

Caffeic acid-assisted cross-linking catalyzed by polyphenol oxidase decreases the allergenicity of ovalbumin in a Balb/c mouse model

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ABSTRACT

Ovalbumin (OVA) is the most abundant egg white protein, but is also a major egg allergen. Desensitization of OVA may be a good way to control an egg allergy. In this study, caffeic acid-assisted cross-linked OVA catalyzed by polyphenol oxidase (PPO) was prepared, the effect of cross-linking on the allergenicity of OVA was tested in a Balb/c mouse model. Mice were orally sensitized with OVA or cross-linked OVA using cholera toxin as adjuvant. Clinical signs of allergy, specific antibody levels, serum histamine levels, mast cell protease-1 (mMCP-1) concentrations, morphological structure of duodenum, and cytokines were determined after mice were challenged with OVA or cross-linked OVA. Both OVA and cross-linked OVA induced allergic diarrhea in Balb/c mice, however, histological symptoms of small intestine were much milder in mice fed with cross-linked OVA than in those fed with OVA. A tendency toward decreased allergen-specific IgE, IgG, IgG1 and IgG2a levels, as well as serum histamine and mMCP-1 concentration were observed in cross-linked OVA group, accompanied by an inhibition of IL-4, IL-5, IL-13 and IFN- γ production in the stimulated spleen cell. It could be concluded that caffeic acid-assisted PPO-catalyzed cross-linking significantly reduced the potential allergenicity of OVA, but may not completely eliminate it.

1. Introduction

Food allergy is recognized as a common worldwide problem and affects an estimated 6–8% of children and 2–5% of adults (Ballmer-Weber and Fernandez-Rivas, 2008; Nowak-Węgrzyn, 2007). Egg is one of the top 8 most allergenic foods in children and infants, with an estimated incidence of 1.6–3.2% (Eggesbo et al., 2001; Houben et al., 2016; Savage et al., 2016). Nowadays, strict dietary avoidance of eggs and egg-containing foods is the best approach to prevent egg allergy, however, due to the unique functional properties of egg proteins, such as foaming, emulsifying, and gelling, the omnipresence of egg proteins in many food products makes it difficult to avoid (McGowan et al., 2015; Mine, 2002). Accordingly, developing hypoallergenic egg products is still a challenging work.

Enzymatic cross-linking is an innovative non-thermal processing method exploited in food industry for improving food texture and functionality (Verhoeckx et al., 2015). The principle of enzymatic cross-

linking is to utilize the enzyme to catalyze the polymerization of proteins primarily through formation of intramolecular and intermolecular cross-links (Djoullah et al., 2016; Giosafatto et al., 2012; Heck et al., 2013; Hiller and Lorenzen, 2009). Transglutaminase, peroxidase, tyrosinases, and laccases are usually the most commonly used for proteins cross-linking (Heck et al., 2013). Recently, cross-linking was demonstrated to change the allergenicity of some allergens. For example, transglutaminase cross-linked sodium caseinate showed a tendency toward decreasing casein-specific IgE levels (van Esch et al., 2013). Cross-linked Ara h2 decreased its allergenicity after being catalyzed by transglutaminase and polyphenol oxidase, respectively (Wu et al., 2016b, 2017). The cross-linked shellfish allergens by tyrosinase or horseradish peroxidase could alter the morphology of proteins and had a high potential for mitigating allergenicity of the shellfish proteins (Fei et al., 2016; Liu et al., 2017). These studies implied that enzymatic cross-linking may be a new technique to reduce the allergenicity of food allergens.

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Egg contains 10–12% of proteins, and four proteins in egg white including ovomucoid (OVM), ovalbumin (OVA), ovotransferrin (OVT), and lysozyme (Lys), and two proteins in egg yolk including a-livetin and yolk glycoprotein 42 (YGP42), were recognized as the allergens in egg (Amo et al., 2010; Jacobsen et al., 2008). Among these allergens, OVM, OVA, and Lys were cross-linked by transglutaminase and peroxidase, respectively, and it was indicated that the molecular weight, structure, rheological properties, functional characteristics, digestibility, and potential allergenicity of these proteins had been changed after cross-linking (Ma et al., 2015; Porta et al., 2013; Schuh et al., 2010; Yaghoubi et al., 2007). In addition, laccase was also used to catalyze egg white to prepare cross-linked aggregates (Jiang et al., 2014). However, tyrosinases have yet to be utilized to catalyze the cross-linking of egg white proteins.

Polyphenol oxidase (PPO), also often called tyrosinase, is a copper-containing enzyme which could insert oxygen in a position ortho-to a tyrosine residue or an existing hydroxyl group in an aromatic ring, followed by the oxidation of o-diphenols to their cognate o-quinones (Mayer, 2006; VanGelder et al., 1997). With regard to the cross-linking reaction catalyzed by PPO, the weakly defined three-dimensional structure and unfolded proteins were easier to be cross-linked, while cross-linking of globular proteins was limited (Hellman et al., 2011). However, cross-linking between globular proteins could be induced by the addition of low molecular weight phenolic compounds, such as caffeic acid (Jus et al., 2012; Thalmann and Lotzbeyer, 2002). This small-molecule phenolic source acted as an assistant mediator for the cross-linking process, which was oxidized by PPO into an o-quinone derivative, followed by generating caffeic-containing protein-protein cross-links (Chung et al., 2005; Thalmann and Lotzbeyer, 2002).

