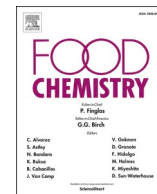




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Insight into the mechanism of D-allose in reducing the allergenicity and digestibility of ultrasound-pretreated α -lactalbumin by high-resolution mass spectrometry

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ABSTRACT

The mechanism of D-allose in reducing the allergenicity and digestibility of ultrasound-pretreated α -lactalbumin (α -LA) was studied. The intensity reduction and peak red shift occurred in fluorescence spectra of glycosylated samples. Enzyme-linked immunosorbent assay and basophil degranulation analysis showed that D-allose significantly reduced the allergenicity of α -LA, and ultrasound-pretreated α -LA showed the lowest allergenicity after glycation. Compared with α -LA, the degree of hydrolysis decreased in glycosylated samples. Size-exclusion high-performance liquid chromatography showed that the glycosylated α -LA was resistant to digestive enzymes. The glycosylated sites and average degree of substitution per peptide molecule were determined using LC Orbitrap MS/MS. These results suggested that the masking of linear allergenic epitopes by glycation could reduce the allergenicity. Therefore, the combination of ultrasound pretreatment and glycation is a potential method to reduce protein allergenicity in food processing and provides a useful approach for the application of rare sugars in food processing.

1. Introduction

Milk is rich in various nutrients, including proteins, fats, vitamins, and minerals, which are typically easily absorbed by the body (Skripak, Matsui, Mudd & Wood, 2007). However, the allergens found in milk are one of the top eight food allergens (Fiocchi et al., 2010). Cow's milk protein allergy (CMPA) mainly occurs in infants and children under 3 years of age (Del Chierico et al., 2012). The most prominent allergens in milk and milk products are caseins, β -lactoglobulin, and α -lactalbumin (α -LA) (Villa, Costa, Oliveira & Mafra, 2018). α -LA has 123 amino acids and eight cysteine residues that exist in the form of four disulfides (Zhang et al., 2019). It is the only globular whey protein that binds calcium ions (Lam & Nickerson, 2015). Over 75% of patients with CMPA

are allergic to α -LA (Host & Halken, 2014). Allergic reactions are caused by the presence of allergic epitopes. If certain methods or means can disrupt these allergic epitopes, the occurrence of allergic reactions can be reduced or even eliminated, which is of great significance to consolidate and expand the application of cow milk in the food field.

Ultrasound is widely used to improve the structural and functional properties of proteins, such as improving the solubility of rice protein (Povey et al., 2011), and increasing the foaming ability of bovine serum albumin (Li, Ma & Guo, 2018). Ultrasound was reported can disrupt the conformational allergenicity epitopes of protein and loosen the protein structure. Glycation can mask the linear epitopes of protein and reduce the IgG/IgE binding capacity of allergic proteins (Wang, Ye, Tu, Hu, Wang & Huang, 2021). Moreover, ultrasound pretreatment combined

Abbreviations: α -LA, α -lactalbumin; CMPA, cow's milk protein allergy; LA, native α -lactalbumin; ULA, ultrasound-pretreated α -LA; Man-LA, α -LA glycosylated with D-mannose; U-Man-LA, ultrasound-pretreatment α -LA glycosylated with D-mannose; Glc-LA, α -LA glycosylated with D-glucose; U-Glc-LA, ultrasound-pretreatment α -LA glycosylated with D-glucose; All-LA, α -LA glycosylated with D-allose; U-All-LA, ultrasound-pretreatment α -LA glycosylated with D-allose; DH, degree of hydrolysis; OPA, o-phthalaldehyde; DTT, D-thiothreitol; SGDS, simulated gastric digestive solution; SIDS, simulated intestinal digestive solution; Trp, tryptophan; Tyr, tyrosine; Lys, lysine; Arg, arginine; DSP, the average degree of substitution per peptide molecule; HCD, high-energy C-trap dissociation.

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with glycation can significantly reduce protein allergenicity (Wang, Tu, Ye, Liu, Wang & Hu, 2021). However, in previous studies, only the IgG/IgE binding ability of the allergic protein was measured to evaluate the effect of glycation on the allergenicity of protein, which is incomplete, because the IgE antibody level in the blood is not the only factor that induces allergic symptoms. Inflammatory mediators (histamine, β -hexosaminidase and interleukin [IL]-6) are released when the protein allergen binds to two or more adjacent IgE antibodies and links with multiple high-affinity IgE receptors (Fc ϵ RI) to form a complex, eventually leading to allergic symptoms (Finkelman, Khodoun & Strait, 2016). The study found that the allergenicity of β -lactoglobulin hydrolysates can be effectively monitored by measuring the amount of mast cell protease released after mast cell activation in the blood (López-Expósito, Chicón, Belloque, López-Fandiño & Berin, 2012). In addition, the changes in the digestive properties of α -LA glycated with D-allose require further investigation. Compared with *in vivo* digestion experiments, *in vitro* simulated digestion is controllable and reproducible, which makes it an alternative choice.

D-allose, a rare and important D-form sugar, exhibits 80% sweetness of that of table sugar (sucrose). It is an ideal table sugar substitute with ultra-low calories and provides very little energy to the body (Moora-dian, Smith & Tokuda, 2017). This rare monosaccharide has anti-tumour (Malm, Hanke, Gill, Carbajal & Baker, 2015), anti-cancer (Noguchi et al., 2016) and other beneficial physiological functions. Compared with fructose and glucose, D-allose exhibits a higher degree of covalent cross-linking and a faster reaction rate in undergoing condensation reactions with amines (Sun, Hatakawa, Puangmanee & Izumori, 2006). Therefore, D-allose has broad application prospects in the food system.

In this study, the effect of glycation on the allergenicity and digestibility of ultrasound-pretreated α -LA was investigated. The potential allergenicity of α -LA glycated with D-allose was evaluated based on indirect competitive ELISA (icELISA) and the KU812 cell degranulation model. Digestibility was investigated by using size-exclusion high-performance liquid chromatography (SE-HPLC) and the degree of hydrolysis (DH). The glycation sites and degree of substitution per peptide value were determined by high-resolution mass spectrometry, and the mechanisms underlying the effect of D-allose in reducing the allergenicity and digestibility of ultrasound-pretreated α -LA were explored. This study provides basic information for the potential application of α -LA in the food industry and dairy products and expands the application of rare sugars in food processing.

