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Reactivity of Fish and Microbial Transglutaminases on Glutaminyl Sites of Peptides Derived from Threadfin Bream Myosin

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Fish liver transglutaminase (FTG), a Ca^{2+}-dependent enzyme, exhibits different characteristics from the Ca^{2+}-independent microbial transglutaminase (MTG), leading to potential differences in their substrate specificity and reactivity. The ability of these enzymes to catalyze isopeptide bond formation by incorporating 5-(biotinamido)pentylamine ($BPNH_2$) into peptides derived by tryptic digestion of threadfin bream (TB)-myosin was investigated to identify reaction sites and substrate specificity using a peptidomic strategy. $BPNH_2$ was incorporated into TB-myosin peptides to a greater extent by MTG than FTG. Peptides derived from TB-myosin heavy chain (MHC) shared highest similarity to amberjack-MHC on the basis of a Mascot database search. Amino acid sequences and modification sites of $BPNH_2$-tagged peptides were identified by tandem mass spectrometry based on the amberjack-MHC sequence. The $BPNH_2$ modification sites catalyzed by both TGases were at the myosin rod. Most of the $BPNH_2$ peptides contained charged amino acids (E, R, K) at the glutaminylamide site of reactive glutamine (Q*). The α-acrylamide site of Q* contained E, F, or L on peptides catalyzed by both enzymes, I, Q, or A on peptides catalyzed only by FTG, and V on a peptide catalyzed only by MTG. These results demonstrate the different structural requirements for glutaminyl substrates between these two enzymes.

KEYWORDS: Fish liver transglutaminase; microbial transglutaminase; glutaminyl substrate; myosin; tandem mass spectrometry; cross-linking

INTRODUCTION

Transglutaminases (TGases) (protein-glutamine:amine γ-glutamyltransferases, EC 2.3.2.13) are enzymes that catalyze the formation of isopeptide bonds between protein-bound glutamine (Q) residues and lysine (K) or protein-bound lysine residues, resulting in cross-linked proteins (*1*). Primary amines, including the ϵ-amino groups of K residues, typically serve as the acyl acceptor or amine donor, whereas the amide groups of the Q residue serve as the acyl donor. TGases can be distinguished into two types based on Ca^{2+} requirement for activation.

Ca^{2+}-dependent TGases require Ca^{2+} for full activation and have molecular masses ranging from 80 to 90 kDa. The most well-characterized enzyme is factor XIIIa, which is involved in stabilization of fibrin clots and in wound healing (*2*). Other TGases in mammals have been reported to be involved in several diseases including celiac disease, autoimmune diseases, fibrosis, inflammation, and cancer (*3*), whereas fish liver TGase (FTG) catalyzed the cross-linking of fish proteins and improved textural properties of various protein gels (*4, 5*). The crystal structure of the FTG from red sea bream liver showed four sequential domains and active site structures similar to those of factor XIIIa (*6*).

Ca^{2+}-independent TGase has been isolated from *Streptoverticillium* sp. and is usually called microbial TGase (MTG). In contrast to factor XIIIa and FTG, the crystal structure of MTG showed a single compact domain, and the molecular mass of MTG (38 kDa) is about half that of the Ca^{2+}-dependent tissue TGases (*7*). The catalytic C residue of FTG is inaccessible to the solvent, requiring a complicated process for activation. In contrast, the catalytic C residue of MTG is sufficiently exposed and readily reacts with substrates (*8*). The active site arrangement of MTG (C-D-H) was reported to superimpose well on the catalytic triad (C-H-D) of factor XIII-like TGases, leading to a different catalytic mechanism, in which the D residue of MTG is assumed to play the role of H in the cysteine protease-like catalytic triad. The negatively charged state of the D residue

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is likely to be advantageous for substrate specificity toward acyl acceptor (positively charged amines) compared to neutral water molecules (*8*) and may explain why MTG exhibits a lower deamidation rate when compared to factor XIII-like TGases (*9*). The differences in active site structure also account for the higher reaction rate of MTG compared to FTG (*10*).

The structural dissimilarities between MTG and Ca^{2+}-dependent TGases contribute to differences in their substrate requirements, which vary with the types of TGase and substrate. For example, the preferred feature of glutaminyl substrates in gliadin peptides for guinea pig liver TGase (GTG) activity was reported to be the consensus sequence Q-X-P (*11*), whereas this consensus sequence was catalyzed only slowly by MTG (*12*). Moreover, GTG exhibited preferred sequences in other proteins that were different from those in gliadin (*13, 14*). Thus, the glutaminyl substrates for TGases should be investigated for each specific protein to gain better understanding of the catalytic reaction of the enzyme.