Previous studies demonstrated that PPO catalyzed cross-linking could decrease the allergenicity of peanut allergens (Chung et al., 2005; Wu et al., 2016b), especially with the assist of caffeic acid (Chung et al., 2005), implying PPO catalyzed cross-linking was an effective way to desensitize food allergens. Accordingly, we hypothesized that the allergenicity of egg white allergens could also be decreased by PPO catalyzed cross-linking, and OVA was chosen to verify this hypothesis because it is the most abundant protein in egg white (Huntington and Stein, 2001) and it is usually used as a model protein for studying antigen-specific immune responses in mice (Basto et al., 2015; Claude et al., 2016; Park et al., 2017). Native OVA has a globular structure (Huntington and Stein, 2001), therefore, in the current study, caffeic acid-assisted cross-linked OVA catalyzed by polyphenol oxidase was prepared. The stability and IgE-binding ability of cross-linked OVA were tested by simulated gastrointestinal digestion and ELISA *in vitro*, and the allergenicity of cross-linked OVA was assessed by allergic clinical signs, pathological examination of intestine, specific antibodies levels, serum histamine levels, mast cell protease-1 (mMCP-1) concentrations, as well as cytokines production in a Balb/c mouse model. This work aimed to evaluate the effect of cross-linking catalyzed by PPO on the allergenicity of egg allergen OVA.

2. Materials and methods

2.1. Materials

OVA with a purity of > 98% was prepared and identified by anion exchange chromatography, SDS-PAGE, and MS, respectively, as previously described (Ma et al., 2009). PPO powders were lab-made from mushroom (*Agaricus bisporus*) according to the method of Wu et al. (2013). Pre-stained protein maker was purchased from MBI Fermentas (Burlington, Canada). All other chemicals were purchased from the Sigma Aldrich Corporation (St Louis, MO, USA).

2.2. Human sera collection

Human sera were collected with informed consent from 8 egg

allergy patients from the First Affiliated Hospital of Guangxi Medical University (Guangxi, China). The availability of the sera samples was approved by the internal ethical committee of the hospital. Subjects with a strong history of severe systemic reactions to egg and total egg IgE of at least 100 IU/mL were recruited in this study. All patients were confirmed to have an egg allergy based on physical examination and objective clinical manifestations. A sera pool was prepared according to the method of Wu et al. (2016b), containing sera from the eight patients in equal volumes and used for further analysis.

2.3. Preparation of cross-linked OVA catalyzed by PPO

PPO was dissolved in phosphate buffer (pH 7.0), and the enzymatic activity was measured according to the method of Gawlik-Dziki (Gawlik-Dziki et al., 2008) with some modifications. Caffeic acid was dissolved in ethyl alcohol to be a 100 mmol/L solution. 1 mg of the lyophilized OVA was dissolved in a 50 mmol/L solution of phosphate buffer (pH 7.0), followed by caffeic acid was added and mixed well to the final concentration of 1.0 mmol/L. After that, PPO was added into the mixture, and the final enzymatic activity was 1000 U/mL. Finally, the volume of the reaction system brought to 1 mL with 50 mmol/L solution of phosphate buffer (pH 7.0). The reaction was carried out at 50 °C for 8 h and stopped by heating at 80 °C for 5 min. The cross-linked OVA was frozen and stored at −20 °C until use.

2.4. Dynamic light scattering (DLS) measurement

Determination of the mean particle size of 1 mg/mL OVA and cross-linked OVA was carried out with a BI-200SM dynamic/Static light scattering spectrometer (Brookhaven Instruments Corp., USA) at a wavelength of 532 nm and a scattering angle of 90° at 25 °C according to the method of Shan (Shan et al., 2014).

2.5. *In vitro* simulated gastrointestinal digestion

Simulated gastrointestinal digestion of OVA and cross-linked OVA was performed as previously described (Amigo-Benavent et al., 2011) with some modifications. Briefly, for the simulated gastric digestion, OVA and cross-linked OVA (1.0 mg/mL) were suspended respectively in simulated gastric solution (35 mmol/L NaCl, pH 2.0, adjusted with 2 mol/L HCl) with porcine pepsin (EC 3.4.23.1, 3440 U/mg, Sigma), at an enzyme: substrate ratio of 1:20 w/w (172 U/mg of protein), and incubated at 37 °C in a shaker for 0, 10, 20, 30, 60 min. The reaction was stopped by raising the pH to 7.0 using 2 mol/L NaHCO₃. For the intestinal digestion, the digested protein solution after the 60-min pepsin digestion was shifted to pH 7.0 with the addition of 1 mol/L CaCl₂, 0.25 mol/L Bis-Tris (pH 6.5) and a 0.125 mol/L bile salts mixture containing the same molar quantities of sodium taurocholate and glycocodeoxycholic acid. After preheating at 37 °C for 15 min, trypsin was added at an enzyme: substrate ratio of 1:3 (w/w) to the mixture. The pH was adjusted to pH 7.5 with 1 mol/L NaOH. After that, the reactions were incubated at 37 °C in a shaker for 5, 10, 30, 60 min, and then stopped by boiling for 10 min at 100 °C in water bath. All protein samples were collected and stored at −20 °C, until analyzed by SDS-PAGE.

2.6. IgE binding assessment

To assess the potential allergenicity of OVA and cross-linked OVA, the IgE binding capacity was measured by indirect Enzyme-Linked Immunosorbent Assay (ELISA) (White et al., 2013; Wu et al., 2016a). The individual wells of a 96-well microtiter plate were coated with OVA and cross-linked OVA samples (100 µL per well, from 0.5 µg/mL to 16 µg/mL) in 50 mmol/L sodium carbonate buffer (CBS, pH 9.6) overnight at 4 °C. The wells were washed thrice with phosphate-buffered saline (PBS, pH 7.4) containing 0.05% Tween 20 (PBST), followed

by blocking with 280 μ L of 3% gelatin(w/v) in CBS for 1 h at 37 °C. The plates were incubated with the pooled sera (diluted to 1:20 in PBS, 100 μ L per well) for 1 h at 37 °C, and then 100 μ L rabbit anti-human IgE-peroxidase (diluted to 1: 5000 in PBS) was added to each well, followed by incubation for 1 h at 37 °C. The wells were washed thrice with PBST between each step. Color was developed by adding 100 μ L of substrate solution (0.1 mg/mL citric acid, 0.2 mL/L Na_2HPO_4 solution, 0.4 mL/L o-phenylenediamine, 0.4 mL/L H_2O_2 in ultrapure water) to each well and incubating for 15 min at 37 °C. The reaction was terminated by adding 50 μ L of 2 mol/L H_2SO_4 . The absorbance at 490 nm was read using a Bio-Rad 1860 microplate reader (Bio-Rad, CA, USA).

