



Comparative study on the effects of nystose and fructofuranosyl nystose in the glycation reaction on the antigenicity and conformation of β -lactoglobulin



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ABSTRACT

Our previous work indicated that the antigenicity of bovine β -lactoglobulin (β -LG) decreased after conjugation with fructo-oligosaccharides (FOS) which was related to its conformational changes. In attempt to unravel further changes of β -LG antigenicity, nystose (GF_3) and 1^F- β -fructofuranosyl nystose (GF_4) of FOS were used to investigate the relationship between conformation and antigenicity. The antigenicity of β -LG after conjugated with GF_3 and GF_4 decreased from 143.4 to 29.5 and 31.6 μ g/mL, respectively. The results of mass spectrometry revealed that the molecular weight of β -LG increased from 18.4 to 19.8 and 19.1 kDa after conjugation with GF_3 and GF_4 , respectively. It was shown that the conformational changes of β -LG after conjugation with GF_3 were bigger than that with GF_4 , including quenching of fluorescence intensity, the red-shift of fluorescence spectra, and the increase in sulfhydryl content. However, there was no significant difference in the antigenicity between β -LG- GF_3 and β -LG- GF_4 conjugates ($P > 0.05$).

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1. Introduction

Bovine β -lactoglobulin (β -LG), the major whey protein in milk with great nutritional properties, has been considered as a valuable dairy ingredient in recent years (Moro, Baez, Busti, Ballerini, & Delorenzi, 2011). To further improve the functionality of and decline the antigenicity of β -LG, numerous chemical treatments, such as phosphorylation (Enomoto et al., 2009; Wong, Li, Wong, Jiang, & Shaw, 2012), polyethylene glycol (Kumar, Sharma, & Kalonia, 2009), and glycation (Corzo-Martinez, Sanchez, Moreno, Patino, & Villamiel, 2012; Jones, Lesmes, Dubin, & Mc Clements, 2010), have been used to modified the protein. Among them, the glycation reaction is the most representative one that does not utilize toxic chemical reagents (Zeng, Zhang, Guan, Zhang, & Sun, 2013).

In addition to reports on the impact of β -LG modification by glycation, structural changes of β -LG during the reaction has also been explored besides the functional properties and antigenicity. (Jimenez-Castano, Villamiel, Martin-Alvarez, Olano, & Lopez-Fandino, 2005; Thomsen et al., 2012). Furthermore, the nature of the saccharides used for glycation showed profound

influence on the structural changes of β -LG. For example, it was shown that the decrease in the antigenicity of β -LG conjugated with phosphoryl oligosaccharides was more obvious than that conjugated with alginic acid oligosaccharide (Hattori et al., 2004); Kobayashi et al. (2001) concluded that conjugation with carboxymethyl dextran of higher molecular weight is effective in reducing the immunogenicity of β -LG. However, it was found that the conjugation with trimethyl chitosan can increase the immunogenicity of protein (Slutter et al., 2010).

In our previous work, it was observed that β -LG conjugated with Fructo-oligosaccharides (FOS) significantly improved its functional properties and decreased its antigenicity (Zhong et al., 2013). Meanwhile, conformational changes of β -LG during the glycation reaction were related to changes in functional properties and antigenicity (Zhong et al., 2013). FOS is mixture of oligosaccharides, which consists of different structures, including 1^F-(1- β -fructofuranosyl)_{n-1} sucrose; such as, 1-kestose ($n = 2$, GF_2), nystose ($n = 3$, GF_3), 1^F- β -fructofuranosyl nystose ($n = 4$, GF_4). Although β -LG glyated with FOS had a significant effect on its antigenicity, it was unclear which 1^F-(1- β -fructofuranosyl)_{n-1} sucrose of FOS played a main role on decreasing β -LG antigenicity. In addition, little information is available on the comparative study about antigenicity and conformational changes of β -LG conjugated with different components in the FOS.

