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# Impacts of glycation and transglutaminase-catalyzed glycosylation with glucosamine on the conformational structure and allergenicity of bovine $\beta$ -lactoglobulin

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$\beta$ -Lactoglobulin ( $\beta$ -LG) is recognized as the major milk allergen. In this study, the effects of transglutaminase (TGase) and glucosamine (GlcN)-catalyzed glycosylation and glycation on the conformational structure and allergenicity of  $\beta$ -LG were investigated. The formations of cross-linked peptides were demonstrated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and GlcN-conjugated modification was identified using matrix-assisted laser desorption/ionization-time of flight-mass spectrometry (MALDI-TOF-MS). Structural analysis revealed that glycosylation and glycation of  $\beta$ -LG induced unfolding of the primary protein structure followed by a loss of the secondary structure. As revealed by circular dichroism (CD) spectroscopy, glycosylated  $\beta$ -LG exhibited the highest increase in the  $\beta$ -sheets from 32.6% to 40.4% (25 °C) and 44.2% (37 °C), and the percentage of  $\alpha$ -helices decreased from 17.7% to 14.4% (25 °C) and 12.3% (37 °C), respectively. The tertiary and quaternary structures of  $\beta$ -LG also changed significantly during glycosylation and glycation, along with reduced free amino groups and variation in surface hydrophobicity. Immunoblotting and indirect enzyme-linked immuno sorbent assay (ELISA) analyses demonstrated that the lowest IgG- and IgE-binding capacities of  $\beta$ -LG were obtained following glycosylation at 37 °C, which were 52.7% and 56.3% lower than that of the native protein, respectively. The reduction in the antigenicity and potential allergenicity of glycosylated  $\beta$ -LG was more pronounced compared to TGase treated- and glycated  $\beta$ -LG, which correlated well with the structural changes. These results suggest that TGase-catalyzed glycosylation has more potential compared to glycation for mitigating the allergenic potential of milk products.

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## 1. Introduction

$\beta$ -Lactoglobulin ( $\beta$ -LG), a component of whey protein, is considered a promising ingredient in the food industry. However, this globular protein is also classified as a major allergen of milk and other dairy products.<sup>1–3</sup> In order to utilize this func-

tional ingredient more effectively, several methods have been used to reduce the antigenicity and allergenicity of  $\beta$ -LG, such as ultrasound,<sup>4</sup> dynamic high-pressure micro-fluidization,<sup>5</sup> fermentation and enzymatic hydrolysis.<sup>6</sup> Similarly, several research studies have been focused on altering the structure of food proteins by various approaches in order to improve their functionality and shelf-life stability and reduce allergenicity.<sup>7–13</sup> However, few methods can be widely applied in actual processing.

Glycation (the Maillard reaction) is referred to as the non-enzymatic covalent interaction between the carbonyl groups of a reducing sugar and the amino groups of amino acids, which takes place following heat treatment and even after storage for a long duration.<sup>14,15</sup> During the glycation process, Schiff bases are formed initially, followed by their Amadori rearrangements and the subsequent glycooxidation. These processes resultantly lead to the formation of advanced glycation end products (*i.e.* methylglyoxal-H1, pyrrole and pentosidine).<sup>15,16</sup> The extent and types of the glycation products are mainly dependent on the ratio of amino groups and reducing sugars, reaction tem-

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perature and time, pH and water activity.<sup>17,18</sup> These reaction products have the potential to modify the functional properties, digestibility, immunogenicity and allergenicity of food products. The sugar and protein interaction leads to the alteration of the protein structure and subsequently modifies the binding epitopes (either mask or expose the previously inaccessible epitopes). Epitopes are the part of an antigen molecule to which an antibody attaches itself; as these binding epitopes are denatured, resultantly the IgG/IgE binding activities of the processed allergens are reduced.<sup>15,16,19,20</sup> Previous studies showed that allergenicity declined significantly when  $\beta$ -LG was conjugated with galactose,<sup>21</sup> chitosan,<sup>22</sup> maltose,<sup>23</sup> nystose and fructofuranosyl nystose.<sup>24</sup>

Recently, significant attention has been paid to enzyme-mediated conjugation (glycosylation) that can site-specifically link the sugar at lower temperatures due to its strict substrate specificity. This mild modification has been used to enhance the sugar-conjugation efficiency and improve the functional properties of proteins.<sup>25–28</sup> Transglutaminase (TGase, EC 2.3.2.13) has been used widely in food protein processing, as it promotes functional properties. This acyltransferase can induce an acyl-transfer reaction between the  $\gamma$ -carboxyamides of glutaminyl residues (acyl donors) and primary amines (acyl acceptors), resulting in the formation of  $\epsilon$ -( $\gamma$ -glutamyl)-lysine isopeptide bonds.<sup>29</sup> Therefore, amino sugars (provide a primary amine) can be regarded as a substrate for the cross-linking of protein and sugar. Glucosamine (GlcN), a natural hydrolysate of crustaceans and other marine foods, can possess primary amines.<sup>30</sup> Previous studies revealed that this hydrophilic sugar could conjugate with various proteins following TGase treatments and lead to the modification of structural and functional properties.<sup>25,31,32</sup> The main antigenic epitopes in  $\beta$ -LG contain glutamine.<sup>33</sup> TGase-catalyzed glycosylation may change linear epitopes, thus affecting the conformational structures and allergenicity. This method is expected to be a promising method for hypoallergenic food processing. However, whether glycation and TGase-catalyzed glycosylation can be used to change the structural properties and allergenic potential of  $\beta$ -LG has remained unclear.

Therefore, the present study was aimed to investigate the influence of TGase and GlcN-catalyzed glycosylation and glycation on the structure and potential allergenicity of  $\beta$ -LG. The changes in the molecular weight (MW) of glycated/glycosylated peptides were characterized using MALDI-TOF-MS, while the changes in the secondary and tertiary structures were analyzed *via* CD spectroscopy, ultraviolet (UV) absorption spectroscopy, intrinsic fluorescence spectroscopy, surface hydrophobicity, and free amino group content analysis. IgG- and IgE-binding capacities of treated  $\beta$ -LG were determined by immunoblotting and indirect ELISA.

