

Combined effect of glycation and sodium carbonate–bicarbonate buffer concentration on IgG binding, IgE binding and conformation of ovalbumin

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Abstract

BACKGROUND: Ovalbumin (OVA) is a major allergen in hen egg. During thermal processing, reducing sugars contained in the hen egg white might easily undergo glycation with OVA, but few studies have been conducted on its corresponding immunoreactivity changes. The aim of the present study was to assess changes of the antigenicity, potential allergenicity and conformation of OVA after glycation in a wet-thermal processing system under different concentrations of sodium carbonate–bicarbonate buffer.

RESULTS: IgE binding of the glycated OVA was increased after glycation, and the higher the sodium carbonate–bicarbonate buffer concentration, the higher the IgE binding capacity. The increase in IgE binding of OVA corresponded well with the disruption of the disulfide bond, which exposed the epitopes initially buried. Antigenicity of the glycated OVA was increased, and the amount of the increase varied among samples treated under different buffer concentrations.

CONCLUSION: Glycation increased the allergenic potential for OVA, with the amount of increase varying with different sodium carbonate–bicarbonate buffer concentrations.

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Keywords: egg allergy; ovalbumin; glycation; allergenicity; protein structure

INTRODUCTION

Different types of chemical modifications, including deamidation, acylation, alkylation, phosphorylation, esterification, amidation, covalent attachment of carbohydrates and fatty acids, and enzymatic modification, may alter protein structure in different ways, potentially changing its antigenicity and allergenicity. Among them, the Maillard reaction, the chemical reaction between reducing sugars and proteins, is perhaps the most suitable choice in the food industry because it is independent of special chemical materials. Recent studies have shown that the Maillard reaction could alter the allergenicity of food proteins by inducing structural changes. In some cases, glycation of allergens decreased IgE binding capacity; for example, the Maillard reaction for 2S albumins (Ara h 2/6) from peanut, and glycation with glucose, induced extensive denaturation, hydrolysis and aggregation of the protein, resulting in reduced IgE reactivity.¹ Structural changes caused by the Maillard reaction were also reported to induce a decrease in the allergenicity of squid tropomyosin.² In other studies, glycation had no effect or increased IgE binding capacity. For instance, allergenicity of scallop tropomyosin³ was increased by structural changes caused by the Maillard reaction, and it has been shown that advanced stages of the Maillard reaction can lead to protein crosslinking, creating novel epitopes which can bind to IgE antibodies and therefore enhance the allergenicity of the protein.^{4,5}

Eggs are included in the 'big eight' foods that are believed to be responsible for 90% of all allergic reactions. Since egg white contain 0.4–0.9% carbohydrates, with glucose accounting for 98% of the total free carbohydrates,⁶ egg white proteins may easily undergo the Maillard reaction during processing. Of the egg allergens, ovalbumin (OVA, 45 kDa) is the most abundant protein in egg white, making up 54% of the total protein,⁷ and it possesses six cysteines with a single disulfide bond between Cys 73 and Cys 120.⁸ Reports on the secondary structure of OVA, however, have been inconsistent (25–40.56% α -helices, 5–34.3% β -sheets and 21.3–62% random coils).^{9–12} These contradictions may have resulted from differences in analysis conditions, including sample preparation and treatment, OVA purity, analytical techniques,

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sample storage and calculation. Actually, numerous works have shown that OVA molecular structure could be strongly modulated by its environment. Hu and Du looked at OVA secondary structure using circular dichroism (CD) under different conditions of pH (pH 2.0–12.0), and it was found that the acid-treated OVA has an indistinguishable spectrum from the untreated one, exhibiting an unchanged secondary structure, while the alkali-treated form showed a decrease in the α -helical content.¹³ Bajaj *et al.* found that the CD profiles of OVA at pH 7.4 and pH 4.0 were quite different, and the changes in tertiary structure were far more drastic compared with those observed in the secondary structure.¹⁴ However, laneselli *et al.* found no significant change in OVA secondary structure without and with added YCl₃,¹⁵ indicating that ionic strength may not affect secondary structure of OVA. Nevertheless, it is well known that CD spectroscopy is very sensitive to changes in protein α -helix, and the UV absorbance of chloride ions greatly interferes with the CD analysis.¹⁶ However, much is still unknown on how OVA conformation is affected by buffering ions, and also very little is known of how such OVA conformational modulation affects its antigenicity and allergenicity.

