Short communication

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Comparison of antigenicity and conformational changes to β-lactoglobulin following Kestose glycation reaction with and without dynamic high-pressure microfluidization treatment.

Running title: Comparison of antigenicity and conformational changes to β-LG

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Abstract

Previous work indicated that conformational changes of β-lactoglobulin (β-LG) induced by dynamic high pressure microfluidization (DHPM) was related to the increase of antigenicity. In this study, β-LG glycated with 1-kestose and combined with DHPM decreased the antigenicity of β-LG. The antigenicity of control, β-LG-kestose (0.1 MPa) and β-LG-kestose (80 MPa) were 100, 79 and 42 μg/mL respectively. The molecular weight of β-LG conjugated to kestose increased from 18.4 to 19.6 kDa and its conformation scarcely changed. Conversely, combined with DHPM treatment (80 MPa), β-LG conjugated to kestose formed two conjugates with molecular weight of 18.8 and 19.8 kDa, respectively. Furthermore, the unfolding of β-LG as a result of the treatments is reflected by a decrease of intrinsic and synchronous fluorescence intensity and changes to the secondary structure. The conformational changes induced by DHPM and glycation treatments synergistically decrease the antigenicity of β-LG due to more masked or disrupted epitopes.

Keywords: β-lactoglobulin; 1-kestose; Antigenicity; Combined treatment; Modulation mechanism

1. Introduction

The dairy protein, β-LG possesses lots of nutritive, biological and functional properties. However, the antigenicity of this protein limits their application in the food industry (Moro, Baez, Busti, Ballerini, & Delorenzi, 2011). Previous works have investigated the effect of various processing treatments on the antigenicity of β-LG, including dynamic high pressure microfluidization (DHPM) (Zhong, et al., 2012; Zhong, Luo, Liu, & Liu, 2014a), heat treatment (Zhong, et al., 2011), glycation (Zhong, Tu, Liu, Luo, & Liu, 2015; Zhong, et al., 2014b; Zhong, et al., 2013), and PEGylation (Zhong, et al., 2016). It was found that the increase in the antigenicity of β-LG was related to the conformational changes induced by DHPM treatment (Zhong, et al., 2012). On the contrary, it was observed that when conjugated with different saccharides the conformation of β-LG changed differently, but a similar decrease in antigenicity occurred (Zhong, et al., 2015). As two different modulated processes of antigenic response of β-LG, the mechanism of its antigenicity change is not completely clear when the protein undergo a combined treatment of glycation reaction and DHPM.

Additionally, the type of saccharide used for glycation plays a significant role in the antigenicity changes of β-LG. Higher molecular weight of carboxymethyl dextran was more effective in declining the immunogenicity of β-LG (Kobayashi, et al., 2001). The antigenicity of β-LG conjugated with phosphoryl oligosaccharides was much lower than that conjugated with alginic acid oligosaccharide (Hattori, et al., 2004). However, there was no significant difference in the antigenicity decrease when β-LG

was conjugated with the two main components of fructo-oligosaccharides (FOS) - nystose and β-fructofuranosyl nystose (Zhong, et al., 2015). As per Kim, Lee, Go, Ahn, and Hong (2018), kestose, the smallest short-chain FOS, activates the gut immune system that induces tolerance against allergic skin inflammations in atopic dermatitis. However, there is no information describing how kestose modulates the antigenicity of β-LG. Further, no studies have reported the combined effect of kestose glycation reaction and DHPM treatment on the antigenicity of β-LG which is investigated in this study.

Previous research has reported DHPM at 80 MPa induces conformational changes in β-LG and therefore used in this study. The DHPM at 80 MPa was combined with and without kestonse glycation reaction to elucidate the mechanism of antigenicity changes of β-LG. In addition, the modulation mechanism was characterized by multi-spectroscopy, including Quadrupole Time-of-Flight (Q-TOF) LC/MS spectrometer, intrinsic fluorescence spectra, synchronous fluorescence spectra, and circular dichroism spectra that helps to determine the conformational changes of the protein.

2. Materials and methods

2.1 Materials

Bovine β-LG standard (L3908), kestose (GF_2) and anti-rabbit IgG (A6154) were obtained from Sigma Chemical Company (St. Louis, MO, USA). All other reagents

were of analytical grade or better.

