

The Mechanism of Decreased IgG/IgE-Binding of Ovalbumin by Preheating Treatment Combined with Glycation Identified by Liquid Chromatography and High-Resolution Mass Spectrometry

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Supporting Information

ABSTRACT: Ovalbumin is one of the most important sensitizing ingredients in allergens of egg albumin, which restricts the application of egg in the field of food processing. Previous research has indicated that glycation could cause the protein to partially expand, which may bring about the destruction of the structural IgG and IgE epitopes and induce the decline of the IgG- and IgE-binding ability of ovalbumin. In this research, the effect of a preheating treatment integrated with glycation on the IgG- and IgE-binding capability and the conformation changes of ovalbumin was studied by detecting the glycated sites and the values of degree of substitution per peptide (DSP) by liquid chromatography and high-resolution mass spectrometry (LC-HRMS). Interestingly, we found that a glycation site (K227) attached by two ribose molecules was detected in glycated ovalbumin with preheating treatment. In addition, a new glycation site (K323) appeared in G-60. The results displayed that preheating treatment could strengthen the changes in the secondary and tertiary structure of ovalbumin by enhancing glycation and further reduce the IgG/IgE-binding ability by integrating with glycation because of the cover of IgG and IgE epitopes. Therefore, preheating treatment integrated with glycation may offer a way for ovalbumin to reduce sensitization.

KEYWORDS: ovalbumin, preheating treatment, glycation, IgG/IgE-binding capability, mass spectrometry

INTRODUCTION

Egg albumin is a most common source of animal protein owing to its outstanding properties, such as low price, nutrition, foaming capacity, and heat setting, which is extensively utilized in the food manufacturing industries. Ovalbumin is a foremost protein in the egg white, which constitutes about 54% of the whole egg white protein.¹ Unfortunately, previous studies have proven that ovalbumin is one of the most important sensitizing ingredients in allergens of egg albumin, which restricts the application of egg in the food processing industry.² It is a 45 kDa globular protein, consisting of 386 amino acids with a PI (isoelectric point) of 4.5, where one set of disulfide bonds (Cys73–Cys120) stabilize an “S” form structure that is composed by the six main strands of the β -sheet.^{3–5} In recent years, the linear epitopes and conformational epitopes were identified via ELISA, and some scholars have concentrated on ovalbumin, finding that a single way to mask or hydrolyze specific sequences of allergenic epitopes of ovalbumin is to reduce allergenicity.^{6,7} A great many handling methods, such as heating,⁸ glycation reaction,⁹ hydrolysis,¹⁰ and irradiation¹¹ have been used to alter ovalbumin to reduce the allergenicity. However, the allergenicity of ovalbumin cannot be adequately decreased to the ideal level by a single treatment.

It is a significant discovery that glycation assembly is an excellent method to upgrade functional properties of the protein, such as allergenicity,⁹ thermal stability,¹² and emulsification.¹³ It is the early reaction of the Maillard reaction, which is the dehydration between the amino and

reducing sugars of the protein and the assembly of glycosyl groups to lysine of protein molecules, ϵ -amino groups of arginine, or nitrogen-terminal amino groups of proteins.⁵ Glycation assembly was reported as an effective way to decrease the sensitization of the protein.¹⁴ The principle of this method is to reduce the IgE binding capacity of β -lactoglobulin by covering up IgE epitopes through glycation with galactooligosaccharides. However, scarce investigation of the connection between allergenicity and glycated sites and the degree of glycation have been reported at the molecular level.

To date, the effect of preheating treatment on allergenicity has hardly been reported. In previous studies, we noticed that preheating could obviously change the conformation of ovalbumin and enhance the properties of proteins, such as the foaming properties.¹⁵ The mechanism is that the partial secondary and tertiary structures of the ovalbumin are induced to expand and improve protein–protein interactions. In addition, it is clear that the IgG/IgE-binding capabilities can be reduced by promoting the number of glycated sites or the degree of glycation.^{7,16} Simultaneously, the improvement of the glycated sites and the degree of glycation are accompanied by structural changes of ovalbumin. Therefore, we speculated that preheating treatment combined with glycation may reduce

Received: August 2, 2018

Revised: September 18, 2018

Accepted: September 25, 2018

Published: September 25, 2018

the IgG/IgE-binding capabilities. Compared with the ultrasound pretreated integrated with glycation to reduce the allergenicity of the protein, preheating integrated with glycation is a more mild process that does not produce the carbonized black substances, which can guarantee the quality of hypoallergenic products.¹⁷ However, little research has been carried out to study the influence of the allergenicity of ovalbumin by preheating combined with glycation. In recent years, Fourier transform ion cyclotron resonance mass spectroscopy (FTICR-MS) has been considered as an useful technology to define the glycated sites and the degree of glycation at the molecular level because of its excellent quality, resolution, and sensitivity.^{17–19} Therefore, in this study, HCD/FTICR-MS was utilized to evaluate the accurate structure of ovalbumin at the molecular level and analyze the glycated sites and the degree of glycation. In addition, pretreatment temperatures of 20, 40, and 60 °C were selected because the preheated ovalbumin after lyophilization can be well-reconstituted. It has been reported that irreversible aggregation of ovalbumin has occurred at approximately 75 °C.²⁰ D-Ribose, which is an important component of biomacromolecules, such as ATP, RNA, and riboflavin, was considered to be a more active sugar than glucose or mannose in nature.²¹ Therefore, it is reasonable for us to select ribose and ovalbumin as the glycation reaction model in this study.

The intention of this study was to clarify the mechanism of different preheating temperatures which could enhance the glycation and explain the connection between the allergenicity and conformation changes of ovalbumin by preheated integrated with glycation via detecting the glycated sites and the degree of glycation at the molecular level using HCD/FTICR-MS. The traditional methods, such as circular dichroism, free amino group content analysis, surface hydrophobicity analysis, and intrinsic fluorescence analysis were applied to estimate the secondary and tertiary structures of the protein. Moreover, the allergenicity was estimated by indirect competitive ELISA. The results of this study may point to an alternative approach to produce ovalbumin desensitization.

MATERIALS AND METHODS

Chemicals and Materials. Ovalbumin (Grade V, A-5503) and D-ribose were acquired from Sigma-Aldrich (St. Louis, MO, USA). 8-Anilino-1-naphthalenesulfonic acid (ANS) was obtained from Beijing Solarbio Science & Technology Co., Ltd. (Beijing, China). *o*-Phthaldialdehyde (OPA) and Freund's incomplete and complete adjuvant were received from Aladin Chemical Co. (St. Louis, MO, USA). All other reagents were of analytical reagent grade. Ultrapure water was made by professional distillation, which was used throughout the experiment.

Preparation of Ovalbumin Samples. Ovalbumin (180 mg) was dissolved in 180 mL of Tris-HCl buffer (pH 7.8, 0.05 M) to give a final concentration of 1.0 mg/mL. The sample solution was divided into nine equal parts. One of them was defined as the blank control, which was named N-Oval. Six equal parts of the same sample were divided into three groups, which were heated at 20, 40, and 60 °C for 1 h, respectively, and named C-20, C-40, and C-60, respectively. The reaction was stopped via an ice bath. One sample from each of the three groups were mixed with ribose at a mass ratio of about 1:1 of ovalbumin. Then, the sample solutions were lyophilized under vacuum conditions for 48 h and then incubated at a temperature of 50 °C with 60% humidity using a saturated KI solution for 6 h, namely, G-20, G-40, and G-60, respectively. A ribose-free ovalbumin sample under the heated at 50 °C with 60% humidity condition was defined as another control and named H-Oval. The last aliquot of the sample was mixed with ribose at a mass ratio of about 1:1 of

ovalbumin, followed by lyophilization, which was incubated at a temperature of 50 °C with 60% humidity for 6 h and named R-Oval. The glycated samples were dialyzed overnight at 4 °C by a centricon centrifugal filter unit (7000 Da cutoff), (Millipore; Bedford, MA) to remove free ribose, lyophilized, and then kept at –20 °C until use. All the glycated ovalbumin samples were homogeneous.

