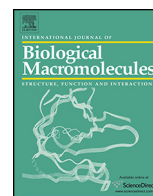




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Cross-linked ovalbumin catalyzed by polyphenol oxidase: Preparation, structure and potential allergenicity

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ABSTRACT

Ovalbumin (OVA) is described as one of the major allergens in hen's egg, and it is the most abundant protein in egg white. Enzyme-mediated covalent cross-linking of food proteins, can influence their structure and allergenicity. The aim of this study was to investigate the potential of polyphenol oxidase from *Agaricus bisporus* to cross-link OVA (CL-OVA) in the presence or absence of caffeic acid, followed by characterizing the structure and allergenicity of CL-OVA. A single-factor experiment was designed to assess the optimum conditions for cross-linking of OVA by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Under the optimal conditions, structural changes in OVA were analyzed by circular dichroism, ultraviolet and fluorescence spectra. It was shown that CL-OVA became unordered and unfolded, and more tyrosine and tryptophan residues and hydrophobic groups were exposed onto the surface of molecules when compared to the native OVA. Enzyme-linked immunosorbent assay indicated that IgG and IgE binding abilities to OVA significantly reduced after cross-linking. All the findings demonstrated that enzymatic cross-linking in the presence of caffeic acid as a mediator may decrease the antigenicity and potential allergenicity, and the changes of the modified OVA were most likely a consequence of some changes or adjustment in the local conformation of OVA and the epitopes of OVA by cross-linking.

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1. Introduction

Hen's egg contains rich proteins that are beneficial to human bodies. However, it is also recognized as one of the most important food that causes allergic reactions, especially in infants and young children [1,2]. Thus far, allergic patients should absolutely avoid ingesting offending food, which is the only direct and dependable way to prevent egg allergy [3]. However, avoiding egg dishes or foods is extremely difficult because of the food diversity and the complexity of components in food products. By contrast, if eggs and egg ingredients products were removed from the diets of egg allergy sufferers, their nutritional balance and dietary pattern are bound to break [4]. Nevertheless, 50%–85% of egg allergic children could tolerate baked or heated eggs, which might be linked to the

types of food that are accepted by patients with allergies to hen eggs [5].

Ovalbumin (OVA) is described as one of the major allergens in egg white, and it is also the most abundant protein in egg white, accounting for approximately 54% of the total protein [6]. Reasonably, reducing the allergenicity of OVA is of vital significance in the treatment of egg allergy. Moreover, OVA-induced allergy is considered mainly an IgE-mediated type I hypersensitivity [7], and the IgE binding epitopes including AA38–49, AA95–102, AA191–200, AA243–24, and AA251–260 presented on OVA were identified in sera from egg allergy patients [8] (Table 1).

It is well known that food processing can induce structural changes, which can influence the allergenicity by disrupting conformational or linear epitopes [9,10]. Stănciuc et al. [11] found OVA at alkaline pH value retained low levels of residual allergenic components heating at temperatures higher than 80 °C. Lechevalier et al. [12] tried to reduce the antigenicity of egg white by dry heat treatment. Compared with physically and chemically processed food proteins, as a new method of nonthermal processing technique in food industry, the improvements and modifications of proteins catalyzed by enzymes were widely studied, resulting in attracting

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Table 1
Information of egg allergic patients' sera.

Serum	Gender	Age (year)	Symptom	Specific IgE to egg white (IU mL ⁻¹)
1	Male	5	urticaria	>200
2	Male	2	asthma	>200
3	Male	1	Bronchial asthma	>100
4	Male	6	eczema	>200
5	Female	2	Bronchial asthma	>200
6	Male	2	eczema	>200
7	Male	1	Mycoplasma infection	>200
8	Female	30	Chronic urticaria	>200

more attention [13]. Actually, some documents are responsible for the enzymatic cross-linking of food proteins that can improve food texture, alter functionality, and influence digestibility and allergenicity [14–16]. Of the enzymes used for cross-linking proteins, polyphenol oxidases (PPOs), as one of the newly used enzymes, were caused wide concern [17,18].

Actually, PPOs are a group of copper-containing enzymes with complex composition, which summarize tyrosinases (EC 1.14.18.1), catechol oxidases (EC 1.10.3.1) and laccases (EC 1.10.3.2) [19,20]. They are oxidoreductases that are widespread in plants, fungus, and prokaryotic and eukaryotic organisms [21]. PPO can catalyze the hydroxylation of monophenol to dihydric diphenols and the subsequent oxidation of these dihydric diphenols to the respective o-quinones [22]. Regarding to proteins, PPO can catalyze the tyrosine (Tyr) residues to o-quinones. These quinones react spontaneously mainly via 1,4-additions with the $-NH_2$ or $-SH$ group of Tyr, lysine (Lys), or cysteine (Cys) residues to form intra- or intermolecular covalent cross-link [23]. Moreover, cross-linking between proteins with strongly defined structure or without Tyr residues can be induced by the small-molecule phenolic compounds as cross-linked mediators, such as caffeic acid. Caffeic acid can be oxidized by PPO into an o-benzoquinone, which reacts spontaneously with the $-NH_2$ or $-SH$ group of proteins to generate a caffeic acid-containing protein–protein cross-link [24,25].