2.7. Sensitization in a mouse model

The mouse model used in this study was similar to previously described works (Hobson et al., 2007; Meng et al., 2016; Rupa and Mine, 2012) with some modifications. A total of 24 female Balb/c mice (6–8 weeks of age) were purchased from the Laboratory Animal Center of Nanchang University (Nanchang, China, permission number “SYXK (Gan) 2015-0001”). All the mice were maintained under standard animal housing conditions and accommodated for a week after arrival at laboratory. The mice were randomly divided into three groups ($n = 8$ /group) and were sensitized intragastrically (i.g.) twice per week for 5 weeks with 1 mg of OVA or cross-linked OVA adjuvanted with 10 μ g of cholera toxin in 100 μ L PBS solution, as outlined in Fig. 1. The control group received PBS instead of protein. All mice in allergic group were orally challenged with 20 mg of OVA or cross-linked OVA, followed by monitoring the clinical signs of allergy for approximately 40 min after challenging by three independent and blind observers according to a scoring system as described earlier (Yang et al., 2009). All animal-use protocols were approved by the Animal Care Committee of Nanchang University under the guidelines of the Care and Use of Laboratory Animals (NIH Publication No. 86-23, Revised 1996).

2.8. Pathological examination of intestine

With regard to the assessment of pathologic alterations, duodenum samples were collected and fixed in neutral-buffered formaldehyde and embedded in paraffin. Five-micrometer sections were stained with hematoxylin and eosin (H and E), and then the sections were examined histologically using a light microscope on the basis of the method of Yang (Yang et al., 2015).

2.9. Measurement of specific antibodies levels

Whole blood from each mouse was collected, followed by separating serum for ELISA test. A standard ELISA protocol was used to measure

OVA specific murine IgE, IgG, IgG1 and IgG2a levels as previously described (Jimenez-Saiz et al., 2011) with slight variations. Since the serum from each mouse was not enough for all the ELISA detections, the levels of specific antibodies were detected using the pooled sera in each group. Briefly, 96-well microtiter plates were coated either with OVA or cross-linked OVA (5 μ g/well). The pooled sera in each group were diluted to 1:5 for IgE, 1: 2000 for IgG and IgG1, 1:100 for IgG2a. HRP-conjugated goat anti-mouse IgE, IgG, IgG1 and IgG2a antibodies (diluted to 1:5000) were applied. The results were reported as optical density (OD).

2.10. Measurement of levels of serum histamine and mouse mast cell protease-1

Histamine concentrations in sera were determined by ELISA assay using a commercial ELISA kit (Histamine ELISA kit, Demeditec Diagnostics GmbH, Germany). Mouse mast cell protease-1 (mMCP-1) was detected by a commercial ELISA kit according to manufacturer's instructions (eBioscience, USA).

2.11. Cell culture and cytokine analysis

Murine splenocyte culture was performed as described previously (Rupa et al., 2007). In brief, individual spleen from each mouse was collected aseptically and single-cell suspensions were prepared. Cells (2×10^6 /well) were cultured at 37 °C in a flat-bottom 96-well plate without any stimulation or in the presence of OVA or cross-linked OVA (2 mg/mL) for 72 h in a 5% CO_2 incubator. Supernatants were collected and stored at –20 °C until analysis. IL-4, IL-5, IL-13 and IFN- γ were determined by commercial ELISA Ready-Set-Go! Kits (Affymetrix, eBioscience, San Diego, CA, USA).

2.12. Statistical analysis

All data were manipulated in Office Excel (Microsoft, USA), and showed as average and standard deviation, unless otherwise stated. Appropriate data were analyzed in SPSS (version 13.0, Chicago, IL, USA) for statistical significance using one-way ANOVA procedures and bivariate Pearson Correlation. Differences were considered significant when p-values were less than 0.05 (Wu et al., 2016a).

3. Results

3.1. Electrophoretic characterization and size determination of cross-linked OVA

It was shown in Fig. 2A that a smearing at the top of the gel was

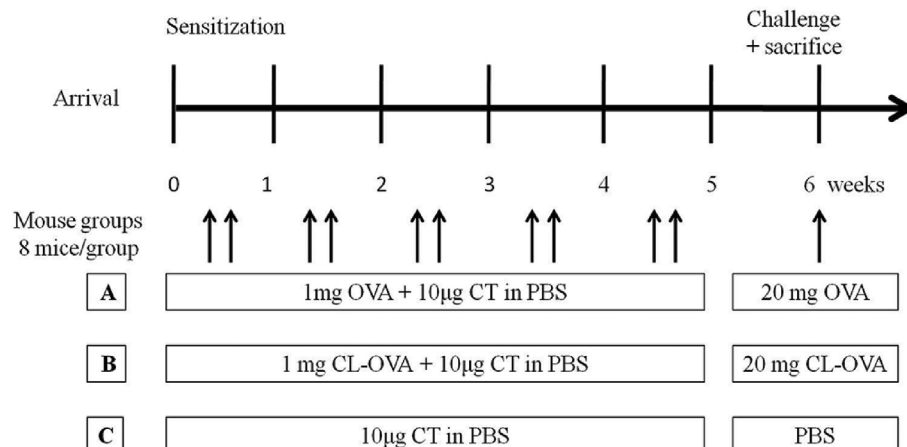


Fig. 1. Oral immunization of Balb/c mice with OVA or cross-linked OVA. Mice were sensitized to 1 mg of OVA or cross-linked OVA and 10 μ g of cholera toxin for 5 weeks, twice per week, followed by oral challenge with 20 mg of OVA or cross-linked OVA. The control group received PBS instead of protein. Clinical signs of allergy were recorded, and all mice were sacrificed 40 min later to collect the blood and spleen. CT, cholera toxin; OVA, ovalbumin; CL-OVA, cross-linked OVA. A, OVA group; B, Cross-linked OVA group; C, CT group as control.