2. Materials and methods

2.1. Materials and chemicals

α -LA (L6010, $\geq 85\%$) and pepsin (p6887, ≥ 3200 units/mg) were obtained from Sigma-Aldrich (St. Louis, MO, USA). D-(+)-glucose (G116303), D-(+)-mannose (M103969, 99%) and D-(+)-allose (A111907, 98%) were purchased from Aladdin (Shanghai, China). Trypsin (T8150, 1:250) was obtained from Solarbio (Shanghai, China). The serums of patients with Cow's milk allergy (CMPA) were obtained from Plasma Lab International (Everett, WA, USA). Goat anti-human IgE-horseradish peroxidase (HRP) conjugate (A9667) was bought from Immunology Consultants Laboratory (Shanghai, China). Rabbit serum (bs-0814R) and goat anti-rabbit IgG-HRP conjugate (bs-0295G-HRP) were from Bioss Company (Beijing, China). All other reagents and solvents used were of analytical and HPLC grade. Ultrapure water was obtained from a water purification system (Millipore, Billerica, MA) and used throughout the study.

2.2. Sample preparation

Native α -LA (160 mg) without any treatment was dissolved in 160 mL of 10 mM phosphate-buffered saline (PBS) at pH 7.4, with 1 mg/mL

of α -LA solution. The solution was treated with a probe sonicator (JY98-III, 20 kHz) equipped with a microtip probe (probe diameter 6 mm) with a 3 s on and 7 s off pulsation at an actual ultrasound intensity of 130 W/cm² for 10 min. The whole ultrasound process was carried out in an ice bath to ensure that the temperature of the sample solution is not elevated (lower than 15 °C). Then, the solution was stored in a refrigerator at 4 °C.

In the case of glycation, 20 mg of D-glucose, D-mannose and D-allose was added to 20 mL of ultrasound-pretreated α -LA solution, separately. After lyophilization, the freeze-dried powders were immediately put into the glycation reaction model with 74.41% relative humidity and 55 °C for reaction. The reaction was stopped by transferring the sample tubes into an ice bath. The free reducing sugar and salts were filtered by a Centricon centrifugal filter unit (3000 Da cutoffs, Millipore, Bedford, MA, USA) and dialyzed. The concentration of all samples was adjusted to 1 mg/mL, and samples were stored at 4 °C for further analyses. Native α -LA was named LA. Native α -LA glycated with D-allose was designated as All-LA. Ultrasound-pretreated α -LA was named U-LA, whilst ultrasound-pretreated α -LA glycated with D-allose was designated as U-All-LA.

2.3. Determination of available amino groups

o-Phthalaldehyde (OPA) solution was used according to the method of (Guan et al., 2010). A total of 0.20 g of OPA and 0.22 g of DTT were dissolved into 5 mL of 95% ethanol. A total of 9.525 g of borax and 0.25 g of SDS were dissolved into 100 mL of ultrapure water. Finally, the two solutions were mixed and transferred into a volumetric flask with ultrapure water to 250 mL, which was called an OPA reagent. A total of 0.1 mL of α -LA sample (1 mg/mL) and 2 mL of freshly prepared OPA reagent were mixed and then incubated for 2 min at 37 °C in the dark. The absorbance of the mixture at 340 nm was immediately measured using the U-2910 spectrophotometer (Hitachi, Tokyo, Japan). With L-lysine concentration (0.025–0.50 mg/mL) as the standard curve. The measurement of all samples was performed in triplicate.

2.4. Fluorescence spectroscopy

The fluorescence spectra of samples were obtained by using a Hitachi F-7000 fluorescence spectrophotometer (Hitachi, Japan). The sample concentration was 1 mg/mL. The intrinsic emission fluorescence spectra parameters were set as follows: excitation wavelength of 280 nm, scanning emission wavelength of 300–450 nm, slit width of 5 nm and scanning speed of 1200 nm/min (Hoz & Netto, 2008).

2.5. IgG/IgE-binding ability

The IgG-binding abilities and IgE-binding rates of samples were measured via indirect competitive ELISA. ELISA was carried out at 37 °C. A 96-well microplate was washed with PBST by using a micro-oscillator. The specific procedures are as follows: antigen coating: 100 μ L of 4 μ g/mL α -LA standard was coated in each well of the 96 enzyme-labeled plates, and the plate was incubated for 2 h. The plate was washed three times and patted dry. Sealing: add 250 μ L of fish skin gelatin sealing solution (mass fraction: 3%), incubate for 1 h, wash the plates three times, and pat dry. External competition: Mix 200 μ L of 8 μ g/mL sample with 200 μ L of rabbit serum (1:320,000) or the 16 μ g/mL sample with 200 μ L serum of CMPA patients (1:250, n = 10) and incubate for 1 h. After the reaction, absorb 100 μ L from each well and add to the plate and incubate for 1 h. Wash the plate three times and pat dry. Goat anti-rabbit IgG-HRP conjugate (100 μ L) or goat anti-human IgE-HRP conjugate (100 μ L), diluted to 1:5000, was inputted for complete recognition by IgG/IgE. The plates were washed three times and patted dry. Finally, 100 μ L of TMB was added for hatching and coloring. The reaction was terminated with 100 μ L of sulphuric acid solution (2 mol/L). The absorbance was detected by using a microplate reader at a wavelength of

450 nm. PBST and serum mixture were used as the positive control. The inhibition rate was calculated as follows:

$$\text{Inhibition}(\%) = \left(1 - \frac{B}{B_0}\right) \times 100\%$$

where B is the absorbance value with the addition of inhibitor antigen and B_0 is the light absorption value without the addition of inhibitor antigen.

2.6. Evaluation of the degranulation model of KU812 cells

Based on the KU812 cell degranulation model, we were able to evaluate the potential allergenic changes of α -LA glycosylated with D-allose. The intact cells in the logarithmic phase were transferred to a 48-well plate (500 μ L/well) for culture for 48 h culture to adapt to the new environment. The number of cells should be 10^6 – 10^8 cells/mL, and 100 μ L of mixed serum from 10 patients with CMPA was added to each well. The samples were placed in a cell incubator for incubation for 24 h, and 50 μ g of samples were added to stimulate for 4 h. Then, the samples were centrifuged for 10 min at 252 g. The supernatant was collected for the degranulation assay. The release of histamine, IL-6 and TNF- γ was evaluated by using a commercial ELISA kit (Meimian, Jiangsu, China).