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As we know, fructo-oligosaccharides are commonly used as a sweetener and a prebiotic dietary supplement in dairy products. They have been shown to have minimal effect on blood sugar levels when used as sweeteners for those who have diabetes. To maximize the efficiency of β -LG-FOS in the food industry, it would be desirable to obtain more detailed information on how β -LG is modified by the components of the FOS. Therefore, the present study was undertaken to further elucidate how FOS changes antigenicity of β -LG related to the conformational changes. For this purpose, GF₃ and GF₄ of FOS were chosen as the glycation materials. The effect of glycation with GF₃ and GF₄ of FOS on antigenicity of β -LG was investigated in relation to conformational changes of β -LG, characterized by Quadrupole Time-of-Flight (Q-TOF) LC/MS spectrometer, sulfhydryl (SH) content, surface hydrophobicity (H_0), intrinsic fluorescence spectra, and circular dichroism spectra.

2. Materials and methods

2.1. Materials and reagents

Bovine β -LG (L3908) was obtained from Sigma Chemical Company (Aldrich, USA). Nystose (GF₃) and fructofructosyl nystose (GF₄) were obtained from Sigma Chemical Co. (St Louis, MO, USA). All other reagents were of analytical grade, and solutions were made in double-distilled water.

2.2. β -LG glycation

β -LG was conjugated with GF₃ and GF₄ in the following conditions according to Hattori et al. (2004), Li, Luo, and Feng (2011) with slight modification. β -LG and GF_n ($n = 3$ or 4 , w/w: 1:0 and 1:4) were dissolved in 600 mL distilled water and then lyophilized. The mixture was incubated at 50 °C at a relative humidity of 79% for 24 h. After dialysis against distilled water and lyophilization, crude β -LG-GF₃ and β -LG-GF₄ conjugates were obtained. Free saccharine was removed by salting out. The crude sample was dissolved in distilled water, and ammonium sulfate was added for a final concentration of 5 M in solution. The precipitate was recovered by centrifuging (48,400g for 30 min, 20 °C). The purified conjugate was obtained after dialysis against distilled water and lyophilization. β -LG was heated at conjugation conditions without sugar to serve as a control. All glycation experiments were performed in triplicate.

2.3. Antigenicity analysis

The antigenicity of different β -LG samples was estimated by indirect competitive ELISA according to Zhong et al. (2011). 96-wells microtitre plates (flat-bottomed; Costar, Corning, USA) were coated with 100 μ L/well of 1 μ g/mL antigen and were incubated overnight at 4 °C. Residual free binding sites were blocked with 1% pig gelatine in PBS/Tween solution (PBST) for 30 min at room temperature. The wells of the plates were washed six times with PBST. Competition was initiated by adding 50 μ L of either the standard or a sample solution containing 0.25–50 μ g/mL of β -LG in PBST and 50 μ L anti- β -LG-IgY (10 μ g/mL) to the wells. The plates were incubated overnight at 4 °C. After removing the solutions, the wells were washed six times with PBST. A 100 μ L solution purified anti-rabbit IgG (Sigma, A6154), diluted 1:10,000 in PBST was added and the plates were incubated for 2 h at 37 °C. After washing, 50 μ L H₂O₂-urea solutions and 50 μ L tetramethylbenzidine-solution were immediately added to each well. The reaction was stopped by adding sulfuric acid (100 μ L, 1 M). Absorption was measured at 450 nm.

The antigenic response of the samples was calculated from a standard curve of β -LG, for which a linear logarithmic correlation was observed in the range of 0.01–400 μ g/mL for β -LG. All analyses were carried out in triplicate and the averaged values were converted to concentration equivalents in micrograms per milliliter (μ g/mL).

2.4. Q-TOF mass spectrometry analysis

The method by Zhong et al. (2013) was used to determine the molecular weight of β -LG and β -LG-GF_n ($n = 3$ or 4) conjugates. Protein was analyzed using the Agilent 6538-accurate-mass Quadrupole Time-of-Flight (Q-TOF) LC/MS spectrometer (Agilent Technologies, Waldbronn, Germany). Samples were diluted in a saturated solution of R-cycano-4-hydroxycinnamic acid in 0.1% TFA with 33% acetonitrile. An aliquot of this mixture was spotted onto a stainless steel target, air-dried, and subjected to mass determination (Medrano, Abirached, Panizzolo, Moyna, & Anon, 2009). The monitored mass range was m/z 100–20,000.

