

Effects of deglycosylation and the Maillard reaction on conformation and allergenicity of the egg ovomucoid

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Abstract: Ovomucoid (OVM), known as the major allergen in egg white, has gained increasing concerns in industrialized countries. Here, we found the deglycosylation and Maillard reaction with galactooligosaccharide (GOS) and fructooligosaccharide (FOS) can induce conformational transformation of OVM from other structures (β -turn, strand, and random coils) to α -helix. We also introduced an approach to reduce the allergenicity of *Gallus domesticus* OVM by Maillard reaction with GOS and FOS. However, the OVM glycosylated by mannan (MOS) and deglycosylated OVM exhibited higher allergenicity than native OVM. Therefore, GOS and FOS, especially GOS, could be applied in the reduction of the potential allergenicity of OVM through glycation. Furthermore, these findings may provide new insights into the development of hypoallergenic egg products.

Practical Application: In this study, the allergenicity and conformation of OVM treated with deglycosylation and glycation (GOS, FOS, and MOS) were investigated. The results would provide a better understanding of the effects of deglycosylation and Maillard reaction with different reducing sugars on the molecular characteristics of OVM and further provide new insights into the development of hypoallergenic egg products.

KEYWORDS

allergenicity, deglycosylation, food safety, Maillard reaction, ovomucoid

1 | INTRODUCTION

Egg is a valuable ingredient in food manufacturing due to its nutritional compositions, including proteins, lipids, vitamins, and minerals. However, the egg is the leading cause of food allergy among young children, affecting 0.9% of all children, 1.3% of children 5 years in the United States (Samady et al., 2020), and over 3.2% of infants in Australian (Sánchez & Sánchez, 2019). Exposing sensitized individuals to eggs can induce IgE-mediated hypersensitivity, which may trigger adverse reactions, such as asthma, urticaria, gastrointestinal spasms, hypotension, and even anaphylaxis. To date, effective prevention for allergic indi-

viduals focuses on a complete avoidance of egg-containing food products. However, it is difficult to completely avoid the ingestion of eggs because of their wide application in many foods. Hence, the development of food processing technologies for reducing the allergenicity of egg allergens is extremely desirable.

Nowadays, several processing methods have been developed to decrease the allergenicity of allergens in eggs, including thermal processing, high-pressure processing, and chemical treatments (Y. Zhu et al., 2018). Most egg allergens such as ovalbumin, ovotransferrin, avidin, and lysozyme were affected on structure and antigenicity under heat treatment, high-pressure processing, and

chemical treatments (e.g., acetic acid treatment and enzymatic hydrolysis). However, ovomucoid (OVM), the most allergenic protein in egg, is a heat-stable glycoprotein containing 186 residues and a molecular weight of 28 kDa. Because of its unique property, the allergenicity and structure of OVM remain stable under thermal treatment, high-pressure processing, and acetic acid treatment (Julià et al., 2007; Lee et al., 2017; Odani et al., 2007). Thus, with the purpose of reducing its allergenicity, an effective, user-friendly, and low-cost food-processing method is required.

The Maillard reaction (also referred as glycation), one of the most common reactions used in the food industry, is attracting a lot of attention for improving protein functionality and obtaining newly modified proteins with high technological interest (Nooshkam et al., 2019). The Maillard reaction is a complex series of chemical and non-enzymatic browning reactions between the ϵ -amino group of amino acid residues and the carbonyl groups (H.-Y. Wang et al., 2011). Generally, the Maillard reaction (glycation) occurs between proteins and reducing sugars, further modifying protein structure, eventually affecting the allergenic potential. Recent studies highlighted the importance of this reaction in the allergenicity of food allergens; however, contradictory results have been reported about its effect on the IgE-binding ability of food allergens. For example, the IgG/IgE binding properties of silver carp parvalbumin were weakened after the Maillard reaction (Zhao et al., 2017), while the IgE-binding of glycated codfish parvalbumin was enhanced (de Jongh et al., 2013). One possible explanation for these may be that glycation-induced changes in protein conformation depend on the properties of allergenic proteins, conjugated carbohydrates, and incubating conditions. Moreover, a recent study shows that the influence of glycation on the allergenicity of allergenic proteins is heavily dependent upon the kinds of reducing sugars (Rao et al., 2018). Gruber et al. reported that the allergenicity of cherry allergen Pru av 1 can be reduced through glycation by glucose (Gruber et al., 2004). Fu et al. (2019) found the allergenicity of shrimp tropomyosin was significantly reduced after the Maillard reaction with galactooligosaccharide (GOS). Hence, it is difficult to predict the effects of glycation with different reducing sugars and deglycosylation on the conformation and allergenicity of allergenic proteins.

In this work, we first purified OVM from *Gallus domesticus* and further identified types of glycans in OVM. Moreover, the allergenicity and conformation of deglycosylated and glycated OVM proteins were investigated. The results would provide a better understanding of the effects of deglycosylation and glycation with different reducing sugars on the molecular characteristics of OVM including conformation stability and allergenicity.

2 | MATERIALS AND METHODS

2.1 | Materials and chemicals

Hydroxymethyl aminomethane (Tris), dodecyl sodium sulfate (SDS), sodium chloride, hydrochloric acid (HCl), sodium tetraborate, β -mercaptoethanol, and reducing sugars (GOS, FOS, and MOS) were purchased from Aladdin, Los Angeles, southern California. All chemicals used were of analytical grade. The DEAE-Sepharose fast flow was purchased from General Electric Company (Fairfield, CT, USA). LAD2 cells were obtained from ATCC (Rockefeller, MD, USA). Eggs were purchased from a local supermarket (CenturyMart, Hangzhou, China). All solutions were prepared using ultrapure water (18.2 M Ω) purified using a Milli-Q system (Millipore, Billerica, MA, USA).

