

Mechanism of the Reduced IgG/IgE Binding Abilities of Glycated β -Lactoglobulin and Its Digests through High-Resolution Mass Spectrometry

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ABSTRACT: Glycation between proteins and reducing sugars is the common chemical modification in food protein, and many studies have focused on the allergenicity of the glycated protein. However, a systemic study on the allergenicity change of its digests is lacking. In this work, we explored the change rule of the digestibility and allergenicity of glycated β -Lg during *in vitro* gastrointestinal digestion and interpreted the mechanism using high-resolution mass spectrometry. Glycation with arabinose increased the resistance of β -Lg to digestive enzyme, with a low hydrolysis value. Indirect competitive ELISA showed that the IgG/IgE binding rates of β -Lg were reduced after glycation and further reduced after digestion, in comparison with the digests of unglycated β -Lg. There are two reasons for this phenomenon. On the one hand, 11 glycated sites were determined in the lowest allergenicity arabinose- β -Lg conjugation (Ara- β -Lg), which was distributed in the IgG and IgE linear allergic epitopes of β -Lg. On the other hand, glycation masking linear allergic epitopes had a more significant effect on reducing allergenicity in comparison to digestive enzyme hydrolysis. These results indicated that the allergenicity of Ara- β -Lg in the human body might be lower than that of unglycated β -Lg.

KEYWORDS: β -lactoglobulin, glycation, digestibility, IgG/IgE binding abilities, high-resolution mass spectrometry

INTRODUCTION

Cow milk, owing to its abundant reserves in protein and other nutrients with high bioavailability, is a source of protein supplement in daily life and a favorable substitute for breast milk.¹ However, cow milk is one of the eight major food allergens triggering allergy symptoms, and the incidence of milk allergy is 2–3% in infants below 1 year old and less than 0.5% in adults.^{2,3} Many developed countries, such as the USA and Canada, apply a strict identification of allergen components in food products, including milk and other dairy products.⁴ β -Lactoglobulin (β -Lg), with a stable barrel structure formed by eight antiparallel β -sheets, is the main allergen in milk, which can lead to an allergic reaction.^{5,6} The allergenic epitopes of β -Lg include linear and conformational allergic epitopes. Therefore, attenuation or even elimination of the β -Lg-mediated allergic reaction through destroying the linear or/and conformational allergic epitopes is important to consolidate and strengthen the wide application of cow milk in food industries.

Many methods, such as heating, high-intensity ultrasound treatment, enzymatic hydrolysis, phosphorylation, and glycation, sought to reduce the allergenicity of protein allergens.^{7–11} Among these methods, glycation normally occurs at the initial stage of the Maillard reaction involving the covalent binding between proteins and reducing sugars and is a prospective method for the creation of a hypoallergenic protein by masking linear allergic epitopes. Several studies have investigated the effect of glycation on protein allergens and confirmed that glycation could reduce their IgG/IgE binding abilities.^{12,13} However, glycation with glucose, ribose, and maltose can boost

the IgE binding abilities of scallop tropomyosin.¹⁴ Therefore, choosing a suitable reducing sugar must be considered to reduce the allergenicity of protein allergens.

Arabinose, a pentose sugar, in comparison with hexose, disaccharide, or oligosaccharides, is prone to glycation. Moreover, arabinose does not react as violently as ribose, which easily produces advanced glycation end products (AGEs).^{15–17} AGEs can strengthen the allergenicity of protein allergens.¹⁸ In addition, arabinose as a reagent of glycation can expand its application in the food industry. Therefore, arabinose is a good choice for glycation with β -Lg because of its potential to improve the degree of glycation under certain conditions without generating AGEs.¹⁹

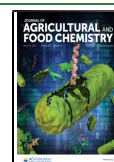
Protein allergens must be digested by the gastrointestinal tract before being absorbed by the human body. An *in vitro* simulated gastrointestinal digestion model is a swift and effective method to replace *in vivo* digestion.²⁰ The digestion degree is greatly affected by factors such as digestion time, pH, the ratio of digestive enzymes to protein allergens, processing methods of the allergen, and the food matrix.^{21–23} Previous research showed that glycation affects the digestion degree of proteins.²⁴ The digestion degree can change the structure and

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molecular weight of proteins, affecting their allergenicity. Corzo-Martinez et al.²⁵ showed that glycation with galactose impaired β -Lg proteolysis, and the hydrolysates of glycated β -Lg could be recognized by more IgG/IgE in comparison with the hydrolysates of unglycated β -Lg. Nakamura et al.¹⁴ indicated that the digestibility of ribose–squid tropomyosin conjugation was decreased, but its digests were lower in allergenicity in comparison to the digests of native squid tropomyosin. Moreover, applying polysaccharides as glycation agents does not significantly affect the digestibility of β -Lg, and the allergenicity between digests of glycated β -Lg and the control showed no significant difference.²⁵ A possible explanation for these inconsistent conclusions could be that, given the complexity of the glycation and digestion, the allergenicity also depends on selecting a suitable reducing sugar. No studies focus on the allergenicity of arabinose- β -Lg conjugation during *in vitro* gastrointestinal digestion. Furthermore, the mechanism of the allergenicity of arabinose- β -Lg conjugation and its digests needs further investigation on a molecular level. In recent years, liquid chromatography–high-resolution mass spectrometry (HPLC-MS) has been used to characterize glycated proteins for an analysis of the glycated sites and the glycation degree.²⁶ Thus, it is an accurate choice to analyze the mechanism of the IgG/IgE binding abilities of glycated β -Lg during *in vitro* gastrointestinal digestion.

In this work, the effect of glycation on the digestibility and allergenicity of β -Lg during *in vitro* gastrointestinal digestion was studied. The digestibility was investigated by using tricine SDS-PAGE, HPLC, and the degree of hydrolysis (DH). The allergenicity of glycated β -Lg and its digests were studied by indirect competitive ELISA. The glycated sites of β -Lg were characterized with an LC Orbitrap MS/MS instrument, and the mechanisms of IgG/IgE binding abilities of both glycated β -Lg and its digests were revealed. Our work will provide an alternative reducing sugar for decreasing the allergenicity of β -Lg.

MATERIALS AND METHODS

Chemicals and Materials. Bovine β -Lg (L3908, $\geq 90\%$, including peptides A and B), pepsin (P7000, 284 units/mg, from porcine gastric mucosa), pancreatin (V900486, including trypsin, amylase, and lipase, from porcine pancreas), disodium edetate dihydrate (E4884, $\geq 99\%$), and 3,3',5,5'-tetramethylbenzidine (87748, $\geq 98\%$) were purchased from Sigma-Aldrich (St. Louis, MO, USA). D-(+)-Arabinose (A115318, $\geq 98\%$) was purchased from Aladdin (Shanghai, China). Rabbit anti- β -Lg IgG (bs-0814R) and goat antirabbit IgG-horseradish peroxidase (HRP) conjugate (bs-0295G) were obtained from Bioss (Beijing, China). The serum of cow's milk allergy (CMA) patients was purchased from Plasma Lab International (Everett, WA, USA). Goat antihuman IgE-HRP conjugate (A9667) was obtained from Immunology Consultants Laboratory (Shanghai, China). The milk protein specific IgE levels of CMA patient serum were 20.886, 23.8, 42, and 91 kU/L, separately (Table S1).