2.2 DHPM Combining with Glycation Treatment

β-LG solutions (1 mg/mL) were treated at the atmospheric pressure (0.1 MPa) and pressure of 80 MPa in a dynamic high pressure microfluidizer processor (model M-110EH-30, Microfluidics, Newton, MA). Samples were collected and glycated with kestose immediately according to the methods of Zhong et al. (2015). All glycation experiments were performed in triplicate. β-LG heated at conjugate conditions without kestose (heated control) was named "β-LG (control)", and unprocessed β-LG-GF_2 conjugate was named "β-LG-GF_2 (0.1MPa)". Samples glycated with GF_2 after DHPM treatment at 80MPa was named "β-LG- GF_2 (80MPa)".

2.3 Antigenicity analysis.

The antigenicity of different samples was determined by an indirect competitive ELISA (Zhong, et al., 2015; Zhong, et al., 2014b). Polyclonal IgG antibodies of rabbit (bs-0814R, Bioss Co., Beijing, China), monospecific for β-LG, were applied, detecting the antigenic response of β-LG (Kleber, Krause, Illgner, & Hinrichs, 2004). The antigenic response of the β-LG samples was calculated from a standard curve of β-LG, for which a linear logarithmic correlation was observed in the range of 0.01 to 400 μg/mL for β-LG. All analyses were carried out in triplicate, and the averaged values were converted to concentration equivalents in micrograms per milliliter (μg/mL).

2.4 Q-TOF mass spectrometry analysis.

The method by Zhong et al. (2013) was used to investigate the molecular weight of different β-LG samples. Samples 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 Circular dichroism spectroscopy analysis.

Circular dichroism (CD) spectroscopy analyses were run for different samples according to Zhong et al. (2015). Far-UV CD spectra of samples at 0.1 mg/mL in 10 mM sodium phosphate buffer, pH 7.0, were measured at 22 ºC 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 regions from 190 to 250 nm. Scans were performed for each solution according to the following conditions: 100 nm/min scan rate, 1 nm step resolution, and 1.0 nm of bandwidth. Structure predictions from CD spectra were obtained using the Contin LL program (de la Hoz & Netto, 2008).

2.6 Intrinsic fluorescence analysis.

Fluorescence analyses were performed for all samples, as described by Zhong et

al. (2014 and 2015). Fluorescence spectra was measured in a Hitachi Spectro-fluorimeter F-4500 (Hitachi, Tokyo, Japan) using 10 mm square quartz cells. 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 450nm 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.

2.7 Synchronous Fluorescence Spectra Analysis

Synchronous fluorescence analyses were performed for different β-LG samples that were carried out in a Hitachi spectrofluorimeter (F-4500, Hitachi, Tokyo, Japan) using 10 mm quartz cells and a protein concentration of 0.15 mg/mL in phosphate buffer solution (10 mM, pH 7.0). The synchronous fluorescence spectra were obtained considering the wavelength intervals $\Delta\lambda$ = 15 nm and $\Delta\lambda$ = 60 nm to evidence the tyrosine and tryptophan residues, respectively ($\Delta\lambda = \lambda_{em} - \lambda_{ex}$) (Varlan & Hillebrand, 2010).

2.8 Statistical analysis.

The one-way ANOVA test for significant effects of treatments and assays was determined using SPSS 12.0.1 (SPSS Inc., Chicago, IL). Main effect differences were considered significant at $P \leq 0.05$. Mean separations were determined by Tukey's procedure for multiple comparisons. All experiments were analyzed in triplicate.

3. Results and discussion

3.1 Changes in molecular weight

The molecular weight of the β-LG-GF_2 conjugates increased due to the formation of a covalent bond between the amino residue of protein and the reducing end of sugar. The molecular weight of β-LG is about 18.4 kDa that increased to 19.6 kDa following conjugation with GF_2 (Fig. 1). Only one conjugate was formed when β-LG was subjected to glycation. Interestingly, after the β-LG was pretreated by DHPM, two β-LG-GF_2 (80 MPa) conjugates were formed, the molecular weight of which increased to 18.8 and 19.8 kDa, respectively (Fig. 2). Since the molecular weight of GF_2 is 504 Da, therefore we conclude that the number of GF_2 conjugated with β-LG in the three conjugations may be three, one and four, respectively. According to Chen et al. (2017), DHPM treatment could improve the glycation level, by forming more glycation sites via conformational changes of the protein.

