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Glycation of β-lactoglobulin combined by sonication pretreatment reduce its allergenic potential

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ABSTRACT

β-lactoglobulin (β-Lg) was treated through different ultrasonic power and subsequently glycated with galactose to investigate its structural changes and immunological properties, and then evaluated by high-resolution mass spectrometry, enzyme-linked immunosorbent assay and basophil histamine release test. Ultrasonication combined with glycation (UCG) modification significantly reduced the IgE/IgG-binding capacity, and the release of β-hexosaminidase, histamine and interleukin-6, accompanied with changes in the secondary and tertiary structures. The decrease in the allergenicity of β-Lg depended not only on the glycation of K47, 60, 83, 91 and 135 within the linear epitopes, but also on the denaturation of conformational epitopes, which was supported by the glycation-induced alterations of the secondary and tertiary structures. This study confirmed that UCG modification is a promising method for decreasing the allergenic potential of allergic proteins, which is likely to develop a practical technology to produce hypo-allergenic milk.

1. Introduction

Cow's milk allergy (CMA), defined as an immunologically mediated reaction against cow's milk antigens [1]. CMA is the most common allergic reaction in infants and children [2], which seriously affects the life of allergic people as well as the growth and development of infants and children. The major allergens in bovine milk are caseins, α-lactalbumin, and β-lactoglobulin (β-Lg), and β-Lg accounts for 10% of total milk protein and 60% of whey protein. Ninety percent of those with an allergy to cow's milk have an allergy to β-Lg [3]. In addition, β-Lg is resistant to acid and pepsin hydrolysis, so there are still intact β-Lg and its peptides in the body after digestion, which could cause allergic reactions [4]. Besides, β-Lg is regarded as the most important allergen in cow's milk.

At present, many methods have been utilized to change the allergenicity of β-Lg. According to different principles, heating [5], glycation [6], high pressure [7], enzymatic hydrolysis [8], lactic acid fermentation [9] and other processing methods can be used to effectively change the allergenicity of β-Lg. Among these, thermal treatment may lead to the loss of nutrient quality. After enzymatic hydrolysis, milk has a certain bitterness and off-flavor, which is unsuitable for children to drink [10]. High pressure conditions maybe not easy to achieve, and the period of lactic acid fermentation is long. Compared with other methods, glycation is believed to be a safe method for protein modification [11]. It usually occurs in the first stage of Maillard reaction and is one of the most common chemical reactions during food processing and storage. Glycation can affect the structural and physicochemical properties of β-Lg, such as antioxidant, emulsifying and foaming properties, especially reducing the allergenic potential of β-Lg [6,12]. However, only glycation cannot effectively reduce allergenicity to a satisfactory level. Therefore, many researchers tend to use some other modification methods such as pulsed electric field, ultrasonic, dynamic high pressure microfluidization to assist glycation to change the allergenic potential of β-Lg [3,13,14].

High-intensity ultrasonication can improve the chemical, physical, and functional properties of various foods [15] in four ways, namely, acoustic cavitation, heating effect, fluid particle oscillation, and acoustic streaming [16]. The action principle is to produce high- and low- pressure regions through sound wave propagation [17]. As an efficient food processing technology, high-intensity ultrasonication has been

Abbreviations: β-Lg, β-lactoglobulin; UCG, Ultrasonication combined with glycation; Gal, galactose; CMA, Cow's milk allergy; β-hex, β-hexosaminidase; IL-6, interleukin-6; DSP, the average degree of substitution per peptide molecule; ELISA, enzyme-linked immunosorbent assay; CD, circular dichroism; PBST, phosphate buffer solution/Tween solution; HPLC, high performance liquid chromatography; ETD MS/MS, electron transfer dissociation mass spectrometry/mass spectrometry.

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successfully applied in the dairy industry, for the purpose of homogenization, inactivation of enzymes, enhancement of extraction process, dehydration, aging and ripening [18]. Our previous studies have shown that ultrasonic-assisted glycation obviously reduced the allergenicity comparing to glycation alone [3]. They usually detect the allergenicity of β-Lg using enzyme-linked immunosorbent assay (ELISA) and western blotting, but the occurrence of allergic reactions is not only related to the level of IgE in the blood, but ultimately to the mediator release from basophils. Thus, basophil histamine release test for food allergens is an indicator of food allergy, which can fully reflect the ability of protein allergenicity. However, the effect of the treated β-Lg by different ultrasonic power and subsequently glycated with galactose by dry heating on the mediator release from basophils has not been reported. Besides, galactose (Gal) is the most important raw material in food processing. As an aldose, Gal has high glycation reaction activity with proteins.

This study was to explore the impact of ultrasonication combined with glycation (UCG) modification on the immunological properties of β-Lg. The IgE/IgG binding capacity of modified β-Lg was evaluated by ELISA, and the effect on the release of cell biological active mediators, such as β-hexosaminidase (β-hex), histamine and interleukin-6 (IL-6) was analyzed through RBL-2H3 cell model. The structural changes of modified β-Lg were characterized by circular dichroism (CD) and fluorescence spectrometry, and the glycation sites and extent were determined by electron-transfer dissociation tandem mass spectrometry (ETD-MS/MS). Our results suggested that the structural changes play an essential role in reducing the IgE antibody binding of β-Lg treated by UCG modification, thus providing more basic information on the potential applications of β-Lg in the food industry.

2. Materials and methods

2.1. Materials

β-Lg from bovine milk (L3908, ≥90%), galactose (Gal, V900922), goat anti-human IgE-horseradish peroxidase (HRP) conjugate (A9667), β-hexosaminidase (β-hex, N9376) and trypsin (T8802, 10,000 units/mg of protein) were from Sigma-Aldrich (St. Louis, MO, U.S.A.). Goat anti-rabbit IgG-HRP conjugate (SE134) and other reagents were obtained from Beijing Solarbio Technology Co., Ltd. (Beijing, China). ELISA kits for histamine and IL-6 were purchased from MeiMian Systems (Jiangsu, China). The RBL-2H3 was obtained from the Chinese Academy of Sciences. The polyclonal anti-bovine β-LG antibody produced in rabbit (Bioss, Beijing, China) was applied to study the IgG-binding capacity of β-Lg.

A total of 10 sera from patients allergic to milk were from Plasma Lab International (Everett, WA, U.S.A.). They had total milk protein-specific IgE levels ranging from 7.12 to 117.43 kUA/L. Human antisera (prepared by mixing the sera of 10 patients at the same volume) was applied to study the IgE-binding capacity of β-Lg.

