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Investigation into allergenicity reduction and glycation sites of glycated β -lactoglobulin with ultrasound pretreatment by high-resolution mass spectrometry

Running Title: Ultrasound pretreatment promoted the allergenicity reduction of glycated β -Lg

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ABSTRACT

Ultrasound treatment could change the conformation of β -lactoglobulin (β -Lg) and improve the glycation reaction in aqueous solution under neutral condition. However, the effect of ultrasound pretreatment on glycation of β -Lg with pentose at dry-state remains ambiguous, and the relationship between glycation and allergenicity of β -Lg with ultrasound pretreatment is unclear. This study aimed to evaluate the effect of ultrasound pretreatment on glycation and allergenicity of β -Lg. Markedly decreased allergenicity of β -Lg was observed after glycation with ribose before and after ultrasound pretreatment with the minimum found at 400 W. Orbitrap LC-MS/MS showed that the glycation degree of some peptides in glycated β -Lg with and without ultrasound pretreatment were different although the content of free amino group and molecular mass were insignificantly different. Therefore, ultrasound pretreatment promoted the reduction in allergenicity by improving the glycation extent of some glycation sites although it hardly enhanced the whole glycation degree of β -Lg.

Keywords: β -lactoglobulin; allergenicity; specific glycation sites; antigenic epitopes; ultrasound pretreatment; glycation

Abbreviations:

β -Lg, β -lactoglobulin; IgE, immunoglobulin E; IgG, immunoglobulin G; DSP, the average degree of substitution per peptide molecule; HCD, high-energy C-trap dissociation; ETD, electron transfer dissociation; ANS, 1-anilinonaphthalene-8-sulfonate; ELISA, enzyme-linked immunosorbent assay; PBS, phosphate buffer solution; PBST, PBS/Tween solution; CBS, carbonate buffer solution; CMA, cow's milk allergy; OPA, ortho-phthalaldehyde; SDS-PAGE, sodium dodecyl sulphate polyacrylamide gel electrophoresis; MALDI TOF, matrix-assisted laser desorption ionization time of flight mass spectrometry; UV, ultraviolet; TFA, trifluoroacetic acid; DTT, DL-dithiothreitol; BSA, bovine serum albumin

1. Introduction

β -lactoglobulin (β -Lg) is the major component in cow's milk protein (56-60% of total bovine whey proteins), which is considered as a valuable dairy ingredient (Moro, Báez, Busti, Ballerini, & Delorenzi 2011). However, it is also one of the major allergens that cause serious allergic reactions in infants or young children (Meng, Li, Wang, Gao, Yang, & Chen 2016). Its monomer consists of 162 amino acids and has a molecular weight of 18.3 kDa. β -Lg is a globular protein with a single polypeptide chain, and it involves 8 strands of anti-parallel β -sheets and 2 α -helices, including 2 disulfide bonds (Cys66-Cys160 and Cys106-Cys119) and 1 free sulfhydryl group at Cys121(Papiz, Sawyer, Eliopoulos, North, Findlay, Sivaprasadarao, et al. 1986). As a member of the lipocalin, native β -Lg has the lipocalin superfamily tertiary (beta-barrel) structure and exhibits strong affinity for hydrophobic and amphiphilic ligands (Niemi, Rytönen-Nissinen, Miettinen, Jänis, Virtanen, & Rouvinen 2015). Previous studies suggested that the hydrophobic profile was the allergenic epitopes of sequence corresponding to AA 41-60, 102-124, and 149-162 recognized by 92%, 97%, and 89% of human sera (Selo, Clement, Bernard, Chatel, Creminon, Peltre, et al. 1999). Secondary fragments of AA 1-8 and 25-40 were recognized by 58% and 72%, respectively, meanwhile AA 9-14, 84-91, 92-100 were also recognized by 40% of human sera (Selo et al. 1999). To date, considerable efforts have been focused on developing an approach to decrease the allergenicity by covering up or hydrolyzing the specific sequences of allergenic epitopes of β -Lg. A lot of processing methods, such as heating (Bloom, Huang, Bencharitiwong, Bardina, Ross, Sampson, et al. 2014), high pressure (López-Expósito, Chicón, Belloque, López-Fandiño, & Berin 2012), hydrolysis (Peram, Loveday, Ye, &

Singh 2013), glycation (Zhong, Tu, Liu, Xu, Liu, & Dun 2014) and phosphorylation (Wong, Li, K Wong, Jiang, & Shaw 2012), have been used to modify the β -Lg to decrease its allergenicity. However, among them, glycation was considered the potential method to destroy the epitopes because of direction and specificity of modification.

Glycation based on the Maillard reaction occurs between proteins and reducing sugars might still be desired to decrease allergenicity through directionally modification in lysine, arginine and amino-terminal end. Numerous reviews have stated that the allergenicity of β -Lg glycosylation with fructo-oligosaccharides decreased by causing a irreversible structural modification, leading to covering up the antigenic epitopes (Zhong, Tu, Liu, Luo, & Liu 2015); however, the precise glycation sites have not been identified and these are the main data needed to explore the relationship between the allergenicity and glycation modification.

In our previous work, we observed that ultrasound pretreatment can significantly improve the glycation extent and increase the glycated sites of BSA through altering the structure (Zhang, Tu, Wang, Huang, Shi, Sha, et al. 2014). Ultrasound pretreatment may be able to provide a new method of glycation to acquire more glycated sites to cover up the antigenic epitopes. Although previous study also showed that ultrasound pretreatment improved the glycation in aqueous solution under neutral condition (Stanic-Vucinic, Prodic, Apostolovic, Nikolic, & Cirkovic Velickovic 2013), the effect of ultrasound pretreatment on dry-state glycation and allergenicity remains unclear. Therefore, the influence of dry-state glycation with ultrasound pretreatment on allergenicity of β -Lg should be further investigated. To prove this effect, it is necessary to perform the experiment in a

stepwise fashion: first, ultrasound pretreatment was employed on the protein to initiate the Maillard reaction of β -Lg; then the glycation extent of glycation site was evaluated and the relationship between the extent of glycation sites and allergenicity was interpreted.

In this work, the effect of ultrasound pretreatment on the glycation extent, IgG/IgE-binding ability and structure of β -Lg was explored. The glycation sites and the average degree of substitution per peptide (DSP) were characterized by combining the approach of high-energy C-trap dissociation (HCD) and electron transfer dissociation (ETD) fragmentation. Our investigation clearly revealed the critical role of glycation with ultrasound pretreatment in covering up the special sequences of antigenic and allergenic epitomes that induce the declined allergenicity.

2. Materials and methods

2.1. Materials

β -lactoglobulin, *D*-(+)-ribose, pepsin were purchased from Sigma-Aldrich (St. Louis, MO, USA). All other reagents were analytical grade. Ultrapure water from water purification system (Millipore, Bedford, MA, USA) was used in this study.

Human sera: the sera of patients allergic to milk were purchased from PlasmaLab International, a series of sera from five CMA patients presenting various symptoms were used according to the positive IgE responses to milk proteins, especially to β -Lg. The total milk protein-specific IgE levels of all sera ranged from 13.8 kU/L to 96.6 kU/L.

2.2. Glycation samples preparation

β -Lg solution (2 mg/mL) was prepared in 0.01 M of sodium phosphate buffer at pH 7.4. The solution was split into five aliquots. One was used as the control. The other four aliquots were treated for 25 min at 100, 250, 400, 550 W by sonicator (JY92- II, Xinzhi Co., Ningbo, China) equipped with a microtip probe 6 mm, respectively. The equal mass of *D*-(+)-ribose (100 mg) was added to 50 mL of native and ultrasonicated β -Lg solution, respectively. The native β -Lg without ultrasound pretreatment was used as the control. All treated and untreated sample solutions were lyophilized. The lyophilized samples were incubated at 79% relative humidity and 60 °C for 1 h. The reaction was stopped by putting the sample tubes into an ice bath. Each sample in the tube was then dissolved in 100 μ L distilled water and filtered by a centrifugal filter unit (3000 Da cutoff, Merck Millipore Ltd., Darmstadt, Germany) to remove salts and free ribose. The concentration of protein was adjusted to 2 mg/mL for subsequent assays.