2.5. Determination of surface hydrophobicity (H_0).

The H_0 was determined with the fluorescence probe 8-anilino-1-naphthalene sulfonic acid (ANS) according to the method of Haskard and Li-Chan (1998). Serial dilutions of protein samples (stock solutions; 1.5%, w/v) were made in 0.01 M phosphate buffer (pH 7.0) to final concentrations of 0.004–0.02% (w/v). ANS[−] solution (8.0 mM) was also prepared in the same buffer. ANS[−] solution (20 μ L) was added to 4 mL of each diluted solution and the fluorescence intensity (FI) of the mixture was measured at 370 nm (excitation) and 470 nm (emission) using an F4500 fluorescence-spectrophotometer (Hitachi Co., Japan). The initial slope of the FI versus protein concentration (mg/mL) plot (calculated by linear regression analysis) was used as an index of H_0 .

2.6. Determination of free sulfhydryls (SH) contents

Free SH content was analyzed using Ellman's reagent (Ellman, 1959; Sava, Van der Plancken, Claeys, & Hendrickx, 2005). Free SH content for β -LG samples was obtained by adding β -LG solution (1 mg/mL) to 0.1 M pH 8.0 sodium phosphate buffer. The above solutions were incubated at 25 °C for 1 h. Fifty microliters of each sample was extracted from the above solutions after 1 h and 25 μ L of 0.01 mol DTNB (5,5'-dithiobis-2-nitrobenzoic acid) was added to it. The solution was then diluted to 10 mL. The UV absorbance at 412 nm was measured at 25 °C. The SH content was calculated using the extinction coefficient of NTB (2-nitro-5-thiobenzoate) at 412 nm, 13,600 M^{−1} cm^{−1}, expressed as μ mol/g of protein.

2.7. Circular dichroism spectroscopy analysis

Circular dichroism (CD) spectroscopy analyses were run for different β -LG samples according to Divsalar, Saboury, Ahmad, and Moosavi-Movahedi (2009). Far-UV CD spectra of β -LG at 0.1 mg/mL in 10 mM sodium phosphate buffer, pH 7.0, were collected at 22 °C using a MOS-450 spectropolarimeter (French Bio-Logic SAS Co., Claix, French). Cylindrical quartz cuvette with path lengths of 0.1 cm was used for collecting data in the far-UV (190–250 nm) regions. Four scans were performed for each sample using the following conditions: 1 nm step resolution, 100 nm/min scan rate, and 1.0 nm of bandwidth. The scans were corrected by subtracting the buffer baseline spectrum. Structure predictions from CD spectra were obtained using the Contin LL program (de la Hoz & Netto, 2008).

2.8. Intrinsic fluorescence analysis

Fluorescence analyses were performed for β -LG samples, as described by [de la Hoz and Netto \(2008\)](#). Fluorescence measurements were carried out in a Hitachi Spectro-fluorimeter F-4500 (Hitachi, Tokyo, Japan) using 10 mm square quartz cells. Sample protein concentration was 2 mg/mL in phosphate buffer (10 mM, pH 7.0). The excitation wavelength was 280 nm and the emission spectrum was scanned from 300 to 450 nm using a 5 nm bandwidth, excitation and emission slits of 2.5 and 5.0 nm, respectively, and a scan speed of 240 nm/min.

2.9. Statistical analysis

All glycation experiments were performed in triplicate. All glycation samples and control samples were analyzed in triplicate. The statistical analysis was established by variance analysis (ANOVA).

