

Effect of tyrosinase-catalyzed crosslinking on the structure and allergenicity of turbot parvalbumin mediated by caffeic acid

Shenglan Tian, Jiaju Ma, Ishfaq Ahmed, Liangtao Lv, Zhenxing Li* and Hong Lin

Abstract

BACKGROUND: Enzymatic treatment of allergenic protein can alter their functional properties under a mild reaction condition due to specificity of enzymes. Phenolic compounds act as mediators and enhance the crosslinking reactions. The study aimed to assess the changes in the structure and immunoglobulin G (IgG) binding capacity of turbot parvalbumin (PV) upon crosslinking with tyrosinase (Tyr) in the absence and presence of caffeic acid.

RESULTS: Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis revealed the appearance of higher molecular weight bands (24, 36 kDa) in the crosslinked PV. The secondary structure of crosslinked PV became loosened and disordered. The results of intrinsic fluorescence and ultraviolet absorption spectral analyses, as well as surface hydrophobicity and free amino group analyses also revealed structural changes. As observed by western blot analysis, the intensity of the PV bands reduced upon Tyr treatment, indicating reduced binding of specific IgG to PV. Moreover, the indirect ELISA (enzyme-linked immunosorbent assay) analysis confirmed that the IgG binding ability of crosslinked PV was reduced 34.94%.

CONCLUSION: Enzymatic treatment mitigated the allergenicity of fish PV, which was closely related to the alterations in the conformational structure. This treatment showed potential for developing hypoallergenic fish products under mild reaction conditions.

© 2019 Society of Chemical Industry

Keywords: turbot parvalbumin; tyrosinase; caffeic acid; crosslinking; IgG binding; structure

INTRODUCTION

Food allergy is an adverse immune response experienced by allergic people on exposure to certain foods. Adverse immune responses to foods affect approximately 5% of young children and 3–4% of adults in Westernized countries and appear to have increased in prevalence.^{1,2} There are eight major sources of food allergens: egg, milk, peanut, tree nuts, fish, shellfish, wheat, and soy.³ Fish is widely appreciated by consumers because of its delicious taste and rich nutritional value. However, allergic reaction occurs in fish-allergic individuals upon ingestion, which may sometimes lead to severe life-threatening anaphylactic reactions. Allergens in turbot caused asthma in three farm workers, and one of the allergens was parvalbumin (PV).⁴ PV is the major fish allergen responsible for adverse reactions occurring in 96–100% of fish-allergic patients.⁵ PV is a kind of small (~12 kDa) calcium-binding protein with a remarkable resistance to heat, denaturing chemicals, and proteolytic enzymes, which is present in high amounts in the white muscles of lower vertebrates.

Until now, no proper treatment is available to overcome the incidence of food induced allergic reactions. However, several types of processing techniques have been adopted to mitigate the allergenic potential of different food commodities. Among them, thermal treatment is the most commonly used method for reducing seafood allergenicity.^{6,7} Furthermore, Maillard reaction

can significantly reduce the IgE binding capacity of fish PV.⁸ In addition, various non-thermal processing methods have been investigated for altering the allergenic potential of food proteins, which may cause aggregation, denaturation, crosslinking, oxidation, and glycosylation, subsequently lead to masking or unmasking of allergenic epitopes.^{9–15}

Enzymatic treatment of allergenic protein offers a remarkable alternative due to the mild reaction conditions and specificity of enzymes for tailoring food functionality. Currently, the food industry employs tyrosinases, transglutaminases, laccases, and peroxidases for enzymatic crosslinking of dietary proteins. Tyrosinase (Tyr) is a copper-containing oxidative enzyme widely distributed in nature,¹⁶ which can oxidize substrates by shuttling electrons from the substrate to molecular oxygen.¹⁷ Tyr can catalyze ortho-hydroxylation of monophenols and subsequent oxidation of o-diphenols to quinones. Tyrosine side chains in proteins can be oxidized by Tyr, while lysyl, tyrosyl, cysteinyl,

* Correspondence to: Z Li, Professor, College of Food Science and Engineering, Ocean University of China, No. 5, Yushan Road, Qingdao, Shandong Province, 266003, P. R. China. E-mail: lizhenxing@ouc.edu.cn

Food Safety Laboratory, College of Food Science and Engineering, Ocean University of China, Ocean University of China, Qingdao, PR China

and histidinyll moieties have been reported to react further with Tyr-oxidized tyrosine residues.^{18,19} Ercili Cura *et al.*²⁰ observed crosslinking reaction after the addition of Tyr in heated milk. Recently, Ahmed *et al.*¹³ investigated the influence of Tyr and caffeic acid (CA) on the allergenicity and structure of major allergens of shrimp. It was found that the protein structure changed greatly, and the immunoglobulin E/immunoglobulin G (IgE/IgG) binding capacity was reduced significantly. The addition of phenolic mediator along with Tyr enhanced the crosslinking efficiency.

The objective of the present study was to determine the changes in the conformational structure and allergenic potential of fish PV upon Tyr and CA crosslinking. The changes in the secondary and tertiary structures of crosslinked PV were analyzed via circular dichroism (CD) spectroscopy, ultraviolet (UV) absorption spectroscopy, intrinsic fluorescence spectroscopy, surface hydrophobicity, and free amino group content. While, the IgG binding capacity of treated PV were determined by western blotting and indirect ELISA (enzyme-linked immunosorbent assay).

MATERIALS AND METHODS

Materials and reagents

The anti-rabbit IgG, horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG antibodies, loading buffer and the standard protein used for performing sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) were purchased from Solarbio Co. (Beijing, China). Mushroom Tyr, bovine serum albumin (BSA), 3,3',5,5'-tetra-methylbenzidine (TMB), o-phthalaldehyde (OPA), 3,4-dihydroxy-L-phenylalanine (L-DOPA) and 1-anilino-8-naphthalene-sulfonate (ANS) were acquired from Sigma Chemical Co. (St Louis, MO, USA). CA was obtained from Shanghai Yuanye Co. (Shanghai, China). The enhanced chemiluminescent (ECL) kit for the western blot analysis was purchased from Thermo Fisher Scientific Inc. (Waltham, MA, USA). Purified turbot PV was extracted in the laboratory of the Food Safety, Ocean University of China. All the chemical reagents used were pure and of analytical grade, unless otherwise stated.

