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## Conformation affects the potential allergenicity of ovalbumin after heating and glycation

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Ovalbumin (OVA), one of the major allergens in hen egg white, and has widespread use in experimental models of allergy. The aim of this research was to assess the effect of glycation and heat treatment on the potential allergenicity of OVA prepared from hen egg white. Secondary and tertiary structures of OVA were also characterised to show the relationship between potential allergenicity and the conformation of OVA after heating and glycation. Glycation significantly reduced the potential allergenicity of OVA tested with egg allergy patients' sera, which was caused by conformation changes. An increased IgG reactivity was measured using rabbit anti-OVA and was supposed to be caused by protein unfolding which exposed hidden epitopes. Heating reduced the potential allergenicity of OVA at the expense of increased IgG reactivity. It is suggested that conformational changes of OVA induced by glycation and controlled heating significantly reduced its potential allergenicity.

**Keywords:** egg allergy; ovalbumin; heating; glycation; antigenicity; allergenicity; protein structure

### Introduction

Ovalbumin (OVA), one of the main egg allergens, is the most abundant globular protein of egg white, making up 54% of the total protein. The OVA protein of chickens is made up of 385 amino acids, and its relative molecular mass is 45 kDa (Nisbet et al. 1981). Its ready availability in large quantities has led to its widespread use as a standard preparation in studies of the structure and properties of proteins, and in experimental models of allergy (Yamasaki et al. 2003). The crystal structure of native hen OVA includes four crystallographically independent OVA molecules (Stein et al. 1991), and Mine and Prithy (2003) have identified five distinct regions that contain dominant allergic immunoglobulin E (IgE)-binding linear epitopes. Although OVA can potentially undergo a Maillard reaction on any of its 20 lysine residues, there is only one lysine residue (K47) on the five epitopes, and two other (K93 and K190) close to the epitopes. Moreover, OVA is found to be N-glycosylated on asparagine-293, and N-terminally acetylated. It also exists in three forms, A1-, A2- and A3-OVA, with two, one and no phosphate groups per molecule, respectively (Huntington & Stein 2001).

Since hen egg white contains 0.4–0.9% carbohydrates, the free amino groups on OVA may easily undergo covalent modification through the Maillard reaction, and it can lead to alterations in protein structure, resulting in changes in their immunoreactivity/allergenicity. The similar phenomenon also exists with other food allergens. The

structural changes caused by the Maillard reaction were found to induce an increase in allergenicity of scallop tropomyosin (Nakamura et al. 2005), whereas the allergenicity of squid tropomyosin was reduced during the Maillard reaction (Nakamura et al. 2006). Taking bovine  $\beta$ -lactoglobulin as another example (Hattori et al. 2004), its conformation around (8)Lys-(19)Trp ( $\beta$ -sheet, random coil, short helix) changed after conjugation with acidic oligosaccharides, and the immunogenicity was reduced. With respect to some plant proteins, buckwheat Fag e 1 (Nakamura et al. 2008) and soy protein (Usui et al. 2004) had reduced allergenicity after the Maillard reaction, which is well reflected by the fact that thermally processed plant products often show non- or less severe effects toward patients than the corresponding fresh plant material. The Maillard reaction for Pru av 1, the major allergen from cherry (Gruber et al. 2004), and glycation with glucose or ribose could induce an irreversible change in the tertiary structure of the protein, resulting in a loss of conformational epitopes of the allergen. As for 2S albumins (Ara h 2/6) from peanut, it was indicated that glycation in conjunction with thermal denaturation generally led to a further decrease of IgE binding capacity (Vissers et al. 2011). Interestingly, an earlier report showed that the allergenicity of peanut allergens was increased after non-enzymatic browning (Maleki et al. 2000).

The Maillard reaction is probably the most promising processing method to improve the functional properties of proteins for food purposes as it is spontaneous, naturally

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occurring and independent of special chemical materials, and it is greatly accelerated by heat. It is necessary to point out that heating can also pose an effect on allergenicity of the processed protein. For instance, thermal processing resulted in structural changes of the peanut allergen Ara h 2, which might have an impact on the protein's molecular interactions with enterocytes, subsequent cellular signalling response and immunogenicity (Starkl et al. 2011).