3.2 CD spectra analysis

β-LG is a predominantly β-sheet protein, as per the CD spectra and the secondary structure content shown in Fig. 3. High amount of β-strands displayed in the CD spectra, is reflected by a negative extreme around 217 nm, a crossing zero dichroism around 204 nm, and a positive maximum around 195 nm. Similarly, the secondary structure of β-LG (control) was composed of 35.6% β-sheets, 14% α-helix, 21.8% turns, and 29.1% random coil. The spectrum of the β-LG-GF_2 (0.1 MPa) displayed a little decrease in ellipticity compared with the control β-LG. Similar results were observed in a previous work where β-LG was conjugated with FOS (Zhong et al.,

2013) and GF_3 (Zhong et al., 2015). In addition, except for a significant increase in α-helix content, there were no obvious differences among the β-sheets, turns, and unordered contents. Some researchers observed a small alteration in the secondary structure of the protein after glycation (Hattori, et al., 2004; Wong, Day, & Augustin, 2011). However, Liu et al. (2012) indicated that glycation increased the α-helix content. Based on the above results, we conclude that after conjugation with GF_2 the secondary structure of β-LG slightly changed.

Surprisingly, DHPM changed the glycation modification process. The spectrum of the β-LG-GF_2 (80 MPa) displayed a notable increase in ellipticity and a shift of the negative maximums to a lower wavelength (from 217 nm to 213 nm) compared with that of β-LG-GF_2 (0.1 MPa), accompanied by an increase in percentage content of β-sheets and unordered from 32.3% and 25% to 38.3% and 30.6%, respectively. Additionally, the α-helix content decreased from 21.5% to 9.97%. According to some researchers (Chen, et al., 2016; Zhong, et al., 2014b), when DHPM treated β-LG conjugates with galactose and GOS, the negative molar residue ellipticity peaks decreases. Chen et al. (2016) suggests that the effect of glycation combined with DHPM treatment on the secondary structure of β-LG depended on many factors, such as the sugar used, the level of glycation and so on. The secondary structure changes of β-LG glycated with GF_2 combined with DHPM treatment in the current study were similar with the changes of β-LG-GOS under similar conditions (Zhong, et al., 2014b), while these changes were different from that conjugated with galactose combined with DHPM (Chen et al., 2016). β-LG consists of 9 antiparallel β-strands and 1 major

α-helix at the C terminus of the protein (Le Maux, Bouhallab, Giblin, Brodkorb, & Croguennec, 2014). The secondary structure changes of β-LG-GF_2 (80 MPa) indicated that the combined treatments of DHPM and glycation induced the unfolding of protein, reflected by the increase of unordered content mainly at the expense of α-helix.

3.3 Intrinsic fluorescence emission spectroscopy

Intrinsic fluorescence of β-LG is mainly due to the excitation of the tryptophan (Trp) and tyrosine (Tyr) residues or some specific interactions between Trp and Tyr, affected by the surrounding environment and any alteration to the conformation (Chen et al., 2016 and Zhong et al., 2013). The fluorescence intensity of β-LG (control) was 373.2. As shown in the Fig. 4a, the fluorescence spectrum of β-LG-GF_2 (0.1 MPa) and β-LG (control) almost overlapped. A similar result was also observed when β-LG conjugated with phosphoryl oligosaccharides (Hattori et al., 2004). In contrast, when β-LG was glycated by FOS, GOS, GF_3, and galactose, an obvious decrease of fluorescence intensity was displayed (Zhong et al., 2012, 2013, 2015; Chen et al., 2016). The fluorescence intensity of β-LG-GF_2 (0.1 MPa) was 374.9, slightly higher than that of control sample. In conclusion, the tertiary structure of β-LG was scarcely influenced by GF_2 during the glycation treatment. After pretreatment at 80 MPa, the fluorescence intensity of β-LG-GF_2 (80 MPa) noticeably declined to 69.98. Compared with β-LG (control), the fluorescence spectrum of β-LG-GF_2 (80 MPa) showed a slight red shift in the maximum fluorescence emission wavelength. Liu et al. (2009) and Hattori et al. (2004) indicated that fluorescence intensity decreased and the shift

in fluorescence wavelength were due to the altered conformation around Trp or Tyr residues. Hence, we further investigated the synchronous fluorescence spectra changes of Trp and Tyr residues.