Preparation of Antisera against Ovalbumin. Polyclonal antibodies against ovalbumin were produced from two rabbits (2.5-month-old, about 1.7 kg), which were subcutaneously immunized with 1.0 mg of N-Oval by injecting it with the equal mass of Freund's complete adjuvant. Next, an equal amount of ovalbumin emulsified with the same mass of Freund's incomplete adjuvant was used to restimulate the rabbits for eight times at 2 week intervals. The antisera titer was detected by ELISA, and when the antisera titer maintained a stable level, the rabbits were taken out to obtain the serum, which was centrifuged (5000g, 8 min). Finally, all antisera were kept at –80 °C until use.

All serum samples that were from patients allergic to ovalbumin were obtained via PlasmaLab International (Everett, WA), which was approved by the Internal Ethical Committee of First Affiliated Hospital of Nanchang University and the Animal Care and Use Committee of Nanchang University [SCXK(Gan)-2014-0005]; moreover, the written informed consent was acquired from all participants. All serum samples were documented by clinical history from six patients (in Table S2 in the SI). The specific IgE levels changed from 15.8 to 92.7 kU_A/L. The antisera titer was measured via the indirect ELISA method before use.

Indirect Competitive ELISA. The IgG/IgE-binding capabilities of ovalbumin samples were measured via the indirect competitive ELISA method with rabbit serum and EA patients' antisera according to the method of Meng with slight modifications.²² First of all, the 96-well microplate was coated for 1 h at 37 °C with the N-Oval (100 µL/well, 2 µg/mL), followed by being washed three times with PBST. Second, the microplate was blocked with 5% skimmed milk powder (250 µL/well) for 0.5 h at 37 °C. Then, 50 µL of standard ovalbumin (0.5, 1, 2, 4, 8, 16, 32, and 64 µg/mL) or diluted ovalbumin samples and the rabbit antisera (50 µL, diluted to 1:51200) or EA patients' antisera (diluted to 1:60) were injected into the microplate, followed by incubation for 0.5 h at 37 °C. Next, the microplate was washed, and the goat antirabbit IgG-HRP conjugate (100 µL, diluted to 1:50000) or goat antihuman IgE-HRP conjugate (100 µL, diluted to 1:5000) was injected and incubated for 20 min at 37 °C. The tetramethyl benzidine solution (100 µL/well) was used to develop the color. Finally, the process was finished by using sulfuric acid solution (50 µL, 2.0 mol/L), and the absorbance was evaluated at 450 nm via using the microplate reader (Multiskan FC, THERMO, USA). The IgG/IgE-binding capability was evaluated from a standard curve of ovalbumin with a linear logarithmic correlation within the scope of 0.5–64 µg/mL. All of the measurements were accomplished in triplicate.

Free Amino Group Analysis. The amount of free amino groups was evaluated via the modified way.²³ *o*-Phthaldialdehyde (400 mg) was dissolved in 10 mL of ethanol (95%), and then SDS (5 g), borax (9.5 g), and β-mercaptoethanol (1 mL) were added into the mixture; the mixture was diluted with distilled water, and the final volume was 500 mL. A 200 µL of sample solution (1.0 mg/mL) was injected to *o*-phthaldialdehyde reagent (4 mL) in which the solution was agitated and incubated for approximately 2 min at room temperature. The absorbance was measured at 340 nm. A calibration curve was obtained using 0–0.5 mg/mL lysine as a standard. Finally, the average of three measurement values were regarded as the results.

Far-UV CD Spectroscopy. The secondary structure of the ovalbumin handled by different conditions was measured by CD (circular dichroism) spectroscopy via using a JASCO J-810 spectropolarimeter (Tokyo, Japan), which was always flushed with liquid nitrogen. Briefly, the sample was dissolved in Tris-HCl buffer (pH 7.8, 0.05 M), with a final concentration of 0.1 mg/mL. Then, the sample was injected into a quartz cuvette with a path length of 1.0 mm at room temperature. The scan speed was 100 nm/min, with a wavelength scope of 190–250 nm. All measured CD spectra results

were baseline diminished for Tris-HCl buffer, and the results of the sample were presented as the mean residue ellipticity (deg cm²/dmol). The different secondary structures of ovalbumin samples were defined by the online Dichroweb software (<http://dichroweb.cryst.bbk.ac.uk/html/process.shtml>).

Determination of the Surface Hydrophobicity (H_0). The H_0 value of ovalbumin was measured using ANS as a fluorescent probe in the light of the method of Shen and Tang with slight modifications.²⁴ The ovalbumin was mixed via using Tris-HCl buffer (pH 7.8, 0.05 M) to obtain different concentrations (1.0–0.125 mg/mL). ANS (20 μ L) was injected to 4 mL of the ovalbumin solution. Meanwhile, fluorescence intensity was gauged at 390 nm (excitation wavelength) and 600 nm (emission wavelength) via using a fluorescence spectrophotometer (F-4500, BioTek, Vermont, USA). The curve intensity was plotted with protein concentration, and the slope of the straight line was the H_0 value of the protein.

Measurement of Intrinsic Fluorescence. A Fluor photometer (F7000, Hitachi, Tokyo, Japan) was used to measure the endogenous fluorescence intensity of the ovalbumin samples in the light of the method of Liu et al. with minor modifications.²⁵ The samples (1 mg/mL) were obtained via utilizing the 0.05 M Tris-HCl buffer (pH 7.8). The protein sample solutions were measured at 290 nm (excitation wavelength), and the emission spectra were measured from 280 to 500 nm. All of the measurements were accomplished in triplicate.

Sample Digestion. Before mass spectrometry, the trypsin was used to digest the ovalbumin samples in the light of the method revealed by Wang et al.¹⁹ In order to leave the digestion and mass spectrometry process undisturbed, the disulfide bridge was disrupted by the dithiothreitol reducing reaction. First, 10 μ L of ovalbumin solution was injected into the Tris-HCl buffer (pH 7.8, 0.05 M), including 60 μ L of 40 mM ammonium bicarbonate solution. Then, 15 μ L of dithiothreitol reducing agent (100 mM) was injected into the sample and preserved for 5 min at 95 °C, which was cooled in an ice bath environment. Second, 12 μ L of iodoacetamide (100 mM) was injected into the test tube, followed by 20 min incubation at room temperature in a dark environment. Then, 4 μ L of 0.5 μ g/ μ L trypsin was added into the ovalbumin samples and hydrolyzed at 37 °C for 15 h. Then, 50% trifluoroacetic acid (2 μ L) was used to quench the process.

Analysis by HPLC HCD MS/MS. A Shimadzu HPLC system was used to investigate the values of DSP and the glycosylated sites according to Chen et al. with minor modifications.²⁶ Then, 50 μ L of ovalbumin solution was infused into a 50 $\times$ 1.00 mm peptide column (Micro-Tech Scientific Inc., Vista, CA) for HPLC with a speed of 0.25 mL/min. The mobile phase included solvent B (99% acetonitrile including 0.1% formic acid) and solvent A (0.1% formic acid in ultrapure water). After they were desalted for 10 min with 5% solvent B, the peptides were washed with a reasonable gradient of 5–60% solvent B for 35 min, which was infused into the LTQ-Qrbitrap Velos mass spectrometer (Thermo Fisher Scientific, Waltham, MA, USA) for FTICR-MS analysis and fragmented by HCD to seek the glycosylated sites.

The glycation degree of the peptide was displayed by the DSP value and the formula as follows:¹⁷

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

where I is the overall number of the intensity of the different glycosylated ovalbumin peptides. In addition, i is the amount of ribose units integrated with the peptide in each type of glycosylated form.

Statistical Analysis. All of the research was completed in triplicate, and the experimental results were shown as the mean value $\pm$ standard deviation. An Origin-Pro 9.0 (OriginLab Corp, Northampton, USA) software was used to analyze the data. The variance and significant difference were obtained using SPSS 22.0 ($P < 0.05$).

