

Effect of laccase-catalyzed cross-linking on the structure and allergenicity of *Paralichthys olivaceus* parvalbumin mediated by propyl gallate

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ABSTRACT

The aim of the present study was to evaluate *Paralichthys olivaceus* parvalbumin (PV) following treatment by laccase (LAC) in the presence of propyl gallate (PG) on the structure and potential allergenicity. The structure of LAC + PG treated PV was analyzed through SDS-PAGE, CD, fluorescence, and allergenicity was analyzed by immunological and cell model. Our results showed that LAC + PG treatment can induce structural changes through PV cross-linking. Western blotting and indirect ELISA analysis revealed the decrease in IgG binding capacity of PV, corresponding with the structural changes. The results of in vitro digestion illustrate that LAC + PG treated PV showed more resistance to gastrointestinal digestion compared to untreated PV. The release rate of β -hexosaminidase and histamine decreased by 35.6% and 66.9%, respectively, with LAC + PG treatment by RBL-2H3 cell assay. Considering the wide utilization of LAC in food industry, our treatment reveals its potential for creation of hypoallergenic fish products under mild reaction conditions.

1. Introduction

Food allergy causes an abnormal response of immune system triggered by the exposure of the body to specific allergenic food proteins. It affects as many as 3–4% of adults and 5% of young children (Sicherer & Sampson, 2010). Most of the allergic reactions are mediated by immunoglobulin E (IgE), and generate mucocutaneous, gastrointestinal, anaphylactic and cardiovascular symptoms, etc (Pedrosa, Boyano-Martínez, García-Ara, & Quirce, 2015).

Food processing, such as enzymatic cross-linking, thermal treatment, and the Maillard reaction, known to have varied effects on food allergens (Paulsen, Christensen, & Andersen, 2006; Nowak-Węgrzyn & Fiochi, 2009; Roth-Walter et al., 2008; Yuan et al., 2017). Enzymatic cross-linking of proteins has been carried out for processing cereal, dairy, meat, and fish products (Stojadinovic, Pieters, Smit, & Velickovic, 2014). Enzymatic cross-linking, along with improving food textural properties, can have advantageous effect on their safety and digestibility by gastrointestinal enzymes (Tantoush et al., 2011). A previous study revealed that the allergenic potential of peanut allergens was influenced via cross-linking with polyphenol oxidase enzyme (Chung, Kato, & Champagne, 2005). Currently, food industry employs LAC, tyrosinases, transglutaminases, and peroxidases for enzymatic cross-

linking of dietary proteins (Stanic et al., 2010). LAC is a widely distributed copper-containing oxidative enzyme, which can oxidize substrates by shuttling electrons from the substrate to molecular oxygen. The proteins containing tyrosine side chains in proteins can be oxidized by LAC, which are currently being investigated as cross-linking agents (Selinheimo, Lampila, Mattinen, & Buchert, 2008). LAC could slightly oxidize PV without presence of mediator. Phenolic compounds (such as PG, caffeic acid) can provide the functional groups (phenolic hydroxyl), which could react with an amino group, and thereby improving the cross-linking efficiency of LAC (Leung et al., 2014). Moreover, PG was considered as one of the food additives, which can be easily oxidized to quinone forms by heat treatment. These quinones can react with amino groups modifying them without cross-linking.

Fish is extensively preferred as a raw material in the food industry due to its delicate flavor and high nutritional importance. The largest consumers of fish products are in China, North America and Japan. Fish allergy is one of the most prevalent forms in food allergy, and allergic reaction occurs in fish-allergic individuals upon ingestion, which may sometimes lead to severe life-threatening anaphylactic reactions. *Paralichthys olivaceus* PV is the major allergen responsible for adverse reactions occurring in 96–100% of fish-allergic patients (Agnieszka Drewnik, 2016). PV is a kind of small (~12 kDa) calcium-binding

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protein with a remarkable resistance to heat, proteolytic enzymes, and denaturing chemicals, which is present in high amounts in the white muscles of lower vertebrates. Several researchers reported that thermal treatment and polymerization could influence the allergenicity of PV (Chen et al., 2013; Abramovitch et al., 2013). Nevertheless, the enzymatic cross-linking effect on PV is not well-defined, therefore it is essential to probe whether enzymatic cross-linking can be used to reduce allergenicity.

The present study attempts to elucidate the structural changes and potential allergenicity of fish PV upon its cross-linking by LAC in the presence of PG. The structure of modified PV was investigated by SDS-PAGE, free amine group, CD, fluorescence spectroscopy. The IgG binding capacity was detected by immunoblot and indirect enzyme-linked immuno sorbent assay (ELISA). The digestion stability was evaluated by simulated gastrointestinal digestion. Additionally, the allergenicity was also examined using a passive rat basophil leukemia (RBL-2H3) cell model.

2. Materials and methods

2.1. Materials and chemicals

PV was extracted from *Paralichthys olivaceus* in our lab. Rabbit polyclonal antibody against TM was generated by the same lab. 1-anilino-8-naphthalene-sulfonate (ANS) and Bovine serum albumin (BSA) were bought from Sigma Chemical Co. (St. Louis, MO, USA). Horseradish peroxidase (HRP)-labelled goat anti-rabbit (IgG) were purchased from Zhongshan Jinqiao Co. (Beijing, China). The enhanced chemiluminescent (ECL) kit for Western blot analysis was procured from Thermo Fisher Scientific Inc. (Waltham, MA, USA). All other chemicals used in this work were of analytical grade.

2.2. LAC + PG cross-linking reaction with PV

The activity of Laccase was measured following the previous method established by d'Acunzo, Barreca, and Galli (2004). The laccase is from *Coriolus versicolor* and the activity is 2.2U/mg. Based on the previous study, the conditions of enzymatic cross-linking reactions were precisely determined (Yuan et al., 2017). Fig. 1A is the reaction formula about cross-linking reaction. PV (2.0 mg/ml in 10 mM PBS pH 7.4) and PG were mixed at a weight ratio of 1:1 (w/w), with (+) or without (−) LAC to initiate the reaction, respectively. Approximately 100 U of LAC/g protein was added to induce the enzymatic reaction. Furthermore, under constant agitation (200 rpm in oscillating incubator) all samples were incubated for 8 h at 37 °C, followed by boiling the samples for 5 min to stop the reaction, and then stored at −40 °C until use after dialyzed.