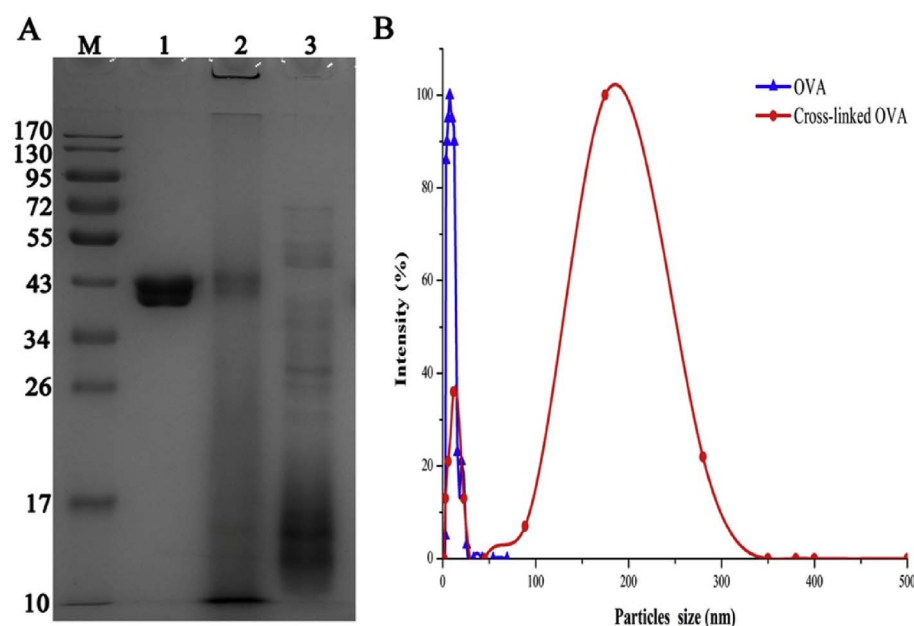


Fig. 2. The polymerization and size distribution of enzymatic cross-linked OVA. (A) SDS-PAGE patterns of OVA and cross-linked OVA. M: marker; 1: native OVA; 2: cross-linked OVA; 3: PPO. (B) Particle size distribution of OVA and cross-linked OVA.

evident in the SDS-PAGE profile after the cross-linking reaction, and the intensity of the OVA band was obviously decreased. The smearing at the top of the gel represented that polymers with high molecular weight were formed after enzymatic treatment, and the formation of polymers were speculated to be intermolecular cross-linked OVA according to the migration of proteins. Cross-linking could change the size of the protein, therefore, in order to confirm the conclusion of intermolecular cross-links of OVA catalyzed by PPO, dynamic light scattering (DLS) was used to check the effect of cross-linking on the mean size of OVA (Fig. 2B). A significant increase in the mean size after cross-linking was observed in Fig. 2B, and the mean size of native OVA and cross-linked OVA was 8.2 nm and 125.6 nm, respectively.

3.2. Digestibility of cross-linked OVA

In order to evaluate the effect of enzymatic cross-linking by PPO on the digestibility of OVA, gastrointestinal digestion under simulated gastric fluid (SGF) and simulated intestinal fluid (SIF) were performed, and the products were analyzed by SDS-PAGE (Fig. 3). It was shown that both OVA and cross-linked OVA were resistant to simulated gastric digestion, indicating a consistent resistance to proteolysis even after 60 min (Fig. 3A and B, line 1–5). The samples obtained after 60 min of

simulated gastric digestion were further subjected to a mimicking duodenal digestion. The SDS-PAGE profiles indicated that a small part of native OVA was rapidly digested into two protein bands after a 5 min incubation, however, intact OVA were still detectable even after 60 min of incubation (Fig. 3A, line 6–9). This suggests that native OVA was only partially degraded following subsequent incubation with trypsin. Moreover, in comparison with native OVA, cross-linked OVA in SIF was easily and rapidly broken down within 5 min, and the polymers above 130 kDa disappeared (Fig. 3B, line 6–9).

3.3. IgE binding assessment of cross-linked OVA

The potential allergenicity of the native and cross-linked OVA was assessed by indirect ELISA using the pooled sera from egg-allergic patients. The cross-linked products had lower IgE binding ability than native OVA (Fig. 4).

3.4. Systemic anaphylactic symptoms in the challenged mice

A Balb/c mouse model of allergy was used to define the allergenicity of cross-linked OVA *in vivo*, and systemic anaphylactic symptoms of the mice were recorded in detail. Five weeks after initial sensitization, the

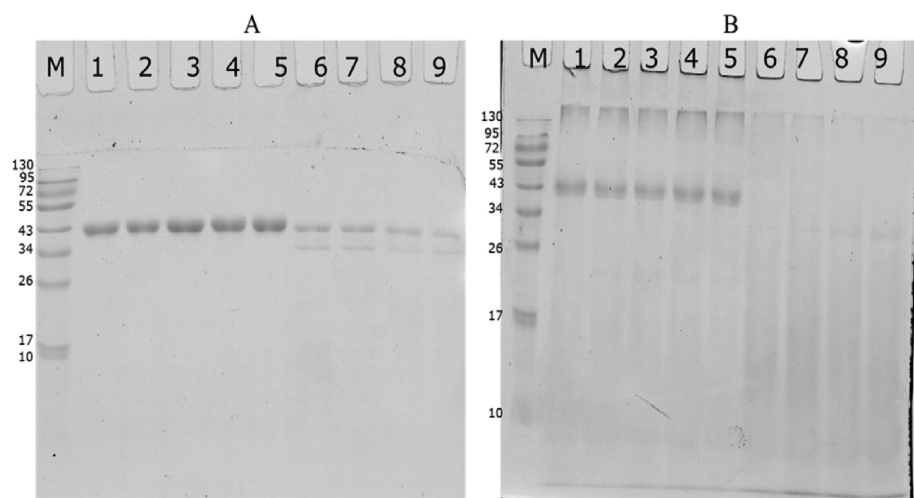


Fig. 3. Effect of SGF and SIF digestion on OVA and cross-linked OVA by SDS-PAGE. A: OVA, B: cross-linked OVA. M: marker; 1–5: SGF digestion for 0, 10, 20, 30, 60min, respectively; 6–9: SIF digestion for 5, 10, 30, 60min, respectively.