Human sera from volunteer patients sensitized to egg white were taken from PlasmaLab (USA) and listed in Table 1. The sIgE values of all sera were determined by ImmunoCAP 100 (Phadia, Sweden). All patients had sIgE to egg white > 0.5 kU/L. Human serum samples from healthy subjects (negative in sIgE with egg white) were obtained from PlasmaLab.

2.2 | Purification and identification of OVM

Briefly, egg white was first separated from the yolk and diluted with isopycnic 10% trichloroacetic acid. After adjusting pH to 3.5, the mixture was allowed to stand for 4 hr, followed by centrifugation at 3000 r/min for 10 min at 4°C (Beckman Coulter, Mississauga, ON, USA). The pH of supernate was adjusted to 3.5 and mixed well with acetone (1/3, v/v) with a slow stirring before standing 4 hr at 0°C. After centrifugation at 3000 r/min for 10 min at 4°C, the precipitant was dried using a vacuum dryer and then dissolved by 20 ml of ultrapure water as a crude extract. The crude extract was then eluted at 1 ml/min by linear gradients using 0.05 M Tris-HCl buffer (pH 9.0) containing 0.3 M NaCl in DEAE F.F. column by AKTApure 25 (General Electric Company, Boston, MA, USA). The protein fractions were pooled and then dialyzed against distilled water several times. Afterward, the OVM was lyophilized and stored at -20°C. The purified OVM was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Each protein sample was dissolved in 5 $\times$ loading buffer (Sangon Biotech, Shanghai, China) and then heated at 100°C for 5 min in a boiling water bath. Protein samples were then loaded into the gel lanes composed of a 12% separating gel and 5% stacking gel. The gel was electrophoresed at a constant voltage of 120 V in

TABLE 1 Characterization of positive sera from patients sensitized to egg white

No.	Ethnicity	Gender	Age	Symptoms	sIgE (kU/L)
1	Caucasian	Male	51	Allergic rhinitis	30
2	Caucasian	Female	49	Allergic rhinitis	12.4
3	Caucasian	Male	36	Allergic rhinitis	7.62
4	Caucasian	Female	63	Allergic rhinitis	42.3
5	Caucasian	Male	19	Allergic rhinitis	26.3
6	Caucasian	Male	31	Allergic rhinitis	3.81
7	Caucasian	Male	36	Allergic rhinitis	4.08

running buffer (0.025 M Tris, 0.2 M glycine, and 0.1% SDS) using a PowerPac (Bio-Rad Lab, Hercules, CA, USA). The SDS-PAGE gel was stained overnight with 0.025% (w/v) Coomassie Brilliant Blue R250 in 40% (v/v) methanol and 10% (v/v) acetic acid and destained in a 40% (v/v) methanol and 10% (v/v) acetic acid solution.

The single band was cut from the SDS-PAGE gel and then hydrolyzed with trypsin at 37°C for 16 hr. After desalting on a C18 tip column, the enzymatic peptides were processed with vacuum freeze-drying. Afterward, the tryptic peptides were dissolved in solvent A (0.1% formic acid) and directly loaded onto a C18 reversed-phase analytical column. The gradient consisted of an increase from 4% to 25% solvent B (100% acetonitrile) over 80 min. Then, 25–35% solvent B was eluted for 4 min, climbed to 90% for 2 min, and held at 80% for the last 2 min. All procedures were performed at a flow rate of 300 nl/min on an EASY-nLC 1000 UPLC system (Thermo Scientific, New York, NY, USA). Then, the tryptic peptides were analyzed by a mass spectrometer (LTQ Orbitrap ETD; Thermo Fisher, Waltham, MA, USA). The mass spectrometry parameters were set as follows: spray voltage, 2.20 kV; *m/z* scan range, 350–1600; resolution of Orbitrap, 30,000; fragmentation type, HCD. The search parameters were set as follows: allowance, up to 2 missed cleavage; precursor and fragment errors, 0.02 Da. The protein identification was obtained using Proteome Discoverer 1.4 (Thermo) based on homology searches, which are against all the sequences in the UniProt-Homo sapiens databases.

2.3 | Glycan analysis of OVM

2.3.1 | O-Glycan

Note that 9 ml of OVM (0.2 mg/ml) was mixed with 10 ml of 0.5 M sodium hydroxide and incubated at 37°C for 16 hr. Then, the pH of the treated OVM sample was adjusted to 7.0 using 1 M hydrochloric acid, followed by SDS-PAGE electrophoresis and UV-vis analysis via a UV2600 UV-vis spectrophotometer (Shimadzu, Kyoto, Japan).

2.3.2 | N-Glycan

The purified OVM was dissolved in 50 mM sodium bicarbonate at 1 mg/ml and then was mixed with 3 µl of PNGase F (5 u/µl) for 8 hr at 37°C to destroy their carbohydrate structures. The treated OVM was then separated by ultrafiltration using 10 kDa centrifugal filter devices (Millipore, Burlington, MA, USA) at 4000 × *g* for 15 min at 4°C, followed by SDS-PAGE electrophoresis.