OVA has a wide-spread use in experimental models of allergy. In one study, the four major egg allergens were either physically or chemically modified, and it was found that OVA can retain its human IgE binding capacity after chemical treatment such as reduction and carboxymethylation, heat or urea.¹⁷ In another study, BALB/c mice were immunized intragastrically by feeding untreated OVA, sham-nitrated OVA or nitrated OVA, and the results indicated that nitration of OVA may be associated with a reduced risk of sensitizing capability via the oral route due to enhanced protein digestibility and/or changes in antibody epitopes.¹⁸ Jiménez-Saiz *et al.* reported that heat treatment and glycation significantly reduced IgE binding capacity of OVA,¹⁹ whereas their glycation procedure was under dry-thermal processing conditions, and the relationship between changes in the potential allergenicity and conformation was not explained.

The purpose of the present work was therefore to define the specific immune response of OVA during glycation under varying concentrations of sodium carbonate–bicarbonate buffer. The conformational change induced by glycation was measured, and correlations with the changes observed in OVA immunoreactivity were also investigated. In the present paper we found that IgE binding ability of the glycated OVA increased with sodium carbonate–bicarbonate buffer concentration. Rate of disulfide bond disruption also increased with sodium carbonate–bicarbonate buffer concentration, and was supposed to be the cause of the increased IgE binding ability by exposing the epitopes initially buried. Antigenicity of the glycated OVA increased due to the formation of molten globules under different buffer concentrations.

MATERIAL AND METHODS

OVA purification protocol

OVA with a purity of >98% was prepared as described previously, by using a single-step ion exchange chromatography on a DEAE-Sephacel Fast Flow column.²⁰ In brief, fresh hen egg white was diluted with two volumes of distilled water, and the pH was adjusted and maintained at 6.0 with 2 mol L⁻¹ hydrochloric acid, followed by gently stirring for 3.5 h at 4 °C to enable ovomucin precipitation. The mixture developed a white and gelatinous precipitate which was later removed by centrifugation at 2000 × *g* at 4 °C for 45 min. The pH of the clarified supernatant was adjusted

to 7.5 with 3 mol L⁻¹ sodium hydroxide, followed by centrifugation at 10 000 × *g* for 30 min at 4 °C to remove insoluble materials. The supernatant was loaded onto a DEAE-Sephacel Fast Flow column previously equilibrated with 0.05 mol L⁻¹ Tris–HCl buffer, pH 7.5, and the column was eluted with a linear gradient of 0–0.12 mol L⁻¹ NaCl in 0.05 mol L⁻¹ Tris–HCl buffer, pH 5.5, at a flow rate of 1.5 mL min⁻¹. OVA with a purity of >98% was pooled and concentrated by ultrafiltration using a 10 kDa molecular cut-off membrane (Millipore, Billerica, MA, USA). The concentrated OVA fraction was desalted by adding distilled water to the membrane, followed by ultrafiltrating three times, and the desalted OVA was lyophilized and stored at –20 °C until use.

Preparation of polyclonal antibodies against OVA

Polyclonal antibodies were derived from three 8-week-old New Zealand rabbits. All the rabbits were accommodated for a week after arrival at the facility, and maintained under standard animal housing conditions with free access to food and water (Institute of Occupational Medicine of Jiangxi, permission number SYXK (Gan) 2008-003). Each rabbit was subcutaneously injected with 0.2 mg OVA resuspended in 1 mL saline water emulsified with an equal amount of complete Freund's adjuvant (Sigma, St Louis, MO, USA) as the primary immunization. Booster immunizations were then given with the same amount of OVA in incomplete Freund's adjuvant (Sigma, St Louis, MO, USA) at a 2-week interval, and the immune response was monitored by defining antibody level using enzyme-linked immunosorbent assay (ELISA). The animals were bled at the 7th day after the last immunization, and the blood was stored at 4 °C overnight to form a clot, followed by collection of antisera. The antisera were stored at –80 °C until use.