Preparation of Glycated β -Lg. Dry-state glycation was used to prepare arabinose- β -Lg conjugation. β -Lg was mixed with arabinose in a ratio of 1/1 (w/w) and a final concentration of 10 mg/mL. After 48 h of freeze-drying, the mixture was incubated for 1 h at 55 °C with 74.41% relative humidity (saturated NaCl solution). All samples were stored at -20 °C until use. β -Lg without any treatment was denoted as N- β -Lg (blank control), that treated by freeze-drying and the same glycation condition was denoted H- β -Lg, and β -Lg glycated with arabinose was denoted Ara- β -Lg. Ara- β -Lg was filtered with centrifugal filters (3 kDa cutoff) to eliminate unreacted arabinose.

Gastrointestinal Digestion *In Vitro*. The stimulated gastrointestinal digestive fluid was created by following the method

described by Moreno et al.²⁷ with some modifications. Pepsin was used to prepare simulated gastric digestive fluid (SGF) with a pepsin/substrate ratio of 1/20 (w/w). After digestion in SGF for 1 h, the pH was adjusted to 7.0, and the samples were digested in simulated intestinal digestive fluid (SIF), with a pancreatin/substrate ratio of 1/25 (w/w). Pancreatin was inactivated by heating at 100 °C for 2 min. All samples were stored at -20 °C and used within a week. N- β -Lg, H- β -Lg, and Ara- β -Lg digested by SGF are denoted N- β -Lg-G, H- β -Lg-G, and Ara- β -Lg-G, respectively; N- β -Lg, H- β -Lg, and Ara- β -Lg digested by both SGF and SIF are denoted N- β -Lg-GI, H- β -Lg-GI, and Ara- β -Lg-GI, respectively.

Tricine SDS-PAGE Analysis. The preparation was carried out according to the method of Haider et al.²⁸ with some modifications. For undigested samples, 12% acrylamide separating gel and 5% acrylamide stacking gel were used. An initial voltage of 80 V was used until the indicated front reached the upper edge of the separating gel, and the voltage was then adjusted to 100 V. A 16.5% amount of separating gel, 10% of spacer gel, and 4% of stacking gel were prepared for digested samples with an initial voltage of 30 V for 3 h and 120 V for 2 h. Thereafter, all gels were bathed in Coomassie brilliant blue R-250 for 20 min and then decolorized until the background was clear.

HPLC Analysis. The molecular weight distribution of these samples was analyzed by high-performance liquid chromatography (HPLC, D-2000, Hitachi, Japan) with a chromatographic column (XBridge BEH 125 Å SEC 7.8 mm $\times$ 300 mm, 3.5 μ m). The chromatographic conditions were as follows: the mobile phase was 60% ultrapure water (containing 0.1% trifluoroacetic acid) and 40% acetonitrile (isocratic elution), the flow velocity was 0.4 mL/min, the injection volume was 10 μ L, the detector wavelength was 220 nm, and the detection time was 60 min. The molecular weight calibration curve was drawn according to the average elution volume of standards (aprotinin, bacitracin, oxidized glutathione, and L-hydroxyproline).

Degree of Hydrolysis Analysis. The degree of hydrolysis (DH) of N- β -Lg-G, H- β -Lg-G, Ara- β -Lg-G, N- β -Lg-GI, H- β -Lg-GI, and Ara- β -Lg-GI was analyzed according to the method described by Nielsen et al.²⁹ Disodium tetraborate decahydrate (9.525 g) and sodium dodecyl sulfate (SDS, 0.25 g) were dissolved in a 200 mL beaker containing 150 mL of distilled water. *o*-Phthalaldehyde (OPA, 200 mg) was dissolved in 5.0 mL of ethanol (95%), and then 0.264 g of DTT (99%) was added and the volume finally fixed to 250 mL. The OPA solution was protected from light. Then, 100 μ L samples were mixed with 2 mL of OPA solution and reacted for 2 min. The absorption value was obtained at 340 nm. Serine was used to prepare a calibration curve. The DH value was calculated as

$$\text{DH}(\%) = \frac{\text{OD}_t - \text{OD}_0}{\text{OD}_{\text{max}} - \text{OD}_0} \times 100\%$$

where OD_t refers to the absorbance of digested samples, OD_0 is the absorbance of native β -Lg, and OD_{max} is the absorbance of a fully digested β -Lg, which was determined from complete hydrolysis of β -Lg using 6 M HCl for 3 h at 100 °C.

Indirect Competitive ELISA. The IgG/IgE-binding rates of N- β -Lg, H- β -Lg, Ara- β -Lg, N- β -Lg-G, H- β -Lg-G, Ara- β -Lg-G, N- β -Lg-GI, H- β -Lg-GI, and Ara- β -Lg-GI were measured via indirect competitive ELISA with the serum of rabbit and CAM patients, respectively. First, a 96-well microplate was coated with 2 μ g/mL of N- β -Lg (100 μ L/well) for 1 h at 37 °C and then washed three times with PBST. Second, the microplate was blocked with 1% fish gelatin solution (250 μ L/well) for 1 h at 37 °C and then washed three times with PBST. Then, 50 μ L of the diluted samples and the serum of rabbit (50 μ L, diluted to 1:320000) or CAM patients (50 μ L, diluted to 1:30) was injected into the plate for 1 h. After washing with PBST five times, the plate was injected with goat antirabbit IgG-HRP conjugate (100 μ L, diluted to 1:2500) or goat antihuman IgE-HRP conjugate (100 μ L, diluted to 1:700) and then incubated for 1 h. Finally, 100 μ L of tetramethylbenzidine (TMB) was added after washing with PBST five times, and the absorbance was evaluated at 450 nm after terminating

the reaction by adding 100 μ L of sulfuric acid solution (12 M). The binding rates were calculated as

$$\text{binding rate(\%)} = \frac{B - B_s}{B - B_0} \times 100\%$$

wherein B indicates the OD value of a well without a sample, B_s is the OD value of a well with a sample, and B_0 is the OD value of a well without rabbit serum or CAM patient serum.

HPLC HCD MS/MS Analysis. An HPLC (Ultimate 3000RSLCnano)-orbitrap fusion mass spectrometer (Thermo Fisher Scientific, USA) was used to investigate the glycosylated sites and the average degree of substitution per peptide molecule (DSP) values of Ara- β -Lg according to Yang et al.⁹ Ara- β -Lg was hydrolyzed by pepsin (diluted in HCl solution, pH was 2.2) at a ratio of 1/1 (w/w) and incubated at 4 $^{\circ}$ C for 10 min. Then, the hydrolyzed Ara- β -Lg was separated by HPLC at a nanoliter flow rate and finally analyzed by MS. The detection mode of MS was positive ion.

