

Pasteurization induced protein interaction decreased the potential allergenicity of ovalbumin and ovomucoid in egg white

Qiang Shi,^{a,b} Wujing Wang,^{a,b} Yong Wu,^c Hongbing Chen,^{a,c} Ping Tong^{a*} and Jinyan Gao^{b*}



Abstract

BACKGROUND: Approximately 9.9% of young children in China suffer from egg allergies. Ovalbumin (OVA) and ovomucoid (OVM) are both the main allergens with higher allergenicity in egg white. The previous studies mainly focused on the effects of pasteurization on the structure and allergenicity of the isolated protein itself. The effects of the interaction between OVA and OVM on their spatial structure and allergenicity under pasteurization are still unclear. Therefore, in this study, the spectroscopic, immunological, and cytological methods were used to investigate the effects on OVA and OVM by their interactions which were induced by the following pasteurization, heating for 10 min at 60, 65, and 70 °C, respectively.

RESULTS: Results indicated that OVA and OVM could form macromolecular aggregates by their interaction at 70 °C, and their solubility was decreased while turbidity was increased. The spatial structures of OVA and OVM were both changed by their interaction, when pasteurization temperature was at 70 °C the exposure of their hydrophobic groups and α -helix content were decreased while their β -sheet was increased. The potential allergenicity of OVA and OVM was also changed, which showed that the immunoglobulin G (IgG)-binding ability of OVA and OVM could be increased, and their IgE-binding ability was decreased a bit. The releases of interleukin 4 (IL-4), IL-6, β -HEX, histamine and tumor necrosis factor alpha (TNF- α) from OVA–OVM-induced KU812 cells were all decreased at 70 °C.

CONCLUSION: Therefore, according to the results, if the liquid egg products were pasteurized for 10 min, the temperature of 70 °C should be carefully considered.

© 2022 Society of Chemical Industry.

Supporting information may be found in the online version of this article.

Keywords: interaction; ovalbumin; ovomucoid; protein conformation; potential allergenicity; pasteurization

INTRODUCTION

A hen egg is rich in nutrients, and is widely used as a major ingredient in the food industry.¹ However, it is also one of the eight major allergenic foods defined by the World Health Organization (WHO), it was reported that 9.9% of young children in China and 2–3% of adults worldwide were allergic.^{2,3} Its majority of the allergens, including ovalbumin (OVA), ovomucoid (OVM), ovotransferrin (OVT), and lysozyme (LYZ), are found in egg white. Among them, OVA is the most abundant one, accounting for 54% of egg white proteins with an isoelectric point of 4.5 and composed of 385 amino acids. It is also a phosphoglycoglobulin with a molecular weight of 44.5 kDa and there are about 3% sugar-based components, particularly, OVA is often used as a model for the characterization of proteins structure, properties, and food allergens.⁴ While, OVM is the most allergenic one, it has excellent thermal stability and stability against enzymolysis, and has a high affinity to bind with immunoglobulin E (IgE) after being simulated in gastrointestinal digestion.⁵

Since egg products are easily contaminated with a variety of pathogenic bacteria, especially *salmonella*, pasteurization is a

common operation to guarantee the microbiological safety of liquid eggs.⁶ The temperatures and times for the pasteurization varied partly depending on the thermal sensitivity of egg white proteins.⁷ These proteins tend to be aggregated when the thermal temperatures are relatively higher, and their physicochemical properties and functional properties, such as foaming and

* Correspondence to: P Tong, State Key Laboratory of Food Science and Technology, Nanchang University, Nanchang, P. R. China, E-mail: tongping@ncu.edu.cn; or J Gao, College of Food Science and Technology, Nanchang University, 235 Nanjing Eastern Road, Nanchang 330047, P. R. China. E-mail: gaoyj2013@ncu.edu.cn

a State Key Laboratory of Food Science and Technology, Nanchang University, Nanchang, P. R. China

b College of Food Science and Technology, Nanchang University, Nanchang, P. R. China

c Jiangxi-OAI Joint Research Institute, Nanchang University, Nanchang, P. R. China

emulsification, can be changed.⁸ Previous studies have mainly focused on the impact of the allergenicity or structure of the isolated or whole egg white proteins by pasteurization, while in the whole egg white proteins, they have ignored the fact that processing might also induce the interaction of food proteins and give rise to the possibility of changed allergenicity.^{9–11} Pasteurization could decrease the allergenicity of OVA, but it had little or no effects on the allergenicity of OVM because of its heating resistance.^{12,13} However, Lechevalier *et al.* reported that the antigenicity of OVM in the liquid whole egg was decreased after being heated for 4–10 min at 60 °C.¹⁰ Shin *et al.* also reported that the antigenicity of OVM could be decreased by heating when egg white was mixed with wheat flour.¹⁴ These inconsistent results reminded us that the changes in the spatial structure and allergenicity of egg white proteins during pasteurization might be both related to the pasteurization operations and the protein–protein interactions as well. However, the effects on the spatial structure and the allergenicity of proteins by the interaction between two proteins induced by pasteurization, especially for the interaction between OVA and OVM which are the most important allergenic proteins in egg white, has not been well elucidated. It is worthy of more research to draw further conclusions. There are about 40 kinds of proteins in egg white, therefore, it is very difficult to create the possibility for a multitude of complex interactions. Thus, OVA and OVM were chosen for the study in order to simplify the effects of interaction on the two proteins during pasteurization. And it was hoped that the results could be helpful to study the more complex cases of food allergens. Based on the earlier considerations, the effects of interaction between OVA and OVM on their spatial structure and potential allergenicity during pasteurization would be researched.

The OVA and OVM were isolated from the white egg and were purified by ion-exchange chromatography. Then the two proteins and their mixture OVA–OVM were separately heated for 10 min at

three different temperatures (60, 65, and 70 °C) to simulate the pasteurization operations. The ratio (w/w) of the two proteins in the mixture was as their original ratio in egg white. The interaction of the two proteins was evaluated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE), the changes in their physicochemical properties were defined by both turbidity and solubility, the effects on their spatial structures were then characterized by ultraviolet (UV), fluorescence and circular dichroism (CD) spectroscopy, and the changes of their antigenicity and potential allergenicity were characterized by the binding ability with IgG/IgE and the cytokines being released from the activated basophilic cells KU812, respectively.

MATERIALS AND METHODS

Materials

White shell hen eggs were purchased from the local market (Nanchang, China). Bovine serum albumin standard products were purchased from Shanghai Bioengineering Co. (Shanghai, China); Coomassie Brilliant Blue R-250, 8-anilino-1-naphthalenesulfonate (ANS), horseradish peroxidase (HRP)-labeled sheep anti-rabbit IgG, and avidin-labeled sheep anti-human IgE were purchased from Sigma-Aldrich Chemical Co. (Burlington, MA, USA); HRP-labeled streptavidin and commercial ELISA kit were purchased from Neobioscience Co. (Shenzhen, China); anti-OVM rabbit polyclonal antibody was purchased from Aviva systems biology Co. (San Diego, CA, USA); rabbit polyclonal antibody against egg allergen OVA was supplied by our own laboratory. All chemical reagents used in this study were of analytical grade.

Human sera

Human sera from 19 patients who were all allergic to eggs (Table 1) were collected together as a human sera pool and each patient of them had egg protein-specific IgE levels ranging from

Table 1. Information of egg-allergic patients' sera

Patient number	Sex	Age	Symptoms	S-IgE to egg white (IU mL ⁻¹)
1	Female	74	Health check-up	1.6
2	Female	11	Bronchial asthma	100
3	Female	23	Contact dermatitis	3.2
4	Female	30	Chronic urticaria	2.4
5	Female	4	Chronic cough	11.5
6	Male	3	Eczema	1
7	Male	30	Eczema	2.2
8	Male	4	Asthma	2.7
9	Male	6	Pneumonia	1.3
10	Female	3	Cough	2.6
11	Male	N ^a	Urticaria	3.2
12	Male	6	Rhinoconjunctivitis	1.9
13	Male	8	Rhinoconjunctivitis	3.3
14	Male	71	ND ^b	1.2
15	Female	23	Eczema	3.8
16	Male	27	Chronic urticaria	1.2
17	Male	31	Urticaria	2.6
18	Male	7	Allergic purpura	2.1
19	Female	3	Abdominal pain	19.9

^a N, unknown.

^b ND, not done.

1 to 100 IU mL⁻¹. All serum samples were from the First Affiliated Hospital of Guangxi Medical University (Nanning, China) and were stored at -80 °C.

