

Mechanism of Reduction in IgG and IgE Binding of β -Lactoglobulin Induced by Ultrasound Pretreatment Combined with Dry-State Glycation: A Study Using Conventional Spectrometry and High-Resolution Mass Spectrometry

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S Supporting Information

ABSTRACT: Bovine β -lactoglobulin (β -Lg) is one of major allergens in cow's milk. Previous study showed that ultrasound treatment induced the conformational changes of β -Lg and promoted the glycation in aqueous solutions, which is, however, less efficient compared with dry-state. In this work, the effect of ultrasound pretreatment combined with dry-state glycation on the IgG and IgE binding of β -Lg was studied. Dry-state glycation with mannose after ultrasound pretreatment at 0–600 W significantly reduced the IgG and IgE binding of β -Lg, with the lowest values observed at 400 W. The decrease in the IgG and IgE binding of β -Lg was attributed to the increase in glycation extent and the changes of secondary and tertiary structure, which reflected in the increase of UV absorbance, α -helix and β -sheet contents, as well as the decrease of intrinsic fluorescence intensity, surface hydrophobicity, β -turn, and random coil contents. Moreover, ultrasound pretreatment promoted the reduction of IgG and IgE binding abilities by improving glycation, reflecting in the increase of the glycation sites and the degree of substitution per peptide (DSP) value determined by Fourier transform ion cyclotron resonance mass spectrometry (FTICR-MS). Ultrasound pretreatment at 400 W showed the most significantly enhanced glycation extent. Besides, the results suggested FTICR-MS could provide insights into the glycation at molecular level, which was conducive to the understanding of the mechanism of the reduction in the IgG and IgE binding of β -Lg. Therefore, ultrasound pretreatment combined with dry-state glycation may be a promising method for β -Lg hypsensitization.

KEYWORDS: β -lactoglobulin, IgG and IgE binding abilities, ultrasound pretreatment, dry-state glycation, FTICR-MS

INTRODUCTION

Bovine β -lactoglobulin (β -Lg), which constitutes about 60% of whey proteins, is the major allergen that causes about 90% IgE-mediated cow's milk allergy (CMA).^{1,2} It is a 162-amino-acid globular protein with a molecular mass of 18.36 kDa.³ The formation of two disulfide bonds (Cys66–Cys160 and Cys106–Cys119) stabilizes a so-called barrel (or calyx) structure that is formed by nine antiparallel sheets.⁴ So far, β -Lg IgG and IgE epitopes, including linear and conformational types, are mainly identified.^{5,6} Recently, numerous methods have been investigated to alter the allergenicity of β -Lg through physical modifications, such as heating,⁷ dynamic high-pressure microfluidization (DHPM),⁸ high hydrostatic pressure,⁹ and high-intensity ultrasound treatment;¹⁰ chemical treatments, such as hydrolysis^{11,12} and glycation;¹³ and genetic modification.^{14,15} However, DHPM and high hydrostatic pressure could increase the allergenicity of β -Lg, whereas high-intensity ultrasound treatment had insignificant effect on the allergenicity of β -Lg. Hydrolysis has some sensory defects (such as bitterness) and might cause some potential disordered metabolism.¹¹ Genetic modification is controversial and inefficient.¹⁶

Among the potential alteration methods, glycation has been considered as a safe way for modifying proteins compared with

other methods.¹⁷ It generally takes place during the first stage of the Maillard reaction between proteins and reducing sugars. As one of most common and important chemical reactions during food processing and storage, glycation is often used to modify the food protein properties, such as antioxidant activity and allergenicity.^{18,19} However, a single modification cannot decrease the allergenicity to a satisfactory result due to the insufficient glycation extent.

High-intensity ultrasound is an emerging nonthermal technology that uses a collective force of high temperature, high pressure, microjet, shear force, shock wave, and turbulence.²⁰ Our previous work showed that ultrasound treatment partially unfolded the structure of ovalbumin (OVA) and promoted the glycation of bovine serum albumin (BSA).^{21,22} Stanic-Vucinic et al.¹⁸ reported that ultrasound promoted the glycation of β -Lg in aqueous solutions. Therefore, ultrasound pretreatment combined with glycation may provide a new method for β -Lg desensitization. Although, the glycation

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efficiency at dry-state is far higher than that in aqueous solutions,²² few studies investigated the influence of ultrasound pretreatment coupled with dry-state glycation on allergenicity of β -Lg. Moreover, the mechanism of the decrease in allergenicity of β -Lg by glycation after ultrasound pretreatment remains unclear.

The purpose of this study was therefore to carry out a detailed interpretation of the relationship between allergenicity and structural changes of β -Lg induced by ultrasonic pretreatment combined with dry-state glycation. In this work, β -Lg was pretreated by high-intensity ultrasound followed by glycation with mannose at dry-state under nondenaturing conditions. The IgG and IgE binding of β -Lg samples was evaluated by inhibition ELISA. The structural changes were characterized by conventional spectrometry, including circular dichroism (CD), ultraviolet (UV), and fluorescence spectrometry. The glycation sites and extent of glycated β -Lg were determined by Fourier transform ion cyclotron resonance mass spectrometry (FTICR-MS). The results of this research enhance our understanding of the mechanism of the decrease in IgG and IgE binding of β -Lg induced by ultrasound pretreatment combined with dry-state glycation, as well as demonstrate that ultrasound pretreatment combined with dry-state glycation may be a promising method for β -Lg hyposensitization.

MATERIALS AND METHODS

Materials. β -Lg (L3908), D-(+)-mannose (M2069), goat antirabbit IgG-HRP conjugate (A9169), goat antihuman IgE-HRP conjugate (A9667), 1-anilino-8-naphthalene-sulfonate (ANS), and 3,3',5,5'-tetramethylbenzidine (TMB) were purchased from Sigma-Aldrich (St. Louis, MO, USA). CMA patients' sera were bought from PlasmaLab International (Everett, WA, USA) and frozen (-80°C) until analysis. Their specific IgE levels ranged from 9.58 to 96.6 kU L^{-1} (detailed information is shown in Supporting Information Figure S1). The polyclonal antisera against β -Lg were obtained from six young (three months old, about 2.0 kg) Japanese male rabbits. H_2O used in this work was Milli-Q water. All other chemicals used were of analytical grade.

β -Lg Samples Preparation. β -Lg solution (1 mg/mL, dissolved in 50 mmol L^{-1} phosphate buffered saline (PBS), pH 8.0) was placed in a beaker to be treated by a sonicator (JY98-IIIDN, Scientz Biotechnology Co. Ltd., Ningbo, China). The sonicator frequency was 20 kHz and the maximum power was 950 W. The solution was irradiated with an ultrasonic wave directly from the probe with a vibrating titanium tip of 15 mm. Samples were treated for 30 min at different powers (0, 200, 400, and 600 W). The ultrasonic intensity at 200, 400, and 600 W was 38–42, 57–59, and 68–72 W cm^{-2} , respectively. The sonicator worked for 3 s after a pause of 7 s. The samples were treated in the ice bath to keep the processing temperature below 20°C . The mannose was then added into the samples (the mass ratio of β -Lg and mannose was 1:2). After mixing evenly, all the samples (100 μL , 10 $\mu\text{g mL}^{-1}$) were lyophilized and incubated for 4 h at 55°C and 0.79 water activity. They were then placed into a freezer to stop the reaction. The remaining mannose was removed by Millipore ultrafiltrator (molecular weight cut off 3000 Da). Finally, all samples were adjusted to 1 $\mu\text{g mL}^{-1}$ and stored at 4°C for less than 48 h. Native β -Lg was a control and named β -Lg-N. The sample without mannose heated under the same glycation conditions was another control and named β -Lg-H. The glycated samples with ultrasound pretreatment at 0, 200, 400, and 600 W were named β -Lg-M, β -Lg-M-200, β -Lg-M-400, and β -Lg-M-600, respectively.

Determination of IgG and IgE Binding Abilities. The IgG and IgE binding of β -Lg samples was evaluated by inhibition ELISA with rabbit antisera and CAM patients' antisera, respectively.²³ First, the 96-well microplate was coated overnight at 4°C with native β -Lg (100 μL per well, 2 $\mu\text{g mL}^{-1}$). After washing with PBST (0.05% Tween-20 in 50 mmol L^{-1} PBS, pH 7.4), the microplate was blocked with 50 mg mL^{-1} skimmed milk for 1 h at 37°C . Then 50 μL of either pooled rabbit antisera (diluted to 1:12 800) or pooled CAM patients' antisera (diluted to 1:8) and 50 μL of β -Lg samples (inhibitors) were added after washing.

After incubation for 1 h at 37°C , samples were washed five times with PBST. Then 100 μL of goat antihuman IgE-HRP conjugate or goat antirabbit IgG-HRP conjugate (diluted to 1:5000 in PBST) was added and incubated for 30 min at 37°C . After washing, 100 μL of TMB solution was added and incubated for 15 min at 37°C . Finally, 50 μL of 2 mol L^{-1} sulfuric acid was added to stop the reaction and the absorbance was measured at 450 nm using a microplate reader (HF2000, Huaan Magnech, Beijing, China). The inhibition rate was calculated using the following equation: $\text{Inhibition} = (1 - B/B_0) \times 100\%$, where B and B_0 are the absorbance values of the well with and without the inhibitor, respectively. IC_{50} is the concentration of inhibitors that causes a 50% inhibition of antibody binding ($\mu\text{g mL}^{-1}$).

Far-UV CD Spectroscopy. Far-UV (190–240 nm) CD spectroscopy was performed to define the secondary structure of β -Lg samples. The CD spectra of β -Lg (0.1 mg mL^{-1} in 50 mmol L^{-1} PBS, pH 8.0) were collected by using a MOS-450 spectropolarimeter (Bio-Logic SAS, Claix, French). The path length, bandwidth, and step resolution were 1.0 mm. The scan speed was 100 nm min^{-1} . The data were shown in terms of molar residue ellipticity ($[\theta]$, $\text{deg cm}^2 \text{dmol}^{-1}$), and the contents of different secondary structures were analyzed with DichroWeb.

