-7107; fax +81-44-244-7198; e-mail tomoko_ootsuka@ajinomoto.com).

MATERIALS AND METHODS

Enzyme. MTGase (25 units/mg) was purified from the culture medium of *Streptoverticillium mobaraense* var. as described before (Ando et al., 1989). GTGase (2.6 units/mg) was purchased from Sigma Chemical Co. (St. Louis, MO). Both enzymes gave a single band on polyacrylamide gel electrophoresis in sodium dodecyl sulfate (SDS-PAGE).

Reagents. Benzyloxy carbonyl-L-Gln-Gly (Z-Gln-Gly) was purchased from Peptide Institute, Inc. (Osaka, Japan). Aliphatic amines were obtained from Nakalai Tesque Co. (Kyoto, Japan), and other amine substrates and amino acid esters were purchased from Sigma. Amino acids were from Ajinomoto Co., Inc. (Tokyo, Japan). All reagents were of guaranteed grade, and the purities were >95% on high-performance liquid chromatography (HPLC).

Apparatus. The HPLC apparatus used consisted of a Waters model 600 multisolvent system, a model 490 programmable multiwavelength detector from Nihon Waters K.K. (Tokyo, Japan), and a model CR7A integrator from Shimadzu Co. (Kyoto, Japan) with a Cosmosil 5C18-AR column (4.6 mm × 250 mm i.d.) from Nacalai Tesque Co.

Measurement of Enzymatic Activity. Enzymatic activity was measured according to the colorimetric hydroxamate procedure (Folk et al., 1966) with slight modification. As a standard product, we used L-aspartic acid β-hydroxamate instead of L-glutamic acid γ-monohydroxamate. The molar absorption coefficient of the ferric complex of L-glutamic acid γ-monohydroxamate is 1.9 times that of L-aspartic acid β-hydroxamate (data not shown). One unit of activity was defined as the amount of enzyme that catalyzes the formation of 1 μmol of L-glutamic acid γ-monohydroxamate per minute.

Incorporation of Primary Amine into Z-Gln-Gly. The reaction buffer contained 60 mM Z-Gln-Gly, 20 mM glutathion (reduced form), 5 mM $CaCl_2$, 60 mM primary amine, and 100 mM Tris-HCl, pH 7.5. The reaction was started with an addition of 50 μL of TGase (6 units/mL) into 350 μL of the reaction mixture, and the mixture was incubated at 37 °C. Aliquots of 5 μL of the reaction mixture were removed at

10.1021/jf000302k CCC: $19.00
Published on Web 11/16/2000

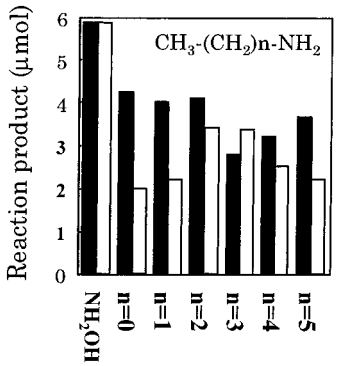

Figure 1. Substrate specificity for aliphatic amines: (■) GTGase; (□) MTGase. $n = 0$, methylamine; $n = 1$, ethylamine; $n = 2$, *n*-propylamine; $n = 3$, *n*-butylamine; $n = 4$, *n*-pentylamine; $n = 5$, *n*-hexylamine.

intervals (after 10, 20, 30, 60, and 120 min), and the reaction was terminated by the addition of 45 μL of 0.1% TFA.

The reaction mixture was subjected to reverse-phase HPLC with a Cosmosil 5C18-AR column. The solvent system was consisted of 0.1% TFA (A) and acetonitrile containing 0.1% TFA (B). The components of the reaction mixture were eluted with a linear gradient of 10% B to 50% B in 60 min at a flow rate of 1 mL/min. The elution profile was monitored at 215 nm.

The quantity of reaction product was estimated from the peak area based on extinction coefficients calculated using the peak area of the remaining Z-Gln-Gly. Each reaction product was purified with reverse-phase HPLC and identified by electrospray ionization mass spectra recorded on a Finnigan Mat TSQ700 mass spectrometer (Thermo quest Co., San Jose, CA). Solvent consisted of 1% acetic acid and 50% methanol at a flow rate of 3 μL/min with a capillary temperature of 150 °C.

