



Effect of heat treatment on the potential allergenicity and conformational structure of egg allergen ovotransferrin

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ABSTRACT

The aim of this research was to assess heat induced changes of the potential allergenicity and the structure of ovotransferrin (OVT). Changes in conformational structure of OVT were characterised by circular dichroism (CD) spectroscopy, ultraviolet (UV) absorption spectroscopy and fluorescence spectroscopy. A reversible unfolding structure was found at 55–60 °C, followed by the loss of secondary structure at 70–80 °C. Cleavage and rearrangements of disulphide bonds occurred when heated above 80 °C. The indirect enzyme-linked immunosorbent assay (ELISA) of heat-treated OVT with egg allergic patients' sera and specific rabbit polyclonal antibodies against native OVT showed that the potential allergenicity of OVT correlated well with the structural changes. Allergenicity increased with the unfolding of its structure, and decreased with the cleavage of disulphide bonds and the rearrangements of disulphide bonds. The results suggested that conformational changes of OVT induced by heat treatment significantly influenced the allergenicity of OVT, and the potential allergenicity of heated OVT is related to individual patient characteristics.

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1. Introduction

Hen egg is one of the most frequent causes of food allergy in infants and young children, with an estimated incidence of 1.6–3.2% (Eggesbo, Botten, Halvorsen, & Magnus, 2001; Heine, Laske, & Hill, 2006). Moreover, egg hypersensitivity was found in two thirds of children with severe atopic dermatitis (Sampson, 2004). Currently, the most effective way to prevent egg allergy is to avoid the offending egg compounds totally. However, the omnipresence of egg-derived components in cooked or manufactured food products renders the approach difficult (Allen, Campbell, & Kemp, 2007), and inadvertent exposure may lead to life-threatening anaphylactic responses. Furthermore, when egg allergy occurs in association with another food allergy, such as cow's milk, the risks of nutritional deficiency represent an additional hurdle for the patients (Grimshaw, 2006). Therefore, the development of egg-derived products that are safe for consumption by allergic consumers is highly warranted (Mine & Yang, 2008).

Heat treatment, a commonly used method in food processing, is usually carried out to enhance texture and flavours or to ensure microbiological safety. At the same time, it may alter, i.e., increase or decrease, the allergenicity of allergens, depending on the severity of the treatment. Effects of thermal processing on food protein allergens have been reviewed in several recent papers (Sanchez & Frémont, 2003; Sathe, Teuber, & Roux, 2005). The basis for this approach has been demonstrated in a clinical study in egg allergy. For example, Urisu et al. (1997) pointed out that patients who were allergic to freeze-dried egg white did not react to cooked egg white. Another report described two patients who developed anaphylactic reactions to raw egg ingestion, whereas no reaction was observed in consumption of cooked egg (Eigenmann, 2000). Similarly, earlier *in vitro* studies showed that thermal treatment of egg white at 90 °C for 10 min led to a 50% decrease of IgE-binding intensity in radioallergosorbent test (RAST) using 16 egg-allergic patients' sera (Anet et al., 1985).

Major egg allergens are mainly contained in egg white (Anet et al., 1985), including ovalbumin (OA), ovomucoid (OVM), ovotransferrin (OVT) and lysozyme (Lys). The effect of heating on allergenicity of single allergens in egg white has been studied. These studies mainly focused on ovalbumin and ovomucoid (Hoffman, 1983; Mine & Zhang, 2002), and rarely on ovotransferrin. Ovotransferrin, also called conalbumin, accounts for 12% of

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the total egg white protein. It consists of 686 amino acids with 15 disulphide bonds and without free sulfhydryl groups. The isoelectric point (pI) and molecular weight (MW) of OVT are 6.5 and 78 kDa, respectively (Mine, 1995; Williams, Elleman, Kingston, Wilkins, & Kuhn, 1982). Moreover, OVT is an iron-binding glycoprotein which contains 2.63% carbohydrate and can bind two ferric ions. It has been reported that the conformational stability of OVT, to some extent, depends on iron binding and release, as well as on the covalent attachment of carbohydrate to the polypeptide backbone of the protein (Hirose, 2000; Wang, Eufemi, Turano, & Giartosio, 1996). However, the dominating form of OVT in egg white is iron-free OVT (Jacobsen et al., 2008). OVT is a glycoprotein, and contains 0.85% mannose and 1.78% *N*-acetylglucosamine. The role of carbohydrate moieties in the allergenic activity of OVT is still a controversial issue. In addition to natural glycosylation, an important heat-induced modification of proteins is the reaction between non-bound reducing sugars and specific amino acids in the protein. This is known as the Maillard reaction, which may introduce neoantigens and affect the allergenicity (Davis, Smales, & James, 2001).

In the present study, we investigated the effect of heating on the potential allergenicity and the structure of OVT, to gain insight into mechanisms that underlie possible changes. The binding of OVT to IgE and IgG were assessed by indirect enzyme-linked immunosorbent assay (ELISA) with egg allergic patients' sera and rabbit polyclonal antibodies. The secondary structure was analysed by circular dichroism (CD) spectroscopy. Ultraviolet (UV) absorbance of the protein was determined by UV absorption spectroscopy, and surface hydrophobicity and sulfhydryl groups were estimated by fluorescence spectroscopy, respectively.

2. Materials and methods

2.1. Ovotransferrin isolation

Ovotransferrin was isolated from "mucin-free" hen egg white, prepared according to a modified method of Croguennec (Croguennec, Nau, Pezennec, Piot, & Brule, 2001), by using a single-step ion exchange chromatography on a DEAE-Sepharose Fast Flow column (Tong, Gao, & Chen, 2010). Briefly, 10-day-old red eggshell hen egg white was diluted with two volumes of distilled water. The mixture was adjusted to pH 6.0 with 2 M hydrochloric acid, then gently stirred and kept at 4 °C for 3 h enabling ovomucin precipitation. The mixture developed a white and gelatinous precipitate which was later removed by centrifugation at 3000g for 3 min (4 °C). The supernatant was adjusted to pH 8.0 with 3 M sodium hydroxide, followed by centrifugation at 10,000g for 30 min at 4 °C in order to remove insoluble materials. The supernatant was "mucin-free" egg white ("mucin-free" EW), which was loaded onto a DEAE-Sepharose Fast Flow column previously equilibrated with 0.05 M Tris-HCl buffer (pH 8.0) at a rate of 1.5 ml/min. Proteins were eluted by linear gradients using 0–0.5 M sodium chloride in 0.05 M Tris-HCl buffer (pH 8.0) at a rate of 1.0 ml/min, and then fractionated and identified by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and electro spraying ionisation-mass spectrometry (ESI-MS).