UV Absorption and Intrinsic Fluorescence Spectroscopy. UV absorption spectrum was performed by a Hitachi spectrophotometer (UV-2910, Hitachi, Tokyo, Japan). The β -Lg samples (0.5 mg mL^{-1} in 50 mmol L^{-1} PBS, pH 8.0) were scanned from 240 to 320 nm at a scan speed of 800 nm min^{-1} . Intrinsic fluorescence was measured by a Hitachi spectrofluorimeter (F-7000, Hitachi, Tokyo, Japan). The concentration of β -Lg was 0.1 mg mL^{-1} . The emission spectra were scanned from 300 to 420 nm at a speed of 1200 nm min^{-1} with the excitation wavelength of 280 nm. The bandwidth, excitation, and emission slits were all 5.0 nm.

Surface Hydrophobicity. According to the method of Matulis and Lovrien²⁴ with modification, the surface hydrophobicity of β -Lg samples was measured using ANS fluorescent probes. The volume ratio of sample (0.25, 0.5, and 1.0 mg mL^{-1}) and ANS solution (0.008 mol L^{-1}) was 200:1. The emission spectrum was scanned from 400 to 600 nm at a speed of 1200 nm min^{-1} with the excitation wavelength of 390 nm. Other parameters were the same as those of intrinsic fluorescence measurement. The surface hydrophobicity of the samples was defined as the ratio of the extrinsic fluorescence intensity to the protein concentration with different concentrations.

HPLC-FTICR-MS Analysis. First, β -Lg (10 μg) was added into the Tris-HCl buffer (pH 7.5, 1 mol L^{-1}) containing 6 mol L^{-1} guanidine. Then 0.25 μL of 0.5 mol L^{-1} DTT was added and incubated for 30 min at 37°C , following by addition of 0.5 μL iodoacetamide (0.5 mol L^{-1}). After dark incubation for 30 min at room temperature, the solution was diluted 10-fold with H_2O . Then 2 μL of 1 mg mL^{-1} trypsin was added and the sample was incubated overnight at 37°C . Next, 2 μL of 10% formic acid (FA) was added to stop the reaction. Then 50 μL of sample solution was injected into a BioSuite C18 column (2.1 mm $\times$ 50 mm, 3 μm ; Waters, Milford, MA) for HPLC-MS. The flow rate of LC was 0.2 mL min^{-1} . The mobile phase consisted of solvent A (0.1% FA in H_2O) and solvent B (100% acetonitrile containing 0.1% FA). The gradient elution was 0–5 min, 5% solvent B; 5–35 min, 5%–50% solvent B gradient; 35–37 min, 60%–80% solvent B gradient; 37–40 min, 80% solvent B; 40–42, 20%–95% solvent A gradient. The effluent was infused into the Bruker 7T-Solarix FTICR-MS (Bruker Daltonics Inc., Billerica, MA). The electron capture dissociation (ECD) fragmentation mode was used to acquire MS/MS spectra. The ionization energy was 1.0 eV and the capillary voltage was 4500 V. The temperature and flow rate of dry gas were 180°C and 8 L min^{-1} , respectively. Software (DataAnalysis and BioTools) was used to identify peptides according to the combination of accurate masses and MS/MS spectra.

To further compare the glycation extent of each peptide, the average degree of substitution per peptide (DSP) was calculated according to the following formula:²⁵

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

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

Statistical Analysis. All the experiments were carried out in triplicate and the results are presented as mean value $\pm$ standard deviation (SD). The analysis was performed using Origin-Pro 2017 (OriginLab Corp., Northampton, MA).

RESULTS AND DISCUSSION

IgG and IgE Binding Capacities Analysis. The IgG and IgE binding abilities of β -Lg samples were determined by inhibition ELISA with anti- β -Lg-sera from rabbit and CMA patients, respectively. The ability of antibody binding to allergen was reflected by the IC_{50} value. Higher IC_{50} value implies lower binding capacity. Figure 1 shows the effect of ultrasound pretreatment combined with glycation on the IgG and IgE binding of β -Lg. As shown in Figure 1A, compared with β -Lg-N ($1.94 \mu\text{g mL}^{-1}$), β -Lg-H showed almost the same IC_{50} value, whereas the glycosylated β -Lg samples exhibited much higher IC_{50} values, with highest value ($18.11 \mu\text{g mL}^{-1}$, about 9-fold with β -

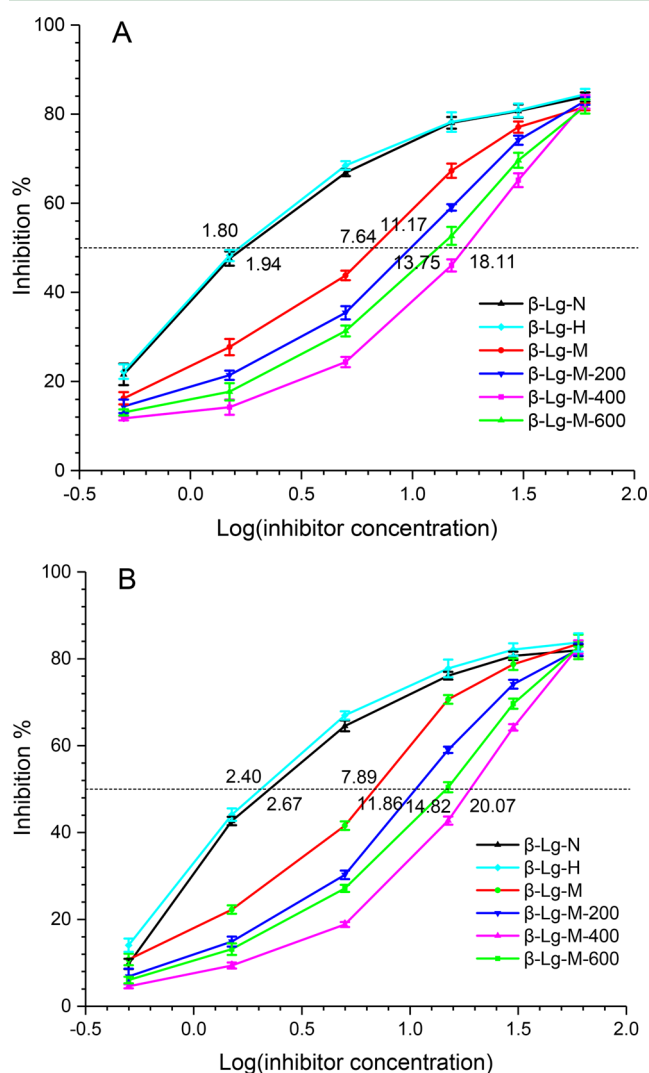


Figure 1. Changes of IgG (A) and IgE (B) binding abilities of β -Lg induced by ultrasound pretreatment coupled with glycation. The IgG and IgE binding abilities of β -Lg samples were determined by inhibition ELISA. Pooled rabbit anti- β -Lg-sera or human anti- β -Lg-sera ($50 \mu\text{L}$ per well) were incubated separately with 1, 5, 10, 10, 40, 48 $\mu\text{g mL}^{-1}$ of corresponding OVA samples as inhibitors.

Lg-N) observed at 400 W. When β -Lg was glycosylated after ultrasound pretreatment at 0–400 W, the IC_{50} value of IgG gradually increased with increasing ultrasound intensity. However, when it was glycosylated after ultrasound pretreatment at 600 W, the IC_{50} value decreased to $13.75 \mu\text{g mL}^{-1}$. Similar results were observed in Figure 4B. At ultrasound powers of 0 ($7.89 \mu\text{g mL}^{-1}$), 200 ($11.86 \mu\text{g mL}^{-1}$), 400 ($20.07 \mu\text{g mL}^{-1}$), and 600 ($14.82 \mu\text{g mL}^{-1}$)W, the IC_{50} values of glycosylated β -Lg were 2.95-, 5.00-, 7.74-, and 5.55-fold higher than that of β -Lg-H ($2.67 \mu\text{g mL}^{-1}$). These results indicated that glycation significantly reduced the IgG and IgE binding of β -Lg and ultrasound pretreatment promoted the reduction. This was also similar to that reported in a previous study,^{13,23,26} wherein they showed that glycation notably decreased the IgG and/or IgE binding of β -Lg.

The decrease in IgG and IgE binding abilities may be attributed to the shielding of some epitopes by the covalent attachment of amino acid residues and reducing sugar. Moreover, the changes in conformational structure induced by glycation may be responsible for it. Although high-intensity ultrasound treatment hardly affected the allergenicity of β -Lg, it caused the significant changes in conformational structure of β -Lg.¹⁰ Our previous work showed that the conformational structure of OVA was partially unfolded by ultrasound treatment.²¹ Moreover, the glycation of BSA was improved and the number of glycation sites was dramatically increased by ultrasound pretreatment.²² These may be the reasons that ultrasound pretreatment resulted in the further reduction of the IgG and IgE binding of glycosylated β -Lg. However, too-high-intensity ultrasound pretreatment could make the proteins refold and some epitopes buried, leading to the decrease in IgG and IgE binding abilities.²¹ Therefore, the decrease in the IgG and IgE binding of β -Lg in this work may result from both the masking of some epitopes and the conformational changes induced by ultrasound pretreatment combined with glycation. To better understand the mechanism of the reduction in IgG and IgE binding of β -Lg by ultrasound pretreatment combined with glycation, the conformation changes of β -Lg were monitored by conventional spectroscopy and the glycation extent and sites were determined by FTICR-MS in the subsequent experiments.