OVA glycation protocol under different sodium carbonate–bicarbonate buffer concentrations

A series of sodium carbonate–bicarbonate buffer solutions at pH 9.6 (0.4, 0.1 and 0.04 mol L⁻¹) were prepared. OVA powder together with glucose powder at a ratio of 25:21 (w/w) were dissolved in distilled water to a protein concentration of 800 mg mL⁻¹, resulting in a molar ratio of primary amino groups (pAA) to glucose of 1:10. The protein solution was then added to the sodium carbonate–bicarbonate buffer under different concentrations at a ratio of 1:1 (v/v), which gave a final buffer concentration of 0.2, 0.05 and 0.02 mol L⁻¹ (C = 0.2, C = 0.05 and C = 0.02), respectively. The prepared samples were put in well-capped flasks and heated in a water bath at 60 °C for 30 min, followed immediately by cooling in ice-water. In order to eliminate the effects of the reaction system during detection, all samples were dialyzed against 5 mmol L⁻¹ bicarbonate buffer at pH 9.6. The samples were then stored at –20 °C until use. Each sample treatment was performed in triplicate.

Patients' Sera

The sera of 11 subjects with specific IgE antibody against hen egg were used in this study. Egg allergy was diagnosed on the basis of detection of the serum IgE specific for egg using AllergyScreen (MEDIWISS Analytic, Moers, Germany) and objective clinical manifestations observed after egg ingestion. The 11 patients included six males and five females, with an average age of 5.8 years. Their specific IgE level was 5.3, 4.8, 11.5, 3.4, 5.7, 1.6, 2.5, 2.1, 1.3, 2.1 and 61.4 kU L⁻¹, respectively. Of the 11 patients, two had asthma, and one had urticaria. These sera were frozen at –80 °C until use. All serum samples were kindly provided by Dr Luo (Department of Paediatrics, First Affiliated Hospital of Guangxi Medical University, China).

IgG and IgE binding ability of the glycosylated OVA

The antigenicity and potential allergenicity of the glycosylated OVA were estimated by the binding ability of OVA to IgG and IgE, respectively, defined by competitive inhibition ELISA with rabbit polyclonal antibodies and egg-allergic patients' sera, respectively. The ELISA protocol was described as follows. The 96-well microtiter plate was pre-coated with the untreated OVA ($5 \mu\text{g mL}^{-1}$) in 50 mmol L^{-1} sodium carbonate buffer (pH 9.6) overnight at 4°C . All OVA samples were diluted in phosphate-buffered saline (PBS), 1/5 serial dilutions, and $60 \mu\text{L}$ antisera (diluted 1:2 500 for the rabbit sera to test the IgG binding ability of OVA, and diluted 1:20 for human sera for testing the IgE binding ability of OVA) was mixed with $60 \mu\text{L}$ of diluted sample and incubated for 1 h at 37°C . The OVA pre-coated plate was washed three times with PBS containing 0.05% Tween 20 (PBST), followed by blocking with $250 \mu\text{L}$ of 5% skimmed milk in PBS for 1 h at 37°C , and washed three times with PBST. After that, $100 \mu\text{L}$ of serum samples, pre-incubated with OVA solutions, was added to the wells and incubated for 1 h. They were again washed three times, and $100 \mu\text{L}$ goat anti-rabbit IgG or anti-human IgE horseradish peroxidase (HRP) conjugates (Sigma, St Louis, MO, USA) was added to each well, followed by incubation for 1 h at 37°C . After washing three times with PBST, the color was developed by adding a substrate solution ($100 \mu\text{L}$) of *o*-phenylene diamine (OPD) for testing the IgG binding ability of OVA, and 3,3',5,5'-tetramethylbenzidine (TMB) (Sigma, St Louis, MO, USA) for testing the IgE binding ability of OVA. The color development lasted for 15 min at 37°C , and the reaction was terminated with $50 \mu\text{L}$ of 2 mol L^{-1} sulfuric acid. The absorbance was read at 490 nm for IgG reactivity and 450 nm for IgE reactivity using a Bio-Rad Microplate Reader (Model 1860, Bio-Rad, Hercules, CA, USA), and the results were expressed as B/B₀, where B₀ and B represented the absorbance of antisera without the inhibitors and the absorbance of the antisera with various concentrations of the inhibitors, respectively.

Conformation analysis

Conformational changes of OVA after glycation were defined as the changes in secondary structure, protein surface hydrophobicity, sulfhydryl group content and UV absorbance by far-ultraviolet circular dichroism (far-UV CD) spectrum, fluorescence spectra and UV absorption spectrum, respectively. All the experiments were repeated three times and the results of each sample were averaged.