2.2. Preparation of antisera against OVT

Antisera against OVT were obtained from two female New Zealand rabbits by injection of purified OVT. Two other rabbits, injected with normal saline (0.9% w/v sodium chloride), were used as control. All rabbits were accommodated for a week after arrival at the facility (Institute of Occupational Medicine of Jiangxi, the permission number is "SYXK (Gan) 2008-003"), and maintained

under standard animal housing conditions with free access to food and water. The rabbits were pre-bled and received 1 ml of OVT solution emulsified with equal volume of complete Freund's adjuvant (Sigma, St. Louis, MO, USA) by subcutaneous injection on the back. Control rabbits ($n = 2$) were injected with normal saline. Two weeks after the primary immunization, a booster was given with the same amount of antigens using incomplete Freund's adjuvant (Sigma, St. Louis, MO, USA). The rabbits were re-stimulated several times as boosters in intervals of two weeks, and titres of the sera (the highest dilution factor of serum that still shows a positive reaction with OVT) were determined by indirect ELISA (Li, Luo, Chen, & Cao, 2008). When the titre rose to a stable value, the animals were bled and the antisera were separated and stored at -80 °C until use.

2.3. Human sera

Human sera were collected from three egg allergic patients. Their specific IgE level for egg and total IgE level were determined by AllergyScreen (MEDIWISS Analytic GmbH, Moers, Germany). The three patients were numbered P1, P2 and P3, with an average age of 6.9 years. Their specific IgE level was 1.2, 0.5 and 0.47 kU/l, respectively. Of the three patients, P3 had asthma. P2's total IgE level was more than 200 kU/l, higher than the other two patients (<200 kU/l, data not shown), although the specific IgE level for egg was not the highest. All serum samples were kindly provided by Dr. Luo Hong from the Department of Pediatrics, First Affiliated Hospital of Guangxi Medical University (Guangxi, China) and stored at -80 °C until use.

2.4. Heat treatment of OVT

A volume of 1 ml of a 0.6 mg/ml OVT solution in phosphate buffer, pH 7.3, was introduced into stoppered tubes. Three tubes for each point were prepared and immersed in a temperature-controlled water bath heated at 8 different temperatures: 55, 60, 70, 80, 85, 90, 95 or 100 °C, respectively. The tubes were removed after heating for 5, 15, 30, 45 or 60 min, and immediately cooled in an ice-water bath to prevent further heating reaction. Samples from the tubes were removed and diluted to the adequate dilution in buffers used for the following studies.

2.5. Effect of heat treatment on the antigenicity and potential allergenicity of OVT

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

A 96-well ELISA microplate was coated with 100 μ l of heated or native purified OVT (1.5 μ g/ml) in 50 mM sodium carbonate buffer (pH 9.6) and incubated overnight at 4 °C. The wells were washed three times with phosphate-buffered saline containing 0.05% Tween 20 (PBST), followed by blocking with 5% skimmed milk in phosphate-buffered saline (PBS) for 1 h at 37 °C. After further washing with PBST for three times, 100 μ l antisera (diluted 1:100,000 for the rabbit sera pool to test the IgG binding ability of OVT, and diluted 1:20 for human sera for testing the IgE binding ability of OVT), were added to the wells and incubated for 1 h at 37 °C. The wells were washed again, and 100 μ l of goat anti-rabbit IgG or anti-human IgE horseradish peroxidase (HRP) conjugates (Sigma, St. Louis, MO, USA), respectively, were added to each well, followed by incubation for 1 h at 37 °C. The plates were further washed three times with PBST, and then developed in a 100 μ l substrate solution of 3,3',5,5'-tetramethylbenzidine (TMB) (Sigma, St. Louis, MO, USA) with incubation for 15 min at 37 °C. The reaction

was terminated by addition of 50 μ l of 2 M sulphuric acid, and the optical density was determined at 450 nm using the Bio-Rad Microplate Reader.

2.6. Structure analysis

Conformational changes of OVT after heat treatment 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 measured three times and the results of each sample were averaged.

2.6.1. Circular dichroism analysis

To define the secondary structure of unheated and heated OVT, Far-UV CD spectroscopy was performed in the spectral range from 190 to 250 nm. Protein concentrations of 0.265 mg/ml were used for analysis. Spectra were recorded on a JASCO J810 spectropolarimeter (Jasco, Groß-Umstadt, Germany) at room temperature. A scan speed at 100 nm per min, a bandwidth of 1 nm, and a response time of 1–4 s were used. The CD spectra were measured three times and the results of each sample were averaged, followed by the molar residue ellipticity (θ) being expressed as $\text{deg}\cdot\text{cm}^2\cdot\text{dmol}^{-1}$, and the contents of different secondary structures were calculated with JASCO secondary structure software.

2.6.2. Measurement of surface hydrophobicity of OVT

Hydrophobicity of OVT was determined on the basis of the method of Takagi (Takagi, Akashi, & Yasumatsu, 1979) using hydrophobic fluorescence probes, 8-anilino-1-naphthalenesulfonate (ANS). An aliquot of 20 μ l of 5.0 mM ANS solution was added to 4.0 ml of 80 μ g/ml of the each protein sample solution. The relative fluorescence was measured with a spectrophotofluorometer (F-4500, HITACHI, Hitachi, Japan). The excitation wavelength was 390 nm, and the emission wavelength was from 400 to 650 nm at a scanning speed of 25 nm/s.