Purification of OVA–OVM and sample preparation

OVA and OVM in egg white were purified by anion exchange chromatography according to the following methods described by Ma *et al.*¹⁵ The purified OVA and OVM were respectively diluted with 0.01 mol L⁻¹ phosphate-buffered saline (PBS) to a concentration of 1.58 and 0.323 mg mL⁻¹, the OVA–OVM were prepared according to the ratio 54:11 (OVA–OVM) and the volume of the mixed solution was 1 mL. All solutions of OVA, OVM, and OVA–OVM were separately heated for 10 min at 60, 65, and 70 °C, respectively. The temperatures being chosen were to simulate the pasteurization temperature of 60, 65, and 70 °C, and the heating time of 10 min adhered to any pasteurization standards for liquid egg white. The sample (25 °C) was used as the control.

Identification of molecular weight of the subunit of OVA–OVM by polyacrylamide gel electrophoresis

The spatial structures of samples were analyzed using SDS-PAGE method. Samples (1.0 mg mL⁻¹) were mixed with 2 × SDS loading buffer containing 10% β-mercaptoethanol and heated to 100 °C for 5 min, and then 10 μL was added into polyacrylamide gel pore. The gel was run for 30 min at 12 mA, then 90 min at 24 mA, next, it was stained with Coomassie Brilliant Blue R-250, and the image was recorded by Quantity One-4.6.2 (BIO-RAD Co., Hercules, CA, USA).

Determination of solubility and turbidity

BCA Protein Quantitative Analysis Kit (Shanghai Bioengineering Co., Shanghai, China) was used to measure the soluble protein content. The supernatants of OVA, OVM and OVA–OVM treated at different temperatures were centrifugated at 10 000 × *g* for 20 min at 4 °C, and then the soluble protein content in them was analyzed. The bovine serum albumin was used as the standard protein for drawing the standard curve, the solubility of samples was calculated with Eqn (1), where the *P*₁ was the soluble protein content in the supernatant of heated samples, and the *P*₀ was that of the control.

$$S = P_1 / P_0 \times 100\% \quad (1)$$

Solution turbidity was measured following the method of Van der Plancken *et al.*, and the turbidity of 0% corresponding to a totally clear solution (transmittance in comparison to the blank was 100%).¹⁶ The transmittance was measured at 650 nm using a ultraviolet-visible (UV-vis) spectrophotometer (TU-1901; Shimadzu Co., Shanghai, China).

Structural analysis of protein

Ultraviolet-visible (UV-vis) spectroscopic analysis

UV-vis analysis was performed by TU-1901 UV-vis spectrophotometry (Shimadzu Co.) to examine the tertiary structure of all samples. The samples were diluted to 0.1 mg mL⁻¹ with 0.01 mol L⁻¹ PBS (pH 7.0), and then detected at wavelength range from 250 to 350 nm and response time of 0.2 s.

Exogenous fluorescence spectrum

The surface hydrophobicity of all samples was evaluated by their fluorescence intensity data, and ANS was used as hydrophobic fluorescence probe. Samples were initially diluted to the

concentration of 0.1 mg mL⁻¹ by PBS buffer. Then 4 mL of each diluted sample was mixed with 40 μL of ANS solution (5 mmol L⁻¹) and incubated in darkness for 1 h at room temperature. Fluorescent spectra of the treated samples were recorded on the F-4500 fluorescence photometer (HITACHI, Tokyo, Japan). The settings for testing were as follows: wavelength of excitation at 390 nm and emission at 400–650 nm, speed at 1200 nm min⁻¹, slit width 0.5 nm, light path 1.0 cm, and response time 0.5 s.

Circular dichroism (CD) spectrum

Far-UV (190–240 nm) CD was performed by MOS-450/AF-CD (Bio-Logic, Paris, France) to detect the secondary structure of samples which should be diluted to 0.1 mg mL⁻¹ with 0.01 mol L⁻¹ PBS (pH 7.0) before being tested. The spectra of samples were recorded at the speed of 100 nm min⁻¹ with a bandwidth of 0.1 nm. And their smoothed spectra were represented as mean residue ellipticity (deg cm² dmol⁻¹) based on the mean residue molecular weight, and the contents of different secondary structural items were calculated online (<https://dichroweb.cryst.bbk.ac.uk/html/home.shtml>).

Antigenicity assessment

In order to analyze the influence of the interaction on samples' antigenicity and potential allergenicity, the ability of their binding IgG and IgE were performed by competitive ELISA according to the procedure described by Meng *et al.* with some modifications.¹⁷ For detecting the influence of interaction on the antigenicity and potential allergenicity of OVA, the samples of OVA and OVA–OVM heated at different temperatures were used as competitive proteins, the anti-OVA rabbit sera (diluted 1:20 000, for IgG-binding ability) and the egg-allergic patients' sera pool (diluted 1:20, for IgE-binding ability) were used as the primary antibody, respectively. For the OVM samples, OVM and OVA–OVM heated at different temperatures were used as competitive proteins, the anti-OVM rabbit sera were sensitized by OVM (diluted 1:15000, for IgG-binding ability), and sera pool of egg-allergic patients (diluted 1:20, for IgE-binding ability) was used as the primary antibody. The sheep anti-rabbit IgG conjugated with HRP (diluted 1:5000) and biotin conjugated with sheep anti-human IgE (diluted 1:5000) were used as the second antibody, respectively. The IgG-/IgE-binding ability was calculated by each samples' optical density (OD) value at 490 nm with the Eqns (2) and (3).

$$\text{IgG binding ability} = (1 - B/B_0) \times 100\% \quad (2)$$

$$\text{IgE binding ability} = (1 - B/B_0) \times 100\% \quad (3)$$

where *B* represents the OD value corresponding to competitive protein and *B*₀ represents the OD value corresponding to non-competitive protein.

Potential allergenicity assessment

KU812 cells were purchased from the cell bank at the Chinese Academy of Sciences (Shanghai, China) and were cultured in RPMI-1640 complete medium at 37 °C in a 5% carbon dioxide (CO₂) humidified atmosphere. Specific description is reported in the Supporting Information, Methods S1.

Data analysis

All experiments were performed in triplicate, and every final value was presented as the mean ± standard deviation (SD). Error bars indicated the SD of three measurements. Statistical analysis of the

data was carried out by one-way analyses of variance (ANOVA), SPSS software (version 13; IBM software, Armonk, NY, USA) was for correlation analysis and the least significant difference (LSD) test for multiple comparisons with a significance level of 0.05.

RESULTS

Effects of the interaction on the solubility and turbidity of OVA–OVM

The solubility of all samples heated at different temperatures was analyzed by BCA kit (Fig. 1(A)) and the solubility at 25 °C was the control. The solubility of OVA was slightly decreased being heated at 60 and 65 °C, being reduced to 94% at 70 °C; the solubility of OVM was also only slightly decreased at 60 and 65 °C, but it dramatically decreased to 92% at 70 °C. The solubility of OVA–OVM continuously decreased with heating temperature increasing, which was 94%, 92% and 86% at 60, 65, and 70 °C, respectively. The results showed that the variation tendencies of OVA–OVM solubility were similar to their own monomer, indicating it could be influenced by their interaction, but the pasteurization temperature played the critical factor.

Turbidity is a useful indicator that can directly show food protein aggregation during thermal processing. The turbidity of all samples heated at different temperatures was analyzed (Fig. 1(B)), and the turbidity at 25 °C was the control. The turbidity of OVA was both increased by 50% being heated at 60 and 65 °C, while there was a 250% increase at 70 °C. Moreover, the turbidity of OVM being heated at 60 °C was comparable to the control, but there were 200% and 400% increases at 65 and 70 °C, respectively. The turbidity of OVA–OVM being heated at 60 °C was similar to the control, while it was increased by 400% at 65 °C and there was a sharp increase at 70 °C. Taken as a whole, the turbidity of OVA–OVM was almost the same as their own monomer when pasteurization temperature was below 65 °C, but was significantly increased at 70 °C, which might be caused by the interaction induced by pasteurization.

Effects of the interaction on the reduced and non-reduced SDS-PAGE patterns of OVA–OVM

All samples heated at different temperatures were separately analyzed by reduced and non-reduced SDS-PAGE (Fig. 2). β -Mercaptoethanol was used as the reductive reagent and could cleave the

disulfide bonds in protein structure to generate free sulfhydryl groups. For the OVA samples, there were more dimers available in the non-reduced state but only one band in the reduced state, which indicated the linkage of disulfide bond could make OVAs form dimers hardly by the pasteurization. For the OVMs, their bands in the reduced and non-reduced states were the same at all pasteurization temperatures, indicating that the free sulfhydryl groups and disulfide bonds in OVM structure were still in their natural state, pasteurization treatment had almost no effects on them. For the OVA–OVM mixture, electrophoretic patterns of samples being heated at 60 and 65 °C were the same as the control, however, when heated at 70 °C, there were some new macromolecular polymers formed with a molecular weight above 170 kDa on the top of non-reduced and reduced SDS-PAGE (Fig. 2(C,F)). The results showed that OVA and OVM could form a kind of macromolecular aggregates by their interaction at 70 °C, and the interaction was partly bound by disulfide bonds.