Interestingly, the usage of PPO was anticipated as a novel approach to produce hypoallergenic foods [26]. Tantoush et al. [16] investigated the cross-linking potential of laccase on β -lactoglobulin (BLG) in the presence of sour cherry phenolics as a source of phenolic mediators, and the allergenicity of the cross-linked proteins was shown to be decreased in all nine cow's milk-allergic patients. Chung et al. [25] found that PPO treatment led to cross-linking of peanut extracts, resulting in the reduction of the allergenic properties of peanut allergens assessed by testing IgE binding ability. Recently, Wu et al. [27] also demonstrated that the modification of Ara h 2 molecules by PPO could decrease its potential allergenicity. However, information on egg allergens cross-linking catalyzed by PPO is not available yet. Accordingly, this work aimed to study whether OVA could be cross-linked catalyzed by PPO, followed by defining the effect of cross-linking on the potential allergenicity and structure changes of OVA.

2. Materials and methods

2.1. Materials

OVA with a purity of more than 98% was prepared by anion exchange chromatography on a DEAE-Sephacel Fast Flow column (16 mm $\times$ 250 mm, Amersham Biosciences, Freiburg, Germany) following the method of Ma et al. [28]. The protein solution was dialyzed against water at pH 7.0 for 48 h using 10 kDa molecular weight cutoff membranes, and then was lyophilized. The lyophilized OVA powder was stored at -20°C . PPO powders from mushroom (*Agaricus bisporus*) were lab-made [18]. All other chem-

icals were purchased from the SigmaAldrich Corporation (St Louis, MO, USA).

2.2. Rabbit antibodies

Anti-OVA polyclonal antibodies IgG used for enzyme-linked immunosorbent assay (ELISA) were obtained in adult New Zealand rabbits according to the method of Ma et al. [29]. The sera were frozen at -80°C until use.

2.3. Patients' sera

A pooled serum sample of eight subjects was used in this study. Subjects with a history of severe systemic reactions to eggs were provided by the First Affiliated Hospital of Guangxi Medical University, China, and were selected with informed consent. Egg allergy was diagnosed based on physical examination and objective clinical manifestations observed after egg ingestion. These sera were frozen at -80°C until use.

2.4. Enzyme assay

Enzyme activity of PPO solution was measured using the method of Coseteng and Lee [30] with certain modifications. The sample contained 2.95 mL of 40 mM catechol solution in PBS (50 mM, pH 7.0) and 0.05 mL of PPO solution. Since the enzyme extract was added, the changes in absorbance at 420 nm were recorded every 30 s up to 3 min using a TU-1901 UV–vis spectrophotometer (Purkinje General Instrument Limited Liability Company, China). Each sample was detected in triplicate unless mentioned otherwise. The blank sample contained 2.95 mL of substrate solution and 0.05 mL of PBS. One unit of enzyme activity was defined as the increase in absorbance of 0.001 per min and per mL of the crude enzyme extract [31].

2.5. Experimental design for cross-linking conditions upon single factor

Ovalbumin (1.0 mg mL^{-1}) catalyzed by PPO of different enzyme activity (200, 400, 600, 800, 1000, and 1200 U mL^{-1}) in the presence of various caffeic acid concentrations (0, 0.1, 0.2, 0.5, 1, 2, 5, and 10 mM), was cross-linked at various temperatures (20, 30, 40, 50, 60, 70, and 80°C), pH (4.0, 5.0, 6.0, 7.0, 9.0, and 10.0), ionic strengths (0.1, 10, 50, 100, and 200 mM, phosphate buffer solution, PBS), and times (1, 2, 4, 8, 12, and 24 h), respectively. When one factor was evaluated, the other factors were kept constant. The mixture were incubated in a constant temperature shaker. After the reaction, the cross-linked products were frozen and stored at -20°C until use.

2.6. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE)

SDS–PAGE gels (10%) were prepared to assess the extent of OVA cross-linking caused by PPO. A PhastSystem Electrophoresis equip-

ment (BIO-RAD, USA) was used. Ten microliter protein samples (1.0 mg mL^{-1}) were mixed with $10 \mu\text{L}$ of reducing loading buffer with beta-mercaptoethanol and heated for 5 min at 100°C . The gels were stained with Coomassie Brilliant Blue R-250 (Sigma), and image intensity was quantified by Quantity One-4.6.2 (BIO-RAD, USA). The cross-linked OVA (CL-OVA) under optimal conditions of cross-linking was used for the following assessment experiments, such as structural characterization and IgG/IgE binding capacity assessment.

2.7. Circular dichroism (CD) spectrum

The changes in secondary structure of untreated OVA and CL-OVA were assessed by Far-UV ($190\text{--}250 \text{ nm}$) CD using a MOS-450/AF-CD (Bio-Logic, France). The spectra were recorded at a rate of 60 nm min^{-1} with a bandwidth of 1 nm at 25°C . The samples were all diluted to 0.1 mg mL^{-1} with PBS (50 mM , $\text{pH } 7.0$). The smoothed spectra were represented as mean residue ellipticity [$\text{deg cm}^2/\text{dmol}$] based on the mean residue molecular weight. The contents of different secondary structures were calculated using secondary structure software (<http://dichroweb.cryst.bbk.ac.uk/html/home.shtml>).