RESULTS AND DISCUSSION

Incorporation of Aliphatic Amines into Z-Gln-Gly. The primary amines, which have a side chain next to the amino group (*tert*-butyric amine and isopropiric amine), and secondary (dimethylamine) and tertiary amines (trimethylamine) were not incorporated by TGase (data not shown). These results suggest that primary amines without steric hindrance are preferable as spacers for functional groups. Aliphatic amines with up to six carbon atoms were examined, and they were recognized as substrates by TGase (Figure 1). The results obtained with GTGase were well correlated with the data reported by Folk (1983).

Incorporation of Amino Acids into Z-Gln-Gly. As TGase does not recognize α-amino groups of amino acids as substrates, essential amino acids could not be incorporated. Lysine with an ϵ-amino group is a good substrate for TGase. Therefore, it was examined whether the enzyme recognizes optical isomers of Lys (Figure 2). Both GTGase and MTGase recognized the two optical isomers as substrates. L-Lysine was incorporated into Z-Gln-Gly slightly more than D-Lys.

The incorporation of amino acid esters into Z-Gln-Gly was also examined. Although the presence of α-carboxyl groups in free amino acids prevents their incorporation into Z-Gln-Gly, Ikura et al. (1981) found that Met ethyl ester was recognized as a substrate by GTGase. In the present study, Gly, Ala, Val, and Met ethyl esters were used as substrates (Figure 3). Glycine ethyl ester was well recognized by both GTGase and MTGase. GTGase could incorporate all four varieties of amino acid esters

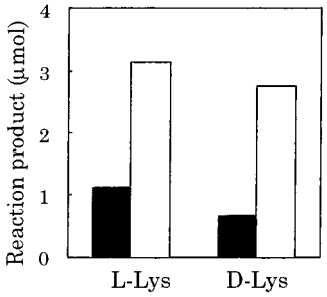

Figure 2. Substrate specificity for D-Lys: (■) GTGase; (□) MTGase.

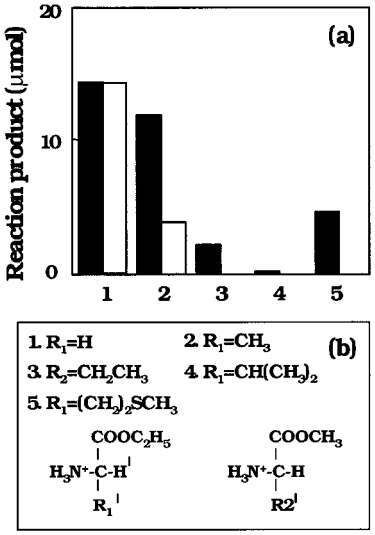

Figure 3. Substrate specificity and structures for amino esters: (a) substrate specificity [(■) GTGase; (□) MTGase]; (b) structures of substrates [(1) Gly ethyl ester; (2) Ala ethyl ester; (3) α-amino-*n*-butyric acid methyl ester; (4) Val ethyl ester; (5) Met ethyl ester].

into Z-Gln-Gly. MTGase, however, did not recognize Val or Met ethyl esters.

The side chain of a Val-containing branch chain would influence the recognition with steric hindrance. α-Amino-*n*-butyric acid methyl ester was examined as a Val ethyl ester analogue substrate that does not have branches in its side chain. The amount of α-amino-*n*-butyric acid methyl ester incorporated by GTGase was ~10 times that of Val ethyl ester, but the incorporation of α-amino-*n*-butyric acid methyl ester by MTGase was not observed. It was suggested that not only the presence of α-carboxyl groups but also steric hindrance prevented the incorporation of amino acids. Also, MTGase was more sensitive to the steric hindrance than GTGase.

Effect of the α-Carboxyl Group. Some of the α-amino groups in amino acids would become a substrate with esterification of their α-carboxyl groups. To elucidate the influence of carboxyl groups in amine substrates, the incorporation of Lys analogue was examined (Figure 4). These amine substrates have a number of carbon atoms distributed from the α-carboxyl group to the amino group, which are recognized by TGase. Lysine and L-ornithine were well incorporated into Z-Gln-Gly by both GTGase and MTGase, whereas the others were not. It was suggested that more than four carbon atoms are required for an amine to be a substrate for TGase.