2.3. Protein electrophoresis

All PV samples were analyzed by SDS-PAGE utilizing the previously established method by Laemmli (1970). Samples (8 µg/well) were mixed with loading buffer (containing β-mercaptoethanol) (3:1) and heated for 7 min at 100 °C. Later, the mixtures were loaded into each well of SDS-PAGE gel (5% stacking gel and 15% resolving gel). After resolving the protein, gels were stained with Coomassie Brilliant Blue R-250, and then were destained till the solution become clear. Images were captured by HP Scanjet G4050 Photo Scanner (Hewlett-Packard Co., USA).

2.4. The effect of IgG binding capacity of cross-linking PV

2.4.1. Western-blot

Western blotting was carried out to evaluate IgG binding capacity

following the method of Ahmed et al. (2018). The membrane was incubated using iBind solution (6 mL iBind 5X Buffer + 300 µL iBind 1000X Addition + 23.7 mL double distilled H₂O). The rabbit polyclonal antibody was added in one of the well as the primary antibody (diluted 1:2000), later incubated with HRP-labelled goat anti-rabbit (diluted 1:10000) as the secondary antibody. Finally, the results were examined using ECL following washing with deionized water.

2.4.2. Indirect-ELISA

Indirect ELISA was further evaluated following the previous described method by Kamath, Rahman, Komoda, and Lopata (2013) with slight modifications. Rabbit polyclonal antibodies (1: 2000) was added into PBS (10 mM, pH7.4) as the primary antibody and the plate was incubated at 37 °C for 2 h. After washing, HRP-labelled goat anti-rabbit (1: 10,000) into PBS (10 mM, pH7.4) as the secondary antibody was added to each well and then incubated at 37 °C for 1.5 h. After washing, TMB was added and the plate was incubated at 37 °C for 20 min. Sulfuric acid (2 M) was added to terminate the reaction. The result was measured at 450 nm using a Thermo microplate reader (MultiscanMK3, Thermo Fisher Scientific Inc., USA).

2.5. Structure analysis

2.5.1. Free amine

Free amine was determined using our previous method (Lv et al., 2019), where L-leucine was taken as the standard, and the absorbance was measured at 340 nm.

2.5.2. Surface hydrophobicity

Surface hydrophobicity of LAC and PG treated PV was analyzed using ANS following the method of Lin et al. (2015) with some modifications. All the sample solutions (1.5 mL) were diluted to 0.01, 0.05, 0.1, 0.2, 0.5 mg/mL, respectively. And the samples were mixed with 15 µL of 5.0 mM ANS solution and react for 5 min. The resulting relative fluorescence of the solutions was measured with a spectro-photofluorometer (F4600, Hitachi, Japan).

2.5.3. Intrinsic fluorescence

Intrinsic fluorescence of LAC and PG treated PV were determined using the method of Yuan et al. (2017). The excitation and emission wavelengths were set at 280 nm and 290–400 nm with 2.5 nm slit width and 1200 nm/s scanning speed using an RF-5301PC fluorescence spectrometer (Shimadzu, Kyoto, Japan).

2.5.4. Secondary structure

Secondary structural changes of all the samples were estimated as described by Wu, Wu, and Hua (2010). The concentration of the samples was finalized at 0.5 mg/mL. The operating parameters include the scan rate of 100 nm/min and bandwidth of 1.0 nm at an interval of 0.25 s. The far-ultraviolet CD spectra range (190 nm–260 nm) with a CD spectrophotometer (Japan Spectroscopic Co. Inc., Japan).

2.6. In vitro digestion assay

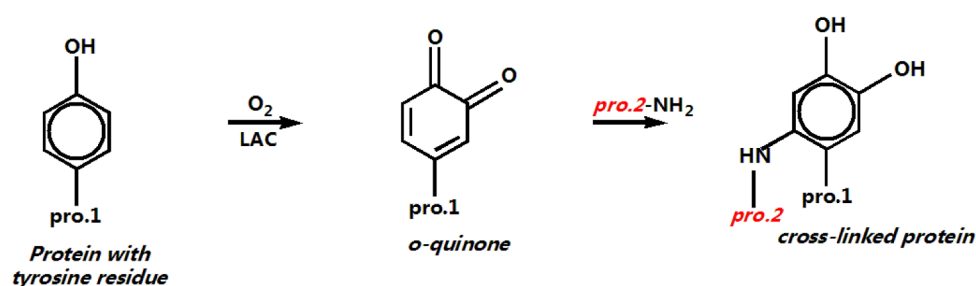
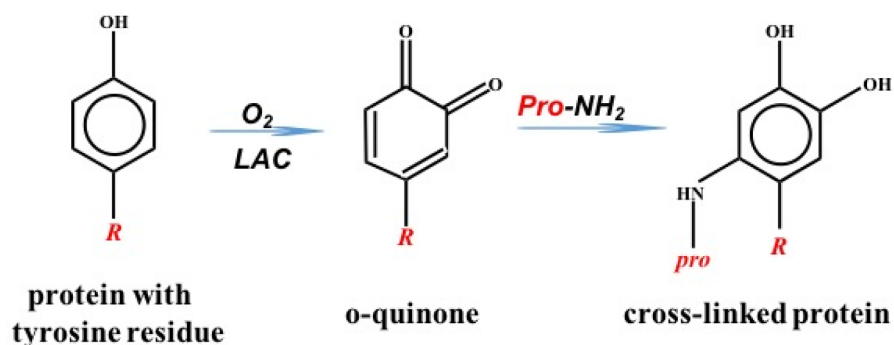
Digestion stability assay of simulated gastric fluid (SGF) and intestinal fluid (SIF) was determined according to the previously described protocol (Lv et al., 2019). The concentration of all the samples was kept at 0.5 mg/mL. The digested samples were then examined by SDS-PAGE and western blot with the same method (2.3. and 2.4.).

2.7. RBL-2H3 cells assays

The rat mast cell line RBL-2H3 were bought from the Chinese Academy of Sciences. The cells were cultured as described previously

A

(A-1)



(A-2)