RESULTS AND DISCUSSION

Free Amino Group Content Analysis. Glycation modification based on the Maillard reaction is the process between carbonyl compounds (reducing sugars) and amino group-containing compounds (amino acids of proteins).²⁷ Studies have shown that lysine residue, arginine residue, and the free amino group at the N-terminal were the major glycosylated sites that reacted with the reducing sugars.⁴ Therefore, the amount of free amino groups was used as an important norm to assess the glycation reaction. Figure 1A shows the changes of the free amino group content of the unglycosylated and glycosylated ovalbumins. The amount of the free amino groups of the unglycosylated ovalbumin (H-Oval, C-20, C-40, and C-60) was slightly lower than N-Oval. A possible explanation was that there was an S–S covalent bond between ovalbumin and steric obstruction, which induced the loss of free amino groups.^{28,29} Simultaneously, there is a significant decrease of the content of free amino groups in glycosylated ovalbumin (R-Oval, G-20, G-40, and G-60), and with the increasing of preheating temperature, the amount of free amino groups gradually decreased. Therefore, we speculated that preheating could promote glycation. However, there is no inevitable link between the allergenicity of ovalbumin and the amount of free amino groups. It was necessary to acquire the exhaustive information on the glycosylated sites of ovalbumin with and without preheating treatment so as to detect the accurate mechanism of the reduced IgG/IgE-binding abilities of G-20, G-40, and G-60.

Tertiary Structure Analysis. In general, the intrinsic fluorescence is typically produced by only three amino acid residues (tyrosine, phenylalanine, and tryptophan), and the fluorescence of the tryptophan residue makes up the main part of the fluorescence spectrum.³⁰ The changes in the fluorescence spectrum of ovalbumin samples under different processing conditions are displayed in Figure 1B. When the samples were excited at 280 nm, the N-Oval displayed a maximum fluorescence emission value (λ_{max}) at 350 nm. Compared to N-Oval, the R-Oval, G-20, G-40, and G-60 had a very visible decrease while H-Oval, C-20, C-40, and C-60 had a very slight decrease. In addition, there was an obvious phenomenon that the glycosylated samples treated with different pretreatment temperatures had a red shift of the maximum Trp emission value to 360 nm and to 364 nm for the G-60 preheated at 60 °C. The results indicated that the tertiary conformation of ovalbumin was significantly changed via glycation between ovalbumin and ribose. Thus, due to glycation modification, conformational changes occurred about the Trp residues (19 Trp and 61 Trp), including masking the glycosylated sites of R-Oval.³¹ Furthermore, owing to the effect of Arg or Lys residues, we speculated that the glycosylated sites were different between ovalbumin with preheating and without preheating treatment.

The surface hydrophobicity (H_0) of the protein is a parameter of the amount of hydrophobic groups on the exterior of the protein in link with a polar aqueous area, which was used to detect the tertiary structure of the protein.³² As shown in Figure 1C, the H_0 values of the H-Oval, C-20, C-40, and C-60 showed a slight upward trend with the increase of the preheating temperature, which may be due to when the ovalbumin molecules were denatured by preheating, and the globular structure gradually unfolded. The hydrophobic groups originally embedded in the curly structure were exposed and combined with the hydrophobic probe ANS to increase the H_0

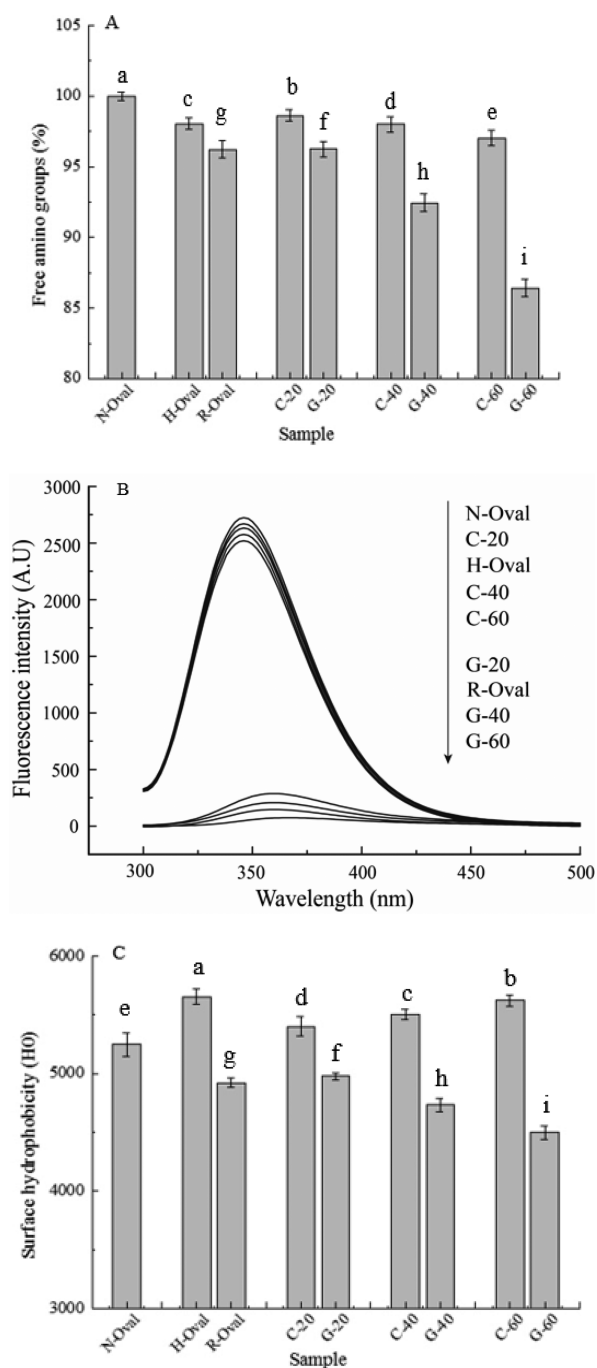


Figure 1. The content of free amino groups (A), intrinsic fluorescence intensity (B), and the surface hydrophobicity (C) of the samples (N-Oval, native ovalbumin; R-Oval, the ovalbumin glycation without preheating treatment; H-Oval, the ovalbumin without ribose incubated at a temperature of 50 °C; C-20, C-40, and C-60, the preheating temperature at 20, 40, and 60 °C, respectively; and G-20, G-40, and G-60, the ovalbumin glycation with the preheating temperature at 20, 40, and 60 °C, respectively). Different letters (a–i) on the top of the bars are significantly different ($p < 0.05$).

values. This indicated that the structural changes of the N-Oval occurred after preheating treatment at different temperatures. However, the H_0 values of the R-Oval, G-20, G-40, and G-60 had significantly decreased with the increase of the preheating temperature, which may be explained by the changes of the tertiary structure of ovalbumin; the glycosylated sites and

preheating treatment expanded the structure of ovalbumin, and hydrophilic ribose molecules were more prone to glycation reaction with the protein. Studies have shown that the potential allergenicity of the protein was changed as a result of the unfolding of the protein structure.⁸ The increase of the allergenicity of the protein can be reflected by the increase of surface hydrophobicity. Therefore, glycation reaction can reduce the surface hydrophobicity of ovalbumin, which induced the IgG- and IgE-binding ability to drop.

Secondary Structure Analysis. The results of the secondary conformation of ovalbumin were displayed in Table S3. Compared to N-Oval, no obvious changes ($p < 0.05$) in the secondary conformation were detected for H-Oval, C-20, C-40, and C-60. However, after glycation modification at 50 °C for 6 h, the total amount of α -helix and random coil of ovalbumin decreased while the β -turn and β -sheet contents improved, which demonstrated that glycation modification altered the secondary structure of ovalbumin. Furthermore, after preheating treatment at 20 °C and then glycation with ribose, the total amount of α -helix and random coil of N-Oval decreased from 57.6% (N-Oval) to 48.5% (G-20) while the total amount of β -turn and β -sheet increased from 42.4% (N-Oval) to 52.9% (G-20). Moreover, preheating treatment at 40 °C and then glycation with ribose induced a greater reduction of the total amount of α -helix and random coil of N-Oval. In particular, compared to N-Oval, after preheating treatment at 60 °C and then glycation with ribose, G-60 had the biggest drop of the total amount of α -helix and random coil of ovalbumin and extra improved β -turn and β -sheet contents. It showed that preheating treatment could promote the change of secondary structure of ovalbumin during the glycation reaction process.