Crosslinking of fish PV by Tyr in the presence of CA

The activity of mushroom Tyr was measured following the method described earlier by Yang and Ouyang.²¹ The conditions of the enzymatic crosslinking reactions were decided based on a previous study²² and pre-experiments. Figure 1(A) displays the reaction formula showing the crosslinking reaction. Fish PV was prepared at a concentration of 2.0 mg mL⁻¹ in 10 mmol L⁻¹ phosphate-buffered saline (PBS, pH 6.5) and incubated with Tyr (600 U g⁻¹ PV) and CA (0.1 mg mg⁻¹ PV). All samples were incubated under constant agitation (200 rpm in an oscillating incubator) for 12 h at 37 °C, followed by boiling the samples for 5 min to stop the reaction. Control samples that lack Tyr or CA were also run in parallel with the test samples. All the samples were kept at -20 °C until further analyses.

Structural characterization of crosslinked PV

Protein electrophoresis

All PV samples were analyzed by SDS-PAGE according to the method depicted by Laemmli.²³ Each protein sample was diluted to 1 mg mL⁻¹ and mixed with loading buffer at 3:1 (v:v) and heated for 7 min at 100 °C to denature proteins. Then the mixtures were loaded into each well of the SDS-PAGE gel (15% resolving gel and 5% stacking gel). Similarly, the standard protein (14–180.0 kDa)

was loaded in the well in order to determine the molecular weight of the crosslinked PV. Electrophoresis was performed in the stacking gel at 80 V for 0.5 h and in the resolving gel at 120 V for 1.5 h. After resolving the proteins, gels were stained with 0.1% Coomassie brilliant blue R-250 and then was destained until the decoloring solution became clear. Images were captured using a HP Scanjet G4050 Photo Scanner (Hewlett-Packard Co., Palo Alto, CA, USA).

CD analysis

The secondary structure of PV was determined by far UV-CD spectra, using a Jasco J-815 CD spectrometer (Japan Spectroscopic Co. Inc., Tokyo, Japan). Each sample was diluted with ultrapure water to a final concentration of 1.0 mg mL⁻¹ and the analysis was performed in the spectral range of 185 to 260 nm. Ultrapure water was used as a blank solution for all samples. The diluted samples were scanned at a speed of 100 nm min⁻¹ using a 1 nm bandwidth at an interval of 2 s. The results were expressed as deg cm² dmol⁻¹ by analyzing the molar residue ellipticity.

UV absorption analysis

All samples were diluted to 0.1 mg mL⁻¹ in 10 mmol L⁻¹ PBS (pH 6.5) and the UV absorbance spectrum was recorded using a UV-1101 spectrometer (Techcomp Co., Shanghai, China) at room temperature. The scan speed was 400 nm min⁻¹ and the scan range was 200–400 nm. Each experiment was repeated three times.

Intrinsic fluorescence analysis

All protein samples were diluted to 0.1 mg mL⁻¹ in 10 mmol L⁻¹ PBS (pH 6.5). Intrinsic fluorescence spectra of samples were acquired on a fluorescence spectrophotometer (F-4600, Hitachi, Tokyo, Japan) at a wavelength of 280 nm (excitation) and 300–400 nm (emission), using 2.5 nm as a constant slit. The PBS was used as a blank solution for all the samples.¹³ The experiments were conducted at room temperature. Triplicate measurements were carried out in order to ensure reproductive results.

Surface hydrophobicity analysis

The surface hydrophobicity of different PV samples was analyzed using F-4600 fluorescence spectrophotometer with ANS as a probe, as described by Lv *et al.*¹¹ All samples were serially diluted to final concentrations of 0.01 to 0.5 mg mL⁻¹ in 10 mM PBS (pH 6.5). Then, 5 µL of 8.0 mM ANS solution prepared in the same buffer was mixed with 1.0 mL protein solution to react for 15 min in the dark. The fluorescence intensity was determined at a wavelength of 370 nm (excitation) and 490 nm (emission), using 2.5 nm as a constant slit. The surface hydrophobicity of PV samples was defined as the initial slope of the curve formed by the fluorescent intensity plotted in line with protein concentration.

Free amino group analysis

The free amino group of each sample was measured as described by Adler-Nissen.²⁴ All protein samples were diluted to 0.25 mg mL⁻¹ in 10 mmol L⁻¹ PBS (pH 7.4) and the same buffer was used as a blank solution for all the samples. Then, 200 µL of each sample solution was mixed with 4 mL of OPA reagent, and allowed to incubate for 2 min at room temperature. The free amino groups were measured at a wavelength of 340 nm by a UV-1101 spectrometer. The content of free amino groups was expressed as mol kg⁻¹ protein, and L-leucine was used as a standard.