With respect to OVA, recent work showed that OVA, after the Maillard reaction under wet-thermal processing procedures with glucose, enhanced its T-cell immunogenicity (Ilchmann et al. 2010). It is a coincidence that people have been searching for some immunotherapeutic approaches that might eliminate (by masking or removal) the IgE binding epitopes while retaining T-cell reactivity, thereby preserving immunogenicity (Babakhin et al. 1995). Moreover, Jiménez-Saiz et al. (2011) indicated that heat treatment and glycation could significantly reduce the IgE binding capacity of OVA, whereas their glycation procedures was under dry-thermal processing conditions and the reasons for the potential allergenicity changes were not yet explained. Therefore, the aims of the present work were to define the IgG and IgE binding capacity of OVA during heating and moderate glycation under wet-thermal processing procedures. The corresponding conformational changes were measured, and the correlations with the changes observed in OVA immunoreactivity were also investigated.

## Materials and methods

### Purification of OVA

The purification procedure of OVA was described previously (Ma et al. 2009). In brief, fresh hen egg white, after diluting with 2 vols of distilled water and adjusting to pH 6.0 with 2 M hydrochloric acid, was gently stirred 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 2000g for 45 min at 4°C. The supernatant was adjusted to pH 7.5 with 0.05 M Tris-HCl buffer, followed by centrifugation at 10,000g for 30 min at 4°C to remove insoluble materials. The supernatant was loaded onto a DEAE-Sepharose Fast Flow column (GE Healthcare, Pittsburgh, PA, USA) previously equilibrated with 0.05 M Tris-HCl buffer, pH 7.5. The column was eluted with a linear gradient of 0–0.12 M NaCl in 0.05 M Tris-HCl buffer, pH 5.5, at a flow rate of 1.5 ml min<sup>-1</sup>. OVA was identified by sodium dodecylsulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and MS (data not shown), and the prepared OVA with a purity of more than 98% was lyophilised and stored at –20°C until use.

### Preparation of antisera against OVA

Polyclonal antibodies were derived from three 8-week-old New Zealand rabbits. All the rabbits were accommodated

for 1 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 injected subcutaneously with 0.2 mg of OVA resuspended in 1 ml saline water emulsified with an equal amount of Freund's complete adjuvant (Sigma, St. Louis, MO, USA) as the primary immunisation. Booster immunisations were then given with the same amount of OVA in incomplete Freund's adjuvant (Sigma) at a 2-week interval. Antibody level was monitored by ELISA. The animals were bled on the seventh day after the last immunisation, and blood from rabbit was stored at 4°C overnight to form a clot, followed by collection of the antisera. The antisera were stored at –80°C until use.

### Preparation of glycated and heated proteins

To obtain glycated OVA (OVA-G), the prepared OVA (0.4 mg ml<sup>-1</sup>) in 5 mM bicarbonate buffer at pH 9.6 was mixed with glucose powder, obtaining a OVA–glucose weight ratio of 25:21, which resulted in a molar ratio of primary amino groups (pAA) to glucose of 1:10. Mixtures of OVA and glucose were then put in well-capped flasks and heated in a water bath at 60°C for 0, 30, 60 and 120 min, respectively. After the heat treatment, samples were immediately cooled in iced water and stored at –20°C prior to analysis. OVA solution (the same as used in glycation described above) in the absence of glucose was treated equally as the heated samples (OVA-H). Each treatment was performed in triplicate.

### Determination of free amino groups

The quantity of free amino groups was determined according to the OPA method (Fayle et al. 2001). The OPA reagent was prepared by dissolving 40 mg OPA in 1 ml of methanol, followed by the addition of 25 ml of 0.1 M borate buffer (pH 9.0), 100 µl mercaptoethanol, and 2.5 ml aqueous SDS solution (20% w/v). The reagent was incubated at 4°C with constant shaking, and 1 ml of it was added to 50 µl aliquot of samples (containing 3 mg ml<sup>-1</sup> OVA). Then the absorbance was read at 340 nm after a minimal delay of 2 min at RT. A calibration curve of glycine was obtained by preparing standards at a final concentration ranging from 0.2 to 2.00 mM glycine. Unless otherwise indicated, all measurements below were performed in triplicate.

### Patients' sera

The sera of four subjects with the specific IgE antibody against hen egg were used. Egg allergy was diagnosed on the basis of the detection of the serum IgE specific for egg using the AllergyScreen (MEDIWISS Analytic GmbH,

Moers, Germany) and objective clinical manifestations observed after egg ingestion. The four patients were numbered P1, P2, P3 and P4; they had an average age of 13 years. Their specific IgE levels were 0.68, 3.2, 2.1 and 3.0 kU l<sup>-1</sup>, respectively. Of the four patients, P1 had chronic urticaria, P2 had eczema, and P3 and P4 had anaphylaxis. Control sera were obtained from two shrimp-allergic patients (S1 and S2) without egg-specific IgE antibody and two normal subjects (N1 and N2) who had shown no adverse reactions to egg. These sera were frozen at -80°C until use. All serum samples were kindly provided by Dr Luo (Department of Paediatrics, The First Affiliated Hospital of Guangxi Medical University, China).