2.3. IgG and IgE binding abilities analysis

The IgG and IgE binding abilities of the β -Lg samples were estimated by indirect competitive ELISA with the rabbit polyclonal antibodies and the human sera from patients allergic to milk (Meng, Li, Wang, Gao, Yang, & Chen 2016). Firstly, 96-well microplate was coated with 100 μ L/well of 2 μ g/mL of standard β -Lg following by incubation at 4 °C overnight. Then residual-free binding sites were blocked with 10 mg/mL of pig gelatin in a PBS/Tween solution (0.05% Tween-20 in pH 7.4 and 0.01 mol/L of PBS) for 1 h in 37 °C bath. Competition was initiated by adding 50 μ L of either the standard or glycation samples (15 μ g/mL) treated ultrasound pretreatment with different powers, and 50 μ L of antisera samples

(1:102400 diluted rabbit sera to evaluate the IgG-binding ability and 1:30 diluted human sera to analyze the IgE-binding ability). Then the plate was incubated for 1h in a 37 °C bath. After removing the solutions, the wells were washed for 6 times with PBST. Then 100 µL of a solution of inhibited antisera (1:20000 diluted HRP-labeled anti-rabbit IgG/1:200 goat anti-Human IgE in PBST) was added and incubated for 15 min at 37 °C bath. After washing, 100 µL of tetramethyl benzidine solution was immediately added into each well. Finally, the reaction was stopped by adding sulfuric acid (100 µL, 2 mol/L) and the absorption was measured at 450 nm using a multi-mode microplate reader (Synergy H1, Bio-Tek, USA). The decline rate was calculated using the following equation: Inhibition (%) = $(1-B/B_0) \times 100\%$, where B and B₀ were the absorbance values of blank control and glycated samples, respectively.

2.4. Determination of free amino groups

The degree of glycation was determined by measuring the free amino groups using the ortho-phthalaldehyde (OPA) method ([Fayle, Healy, Brown, Reid, Gerrard, & Ames 2001](#)).

OPA solution was prepared by mixing 25 mL of 0.1 M sodium borate, 2.5 mL of 20% SDS, 100 µL of 2-mercaptoethanol, and 4 mg of OPA, which was dissolved in 1 mL of methanol.

The final volume was adjusted to 50 mL with distilled water. The samples containing 50 µg of protein were mixed with the OPA reagent, and incubated for 2 min at 37 °C. Absorption was measured at 340 nm against a blank containing the OPA reagent. Working standards were prepared by serial leucine dilutions to final concentrations from 0 to 0.4 mg/mL.

2.5. SDS-PAGE and MALDI-TOF analysis

The molecular mass of the β -Lg was analyzed by 12% sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and MALDI-TOF mass spectrometer (4800 Plus MALDI-TOF/TOF Analyzer, AB Science, CA). Briefly, proteins were diluted in loading buffer consisting of pH 6.8, 60 mM of Tris-HCl, 2% (w/v) SDS, 10% (v/v) glycerol, and 0.025% (w/v) bromophenol blue. The sample solutions were loaded on 12% polyacrylamide gel containing 0.1% (w/v) SDS. Electrophoresis was performed in the electrophoresis buffer (containing 0.025 M of Tris-HCl, 0.192 M of Glycine, and 0.1% SDS) with currents of 15-35 mA. Gels were stained with Coomassie Brilliant Blue R250 and destained in a mixture of 5% ethanol and 7.5% acetic acid. The mass of samples was carried out using a MALDI-TOF/TOF mass spectrometer according to [Zhang et al. \(2014\)](#). Sinapic acid (5 mg/mL) in 50% acetonitrile with 0.1% TFA was used as the matrix. The proteins were diluted to 1:100 with distilled water and mixed at a ratio of 1:1 with the matrix. Mixtures (1.5 μ L) were spotted onto the MALDI target and air-dried before analysis.

2.6. Intrinsic fluorescence and UV absorption spectra

Fluorescence analysis was carried out in a fluorophotometer (F-7000, Hitachi, Tokyo, Japan) with a protein concentration of 0.1 mg/mL in PBS buffer (10 mM, pH 7.4). The excitation wavelength was 280 nm. The emission spectrum was scanned from 300 nm to 450 nm using 5 nm bandwidth at a scan speed of 1200 nm/min.

The UV absorption spectra of samples were measured by a UV spectrophotometer (U-2910, Hitachi, Tokyo, Japan) with a concentration of 1 mg/mL in PBS buffer (10 mM, pH 7.4). UV absorption spectra were scanned from 240-400 nm at a scan speed of 800 nm/min.

2.7. Surface hydrophobicity

The surface hydrophobicity of the samples was determined using ANS fluorescent probes in a fluorophotometer (F-7000, Hitachi, Tokyo, Japan). The samples were diluted to 1 mg/mL, 0.5 mg/mL, and 0.25 mg/mL using PBS buffer (10 mM, pH 7.4), respectively. Then 4 mL of samples were mixed with 20 μ L, and 8 μ M of bis-ANS, and then the fluorescence emission spectrum was obtained from 400 nm to 600 nm, with an excitation at 370 nm, using a 5 nm bandwidth at a scan speed of 600 nm/min. The surface hydrophobicity of the samples was defined as the initial slope of the extrinsic fluorescence intensity to the protein concentration with different concentrations (Xiang, Ngadi, Ochoa-Martinez, & Simpson 2011).

2.8. Sample digestion

The samples were digested by pepsin according to the method of Wang, Tu, Liu, Liu, Huang, and Xiao (2013). They were resuspended in 6 M of urea to 1 mg/mL, and 5 μ L of reducing reagent (100 mM of DTT) was added per 100 μ L of the solution. Then a sample of the solution (2 μ L, 1 mg/mL) was added to a 500 μ L centrifuge tube containing 78 μ L of 50 mM ammonium bicarbonate solution and 20 μ L of DTT. Then, 100 μ L of 2 mg/mL pepsin solution was used to hydrolyze the protein sample in a buffer solution (pH 2.2). The reaction was then quenched by adding 2 μ L of 50% trifluoroacetic acid. After digestion by pepsin for 5 min, 40 μ L of the sample solution was injected into a C18 column (2 cm $\times$ 100 μ m, 5 μ m) for MS.

2.9. LC-MS and peptide identification

LC-MS was used to identify the glycosylated sites and evaluated the glycosylation extent according to

the method of Tu, Zhong, and Wang (2017). The column effluent was injected into an LTQ-Orbitrap Velos mass spectrometer (Thermo Fisher Scientific, MA, USA) for analysis by tandem mass spectrometry (MS/MS) to identify glycosylated sites of protein. Positive ions were used to detect isolates. Twenty fragment maps (MS/MS scans) showing the mass-to-charge ratio of the polypeptide and polypeptide fragments were collected at each full scan. The ions detected in the precursor ion scanning were further subjected to HCD and ETD fragmentation to detect the fragment ions. Dynamic exclusion was enabled with exclusion duration of 90 s. To further compare the glycosylation extent of each peptide, DSP namely the average degree of substitution per peptide of β -Lg was calculated according to the formula:

$$DSP = \frac{\sum_{i=0}^n i \times I(\text{peptide} + i \times \text{ribose})}{\sum_{i=0}^n I(\text{peptide} + i \times \text{ribose})}$$

where I is the sum of the intensities of the glycosylated peptides and i is the number of ribose units attached to the peptide in each glycosylated form.

2.10. Data analysis

The data are expressed as the mean $\pm$ standard deviation. The analysis was performed using Origin-Pro 8.0 (OriginLab Corp., Northampton, MA). The values of DSP $\pm$ standard deviation were determined from 3 separate experiments. Statistical data were determined based on a two-tailed t-test using standard deviations.

3. Results and discussion

3.1. IgG- and IgE-binding capacity analysis

In this work, the IgG and IgE binding abilities of β -Lg samples were estimated by indirect competitive ELISA with the rabbit antisera and the sera from patients allergic to milk, respectively. As shown in Figure 1 (A), the IgG-binding ability of β -Lg and β -Lg-rib glycosylation without ultrasonic treatment was 1016.02 $\mu\text{g/mL}$ and 593.02 $\mu\text{g/mL}$, respectively, and a significant decrease of 61.54% was observed after glycation modification. When β -Lg was ultrasonically pretreated at different powers, the IgG-binding ability decreased as the power increased from 0 W to 550 W, with a maximum decline rate up to 61% at 400 W. However, a decrease in the decline rate was observed at 550 W. Interestingly, the trend of changes in the IgE-binding ability was highly similar to that of the IgG-binding ability. Figure 1 (B) shows that the IgE-binding ability of β -Lg declined by 27.72% after glycation without ultrasonic pretreatment, while it gradually decreased with ultrasound power after glycation with ultrasound pretreatment at 100 W - 400 W. However, a decreased declined rate was observed at 550 W ultrasound pretreatment. Previous studies indicated that glycation could significantly decrease the IgG-binding of β -Lg ([Chen, Tu, Wang, Zhang, Sha, Pang, et al. 2016](#); [Zhong, Cai, Liu, Liu, Xu, & Luo 2016](#)) and ultrasonic treatment could improve the glycation extent and increase the glycation sites ([Zhang et al. 2014](#)). As for our hypothesis, the obvious decline of IgG- and IgE-binding capacity of β -Lg after conjugation with ribose was attributed to the covering up of the special sequences of antigenic epitopes through glycation. Our previous research indicated that ultrasonic treatment could change the conformation of the BSA, which could improve the glycation extent and sites on the protein sequence ([Zhang et al. 2014](#)). Therefore, the conformation of β -Lg might be suffered from unfolding change that improved the glycation extent of the antigenic epitopes. In order to

better understand the reason for the decreasing IgG- and IgE-binding capacity of β -Lg caused by glycation with ultrasonic pretreatment, the conformation and the exact glycated sites of glycated β -Lg with and without ultrasonic pretreatment should be investigated.