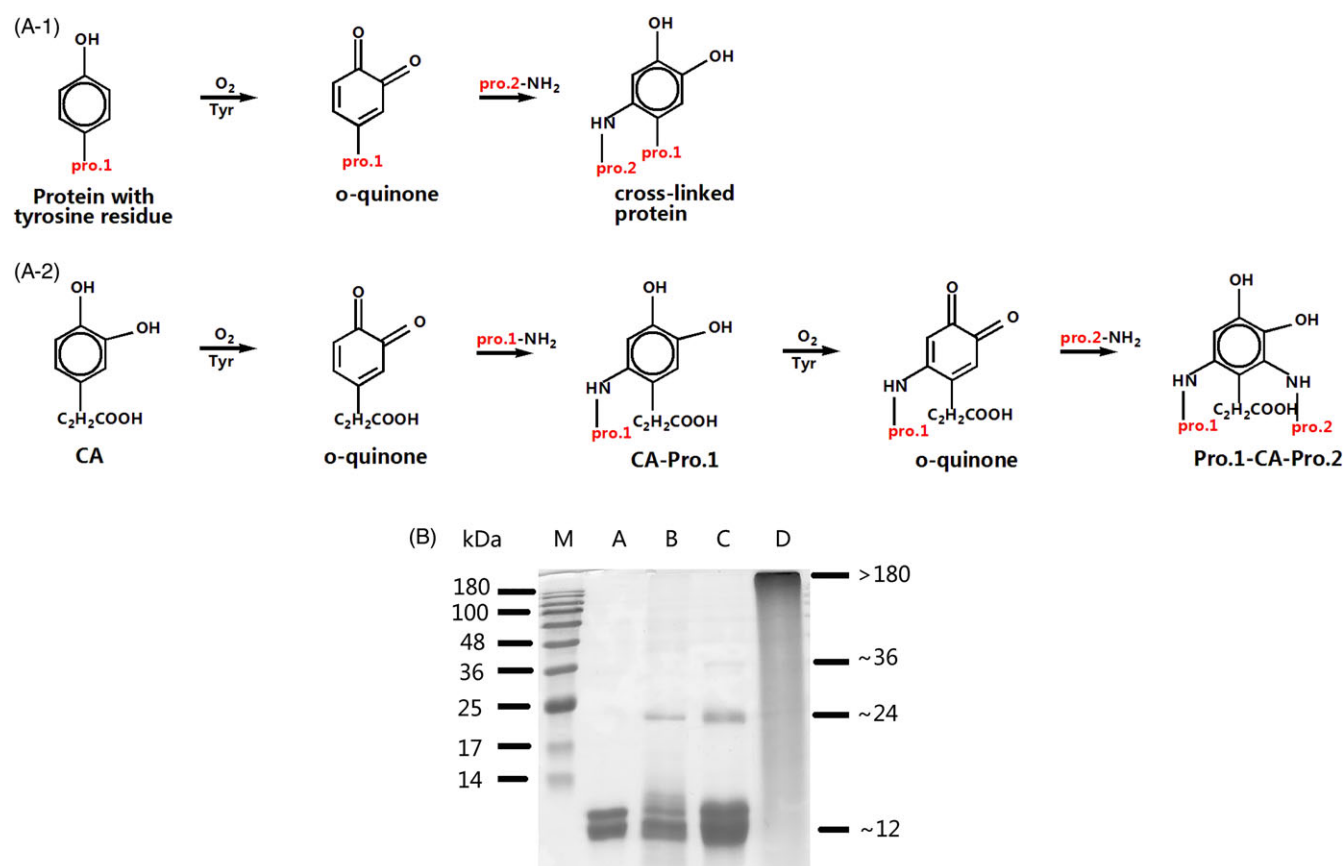


Figure 1. (A) Reactions catalyzed by tyrosinase (Tyr) and/or caffeic acid (CA). (A-1) crosslinking of protein1 and protein2 by Tyr through tyrosine residues; (A-2) crosslinking of protein1 and protein2 by Tyr in the presence of CA. (B) SDS-PAGE analysis of PV crosslinked by Tyr and CA. Lane M, protein marker; lane A, non-treated PV; lane B, Tyr treated PV; lane C, CA treated PV; lane D, Tyr + CA treated PV.

IgG binding analysis of crosslinked PV

Western blotting analysis

Western blotting was conducted to evaluate the IgG binding potentials of all the samples according to the method described earlier by Ahmed *et al.*¹³ with some modifications. In brief, samples were electrophoresed (see earlier) and the pre-run gel was transferred to a polyvinylidene fluoride (PVDF) membrane by the iBlot® 2 Gel Transfer Device (Life Technologies Co., Israel). Then, 2 mL of primary antibody (Rabbit sera, 1:1500 dilution, v:v) and secondary antibody (HRP-labeled goat anti-rabbit IgG, 1:2000 dilution, v:v) were prepared with the ibind solution. Later on, the immuno detections were carried out using iBind™ Western Device (Life Technologies Co., Israel). The immunoassay was then photographed by Tanon-4200SF gel imaging system with the ECL kit. The intensity of the bands indicated the amount of specific IgG binding to PV.

Indirect ELISA analysis

The IgG binding capacity of all treated PV samples was measured by indirect ELISA, as described by Song *et al.*²⁵ and Van Do *et al.*²⁶ with some modifications. Firstly, all PV samples were diluted to 30 µg mL⁻¹ with carbonate buffer solution (pH 9.6) and coated in a 96-well microtiter plate with 100 µL per well to incubate at 4 °C overnight. After rinsing with PBST in triplicate, the plate was blocked by 1% BSA in PBS (10 mmol L⁻¹, pH 7.4) at 37 °C for 2 h to block the non-specific binding. Furthermore, 100 µL of rabbit polyclonal antibody (1:1000 dilution, v:v) was loaded

and incubated at 37 °C for 1.5 h. Then the plate was washed and incubated with HRP-labeled goat anti-rabbit IgG (1:2000 dilution, v:v). After a final rinsing, 100 µL of a TMB solution was added and incubated for 20 min at 37 °C, followed by stopping the reaction by the addition of 50 µL of 2 mol L⁻¹ sulfuric acid. The absorbance of each well was recorded at 450 nm by a microtiter plate reader (MultiscanMK3, Thermo Fisher Scientific Inc., Waltham, MA, USA).

Statistical analysis

One-way analysis of variance (ANOVA) with Duncan's multiple range test was used for analyzing significance among the obtained results with the help of SPSS for Windows (Version 19.0; Chicago, IL, USA). Values differed significantly ($P < 0.05$) were considered, unless otherwise indicated.

RESULTS AND DISCUSSIONS

SDS-PAGE analysis of Tyr crosslinked PV

All PV samples after crosslinking by Tyr or CA can be observed in Fig. 1(B). Turbot PV has two subunits and the molecular weight is near 12 kDa (Fig. 1(B), lane A), consistent with the report of Jiaju *et al.*²⁷ When the PV was treated with Tyr, the bands of native PV decreased in consequence and several PV polymers were observed at ~24 kDa with higher molecular weight bands (Fig. 1(B), lane B). A report showed that shrimp tropomyosin having a molecular weight of 36 kDa treated with Tyr produced higher molecular weight polymers (over 66 kDa).^{13,28} The decrease in the densities, escorted by the appearance of high molecular weight smears

were presumed to be the crosslinks. New bands with a molecular weight of ~24, ~36 kDa and above, were most obvious, as the PV was treated with CA (Fig. 1(B), lane C). When the PV was treated with Tyr and CA, the intensity of the high molecular weight smears became significantly heavier and the bands of native PV could not be recognized clearly (Fig. 1(B), lane D). The possible reason is that CA converted to quinone derivatives and led to crosslinking of PV, rather than only Tyr residues. Stanic *et al.*²⁹ treated β -casein with Tyr and CA, which increased the polymerization and resulted in crosslinking with dominant molecular weight above 220 kDa.