2.8. Ultraviolet (UV) spectrum

UV spectrum was performed by TU-1901 UV-vis spectrophotometry to analyze the tertiary structure of untreated OVA and CL-OVA. The samples were diluted to 0.2 mg mL^{-1} with 50 mM PBS ($\text{pH } 7.0$), followed by detecting with a wavelength range from 250 to 350 nm at a rate of 120 nm min^{-1} .

2.9. Fluorescence spectrum

The surface hydrophobicity of OVA was evaluated by measuring the fluorescence intensity of OVA in the presence of hydrophobic fluorescence probes, 8-anilino-1-naphthalenesulfonate (ANS). The ANS binding tests were performed in an F-4500 fluorescence photometer (HITASCHI, Japan) by incubating 5 mL of (un)-treated protein samples (0.1 mg mL^{-1}) for 1 h at 25°C in the dark with $30 \mu\text{L}$ of 5 mM ANS in 50 mM PBS ($\text{pH } 7.0$). The fluorescence of ANS was excited at 390 nm and emission was collected between 400 and 650 nm at a scan speed of 1200 nm min^{-1} .

2.10. Spatial structure of OVA

The spatial structure of a protein is widely used to define the spatial location of amino acid residues and peptides. The spatial structure of OVA was built on the PyMOL soft (<http://www.pymol.org/>) using OVA (PDB database, <http://www.rcsb.org/pdb/explore/explore.do?structureId=1JTI>) [32] as a model. The identified IgE binding epitopes and possible cross-linking sites located in allergic epitopes were marked on the structure of OVA.

2.11. IgG binding capacity assessment

For analysis of the antigenicity of OVA and CL-OVA, an IgG binding capacity test was performed by competitive inhibition ELISA according to Ma et al. [29,33]. A 96-well microliter plate was pre-coated with the untreated OVA ($1.5 \mu\text{g mL}^{-1}$) in 50 mM sodium carbonate buffer ($\text{pH } 9.6$) overnight at 4°C . The anti-OVA polyclonal antibodies ($1:25,000$ dilution) and varying concentrations of OVA/CL-OVA ranging from 0.125 to $2 \mu\text{g mL}^{-1}$ at a volume ratio of $1:1$ were added and incubated for 1 h at 37°C after blocking with $250 \mu\text{L}$ of 5% skimmed milk in PBS for 1 h at 37°C . When the reaction was terminated by adding $50 \mu\text{L}$ of $2 \text{ M H}_2\text{SO}_4$, the optical density (OD) was read at 490 nm using a Bio-Rad 1860 microplate reader

(Bio-Rad, CA, USA). The IgG binding rate was expressed as OD_{490} (experimental group)/ OD_{490} (positive control), where the experimental group and positive control represented the absorbance of the antisera with/without various concentrations of inhibitors.

2.12. IgE binding capacity assessment

To assess the potential allergenicity of OVA and its cross-linked products, the IgE binding capacity test was measured by indirect ELISA [33]. The individual wells of a 96-well microtiter plate were pre-coated with OVA or CL-OVA ($100 \mu\text{L}$ each; $0.5\text{--}16 \mu\text{g mL}^{-1}$) in coating buffer overnight at 4°C . The corresponding OD values of 490 nm were used to analyze the IgE binding capacity of OVA and CL-OVA.

2.13. Statistical analysis

All data were processed in Origin (version 8.0) and reported as the average and standard deviation, unless mentioned otherwise. Appropriate data were analyzed for statistical significance using analysis of variance procedures (SPSS, version 13.0, Chicago, IL, USA) and differences were considered significant when the p -values were less than 0.05 .

3. Results and discussion

3.1. Effect of caffeic acid concentration and enzymatic activity on cross-linking

Since enzymatic cross-linking of native OVA is limited possibly because of its spatial hindrance for enzymes [34], it was postulated that the addition of phenolic compound caffeic acid to the system of OVA catalyzed by PPO may contribute to cross-linking reaction, followed by verifying this hypothesis. Fig. 1A shows the change in the degree of cross-linking for OVA with or without caffeic acid. No or few bands of polymers were produced in the only PPO-treated OVA (Fig. 1A, Lane 9), whereas the PPO/caffeic acid-treated OVA produced smears of high molecular weight ($> 90 \text{ kDa}$) along with a decrease of OVA signaling of 45 kDa . OVA with only PPO-treated did not form intra- or intermolecular covalent cross-links, and it may be prevented by the steric hindrance or repulsion of OVA. While, when caffeic acid was added into this system, the polymers represented the formation of intermolecular cross-links were appeared. A possible reason for that was caffeic acid rather than tyrosine residues in the protein molecules may be involved in the cross-linking, generating caffeic acid-containing protein–protein cross-links. Thereby it indicated that caffeic acid as a mediator to cross-link OVA molecules has an crucial role in OVA the cross-linking system catalyzed by PPO.