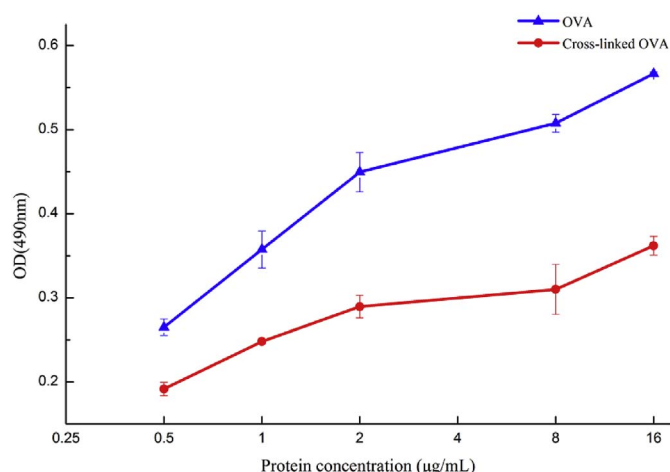


Fig. 4. IgE binding ability of OVA and cross-linked OVA tested by indirect ELISA.

mice were challenged orally. A slight diarrhea was found in all the mice, and scratching and rubbing around the snout and head were found in OVA group and cross-linked OVA group. Histological examination of the small intestines also revealed that OVA and cross-linked OVA induced pathological changes (Fig. 5). In the control group, villi structure of the small intestine was tidiness and intact (Fig. 5C), while in the OVA group, striated border of the small intestine villi disappeared, and vacuolar degeneration of cytoplasm and partial necrosis of the enterocyte could be found compared with the control group (Fig. 5A). In the cross-linked OVA group, the disorder of the enterocyte also existed, while the inflammation of the small intestine

was mild (Fig. 5B), indicating that the potential allergenicity of cross-linked OVA was lower than native OVA.

3.5. Allergic responses to cross-linked OVA

The immunogenicity and allergenicity of OVA and cross-linked OVA were tested in a mouse model, and the allergic response changes between the OVA group and the cross-linked OVA group were defined well. Firstly, cross-linked OVA significantly decreased the levels of specific IgE, IgG, IgG1 and IgG2a in the mouse sera (Fig. 6 A–D, and for Fig. 6A and C, $p < 0.001$, for Fig. 6B and D, $p < 0.01$). Secondly, the levels of mMCP-1 in mice sensitized by cross-linked OVA were significantly decreased compared with the mice sensitized by OVA (Fig. 6E, $p < 0.001$). In addition, the concentration of histamine in mouse plasma of the cross-linked OVA group was significantly lower than that in the OVA group (Fig. 6F, $p < 0.05$). However, when compared to the control group, levels of specific IgE, IgG, IgG1 and IgG2a, and mMCP-1, as well as the concentration of histamine in mouse plasma were all still significantly higher than that in the control group (For Fig. 6E and F, $p < 0.001$, for Fig. 6A, B and 6C, $p < 0.01$, and for Fig. 6D, $p < 0.05$).

3.6. Cytokine ratios

In this study, we detected IL-4, IL-5 and IL-13 as Th2-type cytokines, IFN- γ as Th1-type cytokine. Levels of IL-4, IL-5, IL-13 and IFN- γ were significantly elevated in the OVA group compared with the control group ($p < 0.001$, Fig. 7). Cross-linked OVA led to a significant decrease in IL-4, IL-5, IL-13 and IFN- γ production in the stimulated splenocyte compared to OVA, but still caused a significantly higher