3.2. Free amino groups

Generally, the potential glycation sites of proteins during Maillard reaction are lysine, arginine, and the free amino at N-terminal. Previous research indicated that lysine was the main glycated site with reducing sugar (Huang, Tu, Wang, Zhang, Shi, & Xiao 2013; Wang, Tu, Liu, Liu, Huang, & Xiao 2013), while arginine is infrequent glycation site because of the lower freedom of the amino group by steric effect from neighboring atoms (Zhang et al. 2014). Therefore, the content of free amino groups was an index to evaluate the glycation extent of the protein. As presented in Figure 2(A), the content of the free amino groups was significantly decreased after glycation. However, there was no significant difference between with and without ultrasonic pretreatment. It indicated that ultrasound pretreatment did not improve the whole glycation extent of β -Lg. Because the ribose is the most reactive sugar for Maillard reaction and dry-state glycation is also more effective than wet-state glycation, the ultrasound treatment had little effect on the glycation extent of β -Lg. The result is opposite to the previous study, which they showed that ultrasound treatment could promote the glycation of BSA. The different results were caused by different proteins and sugars used in glycation system. Therefore, there is no linear relation between IgG-/IgE-binding ability and the content of the free amino groups through the correlation analysis. It was essential to obtain detailed information about the glycated sites of β -Lg with and without ultrasonic pretreatment in order

to explore the exact mechanism of the decreased IgG- and IgE-binding of β -Lg-*rib* glycation with ultrasonic pretreatment.

3.3. Surface hydrophobicity

Surface hydrophobicity is an important index impacting the hydrophobic interaction that has been recognized to relate to the stability, conformation, function and antigenicity of protein (Chandrapala, Zisu, Palmer, Kentish, & Ashokkumar 2011). In Figure 2(B), the surface hydrophobicity of β -Lg decreased after glycation and the decrease was also observed after ultrasonic treatment at 100, 250, 400 and 550 W. The H_0 value of the native β -Lg was 1668.6, while that of β -Lg-*rib* without ultrasonic pretreatment significantly reduced to 1581.69. Zhong, Tu, Liu, Luo, and Liu (2015) and Chen et al. (2016) reported that the glycated conjugation of β -Lg had decreased hydrophobicity compared to the native β -Lg. However, the glycated β -Lg with ultrasonic pretreatment at 100, 250, 400, and 550 W was decreased to 1503.75, 1447.5, 1387.5, 1448.6, respectively, indicating that the unfolding and structural changes of the native β -Lg after ultrasonic treatment at different powers occurred, including the changes in the glycation sites. These results may result from the unfolding of β -Lg caused by minor structural changes, and the glycation modification occurred at different amino acid residues. The surface hydrophobicity was related to the IgG- and IgE-binding ability of proteins. Native β -Lg has the lipocalin superfamily tertiary (beta-barrel) structure and high affinity for hydrophobic ligands, which makes it potentially highly allergenic (Niemi, Rytkönen-Nissinen, Miettinen, Jänis, Virtanen, & Rouvinen 2015). Therefore, glycation modification can lead to the conformation change and decrease the surface

hydrophobicity of β -Lg, which then declined the IgG- and IgE-binding ability.

3.4. Molecular weight

The Maillard reaction could increase the molecular weight and promote the conjugation of the protein to dimer, trimer or polymer. It has been reported that protein interacts with sugar through the formation of the covalent bond between amino residue and aldehyde group. Thus, SDS-PAGE and MALDI-TOF MS were used to measure the molecular weight of glycated β -Lg. The molecular weight of native β -Lg was 18.3 kDa, while the dimer and trimer, with molecular weight of 36.71 and 55.01, 73.04, and 91.80 kDa were obviously observed in Figure 2(C). As shown in the SDS-PAGE image and MALDI-TOF mass spectra, the molecular weight of β -Lg-rib increased due to the Maillard reaction between protein and sugar, which was obvious in comparison to native β -Lg. Likewise, some dimer, trimer, and polymer bands and new peaks were observed in the SDS-PAGE image and MALDI-TOF mass spectra, which indicated that covalent linkage reactions that involved sugar cross-links occurred. The molecular weight of β -Lg was increased from 18.3 kDa of the native to 20.9 kDa of 400 W for 25 min. However, there was no obvious change between that with and without ultrasonic pretreatment. This result was in contrast with our previous research which found ultrasonic treatment can greatly improve protein glycation by altering the structure of BSA throughout all 3 of the domains ([Zhang et al. 2014](#)). We presumed that ultrasonic treatment may change the conformation of β -Lg; however, there was no relationship between the glycation extent and the conformation due to structural flexibility and low molecular mass of β -Lg. In contrast, the conformation change may cause the change of glycated sites and the

glycation extent of each site.

Likewise, a number of aggregation bands corresponding to the new oligomeric forms were observed, which indicated that covalent linkage reactions occurred in this system. These aggregations were observed in the SDS-PAGE bands when they were studied with the β -Lg incubated with galactose (Chen et al. 2016; Corzo-Martínez, Moreno, Olano, & Villamiel 2008). However, no significant difference in the aggregation forms was observed in the SDS-PAGE bands. It is well known that β -Lg associates as a non-covalent dimer under physiological and a rapid equilibrium between native β -Lg dimer and monomer (Moro, Báez, Busti, Ballerini, & Delorenzi 2011). MALDI-TOF MS was used to measure the molecular weights of the aggregation of β -Lg-rib conjugations under different ultrasonic powers. Figure 2(D) shows that the MALDI-TOF MS spectrometry analysis clearly revealed the molecular weight of β -Lg-rib conjugations, and the polymers also clearly contain dimers, trimers, tetramers, and pentamers that exist in native β -Lg and β -Lg-rib conjugations. Otherwise, the significant increase was observed in the spectra of the monomer and polymer with a 2200 to 2500 Da shift. Therefore, we concluded that there were some covalent polymers, including the disulfide bond and other covalent bands in native β -Lg, and the β -Lg-rib conjugations may be generated through Maillard reaction or native polymer glycation modification. However, the molecular weights of the native β -Lg and ultrasonicated β -Lg shifted to 20900 Da, much higher than the original molecular weights of the native β -Lg. Interestingly, compared to the BSA-galactose (Zhang et al. 2014), the molecular weights that increased the glycation of ultrasonicated β -Lg was smaller. Thus, it is speculated that the molecular weight of β -Lg was very small and did not have a compact global structure; leading to insignificant difference in

the improvement of the glycation β -Lg before and after ultrasonic treatment based on the unfolding change after ultrasonic treatment.

3.5. UV and intrinsic fluorescence spectroscopy

UV absorption and intrinsic fluorescence spectra were used to verify the change of conformation and the main amino acid. Figure 3(A) shows that the maximum UV absorbance of native β -Lg and β -Lg-rib was observed at 278 nm and 280 nm, respectively. Compared to the native β -Lg, the glycated β -Lg-rib showed an increase in UV absorbance and exhibited a red shift in the maximum absorption peak, which suggested that tryptophan, tyrosine, and phenylalanine were exposed to the surface of the protein molecule. It may be attributed to the partial unfolding caused by glycation. However, there was no obvious difference in the maximum absorption peak compared with the β -Lg-rib with and without different ultrasonic treatments. It suggested that ultrasound pretreatment had little influence on the UV absorption of glycated β -Lg.

Fluorescence spectrum was used to characterize the conformation changes of β -Lg. Figure 3(B) shows the Trp fluorescence emission spectra of native β -Lg and β -Lg-rib with and without different ultrasonic powers. When excited at 280 nm, the native β -Lg exhibited a fluorescence emission maximum ($\lambda_{\max}$) at 350 nm. The intensity of Trp-related fluorescence was decreased in the glycated β -Lg-rib treated with different ultrasonic powers and this was accompanied by a large red shift of the Trp emission maximum to 462, and to 466 for glycated β -Lg-rib pretreated at 400 W. It implied that the tertiary structure of β -Lg was dramatically changed by glycation between β -Lg and ribose. Thus, conformation changes,

including covering up of the glycated β -Lg-rib occurred around the Trp residues (19 Trp and 61 Trp) due to the glycation modification. Jiménez-Castaño, López-Fandiño, Olano, and Villamiel (2005) also found that the fluorescence intensity of β -Lg conjugation was lower than that of the native protein, which may be attributed to the shielding effect of ribose bound to the Trp residues (19 Trp and 61Trp). β -Lg-rib conjugation treated by 400 W gave the lowest fluorescence intensity. Taking the blocking of Lys or Arg residues into consideration, it is believed that the differences in the glycated sites occurred between β -Lg with and without ultrasonic treatment.