## 2. Materials and methods

### 2.1. Materials and reagents

Purified bovine  $\beta$ -LG and GlcN hydrochloride were obtained from Yuanye Biological Co. (Shanghai, China). Glycoprival

TGase ( $\text{Ca}^{2+}$ -independent) was purchased from Pangbo Biological Engineering Co. (Nanning, China). Bovine serum albumin (BSA), *o*-phthalaldehyde (OPA), DL-dithiothreitol (DTT), 1-anilino-8-naphthalene-sulfonate (ANS), and 3,3',5,5'-tetramethylbenzidine (TMB) were purchased from Sigma Chemical Co. (St Louis, MO, USA). The standard protein and the loading buffer for conducting SDS-PAGE were acquired from Solarbio Co. (Beijing, China). Rabbit polyclonal IgG antibody against  $\beta$ -LG was generated by Huada Protein Innovation Co., Ltd (Beijing, China), while horseradish peroxidase (HRP)-labeled goat anti-rabbit IgG and anti-human IgE were obtained from Zhongshan Jinqiao Co. (Beijing, China). The enhanced chemiluminescence (ECL) kit for immunoblotting was from Thermo Fisher Scientific Inc. (Waltham, MA, USA). All other reagents were of analytical grade.

### 2.2. Human sera

Human sera pooled from 4 patients allergic to bovine milk were provided by the Affiliated Hospital of Medical College of Qingdao University, Qingdao, China. Specific IgE level was found to be higher than  $1 \text{ IU mL}^{-1}$  and positive skin prick tests to bovine milk was observed, as evident *via* Allergy Screen (MEDIWISS Analytic GmbH, Moers, Germany). The patients enrolled in the study provided written consents about the usage of their sera for experimental purposes. The Medical Ethics Committee of Qingdao Municipal Hospital (File No, QD-PF-20140, 314551) certified the approval regarding the collection procedure, storage and sera usage for the purpose of protecting the interest and right of the subjects. All serum samples were stored at  $-80^\circ\text{C}$  until further analysis.

### 2.3. Preparation of glycosylated $\beta$ -LG

$\beta$ -LG solution was prepared by diluting to  $1.0 \text{ mg mL}^{-1}$  using  $10 \text{ mM}$  PBS (phosphate-buffered saline) containing  $10 \text{ mM}$  DTT and had a pH of 7.5. GlcN was added to the protein solution at a ratio of 1 : 1 (w/w). The glycosylation or glycation reaction was initiated with (+) or without (–) TGase, respectively. The activity level of TGase in the mixed system was  $10 \text{ U g}^{-1}$  protein. The group of samples was incubated for 6 h with continuous agitation (180 rpm) at  $25^\circ\text{C}$  and  $37^\circ\text{C}$ , respectively. Temperature was varied in order to know its effect on the reaction mixture and the subsequent glycation and glycosylation processes. The experimental conditions were chosen in order to provide sufficient time for reaction based on preliminary studies.<sup>26,31</sup> The reaction was terminated by rapid cooling in an ice bath for 15 min. Unreacted GlcN was removed by dialysis against  $10 \text{ mM}$  PBS at  $4^\circ\text{C}$  for 24 h with a MW cut-off membrane of 500 Da (Millipore Corporation, Billerica, MA, USA). TGase treated  $\beta$ -LG (without sugar) and native protein were used as controls. All prepared samples were stored at  $-40^\circ\text{C}$  until further analysis.

### 2.4. SDS-PAGE analysis

SDS-PAGE analysis was carried out to confirm cross-linking as described by Lv *et al.*<sup>34</sup> All the samples were diluted ( $0.25 \text{ mg}$  protein per  $\text{mL}$ ) and mixed with loading buffer (25% glycerol,

2% SDS, 14.4 mM  $\beta$ -mercaptoethanol, and 0.1% bromophenol blue in 1 M Tris-HCl, pH 6.8) at a ratio of 4:1 (v/v). After heating in boiling water for 7 min, exactly 10  $\mu$ L of each mixture was loaded into each well. Electrophoresis was performed using a 5% (w/v) stacking gel and 15% (w/v) resolving gel, with constant voltages of 80 V and 120 V, respectively. The gel was stained with Coomassie Brilliant Blue R-250 for 2 h and destained with 10% aqueous methanol (v/v) and 10% acetic acid (v/v) for 24 h. Gel images were captured using a Tanon-4200SF (Tanon Science & Technology Co., Ltd, Shanghai, China).

## 2.5. Determination of glycation/glycosylation by MALDI-TOF-MS

A previously described protocol was used for MALDI-TOF-MS analysis.<sup>28,35</sup> After electrophoresis, the gel pieces containing the target proteins were fragmented and put into sterile tubes, followed by washing with 100 mM ammonium bicarbonate/acetonitrile (1:1, v/v) solution and dried in a SpeedVac (AES 2000, Savant Instruments Inc., Farmingdale, NY, USA). The dried gel bands were subjected to overnight proteolytic digestion at 37 °C with 0.2  $\mu$ g of trypsin. After incubation, peptides were extracted with 30  $\mu$ L of 100 mM ammonium bicarbonate followed by extraction with a 30  $\mu$ L solution containing 50% acetonitrile (ACN) and 5% formic acid in water twice and dried to 15  $\mu$ L in a SpeedVac. Next, the samples (obtained as described above) were diluted tenfold in 50% ACN/water + 0.1% trifluoroacetic acid (TFA). Afterwards, 1  $\mu$ L of each sample was mixed with 1  $\mu$ L of  $\alpha$ -cyano-4-hydroxycinnamic acid (10 mg mL<sup>-1</sup> in 50% ACN/water + 0.1% TFA). Next, 1  $\mu$ L of the sample/matrix solution was spotted onto a stainless steel target plate and allowed to dry in air. All mass spectra were obtained using a Bruker Ultraflex MALDI-TOF/TOF-MS (Bruker Daltonics GmbH, Bremen, Germany).

## 2.6. CD analysis

The far-UV CD spectra were analyzed to detect alterations in the secondary structures of modified protein as described by Zhong *et al.*<sup>24</sup> The experiment was performed in the spectral range of 190–250 nm using a JASCO J-815 CD spectrometer (Japan Spectroscopic Co. Inc., Tokyo, Japan). Each sample was diluted to 1.0 mg mL<sup>-1</sup> with distilled water and collected in a rectangular quartz cuvette with a path length of 1 cm. Distilled water was used as a blank solution for all the samples. The operation indexes were as follows: bandwidth 1.0 nm; scan speed 100 nm min<sup>-1</sup>; response time 2 s. This process was performed at least twice and the average results of ellipticities of the molar residues were evaluated and expressed in units of mdeg. The contents of different secondary structures were calculated with the help of JASCO software.

## 2.7. UV absorption analysis

UV absorption spectra analysis was carried out using a UV-1101 spectrometer (Techcomp Co. Shanghai, China) at ambient temperature. All samples were diluted to a final concentration of 0.3 mg mL<sup>-1</sup> in 10 mM PBS (pH 7.0) and

scanned from 220–320 nm at room temperature. A rectangular quartz cuvette having 1 cm path-length was used. PBS was used as a blank solution for all the samples. Each sample was analyzed in triplicate.