2.6.3. Determination of sulfhydryl groups

Sulfhydryl group (SH) content on the surface of unheated and heated OVT was determined by the method of Ellman (1959). A volume of 1 ml of each OVT sample solution was added to 4 ml of 0.1 M phosphate buffer (pH 8.0) mixed with 0.05 ml of 0.01 M

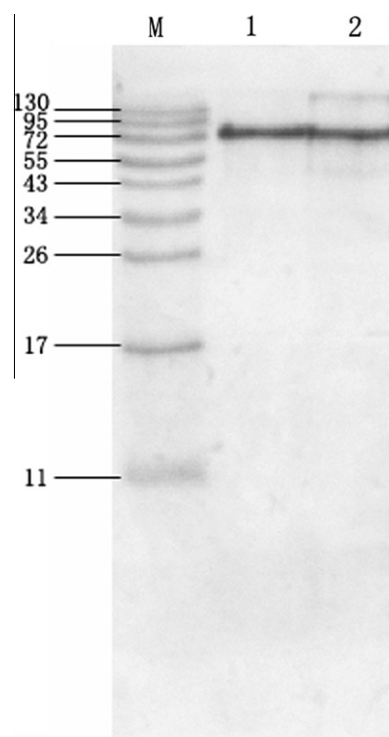


Fig. 1. SDS-PAGE patterns of purified and commercial OVT (Stained with Coomassie brilliant blue R250). Lane M, prestained marker; lane 1, purified OVT; lane 2, commercial OVT (Sigma, St. Louis, MO, USA, purity >95%). The loading amount of each OVT sample was 3 μ g.

5,5-dithiobis-2-nitrobenzoic acid (DTNB), 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 molar extinction coefficient of $13,600 \text{ M}^{-1} \text{ cm}^{-1}$, and expressed as $\mu\text{mol/g}$ of protein, as described by Yin, Tang, Wen, Yang, and Li (2008).

2.6.4. UV absorption spectra analysis

UV absorption spectra analysis was performed by UV-2450 spectrophotometer (Shimadzu Corporation, Kyoto, Japan). The UV absorption spectra were scanned from 220 to 420 nm.

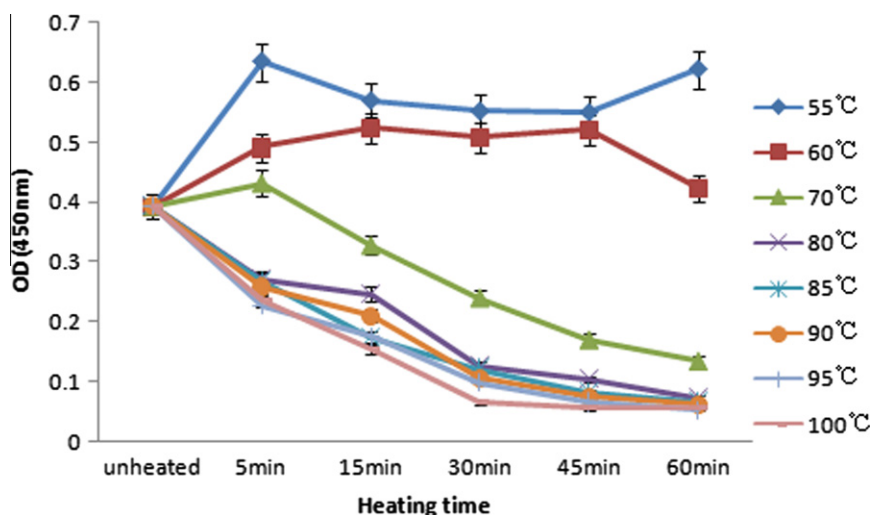


Fig. 2. Effects of various heating temperatures and durations on the IgG-binding to OVT measured by indirect ELISA using rabbit polyclonal antibody. OVT was treated by heating as indicated in the materials and methods. The unheated sample was used as the control. Data are represented as mean $\pm$ standard deviation ($\bar{X} \pm \text{SD}$).