2.4 | Preparation of deglycosylated and glycosylated OVM

Preparation of deglycosylated OVM was carried out according to Z. Zhang, Xiao, Zhang, et al. (2019). The 200 µg of OVM was mixed with 10 µl of 10 × glycoprotein denaturing buffer and 90 µl of ultrapure water, followed by heating in a water bath for 10 min. After cooling to 25°C, 20 µl of 10 × glycoBuffer 2, 20 µl of 10% NP-40, 10 µl of PNGase F, 20 µl of α2-3,6,8 neuraminidase, ultrapure water, and 20 µl O-glycosidase were added to reach 200 µl. After incubation at 37°C for 18 hr, the deglycosylated OVM was separated by ultrafiltration using 10 kDa centrifugal filter devices at 4,000 × *g* for 15 min at 4°C. The deglycosylated OVM was then dialyzed in ultrapure water (10 kDa; Bio Basic Inc., Amherst, NY, USA), lyophilized, and stored at −20°C.

Preparation of glycosylated OVM was carried out according to Z. Zhang et al. (2020). The purified OVM was dissolved in a solution (1 mM sodium bicarbonate and 5 mM β-mercaptoethanol) containing fructooligosaccharide (FOS), galactooligosaccharide (GOS), or mannan (MOS) (10.8%, w/v) at 0.2 mg/ml. After lyophilizing, mixtures of OVM and functional oligosaccharides were incubated at 60°C for 48 hr to form OVM-FOS, OVM-GOS, and OVM-MOS, respectively. The glycosylated OVM was then dialyzed in ultrapure water, lyophilized, and stored at −20°C.

Native, glycosylated, and deglycosylated OVM were analyzed by SDS-PAGE electrophoresis. The molecular masses of proteins were calculated by AlphaView SA 3.4.0 software.

2.5 | Glycation degree of glycated OVM

2.5.1 | o-Phthalaldehyde assay

To investigate the free amino groups of OVM proteins, an o-phthalaldehyde (OPA) assay was carried out according to Iwan et al. (2011). The OPA reagent was prepared by mixing OPA (80 mg in 2 ml ethanol), sodium tetraborate (5 mmol), SDS (1 g), and β -mercaptoethanol (0.2 ml) into 100 ml and used within 3 hr. Note that 200 μ l of OVM proteins were then mixed well with 4 ml OPA reagent before incubating at 25°C for 5 min. After that, the optical density of each sample was measured at 340 nm with a microplate reader (Spectra Max i3x; Molecular Devices, San Jose, CA, USA). Native OVM was regarded as a control with 100% free amino groups.

2.5.2 | Available lysine content assay

The available lysine content was measured according to Fu et al. (2019). The OVM proteins (10 mg) were mixed well with 100 μ l of sodium acetate solution (16.4%, w/v) and vortex-shake for 20 min. Then, 10 μ l of propionic anhydride was added and shaken for 20 min. Note that 2 ml of dye solution (0.135% AO-12 in buffer solution [2% oxalic acid dehydrate, 0.34% potassium dihydrogen phosphate, and 6% glacial acetic]) was added into the mixture and shaken for 60 min to form a protein–dye complex. After centrifugation at 5000 g for 10 min, the absorbance of diluted supernate was measured at 475 nm, with the buffer solution as blank.

2.6 | Secondary structure of OVM proteins

The secondary structure of native, glycated, and deglycosylated OVM proteins was determined using a J-1500 CD spectrometer (Jasco, Tokyo, Japan). The freeze-dried OVM proteins were dissolved in 10 mM phosphate buffer (pH 7.2) at 0.1 mg/ml and then transferred in a cell of 0.1 cm path length. Each sample was scanned from 190 to 240 nm. Secondary structure data were analyzed using the DichroWeb (<http://dichroweb.cryst.bbk.ac.uk>).

2.7 | IgE-binding and biological activity analysis of OVM proteins

2.7.1 | Indirect ELISA

The OVM proteins (native, glycated, and deglycosylated OVM) were immobilized in 96-well microtiter plates

overnight at 4°C. After washing three times, each well was blocked with 5% BSA for 1 hr at 37°C. After washing three times, anti-egg white IgE antiserum (pooled sera from seven patients) was added to each well and incubated with 100 μ l of for 1.5 hr at 37°C. After washing three times, 100 μ l of peroxidase-conjugated goat anti-human IgE antibodies (Sigma-Aldrich, Munich, Germany) was added into each well, followed by incubating for 1.5 hr at 37°C. Then, 100 μ l of TMB solution was added to the plate and incubated for 15 min at 37°C, and the color development reaction was stopped by 50 μ l of 2 M H₂SO₄ solution. The optical density of each well was measured at 450 nm with a microplate reader (Spectra Max i3x; Molecular Devices).

2.7.2 | Competitive ELISA (icELISA)

The native OVM was immobilized in 96-well microtiter plates overnight at 4°C, followed by washing with 200 μ l of PBS and incubating with 200 μ l of 5% BSA for 2 hr at 25°C. Then, 100 μ l of OVM proteins (deglycosylated and glycated OVM proteins) at different concentration (0.01, 0.05, 0.1, 0.2, 0.5, and 1 μ g/ml) were mixed with 100 μ l of anti-egg white IgE antiserum with a dilution ratio of 100 times in another plate for 1.5 hr at 37°C. Afterward, a 200 μ l mixture of OVM proteins and antiserum was added and incubated with native OVM for 1 hr at 37°C. After washing three times, 100 μ l of peroxidase-conjugated goat anti-human IgE antibodies were added into each well, followed by incubating for 1.5 hr at 37°C. Then, 100 μ l of TMB solution was added to the plate, followed incubation for 15 min at 37°C. Color development reaction was stopped by 50 μ l of 2 M H₂SO₄ solution. The optical density of each well was measured at 450 nm with a microplate reader. The results were expressed as OD/OD₀, where OD is the mean absorbance of the known concentration of inhibitor, and OD₀ represents the maximal absorbance obtained in the absence of inhibitor.