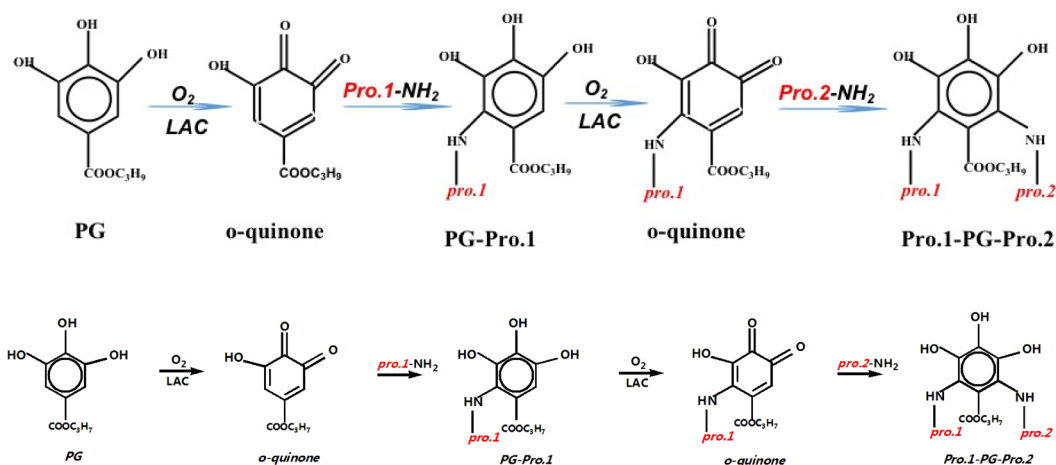
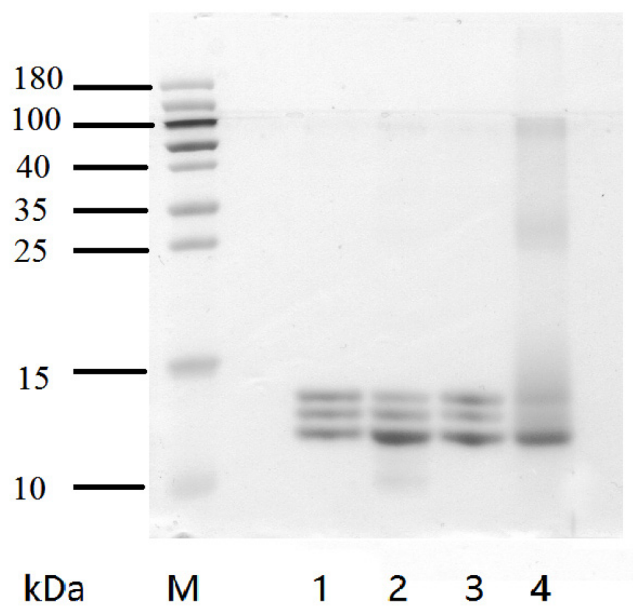
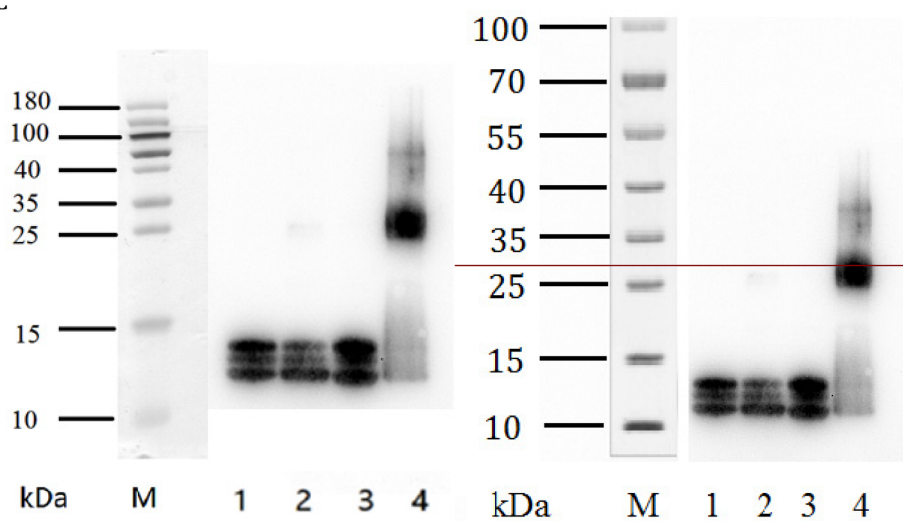


Fig. 1. (A) Reactions catalyzed by LAC and/or PG. (A-1) cross-linking of protein by LAC through tyrosine residues; (A-2) cross-linking of protein1 and protein2 by LAC in the presence of PG; (B) SDS-PAGE analysis of PV cross-linking by LAC and PG; (C) Western blotting analysis of PV cross-linking by LAC and PG. Lane M: Protein marker; Lanes 1–4 represent non-treated PV, LAC treated PV, PG treated PV, LAC + PG treated PV, respectively. (D) Indirect ELISA analysis of PV cross-linking by LAC and PG. All data are expressed as means $\pm$ standard deviation ($X \pm SD$). Different letters above the columns indicate significant differences ($p < 0.05$).

B



C



D

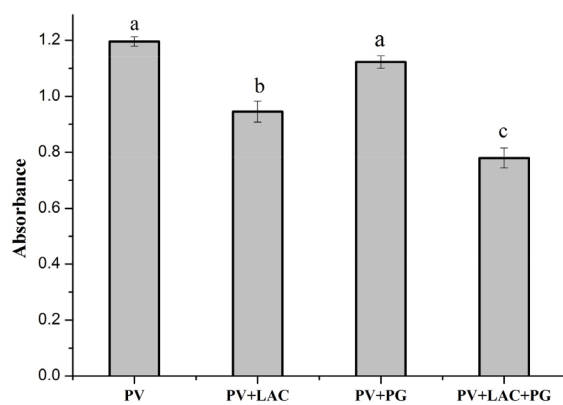


Fig. 1. (continued)

(Song, Li, Gao, Pavase, & Lin, 2016). The cells were passively sensitized with mouse serum IgE. The release of β -hexosaminidase from RBL-2H3 cells were measured with LAC, PG, and LAC + PG treated PV at 37 °C. The release of histamine, prostaglandin D2, cysteinyl leukotriene IL-13 and IL-4 were required for 15 min and investigated using ELISA assay kits according to the manufacturer's instructions.

2.8. Statistical analysis

The obtained data were presented as the mean $\pm$ SD of triplicate determinations, and examined by applying General Linear Model and ANOVA of Duncan's test using the SPSS Statistics 18 software (SPSS Inc., Chicago, USA). Statistical significance was defined at $p < 0.05$.

3. Result

3.1. SDS-PAGE analysis of LAC + PG treated PV

As shown in Fig. 1(B), all the samples were observed after LAC, PG or LAC + PG treatment. *Paralichthys olivaceus* PV has three isoforms and the molecular weight is approximately 12–14 kDa (Fig. 1B, Lane 1). When PV was treated with LAC + PG, only half of PV polymers were noticed at approximately 35 kDa, and higher molecular bands were obtained (Fig. 1B, Lane 4), while the intensity of the PV band at approximately 12–14 kDa become lighter (Fig. 1B, Lane 4) at the same time. However, only two PV isoforms are cross-linked and third isoform almost remained unchanged. Moreover, no polymers were observed by SDS-PAGE when LAC or PG alone was mixed with fish PV, respectively (Fig. 1B, Lane 2,3).