Effects of the interaction on the structural changes of OVA–OVM

The tertiary structural changes of all samples heated at different temperatures were analyzed (Fig. 3(A–C)). Compared with the control, the spectral shape of OVA heated at 60 and 65 °C was similar to its control, and the maximum absorption value was approximately 0.09, indicating that the tertiary structure of OVA displayed negligible changes as the temperature was below 65 °C, but it was increased to 0.19 as the temperature was at 70 °C, indicating that the tertiary structure of OVA was further expanded. The UV spectra of OVM heated at 60, 65, and 70 °C showed almost no changes and were the same as its control, and the maximum absorption value was about 0.2, indicating that the tertiary structure of OVM exhibited insignificant changes with temperature increasing. However, the maximum absorption of OVA–OVM increased with the temperature increasing, which was 0.091, 0.096, and 0.102 at 60, 65, and 70 °C, respectively. The tertiary structural changes of OVA–OVM were various under different pasteurization temperatures compared with their own monomer.

The hydrophobicity of all samples heated at different temperatures was analyzed by exogenous fluorescence spectroscopy (Fig. 3(D–F)). Compared with the control, there was a small

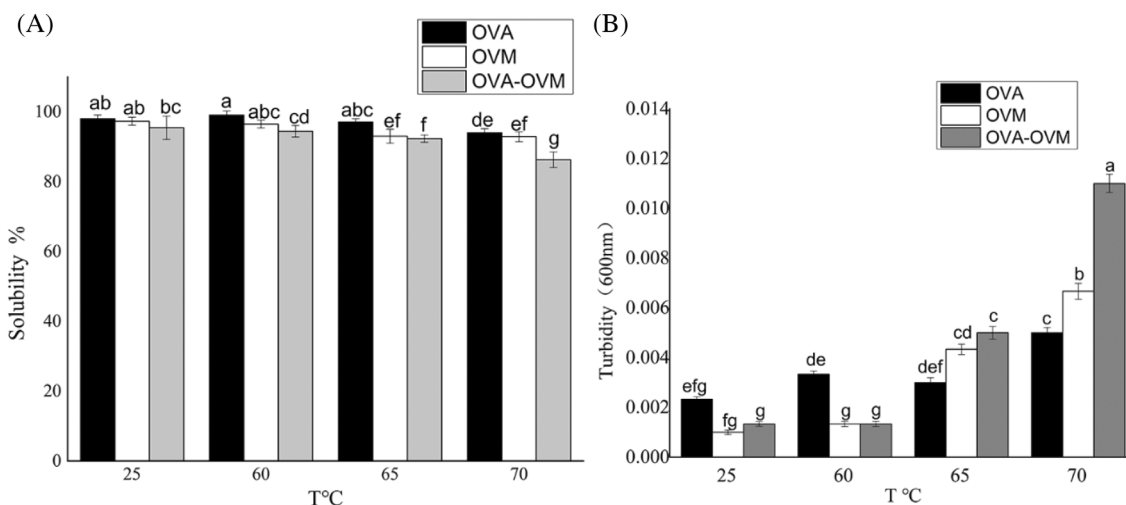


Figure 1. Solubility (A) and turbidity (B) of OVA, OVM, and OVA–OVM at 25, 60, 65, and 70 °C, respectively. All measurements were conducted at 25 °C.

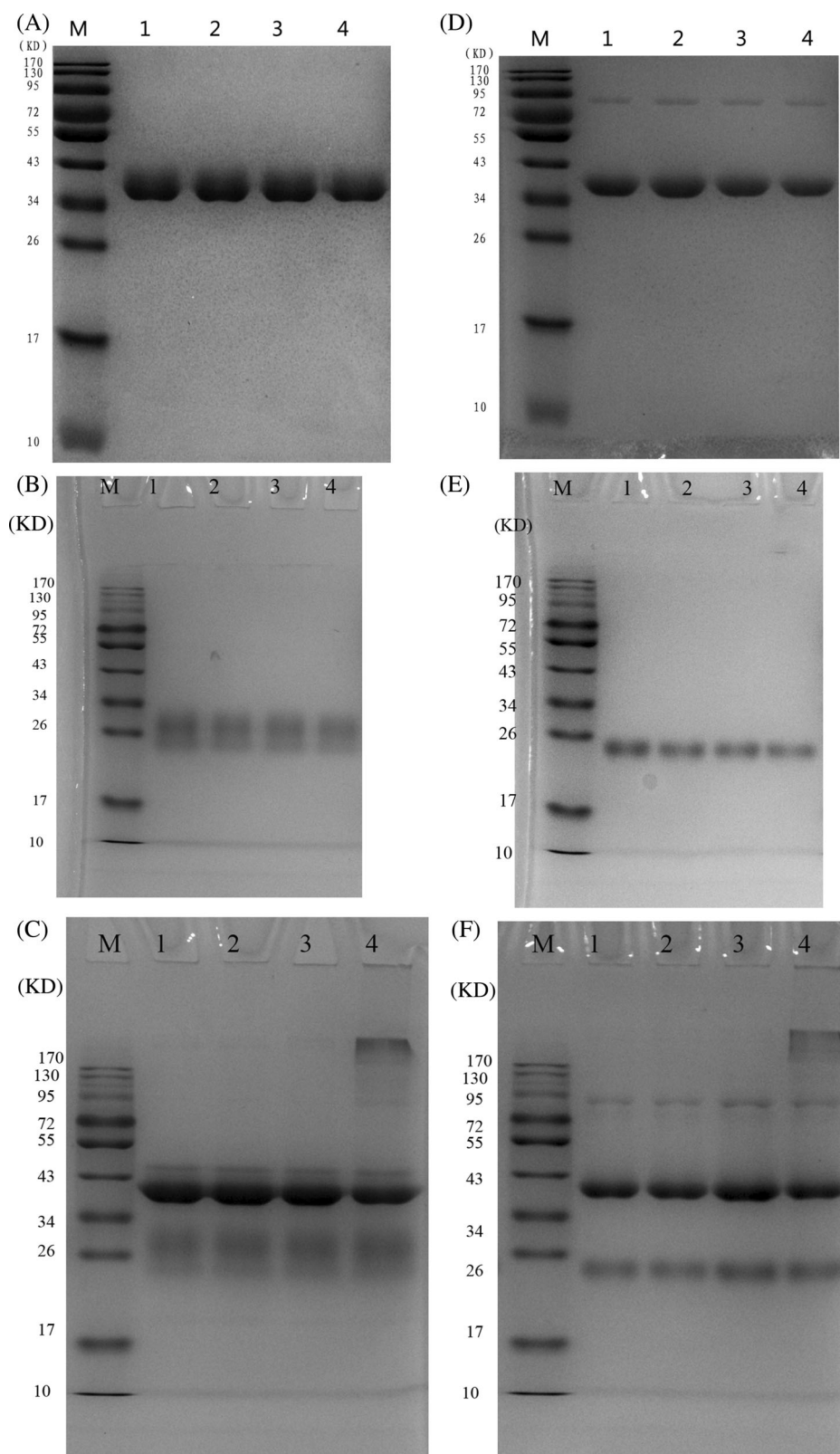


Figure 2. (A–C) Reduced SDS-PAGE patterns of OVA (A), OVM (B) and OVA–OVM (C). (D–F) Non-reduced SDS-PAGE patterns of OVA (D), OVM (E) and OVA–OVM (F). Lane M: Marker, 1: 25 °C, 2: 60 °C, 3: 65 °C, 4: 70 °C.

difference among the maximum fluorescence intensity of samples, the hydrophobicity of OVAs were slightly increased at 70 °C; however, the OVMs showed a visible increase when heated at 60 and 65 °C, but a sharp decrease at 70 °C; and the OVA–OVM

mixture displayed a 500% increase at 65 °C and 140% at 70 °C. Therefore, the maximum fluorescence intensity change of OVA–OVM was neither the same as OVA or OVM at different pasteurization temperatures.