2.7.3 | Degranulation assay

The LAD2 human mast cells were cultured in serum-free RPMI 1640 medium (GIBCO, Los Angeles, southern California) supplemented with 9% (v/v) fetal bovine serum and 1% (v/v) penicillin-streptomycin. Cells (1 $\times$ 10⁶ cells/ml) were incubated in a 24-well plate with 10 μ l of anti-egg white IgE antiserum (2 ng/ml) in triplicate for 2 hr at 37°C. After centrifugation at 900 $\times$ g for 5 min at 4 °C, cell pellets were washed and resuspended in 4-(2-hydroxyethyl)–1-piperazineethanesulfonic acid (HEPES) buffer (10 mM HEPES, 137 mM NaCl, 2.7 mM KCl, 0.38 mM

FIGURE 1 Identification of purified protein by LC-MS/MS

Purified protein	FPNATDKEG	
<i>Gallus domesticus</i>	MAMAGVFVLF SFVLCGFLPD AAFGAEVDCS RFPNATDKEG	40
Purified protein	KDVLVCNK	
<i>Gallus domesticus</i>	KDVLVCNKDL RPICGTDGVT YTNDCLLCAY SIEFGTNISK	80
Purified protein	VMVL CNRAFPVCG	
<i>Gallus domesticus</i>	EHDGECKETV PMNCSSYANT TSEDGKVMVL CNRAFPVCG	120
Purified protein	TDGVITYDNEC LLCAHKVEQG ASVDRKRDGG CRKELAAVSV	
<i>Gallus domesticus</i>	TDGVITYDNEC LLCAHKVEQG ASVDRKRDGG CRKELAAVSV	160
Purified protein	DCSEYKPKDC TAEDRPLCGS DNKTYGNKCN FCNAVVESNG	
<i>Gallus domesticus</i>	DCSEYKPKDC TAEDRPLCGS DNKTYGNKCN FCNAVVESNG	200
Purified protein	TLTSLSHFGK	
<i>Gallus domesticus</i>	TLTSLSHFGKC	210

Na₂HPO₄·7H₂O, 5.6 mM glucose, 1.8 mM CaCl₂·H₂O, 1.3 mM MgSO₄·7H₂O, 0.4% bovine serum albumin [BSA], pH 7.4). Then, the cell suspensions were stimulated with OVM proteins (native, deglycosylated, and glycosylated OVM proteins) for 30 min at 37°C. Meanwhile, the cells were supplemented with keyhole limpet hemocyanin (KLH) as a negative control. After centrifugation at 900 × g for 5 min at 4°C, the supernatants were collected to determine the β-hexosaminidase levels in the cell supernatants according to Y. Wang et al. (2019).

2.8 | Statistical analysis

All determinations were performed at least in triplicate. Statistical significance of the data was determined by one-way analysis of variance using SPSS software (19.0, SPSS Inc., Chicago, IL, USA). A *p*-value of < 0.05 was considered statistically significant. GraphPad Prism version 7.0, Origin 2018 was used for data analysis.

3 | RESULTS AND DISCUSSION

3.1 | Purification and identification of OVM

The OVM was purified from *Gallus domesticus* using column chromatography on a DEAE-Sephacel. As shown in Figure S1, two elution peaks were observed after crude OVM was injected into a DEAE-Sephacel column in the chromatographic profile. In fractions of elution peak 2, eluted at 0.1 M NaCl, a single band with an apparent molecular mass of around 30 kDa was observed in SDS-PAGE

electrophoresis. The protein band was successfully authenticated as OVM through LC/MS-MS. As shown in the total ion chromatogram (Figure S2), multiple peaks ranging from 5 to 117 min were observed and further analyzed and confirmed by MS/MS. The effective peaks were compared with homologous proteins in the entire NCBI protein. As shown in Figure 1 and Table S1, the purified protein exhibited 35 peptides matched with OVM sequences in *Gallus domesticus*. Hence, the purified protein was confirmed as OVM in *Gallus domesticus* (Genbank accession number: P01005).

Generally, two types of glycans, O-glycans and N-glycans, are observed in glycoproteins. Currently, five N-glycosylation sites of OVM have been found on asparagine residues with a sequence of Asn-X-Ser/Thr (Hägglund et al., 2004). To investigate the type of glycans in OVM, PNGase F and β-elimination were applied to release N-glycans and O-glycans of OVM, respectively. Figure 2A shows the molecular weight of native OVM and that of enzymatically and chemically deglycosylated OVM. Compared to the native OVM (lane 1), the molecular weight of de-N-glycosylated OVM (OVM-DNG, lane 2) had an obvious reduction, showing that OVM had a molecular weight of N-glycans. Besides, the molecular weight of the de-O-glycosylated OVM (OVM-DOG, lane 3) was also decreased. The OVM-DOG treated by NaOH and native OVM was then analyzed by UV-vis spectrum. The serine and threonine on O-glycans are unstable and easy to transform to α-amino acids under alkaline conditions, which had characteristic UV absorption at 240 nm (Breiteneder & Mills, 2005). As shown in Figure 2B, the UV absorbance at 240 nm of the OVM-DOG was significantly increased and UV absorption at 220 nm and 280 nm shifted compared with the native OVM, demonstrating that O-glycans existed in the OVM.