Aliphatic amines with ω-carboxyl groups were examined as substrates. In the case of these substrates, more

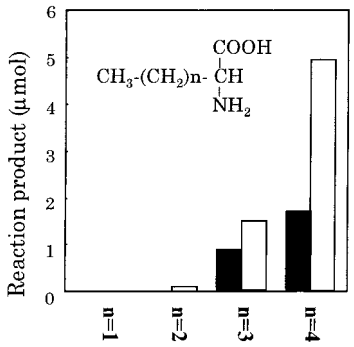

Figure 4. Effect of length of carbon atom chain of Lys analogue: (■) GTGase; (□) MTGase. $n = 1$, L-2,3-diaminopropionic acid; $n = 2$, 2,4-diamino-*n*-butyric acid; $n = 3$, L-ornithine; $n = 4$, Lys.

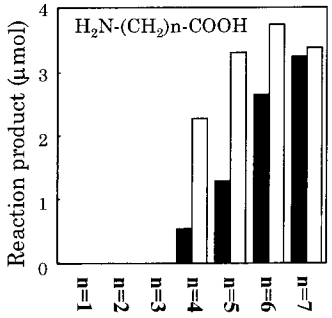

Figure 5. Substrate specificity for primary amine with a carboxyl residue: (■) GTGase; (□) MTGase. $n = 1$, Gly; $n = 2$, γ-Ala; $n = 3$, γ-amino-*n*-butyric acid; $n = 4$, 5-aminovaleric acid; $n = 5$, ϵ-amino-*n*-caproic acid; $n = 6$, 7-aminoheptanoic acid; $n = 7$, ω-aminocaprylic acid.

than four carbon atoms from carboxyl groups to amino groups were also required for recognition (Figure 5).

These results suggest that aliphatic amines with more than four carbon atoms would be good spacers for the incorporation of functional groups into proteins by TGase.

Incorporation of Functional Groups into Z-Gln-Gly. Incorporation of various functional groups into Z-Gln-Gly by TGase was inverstgated. Carbonyl, phosphate, sulfo groups, and saccharides were incorporated into Z-Gln-Gly (Figure 6).

On addition, with Lys as spacer it was possible to incorporate dipeptides or longer peptides such as Ala-Ala-Ala-Ala-Lys, and ϵ- or γ-poly-Lys was also incorporated into Z-Gln-Gly by MTGase (data not shown). These results suggest that protein functionality could be improved using this method.

However, when primary amines are incorporated into proteins, cross-linking simultaneously occurs. Therefore, to incorporate them efficiently, the control of cross-linking is needed.

When a functional group with a negative charge is incorporated, an *n*-alkylamine with a chain of more than four carbon atoms was required as a spacer. In the reaction, at first the enzyme forms a thioester with the Gln residue in Z-Gln-Gly. Nucleophilic attack by a primary amine to the thioester then occurs. It is speculated that there is a region that captures negative charges near the active site, and the distance from this region to the thioester is about the length of a four carbon atom chain. The quantity of incorporated 7-aminoheptanoic acid (Figure 5, $n = 6$) was about the same as that of incorporated *n*-hexylamine (Figure 1, $n = 5$). This suggests that the region does not accelerate the

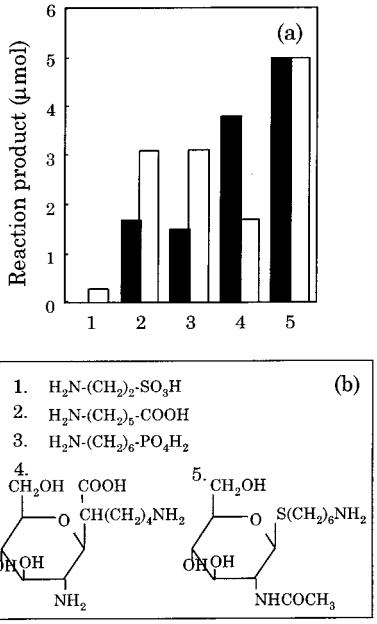

Figure 6. Substrate specificity and structues for amines with various functional groups: (a) substrate specificity [(■) GTGase; (□) MTGase]; (b) structures of substrates [(1) taurine; (2) ϵ-aminocaproic acid; (3) 6-amino-1-hexyl phosphate; (4) *N*-(ϵ-aminoacproyl)-β-D-glucopyranosylamine; (5) 6-aminohexyl-*N*-acetyl-β-D-thioglucosamine].

incorporation of primary amines that have a negative charge. On addition, the substrates with bulky side chain could not be incorporated into Z-Gln-Gly. Therefore, we speculate that the space around the thioester is narrow and sensitive to steric hindrance.