CD analysis

To define the secondary structure of untreated and glycosylated OVA, far-UV CD spectroscopy was performed on a JASCO J810 spectropolarimeter (Jasco, Groß-Umstadt, Germany) at room temperature. A protein concentration of 0.2 mg mL^{-1} was used for measurement. A scan speed at 100 nm min^{-1} , a response time of 1–4 s and a bandwidth of 1 nm were used. The obtained spectrum represented an average of three consecutive scans. Results of each sample were averaged, and the mean residue ellipticity (θ) was expressed as $\text{deg cm}^2 \text{ dmol}^{-1}$. The contents of different secondary structures were calculated using JASCO secondary structure software.

Evaluation of surface hydrophobicity

The surface hydrophobicity of OVA was evaluated by measuring the fluorescence intensity (FI) of OVA in the presence of hydrophobic fluorescence probes, 8-anilino-1-naphthalenesulfonate (ANS) according to the method of Takagi

*et al.*²¹ An aliquot of $10 \mu\text{L}$ of 8.0 mmol L^{-1} ANS solution was added to 4.0 mL of each of the protein sample solutions. The relative fluorescence was measured with a spectrophotofluorometer (FS, F-4500, Hitachi, Japan). The excitation wavelength was 390 nm and the emission wavelength was from 400 to 650 nm at a scanning speed of 10 nm min^{-1} .

Determination of sulfhydryl groups

Sulfhydryl group (SH) content on the surface of untreated and glycosylated OVA were determined by the method of Ellman.²² A volume of 1 mL of each OVA sample solution was added to 0.05 mL of 0.01 mol L^{-1} 5,5-dithiobis-2-nitrobenzoic acid (DTNB) mixed with 4 mL of 0.1 mol L^{-1} phosphate buffer pH 8.0 (exposed sulfhydryl) or the phosphate buffer was added with 0.5% SDS (total sulfhydryl), followed by incubation for 20 min at room temperature. The absorbance of the mixture was measured at 412 nm. The SH contents were calculated by the use of a molar extinction coefficient of $13\,600 \text{ L mol}^{-1} \text{ cm}^{-1}$ and expressed as $\mu\text{mol g}^{-1}$ protein, as described by Yin *et al.*²³

UV absorption spectrum analysis

UV absorption spectrum analysis was performed using a UV-2450 spectrophotometer (Shimadzu Corp., Kyoto, Japan). The UV absorption spectrum of protein samples was scanned from 250 to 420 nm.

Statistical analysis

Appropriate data were analyzed for statistical significance using analysis of variance procedures (SPSS for Windows, version 13.0, Chicago, IL, USA) and Fisher's least significant difference (LSD, $P < 0.05$) test.

RESULTS:

Effect of sodium carbonate–bicarbonate buffer concentration during glycation on OVA-IgG and OVA-IgE binding capacity

To determine whether sodium carbonate–bicarbonate buffer concentration has an effect on the antigenicity of OVA during glycation, IgG binding to glycosylated OVA was tested by competitive inhibition ELISA assays with rabbit antisera against untreated OVA. As outlined in Fig. 1, major differences in OVA antigenicity were observed between untreated OVA and the glycosylated forms. The shift of the inhibition curves toward lower inhibition concentration indicated that glycation reaction induced an increase in the IgG reactivity of OVA. Lower buffer concentration during glycation caused OVA to have a similar, albeit slightly increased, antigenicity.

To gain insight into the influence of buffer concentration during glycation on the potential allergenicity of OVA, IgE binding to glycosylated OVA was tested by competitive inhibition ELISA assays with sera from patients with egg allergy. Figure 2 shows inhibition of the IgE binding to glycosylated OVA under different buffer concentrations. Glycation reduced IC₅₀ regardless of the buffer concentration, indicating increased potential allergenicity, and the increase was more significant as buffer concentration increased.