2.2. Sample preparation

Native β-Lg was dissolved in double distilled water to concentration of 1 mg/mL. A 3 mL of β-Lg solution was added into 5 mL screw glass bottles, the solution was treated by probe sonicator (Misonix Qsonica Q700 Sonicator, USA, 20 kHz) with a 7 s on and 3 s off pulsation at an actual ultrasonic intensity of 0, 60, 90, 120 and 150 W/cm^2 for 15 min. The whole treatment process was carried out in ice-bath to ensure the sample temperature (lower than 15 °C). Then, a 3 mg of Gal was dispersed in 3 mL of native and treated β-Lg solution. Samples were lyophilized to powder, followed by incubation at 55 °C and 65% relative humidity (saturated potassium chloride solution) for 4 h. The reaction was stopped in an ice bath. The samples were dissolved in double distilled water to concentration of 1 mg/mL, and then filtered with Millipore Ultrafiltration centrifugal tube (molecular weight cut off 3000 Da) to remove unreacted Gal. The samples were diluted into 5.0 mg/mL for future use. Native β-Lg was named β-Lg. The modified samples with glycation and ultrasonication at 0, 60, 90, 120, and 150 W/cm^2 were named β-Lg-G, β-Lg-G-60, β-Lg-G-90, β-Lg-G-120, and β-Lg-G-150, respectively.

2.3. IgE/IgG binding capacity of β-Lg

Inhibition ELISA assays were used to estimate the IgE/IgG-binding capacity of β-Lg by the method of Liu et al. [19] with human antisera and rabbit antisera. The 96-well microtiter plates were coated with 2 μg/mL of native β-Lg samples (100 μL/well), followed by incubation for 1 h at 37 °C. The plates were washed 3 times with the addition of PBST (prepared by dissolving 0.05% Tween-20 in 10 mM pH 7.4 PBS), then blocking with the addition of 2% fish gelatin (dissolved in carbonate buffer) for 1 h at 37 °C, and repeat the washing step as previous. The incubation at 37 °C for 2 h was initiated after finishing the addition of 50 μL of antisera samples (1:10 diluted human sera or 1:50000 diluted rabbit sera) and 50 μL of the treated samples. After incubation, the solution was removed and the plate was washed. A total of 100 μL of purified goat anti-human IgE-HRP conjugate (diluted to 1:5000 in PBST) or goat anti-rabbit IgG-HRP conjugate (diluted to 1:20000 in PBST) was added and then incubated at 37 °C for 1 h. After incubation, tetramethylenbenzidine solution (100 μL) was immediately added to each well and the reaction was terminated by the addition of sulfuric

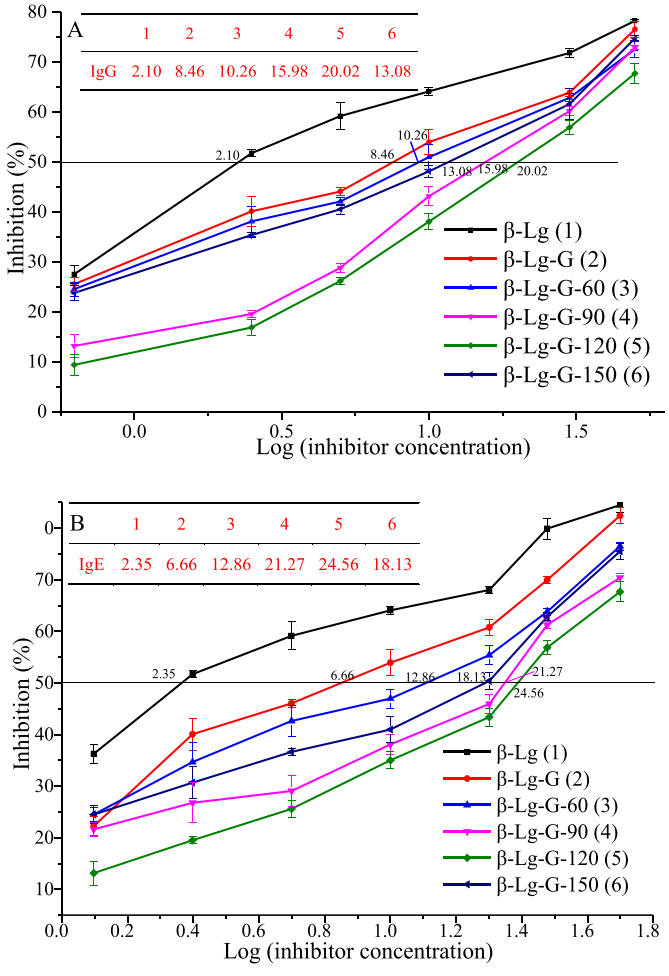

Fig. 1. Changes of IgG (A) and IgE (B) binding abilities of glycated β-Lg were determined by inhibition ELISA. IC_{50} is the concentration of inhibitors that causes a 50% inhibition of antibody binding (μg/mL). Pooled rabbit anti-β-Lg-sera were incubated separately with 0.625, 2.5, 5, 10, 30, 50 μg/ mL of glycated-β-Lg as inhibitors or human anti-β-Lg-sera (50 μL per well) were incubated separately with 1.25, 2.5, 5, 10, 20, 30, 50 μg/ mL of glycated-β-Lg as inhibitors.

acid (50 μL, 2 mol/L). Finally, the absorption was monitored at 450 nm by a microplate reader (BioTek Instruments Co., Ltd., Winooski, VT, U.S.A.). The decline rate was calculated as percent inhibition (%) = $[1 - (B/B_0)] \times 100$, where B and B_0 are the absorbance values of the well with and without modified samples, respectively. Each sample was performed in triplicate.

2.4. Allergenicity in RBL-2H3 cells

The RBL-2H3 cells was cultured by the medium of RPMI-1640, with 10% fetal bovine serum (FBS) and 1×10^5 U/L penicillin/streptomycin at 37 °C in a humidified 5% CO_2 incubator, adopting a previously established method by Appel et al. [20]. The RBL-2H3 cells were seeded in 24-wells plates with $5*10^5$ cells for 24 h, passively activated with human serum IgE from milk-allergic children for 24 h, then stimulated by 50 μg/well samples for 4 h. Cells treated by PBS buffer were employed as a negative control. The release of β-hex was performed as described by Kuehn et al. [21]. The release of histamine and IL-6 were analyzed by ELISA assays, following the manufacturer's instructions [22].

2.5. Intrinsic fluorescence emission spectroscopy

The intrinsic emission fluorescence spectra of the protein samples (1 mg/mL) were obtained using a Hitachi F-7000 fluorescence spectrophotometer (Hitachi, LTd, Tokyo, Japan) following a previous report [23]. Samples (1 mg/mL) were prepared with PBS (50 mM, pH 7.4). The protein solution was excited at 290 nm, and the emission spectra was recorded from 300 nm to 400 nm (both at a constant slit of 2.5 nm).

2.6. Circular dichroic spectra (CD)

Protein secondary structure was studied using circular dichroism (CD) according to Chen et al. [13]. CD of the samples (0.1 mg/mL) was measured using a MOS-450 spectropolarimeter (French Bio-Logic SAS Co., Claix, France) with a 1.0 mm path length quartz cuvette. Each spectrum was recorded in the wavelength range of 190–250 nm at a scan speed of 100 nm/min. All observed CD spectra were corrected by subtracted buffer baseline spectrum, and the CD data were expressed as mean residue ellipticity. Structure predictions from CD spectra were analyzed by online Dichroweb software (http://dichroweb.cryst.bbk.ac.uk/html/process.shtml).