Secondary Structure Analysis. The changes in CD spectra of β -Lg induced by ultrasound pretreatment combined with glycation are shown in Figure 2 and Table 1. There were no significant changes in the secondary structure of β -Lg after heating without mannose at dry-state ($p < 0.05$), suggesting that dry-state heating under nondenatured conditions (55°C for 4 h) had little influence on the secondary structure of β -Lg. This may result from the limited movement of protein molecules due to the low water activity. Similar results were found by Matsudomi et al.,²⁷ who showed that OVA dry-heated at 80°C for 7 days had nearly the same secondary structure as untreated OVA. After glycation, the α -helix and β -sheet contents of β -Lg increased from 52.9% (β -Lg) to 58.4% (β -Lg-M-400) while the β -turn and random coil contents decreased from 47.1% (β -Lg) to 41.6% (β -Lg-M). This suggested that glycosylated β -Lg was partially unfolded and reorganized to be a more stable structure. Similar results were also reported that glycation increased the α -helix and β -sheet contents and decreased β -turn and random coil contents.¹³ Glycation could change conformation of protein to a more stable state at the expense of β -turn or unordered structure.²⁸ It may result in the increase of α -helix and β -sheet contents and the decrease of β -turn and random coil contents due to the glycation. However, the result was opposite to other studies,^{18,29} in which

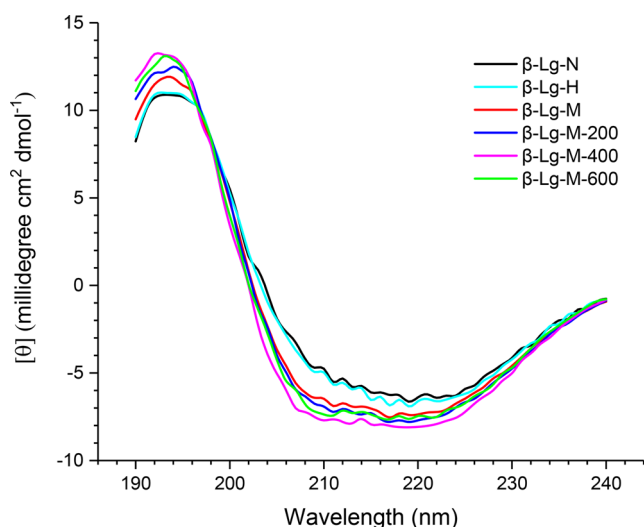


Figure 2. Changes in CD spectra of β -Lg induced by ultrasound pretreatment combined with glycation.

Table 1. Effect of Ultrasound Pretreatment Combined with Glycation on the Content (%) of β -Lg Secondary Structures^a

sample	α -helix	β -sheet	β -turn	random coil
β -Lg	13.3 $\pm$ 0.3a	39.6 $\pm$ 0.2a	18.5 $\pm$ 0.4a	28.6 $\pm$ 0.5a
β -Lg-H	12.4 $\pm$ 0.6a	40.2 $\pm$ 0.5a	19.2 $\pm$ 0.6a	28.2 $\pm$ 0.4a
β -Lg-M	18.5 $\pm$ 0.2b	39.9 $\pm$ 0.5a	16.1 $\pm$ 0.2b	25.5 $\pm$ 0.7b
β -Lg-M-200	19.4 $\pm$ 0.3c	40.1 $\pm$ 0.4a	16.2 $\pm$ 0.7b	24.3 $\pm$ 0.3b
β -Lg-M-400	21.6 $\pm$ 0.6d	41.2 $\pm$ 0.3b	15.2 $\pm$ 0.4b	22.0 $\pm$ 0.4c
β -Lg-M-600	20.2 $\pm$ 0.3c	39.8 $\pm$ 0.5a	16.1 $\pm$ 0.6b	23.9 $\pm$ 0.2b

^aValues followed by different letters (a–d) in the same column are significantly different ($p < 0.05$).

they showed no significant changes induced by glycation. This may be caused by the different glycation conditions, such as the sugar used, water activity, pH, reaction temperature, and time. Moreover, ultrasound pretreatment induced further increase of α -helix and β -sheet contents and decrease of β -turn and random coil contents, which may be attributed to the promotion of glycation by ultrasound pretreatment. The results implied the secondary structure of β -Lg was significantly changed by ultrasound pretreatment combined with dry-state glycation.

UV Absorption and Intrinsic Fluorescence Spectra Analysis. The changes in UV absorption spectra of β -Lg samples induced by ultrasound pretreatment combined with glycation are shown in Figure 3A. Compared to β -Lg-N, β -Lg-H had a very slight increase in the maximum UV absorbance at 280 nm, whereas β -Lg-M had a notable increase from 0.47 (β -Lg) to 0.55 (β -Lg-M). Moreover, the glycosylated samples with ultrasound pretreatment had higher maximum UV absorbance at 280 nm and β -Lg-M-400 had the highest value. This implied that glycation could partially unfold the structure of β -Lg and make aromatic amino acid (mainly Trp) residues exposed on the surface of β -Lg molecule. The results may be attributed to the destruction of the interactions that maintain the rigid native protein tertiary structure by ultrasound pretreatment coupled with dry-state glycation. Previous study also showed similar results that ultrasound-induced glycation of β -Lg made the tertiary structure of β -Lg surrounding Trp19 markedly loosened.¹⁸

The intrinsic tryptophan fluorescence spectra of native and glycosylated β -Lg are shown in Figure 3B. Similarly, compared with

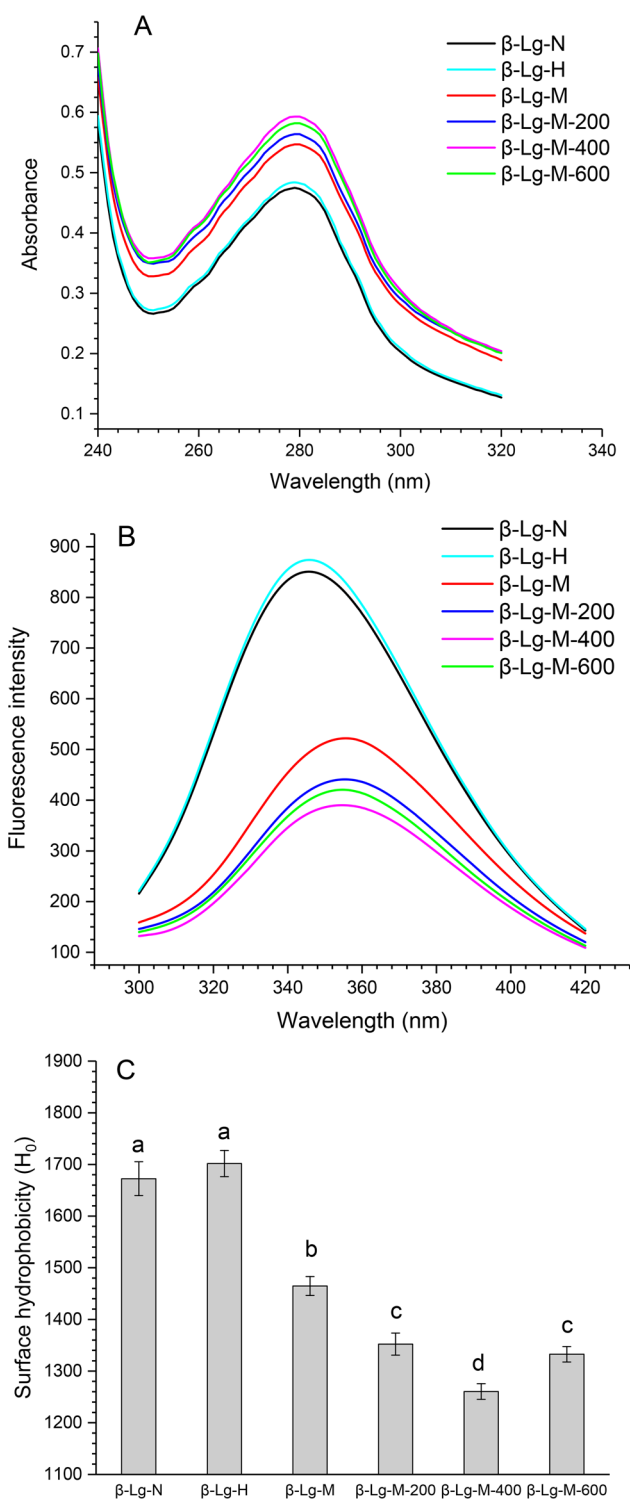


Figure 3. Changes in UV absorption (A) and intrinsic fluorescence (B) spectra, and surface hydrophobicity (C) of β -Lg induced by ultrasound pretreatment combined with glycation. Letters (a–d) in the bars mean significantly different ($p < 0.05$).

β -Lg-N, β -Lg-H had a very slight increase in the intensity of fluorescence emission maximum (λ_{max}). However, when β -Lg was glycosylated, the λ_{max} intensity decreased from 842 (β -Lg-N) to 513 (β -Lg-M), and the glycosylated β -Lg after ultrasound pretreatment showed lower λ_{max} intensity, with a lowest value λ_{max} intensity of 386 (β -Lg-M-400), indicating that conformational structure of β -Lg was dramatically changed by ultrasound