Effect of glycation under different sodium carbonate–bicarbonate buffer concentrations on the conformation of OVA

Far-UV CD spectroscopy was used to characterize the impact of glycation under different buffer concentrations on the secondary

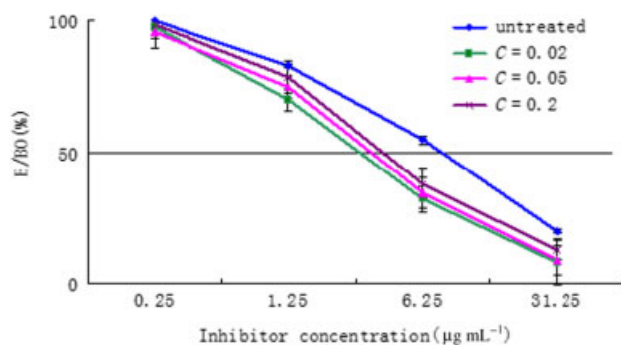


Figure 1. Changes in IgG binding ability of OVA after glycation under different buffer concentrations ($C = 0.02, 0.05$ and 0.2) defined by inhibition ELISA. Data points represent the mean of triplicate determinations, with error bars indicating the standard deviations.

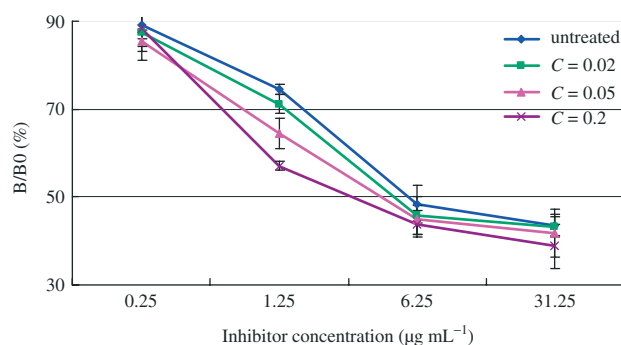


Figure 2. Changes in IgE binding ability of OVA after glycation under different buffer concentrations ($C = 0.02, 0.05$ and 0.2) defined by inhibition ELISA. Data points represent the mean of triplicate determinations, with error bars indicating the standard deviations.

structure of the protein. CD spectra of the OVA samples after glycation under various buffer concentrations (Fig. 3a) showed that major changes were on a positive molar residue ellipticity at 195 nm and a negative molar residue ellipticity at 216 nm, indicating changes in β -sheet content. Changes of secondary structure contents of the untreated and glycated OVA calculated by JASCO secondary structure software are shown in Fig. 3(b). It was shown that glycation reduced the content of the β -sheet.

As shown in Fig. 4(a), the sulfhydryl group content on the surface of OVA samples increased upon buffer concentration during glycation. Since OVA has one disulfide bond per molecule and four sulfhydryl groups buried in the interior of the native core of the protein, they could not be accessible to chemical reagents such as DTNB. However, conserved cysteine residues may occur; thus the sulfhydryl group content of native OVA could still be tested.

Figure 4(b) indicates that the total number of sulfhydryl groups reacting with DTNB increased after glycation, and the increase was more significant as the buffer concentration increased.

Moreover, structural modifications of OVA upon glycation under different buffer concentrations were characterized for surface hydrophobicity and UV absorption spectrum. OVA surface hydrophobicity was investigated using the probe ANS (1-anilino-8-naphthalenesulfonate). As shown in Fig. 5(a), glycation under low buffer concentration ($C = 0.02$) increased fluorescence intensity further. After glycation, it seemed that the lower the buffer concentration was, the higher the fluorescence intensity would be.

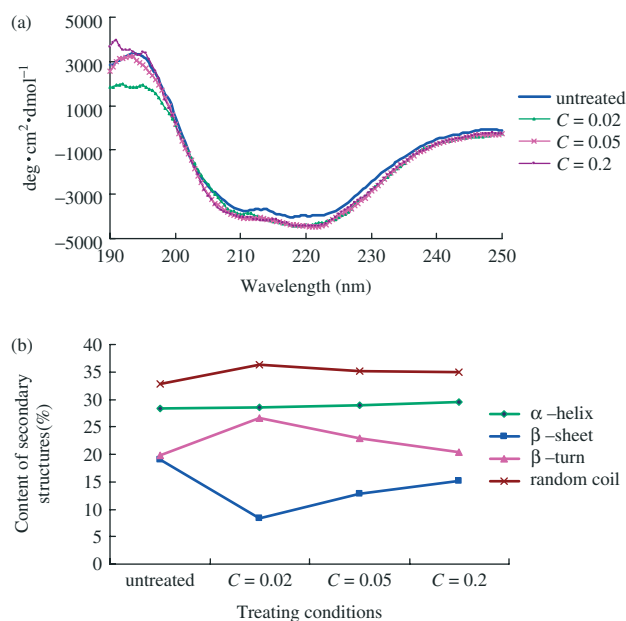


Figure 3. Effect of glycation under different buffer concentrations on the secondary structure of OVA measured by far-UV CD spectroscopy. (a) Far-UV CD spectra of untreated OVA and OVA glycated under different buffer concentrations. (b) The content of each secondary structure of untreated OVA and OVA glycated under different buffer concentrations.