RESULTS AND DISCUSSION

Digestibility Analysis. Tricine SDS-PAGE Profiles. Glycation can raise the molecular weight, can alter the structure of the protein, and may cause the aggregation of protein molecules. During digestion, proteins are hydrolyzed into peptide fractions, and glycation can affect the hydrolysis of the digestive enzyme.³⁰ The SDS-PAGE profiles are shown in Figure 1. Similarly to N- β -Lg, H- β -Lg had an obvious band between 14.4 and 20.1 kDa (Figure 1A), suggesting that heat treatment did not change the molecular weight of β -Lg under the experimental conditions. However, the band of Ara- β -Lg was obviously moved up, which indicated that covalent cross-linking occurred between β -Lg and arabinose. Corzo-Martinez et al.³¹ also reported that a protein reacted with galactose/tagatose to form conjugates by an analysis with SDS-PAGE. As shown in Figure 1A, β -Lg aggregated and the degree of aggregation was enhanced after glycation modification. A similar phenomenon had also been discovered by Chen et al.³²

Figure 1B shows the tricine SDS-PAGE profile of N- β -Lg-G, H- β -Lg-G, and Ara- β -Lg-G. In comparison with Figure 1A, the number of electrophoretic bands increased (two changed to five bands), mainly distributed within 3.3–5.8 kDa (bands 1 and 2) and 14.4–20.1 kDa (bands 3–5). Pepsin mainly hydrolyzes peptide bonds between hydrophobic and aromatic amino acid residues.³³ As shown in Figure 5A, peptide bonds W₁₉–Y₂₀, V₄₁–Y₄₂, V₈₁–F₈₂, and L₁₀₄–F₁₀₅ in β -Lg were possible hydrolysis sites of pepsin. When pepsin hydrolyzed peptide bonds V₄₁–Y₄₂ and L₁₀₄–F₁₀₅, the molecular weights of peptide fractions L₁–V₄₁ and Y₄₂–L₁₀₄/F₁₀₅–I₁₆₂ were $\sim$ 4 and $\sim$ 6 kDa, respectively, which was consistent with two bands (bands 1 and 2) distributed in the range 3.3–5.8 kDa. Bands 3 and 4 emerged possibly because of the hydrolysis of W₁₉–Y₂₀ or V₄₁–Y₄₂. Obviously, the undigested intact β -Lg was still observed in the SDS PAGE profile, with a band appearing near 20.1 kDa (band 5). Therefore, β -Lg was partially resistant to pepsin hydrolysis, which was also observed by Corzo-Martinez et al.²⁵

Trypsin mainly hydrolyzes peptide bonds at the carboxyl side of basic amino acids, and β -Lg has a total of 18 arginine and lysine (K8, K14, R40, K47, K60, K69, K70, K75, K77, K83, K91, K100, K101, R124, K135, K138, K141, and R148). Further hydrolysis by trypsin might convert N- β -Lg-GI and H- β -Lg-GI into smaller peptide fractions or even single amino acids; thus, no bands were found. However, a wide band distribution in the range 5.8–14.4 kDa was discovered in Ara- β -Lg-GI (Figure 1C). These findings implied that glycosylated β -

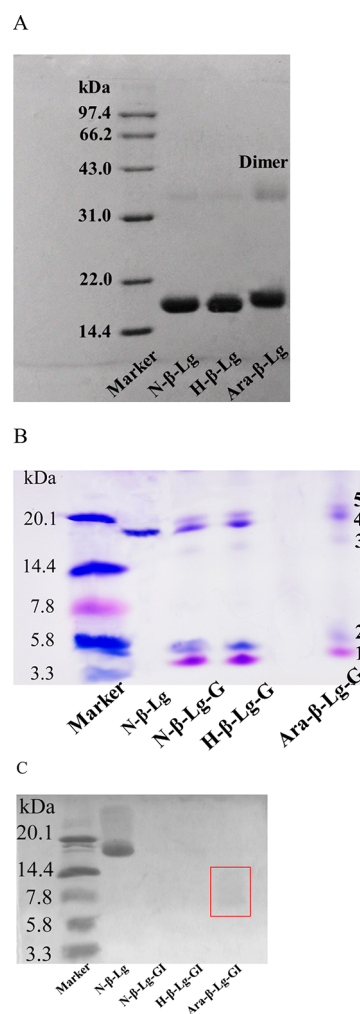


Figure 1. SDS-PAGE profiles of N- β -Lg, H- β -Lg, and Ara- β -Lg (A), tricine SDS PAGE profile of N- β -Lg-G, H- β -Lg-G, and Ara- β -Lg-G (B), and tricine SDS PAGE profile of N- β -Lg-GI, H- β -Lg-GI, and Ara- β -Lg-GI (C). N- β -Lg, H- β -Lg, and Ara- β -Lg indicate β -Lg without any treatment, β -Lg treated by freeze-drying and the same glycation condition, and β -Lg glycosylated with arabinose, respectively. N- β -Lg-G, H- β -Lg-G, and Ara- β -Lg-G indicate samples digested by simulated gastric digestive fluid. N- β -Lg-GI, H- β -Lg-GI, and Ara- β -Lg-GI indicate samples digested by simulated gastric and intestinal digestive fluids.

Lg resisted the trypsin digestion due to the covalent cross-linking of lysine/arginine with arabinose. The absence of bands for N- β -Lg-GI and H- β -Lg-GI might be due to the predominance of ultralow-molecular-weight peptides (<3.3 kDa), which could easily run out of the gel. This conjecture needed further confirmation. Therefore, the molecular weight distribution of all samples was analyzed by HPLC.