Although PPO/caffeic acid was more effective than PPO only, the PPO activity was also one of the important factors for cross-linking reaction. To elucidate the effect of PPO activity on cross-linking of OVA, the PPO activity of $200\text{--}1200 \text{ U mL}^{-1}$ in the reaction mixture was added progressively. Clearly, different amounts of protein aggregates were observed at the designed PPO activities in the presence of caffeic acid. Moreover, within 1000 U mL^{-1} , the higher the PPO activity, the better the cross-linking effect. Further increase of enzyme activity to 1200 U mL^{-1} did not lead to an increase of the cross-linked degree.

It was demonstrated that the globular proteins with a confined tertiary structure were hardly achieved on the native substrates [35]. Thalmann et al. [36] used tyrosinase to induce cross-linking of three model proteins including α -lactalbumin, β -lactoglobulin, and lysozyme in the presence of caffeic acid and succeeded, however, they had no further work on cross-linked products. Chung et al. [25] also found that caffeic acid could enhance the cross-linking of Ara h

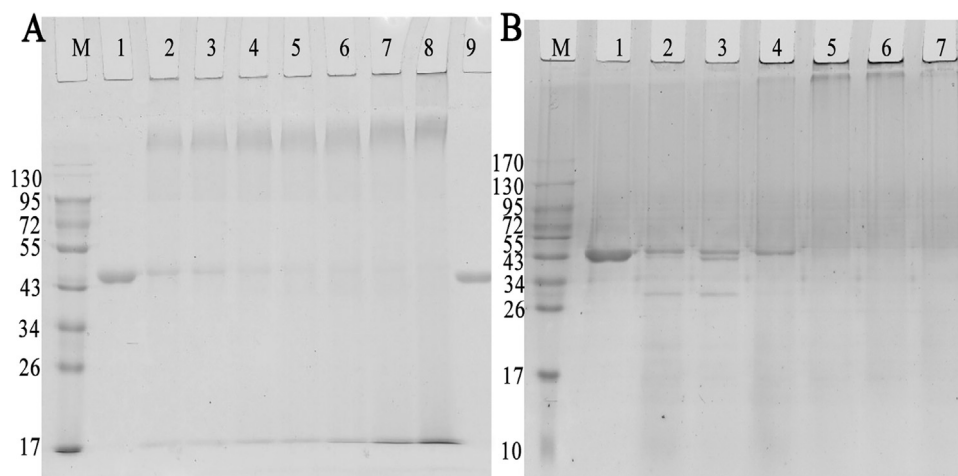


Fig. 1. SDS-PAGE analysis of the cross-linked OVA. (A) Effect of caffeic acid concentration on cross-linking of OVA (caffeic acid 0–10 mM, PPO activity 1000 U mL⁻¹, pH7.0, 50 mM, 50 °C for 8 h). The line 1 was the untreated OVA, and lines 2–9 were the OVA cross-linked in the presence of caffeic acid at 0.1, 0.2, 0.5, 1, 2, 5, 10, and 0 mM, respectively; and (B) effect of PPO activity on cross-linking (PPO activity 200–1200 U mL⁻¹, caffeic acid 1 mM, pH7.0, 50 mM, 50 °C for 8 h). The line 1 was untreated OVA, and lines 2–7 were the OVA cross-linked by PPO activity at 200, 400, 600, 800, 1000, and 1200 U mL⁻¹, respectively.

1 and Ara h 2 by PPO, while the influence of caffeic acid concentration on cross-linking was not available. In this work, the smears of polymers were increased and OVA bands were fainter with increasing concentration of caffeic acid from 0.1 mM to 1 mM (Fig. 1A). For higher concentrations of mediators, cross-linking of OVA was inhibited. Moreover, the PPO-catalyzed cross-linking reaction led to a maximum degree of polymerization with a caffeic acid concentration of 1 mM (data not shown). Regarding to the effect of PPO activity on cross-linking, when the enzymatic activity reached to 1000 U mL⁻¹, most of OVA was cross-linked, the maximum polymers appeared, and certain polymers with high molecular weight were unable to enter into the stacking gel. However, the intensity of the polymers decreased when the PPO activity was 1200 U mL⁻¹. Thus, the optimal concentration of caffeic acid and PPO activity for OVA cross-linking were 1 mM and 1000 U mL⁻¹, respectively.

3.2. Effect of temperature and pH on cross-linking

To define the influence of temperature on cross-linking of OVA, temperature range from 20 °C to 70 °C was selected (Fig. 2A). At 20 °C, faint bands representing the polymers were formed. When the temperatures for the PPO/caffeic acid-based cross-linking reaction of OVA were increased from 20 °C to 50 °C, the gradual increasing polymers formed by intermolecular covalent cross-links appeared in the gel, and the bands of OVA monomers became fainter and fainter. With a further increase in temperature to 70 °C, the high molecular-weight smears decreased slightly. The appearance of this situation may be due to partial inactivation of PPO at an excessively high temperature. Besides, the formation of the dimers and polymers had closed relation with pH in this system. Clearly, PPO/caffeic acid-induced cross-linking could be demonstrated successfully within the range pH 6–8 (Fig. 2B). However, in acidic conditions, neither OVA monomers nor polymers were almost observed. A possible reason for this condition could be that the isoelectric point (pI) value of OVA is 4.5 [37]. Acidic pH region might lead to a decrease of the OVA solubility and even precipitation of OVA monomers, resulting in no cross-linking reaction. Moreover, in alkaline environment, the density of OVA band was increased, and the cross-linking effect became poor with pH increasing. In addition, OVA was purified under closely neutral conditions. Therefore, the neutral condition was selected as optimum throughout the whole work.