Protein structure analysis

Far-UV CD spectra was used to characterize the effect of Tyr and CA on the secondary structure of PV,^{30,31} as shown in Fig. 2. The results showed that the secondary structure of PV changed into a more loose and disordered aperiodical coil with higher random curl content. The negative peak near 210 nm indicates that native PV had a higher content of β -sheets. The two negative peaks at 210 and 220 nm were slightly weakened, and the positive peak was slightly increased, which indicates a decrease in the α -helix and an increase in aperiodical coil. Further analyses performed with CDNN software showed that the relative percentage of α -helix decreased from 26.90% to 16.60%. After crosslinking treatment, the aperiodical coil increased from 35.20% to 45.30%. The reason may be that the secondary structure of PV was changed when the free amino acid of PV reacted with Tyr, and the presence of CA accelerated this reaction. Therefore, the secondary structure was destroyed and generated some irregular fragments.

UV absorption spectroscopy is an important indicator reflecting the tertiary structure of proteins. The UV absorption spectra of natural proteins mainly depend on tyrosine and tryptophan residues.³² As shown in Fig. 3(A), the maximum absorption peak of native PV was around 258 nm and it was determined by its amino acid composition. Unlike most proteins, PV contains a small amount of tryptophan but contains a lot of phenylalanine. The amino acid composition of PV resulted in a maximum absorption peak around 260 nm, which was consistent with the report of Strehler *et al.*³³ The UV absorption spectra of PV treated with Tyr appeared at 258 nm, which was the same as the native PV. There was an obvious red shift in the UV absorbance peaks of PV treated with CA and PV treated with Tyr and CA, which indicated that the addition of CA enhanced the crosslinking reaction. In addition, CA may react with other amino acid side chains of PV and may affect the aromatic amino acid microenvironment and tertiary structure of PV.

Intrinsic fluorescence intensity is an important characterization of the tertiary structure of proteins. Protein contains aromatic amino acids, tryptophan, tyrosine and phenylalanine which can emit fluorescence.^{34,35} The intrinsic fluorescence of protein when excited at 295 nm is mainly due to the presence of the tryptophan residues. However, when the excitation wavelength is 280 nm, the fluorescence intensity appeared by the interaction of tyrosine and tryptophan, in which the fluorescence intensity of tryptophan is the highest.³⁶ The fluorescence intensity of phenylalanine is very low, so the effect of phenylalanine cannot be detected under most conditions. As shown in Fig. 3(B), the native PV showed the highest fluorescence intensity at 337 nm. After crosslinking, the intrinsic fluorescence intensity of the samples was reduced by different magnitudes. The maximum fluorescence intensity of PV treated by Tyr was 96.15 that was approximately 49.37% lower than the non-treated PV. The intrinsic fluorescence intensity of

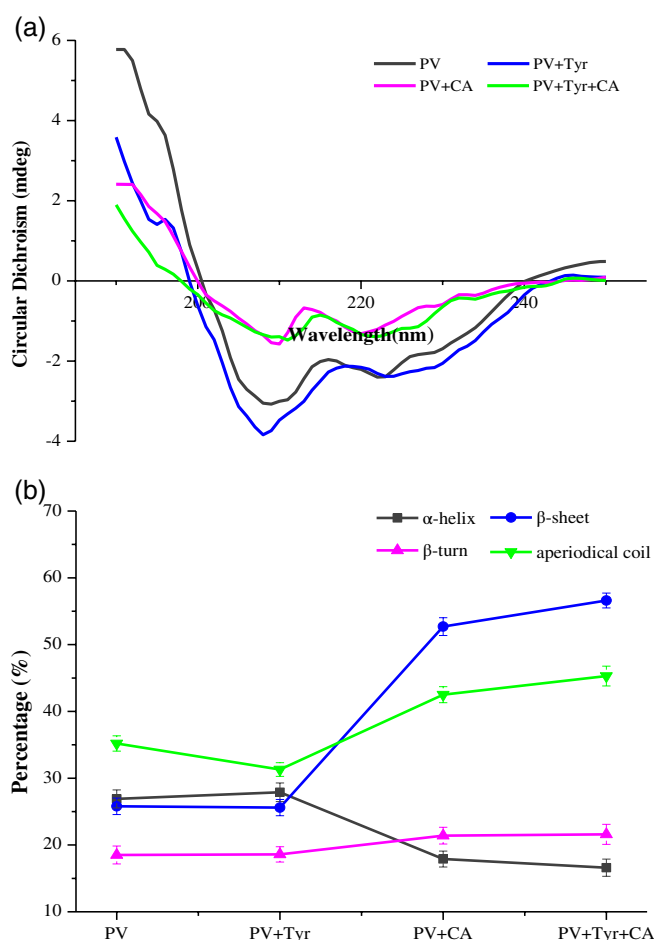


Figure 2. Secondary structure changes of treated parvalbumin (PV). (A) Far-ultraviolet circular dichroism spectroscopy. (B) The percentage of each secondary structure of treated PV.

PV treated by CA was approximately 57.22% higher than that of non-treated PV. The intrinsic fluorescence intensity of PV treated by Tyr and CA was approximately 68.67% lower than that of native PV. Compared with the control group, there was a red shift in the experimental groups, indicating that the tertiary and quaternary structure of PV changed. When crosslinking occurred, the original structure of PV changed and tended to unfold. The chromophoric groups and their residues were more exposed to the aqueous environment and underwent fluorescence quenching, leading to the decrease in fluorescence intensity. A report used polyethylene glycol to modify BSA, which caused small unfolding of the protein.³⁷ But Yuan *et al.*³⁸ reported that the endogenous fluorescence intensity of β -LG increased following transglutaminase and glucosamine treatment. These maybe caused by different crosslinking mechanisms and different responses to allergens.

