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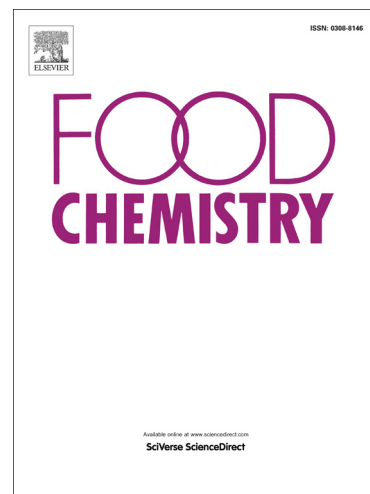
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Running title: Tyrosinase influenced the allergenicity and structure of shrimp
TM

**Effect of tyrosinase-aided crosslinking on the IgE binding potential and
conformational structure of shrimp (*Metapenaeus ensis*) tropomyosin**

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25 Abstract

26 The present study was performed to determine crosslinking and oxidative reactions
27 catalyzed by tyrosinase (Tyr), caffeic acid (CA) and their combination with respect to IgE
28 binding potential and conformational structure of shrimp tropomyosin (TM). Cross-links and
29 IgE binding potentials were analyzed by SDS-PAGE, western blot and indirect ELISA. While
30 structural changes were characterized using surface hydrophobicity, ultraviolet (UV),
31 fluorescence and circular dichroism (CD) spectroscopies. Maximum reduction in the IgG
32 (37.19%) and IgE binding potentials (49.41%) were observed when treated with 2000 nkat/g
33 Tyr + CA, as indicated by ELISA analyses. These findings correlated well with the
34 denaturation of protein, as evident by slight blue shift and alterations in the ellipticities
35 observed via structural analyses. The results demonstrated that addition of CA mediator with
36 Tyr pronouncedly enhanced crosslinking, and altered the conformational structure, thereby
37 mitigated allergenicity of TM, thus showing promise in developing novel food structures with
38 reduced allergenic potential.

39 **Keywords:** Shrimp Tropomyosin, Tyrosinase, Caffeic acid, Cross-linking, IgE-binding,
40 Protein structure

41 1. Introduction

42 Food allergy refers to an anomalous or overstated response of immune system elicited on
43 exposure to specific food proteins or antigens. Food allergic reactions are caused either by
44 antibodies in the blood or cells in the immune system (Waserman & Watson, 2011), and is the
45 most common disorder that has become rather prevalent in the recent past. Over 90% of
46 allergic reactions are associated with IgE that resulted in mucocutaneous, respiratory,
47 gastrointestinal, anaphylactic and cardiovascular (95.7%, 23.9%, 16.3%, 11.9% and 3.3%,
48 respectively) disorders (Takács et al., 2014). New cases of food allergies have been witnessed

49 due to the innovation in food formulation and novel protein, thus posing significant public
 50 health concerns and recurrently lead to anaphylaxis (Clark, Espinola, Rudders, Banerji, &
 51 Camargo, 2011; Wai et al., 2014).

52 Shellfish allergy is an IgE-mediated type 1 allergy that accounts 0.6% prevalence in the
 53 world population and is predominantly common in Asia (Sicherer, 2011). In recent times, its
 54 prevalence is mounting due to the rising trend in shellfish production and consumption.
 55 Tropomyosin is the major allergen identified in shellfish (i.e. *Metapenaeus* and *Penaeus* spp)
 56 based on molecular characterization and is associated with actin filament of muscle cells and
 57 in some non-contractile cells (Albrecht et al., 2008; Wai et al., 2014). Biochemically, TM is a
 58 34-38 kDa protein with a coiled-coiled secondary structure that accounts for 6% of total
 59 shrimp protein. The most plentiful amino acids present in TM epitopes are glutamic acid,
 60 tyrosine, arginine, phenylalanine and serine (Leung et al., 2014). Tropomyosin demonstrates
 61 specific serological IgE reactivity response against shrimp allergic patient sera (Wei et al.,
 62 2014). This protein is considered as the major cross-reactive shellfish panallergen, mainly
 63 because of the high amino acid sequence homology among the crustaceans TM (93.8%) and
 64 mollusks TM (77.2%) (Chu, Wong, & Leung 2000; Leung et al., 2014; Lv, Lin, Li, Ahmed, &
 65 Chen, 2017a; Lv, Lin, Li, Wang, Ahmed, & Chen, 2017b; Reese, Ayuso, & Lehrer, 1999).
 66 Therefore, Met e 1 is an ideal model allergen to be studied for understanding and reducing
 67 shrimp allergy but possibly other shellfish allergies induced by TM.