#### **Effect of heating and glycation on the antigenicity and potential allergenicity of OVA**

The antigenicity and potential allergenicity of OVA were estimated by the binding ability of OVA to IgG and IgE, respectively, by inhibition ELISA with rabbit polyclonal antibodies and indirect ELISA with egg allergic patients' sera.

#### *Competitive ELISA to determine IgG antibody response*

Heated and glycated OVA were subjected to competitive ELISA to investigate the changes in the specific IgG reactivity of OVA. The 96-well microtitre plate was coated with the untreated OVA (5 µg ml<sup>-1</sup>) in PBS overnight at 4°C. The OVA-coated plate was washed three times with PBS containing 0.05% Tween 20 (PBST), followed by blocking with 250 µl of 5% skimmed milk in PBS for 1 h at 37°C and washed three times with PBST. Serial five-fold dilutions of untreated, heated and glycated OVA were first pre-incubated with the same volume of diluted rabbit polyclonal sera (diluted to 1:10,000 in PBS) for 1 h at 37°C, then transferred to the wells (100 µl) and incubated for 1 h. Then it was again washed three times and 100 µl of goat anti-rabbit IgG conjugated with horseradish peroxidase (HRP) (Sigma) diluted with PBS to 1:10,000 were transferred to the wells and incubated for 1 h at 37°C to detect the bound immunogen. After washing three times with PBST, the colour was developed by adding a substrate solution (100 µl) of *o*-phenylene diamine (0.5 mg ml<sup>-1</sup>), 0.03% hydrogen peroxide in 0.1 M citrate buffer (pH 5.5) to each well for 15 min at 37°C, and the reaction was terminated with the addition of 50 µl of 2 M sulphuric acid. The absorbance was read at 490 nm using a Bio-Rad Microplate Reader (Model 1860, BioRad, Hercules, CA, USA).

The loss of the specific IgG-binding ability resulting from the treatment with the inhibitors was represented by calculating the inhibition rate using the formula:

$$\text{Inhibition rate (\%)} = (1 - Y/X) \times 100 \quad (1)$$

where  $X$  is the absorbance of rabbits' sera without the inhibitors; and  $Y$  is the absorbance of the rabbits' sera treated with various concentrations of the inhibitors, respectively. The concentration of protein (inhibitor) needed to inhibit 50% of IgG binding (IC<sub>50</sub>) was calculated from the inhibition curves.

#### *Indirect ELISA to determine the IgE binding capacity of OVA*

To determine the IgE binding capacity of OVA, the ELISA microplate was pre-coated with heated or glycated OVA (5 µg ml<sup>-1</sup>) in PBS overnight at 4°C. The blocking and washing procedures were similar to the IgG response determination above, followed by incubation at 37°C for 2 h with human serum (diluted to 1:20 in PBS). The plate was washed and incubated at 37°C for 1 h with anti-human IgE-peroxidase antibody produced in goat (Sigma) diluted to 1:5000 in PBS. After washing three times with PBST, the plate was developed with 3,3',5,5'-tetramethylbenzidine (TMB) (Sigma) substrate solution for 15 min at 37°C. The reaction was terminated by the addition of 50 µl of 2 M sulphuric acid; and the plate was read at 450 nm using the Bio-Rad Microplate Reader.

#### **Conformation analysis**

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

#### *Circular dichroism (CD) analysis*

To define the secondary structure of untreated, glycated and heat-treated OVA, far-UV CD spectroscopy was performed on a JASCO J810 spectropolarimeter (Jasco, Groß-Umstadt, Germany) at RT. Protein concentrations of 0.2 mg ml<sup>-1</sup> were used for measurements. A scan speed of 100 nm per min, 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 ( $\theta$ ) was expressed as deg · cm<sup>2</sup> · dmol<sup>-1</sup>. The contents of different secondary structures were calculated with 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. (1979). An aliquot of 10  $\mu$ l of 8.0 mM ANS solution was added to 4.0 ml of each of the protein sample solutions. The relative fluorescence was measured with spectrophotofluorometer (F-4500, HITACHI, Tokyo, 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<sup>-1</sup>.