Molecular Weight Distribution. Ultralow-molecular-weight peptides were easy to remove from the tricine SDS-PAGE gel. Therefore, identifying the molecular weight distribution of all peptide fractions during *in vitro* gastrointestinal digestion is essential. The peak areas of four peptide groups were quantified corresponding to different regions of the chromatogram (F1–F4) relative to all peaks (Sum) (Table 1A). The corresponding molecular weights of F1–F4 were 5–18.3, 3–5, 1–3, and <1 kDa, respectively. N- β -Lg, H- β -Lg, and Ara- β -Lg had only one peak, with an elution time of $\sim$ 14.30 min (Figure 2). These findings indicated that the glycation conditions did

Table 1. Relative Areas of Peptide Fractions from HPLC Chromatograms (A),^a Free Amino Groups and DH Values during *In Vitro* Simulated Digestion (B),^b and Glycated Peptides, Glycated Sites, and DSP Values of β -Lg Glycated with Arabinose (C)^c

Section A						
	content (%)					
	A1	B1	C1	A2	B2	C2
F1/sum	16.83	19.52	18.33	2.03	1.71	4.04
F2/sum	28.56	29.22	29.06			10.32
F3/sum	9.88	8.64	9.23	14.84	15.48	12.15
F4/sum	44.73	42.62	43.38	83.13	82.81	73.49

Section B		
sample	free amino groups (mg/mL)	DH (%)
N- β -Lg	0.104 $\pm$ 0.000 ^a	
H- β -Lg	0.099 $\pm$ 0.003 ^a	
Ara- β -Lg	0.045 $\pm$ 0.002 ^b	
N- β -Lg-G	0.140 $\pm$ 0.000 ^a	23.95 $\pm$ 0.005 ^b
H- β -Lg-G	0.143 $\pm$ 0.002 ^a	28.07 $\pm$ 0.01 ^a
Ara- β -Lg-G	0.089 $\pm$ 0.000 ^b	21.15 $\pm$ 0.002 ^c
N- β -Lg-GI	0.201 $\pm$ 0.004 ^a	62.36 $\pm$ 0.027 ^a
H- β -Lg-GI	0.207 $\pm$ 0.004 ^a	67.10 $\pm$ 0.024 ^a
Ara- β -Lg-GI	0.126 $\pm$ 0.000 ^b	38.10 $\pm$ 0.040 ^b

Section C						
peptide location	m/z	ppm	sequence	glycated peptide m/z	glycated site	DSP (%)
2-11	553.3013 ²⁺	2.85	(L)IVTQTMKGLD(I)	619.3209 ²⁺	K8	26.38 $\pm$ 0.002 ^d
45-53	493.2453 ²⁺	-0.146	(E)ELKPTPEGD(L)	559.2662 ²⁺	K47	13.78 $\pm$ 0.008 ^e
58-64	437.7245 ²⁺	-0.022	(L)LQKWENG(E)	503.7453 ²⁺	K60	27.49 $\pm$ 0.004 ^d
61-73	745.3795 ²⁺	3.320	(K)WENGECAQKKIAE(K)	877.4177 ²⁺	K69, K70	34.70 $\pm$ 0.12 ^c
99-104	414.2551 ²⁺	1.73	(D)YKKYLL(F)	546.2969 ²⁺	K100, K101	43.02 $\pm$ 0.002 ^b
123-129	408.2167 ²⁺	0.448	(L)VRTPEVD(D)	475.2343 ²⁺	R124	2.78 $\pm$ 0.005 ^f
134-142	525.3031 ²⁺	-0.046	(L)EKFDKALKA(L)	723.8530 ²⁺	K135, K138, K141	48.87 $\pm$ 0.13 ^a

^aA1, B1, C1, A2, B2, and C2 are N- β -Lg-G, H- β -Lg-G, Ara- β -Lg-G, N- β -Lg-GI, H- β -Lg-GI, and Ara- β -Lg-GI, respectively. F1–F4 are 5–18.3, 3–5, 1–3, and <1 kDa, respectively. ^bData are mean $\pm$ standard deviation; different letters (a–c) mean significant differences ($p > 0.05$). ^cData are mean $\pm$ standard deviation; different letters (a–f) mean significant differences ($p > 0.05$).

not cause β -Lg degradation, which was consistent with the result in Figure 1A.

Overall, N- β -Lg-G, H- β -Lg-G, and Ara- β -Lg-G had 16.8–19.5% of 5–18.3 kDa peptide fractions (F1/Sum, Table 1A), indicating that they were partially resistant to pepsin hydrolysis, which was also proven by tricine SDS-PAGE profiles (Figure 1B). The F2/Sum contents of N- β -Lg-G, H- β -Lg-G, and Ara- β -Lg-G were 28.56%, 29.22%, and 29.06%, respectively. The corresponding molecular weight of F2/Sum was 3–5 kDa, which was consistent with bands 1 and 2 discovered at 3.3–5.8 kDa (Figure 1B). Meanwhile, F4/Sum had a high content of 42–45%, and it was not found in Figure 1B because of its low molecular weight (<1 kDa). However, comparing the hydrolysis degrees (DHs) of N- β -Lg-G, H- β -Lg-G, and Ara- β -Lg-G was difficult.

After gastrointestinal digestion, the samples were further hydrolyzed, and N- β -Lg-GI and H- β -Lg-GI had high percentages of 1–3 kDa (~15%) and <1 kDa (~83%). Meanwhile, Ara- β -Lg-GI had a high percentages of 5–18.3 kDa (~4%) and 3–5 kDa (~10%). Interestingly, peptide fractions of 3–5 kDa were not discovered in N- β -Lg-GI and H- β -Lg-GI (Table 1A). The findings showed that β -Lg glycated with arabinose could mask lysine/arginine, resulting in resistance to the trypsin digestion, which followed the result of Figure 1C that only one wide band distribution at 5.8–14.4 kDa was detected in Ara- β -Lg-GI. Therefore, we further determined the free amino group to study the DH values of these digests.

Free Amino Groups and Degree of Hydrolysis. The free amino group contents increased as a result of hydrolyzing β -Lg to peptide fractions. As shown in Table 1B, the free amino group content of Ara- β -Lg was 0.045 mg/mL, which was lower than those of N- β -Lg (0.104 mg/mL) and H- β -Lg-G (0.099 mg/mL), indicating that β -Lg was glycated with arabinose ($p < 0.05$). These results were consistent with Figure 1A. Hu et al.³⁴ indicated that the content of free amino groups of ovalbumin decreased with an increase of glycation.

After SGF digestion, the free amino group contents of N- β -Lg-G, H- β -Lg-G, and Ara- β -Lg-G were 0.140, 0.143, and 0.089 mg/mL, respectively, and Ara- β -Lg-G had the lowest content ($p < 0.05$). Furthermore, the DH values of N- β -Lg-G, H- β -Lg-G, and Ara- β -Lg-G were 23.95%, 28.07%, and 21.15% ($p < 0.05$), respectively, indicating that glycation might lead to a steric hindrance to prevent pepsin from hydrolyzing peptide bonds near modified lysine or arginine.³⁵ Zhao et al.²⁴ also proved that β -Lg glycated with α -dicarbonyl compounds reduced the digestibility of the *in vitro* gastric stage. The DH value of Ara- β -Lg-GI (38.10%) was lower than those of N- β -Lg-GI (62.36%) and H- β -Lg-GI (67.1%) ($p < 0.05$), and the free amino group content of Ara- β -Lg-GI was 0.126 mg/mL. The glycation sites of β -Lg and the hydrolysis sites of trypsin are both lysine and arginine, and the DH value of Ara- β -Lg-GI was the lowest, possibly because glycation prevented trypsin from hydrolyzing Ara- β -Lg. All in all, β -Lg glycated with arabinose effectively prevented the hydrolysis of pepsin and trypsin, thus reducing its digestibility.