The fluorescence intensity of β -Lg-rib conjugation decreased in the untreated and ultrasonically treated and the minimum was observed at 400 W, which indicated structural change was caused by ultrasonic treatment and glycation modification. Ultrasonic treatment of β -Lg has been reported to induce a red shift in the emission maximum of the intrinsic fluorescence of tryptophan from 336 nm to 348 nm without the increase of fluorescence intensity (Stanic-Vucinic, Stojadinovic, Atanaskovic-Markovic, Ognjenovic, Grönlund, van Hage, et al. 2012), and the temperature can improve the fluorescence intensity because the partial exposure of Trp19 (Stănciuc, Aprodu, Răpeanu, & Bahrim 2012). In the case of the glycation modification, the fluorescence intensity of glycation β -Lg was slightly lower than that of the native β -Lg. Some reports also found that the fluorescence intensity of β -Lg-dextran (Jiménez-Castaño, López-Fandiño, Olano, & Villamiel 2005) and β -Lg-carboxymethyl dextran (Hattori., Koichi Nagasawa, Koki Ohgata, Noriko Sone, Ayumu Fukuda, Hiroshi Matsuda, et al. 2000) was lower than that of the native protein. Exposure of β -Lg to high intensity ultrasound can lead to the formation of nonnative oligomers and

dimers, β -sheet to α -helix conversion, and the exposure of tryptophan (Trp) residues to provide a less compact and more dynamic structure compared to the untreated β -Lg.

3.6. Identification of glycation sites and conformational changes

As we know, the antigenicity of β -Lg depends on both linear and conformational epitopes that widely spread along 162 amino acids (Clement, Boquet, Frobert, Bernard, Negroni, Chatel, et al. 2002). Therefore, to explore the mechanism of the decreased IgG- and IgE-binding ability of the glycation β -Lg with and without ultrasonic treatment, it is very important to monitor the precise glycation sites on the linear epitopes and figure out the difference between the native β -Lg and β -Lg-rib with and without ultrasonic treatment. Previous studies have suggested that the hydrophilic profile is the antigenic epitome of sequences corresponding to AA 41-60, 102-124, and 149-162 recognized by 92, 97, and 89% of human sera. A second fragment of AA 1-8 and 25-40 was recognized by 58% and 72%, while a third fragment comprised of peptides 9-14, 84-91, and 92-100 was still detected by more than 40% of sera (Selo et al. 1999). However, lysine and arginine amino groups and the N-terminal are possible sites of glycation and the glycation forms of the peptides can easily be determined from the mass shift induced by glycation (Wang, Tu, Liu, Liu, Huang, & Xiao 2013). It was essential to obtain the complete peptide map with all of the amino acids of β -Lg to compare them with the antigenic epitomes of sequences to fully explore the relationship between the glycation sites and IgG- and IgE-binding capacity. Pepsin was selected as the protease to digest the native and glycation β -Lg to guarantee higher protein coverage (Table S1, Figure S1 and S2 in the supplement material). Furthermore, all of

the glycated peptides and the corresponding unglycated peptides were eluted at the same retention time, which were used to calculate the DSP of the glycation peptides.

Ribose is most reactive reducing sugars in the glycation of proteins (Wei, Han, Zhou, Liu, Chen, & He 2012). The glycation sites and extent are the most important information about the glycation. A MS survey scan for the glycated β -Lg was performed using pepsin digestion. The results showed that the glycation sites were similar in glycated β -Lg without and with ultrasonic treatment at 400 W, and some derivative products were detected in both samples. For example, the m/z peak of 609.84^{2+} was identified as the unglycated peptides 1-11, and the mono-glycation and dual-glycation form were 675.86^{2+} and 741.88^{2+} , respectively. The MSMS spectra are shown in Figure 4. At the same time, the derivative product losing H_2O was detected with the m/z peaks of 732.88^{2+} . Table 1 lists all of the glycated peptides and the glycation sites without and ultrasonic treatment at 400 W. There were 14 lysines, 1 arginine, and 1 N-terminal leucine that were glycated without and with ultrasonic treatment (the three-dimensional structure was shown in Figure S3 in the supplement material). There were 7 and 3 derivative products that lost H_2O with mono- and dual- glycated peptides at lysine and arginine that were detected without and with ultrasonic treatment at 400 W, respectively.

Compared with the free amino acid content and the glycation site, the glycation extent was similar between with and without ultrasonic treatment. Therefore, to further understand the relationship between the glycation sites and antigenicity, the DSP (the average degree of substitution per peptide compared with the glycated content of each peptide) of each glycated peptide was calculated and the values were shown in Figure 5(A). Taken together, both the increased DSP of peptides 1-11, 10-19, and 96-104 and the similar DSP of other antigenic

epitopes of sequences were the main reasons for the decreased antigenicity ability of the glycosylated β -Lg with ultrasonic treatment at 400 W. Unexpectedly, the conformation change occurred with the glycosylated DSP without new glycosylation sites. Another reason was that the molecular weight of β -Lg is low, the 162 amino acids which can almost catch all ribose. In addition, comparison of the glycosylation sites and DSP of each peptide in a three-dimensional structure of β -Lg with and without ultrasonic treatment at 400 W (Figure 5(B&C)) suggested that the increase of DSP occurred in the helix and turned one of the surfaces of the global molecule after ultrasonic treatment at 400 W. Otherwise the higher DSP occurred in the sheet and turned another surface. However, the sheet in the core of β -Lg molecule with and without ultrasonic treatment was also glycosylated by ribose.

4. Conclusion

It was shown that the IgG and IgE binding of β -Lg dramatically increased after glycosylation with ribose after ultrasound pretreatment due to the mask of IgG and IgE epitopes and conformational changes of β -Lg. However, ultrasound pretreatment had an influence on glycosylation sites rather than glycosylation extent. Moreover, Orbitrap LC-MS/MS is a useful tool for analysis of glycosylation sites and extent. In the future, other experiments *in vivo* should be used to examine the decreased allergenicity by glycosylation with ultrasound pretreatment.

Acknowledgments

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References

- Bloom, K. A., Huang, F. R., Bencharitiwong, R., Bardina, L., Ross, A., Sampson, H. A., & Nowak - Węgrzyn, A. (2014). Effect of heat treatment on milk and egg proteins allergenicity. *Pediatric Allergy and Immunology*, 25(8), 740-746.
- Chandrapala, J., Zisu, B., Palmer, M., Kentish, S., & Ashokkumar, M. (2011). Effects of ultrasound on the thermal and structural characteristics of proteins in reconstituted whey protein concentrate. *Ultrasonics Sonochemistry*, 18(5), 951-957.
- Chen, Y., Tu, Z., Wang, H., Zhang, L., Sha, X., Pang, J., Yang, P., Liu, G., & Yang, W. (2016). Glycation of β -lactoglobulin under dynamic high pressure microfluidization treatment: Effects on IgE-binding capacity and conformation. *Food Research International*, 89, Part 1, 882-888.
- Clement, G., Boquet, D., Frobert, Y., Bernard, H., Negroni, L., Chatel, J.-M., Adel-Patient, K., Creminon, C., Wal, J.-M., & Grassi, J. (2002). Epitopic characterization of native bovine β -lactoglobulin. *Journal of Immunological Methods*, 266(1-2), 67-78.
- Corzo-Martínez, M., Moreno, F. J., Olano, A., & Villamiel, M. (2008). Structural characterization of bovine β -lactoglobulin– galactose/tagatose Maillard complexes by electrophoretic, chromatographic, and spectroscopic methods. *Journal of Agricultural and Food Chemistry*, 56(11), 4244-4252.
- Fayle, S. E., Healy, J. P., Brown, P. A., Reid, E. A., Gerrard, J. A., & Ames, J. M. (2001). Novel approaches to the analysis of the Maillard reaction of proteins. *Electrophoresis*,

22(8), 1518-1525.

Hattori, M., Koichi N., Koki O., Noriko S., Ayumu F., Hiroshi M., & Takahashi, a. K. (2000).

Reduced Immunogenicity of β -Lactoglobulin by Conjugation with Carboxymethyl Dextran. *Bioconjugate Chemistry*, 11(1), 84-93.

Huang, X., Tu, Z., Wang, H., Zhang, Q., Shi, Y., & Xiao, H. (2013). Increase of ovalbumin glycation by the Maillard reaction after disruption of the disulfide bridge evaluated by liquid chromatography and high resolution mass spectrometry. *Journal of Agricultural and Food Chemistry*, 61(9), 2253-2262.

Jiménez-Castaño, L., López-Fandiño, R., Olano, A., & Villamiel, M. (2005). Study on β -lactoglobulin glycosylation with dextran: effect on solubility and heat stability. *Food Chemistry*, 93(4), 689-695.

