



Effect of transglutaminase-catalyzed glycosylation on the allergenicity and conformational structure of shrimp (*Metapenaeus ensis*) tropomyosin



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ABSTRACT

Tropomyosin (TM), a myofibrillar protein, is a major allergen in shrimp. The aim of this study was to evaluate the effect of transglutaminase (TGase)-catalyzed glycosylation on the potential allergenicity and conformational structure of TM in *Metapenaeus ensis*. Results showed that glycosylation of TM induced unfolding of the primary protein structure followed by loss of the secondary structure. Cleavage of certain free amino groups was observed during TGase-catalyzed glycosylation. The glycosylation rate correlated with reaction temperature. Western blotting and indirect ELISA with TM-specific polyclonal antibodies from rabbit and sera from patients allergic to shrimp demonstrated that antigenicity and potential allergenicity of TM decreased, which correlated well with the conformational changes in its structure. Considering TGase is widely utilized in the food industry, these results indicate that TGase-catalyzed glycosylation has the potential to serve as a mild method for reducing the allergenicity of shrimp products.

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1. Introduction

Shrimp is a popular seafood consumed around the world because of its delicious and comprehensive nutritional properties. However, shrimp is categorized as one of the “big eight” food allergens (Lehrer, Ayuso, & Reese, 2003; Rona et al., 2007). Currently, there are no effective methods for overcoming food allergy emergencies. Many attempts have been made to reduce the allergic activity of shrimps, such as microwaving (El Mecherfi et al., 2011), high-intensity ultrasound (Li, Yu, Ahmedna, & Goktepe, 2013), heat processing (Kamath, Rahman, Komoda, & Lopata, 2013) and high hydrostatic pressure processing (Li, Zhu, Zhou, & Peng, 2012). However, only a few methods can be extensively applied in the food industry.

The Maillard reaction (glycation) has become a promising approach for modifying functional properties of proteins, including allergenicity. Previous studies have shown that the allergenicity of crustacean (Nakamura, Watanabe, Ojima, Ahn, & Saeki, 2005), milk (Corzo-Martínez, Soria, Belloque, Villamiel, & Moreno, 2010), peanut (Vissers et al., 2011), wheat (Yang, Li, Li, & Wang, 2013), soybeans (Bu et al., 2015) and other food-derived protein can be affected by the Maillard reaction with reducing sugar. Although reduction of allergenicity is a greater possibility now, the application of current methods in the food industry is limited due to irrepressible chemical reactions (Brands, Alink, van Boekel, & Jongen, 2000), generating undesired compounds (Zhu, Damodaran, & Lucey, 2008) and forming anti-nutrients (Zhang, Ames, Smith, Baynes, & Metz, 2008). Recently, Jiang and Zhao (2010, 2011) reported a new enzyme-mediated method to covalently link glucosamine to proteins and improve their functional properties. Enzyme-mediated conjunction (glycosylation) may be useful in

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the food industry because of its site-specific modification and mild reaction conditions. However, no effort has been made to examine and emphasize the effect of such an approach in food allergens.

The enzyme transglutaminase (TGase, EC 2.3.2.13) is a type of acyltransferase. It can catalyze acyl transfer from the γ -carboxamide of glutamyl (Gln) residues (acyl donors) to primary amines (acyl acceptors) and induce the formation of ϵ -(γ -glutamyl)-lysine cross-linking. Since it possesses a primary amine, glucosamine (GlcN, a natural amino sugar present in the crustacean chitosan) can conjugate with various proteins or hydrolysates and induce changes in their functional properties and structures (Jiang & Zhao, 2010; Hrynets, Ndagijimana, & Betti, 2014; Gottardi, Hong, Ndagijimana, & Betti, 2014). Compared to non-enzymatic glycation, TGase-catalyzed glycosylation is considered a powerful method for increasing the glyco-conjugation efficiency under mild conditions. The protein or peptide can be site-specifically modified because of its substrate specificity; moreover, the adverse compounds produced via the glycation pathways are no longer present.

Tropomyosin (TM) comprises 5–7% of shrimp muscle and is known to be the major allergen that can react in at least 80% of shrimp-allergic patients. TM is a hydrophilic and heat-stable protein with a molecular weight of 36 kDa (Liu et al., 2010; Yang et al., 2010). Previous studies have shown eight linear epitopes in TM that are responsible for its allergenicity (Ayuso, Lehrer, & Reese, 2002; Zheng, Lin, Pawar, Li, & Li, 2011). Further analysis showed that six epitopes contain Gln. This indicates that these epitopes could be glycosylated by GlcN through catalysis by TGase. We hypothesized that these modifications would change the structure and potential allergenicity of shrimp TM.

The objective of the present study was to determine the changes in conformational structure and potential allergenicity of shrimp TM upon TGase-catalyzed glycosylation. Structural modifications were detected by circular dichroism (CD) spectroscopy, ultraviolet (UV) absorption spectroscopy and intrinsic fluorescence spectroscopy; the antigenicity and potential allergenicity were evaluated by western blotting and indirect enzyme-linked immuno sorbent assay (ELISA).

2. Materials and methods

2.1. Materials and chemicals

TM was purified from shrimp (*Metapenaeus ensis*) in the Food Safety Lab (Ocean University of China), as described by Wang et al. (2012). Rabbit polyclonal antibody against TM was generated by the same laboratory. D-(+)-Glucosamine hydrochloride (GlcN), bovine serum albumin (BSA), 1-anilino-8-naphthalene-sulfonate (ANS), 3,3',5,5'-tetramethylbenzidine (TMB) and o-phthalaldehyde (OPA) were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Transglutaminase (TGase, Ca^{2+} -independent) was purchased from Pangbo Biological Engineering Co. (Nanning, China). DL-Dithiothreitol (DTT) was purchased from Yuanye Co. (Shanghai, China). Horseradish peroxidase (HRP)-labelled goat anti-rabbit IgG and anti-human IgE were purchased from Zhongshan Jinqiao Co. (Beijing, China). All other chemicals used in this study were of analytical grade.

