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Identification of glutaminyl sites on β-lactoglobulin for threadfin bream liver and microbial transglutaminase activity by MALDI-TOF mass spectrometry

Bung-Orn Hemung [a], Eunice C.Y. Li-Chan [b], Jirawat Yongsawatdigul [c,*]

[a] School of Science and Technology, Nong Khai Campus, Khon Kaen University, Nong Khai 43000, Thailand
[b] Food, Nutrition and Health Program, Faculty of Land and Food Systems, The University of British Columbia, Vancouver, British Columbia, V6T 1Z4, Canada
[c] School of Food Technology, Institute of Agricultural Technology, Suranaree University of Technology, Nakhon Ratchasima 30000, Thailand

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ABSTRACT

The cross-linking of β-lactoglobulin (BLG) was efficiently catalysed by microbial transglutaminase (MTG) but not by fish (threadfin bream) liver transglutaminase (FTG). BLG cross-linking was inhibited by 2 mM 5-(biotinamido) pentylamine ($BPNH_2$) and MTG incorporated $BPNH_2$ into BLG ~5 times more than was FTG. The glutaminyl sites for the incorporation of $BPNH_2$ into BLG by FTG and MTG were identified using matrix-assisted laser desorption/ionisation-time of flight mass spectrometry (MALDI-TOF MS). MALDI-TOF MS analyses showed that MTG and FTG incorporated 4 and 1 residues of $BPNH_2$ per molecule of BLG, respectively. The $BPNH_2$-tagged BLG was digested by trypsin and $BPNH_2$-tagged peptides were selectively purified by avidin-affinity chromatography. Amino acid sequences of $BPNH_2$-tagged peptides were identified by comparing their MALDI-TOF mass spectra with the theoretical mass profiles from the MASCOT database. The $BPNH_2$-modification sites catalysed by MTG were glutamine (Q)13, Q68, Q15 or Q20, Q155 or Q159, whilst FTG only incorporated $BPNH_2$ into BLG at Q68. The different reactivities between FTG and MTG might be due to the different accessibilities of these TGases to the Q residues as well as to differences in substrate specificities.

1. Introduction

Transglutaminases (TGases) are enzymes that catalyse the formation of isopeptide bonds through the acyl transfer reaction between protein-bound glutamine and primary amines, including protein-bound lysine (Folk, 1980). The amide group of glutamine (Q) is an acyl donor, whilst lysine (K) or other primary amines typically serve as an acyl acceptor. Tissue TGases are Ca^{2+}-dependent enzymes and are typically found in blood (factor XIIIa), muscle, and liver of human, guinea pig, and fish (red sea bream and threadfin bream) (Ahvazi, Kim, Kee, Nemes, & Steinert, 2002; Folk, 1980; Hemung & Yongsawatdigul, 2008; Muszbek, Yee, & Hevessy, 1999; Yasueda, Kumazawa, & Motoki, 1994). These Ca^{2+}-dependent TGases are involved in various physiological functions, including blood clotting, tissue repair, and signal transduction (Aeschlimann & Paulsson, 1994). Participation of tissue TGases in many pathological diseases, including autoimmune diseases, cancer and neurodegenerative disorders, has been reported (Kim, Jeitner, & Steinert, 2002). In addition, these enzymes have a potential to improve textural and functional properties of food proteins (De Jong & Koppelman, 2002). The molecular weights (Mw) of Ca^{2+}-dependent TGases have been reported to be 80–90 kilodaltons (kDa) (Wu & Zern, 2004). The crystal structure of these enzymes consists of four sequential domains, and the catalytic triad (C–H–D) at the core domain is similar to that of cysteine proteases (Noguchi et al., 2001). The catalytic C residue forms a hydrogen bond with a Y residue, suppressing the enzyme activity. Addition of Ca^{2+} induces conformational changes which expose the catalytic C residue to the substrates (Noguchi et al., 2001).