2.7. Simulated gastrointestinal digestion and DH analysis

The digestion behavior of glycosylated α -LA and LA was simulated following the method of Minekus et al. (2014) with minor changes. In detail, the enzymatic activity ratio of pepsin to sample protein was 182:1 (units/mg protein). After digestion in simulated gastric digestive solution (SGDS, pH 2) for 2 h at 37°C, the pH was adjusted to 7.0 to stop the reaction, and the samples were digested in simulated intestinal digestive solution (SIDS, pH 6.5) for 2 h at 37°C, the enzyme activity ratio of trypsin to protein was 35:1 (units/mg protein), the reaction was terminated in a boiling water bath for 3 min. A part of the sample was taken out after 1 h of gastric digestion and 1 h of gastrointestinal digestion, respectively, and subsequent analysis was performed after the termination of digestion. Samples digested with SGDS were named G-LA-1 h, G-LA-2 h, G-All-LA-1 h, G-All-LA-2 h, G-U-All-LA-1 h and G-U-All-LA-2 h, and samples digested by SGDS and SIDS were named GI-LA-1 h, GI-LA-2 h, GI-All-LA-1 h, GI-All-LA-2 h, GI-U-All-LA-1 h and GI-U-All-LA-2 h. The DH of all samples was determined according to the change of free amino content and calculated as follows:

$$\text{DH}(\%) = \frac{h_t}{h_{\max}} \times 100\%$$

where h_t is the millimoles of free amine groups per gram of protein in the test sample and $h_{\max}$ is the millimoles of free amino groups per gram of protein that are completely hydrolyzed (8.6 mmol/g for α -LA) (Deng, Butré & Wierenga, 2018).

2.8. Size-exclusion high-performance liquid chromatography (SE-HPLC)

The molecular weight distribution of samples was analyzed using SE-HPLC (Hitachi, Japan). The chromatographic column used was XBridge BEH 125A SEC (7.8 mm $\times$ 300 mm $\times$ 3.5 μ m). The chromatographic conditions were as follows: mobile phase: ultra-pure water 60% (including 0.1% trifluoroacetic acid), acetonitrile 40% flow rate: 0.4 mL/min sample size: 10 μ L detector wavelength: 220 nm detection time: 30–60 min elution time: 30–60 min. The calibration curve, $\log(\omega) = -0.1986t + 6.8741$, with $R^2 = 0.9982$, was prepared using standards, such as L-hydroxyproline (131 Da), oxidized glutathione (613 Da), cytochrome c (12384 Da), and α -lactalbumin (14200 Da).

2.9. HPLC-HCD-MS/MS analysis

A liquid chromatography-Orbitrap fusion mass spectrometer (Thermo Fisher Scientific, USA) was used to investigate the glycosylated sites and average degree of substitution per peptide molecule (DSP) values of All-LA and U-All-LA. All-LA and U-All-LA were hydrolyzed by pepsin (diluted in HCl solution, pH 2.2) at a ratio of 1/1 (w/w) and incubated at 4 °C for 10 min. Then, the hydrolysate was separated via Ultimate 3000 RSLC nano liquid chromatography with 0.1% formic acid buffer solution (A) and 0.1% formic acid-acetonitrile buffer solution (B), and the chromatographic column was balanced with 4% B. The hydrolysate entered the upper sample column (100 μ m $\times$ 2 cm, Thermo Fisher Scientific, USA) and was then separated by the analytical column (75 μ m $\times$ 15 cm, Thermo Fisher Scientific, USA) at a flow rate of 300 nL/min. The gradient elution was set as follows 0–6 min, 4% solvent B 6–50 min, 4–25% solvent B linear gradient 50–58 min, 25–40% solvent B linear gradient 58–61 min, 40–85% solvent B linear gradient 61–66 min, 85% solvent B and solvent B back at 4%.

The effluents of samples were injected into an LTQ-Orbitrap Fusion mass spectrometer (Thermo Fisher Scientific, Waltham, MA, U.S.A.) for tandem mass spectrometry (MS/MS²) determination to ascertain α -LA glycosylation. The positive ions detected in the scanning for precursor ions were then fragmented with high-energy C-trap dissociation (HCD) to acquire the fragment ions.

To further compare the extent of glycosylation of each peptide, the DSP of α -LA was calculated (Yang et al., 2020):

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

where I is the sum of the intensity of different glycosylated α -LA peptides and i is the amount of D-allose units attached to the peptide in each glycosylated form.

2.10. Statistical analysis

All measurements were performed in triplicate, and the results were expressed as mean $\pm$ standard deviation. Data were analyzed by analysis of variance using SPSS version 25 (SPSS Inc., Chicago, IL), and the separation of means was tested by Duncan's multiple range test at a probability level of $p < 0.05$.

3. Results and discussion

3.1. Free amino groups content

Generally, the potential glycosylation sites of proteins during the Maillard reaction are lysine (Lys), arginine (Arg) and free amino at the N-terminal. Previous studies indicated that Lys is the main glycosylated site with reducing sugar (Huang, Tu, Wang, Zhang, Shi & Xiao, 2013), whilst Arg is an infrequent glycosylation site because of the lower freedom of the amino group by steric effect from neighboring atoms. The decrease of free amino group content can be used to reflect the extent of the Maillard reaction on a macroscale level (Liu et al., 2018). As shown in Fig. 1A, the content of the free amino groups significantly decreased and ultrasound pretreatment promoted the reduction ($p < 0.05$). In the presence of glycosylation, D-allose is the most reactive sugar for Maillard reaction by ultrasound treatment, resulting in the lowest free amino content of U-All-LA. An increase in the degree of glycosylation decreases the number of free amino groups. More N-terminal was exposed to the molecular surface of LA after ultrasound treatment, which promotes the crosslinking reaction between free amino groups of protein and carbonyls of sugar (Wang et al., 2020).

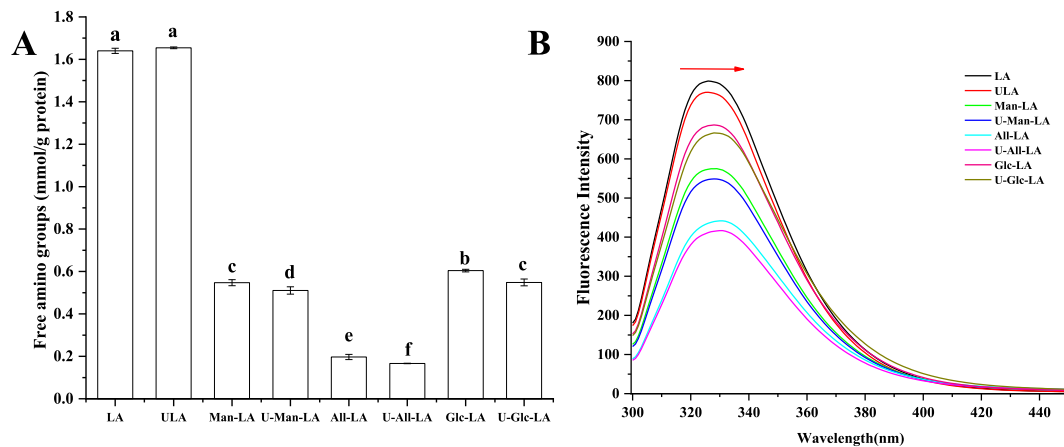


Fig. 1. The free amino groups contents (A) and intrinsic fluorescence spectra (B) glycated α -LA (with/without ultrasound pretreatment) with different reducing sugars. Letters (a–f) above the bars demonstrate significant differences ($p < 0.05$). (LA, native α -lactalbumin (α -LA); ULA, ultrasound-pretreated α -LA; Man-LA, α -LA glycated with D-mannose; U-Man-LA, ultrasound-pretreatment α -LA glycated with D-mannose; Glc-LA, α -LA glycated with D-glucose; U-Glc-LA, ultrasound-pretreatment α -LA glycated with D-glucose; All-LA, α -LA glycated with D-allose; U-All-LA, ultrasound-pretreatment α -LA glycated with D-allose).