#### Determination of sulfhydryl groups

Sulfhydryl group (SH) content on the surface of untreated and glycated OVA was determined by the method of Ellman (1959), with some modifications. A volume of 1 ml of each OVA sample solution was added to 0.05 ml of 0.01 M 5,5-dithiobis-2-nitrobenzoic acid (DTNB) mixed with 4 ml of 0.1 M 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 RT. The absorbance of the mixture was measured at 412 nm. The SH contents were calculated by the use of molar extinction coefficient of 13 600 M<sup>-1</sup> cm<sup>-1</sup>, as described by Fernandez-Diaz et al. (2000).

#### Statistical analysis

Data were analysed for statistical significance using analysis of variance (ANOVA) procedures (SPSS for Windows 2003, version 13.0; Microsoft Corp., Chicago, IL, USA) and Fisher's least significant difference (LSD,  $p < 0.05$ ) test.

### Results

#### Determination of free amino groups

At an early stage of the Maillard reaction, the protein containing free amino groups reacted with carbonyl

groups of sugars to cause the loss of free amino groups. The prepared OVA was incubated with glucose for 0–120 min, and the loss of free amino groups in OVA-G was analysed by using the OPA method. The content of free amino groups in OVA-G significantly decreased after 30 min of incubation and then displayed less change after 60 min of incubation (Figure 1). The reason might be that during the first 30 min of the Maillard reaction, glucose reacted with most of the reactive amino groups available. Furthermore, the N-terminal of OVA is acetylated; thus, all free amino groups of OVA that took part in the Maillard reaction originate from the amino groups of its 20 lysine residues. Since OVA is a globular protein with an ellipsoidal shape, most of the lysine residues are buried, restricting the glucose availability. The numbers of modified amino groups were calculated from the percentage of modified amino groups and the number of total amino groups. The average number of sugars linked per OVA molecule after 60 min of the Maillard reaction was about three, and no changes in free amino groups were observed in the OVA-H (data not shown).

#### Effect of heating and moderate glycation on the antigenicity and the potential allergenicity of OVA

The calculated IC<sub>50</sub> of untreated OVA (0 min) was the highest, and IC<sub>50</sub> gradually decreased when glycation or heating occurred (Figure 2). That is to say, untreated OVA had a clear but relatively low antigenicity, and heating with or without glucose increased its IgG binding capacity.

All the patients allergic to egg showed a high level of IgE binding capacity, whereas all patients with shrimp allergy and the two non-allergic subjects did not show much binding to the OVA (Figure 3). It clearly indicated a specific interaction of OVA with IgE antibodies from egg-sensitive patients.

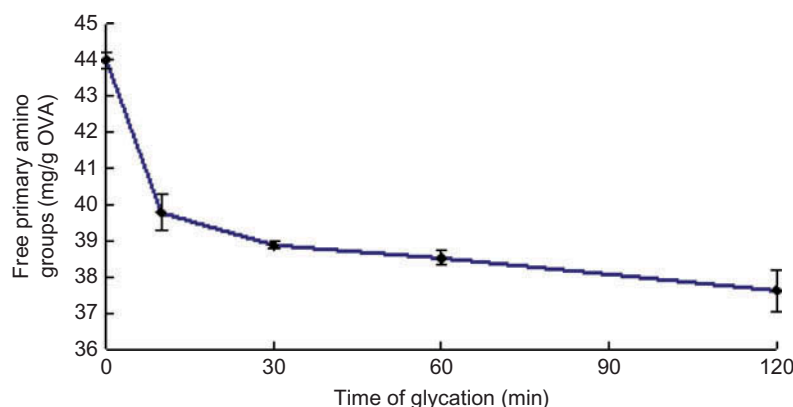


Figure 1. Changes in free primary amino groups of OVA-G after glycation for 0–120 min. Data points represent the mean of triplicate determinations with error bars indicating standard deviations (SDs).