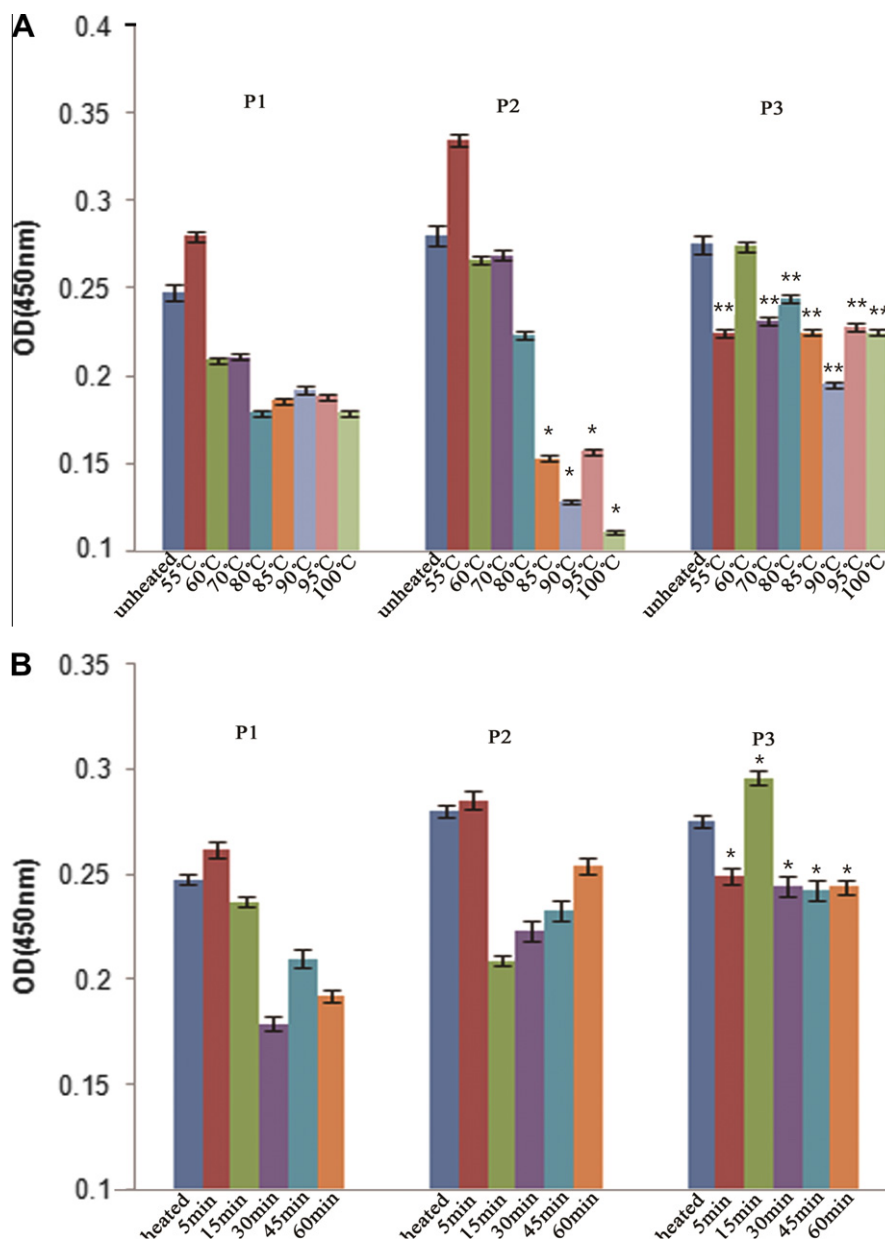


Fig. 3. Effect of heat treatment on the IgE binding to OVT determined by ELISA. (A) Effect of heating for 30 min at different temperature on the IgE binding to OVT. (B) Effect of heating at 80 °C for 5–60 min on the IgE binding to OVT. P1–P3, egg allergy patients numbered 1, 2 and 3. Data are represented as mean $\pm$ standard deviation ($\bar{X} \pm \text{SD}$). Asterisks indicate that the IgE binding to OVT at these temperatures or times are significantly different from that to unheated OVT ($p < 0.05$). Double asterisks means the significance level was $p < 0.001$.

2.7. Statistical analysis

Appropriate data were analysed for statistical significance using ANOVA procedures (SPSS for Windows, version 13.0, Chicago, IL, USA) and Fisher's least significant difference (LSD, $p < 0.05$) test.

3. Results

3.1. Purity and identity of isolated OVT

In this study, OVT was isolated from egg white by using a single step anion exchange chromatography procedure carried out on a DEAE-Sepharose Fast Flow column. The purity of isolated OVT, compared with commercial OVT (Sigma, St. Louis, MO, USA, purity >95%), was analysed with SDS-PAGE and is shown in Fig. 1. Considering that identical amounts of 3 μg protein were loaded onto the

gels, the purity of the isolated OVT is much higher than that of the commercial one. In addition, Electro spraying ionisation–mass spectrometry (ESI–MS) revealed that the purified OVT is homogeneous with the reported OVT and the MW and pI value of purified protein were 77,726 Da and 6.85, respectively (data not shown). In the view of pI values of 6.4, 6.6 and 6.8 for OVT containing 2, 1 and no ferric ions, as reported by Desert et al. (2001) we could conclude that the purified OVT with our method was iron-free and represented the main form as occurring in egg white (Jacobsen et al., 2008).

3.2. Effect of heat treatment on the antigenicity and the potential allergenicity of OVT

To determine whether heating has an effect on the antigenicity and potential allergenicity of OVT, the IgG and IgE binding to heated OVT were tested by indirect ELISA assays with rabbit anti-

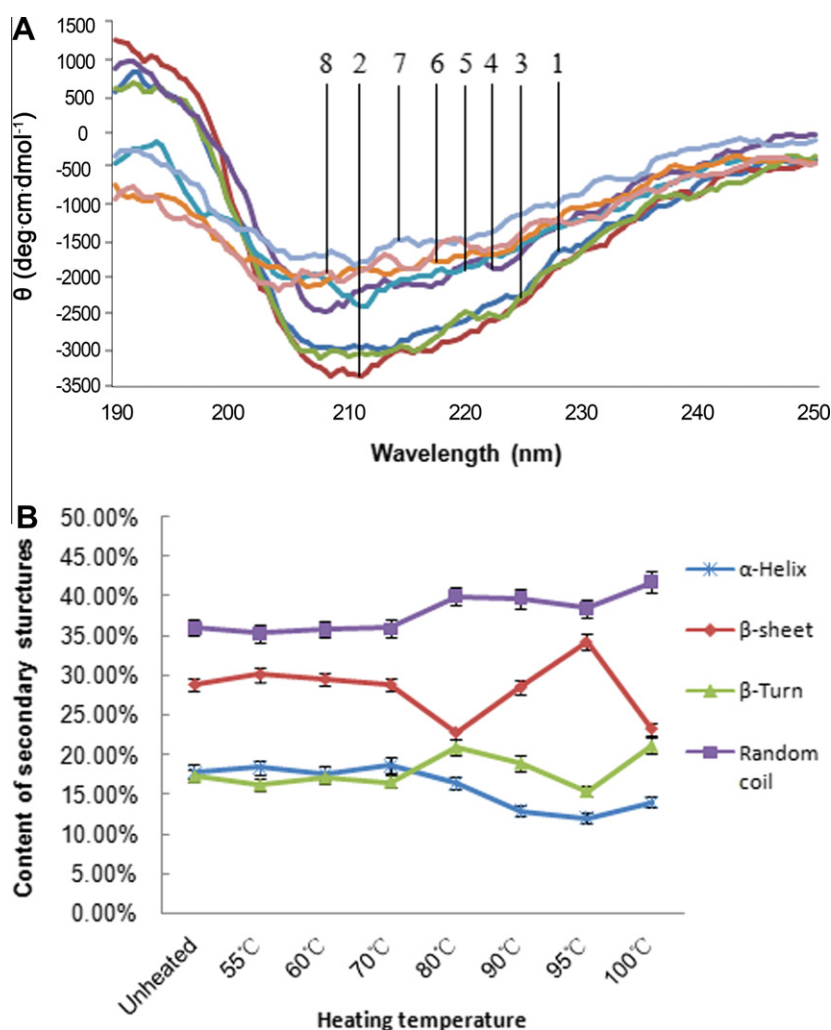


Fig. 4. Effect of heat treatment on the secondary structure of the OVT at different heating temperatures for 30 min measured by Far-UV CD spectroscopy. (A) Far-UV CD spectra of unheated and heated OVT at different temperatures for 30 min. 1–8 represent samples of unheated OVT and of heated OVT treated under a continuous temperature of 55, 60, 70, 80, 90, 95 and 100 °C, respectively. (B) The content of each secondary structure of unheated and heated OVT at different temperatures for 30 min.