3. Results and discussion

3.1. The effect of conjugation with GF₃ and GF₄ on antigenicity of β -LG

Our previous study indicated that the conjugation of FOS significantly decreased the antigenicity of β -LG ([Zhong et al., 2013](#)). Similarly, the antigenicity of β -LG clearly declined after conjugation with GF₃ and GF₄. As shown in the [Table 1](#), the antigenicity of the control β -LG was 143.4 μ g/mL. A significant decrease in the antigenicity of β -LG was observed after the glycation modification ($P < 0.05$). The values of antigenicity of β -LG conjugated with GF₃ and GF₄ in the FOS were 29.5 and 31.6 μ g/mL, respectively. Interestingly, there was no significant difference between the

antigenicity of β -LG conjugated with GF₃ and GF₄ ($P > 0.05$). Our previous work indicated that the antigenicity of β -LG was related to conformational changes ([Liu et al., 2011](#); [Zhong et al., 2011, 2012, 2013](#)). However, since there was no significant difference in the antigenicity between β -LG-GF₃ and β -LG-GF₄ in this study, we demonstrated that further studies would be required to gain more information regarding conformational changes of β -LG following conjugation with GF₃ and GF₄.

3.2. Changes in molecular weight

The altered molecular weight due to conjugation of β -LG with GF₃ and GF₄ was analyzed by mass spectrometry. It was reported that proteins and sugars interact, forming a covalent bond between the amino residue of protein and the reducing end of sugar ([Kato, Minaki, & Kobayashi, 1993](#)). Thus, if the protein and sugar were successfully conjugated, the molecular weight of the resulting β -LG-sugar conjugates would be increased compared to the protein or sugar alone. As shown in [Figs. 1 and 2](#), the molecular weight of β -LG following conjugation with GF₃ and GF₄ was increased from 18.4 to 19.8 and 19.1 kDa, respectively. This indicated that β -LG was successfully conjugated with GF₃ and GF₄ by formation of a covalent bond. In our previous work, the molecular weight of the β -LG-FOS conjugate was 19.9 kDa, which was about 1.5 kDa higher than that of control β -Lg ([Zhong et al., 2013](#)). The molecular weight of GF₃ and GF₄ were 666 and 828 Da, respectively. Thus, it was concluded that the number of GF₃ and GF₄ conjugated with β -Lg may be three and one, respectively. According to [Zhang et al. \(2014\)](#), the saccharide size had a significant effect on degree of glycation. It was reported that when saccharide size increased, the glycation degree declined. This may directly influence β -LG conjugations ([Zhang et al., 2014](#)).

Table 1

The antigenicity, surface SH contents, and surface hydrophobicity (H_0) of control β -LG and β -LG after conjugated with GF₃ and GF₄.

Sample	Antigenicity (μ g/mL)	Surface SH contents (μ mol/g)	Surface hydrophobicity (H_0)
β -LG (control)	143.4 $\pm$ 2.1a	52.44 $\pm$ 1.97a	1234.8 $\pm$ 9.52a
β -LG-GF ₃	29.5 $\pm$ 0.81b	55.67 $\pm$ 0.41b	986.5 $\pm$ 9.96b
β -LG-GF ₄	31.6 $\pm$ 0.8b	58.78 $\pm$ 1.71c	1285.3 $\pm$ 12.95c

^{a-c}Different letters denote significant differences across different treatments ($P \leq 0.05$).