3.4 Synchronous fluorescence spectroscopy

According to Varlan and Hillebrand (2010), the synchronous fluorescence spectra were obtained considering the wavelength intervals $\Delta\lambda$ = 15 nm and $\Delta\lambda$ = 60 nm that indicates the tyrosine and tryptophan residues, respectively ($\Delta\lambda = \lambda em - \lambda ex$). As shown in the Fig. 4b and 4c, the changes in the fluorescence spectra of Trp and Tyr residues were similar with the intrinsic fluorescence spectra of β-LG (Fig. 4a). The fluorescence spectrum of Tyr residues in β-LG-GF_2 (0.1 MPa) and β-LG (control) almost overlapped completely (Fig. 4b). The fluorescence intensity of Trp and Tyr residues slightly increased from 618.0 and 1111 (control) to 674.7 and 1121 in the presence of GF_2 without DHPM pretreatment. Similarly, the conformation around the Trp and Tyr residues remained unchanged.

However, the intensity values of Trp and Tyr residues in β-LG-GF_2 (80 MPa) sample decreased to 104.1 and 183.4 (Fig. 4c) respectively from 674.7 and 1121, similar to the results obtained with intrinsic fluorescence spectra of β-LG-GF_2 (80 MPa). It is well know that one β-LG molecule has two tryptophan residues (Trp19 and Trp61), located at the bottom of the calyx and close to the entrance of calyx, and four Tyr residues at positions 20, 42, 99, and 102 (Keppler, Sonnichsen, Lorenzen, & Schwarz, 2014). Tyr20 and Tyr42 are located in the groove between the β-strand B and helical turn 153-157, and Tyr102 is located in the groove between the α-helix and

the β-barrel. It was reported that the carbohydrate-binding sites in the β-LG were mainly identified in residue Lys (Hattori et al., 2004). In addition, Chen et al. (2017) indicated DHPM could improve the glycation level and changed the glycation sites induced by the alteration of conformation. The decrease of fluorescence intensity in the case of β-LG-GF_2 (80 MPa) was considered to be due to a masking influence by GF_2 chain bound to Lys60, 100, or 101 in the conjugate.

3.5 Antigenicity analysis

FOS is a mixture of oligosaccharides, consisting of different structures including 1^F-(1-β-fractofranosyl)$_{n-1}$ sucrose; such as, 1-kestose (n=2, GF_2), nystose (n=3, GF_3), 1^F-β-fractofranosyl nystose (n=4, GF_4). In previous studies, we observed that the conjugation of FOS, GF_3 and GF_4 significantly declined the antigenicity of β-LG (Zhong et al., 2013 and 2015). A similar result was obtained with GF_2. From Fig. 5 it is clear that the antigenicity of the control β-LG was 100 μg/mL. After the glycation treatment with GF_2, an obvious decrease in the antigenicity of β-LG was observed ($p < 0.05$) with antigenicity value of β-LG-GF_2 (0.1 MPa) being 79 μg/mL. Compared to our previous results (Zhong et al., 2013 and 2015), the rate of decline in the antigenicity of β-LG-GF_2 (21%) was far lower than that of β-LG-FOS (81.5%), β-LG-GF_3 (79.4%) and β-LG-GF_4 (78.0%). Therefore, due to the different nature of saccharides, it can be said that GF_2 was not the main component in the FOS affecting directly the antigenicity of β-LG.

Interestingly, we found that DHPM and glycation treatments have a synergistic effect on the decrease of antigenicity. After pre-treated by DHPM, the antigenicity of

β-LG-GF_2 (80 MPa) further declined to 42 μg/mL from 79 μg/mL, which was approximately half of β-LG-GF_2 (0.1 MPa). Similar results were obtained by Zhong et al. (2014b) and Chen et al. (2016). Zhong et al., (2012) and Zhong et al., (2013) indicate that not only the conformational changes of protein during treatments but also the glycation process affected by nature of saccharides both influence the decrease in antigenicity of β-LG. Zhong et al., (2015) also observed that different conformational changes in β-LG during the glycation treatment could result in a similar decrease in antigenicity. Then the obvious question arises as to what happens to the antigenicity of β-LG during a combined treatment of DHPM and glycation.