The amino acid sequence around the active site differs in MTGase from that in GTGase, but the hydropathy analysis revealed that the area around the active site located on the β-sheet connecting the α-helix to the β-sheet structure is similar to that of GTGase (Kanaji et al., 1993). A study of three-dimensional structure and function is needed to reveal the extent to which MTGase differs from GTGase in catalytic structure and amino acid sequence.

LITERATURE CITED

Ando, H.; Adachi, H.; Umeda, K.; Mitsuoka, A.; Nonaka, M.; Uchio, R.; Tanaka, H.; Motoki, M. Polymerization and characteristics of a novel transglutaminase derived from microorganisms. *Agric. Biol. Chem*. **1989**, *53*, 2613−2617.

Colas, B.; Caer, D.; Fournier, E. Transglutaminase-catalyzed glycosylation of vegetable proteins. Effect on solubility of pea legumin and wheat gliadins, *J. Agric. Food Chem*. **1993**, *41*, 1811−1815.

Folk, J. E. Mechanism and basis for specificity of transglutaminase catalyzed ϵ-(γ-glutamyl)lysine bond formation. *Adv. Enzymol*. **1983**, *54*, 1−56.

Folk, J. E.; Cole, P. W. Transglutaminase, mechanistic features of the active site as determined by kinetic and inhibitor studies. *Biochim. Biophys. Acta* **1966**, *122*, 244−264.

Ikura, K.; Kometani, K.; Yoshikawa, M.; Sasaki, R.; Chiba, H. Cross-linking of casein components by transglutaminase. *Agric. Biol. Chem*. **1980**, *44*, 1567−1573.

Ikura, K.; Yoshikawa, M.; Sasaki, R.; Chiba, H. Incorporation of Amino Acids into Food Proteins by Transglutaminase. *Agric. Biol. Chem*. **1981**, *45*, 2587−2592.

Ikura, K.; Sasaki, R.; Motoki, M. Use of transglutaminase in quality-improvement and processing of food proteins. *Agric. Food Chem*. **1992**, *2*, 389−407.

Kanaji, T.; Ozaki, H.; Takano, T.; Ide, H.; Motoki, M.; Shimonishi, Y. Primary structure of microbial transglutaminase from *Streptoverticillium* sp. Strain s-8112. *J. Biol. Chem.* **1993**, *268*, 11565−11572.

Kuraishi, C.; Sakamoto, J.; Soeda, T. The usefulness of transglutaminase for food processing. In *Biotechnology for Improved Foods and Flavors*; ACS Symposium Series 637; Takeoka, G. R., Teranishi, R., Williams, P. J., Kobayashi, A., Eds.; American Chemical Society: Washginton, DC, 1996; pp 29−38.

Motoki, M.; Nio, N. Cross-linking between different food proteins by transglutaminase. *J. Food Sci.* **1983**, *48*, 561−566.

Nonaka, M.; Matsuura, Y.; Motoki, M. Incorporation of lysine- and lysinedipeptides into α_{s1}-casein by Ca^{2+}-independent microbial transglutaminase *Biosci., Biotechnol., Biochem.* **1996**, *60*, 131−133.

Seguro, K.; Kumazawa, Y.; Ohtsuka, T.; Toiguchi, S.; Motoki, M. Microbial Transglutaminase and ϵ-(γ-Glutamyl)lysine Cross-link Effects on Elastic Properties of Kamaboko Gels. *J. Food Sci.* **1995**, *60*, 305−311.

Soeda, T.; Susa, Y.; Sakamoto, J.; Yoshimura, T.; Yamato, T. Quality improvement of edible meat and marine products using transglutaminase. *Jpn. Fudo Saiensu* **1995**, *34* (7), 37−43.

Yan, S. C. B. Covalent attachment of oligosaccharide-asparagine derivatives: Incorporation into glutamine residues with the enzyme tranglutaminase. *Methods Enzymol.* **1987**, *138*, 413−418.

Yan, S. C. B.; Wold, F. neoglucoproteins: In vitro introduction of glycosyl units at glutamine in β-casein using transglutaminase. *Biochemistry* **1984**, *23*, 3759−3769.

Received for review March 6, 2000. Revised manuscript received August 21, 2000. Accepted August 24, 2000. This work was supported by the Material Innovation Project of the Society for Techno-innovation of Agriculture, Forestry and Fisheries in Japan.

JF000302K