2.2. Human serum

Sera samples were obtained from Qingdao Municipal Hospital (Qingdao, China). All patients were allergic to shrimp, with a clear clinical history, and presented asthma and chronic urticarial symptoms. The patients were diagnosed by skin prick tests, and the specific IgE level was determined by Allergy Screen (MEDIWISS

Analytic GmbH, Moers, Germany). Sera with specific IgE levels higher than 0.5 kU/L were pooled and stored at -80°C until analysis. The research work was approved by the Medical Ethics Committee of Qingdao Municipal Hospital (File No, QD-PF-20140314551). Moreover, all patients were aware of the usage of their sera for the experimental purpose.

2.3. Preparation of glycosylated TM

TM was prepared at a concentration of 1.0 mg/ml in 10 mM phosphate-buffered saline (PBS, pH 7.5) containing 5 mM DTT. TM and GlcN were mixed at a weight ratio of 1:1 (w/w), with (+) or without (–) TGase to initiate the reaction (glycosylation or glycation, respectively). Approximately 10 U of TGase/g protein was added to induce the enzymatic reaction. All samples were incubated under constant agitation (180 rpm) for 6 h at 25°C or 37°C . The experimental conditions were selected to minimize non-enzymatic glycation and were conducted for a sufficient amount of time for glycosylation to occur, as described by Hrynets, Ndagijimana, and Betti (2013). The reaction was terminated by heating the sample at 85°C in a water bath for 5 min, and then the samples were rapidly cooled to ambient temperature. Excess GlcN was removed by dialysis against 10 mM PBS (pH 7.5) in a dialysis bag (cut-off 500 Da) (Millipore Corporation, Billerica, MA, USA). A cross-linked TM sample was also prepared under the same conditions, except that no GlcN was added. Cross-linked TM and original TM were both used as controls. All prepared samples were stored at -40°C until further analysis.

2.4. Protein structure characterization

2.4.1. Protein electrophoresis

Protein electrophoresis was performed as described by Laemmli (1970). Each protein sample was mixed 4:1 with loading buffer (containing 5% 2-mercaptoethanol) and heated in boiling water for 7 min before electrophoresis. Exactly 2.5 μg of sample was loaded onto each well (stacking gel, 5%; resolving gel, 15%). The electrophoretic conditions were 80 V for the stacking gel and 120 V for the resolving gel. Next, the gel was stained with Coomassie Brilliant Blue R-250 and photographed using a Tanon-4200SF (Tanon Science & Technology Co., Ltd., Shanghai, China).

2.4.2. CD analysis

The secondary structure of TM was determined by far-ultraviolet CD spectra, using a Jasco J-815 CD spectrometer (Japan Spectroscopic Co. Inc., Japan). Each sample was diluted with ultrapure water to a final concentration of 1.0 mg/ml and scanned at a rate of 50 nm/min and a wavelength range of 185–260 nm. The final CD data were expressed in units of mdeg ($\text{deg}\cdot\text{cm}^2\cdot\text{dmol}^{-1}$), and the percentage of different secondary structures was calculated using JASCO software.

2.4.3. UV absorption analysis

All treated TM samples were diluted to 0.5 mg/ml in 10 mM PBS and the UV absorbance spectrum was recorded using a UV-1101 spectrometer (Techcomp Co. Shanghai, China) at room temperature over a scan range of 220–320 nm.

2.4.4. Intrinsic fluorescence analysis

Intrinsic fluorescence spectra of samples were acquired on a fluorescence spectrophotometer (F-4600, Hitachi, Tokyo, Japan) at a wavelength of 295 nm (excitation) and 300–400 nm (emission), using 2.5 nm as a constant slit. Protein samples were diluted to 0.5 mg/ml in 10 mM phosphate buffer (PB, pH 7.0). The intrinsic fluorescence of TM samples was assessed in a rectangular quartz cuvette with a 1 cm path-length at room temperature.

2.4.5. Surface hydrophobicity analysis

The surface hydrophobicity of different TM samples was analyzed using a F-4600 fluorescence spectrophotometer with ANS as a probe, as described by Lin et al. (2015). All samples were serially diluted to final concentrations of 0.01–0.5 mg/ml in 10 mM PB; 20 μ l of 8.0 mM ANS solution prepared in the same buffer was added to 4.0 ml of each sample. The fluorescence intensity was determined at a wavelength of 370 nm (excitation) and 490 nm (emission). The surface hydrophobicity of TM samples was defined as the initial slope of the curve formed by the fluorescent intensity plotted in line with protein concentration.

2.4.6. Free amino group analysis

The free amino group of each sample was measured as described by Church, Swaisgood, Porter, and Catignani (1983). Briefly, 200 μ l of each sample solution (final concentration of 0.5 mg/ml) was added into 4 ml of OPA reagent, which was prepared daily. After being mixed equally and incubated for 2 min at room temperature, free amino groups were measured at a wavelength of 340 nm, using a UV-1101 spectrometer. The content of free amino groups was expressed as mol/kg protein, and l-leucine was used as the standard.