2.7. Identification of glycation sites

The glycation sites of modified β-Lg were identified by our previous method [24]. Protein was digested according to the filter-aided sample preparation (FASP) method. After FASP method, the peptides were separated with Ultimate 3000RSLCnano high-performance liquid chromatography (HPLC, Thermo Fisher Scientific, Waltham, MA, U.S.A.) using a RP-C18 column, and then the column effluent was performed by Thermo Fisher Q Exactive Mass Spectrometer. The electron-transfer dissociation (ETD) fragmentation mode was used to acquire MS/MS spectra. Detection mode was executed by our previous method for sample analysis.

We applied the degree of substitution per peptide of each site (DSP) to analyze the glycation extent. DSP can be estimated using the following equation:

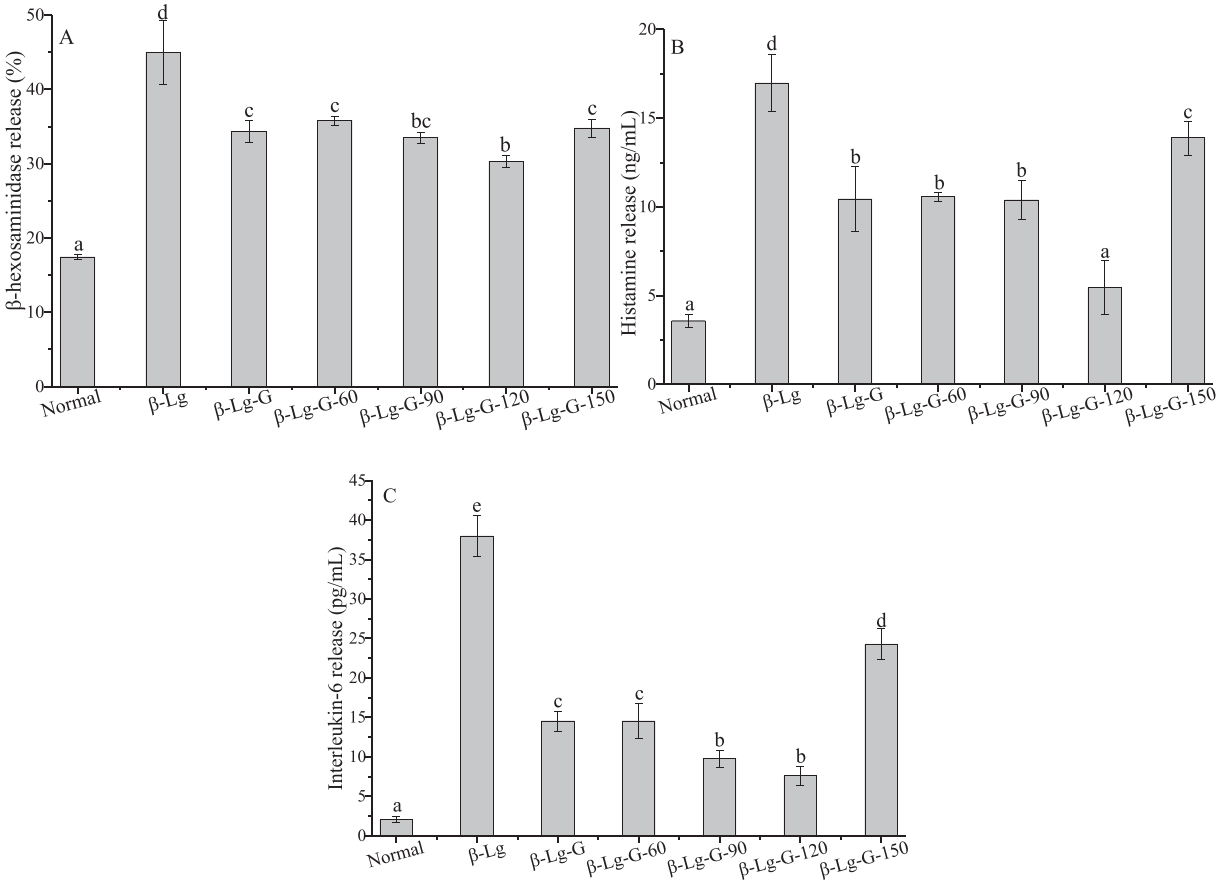

Fig. 2. Effects of glycated β-Lg in IgE-mediated allergic responses in RBL-2H3 cells sensitized with sera from patients allergic to milk. A, The release of β-hexosaminidase; B, The release of histamine. C, The release of interleukin-6 (IL-6). Letters (a–e) in the bars mean significantly different ($p < 0.05$).

$$DSP = \frac{\sum_{i=0}^{n} i \times I_{peptide+i\times galactose}}{\sum_{i=0}^{n} I_{peptide+i\times galactose}}$$

where I is the sum of the intensity of the glycated peptide and i is the number of Gal units attached to the peptide in each glycated form.

2.8. Statistical analysis

All experiments were performed in triplicate and the data obtained were analyzed by one-way analysis of variance (One-Way ANOVA) using SPSS for windows version 20 (SPSS Inc., Chicago, IL). Values were expressed as means ± SD.

3. Results and discussion

3.1. Analysis of the IgE/IgG-binding capacity

The IgE/IgG-binding abilities of β-Lg, β-Lg-G, β-Lg-G-60, β-Lg-G-90, β-Lg-G-120, and β-Lg-G-150 were showed in the Fig. 1. The IC_{50} value of β-Lg-G-60, β-Lg-G-90, β-Lg-G-120, and β-Lg-G-150 shifted to 10.26, 15.98, 20.02, and 13.08 μg/mL, respectively, much higher than that of β-Lg and β-Lg-G, which were 2.10 and 8.46 μg/mL (Fig. 1A). A similar trend was observed in the Fig. 1B. At ultrasound powers of 0 (6.66 μg/mL), 60 (12.86 μg/mL), 90 (21.27 μg/mL), 120 (24.56 μg/mL) and 150 W/cm^2 (18.13 μg/mL), the IC_{50} values of β-Lg-G, β-Lg-G-60, β-Lg-G-90, β-Lg-G-120, and β-Lg-G-150 were 2.83-, 5.47-, 9.05-, 11.45-, and 7.71-times that of β-Lg (2.35 μg/mL). These results indicated that the IC_{50} value of glycated β-Lg pretreated by different ultrasonic powers is higher than that of native β-Lg and β-Lg-G. It implies that glycation can reduce the IgG/IgE-binding abilities of β-Lg and ultrasonic pretreatment promotes the reduction.