The temperature and pH were two key factors that greatly influence the catalytic activity of enzymes [38]. Wu et al. [18] found the optimum temperature and pH ranges for PPO (activity) derived from *agaricus bisporus* were 20 °C and 6.5–7.0, respectively. However, the result was disagreed with our work on the cross-linking temperature. It was taken into consideration that the optimal temperature might be related with different substrate [39], and it was not the only limiting factor for the PPO-induced cross-linking reaction. Therefore, temperature for PPO activity might have no direct effect on the optimal temperature in the cross-linking system. Logically, the best temperature and pH of cross-linking reaction for OVA was 50 °C and 7.0 in our work, respectively.

3.3. Effect of ionic strength and time on cross-linking

Under the designed conditions, the appearance of dimers with a molecular weight of roughly 90 kDa and polymers with higher molecular weight, and the concomitant disappearance of OVA monomers of about 45 kDa were observed under different ionic strengths (Fig. 3A). The intensity of bands with relative molecular mass > 95 kDa turned slightly increased, and the degree of polymerization was increased with the increase of ionic strength up to 50 mM. Moreover, the degree of polymerization was reduced when the ionic strength was higher than 50 mM. Therefore, 50 mM was the optimal ionic strength in cross-linking system. However, it seemed that the ionic strength was not a key factor on cross-linking reaction.

The effect of time on cross-linking of OVA was defined by SDS-PAGE patterns presented in Fig. 3B. It was clearly indicated that different amounts of protein aggregates were formed after reacting for different time. Cross-linking reaction had already happened and polymers were generated after 1 h of reaction, and OVA monomers decreased progressively with the increasing time at the ranges of 1–8 h. When the reaction time was kept for 12 h and 24 h, the smears of polymer were observed obviously, and there was no significant improvement when compared with that at 8 h of reaction time. Thus, 8 h was selected as the optimal reaction time.

Collecting all the results, the maximum degree of polymerization for 1.0 mg mL⁻¹ OVA diluted by PBS with an ionic strength of 50 mM at pH 7.0 was achieved at a PPO activity of 1,000 U mL⁻¹ in the presence of 1 mM concentration of caffeic acid, where the mixture of the cross-linking system was incubated at 50 °C for 8 h.

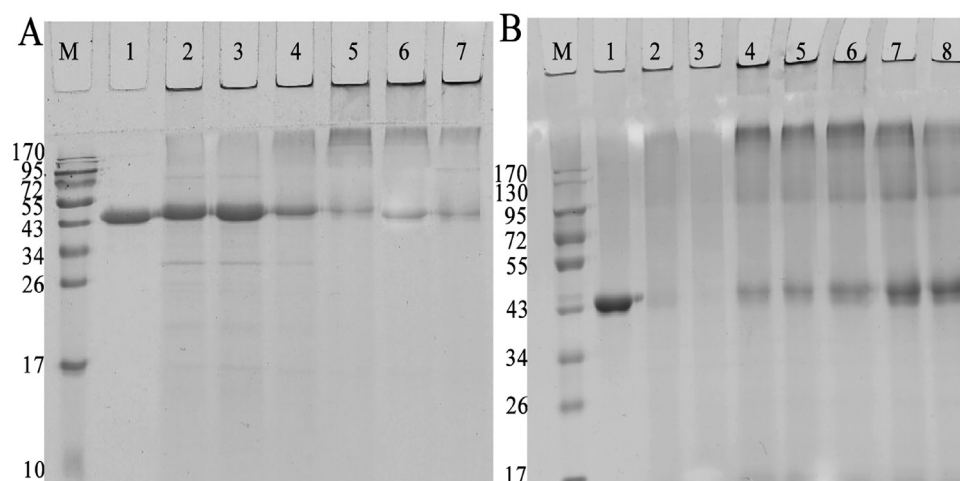


Fig. 2. SDS-PAGE analysis of the cross-linked OVA. (A) Effect of temperatures on cross-linking (20–70 °C, caffeic acid 1 mM, PPO activity 1000 U mL⁻¹, pH 7.0, 50 mM for 8 h). The line 1 was the untreated OVA, and lines 2–7 were the OVA cross-linked at 20, 30, 40, 50, 60, and 70 °C, respectively; and (B) effect of pH on cross-linking (pH 4.0–10.0, caffeic acid 1 mM, PPO activity 1000 U mL⁻¹, 50 °C, 50 mM for 8 h). The line 1 was the untreated OVA, and lines 2–8 were the OVA cross-linked at pH 4.0, 5.0, 6.0, 7.0, 8.0, 9.0, and 10.0, respectively.