## 2.8. Intrinsic fluorescence analysis

The intrinsic fluorescence of each sample was measured using an F-4600 fluorescence spectrophotometer (Hitachi, Tokyo, Japan) with a scanning speed of 12 000 nm min<sup>-1</sup> as described by Wu *et al.*<sup>36</sup> The excitation and emission wavelengths were 295 nm (slit width: 2.5 nm) and 320–400 nm (slit width: 2.5 nm), respectively. 10 mM PBS (pH 7.0) was used to dilute the samples and as a blank control.

## 2.9. Surface hydrophobicity analysis

The analysis of the surface hydrophobicity of treated  $\beta$ -LG samples was performed using ANS as a fluorescent probe following the protocol of Haskard and Li-Chan.<sup>37</sup> Each sample was serially diluted to concentrations of 0.01, 0.05, 0.1, 0.2, and 0.5 mg mL<sup>-1</sup> in 10 mM PBS (pH 7.0). In addition, 8.0 mM ANS solution was prepared in the same buffer. Before detection, 4.0 mL of each sample and 20  $\mu$ L of ANS solution were mixed and incubated in the dark for 15 min. The fluorescence intensities of all the samples were measured at 370 nm (excitation) and 490 nm (emission) using an F-4600 fluorescence spectrophotometer (Hitachi, Tokyo, Japan). All the measurements were performed in triplicate. The initial slope of the plot ( $S_0$ ) of the protein relative fluorescence intensity ( $F$ ) versus protein concentration ( $C$ ), as calculated by linear regression ( $r^2 = 0.99$ ), was used as an index of the surface hydrophobicity of the treated samples:

$$S_0 = \frac{\Delta F}{\Delta C}$$

## 2.10. Free amino group analysis

The free amino group contents of the treated samples were measured according to the OPA method.<sup>38</sup> Briefly, 200  $\mu$ L of each sample was mixed equally with 4 mL of OPA solution and incubated at 25 °C for 2 min. The content of free amino groups was measured at a wavelength of 340 nm using a UV-1101 spectrometer. All results were expressed as mol kg<sup>-1</sup> protein, and L-leucine was used as a standard.

## 2.11. Immunoblotting analysis

Immunoblotting analysis was performed to evaluate the IgG and IgE-binding potential of protein samples, as described by Yuan *et al.*<sup>31</sup> Briefly, protein bands on resolving gels were electrophoretically transferred onto a polyvinylidene fluoride membrane (Millipore Corporation). Next, the membrane was immersed in 3% BSA for 3 h to block nonspecific binding, followed by incubation at 4 °C overnight with primary antibodies, namely IgG (rabbit polyclonal antibodies, 1:15 000 dilution, v/v) or IgE (allergic human sera, 1:10 dilution, v/v) antibodies. After rinsing five times with PBST (10 mM PBS, containing 0.1% Tween 20, pH 7.4), the membrane was incubated for 2 h

with HRP-labeled goat anti-rabbit IgG (1 : 3000 dilution, v/v) or HRP-labeled goat anti-human IgE (1 : 50 dilution, v/v), as secondary antibodies. Upon a final rinse, the antibody-bound proteins on the blot were detected using an ECL substrate and photographed with a Tanon-4200SF (Tanon Science & Technology Co., Ltd, Shanghai, China). An optimal image was chosen under multiple exposure times.

### 2.12. Indirect ELISA analysis

The IgG- and IgE-binding potential of different protein samples was evaluated as described by Lin *et al.*<sup>39</sup> using IgG (rabbit polyclonal antibodies) and IgE (sera from allergic patients) antibodies. Each sample was diluted to 5  $\mu\text{g mL}^{-1}$  with 50 mM carbonate buffer solution (pH 9.6) and coated in a polystyrene 96-well plate (100  $\mu\text{L}$  per well). After coating and blocking, the plate was washed three times with PBST (10 mM PBS, containing 0.1% Tween 20, pH 7.4) and incubated with 100  $\mu\text{L}$  of rabbit polyclonal antibodies (1 : 40 000 dilution) or allergic human sera (1 : 100 dilution) at 37 °C for 1.5 h. Next, the plate was washed and incubated with HRP-labeled goat anti-rabbit IgG (1 : 10 000 dilution) or HRP-labeled goat anti-human IgE (1 : 1000 dilution) at 37 °C for 1 h. After the chromogenic reaction with TMB, the optical density (OD value) was measured at a wavelength of 450 nm using a MultiscanMK3 microplate reader (Thermo Fisher Scientific Inc., Waltham, MA, USA).

### 2.13. Statistical analysis

One-way analysis of variance with Duncan's multiple range tests was performed using SPSS version 19.0 (SPSS Inc., Chicago, IL, USA). Differences were considered significant when  $p < 0.05$ . All data are presented as the means  $\pm$  standard deviation of three independent experiments.

## 3. Results

### 3.1. SDS-PAGE of glycosylated and glycated $\beta$ -LG

The SDS-PAGE analysis of modified  $\beta$ -LG treated with TGase and/or GlcN is presented in Fig. 1. When  $\beta$ -LG was cross-

linked with TGase, new bands with higher MW were observed on top of the resolving gel, and the intensity of the original protein bands was weaker (Fig. 1A and B, Lane 2), indicating that peptide cross-links were generated. In the case of "TGase + GlcN" treated  $\beta$ -LG, the intensity of the original protein bands was very weak, while the intensity of the new protein bands enhanced substantially (Fig. 1A and B, Lane 4). Additionally, no new polymer bands appeared when  $\beta$ -LG was mixed with GlcN alone (Fig. 1A and B, Lane 3), as the MW remained unchanged. With increasing incubation temperature to 37 °C, "TGase + GlcN" treated  $\beta$ -LG showed higher-intensity polymer bands than those at 25 °C (Fig. 1B).

### 3.2. Evaluation of glycation/glycosylation by MALDI-TOF-MS

The MW changes of glycated/glycosylated peptides were analyzed using MALDI-TOF-MS as shown in Fig. 2A–E. The results revealed that non-conjugated, glycated, and glycosylated peptides coexisted in the mixture solution after incubation. Compared to the native peptide, the MW of the glycated peptide (treated with GlcN alone) was altered by adding a mass shift of 161.2 Da because of the release of 1 mol of water. Similarly, the glycosylated peptide (treated with "TGase + GlcN") was identified by a mass shift of 162.1 Da added because of the loss of 1 mol of ammonia.