The antigenicity of β-LG is related to the 3D structure of protein, depended on the conformational epitopes and the linear epitopes (Kleber, et al., 2004). As shown in Fig. 6, although the conformation of β-LG barely changed when conjugated with GF_2, the decline in the antigenicity was mostly due to the epitopes masked or disrupted by GF_2 during the glycation treatment. As Chen et al. (2017) indicated, DHPM treatment could improve the glycation level and change the glycation sites. After DHPM pretreatment, an additional glycated site (Lys 60) was observed in the β-LG. Similar results were also observed in this current study. The unfolding of β-LG induced by DHPM contributed to exposure of more reactive sites (e. g. Lys60), as reflected by the fluorescence spectra results and the molecular weight changes, and subsequently resulted in two different conjugates. And the number of GF_2 in one conjugate was one more than that of β-LG-GF_2 (0.1 MPa). Zhang et al. (2014) suggested that when the main epitopes were glycated, the antigenicity declined the most. Similarly, the best

recognized epitope AA41-60 may be shielded or disrupted by the conjugation, resulting in reducing the antigenicity obtained in this study. Furthermore, the epitopes AA41-60 and AA102-124 form a protruding loop between β-strands C and D on the surface of the molecule (Wal, 2001). Also, the β-sheets of β-LG include certain epitopes, particularly two very conformation-sensitive epitopes (Clement, et al., 2002). During the coupled treatments, the changes of β-sheets could influence those epitopes and contribute to less antigenicity. Previous research has reported that the conformational changes of β-LG maybe directly related to its antigenicity. For example, the unfolding of β-LG induced by DHPM at 80 MPa contributed to exposure of some hidden epitopes that eventually increased the antigenicity (Zhong et al, 2012). Some researchers also suggest that conformational changes could affect the glycation degree and site, and eventually impact the antigenicity of protein (Zhang et al., 2014; Chen et al., 2017). In conclusion, in the current study, conformational changes induced by DHPM and the glycation process have a synergistic effect on the decline of antigenicity, mainly due to masking or disruptions of the important epitopes.

4. Conclusions

In this study it was observed that conformational changes induced by DHPM and the glycation process together have a synergistic effect on the decrease of antigenicity of β-LG. During the glycation treatment without DHPM, one type of conjugates was formed. A decline in the antigenicity of β-LG-GF_2 without DHPM treatment mainly resulted from epitopes masked or disrupted by GF_2, with hardly any changes to the

conformation of protein. On the other hand, two types of conjugates were obtained after glycation combined with DHPM treatment. During the combined treatment of DHPM and glycation, the unfolding of β-LG induced by DHPM contributed to more epitopes being masked or disrupted by GF_2, resulting in further decline in the antigenicity of β-LG.

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Figure Captions:

Figure 1. The Q-TOF/Mass spectrum of β-LG-GF_2 (0.1 MPa).

Figure 2. The Q-TOF/Mass spectrum of β-LG-GF_2 (80 MPa).

Figure 3. CD spectra analysis and the percentage content of secondary structures of β-LG (control), β-LG-GF_2 (0.1 MPa) and β-LG-GF_2 (80 MPa).

Figure 4. a. Intrinsic fluorescence spectroscopic analysis of β-LG (control), β-LG-GF_2 (0.1 MPa) and β-LG-GF_2 (80 MPa); b. Synchronous fluorescence spectrum of tryptophan residues in the β-LG (control), β-LG-GF_2 (0.1 MPa) and β-LG-GF_2 (80

MPa), λ = 60 nm; c. Synchronous fluorescence spectrum of tyrosine residues in the β-LG (control), β-LG-GF_2 (0.1 MPa) and β-LG-GF_2 (80 MPa), λ = 15 nm.