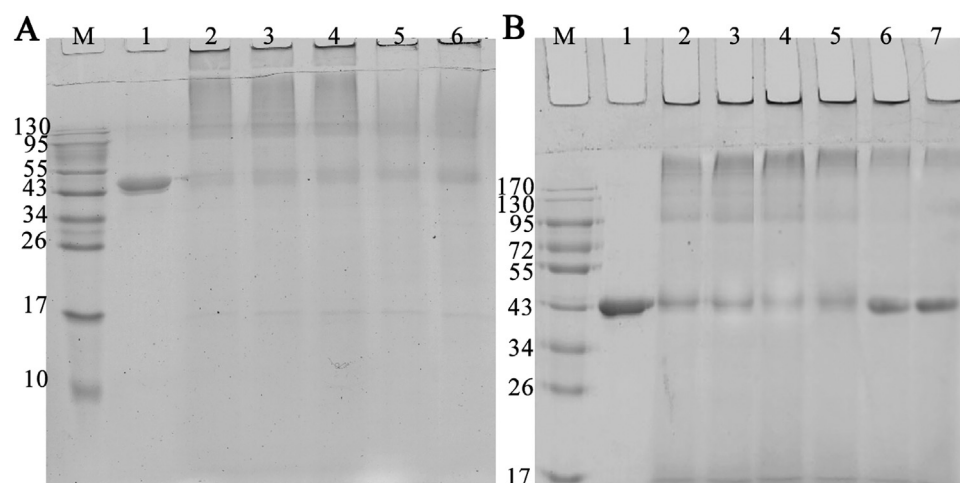


Fig. 3. SDS-PAGE analysis of the cross-linked OVA. (A) Effect of ionic strength on cross-linking (ionic strength 0.1–100 mM, caffeic acid 1 mM, PPO activity 1000 U mL⁻¹, pH 7.0, 50 °C for 8 h). The line 1 was the untreated OVA, and lines 2–8 were the OVA cross-linked at 200, 100, 50, 1, and 0.1 mM of PBS, respectively; and (B) effect of time on the cross-linking (1–24 h, caffeic acid 1 mM, PPO activity 1000 U mL⁻¹, pH 7.0, 50 mM 50 °C). The line 1 was the untreated OVA, and lines 2–8 were the OVA cross-linked for 24, 12, 8, 4, 2, and 1 h, respectively.

3.4. Effect of cross-linking on the structure of OVA

CD spectroscopy was applied to characterize the differences between untreated OVA and CL-OVA about the secondary structure. The native OVA presented typical α -helix and β -structure (Fig. 4A). Clearly, a loss of α -helix and β -structure content and an increase in random coil structures occurred after cross-linking from the estimated constituent of the secondary structure (Table 2). Actually, other study also indicated the changes of protein secondary structures induced by enzymatic cross-linking. The milk casein conformation was altered by tea polyphenol with a major reduction of α -helix and β -sheet and increase of β -turn structure and random coil, suggesting further protein unfolding [40]. Besides, Jia et al. [41] pointed out that laccase-induced rice proteins in the presence of ferulic acid presented decrease in α -helix and β -turn with concomitant increased β -sheet and random coil structure. Nevertheless, our work demonstrated that cross-linking by PPO could change OVA secondary structure, and cross-linked proteins became unordered from original highly organized and tightly regulated state.

Regarding to the UV spectrum, the maximum UV absorption peak of native OVA was found at 279 nm (Fig. 4B), indicating that the UV absorption spectrum of OVA was mainly dependent on Tyr and tryptophan (Trp) residue side-chain groups. After cross-linking of OVA by PPO/caffeic acid, its UV absorption intensity was obviously increased, suggesting that cross-linking made the structure of OVA unfolded and the exposure of more aromatic amino acids on the surface of the protein molecules. Moreover, the wavelengths that correspond to the maximum of UV absorption of untreated OVA and CL-OVA were 279 nm and 272 nm, respectively. A 7 nm blue-shift was registered through cross-linking, implying the Tyr and Trp residues exposed to a less polar microenvironment after cross-linking.

Currently, exogenous fluorescence spectrum is used to detect surface hydrophobicity of proteins, and the non-covalent binding of ANS as an efficient fluorescent probe to the nonpolar groups of protein can induce a blue shift and increase of fluorescence intensity [11]. In this work, the wavelengths corresponding to the maximum fluorescence intensity ($\lambda_{\max}$) were 495 nm and 476 nm for untreated OVA and CL-OVA, respectively. Such a significant blue