3.2. Intrinsic fluorescence spectroscopy

The intrinsic fluorescence intensity can accurately reflect the change of protein conformational characteristics (Yang, Tu, Wang, Zhang & Song, 2018). As shown in Fig. 1B, the intrinsic fluorescence emission maximum ($\lambda_{\max}$) of glycated samples decreased markedly from 799.1 (LA) to 770.2 (ULA), 686.7 (Glc-LA), 575.2 (Man-LA), 441.7 (All-LA), 666.49 (U-Glc-LA), 548.7 (U-Man-LA), 416.9 (U-All-LA). With the gradual unfolding of the protein, the aromatic amino group was gradually exposed to hydrophilic solvents, which eventually led to the quenching of intrinsic fluorescence (Wang, Ye, Tu, Hu, Wang & He, 2021).

Yang et al., 2020)The space shielding effect produced by monosaccharides and the extended dimension of a protein matrix induced by unfolding may also diminish its $\lambda_{\max}$ value (Yang, Liu & Wang, 2019). The red-shifts occurred in glycated samples, indicating that glycation gradually increases the polarity of the microenvironment in which the chromophores are located, which reduced electron transition ability. All these indicate that the tertiary structure of LA has changed.

3.3. Analysis of IgG/IgE -binding abilities

The IgG/IgE -binding abilities of all samples were determined

through indirect competitive ELISA. As shown in Fig. 2A and B, compared with LA, the IgG/IgE binding capacity of Glc-LA, Man-LA, U-Glc-LA, U-Man-LA, All-LA, and U-All-LA was significantly reduced, and the glycation samples with ultrasound pretreatment were further reduced. U-All-LA had the lowest IgG/IgE binding capacity, with a 43.10% reduction in IgG binding rate and a 53.32% reduction in IgE binding rate, which was consistent with the results in Fig. 1A. α -LA glycated with D-allose had the lowest number of free amino groups. This indicates that D-allose has the strongest ability to reduce α -LA IgG/IgE binding compared with glucose and mannose.

3.4. Evaluation of the degranulation model of KU812 cells

Based on the KU812 cell degranulation model, the effect of D-allose on the allergenicity of ultrasound-pretreated α -LA was further studied to verify the results of the ELISA experiment, aiming to comprehensively evaluate the allergenicity changes of D-allose to α -LA. β -Hexosaminidase is a signature proteolytic enzyme released by basophil degranulation. When the body is allergic, the content of β -hexosaminidase in plasma increases significantly. Fig. 3A shows the changes of β -hexosaminidase after KU812 cells degranulation. The content of β -hexosaminidase in LA, All-LA and U-All-LA showed a decreasing trend. The content of β -hexosaminidase in U-All-LA was decreased by 23.198% ($p < 0.05$).

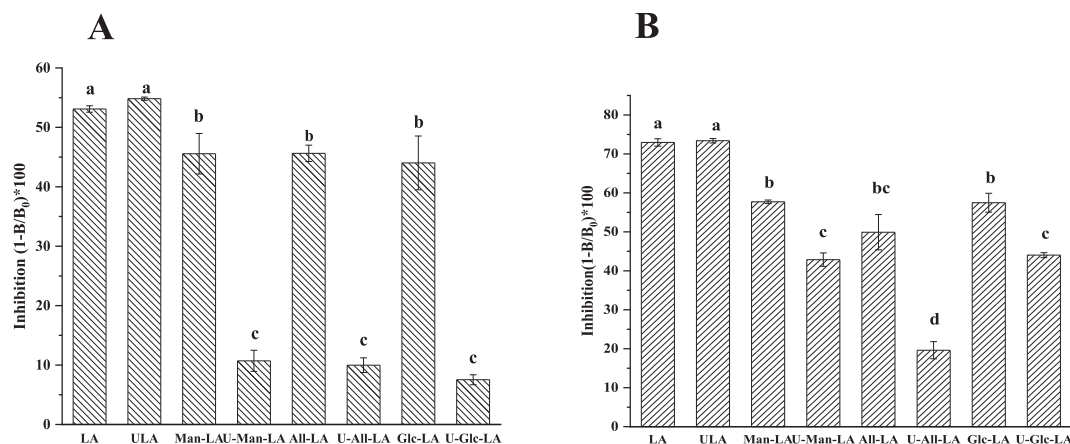


Fig. 2. The IgG binding rates (A) and IgE binding rates (B) of glycated α -LA (with/without ultrasound pretreatment) with different reducing sugars. Letters (a–f) above the bars demonstrate significant differences ($p < 0.05$). (LA, native α -lactalbumin (α -LA); ULA, ultrasound-pretreated α -LA; Man-LA, α -LA glycated with D-mannose; U-Man-LA, ultrasound-pretreatment α -LA glycated with D-mannose; Glc-LA, α -LA glycated with D-glucose; U-Glc-LA, ultrasound-pretreatment α -LA glycated with D-glucose; All-LA, α -LA glycated with D-allose; U-All-LA, ultrasound-pretreatment α -LA glycated with D-allose).