The reduction in the IgG/IgE-binding abilities of β-Lg may be due to the structural changes induced by the modification, such as the cross-linking [25], heat and chemical treatment, etc. Alterations in the structure of β-Lg amino acid side chain result in the partial shielding of some linear epitopes, leading to loss the IgG/IgE-binding abilities [19]. Therefore, UCG modification can reduce the IgE/IgG-binding capacity of β-Lg by changing the β-Lg structure. When ultrasonic power was 150 W/cm^2, the IC_{50} values of β-Lg-G-150 was decreased. It may result from more compact structure of β-Lg caused by excessive ultrasonic pretreatment, leading to the shelter of the reactive groups and inhibiting reaction [26]. Moreover, the mediators release from basophils may be responsible for the occurrence of type I allergic reactions. The histamine level was evaluated using an RBL-2H3 cell model in the subsequent experiment.

3.2. The impact of β-Lg on IgE sensitized RBL-2H3 cells

The effects of β-Lg, β-Lg-G, β-Lg-G-60, β-Lg-G-90, β-Lg-G-120, and β-Lg-G-150 on the release of β-hex, histamine and IL-6 were shown in Fig. 2. The results showed that glycation reduced the release of β-hex, histamine and IL-6. The release of β-hex in cells exposed to β-Lg was 44.96%, which was significantly higher than that of β-Lg-G (Fig. 2A). The results indicated that glycation could affect the decreasing on the release of mediators related to allergic symptoms. When β-Lg was glycated after ultrasound pretreatment at 0–120 W/cm^2, the release of β-hex gradually decreased with the increasing of ultrasound intensity. However, when the power was 150 W/cm^2, the release of β-hex increased to 34.77%. This indicates the mediator release of allergenic β-Lg treated by UCG modification is lower than that of native β-Lg and β-Lg-G. A similar trend was found in the Fig. 2B and C. It is suggested that glycation can decrease the release of mediators related to allergic symptoms, and ultrasound pretreatment can promote the reduction. The possible explanation is that the antigenic epitopes of β-Lg was masked after glycation, which affects the specific binding of β-Lg to IgE epitopes on the membrane of RBL-2H3 cells, causing the reduction in the release of mediators. It was also similar to that reported in a previous study, wherein they showed that glycation notably decreased the release of mediators [27]. UCG modification can not only obviously reduced the IgE/IgG-binding capacity comparing to glycation alone, but also can reduce the immunological activities of allergens at the cellular level. Liu et al. [19] reported that ultrasonic-assisted with glycation can reduce the IgE/IgG-binding abilities of α-Lactalbumin though changing the structure. As shown in Figs. 1 and 2, a decrease of IgE/IgG-binding capacity and the release of β-hex, histamine and IL-6 of β-Lg under UCG modification was also observed. To understand the relationship between structural changes and allergenicity, conventional spectrometry and high-resolution mass spectrometry was conducted in the subsequent experiments.

3.3. Intrinsic fluorescence emission spectroscopy

The intrinsic fluorescence spectra of β-Lg, β-Lg-G, β-Lg-G-90, β-Lg-G-120, and β-Lg-G-150 were presented in Fig. 3A. When excitation occurred at 290 nm, a high intensity intrinsic tryptophan florescence at 334.4 nm was observed in native β-Lg. After glycation, the intrinsic fluorescence intensity of β-Lg-G, β-Lg-G-90, β-Lg-G-120, and β-Lg-G-150 was decreased. The UCG modified β-Lg showed lower fluorescence emission maximum (λ_{max}) intensity, and the β-Lg-G-120 exhibited the lowest λmax intensity, indicating that UCG modification had significant influence on the conformational structure of β-Lg. This might be

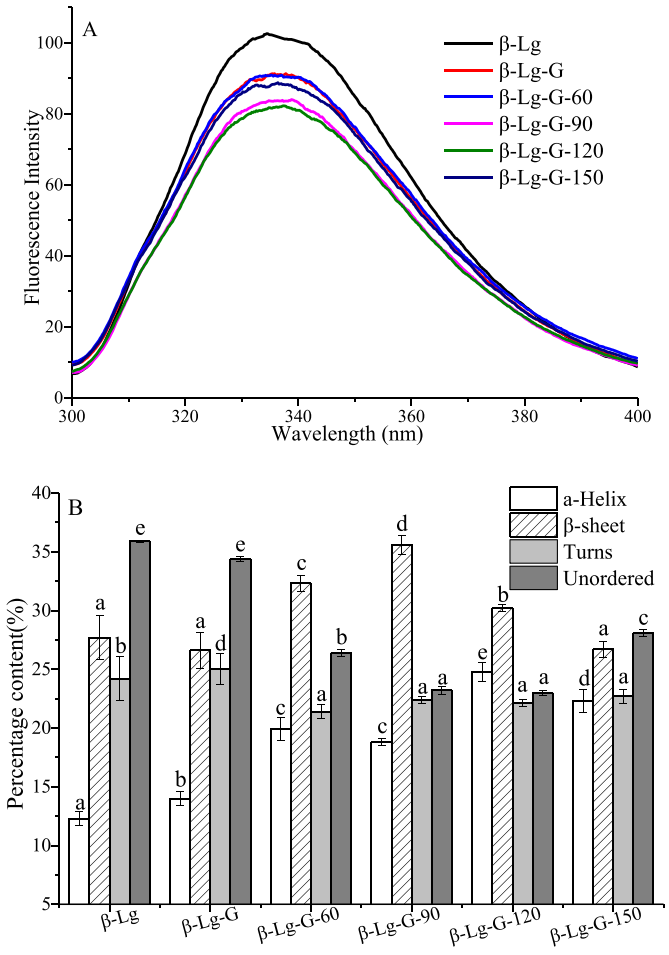

Fig. 3. Changes in intrinsic fluorescence (A) spectra, and the percentage content of secondary structure (B) of β-Lg induced by ultrasound pretreatment combined with glycation. Letters (a-e) in the bars mean significantly different ($p < 0.05$).