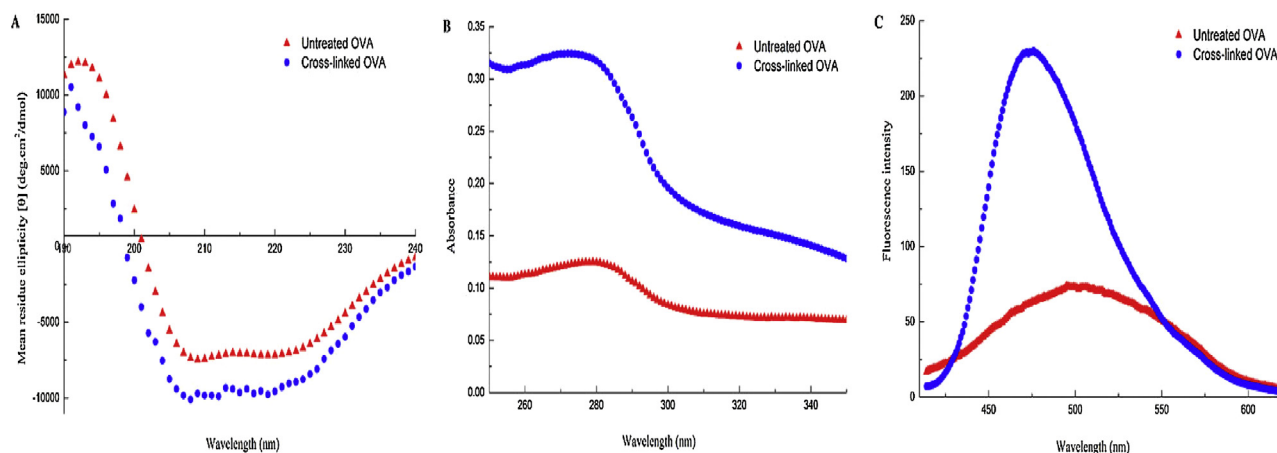


Fig. 4. Far-UV CD spectrum, UV absorption spectrum and ANS fluorescence spectrum of OVA. (A) Effect of cross-linking on the CD spectrum of OVA; (B) effect of cross-linking on the UV absorption spectrum of OVA; and (C) effect of cross-linking on the ANS fluorescence spectrum of OVA.

shift caused by PPO/caffeic acid suggested a more nonpolar environment in the system due to the exposure of certain nonpolar groups. Besides, the fluorescence intensity of CL-OVA was greatly increased when compared to its native state. Since ANS has higher affinity for partially unfolded state compared to native and denatured states of the protein [42], the increased fluorescence intensity indicated that the CL-OVA structure was partial unfolded, leading to the exposure of hydrophobic regions and the increase of surface hydrophobicity.

3.5. Effect of cross-linking on IgG and IgE binding abilities

To assess whether cross-linking affected the antigenicity and potential allergenicity of OVA, IgG and IgE binding abilities were tested by competitive inhibition and indirect ELISA assays, respectively. Obviously, the IgG binding ratio of CL-OVA was higher than that of OVA at various protein concentrations (Fig. 5A). Since high binding ratio meant that the antigenicity of the competitive protein was lower at the same concentration, our test indicated that the cross-linked products had lower antigenicity than native OVA.

The IgE binding ability to CL-OVA was reduced (Fig. 5B), implying that cross-linking could significantly decrease the potential allergenicity of OVA, and it might be an effective method for OVA desensitization.

Moreover, antigenicity and allergenicity are closely related to specific peptides, namely, epitopes [43]. The epitopes include linear and conformational epitopes. The primary reason for the reduced IgG and IgE binding activity of CL-OVA catalyzed by PPO was the cross-linking restriction sites, including Tyr, Lys, and Cys residues [23]. From the spatial molecular model of OVA (Fig. 6), the 5 IgE binding linear epitopes are all exposed on the surface and a total of 10 Tyr, 20 Lys and 4 Cys residues in one OVA molecule provides the potential cross-linking sites. During the cross-linking, the polymers are appeared in the presence of caffeic acid via intermolecular cross-linking at the potential cross-linking sites. Moreover, one Tyr and one Lys are located in AA 38–49, while one Tyr is located in AA95–102. Accordingly, when the cross-linking restriction site was located in the allergic epitope, the linear epitope was changed,

modified, and probably even destroyed, leading to the decrease of IgE and IgG binding activity of CL-OVA. On the other hand, the cross-linking processing reduced the formation of polymers, resulting in the hide of linear epitopes.

Furthermore, cross-linking was known to modify from OVA monomer to polymer (seen from the figures of SDS-PAGE), and its secondary structure became disordered, aromatic amino acid residues and hydrophobic regions exposed on the molecular surface. Due to important modification of OVA conformation, changes or destruction of the conformational epitopes may be happened, or the epitopes in OVA polymers were not sterically accessible to IgG and IgE.

Reasonably, the antigenicity and potential allergenicity of CL-OVA had a significant decrease when compared with native OVA. The results obtained in the present study were in agreement with certain study, and enhanced the consequences of cross-linking catalyzed by enzymes on allergenicity of proteins already observed [16,17,25,27].

4. Conclusions

This study indicated that the cross-linking reaction of egg allergen OVA catalyzed by PPO was influenced by many factors. The optimum parameters for the cross-linking of 1.0 mg mL⁻¹ OVA included that a PPO activity was 1000 U mL⁻¹ in the presence of a 1 mM concentration of caffeic acid, the ionic strength and pH of the system were 50 mM and pH 7.0, respectively, and the reaction was carried out at 50 °C for 8 h. After OVA was cross-linked under the optimal conditions, its conformational structure was changed, particularly the tertiary structure. Moreover, the antigenicity and potential allergenicity of CL-OVA was significantly lower than that of its native state. However, the effect of cross-linking on the allergenicity of OVA should be validated *in vivo* in the future. Anyway, in this work, we confirmed that OVA could be cross-linked by PPO from *Agaricus bisporus* in the presence of caffeic acid, and PPO/caffeic acid treatment on OVA might provide a promising direction to develop hypoallergenic egg products.