68 Modifications of protein structures have recently acquired ample importance for
 69 developing food matrices with improved structures, controlled nutrient delivery, reduced
 70 allergenicity and improved shelf-life stability (Ahmed et al., 2017; Feng, Fu, & Yang, 2017a;
 71 Feng, Zhu, Liu, Lai, & Yang, 2017b; Li et al., 2016; Lv et al., 2017a, 2017b; Sow, Peh,
 72 Pekerti, Fu, Bansal, & Yang, 2017; Yuan, Lv, Li, Mi, Chen, & Lin, 2017). The development of
 73 hypoallergenic food eases patient's life by effectively reducing allergic potential of food

products (Li, Lin, Limin, & Jamil, 2007; Lin, Li, Lin, Song, Lv, & Hao, 2015; Nayak, Li, Ahmed, & Lin, 2017). Enzymatic crosslinking of allergic proteins by transglutaminases, peroxisases and polyphenol oxidases (i.e. tyrosinase) have shown promise in reducing allergenicity (Chung, Kato, & Champagne, 2005; Fei et al., 2016; Stanic et al., 2010; Tantoush et al., 2011; Yuan et al., 2017).

Tyrosinase, a copper containing enzyme, is a bi-functional enzyme that catalyzes ortho-hydroxylation of monophenols (cresolase or monophenolase activity) to *o*-diphenols that subsequently oxidized (catecholase or diphenolase activity) to the corresponding *o*-quinones. These can further react non-enzymatically with each other and/or with primary amino or thiol groups, due to their highly reactive nature. It has the potential to oxidize tyrosine residues, thereby led to the formation of covalent tyrosine-lysine, tyrosine-cysteine or tyrosine-tyrosine crosslinks (Selinheimo, Lampila, Mattinen, & Buchert, 2008; Vate & Benjakul, 2016a). Tyrosinase is also capable of reacting with various diphenolic and monophenolic compounds, such as caffeic acid, tannic acid, catechol, phenol and or phloretic acid (Chung et al., 2005; Stanic et al., 2010; Tantoush et al., 2011). However, no works have been reported yet on the influence of Tyr and CA on the allergenicity of shrimp TM (Met e 1). Therefore, the aim of the present study was to elucidate the changes in shrimp TM epitopes, and its potential antigenicity and allergenicity via Tyr-aided crosslinking in the presence or absence of CA.

2. Material and methods

2.1. Materials and reagents

Mushroom Tyr, bovine serum albumin (BSA), 3,3',5, 5'-tetramethylbenzidine (TMB), 3,4-dihydroxy-L-phenylalanine (L-DOPA) and 1-anilino-8-naphthalene-sulfonate (ANS) were acquired from Sigma Chemical Co. (St. Louis, MO, USA). The standard protein used for performing sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was purchased from Solarbio Co. (Beijing, China). While, anti-rabbit IgG and horseradish

peroxidase (HRP)-conjugated goat anti-human IgE antibodies were obtained 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). Purified shrimp TM was acquired from the laboratory of Food Safety, Ocean University of China. All the chemical reagents used were pure and of analytical grade, unless otherwise stated.