Identification of Glycation Sites. The peptide mapping of N-Oval by HCD/FTICR-MS is shown in Table S1, which had a sequence coverage of the peptide map of more than 95.5%. HCD/FTICR-MS is an excellent method to detect the low quality fragments and the precise peptide sequences.³³ Theoretical analysis demonstrated that if a peptide is modified with one ribose molecule, the mass will increase 132 Da, reflected in an m/z shift of 132, 66, and 44, respectively, at 1, 2, and 3 charges, accordingly. In addition, glycation with two or three ribose molecules will double or triple the m/z shift. For G-20, the glycosylated forms of the peptide can be clearly detected from the mass change caused via glycation with ribose, which is shown in Figure 2A. The m/z of unglycosylated peptide 224–233 was 581.3302²⁺ while the homologous m/z of its glycosylated peptides were 647.3515²⁺ and 713.3538²⁺ with an m/z shift of 66 and 132 Da, respectively, which indicated that it had a monoglycosylated form and a doubly glycosylated form of peptides. In addition, Figure 2B displayed the HCD MS/MS spectrum of the monoglycosylated-form peptide with an m/z value of 647.3515²⁺, which illustrated a series of b and y ions. The glycosylated site, K227, was detected by the mass change between b3 and b5 ions, or between y6 and y8, which was the composed mass of lysine and ribose. Interestingly, on the basis of the HCD MS/MS spectrum of this glycosylated peptide with m/z value of 713.3538²⁺ in Figure 2C, K227 combined with two ribose molecules, which revealed that one glycosylated site was integrated by two ribose molecules. The same phenomenon was shown in G-40 and G-60. This is an interesting phenomenon that one glycosylated site (K227) integrated by two ribose molecules was defended in the glycosylated ovalbumin after preheating treatment. Previous research revealed that

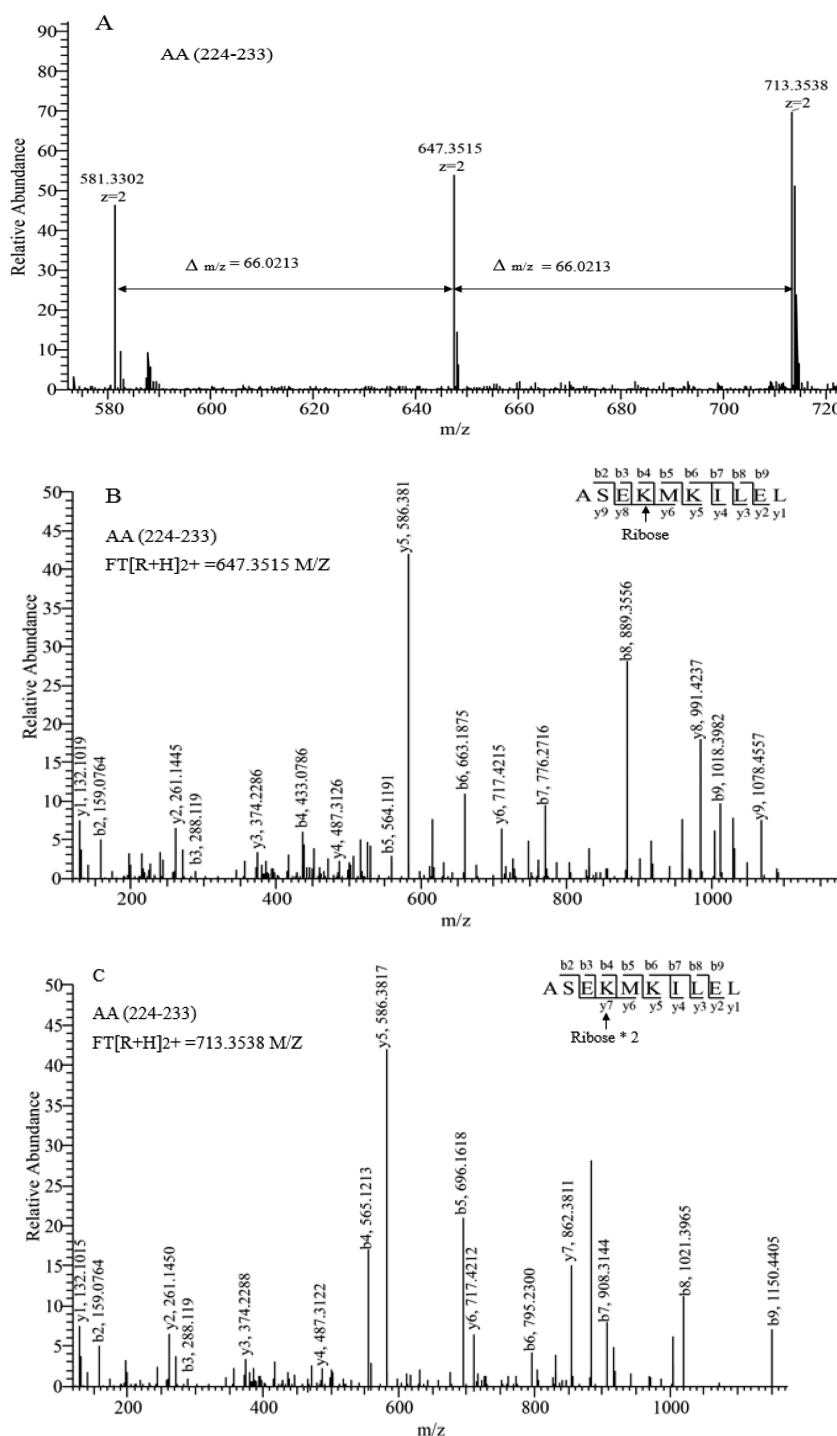


Figure 2. Determination of glycation sites in the glycation peptide 224–233. (A) Mass spectrum of the peptide 224–233 with one glycation site. (B) HCD MS/MS spectrum of the monoglycated peptide with m/z of 647.3515 $^{2+}$. (C) HCD MS/MS spectrum of dual-glycated peptide with m/z of 713.3538 $^{2+}$. The mass differences are shown with the numbers and arrows. “Ribose” and “Ribose*2” mean glycation site is added by one and two ribose molecules, respectively. The b and y ions are rated by the numbers and lines.

glycation reaction could generate the reactive intermediates, which may combine with ribose in a hydrophilic environment.³⁴ In this study, we found that a preheating treatment could transform a hydrophobic environment into a hydrophilic environment of the ovalbumin molecule, which may promote the form of the reactive substances during the glycation reaction process. The two ribose molecules were combined with one glycation site, which induced the steric hindrance and caused increased difficulty for IgG and IgE epitopes to be

identified as the linear epitopes of the ovalbumin, resulting in the further obvious decrease on the IgG/IgE-binding capability of ovalbumin by preheating treatment combined with glycation.⁷

The amount of glycation sites is an important indicator to measure the extent of glycation reaction. As shown in Table 1, the glycation sites of R-Oval, including K47, K62, K93, K190, K207, K227, and K370, were detected by HCD MS/MS. As expected, six monoglycated peptides and one dual-glycated