Figure 5. The antigenicity of β-LG (control), β-LG-GF_2 (0.1 MPa), β-LG-GF_2 (80 MPa). $^{a-c}$Different letters denote significant differences across different treatments ($P \leq 0.05$)

Figure 6. A model of the initial modulation mechanism

Figure 1

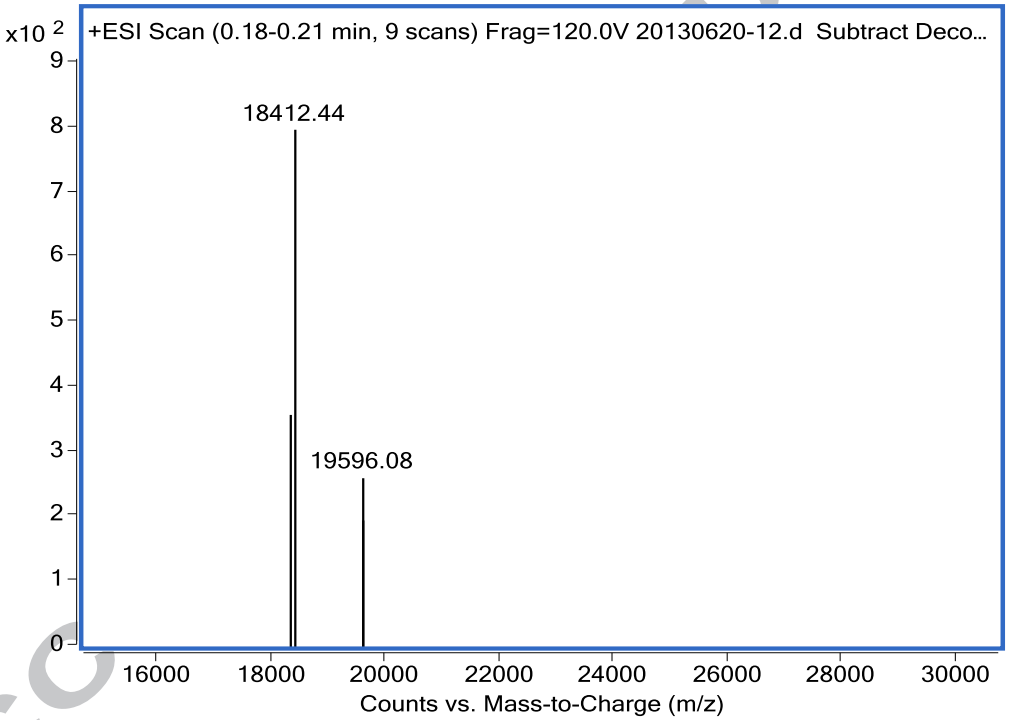


Figure 2

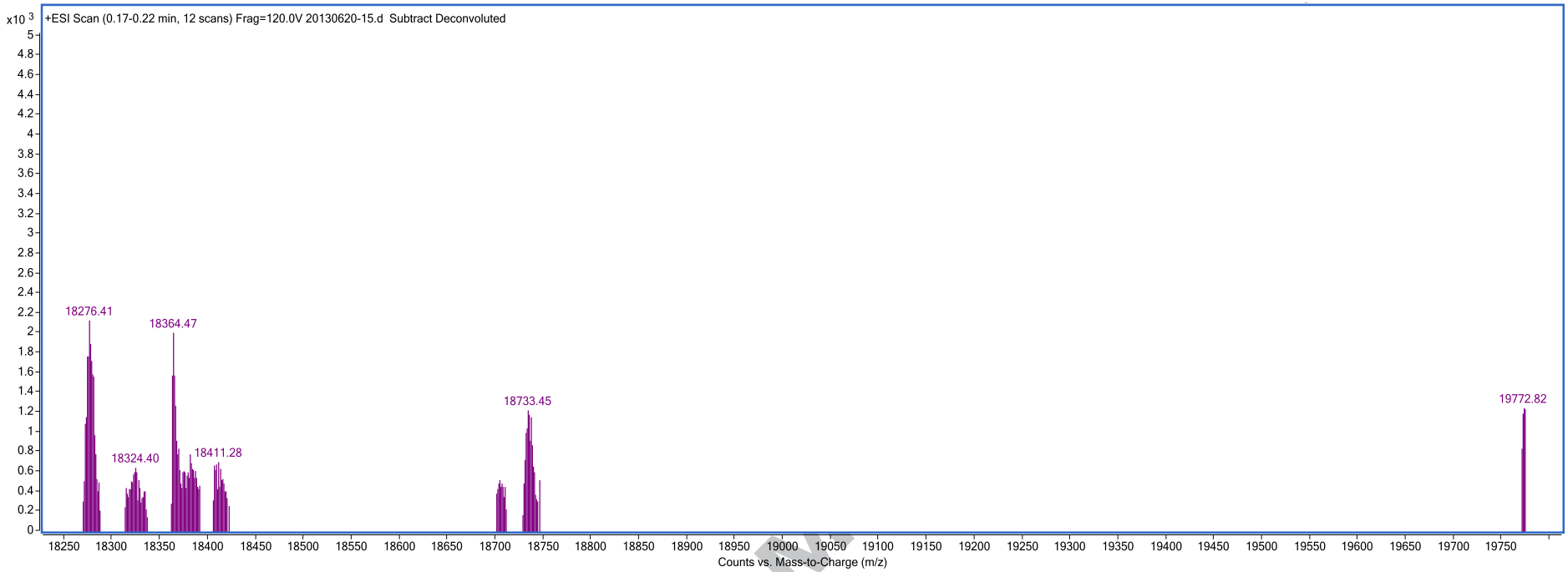


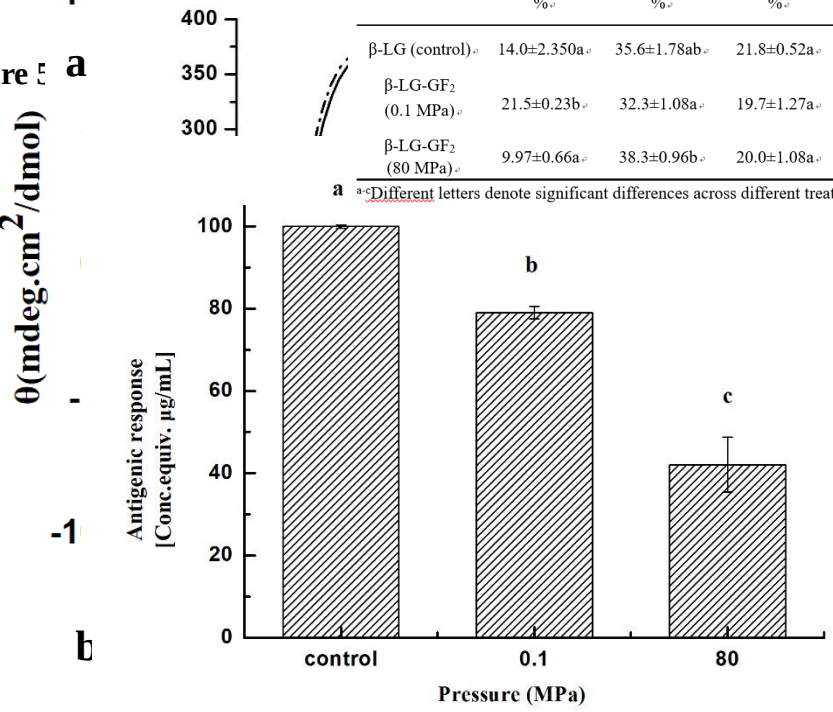
Figure 3