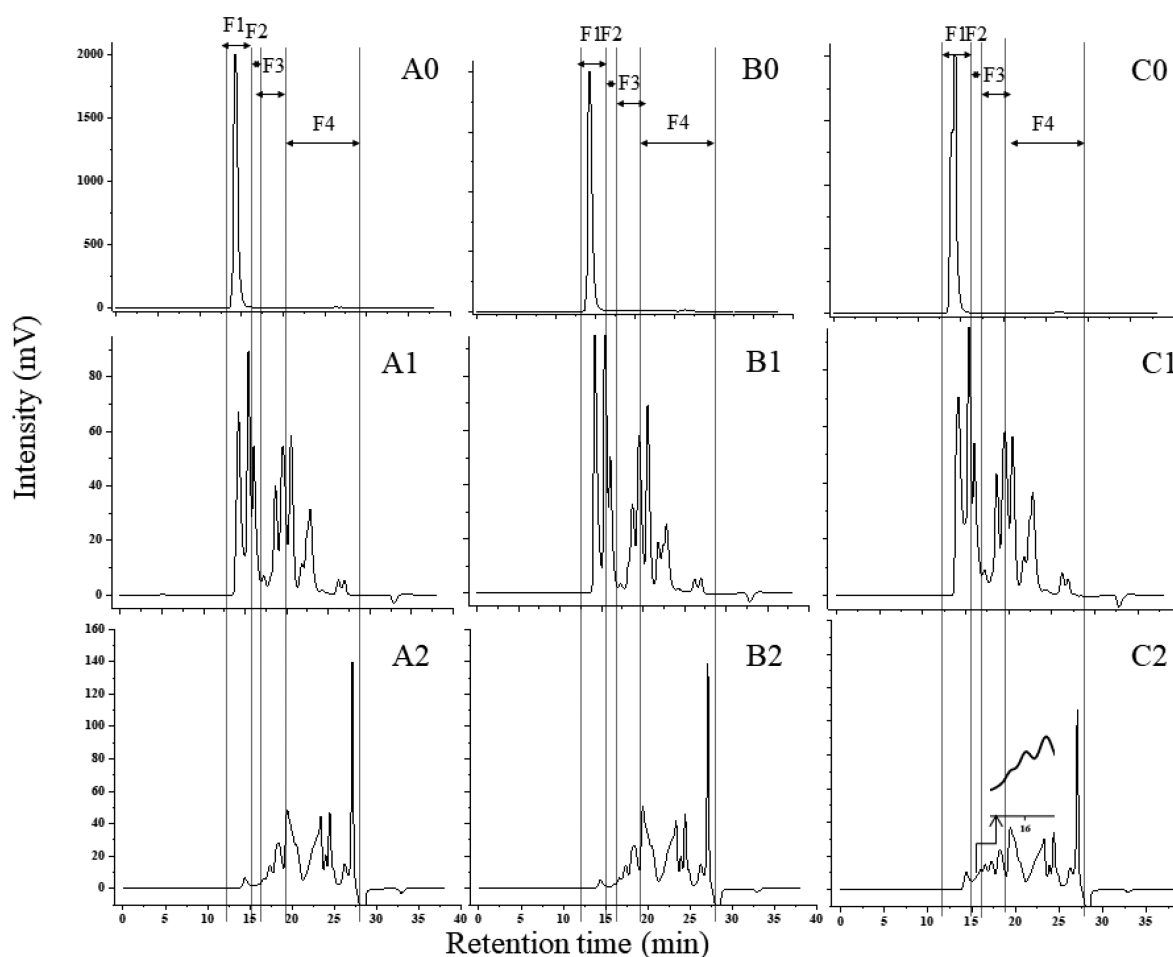


Figure 2. Effect of glycation on the molecular weight distribution of β -Lg during *in vitro* gastrointestinal digestion: N- β -Lg (A0), H- β -Lg (B0), Ara- β -Lg (C0), N- β -Lg-G (A1), H- β -Lg-G (B1), Ara- β -Lg-G (C1), N- β -Lg-GI (A2), H- β -Lg-GI (B2), and Ara- β -Lg-GI (C2). F1–F4 are 5–18.3, 3–5, 1–3, and <1 kDa, respectively.

IgG/IgE Binding Abilities. The IgG and IgE binding abilities of all samples were determined via indirect competitive ELISA. As shown in Figure 3, the IgG binding rate of N- β -Lg was 83.49%, whereas it was increased by 7.96% for H- β -Lg (91.45%). Interestingly, after β -Lg reacted with arabinose, the IgG binding rate of Ara- β -Lg decreased by 17.76% (65.73%). These results indicated that glycation could reduce the IgG binding ability possibly by masking the linear allergic epitopes of β -Lg. Liao et al.³⁶ reported that glycation reduces the IgG binding ability of ovalbumin by masking the linear allergic epitopes.

After SGF digestion, the IgG binding rates of N- β -Lg-G, H- β -Lg-G, and Ara- β -Lg-G decreased. Among them, Ara- β -Lg-G had the lowest IgG binding rate of 20.25%, and no significant difference was found between N- β -Lg and H- β -Lg ($p > 0.05$). The binding rates of N- β -Lg-GI, H- β -Lg-GI, and Ara- β -Lg-GI decreased again and followed the order N- β -Lg-GI $\approx$ H- β -Lg-GI $>$ Ara- β -Lg-GI ($p > 0.05$). These results showed that the IgG binding rate of the digests of glycated β -Lg was the lowest, manifesting that the glycation on lysine/arginine could better reduce the allergenicity of β -Lg during *in vitro* gastrointestinal digestion.

The IgE binding rates of all samples are shown in Figure 3. In general, the changes in the IgE binding rates of patients 1–4 (P1–P4) were similar to those of IgG, and Ara- β -Lg had lower IgE binding rates in comparison to N- β -Lg and H- β -Lg ($p <$

0.05). After SGF digestion, the IgE binding rate was reduced and Ara- β -Lg-G had the lowest value ($p < 0.05$), which was similar to the IgG binding abilities. The IgE binding rates decreased again, and Ara- β -Lg-GI had the lowest value ($p < 0.05$). The differences among the IgE binding rates of P1–P4 might be due to the different serums of CMA patients. These results indicated that glycation might mask some linear allergic epitopes, making the attack of IgE unrecognizable. Moreover, the IgE binding rates of the digests of glycated β -Lg were further reduced after digestion. Nakamura et al.¹⁴ also reported that the lower allergenicity in squid tropomyosin by glycation with ribose remained after *in vitro* digestion in comparison with the digests of unglycated squid tropomyosin.