Table 1. Glycated Peptides of Ovalbumin

no.	<i>m/z</i>	Δ ppm	st.	end	sequence	glycated peptides <i>m/z</i>	glycated site
R-Oval							
1	477.2450 ²⁺	0.288	42	50	(M)VYLGAKDST(R)	543.2723 ²⁺	K47
2	589.2910 ²⁺	1	61	71	(F)DKLPGFGDSIE(A)	655.3120 ²⁺	K62
3	465.2378 ²⁺	−0.39	89	96	(L)NQITKPND(V)	531.2592 ²⁺	K93
4	377.6660 ²⁺	−0.57	189	194	(A)FKDEDT(Q)	443.6871 ²⁺	K190
5	473.7361 ²⁺	−0.173	204	211	(E)QESKPVQM(M)	539.7575 ²⁺	K207
6	581.3302 ²⁺	−1.42	224	233	(M)ASEKMKILEL(P)	647.3515 ²⁺	K227
7	523.7758 ²⁺	−0.548	367	375	(L)FCIKHIATN(A)	589.7968 ²⁺	K370
G-20							
1	477.2499 ²⁺	−1.38	42	50	(M)VYLGAKDST(R)	543.2722 ²⁺	K47
2	589.2896 ²⁺	−1.38	61	71	(F)DKLPGFGDSIE(A)	655.3107 ²⁺	K62
3	465.2374 ²⁺	−1.25	89	96	(L)NQITKPND(V)	531.2584 ²⁺	K93
4	377.6659 ²⁺	−1.13	189	194	(A)FKDEDT(Q)	443.6870 ²⁺	K190
5	473.7363 ²⁺	−0.972	204	211	(E)QESKPVQM(M)	539.7574 ²⁺	K207
6	581.3302 ²⁺	−1.42	224	233	(M)ASEKMKILEL(P)	647.3509 ²⁺	**K227
7	523.7756 ²⁺	−1.57	367	375	(L)FCIKHIATN(A)	589.7967 ²⁺	K370
G-40							
1	477.2511 ²⁺	1.14	42	50	(M)VYLGAKDST(R)	543.2723 ²⁺	K47
2	589.2900 ²⁺	−0.018	61	71	(F)DKLPGFGDSIE(A)	655.3107 ²⁺	K62
3	465.2374 ²⁺	−1.22	89	96	(L)NQITKPND(V)	531.2595 ²⁺	K93
4	377.6658 ²⁺	−1.73	189	194	(A)FKDEDT(Q)	443.6869 ²⁺	K190
5	473.7373 ²⁺	1.14	204	211	(E)QESKPVQM(M)	539.7585 ²⁺	K207
6	581.3308 ²⁺	−0.214	224	233	(M)ASEKMKILEL(P)	647.3522 ²⁺	**K227, K229
7	523.7769 ²⁺	1.3	367	375	(L)FCIKHIATN(A)	589.7978 ²⁺	K370
G-60							
1	477.2507 ²⁺	0.299	42	50	(M)VYLGAKDST(R)	543.2720 ²⁺	K47
2	589.2913 ²⁺	1.51	61	71	(F)DKLPGFGDSIE(A)	655.3123 ²⁺	K62
3	465.2376 ²⁺	−0.175	89	96	(L)NQITKPND(V)	531.2593 ²⁺	K93
4	377.6659 ²⁺	−1.42	189	194	(A)FKDEDT(Q)	443.6871 ²⁺	K190
5	473.7371 ²⁺	0.719	204	211	(E)QESKPVQM(M)	539.7582 ²⁺	K207
6	581.3302 ²⁺	−1.42	224	233	(M)ASEKMKILEL(P)	647.3509 ²⁺	**K227, K229
7	566.8149 ²⁺	1.5	318	328	(S)SAESLKISQAV(H)	632.8363 ²⁺	K323
8	523.7762 ²⁺	1.43	367	375	(L)FCIKHIATN(A)	589.7975 ²⁺	K370

^aThe double-star (**) means that the glycated site was added by two ribose molecules. The increased glycated sites and double-ribose-added glycated sites in glycated ovalbumin with preheating treatment are shown in bold.

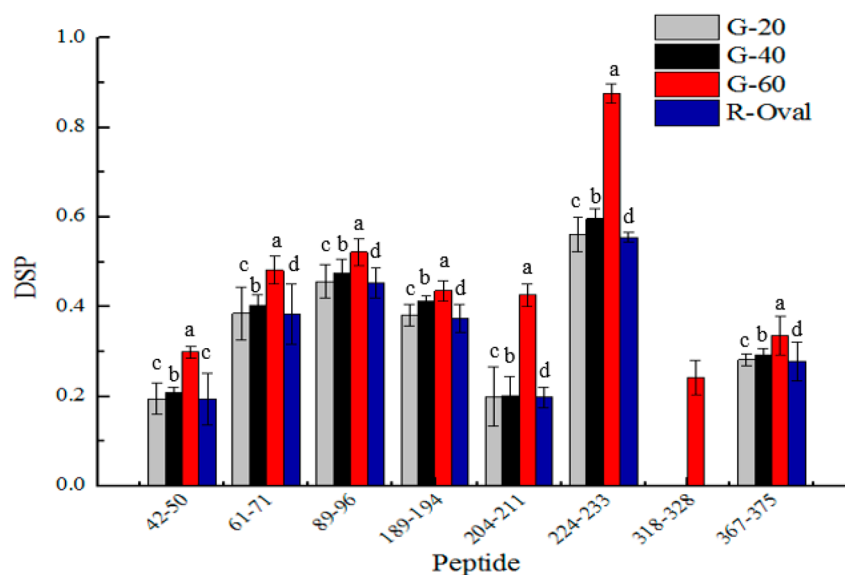


Figure 3. DSP values of each of the glycated peptides in R-Oval (blue bars), G-20 (slashed bars), G-40 (black bars), and G-60 (red bars) (G-20, G-40, and G-60, the ovalbumin glycation with the preheating temperature at 20, 40, and 60 °C, respectively). Different letters (a–d) on the top of the bars are significantly different ($p < 0.05$).