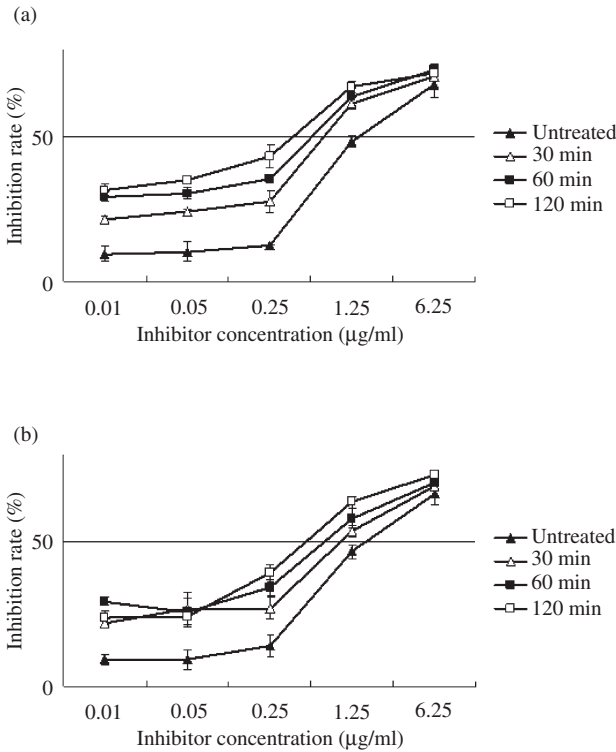


Figure 2. Changes in antigenicity of OVA after conjugating with glucose for 0, 30, 60 and 120 min, respectively (a) and heating for 0, 30, 60 and 120 min, respectively (b) defined by inhibition ELISA. Data points represent the mean of triplicate determinations with error bars indicating standard deviations (SDs).

To gain insight into the influence of glycation and heating on IgE binding capacity to OVA, indirect ELISA, instead of inhibition ELISA, was performed to reduce the amount of human sera consumed. We have verified that untreated, heated and glycated OVAs bind to the plate wells at similar levels by performing indirect and inhibition ELISA using the rabbit sera and results showed no important differences. Results showed that

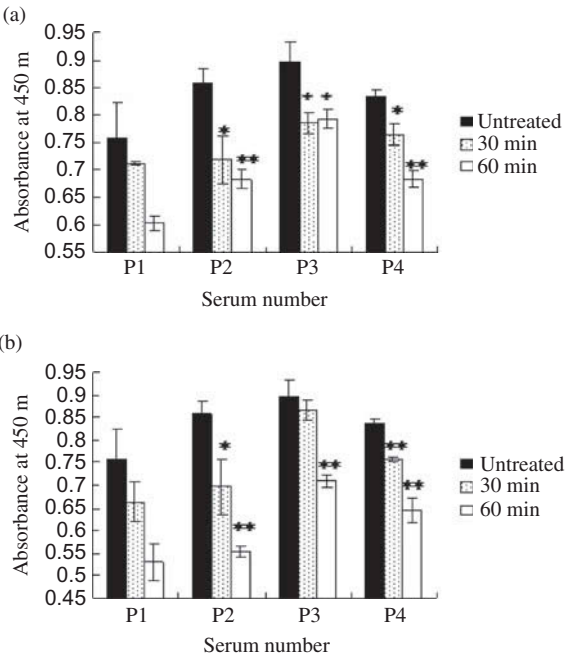


Figure 4. Indirect ELISA assays for the influence of glycation on the IgE binding activity of OVA (500 ng/well) using sera from egg-sensitive patients (a) and heating on the IgE binding activity of OVA (500 ng/well) using sera from egg-sensitive patients (b). Data are represented as mean  $\pm$  standard deviation ( $X \pm SD$ ). Asterisks (\*) indicate that the IgE binding to OVA at these treating conditions are significantly different from that to untreated OVA ( $p < 0.05$ ). Double asterisks (\*\*) mean the significance level was  $p < 0.001$ .

heating in the presence or absence of glucose significantly reduced the IgE binding ability of OVA, and the IgE reactivity gradually reduced when prolonging the heating time (Figure 4). Nevertheless, the result derived from serum P3 showed that glycation for 30 min reduced IgE binding capacity of OVA similar to glycation for 60 min. Obviously, there are individual differences and the epitope

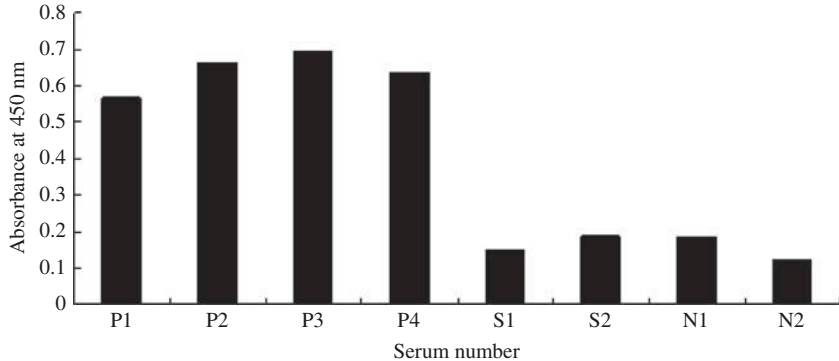


Figure 3. Indirect ELISA assays for specific IgE binding activity of OVA (500 ng/well) using sera from egg-sensitive (P1–P4), shrimp-sensitive (S1 and S2), and non-allergic subjects (N1 and N2).

sites recognised by human IgE are different among patients.