Surface hydrophobicity can characterize the three-dimensional conformation of proteins. ANS, itself, does not show any fluorescence but on binding non-covalently with hydrophobic residues (such as tryptophan) of a protein like PV, the fluorescence intensity is greatly enhanced.³⁹ As shown in Fig. 4(A), the surface hydrophobicity of non-treated PV was 174.59. There was a decline in surface hydrophobicity of treated PV among the experimental groups. The surface hydrophobicity of PV treated by Tyr was 114.59, and was 34.47% lower than the non-treated PV, while the surface

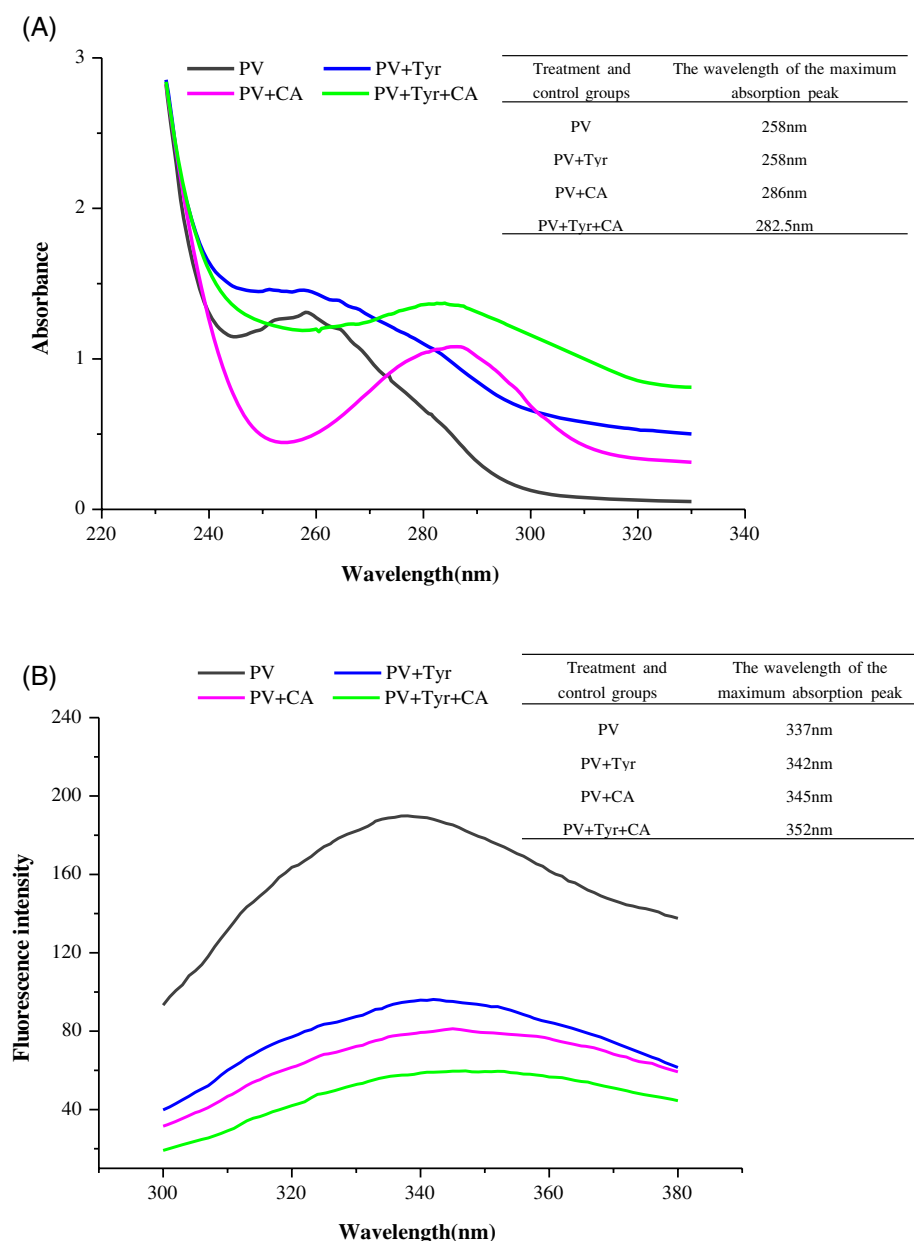


Figure 3. Effect of tyrosinase (Tyr) and caffeic acid (CA) on the ultraviolet (UV) absorption and intrinsic fluorescence of parvalbumin (PV). (A) UV absorption spectrum. (B) Fluorescence emission spectra.

hydrophobicity of PV treated by CA was 167.4, and was 4.1% lower than the non-treated PV. The surface hydrophobicity of PV treated by Tyr and CA was 67.04, and was 59.95% lower than the non-treated PV. The reason may be that the tertiary structure of PV changed after crosslinking, which resulted in the formation of some partially hydrophilic products that accumulated on the surface of PV, leading to a decrease in the surface hydrophobicity. This is in agreement with the findings of phenolic oxidation reported by Ou *et al.*⁴⁰

Changes in free amino groups depend on the degree of protein hydrolysis.⁴¹ As shown in Fig. 4(B), the content of free amino groups of native PV was 2.92 mol kg^{-1} protein, and there was an obvious decline in free amino groups of PV treated by Tyr or CA. The free amino groups of PV treated by Tyr and CA were 1.46 mol kg^{-1} protein, which was 50% lower than the native PV. This indicated that enzymatic crosslinking can continuously

consume free amino groups of PV, resulting in a decrease in the contents of free amino groups. It is also possible that Tyr reacted with tyrosine residues of PV and generated o-benzoquinones. These products can react with amino acid residues of PV and cause intramolecular or intermolecular crosslinking of PV, leading to the decrease of free amino groups. Phenolic compounds can decrease amino group content of fish myofibrillar protein continuously which was treated with different phenolic compound levels (from 1% to 5%) ($P < 0.05$).⁴²

IgG binding analysis of crosslinked PV

Western blotting can be used to estimate the IgG binding ability of the untreated and treated PV. As shown in Fig. 5, the antigenicity of PV treated by Tyr or CA were evaluated using a rabbit IgG antibody against fish PV. The western blotting results were