sera against native OVT and egg allergic patients' sera. Fig. 2 shows that the IgG binding to OVT was enhanced when heated at 55, 60 or 70 °C for 5 min. The maximal increase was calculated to be 60% of the initial value (data not shown). When heated longer at 55 and 60 °C, the IgG binding to OVT remained higher than that of the untreated protein, while it decreased after heating at 70 °C and became lower than that of unheated OVT. Moreover, when the heating temperature was equal to or higher than 80 °C, the IgG binding to OVT decreased significantly and it decreased with increasing temperature. However, the changes in IgG binding to OVT varied little after heating for 30 min, and there was no obvious further decrease of the IgG binding to OVT when the heating temperature was above 80 °C.

Fig. 3 showed that the binding of IgE from different patients to OVT was different, and the same temperature treatment had different effects on the interaction between OVT and IgE from different patients. The binding of serum IgE of P1 and P2 increased when OVT was heated at 55 °C, while that of P3 decreased (Fig. 3A). With increasing heating temperature, the serum IgE of P2 binding to heat-treated OVT decreased gradually (heating at 95 °C was an exception), and a significant decrease appeared when the heating temperature was above 80 °C. Binding of serum IgE from P3 to heated OVT showed a significant decrease compared with IgE-binding for the unheated protein, except for OVT heated at 60 °C.

Moreover, the binding of IgE from P1 to OVT also decreased when the treated temperature was increased. However, the changes were not significant, and there seemed to be no obvious change after treatment at 80 °C (Fig. 3A). Furthermore, the effect of heating time at 80 °C on the IgE binding to heat-treated OVT was shown in Fig. 3B. Interestingly, individual patient differences appeared again, and the binding of serum IgE from P1 and P2 to heated OVT at 80 °C for 5 min increased compared with the binding to unheated protein. When prolonging the heating time at 80 °C from 15 to 60 min, the binding of IgE from P2 to heated OVT increased gradually, but was still lower than that of unheated OVT. The binding of serum IgE from P3 to OVT increased compared with the binding unheated one when heated at 80 °C for 15 min, while it was lower, but almost constant, at other heating points (5, 30, 45 and 60 min).

3.3. Effect of heat treatment on the structure of OVT

CD spectroscopy was used to characterise the impact of heating on the secondary structure of the protein. When heated at 55 °C for 30 min, the positive and negative molar residue ellipticity peaks of this sample increased slightly, which showed that the amounts of α -helix and β -sheet had increased. The CD spectrum of the OVT treated at 60 °C, however, was almost identical to that of the unheated protein. When OVT was heated at 70 °C, the intensity of

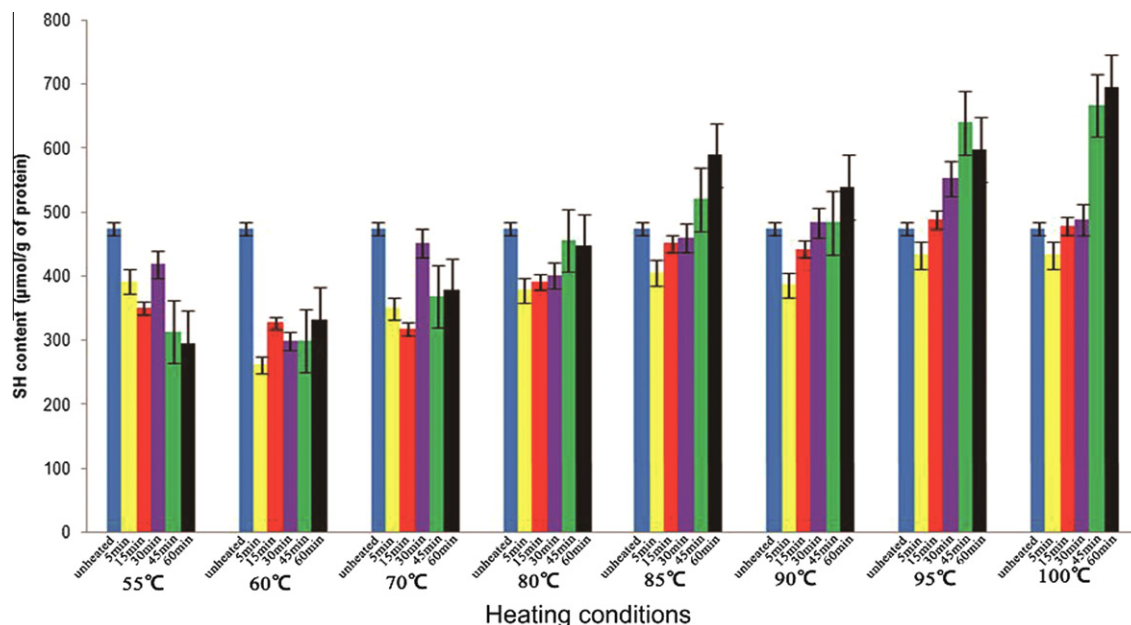


Fig. 5. Effect of different heating temperature and durations on the variation of the sulfhydryl group (SH) content of OVT measured by Ellman's method at 412 nm. Data are represented as mean $\pm$ standard deviation ($\bar{X} \pm SD$).

the negative molar residue ellipticity peak began to decrease, and at 80 °C, the positive molar residue ellipticity peaks started to disappear. Moreover, all the CD spectra of the heated OVT samples became much more unsmooth (Fig. 4A). Consequently, the CD spectra showed that there may be a reversible unfolding between 55 and 60 °C, and the secondary structure of OVT was significantly changed when the heating temperature was higher than 70 °C, which was proved by the changes of secondary structure contents of the unheated and heated OVT calculated by JASCO secondary structure software (Fig. 4B).