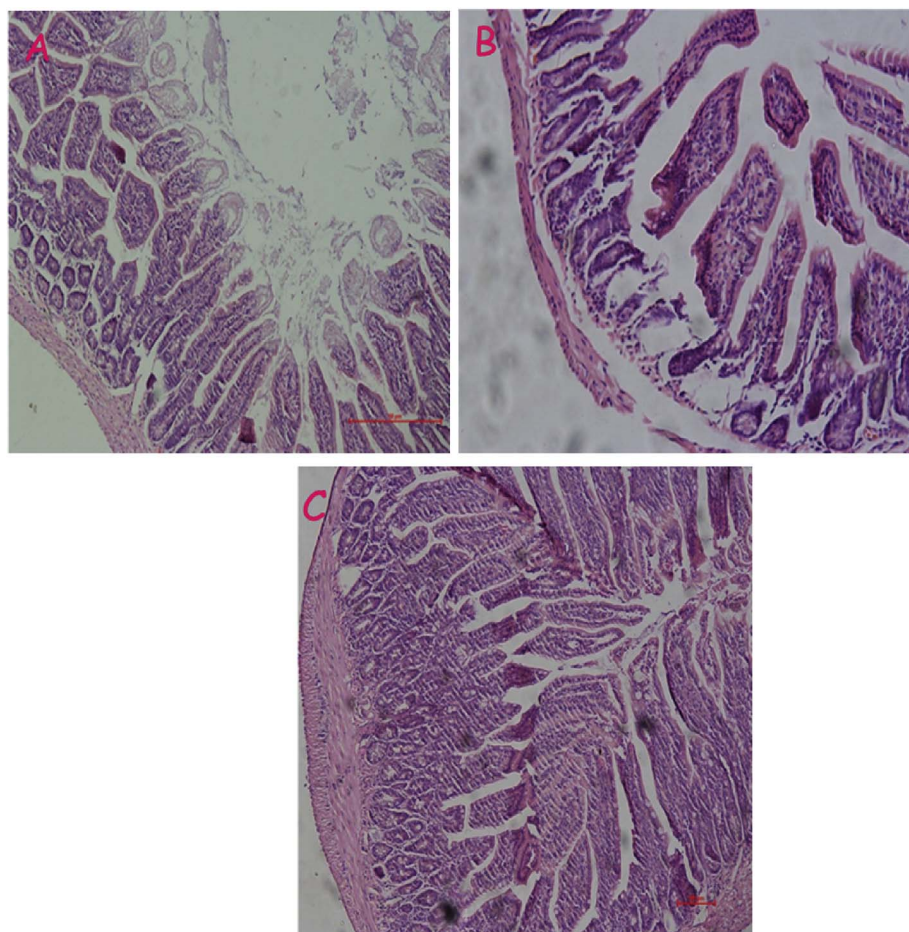


Fig. 5. Typical histological alterations in the intestine of the orally challenged mouse. Duodenum samples were collected and fixed in neutral-buffered formaldehyde and embedded in paraffin. Five-micrometer sections were stained with hematoxylin and eosin, and then the sections were examined histologically at $\times 10$ magnification using a light microscope. A, duodenum of OVA group; B, duodenum of cross-linked OVA group; C, duodenum of control group.

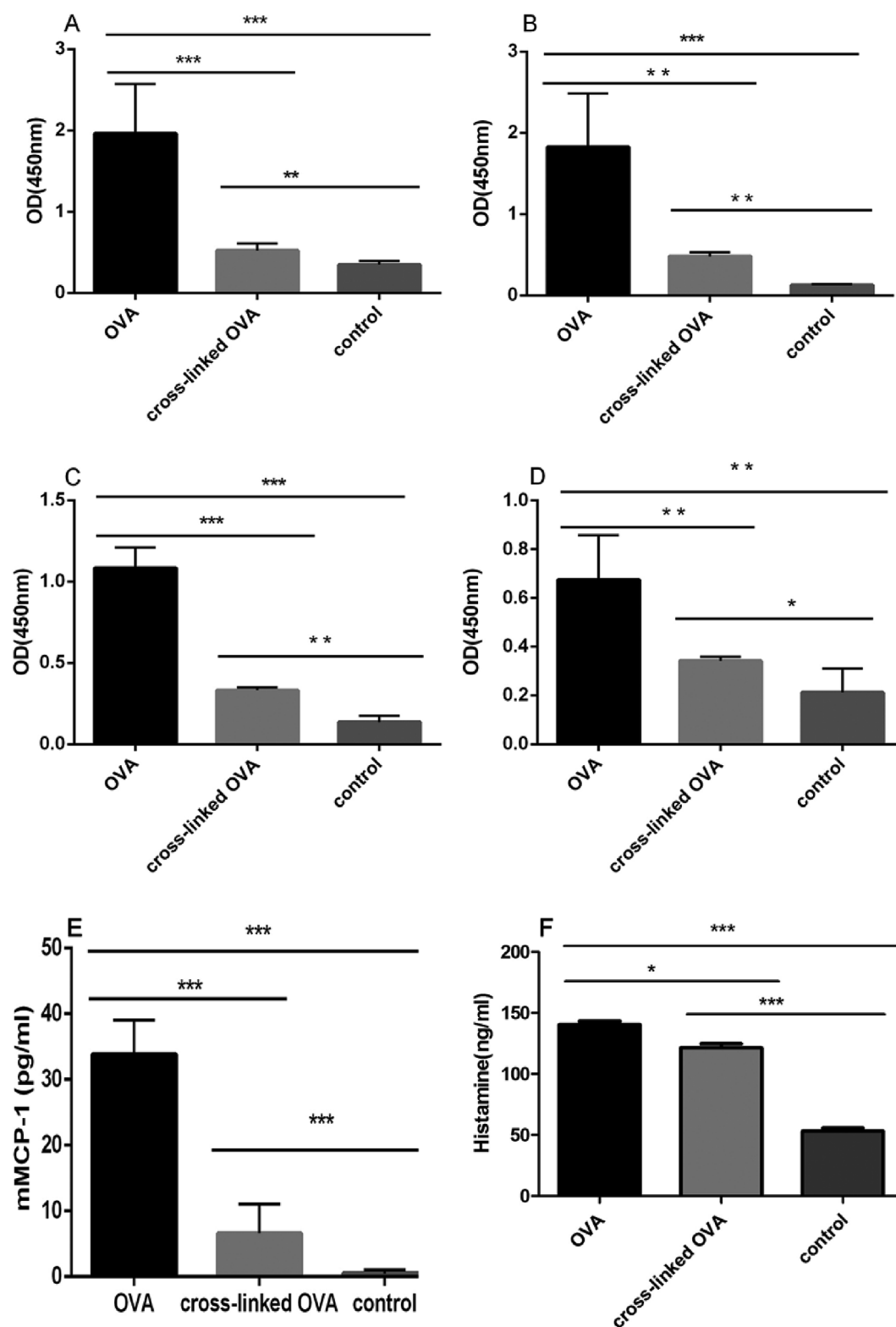


Fig. 6. Special antibody levels of IgE (A), IgG (B), IgG1 (C) and IgG2a (D), as well as concentration of mMCP-1 (E) and histamine (F) in the sera of Balb/c mice. OVA-specific IgE, IgG, IgG1 and IgG2a activity was measured by ELISA in triplicates for each group. HRP-conjugated goat anti-mouse IgE, IgG, IgG1 and IgG2a antibodies were applied. The results were reported as optical density (OD). The concentration of mMCP-1 and histamine in sera was detected by the commercial ELISA kits according to manufacturer's instructions, respectively. Significance was taken at $P < 0.05$. ***: $p < 0.001$, **: $p < 0.01$, *: $p < 0.05$.