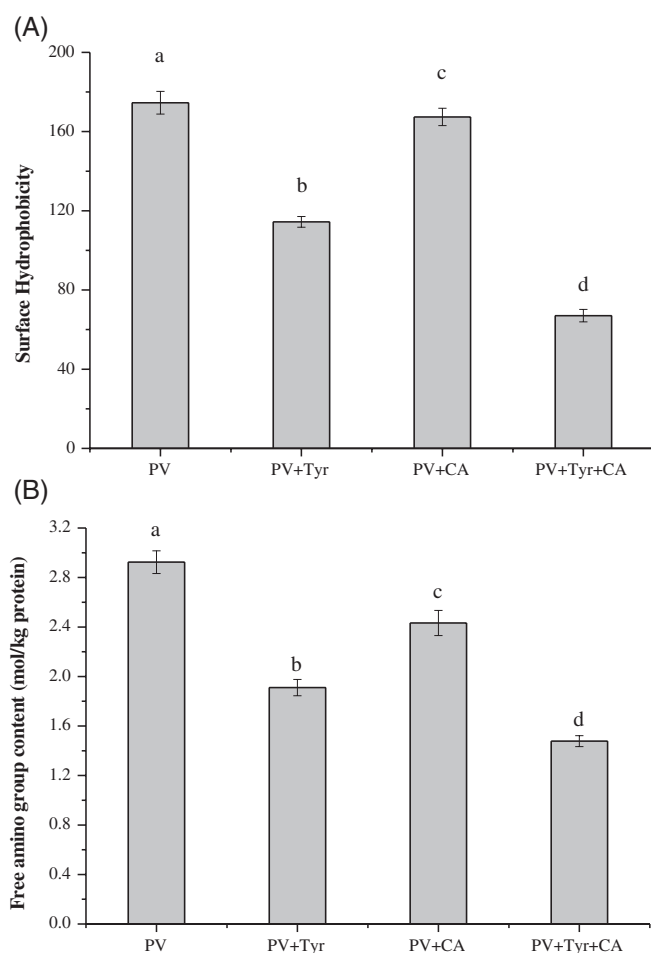


Figure 4. Effect of tyrosinase (Tyr) and caffeic acid (CA) on the surface hydrophobicity and free amino groups of parvalbumin (PV). (A) Surface hydrophobicity. (B) The content of free amino groups. All data are expressed as means $\pm$ standard deviation ($X \pm SD$). Different letters above the columns indicate significant differences ($P < 0.05$).

highly consistent with the SDS-PAGE results, and the PV bands appeared near 12 kDa, consistent with previous reports.⁴³ In treatment groups, there were some new bands that appeared at ~ 24 , ~ 36 and, ~ 48 kDa, indicating crosslinking of PV. The PV bands in lane B and D were shallower than those in lane A and C, indicating that the IgG binding ability of PV were substantially decreased after being treated. After crosslinking, some products with higher molecular weight were still recognized by IgG antibodies, indicating that the crosslinked polymers also had a certain sensitization ability. Moreover, caffeic acid helped to enhance the crosslinking of PV catalyzed by Tyr. Chung *et al.*⁴⁴ treated the roasted peanut extracts with and without PPO (polyphenol oxidase), PPO/CA and CA, respectively. Results showed that, in all cases, crosslinks were formed and a decrease in the levels of two peanut allergens, Ara h 1 and Ara h 2, were observed. Among the three treatments, PPO/CA was the most effective in reducing IgE binding capacity of peanut allergens. More recently, Ahmed *et al.*¹³ also observed crosslinks formation and allergenicity reduction, following treatment of shrimp tropomyosin with Tyr/CA.

Examination of the reactivity of native and crosslinked PV was analyzed by indirect ELISA using rabbit serum specific IgG, therefore we can judge whether crosslinking affected the

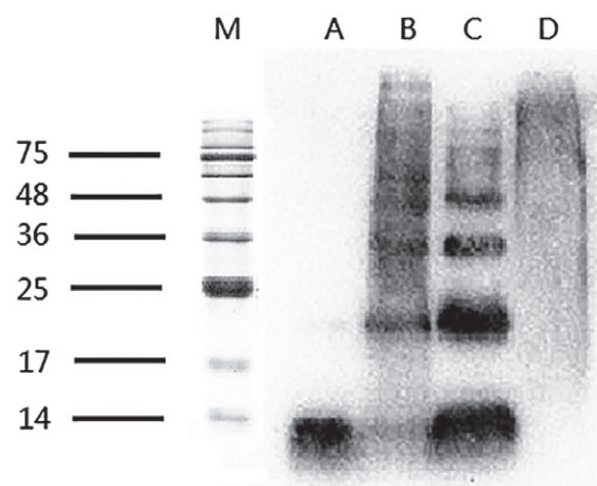


Figure 5. Western-blotting analysis of parvalbumin (PV) crosslinked by tyrosinase (Tyr) and caffeic acid (CA). Lane M, protein marker; lane A, non-treated PV; lane B, Tyr treated PV; lane C, CA treated PV; lane D, Tyr + CA treated PV.

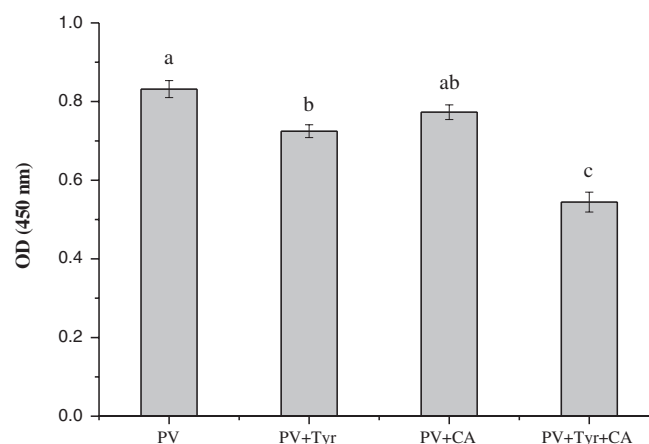


Figure 6. Indirect ELISA analysis of parvalbumin (PV) crosslinked by tyrosinase (Tyr) and caffeic acid (CA). All data are expressed as means $\pm$ standard deviation ($X \pm SD$). Different letters above the columns indicate significant differences ($P < 0.05$).