Table 2
Secondary structure contents of untreated OVA and cross-linked OVA.

Sample	α -helix content (%)	β -sheet content (%)	β -corner content (%)	random curl content (%)
Untreated OVA	28.3	24.7	21.1	25.9
Cross-linked-OVA	27.7	18.4	20.1	35.2

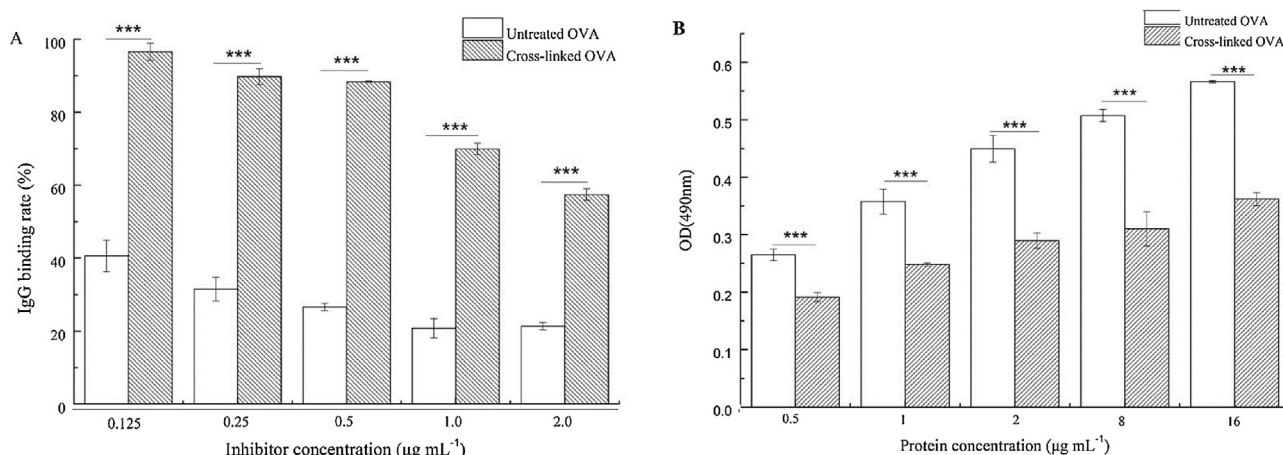


Fig. 5. IgG and IgE binding abilities of OVA and CL-OVA. (A) Effect of cross-linking on the IgG binding ability of OVA tested by inhibition ELISA; and (B) effect of cross-linking on the IgE binding ability of OVA tested by indirect ELISA. Data are given as mean $\pm$ SD ($n = 3$). ***: $p < 0.001$.

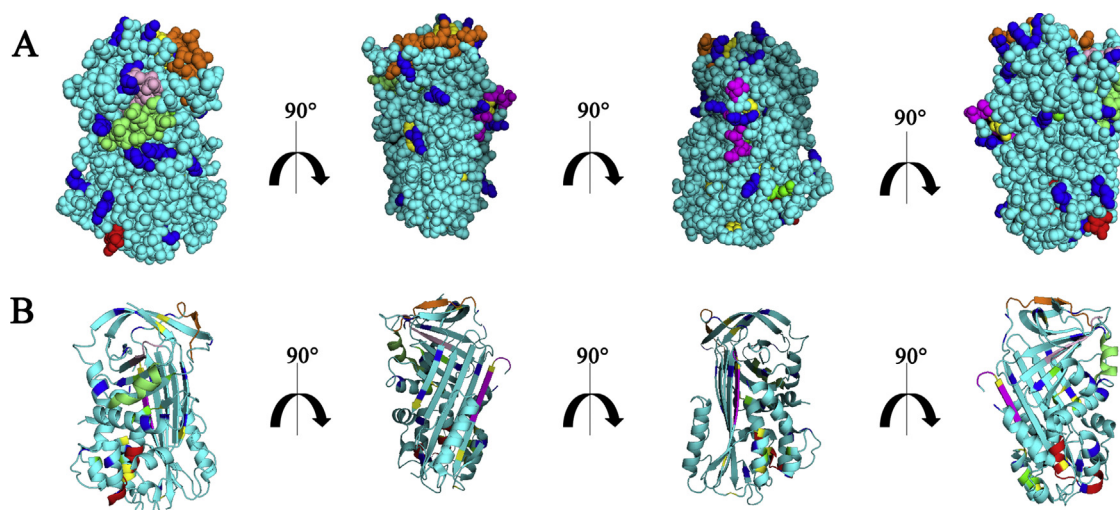


Fig. 6. Ribbon (A) and globular (B) presentations of five IgE binding epitopes on egg OVA. The five epitopes including AA38–49, AA95–102, AA191–200, AA243–248, AA251–260 are marked in red, magenta, orange, lightpink, and lime, respectively; Tyr, Lys and Cys residues are marked in yellow, blue and green, respectively. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

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