Based on this calculation, three glycated peptides were produced following the Maillard reaction at 25 °C, with a MW of 1729.9 Da, 2707.5 Da and 2846.4 Da (Fig. 2B). After incubation at 37 °C, three glycated peptides with a MW of 1729.9 Da, 2089.2 Da and 2092.2 Da were acquired. In addition, two lysine sites were modified *via* saccharification, wherein the peptide had a molecular mass of 2092.2 Da (Fig. 2C). For glycosylation treatments, two glycated peptides (1715.9 Da and 2092.2 Da) and glycosylated peptides (2707.5 Da and 2773.6 Da) were generated at 25 °C (Fig. 2D). Furthermore, we obtained three glycated peptides 1715.9 Da, 2755.6 Da and 2833.5 (Da) and three glycosylated peptides (2669.9 Da, 2707.6 Da and 2847.6 Da) in the reaction at 37 °C (Fig. 2E); among them, two glutamine sites derived from the 2847.6 Da peptide were modified during the glycosylation reaction.

### 3.3. Secondary structures of glycosylated and glycated $\beta$ -LG

Far-UV CD spectra were used to investigate the alteration in the secondary structures of the modified  $\beta$ -LG. The secondary structure of protein contains  $\alpha$ -helices,  $\beta$ -sheets,  $\beta$ -turns, and random structures. The positive peak represents the  $\beta$ -sheets, and the negative peak represents the presence of the  $\alpha$ -helix. As shown in Fig. 3A and B, the negative peaks at approximately 210 nm indicated the presence of  $\alpha$ -helices in native  $\beta$ -LGs. Following the "TGase + GlcN" treatment, new positive peaks appeared at approximately 195 nm, which were related to the  $\beta$ -sheets that increased from 32.6% to 40.4% (25 °C) and 44.2% (37 °C), respectively. Moreover, the percentage of  $\alpha$ -helices decreased from 17.7% to 14.4% (25 °C) and 12.3% (37 °C). Similar results were observed when protein was incubated with TGase alone. For the GlcN-conjugated samples, the  $\alpha$ -helix content increased from 17.7% to 19.3% (25 °C) and

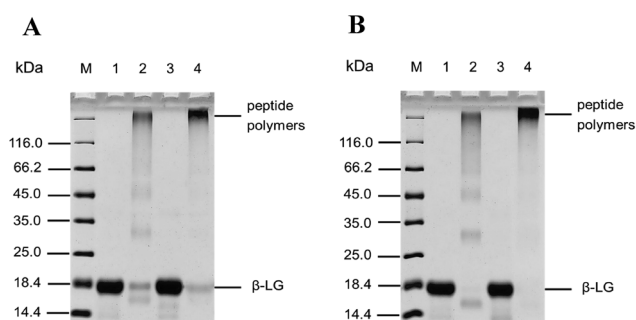


**Fig. 1** SDS-PAGE analysis of glycosylated and glycated  $\beta$ -LG. (A) Incubated at 25 °C. (B) Incubated at 37 °C. Lane M, protein marker; Lane 1, untreated  $\beta$ -LG; Lane 2,  $\beta$ -LG treated with TGase; Lane 3,  $\beta$ -LG treated with GlcN; Lane 4,  $\beta$ -LG treated with TGase + GlcN.

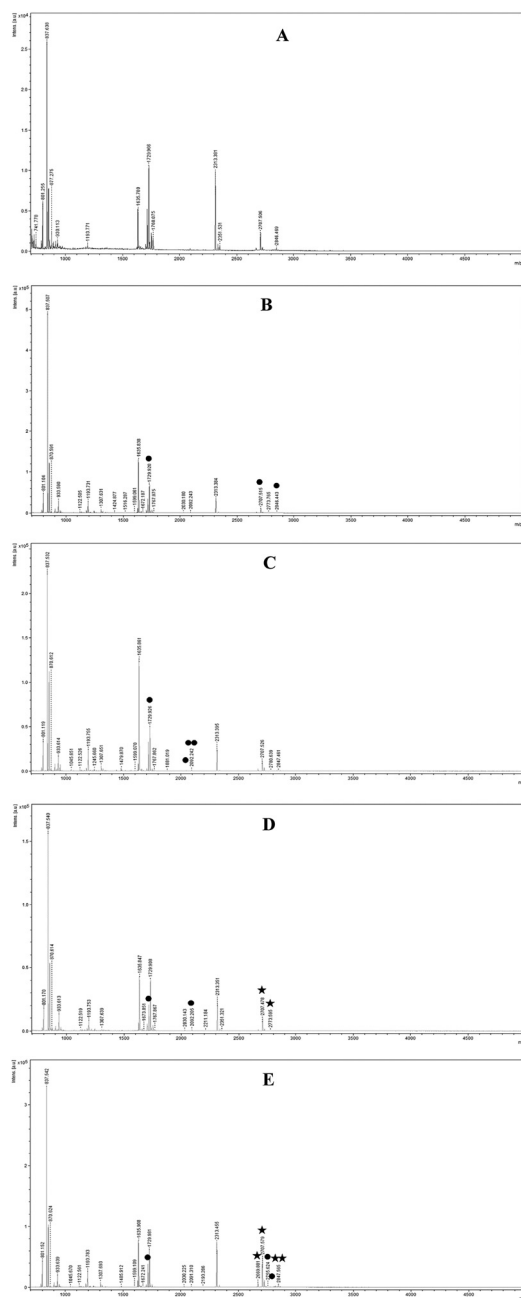


**Fig. 2** MALDI-TOF-MS analysis of glycosylated/glycosylated  $\beta$ -LG. (A) untreated protein. (B) Glycosylated with GlcN at 25 °C. (C) Glycosylated with GlcN at 37 °C. (D) Glycosylated with GlcN + TGase at 25 °C. (E) Glycosylated with GlcN + TGase at 37 °C. The filled circles (●) and pentacles (★) refer to glycosylated or glycosylated peptides, respectively.

20.4% (37 °C) respectively, while  $\beta$ -sheets decreased from 32.6% to 31.8% and 29.1%, respectively. Under each treatment, the proportion of  $\beta$ -turns and random coils varied slightly.

### 3.4. UV absorption of glycosylated and glycosylated $\beta$ -LG

The multistage structural changes of  $\beta$ -LG were detected by UV absorption analysis. As shown in Fig. 4A and B, a 3.0 nm blue