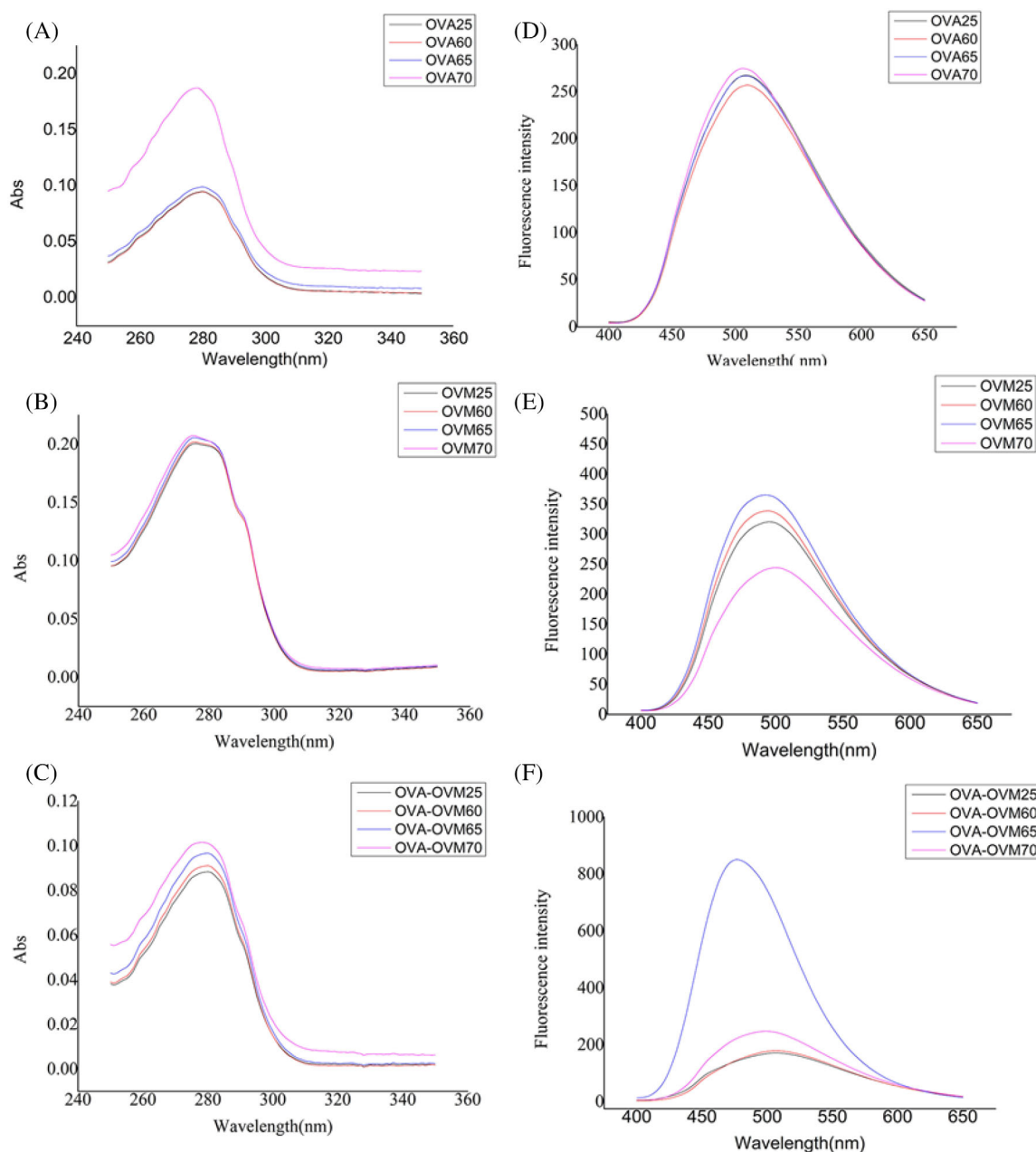


Figure 3. (A–C) UV-vis absorption spectra of OVA (A), OVM (B), and OVA–OVM (C) at 25, 60, 65, and 70 °C. (D–F) ANS fluorescence spectra of OVA (D), OVM (E), and OVA–OVM (F) at 25, 60, 65, and 70 °C, respectively.

The secondary structures of samples were detected via their CD spectroscopies and were analyzed online (Fig. 4 and Tables 2–4). Compared with the control, the peak shape of OVA showed a typical α -helix and β -sheet structure (Fig. 4), but the spatial structure of OVA became disordered to a certain extent after being pasteurized. The negative peak value of OVM was increased and the curve was significantly fluctuated after being pasteurized at 70 °C. Moreover, the peak shape of OVA–OVM was more similar to OVA than OVM as a whole. The contents of the samples' secondary structure were also affected. For the OVA, the α -helix content was decreased by 0.7% at 60 °C but was increased by 1.6% and 0.7% at 65 and 70 °C, respectively; the β -turn content was also increased at 60, 65, and 70 °C, while the random coil content was increased to the maximum at 70 °C. For the OVM, its

secondary structure changed more severely at 70 °C, the α -helix content was decreased by 6.6% and the random coil content was also decreased, while the β -sheet and β -turn contents were both increased. For the OVA–OVM, the α -helix content was decreased while the β -sheet was increased as the temperatures became higher, but the β -turn and random coil contents were both less changed.

IgG-binding ability and IgE-binding ability of OVA–OVM

The IgG-binding ability of all samples was assessed by competitive inhibition ELISA assays to evaluate the effects on their antigenicity at different temperatures, and the sample at 25 °C was the control. For OVA monomer, the IgG-binding ability was decreased to 43% at 60 °C, 42.4% at 65 °C and 40.4% at 70 °C (Fig. 5(A)). For

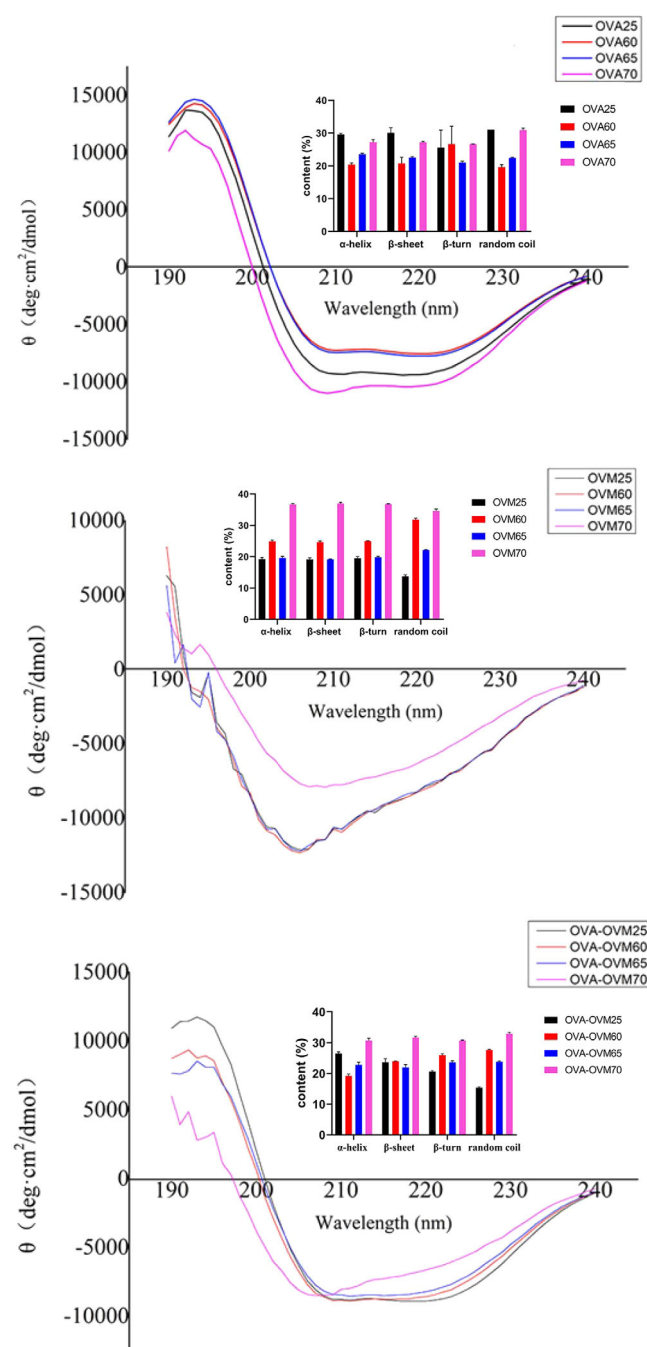


Figure 4. Far-UV CD spectra of OVA (A), OVM (B), and OVA–OVM (C) at 25, 60, 65 and 70 °C, respectively. The contents of each secondary structure of the samples are shown in the top right corner.

OVA complex (OVA–OVM), the IgG-binding ability was significantly higher than that of OVA, indicating that the binding ability of OVM to IgG could be reduced with heating temperature increasing. For OVM monomer, the IgG-binding ability was decreased by 1.1%, 0.1% and 1.4% (Fig. 5(B)) after being heated at 60, 65, and 70 °C, respectively. However, the IgG-binding ability of OVM complex (OVM–OVA) was higher than that of OVM at 65 and 70 °C, indicating that IgG-binding ability of OVM could be enhanced by the interaction between OVM and OVA at 65 and 70 °C.