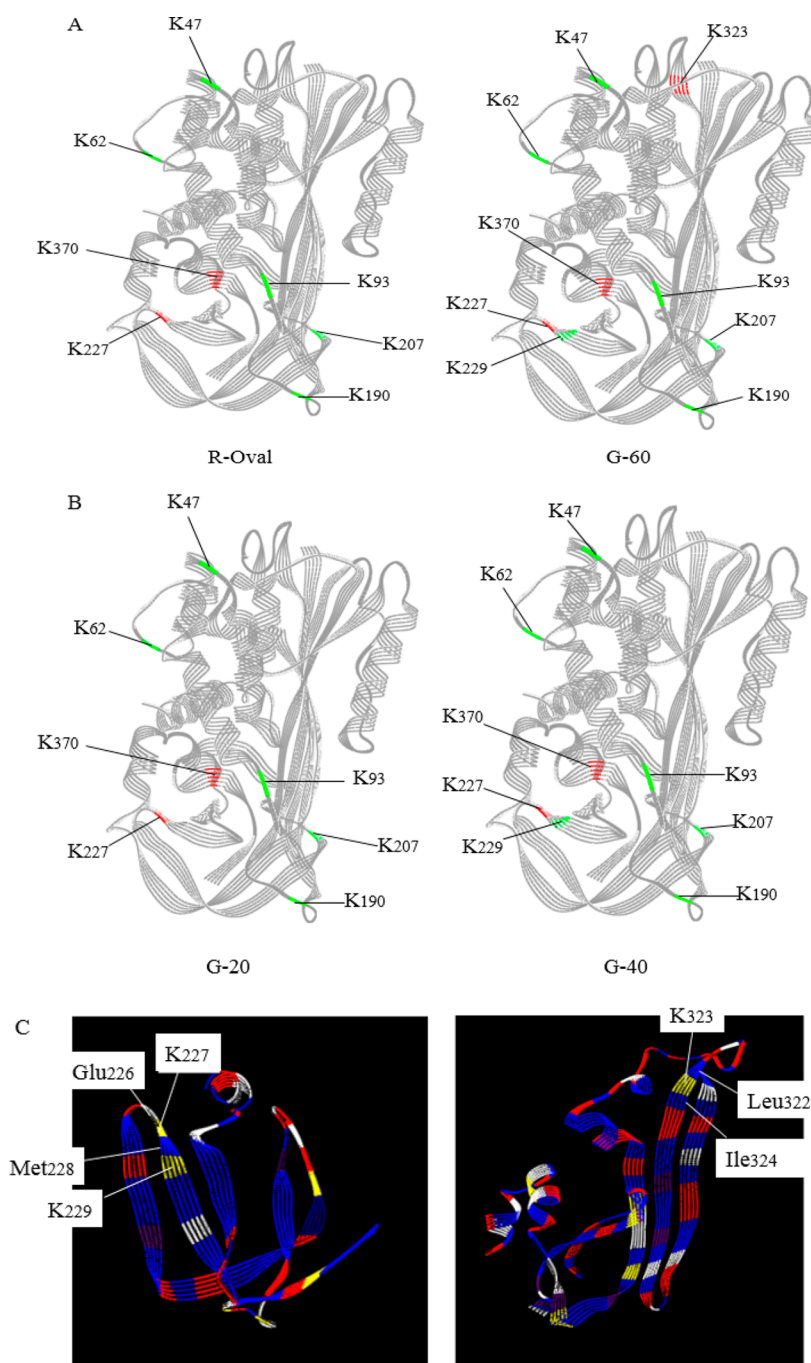


Figure 4. Ribbon diagram (PDB: 1JT1) in R-Oval and G-60 (A) and in G-20 and G-40 (B). The partial amino acid residues in G-60 (C). The lysine residues are color coded as follows: gray = the framework of the ovalbumin; red = high glycated amino acid residue content; green = low glycated amino acid residue content. In the partial ribbon diagram, the color code is as follows: yellow = lysine residue; red = hydrophilic amino acid residue; white = acid amino acid residue; blue = hydrophobic amino acid residue; purple = basic amino acid residue (G-20, G-40, and G-60, the ovalbumin glycation with the preheating temperature at 20, 40, and 60 °C, respectively).

peptide were detected in G-20 from the mass spectrometry (MS) scan. However, G-40 had eight glycated sites, including K47, K62, K93, K190, K207, K227, K229, and K370. This indicated that the conformation of ovalbumin was expanded by preheating treatment at 40 °C. Interestingly, compared to R-Oval, two new glycated sites named K229 and K323 appeared in G-60. A possible reason for this was that with the increase of preheating temperature, the ovalbumin underwent a deeper glycation modification.

Glycation Extent Analysis. To further understand the changes in the conformation of the glycated ovalbumin, the number of glycated sites and the glycated degree per glycated site were evaluated. Compared to R-Oval, the result in Table 1 showed that preheating treatment at 20 °C did not increase the number of glycated sites. Therefore, it is essential to compute the DSP value to assess the glycated degree of the ovalbumin samples. The situation of DSP of the glycation peptides obtained by glycated ovalbumin with and without preheating treatment were displayed in Figure 3. According to the DSP

value, K227 of peptide 224–233 is the most reactive ribose glycosylated site in G-20, with a DSP value of 0.586. The possible reason was that K227 is located in the outer exterior of the hydrophilic area in the 3D conformation of the ovalbumin model, which is composed of an amount of hydrophilic amino acids (see Figure 4B and C). In addition, K227 had an adjacent hydrophilic amino acid named Glu226, which can catalyze lysine during glycation.²⁶ Ribose could easily enter the hydrophilic region and undergo glycation reaction with lysine. The equivalent DSP value occurred in R-Oval. However, the DSP value of K227 and K229 of peptide 224–233 in G-40 is higher than that in R-Oval. Meanwhile, it is obvious that G-60 had the highest DSP values (0.895), higher than R-Oval, G-20 and G-40, which were significantly enhanced by preheating treatment at 60 °C as a whole. Moreover, the peptide (318–328) was unglycosylated in N-Oval. Interestingly, it had a high DSP value of 0.278 in the glycosylated sample with preheating treatment at 60 °C for 1 h.

IgG/IgE-Binding Abilities Analysis. In this study, the IgG/IgE-binding capabilities of ovalbumin samples were measured by indirect competitive ELISA, respectively. As shown in Figure 5A, the IgG-binding capability of N-Oval and R-Oval was 984.31 and 602.42 $\mu\text{g/mL}$, respectively, and an obvious reduction of the IgG-binding ability was noticed after glycation modification. H-Oval displayed nearly the same IgG binding ability to N-Oval, while C-20, C-40, and C-60 had a very slight increase of IgG binding ability as compared to N-Oval. However, G-20 and G-40 had a much higher decrease (about 35.5% and 42.9%, respectively) of IgG binding ability as compared to N-Oval. In particular, G-60 demonstrated only about 41% of the IgG binding ability of N-Oval. Interestingly, the same trend was revealed in Figure 5B. H-Oval had almost the same IgE binding ability as N-Oval (1002.32 $\mu\text{g/mL}$), whereas it improved after preheating treatment. However, it decreased by approximately 40% after glycation with ribose, and the further reduction of the IgG-binding ability of G-40 and G-60 were observed (about 44% and 59.7%, respectively).

Previous studies have demonstrated that glycation modification could obviously decrease the IgG/IgE-binding capabilities of the protein, such as ovalbumin and β -lactoglobulin.^{35,36} In this study, we found that preheating treatment could enhance the extent of glycation and improve the number of the glycosylated sites. Taking into account our assumptions, the apparent decrease of IgG/IgE-binding ability of ovalbumin after combination with ribose was due to the masking up of the special sites of allergen epitopes via glycation. Our study indicated that the structure of ovalbumin was changed more deeply with enhancing the preheating treatment temperature, which could increase the glycation degree and glycosylated sites of ovalbumin. In particular, G-60 had the best effect to enhance the glycation modification and reduce IgG/IgE-binding capabilities of ovalbumin. Therefore, the conformation of ovalbumin might be subjected to extension change that increased the glycation degree of the allergen epitopes.

In this study, the conformational changes of ovalbumin at the macro level was calculated by traditional methods. We found that glycation modification could induce the structure of ovalbumin more stable, which induced the improvement of β -turn and β -sheet contents. In addition, glycation modification could arouse exposure of Trp residue and cover some hydrophobic groups via the amino acid residues modification by covalent attachment of ribose, which caused an obvious

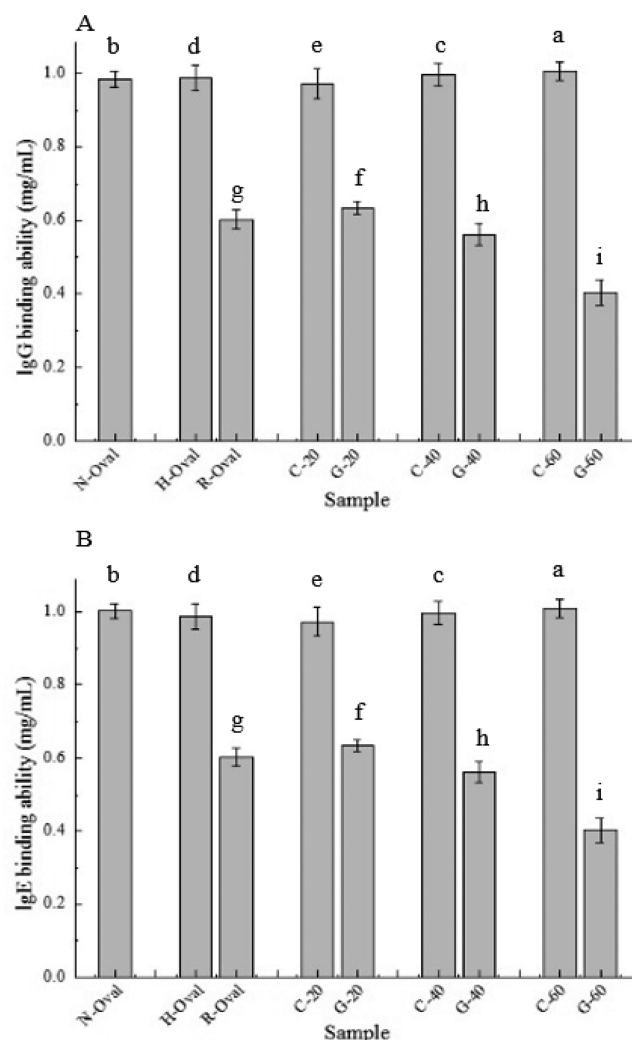


Figure 5. IgG- (A) and IgE-binding (B) abilities of ovalbumin samples were measured by indirect competitive ELISA. (N-Oval, native ovalbumin; R-Oval, the ovalbumin glycation without preheating treatment; H-Oval, the ovalbumin without ribose incubated at a temperature of 50 °C; C-20, C-40, and C-60, the preheating temperature at 20, 40, and 60 °C, respectively; G-20, G-40, and G-60, the ovalbumin glycation with the preheating temperature at 20, 40, and 60 °C, respectively). Different letters (a–i) on the top of the bars are significantly different ($p < 0.05$).

reduction of the surface hydrophobicity.³⁷ It demonstrated that the partial unfolding of ovalbumin structure by glycation may lead to the destruction of the structural IgG and IgE epitopes and induce the decrease of IgG/IgE-binding ability of ovalbumin. It is worth noting that preheating treatment strengthened the transformation in the secondary and tertiary structure of ovalbumin by enhancing glycation and further reducing the IgG/IgE-binding capability. Therefore, we speculate that the damage of structural epitopes created through preheating treatment conjugated with glycation modification was another important factor for the decrease of IgG- and IgE-binding ability of ovalbumin and decreased the H_0 value also devoted to the reduction.