Myosin, a major component of muscle proteins, consists of two myosin heavy chains (MHC) with molecular masses of about 200 kDa and four light chains (LC) with molecular masses of 15−20 kDa (*15*). The N-terminal region of each MHC folds together with two LC into subfragment-1 (S1), whereas the remaining parts of the two MHC participate in a coiled-coil rod structure known as myosin rod. The limited digestion of myosin rod by proteases results in two distinct portions, subfragment-2 (S2) and light meromyosin (LMM) (*16*). MHC has been reported to be the favored substrate for both Ca^{2+}-dependent and Ca^{2+}-independent TGases (*17*). However, the glutaminyl sites on myosin and amino acids neighboring those glutaminyl sites on myosin have not been reported. Identification of those glutaminyl substrates for fish and microbial TGases would lead to in-depth information on their potential cross-linking sites, which can provide valuable knowledge for enzymatic modification of muscle proteins.

Identification of glutaminyl substrates for TGases on the myosin molecule has been a challenge due to the extremely large size and poor solubility of the cross-linked products (*18*). However, introduction of mass spectrometry (MS)-based analysis and the development of tandem mass spectrometry (MS/MS) have led to the new peptidomic strategy that enables rapid identification of peptides from complex mixtures (*12*). Furthermore, labeling glutaminyl substrates with a biotin probe provides a tool for selective purification of the biotinylated substrates by affinity chromatography based on the avidin−biotin interaction (*14*). Thus, modification of myosin peptides with an affinity probe followed by a peptidomic approach could facilitate identification of the TGase-mediated modification of myosin peptides. Therefore, our objective was to apply a peptidomic approach to determine the reactivity of fish liver and microbial TGases on glutaminyl substrates derived from threadfin bream myosin.

MATERIALS AND METHODS

Materials. Monodansylcadaverine (MDC), *N*,*N*′-dimethylated casein (DMC), 2-(4′-hydroxyazobenzene)benzoic acid (HABA), sodium dodecyl sulfate (SDS), and trypsin were purchased from Sigma Chemicals (St. Louis, MO). Dithiothreitol (DTT) was obtained from ICN Biomedicals (Aurora, OH). 5-(Biotinamido)pentylamine ($BPNH_2$), prepacked monomeric avidin column, bicinchoninic acid (BCA) protein assay kit, and bovine serum albumin (BSA) were purchased from Pierce (Rockford, IL). Other chemicals and reagents used were of analytical grade.

TGase Preparation and Activity Assay. FTG was partially purified from threadfin bream (TB) liver tissue using DEAE-Sephacel, hydroxyapatite, Sephacryl-200, and Hi-Trap heparin chromatography as described previously (*5*). Partially purified TGase preparation was mixed with 20% sucrose at a ratio of 1:1, lyophilized, and kept at −20 °C until use. Lyophilized FTG was dissolved with cold deionized water (DI water) and was diafiltered with buffer (20 mM Tris-Cl, pH 7.5, containing 2 mM DTT) to eliminate sucrose using Nanosepcentrifugal devices with a 10 kDa molecular weight cutoff (MWCO) membrane (Pall Life Science, Ann Arbor, MI). The retentate was used as FTG.

MTG from *Streptoverticillium mobaraense* was supplied by Ajinomoto Co., Inc. (Tokyo, Japan). The enzyme was dissolved with DI water to obtain a concentration of 5 mg/mL and then diafiltered with the same buffer and protocol as described for FTG preparation.

TGase activity was measured on the basis of the incorporation of MDC into DMC (*20*). The reaction mixtures contained 1 mg/mL DMC, 15 μM MDC, 70 mM Tris-Cl (pH 7.5), 5 mM $CaCl_2$, 3 mM DTT, and 100 μL of TGase solution. $CaCl_2$ was omitted in the reaction for MTG. After incubation at 37 °C for 10 min, ammonium sulfate was added to obtain a final concentration of 42 mM to stop the reaction. The fluorescence intensity of sample (FI_S) was measured at excitation and emission wavelengths of 350 and 480 nm, respectively, using a Shimadzu RF-540 spectrofluorometer (Shimadzu Corp., Kyoto, Japan). Blanks were prepared in a similar manner except that enzyme was substituted with DI water and FI (FI_b) was measured without incubation. One unit of TGase activity was defined as the amount of enzyme that catalyzed the incorporation of 1 nmol of MDC into DMC within 1 min at 37 °C using enhancing factors of 3.2 and 1.5 determined experimentally for FTG and MTG, respectively.