2.5. Protein antigenicity and potential allergenicity analysis

2.5.1. Western blotting analysis

Western blotting analysis was carried out according to a method previously reported by Song et al. (2015). In brief, samples were electrophoresed as described in previous Section 2.4.1. Then the proteins were transferred to a polyvinylidene fluoride membrane (Millipore Corporation). After blocking with blocking buffer (10 mM PBS containing 5% skim milk and 0.05% Tween 20, pH 7.4), the membrane was incubated with rabbit antibody against shrimp TM (1:10,000 dilution in 50% blocking buffer, v/v) or pooled sera from allergic patients (1:10 dilution in 50% blocking buffer, v/v) at ambient temperature overnight. Then the membrane was treated with HRP-labelled goat anti-rabbit IgG (1:5000 dilution in 50% blocking buffer, v/v) or anti-human IgE (1:100 dilution in 50% blocking buffer, v/v) for 1.5 h. IgG/IgE-binding characteristics were determined by enhanced chemiluminescence with different exposure times, using a Tanon-4200SF, and the optimal image was selected.

2.5.2. IgG/IgE-binding capacity analysis

The IgG/IgE-binding capacity of different TM samples was assayed via indirect ELISA based on the method described by Li,

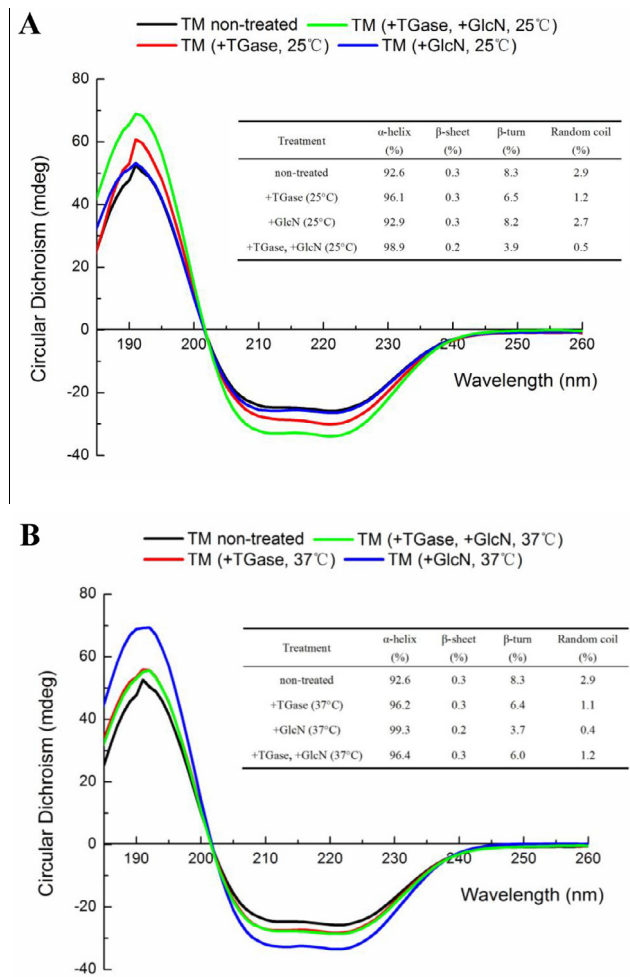


Fig. 2. Secondary structure changes of glycosylated TM, measured by Far-UV CD spectroscopy. (A) Incubated at 25 °C. (B) Incubated at 37 °C.

Lu, Khan, Lin, and Zhang (2014). In brief, TM samples were diluted to 5 μ g/ml with carbonate buffer solution (pH 9.6) and coated in a 96-well microtiter plate with 100 μ l per well. After blocking with blocking buffer (1% BSA in PBST), rabbit polyclonal antibody (1:15,000 dilution in blocking buffer, v/v) or pooled patients' sera (1:100 dilution with blocking buffer, v/v) was loaded and

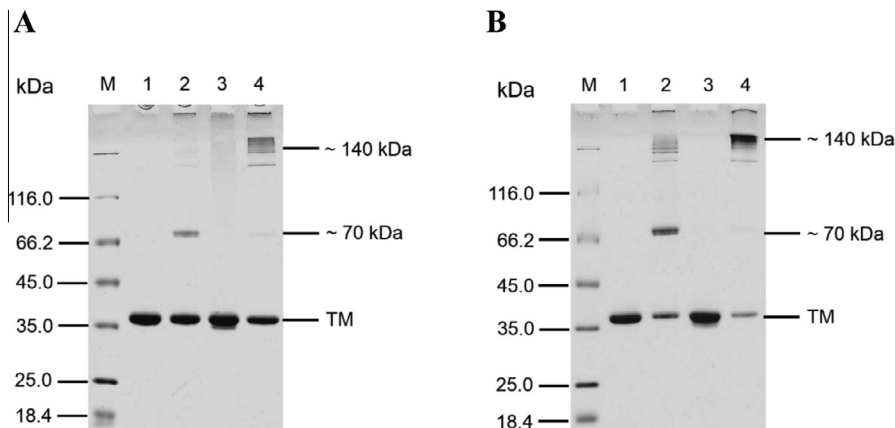


Fig. 1. SDS-PAGE analysis of glycosylated TM. (A) Incubated at 25 °C. (B) Incubated at 37 °C. M, protein marker; 1, non-treated TM; 2, TM treated with TGase; 3, TM treated with GlcN; 4, TM treated with TGase and GlcN.

incubated at 37 °C for 1.5 h. The plate was washed and incubated with HRP-labelled goat anti-rabbit IgG (1:5000 dilution in blocking buffer, v/v) or anti-human IgE (1:500 dilution in blocking buffer, v/v). After a final wash, HRP activity was assayed by adding 100 μ l of a substrate solution of TMB, incubating at 37 °C for 20 min, and terminating the reaction by addition of 50 μ l of 2 M sulfuric acid. The absorbance of each well was read at 450 nm by a microtiter plate reader (MultiscanMK3, Thermo Fisher Scientific Inc., Waltham, MA, USA).