antigenicity of PV. As shown in Fig. 6, the absorbance value of PV treated by Tyr or CA was 13.25% or 7.23% lower than the native PV, respectively. Treatment of PV with Tyr and CA led to the largest decrease (34.94%) in the IgG binding capacity among all groups. The presence of CA may increase the substrate of Tyr, which greatly increases the crosslinking efficiency, resulting in a significant decrease in IgG binding capacity. The reduction in the allergenicity of turbot PV via Tyr and CA are consistent with the changes in the structural properties. Structural changes might cause the modification of amino acid residues and binding epitopes, which agreed with the results reported in recent studies.^{13,25,45} Fei *et al.*⁴⁵ did some research on crosslinked thermal polymerized arginine kinase (AK) and analyzed the potential allergenicity. According to the results of inhibition ELISA, the half inhibitory concentration of AK after crosslinking changed from 1.13 to 228.36 $\mu\text{g mL}^{-1}$ and the binding activity of crosslinked thermal polymerized AK reduced. Addition of CA along with oxidative enzymes resulted in higher reduction of allergenicity, which is due to the increased crosslinking and formation of o-quinones, or other

intermediates, thereby modifying the tertiary structures of the allergens.^{13,44,46}

CONCLUSIONS

This study showed that fish PV treated by Tyr or CA led to crosslinking under mild conditions and generated high-molecular weight polymers. Furthermore, crosslinking induced alteration in the PV structure and resulted in the reduction of IgG binding capacity. Far-UV CD analysis revealed that the content of α -helix in PV decreased after crosslinking, and the content of β -sheet and irregular coils increased, resulting in a looser and disordered secondary structure. In fact, the amino acid microenvironment and the tertiary and quaternary structures of PV changed significantly, as evident by UV, intrinsic fluorescence and surface hydrophobicity analysis. Moreover, the results of western blotting and indirect ELISA showed that the IgG binding ability of PV crosslinked by Tyr and CA was significantly reduced. This phenomenon was closely related to the changes of conformational structure. Therefore, Tyr and CA crosslinking of PV can be a useful way to reduce the allergic hazards of fish in the food industry.

ACKNOWLEDGEMENTS

This work was supported by the National Natural Science Foundation of China (31871719), and Fundamental Research Funds for the Central Universities (201762004).