Another type of TGase is Ca^{2+}-independent TGase derived from microorganisms, such as *Streptoverticillium* sp. (Ando et al., 1989). The Mw of microbial TGase (MTG) is about 38 kDa, which is half that of tissue TGases, and the crystal structure of MTG shows a single compact domain (Kashiwagi et al., 2002). At the active site of MTG, a D residue occupies the position corresponding to the H residue in tissue TGases, and plays a more catalytically important role than does the H residue. The catalytic C residue of MTG is sufficiently exposed to the solvent and has only weak van der Waals contact with a F residue, in contrast to the hydrogen bond with a Y residue found in tissue TGases (Kashiwagi et al., 2002). Thus, MTG activity does not require Ca^{2+} activation. The molecular dissimilarities between MTG and tissue TGases result in their different reactivities on synthetic peptide substrates (Ohtsuka, Ota, Nio & Motoki, 2000a). However, the use of synthetic peptide substrates may not accurately reflect the enzyme reactivity and/or activity toward protein substrates. This is because peptide substrates would be more accessible to the enzyme, whilst the accessibility of

* Corresponding author. Tel.: +66 44 224 359; fax: 66 44 224 150.
E-mail address: jirawat@sut.ac.th (J. Yongsawatdigul).

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TGases to protein substrates may be impeded by steric hindrance. Comparison of the reactivity of different TGases toward protein molecules has not been widely studied.

A food protein with known amino acid sequence would be an ideal substrate to elucidate reactivity of TGases that may be of relevance for potential food applications. β-Lactoglobulin (BLG) is a globular protein found in milk whey. It consists of 162 amino acid residues, has a Mw of ~18 kDa, and is present in milk as two genetic variants, A and B, which differ by two amino acids (Quin, Bewley, Creamer, Baker, & Jameson, 1999). Nine Q residues and 2 disulphide bonds are found in each BLG monomer (Sakurai & Goto, 2002). Nieuwenhuizen, Dekker, Groneveld, Koster, and De Jong (2004) demonstrated that MTG incorporated 6-aminohexanoic acid into native BLG. Cross-linking of BLG by MTG has also been reported (Wada et al., 2001), and the susceptibility of BLG to cross-linking by MTG could be enhanced by treatment with the reducing reagent, dithiothreitol (DTT) (Motoki & Nio, 1983).

Nuclear magnetic resonance (NMR) was used to compare the reactivities of different TGases toward ovalbumin (Shimba, Yokoyama, & Suzuki, 2002). However, this technique, was based on the $^{1}H-^{15}N$ heteronuclear single quantum coherence method (HSQC), could only differentiate between the glutaminyl sites for MTG and other Ca^{2+}-independent TGases and it could not identify the exact reactive site due to the large molecular size of the protein substrate. On the other hand, matrix-assisted laser desorption/ionisation-time of flight mass spectrometry (MALDI-TOF MS) has been applied to characterise the MTG-mediated deamidation of the Q-rich protein, dB1 (Piersma, Pijpekamp, Wijngaards, Gruppen, & Boumans, 2002), and to detect the degree of amine incorporation into BLG catalysed by MTG, as well as to identify the modification sites in the BLG sequence based on the change in molecular mass of specific peptides derived from proteolytic digestion (Nieuwenhuizen et al., 2004). Since the labelling of Q residues with a biotin probe could provide a means for selective purification of the biotinylated substrates by avidin-affinity chromatography (Rouppolo et al., 2003), biotinylation of a protein with known amino sequence, combined with MALDI-TOF MS analysis, could be a promising approach to characterise TGase-mediated modification.

The objective of this study was to compare the cross-linking ability of two TGases, namely threadfin bream liver TGase and MTG, using BLG as the protein substrate. MALDI-TOF MS analysis was also performed to identify the reactive sites of different TGases on the BLG sequence based on incorporation of the 5-biotinamidopentylamine. The outcome would lead to a better understanding of the catalytic activity of these TGases, which would ultimately lead to suitable application of these TGases in food protein modification.

2. Materials and methods

2.1. Chemicals

Monodansylcadaverine (MDC), *N, N′*-dimethylated casein (DMC), 2-(4′-hydroxy azobenzene) benzoic acid (HABA), trypsin, and BLG were purchased from Sigma Chemicals (St Louis, MO). Dithiothreitol (DTT) was obtained from ICN Biomedicals (Aurora, OH). About 5-(Biotinamido) pentylamine ($BPNH_2$) and pre-packed monomeric avidin column kit were purchased from Pierce (Rockford, IL). All chemicals and reagents were analytical grade.