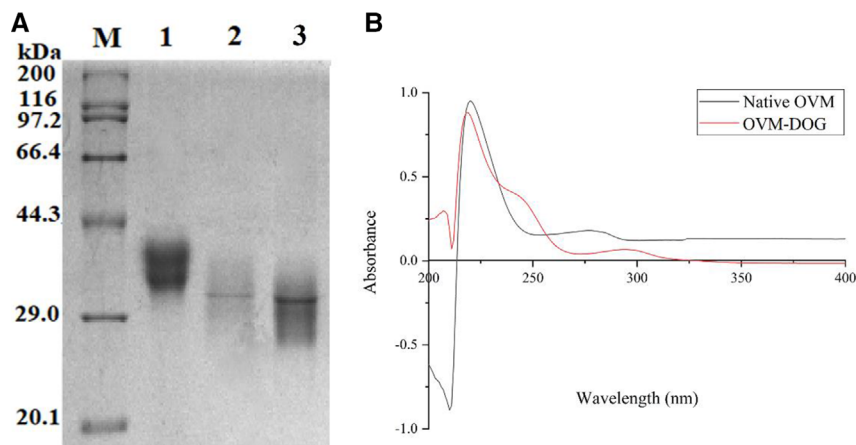


FIGURE 2 Characterization of native ovomucoid (OVM). (A) SDS-PAGE electrophoresis of OVM proteins. (B) UV-vis spectrum of O-deglycosylated OVM (OVM-DOG). Note A: M, marker; lane 1, native OVM; lane 2, N-deglycosylated OVM (OVM-DNG); lane 3, O-deglycosylated OVM (OVM-DOG)

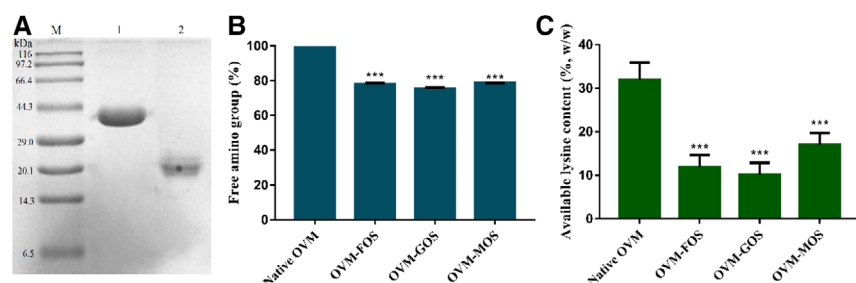


FIGURE 3 Characterization of deglycosylated and glycosylated ovomucoid (OVM) proteins. (A) SDS-PAGE electrophoresis of deglycosylated OVM. (B) The free amino group of glycosylated OVM proteins. (C) Available lysine content of glycosylated OVM proteins. Note A: M, marker; lane 1, native OVM; lane 2, N-&O-deglycosylated OVM. *Significant differences as compared with native OVM, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.005$. Error bars refer to standard deviation

3.2 | Glycoprotein detection

Deglycosylated OVM was prepared by removing the N-glycans and O-glycans of native OVM using PNGase F and O-glycosidase, respectively. As shown in Figure 3A, the molecular weight of N-&O-deglycosylated OVM (lane 2) was smaller than that of native OVM (lane 1), OVM-DNG (Figure 2A, lane 2), and OVM-DOG (Figure 2A, lane 3), showing N-&O-deglycosylated OVM was successfully obtained.

Glycation is a chemical reaction between free amino groups of protein and reducing sugars (Gupta et al., 2018). The main sites of protein glycation are the lysine side chain, arginyl guanidine groups, and N-terminal amino group of proteins (N. Zhang et al., 2018). Besides, lysine mostly participated in the generation of CML (N^{ϵ} -carboxymethyl lysine), a common marker of advanced glycation end products (AGEs), which are the products of protein glycation (Goldin et al., 2006). The purified OVM was glycosylated using FOS, GOS, and MOS. To assess the glycation degree of glycosylated OVM proteins, changes in the number of primary amino groups and available lysine residues contents were determined. Compared with native OVM, the OPA analysis showed an obvious decrease of primary amino groups in all glycosylated OVM proteins, with the proportions of the free amino groups of glycosylated OVM proteins reached nearly 80% (Figure 3B). Besides, a significant decrease of the available lysine content was observed

after glycation, compared with the native OVM. The available lysine content decrease was as follows: OVM > OVM-MOS > OVM-FOS > OVM-GOS (Figure 3C), showing a similar trend to the free amino groups. These results suggested that OVM was highly glycosylated by GOS, followed by FOS and MOS.

GOS and FOS are small saccharides with a low degree of polymerization ($DP \leq 3$), while MOS is considered as a saccharide with high DP ($DP \geq 3$). The small saccharides are easier to glycosylate protein than longer saccharides because smaller saccharides have less steric hindrance and higher accessibility to protein, resulting in a higher glycation degree of protein (Z. Zhang, Xiao, & Zhou, 2019). Z. Zhang et al. (2020) have found that the higher is glycation degree of glycosylated shrimp tropomyosin, the lower is molecular size of saccharides.