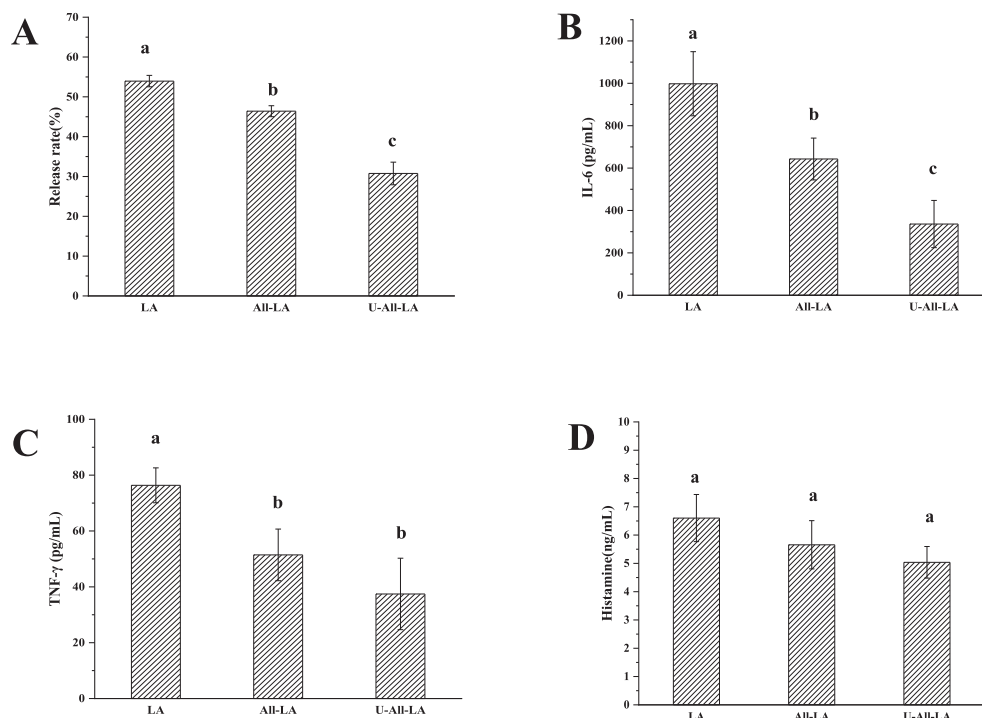


Fig. 3. The changes in the β -HEX (A), IL-6 (B), TNF- γ (C), and histamine (D) of antigen-induced KU812. Letters (a–c) above the bars demonstrate significant differences ($p < 0.05$). (LA, native α -LA; All-LA, α -LA glycated with D-allose; U-All-LA, ultrasound-pretreatment α -LA glycated with D-allose).

compared with that in LA, suggesting that D-allose-modified α -LA has much weaker β -hexosaminidase secretion capacity, especially ultrasound-pretreated glycated α -LA. As shown in Fig. 3B and C, similar

phenomena were found in the release of IL-6 and TNF- γ . In general, the higher the degree of glycation, the better the reduction of allergenicity. D-allose modified α -LA inhibits cell degranulation and reduces potential

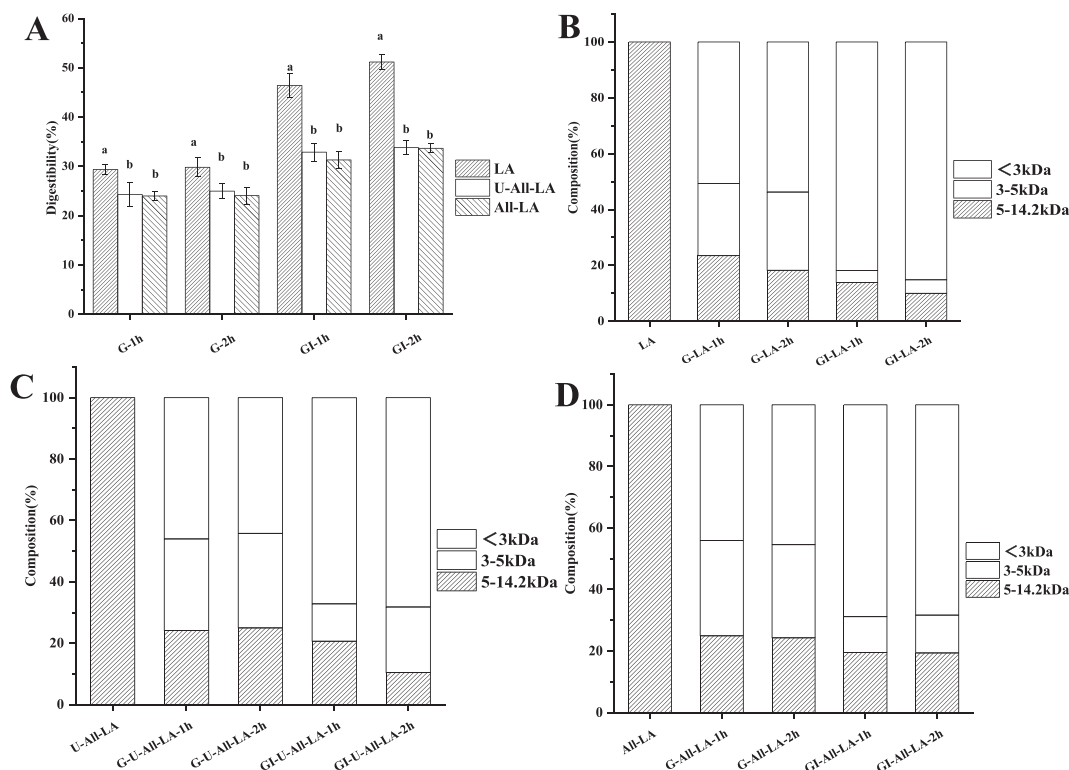


Fig. 4. The values of DH (A), the relative areas of peptide fractions from SE-HPLC chromatograms of α -LA (B), ultrasound-pretreatment α -LA glycated with D-allose (C), α -LA glycated with D-allose (D). Letters a-b in the table indicate significant differences ($p < 0.05$). (LA, native α -LA; All-LA, α -LA glycated with D-allose; U-All-LA, ultrasound-pretreatment α -LA glycated with D-allose; Samples digested with SGDS were named G-LA-1h, G-LA-2h, G-All-LA-1h, G-All-LA-2h, G-U-All-LA-1h, G-U-All-LA-2h, and samples digested by SGDS and SIDS were named GI-LA-1h, GI-LA-2h, GI-All-LA-1h, GI-All-LA-2h, GI-U-All-LA-1h, GI-U-All-LA-2h.)

allergenicity, which was consistent with ELISA results. However, Fig. 3D shows that D-allose modified α -LA has no significant effect on histamine release ($p > 0.05$).

3.5. Digestibility analysis

The DH of G-LA was significantly different from that of G-All-LA and G-U-All-LA, and G-LA-2 h had the highest value ($p < 0.05$) (Fig. 4A), indicating that glycation might lead to a steric hindrance to prevent pepsin from hydrolyzing peptide bonds near modified Lys or Arg (Zhao et al., 2017). Although the structure of the glycated protein is partially unfolded, which appears to be more conducive to the contact of pepsin, the glycated protein is less conducive to gastric digestion than the control protein due to the greater steric hindrance produced by the access of sugar molecules. Similar results were obtained during the intestinal digestion of glycated samples.