2.2. TGase preparation and activity assay

Fish TGase (FTG) was partially purified from threadfin bream (*Nemipterus* sp.) liver tissue, using DEAE-Sephacel, hydroxyapatite, Sephacryl-200, and hi-trap heparin chromatography, as previously described by Hemung and Yongsawatdigul (2008). Partially purified FTG solution was mixed with 20% sucrose at a ratio of 1:1, lyophilised, and kept at −20° C. Before use, lyophilised FTG was dissolved with cold de-ionised water (DI-water) and diafiltrated with Tris–buffer (20 mM Tris–Cl, pH 7.5 containing 2 mM DTT) to eliminate residual sucrose using a Nanosep® centrifugal device with 10-kDa-MWCO membrane (Pall Life Science, Ann Arbor, MI). The retentate was referred to as FTG.

MTG from *Streptoverticillium mobaraense* (TG-K) was supplied by Ajinomoto Co., Inc (Tokyo, Japan). The enzyme was dissolved with DI-water at a concentration of 5 mg/ml and was diafiltrated with the same buffer as described above for FTG.

TGase activity was measured by the incorporation of MDC into DMC according to the method of Takagi, Saito, Kikuchi, and Inada (1986), with slight modifications. The reaction mixtures contained 1 mg/ml of 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 catalysed by MTG. After incubation at 37 °C for 10 min, ammonium sulphate was added to obtain a final concentration of 42 mM in order 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. The unit (U) of TGase activity was defined as the amount of enzyme that catalysed the incorporation of 1 nmol of MDC into DMC within 1 min at 37 °C using enhancing factors of 3.2 and 1.5 for FTG and MTG, respectively.

2.3. BLG cross-linking catalysed by TGases

The TGase-catalysed cross-linking of BLG was performed in the reaction mixture containing 5 mg/ml of BLG, 20 mM Tris–Cl (pH 7.5), 5 mM $CaCl_2$, 5 mM DTT, and 5 U/ml of TGase (FTG or MTG). The enzyme was replaced with DI water in the control and $CaCl_2$ was omitted in the reaction catalysed by MTG. All samples were incubated at 40 °C for 2 h. The reaction was stopped by adding an equal volume of 10% SDS solution and protein patterns of BLG were analysed by SDS–PAGE performed under reducing conditions, using the PhastSystem on 10–15% SDS–polyacrylamide gradient gels (GE Healthcare, Uppsala, Sweden).

2.4. Incorporation of $BPNH_2$ into BLG by TGases

TGase was also used to catalyse the incorporation of $BPNH_2$ (2 mM) into BLG, using the conditions described above. After incubation at 40 °C for 4 h, an aliquot of the reaction mixture (50 μl) was added to an equal volume of 10% SDS solution, and protein patterns were analysed by SDS–PAGE. The rest of the sample reaction mixture was boiled for 10 min to terminate TGase activity, and then loaded onto a PD-10 column (GE healthcare, Uppsala, Sweden) equilibrated with 0.1 M NaCl, 50 mM ammonium bicarbonate, pH 8.0, to remove free $BPNH_2$. BLG or $BPNH_2$-incorporated BLG was eluted from the column by the same buffer and fractions containing BLG species, as indicated by high absorbance at 280 nm (A_{280}), were pooled and kept for biotin quantification and MALDI-TOF MS analysis.