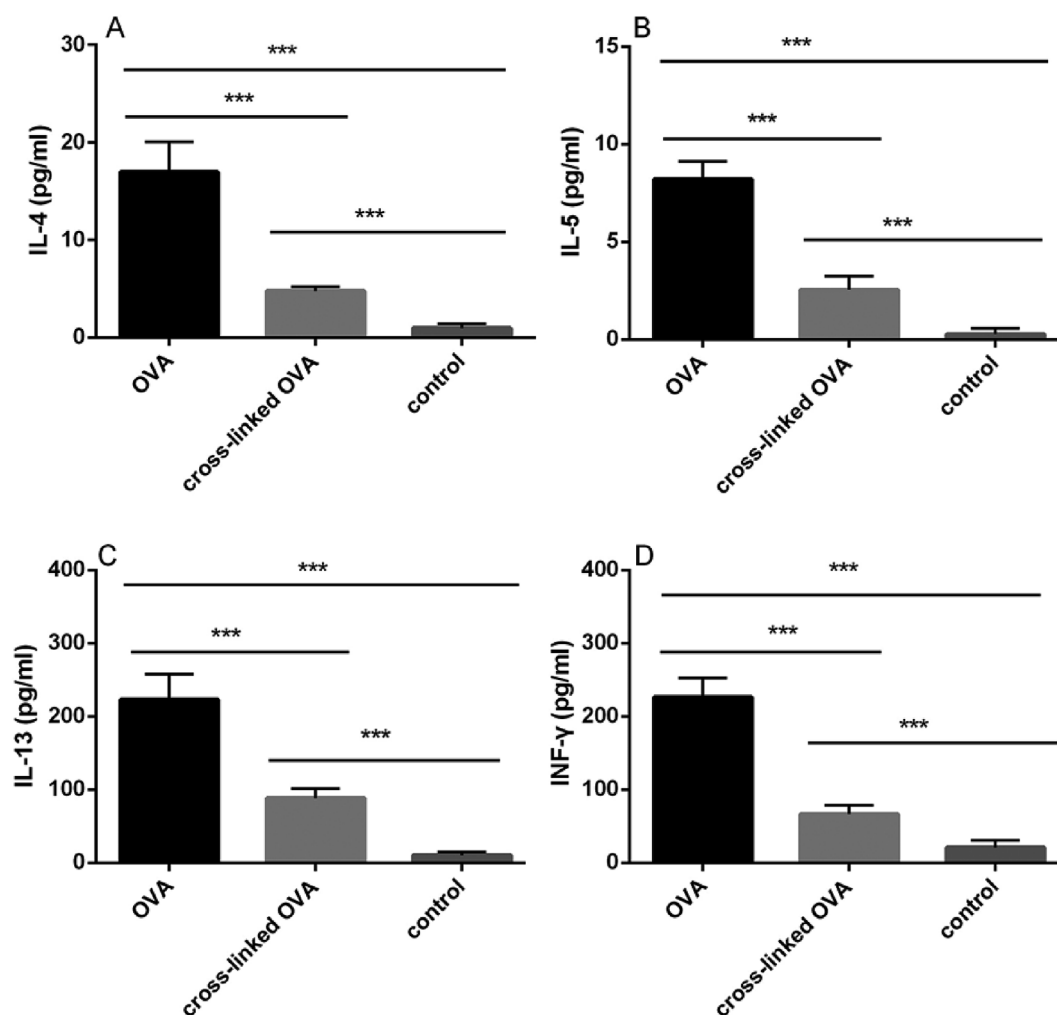


Fig. 7. Cytokines producing of the splenocyte stimulated by OVA or cross-linked OVA: levels of IL-4 (A), IL-5 (B), IL-13 (C) and IFN- γ (D). Spleen was collected from each mouse, and splenocytes were isolated and cultured at 2×10^6 /well in triplicate wells as unstimulated (control) or stimulated with OVA or cross-linked OVA for 72 h. The cytokine concentration of cell culture supernatants for IL-4, IL-5, IL-13 and IFN- γ was determined by ELISA. Significance was taken at $P < 0.05$. ***: $p < 0.001$, **: $p < 0.01$, *: $p < 0.05$.

release of these cytokines compared to the controls ($p < 0.001$, Fig. 7).

4. Discussions

By using caffeic acid-assisted PPO-catalyzed cross-linked reaction, the effect of cross-linking on the protein aggregation, digestibility, and capacity to induce allergic sensitization in a mouse model for OVA allergy was well investigated.

In the PPO catalyzed cross-linking reaction, PPO catalyzed the oxidation of tyrosine residues to an o-quinone derivative, and then the intermolecular cross-links among proteins were formed through the quinone (Chung et al., 2005; Thalmann and Lotzbeyer, 2002). However, such reaction was limited for globular proteins (Hellman et al., 2011). Caffeic acid can eliminate this kind of limitation by acting as an assistant mediator, which was easily oxidized by PPO into an o-quinone derivative, followed by generating a caffeic-containing protein-protein cross-link (Chung et al., 2005; Heck et al., 2013; Jus et al., 2012; Thalmann and Lotzbeyer, 2002). No PPO-based enzymatic cross-linking of OVA was detected in native OVA (data not shown). However, when caffeic acid was added into the reaction, the cross-linking reaction was generated. Accordingly, intermolecular cross-linked OVA catalyzed by PPO with the assistant of caffeic acid was prepared to perform the presented works. The intermolecular cross-links could change the size and aggregation of proteins after the cross-linking reaction (Radosavljevic et al., 2014). In the present study, the smearing at the

top of the gel in the SDS-PAGE (Fig. 2A) and the particles size (Fig. 2B) indicated polymers with high molecular weight were formed and a significant increase in the mean size after cross-linking was found. The results demonstrated that the intermolecular aggregation of OVA existed under the enzymatic catalysis of PPO, producing polymers.