3.2. IgG binding capacity analysis

The potential allergenicity of LAC and PG treated PV was evaluated using western blotting (Fig. 1C). The IgG binding capacity of the cross-linked of first two PV isoforms treated with LAC + PG are highly recognized, and the new cross-linked bands showed some binding. Moreover, only third isoform was poorly recognized. Further, PV with LAC treatment only resulted in low-extent formation of cross-linking. The IgG binding capacity of LAC + PG treated PV was almost well corresponding to the SDS-PAGE results.

To examined the potential allergenicity of PV with different treatment further, indirect ELISA was performed to evaluate the IgG binding capacity of PV using the same antibodies as used for western blot (Fig. 1D). IgG binding capacity remain significantly unchanged when PV was treated by PG, while IgG binding capacity decreased 20.9% with LAC treatment. Compared with PG or LAC treated PV, IgG binding capacity decreased 34.8% with LAC + PG treatment. The results revealed that LAC + PG treatment caused a significant decrease on the IgG binding capacity of PV.

3.3. Effect of LAC + PG treated PV on conformational structure

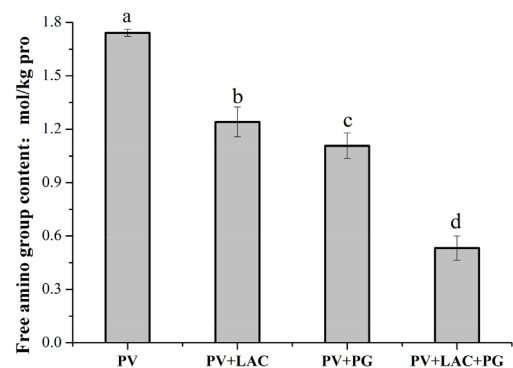
Changes in free amino groups depend on the degree of protein hydrolysis. As shown in Fig. 2A, the content of free amino content of PV was 1.73 mol/kg protein, and there was an obvious decline (28.3% or 31.8%) in free amino groups of PV by LAC or PG treatment. The free amino groups of PV by LAC + PG treatment were 0.58 mol/kg protein. Compared with the native PV, LAC + PG treated PV decreased 66.5%.

Surface hydrophobicity was applied as a probe to determine the changes in the three-dimensional conformational structure of the protein. The observed surface hydrophobicity of PV was 603.36 as shown in Fig. 2B. The surface hydrophobicity of LAC treated PV showed a significant increment from 603.36 to 768.23, and was 27.3% higher

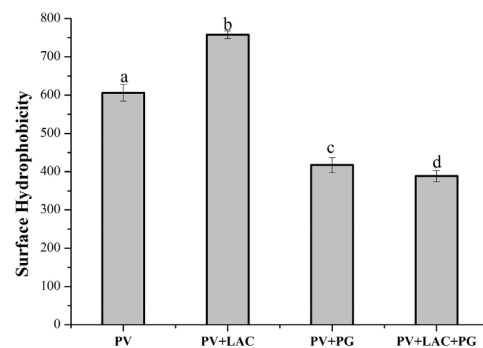
than the non-treated PV, while the surface hydrophobicity of PG treated PV was 428.58, and was 29% lower than the non-treated PV. The surface hydrophobicity of LAC + PG treated PV was 406.69, and was 29.3% lower than the non-treated PV.

The intrinsic fluorescence also represents the structural character. Protein contains aromatic amino acids, such as tryptophan and

A



B



C

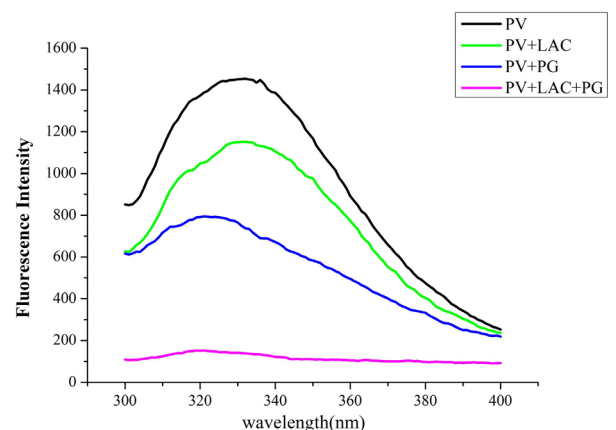
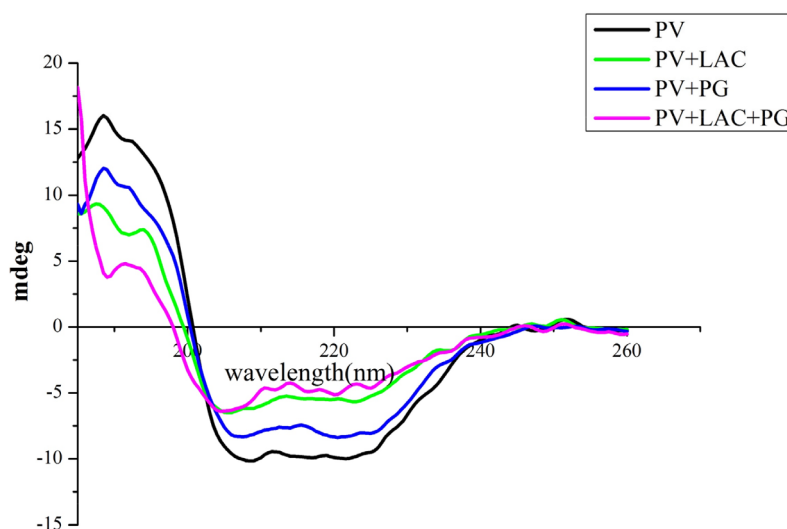


Fig. 2. Effect of LAC and PG on (A) Free amino group, (B) Surface Hydrophobicity, (C) intrinsic fluorescence and (D) CD spectroscopy of PV. The chart shows the proportion of different secondary structure. All data are expressed as means $\pm$ standard deviation ($\bar{x} \pm SD$). Different letters above the columns indicate significant differences ($p < 0.05$).

D



	PV	PV+LAC	PV+PG	PV+LAC+PG
<u>α-helix</u>	<u>31.10%</u>	<u>19.50%</u>	<u>30.70%</u>	<u>16.90%</u>
<u>β-sheet</u>	<u>27.70%</u>	<u>46.90%</u>	<u>21.30%</u>	<u>43.80%</u>
<u>β-turn</u>	<u>10.30%</u>	<u>1.00%</u>	<u>13.70%</u>	<u>3.90%</u>
<u>aperiodical coil</u>	<u>30.90%</u>	<u>32.60%</u>	<u>34.30%</u>	<u>35.40%</u>

Fig. 2. (continued)

tyrosine, which can emit fluorescence. The intensities of LAC and PG treated PV are shown in Fig. 2C. A maximum fluorescence intensity of PV was at 337 nm. Moreover, the intensity decreased and the maximum peak showed a slight blue shift (from 337 to 322 nm) by different treated PV. The lowest intensity (about 87.3% of the native TM) was observed in case of LAC + PG treatment.