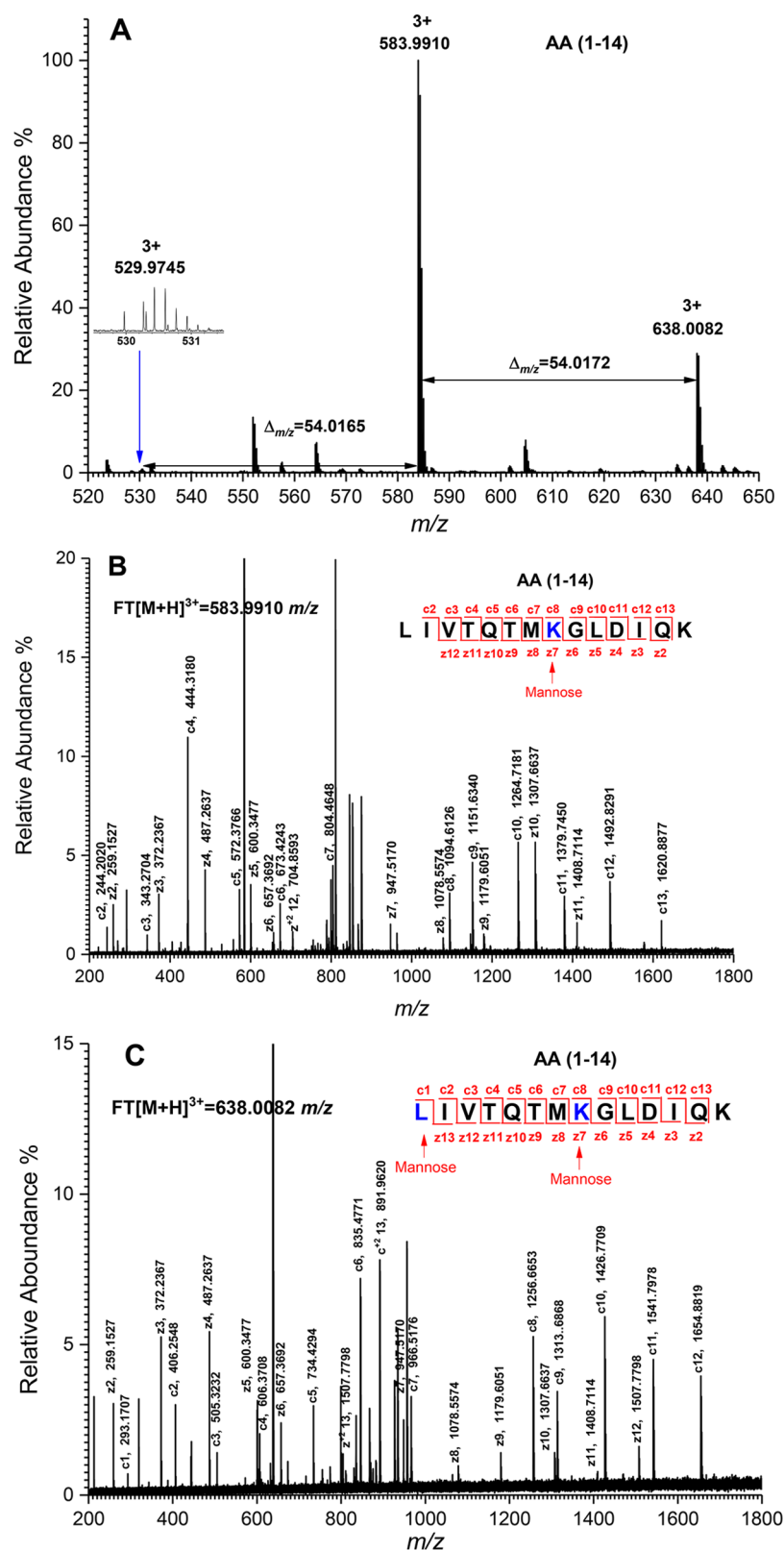


Figure 4. Mass spectrum of the peptide 1–14 (A) and ECD MS/MS spectra of the glycosylated peptide 1–14 with m/z of 583.9910³⁺ (B) and 638.0082³⁺ (C) from the glycosylated β -Lg after ultrasound pretreatment at 400 W. The m/z differences are indicated with numbers and arrows. The sequence of the peptide is shown on the top of figure. The determined glycation site (blue bold) is shown by an arrow with mannose. The c and z ions are indicated by the number.

pretreatment combined with glycation. This may result from the quenching of fluorescence intensity induced by the exposure of Trp residues to solvent. Moreover, a red shift of $\lambda_{\max}$ from 340

(native β -Lg) to 350 nm (glycosylated β -Lg) implied the polarity of β -Lg was changed. This result was also similar to some other reports,^{23,30} wherein they showed that glycation significantly

decreased the intrinsic fluorescence intensity and induced a red shift of λ_{max} in fluorescence spectra of β -Lg.

Surface Hydrophobicity. The surface hydrophobicity of β -Lg samples is shown in Figure 3C. β -Lg-H had no significant difference in the surface hydrophobicity with β -Lg-N ($p < 0.05$). When β -Lg was glycosylated with mannose at ultrasound pretreatment at 0–600 W, the surface hydrophobicity declined from 1673 (β -Lg-N) to 1465 (β -Lg-M), 1352 (β -Lg-M-200), 1280 (β -Lg-M-400), and 1333 (β -Lg-M-600), respectively. This result was in accordance with previous reports that glycosylation significantly reduced the hydrophobicity of β -Lg.^{23,30} It may be attributed to the masking of some hydrophobic groups induced by amino acid residues modification through covalent attachment of mannose. Furthermore, ANS could strongly bind cationic groups, such as lysine (Lys) and arginine (Arg) residues.³¹ The interaction of Lys and/or Arg residues and mannose induced by glycosylation also led to the decrease of surface hydrophobicity of β -Lg. Ultrasound pretreatment could promote conjugation of Lys and/or Arg residues with mannose, make ANS bind less cationic groups, and finally lead to the further reduction of surface hydrophobicity. Accordingly, glycosylated β -Lg with ultrasound pretreatment had lower surface hydrophobicity than that without ultrasound pretreatment.

Glycation Site Determination. To acquire MS/MS spectra, ECD fragmentation mode produced sufficient *c* and *z* ions and was used in this work due to the retention of glycation sites during ion dissociation. Trypsin was chosen as the digestion enzyme due to the specific cleavage of amide bonds following amino acids Lys and Arg; it cannot cleave the site when the Lys or Arg is modified by glycation. To reduce aggregation, the glycosylation was performed at 55 °C for 4 h (pH 8.0) under dry-state. No apparent protein aggregation occurred under these conditions. The result above showed that dry-heating without mannose at 55 °C for 4 h had no significant effect on the secondary and tertiary structure, indicating the glycosylation conditions used in this work were non-denaturing.

Theoretically, if a peptide is monoglycosylated and dual-glycosylated by mannose, the mass will increase by 162.0528 and 324.1056 Da, respectively. A single glycosylation event induces an m/z shift of 81.0264, 54.0176, 40.5132, and 32.4106, respectively, at 2, 3, 4, and 5 charges. Likewise, two glycosylation events will double the m/z shift. As shown in Figure 4A, the m/z of unglycosylated peptide 1–14 was 529.9745³⁺ while the corresponding m/z of its glycosylated forms were 583.9910³⁺ and 638.0082³⁺, with an m/z shift of 54.0165 and 108.0337, respectively, indicating it had monoglycosylated and dual-glycosylated peptides. Figure 4B shows the ECD MS/MS spectrum of the monoglycosylated peptide with m/z of 583.9910³⁺, which exhibited a series of *c* and *z* ions (*c*2–*c*13 and *z*2–*z*12). The glycation site, Lys8, was determined by the mass difference between *c*7 and *c*8 ions, or between *z*6 and *z*7, which is the combined mass of lysine and mannose residues. Similarly, the glycation sites, Leu1 and Lys8, were determined according to the ECD MS/MS spectrum of the dual-glycosylated peptides with m/z of 638.0082³⁺ (Figure 4C). It is noted that some *c* and *z* ions generated from glycosylated peptide may be missing due to the low intensity of ion peaks. The glycation site should be determined by mass spectrum and ECD MS/MS spectrum with *c* and/or *z* ions generated by cleavage of N–C bonds within the peptide backbone before and after the glycation sites. Figure 5A shows the mass spectrum of the peptide 41–60 with m/z of 771.7540³⁺ and the monoglycosylated peptide with m/z of 825.7706³⁺. The glycosylated peptide has an m/z shift of 54.0168, indicating that one mannose molecule was added to the peptide. However, because

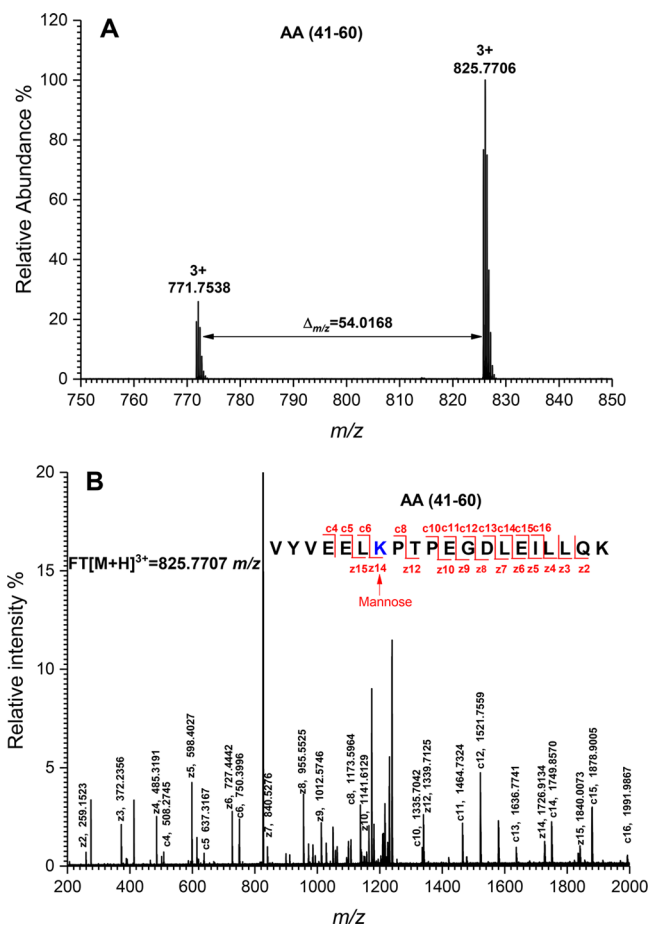


Figure 5. Mass spectrum of the peptide 41–60 (A) and ECD MS/MS spectrum of the glycosylated peptide 41–60 with m/z of 825.7707³⁺ (B) from the glycosylated β -Lg after ultrasound pretreatment at 400 W. The m/z differences are indicated with numbers and arrows. The sequence of peptide is shown on the top of figure. The determined glycation site (blue bold) is shown by an arrow with mannose. The *c* and *z* ions are indicated by the number.

ECD does not usually cleave N-terminally to proline,³² some *c* and *z* ions, such as *c*7, *c*9, *z*11, and *z*13, were missing.