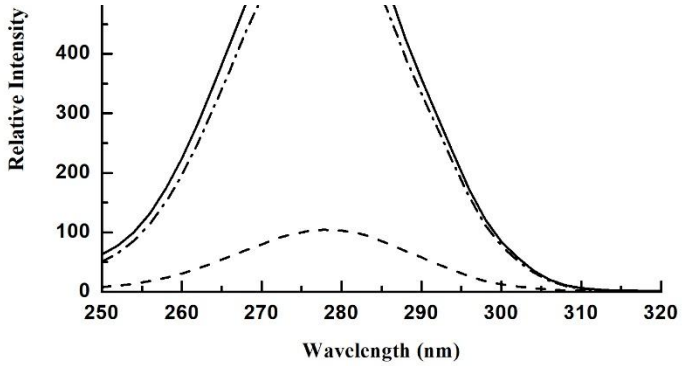
Figure 4

Sample	Secondary structure			
	α-helix	β-stands	Turns	Unordered
	%	%	%	%
β-LG (control)	14.0±2.350a	35.6±1.78ab	21.8±0.52a	29.1±1.42ab
β-LG-GF_2 (0.1 MPa)	21.5±0.23b	32.3±1.08a	19.7±1.27a	25.0±0.15a
β-LG-GF_2 (80 MPa)	9.97±0.66a	38.3±0.96b	20.0±1.08a	30.6±1.43b

a-cDifferent letters denote significant differences across different treatments (P≤0.05)