The DH values of GI-All-LA and GI-U-All-LA were lower than those of GI-LA. Digestion mainly occurred in the first hour, and the prolongation of digestion time had no significant effect on the DH of glycated α -LA. The glycation sites of α -LA and the hydrolysis sites of trypsin are both Lys and Arg. Therefore, in addition to the loss of tryptic cleavage sites, steric hindrance plays a critical role in the reduced digestibility of these glycated proteins. On the whole, α -LA glycated with D-allose effectively prevented the hydrolysis of pepsin and trypsin, thus reducing its digestibility.

The peptide fragments of LA, All-LA, U-All-LA, G-LA-1 h, G-LA-2 h, G-All-LA-1 h, G-All-LA-2 h, G-U-All-LA-1 h, G-U-All-LA-2 h, GI-LA-1 h, GI-LA-2 h, GI-All-LA-1 h, GI-All-LA-2 h, GI-U-All-LA-1 h, and GI-U-All-LA-2 h were mapped via SE-HPLC. Molecular weight distribution was inferred from the molecular weight calibration curve. The peak areas of the SE-HPLC were quantized into three peptide groups following different retention times. The corresponding molecular weights were < 3 , 3–5, and 5–14.2 kDa, respectively (Fig. 4B, C and D).

After *in vitro* simulated gastric digestion, G-LA-1 h, G-All-LA-1 h and G-U-All-LA-1 h had about 24% of 5–14.2 kDa peptide fractions, indicating that they were partially resistant to pepsin hydrolysis and might still retain their integrity. The < 3000 kDa peptide fractions of G-LA-1 h,

G-All-LA-1 h and G-U-All-LA-1 h were 50.687%, 44.098% and 46.015%, respectively. Although ultrasound-pretreated α -LA was unfolded, it had no significant effect on the digestibility of α -LA glycated with D-allose, which was consistent with the hydrolysis degrees of LA, All-LA and U-All-LA. The molecular weights of GI-LA-2 h, GI-All-LA-2 h and GI-U-All-LA-2 h were mainly < 3 kDa (85.168%, 68.347% and 68.217%, respectively), compared with gastric digestion, which increased by about 31.418%, 22.908% and 23.989%, respectively. The molecular weights of GI-LA-2 h, GI-All-LA-2 h and GI-U-All-LA-2 h were distributed in the range of 5–14.2 kDa (9.974%, 19.366% and 10.412% respectively). The findings showed that the intestinal digestion efficiency of α -LA glycated with D-allose is lower than that of blank α -LA at the same stage. Thus, the steric hindrance and the reduced sensitivity of trypsin to the active site of Lys and Arg result in the relatively high stability to trypsin hydrolysis, which is consistent with the result of the DH value.

3.6. Glycation sites and DSP values

LC-HCD-MS/MS was performed to precisely identify the glycation sites and the peptide sequence of All-LA and U-All-LA to obtain a comprehensive understanding of α -LA glycated with D-allose. In theory, if a peptide was glycosylated by D-allose, the corresponding m/z of the peak with charges of 1, 2, 3 or 4 will appear with a mass shift of 162.0528, 81.0264, 54.0176 or 40.5132 accordingly. Similarly, the m/z shift would double with the occurrence of two glycation sites in a peptide. The results of the mass spectra analysis are shown in Fig. 5(1). The m/z peaks of non-glycated peptides 1–9, 98–103 and 118–123 were 548.7717²⁺, 693.3939⁺ and 396.2096²⁺, whereas the relative m/z peaks of the glycated peptide were 629.7981²⁺, 855.4470⁺ and 477.2366²⁺, respectively. The m/z shift of these peaks was 81.0264, 162.0531 and 81.0270 Da, respectively. This result indicated that these peptides had monoglycated or dual-glycated peptides. Fig. 5E shows the HCD MS/MS spectrum of the monoglycated peptide 118–123 of U-All-LA with the sequence of (Q)WLCEKL (-) at m/z 477.2361²⁺. Lys122 was the glycation site, as proved by the mass difference between b4 and b5 ions (or between y1 and y2), which indicated the combination of Lys residue and D-allose. Similarly, the glycation sites, Lys 5, were

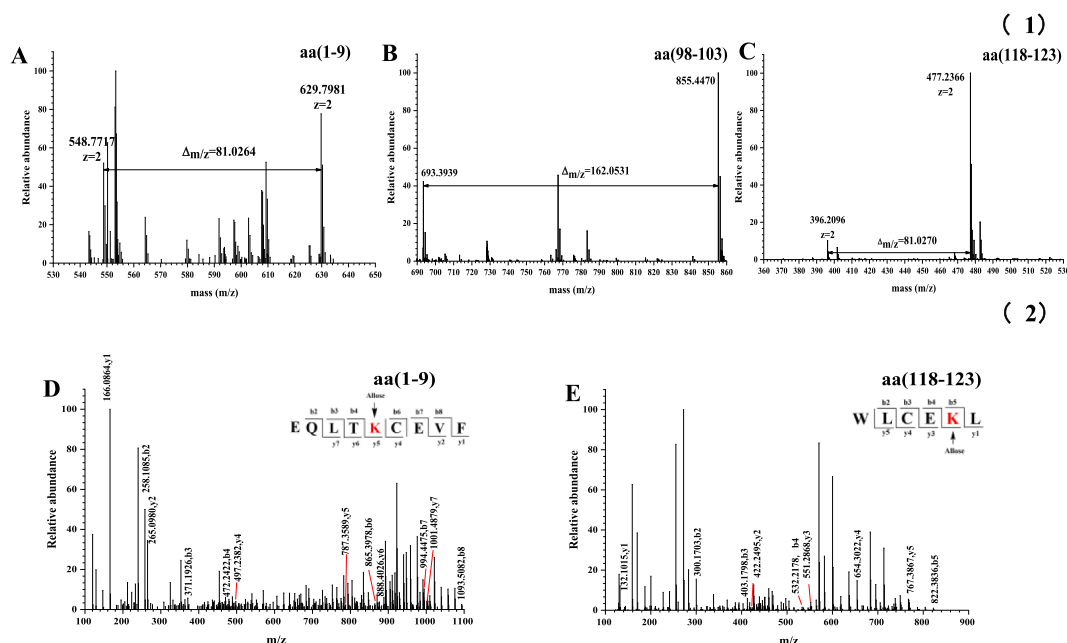


Fig. 5. MS spectra of glycated peptides 1–9 with peak at m/z 548.7717²⁺ (A), glycated peptides 98–103 with peak at m/z 693.3939⁺ (B), and glycated peptides 118–123 with peak at m/z 396.2096²⁺ (C). HCD MS/MS spectra of glycated peptide 1–9 (EQLTKCEVF) with peak at m/z 629.7981²⁺ (D), glycated peptide 118–123 (WLCEKL) with peak at m/z 477.2361²⁺ (E). The sequence of each peptide is shown at the top of the spectrum. Mainly, b and y ions are shown in the HCD MS/MS spectrum.

determined according to the HCD MS/MS spectrum of the peptide 1–9 after matching with the b and y ions (Fig. 5D).