### Effect of heating and glycation on the conformation of OVA

The effect of heating and glycation on OVA secondary structure was assessed by far-UV CD spectroscopy (Figure 5a). Figure 5a showed no major change in the secondary structure, but it can be clearly seen that, after glycation for 30 min, the positive and negative molar residue ellipticity peaks of this OVA sample increased, which showed that the amounts of the  $\alpha$ -helix and  $\beta$ -sheet had increased. Changes in the secondary structure contents of the untreated, heated and glycated OVA were tested by JASCO secondary structure software (Figure 5b). It was shown that in the presence of glucose there was a slight increase in the  $\beta$ -sheet content of OVA by wet heating for 30 min, which increased further by wet heating for 60 min, and the  $\beta$ -turn content, in contrast, gradually decreased. A similar result was also reported by Sun et al. (2004) who indicated that glycated OVA had slightly lost

its  $\alpha$ -helical structure with a concomitant increase in the  $\beta$ -sheet proportion.

The effect of heating and glycation on the surface hydrophobicity of OVA was evaluated by measuring the fluorescence intensity of OVA using the ANS binding method. It was shown that untreated OVA exhibited low fluorescence intensity, and glycation for 30 min significantly increased it, to about two-fold over that of untreated OVA, followed by OVA glycation for 60 min, OVA heating for 60 min and OVA heating for 30 min (Figure 6). It suggested that glycation of OVA through wet heating in the presence of glucose caused the more buried hydrophobic residues to become exposed rather than that of OVA wet heated in the absence of glucose. Moreover, OVA glycated for 30 min exposed more buried hydrophobic residues than OVA after 60 min of glycation, suggesting a refold of the OVA molecule. The result of the OPA method between OVA-H and OVA-G also indicated glucose conjugation with OVA under the low temperature wet-glycation process.

As shown in Figure 7a, the sulfhydryl group contents on the surface of OVA samples first increased and then

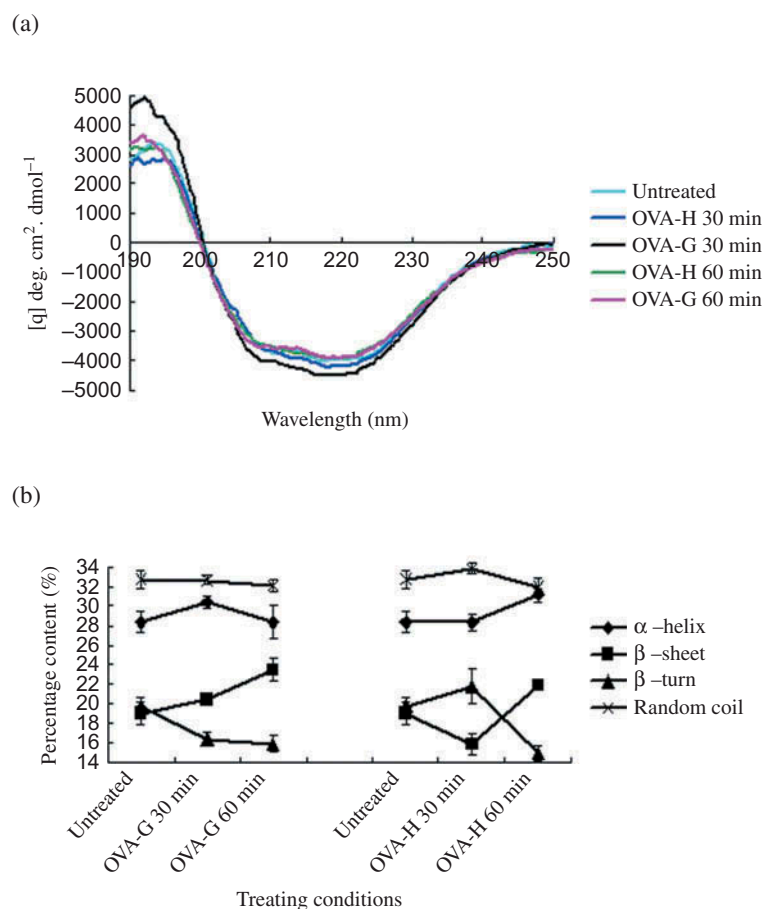


Figure 5. Circular dichroism (CD) spectra of untreated, heated and glycated OVA (a) and the percentage content of OVA secondary structures by far-ultraviolet (UV) CD of untreated versus glycated OVA calculated with JASCO secondary structure software (b).