3.3 | Secondary structure of OVM proteins

The conformation analysis of deglycosylated OVM and glycosylated OVM proteins was determined by CD spectroscopy (Figure 4A). As shown in Figure 4B, native OVM consisted of α -helix (45.3%), β -turn (16.8%), strand (3.6%), and random coils (34.2%). After deglycosylation, deglycosylated OVM consisted of α -helix (68.7%), β -turn (10.3%), strand (0.6%), and random coils (20.3%).

FIGURE 4 Secondary structure of ovomucoid (OVM) proteins. (A) CD spectrums of OVM proteins. (B) Secondary structure of OVM proteins

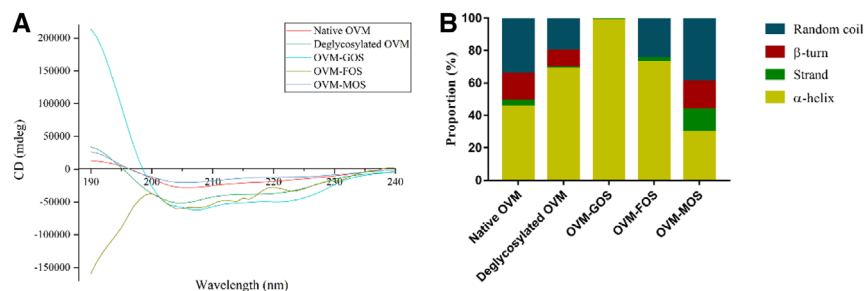
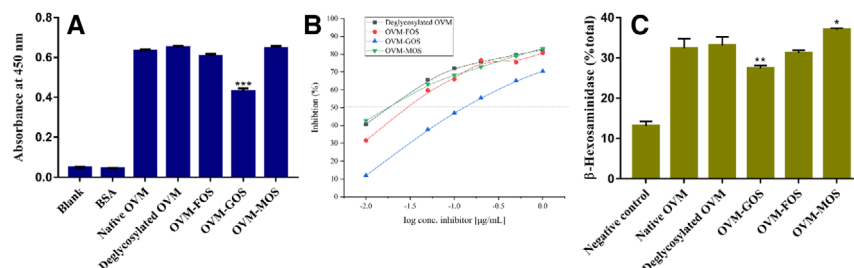


FIGURE 5 Effects of deglycosylation and glycation of ovomucoid (OVM) on allergenicity. (A) Indirect ELISA of OVM proteins. (B) icELISA of OVM proteins. (C) Effects of OVM proteins on LAD2 Cells. *Significant differences as compared with native OVM, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.005$. Error bars refer to standard deviation



Compared with the native OVM, the α -helix contents of glycosylated OVM proteins significantly increased from 45.3% to 98.5% (OVM-GOS), 72.8% (OVM-FOS), and decreased from 45.3% to 29.6% (OVM-MOS), while the β -turns contents decreased from 16.8% to 0% (OVM-GOS), 0% (OVM-FOS), and increased from 16.8% to 17.4% (OVM-MOS), and random coils decreased from 34.2% to 0% (OVM-GOS), 24.7% (OVM-FOS), and increased from 34.2% to 39% (OVM-MOS), respectively. These results demonstrated that deglycosylation of OVM can lead to the transformation of β -turn, strand and random coils to α -helix, further increasing conformation stability of OVM. Besides, the α -helix contents of OVM-GOS and OVM-FOS were significantly increased due to the transformation of other secondary structures to α -helix. Interestingly, the secondary structural change of OVM-MOS was certainly the opposite. The MOS-induced transformation from α -helix to other structures in OVM-MOS. These results indicated that the deglycosylated OVM and glycosylated OVM proteins with higher glycation degree can induce the transformation from other secondary structures to α -helix.

however, there were no significant differences in their IgE-binding abilities. These results may be due to the exposure of interior epitopes of OVM during deglycosylation and the generation of glycation products, which could function as potent epitopes (Toda et al., 2014). Conversely, the IgE-binding abilities of OVM-FOS and OVM-GOS decreased because of the mask of the epitopes of OVM by glycation (Rao et al., 2018). Besides, the IgE-binding capacity of OVM-FOS was stronger than that of OVM-GOS, showing the decrease of IgE-binding capacity of glycosylated OVM with the increase of its glycation degree.

Besides, these ELISA results further confirmed by the icELISA results. As in Figure 5B and Table S2, the IC_{50} of OVM proteins decreased as follows: OVM-GOS, OVM-MOS, OVM-FOS, and deglycosylated OVM, suggesting deglycosylated OVM and OVM-FOS had stronger IgE-binding capacities and deglycosylated OVM > OVM-FOS > OVM-MOS > OVM-GOS. The icELISA results demonstrated that the deglycosylated OVM had the highest IgE-binding capacity, while the GOS-OVM had the weakest IgE-binding capacity.

3.4 | Evaluation of potential allergenicity

3.4.1 | In vitro potential allergenicity of OVM proteins

To investigate the allergenicity of deglycosylated and glycosylated OVM, ELISA and degranulation assay was performed using sera from patients sensitized to egg white. In Figure 5A, the deglycosylated OVM and OVM-MOS exhibited slightly higher IgE-binding abilities than native OVM;

3.4.2 | Effects of OVM proteins on LAD2 cells

The effects of native OVM, deglycosylated OVM, and glycosylated OVM proteins on the release of β -hexosaminidase from LAD2 mast cells were further investigated. It is critical to confirm the effect of OVM proteins on the activation of human mast cells (LAD2). The β -hexosaminidase, a common marker of the degranulation of mast cells, is induced and released by degranulation of proinflammatory mediators after mast cells are stimulated by allergens.