2.5. Quantification of biotin in $BPNH_2$-incorporated BLG

Biotin content of the $BPNH_2$-incorporated BLG was determined according to Green (1970) with slight modifications. A stock solution of HABA (25 mM HABA in 0.01 M NaOH) was prepared and kept at −20 °C throughout the study. HABA–avidin complex was formed by mixing HABA stock solution with avidin to obtain a final

concentration of 0.75 mM HABA and 2 mg/ml of avidin in 50 mM ammonium bicarbonate, pH 8.0. The 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 reading A_{500} (A_2). Biotin content was calculated according to the equation developed by Green (1970), using an extinction coefficient of 34 mM^{-1} cm^{-1} as described in the following equation:

$$[Biotin]mM = \frac{A_1 - \frac{A_2 \times (V_2 + V_1)}{V_1}}{34}.$$

2.6. Purification of $BPNH_2$-tagged peptides

$BPNH_2$-incorporated BLG (2 mg/ml) was digested with trypsin in 0.1 M NaCl, 50 mM ammonium bicarbonate, pH 8.0, at the ratio of BLG to trypsin of 25:1 at 37 °C overnight. The reaction was stopped by boiling for 10 min. Subsequently, the digest was loaded onto a monomeric avidin column equilibrated with phosphate buffer saline, PBS (0.1 M NaCl, 0.1 M phosphate buffer, pH 7.2). After incubation at room temperature for 1 h, unbound peptides were washed with PBS buffer until the A_{280} reached the baseline of approximately zero. Bound peptides were then selectively eluted by elution buffer (2 mM D-biotin in PBS) and the fractions containing high A_{280} were collected and dialysed against DI-water, using a Spectra/Por® Biotech Cellulose dialysis membrane with molecular weight cut-off 500 Da (Spectrum® Laboratories, Inc., Roncho Dominguez, CA). Dialysed peptides were lyophilised and submitted to MALDI-TOF MS analyses.

2.7. MALDI-TOF MS analysis

Mass spectra of $BPNH_2$-incorporated BLG were analysed on a Voyager-DE STR mass spectrometer (Applied Biosystems, Foster, CA) equipped with a nitrogen laser emitting pulsed UV light at 337 nm and operated at an acceleration potential of 25 kV in the linear mode.

$BPNH_2$-tagged BLG solution (2 mg/ml) was mixed with 3, 5 dimethoxy-4-hydroxycinnamic acid (sinapinic acid) at the ratio 1:1 (v/v). The matrix solution was prepared by dissolving 10 mg of sinapinic acid in 1 ml of water:acetonitrile (50:50) containing 0.1% TFA. The mixture (1 μl) was allowed to crystallize under atmospheric pressure at room temperature before acquiring the spectra in positive ion mode for 100 shots.

$BPNH_2$-tagged peptides from BLG, isolated by avidin column chromatography of the BLG tryptic digests, were also analysed using MALDI-TOF MS. The peptide solution (1 μl) was mixed with 1 μl of the α-cyano-4-hydroxycinnamic acid (CHCA) solution prepared by dissolving 10 mg of CHCA in 1 ml of water:acetonitrile (50:50) containing 0.1% TFA. After crystallization, the mass spectra were recorded in linear mode with an acceleration potential of 20 kV. The spectra were averaged from 75 shots.

2.8. Identification of $BPNH_2$-tagged peptides

The predicted MS data of tryptic peptides that would be derived from unmodified BLG-A and BLG-B were generated by submitting their amino acid sequences to *in silico* digestion by trypsin, up to 2 missed cleavages, using the ProteinProspector 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 Q-containing peptide, was calculated and compared to the experimental mass obtained in the MALDI-TOF MS data of $BPNH_2$-tagged peptides, in order to obtain the possible amino acid sequences of $BPNH_2$-tagged peptides. The matches for modifications were documented as output of the analyses.

3. Results and discussion

3.1. Cross-linking of BLG catalysed by TGases

Cross-linking of BLG in the presence of DTT by MTG was noticeable by formation of polymer with Mw of about 116 kDa when the reaction was performed for 4 h (Fig. 1). Tang and Ma (2007) observed cross-linking of BLG induced by MTG in the presence of DTT within 6 h. These results indicated that BLG had a potential for being both acyl donor and acyl acceptor for MTG. However, in the absence of DTT cross-linking of BLG catalysed by MTG required prolonged incubation for 22 h (Wada et al., 2001). Enhanced cross-linking of BLG by MTG, upon inclusion of DTT, may be attributed to reduction of the two disulphide linkages within BLG, leading to exposure of the potential substrate sites for MTG.