Similarly, other glycation sites, including Lys14, Lys60, Lys69, Lys70, Lys75, Lys77, Lys83, Lys91, Lys100, Lys135, Lys138, and Lys141, were determined by ECD MS/MS (as shown in Figure S1). As we know, the main glycation sites are lysine, arginine, and N-terminus of protein. There are 19 potential glycation sites in β -Lg, including one N-terminal leucine (Leu), 15 Lys, and 3 Arg. Table 2 shows the changes in glycation sites of β -Lg by ultrasound pretreatment with glycation. The glycosylated β -Lg without ultrasound pretreatment had 12 glycosylated sites, including Lys47, Lys60, Lys69, Lys70, Lys75, Lys77, Lys83, Lys91, Lys100, Lys135, Lys138, and Lys141. However, 3 additional glycosylated sites (Leu1, Lys8, and Lys14) were found to be glycosylated after ultrasound pretreatment. In the native state, β -Lg usually exists as a noncovalent dimer.³³ The formation of the dimer may make some glycation sites inaccessible to mannose. Ultrasound pretreatment may destroy these interactions, make these glycation sites exposed, and finally promote glycation. Interestingly, the result showed no Arg was modified in glycosylated samples with and without ultrasound pretreatment. This may result from the reduced freedom of Arg amine group by spatial effects from neighboring atoms.²² The result conforms with

Table 2. Glycated Peptides of Glycated β -Lg with Ultrasound Pretreated at Difference Intensities

peptide location	<i>m/z</i> (glycated)	Δm (ppm)	sequence ^a	glycation site ^b
1–14	638.0082 ³⁺	−4.87	(−)LIVTQTMKGLDIQK(V)	L1, K8
9–40	881.7032 ⁴⁺	−2.27	(K)GLDIQKVAGTWYSLAMAASDISLLDAQSAPLR(V)	K14
41–60	825.7707 ³⁺	−2.84	(R)YVVEELKPTPEGDLEILLQK(W)	K47
41–70	774.5906 ⁵⁺	−3.95	(R)YVVEELKPTPEGDLEILLQKWENGEC* AQKK (I)	K60, K69
61–75	533.0050 ⁴⁺	−3.21	(K)WENGEC* AQKK IAEK(T)	K69, K70
71–83	446.2582 ⁴⁺	−2.02	(K)IAEKTIPAVFK(I)	K75, K77
76–91	491.5228 ⁴⁺	−2.64	(K)IPAVFKIDALNENK(V)	K83
84–101	604.5625 ⁴⁺	−3.35	(K)IDALNENKVLVLDTDYKK(Y)	K91, K100
125–141	568.7770 ⁴⁺	−3.43	(R)TPEVDDEALEKFDKALK(A)	K135, K138
139–148	437.9181 ³⁺	−3.89	(K)ALKALPMHIR(L)	K141

^aC* means the alkylated cystine residue by iodoacetamide. ^bThe increased glycation sites after ultrasound pretreatment are exhibited in bold.

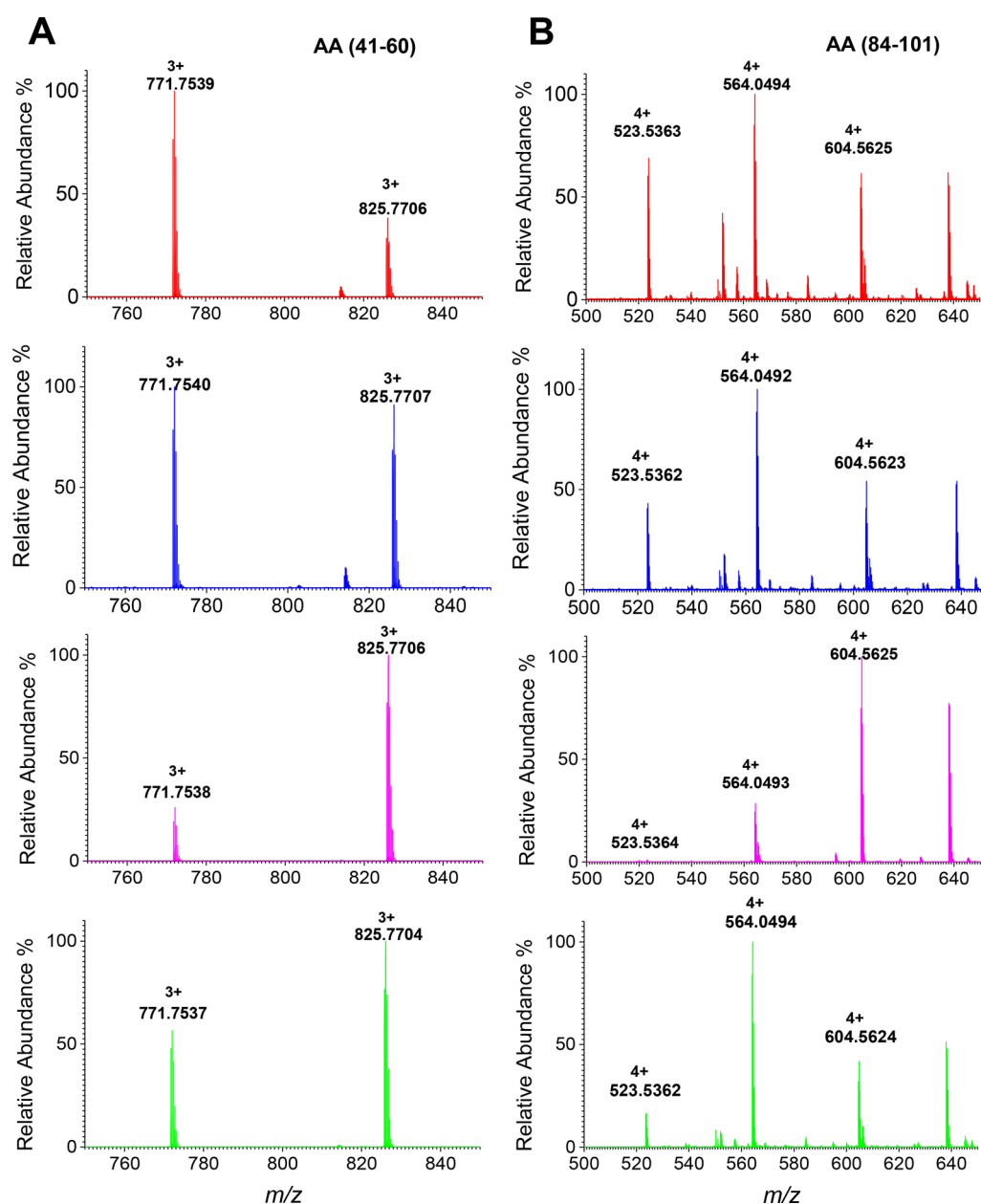


Figure 6. Mass spectra of the glycated peptides 41–60 (A) and 84–101 (B). The red, blue, magenta, and green spectra refer the peptides from glycated β -Lg with ultrasound pretreatment at 0, 200, 400, and 600 W, respectively.

previous studies, wherein they showed that glycation sites were predominantly Lys rather than Arg.^{25,34}

Glycation Extent Analysis. The glycation extent was estimated by both the number of glycation sites and the

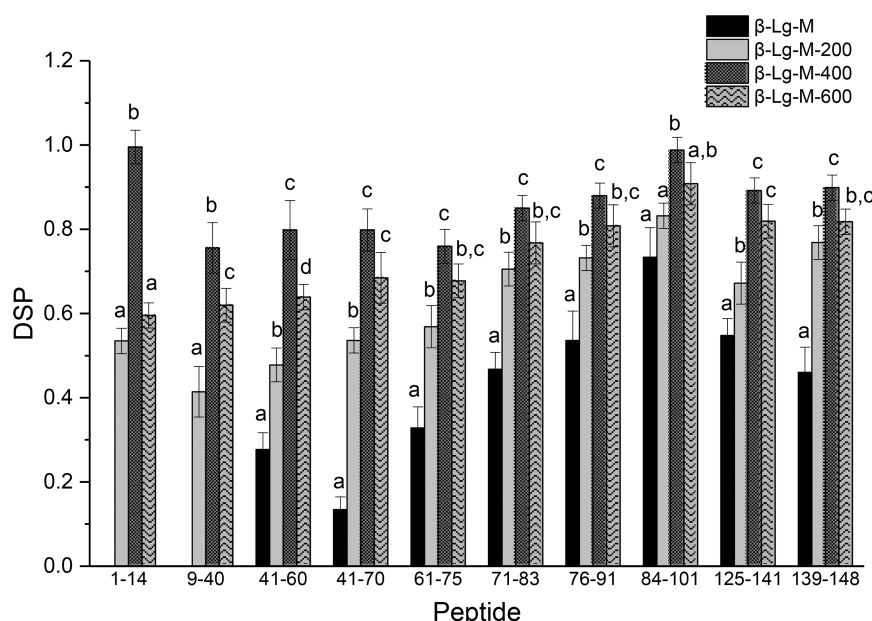


Figure 7. DSP values of the glyated peptides from the glyated β -Lg with ultrasound pretreatment at 0, 200, 400, and 600 W. Letters (a–d) in the bars mean significantly different ($p < 0.05$).

percentage of modified Lys residues at each glycation site. It is clear that ultrasound pretreatment increased the number of glycation sites. However, it is necessary to calculate DSP to evaluate the glycation extent of glyated β -Lg with ultrasound pretreatment at different intensities. As shown in Figure 6A, the glyated β -Lg with ultrasound pretreatment at 0–600 W had different glycation extent compared to the monoglycated peptide 41–60. The relative abundance of the glyated form with m/z of 825.7706³⁺ increased from 38.3% (β -Lg-M) to the highest value of 100% (β -Lg-M-400 and β -Lg-M-600), whereas the relative abundance of the unglyated form with m/z of 771.7539³⁺ decreased from 100% (β -Lg-M) to the lowest value of 25.3% (β -Lg-M-400). This suggests that ultrasound pretreatment improved the glycation degree of this peptide. Another example is displayed in Figure 6B. Two mannose residues were added to the peptide 84–101, which was confirmed by the double-increased m/z of 40.5131. The decrease in relative abundance of the ion peak with m/z of 523.5363⁴⁺ and the increase in relative abundance of the ion peak with m/z of 564.0494⁴⁺ and 604.5625⁴⁺ indicated that glyated β -Lg with ultrasound pretreatment had higher glycation extent than that without ultrasound pretreatment.