In order to further comprehend the mechanism of the reducing IgG/IgE-binding ability of ovalbumin occurring from glycation with preheating treatment, the structure and the precise glycosylated sites of the glycosylated ovalbumin with and without preheating treatment should be studied. HCD/

FTICR-MS was used to estimate the conformational changes of ovalbumin at a molecular level. As shown in Figure 4A and B, R-Oval and G-20 had seven glycosylated sites while G-40 had eight glycosylated sites, which were named K47, K62, K93, K190, K207, K227, K229, and K370. As expected, compared to G-40, a new glycosylation site appeared in G-60 (named K323), which induced a major decrease of the IgG/IgE-binding capabilities of ovalbumin. Furthermore, the glycosylation extent of ovalbumin was overall enhanced by preheating treatment (see Figure 3). Preheating treatment partially unfolded the conformation of ovalbumin, which induced the appearance of some potential glycosylated sites and enhanced the glycosylation level.

In addition, we also found that H-Oval had a slight effect on the IgG/IgE-binding ability. The possible reason was the movement limitation of the ovalbumin molecule as a result of the low water activity, resulting in insignificant changes in the secondary and tertiary structure of ovalbumin.³⁸ However, preheating treatment without integration with glycosylation insignificantly improved the IgG/IgE-binding capability of ovalbumin. Preheating treatment induced the conformation of ovalbumin to partially unfold, reflecting in the increase in β -sheet and β -turn contents and the reduction in the total amount of α -helix and random coil, intrinsic fluorescence intensity, and surface hydrophobicity. At present, studies have shown that linear IgG and IgE epitopes were transmitted through the entire amino acid sequence of ovalbumin.^{39,40} The partial unfolding aroused via preheating treatment may lead to the exposure of linear and structural IgG and IgE epitopes originally masked in ovalbumin molecules. Therefore, H-Oval was insignificantly enhanced.

In conclusion, this research has shown that the mechanism of preheating treatment could increase glycosylation of ovalbumin. Moreover, the IgG/IgE-binding capability of ovalbumin was dramatically reduced by preheating treatment combined with glycosylation because of the cover of IgG and IgE epitopes and the structural changes of ovalbumin. It revealed that preheating treatment advanced the reduction of IgG- and IgE-binding capabilities by enhancing glycosylation. The research also illustrated that HCD/FTICR-MS was an excellent method for analyzing the structural changes of the protein at a molecular level. Therefore, preheating treatment integrated with glycosylation may offer an effective way for ovalbumin to reduce sensitization. However, in the future research, other tests in vivo should be applied to investigate the reduced allergenicity via preheating treatment integrated with glycosylation.

■ ASSOCIATED CONTENT

● Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jafc.8b04165.

Peptide mapping of N-Oval (Table S1); information of EA patients (Table S2); and the content (in percent) of secondary structures of ovalbumin treated under different conditions (Table S3) (PDF)

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Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

This work was supported by the National Natural Science Foundation of China (21878135 and 21706111), the earmarked fund for the China Agriculture Research System (CARS-45), and the Excellent Youth Foundation of Jiangxi Province (20162BCB23017).

■ ABBREVIATIONS USED

EA, egg allergy; ANS, 8-anilino-1-naphthalenesulfonic acid; LC-HRMS, liquid chromatography and high-resolution mass spectrometry; HCD, high-energy C-trap dissociation; DSP, average degree of substitution per peptide molecule; N-Oval, native ovalbumin; C-20, the ovalbumin solution was heated at 20 °C for 1 h; C-40, the ovalbumin solution was heated at 40 °C for 1 h; C-60, the ovalbumin solution was heated at 60 °C for 1 h; G-20, the ovalbumin solution was heated at 20 °C for 1 h and then mixed with ribose in a ratio of 1:1 of ovalbumin, which was lyophilized, followed by incubation at a temperature of 50 °C with 60% humidity for 6 h; G-40, the ovalbumin solution was heated at 40 °C for 1 h and then mixed with ribose in a ratio of 1:1 of ovalbumin, which was lyophilized, followed by incubation at a temperature of 50 °C with 60% humidity for 6 h; G-60, the ovalbumin solution was heated at 60 °C for 1 h and then mixed with ribose in a ratio of 1:1 of ovalbumin, which was lyophilized, followed by incubation at a temperature of 50 °C with 60% humidity for 6 h; H-Oval, the ovalbumin without ribose incubated at a temperature of 50 °C with 60% humidity for 6 h; R-Oval, the ovalbumin was mixed with ribose at a mass ratio of about 1:1 of ovalbumin, followed by lyophilization, which was incubated at a temperature of 50 °C with 60% humidity for 6 h; ELISA, indirect enzyme-linked immunosorbent assay.

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Table S1 Peptide mapping of N-Oval

No.	m/z	MH+	MH+	Δ ppm	Start	End	Sequence
		experimental	theoretical				
1	404.2139	404.2139	404.214	-0.185	2	6	<u>(M)GSIGA(A)</u>
2	928.3891	928.3891	928.3903	-1.29	4	12	<u>(S)IGAASMEFC(F)</u>
3	527.2498	527.2498	527.25	-0.428	13	16	<u>(C)FDVF(K)</u>
4	536.3075	536.3075	536.3079	-0.699	16	19	<u>(V)FKEL(K)</u>
5	591.335	591.335	591.3357	-1.45	20	24	<u>(L)KVHHA(N)</u>
6	375.1873	375.1873	375.1874	-0.335	26	28	<u>(N)ENI(F)</u>
7	957.4573	957.4573	957.4573	0.0436	29	36	<u>(I)FYCPIAIM(S)</u>
8	492.2482	492.2482	492.2486	-0.905	37	41	<u>(M)SALAM(V)</u>
9	477.2507 ⁺²	953.4941	953.4938	0.299	42	50	<u>(M)VYLGAkdST(R)</u>
10	783.4163 ⁺²	1565.8253	1565.8282	-1.63	42	55	<u>(M)VYLGAkdSTRTOIN(K)</u>
11	522.6143 ⁺³	1565.8283	1565.8282	0.0962	42	55	<u>(M)VYLGAkdSTRTOIN(K)</u>
12	381.7343 ⁺²	762.4613	762.4621	-0.998	55	60	<u>(I)NKVVR(F)</u>
13	589.2904 ⁺²	1177.5735	1177.5735	-0.0181	61	71	<u>(F)DKLPGFGDSIE(A)</u>
14	547.2726	547.2726	547.2722	0.698	69	73	<u>(D)SIEAQ(C)</u>
15	656.3353	656.3353	656.3362	-1.39	76	81	<u>(G)TSVNVH(S)</u>
16	656.3353	656.3353	656.3362	-1.39	79	84	<u>(V)NVHSSL(R)</u>
17	379.7111 ⁺²	758.4149	758.4155	-0.797	85	90	<u>(L)RDILNQ(I)</u>
18	465.2374 ⁺²	929.4675	929.4687	-1.25	89	96	<u>(L)NQITKPN(D)</u>
19	515.2496	515.2496	515.25	-0.827	97	100	<u>(D)VYSF(S)</u>
20	646.3878	646.3878	646.3883	-0.697	101	106	<u>(F)SLASRL(Y)</u>
21	577.2973 ⁺²	1153.5873	1153.5888	-1.28	106	114	<u>(R)LYAEERYPI(L)</u>
22	844.4808	844.4808	844.4815	-0.808	113	119	<u>(Y)PILPEYL(Q)</u>
23	363.1699	363.1699	363.1697	0.641	119	121	<u>(Y)LQC(V)</u>
24	1083.5543 ⁺²	2166.1013	2166.1012	0.057	120	138	<u>(L)QCVKELYRGGLEPINFQTA(A)</u>
25	487.7484 ⁺²	974.4895	974.4901	-0.642	137	145	<u>(Q)TAADQARE(L)</u>
26	929.9858 ⁺²	1858.9643	1858.9658	-0.769	144	159	<u>(R)ELINSWVESQTNGIIR(N)</u>
27	613.3673 ⁺²	1225.7273	1225.7263	0.837	157	167	<u>(G)IIRNVLPSSV(D)</u>
28	575.2953 ⁺²	1149.5833	1149.582	1.15	166	176	<u>(S)SVDSQTAMVLV(N)</u>
29	416.2499	416.2499	416.2504	-1.11	175	178	<u>(V)LVNA(I)</u>
30	449.2755	449.2755	449.2758	-0.772	178	181	<u>(N)AIVF(K)</u>
31	431.7630 ⁺²	862.5187	862.5185	0.217	179	185	<u>(A)IVFKGLW(E)</u>
32	533.2711	533.2711	533.2718	-1.36	185	188	<u>(L)WEKA(F)</u>
33	477.2137 ⁺²	953.4201	953.4211	-0.99	188	195	<u>(K)AFKDED(TQ)</u>
34	377.6659 ⁺²	754.3245	754.3254	-1.13	189	194	<u>(A)FKDED(TQ)</u>
35	603.7996 ⁺²	1206.5919	1206.5936	-1.36	195	204	<u>(T)QAMPFRVTEQ(E)</u>
36	408.2166 ⁺²	815.4259	815.4258	0.202	204	210	<u>(E)QESKPVQ(M)</u>
37	473.7371 ⁺²	946.4669	946.4662	0.719	204	211	<u>(E)QESKPVQ(M)</u>
38	572.2199	572.2199	572.2207	-1.43	211	214	<u>(Q)MMYQ(I)</u>