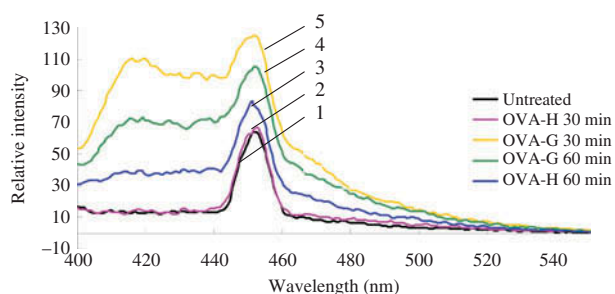


Figure 6. (colour online). Fluorescence intensity of the untreated, heated and glycated OVA. The numbers 1–5 stand for: untreated OVA, OVA heated for 30 min, OVA heated for 60 min, OVA glycated for 60 min, and OVA glycated for 30 min, respectively. Data represents the mean of triplicate determinations.

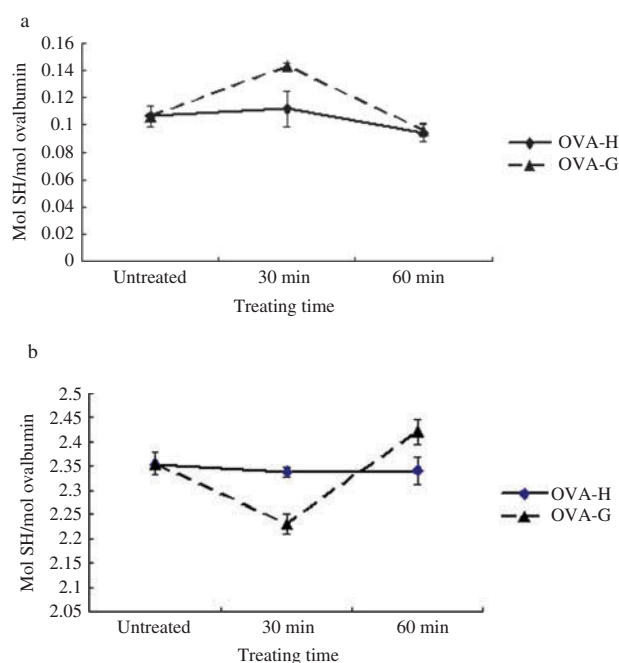


Figure 7. Effect of heating and glycation on the variation of the surface sulfhydryl group (SH) content (a) and the total sulfhydryl group (SH) content of OVA (b) measured by Ellman's method at 412 nm.

decreased during glycation, while heating induced only minor changes. Figure 7b indicates that the total number of sulfhydryl groups reacting with DTNB decreased and then increased after glycation. Heating, nevertheless, did not cause important changes.

## Discussion

This investigation was conducted to explore the effect of moderate glycation under wet thermal processing conditions on the conformation, antigenicity and potential allergenicity of OVA. Mild heating (60°C), lower than the

denaturation temperature of the protein 78.3°C (Li et al. 2005), was chosen to limit thermal denaturation and aggregation effects. This temperature was also chosen in consideration of the temperature often used in accelerated storage trials and common heat treatments such as pasteurisation.

The mechanism for the allergenicity changes during the Maillard reaction could be attributed to the shielding of the epitopes by the covalent attachment of reducing sugar chains. Moreover, Maillard reactions can alter the structure of the allergen and destroy conformational epitopes or expose inner hidden epitopes which can bind to IgE antibodies. Actually, the EFSA opinion related to the evaluation of allergenic foods for labelling purposes has already acknowledged that the data available do not indicate whether and how food processing predictably influences allergenicity. In this study, IgE reactivity of OVA decreased with a prolonged time of glycation, which was in accordance with the previous work (Huang et al. 2009). We first tried to understand the relationship between the reduced IgE reactivity of OVA with the covalent attachment of reducing sugar chains. It indicated that glucose attachment was almost accomplished during the first 30 min of glycation (Figure 1). Longer periods of reaction induced only a minor sugar conjugation, while IgE reactivity lowered even from 30 to 60 min of treatment. Thus, the reduced allergenicity of OVA-G was not induced by the blocking of lysine residue with glucose conjugation. The same phenomenon was also observed by Nakamura et al. (2006), who blocked the lysine residues in squid tropomyosin (TM) using 2,4,6-trinitrobenzenesulfonic acid and found that the modification of the lysine residues in TM had no effect on its allergenicity. We then tried to make it clear whether the reduced IgE reactivity of OVA was caused by structural changes. With respect to the secondary structure change of OVA, prolonged glycation increased the content of  $\beta$ -sheets, while the amount of  $\beta$ -turns decreased. Moreover, OVA contains five immunodominant IgE allergenic epitopes that mainly consist of a  $\beta$ -sheet and a  $\beta$ -turn on the secondary structural level (Mine & Prithy 2003), and four of them exposed on the protein surface. Consequently, we can predict that the glycation treatment buried some of the immunodominant IgE allergenic epitopes by inducing  $\beta$ -sheet and  $\beta$ -turn changes, resulting in reduced OVA allergenic reactivity. Interestingly, the transition to a  $\beta$ -sheet by glycation also existed in other proteins. For example, a prolonged incubation with carbohydrate (glucose, fructose or ribose) induced a transition in human serum albumin from a helical to a  $\beta$ -sheet structure (Sattarahmady et al. 2008). Moreover, Ara h 1 purified from roasted peanuts (R-Ara h 1) appeared to retain more native-like  $\beta$ -sheet structures than the boiled protein, and such structural features account for R-Ara h 1 retaining its allergenic IgE binding capacity (Blanc et al. 2011).