On the other hand, BLG cross-linking was not observed when FTG was added (Fig. 1), which is in agreement with the reported results for guinea pig liver TGase (GTG) (Coussons, Price, Kelly, & Fothergill-Gilmore, 1991). It has been proposed that the cross-linking of BLG by Ca^{2+}-dependent TGases, such as FTG and GTG, is limited by the availability of reactive Q or K residues in the aggregated state of BLG induced by Ca^{2+} (Færgemand, Otte, & Qvist, 1997). Ca^{2+}-induced aggregation of BLG was also promoted by addition of DTT (Simons, Kosters, Visschers, & de Jongh, 2002). Suppression of BLG cross-linking by Ca^{2+} was confirmed when the cross-linking of BLG by MTG was performed in the presence and absence of Ca^{2+} (5 mM) (Færgemand & Qvist, 1999). The presence of non-covalently stabilized aggregates of unfolded BLG molecules, under such conditions, retarded the formation of covalent cross-links (Færgemand et al., 1997). TGase may not be able to polymerise BLG as efficiently in the presence of Ca^{2+} because some reactive residues were hidden within the aggregates induced by Ca^{2+}. These factors may explain the limited cross-linking of BLG by FTG observed in the present study.

3.2. Incorporation of $BPNH_2$ into BLG by TGases

Cross-linking of BLG, catalysed by either MTG or FTG, was not observed in the presence of 2 mM $BPNH_2$ (Fig. 1), implying that

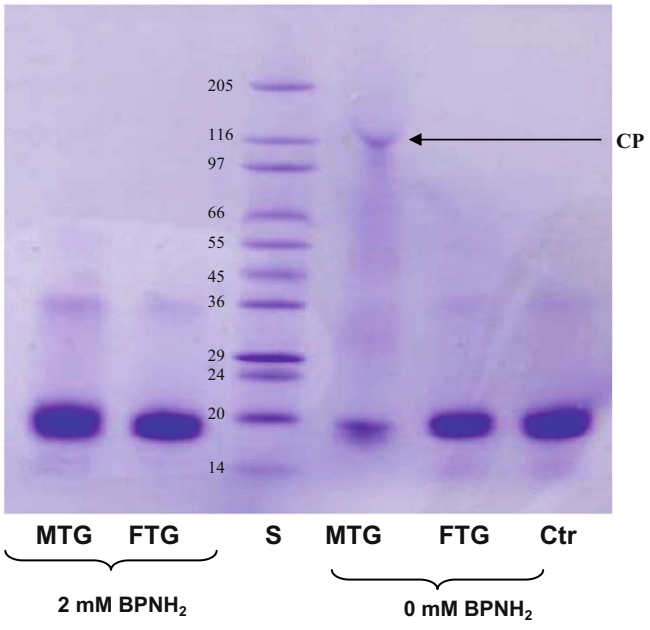

Fig. 1. SDS–PAGE of BLG incubated with MTG and FTG in the presence and absence of 2 mM $BPNH_2$. (CP = Cross-linked polymers, S = Molecular weight standards; Ctr = Control incubated without TGase).

the incorporation of $BPNH_2$ was likely to inhibit BLG cross-linking. Similar inhibitory effects of small amines on BLG cross-linking mediated by TGase have also been reported (Nieuwenhuizen et al., 2004).

Quantification of biotin bound to BLG was conducted as a measurement of the degree of the $BPNH_2$ incorporation catalysed by TGases. The result was 0.829 ± 0.035 mole biotin/mol of BLG when $BPNH_2$ was incorporated into BLG by FTG, which is within the range reported for incorporation of amines into BLG by GTG. For example, 0.6 to 1.5 moles of putrescine were incorporated per mole of BLG by GTG after 2 to 24 h of incubation, respectively, at 25 °C in the presence of DTT (Coussons, Kelly, Price, Smith, & Sawyer, 1992).