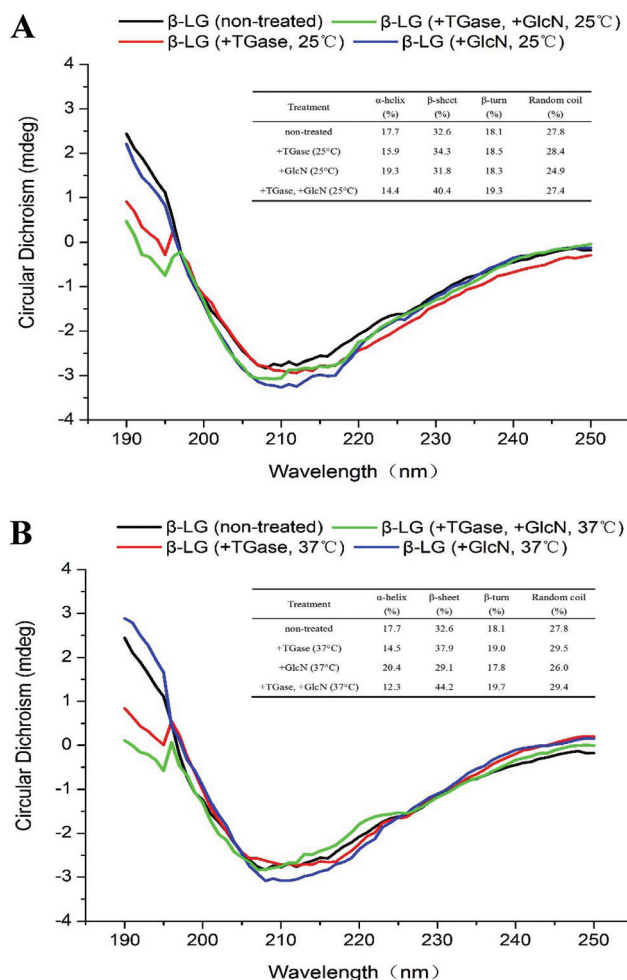


**Fig. 3** Secondary structural changes of glycosylated and glycosylated  $\beta$ -LG, measured by far-UV CD spectroscopy. (A) Incubated at 25 °C. (B) Incubated at 37 °C.

shift in the maximum absorbance peak occurred when the protein was treated with TGase and GlcN at both 25 °C and 37 °C. GlcN-induced glycation led to a 2.0 nm and 2.5 nm blue shift when incubated at 25 °C and 37 °C, respectively. In contrast, cross-linking samples without sugar did not change the wavelength of the maximum peak. The varieties of UV absorption suggested that specific amino acids of  $\beta$ -LG (such as tyrosine and tryptophan) were exposed following treatment.

### 3.5. Intrinsic fluorescence of glycosylated and glycosylated $\beta$ -LG

The intrinsic fluorescence of  $\beta$ -LG was excited at 295 nm, so that the fluorescence intensity was totally attributed to tryptophan residues, as well as eliminating the interference of tyrosine. As shown in Fig. 4C and D, non-treated  $\beta$ -LG showed a much lower fluorescence intensity than the other samples. The fluorescence intensity of the “TGase + GlcN” treated sample reached a maximum that was approximately 200% (25 °C) and 250% (37 °C) of the non-treated sample, respectively. Furthermore, TGase-treated  $\beta$ -LG exhibited a slight red

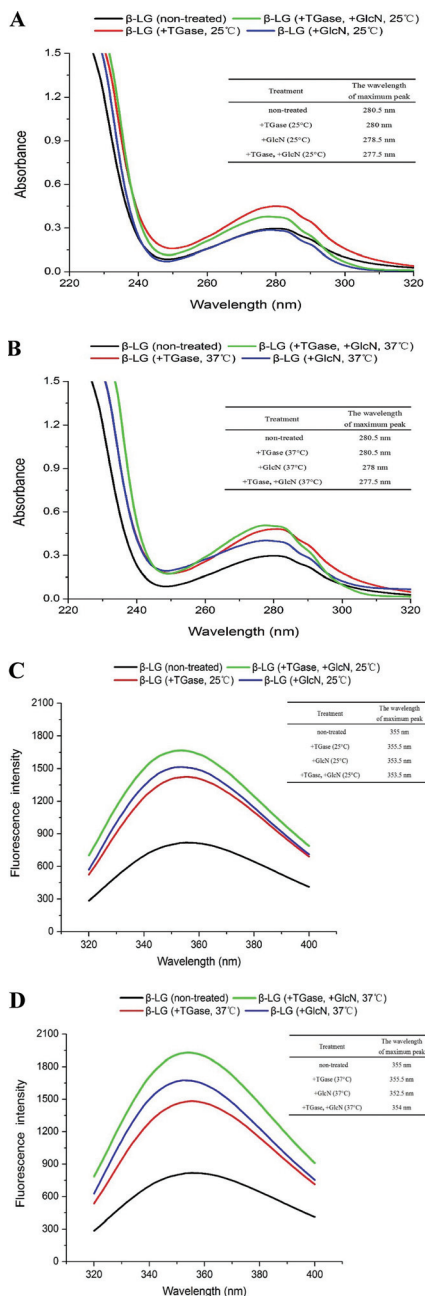


**Fig. 4** Effect of glycosylation and glycation on the UV absorption and intrinsic fluorescence of  $\beta$ -LG. (A and B) UV absorption spectroscopy incubated at 25 °C and 37 °C, respectively. (C and D) Fluorescence emission spectroscopy incubated at 25 °C and 37 °C.

shift in the maximum absorbance peak, while the reaction with GlcN or “TGase + GlcN” triggered a mild blue shift. Although the wavelength of the maximum absorption was not altered significantly, the clear increase in the maximum fluorescence intensities may have been caused by the conformational changes in the microenvironment, where tryptophan residues are susceptible to the microenvironment due to protein unfolding and the binding of GlcN to the other peptide sites.

### 3.6. Surface hydrophobicity of glycosylated and glycated $\beta$ -LG

The surface hydrophobicity of  $\beta$ -LG samples was evaluated under all conditions. As shown in Fig. 5A, the hydrophobicity value of non-treated  $\beta$ -LG was 890.5. When incubated at 25 °C, enzymatic cross-linking resulted in a higher surface hydrophobicity of 1005.5. However, conjugation of GlcN significantly reduced the hydrophobicity to 797.3, while for the “TGase + GlcN” treated sample it was 931.9.

### 3.7. Free amino groups

As shown in Fig. 5B, the content of free amino groups decreased following any modifications. The contents were reduced to 2.31 mol kg<sup>-1</sup> protein from 2.74 mol kg<sup>-1</sup> protein after cross-linking with TGase at 25 °C, while those of GlcN and “TGase + GlcN” treated samples were 2.07 mol kg<sup>-1</sup> protein and 1.97 mol kg<sup>-1</sup> protein, respectively. We also observed that higher temperatures led to a greater loss of free amino groups. These data indicate that free amino groups were unbound and decreased faster during the Maillard reaction and enzymatic glycosylation.