2.6. Statistical analysis

One-way analysis of variance (ANOVA) with Duncan's multiple range test was carried out to analyze significant differences in the data (using SPSS for Windows, version 19.0; Chicago, IL, USA). Values of $p < 0.05$ were considered significant unless otherwise stated.

3. Results

3.1. SDS-PAGE analysis of glycosylation modified TM

As shown in Fig. 1, glycosylation and cross-linking of TM samples were observed after treatment with TGase or "TGase + GlcN". When TM was treated with TGase, several TM polymers were observed at approximately 70 kDa, 140 kDa, and higher molecular bands (Fig. 1A, Lane 2). No polymers were observed by SDS-PAGE when GlcN alone was mixed with shrimp TM (Fig. 1A, Lane 3).

However, when TGase and GlcN were mixed with shrimp TM, several TM polymer bands were significantly heavier than those observed when TM was treated with only TGase (Fig. 1A, Lane 4). In a comparison of the effect of temperature on reaction rate, the glycosylation and cross-linking of shrimp TM at 37 °C showed higher-intensity TM polymer bands than those at 25 °C (Fig. 1A, B).

3.2. Effect of TGase-catalyzed glycosylation on conformational structure of TM

3.2.1. CD spectra

Far-ultraviolet CD spectra was used to determine the effect of GlcN-mediated glycosylation via TGase, under different conditions, on the secondary structure of shrimp TM. As shown in Fig. 2A, B, non-treated TM showed two negative peaks at 208 nm and 220 nm, indicating that the dominant secondary structure of TM is an α -helix. Following TGase treatment, there was a rapid decrease in the peaks because of cross-linking and glycosylation with GlcN. Further calculated results using JASCO secondary structure software are also shown in the figure. After being treated with TGase, the proportion of different secondary structures of TM was not significant, although the α -helix structure increased. It was also noticed that the content of random coil decreased along with the cross-linking and glycosylation with GlcN.

3.2.2. UV absorption spectra

Structural changes in the conformation of shrimp TM were reflected by alterations in UV absorption. As shown in

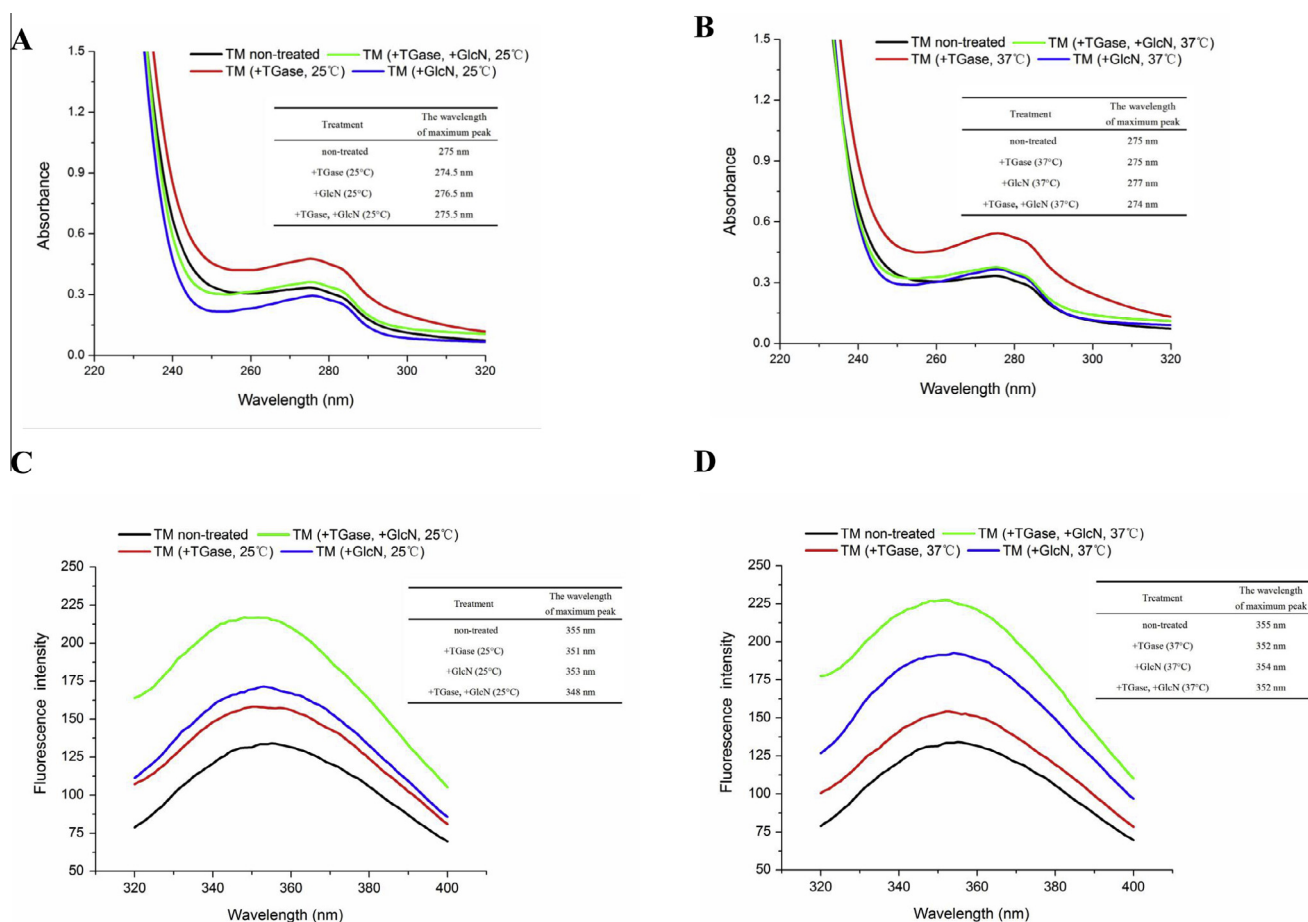


Fig. 3. Effect of TGase-catalyzed glycosylation on the UV absorption and intrinsic fluorescence of TM. (A) UV absorption spectrum, incubated at 25 °C. (B) UV absorption spectrum, incubated at 37 °C. (C) Fluorescence emission spectra, incubated at 25 °C. (D) Fluorescence emission spectra, incubated at 37 °C.