In Figure 5C, incubation of LAD2 mast cells with OVM-GOS significantly ($p < 0.01$) decreased the levels of β -hexosaminidase compared with the native OVM. Besides, the levels of β -hexosaminidase decreased as follows: OVM-MOS > deglycosylated OVM > native OVM > OVM-FOS > OVM-GOS, consistent with the phenomenon observed by ELISA.

In this study, we investigated the effect of deglycosylation and glycation with different reducing sugar on the secondary structure, IgE-binding ability, and degranulation capacity of egg OVM. Our results demonstrated that α -helix contents of deglycosylated OVM, OVM-FOS, and OVM-GOS significantly increased, while that of OVM-MOS decreased. After N-&O-deglycosylation and glycation, the deglycosylated OVM and OVM-MOS showed higher IgE-binding abilities and degranulation capacities than native OVM. Encouragingly, the potential allergenicity of OVM-FOS and OVM-GOS was reduced. These results were supported by many findings in previous studies. The deglycosylation could stabilize the conformation of OVM, resulted in the exposure of more hydrolysis sites and interior epitopes of OVM, further leading to the higher allergenicity. Z. Zhang, Xiao, Zhang, et al. (2019) found that deglycosylation can enhance the allergenicity of shrimp tropomyosin. Besides, the effects of glycation on the allergenicity of allergenic proteins are heavily dependent upon the kinds of saccharides (Rao et al., 2018). Glycation can not only weaken the allergenicity of allergenic proteins through destroying and masking epitopes but also enhances the allergenicity of allergenic proteins via the generation of glycation products as new potent epitopes (Mueller et al., 2013; X. Zhu & Lin, 2006). Fu et al. (2019) indicated that after the reaction with GOS, the allergenicity of *Penaeus chinensis* tropomyosin was reduced by up to 60%.

4 | CONCLUSIONS

In summary, this work investigated the effects of deglycosylation and the Maillard reaction on conformation and potential allergenicity of egg allergen OVM. A noticeable reduction in potential allergenicity of OVM was mainly observed after glycation with FOS and GOS, which could mask its epitopes. As to the N-&O-deglycosylated OVM and MOS glyated OVM, the potential allergenicity of treated OVM was increased. Therefore, further work is underway to highlight the application of FOS and GOS for the desensitization of OVM-induced food allergy through the Maillard reaction. This research will thus enhance our understanding of the effect of deglycosylation and the Maillard reaction on potential allergenicity of OVM and will provide new insights into the immunotherapy for egg-

allergic individuals and the development of hypoallergenic egg products.

ACKNOWLEDGMENTS

This study was financially supported by the Zhejiang Provincial Natural Science Foundation of China (grant number LR19C200001) and the National Natural Science Foundation of China (grant number 31871735).

AUTHOR CONTRIBUTIONS

Investigation: Junjie Ma. Writing-original draft: Jinru Zhou. Investigation: Lerong Chen. Data curation: Hong Zhang. Writing-review & editing: Yanbo Wang. Conceptualization; Funding acquisition: Linglin Fu.

CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

How to cite this article: Ma, J., Zhou, J., Chen, L., Zhang, H., Wang, Y., & Fu, L. Effects of deglycosylation and the Maillard reaction on conformation and allergenicity of the egg ovomucoid. *J Food Sci.* 2021;1–9. <https://doi.org/10.1111/1750-3841.15791>

Effects of Deglycosylation and Maillard Reaction on Conformation and Allergenicity of the Egg Ovomucoid

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Supporting information

Figure S1. The chromatographic profile of the purification process using DEAE-sephrose column and SDS-PAGE electrophoresis of purified fractions.

Note: A: M, marker; lane 1, elution peak 1; lane 2, elution peak 2.

Figure S2. The LC/MS-MS analysis of purified OVM by diagram of total ion flow in the enzymatic hydrolysate.

Table S1. Tryptic peptide matches of the ovomucoid determined by LC-MS/MS.

Table S2. The 50% inhibiting concentration of OVM proteins.