The DSP values of all glyated peptides with ultrasound pretreatment at 0–600 W are shown in Figure 7. On the basis of the DSP value, K91 and K100 were the most reactive mannose glycation sites in native β -Lg with DSP value of 0.75. After ultrasound pretreatment, its DSP value further increased to near 1.0. The DSP values were significantly promoted by ultrasound pretreatment as a whole. The highest DSP values of glyated peptides were found at 400 W. Although some peptides, 1–14 and 9–40, were unglyated in the native form, they had high DSP value (≥ 0.7) after ultrasound pretreatment at 400 W. This may be attributed to the exposure of these regions caused by ultrasound pretreatment,¹⁰ which made some Lys residues accessible to reactions with reducing sugars. However, too-high-intensity ultrasound treatment may lead to the refolding of β -Lg molecules,¹⁸ masking some regions, and finally causing the

decrease in the glycation extent. That may be the reason that β -Lg-M-600 had lower glycation degree than β -Lg-M-400.

Mechanism of the Decrease in IgG and IgE Binding of β -Lg by Ultrasound Pretreatment Combined with Dry-State Glycation. Ultrasound pretreatment coupled with glycation significantly reduced the IgG and IgE binding of β -Lg, which was closely related to its structural changes. To explore the mechanism, combined with the conventional spectrometry, FTICR-MS was applied for structural characterization at the molecular level. The results above showed that the structural changes were responsible for the reduction of IgG and IgE binding abilities of β -Lg.

As we know, the conformational epitopes play an important role in the food allergy.³⁵ β -Lg has 3 conformational epitopes, in which the 15 common residues appear mainly as an α -helix.³⁶ The secondary structure could significantly affect the IgG and IgE binding capacities. Usually, IgG and IgE easily recognize and bind with the epitopes in the β -turn and random coil structures rather than the α -helix and β -sheet structure.³⁷ After glycation with mannose, the α -helix and β -sheet contents of β -Lg increased, whereas the β -turn and random coil contents decreased. Therefore, the IgG and IgE binding of β -Lg decreased after glycation with mannose. Moreover, the ultrasound pretreatment promoted the changes, leading to the further reduction of IgG and IgE binding of β -Lg.

Furthermore, the tertiary structure of protein determines the integrity of conformational epitopes. The ultrasound pretreatment combined with glycation induced the unfolding of β -Lg, reflected in the increase in UV absorbance at 280 nm and the decrease in the intrinsic fluorescence intensity as well as a red shift of λ_{max} . The changes in the tertiary structure of β -Lg may lead to the destruction of the conformational epitopes, and finally cause the reduction in IgG and IgE binding abilities.

Besides, the hydrophobic force is mainly responsible for maintaining the binding of antibody and antigen.³⁸ The surface hydrophobicity of glyated β -Lg was dramatically decreased, which may decrease of IgG and IgE binding abilities. Therefore, the decrease in the surface hydrophobicity of glyated β -Lg was

conductive to the reduction of the IgG and IgE binding abilities. In addition, the promoted decrease in the surface hydrophobicity of glycosylated β -Lg by ultrasound pretreatment resulted in the further reduction of the IgG and IgE binding capacities.

Numerous studies have been done to identify the linear IgG and IgE epitopes of β -Lg.^{5,6,36,39–41} It was summarized that the IgG and IgE epitopes spread through the whole amino acid sequences of β -Lg. To ensure the universality of IgG and IgE epitopes, the pooled antisera used in this study were obtained from 6 rabbits and 8 CAM patients, in which the patients' ages and allergy symptoms were different (shown in Table S1). The glycation modified or masked the IgG and IgE epitopes by the covalent attachment of mannose,⁴² resulting in the non-recognition of β -Lg by IgG and IgE. Finally, it caused the decrease in the IgG and IgE binding of β -Lg. In this case, 12 glycation sites (Lys47, Lys60, Lys 69, Lys70, Lys75, Lys 77, Lys83, Lys91, Lys100, Lys135, Lys138, and Lys141) were determined in the glycosylated β -Lg without ultrasound pretreatment (shown in Figure 8). The IgG and IgE were unable to

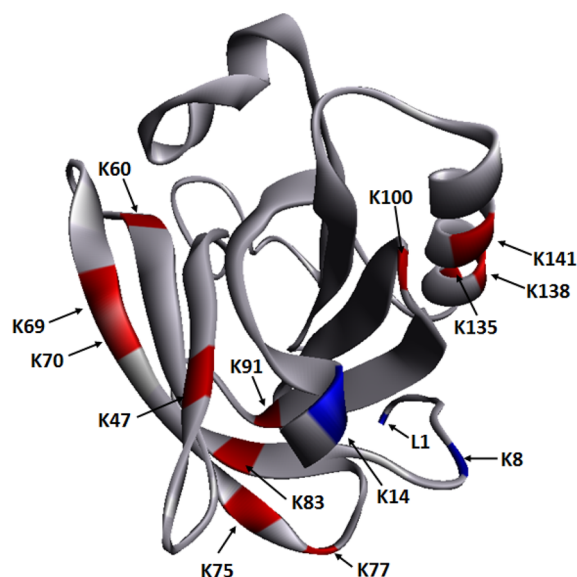


Figure 8. Ribbon diagram of the glycosylated β -Lg (PDB 1BSQ). The glycation sites are colored as follows: gray, framework of β -Lg; red, glycation sites of the native β -Lg; blue, additional glycation sites of the β -Lg after ultrasonication at 400 W.

recognize the epitopes due to the modification of the glycation site in β -Lg molecule. Moreover, 3 additional glycation sites (Leu1, Lys8, and Lys14) were identified in the glycosylated β -Lg with ultrasound pretreatment. It made more epitopes unrecognized by IgG and IgE. Also, the DSP value showed ultrasound pretreatment promoted the glycation extent. Therefore, ultrasound pretreatment promoted the reduction of the IgG and IgE binding abilities by improving glycation extent.

In this work, it was shown that ultrasound pretreatment combined with dry-state glycation significantly decreased the IgG and IgE binding of β -Lg, which was attributed to the covalent binding of mannose to β -Lg and to the succeeding structural changes of the protein. Moreover, ultrasound pretreatment promoted the reduction of IgG and IgE binding abilities by improving glycation, reflecting in the increase of the glycation sites and DSP value. The result also demonstrated that FTICR-MS was extremely valuable for analyzing the protein modifications at the molecular level, which is conducive to the

understanding of the mechanism of the allergenicity decrease in food proteins induced by processing. However, some experiments in vivo, such as double-blind double-blind placebo-controlled trial (DBPC), should be conducted in the future to ensure reduction of allergenicity.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jafc.7b02842.

Table S1 and Figure S1 (PDF)

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■ ABBREVIATIONS USED

β -Lg, β -lactoglobulin; IgE, immunoglobulin E; IgG, immunoglobulin G; ELISA, enzyme-linked immunosorbent assay; OVA, ovalbumin; BSA, bovine serum albumin; CD, circular dichroism; UV, ultraviolet; DTT, dithiothreitol; ANS, 1-anilinonaphthalene-8-sulfonate; PBS, phosphate buffer solution; PBST, PBS with 0.05% Tween; DSP, degree of substitute per peptide; HPLC, high-performance liquid chromatography; FTICR-MS, Fourier transform ion cyclotron resonance mass spectroscopy; ECD, electron capture dissociation