Figure 5





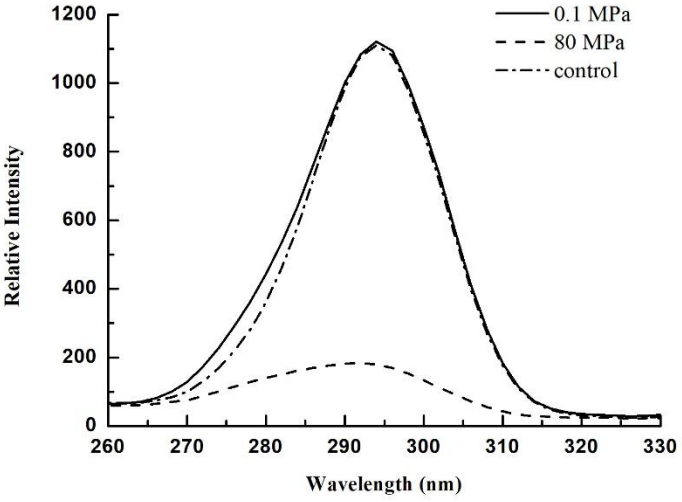
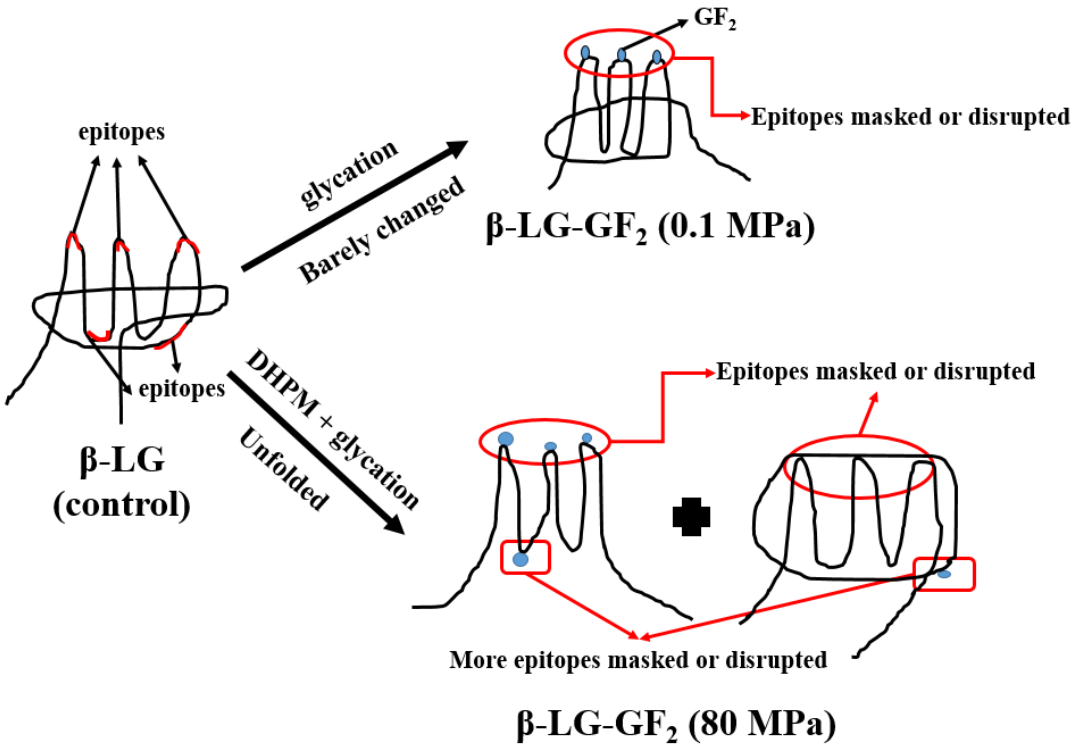


Figure 6



Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

Highlights

- Antigenicity of conjugate formed at 80 MPa was half the value of that under 0.1 MPa
- One conjugate formed using 0.1 MPa and its conformation scarcely changed.
- Two conjugates formed using 80 MPa and the unfolding of their conformation occurred
- Two treatments have a synergistic effect on the decrease of its antigenicity.