Figure S1

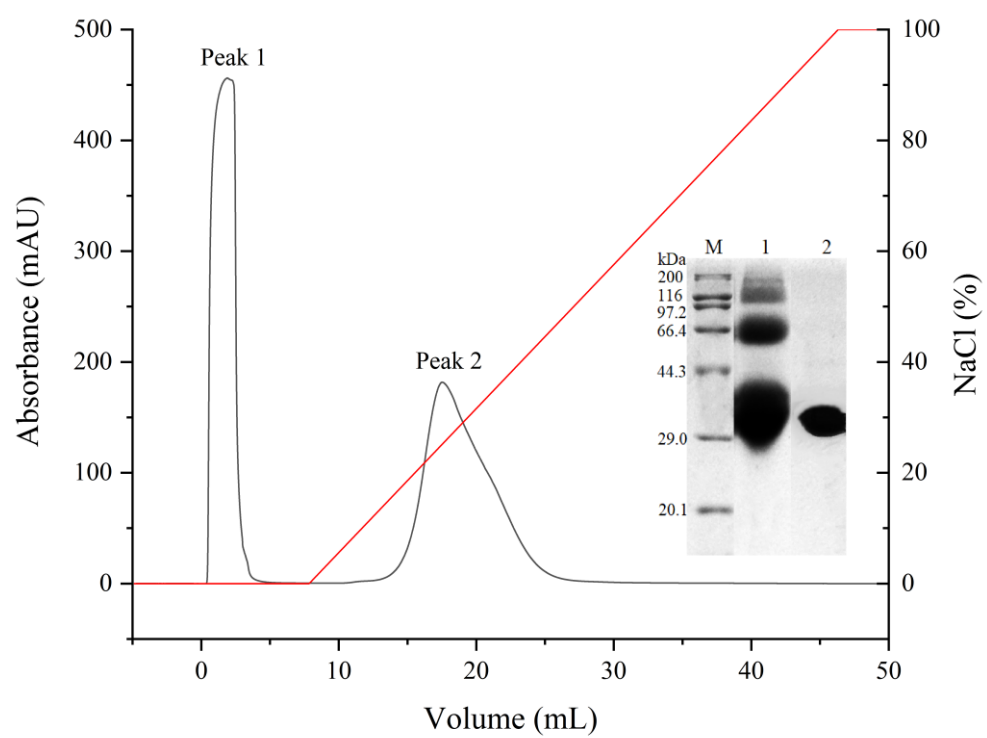


Figure 2

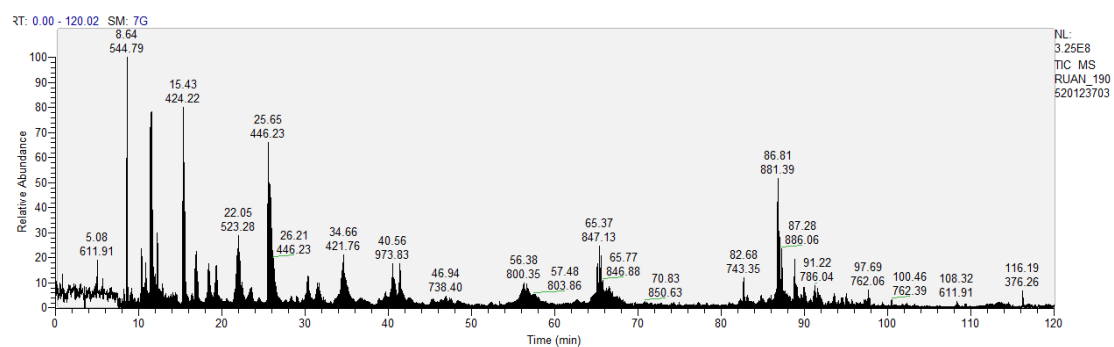


Table S1. Tryptic peptide matches of the ovomucoid determined by LC-MS/MS.

Peptide	Sequence	Mr	Delta
T1	KELAAVSVDCSEYKPDCTAEDRPLCGSDNK	3511.57	-0.15
T2	TYGNKCNFCNAVVESNGTLTLSHFGK	2918.34	-3.01
T3	ELAAVSVDCSEYKPDCTAEDRPLCGSDNK	3383.47	-3.71
T4	CNFCNAVVESNGTLTLSHFGK	2355.08	-2.27
T5	AFNPVCGTDGVTYDNECLLCAHK	2641.13	-3.27
T6	AFNPVCGTDGVTYDNECLLCAHKVEQGASVDR	3710.70	0.70
T7	FPNATDKEGKDVLVCNK	1934.95	-4.47
T8	AFNPVCGTDGVTYDNECLLCAHKVEQGASVDK	3554.60	1.00
T9	TYGNKCNFCNAVVESNGTLTLSHFGKC	3078.38	-1.07
T10	EGKDVLVCNK	1161.59	-2.09
T11	VEQGASVDR	1089.55	-1.25
T12	VEQGASVDR	1088.56	-3.94
T13	CNFCNAVVESNGTLTLSHFGKC	2515.11	-0.29
T14	FPNATDKEGK	1107.52	-2.05
T15	TYGNKCNFCNAVVESNGTLTLSHFGK	2920.31	-2.59
T16	ELAAVSVDCSEYKPDCTAEDRPLCGSDNKTYGNK	3946.74	-2.08
T17	FPNATDK	792.38	-1.95
T18	ELAAVSVDCSEYKPDCTAEDRPLCGSDNKTYGNK	3947.72	-3.29
T19	KELAAVSVDCSEYKPDCTAEDRPLCGSDNKTYGNK	4074.83	-3.24
T20	VMVLCNR	907.44	-0.57
T21	CNFCNAVVESNGTLTLSHFGK	2356.06	-4.09
T22	VEQGASVDK	932.46	-2.39
T23	VMVLCNR	891.45	-2.25
T24	DVLVCNK	847.43	-0.51
T25	FPNATDKEGK	1106.54	-0.76
T26	ELAAVSVDCSEYKPDCTAEDRPLCGSDNK	3384.47	3.67
T27	VMVLCNR	892.43	-4.51
T28	EGKDVLVCNK	1162.57	-1.40
T29	FPNATDKEGKDVLVCNK	1935.94	-2.02
T30	VMVLCNR	908.43	-1.92
T31	CNFCNAVVESNGTLTLSHFGKC	2516.09	-2.63
T32	TYGNKCNFCNAVVESNGTLTLSHFGK	2919.33	-1.46
T33	AFNPVCGTDGVTYDNECLLCAHKVEQGASVDR	3711.66	-4.74
T34	VEQGASVDKRHDGGCR	1770.82	-1.82
T35	CNFCNAVVESNGTLTLSHFGK	2357.06	4.63

Table S2. The 50% inhibiting concentration of OVM proteins.

Sample	IC ₅₀ (μg/mL)
Deglycosylated OVM	0.01869
OVM-FOS	0.02216
OVM-GOS	0.05402
OVM-MOS	0.03889