The IgE-binding ability of all samples was measured by competitive inhibition ELISA assays to evaluate the effects on their potential allergenicity and the sample at 25 °C was the control. The IgE-binding ability of OVA was 17.2% at 60 °C, 16.4% at 65 °C, and 15.1% at 70 °C; that of OVM was decreased to 15.3% at 60 °C, 15.9% at 65 °C and 9.8% at 70 °C; that of OVA–OVM was significantly decreased at 70 °C compared to the one of OVA or OVM. Hence, IgE-binding ability of OVA–OVM could be significantly reduced by the interaction of OVA and OVM, but the degree of reduction was different.

Cytokine release of OVA–OVM-induced KU812 cells

All cytokines released in the culture supernatants were determined by ELISA kit (Fig. 6). Compared with the control, the release of interleukin 4 (IL-4) from OVA-induced KU812 cells was decreased at 70 °C (Fig. 6(A)); that from OVM-induced had no significant change; while that of OVA–OVM-induced was decreased with heating temperature increasing, and there was a maximum decrease at 70 °C compared to monomer, indicating that the release of IL-4 from basophilic granulocytes could be further reduced by the interaction of OVA and OVM at 70 °C.

There was no significant change in the release of IL-6 from OVA-induced KU812 cells after being pasteurized (Fig. 6(C)); that from OVM-induced KU812 cells was decreased substantially at 60, 65, and 70 °C; and that from OVA–OVM-induced KU812 cells was decreased with heating temperature increasing (Fig. 6(D)), and it would be significantly decreased at 70 °C compared to monomer, indicating that the release of IL-6 from basophilic granulocytes could be reduced by the interaction between OVA and OVM.

For the release of histamine from OVA-induced KU812 cells, there was no noticeable change observed after being pasteurized (Fig. 6(E)), but that from OVM-induced was significantly decreased just at 70 °C, and that from OVA–OVM-induced KU812 cells was decreased with heating temperature increasing (Fig. 6(F)), and were substantially decreased at 65 and 70 °C compared to monomer.

The release of tumor necrosis factor alpha (TNF-α) from OVA-induced KU812 cells was decreased profoundly after being pasteurized at 70 °C (Fig. 6(G)), showing that the release of pro-

Table 2. Secondary structure content of ovalbumin (OVA)

Sample	Heating parameters (°C min ⁻¹)	α-Helix content (%)	β-Sheet content (%)	β-Turn content (%)	Random coil content (%)
OVA	25 °C/10 min	29.60 ± 0.30 ^a	20.35 ± 0.55 ^b	23.55 ± 0.25 ^{ac}	27.20 ± 0.80 ^b
	60 °C/10 min	30.10 ± 1.50 ^a	20.75 ± 1.85 ^b	22.55 ± 0.25 ^b	27.20 ± 0.30 ^b
	65 °C/10 min	25.55 ± 5.35 ^b	26.65 ± 5.45 ^{ac}	21.00 ± 0.40 ^c	26.65 ± 0.05 ^b
	70 °C/10 min	31.00 ± 0.00 ^a	19.60 ± 0.80 ^b	22.45 ± 0.15 ^b	30.90 ± 0.60 ^{ac}

Note: Different lowercase letters of the same protein in the same column indicated a significant difference ($P < 0.05$).

Table 3. Secondary structure content of ovomucoid (OVM)

Sample	Heating parameters (°C min ⁻¹)	α -Helix content (%)	β -Sheet content (%)	β -Turn content (%)	Random coil content (%)
OVM	25 °C/10 min	19.25 ± 0.55 ^a	24.90 ± 0.40 ^b	19.60 ± 0.50 ^b	36.70 ± 0.30 ^a
	60 °C/10 min	19.15 ± 0.45 ^a	24.65 ± 0.35 ^b	19.15 ± 0.15 ^c	36.95 ± 0.45 ^a
	65 °C/10 min	19.50 ± 0.50 ^a	25.00 ± 0.10 ^b	19.80 ± 0.30 ^b	36.80 ± 0.10 ^a
	70 °C/10 min	13.70 ± 0.50 ^b	31.85 ± 0.45 ^{ac}	22.15 ± 0.15 ^{ac}	34.60 ± 0.60 ^b

Note: Different lowercase letters of the same protein in the same column indicated a significant difference ($P < 0.05$).

Table 4. Secondary structure content of ovalbumin–ovomucoid (OVA–OVM)

Sample	Heating parameters (°C min ⁻¹)	α -Helix content (%)	β -Sheet content (%)	β -Turn content (%)	Random coil content (%)
OVA–OVM	25 °C/10 min	26.50 ± 0.50 ^{ac}	19.15 ± 0.65 ^d	22.80 ± 0.80 ^a	30.75 ± 0.65 ^c
	60 °C/10 min	23.65 ± 1.15 ^b	23.95 ± 0.05 ^c	21.95 ± 0.95 ^b	31.65 ± 0.35 ^b
	65 °C/10 min	20.60 ± 0.30 ^c	25.90 ± 0.50 ^b	23.60 ± 0.50 ^a	30.70 ± 0.10 ^c
	70 °C/10 min	15.35 ± 0.25 ^d	27.60 ± 0.20 ^{ac}	23.75 ± 0.25 ^a	32.85 ± 0.45 ^{ac}

Note: Different lowercase letters of the same protein in the same column indicated a significant difference ($P < 0.05$).

inflammatory cytokines from OVA would be reduced at this pasteurization temperature; in contrast, that from OVM-induced KU812 cells displayed some negligible change after being pasteurized; that from OVA–OVM-induced KU812 cells was decreased with heating temperature increasing (Fig. 6(H)), and were significantly decreased at 65 and 70 °C compared to monomer.

There was no significant change in the release of β -HEX from monomer-induced KU812 cells after being pasteurized (Fig. 6(I)), while that of OVA–OVM-induced was substantially lower than that of monomer after being heated at 70 °C (Fig. 6(J)).

Hence, the release of cytokines from basophils could be reduced by the interaction between OVA and OVM induced by pasteurization at 70 °C.

DISCUSSION

Both solubility and turbidity were related to the thermo-sensitive stability of proteins.¹⁸ According to the results, as the pasteurization temperature was increased, the solubility of all the samples was decreased, while their turbidity was increased. Moreover, especially for the OVA–OVM, its influence was dependent on the interaction between OVA and OVM, but more closely related to the pasteurization temperature. This was mainly due to the nature of OVA and OVM. The OVA contains four free sulfhydryl groups which are located in the hydrophobic core, two of them, respectively located between Cys73 and Cys120, could form disulfide bonds.¹⁹ The OVM is composed of three tandem domains and each one is bound by interior disulfide bonds, which could promote OVM against thermal denaturation and digestion.²⁰ Pasteurization operation could not only cause globular protein structure to be unfolded but also lead to the distribution of some charges on the molecular surface being rearranged, and damage the hydrophilic/hydrophobic balance, as a result of the solubility was decreased.²¹ The unfolded structures of OVA and OVM caused by pasteurization could also make the hydrophobic groups more exposed and the molecular motion speed was accelerated, thus the chances of their thermal aggregation were increased. The difference between reduced and non-reduced

electrophoresis was dependent on the presence of β -mercaptoethanol, which was a kind of reducing agent that could break down disulfide bonds into free sulfhydryl groups, resulting in some proteins aggregations bound by the disulfide bonds being split. In the non-reduced electrophoresis of OVA, there was one polymer band with a molecular weight of about 95 kDa, which could be the dimer of the OVA via disulfide bonds being aggregated during pasteurization. For the OVM, the reduced and non-reduced electrophoresis pattern were the same, indicating that its molecular weight was not affected by pasteurization even after being heated at 70 °C. However, for the OVA–OVM, in its reduced and non-reduced electrophoresis, there were both a new band with molecular weight of 170 kDa after being pasteurized at 70 °C. The reason for the differences was mainly related to the intermolecular sulfhydryl–disulfide bonds in the structure of OVA and OVM. The OVA structure contains one disulfide bond and four free sulfhydryl groups, while OVM structure contains eight disulfide bonds with no free sulfhydryl groups. Pasteurization temperature at 70 °C could promote the unfolding of monomer, which could increase free sulfhydryl groups formation and enhance the possibility of some new disulfide occurrence, finally leading to some polymers of OVA–OVM formation.

The pasteurization temperature was a dominating factor that affected the interaction between OVA and OVM. The maximum UV absorption of OVA was increased after being pasteurized at 70 °C, indicating that the aromatic rings in OVA side chains were changed and made the tertiary structure of OVA more unfolded. While the spectral shapes and maximum absorption of OVM at any heating temperatures were almost similar to each other and the tertiary structure of OVM was not significantly changed. However, the maximum absorption of OVA–OVM was increased with the increasing heating temperature, indicating that the aromatic rings in their side chains could be profoundly affected by the interaction of OVA and OVM, and the tertiary structure would be more unfolded with temperature increasing.