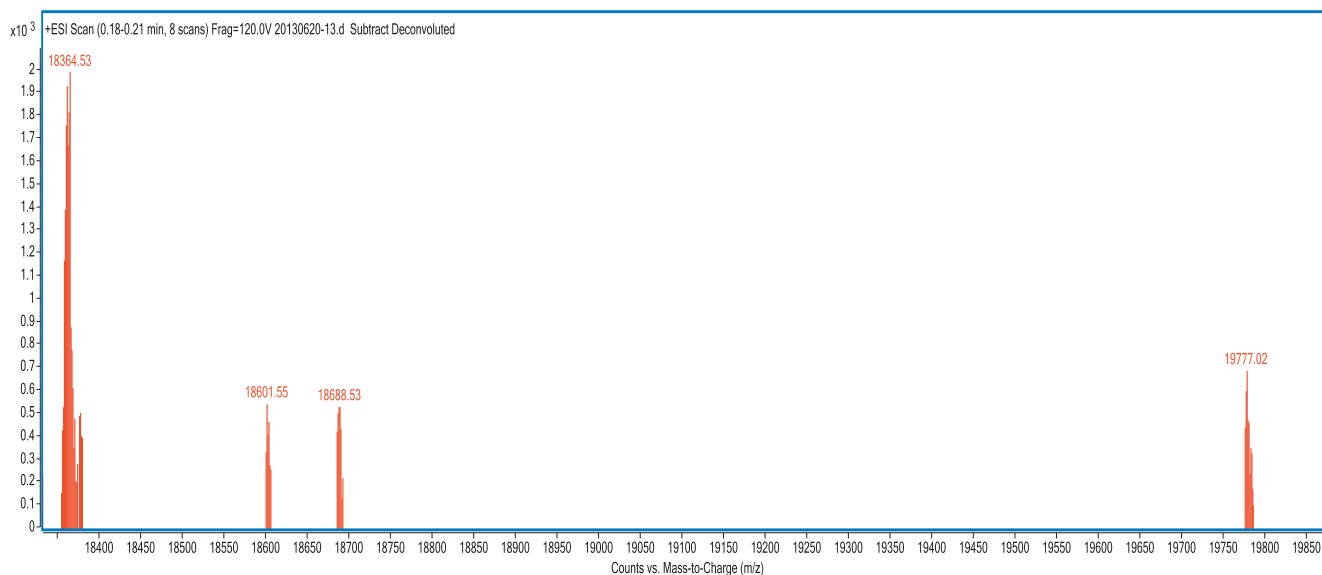


Fig. 1. The Q-TOF/mass spectrum of β -LG conjugated with GF₃.

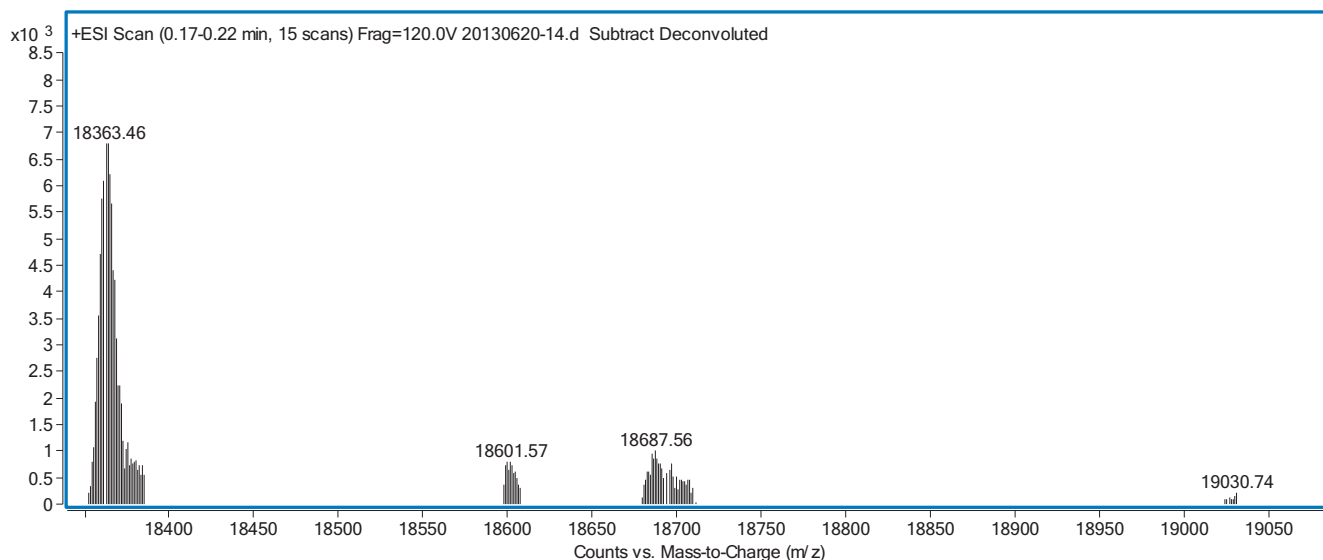


Fig. 2. The Q-TOF/mass spectrum of β -LG conjugated with GF_4 .