López-Expósito, I., Chicón, R., Belloque, J., López-Fandiño, R., & Berin, M. (2012). In vivo methods for testing allergenicity show that high hydrostatic pressure hydrolysates of β -lactoglobulin are immunologically inert. *Journal of Dairy Science*, 95(2), 541-548.

Meng, X., Li, X., Wang, X., Gao, J., Yang, H., & Chen, H. (2016). Potential allergenicity response to structural modification of irradiated bovine α -lactalbumin. *Food & Function*, 7(7), 3102-3110.

Moro, A., Báez, G. D., Busti, P. A., Ballerini, G. A., & Delorenzi, N. J. (2011). Effects of heat-treated β -lactoglobulin and its aggregates on foaming properties. *Food Hydrocolloids*, 25(5), 1009-1015.

Niemi, M. H., Rytkönen-Nissinen, M., Miettinen, I., Jänis, J., Virtanen, T., & Rouvinen, J. (2015). Dimerization of lipocalin allergens. *Scientific Reports*, 5, 13841.

- Papiz, M., Sawyer, L., Eliopoulos, E., North, A., Findlay, J., Sivaprasad arao, R., Jones, T., Newcomer, M., & Kraulis, P. (1986). The structure of b-lactoglobulin and its similarity to plasma retinol-binding protein. *Nature*, 324(6095), 383-385.
- Peram, M. R., Loveday, S. M., Ye, A., & Singh, H. (2013). In vitro gastric digestion of heat-induced aggregates of β -lactoglobulin. *Journal of Dairy Science*, 96(1), 63-74.
- Selo, I., Clement, G., Bernard, H., Chatel, J., Creminon, C., Peltre, G., & Wal, J. (1999). Allergy to bovine b-lactoglobulin: specificity of human IgE to tryptic peptides. *Clinical and Experimental Allergy*, 29(8), 1055-1063.
- Stănciuc, N., Aprodu, I., Râpeanu, G., & Bahrim, G. (2012). Fluorescence spectroscopy and molecular modeling investigations on the thermally induced structural changes of bovine β -lactoglobulin. *Innovative Food Science & Emerging Technologies*, 15, 50-56.
- Stanic-Vucinic, D., Prodic, I., Apostolovic, D., Nikolic, M., & Cirkovic Velickovic, T. (2013). Structure and antioxidant activity of β -lactoglobulin-glycoconjugates obtained by high-intensity-ultrasound-induced Maillard reaction in aqueous model systems under neutral conditions. *Food Chemistry*, 138(1), 590-599.
- Stanic-Vucinic, D., Stojadinovic, M., Atanaskovic-Markovic, M., Ognjenovic, J., Grönlund, H., van Hage, M., Lantto, R., Sancho, A. I., & Velickovic, T. C. (2012). Structural changes and allergenic properties of β -lactoglobulin upon exposure to high-intensity ultrasound. *Molecular Nutrition & Food Research*, 56(12), 1894-1905.
- Tu, Z.-c., Zhong, B.-z., & Wang, H. (2017). Identification of glycosylated sites in ovalbumin under freeze-drying processing by liquid chromatography high-resolution mass spectrometry. *Food Chemistry*, 226(1), 1-7.

- Wang, H., Tu, Z.-C., Liu, G.-X., Liu, C.-M., Huang, X.-Q., & Xiao, H. (2013). Comparison of glycation in conventionally and microwave-heated ovalbumin by high resolution mass spectrometry. *Food Chemistry*, 141(2), 985-991.
- Wei, Y., Han, C. S., Zhou, J., Liu, Y., Chen, L., & He, R. Q. (2012). d-ribose in glycation and protein aggregation. *Biochimica et Biophysica Acta (BBA) - General Subjects*, 1820(4), 488-494.
- Wong, K.-L., Li, H., K Wong, K.-K., Jiang, T., & Shaw, P.-C. (2012). Location and reduction of icarapin antigenicity by site specific coupling to polyethylene glycol. *Protein and Peptide Letters*, 19(2), 238-243.
- Xiang, B. Y., Ngadi, M. O., Ochoa-Martinez, L. A., & Simpson, M. V. (2011). Pulsed electric field-induced structural modification of whey protein isolate. *Food and Bioprocess Technology*, 4(8), 1341-1348.
- Zhang, Q., Tu, Z., Wang, H., Huang, X., Shi, Y., Sha, X., & Xiao, H. (2014). Improved glycation after ultrasonic pretreatment revealed by High-Performance Liquid Chromatography–Linear Ion Trap/Orbitrap High-Resolution Mass Spectrometry. *Journal of Agricultural and Food Chemistry*, 62(12), 2522-2530.
- Zhong, J., Cai, X., Liu, C., Liu, W., Xu, Y., & Luo, S. (2016). Purification and conformational changes of bovine PEGylated β -lactoglobulin related to antigenicity. *Food Chemistry*, 199(15), 387-392.
- Zhong, J., Tu, Y., Liu, W., Luo, S., & Liu, C. (2015). Comparative study on the effects of nystose and fructofuranosyl nystose in the glycation reaction on the antigenicity and conformation of β -lactoglobulin. *Food Chemistry*, 188(1), 658-663.

Zhong, J., Tu, Y., Liu, W., Xu, Y., Liu, C., & Dun, R. (2014). Antigenicity and conformational changes of β -lactoglobulin by dynamic high pressure microfluidization combining with glycation treatment. *Journal of Dairy Science*, 97(8), 4695-4702.

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Tables

Table 1 β -lactoglobulin glycation peptides

No.	m/z	St.	End	Δ ppm	Sequence	β -Lg-rib without ultrasonic treatment			β -Lg-rib with 400W ultrasonic treatment		
						modification m/z	Sites		modification m/z	Sites	
1	609.8423 ²⁺	1	11	0.893	(-)LIVTQTMKGLD(I)	675.86 ²⁺ , 741.88 ²⁺	(732.88 ²⁺), L1, K8		675.86 ²⁺ , 741.88 ²⁺	L1, K8	
2	565.8144 ²⁺	10	19	0.962	(G)LDIQKVAGTW(Y)	631.83 ²⁺ , 697.86 ²⁺	(688.85 ²⁺), K14		631.83 ²⁺ , 697.86 ²⁺	K14	
3	745.3758 ²⁺	42	54	1.51	(V)YVEELKPTPEGDL(E)	811.40 ²⁺ , 877.72 ²⁺	(868.41 ²⁺), K47		811.40 ²⁺ , 877.72 ²⁺	(868.41 ²⁺), K47	
4	549.7870 ²⁺	45	54	-0.917	(E)ELKPTPEGDL(E)	615.81 ²⁺ , 681.83 ²⁺	(672.82 ²⁺), K47		615.81 ²⁺ , 681.83 ²⁺	(672.82 ²⁺), K47	

5	437.7244 ²⁺	58	64	-0.251	(L)LQKWENG(E)	503.75 ²⁺	K60	503.75 ²⁺	K60
6	595.316 ²⁺	64	74	0.495	(N)GECAQKKIIAE(K)	661.32 ²⁺	K69/K70	661.32 ²⁺	K69/K70
7	466.273 ²⁺	66	73	-0.0267	(E)C(Carbamidomethyl)A QKKIIA(E)	532.27 ²⁺ , 598.59 ²⁺	K69/K70	532.27 ²⁺ , 598.59 ²⁺	K69/K70
8	516.8083 ²⁺	74	82	0.499	(A)EKTIPAVF(K)	582.83 ²⁺ , (639.85 ²⁺), 648.85 ²⁺ , (705.87 ²⁺), 714.87 ²⁺	K75, K77	582.83 ²⁺ , 648.85 ²⁺ , 714.87 ²⁺	K75, K77
9	401.7191 ²⁺	83	89	0.516	(F)KIDALNE(N)	467.74 ²⁺ , 533.76 ²⁺	K83	467.74 ²⁺ , 533.76 ²⁺	K83
10	734.9407 ²⁺	83	95	0.523	(F)KIDALNENKVLVL(D)	800.96 ²⁺ , 866.98 ²⁺	K83, K91	800.96 ²⁺ , 866.98 ²⁺	K83, K91
11	579.8066 ²⁺	96	104	1.56	(L)DTDYKKYLL(F)	645.83 ²⁺ , 711.85 ²⁺ , (768.86 ²⁺), 777.87 ²⁺	K100, K101	645.83 ²⁺ , 711.85 ²⁺	K100, K101
12	966.4418	129	136	0.343	(V)DDEALEKF(D)	1032.54 ²⁺	K135	1032.54 ²⁺	K135
13	525.3031 ²⁺	134	142	-0.046	(L)EKFDKALKA(L)	591.74 ²⁺ , 657.34 ²⁺	K135, K138, K141	591.74 ²⁺ , 657.34 ²⁺	K135, K138, K141

						723.37 ²⁺ , 789.39 ²⁺		723.37 ²⁺ , 789.39 ²⁺	
14	440.2655 ²⁺	143	149	0.477	(A)LPMHRL(S)	(497.23 ²⁺), 505.28 ²⁺	R148	(497.23 ²⁺), 505.28 ²⁺	R148

The m/z in bracket is the derivation products of losing H₂O of two H₂O.