REFERENCES

- Boye JL, Food allergies in developing and emerging economies: need for comprehensive data on prevalence rates. *Clin Transl Allergy* **2**:25 (2012).
- Sicherer SH and Sampson HA, Food allergy: a review and update on epidemiology, pathogenesis, diagnosis, prevention, and management. *J Allergy Clin Immunol* **141**:41–58 (2018).
- Berni Canani R, Nocerino R, Pezzella V, Leone L, Cozzolino T, Aitoro R *et al.*, Diagnosing and treating food allergy. *Curr Pediatr Rep* **1**:189–197 (2013).
- Carra CP, Martín-Lázaro J, Ledesma A and de la Torre F, Occupational asthma caused by turbot allergy in 3 fish-farm workers. *J Invest Allergol Clin Immunol* **20**:349–351 (2010).
- Drewnik A and Kowalski ML, Fish allergy. *Alergia Astma Immunologia* **21**:88–95 (2016).
- Lasekan AO and Nayak B, Effects of buffer additives and thermal processing methods on the solubility of shrimp (*Penaeus monodon*) proteins and the immunoreactivity of its major allergen. *Food Chem* **200**:146–153 (2016).
- Mejrhrit N, Azdad O, Chda A, El Kabbaoui M, Bousfiha A, Bencheikh R *et al.*, Evaluation of the sensitivity of Moroccans to shrimp tropomyosin and effect of heating and enzymatic treatments. *Food Agric Immunol* **28**:969–980 (2017).
- Li Z, Jiang M, You J and Feng L, Impact of Maillard reaction conditions on the antigenicity of parvalbumin, the major allergen in grass carp. *Food Agric Immunol* **25**:486–497 (2014).
- Luo C, Hu C, Gao J, Li X, Wu Z, Yang A *et al.*, A potential practical approach to reduce Ara h 6 allergenicity by gamma irradiation. *Food Chem* **136**:1141–1147 (2013).
- Lv L, Lin H, Li Z, Ahmed I, Mi N and Chen G, Allergenicity of acrolein-treated shrimp tropomyosin evaluated using RBL-2H3 cell and mouse model. *J Sci Food Agric* **98**:4374–4378 (2018).
- Lv L, Lin H, Li Z, Wang J, Ahmed I and Chen H, Changes of structure and IgE binding capacity of shrimp (*Metapenaeus ensis*) tropomyosin followed by acrolein treatment. *Food Funct* **8**:1028–1036 (2017).
- Lv L, Lin H, Li Z, Ahmed I and Chen G, Determining the effect of malondialdehyde on the IgE-binding capacity of shrimp tropomyosin upon *in vitro* digestion. *J Sci Food Agric* **97**:4588–4594 (2017).
- Ahmed I, Lv L, Lin H, Li Z, Ma J, Guanzhi C *et al.*, Effect of tyrosinase-aided crosslinking on the IgE binding potential and conformational structure of shrimp (*Metapenaeus ensis*) tropomyosin. *Food Chem* **248**:287–295 (2018).
- Vanga SK, Singh A and Raghavan V, Review of conventional and novel food processing methods on food allergens. *Crit Rev Food Sci Nutr* **57**:2077–2094 (2017).
- Zhang Y, Deng Y and Zhao Y, Structure-based modelling of hemocyanin allergenicity in squid and its response to high hydrostatic pressure. *Sci Rep* **7**:40021 (2017).
- Sampaio S, Taddei P, Monti P, Buchert J and Freddi G, Enzymatic grafting of chitosan onto *Bombyx mori* silk fibroin: kinetic and IR vibrational studies. *J Biotechnol* **116**:21–33 (2005).
- Selinheimo E, Lampila P, Mattinen M and Buchert J, Formation of protein-oligosaccharide conjugates by laccase and tyrosinase. *J Agric Food Chem* **56**:3118–3128 (2008).
- Ito S, Kato T, Shinpo K and Fujita K, Oxidation of tyrosine residues in proteins by tyrosinase. Formation of protein-bonded 3,4-dihydroxyphenylalanine and 5-S-cysteinyl 3,4-dihydroxyphenylalanine. *Biochem J* **222**:407–411 (1984).
- Bittner S, When quinones meet amino acids: chemical, physical and biological consequences. *Amino Acids* **30**:205–224 (2006).
- Ercili Cura D, Lille M, Partanen R, Krus K, Buchert J and Lantto R, Effect of *Trichoderma reesei* tyrosinase on rheology and microstructure of acidified milk gels. *Int Dairy J* **20**:830–837 (2010).
- Yang D and Ouyang M, Antioxidant and anti-tyrosinase activity from olea leaf extract depended on seasonal variations and chromatography treatment. *Int J Org Chem* **02**:391–397 (2012).
- Thalmann C and Lötzbeyer T, Enzymatic cross-linking of proteins with tyrosinase. *Eur Food Res Technol* **214**:276–281 (2002).
- Laemmli U, Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**:680–685 (1970).
- Adler-Nissen J, Determination of the degree of hydrolysis of food protein hydrolysate by trinitrobenzenesulfonic acid. *J Agric Food Chem* **27**:1256–1262 (1979).
- Song Y, Li Z, Lin H, Du S, Hao Z, Lin H *et al.*, Effect of malondialdehyde treatment on the IgE binding capacity and conformational structure of shrimp tropomyosin. *Food Chem* **175**:374–380 (2015).
- Van Do T, Hordvik I, Endresen C and Elsayed S, The major allergen (parvalbumin) of codfish is encoded by at least two isotypic genes: cDNA cloning, expression and antibody binding of the recombinant allergens. *Mol Immunol* **39**:595–602 (2003).
- Jiaju M, Pavase TR, Zhen-Xing L and Lin H, Optimisation of an extraction technique of fish allergens suitable for detection and diagnosis. *Czech J Food Sci* **35**:24–31 (2017).
- Liu G, Hu M, Sun L, Han X, Liu Q, Alcocer M *et al.*, Allergenicity and oral tolerance of enzymatic cross-linked tropomyosin evaluated using cell and mouse models. *J Agric Food Chem* **65**:2205–2213 (2017).
- Stanic D, Monogioudi E, Dilek E, Radosavljevic J, Atanaskovic-Markovic M, Vuckovic O *et al.*, Digestibility and allergenicity assessment of enzymatically crosslinked β -casein. *Mol Nutr Food Res* **54**:1273–1284 (2010).
- Konno T, Conformational diversity of acid-denatured cytochrome c studied by a matrix analysis of far-UV CD spectra. *Protein Sci* **7**:975–982 (2010).
- Hennessey JP and Johnson WC, Information content in the circular dichroism of proteins. *Biochemistry* **20**:1085–1094 (1981).
- Goldfarb AR, Saidel LJ and Mosovich E, Ultraviolet absorption spectra of proteins. *Science* **114**:397–404 (1951).
- Strehler EE, Eppenberger HM and Heizmann CW, Isolation and characterization of parvalbumin from chicken leg-muscle. *FEBS Lett* **78**:127–133 (1977).
- Borkman RF and Lerman S, Fluorescence spectra of tryptophan residues in human and bovine lens proteins. *Exp Eye Res* **26**:705–713 (1978).
- Hospes M, Hendriks J and Hellingwerf KJ, Tryptophan fluorescence as a reporter for structural changes in photoactive yellow protein elicited by photo-activation. *Photochem Photobiol Sci* **12**:479–488 (2013).
- Islam Rabbi SNI, Sultan MZ, Sohel MD and Sultan MZ, Study of interaction between febuxostat and bovine serum albumin by fluorescence spectroscopy. *J Bioanal Biomed* **07**:164–170 (2015).
- Rawat S, Raman Suri C and Sahoo DK, Molecular mechanism of polyethylene glycol mediated stabilization of protein. *Biochem Biophys Res Commun* **392**:561–566 (2010).
- Yuan F, Lv L, Li Z, Mi N, Chen H and Lin H, Effect of transglutaminase-catalyzed glycosylation on the allergenicity and conformational

- structure of shrimp (*Metapenaeus ensis*) tropomyosin. *Food Chem* **219**:215–222 (2017).
- 39 Farruggia B, Nerli B, Di Nuci H, Rigatusso R and Pico G, Thermal features of the bovine serum albumin unfolding by polyethylene glycols. *Int J Biol Macromol* **26**:23–33 (1999).
- 40 Ou S, Wang Y, Tang S, Huang C and Jackson MG, Role of ferulic acid in preparing edible films from soy protein isolate. *J Food Eng* **70**:205–210 (2005).
- 41 Church FC, Swaisgood HE, Porter DH and Catignani GL, Spectrophotometric assay using o-phthalaldehyde for determination of proteolysis in milk and isolated milk proteins1. *J Dairy Sci* **66**:1219–1227 (1983).
- 42 Prodpran T, Benjakul S and Phatcharat S, Effect of phenolic compounds on protein cross linking and properties of film from fish myofibrillar protein. *Int J Biol Macromol* **51**:774–782 (2012).
- 43 Vando T, Hordvik I, Endresen C and Elsayed S, Characterization of parvalbumin, the major allergen in Alaska pollack, and comparison with codfish allergen M. *Mol Immunol* **42**:345–353 (2005).
- 44 Chung S, Kato Y and Champagne ET, Polyphenol oxidase/caffeic acid may reduce the allergenic properties of peanut allergens. *J Sci Food Agric* **85**:2631–2637 (2005).
- 45 Fei D, Liu Q, Chen F, Yang Y, Chen Z, Cao M et al., Assessment of the sensitizing capacity and allergenicity of enzymatic cross-linked arginine kinase, the crab allergen. *Mol Nutr Food Res* **60**:1707–1718 (2016).
- 46 Garcia A, Wichers JH and Wichers HJ, Decrease of the IgE-binding by Mal d 1, the major apple allergen, by means of polyphenol oxidase and peroxidase treatments. *Food Chem* **103**:94–100 (2007).