Fig. 3A, B, a 1.5- and 2.0-nm red shift in the absorbance peak occurred in GlcN-treated samples, which were incubated at 25 °C and 37 °C, respectively. TGase-induced cross-linking resulted in a slight blue shift when the reaction system was incubated at 25 °C and showed the same wavelength at 37 °C. Furthermore, TGase together with GlcN caused a 0.5-nm red shift at 25 °C and a 1.0-nm blue shift at 37 °C. In addition to the absorbance peak shift in wavelength, the UV absorption peak varied when shrimp TM was glycosylated under different conditions, indicating that specific amino acids such as tryptophan (Trp), tyrosine (Tyr) and phenylalanine (Phe) were exposed following glycosylation.

3.2.3. Intrinsic fluorescence

The intrinsic fluorescence spectra of TM exposed to different treatments are shown in Fig. 3C, D. Untreated samples showed an original fluorescence emission spectrum of Trp residues in protein. Following excitation at 295 nm, TM exhibited a fluorescence emission maximum (kmax) at 355 nm. The intrinsic fluorescence intensity of glycosylated samples reached a maximum that was approximately 85% higher than that of non-treated TM; moreover, glycosylation led to a blue-shifted wavelength of the maximum emission (from 355 to 348 nm). Although the reaction temperature did not significantly affect the intrinsic fluorescence spectra, the increase in maximum fluorescence intensity may have been caused by changes in the microenvironment where Trp residues were exposed because of protein unfolding and binding of GlcN to sites in other peptides.

3.2.4. Surface hydrophobicity

Different patterns of the surface hydrophobicity of TM were observed following different treatments. As shown in Fig. 4A, TGase-induced cross-linking led to an increased surface hydrophobicity from 71.45 to 97.11, when incubated at 25 °C. By contrast, the “TGase + GlcN” treated sample showed a similar result to the non-treated one. However, the hydrophilicity of TM increased after treatment with GlcN, while the surface hydrophobicity decreased significantly (from 71.45 to 54.03) due to conjugation of activated sugar (containing –OH and –NH₂). It is worth noting that the increase of surface hydrophobicity was closely related to the rise of reaction temperature (Fig. 4B), since the hydrophobicity may be influenced by the rate of protein cross-linking.

3.2.5. Free amino group

The content of free amino groups of TM samples decreased under all treatment conditions. The content was reduced by 27.6% when TM reacted with TGase and GlcN at 25 °C. It was observed that higher temperatures triggered a further reaction in free amino groups (Fig. 4C, D).

3.3. Effect of TGase-catalyzed glycosylation on the antigenicity and potential allergenicity of TM

3.3.1. IgG and IgE immunoblots

To estimate the effect of glycosylation on antigenicity and potential allergenicity, western blotting analysis was performed