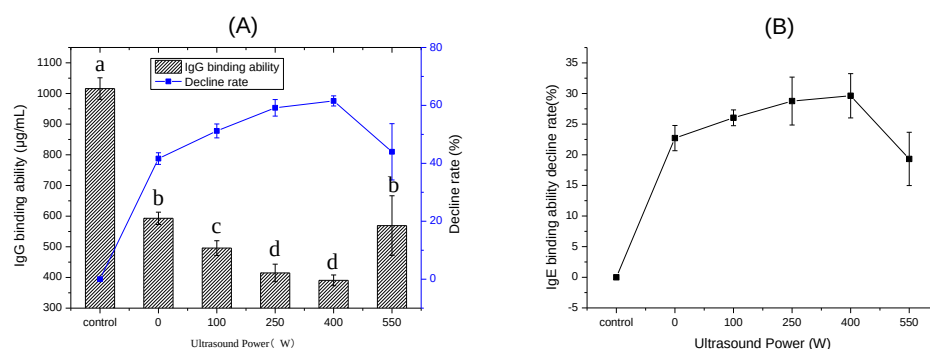


Figure 1. IgG- (A) and IgE-binding (B) capacity of the native β -Lg and β -Lg-rib glycosylation from different ultrasonic treatments combined with glycation. Means with different letters (a-d) on the same patients are significantly different ($p < 0.05$). Control: native β -Lg; 0 W: β -Lg glycosylation without ultrasonic treatment; 100 W, 250 W, 400 W, and 550 W: β -Lg glycosylation with 100 W, 250 W, 400 W, and 550 W treatment, respectively.

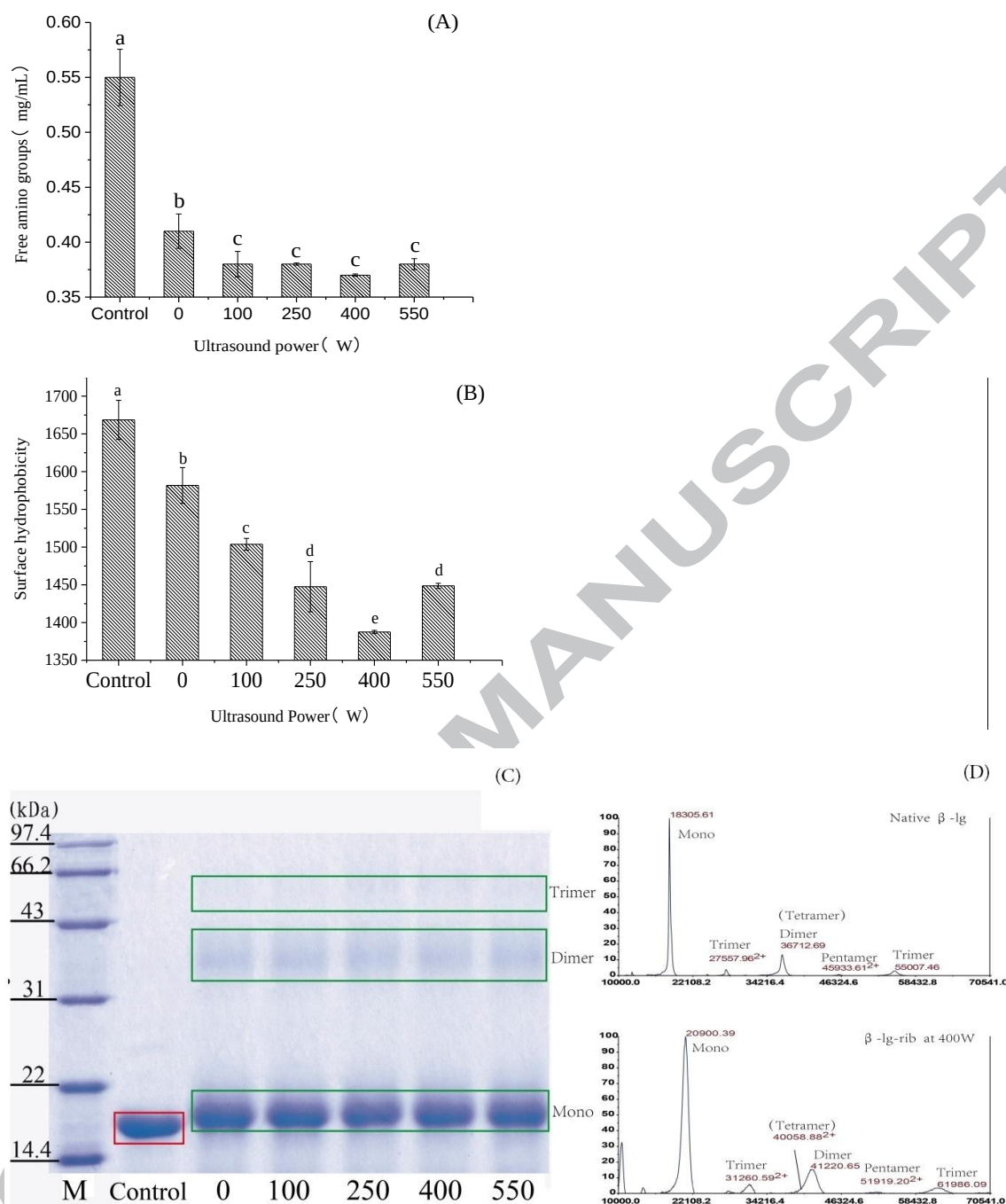


Figure 2. Effects of ultrasound combined with glycation on the free amino groups (A), surface hydrophobicity (B), SDS-PAGE (C) and MALDI-TOF mass spectra (D) of native β -Lg and β -Lg-rib from different ultrasonic treatments combined with glycation. Means with different letters (a-e) on the different samples are significantly different ($p < 0.05$). Control: native β -Lg; 0 W: β -Lg-rib glycosylation without ultrasonic treatment; 100 W, 250 W, 400 W, and 550 W: β -Lg-rib glycosylation with 100 W, 250 W, 400 W, and 550 W treatment, respectively.

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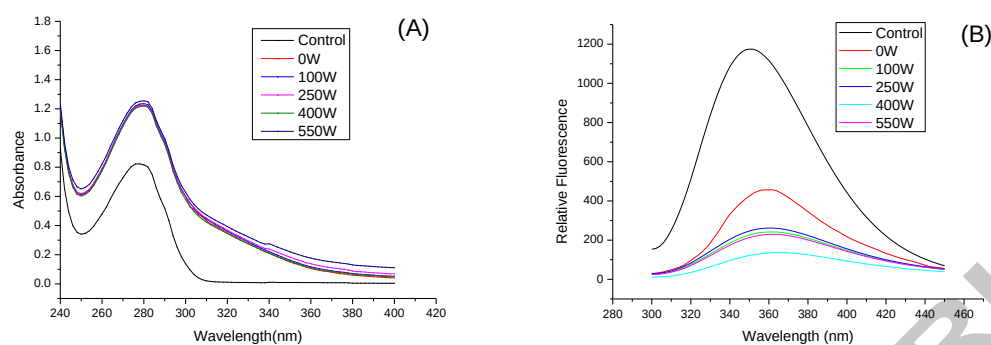


Figure 3. UV absorption and fluorescence of the native β -Lg and β -Lg-rib from different ultrasonic treatment combined with glycation. Control: native β -Lg; 0 W: β -Lg-rib glycosylation without ultrasonic treatment; and 100 W, 250 W, 400 W, and 550 W: β -Lg-rib glycosylation with 100 W, 250 W, 400 W, and 550 W treatment, respectively.