3.3. Changes in H_o and SH contents

The functional properties of protein are affected by their surface hydrophobicity, which depend on amino acid sequence and structural configuration (Zhang, Li, & Mittal, 2010). The H_o values of the samples studied here are shown in Table 1. The H_o value of control β -LG was 1234.8. Binding of GF_3 to β -LG significantly reduced the H_o to 986.5. Corzo-Martinez, Moreno, Olano, and Villamiel (2008) also reported that the glycosylated conjugate of β -LG had decreased hydrophobicity compared to the native β -LG. The decline in H_o may be related to the blocking of lysine and/or arginine residues in the glycation conjugates due to increased amount of hydrophilic groups by conjugation between carbohydrates and the ϵ -amino group of lysine during glycation (Van Teeffelen, Broersen, & de Jongh, 2005). However, the H_o of β -LG- GF_4 conjugates was increased from 1234.8 to 1285.3. β -LG contains a high proportion of hydrophobic amino acid chains, preferentially turned towards the inside of the molecule. Chandrapala, Zisu, Kentish, and Ashokkumar (2012) reported that the surface hydrophobicity of β -LG was expected to increase significantly when the molecule unfolded. Surface hydrophobicity change may be induced by the cleavage of hydrogen bonds and van der Waals forces and the exposure of hydrophilic amino acid residues to the inner site of proteins.

The free SH content of glycosylated β -LG samples is shown in Table 1. Conjugation treatment with GF_3 and GF_4 increased the free SH content of β -LG. The conjugation treatment used here had a significant influence on free SH content ($P < 0.05$). The SH content of control β -LG was 52.44 $\mu\text{mol/g}$ protein. After conjugation with GF_3 and GF_4 , the SH content was increased to 55.67 and 58.78 $\mu\text{mol/g}$ protein, respectively. A possible explanation is that a mild unfolding of the β -LG molecule resulted in the exposure of more free thiol groups (Monahan, German, & Kinsella, 1995).

3.4. Changes in secondary structure

Circular dichroism (CD) spectra for the control β -LG and β -LG conjugates are shown in Fig. 3a. β -LG is a protein composed predominantly of β -sheets with a markedly high helical propensity (Shibayama, 2008). The CD spectra displayed a negative extreme around 217 nm, a crossing zero dichroism at 203 nm, and a positive maximum around 195 nm, suggesting a high content of

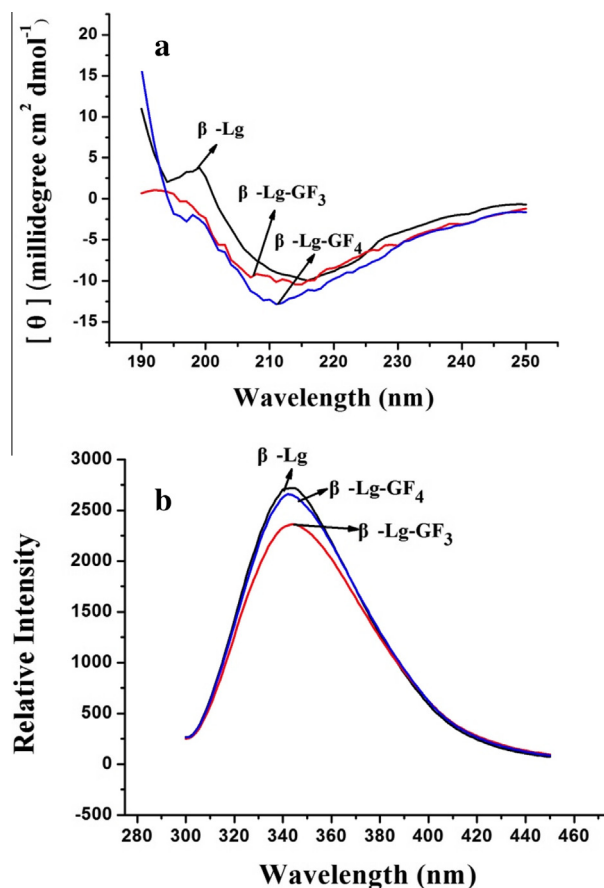


Fig. 3. (a) CD spectra analysis of control β -LG and β -LG after conjugated with GF_3 and GF_4 . (b) Intrinsic fluorescence spectroscopic analysis of control β -LG and β -LG after conjugated with GF_3 and GF_4 .