The sulfhydryl group contents on the surface of the OVT changed following heat treatment in a time-dependent manner at each heating point. The sulfhydryl group contents of the OVT decreased when it was heated for 5 min at each temperature. After prolonged heating at a temperature below 80 °C, the sulfhydryl group contents of the OVT was still lower than that of unheated protein, while it increased after heating at temperatures above 80 °C and became higher than that of unheated OVT after treatment for more than 30 min (Fig. 5).

The fluorescence intensities induced by ANS and UV absorptions of heated OVT increased with increasing temperature and time of heating (Fig. 6A and B). Accordingly, the hydrophobicity and the UV absorption groups of OVT were increased by heating. In addition, a slight blue shift of the UV absorbing peak was observed after heating.

4. Discussion

Heat treatment may affect the allergenicity and antigenicity of the OVT protein. In our study, the potential allergenicity and antigenicity of OVT after heat treatment were determined by the binding of IgE and IgG *in vitro* using egg allergic patients' sera and rabbit anti-OVT sera, respectively. Results showed that the antigenicity of OVT increased with treatment at low temperature, while it decreased significantly when the temperature was above 80 °C. This heat-induced alteration tendency of antigenicity was similar to the previous studies on bovine α -lactalbumin and β -lactoglobulin (Bu, Luo, Zheng, & Zheng, 2009). The potential allergenicity of differently heat-treated OVT towards the serum IgE from different patients showed individual differences. Serum from patient 2

showed a gradually decreasing reactivity upon increasing heating temperature, and serum IgE from patient 3 showed a modest, but significant, decrease in reactivity, while serum from patient 1 has an intermediate, but not significant, response to heat-treated OVT. The individual differences may be explained by assuming that the original material to which these individuals were sensitised differed: some people may have developed the allergy only to raw eggs, while others may have developed egg allergy to partially cooked or even strongly cooked egg products.

Binding of OVT, after heating at 80 °C for different time intervals, to serum IgE of patient 2 showed a small increase when heated for 5 min, then decreased while there was an increased tendency for heating time from 15 to 60 min, although the immunoreactivity was weaker than that of unheated OVT. A possible explanation for this phenomenon may be that this particular patient is more sensitive to Maillard products, which may introduce neoantigens and a subsequent increase in allergenicity (Davis et al., 2001). In addition, the immunoreactivity of OVT binding to serum IgE of patient 3, who has asthma, showed significant changes with the heating temperature and time, which may imply that another allergy also affect egg allergy.

Alterations in OVT protein structure induced by heat treatment were also observed. The nature and extent of these alterations depend on the temperature and the duration of the heating processing. For the secondary structure of OVT, the α -helix and β -sheet content of OVT, increased and β -turn and random coil structure decreased when heated for 30 min at 55 °C, which meant the structure of OVT started to unfold. Interestingly, when the temperature was increased to 60 °C, the secondary structure of the OVT was nearly the same as the unheated protein, and it implied that the unfolding of the structure of OVT at 55 °C may be reversible. Since unfolding of the protein structure may result in more antigenic epitopes buried inside the native molecule being exposed on the surface of the molecule (Davis & Williams, 1998), it may explain why the potential allergenicity of the OVT treated at 55 and 60 °C was higher than that of unheated protein. When the temperature was raised to 70 °C, the secondary structure did not seem to change, while the intensity of the negative molar residue ellipticity peak of CD spectrum began to decrease. Subsequently, the secondary structure significantly changed when the