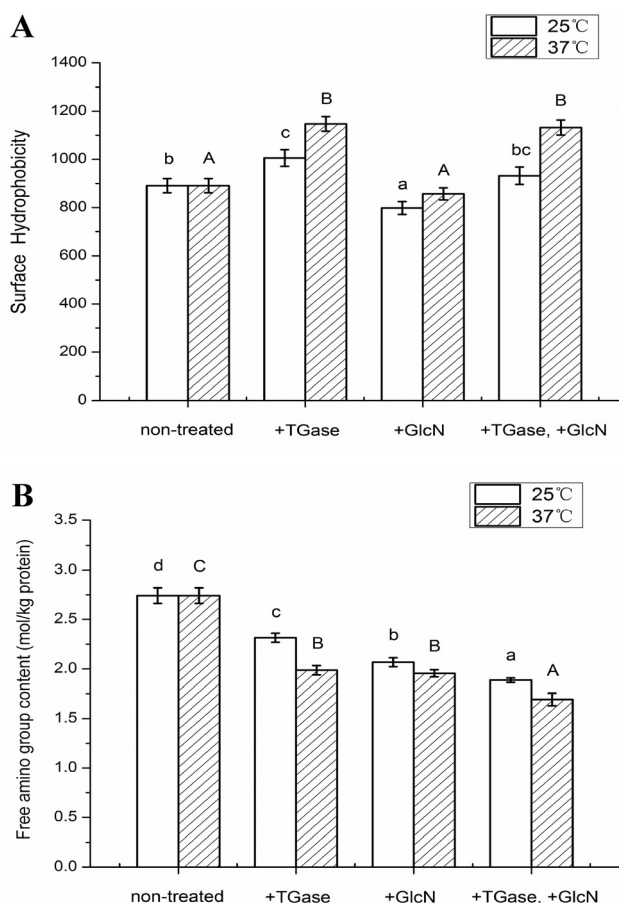


**Fig. 5** Effect of glycosylation and glycation on the surface hydrophobicity and free amino groups of  $\beta$ -LG. (A) Surface hydrophobicity incubated at 25 °C and 37 °C. (B) Free amino group content incubated at 25 °C and 37 °C. Different letters above the columns indicate significant differences ( $p < 0.05$ ). Error bars represent the standard deviation of the mean ( $X \pm SD$ ).

### 3.8. Immunoblotting

Immunoblotting was performed to evaluate the effects of glycation and glycosylation on the IgG- and IgE-binding capacities, using rabbit polyclonal antibodies or serum from patients with milk allergy. Upon treatment of  $\beta$ -LG with TGase and TGase + GlcN, new bands of high MW appeared, indicating cross-linked and glycosylated products. The density of immune-bands of native  $\beta$ -LG at 18.4 kDa became weaker, which is consistent with the SDS-PAGE results (Fig. 6, Lane 2 and 4). Moreover, the high MW protein aggregates were detected by specific antibodies up to certain extent, while the native  $\beta$ -LG bands at 18.4 kDa showed less IgG-, especially IgE-binding upon treatment. This shows that most of the  $\beta$ -LG was modified through crosslinking and glycosylation. On the other hand, the GlcN treated sample showed minor changes compared to the control sample and binds to specific antibodies (Fig. 6, Lane 3). With the rise in the incubation temperature

(37 °C), the immunoblotting results showed that the high MW protein bands were at similar positions; however the intensity of native  $\beta$ -LG bands decreased a bit more and showed very reduced IgG and IgE binding potentials (Fig. 6B and D).

### 3.9. Indirect ELISA

Indirect ELISA was performed to quantitatively evaluate the reduction in the IgG and IgE binding activities of treated  $\beta$ -LG. A significant ( $p < 0.05$ ) reduction in the IgG-binding activity of  $\beta$ -LG was noticed following treatment with TGase, GlcN and their combination, as indicated in Fig. 7A. By treating with TGase + GlcN at 37 °C,  $\beta$ -LG was glycosylated, which caused the highest reduction in the IgG-binding activity (52.7%) compared to the other treatments.  $\beta$ -LG glycation was observed in the presence of GlcN alone and the IgG-binding capacity significantly decreased compared to the control. However, treatment of  $\beta$ -LG with only TGase or GlcN exhibited a

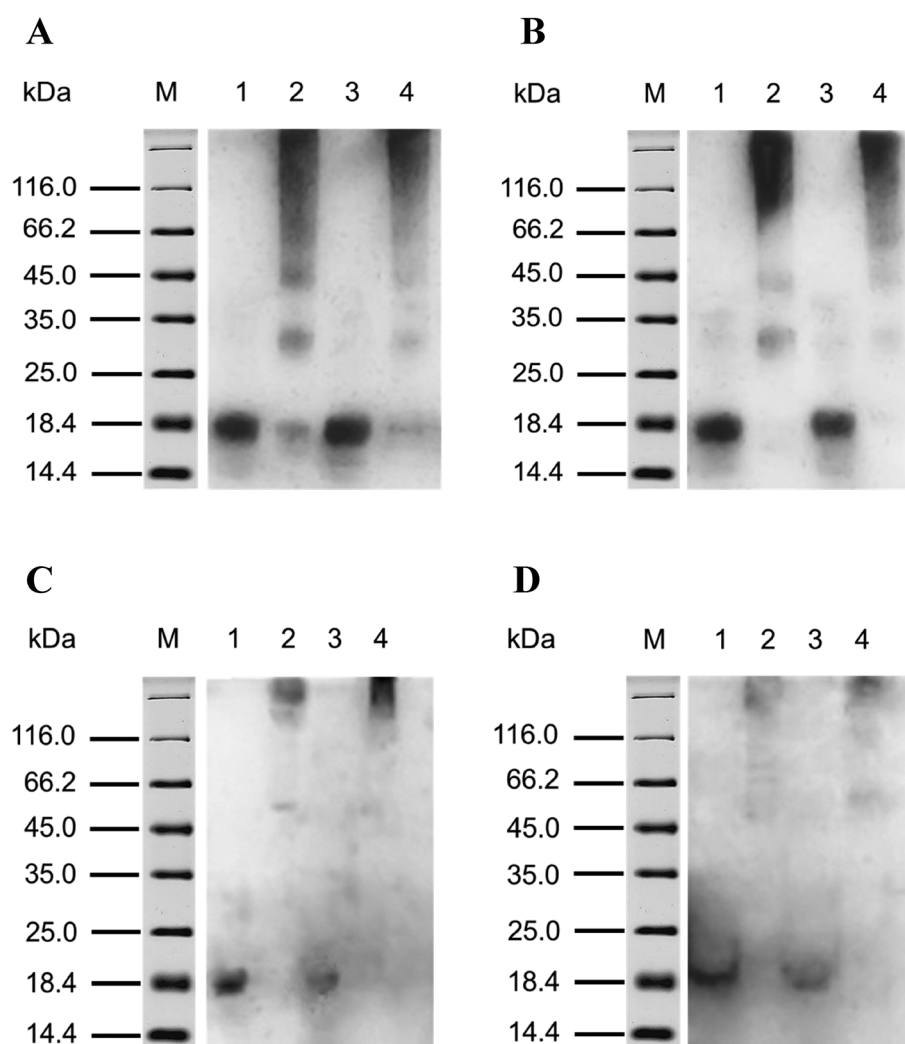


**Fig. 6** Determination of the glycosylated and glycated  $\beta$ -LG, as measured by immunoblotting. (A and B) Investigation of specific IgG-binding activities of the treated  $\beta$ -LG using rabbit polyclonal antibodies. (C and D) Investigation of IgE-binding activities of the treated  $\beta$ -LG using pooled sera from patients allergic to  $\beta$ -LG. (A and C). Incubated at 25 °C. (B and D). Incubated at 37 °C. Lane M, protein marker; Lane 1, un-treated  $\beta$ -LG; Lane 2,  $\beta$ -LG treated with TGase; Lane 3,  $\beta$ -LG treated with GlcN; Lane 4,  $\beta$ -LG treated with TGase + GlcN.