TB-MHC Sequencing by MS/MS Analysis of Tryptic Digest. TB-myosin was prepared as previously described (*19*) and subjected to SDS-PAGE on 10−15% acrylamide gradient gel under reducing conditions using the Phast System (GE Healthcare, Uppsala, Sweden), followed by staining with Coomassie brilliant blue. The protein band at a molecular mass of about 200 kDa corresponding to TB-MHC was excised and digested with trypsin (*21*), and the resulting tryptic peptides were extracted with extraction solution (1% TFA, 0.5% acetic acid, and 3% ACN). The extracted peptides were separated on a Jupiter C_{18} column (1.0 × 150 mm; Phenomenex Inc., Torrance, ON, Canada) equilibrated with solvent A (0.05% TFA and 2% ACN in water) and eluted with a linear gradient of 0−60% solvent B (0.045% TFA and 80% ACN in water). The mass profile of the separated peptides was determined using a PE-Sciex API 300 triple-quadrupole mass spectrometer (Sciex, Thornhill, ON, Canada) at the Proteomic Core Facilities (Michael Smith Laboratories/Biotechnology Laboratory, The University of British Columbia, Vancouver, BC, Canada). The full-scan spectrum was followed by tandem mass spectrum analysis using a data-dependent MS/MS mode of mass spectrometer. Amino acid sequences of the peptides were obtained by submitting the MS/MS data for protein identification using the Mascot search engine available at http://www.matrixscience.com, which is based on a probability-based scoring algorithm (*22*). The protein sequence was identified on the basis of the tryptic peptide sequences by searching for nonredundant proteins in the database within a class of Actinopterygii using the following parameters: molecular mass of 200 kDa, specifically digested by trypsin, with up to two missed cleavages.

$BPNH_2$ Modification of TB-Myosin Peptides. TB-myosin was digested with trypsin in 50 mM ammonium bicarbonate, pH 8.0, at 37 °C overnight, using a trypsin/myosin ratio of 1:10. After termination of the digestion by boiling for 10 min, the solution containing TB-myosin peptides was evaporated using a centrifugal evaporator. Modification with $BPNH_2$ was carried out by incubating the TB-myosin peptides (5 mg/mL) with 2 mM $BPNH_2$ and 5 units/mL TGase (FTG or MTG) in the presence of 20 mM Tris-HCl, pH 7.5, 5 mM DTT, 5 mM $CaCl_2$, 2 mM $BPNH_2$, and 5 units/mL TGase at 40 °C for 4 h, except that $CaCl_2$ was omitted for the reaction catalyzed by MTG. Subsequently, the sample was boiled for 10 min to stop the reaction and then dialyzed against DI water using Spectra/PorBiotech Cellulose Dialysis membrane with a MWCO of 500 Da (SpectrumLaboratories, Inc., Roncho Dominguez, CA) to remove free $BPNH_2$. The dialysate was kept for biotin quantification and $BPNH_2$-tagged peptide purification.

Table 1. Significant Hits of Peptides from TB-MHC Based on a Mascot Search[a]

accession no.	score (matched/searched peptides)	protein	species
Q9IBD7_SERDU	4815 (116/556)	myosin heavy chain	*Seriola dumerili* (amberjack)
Q6SNT2_9PERO	4602 (116/556)	fast skeletal muscle myosin heavy chain	*Siniperca chuatsi* (Chinese perch)
Q90YF6_PARFO	4277 (102/556)	myosin heavy chain	*Paracirrhites forsteri* (blackside hawkfish)
Q2HX57_CYPCA	4160 (97/556)	myosin heavy chain embryonic type 2	*Cyprinus carpio* (common carp)
Q6IQX1_BRARE	4189 (104/556)	muscle myosin, heavy polypeptide 2	*Brachydanio rerio* (zebra fish)
Q90337_CYPCA	3358 (82/556)	myosin heavy chain	*Cyprinus carpio* (common carp)
P87344_THECH	3038 (77/556)	myosin heavy chain (fragment)	*Theragra chalcogramma* (Alaska pollock)
Q2PMW9_DICLA	2912 (67/556)	myosin heavy chain	*Dicentrarchus labrax* (European sea bass)
Q91520_THUTH	2816 (66/556)	myosin heavy chain (fragment)	*Thunnus thynnus* (bluefin tuna)

[a] Significance threshold $p < 0.05$.

Biotin Quantification. The biotin content in the peptide mixture, representing the degree of biotinylation of TB-myosin peptides catalyzed by TGase, was determined using the HABA reaction (*23*). HABA stock solution (25 mM HABA in 0.01 M NaOH) was prepared and kept at −20 °C throughout the study. HABA stock solution was mixed with avidin to obtain HABA−avidin complex solution at a final concentration of 0.75 mM HABA and 2 mg/mL avidin in 50 mM ammonium bicarbonate, pH 8.0. Absorbance at 500 nm (A_{500}) of HABA−avidin complex (0.9 mL, V_1) was recorded as A_1. Then, 0.1 mL (V_2) of sample solution was added before A_{500} (A_2) was read. Biotin content was calculated using an extinction coefficient of 34 according to the following equation developed by Green (*23*):

$$[\text{biotin}]\ \text{mM} = \frac{A_1 - \frac{A_2 \times (V_2 + V_1)}{V_1}}{34} \quad (1)$$