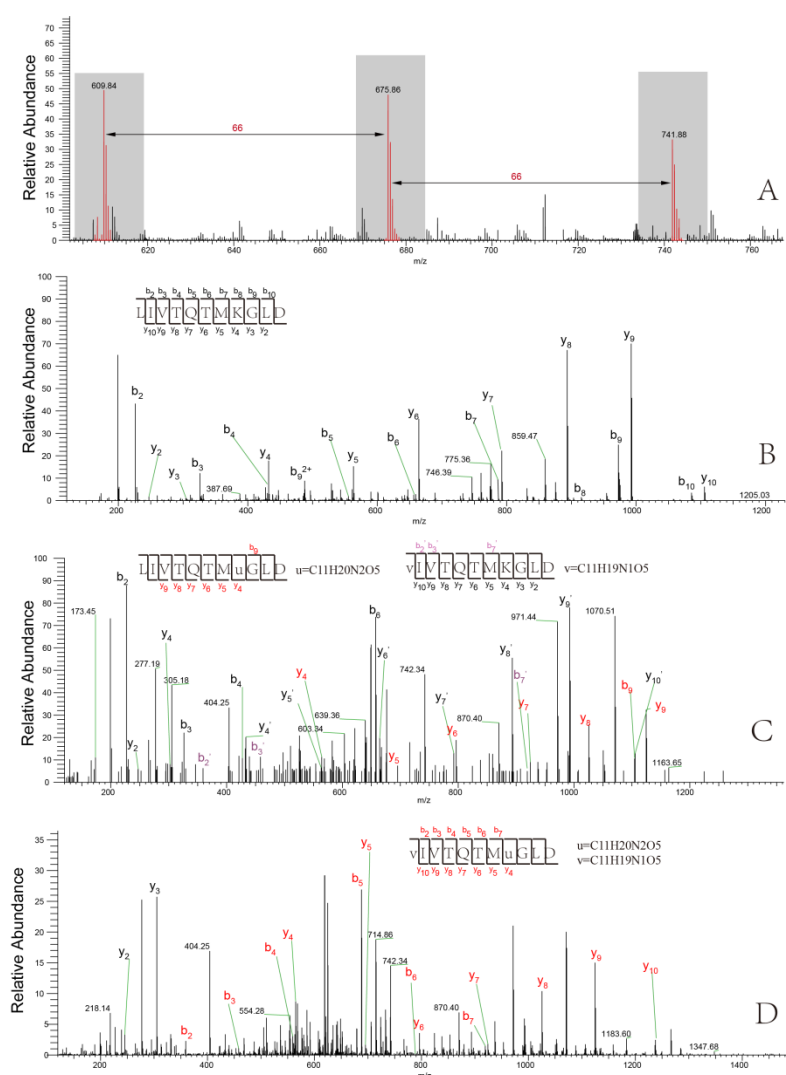


Figure 4. MS spectrum of glycosylated peptide 1-11 at m/z 609.84²⁺(A) and the HCD MS/MS spectrum of this peptide (B) and mono-glycosylated peptide 1-11 at m/z 675.86²⁺(C) and dual-glycosylated peptide 1-11 at m/z 741.88²⁺(D). The sequence of each peptide is shown at the top of the spectrum. Mainly, characteristic b and y ions are shown in red in the HCD MS/MS spectrum.

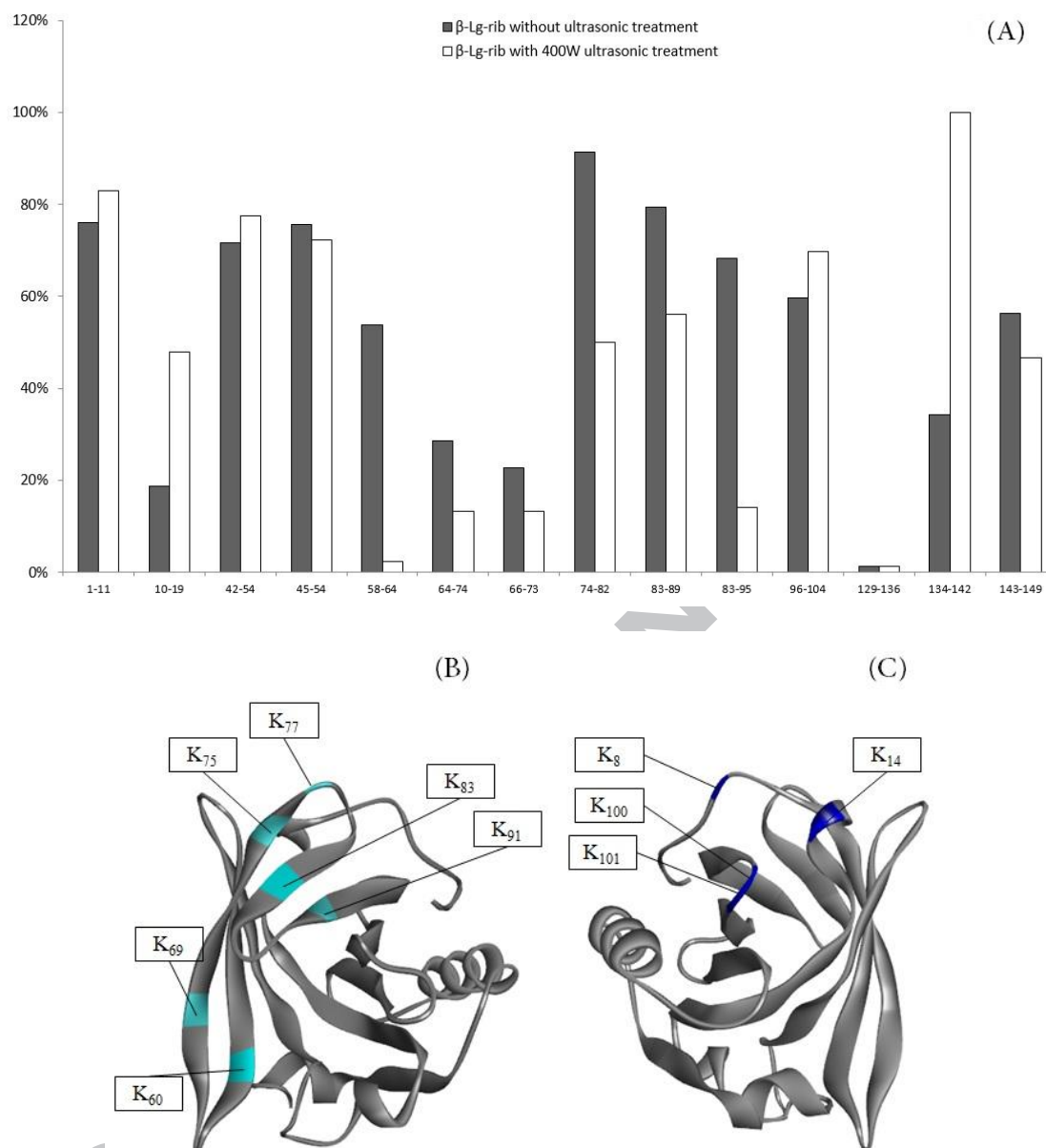


Figure 5. The DSP value of each glycated peptide from the β -Lg-rib from without (gray bars) and with 400W ultrasonic pretreatment (white bars) (A) and ribbon diagram of the β -Lg-rib (PDB 5io5) from without (B) and 400W ultrasonic pretreatment (C). The lysines are color-coded as follows: gray, framework of β -Lg; light blue, the higher DSP of lysines in β -Lg-rib without ultrasonic pretreatment; and blue, the higher DSP of lysines in β -Lg-rib from with 400W ultrasonic pretreatment.

Supplementary Materials

Table S1 Peptide mapping of nature β -Lg

No.	m/z	MH ⁺	MH ⁺	Δ ppm	Start	End	Sequence
1	609.8423 ²⁺	1218.6773	1218.6762	0.893	1	11	(-)LIVTQTMKGLD(I)
2	565.8144 ²⁺	1130.6215	1130.6204	0.962	10	19	(G)LDIQKVAGTW(Y)
3	563.2857	563.2857	563.2858	-0.106	21	26	(Y)SLAMAA(S)
4	535.3038 ²⁺	1069.6003	1069.6	0.27	32	41	(L)LDAQSAPLRV(Y)
5	745.3758 ²⁺	1489.7443	1489.7421	1.51	42	54	(V)YVEELKPTPEGDL(E)
6	549.7870 ²⁺	1098.5667	1098.5677	-0.917	45	54	(E)ELKPTPEGDL(E)
7	437.7244 ²⁺	874.4415	874.4417	-0.251	58	64	(L)LQKWENG(E)
8	595.3162 ²⁺	1189.6251	1189.6245	0.495	64	74	(N)GECAQKKIIAE(K)
9	466.2733 ²⁺	931.5393	931.5393	-0.0267	66	73	(E)C(Carbamidomethyl)AQKKIIA(E)
10	516.8083 ²⁺	1032.6093	1032.6088	0.499	74	82	(A)EGTKIPAVF(K)
11	401.7191 ²⁺	802.4309	802.4305	0.516	83	89	(F)KIDALNE(N)
12	734.9407 ²⁺	1468.8741	1468.8734	0.523	83	95	(F)KIDALNENKVLVL(D)
13	579.8066 ²⁺	1158.6059	1158.6041	1.56	96	104	(L)DTDYKKYLL(F)