CD spectroscopy was used to analyze the secondary structure of protein. The secondary structure comprises of α -helix, β -sheet, β -turn, and random structure (Li, Lin, Limin, & Jamil, 2007). The spectra of LAC and PG treated PV showed a positive peak (~ 196 nm) and two negative peak (~ 212 nm and ~ 223 nm) (Fig. 2D). The negative peak represented α -helix, and the positive peaks represent the β -sheet of protein. The intensity of the positive peaks and the negative peaks steadily decreased when PV was treated by LAC, PG or LAC + PG.

3.4. The stability of cross-linking PV in simulated digestion in vitro

The SDS-PAGE results showed that the band of first isoform in PV began to digest slightly at 1 min. The first isoform was completely digested and produced small fragments (~ 6.5 kDa) by pepsin within 5 min in Fig. 3A-1. Moreover, only second isoform of PV was kept within 15 min and three isoforms of PV were absolutely digested until 30 min. The first isoform of PV with LAC treatment has completely digested and produced small fragments (~ 6.5 kDa) at 15 min. The isoforms of PV

with LAC treatment can still be seen at 60 min (Fig. 3B-1). The PV with PG treatment showed signs of digestion by SGF at 15 min, and only one isoform was kept and produced small fragments (~ 6.5 kDa) at 30 min. The PV with PG treatment was completely digested at 60 min (Fig. 3C-1). LAC + PG treated PV (Fig. 3D-1) can still have bands after digestion at 150 min. However, the bands of first and second isoforms of PV began to digest by trypsin at 1 min, and the third isoform digested at 150 min (3E-1). The first and second isoforms of LAC treated PV completely digested by trypsin and produced small fragments (~ 6.5 kDa, ~ 3.3 kDa) at 1 min, and the small fragments also completely digested at 60 min. The third isoform of LAC treated PV still was kept at 180 min (3F-1). The first and second isoforms of PG treated PV began to digest by trypsin at 1 min, and the third isoform digested at 15 min. The PV was barely visible after digestion at 180 min (3G-1). The isoforms of LAC + PG treated PV began to digest at 1 min (3H-1) and produced small fragments (~ 6.5 kDa). LAC + PG treated PV completely digested at 90 min.

PV-IgG binding capacity was in corresponding to the obtained result of SDS-PAGE with SGF and SIF digestion (Fig. 3A-2, B-2, C-2, D-2). Compared to the native PV, different treated PV exhibited more resistance to digestion by pepsin, and PG and LAC + PG treated PV by trypsin easily was digested. However, no IgG binding capacity was detected for smaller PV fragments (approximately 6.5 and 4.0 kDa).