The intermolecular aggregation of cross-linked OVA may affect the digestibility of OVA. In the current study, OVA was resistant to pepsin, and intact OVA still persisted for at least 1 h of digestion in SIF. This was in agreement with previous studies where OVA was reported to be very resistant to pepsin, and still existed after 2 h of the gastrointestinal digestion (Lopez-Exposito et al., 2008; Martos et al., 2010). After PPO/caffeic treatment, cross-linked OVA was still resistant to simulated gastric digestion, however, cross-linked OVA in SIF was easily and rapidly broken down, suggesting that the cross-linking catalyzed by PPO gave rise to an increase in trypsin digestibility of cross-linked OVA. This is different from the digestibility of the cross-linked peanut allergen treated by PPO (Wu et al., 2016b), as well as transglutaminase-mediated cross-linking of OVA (Giosafatto et al., 2012; Ma et al., 2015), where they were shown that cross-linked allergens were more resistant to both gastric and duodenal digestion. This may be due to the different allergen and different enzyme for cross-linking. Nevertheless, the increased digestibility of cross-linked OVA to SIF implied that the allergenicity of cross-linked OVA may be lower than that of native one.

The allergenicity of cross-linked OVA was first assessed by IgE binding to sera from egg allergic patients, and the weak IgE binding

ability of cross-linked OVA indicated that the potential allergenicity of cross-linked OVA was reduced after the PPO/caffeic treatment. Afterwards, the allergenicity of cross-linked OVA was assessed in a Balb/c mouse model. Balb/c mice were sensitized with native OVA and cross-linked OVA, respectively. Both native OVA and cross-linked OVA induced allergic diarrhea in Balb/c mice, and the histological symptoms of the small intestines were much milder in cross-linked OVA fed mice than in those fed with native OVA. When the levels of antigen-specific antibodies in mice sera were detected, cross-linked OVA-specific IgE, IgG, IgG1 and IgG2a were all significantly decreased compared to OVA group ($p < 0.01$). Similarly, the significantly lower levels of mMCP-1 and histamine in the cross-linked OVA group were also detected as compared to the OVA group ($p < 0.05$). All the results demonstrated that cross-linked OVA relieved allergy symptoms in mice compared to native OVA.

Moreover, the question whether the differences in native OVA and cross-linked OVA-specific antibodies responses were also associated with cytokine production was brought up, and the cytokines production from splenocytes of Balb/c mice were assessed after *in vitro* stimulation by native OVA and cross-linked OVA, respectively. In mice, as well as in humans, the Th2 cell immune response is a mark of hypersensitivity disorders and characterized by IgE and IgG1 production, as well as production of allergy-promoting cytokines, such as IL-4, IL-5 and IL-13, while the Th1 immune response is characterized by increased production of IgG2a antibodies in mice and Th1-type cytokines, such as IFN- γ (Halstensen, 1997; Mihajlovic et al., 2016). In the present study, a significantly higher production of Th2-type cytokines in splenocytes existed in the OVA-sensitized mice, but surprisingly, an up-regulation of Th1-type cytokine IFN- γ in OVA-treated mice compared with the cross-linked OVA group and the control group was observed. The up-regulation of IFN- γ in OVA-treated mice could be a result of severe allergic responses, because IFN- γ was produced to moderate the harmful inflammation (Kelchtermans et al., 2008). This phenomenon fit well with the OVA-specific IgG2a level, as IFN- γ could promote the maturation of B-lymphocytes and the production of IgG2a (Kelchtermans et al., 2008; Velez et al., 2015). After PPO/caffeic treatment, cross-linked OVA significantly reduced the cytokines production in splenocytes, which was consistent with previous studies (Fei et al., 2016; Liu et al., 2017), indicating that cross-linking mitigated the allergenicity of OVA.

Both the *in vitro* and *in vivo* assays showed that cross-linked OVA had the potential for alleviating the allergenicity of OVA. The mechanism of how cross-linking reduces the allergenicity of OVA may be explained by the conformational and morphological changes of OVA after PPO/caffeic treatment, destroying or covering the IgE binding epitopes of OVA caused by the aggregation of proteins. In addition, the steric hindrance of the macromolecules may also influence the binding to antibodies or the original method of antigen presentation, which could also induce the decreased allergenicity. Therefore, in the future, it would be beneficial to examine the cross-linking sites and the IgE binding epitope changes after cross-linking.

It was noteworthy that, although the allergenicity of OVA was decreased by PPO/caffeic cross-linking treatment, the levels of specific antibodies, mMCP-1, histamine as well as the cytokines in cross-linked OVA group were still significantly higher than those in the control group. It implied that the allergenicity of OVA could not be eliminated by PPO/caffeic cross-linking treatment, and cross-linked OVA may still induce allergic reactions to mouse, however, the clinical symptoms were much lower than native OVA.

In conclusion, caffeic acid-assisted PPO-catalyzed cross-linking treatment of OVA was an effective method to significantly reduce the potential allergenicity of OVA. The cross-linking reaction occurred through the intermolecular aggregation of OVA and polymers with high molecular weight were formed. The gastrointestinal digestion of cross-linked OVA exhibited resistance to SGF, but had an increase in trypsin digestibility. After the PPO/caffeic treatment, cross-linked OVA revealed an obviously weaker IgE binding ability and significantly lower

allergic sensitization in mice compared to OVA, however, cross-linking could not eliminate the allergenicity of OVA. In future studies, it is necessary to further validate how caffeic acid-assisted PPO-catalyzed cross-linking reduces the allergenicity of OVA, and whether the allergenicity of OVA can be further reduced by varying cross-linking conditions.

Conflict of interest

None.

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