The fluorescence intensity of OVA was slightly increased with the heating increasing temperature, while that of OVM was increased at 60 and 65 °C, but decreased at 70 °C. This was due

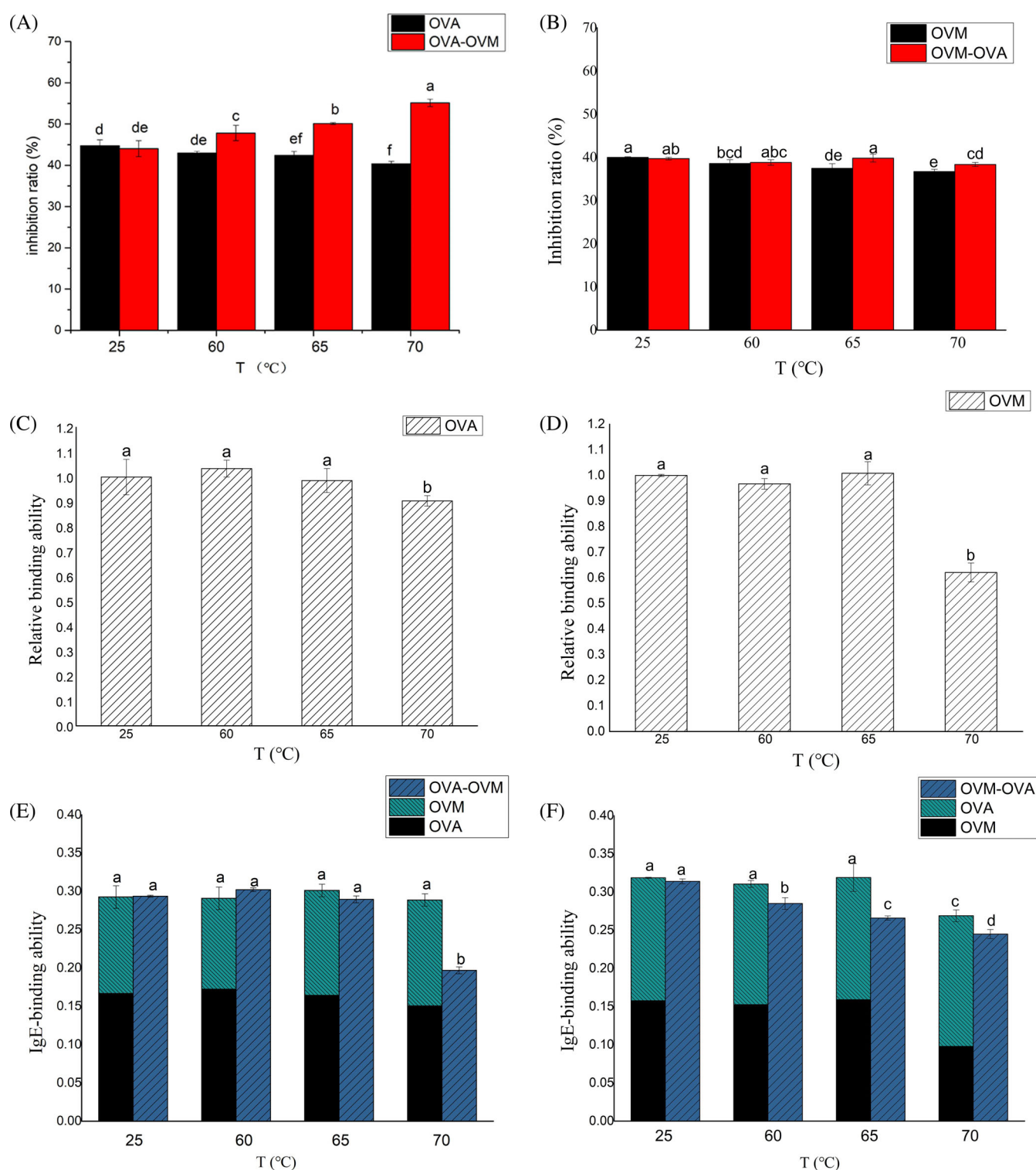


Figure 5. Effects of interaction of OVA–OVM heated at 25, 60, 65 and 70 °C for 10 min on IgG and IgE-binding abilities of OVA and OVM. (A, B) Effects of interaction of OVA–OVM at different temperatures on IgG-binding ability of OVA (A) and OVM (B). (C, D) Effects of heating at different temperatures on IgE-binding ability of OVA (C) and OVM (D). (E, F) The influence of OVA–OVM interaction on IgE-binding ability of OVA (E) and OVM (F). Different letters denote that the variance of the two samples was significant.

to the different denaturation temperatures of OVA and OVM, they are separately at 84 and 77 °C.^{22,23} When the pasteurization temperatures were below the denaturation temperature of OVA, its structure was slowly unfolded, and the internal hydrophobic groups were gradually exposed, leading to an increase in

fluorescence intensity. Since the denaturation temperature of OVM was lower than that of OVA, a large number of hydrophobic groups in its structure were exposed when the heating temperature was close to its denaturation temperature such as 60 or 65 °C. The maximum fluorescence intensity of OVA–OVM was

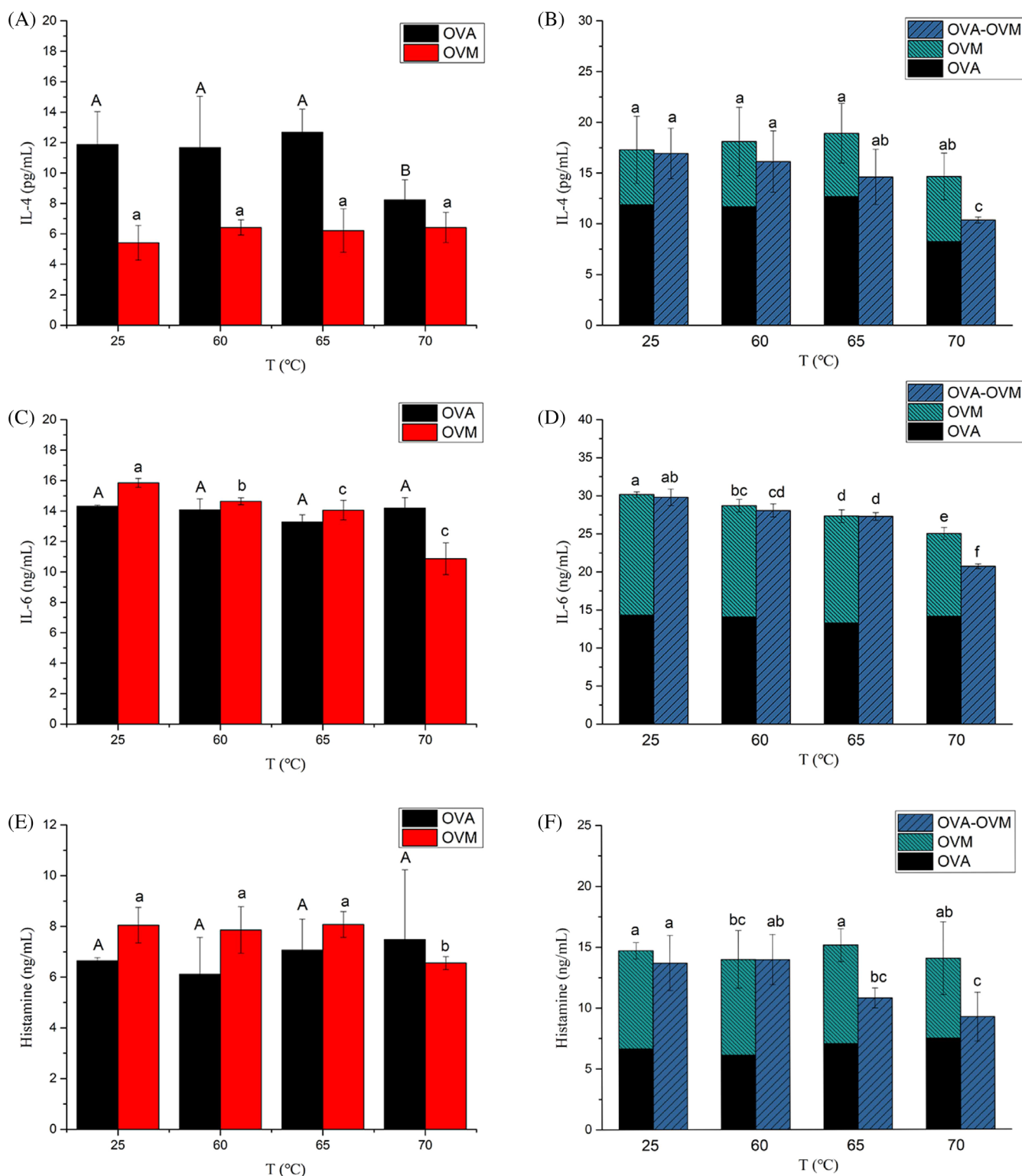


Figure 6. Releases of IL-4 (A, B), IL-6 (C, D), histamine (E, F), TNF- α (G, H) and β -HEX (I, J) from KU812 cells stimulated with OVM, OVA and OVA-OVM treated at 25, 60, 65 and 70 °C, respectively.

dramatically increased at 65 °C, while sharply decreased at 70 °C. It should be noted that the molecular moving speed and the chance of collisions were accelerated when the pasteurization temperature was up to 70 °C, leading to an enhancement of the molecular aggregation of the two proteins by their interaction

through the hydrophobic groups present, finally the fluorescence intensity was decreased.