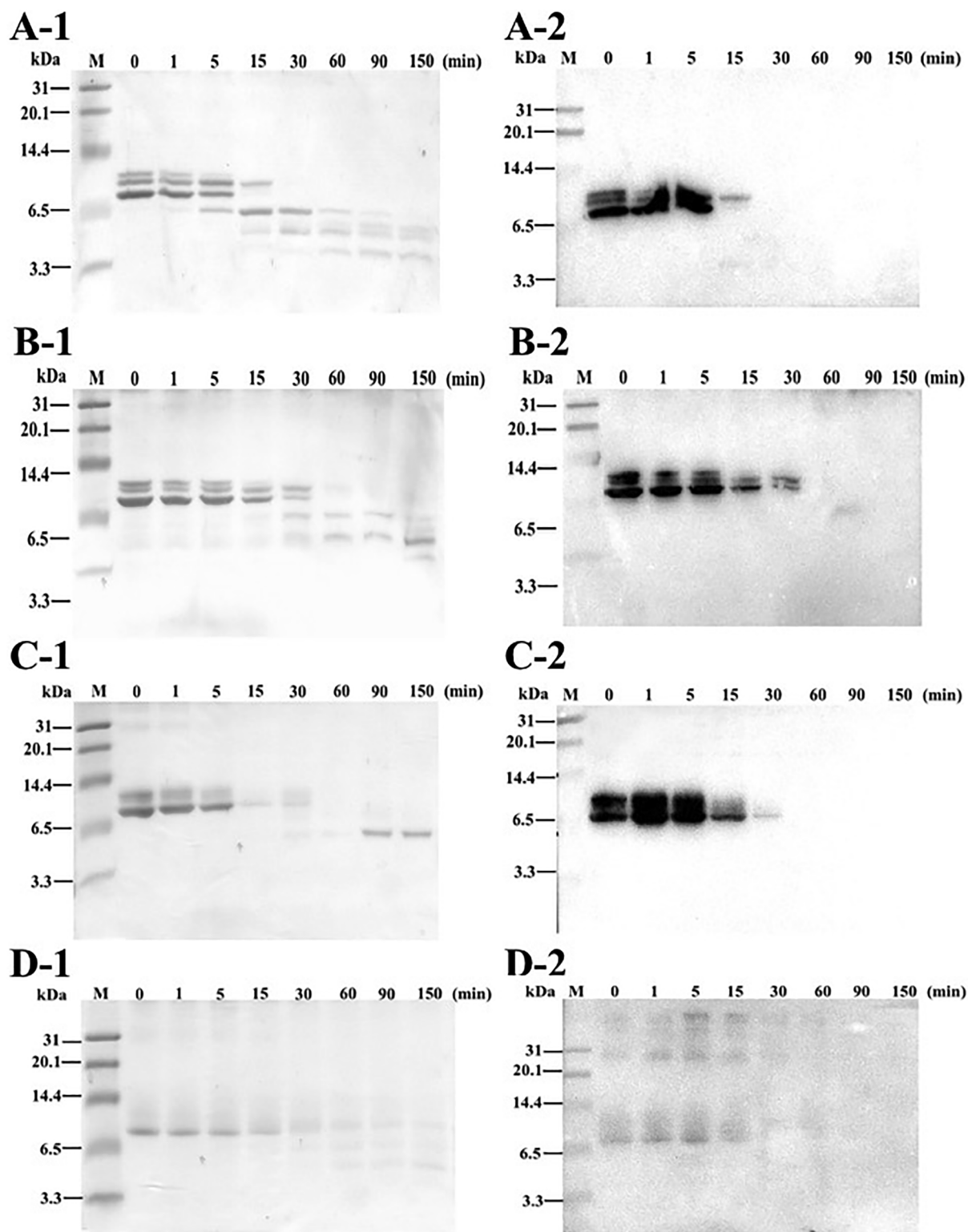


Fig. 3. Effect of simulated gastrointestinal fluid digestion on PV cross-linking by LAC and PG. Enzymatic digestion was performed, followed by SDS-PAGE. (A-1) The fish PV by pepsin; (B-1) LAC treated PV by pepsin; (C-1) PG treated PV by pepsin; (D-1) LAC + PG treated PV by pepsin. (E-1) The fish PV by trypsin; (F-1) LAC treated PV by trypsin; (G-1) PG treated PV by trypsin; (H-1) LAC + PG treated PV by trypsin. Enzymatic digestion was performed, followed by western blot analyses. (A-2) The fish PV, (B-2) LAC treated PV, (C-2) PG treated PV and (D-2) LAC + PG treated PV were detected using polyclonal antisera against shrimp TM with pepsin. (E-2) The fish PV, (F-2) LAC treated PV, (G-2) PG treated PV and (H-2) LAC + PG treated PV were detected using polyclonal antisera against shrimp TM with trypsin. Molecular masses of the protein markers (M) are shown on the left edges.

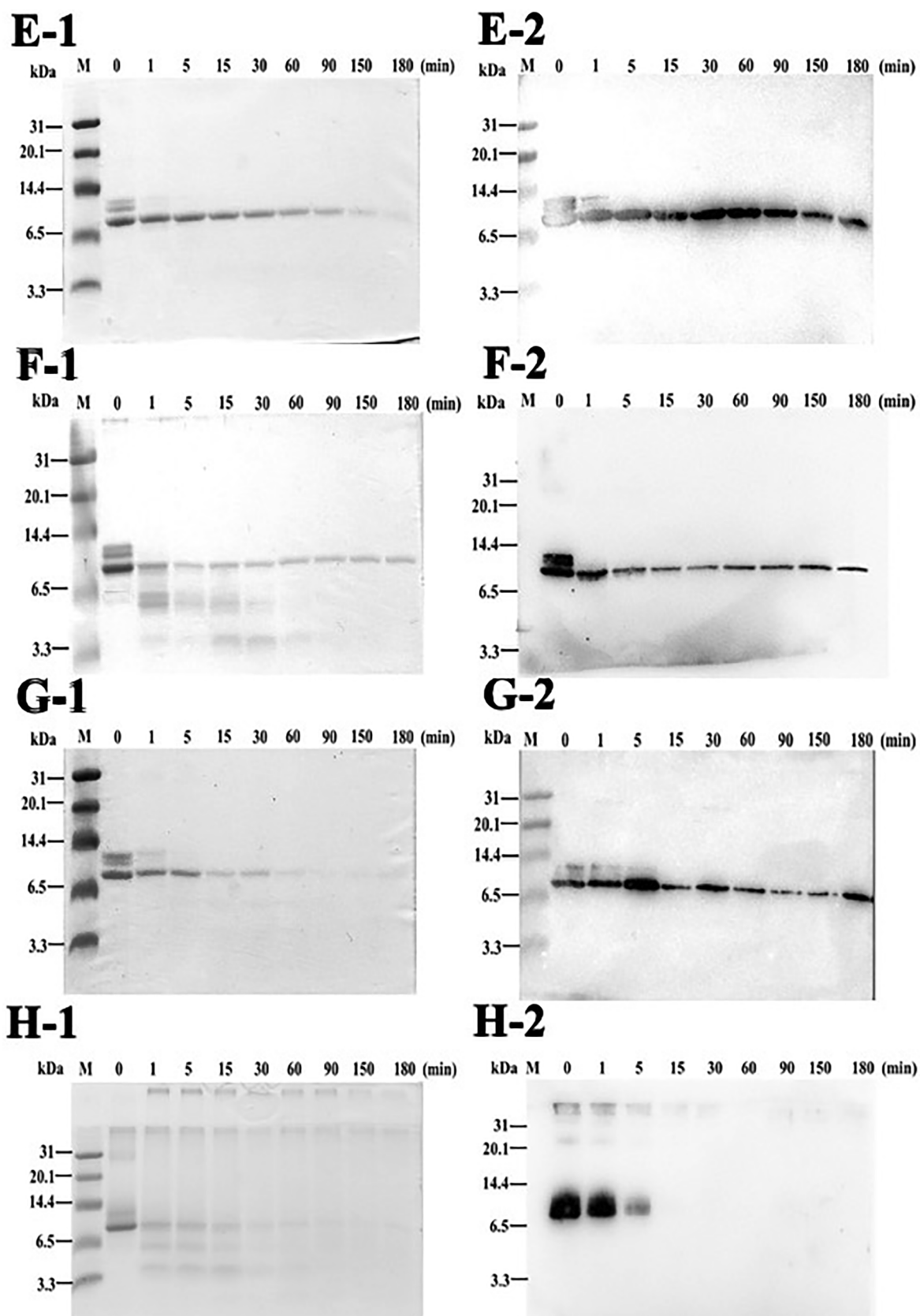


Fig. 3. (continued)

3.5. The impact of cross-linking PV by RBL-2H3 cells

RBL-2H3 cells are commonly used as IgE mediated mast cell model to determine release of β -hexosaminidase, cysteinyl leukotriene, histamine, and prostaglandin D₂, which are shown in Fig. 4. Compared to PV (Fig. 4A), LAC + PG treated PV induced a lower release of β -hexosaminidase (35.6%). In addition, LAC + PG treated PV also decreased histamine release (66.9%) (Fig. 4B), while the releases of cysteinyl leukotriene, tryptase and prostaglandin D₂ also showed a similar reduction (Fig. 4C, D, E).