Purification of $BPNH_2$-Tagged Peptides. $BPNH_2$-tagged peptides were loaded onto a prepacked monomeric avidin column equilibrated with PBS (0.1 M NaCl, 0.1 M phosphate buffer, pH 7.2). The column was incubated at room temperature for 1 h to enhance binding. Subsequently, unbound peptides were washed with PBS buffer until the A_{280} reached the baseline of approximately zero. Bound peptides were selectively eluted by elution buffer (2 mM D-biotin in PBS), and the fractions containing high A_{280} were collected and dialyzed against DI water using a Spectra/PorBiotech Cellulose Dialysis membrane with a MWCO of 500 Da (SpectrumLaboratories, Inc.). Dialyzed samples were lyophilized and submitted for LC-MS and LC-MS/MS analyses.

Identification of $BPNH_2$-Tagged Peptides by LC-MS/MS. $BPNH_2$-tagged peptides were separated on a Jupiter C_{18} column (1.0 × 150 mm; Phenomenex Inc.,) equilibrated with solvent A (0.05% TFA and 2% ACN in water) and eluted with a linear gradient of 0−60% solvent B (0.045% TFA and 80% ACN in water) over 60 min at a flow rate of 50 μL/min. ESI-MS spectra of $BPNH_2$-tagged peptides were analyzed on a PE-Sciex API 300 triple-quadrupole mass spectrometer (Sciex) equipped with an electrospray source. The ion source voltage was used at 5 kV with the orifice voltage of 50 V. For LC-MS mode, the quadrupole mass analyzer was scanned over an *m*/*z* range of 500−3000 atomic mass units with a step size of 0.5 Da and a dwell time of 1.0 ms/step. For LC-MS/MS mode, the spectra were obtained by selectively introducing the precursor ions with the *m*/*z* value of interest from the first quadrupole into the collision cell before fragmenting in the second quadrupole, and the fragmented ions were subsequently analyzed by the third quadrupole. The scan range was 50−1900 atomic mass units with the same step size of 0.5 Da.

The amino acid sequences of $BPNH_2$-tagged peptides were identified by MS/MS on the basis of the amberjack-MHC sequence. The amino acid sequence of amberjack-MHC was submitted to in silico digestion by trypsin, with up to two missed cleavages, using the Protein Prospector MS-digest program at http://prospector.ucsf.edu. The Q-containing peptides were selected as potential candidates for $BPNH_2$ modification. The predicted mass after $BPNH_2$ modification for each peptide was calculated and compared to the experimental mass of $BPNH_2$-tagged peptides of TB-MHC obtained by LC-MS analysis. The sequences of matched peptides were recorded as the possible amino acid sequences of $BPNH_2$-tagged peptides. These putative amino acid sequences were then confirmed by LC-MS/MS.

RESULTS

FTG and MTG incorporated $BPNH_2$ into TB-myosin peptides to levels corresponding to 19.90 ± 0.69 and 122.36 ± 0.41 μmol of biotin/g of peptide, respectively. In addition, the dried weight of $BPNH_2$-tagged peptides recovered from 20 mg of peptide substrate was 9.1 and 1.3 mg when catalyzed by MTG and FTG, respectively. These results indicated the greater ability of MTG than FTG to incorporate $BPNH_2$ into TB-myosin peptides.

A probability-based protein identification approach was applied to compare the TB-MHC sequence to other MHC sequences from the Mascot database. Peptides derived from TB-MHC were matched to those of MHC from several fish species as shown in **Table 1**. The highest score was obtained for MHC from amberjack, and the amino acid sequence deduced from the nucleotide encoding is shown in **Figure 1**.

$BPNH_2$-tagged peptides catalyzed by FTG and MTG were analyzed by LC-MS, which were scanned at *m*/*z* of 500−3000. The amino acid sequences and $BPNH_2$ modification sites of those $BPNH_2$-tagged peptides were identified by LC-MS/MS using amberjack-MHC sequence as a model. These putative amino acid sequences were then confirmed by LC-MS/MS. For example, $BPNH_2$-tagged peptide at a molecular mass of 954.5 Da was found in both experimental and theoretical masses. The mass of this peptide without $BPNH_2$ modification was obtained by subtracting 311.1 Da (mass of $BPNH_2$ after releasing ammonia, 328.1 − 17.0) from the experimental mass (954.5 Da). The resulting mass (643.4 Da) likely corresponded to the sequence of KQLQK, locating at the position of 1814−1818 within the LMM subfragment of amberjack-MHC. To confirm if this was the correct sequence, the doubly charged ion of $BPNH_2$-tagged peptide (KQLQK + 1 $BPNH_2$) at *m*/*z* of 478.1 was selected for MS/MS analysis. The fragmentation process generates a complex set of fragmented ions mainly belonging to the *b* and *y* ion series. Loss of ammonia from the *b* and *y* ion series generates the $b - (NH_3)$ and $y - (NH_3)$ series. Theoretical masses of the fragmented ions of peptide KQLQK are shown in **Table 2**. The experimental spectrum (MS/MS spectrum) of this $BPNH_2$-tagged peptide (KQLQK + 1 $BPNH_2$) is shown in **Figure 2**, and the related patterns of either *b* or *y* ions for KQLQK without $BPNH_2$ modification could be depicted directly. Masses of b_1, b_2, b_3, $(y_3) - NH_3$, and y_4 ions were observed at *m*/*z* of 129.1, 257.1, 370.2, 257.1, and 147.1, respectively. The signals corresponding to $(y_1 - NH_3)$, y_2, and y_3 were not observed, whereas other new signals were shown at *m*/*z* of 810.5, 699.4, and 586.4, corresponding to $(y_1 - NH_3) + 1\ BPNH_2$, $(y_2) + 1\ BPNH_2$, and $(y_3) + 1\ BPNH_2$, respectively. These results strongly indicated that $BPNH_2$ was incorporated