Glycation Sites and DSP Values. The glycation sites were determined simultaneously by MS and HCD MS/MS spectroscopy. Theoretically, if a peptide was glycated by one arabinose molecule, the mass would increase by 132 Da, resulting in m/z shifts of 66 and 44, at 2+ and 3+ charges, respectively. Similarly, the m/z shift would double with the occurrence of two glycation sites in a peptide. Figure 4(1)A shows the MS spectrum of the peptide 45–53 with m/z 493.2453²⁺ and the monoglycated peptide with m/z 559.2662²⁺. The glycated peptide had an m/z shift of 66.0209, indicating that one arabinose molecule was added to the peptide. Figure 4(1)B shows the HCD MS/MS spectrum of the monoglycated peptide 45–53, which exhibited

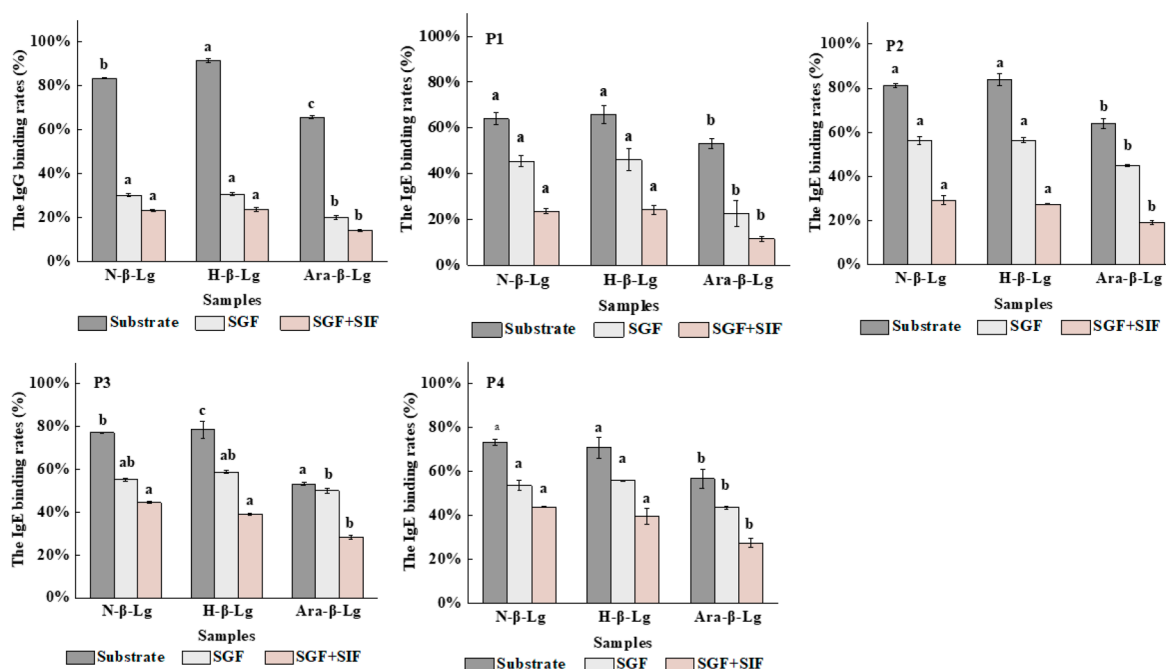


Figure 3. IgG/IgE binding abilities of glycosylated β -Lg and its digests. Substrate denotes N- β -Lg, H- β -Lg, and Ara- β -Lg, SGF denotes N- β -Lg, H- β -Lg, and Ara- β -Lg digested by simulated gastric digestive fluid, and SGF+SIF denotes N- β -Lg, H- β -Lg, and Ara- β -Lg digested by simulated gastric and intestinal digestive fluids. P1–P4 represent the milk protein specific IgE levels of the serum of CMA patients of 20.886, 23.8, 42, and 91 kU/L, respectively. Letters (a–c) at the top of the bars demonstrate significant differences ($p < 0.05$).

a series of b and y ions (b_2 – b_9 and y_1 – y_8). The glycation site, Lys 47, was determined by the mass difference between y_6 and y_8 ions, or b_2 and b_3 ions, which was the combined mass of lysine and arabinose. As shown in Figure 4(2)A, the m/z of unglycated peptide 99–104 was 414.2251^{2+} , while the corresponding m/z values of its glycosylated forms were 480.2756^{2+} and 546.2969^{2+} , with m/z shifts of 66.0505 and 132.0718, respectively, indicating that it contained mono-glycated and diglycated peptides. Similarly, the glycation sites, Lys 100 and Lys 101, were determined according to the HCD MS/MS spectrum of the peptide 99–104 after matching with the b and y ions (Figure 4(2)B,C). It is worth noting that some b and y ions may be lost due to the low intensity of ion peaks. Interestingly, Arg124 of Ara- β -Lg (AA123–129) also underwent glycation (Table 1C and Figure 4(3)).

The m/z peaks of other glycosylated peptides, such as AA2–11, AA58–64, AA61–73, and AA134–142, were 553.3013^{2+} , 437.7245^{2+} , 745.3795^{2+} , and 525.3031^{2+} , respectively, while the relevant m/z peaks of glycosylated peptides were 619.3209^{2+} , 503.7453^{2+} , 877.4177^{2+} , and 723.8530^{2+} , with m/z shifts of 66.0196, 66.0208, 132.0382, and 198.5499, respectively. β -Lg has 1 leucine (Leu, N-terminus), 15 lysines (Lys), and 3 arginines (Arg), producing 19 potential glycation sites. Table 1C and Figure 4(3) shows 11 glycation sites of Ara- β -Lg, including Lys8, Lys47, Lys60, Lys 69, Lys70, Lys100, Lys101, Arg124, Lys135, Lys138, and Lys141. The glycation sites were 10 of Lys and 1 of Arg. The results showed that glycation mainly occurred on Lys, which was in accordance with a previous study.^{37,38}

Although the number of glycation sites in Ara- β -Lg has been given, the DSP value of glycosylated β -Lg needs to be further determined. The DSP values of each glycosylated peptide of Ara- β -Lg are shown in Table 1C. In general, the DSP values of peptides 99–104 and 134–142 were 43.02% and 48.87%, respectively, with DSP values of up to 40%. The DSP values of

peptides 2–11, 58–64, and 61–73 were up to 25%. Interestingly, Arg was glycosylated with a DSP value of only 2%. These findings suggested that Lys100, Lys101, Lys135, Lys138, and Lys141 were the main glycosylated sites in β -Lg and that Arg was not easy to glycate.