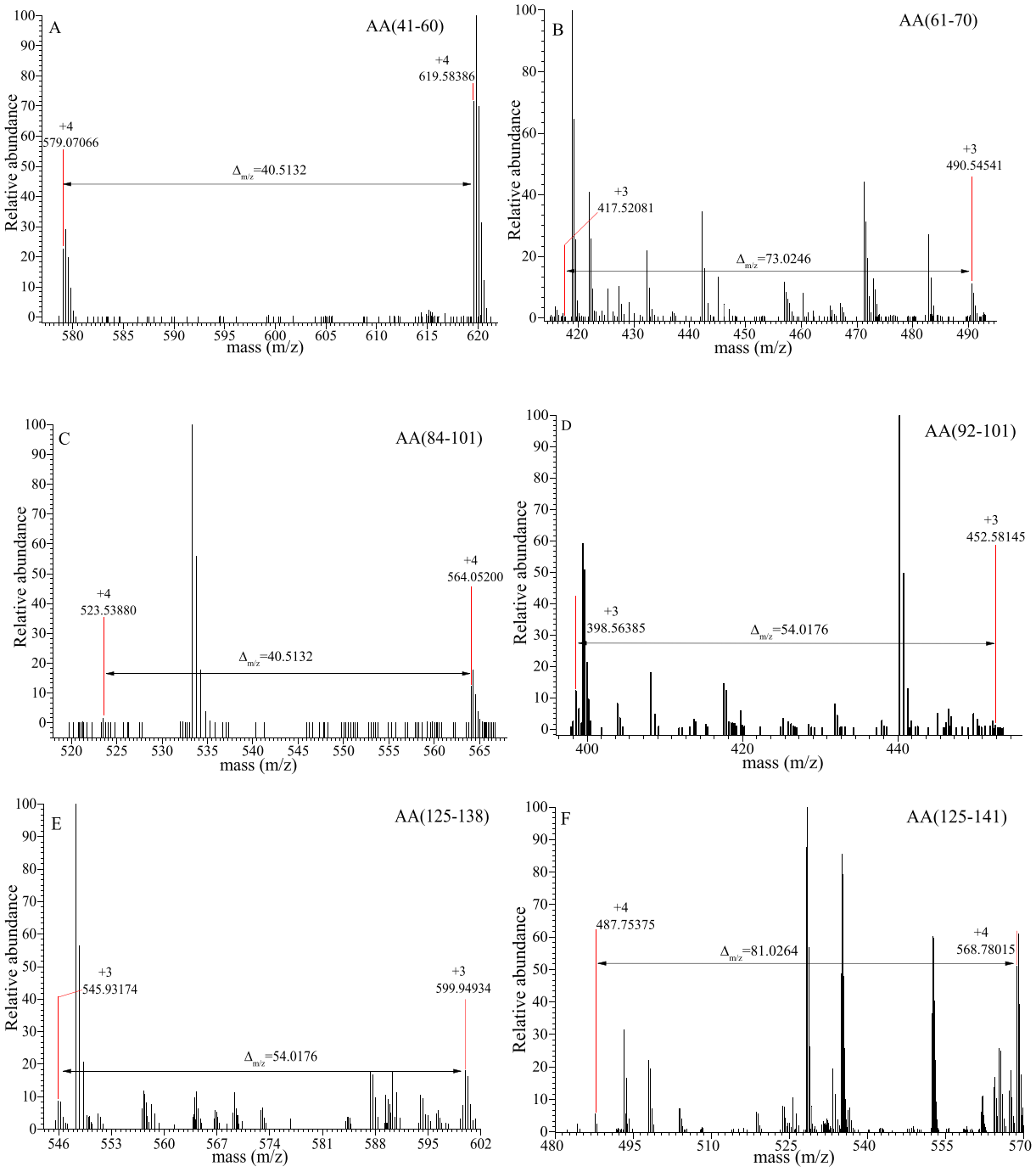

Fig. 4. Mass spectra for the glycated peptides of β-LG-galactose pretreated at 120 W/cm^2. (A) peptide 41–60 at *m/z* 579.07066^{4+}, (B) peptide 61–70 at *m/z* 417.52081^{3+}, (C) peptide 84–101 at *m/z* 523.53880^{4+}, (D) peptide 92–101 at *m/z* 398.56404^{3+}, (E) peptide 125–138 at *m/z* 545.93174^{3+}, (F) peptide 136–148 at *m/z* 385.72703^{4+}. The determined peptides are labelled by residue numbers. The *m/z* differences between glycated and non-glycated peptides are indicated above the arrows.

due to the shielded area around the Trp residues induced by galactose chain [28]. Moreover, a red shift of λmax from 334.4 (β-Lg) to 337.4 (β-Lg-G-120) implied the changes of β-Lg polarity. The lower value of intrinsic fluorescence intensity observed in β-Lg-G-60, β-Lg-G-90, β-Lg-G-120 and β-Lg-G-150 suggested that the shielding effect was more obvious than that of β-Lg-G. This finding could be attributed to the unfolding of β-Lg caused by ultrasonic pretreatment, resulting in a loose structure of β-Lg [29], which led to conformational changes around the Trp residues. It may be that the covalent binding of Gal and β-Lg that will mask some epitopes around lysine residues, causing the masking of allergenic sites and conformational epitopes and thus reduced the allergenic potential of β-Lg. The results showed that the conformational changes were responsible for the decrease of IgG/IgE-binding capacity of β-Lg.

3.4. Secondary structure analysis

The secondary structure contents of β-Lg, β-Lg-G, β-Lg-G-60, β-Lg-G-90, β-Lg-G-120, and β-Lg-G-150 were exhibited in Fig. 3B. It revealed that the secondary structure of the native β-Lg consisted of 12.3% α-helix, 27.7% β-sheet, 24.2% turns, and 35.9% random coil. After glycation modification, the α-helix and β-sheet contents of β-Lg increased, and