MTG incorporated $BPNH_2$ into BLG, resulting in 4.23 ± 0.026 mole biotin/mol of BLG. In comparison, MTG catalysed the incorporation of 1.5 moles of *N*-lauroyl-L-glutamyl-lysine per mole of BLG in the absence of DTT at 37 °C and pH 7.5 (Wada et al., 2001), whilst 2 and 3 moles of 6-aminohexanoic acid were incorporated into BLG-A and BLG-B, respectively (Nieuwenhuizen et al., 2004). Our results indicated that MTG incorporated the amine $BPNH_2$ into BLG to a higher degree than reported previously. This might be due to different reactivities of the various amines and/or to the inclusion of DTT in the reaction conditions in the present study. It has been reported that a significant increase in the degree of amine incorporation catalysed by TGase was observed in the presence of DTT (Coussons et al., 1992).

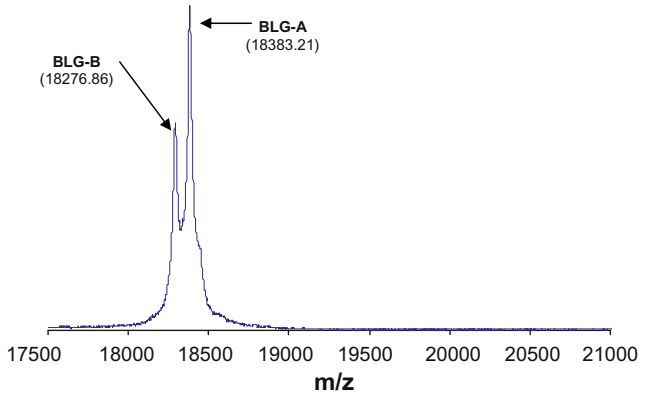

Fig. 2. MALDI-TOF mass spectrum of intact BLG. Peaks assigned to BLG-A and BLG-B are shown by arrows.

3.3. Changes in MALDI-TOF mass spectra of $BPNH_2$-modified BLG

The MALDI-TOF mass spectrum for BLG before modification (i.e. without $BPNH_2$ incorporation) showed two peaks with *m*/*z* of 18383.21 and 18276.86 (Fig. 2), which were likely to be the variants BLG-A and BLG-B, respectively. The difference in masses between BLG-A and BLG-B is attributed to the amino acid substitutions at positions 64 (D to G) and 118 (V to A) (Sakurai & Goto, 2002). In addition, BLG-A exhibited higher intensity than did BLG-B, suggesting a higher proportion of BLG-A than BLG-B in the BLG sample used in the present study. This is consistent with the content of 60% of genetic variant A of BLG and 40% for variant B, as reported by Quin et al. (1999).

When BLG was incubated with $BPNH_2$ in the presence of MTG, new peaks were observed in the MALDI-TOF spectrum (Fig. 3a), which could be explained by the incorporation of $BPNH_2$ into BLG-A and BLG-B. The peaks, observed at *m*/*z* of 18,679.12, 18,994.92, 19,317.15 and 19,626.87, correspond to the masses of BLG-A incorporated with 1, 2, 3, and 4 molecules of $BPNH_2$, respectively. In addition, BLG-B peaks with incorporation of 1, 2, 3, and 4 molecules of $BPNH_2$ were observed at *m*/*z* of 18587.61, 18908.87, 19221.58, and 19512.81, respectively. Based on the mass spectra, MTG incorporated up to 4 molecules of $BPNH_2$ into BLG. Moreover, higher intensity was observed for $BPNH_2$-modified BLG-A than that for $BPNH_2$-modified BLG-B (Fig. 3a). This indicated that BLG-A was a more favourable substrate for MTG than was BLG-B. Oliveira et al. (2001) compared differences in crystal structure between genetic variants A and B of BLG, and found that BLG-A showed higher mobility than did BLG-B. It could be further speculated that the higher flexibility of BLG-A would allow the Q residues to be more accessible and reactive to MTG than would those in the more rigid structure of BLG-B.