Conformational changes of the OVA can be inferred by alterations of the surface and total sulfhydryl contents and the surface hydrophobicity of the protein. The glycation reaction resulted in an increased hydrophobicity due to protein unfolding as was measured by an increase in fluorescence intensity after the treatment. Prolonged time of glycation decreased the hydrophobicity. The same phenomenon also existed in human serum albumin (Zoellner et al. 2001; Sattarahmady et al. 2007). The decrease in hydrophobicity of the longer-term modified OVA could be caused by aggregation or partial refolding of the molecule. 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 a chemical reagent such as DTNB. However, conserved cysteine residues may occur, thus the sulfhydryl group content of native OVA could still be tested. The increase of the sulfhydryl group contents on the surface of OVA after glycation for 30 min may be caused by the unfolding of the protein structure, which exposed the conserved cysteine residues, while the decrease of the sulfhydryl group contents on the surface of OVA after glycation for 60 min may be caused by partial refolding of the molecule. The decrease of the total SH groups after glycation for 30 min was probably due to a rearrangement of cysteine residues and the oxidation of SH groups (Van der Plancken et al. 2005). On the contrary, after glycation for 60 min the total SH content was increased, most likely due to disruption of the disulfide bond. As OVA has a sole disulfide bond between Cys 73 and Cys 120, disruption of the disulfide bond means conformation around Cys 73 and Cys 120 was changed.

Antigenicity of OVA, following a trend of gradual increase after glycation, could be explained by the structural changes. The increase of antigenicity of OVA after glycation might be induced by conformational release – the exposure of inner epitopes by the unfolding of a protein – as reported by Kim et al. (2002). The structural changes could expose hydrophobic groups onto the surface.

Heating resulted in decreased IgE binding of OVA, whereas the IgG binding was increased, which is in agreement with previous work (Kim et al. 2002). These authors indicated that human IgE did not recognise the heated OVA well, and mouse IgG retained more binding capacity under those conditions. Similarly, Mine and Zhang (2002) described that heat treatment lowered the binding of OVA to human IgE, while the binding activities of rabbit IgG antibody to heated OVA was increased. As indicated in the secondary structure change of OVA, heating also caused  $\beta$ -sheet and  $\beta$ -turn changes. Similar to the results in glycation, we could say that heating buried some of the immunodominant IgE allergenic epitopes by inducing  $\beta$ -sheet and  $\beta$ -turn changes, resulting in reduced OVA allergenic reactivity. Since human and rabbit polyclonal antibodies

did not show analogous affinities to the same peptides (Elsayed & Stavseng 1994), and OVA IgG and IgE binding epitopes were only partially overlapped (Elsayed & Stavseng 1994; Mine & Prithy 2003), the changes in OVA structure, though resulting in buried IgE allergenic epitopes, may actually expose more antigenic epitopes and thus increase the IgG binding capacity. Therefore, the IgE epitopes burying can concur with IgG epitopes exposure, resulting in different antigenicity and IgE binding capacity changes.

Studies of other allergens, particularly in the search for hypoallergenic allergens, support the view that structural changes of proteins may affect the allergenicity. In this work it was found that alteration of OVA conformation after heating and glycation did affect its potential allergenicity. However, sera from only four patients with egg allergy were available and several factors such as geographical location, genetics and so on were not taken into account. Moreover, glycation of OVA did not completely abrogate potential allergenicity; and it may be possible to reduce it further through longer treatment or higher temperature wet-heating regimes. Nevertheless, this work might be useful for understanding the effects of processing on loss of immunoreactivity and potential allergenicity.

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