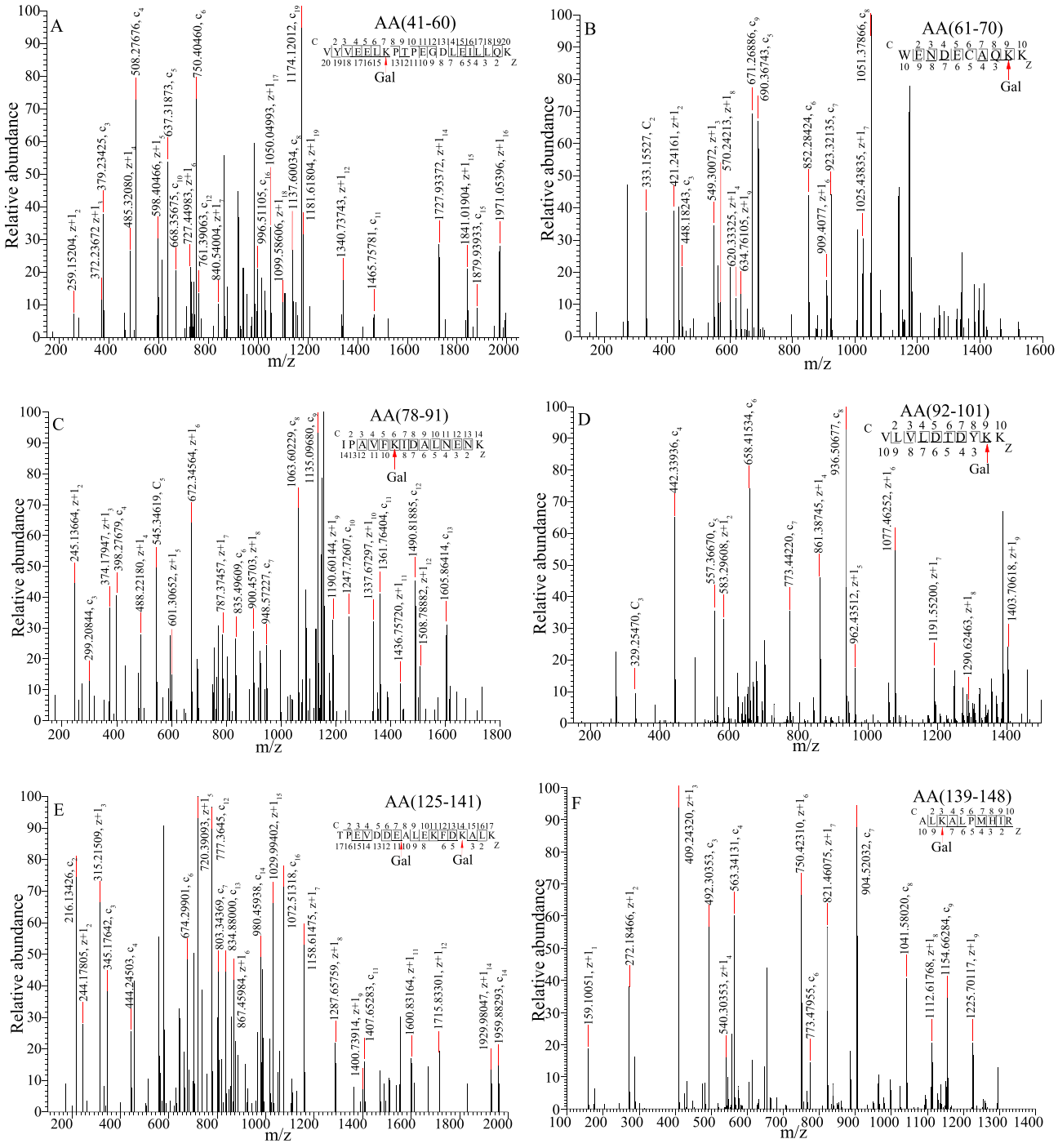

Fig. 5. The ETD MS/MS spectra of the glycated peptides. (A) the glycated peptide 41–60 (VYVEELKPTPEGDLEILLQK) with *m/z* of 619.58386^{4+}, (B) the glycated peptide 61–70 (WENDEC(carbamidomethyl)AQKK) with *m/z* of 490.54541^{3+}, (C) the glycated peptide 78–91 (IPAVFKIDALNENK) with *m/z* of 578.64941^{3+}, (D) the glycated peptide 92–101 (VLVLDTDYKK) with *m/z* of 452.58145^{3+}, (E) the glycated peptide 125–141 (TPEVDDEALEKFDKALK) with *m/z* of 568.78070^{4+}, (F) the glycated peptide 139–148 (ALKALPMHIR) with *m/z* of 437.91998^{3+}. The sequence of per peptide is depicted on the top of the spectrum. The identified glycated sites are indicated by a line with galactose. The c and z ions are shown by the numbers and lines.

the β-turn and random coil contents decreased. This suggested that glycated β-Lg was partially unfolded and reorganized to be a more stable structure [3]. Moreover, UCG modification induced further increase of α-helix contents and decrease of random coil contents, which may be due to the glycation accelerated by ultrasonic pretreatment. The linear epitope may be masked, and the conformational epitope may be destroyed when the secondary structure of β-Lg is changed, which could be the reason of the decrease in IgG/IgE binding and the reduction in mediator release.

3.5. Location and number of glycation site determination

The profiles of MS analysis of glycation sites were shown in Fig. 4. The *m/z* peaks of non-glycated peptides 41–60, 61–70, 84–101, 92–101, 125–138 and 125–141 were 579.07066^{4+}, 417.52081^{3+}, 523.53880^{4+}, 398.56385^{3+}, 545.93174^{3+} and 487.75375^{4+}, whereas the relative *m/z* peaks of the glycated peptide were 619.58386^{4+}, 490.54541^{3+}, 564.05200^{4+}, 452.58145^{3+}, 599.94934^{3+} and 568.78015^{4+}, separately. The *m/z* shift of these peaks was 40.5132, 73.0246, 40.5132, 54.0176,

Table 1
Summary of the glycated peptides in the β-Lg-G, β-Lg-G-60, β-Lg-G-90, β-Lg-G-120 and β-Lg-G-150.

Sample	Peptide location	*m/z* glycated peptide	Δm (ppm)	Sequence	Glycated site
β-Lg-G	61–75	547.25940^{4+}	−0.57	(K)WENDEC*AQKKIIAEK(T)	K69 K70
	78–91	578.64862^{3+}	−0.44	(K)IPAVFKIDALNENK(V)	K83
	84–101	564.05231^{4+}	0.57	(K)IDALNENKVLVLDTDYKK(Y)	K91
	92–101	452.50109^{3+}	−1.29	(K)VLVLDTDYKK(Y)	K100
	125–138	599.94751^{3+}	0.13	(R)TPEVDDEALEKFDK(A)	K135
	125–141	528.26563^{4+}	0.51	(R)TPEVDDEALEKFDKALK(A)	K138
	139–148	437.91934^{3+}	−1.25	(K)ALKALPMHIR(L)	K141
β-Lg-G-60	61–75	547.26001^{4+}	0.55	(K)WENDEC*AQKKIIAEK(T)	K69 K70
	78–91	578.64905^{3+}	0.30	(K)IPAVFKIDALNENK(V)	K83
	84–101	564.05084^{4+}	1.41	(K)IDALNENKVLVLDTDYKK(Y)	K91
	92–101	452.58136^{3+}	0.46	(K)VLVLDTDYKK(L)	K100
	125–138	599.94739^{3+}	0.45	(R)TPEVDDEALEKFDK(A)	K135
	125–141	528.26581^{4+}	−1.18	(R)TPEVDDEALEKFDKALK(A)	K138
	139–148	437.91965^{3+}	−0.76	(K)ALKALPMHIR(L)	K141
β-Lg-G-90	41–60	619.58411^{4+}	1.16	(R)VYVEELKPTPEGDLEILLQK(W)	K47
	61–70	490.54520^{3+}	−0.22	(K)WENDEC*AQKK(I)	K69
	61–75	547.26025^{4+}	−1.82	(K)WENDEC*AQKKIIAEK(T)	K69 K70
	78–91	578.64819^{3+}	−1.18	(K)IPAVFKIDALNENK(V)	K83
	84–101	564.05194^{4+}	−0.08	(K)IDALNENKVLVLDTDYKK(L)	K91
	92–101	452.58136^{3+}	−0.87	(K)VLVLDTDYKK(Y)	K100
	125–138	599.94751^{3+}	0.13	(R)TPEVDDEALEKFDK(A)	K135
	125–141	528.26685^{4+}	0.75	(R)TPEVDDEALEKFDKALK(A)	K138
	139–148	437.92050^{3+}	0.50	(K)ALKALPMHIR(L)	K141
β-Lg-G-120	41–60	619.58386^{4+}	0.76	(R)VYVEELKPTPEGDLEILLQK(W)	K47
	41–75	818.40051^{5+}	1.32	(R)VY…KPT…QKWENDEC*AQKK(I)	K47 K60 K69
	61–70	490.54541^{3+}	0.22	(K)WENDEC*AQKK(I)	K69
	61–75	547.26013^{4+}	1.51	(K)WENDEC*AQKKIIAEK(T)	K69 K70
	78–91	578.64941^{3+}	0.93	(K)IPAVFKIDALNENK(V)	K83
	84–101	564.05200^{4+}	0.02	(K)IDALNENKVLVLDTDYKK(L)	K91
	92–101	452.58145^{3+}	−0.09	(K)VLVLDTDYKK(Y)	K100
	125–138	599.94934^{3+}	3.19	(R)TPEVDDEALEKFDK(A)	K135
	125–141	568.78070^{4+}	1.87	(R)TPEVDDEALEKFDKALK(A)	K135 K138
	139–148	437.91998^{3+}	0.01	(K)ALKALPMHIR(L)	K141
β-Lg-G-150	61–75	547.26013^{4+}	0.77	(K)WENDEC*AQKKIIAEK(T)	K69 K70
	78–91	578.65009^{3+}	2.09	(K)IPAVFKIDALNENK(V)	K83
	84–101	564.05377^{4+}	2.30	(K)IDALNENKVLVLDTDYKK(L)	K91
	92–101	452.58188^{3+}	−0.27	(K)VLVLDTDYKK(Y)	K100
	125–138	599.94812^{3+}	1.15	(R)TPEVDDEALEKFDK(A)	K135
	125–141	568.78015^{4+}	0.91	(R)TPEVDDEALEKFDKALK(A)	K135 K138
	139–148	437.92032^{3+}	0.78	(K)ALKALPMHIR(L)	K141