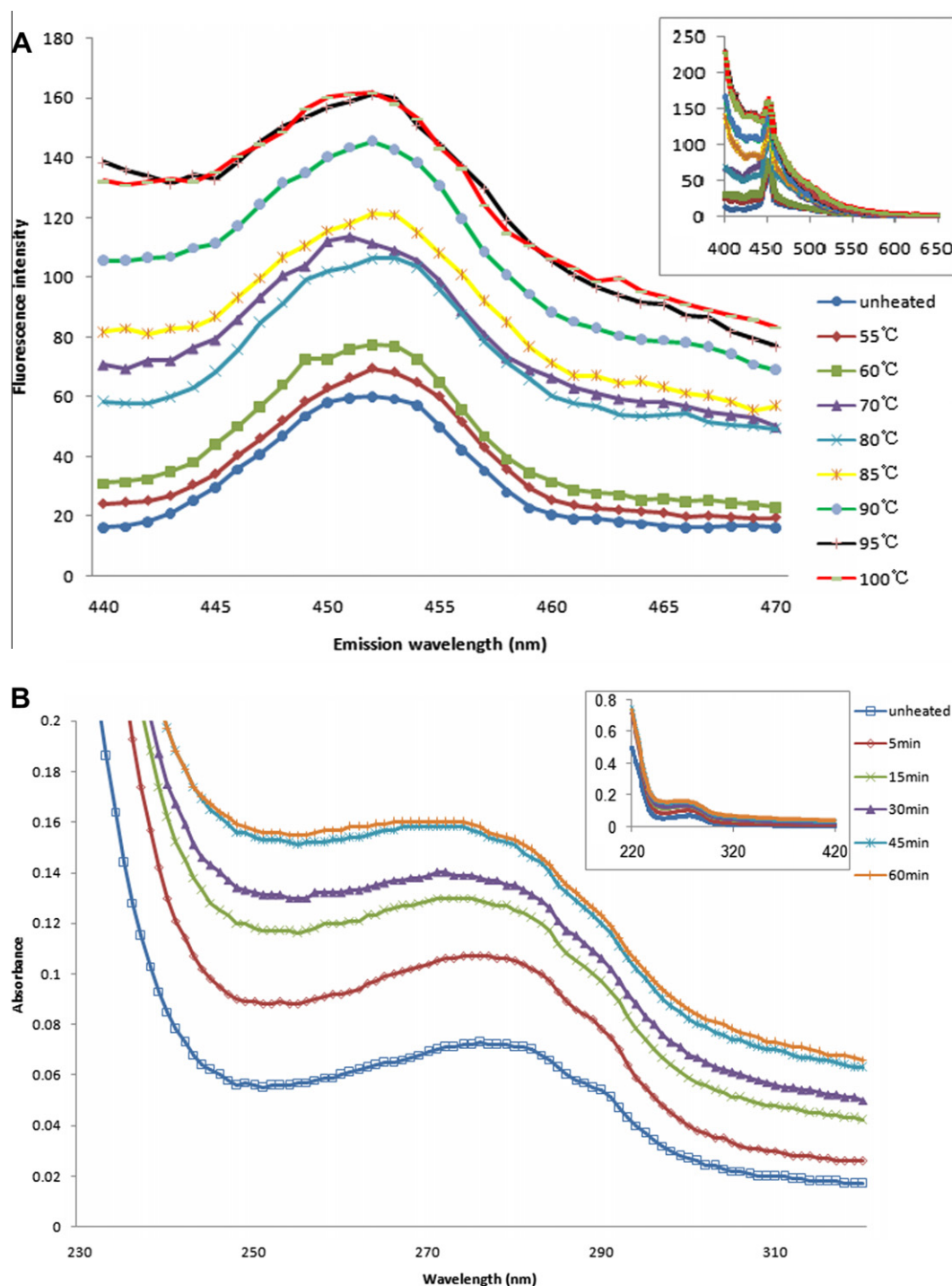


Fig. 6. Effect of heat treatment on the intensity of fluorescence and UV absorption of OVT. (A) The changes of the hydrophobicity of OVT after heating for 30 min was measured using hydrophobic fluorescence probes ANS. The excitation wavelength was 390 nm, and the emission wavelength was from 400 to 650 nm at a scanning speed of 25 nm/s. As the fluorescence spectra were similar after the emission wavelength increased at 500 nm, we simply chose the major emission peaks to show in the main figure. The whole fluorescence spectra were shown on the top right corner. (B) UV absorption of unheated and OVT heated at 80 °C. The whole UV absorption spectrum was scanned from 220 to 420 nm and is shown in the top right corner, while the major absorption peaks spectra were from 250 to 300 nm.

temperature was raised to 80 °C, which indicated that the secondary structure of OVT was destroyed at around 70–80 °C, which was in agreement with previous studies (Davis & Williams, 1998).

The change of sulphhydryl group contents is related to the potential allergenicity variation of the heat-treated OVT. OVT contains 15 disulphide bridges, and of these, six bridges exist in the N-domain and nine in the C-domain (Williams et al., 1982). Most of these are buried in the protein structure, and there is no free cysteine residue (Thornton, 1981). However, conserved cysteine

residues may occur, particularly in the kringle bridges of both the N- and C-lobe of the molecule. Thus, the sulphhydryl group content could be tested on the surface of OVT using Ellman's method. The decrease of the sulphhydryl group contents on the surface of OVT by heating at 55–70 °C may be caused by the unfolding of the structure, which destroyed the conserved cystine residues, and exposed the intra-molecular disulphide bonds. Moreover, there were indications that disulphide bonds play an important role in the integrity of the IgG or IgE-binding epitopes (Marzban

et al., 2009), which could be related to the allergenicity of OVT. With the increase of heating temperature or time, the disulphide bonds were cleaved (Davis and Williams, 1998), resulting in an increase of the sulfhydryl group content on the surface of OVT, accompanied by conformational changes of OVT, resulting in a decrease of the allergenicity of OVT.

Conformational changes of the protein can also be inferred by alterations of surface hydrophobicity and UV absorption of OVT. Since the increased intensity of fluorescence indicated that the protein was unfolded and more hydrophobic regions became exposed after heating, the hydrophobicity of the heated OVT increased. This illustrated that the tertiary and quaternary structure of OVT were changed by heating. Moreover, the maximum UV absorption peak of unheated OVT was found at around 275 nm, indicating that the UV absorption spectrum of OVT was mainly dependent on tyrosine (Tyr) and tryptophan (Trp) residue side-chain groups. The UV absorption intensity of heated OVT samples increased with increasing heating temperature or time. The increase of the absorption intensity suggested that Tyr and Trp residues were exposed on the surface of the protein molecule, as a consequence of unfolding of the structure. A slight blue shift of the absorption peak was observed as the heating time increased, too, which showed that the polarity of the microenvironment of Tyr and Trp had changed after heating, and peptide bonds may be destroyed, which could change the primary structure of OVT.

The potential allergenicity changed remarkably as a result of heat-induced conformational changes. The increase of antigenicity and potential allergenicity of OVT heated at low temperature may result from the unfolding of the structure, which could expose hydrophobic groups, the intermolecular-disulphide bonds and more antigenic epitopes buried inside the native molecule onto the surface of the molecule, while the decrease of antigenicity and potential allergenicity of heated OVT may be caused by the cleavage and rearrangements of disulphide bonds. Thus, the conformational changes of OVT after heating significantly influenced the potential allergenicity of OVT. Heating did not completely destroy the recognition by OVT of serum IgE from egg allergic patients, suggesting the probable persistence of linear epitopes recognised by IgE, and that heat destroyed predominantly the conformational epitopes on OVT.

In conclusion, any process that modifies the structure of a protein may interfere with its ability to be recognised by antibodies. Alteration of the conformational structure of heat-labile proteins, and loss of conformational epitope(s), may explain the reduction or abolition of allergenic potential for these proteins. Moreover, a portion of protein, which may be sufficient for IgE-reactivity, may remain in a more or less native state, depending on the severity of the treatment. The eventual clinical relevance of this may depend on the processing state of the product to which the patient was initially sensitised. For some egg allergy patients, heat treatment may be a good way to reduce the harm of egg by controlling heating temperature and time.