14	529.1783	529.1783	529.1785	-0.411	105	108	(L)FCME(N)
15	617.7664 ²⁺	1234.5249	1234.5256	-0.574	107	117	(C)MENSAEPEQSL(V)
16	678.3309	678.3309	678.3313	-0.633	117	122	(S)LVCQCL(V)
17	522.2721 ²⁺	1043.5369	1043.5368	0.151	122	130	(C)LVRTPEVDD(E)
18	966.4418	966.4418	966.4415	0.343	129	136	(V)DDEALEKF(D)
19	525.3031 ²⁺	1049.5989	1049.599	-0.046	134	142	(L)EKFDKALKA(L)
20	440.2655 ²⁺	879.5237	879.5233	0.477	143	149	(A)LPMHIRL(S)
21	403.7058 ²⁺	806.4051	806.4043	0.999	150	156	(L)SFNPTQL(E)
22	408.1715 ²⁺	815.3357	815.3352	0.607	157	162	(L)EEQC(Carbamidomethyl)HI(-)

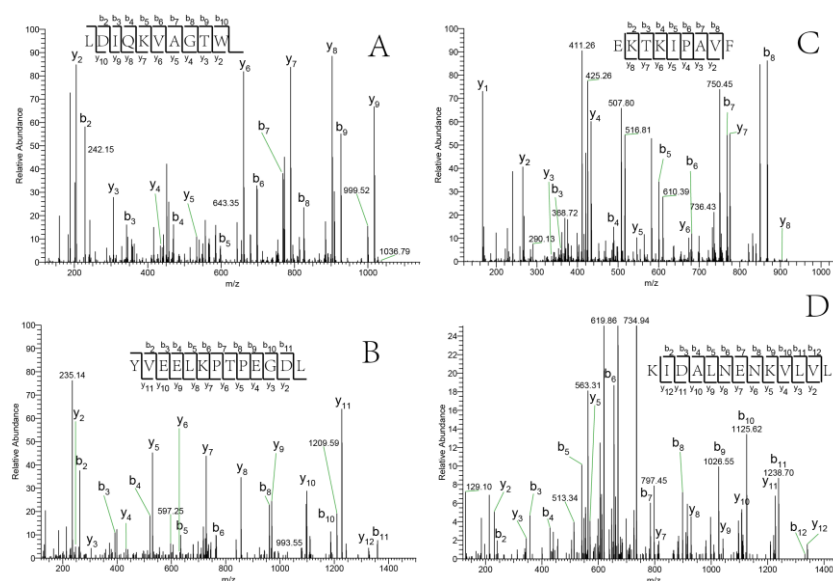


Figure S1. HCD MSMS of native peptides 10-19(A), 42-54(B), 74-82 (C), 83-95 (D)

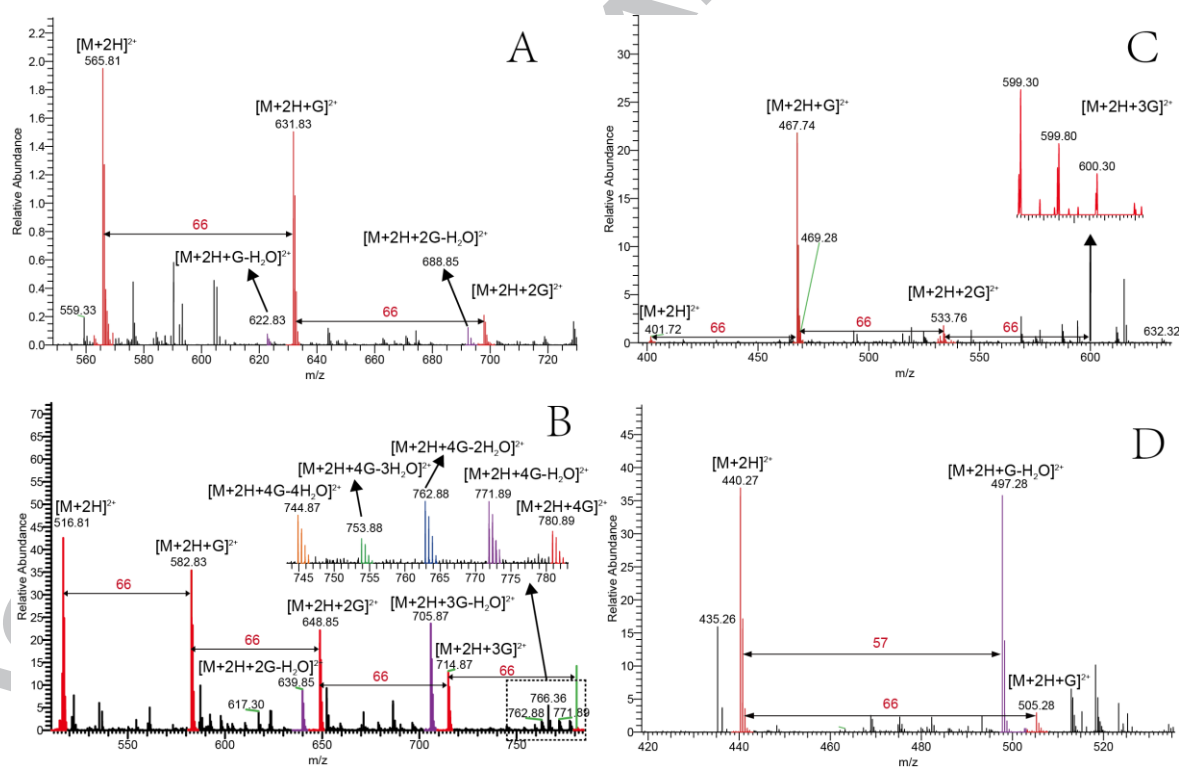


Figure S2. MS spectra with peaks of glycosylated peptides 10-19 at m/z 565.81442+(A), 74-82 at m/z 565.81442+ (B), 83-89 at m/z 41.71913+ (C), and 143-149 at m/z 440.26552+ (D).

Glycation is indicated by a mass increase of 132.0417 and loss H_2O of 114.0311.

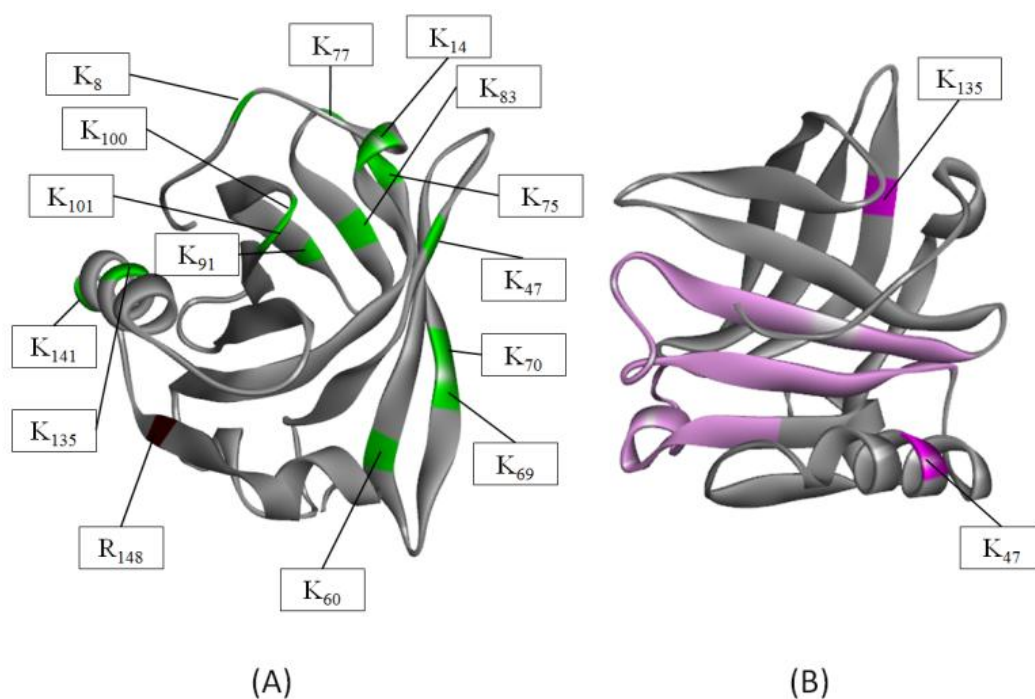


Figure S3. Ribbon diagram of the β -Lg-rib (PDB 5io5). The glycated lysines are colour coded as follows: grey, framework of β -Lg; green, the glycated sites of β -Lg-rib (A); purple, the glycation peptides and sites with similar DSP of β -Lg-rib from between without and with 400W ultrasonic pretreatment (B).

Highlights:

1. Glycation with ultrasound pretreatment obviously decreased allergenicity of β -Lg.
2. Ultrasound pretreatment promoted the reduction of allergenicity of glycated β -Lg.
3. Ultrasound pretreatment had little effect on the glycation degree of β -Lg.
4. Ultrasound pretreatment at 400 W increased the glycation degree of some peptides.
5. The number of glycation sites of β -Lg was kept after ultrasound pretreatment.