54.0176 and 81.0264 Da respectively. As we know, if a peptide was glycated by Gal, the corresponding *m/z* of the peak with the charges of 5, 4, 3, 2, or 1 will appear with a mass shift of 32.4106, 40.5132, 54.0176, 81.0264, or 162.0528 Da respectively. This result showed that peptides were glycated with Gal. For example, as shown in Fig. 4A, the *m/z* of non-glycated peptide 41–60 was 579.07066^{4+}, and the matching *m/z* of its glycated forms was 619.58386^{4+}, with an *m/z* shift of 40.5132, indicating it contains mono-glycated peptide. The *m/z* of non-glycated peptide 125–141 was 487.75375^{4+}, while the matching *m/z* of its glycated forms was 568.78015^{4+}, and the *m/z* shift was 81.0264 Da, indicating it contains dual-glycated peptides (Fig. 4D).

Similarly, we used HPLC-ETD-MS/MS to obtain the detailed map of all glycation sites. Fig. 5 shows the ETD MS/MS spectrum of β-Lg-G-120. The ETD MS/MS spectrum of the mono-glycated peptide ^{41}VYVEELKPTPEGDLEILLQK^{60} exhibits a series of c and z ions (c2-c20 and z2-z20) with *m/z* of 619.58386^{4+}. K47, a glycation site, was determined by the difference in mass between c6 and c8 ions, or between z13 and z15, that is the sum mass of lysine and galactose residues (Fig. 5A). The ETD fragmentations of glycated peptides with *m/z* of 490.54541^{3+}, 578.64941^{3+}, 452.58145^{3+} and 437.91998^{4+} were shown in Fig. 5B, C, D, F. A series of c and z ions were detected, which matched correctly with the peptide fragments ^{61}WENDEC (carbamidomethyl)AQKK^{70}, ^{78}IPAVFKIDALNENK^{91}, ^{92}VLVLDTDYKK^{101}, and ^{139}ALKALPMHIR^{148}. The results confirmed the galactose was linked to K69, K83, K100 and K141. Similarly, Fig. 5E presents the ETD-MS/MS of the dual-glycated peptides ^{125}TPEVDDEALEKFDKALK^{141} with the *m/z* of 568.78070^{4+}, which was identified as K135, K138, respectively.

Glycated peptide and glycation sites of β-Lg-G, β-Lg-G-60, β-Lg-G-90, β-Lg-G-120 and β-Lg-G-150 were shown in Table 1. β-Lg-G contains eight glycation sites (K69, K70, K83, K91, K100, K135, K138 and K141). After treated by ultrasonic at 60 and 150 W/cm^2, the sites were same as that of β-Lg-G totally. When the ultrasonication at 90 W/cm^2, one additional glycated site (K47) were found to be glycated. Interestingly, the maximum glycation sites of K47, K60, K69, K70, K83, K91, K100, K135, K138 and K141 were detected in samples ultrasonicated at 120 W/cm^2. The two additional sites (K47 and K60) were found in treated β-Lg after UCG modification (Fig. 6A). The increase in glycation sites may be due to the loose structure of β-Lg caused by ultrasonic treatment, accelerating the glycation reaction and exposing more active sites [29,30]. When the ultrasonication at 150 W/cm^2, the sample is less glycated than sample sonicated with 90 and 120 W/cm^2. A possible cause of this is the ultrasonication-induced β-Lg aggregation [29]. The aggregation of β-Lg might lead to the reburial of some Lys residues inside the intermolecular and finally result in the less glycated of β-Lg.

3.6. Glycation extent analysis

The average degree of substitution per peptide molecule (DSP) was used to evaluate the glycation extent of modified β-Lg. Fig. 6B shows the DSP value for all glycated peptides of β-Lg-G, β-Lg-G-60, β-Lg-G-90, β-Lg-G-120 and β-Lg-G-150. After ultrasonic pretreatment, the glycated peptide exhibited a higher DSP than untreated samples. For example, K83 were the most reactive sites in β-Lg-G with DSP value of 0.69, the DSP value of β-Lg-G-60, β-Lg-G-90, β-Lg-G-120 and β-Lg-G-150

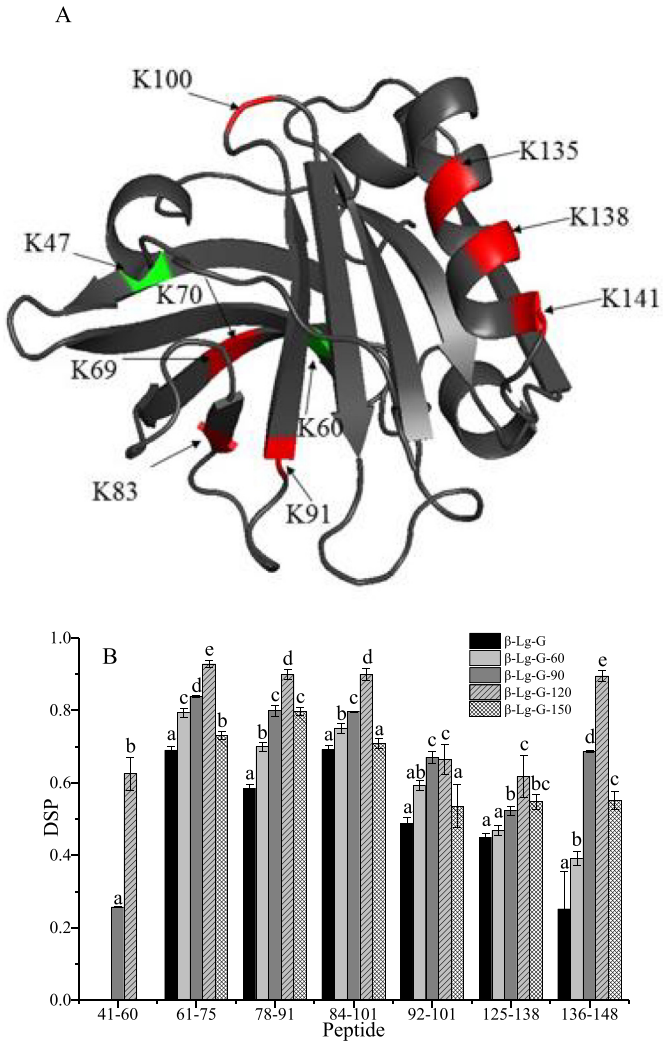

Fig. 6. Ribbon diagram (A) of the glycated β-Lg (PDB 1BSQ). The glycation sites are colored as follows: gray, framework of β-Lg; red, glycation sites of the native β-Lg; green, additional glycation sites of the β-Lg after ultrasonication at 120 W/cm^2. DSP values (B) of the glycated peptides from the glycated β-Lg with ultrasound pretreatment at 0, 60, 90, 120 and 150 W/cm^2. Letters (a–e) in the bars mean significantly different ($p < 0.05$).