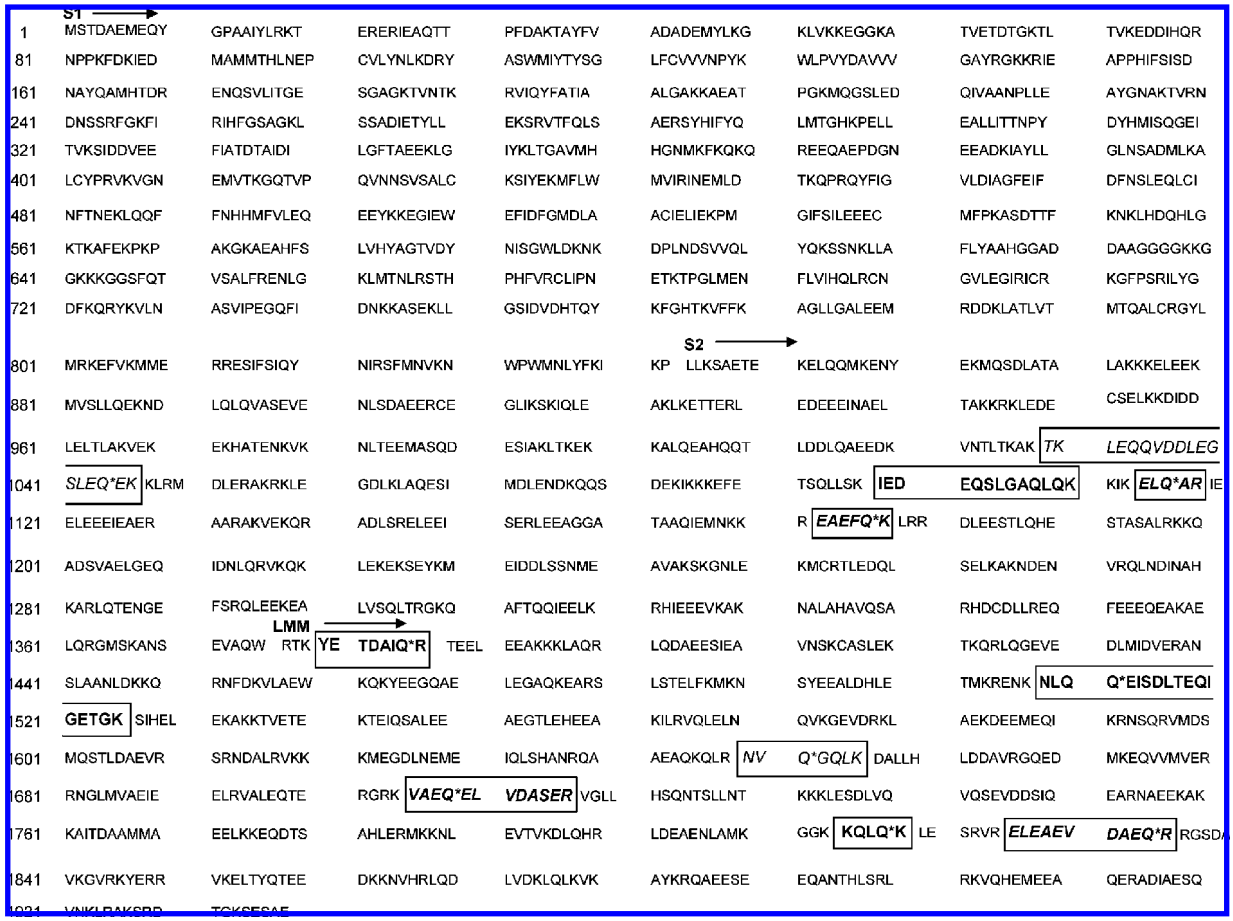

Figure 1. Amino acid sequence of amberjack-MHC. Letters in boxes indicate putative amino acid sequence of $BPNH_2$-tagged peptides from TB-myosin catalyzed by MTG (italic letters), FTG (bold letters), and both MTG and FTG (bold + italic letters). $BPNH_2$-tagged Q residues are indicated by an asterisk. Starting points of S1, S2, and LMM are indicated by arrows.