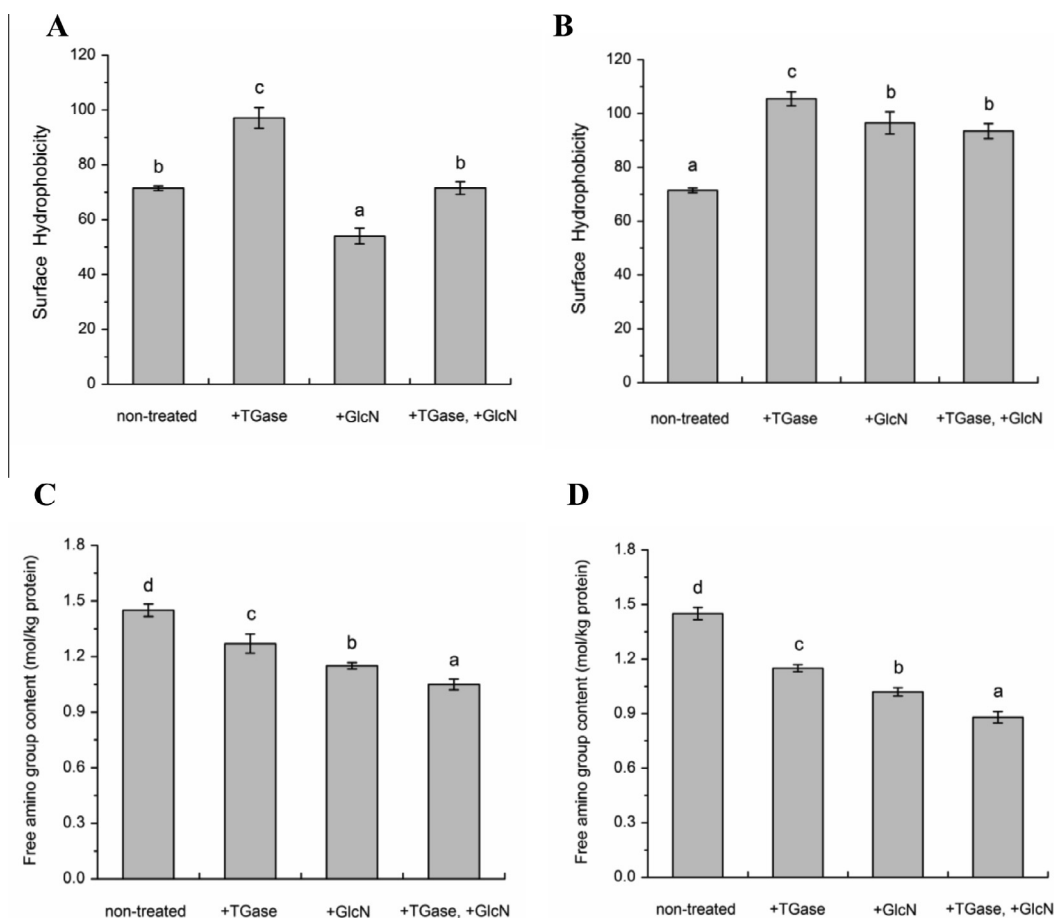


Fig. 4. Effect of TGase-catalyzed glycosylation on the surface hydrophobicity and free amino groups of TM. (A) Surface hydrophobicity, incubated at 25 °C. (B) Surface hydrophobicity, incubated at 37 °C. (C) The content of free amino groups, incubated at 25 °C. (D) The content of free amino groups, incubated at 37 °C. All data are expressed as means $\pm$ standard deviation ($\bar{X} \pm SD$). Different letters above the columns indicate significant differences ($p < 0.05$).

to determine the binding capacities of IgG and IgE. As shown in Fig. 5, the antigenicity of TMs processed by glycosylation or glycation were evaluated using a rabbit IgG antibody against shrimp TM. New bands were observed when TM was treated with TGase or “TGase + GlcN”, indicating cross-linking of TM; however, when band intensity was compared, no significant differences were observed. Since the molecular weight of GlcN was low and the reaction time was short, we did not observe significant diffusion when TM was glycosylated with GlcN (Fig. 5A, B, Lane 3).

Similar patterns were observed when the potential allergenicity of treated TM was evaluated by western blotting using pooled sera from shrimp-allergic patients (Fig. 5C). When TM was treated with TGase, the IgE-binding band at 36 kDa disappeared and cross-linked TM appeared at approximately 70 kDa and 140 kDa with weaker intensity than observed for untreated protein, while “TGase + GlcN” treated samples also had a strong resemblance of these phenomena. Moreover, glycosylated TM with GlcN showed similar bands as the control but with weak intensity. When temperature rose from 25 °C to 37 °C, it was noticed that protein bands were at similar positions, however, the band intensity of glycosylated samples markedly reduced (Fig. 5D, Lane 4).

3.3.2. Indirect ELISA

To determine whether TGase-catalyzed glycosylation affects the antigenicity and potential allergenicity of TM, indirect ELISA was performed to evaluate IgG- and IgE- binding capacity to glycosylated TM by using a rabbit antibody against native TM as well as pooled sera from patients with shrimp allergy. As shown in Fig. 6A, IgG-binding capacity to TM decreased significantly ($p < 0.05$) when the protein was modified with TGase or GlcN. In the presence of TGase and GlcN, TM was glycosylated and IgG-binding capacity was decreased by 36.9%, showing the largest decrease among all groups. TM glycation was observed in the presence of GlcN alone

and IgG-binding capacity significantly decreased compared to that in the controls. However, this binding capacity showed the smallest decrease compared to that in other reaction groups. At 37 °C, the reduction in IgG-binding capacity was significantly ($p < 0.05$) higher than reactions at 25 °C (Fig. 6A, B). Moreover, TM glycosylation induced the highest decrease in IgG binding.

Fig. 6C, D shows the effect of glycosylation on the binding capacity of IgE from pooled sera at different temperature (25 °C and 37 °C). The initial IgE binding of non-treated TM was significantly higher ($p < 0.05$) than that of glycosylated and glycosylated TM. Concerning treated samples, the IgE-binding capacity was highest in “TGase + GlcN”-treated samples, followed by TGase-only, and then by GlcN-only samples. The difference between treatment groups was statistically significant. Among treatments, glycosylated TM showed the lowest IgE-binding capacity (45.27% lower than that of control) compared to those under other reaction conditions. Reaction temperature significantly ($p < 0.05$) influenced IgE-binding capacity. IgE binding decreased by 9% when the temperature was increased from 25 °C to 37 °C.

4. Discussion

According to previous reviews, TGase catalyzes acyl-transfer reactions between peptide-bound glutamine residues (acyl donors) with primary amines (acyl acceptors) (DeJong & Koppelman, 2002; Song & Zhao, 2014a, 2014b). Since GlcN contains primary amines, TGase can induce protein glutaminyl residue modifications with GlcN residues. This modification leads to structural changes in shrimp TM, thus affecting its antigenicity and allergenicity. In this study, linear and conformational structural changes in TM were evaluated. We demonstrated that the dominant structure of shrimp TM is an α -helix, which agrees with the findings of Liu et al. (2010). Compared to natural TM, glycosylated TM showed fewer β -turn structures. Furthermore, random coils decreased while α -helices increased with TGase-catalyzed glycosylation with GlcN. These results partly agree with a previous report by Song and Zhao (2014a, 2014b), who found that cross-linking of the soybean protein by TGase resulted in decreased α -helix and β -structure. This may be because Gln is an acidic amino acid. In conjugation with GlcN, which is a basic group, the modified amino acid was electrically neutral and could form the α -helix structure (Huyghues Despointes, Scholtz, & Baldwin, 1993).