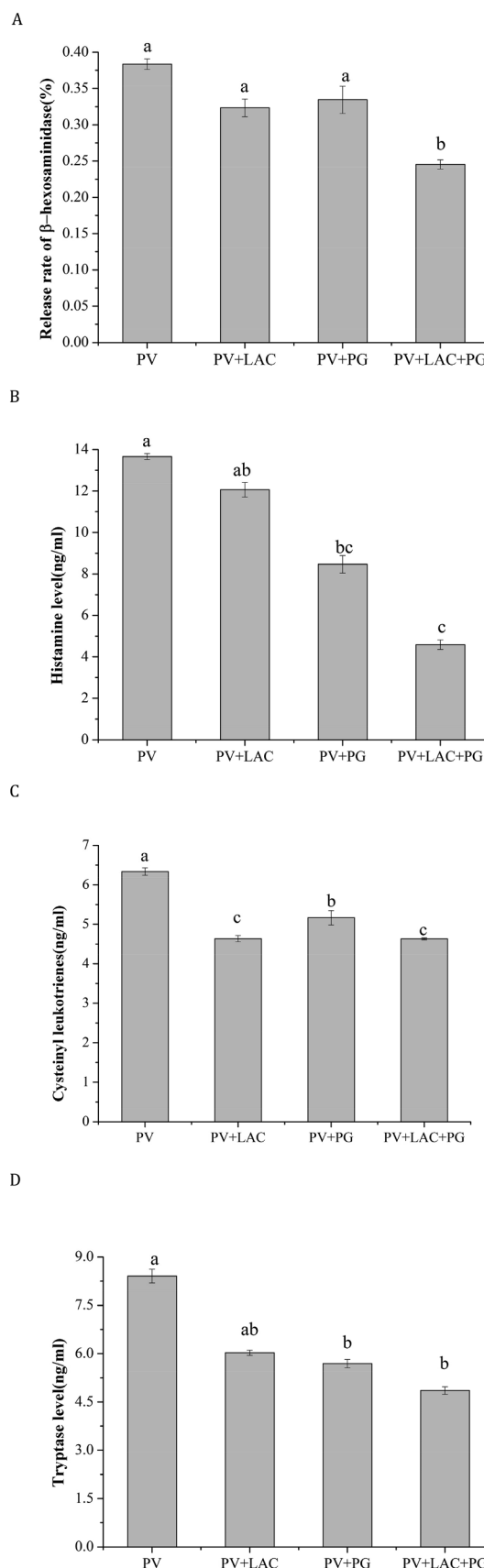
RBL-2H3 is also known to produce cytokines (i.e. IL-4, IL-13), which are known to play a key pathologic role in the onset of various allergic symptoms including IgE-mediated diseases such as atopic rhinitis, asthma and atopic dermatitis. The cytokines were measured to demonstrate the influence of different treatment on fish PV (Fig. 4F, G). IL-4 release showed a significant reduction (56.3%) with LAC + PG treatment ($p < 0.5$), and IL-13 release also reduced (37.5%) with similar trends.

4. Discussion

LAC + PG crosslinking may induce structural changes in *Paralichthys olivaceus* PV that influence the allergenicity. The SDS-PAGE result was accomplished to find the formation of cross-linking following two PV isoforms with LAC + PG treatment. β -mercaptoethanol was mixed with the samples before loading the protein samples into the gel, in order to break the disulfide bonds in PV. Therefore, the bands can only be caused by the cross-linking of PV via non-disulfide linkage. High molecular weight cross-links were formed with LAC + PG treatment, where non-disulphide covalent linkages were responsible for causing cross-linking. The main cause is that PG converted to quinone derivatives and led to the cross-linking of PV, rather than only Tyr residues. Stanic et al. (2010) report that β -casein with similar treatment resulted in cross-linking. But the third isoform almost remained unchanged, which might be that the third isoform didn't contain the free amino group required for the reaction. Moreover, only half of PV was cross-linked and the degree of the cross-linking may be relative to the concentration of LAC, PG, and the reaction time.

Noteworthy, the enzymatic cross-linking can change the IgG binding capacity of PV with LAC + PG treatment. Structural changes are responsible for influencing the IgG binding capacity by the formation of cross-linking. The possible reason is that the alteration of amino acid side chains in PV denatures certain epitopes, which lead in the change of IgG binding capacity. Moreover, the cross-links were recognized by IgG, and the product of cross-linking may still keep certain epitopes. Interestingly, PV with LAC treatment only resulted in low-extent formation of cross-linking. The possible reason is that LAC is cross-linked by the tyrosine of PV, but the content of tyrosine in PV is extremely low. Therefore, the degree of the reaction is low. In addition, indirect ELISA results showed that the IgG binding capacity decrease with different treated PV, especially for LAC + PG treatment. The result revealed that such changes could possibly decrease potential allergenicity of PV.

The reduction of fish PV potential allergenicity is greatly responsible for the decrease of free amine content. Some amino acid residues, for example Lys, contain free amino groups, which are involved in enzymatic cross-linking reactions (LAC + PG treatment) as acyl acceptors and donors, respectively. Hence, such amino acids may be components of the fish PV allergenic epitopes and cross-linking reduced the levels of these free amino groups. The cross-linking can convert ϵ -amino group of the lysine residue, thereby causing the decrease in



(caption on next page)

Fig. 4. Effects of LAC and PG treated PV in IgE-mediated allergic responses in RBL-2H3 cells sensitized with sera from patients allergic to shrimp. A, The release of β -hexosaminidase; B, The release of histamine; C, The release of cysteinyl leukotriene; D, The release of tryptase; E, The release of prostaglandin D2; F, The release of IL-4; G, The release of IL-13. * $p < 0.05$, ** $p < 0.01$. The data represent the mean $\pm$ SD from three independent experiments.

free amino group contents (Liu, Xiong, & Butterfield, 2000) and further affecting its IgG binding capacity. Moreover, PG is relatively unstable and easily oxidized by heat treatment under mild alkaline conditions. Therefore, some oxidation to quinone forms can be expected during its incubation at 37°C at pH 7.4. These quinones can react with amino groups modifying them without cross-linking. In addition, LAC could slightly oxidize PV without the presence of the mediator. Therefore, the amino groups of PV decrease with treatment of LAC only and PG only.

Surface hydrophobicity and intrinsic fluorescence analysis were used to assess the structural changes of different treated PV samples. Different treatment could lead to the protein partial unfolding or aggregation, which changed the exposure of hydrophobic groups. The detected surface hydrophobicity could be instigated by the equilibrium between aggregation via hydrophobic interactions and the exposure of hydrophobic groups. In addition, the decrease of surface hydrophobicity in LAC + PG treated PV in the present study could be attributed to the formation of hydrophilic groups (i.e., protein carbonyls groups), protein aggregation by hydrophobic interactions and structural changes (the phenolic hydroxyl structure introduces a hydrophilic group (–OH)) of the exposed hydrophobic residues of fish PV. It is in agreement with the findings of phenolic oxidation reported by Ou, Kwok, and Kang (2004). This indicates that the quaternary and tertiary structures of PV were modified via different treatment (Lv et al., 2016; Williams, Badley, Davis, Puijk, & Meloen, 1998). The intrinsic fluorescence intensity of different treated PV decrease, especially for LAC + PG treatment. Furthermore, the intrinsic fluorescence peak of PV was at ~ 337 nm, which reveals that aromatic amino acid residues play an important role. LAC could react with tyrosine of PV, which could decrease the content of the aromatic amino acids. Therefore, the intensity of the intrinsic fluorescence decrease. PG is easily oxidized to quinone forms by heat treatment. These quinones can react with amino groups modifying them. The fluorescence of tyrosine and tryptophan are shielded, and the spatial structure of the protein changes, which affects the intensity of the intrinsic fluorescence. Zhu et al. (2015) reported that the react of PG and protein could affect the microenvironment of tryptophan residues, causing fluorescence quenching of protein. A slight blue shift was observed following LAC, PG, and LAC + PG treatment due to the change in the polarity of the aromatic amino acids' microenvironment. Thus, such variations lead to the lower exposure of amino acids to the aqueous environment as a result of the coagulation of protein and modification of specific amino acid residues (King & Li, 1999). The results further demonstrated the reduction of IgG binding capacity with LAC + PG treatment.