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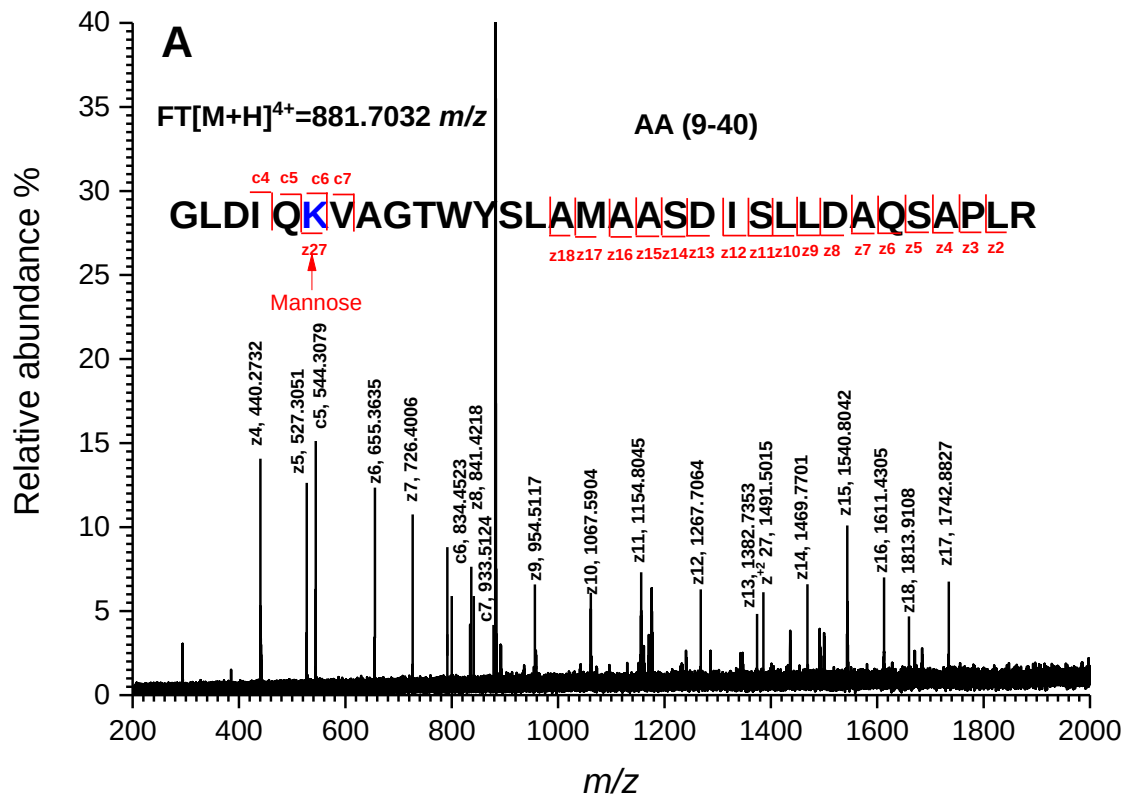
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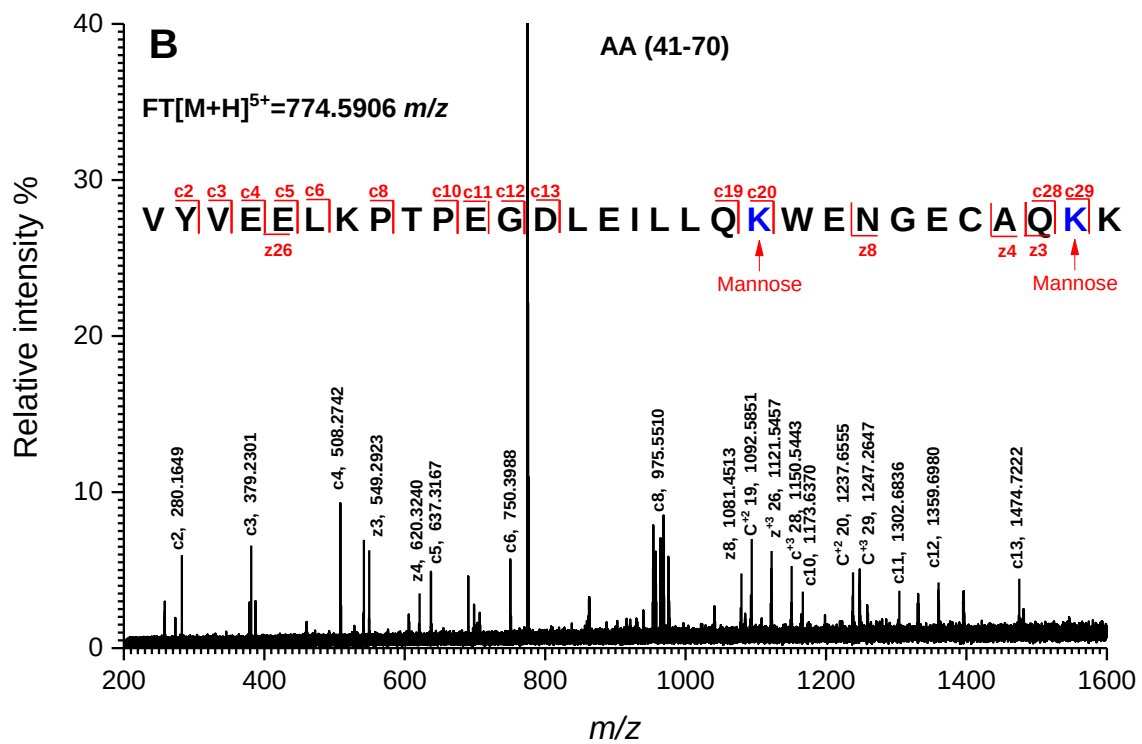
1 **Table S1.** Information of CMA patients

No.	Gender	Age (year)	Clinical symptoms	Other allergy	IgE (kU L ⁻¹)
1	Male	5	AS, EC	Egg allergy	20.3
2	Male	1	AS	Animal allergy	13.8
3	Female	20	HI, EC, RH	Animal allergy	96.1
4	Female	3	AS, HI	Wheat allergy	94.8
5	Male	27	HI, NW	Most food allergy	9.58
6	Female	47	HI, CW	Most food allergy	93.1
7	Male	34	EC, RH	Honey allergy	24.7
8	Female	2	AS, NW	None	96.6

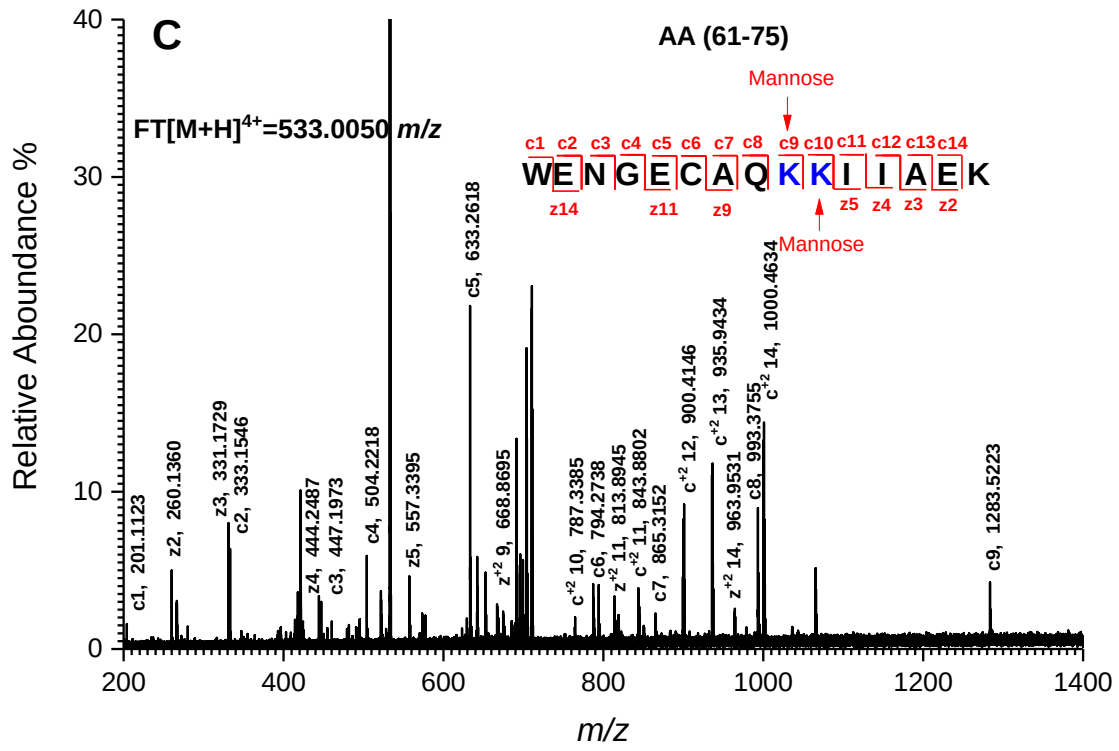
2 AS, asthma; HI, hives; EC, eczema; RH, rhinitis; NW, nose wheezing; CW, chest wheezing