increased to 0.69, 0.78, 0.80, 0.90 and 0.72 respectively. The highest DSP values of 1 were detected in the sample ultrasonicated at 120 W/cm^2. Although 41–60 peptides of β-Lg-G was non-glycated, it also had high DSP value (0.26 and 0.69) in the β-Lg-G-90 and β-Lg-G-120. This is because ultrasonic treatment exposes the glycation sites of β-Lg, which made some Lys residues accessible to react with Gal. However, too strong ultrasonic treatment (power 150 W/cm^2) can cause the aggregation of the β-Lg molecule [29], embedding Lys residues in the interior of β-Lg molecules, which explained the lower glycation degree of β-Lg-G-150 compared to that of β-Lg-G-120.

3.7. Mechanism of the reduction in the allergenicity of β-Lg by UCG modification

In this study, UCG modification significantly reduced the IgG/IgE-binding capacity of β-Lg and the release of β-hexosaminidase, histamine and interleukin-6 in cells, which was closely related to its structural changes. The structural changes of β-Lg by UCG modification was investigated to understand the mechanism of the reduction in the allergenicity of β-Lg.

The effect of glycation reaction on allergenicity of β-Lg occurs by changing linear or conformational epitopes of β-Lg [31]. Studies have shown that allergen epitopes of β-Lg include conformational epitopes and linear epitopes, of which linear epitopes account for 10% and conformational epitopes account for 90% [32]. After UCG modification, the tertiary structure of β-Lg undergoes a significant change, reflected on the decrease of intrinsic fluorescence intensity and the red shift of λ_{max}. This change causes the reduction in allergenicity of β-Lg, thus further affects the reduction in the release of allergic mediators of basophils. After glycation with galactose, the α-helix and β-sheet contents of β-Lg increased, whereas the β-turn and random coil contents decreased. This kind of change also has certain contribution to the reduction in allergenicity of β-Lg. Moreover, the ultrasound pretreatment promoted the changes, leading to the further reduction of allergenicity of β-Lg.

Saccharides can affect protein allergy in the glycation reaction, mainly by disrupting their conformational epitopes and masking the linear epitopes. The potentially identified β-Lg allergenic epitopes are the fragments 1–8, 9–14, 25–40, 41–60, 78–83, 84–91, 92–100, 102–124, 125–135 and 149–162 of the peptides [33–35]. These sequences contain one or more lysine residues (K8, K14, K47, K60, K83, K91, K100 and K135). In this study, the antigenic sites of β-Lg were modified and masked since K83, K91, K100 and K135 covalently bonded with galactose, further resulting in peptide fragments 84–91, 92–100, 102–124, 125–135 cannot bond with IgE and IgG. Finally, it caused the decrease in the IgE/IgG-binding abilities of β-Lg and the release of allergic mediators of basophils. Eight glycation sites (K69, K70, K83, K91, K100, K135, K138 and K141) were determined in the glycated β-Lg without ultrasound pretreatment (Table 1), finally led to a higher IC_{50} value compared to non-glycated samples (Fig. 1). One additional glycation sites (K47) were found after pretreatment of β-Lg with ultrasound power of 90 W/cm^2 (Table 1). The most recognizable linear epitope area of β-Lg include K47. K47 was glycated, which can change the amino acid sequence of β-Lg and reduce the capacity of IgE/IgG-binding. When ultrasound power at 120 W/cm^2, two additional glycated sites K47 and K60 were identified, which resulted in the change of linear epitopes, finally reduced the IgE/IgG-binding capacity of β-Lg and the release of biological active mediators, which reflected in the release of β-hex, histamine and IL-6.

The glycation extent is also responsible for allergenicity of protein (Liu et al., 2018). Although β-Lg-G and β-Lg-G-60 had the same number of glycation sites (Table 1), β-Lg-G-60 had higher DSP and IC_{50} values (Figs. 1 and 6B). Ultrasonication treatment can cause exposure of the glycation reaction region, these reaction regions were well-exposed with increased ultrasonic power, which accelerated the glycation reaction, and ultimately reduces the binding capacity of β-Lg to IgE/IgG. This also indicates that glycation extent could affect the allergenicity of β-Lg. Therefore, UCG modification reduced the IgE/IgG binding capacity of β-Lg, mainly by changing the structure, in details, the masking of the allergenic sites and the structural change of β-Lg lead by the covalent binding of β-Lg and Gal, finally affected the conformational epitopes of β-Lg.

In this work, it was exhibited that UCG modification could effectively decrease the IgG/IgE-binding capacity of β-Lg, and reduce the release inflammatory mediators such as histamine attributed to the covalent binding of galactose to β-Lg, also result in the change of protein conformational epitopes and the masking of linear epitopes. Our results show that the combination of IgE/IgG binding activity, basophil histamine release test and high-resolution mass spectrometry can be used to further verify that ultrasound combined with glycation can reduce the β-Lg allergenic potential. Moreover, ultrasound pretreatment promotes the reduction of the allergenicity by improving the extent of glycation, which is reflected in the increase of glycation sites and DSP value. Therefore, UCG modification may provide new methods for reduction of β-Lg allergenic potential. This strategy provides a valuable support for the development of hypoallergenic dairy products to ensure the food safety of

dairy products obtained by future processing technologies. And, UCG modification was revealed as a good technology for reducing allergenic potential of proteins. This study will provide the theoretical basis for the preparation and safety evaluation of hypoallergenic dairy products.

Ethics statement

This work did not include any human subjects and animal experiments.

Author contributions section

Yanhong Shao: the first author, she brewed and designed experiments, conducted experimental research, collected data, analyzed and interpreted data, and wrote articles.

Yao Zhang: the second author, he conducted the part of the experimental study (Section 2.3).

Minfang Zhu: the third author, she embellished the language in the article.

Jun Liu: the fourth author, the corresponding author of this paper, he critically reviewed the intellectual content of the article.

Zongcai Tu: the fifth author, the corresponding author of this paper, he given the Lab platform support to the research of this paper.

Declaration of competing interest

The authors have no conflict of interest to declare.

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