Table 2. Principal Ion Series of Peptide KQLQK upon Fragmentation by MS/MS

		b series			*y* series		
AA	mass	series	*b*	*b* − (NH_3)	series	*y*	*y* − (NH_3)
K	128.095	1	129.102	112.076			
Q	128.059	2	257.161	240.134	1	516.314	499.288
L	113.084	3	370.245	353.218	2	388.255	371.230
Q	128.059	4	498.304	481.277	3	275.171	258.145
K	128.095				4	147.113	130.086

into the fourth Q residue as NH_2-K-Q-L-Q-($BPNH_2$)-K-COOH. The $BPNH_2$ modification sites on other $BPNH_2$-tagged peptides catalyzed by FTG and MTG were determined in a similar fashion, as shown in **Tables 3** and **4**, respectively. The location of all identified sequences is also depicted on the amino acid sequence of amberjack-MHC as shown in **Figure 1**. However, not all $BPNH_2$-tagged peptides could be identified by this approach due to the lack of information on the exact amino acid sequence of TB-myosin.

DISCUSSION

The results of this study clearly showed that MTG incorporated $BPNH_2$ into TB-myosin peptides to a much greater extent than did FTG. The different amounts of $BPNH_2$-tagged peptides and biotin content catalyzed by MTG and FTG were observed even though the reactions were conducted using the same level of TGase activity (5 units/mL) based on assay of MDC incorporation into DMC. These results suggest differences in reactivity and substrate specificity between FTG and MTG in incorporating MDC or $BPNH_2$ into DMC or TB-myosin peptides. To elucidate these differences, the identification of the reactive glutaminyl sites on TB-myosin peptides for MTG and FTG was performed. Variation in substrate specificities of TGases among acyl donor substrates and acyl acceptor substrate have been reported previously (*24, 25*).

The growing body of nucleotide and protein sequences reported in public domain databases has made rapid protein identification feasible by combining mass spectrometry data with database search (*22*). This approach is applicable for proteins in which the sequences are already known. The partial or complete amino acid sequences of the target protein can be used to search databases to reveal similar or homologous sequences. The probability-based protein identification of TB-MHC was determined to obtain the putative amino acid sequence, and the highest score was obtained for MHC from amberjack. Amberjack (*Seriola dumerili*) is a fish living in warm habitat at the temperature 25−30 °C, which is close to that of TB (*26*). Changes in amino acid composition of MHC induced by water temperature were found in carp (*27*). In addition, habitat temperature also induced expression of either S1 or LMM isoform (*28, 29*). The different amino acid composition might be a molecular mechanism for thermal adaptation of myosin.

Currently, protein or peptide modification sites at the amino acid level can be successfully identified by LC-MS/MS when their amino acid sequences are well established. Because neither DNA nor amino acid sequence of TB-myosin is presently available, the identification of $BPNH_2$-tagged peptides from TB-

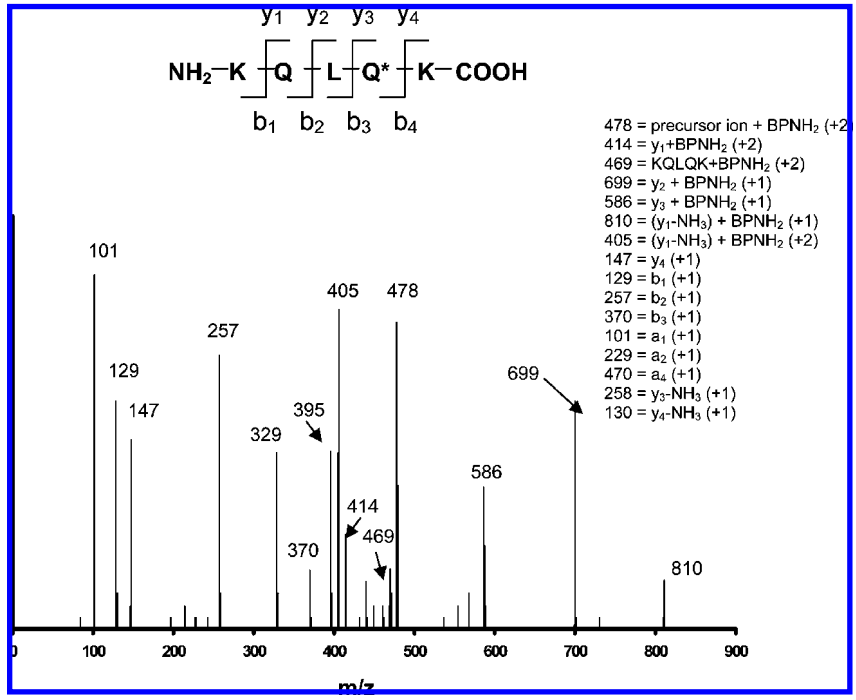

Figure 2. Tandem mass spectrum obtained from fragmentation of the precursor ion at *m*/*z* of 478.1. The peptide sequence and interpretation of *b* and *y* ions are shown. Q* indicates the $BPNH_2$-tagged Q residue.