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References

- Allen, C. W., Campbell, D. E., & Kemp, A. S. (2007). Egg allergy: Are all childhood food allergies the same? *Journal of Paediatrics and Child Health*, 43(4), 214–218.
- Anet, J., Back, J. F., Baker, R. S., Barnett, D., Burley, R. W., & Howden, M. E. (1985). Allergens in the white and yolk of hen's egg. A study of IgE binding by egg proteins. *International Archives of Allergy and Immunology*, 77(3), 364–371.
- Bu, G. H., Luo, Y. K., Zheng, Z., & Zheng, H. (2009). Effect of heat treatment on the antigenicity of bovine α -lactalbumin and β -lactoglobulin in whey protein isolate. *Food and Agricultural Immunology*, 20(3), 195–206.
- Croguennec, T., Nau, F., Pezennec, S., Piot, M., & Brule, G. (2001). Two-step chromatographic procedure for the preparation of hen egg white ovotransferrin. *European Food Research and Technology*, 212(3), 296–301.
- Davis, P. J., Smales, C. M., & James, D. C. (2001). How can thermal processing modify the antigenicity of proteins? *Allergy*, 56(Suppl. 67), 56–60.
- Davis, P. J., & Williams, S. C. (1998). Protein modification by thermal processing. *Allergy*, 53(Suppl. 46), 102–105.
- Desert, C., Guerin-Dubiard, C., Nau, F., Jan, G., Val, F., & Mallard, J. (2001). Comparison of different electrophoretic separations of hen egg white proteins. *Journal of Agricultural and Food Chemistry*, 49(10), 4553–4561.
- Eggesbo, M., Botten, G., Halvorsen, R., & Magnus, P. (2001). The prevalence of allergy to egg: A population-based study in young children. *Allergy*, 56(5), 403–411.
- Eigenmann, P. A. (2000). Anaphylactic reactions to raw eggs after negative challenges with cooked eggs. *Journal of Allergy and Clinical Immunology*, 105(3), 587–588.
- Ellman, G. L. (1959). Tissue sulfhydryl groups. *Archives of Biochemistry and Biophysics*, 82(1), 70–77.
- Grimshaw, K. E. (2006). Dietary management of food allergy in children. *Proceedings of the Nutrition Society*, 65(4), 412–417.
- Heine, R. G., Laske, N., & Hill, D. J. (2006). The diagnosis and management of egg allergy. *Current Allergy and Asthma Reports*, 6(2), 145–152.
- Hirose, M. (2000). The structural mechanism for iron uptake and release by transferrins. *Bioscience, Biotechnology, and Biochemistry*, 64(7), 1328–1336.
- Hoffman, D. R. (1983). Immunochemical identification of the allergens in egg white. *Journal of Allergy and Clinical Immunology*, 71(5), 481–486.
- Jacobsen, B., Hoffmann-Sommergruber, K., Have, T. T., Foss, N., Briza, P., Oberhuber, C., et al. (2008). The panel of egg allergens, Gal d 1–Gal d 5: Their improved purification and characterization. *Molecular Nutrition & Food Research*, 52(Suppl. 2), S176–S185.
- Li, X., Luo, Z. L., Chen, H. B., & Cao, Y. S. (2008). Isolation and antigenicity evaluation of β -lactoglobulin from buffalo milk. *African Journal of Biotechnology*, 7(13), 2258–2264.
- Marzban, G., Herndl, A., Pietrozotto, S., Banerjee, S., Obinger, C., Maghuly, F., et al. (2009). Conformational changes of Mal d 2, a thaumatin-like apple allergen, induced by food processing. *Food Chemistry*, 112, 803–811.
- Mine, Y. (1995). Recent advances in the understanding of egg-white protein functionality. *Trends in Food Science & Technology*, 6(7), 225–232.
- Mine, Y., & Yang, M. (2008). Recent advances in the understanding of egg allergens: basic, industrial, and clinical perspectives. *Journal of Agricultural and Food Chemistry*, 56(13), 4874–4900.
- Mine, Y., & Zhang, J. W. (2002). Comparative studies on antigenicity and allergenicity of native and denatured egg white proteins. *Journal of Agricultural and Food Chemistry*, 50(9), 2679–2683.
- Sampson, H. A. (2004). Update on food allergy. *Journal of Allergy and Clinical Immunology*, 113(5), 805–819.
- Sanchez, C., & Frémont, S. (2003). Consequences of heat treatment and processing of food on the structure and allergenicity of component proteins. *Revue Française d'Allergologie et Immunologie Clinique*, 43(1), 13–20.
- Sathe, S. K., Teuber, S. S., & Roux, K. H. (2005). Effects of food processing on the stability of food allergens. *Biotechnology Advances*, 23(6), 423–429.
- Takagi, S., Akashi, M., & Yasumatsu, K. (1979). Determination of hydrostatic region in soybean globulin. *Nippon Shokuhin Kogyo Gakkaishi*, 26, 133–138.
- Thornton, J. M. (1981). Disulphide bridges in globular proteins. *Journal of Molecular Biology*, 151(2), 261–287.
- Tong, P., Gao, J. Y., & Chen, H. B. (2010). One-step purification of ovotransferrin from hen egg white using ion-exchange chromatography with high purity. *Science and Technology of Food Industry*, 6, 100–102.
- Urisu, A., Ando, H., Morita, Y., Wada, E., Yasaki, T., Yamada, K., et al. (1997). Allergenic activity of heated and ovomucoid-depleted egg white. *Journal of Allergy and Clinical Immunology*, 100(2), 171–176.
- Wang, C., Eufemi, M., Turano, C., & Giartosio, A. (1996). Influence of the carbohydrate moiety on the stability of glycoproteins. *Biochemistry*, 35(23), 7299–7307.
- Williams, J., Elleman, T. C., Kingston, I. B., Wilkins, A. G., & Kuhn, K. A. (1982). The primary structure of hen ovotransferrin. *European Journal of Biochemistry*, 122(2), 297–303.
- Yin, S. W., Tang, C. H., Wen, Q. B., Yang, X. Q., & Li, L. (2008). Functional properties and in vitro trypsin digestibility of red kidney bean (*Phaseolus vulgaris* L.) protein isolate: Effect of high-pressure treatment. *Food Chemistry*, 110, 938–945.