The most significant change in the secondary structure of OVA was the random coil content at 70 °C, while in OVM the content was α -helix at 65 °C and β -sheet at 70 °C. Nevertheless, the

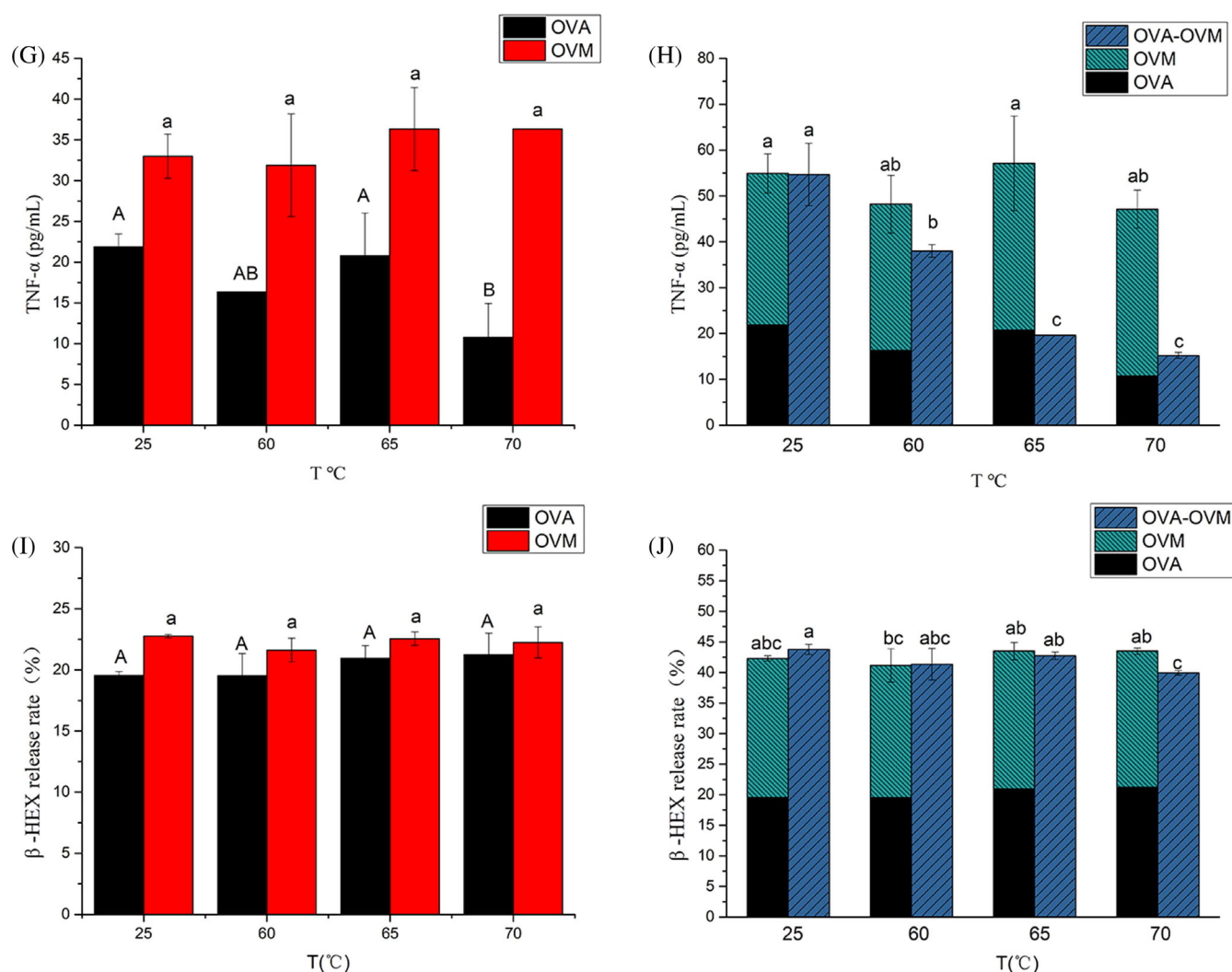


Figure 6 (Continued)

structure of OVA–OVM displayed dynamic changes with heating temperature increasing, in which the reduced α -helix and the increased β -sheet were both significant, as a result, its original ordered secondary structure was broken. Hydrogen bonding was the primary force that maintained the secondary structure of proteins, in which α -helix and β -sheet were the most typical and abundant structural elements in the protein. The hydrogen bonds in the secondary structure could be influenced by pasteurization. OVA was a phosphoglycoprotein containing about 3% carbohydrates, while OVM was a glycoprotein, about 20–25% carbohydrates with a lot of hydroxyl groups, and could benefit from the formation of hydrogen bonds between OVA and OVM.²⁴ The hydrogen bonds originally maintaining the secondary structure of the two proteins might be broken down or re-formed with other groups, resulting in significant changes in α -helix and β -sheet of OVA–OVM at various pasteurization temperatures. Therefore, the secondary structural types of OVA–OVM could be changed a lot because of the interaction induced by pasteurization.

IgG-binding ability of OVA and OVM was slightly decreased with temperature increasing, while the binding ability of OVA–OVM was overall gradually increased. This could possibly be since

the globulin structure of OVA and OVM could be partly folded when pasteurization temperature was increased and their IgG-binding sites would be partially buried. This was especially evident when the temperature was above 65 °C, the number of IgG-binding sites was significantly decreased, resulting in OVA–OVM inability to bind to the variable region of IgG (Fab) through hydrophobic interaction or hydrogen bonds. But for OVA–OVM, the interaction between the two proteins induced by pasteurization could help some new IgG-binding sites be exposed. Therefore, the IgG-binding ability of OVA–OVM was significantly higher than that of the pasteurized monomer alone at the same temperature.

As for the IgE-binding ability of all samples, they all decreased with heating temperature increasing, especially at 70 °C. The dominating reason could be the same as that of the IgG-binding ability. Golias *et al.* has stated that the secondary structure, digestibility, and antigenic epitopes formation of OVA could be changed by heating at 70 °C, which ultimately led to its allergenicity being reduced.²⁵ Jiménez-Saiz *et al.* also mentioned that when OVA was heated its allergenicity tested by egg allergy patients sera would be reduced, and its immunogenicity tested by rabbit sera was reduced as well.¹² Moreover, the IgE-binding ability of

OVA–OVM was decreased with heating temperature increasing due to the change of the spatial structures of the two proteins. Since the IgE antibody of OVA was usually recognized by part of its conformational epitopes, the antibody of OVM was mainly recognized by the linear epitopes, finally, their different epitopes might bring some various changes during pasteurization.

To further understand the influence on OVA and OVM potential allergenicity by the interaction of OVA and OVM, the cytokines related to allergenicity were tested. There were no significant changes in the releases of histamine, IL-6, and β -HEX from OVA-induced KU812 cells, but the releases of TNF- α and IL-4 were significantly decreased after being pasteurized at 70 °C; for OVM, the release of histamine from the induced KU812 cells was dramatically decreased at 70 °C, while the releases of TNF- α , IL-4, and β -HEX were not significantly changed, but the release of IL-6 was decreased with temperature increasing. As previously mentioned, the spatial structures of OVA and OVM were altered at different degrees by the various pasteurization temperatures, which resulted in the burial or destruction of IgG and IgE binding epitopes and inhibition of cytokine releases from the induced basophil.^{26,27} It was shown that the releases of TNF- α and IL-4 from OVA were decreased significantly after being heated at 70 °C, which might be because of the unfolding of the spatial structure of OVA, or because of the increasing destruction or burial of conformational epitopes, therefore, the cytokines induced by OVA were decreased. Claude *et al.* also reported that the aggregated OVA showed a lower ability of Ig-binding and basophil activation.²⁸ The decrease in cytokines release induced by OVM might be owing to the exposure of hydrophobic groups of OVM, resulting in the destruction or burial of epitopes. However, the secondary and tertiary structures of OVM were less affected at being pasteurized at 60 and 65 °C, and the releases of cytokines showed no significant changes except for IL-6. The results implied that the IgE-binding ability of OVA or OVM although could not be entirely damaged by the pasteurization operations, their ability to trigger basophil activation and degranulation would be reduced. However, for the OVA–OVM, the release of histamine and TNF- α were significantly reduced at 65 and 70 °C, and the release of IL-4, IL-6 and β -HEX were all dramatically reduced at 70 °C, the reasons were all because of the interaction of OVA and OVM induced by pasteurization.