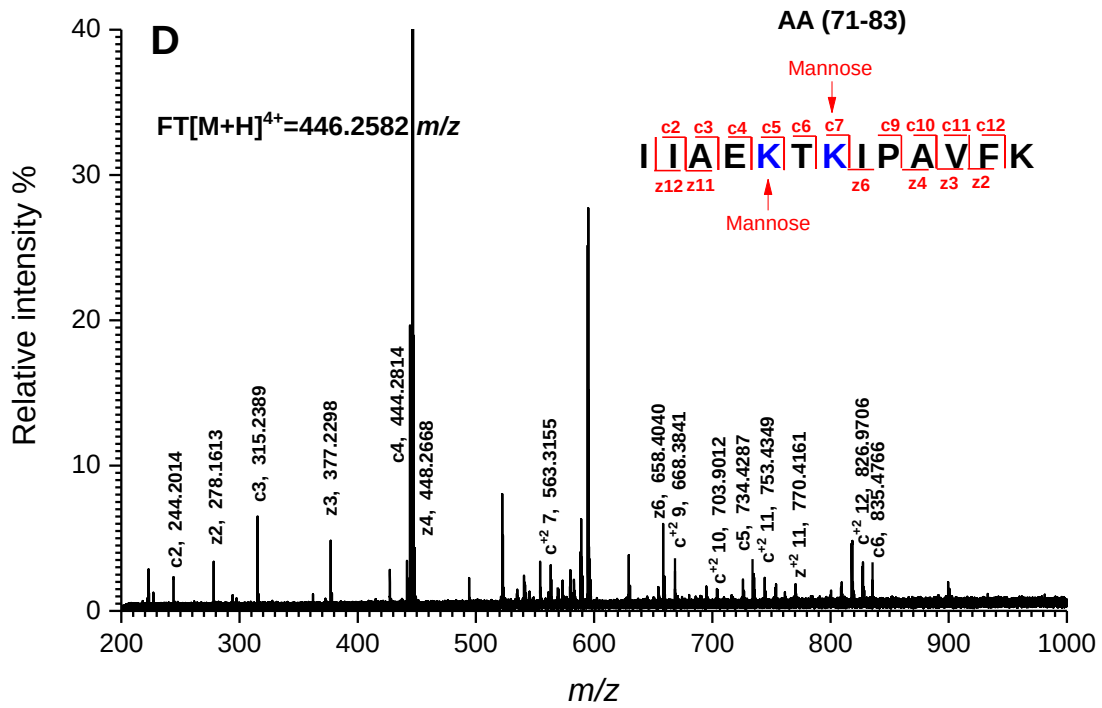
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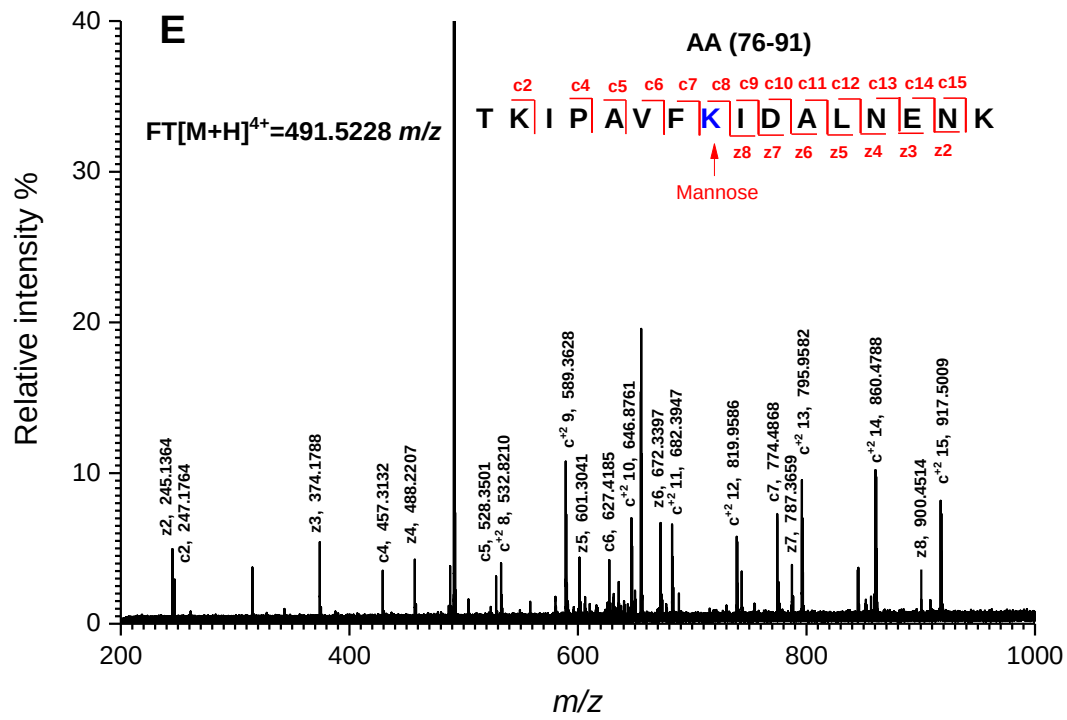
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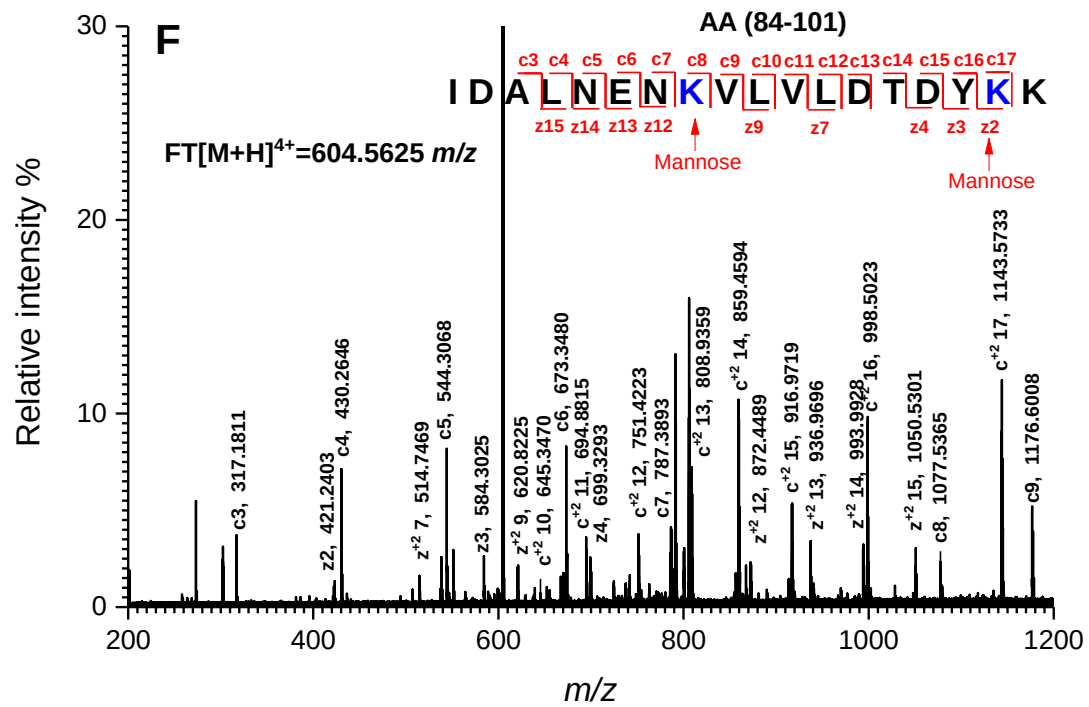
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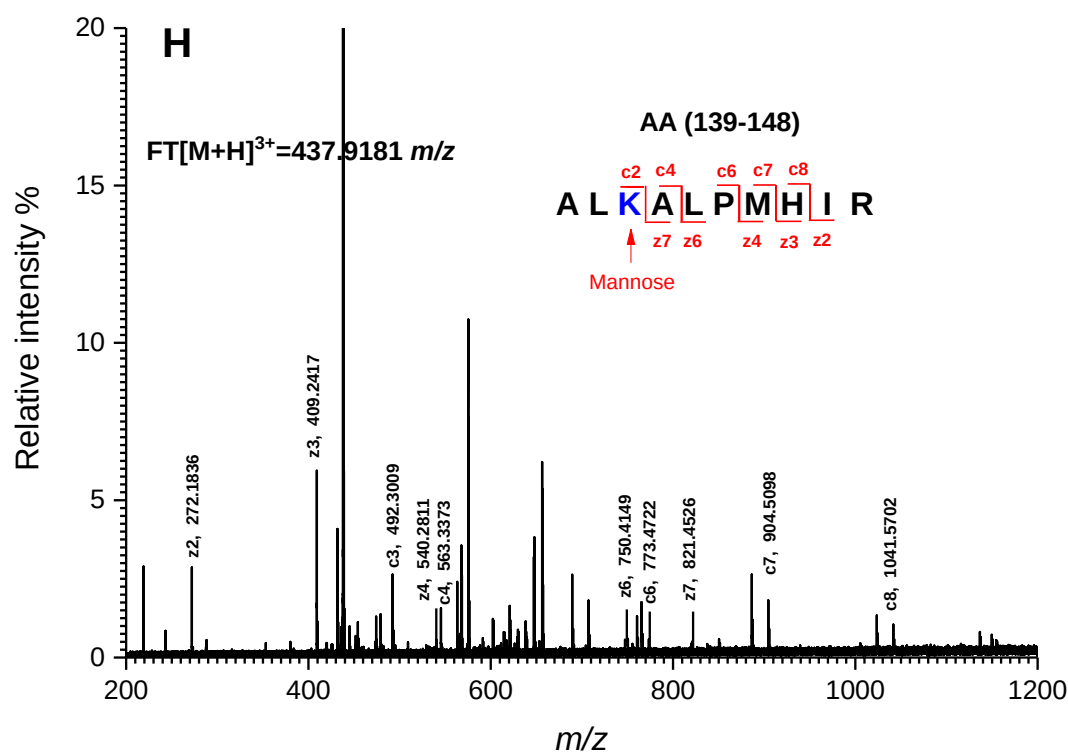
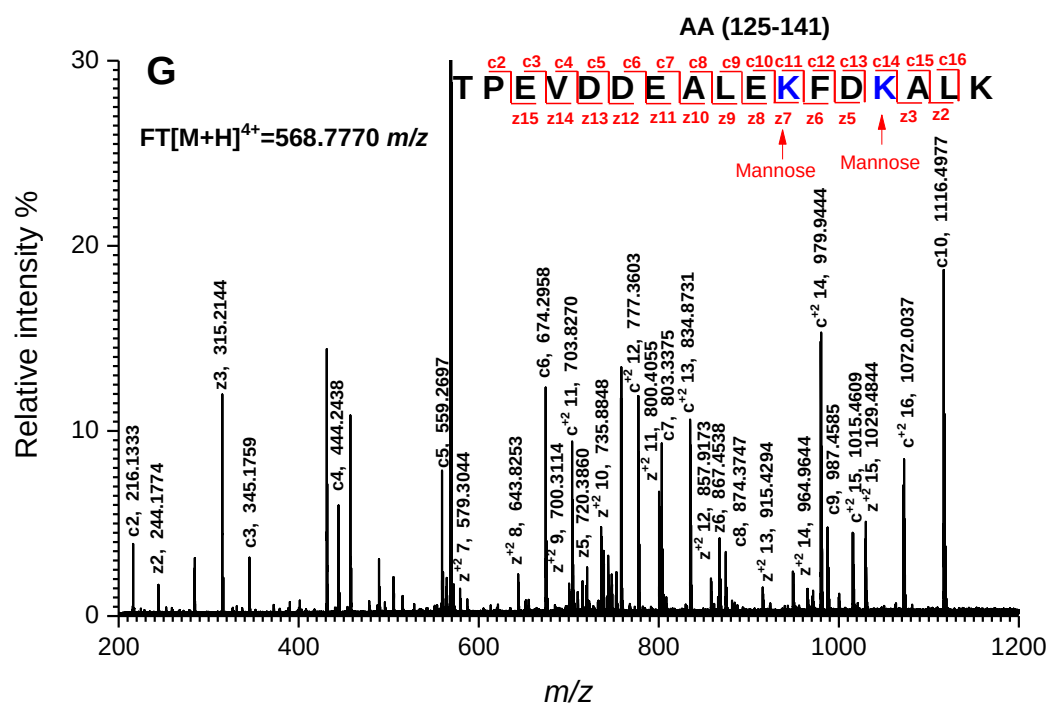


Figure S1. ECD MS/MS spectra of the glycated peptides 9-40 (A), 41-70(B), 61-75 (C), 71-83(D), 76-91 (E), 84-101 (F), 125-141(G) and 139-148 (H). The sequence of peptide is shown on the top of figure. The determined glycation site (blue bold) is shown by an arrow with mannose. The c and z ions are indicated by the number.