Of note, the potential glycation groups are the *N*-terminal α -amino group and the ϵ -amino groups of Lys and Arg at the early stage of MR (Zhang et al., 2018). As we know, the α -NH₂ group in the *N*-terminus and the ϵ -NH₂ groups of 12 Lys compounds in α -LA, 1 Arg, produce 14 potential glycation sites. Table S1 and Fig. 6A show the glycation sites of U-All-LA. All-LA contains nine glycation sites, including Lys5, Lys13, Lys16, Lys58, Lys62, Lys 79, Lys108, Lys114 and Lys122. We used ultrasound at 130 W/cm² to pretreat α -LA. The glycation sites were 10 of Lys compounds, including Lys5, Lys13, Lys16, Lys58, Lys62, Lys 79, Lys98, Lys108, Lys114 and Lys122. The increase of glycation sites was probably because the structure of α -LA loosened with ultrasound treatment, which accelerated glycation and exposed more reactive sites (Li, Xue, Chen, Ding & Wang, 2014). The results showed that glycation mainly occurred on Lys but not on Arg and *N*-terminal amino acid, which was in accordance with a previous study (Chen, Tu, Wang, Liu,

Liao & Zhang, 2018). In the tertiary structure diagram of the protein (Fig. 6A), K16, K79, K114 and K122 are located on the surface of the globular structure of the protein. These sites easily contact with monosaccharides in the solvent, so glycation reaction easily occurs. The glycation site of galactose-modified α -LA was Lys13/16/58/62/94/108 (Wang, Tu, Ye, Liu, Wang & Hu, 2021), but the glycation site of D-allose-modified α -LA was 10, an increase of four sites, confirming the greater glycation activity of D-allose.

Although the glycation sites and amounts of glycated α -LA are known, further analysis is needed to determine the glycation degree of each peptide. The DSP values of each glycated peptide of All-LA and U-All-LA are shown in Table S1. Six glycation sites had high DSP values of up to 77.544%, namely, Lys58, Lys62, Lys79, Lys108, Lys114 and Lys122. The results showed that the DSP value of glycated peptides was increased by ultrasound treatment compared with glycation alone, which might be because the protein structure unfolded after ultrasound pretreatment, thus promoting its glycation, as previously reported

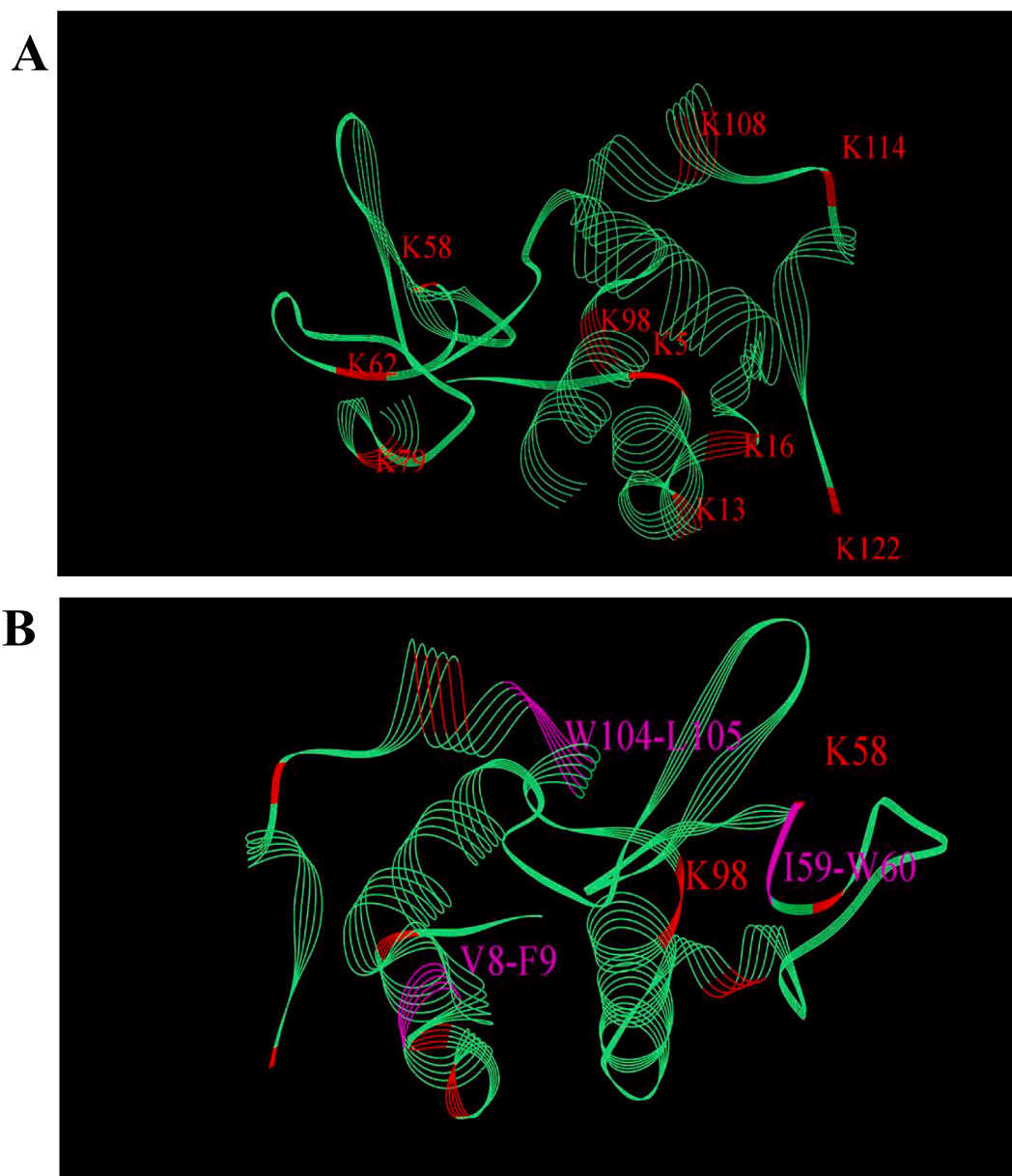


Fig. 6. The framework of α -LA is shown in fluorescent green, glycated Lys is shown in red (A), the possible hydrolysis peptide bonds V8–F9, I59–W60, and W104–L105 of pepsin are shown in fuchsia (B). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

(Zhang et al., 2014).