CONCLUSIONS

The interaction of OVA and OVM induced by pasteurization would indeed change their spatial structure and potential allergenicity, and the interaction had obviously occurred at 70 °C. Specifically, when the temperature was at 70 °C, the spatial structure of OVA was unfolded, its hydrophobic groups were partially exposed and its random coil content was increased, while the hydrophobic groups of OVM were buried and its β -sheet content was increased. Furthermore, the IgG-binding ability of OVA and OVM could be significantly increased, and their IgE-binding ability was decreased. The releases of IL-4, IL-6, β -HEX, histamine and TNF- α from OVA–OVM-induced KU812 cells were all decreased at 70 °C. Altogether, these results suggested that if liquid egg products are to be pasteurized for 10 min, the temperature of 70 °C should be a good choice because it could ensure food safety and security, but also less egg allergy. This study provides a basis for further study on the allergenicity of egg white proteins during multicomponent food mixture processing.

ACKNOWLEDGEMENTS

This work was supported by the National Key Research and Development Program of China (No. 2018YFD0400304) and the National Natural Science Foundation of China (No. 31660436).

CONFLICTS OF INTEREST STATEMENT

The authors declare no conflict of interest.

SUPPORTING INFORMATION

Supporting information may be found in the online version of this article.

REFERENCES

- 1 Sugano M and Matsuoka R, Nutritional viewpoints on eggs and cholesterol. *Foods* **10**:494–514 (2021).
- 2 Luo W, Hu H, Tang W, Zou X, Huang H, Huang Z *et al.*, Allergen sensitization pattern of allergic adults and children in southern China: a survey based on real life data. *Allergy, Asthma Immunol Res* **15**:1–9 (2019).
- 3 Brasca M, Morandi S, Silvestri T, Rosi V, Cattaneo S and Pellegrino L, Different analytical approaches in assessing antibacterial activity and the purity of commercial lysozyme preparations for dairy application. *Molecules* **18**:6008–6020 (2013).
- 4 Sheng L, Huang M, Wang J, Xu Q, Hammad H and Ma M, A study of storage impact on ovalbumin structure of chicken egg. *J Food Eng* **219**: 1–7 (2018).
- 5 Abeyrathne E, Lee H and Ahn DU, Egg white proteins and their potential use in food processing or as nutraceutical and pharmaceutical agents—a review. *Poult Sci* **92**:3292–3299 (2013).
- 6 Howard ZR, O'Bryan CA, Crandall PG and Ricke SC, Salmonella Enteritidis in shell eggs: current issues and prospects for control. *Food Res Int* **45**:755–764 (2012).
- 7 Wang J, Liu X, Li S, Ye H, Luo W, Huang Q *et al.*, Ovomucin may be the key protein involved in the early formation of egg-white thermal gel. *Food Chem* **366**:130596 (2022).
- 8 Jing H, Sun J, Mu Y, Obadi M, McClements DJ and Xu B, Sonochemical effects on the structure and antioxidant activity of egg white protein-tea polyphenol conjugates. *Food Funct* **11**:7084–7094 (2020).
- 9 Lechevalier V, Guérin-Dubiard C, Anton M, Beaumont V, Briand ED, Gillard A *et al.*, Effect of dry heat treatment of egg white powder on its functional, nutritional and allergenic properties. *J Food Eng* **195**:40–51 (2017).
- 10 Lechevalier V, Guérin-Dubiard C, Anton M, Beaumont V, Briand ED, Gillard A *et al.*, Pasteurisation of liquid whole egg: optimal heat treatments in relation to its functional, nutritional and allergenic properties. *J Food Eng* **195**:137–149 (2017).
- 11 Zhu Y, Vanga SK, Wang J and Raghavan V, Impact of food processing on the structural and allergenic properties of egg white. *Trends Food Sci Technol* **78**:188–196 (2018).
- 12 Jiménez-Saiz R, Belloque J, Molina E and López-Fandino R, Human immunoglobulin E (IgE) binding to heated and glycated ovalbumin and ovomucoid before and after in vitro digestion. *J Agric Food Chem* **59**:10044–10051 (2011).
- 13 Martos G, Lopez-Exposito I, Bencharitiwong R, Berin MC and Nowak-Węgrzyn A, Mechanisms underlying differential food allergy response to heated egg. *J Allergy Clin Immunol* **127**:990–997 (2011).
- 14 Shin M, Lee J, Ahn K, Lee SI and Han Y, The influence of the presence of wheat flour on the antigenic activities of egg white proteins. *Allergy, Asthma Immunol Res* **5**:42–47 (2013).
- 15 Ma X, Liang R, Yang X, Gou J, Li Y and Lozano-Ojalvo D, Simultaneous separation of the four major allergens of hen egg white. *J Chromatogr B* **1152**:122231 (2020).
- 16 Van der Plancken I, Van Loey A and Hendrickx ME, Effect of heat-treatment on the physico-chemical properties of egg white proteins: a kinetic study. *J Food Eng* **75**:316–326 (2006).
- 17 Meng X, Li X, Wang X, Gao J, Yang H and Chen H, Potential allergenicity response to structural modification of irradiated bovine α -lactalbumin. *Food Funct* **7**:3102–3110 (2016).

- 18 Liu Y, Oey I, Bremer P, Carne A and Silcock P, Effects of pH, temperature and pulsed electric fields on the turbidity and protein aggregation of ovomucin-depleted egg white. *Food Res Int* **91**:161–170 (2017).
- 19 Sheng L, Tang G, Wang Q, Zou J, Ma M and Huang X, Molecular characteristics and foaming properties of ovalbumin-pullulan conjugates through the Maillard reaction. *Food Hydrocolloids* **100**: 105384 (2020).
- 20 Watson R and Meester F, Physiological, biochemical, and allergenic properties of egg allergens, in *Handbook of Eggs in Human Function*, ed. by Watanabe H, Takushi S, Nakajima-Adachi H, Hachimura S, Vieths S and Toda M. Wageningen Academic Publishers, Wageningen, pp. 124–127 (2015).
- 21 Wang Z, Li Y, Jiang L, Qi B and Zhou L, Relationship between secondary structure and surface hydrophobicity of soybean protein isolate subjected to heat treatment. *J Chem* **2014**:475389 (2014).
- 22 Julià S, Sánchez L, Pérez MD, Lavilla M, Conesa C and Calvo M, Effect of heat treatment on hen's egg ovomucoid: an immunochemical and calorimetric study. *Food Res Int* **40**:603–612 (2007).
- 23 Arzeni C, Pérez OE and Pilosof AM, Functionality of egg white proteins as affected by high intensity ultrasound. *Food Hydrocolloids* **29**:308–316 (2012).
- 24 Zhang Y, Wang W, Zhou R, Yang J, Sheng W, Guo J *et al.*, Effects of heating, autoclaving and ultra-high pressure on the solubility, immunoreactivity and structure of major allergens in egg. *Food Agric Immunol* **29**:412–423 (2018).
- 25 Golias J, Schwarzer M, Wallner M, Kverka M, Kozakova H, Srutkova D *et al.*, Heat-induced structural changes affect OVA-antigen processing and reduce allergic response in mouse model of food allergy. *PLoS One* **7**:e37156 (2012).
- 26 Stănciuc N, Crețu AA, Banu I and Aprodu I, Advances on the impact of thermal processing on structure and antigenicity of chicken ovomucoid. *J Sci Food Agric* **98**:3119–3128 (2018).
- 27 Stănciuc N, Banu I, Turturică M and Aprodu I, pH and heat induced structural changes of chicken ovalbumin in relation with antigenic properties. *Int J Biol Macromol* **93**:572–581 (2016).
- 28 Claude M, Lupi R, Bouchaud G, Bodinier M, Brossard C and Denery-Papini S, The thermal aggregation of ovalbumin as large particles decreases its allergenicity for egg allergic patients and in a murine model. *Food Chem* **203**:136–144 (2016).