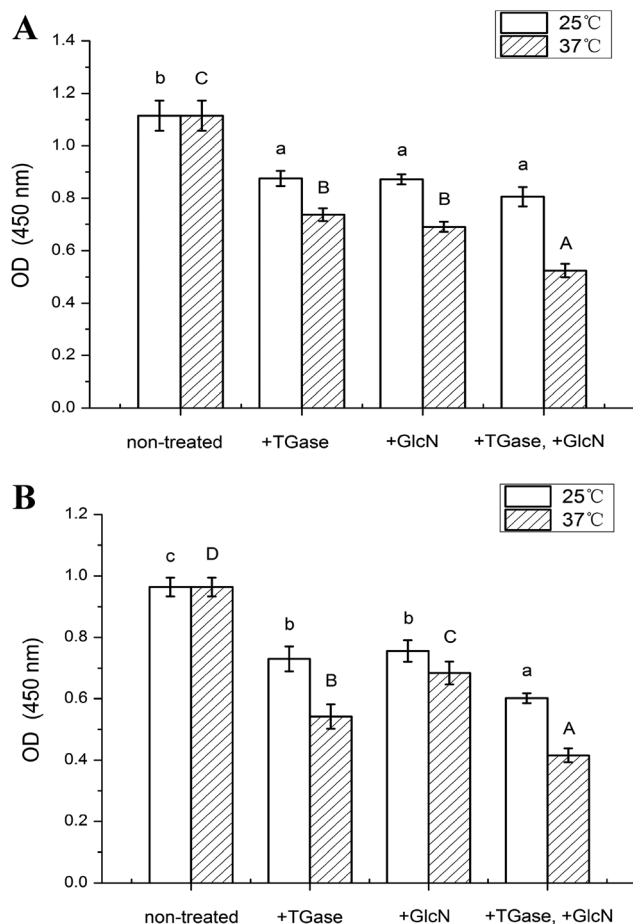


**Fig. 7** Indirect ELISA analysis of glycosylated and glycated  $\beta$ -LG. (A) Investigation of specific IgG-binding activities of the treated  $\beta$ -LG using rabbit polyclonal antibodies. (B) Investigation of IgE-binding activity of the treated  $\beta$ -LG using pooled sera from patients allergic to  $\beta$ -LG. Different letters above the columns indicate significant differences ( $p < 0.05$ ). Error bars represent the standard deviation of the mean ( $\bar{X} \pm \text{SD}$ ).

slightly lower IgG-binding capacity. Fig. 7B shows the effect of glycation and glycosylation on the IgE-binding capacity of  $\beta$ -LG at different temperatures (25 °C and 37 °C). The initial IgE-binding of the native protein was significantly higher ( $p < 0.05$ ) than that of glycated and glycosylated  $\beta$ -LG. TGase or GlcN treated  $\beta$ -LG showed somewhat lower IgE-binding capacity. While, TGase + GlcN treated  $\beta$ -LG showed the largest decline in the IgE-binding activity (56.3%) among all groups. In general, the reduction was more obvious when the incubation temperature was increased from 25 °C to 37 °C. Moreover, the treatment of TGase in conjunction with GlcN led to the highest decrease in allergenicity, which was correlated with the rate of glycosylation.

## 4. Discussion

According to DeJong and Koppelman,<sup>29</sup> glutamine residues and primary amines were defined as acyl donors and acyl

acceptors, respectively. Because GlcN contains primary amines,  $\epsilon$ -( $\gamma$ -glutamyl)-lysine cross-links were formed between sugar and protein *via* TGase. Notably, intra- and inter-linkage of  $\beta$ -LG is inevitable during this stage. Thus, TGase can catalyze a series of modifications with protein glutamyl and GlcN residues.

MALDI-TOF-MS was applied to understand the effect of glycation and glycosylation on the protein and peptide levels. Several researchers prefer this method to precisely analyze the MW of food protein and peptides upon various treatments, which otherwise could be destroyed by other ionization techniques.<sup>35,40,41</sup>

According to Hrynets *et al.*<sup>35</sup> a mass increase of 161.2 Da between the natural protein and glycosylated sample revealed that various Maillard compounds were generated. Therefore, the mass shift of peptides was determined to be +161.2 Da [(peptide + GlcN) – H<sub>2</sub>O]. In the case of TGase-induced glycosylation, the mass increase between the non-treated  $\beta$ -LG and glycosylated protein was considered to be 162.1 Da due to the liberation of ammonia when GlcN was conjugated to the peptide under enzymatic conditions.

The results of MALDI-TOF-MS showed that the Maillard reaction was not suppressed completely in the enzymatic reaction system, indicating that competition between glycosylation and glycation occurred when TGase was used. Generally, when the enzyme was added to the mixture of protein and GlcN, the extent of non-enzymatic glycation was reduced. This phenomenon may be ascribed to the partial steric hindrance of enzymatic polymers, as the modification of glutamine residues may block nearby lysine residues for further glycation.<sup>45</sup> A higher temperature of 37 °C led to an accelerated reaction rate in both glycation and glycosylation, because TGase shows better enzyme activity near the optimal temperature (40–50 °C). Moreover, the Maillard reaction is also generally promoted at higher temperatures.<sup>46</sup> In this study, glycosylation was not found to dominate the mixed system. However, the formation of the cross-linked product during glycosylation inhibited the Maillard reaction at mild temperature. This is because Maillard primary products such as Schiff base and Amadori rearrangement often have unstable structures and are easy to translate into advanced glycation end products or other harmful substances.<sup>47</sup> In contrast, the formation of stable isopeptide bonds improved the stability of glycosylated conjugates and relatively reduced the generation of undesired Maillard by-products. It is reported that large MW proteins and peptides are retained during food processing and preservation, which might be due to the hindering of leaching and slowing down of protein degradation following various treatments. As a consequence, the retention of nutrients and sustainability of food improved during preservation.<sup>42–44</sup>