39	740.397	740.397	740.3978	-1.02	213	218	<u>(M)YQIGLF(R)</u>
40	355.6866 ⁺²	710.3659	710.3654	0.727	218	223	<u>(L)FRVASM(A)</u>
41	581.3309 ⁺²	1161.6545	1161.6548	-0.214	224	233	<u>(M)ASEKMKILEL(P)</u>
42	642.3050 ⁺²	1283.6027	1283.601	1.33	232	243	<u>(L)ELPFASGTMSML(V)</u>
43	362.2103	362.2103	362.2108	-1.39	242	244	<u>(S)MLV(L)</u>
44	1041.5815	1041.5815	1041.5827	-1.11	243	252	<u>(M)LVLLPDEVSG(L)</u>
45	646.3398	646.3398	646.3406	-1.29	251	256	<u>(V)SGLEQL(E)</u>
46	835.456	835.456	835.456	0.00486	256	262	<u>(Q)LESINF(E)</u>
47	536.3075	536.3075	536.3079	-0.699	262	265	<u>(N)FEKL(T)</u>
48	649.3194	649.3194	649.3192	0.357	265	269	<u>(K)LTEWT(S)</u>
49	824.361	824.361	824.3607	0.345	268	274	<u>(E)WTSSNMV(E)</u>
50	507.2125	507.2125	507.2119	1.13	273	276	<u>(N)VMEE(R)</u>
51	619.3812	619.3812	619.3814	-0.282	280	284	<u>(I)KVYLP(R)</u>
52	662.3479	662.3479	662.3476	0.38	284	288	<u>(L)PRMKM(E)</u>
53	536.2386	536.2386	536.2385	0.233	288	291	<u>(K)MEEK(Y)</u>
54	477.2507 ⁺²	953.4941	953.4938	0.299	290	297	<u>(E)EKYNLTSV(L)</u>
55	575.3038 ⁺²	1149.6003	1149.6006	-0.265	293	303	<u>(Y)NLTSVLMAMGI(T)</u>
56	742.3257	742.3257	742.3254	0.437	304	310	<u>(I)TDVFSSS(A)</u>
57	578.2775	578.2775	578.278	-0.919	309	314	<u>(S)SSANLS(G)</u>
58	521.256	521.256	521.2566	-1.09	314	319	<u>(L)SGISSA(E)</u>
59	566.8149 ⁺²	1132.6225	1132.6208	1.5	318	328	<u>(S)SAESLKISQAV(H)</u>
60	581.2852 ⁺²	1161.5631	1161.5647	-1.37	327	337	<u>(Q)AVHAAHAEINE(A)</u>
61	487.7484 ⁺²	974.4895	974.4901	-0.642	338	347	<u>(E)AGREVVGSAAE(A)</u>
62	490.214	490.214	490.2144	-0.752	347	351	<u>(A)EAGVD(A)</u>
63	563.2665	563.2665	563.2671	-1.12	352	357	<u>(D)AASVSE(E)</u>
64	639.8171 ⁺²	1278.6269	1278.6266	0.266	358	367	<u>(E)EFRADHPFLF(C)</u>
65	466.7552 ⁺²	932.5031	932.5022	0.953	367	374	<u>(L)FCIKHIAT(N)</u>
66	523.7765 ⁺²	1046.5457	1046.5452	0.537	367	375	<u>(L)FCIKHIATN(A)</u>
67	449.2755	449.2755	449.2758	-0.772	376	379	<u>(N)AVLF(F)</u>
68	426.2381	426.2381	426.2387	-1.48	378	380	<u>(V)LFF(G)</u>
69	383.1887 ⁺²	765.3701	765.3712	-1.43	380	386	<u>(F)FGRCVSP(-)</u>

Table S2 Information of EA patients

No.	Age (year)	Gender	Clinical symptoms	Other allergy	IgE (kU _A /L)
1	53	Female	EC, UR	Milk allergy	32.7
2	17	Male	AR	Wheat allergy	24.4
3	30	Male	AR, UR	Most food allergy	23.9
4	8	Female	CW	None	15.8

5	25	Male	CW, EC	Animal allergy	70.8
6	44	Female	UR, EC	Animal allergy	92.7

EC, eczema; UR, urticaria; AR, allergic rhinitis; CW, chest wheezing

Table S3 The content (%) of secondary structures of ovalbumin treated under different conditions.

samples	α -helix (%)	β -sheet (%)	β -turn (%)	random coil (%)
N-Oval	30.3 ± 0.2^a	28.1 ± 0.1^c	14.3 ± 0.5^b	27.3 ± 0.4^a
H-Oval	30.6 ± 0.4^a	28.0 ± 0.3^c	14.4 ± 0.3^b	27.0 ± 0.2^a
R-Oval	25.4 ± 0.3^c	34.3 ± 0.5^a	18.6 ± 0.4^a	21.7 ± 0.2^b
C-20	29.7 ± 0.1^a	28.7 ± 0.3^c	14.4 ± 0.3^b	27.2 ± 0.3^a
C-40	29.5 ± 0.4^a	28.6 ± 0.3^c	14.6 ± 0.5^b	27.3 ± 0.4^a
C-60	29.2 ± 0.5^a	29.3 ± 0.2^c	14.9 ± 0.4^b	26.6 ± 0.7^a
G-20	26.6 ± 0.3^b	32.7 ± 0.1^b	18.8 ± 0.3^a	21.9 ± 0.4^b
G-40	25.4 ± 0.2^c	34.8 ± 0.2^a	19.1 ± 0.1^a	20.7 ± 0.3^c
G-60	24.5 ± 0.3^c	35.7 ± 0.3^a	19.7 ± 0.2^a	20.1 ± 0.5^c

Values followed by different letter (a-c) in the same column are significantly different ($p < 0.05$).