Compared to natural PV, far-ultraviolet CD spectra showed that the intensity decreased with LAC, PG or LAC + PG treatment. The result illustrated that the treatment decrease α -helix and β -sheet. Moreover, random coils increased when α -helix decreased after LAC + PG treatment. The main reason is that the amino acid side chains were modified, and the original van der Waals interactions were disrupted, and hence affecting the secondary structure (McClain, Gurnon, & Oakley,

2002). LAC + PG treatment sufficiently demonstrated that the IgG binding capacity of PV decreased by considerably disordering the epitopes.

In this study, the digestibility of different treated PV was examined by simulated gastrointestinal fluids. The digestion of cross-linking PV samples by gastrointestinal fluids were quite different, as shown in Fig. 3. These variations might be caused due to the different cleavage specificity of these two proteases. Pepsin has broad specificity, with only slight preference to hydrophobic, preferably aromatic residues, while trypsin cleaves at Arg and Lys residues (hydrophilic amino acids) at the P1 position (Chen, Barrett, Rawlings, & Woessner, 2004). The protein is easily hydrolyzed when the aromatic amino is located on the surface of the α -protein. LAC is cross-linked with tyrosine in PV, causing the change of the structure. The digestion stability of LAC or PG treated PV by pepsin improved, but the digested products were not recognized by the IgG antibody. The band of LAC + PG treated PV by pepsin also could not be recognized by the IgG antibody in the late digestion stage. Compared to the native PV, different treated PV exhibited more resistance in the SGF. The possible explanation is that the accessible digestion sites were partly altered or masked, which might also have altered the allergic epitopes of PV. However, PV was more resistant to be digested by trypsin compared to different treated PV. The results meant that Arg and Lys might be exposed with LAC, PG and LAC + PG treatment. Hence, such changes might cause the improvement of different treated PV to trypsin digestion. Tao, Liang, Xiao, Liu, and Guan (2016) reported that the digestive stability of PV in the SGF is low, while the digestive stability of PV in the SIF is high in *Cyprinus carpio* and *Hypophthalmichthys molitrix*. Proteases, especially pepsin, could lessen the IgG binding capacity of fish PV effectively. The PV proteolytic fragments (~ 6.5 and 4.0 kDa) showed no IgG binding by SGF and SIF digestion. The main reason was that these digested fragments have denatured IgG binding epitopes after the digestion.

IgE-mediated RBL-2H3 cell assay was conducted for the purpose of evaluating the release of mediators from stimulating cells. RBL-2H3 cells are basically employed as a model for assessing type I allergic reactions to observe the potential of allergens. The present findings specified that LAC + PG treated PV could significantly decrease the release of mediators related to allergic symptoms. These results are similar with the research of arginine kinase, which could decrease degranulation with enzyme cross-linking (Fei et al., 2016). The reason might be that RBL-2H3 cells to the allergen altered IgE epitopes of PV by enzymatic modification, hampering basophils degranulation successfully.

5. Conclusion

This study revealed that LAC + PG induced structure changes of fish PV destroying the binding epitopes, which resulted in the decrease of potential allergenicity. Our findings recommend that LAC + PG may diminish the risk of fish allergy in human, although clinical trials are essential for validation.

Declaration of Competing Interest

None.

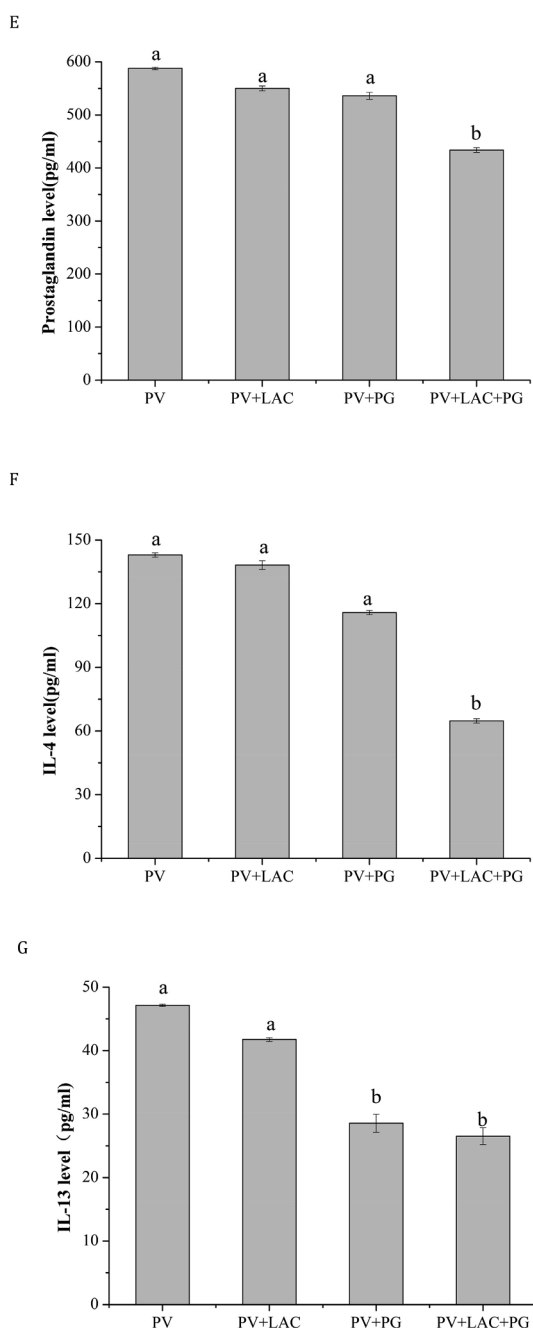


Fig. 4. (continued)

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