The results of CD spectra, UV absorption, and intrinsic fluorescence revealed the structural changes of modified  $\beta$ -LG. When compared to native  $\beta$ -LG, glycosylated  $\beta$ -LG showed an increase in  $\beta$ -sheets and fewer  $\alpha$ -helix structures. However, the percentages of  $\beta$ -turns and random coils were not significantly altered. According to previous studies, TGase catalyzes acyl-

transfer reactions between acyl donors (peptide-bound glutamine residues) and acyl acceptors (primary amines).<sup>27,29</sup> TGase can induce protein glutamyl residue modifications with GlcN residues, as GlcN contains primary amines. This modification leads to structural changes in  $\beta$ -LG, subsequently influencing its antigenicity and allergenicity. The present results are in agreement with the previous report of Yuan *et al.*<sup>31</sup> who observed that glycosylation and glycation of food allergen induced alteration in the secondary structure. These findings fairly agree with a previous study of Song and Zhao,<sup>27</sup> who observed that the cross-linking of soybean protein by TGase caused a reduction in the  $\alpha$ -helix and  $\beta$ -structure. The amino acid side chains of  $\beta$ -LG were polymerized by treating with TGase and GlcN, thus leading to the alteration in the secondary structure.<sup>31,48–50</sup>

The UV absorption and intrinsic fluorescence intensity were increased significantly after incubation, particularly at 37 °C. These results suggest that after cross-linking with sugar chains or other peptides,  $\beta$ -LG was unfolded and more aromatic amino acid residues (tryptophan and tyrosine) were exposed to the surface.<sup>51</sup> Because these amino acid residues are sensitive to changes in the surroundings, the slight blue shift during glycation and glycosylation indicated that the polarity of the microenvironment of tryptophan and tyrosine had changed, which was accordant with the altered conformation of  $\beta$ -LG.<sup>31,52–54</sup>

Although hydrophilicity was significantly enhanced by GlcN-conjugation, enzymatic cross-linking resulted in an increase in surface hydrophobicity, which is consistent with previous studies.<sup>55</sup> OH and NH<sub>2</sub> are hydrophilic groups in saccharides, and thus the incorporation of carbohydrate decreases the surface hydrophobicity. Glutamine is a kind of hydrophilic amino acid that is consumed during TGase mediated cross-linking. Furthermore, the enzymatic reaction led to unfolding of  $\beta$ -LG and exposure of buried hydrophobic residues.<sup>54</sup> These results agreed with those of UV absorption and intrinsic fluorescence intensity analysis.

The reduction in the surface hydrophobicity may be attributed to the incorporation of activated carbon (containing hydrophilic –OH and –NH<sub>2</sub>) during glycation, while the significant increase may be related to the unfolding and subsequent aggregation of  $\beta$ -LG. Interestingly, the increase in surface hydrophobicity was in accordance with the rise in temperature from 25 °C to 37 °C, as the surface hydrophobicity may increase significantly with the rate of cross-linking and glycosylation.<sup>26,27,56</sup>

Several amino acid residues such as glutamine and lysine contain free amino groups and involve the Maillard reaction and TGase-catalyzed cross-linking. These amino acids are also essential components of the allergic epitopes in bovine  $\beta$ -LG. After either treatment, free amino group contents decreased significantly. These results indicate that acyl transfer reactions occurred between primary amines from GlcN and glutamine from  $\beta$ -LG. Furthermore, free amino groups of protein can react with GlcN through an amino-carbonyl reaction.<sup>57</sup> Thus, the amino group losses provide preliminary evidence that the

amino acids of peptides were modified, which may have affected the conformational structures of  $\beta$ -LG.

Immunoblotting results showed a reduction in the IgG and IgE binding potentials of native  $\beta$ -LG upon treatments, which is in accordance with previous studies.<sup>31,58</sup> The high MW protein molecules revealed some binding to specific antibodies, while the native allergenic protein band revealed reduced binding potentials. Tong *et al.*<sup>54</sup> and Ahmed *et al.*<sup>59</sup> also observed reduced IgG and IgE binding to animal antibodies and pooled allergic patients' sera at the native allergenic protein following treatments. However, the high MW polymers were still bound to the specific antibodies, which might be due to the retainability of some linear epitopes even after modification. Likewise, several researchers also observed varying IgG/IgE binding patterns to allergenic protein and the new polymer bands upon various processing methods.<sup>34,53,59–61</sup> Crosslinking, glycosylation and glycation might induce partial unfolding and subsequent exposure of some epitopes on the protein surface, which still possess the capacity to bind to the specific antibodies. Due to unfolding, the tertiary and quaternary structures denatured,<sup>31,34,39,50,51</sup> which elucidates the reason for the reduced IgG/IgE binding capacities of the treated  $\beta$ -LG. Nonetheless, despite the binding of high MW polymers to the antibodies, the polymers may not be as allergenic as the native allergens due to the alteration of the binding epitopes.

The reduction in immunogenic activity was confirmed *via* indirect ELISA analysis, as the decrease in allergenicity may be attributed to the structural changes *via* glycation or glycosylation. During the reaction, the partial unfolding of protein may have led to the exposure of antigen peptides, influencing the modification of free amino acid residues such as lysine and glutamine. Epitope alteration and high MW product formations may have affected the binding capacity to polyclonal antibodies. Moreover, these new peptides may differ in antigenicity.<sup>31,61,62</sup> However, these treatments cannot completely eliminate IgG/IgE recognition of  $\beta$ -LG, suggesting that there are linear epitopes present that cannot be modified by TGase and GlcN, or that the modified epitopes retain allergenicity.<sup>34,59,60</sup> The data also showed that higher temperatures resulted in lower antigenicity and allergenicity, indicating that the efficiency of glycation or glycosylation was promoted, which agreed with the results of MALDI-TOF-MS and the content of free amino groups.

## 5. Conclusions

In this study, we explored the changes in the conformational structure and potential allergenicity of glycated and TGase-mediated glycosylated  $\beta$ -LG with GlcN. Glycation and TGase-catalyzed glycosylation modified the linear and conformation structures of  $\beta$ -LG. The partial unfolding and aggregation of the protein conformation were reflected by the decreased  $\beta$ -sheets, enhanced intrinsic fluorescence, blue-shift in UV absorption spectra, and alterations in surface hydrophobicity

and free amino groups. The MALDI-TOF-MS results showed that the glutamine residues of the immuno-activated peptides were modified after glycosylation. Subsequently, the allergenicity of the treated protein reduced substantially due to the alteration in the IgG/IgE binding epitopes. The most significant decrease in allergenicity was observed following the treatment of  $\beta$ -LG with TGase and GlcN at 37 °C. Compared to glycation, enzymatic glycosylation can site-specifically modify the allergen peptides under mild conditions, and thus has the potential to be applied in hypoallergenic food processing.

## Conflicts of interest

There are no conflicts to declare.

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