Table 3. Identification of $BPNH_2$-Tagged Q* Residues of TB-Myosin Peptides Catalyzed by FTG[a]

no.	*M*	*M**	subfragment	site	sequence
1	**615.32**	**926.46**	**S2**	**1114−1118**	**ELQ*AR**
2	**750.35**	**1061.49**	**S2**	**1172−1177**	**EAEFQ*K**
3	1457.71	1768.85	S2	1098−1110	IEDEQSLGAQ*LQK
4	643.38	954.52	LMM	1814−1818	KQLQ*K
5	2002.04	2313.18	LMM	1508−1525	NLQQ*EISDLTEQIGETGK
6	994.51	1305.65	LMM	1379−1386	YETDAIQ*R
7	**1344.66**	**1655.80**	**LMM**	**1705−1716**	**VAEQ*ELVDASER**
8	**1287.55**	**1598.69**	**LMM**	**1825−1835**	**ELEAEVDAEQ*R**

[a] Q*, $BPNH_2$ modified Q; *M*, mass of singly charged peptide; *M**, mass of singly charged $BPNH_2$-tagged peptide. Bold entries indicate $BPNH_2$-tagged peptides that were also obtained by the action of MTG (see **Table 4**).

Table 4. Identification of $BPNH_2$-Tagged Q* Residues of TB-Myosin Peptides Catalyzed by MTG[a]

no.	*M*	*M**	subfragment	site	sequence
1	**615.32**	**926.46**	**S2**	**1114−1118**	**ELQ*AR**
2	**750.35**	**1061.49**	**S2**	**1172−1177**	**EAEFQ*K**
3	1858.95	2170.09	S2	1029−1046	TKLEQQVDDLEGSLEQ*EK
4	**1344.66**	**1655.80**	**LMM**	**1705−1716**	**VAEQ*ELVDASER**
5	785.47	1096.61	LMM	1649−1655	NVQ*GQLK
6	**1287.55**	**1598.69**	**LMM**	**1825−1835**	**ELEAEVDAEQ*R**

[a] Abbreviations are the same as in **Table 3**. Bold entries indicate $BPNH_2$-tagged peptides that were also obtained by the action of FTG (see **Table 3**).

myosin was investigated on the basis of the highest similarity to amino acid sequences of amberjack-MHC. This approach has been applied successfully to localize the acyl donor for TGase on β-casein (*14*). Amino acid sequences of $BPNH_2$-tagged peptides can be used to localize the probable modification sites on the myosin molecule because amino acid sequences of MHC have been reported to share high similarity (*30*).

It can be noted that all identified peptides were located at the myosin rod (**Tables 3** and **4**). Those sequences were at the extremely conserved region of myosin rod. Myosin rod from carp was the preferential site for endogenous TGase (*31*). Kunioka et al. reported that the incorporation of MDC into heavy meromyosin (HMM) from rabbit skeletal muscle took place primarily in the S2 region in the presence of GTG (*32*). The biotinylated HMM and avidin-coated fluorescent polyacrylamide nanoparticles also revealed that the biotinylated site on S2 was very close to the C terminus (near the S2/LMM junction) (*32*). Our results showed that FTG incorporated $BPNH_2$ into YETDAIQ*R, locating at LMM near the S2/LMM junction (**Figure 1**). It was reported that MTG quickly cross-linked myosin rod from rabbit filamentous myosin (*33*). On the basis of these studies, it might be inferred that glutaminyl substrates on the TB-myosin molecule for TGases are in the myosin rod. Furthermore, the reactive Q residues (Q*) for FTG were consistent with the rule proposed by Folk and Cole (*34*), that Q can serve as a substrate for TGases when it is located at at least the third amino acid position from the NH_2 terminus and at at least the second amino acid position from the COOH terminus (*34*).

When the "window" of five amino acids around Q* of $BPNH_2$-tagged peptides was designated (−2) − (−1) − Q* − (1) − (2), it could be observed that charged residues (K, E, R) were predominant (six of eight peptides) at the 1-position of peptides catalyzed by FTG (**Table 3**). In addition, E and L residues were found more frequently at the −1-position when compared to F, A, I, and Q residues (**Table 3**). It was suggested that amino acids at the α-acrylamide site of Q* act as a strong binding region to enzyme molecule (*35*). It could be speculated that E, L, F, A, I, and Q residues may play a role in binding to the FTG molecule.