Surface hydrophobicity, UV absorption, and intrinsic fluorescence reflected the structural changes in modified TM samples. The increase in fluorescence intensity indicated that TM was unfolded and more hydrophobic regions were generated because of GlcN or TM peptides cross-linking with the side chains of the protein amino acids. This indicates that the tertiary and quaternary structures of TM were altered via glycosylation. Furthermore, a maximum UV absorption peak of natural TM was observed at approximately 275 nm, indicating that Trp and Tyr residue side chain groups contribute to the UV absorption spectrum of TM (Kolakowski, Dumay, & Cheftel, 2001). The UV absorption intensity did not change significantly, indicating that Trp and Tyr were not exposed to the surface during treatment. This result agreed with the CD spectrum changes. The intrinsic fluorescence of natural TM was observed at approximately 350 nm, which indicated that the intrinsic fluorescence of TM was mainly dependent on aromatic amino acid residues. The intrinsic fluorescence intensity of glycosylated samples suggests that intrinsic fluorescence may be exhibited on the surface of TM. A minor blue shift in the intrinsic fluorescence peak was observed following glycation or glycosylation, indicating that the polarity of the aromatic amino acids' microenvironment had changed after glycosylation. The microenvironmental changes induced an intrinsic fluorescence blue shift,

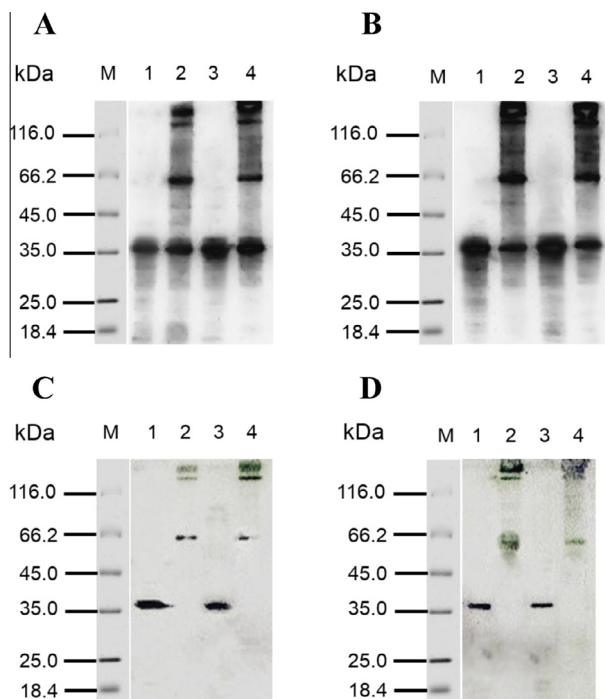


Fig. 5. Determination of the glycosylated TM to different treatments, as measured by western blotting. (A) and (B) to investigate IgG (rabbit polyclonal antibody)-binding capacity of TM. (C) and (D) to investigate IgE (human sera)- binding capacity of TM. (A) and (C), incubated at 25 °C; (B) and (D), incubated at 37 °C. M, protein marker; 1, non-treated TM; 2, TM treated with TGase; 3, TM treated with GlcN; 4, TM treated with TGase and GlcN.