β -strands. This is in accordance with the secondary structure content shown in Table 2. The spectrum of the β -LG conjugates displayed an increase in ellipticity and a shift in wavelength compared with the control β -LG. Similar results were observed in our previous work where β -LG was conjugated with FOS (Zhong et al., 2013). In addition, the α -helix content significantly declined

Table 2

The percentage content of secondary structures of control β -LG and β -LG after conjugated with GF₃ and GF₄.

Sample	Secondary structure			
	α -Helix (%)	β -Strands (%)	Turns (%)	Unordered (%)
β -LG (control)	14.4 $\pm$ 0.60a	33.5 $\pm$ 0.76a	21.6 $\pm$ 1.11ab	30.7 $\pm$ 1.01a
β -LG-GF ₃	11.6 $\pm$ 1.02b	32.5 $\pm$ 1.19a	24.3 $\pm$ 1.46a	31.5 $\pm$ 0.87a
β -LG-GF ₄	21.3 $\pm$ 0.97c	23.8 $\pm$ 0.80b	19.5 $\pm$ 1.22b	35.2 $\pm$ 1.16b

^{a–c}Different letters denote significant differences across different treatments ($P \leq 0.05$).

from 14.4% to 11.6% following conjugation with GF₃, but increased to 21.3% after conjugation with GF₄. Furthermore, the percentage of β -strands also decreased from 33.5% to 32.5% and 23.8%. Considine, Patel, Singh, and Creamer (2007) reported that the trough at 216 nm gradually broadened and deepened, accompanied by β -LG unfolding. Therefore, it was shown from Fig. 3 and Table 2 that β -LG may be partially unfolded to a different degree after conjugation with GF₃ and GF₄.

3.5. Changes in tertiary structure

A fluorescence spectroscopy technique was used to characterize the conformational changes of β -LG induced by conjugation treatment, as shown in Fig. 3b. Conjugation with GF₃ and GF₄ contributed to a decrease in relative fluorescence intensity. The relative fluorescence of β -LG-GF₃ conjugates was much lower than that of the control sample and β -LG-GF₄ conjugates. In addition, the fluorescence emission peak wavelength was mildly red-shifted from 340 nm (control β -LG) to 345 nm (β -LG-GF₃ conjugates) and 343 nm (β -LG-GF₄ conjugates). Similar results were also observed in our previous study (Zhong et al., 2013). The relative fluorescence of β -LG after conjugation with FOS decreased and the wavelength was red shifted. The intrinsic fluorescence of β -LG is mainly attributed to tryptophan (Trp) residues, which are sensitive to the surrounding environment. According to Liu et al. (2009), Zhong et al. (2013), when protein partially unfolds, fluorescence decreases due to the conformational changes around Trp residues. Hattori et al. (2004) revealed that the shift of fluorescence wavelength indicated the altered conformation of β -LG around the Trp residues.

3.6. The conformational changes of β -LG conjugated with GF₃ and GF₄ in relation to antigenicity

β -LG, the major protein in whey, has a very compact globular structure in which intramolecular bonds maintain the structural integrity of protein. Initial report by Clement et al. (2002) showed that the structure of β -LG was necessary for its immunoreactivity. Similarly, our previous study revealed that the conformational changes of β -LG under different treatment conditions was related to antigenicity of β -LG. Zhong et al. (2012) displayed that aggregation and conformational changes of β -LG under dynamic high pressure microfluidization was related to its antigenicity. Zhong et al. (2013) reported that the partial unfolding of β -LG occurred after conjugation with FOS, contributing to decrease in antigenicity. However, FOS is mixture of oligosaccharides, which have the structure 1^F -(1- β -fractofranosyl)_{*n*-1} sucrose; that is, 1-kestose ($n = 2$, GF₂), nystose ($n = 3$, GF₃), 1^F - β -fractofranosyl nystose ($n = 4$, GF₄), and so on. It was unclear which 1^F -(1- β -fractofranosyl)_{*n*-1} sucrose of FOS played a main role on decreasing antigenicity. In attempt to further unravel how FOS changed antigenicity of β -LG, GF₃ and GF₄ of FOS were chosen to investigate the relationship between conformation and antigenicity.