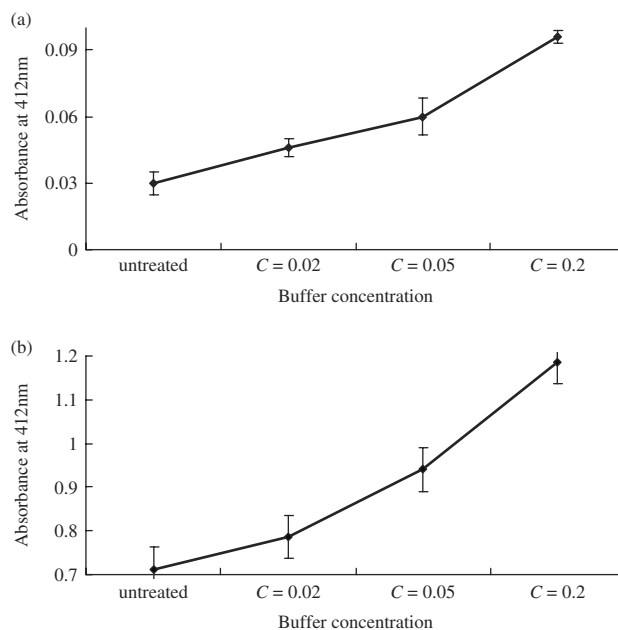


Figure 4. Effect of glycation under different buffer concentrations on variation of the surface sulfhydryl group (SH) content (a) and total SH content of OVA (b) measured by Ellman's method at 412 nm.

Figure 5(b) illustrates the effect of glycation under different buffer concentrations on the UV absorption spectrum of OVA. From the figure we could find that the significant change in UV absorption was at 270–290 nm. After glycation for 30 min, the order of UV absorption spectra intensity was as follows: untreated OVA $< C = 0.05 < C = 0.02 < C = 0.2$. The value for OVA after glycation under high buffer concentration condition ($C = 0.2$) was about two times that of untreated sample.

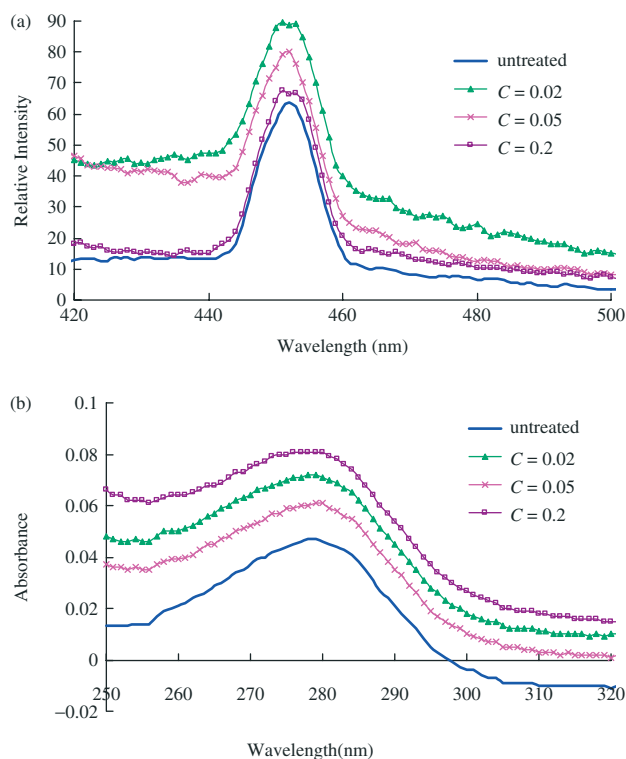


Figure 5. Effect of glycation on the intensity of fluorescence and UV absorption of OVA. (a) The changes of the hydrophobicity of OVA after glycation under different buffer concentration. Hydrophobicity was measured using hydrophobic fluorescence probes (ANS). The excitation wavelength was 390 nm, and the emission wavelength was from 400 to 650 nm. (b) UV absorption of untreated OVA and OVA glycated for 30 min under different buffer concentrations.