FTG only incorporated 1 molecule of $BPNH_2$ into both BLG-A and BLG-B (Fig. 3b). In addition, BLG-B seemed to be the preferred substrate for FTG, rather than BLG-A, since the $BPNH_2$-modified BLG-B exhibited higher intensity than did the $BPNH_2$-modified BLG-A. Simons et al. (2002) demonstrated that the flexible form of BLG underwent aggregation by Ca^{2+} to a greater extent than

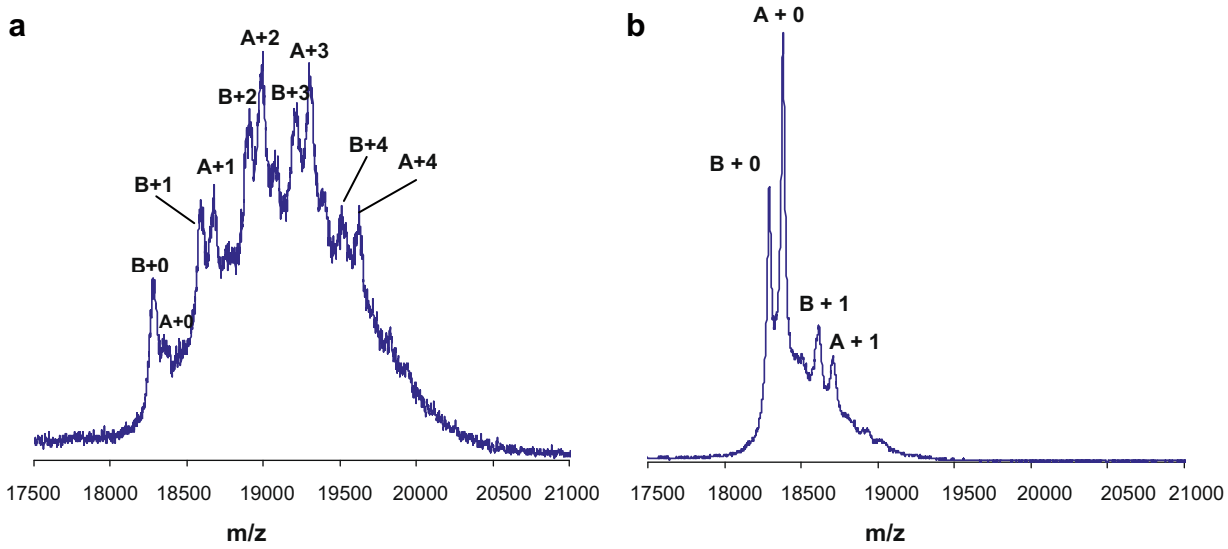

Fig. 3. MALDI-TOF mass spectra of $BPNH_2$-modified BLG catalysed by MTG (a) and FTG (b). Peaks labelled as A + 0, A + 1, A + 2, A + 3, A + 4 were assigned as BLG-A with 0, 1, 2, 3, and 4 $BPNH_2$ molecules, respectively. Peaks labelled with B + 0, B + 1, B + 2, B + 3, B + 4 were assigned as BLG-B with 0, 1, 2, 3, and 4 $BPNH_2$ molecules, respectively.

did the compact form (native BLG). Since BLG-A had a higher molecular flexibility than had BLG-B, it would be more prone to self-aggregation induced by Ca^{2+} than BLG-B. Thus, Q residues in the Ca^{2+}-induced aggregates of BLG-A would likely be less accessible for FTG catalysis than would the Q residues in BLG-B.

Based on the results from biotin quantification and MALDI-TOF spectra, MTG-catalysed amine incorporation into BLG to a greater extent than did FTG. These results might be partly explained by the limited accessibility of FTG to reactive Q residues, due to the larger Mw of FTG and the formation of BLG aggregates induced by Ca^{2+}. Although MTG and FTG were used with the same unit of activity, based on the ability of the enzyme to incorporate MDC into DMC, the catalytic activity and specificity of the enzymes may be different when different substrates, namely $BPNH_2$ and BLG, are used. The different specificities toward various amines of MTG have been previously reported (Ohtsuka, Sawa, Kawabata, Nio & Motoki, 2000b). In addition, GTG also incorporated MDC into BLG to a different extent than putrescine (Ohtsuka, Ota, Nio, & Motoki, 2000a). Thus, the different reactivity between MTG and FTG may also be attributed to the different substrate specificities.