Mechanism of Reduced IgG/IgE Binding Abilities of Glycosylated β -Lg and Its Digests during *In Vitro* Gastro-intestinal Digestion. In this study, arabinose plays an important role in reducing the IgG/IgE binding abilities of β -Lg, by masking linear allergic epitopes. Järvinen et al.³⁹ found that the main IgG linear allergic epitopes of β -Lg include AA51–64, AA67–88, AA129–144, and AA139–156. Six glycation sites of Ara- β -Lg were located on the main IgG linear allergic epitopes AA51–64 (K60), AA67–88 (K69, K70), and AA129–144 (K135, K138, and K141), respectively. The IgG binding rate of Ara- β -Lg was the lowest, indicating that glycation with arabinose could mask the IgG linear allergic epitopes of β -Lg, blocking IgG recognition. The main IgE linear allergic epitopes of β -Lg are AA1–8, AA9–14, AA25–40, AA41–60, AA92–100, AA102–124, and AA149–162.⁴⁰ AA1–8, AA41–60, AA92–100, and AA102–124 were glycosylated with arabinose on K8, K47, K100, and R124, respectively. The lowest IgE binding rate of Ara- β -Lg could be due to the masked IgE linear allergic epitopes.

An analysis of tricine SDS-PAGE profiles, HPLC, and DH values demonstrated that N- β -Lg-G, H- β -Lg-G, and Ara- β -Lg-G were hydrolyzed by pepsin into peptide fractions, which might destroy the linear and conformational allergic epitopes, thereby decreasing the IgG/IgE binding rates of these digests. Figure 5B,D shows a stretch of the peptide F₁₀₅–I₁₆₂ hydrolyzed by pepsin with one IgG linear allergic epitope (AA129–144). Glycation with arabinose masked the IgG linear allergic epitope of Ara- β -Lg-G, so that the IgG linear allergic epitope disappeared, resulting in weaker IgG binding ability (Figure 5D). In contrast, the linear allergic epitope of N- β -Lg-G was

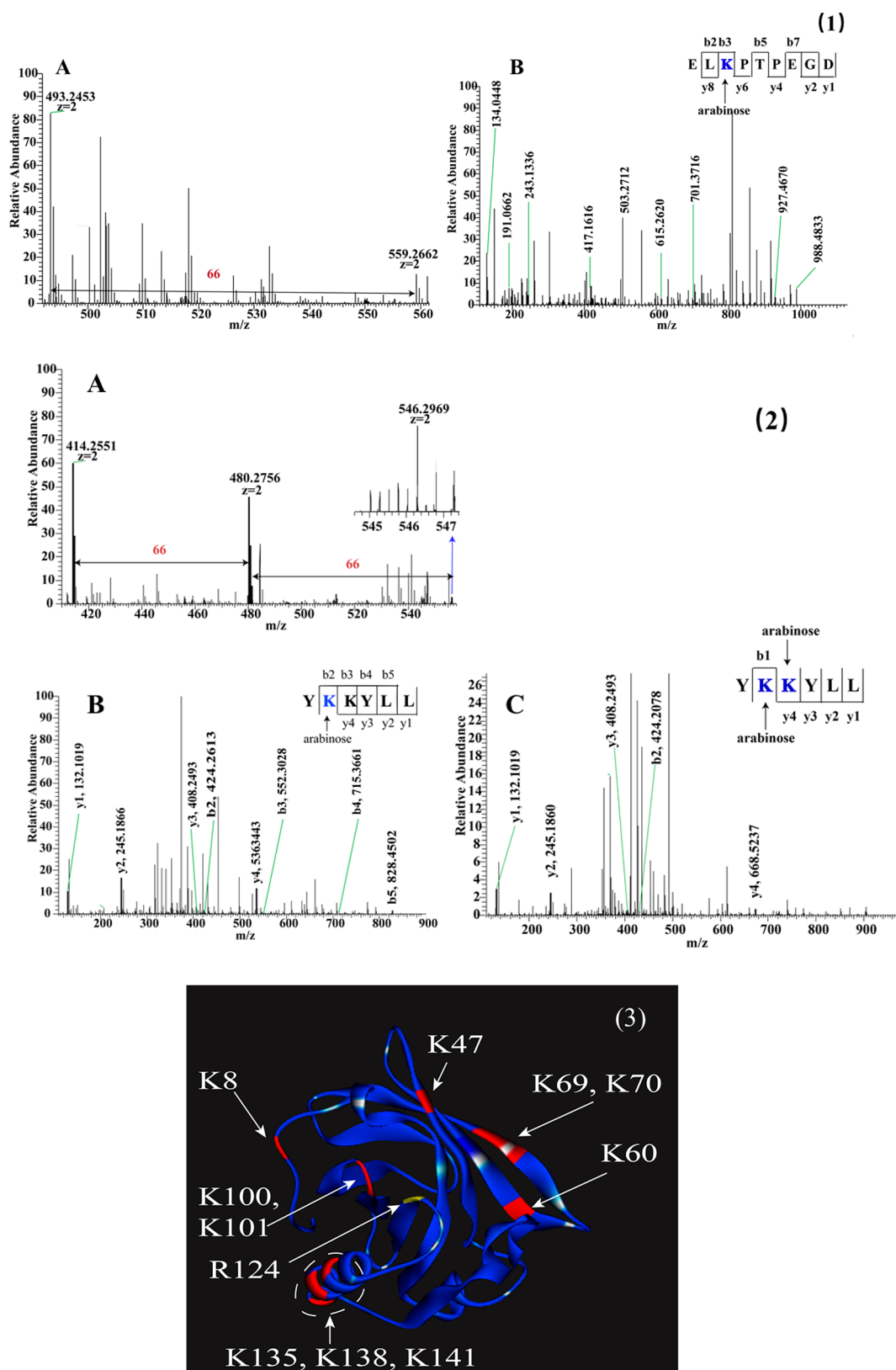


Figure 4. Determination of glycation sites of Ara- β -Lg in the peptides 45-53 (1) and 99-104 (2). Mass spectra for the glycated peptides are displayed, and the mass differences are shown with red numbers and black arrows (A). HCD MS/MS spectra of the glycated peptides are displayed, and the determined glycation sites (blue K) are shown by black arrows with arabinose. The b and y ions are indicated by the numbers and lines (B, C). Ribbon diagram of Ara- β -Lg (Protein Data Bank (PDB) code 1BSQ) (3). The framework of β -Lg is shown in navy blue, glycated Lys is shown in red, and glycated Arg is shown in yellow.