DISCUSSION

In our study, the potential allergenicity and antigenicity of OVA after glycation under different sodium carbonate–bicarbonate buffer concentrations were determined by the binding of IgE and IgG *in vitro* using egg-allergic patients' sera and rabbit anti-OVA sera, respectively. It was found that after glycation potential allergenicity of OVA increased in a buffer concentration-dependent manner. We then defined the conformational changes of OVA after glycation, and the correlations with the changes observed in OVA immunoreactivity were also investigated.

The Maillard reaction may convert the disulfide bonds into sulfhydryl groups, destroying the force that maintains OVA conformation so as to expose the initially buried epitopes and change its potential allergenicity. In this work, the increase in the total number of sulfhydryl groups after glycation indicated that the sole disulfide bond in OVA molecule – Cys 73 and Cys 120 – was disrupted. The disruption of this disulfide bond was confirmed by the fact that hydrophobicity and secondary structure changes of glycated OVA involved the respective changes between Cys 73 and Cys 120. Hydrophobicity analysis, as determined by the method of Kyte and Doolittle,²⁴ identified Cys 73 to Cys 120 as relatively hydrophilic regions of the protein (Fig. 6). This correlated well with what we found in Fig. 5(a), which showed that only a limited number of hydrophobic groups became solvent exposed. As for the secondary structure between Cys 73 and Cys 120, α -helix, β -sheet and random coil were found to be involved from the 3D structure of OVA shown in Fig. 7. Obviously, this demonstrated that what we found from Fig. 3(a) was possible; that is, changes

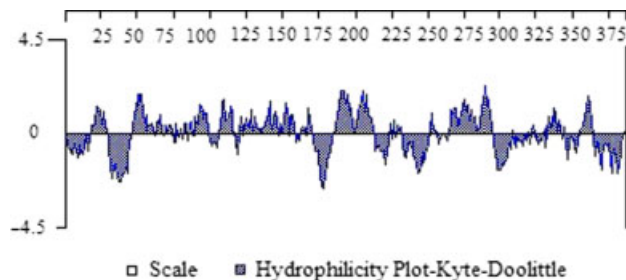


Figure 6. Hydrophilicity plot of ovalbumin defined by the method of Kyte and Doolittle.

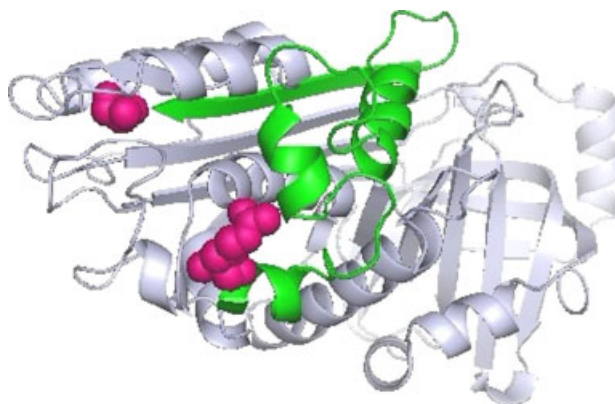


Figure 7. One OVA molecule from the crystal structure of native hen ovalbumin (1OVA) with Cys 73 and Cys 120 highlighted with spheres and the structure between Cys 73 and Cys 120 marked in another color.

in the secondary structure of glycated OVA involved the decrease of β -sheet content. Coincidentally, it was reported that fragments 41–172 and 301–385 exhibited human IgE binding activity,²⁵ and Cys 73 to Cys 120 exists within the fragments of 41–172. Therefore, it may be concluded that the increase of potential allergenicity of glycated OVA was caused by the disruption of the disulfide bond between Cys 73 and Cys 120, which exposed initially buried epitopes. This conclusion is also supported by the fact that higher buffer concentration increased the total number of sulfhydryl groups more, and the potential allergenicity of OVA was increased accordingly. Additionally, the importance of intact disulfide bonds to the allergic response has also been reported by other studies, in which the disulfide bonds were disrupted by heat, site-directed mutagenesis or biochemical treatment.^{26–28}