3.7. Mechanism underlying the effect of D-allose in reducing allergenicity and digestibility of ultrasound-pretreated α -LA

In this study, D-allose significantly reduced the allergenicity and digestibility of ultrasound-pretreated α -LA by masking linear allergic epitopes and obstructing digestion sites of digestive enzymes. The main IgG linear allergic epitopes of α -LA are aa7-18, aa51-61 and aa89-108. Five glycation sites of U-All-LA were located on the main IgG linear allergic epitopes aa7-18 (K13, K16), aa51-61 (K58) and aa89-108 (K98, K108). The main IgE linear allergic epitopes of α -LA include aa1-16, aa13-26, aa47-58 and aa93-102 (Järvinen, Chatchatee, Bardina, Beyer & Sampson, 2001). Five glycation sites of U-All-LA were located on the main IgE linear allergic epitopes aa 1–16 (K5), aa 13–26 (K13, K16), aa 47–58 (K58) and aa (K98). The glycation reaction between Lys and D-allose results in the modification of linear epitopes with a remarkable reduction of allergenicity. In this study, we applied ultrasound to pretreat α -LA, and one additional glycation site (K98) was found. The K98 site is located on IgG and IgE linear allergy epitopes. After ultrasound pretreatment, the DSP value of each glycation site increased to different degrees (Table S1). Thus, α -LA underwent a conformational change brought about by the expose of the reaction region after ultrasound treatment, which improved the glycation extent of α -LA, eventually reducing its potential allergenicity. Although the epitope area of α -LA does not include K62, K79, K114 and K122, it can also alter the aa sequence of α -LA, which may be related to the IgG/IgE-binding rate.

Pepsin prefers to hydrolyze peptide bonds between aromatic or hydrophobic amino acids with either carboxylic or amino terminations. Proteins are hydrolyzed by pepsin into peptides of varying sizes. As shown in Fig. 6B, peptide bonds in V8–F9, I59–W60 and W104–L105 might be cleaved by pepsin. When the peptide F9–I59 was hydrolyzed by pepsin, the proximity of the K58 glycation site to the pepsin cleavage site creates steric hindrance that hinders the recognition of pepsin, so that the digestibility of All-LA and U-All-LA is lower than that of the control protein. This result is similar to the degree of DH. The peptide W60–W104 was hydrolyzed by pepsin, the glycation site of K98 is far away from the cleavage site of pepsin, which cannot block the hydrolysis of pepsin, so the digestibility of All-LA and U-All-LA is not much different.

Lys and Arg are the major glycation sites, but they are also cleavage sites for trypsin. After the protein is digested in the gastrointestinal tract, it is broken down into smaller peptides. However, D-allose partially blocks trypsin recognition. All-LA and U-All-LA still retain a relatively intact peptide segment compared with LA, showing resistance to trypsin. Thus, α -LA glycosylated with D-allose could reduce digestibility.

These results are attributable to the proximity of the D-allose glycation site to the linear allergic epitope, which blocks the recognition of the linear allergic epitope by IgG/IgE antibodies. The degranulation experiment of KU812 cells also showed that D-allose modified α -LA significantly reduced cell degranulation, thereby reducing the allergenicity of D-allose glycosylated α -LA. Meanwhile, the access of monosaccharides also affects the cleavage of peptide bonds near glycation sites by digestive enzymes, thereby reducing the digestibility of α -LA glycosylated with D-allose.

4. Conclusions

Glycation can reduce the allergenicity of α -LA by masking the linear allergic epitopes and destroying the conformational allergic epitopes of α -LA. Ultrasound pretreatment promotes the reduction of allergenicity of α -LA glycosylated with D-allose. Glycation can also reduce digestibility by affecting the cleavage of peptide bonds near glycation sites by digestive enzymes. Therefore, combining ultrasound pretreatment with glycation is a potential method to reduce the allergenicity of α -LA in food processing. This study also provides some valuable information for the use

of rare sugars in food processing. However, *in vivo* studies, such as double-blind placebo-controlled trials, need to be considered to further confirm the reduced allergenicity of glycosylated α -LA.

Declaration of Competing Interest

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

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.foodchem.2021.131616>.

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Table S1. The summary of the glycosylated sites and the DSP values of glycosylated α -LA.Letters a-k in the table indicate significant differences ($p < 0.05$)

native peptide	peptide location	Δ ppm	sequence	glycosylated peptide	DSP (%)	glycosylated site
All-LA				All-LA		
548.7718 ²⁺	1-9	1.820	(-) EQLTKCEVF (R)	629.7972 ²⁺	59.620±0.076 ⁱ	K5
447.2584 ²⁺	12-19	-0.054	(E)LKDLKGYG(G)	609.3110 ²⁺	38.972±0.021 ^j	K13/K16
655.3044 ⁴⁺	53-74	3.170	(L)FQINNKIWCKN DQDPHSSNICN(I)	736.3287 ⁴⁺	77.544±2.758 ^g	K58/K62
750.3300 ²⁺	71-83	1.490	(S)NICNISCDDKFLD D(D)	831.3553 ²⁺	84.511±2.858 ^f	K79
514.7714 ²⁺	104-112	0.900	(Y)WLAHKALCS(E)	593.7973 ²⁺	88.318±0.021 ^{c,d}	K108
466.2480 ²⁺	113-119	0.392	(S)EKLDQWL(C)	547.2742 ²⁺	82.834±0.540 ^f	K114
396.2098 ²⁺	118-123	0.380	(Q)WLCEKL (-)	477.2361 ²⁺	85.814±0.242 ^{e,d}	K122
U-All-LA				U-All-LA		
548.7717 ²⁺	1-9	1.640	(-) EQLTKCEVF (R)	629.7972 ²⁺	61.383±1.161 ⁱ	K5
447.2584 ²⁺	12-19	0.265	(E)LKDLKGYG(G)	609.3110 ²⁺	32.137±0.033 ^k	K13/K16
655.3043 ⁴⁺	53-74	3.020	(L)FQINNKIWCKN DQDPHSSNICN(I)	736.3287 ⁴⁺	91.124±2.474 ^{b,c}	K58/K62
598.7708 ²⁺	78-87	2.160	(C)DKFLDDDLTD(D)	679.7959 ²⁺	96.733±0.460 ^a	K79
693.3939 ⁺	98-103	1.300	(D)KVGINY(W)	855.4458 ⁺	72.000±0.025 ^h	K98
514.7714 ²⁺	104-112	0.900	(Y)WLAHKALCS(E)	595.7973 ²⁺	89.851±0.034 ^c	K108
409.7058 ²⁺	113-118	0.035	(S)EKLDQWL(L)	490.7322 ²⁺	93.574±0.085 ^b	K114
396.2096 ²⁺	118-123	-0.125	(Q) WLCEKL (-)	477.2361 ²⁺	90.298±0.466 ^c	K122