In this study, the antigenicity of β -LG significantly decreased from 143.4 (control sample) to 29.5 and 31.6 μ g/mL respectively

after conjugation with GF₃ and GF₄ (Table 1). However, there was no significant difference in antigenicity between the two β -LG conjugates. Our previous results showed that different conformational changes resulted in differently altered antigenicity of β -LG (Zhong et al., 2012). Interestingly though, it can be shown from results above that there were obvious different conformational changes of β -LG after conjugation with GF₃ and GF₄. Thereafter, it was of interest to determine the ability of conformational changes of β -LG modulate its antigenicity during the conjugation treatment.

The results regarding the changes of conformation obtained using different methods can be mainly represented as one-state processes. After conjugation with GF₃ and GF₄, β -LG may be partial unfolded to different degree, which was reflected by the increase of free SH and changes in secondary structure and tertiary structure. Nevertheless, there were obvious differences between the conformational changes of β -LG-GF₃ and β -LG-GF₄ conjugates. For example, the relative fluorescence of β -LG-GF₃ conjugates was much lower than that of the control sample and β -LG-GF₄ conjugates. According to Hattori et al. (2004), the carbohydrate-binding sites in the β -LG were mainly identified to be Lys residues. In addition, they observed that the significant decrease of fluorescence intensity of glycation conjugates was due to a shielding effect by carbohydrate chain when it was bound to ⁶⁰Lys in this conjugate. Thus, it was indicated here that the GF₃ might be bound to ⁶⁰Lys in this conjugate but GF₄ might be not. It was demonstrated that glycation with different saccharides could significantly alter the conformation of β -LG.

Furthermore, conformational changes could impact the glycation degree and glycation site, and eventually influence the antigenicity of the glycosylated protein (Zhang et al., 2014). Mass spectrometry results suggested three GF₃ and one GF₄, respectively may be conjugated with β -LG. Although conformational changes impacted degree of glycation, it ultimately had little impact on the antigenicity of the two conjugates. It was suggested that the glycation site might be the more important factor for decreased antigenicity. Similarly, it was reported that the degree of antigenicity reduction depended on whether the main epitope was glycosylated or not (Zhang et al., 2014).

As we know, the antigenicity of β -LG depends on the epitopes, which are widely spread along the 162 amino acid (AA) (Clement et al., 2002) and throughout the β -LG conformation (Selo et al., 1999). The main epitopes, by more than 90% of allergic individuals, were fragments AA 41–60, AA 102–104, and AA 149–162, which included some binding sites (e.g., Lys 60, Lys 100 and Lys 101). When the main epitope was glycosylated, the antigenicity decreased the most (Zhang et al., 2014). Although the number of GF₃ conjugated with β -LG was more than that of GF₄, the other two binding sites of conjugated GF₃ may not mask some epitope. May be the reason which was responsible for the different conformational changes of β -LG accompanied with the similar antigenicity changes between the different conjugates. Therefore, the details of the final glycosylated site and how to conjugate the effective sites through glycation need to be investigated in more depth.

4. Conclusions

In this study, we investigated the relationship between the conformational changes and antigenicity of β -LG conjugated with GF₃ and GF₄. Our results indicated that the antigenicity of β -LG decreased greatly after conjugation with GF₃ and GF₄. In addition, there were no significant differences between β -LG-GF₃ and β -LG-GF₄ conjugates. However, results showed that the different conformational changes of β -LG following conjugation with GF₃ and GF₄ occurred. Mass spectrometry results revealed that the molecular weight of β -LG increased from 18.4 to 19.8 and

19.1 kDa after conjugation with GF₃ and GF₄, respectively, by covalent bond. The varying degrees of partial unfolding of β -LG after conjugation with GF₃ and GF₄ was reflected by decreased fluorescence intensity, the red-shift in the fluorescence spectra, and the increase of SH content of each sample in different degree. The different conformational changes of β -LG-GF₃ and β -LG-GF₄ may also be related to the similar decrease behavior in antigenicity, possibly due to the different binding sites.

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