3.4. Identification of $BPNH_2$-modified BLG

The experimental MALDI-TOF mass spectra of the $BPNH_2$-tagged peptides derived from tryptic digestion of $BPNH_2$-modified BLG were compared to the theoretical spectra of tryptic peptides derived from non-modified BLG generated by *in silico* digestion. The putative $BPNH_2$-modified peptides were calculated, based on a 311 Da mass increment of the Q-bound peptides, corresponding to the mass of $BPNH_2$ after releasing ammonia. A similar approach was used to localise the hydroxylation of Q-rich protein, dB1, catalysed by MTG (Piersma et al., 2002).

The results indicate that 4 residues in BLG served as the acyl donor for MTG, namely Q13, Q68, Q115 or Q120, and Q155 or Q159 (Table 1). The accessibility of Q to the solvent may be a critical factor for MTG-catalysed modification. The Q68 is located in a β-strand with accessible surface of 32%, whilst Q13 and Q155 are located in an α-helix with 34% accessibility to solvent (Nieuwenhuizen et al., 2004). The two Q residues, Q115 and Q159, are in a random-coil region with 26% to 34% solvent accessible surface. The Q120 is in a region with less than 3% accessible surface, due to a disulphide bond between C106 and C119. However, the disulphide linkage between the C106 and C119 was speculated to be reduced since the TGase reaction was conducted in the presence of DTT. Consequently, the Q120 would be exposed and had a potential to be modified by MTG.

MTG-catalysed BLG modification would likely depend, not only on the accessibility of Q to the solvent, but also the specificity toward substrates. It has been reported that 6-aminohexanoic acid was incorporated at Q35, Q59, Q68, and Q155 or Q159 by MTG (Nieuwenhuizen et al., 2004), which are different from the $BPNH_2$-modification sites in the present study. These results suggest that MTG exhibited different specificities toward different amines, 6-aminohexanoic acid or $BPNH_2$.

The reactive site for FTG was identified for only one site, namely at Q68 (Table 1), which was also modified by MTG. The larger molecular size of FTG might reduce the enzyme's accessibility to other Q residues. Since Ca^{2+} was included in the reaction catalysed by FTG, it might induce structural changes and self-aggregation of BLG, hampering the accessibility of FTG to Q residues. However, GTG catalysed the putrescine-modification at Q155 and Q159 (Coussons et al., 1992), which were reactive sites for MTG but not FTG in our study. The different reactivities of GTG and FTG toward Q155 and Q159 may be indicative of their differences in substrate specificity rather than the accessibility of these residues in BLG, since Ca^{2+} was added to the reaction for both GTG and FTG, which are Ca^{2+}-dependent TGases.

Table 1
Masses of $BPNH_2$-tagged peptides catalysed by either MTG or FTG and their putative sequences.

TGases	M	M+ $BPNH_2$	Putative sequence	Reactive Q position
MTG	672.77	983.45	^{9}GLDIQK^{14}	Q13
	1064.14	1375.11	^{61}WENGECAQK^{69}	Q68*
	1122.17	1432.77	^{61}WENDECAQK^{69}	Q68**
	2666.12	2976.42	^{102}YLLFCMENSAEPEQSLACQCLVR^{124}	Q115 or Q120*
	2777.30	3089.57	^{139}ALKALPMHIRLSFNPTQLEEQCHV^{162}	Q155 or Q159
FTG	1064.14	1375.11	^{61}WENGECAQK^{69}	Q68*
	1122.17	1432.77	^{61}WENDECAQK^{69}	Q68**

M = Theoretical mass of peptides.
Q = Putative reactive $BPNH_2$-modification sites.
* and ** refer to the sequences for BLG-B and BLG-A, respectively.

4. Conclusions

MTG catalysed both cross-linking and $BPNH_2$ modification of BLG to a greater extent than did FTG. MALDI-TOF MS could be used to monitor the stoichiometric modification of BLG. The locations of the $BPNH_2$-modification sites were identified successfully by comparing the mass profiles of $BPNH_2$-tagged peptides to those from *in silico* digestion of BLG. The different abilities of FTG and MTG to incorporate amines into BLG may be attributed to the different substrate specificities of TGases, the different accessibilities of these enzymes to Q residues, and BLG conformation induced by Ca^{2+}.

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