Amino acids in the myosin rod display a characteristic seven-residue repeat pattern (heptad repeat), designated *a*, *b*, *c*, *d*, *e*, *f*, and *g* (*36*). This heptad repeat is also arranged into 28-residue repeat zones. The hydrophobic residues often locate at the *a*- and *d*-positions, forming a hydrophobic core, and stabilize the coiled coil structure via hydrophobic interactions, whereas the *c*- and *g*-positions are frequently occupied by charged residues able to form salt bridges (*37, 38*). Indeed, amino acids at the −1- or −2-position of most $BPNH_2$-tagged peptides (six of eight

peptides) (**Table 3**) were arranged at the *a*- or *d*-position of the 28 residue repeat zones. The results imply that Q* residues are located near the hydrophobic core within the intact myosin rod. Coussons and Price (*39*) proposed that the accessibility to enzyme was the first requirement for Q to be a reactive residue for TGase. Thus, Q* residues on the intact myosin molecule could serve as glutaminyl substrates for TGase when those Q* are exposed from the hydrophobic core to the solvent.

The general patterns of neighboring residues for Q* catalyzed by MTG have not been reported. Some of the $BPNH_2$-tagged peptides and $BPNH_2$ modification sites catalyzed by MTG were the same as those catalyzed by FTG. These results are in agreement with those reported by Sato et al. (*17*), who revealed that MTG competed with endogenous TGase for the same reactive residues on fish muscle proteins. Ohtsuka et al. (*24*) reported that synthetic peptides containing amino acid residues other than G and positively charged residues at the −1-position are good substrates for MTG. Kashiwagi et al. (*8*) demonstrated that the hydrophobic residues (V, Y, T, I, and F) and nonacidic hydrophilic residues (R and N) are concentrated in the possible acyl donor binding site of MTG. Our results showed that E, L, and F residues were found at the −1-position of $BPNH_2$-tagged peptides catalyzed by both MTG and FTG (**Tables 3** and **4**). Thus, these residues may play a role in binding to the acyl donor binding site of both FTG and MTG.

Although MTG and FTG shared some common glutaminyl sites (**Tables 3** and **4**), different glutaminyl sites between these enzymes were also observed. This was evident by the fact that the −1-position of $BPNH_2$-tagged peptides for only FTG was occupied by I, Q, or A residues. Moreover, the V residue was found at this position of $BPNH_2$-tagged peptides catalyzed by only MTG. Our results showing both common and unique glutaminyl sites catalyzed by MTG and FTG are in agreement with those reported previously by Shimba et al. (*10*), who identified the glutaminyl substrates for TGases on ovalbumin using nuclear magnetic resonance. They demonstrated that three common glutaminyl sites were catalyzed by MTG, GTG, and red sea bream liver TGase (*10*). However, three other glutaminyl sites were substrates for only MTG, whereas another distinct site was observed for only GTG and red sea bream liver TGase. These results suggested the different specificities between MTG and other Ca^{2+}-dependent TGases. The different glutaminyl substrate specificities among GTG, red sea bream liver TGase, and MTG toward the synthetic peptides have also been reported (*24*). On the basis of these reports and our present results, it can be inferred that FTG exhibits different substrate specificities from MTG.

Noguchi et al. (*6*) reported that the catalytic residue of red sea bream liver TGase (C-272) is inaccessible to the solvent and is situated between Y-515 and C-333. The Oη of Y-515 on the barrel domain, covering the active site, forms a hydrogen bond with Sγ of catalytic C-2272 (*6*). Conformational changes arising from the binding of Ca^{2+} and acyl donor substrate are thus required to remove the covering Y residue from the active site enabling formation of an acyl−enzyme intermediate (*6*). In contrast, the catalytic group of MTG (C-64) is sufficiently exposed to the solvent and ready to react with substrate (*8*). Moreover, the flexibility of the side wall of active site cleft of MTG also decreases the steric hindrance between enzyme and substrates. Therefore, such molecular differences may contribute to differences in catalytic reactivity between MTG and FTG.

In conclusion, amino acid sequences of peptides derived from TB-MHC shared the highest similarity to those of amberjack-MHC based on Mascot search. This sequence similarity allowed the peptidomic strategy with MS/MS to successfully identify the TGase-mediated modification of peptides derived from TB-myosin, even though the exact amino acid sequence of TB-MHC has not been investigated. Glutaminyl sites for both MTG and FTG were located at the myosin rod. Peptides with E, L, or F residues at the α-acrylamide site of Q* were catalyzed by both enzymes. However, peptides serving as a substrate for only FTG contained I, Q, or A residues at that site, whereas a peptide containing V at the same position was catalyzed by only MTG. Although these TGases shared some common Q*, they exhibited different substrate specificities depending on the specific amino acids neighboring the Q*.

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