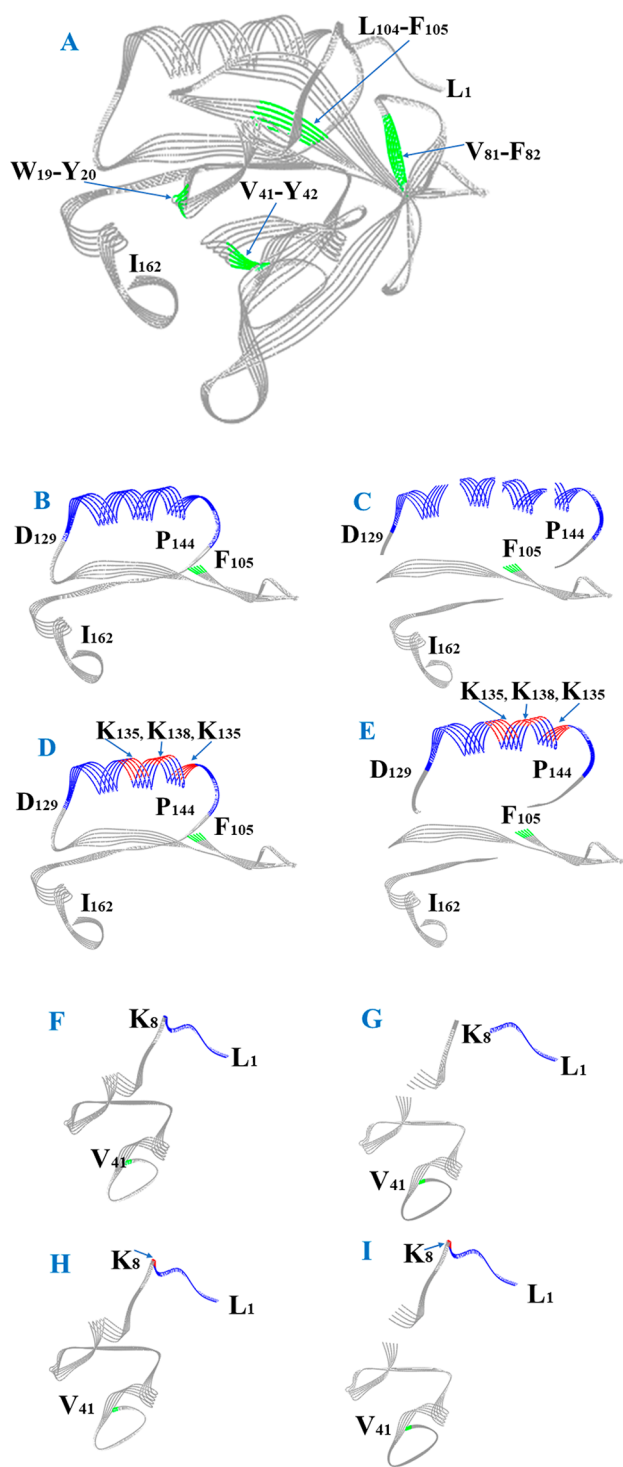


Figure 5. Map of β -Lg (A). The possible hydrolysis peptide bonds W₁₉–Y₂₀, V₄₁–Y₄₂, V₈₁–F₈₂, and L₁₀₄–F₁₀₅ of pepsin are shown in green. The peptide segment F₁₀₅–I₁₆₂ hydrolyzed by pepsin of N- β -Lg-G (B). The peptide segment F₁₀₅–I₁₆₂ hydrolyzed by pepsin and trypsin of N- β -Lg-GI (C). The peptide segment F₁₀₅–I₁₆₂ hydrolyzed by pepsin of Ara- β -Lg-G (D). The peptide segment F₁₀₅–I₁₆₂ hydrolyzed by pepsin and trypsin of Ara- β -Lg-GI (E). The peptide segment L₁–V₄₁ hydrolyzed by pepsin of N- β -Lg-G (F). The peptide segment L₁–V₄₁ hydrolyzed by pepsin and trypsin of N- β -Lg-GI (G). The peptide segment L₁–V₄₁ hydrolyzed by pepsin of Ara- β -Lg-G (H). The peptide segment L₁–V₄₁ hydrolyzed by pepsin and trypsin of Ara- β -Lg-GI (I). The IgG/IgE linear allergic epitopes are shown in blue, and the glycated sites are shown in red.

intact, retaining its allergenicity (Figure 5B). Peptide L₁–V₄₁ hydrolyzed by pepsin with one IgE linear allergic epitope (AA1–8) is shown in Figure 5F,H. The IgE linear allergic epitope was still intact in N- β -Lg-G. The IgE linear allergic epitope of Ara- β -Lg-G was intact but was glycosylated with arabinose, resulting in a lower IgE binding ability in comparison with N- β -Lg-G.

Trypsin mainly hydrolyzes peptide bonds at the carboxyl side of arginine and lysine, but lysine/arginine covalently combines with arabinose after glycation modification, thus preventing trypsin hydrolysis. Figure 5C shows that, after trypsin digestion, the IgG linear allergic epitope of N- β -Lg-GI was destructive, and Ara- β -Lg-GI retained its complete glycosylated linear epitope (Figure 5E). However, the IgG binding rate of Ara- β -Lg-GI was the lowest. This result may be attributed to the fact that trypsin preferentially hydrolyzes the unglycosylated lysine. When the hydrolysis tends to be smooth, some linear allergic epitopes (AA129–144) remained intact in N- β -Lg-GI, while that of Ara- β -Lg-GI was masked due to glycation with a high DSP value (43.02%), leading to lower allergenicity. All in all, glycation masking linear allergenic epitopes had a more significant effect on reducing allergenicity in comparison to digestive enzyme hydrolysis. As shown in Figure 5G,I, the IgE linear allergic epitopes of N- β -Lg-GI and Ara- β -Lg-GI were retained after trypsin hydrolysis, but the glycation on K8 prevented IgE from being distinguished, leading to a lower IgE binding rate in Ara- β -Lg-GI. All in all, β -Lg glycosylated with arabinose might have lower allergenicity in comparison to that of unglycosylated β -Lg in the human body.

In conclusion, glycation with arabinose can reduce the IgG/IgE binding rate of β -Lg, by masking the linear allergic epitopes of β -Lg. Moreover, the IgG/IgE binding rates of its digests were further reduced in comparison with the digests of unglycosylated β -Lg. Therefore, β -Lg glycosylated with arabinose is a potential method to reduce its allergenicity in food processing. However, *in vivo* experiments, such as double-blind placebo-controlled trials, should be considered to confirm the reduced allergenicity of glycosylated β -Lg.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at <https://pubs.acs.org/doi/10.1021/acs.jafc.1c00205>.

Patient serum levels (PDF)

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Notes

The authors declare no competing financial interest.

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ABBREVIATIONS

β -Lg; β -lactoglobulin; Ara- β -Lg; arabinose- β -Lg conjugation; IgE; immunoglobulin E; IgG; immunoglobulin G; ELISA; enzyme-linked immunosorbent assay; HPLC; high-performance liquid chromatography; Tricine-SDS-PAGE; tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis; OPA; o-phthalaldehyde; HPLC MS; liquid chromatography high-resolution mass spectrometry; SGF; simulated gastric digestive fluid; SIF; simulated intestinal digestive fluid; DSP; average degree of substitution per peptide molecule; DH; degree of hydrolysis.

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Table S1 The information of patients' serum

Patients	Blood number	IgE activity ^a (kU/L)	Clinical history
1	BD 1881	20.886	Allergic rhinitis
2	PL 20891	23.8	Allergic rhinitis, asthma
3	PL 19230	42	Allergic rhinitis
4	PL 23320	91	Allergic rhinitis

^aIgE activity was measured by ImmunoCAP against the milk.