The unfolding of the OVA molecule has a different profile upon glycation under different sodium carbonate–bicarbonate buffer concentrations. The maximum UV absorption peak of untreated OVA was found to be around 280 nm, indicating that the UV absorption spectrum of OVA was mainly dependent on tyrosine (Tyr) and tryptophan (Trp) residue side chain groups. After glycation, OVA samples showed the highest UV absorption intensity at the buffer concentration of $C = 0.2$, while the lowest UV absorption intensity was at a buffer concentration of $C = 0.05$. The increase in absorption suggested that Trp and Tyr residues were exposed on the surface of the protein molecule, as a consequence of unfolding of the structure. That is to say, glycation under the buffer concentration of $C = 0.2$ caused the protein to be unfolded to a greater extent, followed by $C = 0.02$ and $C = 0.05$. Moreover, the glycation reaction increased hydrophobicity, defined by an increase in fluorescence intensity. After glycation for 30 min, OVA

samples showed the highest hydrophobicity at $C = 0.02$, followed by concentrations $C = 0.05$ and $C = 0.2$. Since the increased intensity of fluorescence indicated that the protein was unfolded and more hydrophobic regions were exposed,²⁹ glycation under the buffer concentration of $C = 0.02$ seemed to cause the protein to be unfolded to a greater extent, followed by $C = 0.05$ and $C = 0.2$. Furthermore, an increase in sulfhydryl group contents on the surface indicated that more sulfhydryl groups were exposed as a result of protein unfolding. The sulfhydryl group contents on the surface of OVA after glycation for 30 min increased with the buffer concentration, indicating that glycation under the buffer concentration of $C = 0.2$ caused the protein to be unfolded to a greater extent, followed by $C = 0.05$ and $C = 0.02$.

UV absorption spectroscopy and fluorescence spectroscopy indicated that different extents of unfolding among the three buffer concentrations could be explained by refolding of the OVA molecule to arrive at the molten globule conformation. Several studies have indicated the formation of protein folding intermediates (molten globules) which could be either marginally stable, highly disordered conformations, or stable states with nearly native structures.^{30–32} These conformations are usually characterized by a relatively high content of secondary structures and a largely disordered tertiary structure.³³ In this work, apart from glucose conjugation, ionic strength in the different buffer concentrations also contributed to the forming of folding intermediates. At pH 9.6, the OVA molecule is negatively charged, and the effective protein–protein interaction is repulsive due to the net negative charge of acidic residues on the protein surface. This repulsive interaction can be significantly reduced when the buffer concentration increases, where the cations could bind to the acidic residues on the protein surface and the number of cations bound gradually increased with buffer concentration. When the buffer concentration was increased to a fixed point, the binding ions neutralized the overall surface charge, and the van der Waals and hydrophobic interactions became the most important forces. Upon further increase of the buffer concentration, the protein surface would absorb more cations, resulting in the inversion of the surface charge; i.e. the proteins become positively charged. The discrepancy in charges and forces that hold the protein secondary and tertiary structure might cause the OVA molecule to refold in a different way, forming different folding intermediates (molten globules). These different ways of refolding might affect UV absorption intensity, hydrophobicity and sulfhydryl group contents on the surface of the OVA differently.

The antigenicity differences of OVA after glycation under different buffer concentrations could be explained by the formation of different folding intermediates, as it was reported that the different conformations of OVA exhibit different epitopes.²⁵ In our study the antigenicity of OVA was found to be increased, and the sodium carbonate–bicarbonate buffer concentration affected the extent of the increase. This glycation-induced alteration tendency of antigenicity was in agreement with Jiménez-Saiz *et al.*, who reported an increase in IgG binding ability of OVA after glycation under dry-thermal processing conditions.¹⁹ Moreover, the antigenicity increase of OVA was more significant under low buffer concentration, while potential allergenicity increased more under high buffer concentration. Undoubtedly, the reactivity against IgG and IgE after glycation did not follow the same trend, and this is the reason why allergenic epitopes only partially overlap antigenic determinants.⁷

In conclusion, OVA exhibited structural differences after glycation under different buffer concentrations, which then

exerted an effect on OVA antigenicity and potential allergenicity. In the search of previous works on hypoallergenic allergens, much effort was made on the impact of processing on the allergenicity, but not much attention was paid to the environment of the processing. Nevertheless, we demonstrated that buffer concentration works in the changing of IgG and IgE reactivity during glycation.

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