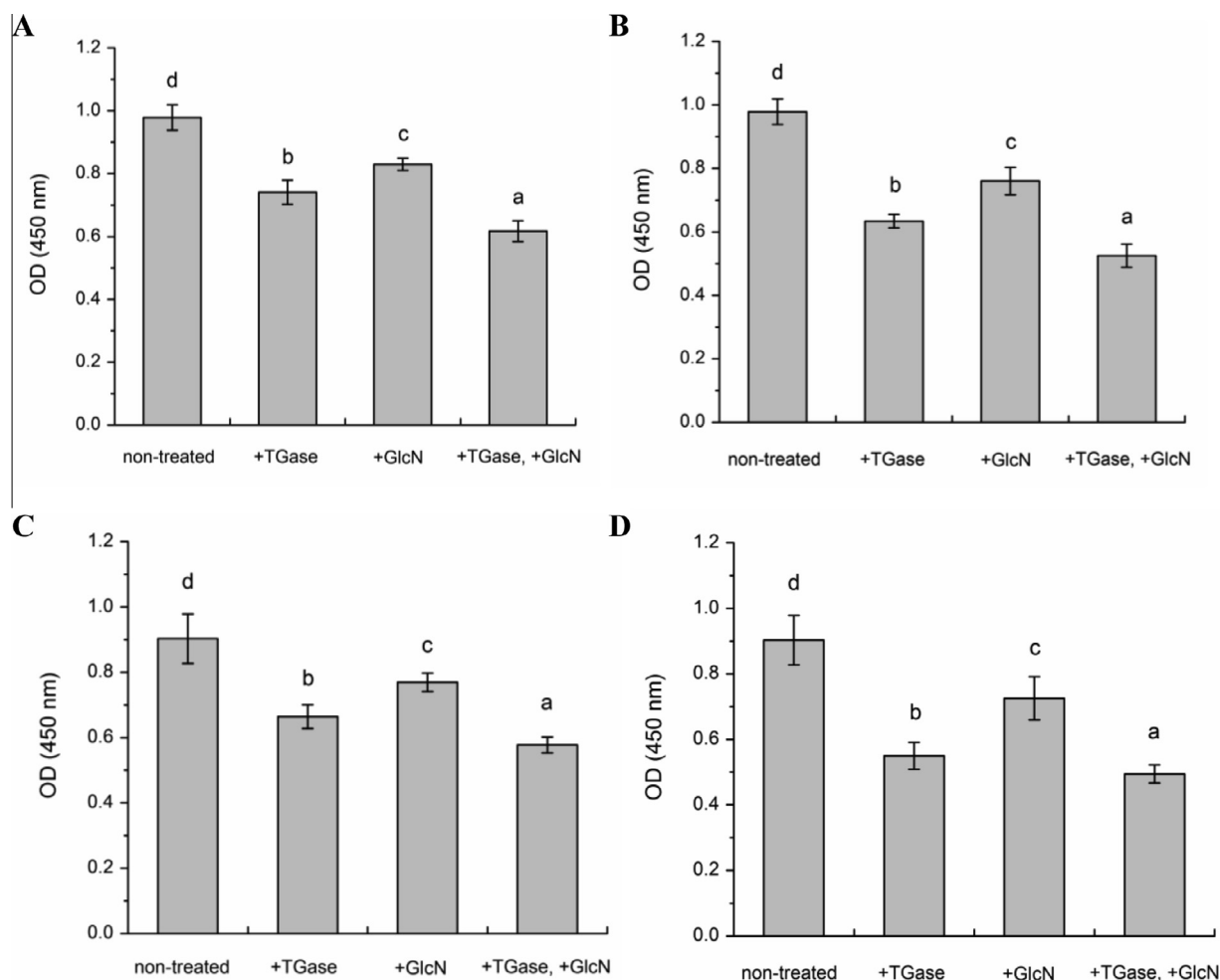


Fig. 6. Determination of the glycosylated TM to different treatments, as measured by indirect ELISA. (A) and (B) to investigate IgG (rabbit polyclonal antibody)- binding capacity of TM. (C) and (D) to investigate IgE (human sera)- binding capacity of TM. (A) and (C), incubated at 25 °C; (B) and (D), incubated at 37 °C. All data are expressed as means $\pm$ standard deviation ($X \pm SD$). Different letters above the columns indicate significant differences ($p < 0.05$).

which was also observed in ovotransferrin when it was heated at 80 °C (Tong et al., 2012).

The changes in the content of free amino groups were associated with the antigenicity and potential allergenicity of modified TM. Several amino acid residues such as Gln and Lys contain free amino groups and involve enzymatic cross-linking reactions as acyl donors and acceptors, respectively. These amino acids are also important components of the allergic epitopes of shrimp TM. Previous studies demonstrated that seven epitopes from shrimp TM contain Gln or Lys (Zheng et al., 2011). During these reactions, free amino groups decreased. There are two possible reasons for this observation. First, TGase can catalyze acyl transfer reactions between the γ -carboxamide and primary amines, and these reactions can cause ϵ -(γ -glutamyl)-lysine cross-linking between Gln and Lys, which contain ϵ -NH₂. Second, the primary amines can react with reducing sugars such as GlcN through a Maillard reaction (Nakamura et al., 2005; Hrynets et al., 2013). Next, the primary amines from GlcN can cross-link with Gln from protein and cause glycosylation modification of proteins. These amino group losses result in amino acid modification of TM, thus affecting its IgG/IgE-binding capacity.

The structural changes in TM altered the antigenicity and potential allergenicity. In this study, a specific polyclonal antibody from rabbit and pooled sera from allergic patients were used to evaluated IgG- and IgE- binding capacity, respectively. The indirect

ELISA results demonstrated that antigenicity decreased with glycation or glycosylation. Glycosylated shrimp TM showed the lowest antigenicity. These results indicate that amino acid modification-induced structure shifting affect IgG-binding capacity. The observed glycation and TGase-catalyzed protein cross-linking induced changes in antigenicity, which were similar to those in previous studies of squid tropomyosin (Nakamura et al., 2006). Moreover, shrimp TM treated at 37 °C showed lower antigenicity than samples treated at 25 °C. As higher temperature resulted in a faster reaction, the efficiency of glycation or glycosylation were both promoted, which was similar to the result of natural actomyosin (Hrynets et al., 2014). Large modifications resulted in further structural changes, affecting antigenicity. The potential allergenicity of treated shrimp TM towards the serum IgE from pooled sera showed changes similar to that in the IgG-binding capacity.

As determined by western blotting, the intensity of IgG/IgE-binding bands showed similar trends to the ELISA results. Cross-linked shrimp TM showed bands at double or triple the molecular weight, and the IgG/IgE-binding capacity was retained. Compared with SDS-PAGE, the antibody-binding intensities did not show significant differences among treatments. This indicates that modification of shrimp TM via glycation or glycosylation affects the antigenicity and potential allergenicity, but some epitopes can still bind to specific antibodies, exhibiting

IgG/IgE-binding capacity. There are several possible reasons for this phenomenon. First, the ELISA antigen is in a natural state but is denatured during western blotting. This denaturation can cause different epitopes to be exposed on the protein surface, and these epitopes can then bind to IgG or IgE. Second, glycation or glycosylation may form new carbohydrate or glycosylation epitopes (Toda, Heilmann, Ilchmann, & Vieths, 2014), and these new epitopes can induce differences in IgG- and IgE-binding capacity. Other reasons may be related to differences in the binding sites of IgG and IgE or different methods of binding between the two assays.

5. Conclusion

This study showed that TGase-catalyzed glycosylation could induce structure changes of shrimp tropomyosin. These modifications can result in the reduction of IgG/IgE-binding capacity. It indicated that alterations in linear and conformational structures would cause epitopes destruction, which was responsible for potential allergenicity. As compared with other methods, such as heating, oxidation, and non-enzymatic browning, TGase-catalyzed glycosylation can induce site-specific modification under mild conditions and is more suitable for use in food processing. Although clinical evidence for potential changes in the allergenicity of glycosylated TM is needed, TGase plus GlcN appears to be useful for reducing the allergic hazards of shrimp in food industry.

Conflict of interest

The authors declare that there are no conflicts of interest.

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