2.2. Serum samples

Serum samples were obtained from patients with authenticated clinical history of allergic responses to shrimp from Qingdao Municipal Hospital (Qingdao, China). Specific IgE levels of above 5 kU/l and positive skin prick tests to shrimp, as evident via Allergy Screen (MEDIWISS Analytic GmbH, Moers, Germany). None of the recruited human subjects has any other allergies. All the patient sera were kept at -80°C until analyses. Written consents were obtained from the patients enrolled in the study, who were aware of the usage of their sera for experimental purposes. Furthermore, the Medical Ethics Committee of Qingdao Municipal Hospital (File No, QD-PF-20140314551) certified approval regarding collection procedure, storage and sera usage in order to protect the interest and right of the subjects.

2.3. Tyrosinase assay

The activity of mushroom Tyr was determined following the method described earlier by Yang and Ouyang (2012). Tyrosinase activity was measured spectrophotometrically by measuring dopachrome formation rate at 475 nm using 2.5 mM L-DOPA (Sigma Aldrich, St. Louis, MO, USA) as substrate (pH 6.6) at room temperature. Enzyme activity is expressed in nanokatal (nkat), which is the amount of enzyme needed to convert one nmol of substrate per second in the assay condition.

2.4. Crosslinking of shrimp TM with Tyr

The conditions employed for enzymatic cross-linking reactions were selected based on the previous study reported by Stanic et al. (2010), with some modifications. Shrimp TM was dissolved in 50 mM sodium phosphate buffer, pH 8.0 (2 mg/mL) and incubated with Tyr using five different enzyme dosages: 0 (Control), 20, 100, 400, 1000 and 2000 nkat/g. Caffeic acid (1mM) was added as a mediator in another set of samples along with the aforementioned proportion of Tyr. The reaction mixtures were incubated at 37 °C for 24 h. The vessels of the reaction mixtures were kept open and constant stirring was maintained during the samples incubation for ensuring sufficient aeration. The reactions were terminated after the incubation period by boiling for 10 min. Reference samples (control) lacking enzyme was also run in parallel with the treated samples. All reactions were carried out in duplicates and the crosslinking of TM was monitored via SDS-PAGE analysis. Later on, all the samples were kept at -40 °C until further analyses.

2.5. SDS-PAGE analysis of Tyr crosslinked shrimp TM

Cross-linked and control TM samples were analyzed via SDS-PAGE according to the method depicted by Laemmli (1970). Samples and bromophenol blue buffer (25% glycerol, 2% SDS, 14.4 mM β -mercaptoethanol, and 0.1 % bromphenol blue in 1 M Tris-HCl, pH 6.8) were mixed in a 4:1, respectively. After heating (100 °C for 7 min), the mixtures were loaded into each well of the SDS-PAGE gel (12% resolving gel and 5% stacking gel). Similarly, protein marker having 14.4-116.0 kDa was loaded in the well in order to determine the molecular weight of the cross-linked TM. Electrophoresis were carried out at 80 V for stacking gel and 120 V for resolving gel. After resolving the proteins, gels were stained with 0.125% Coomassie brilliant blue R-250 (Bio-Rad, Richmond, CA, USA) and de-stained to get clear solution. Images were captured and analyzed with gel imaging and analysis software,

146 Tanon-4200SF (Tanon Science & Technology Co., Shanghai, China).

147 2.6. *Effect of Tyr on the antigenicity and allergenicity of shrimp TM*

148 2.6.1. *Western blot analysis*

149 Western blotting was conducted to evaluate the IgG and IgE binding potentials of all the
 150 samples, according to the method described earlier by Lv et al. (2017b), with some
 151 modifications. Firstly, samples were electrophoresed (section 2.5) and transferred the pre-run
 152 gel on to the polyvinylidene fluoride (PVDF) membrane of the stack of the iBlot® 2 Gel
 153 Transfer Device (Life Technologies Co., Israel) and ensured that the electrical contacts were
 154 aligned properly. Then closed the lid and selected the recommended pre-set templates to run
 155 the gel. After that, carefully discarded the gel and placed the transfer membrane in a blocking
 156 solution. Later on, the immunal detections were carried out using iBind™ Western Device
 157 (Life Technologies Co., Israel), which performs all the detection in a sequential process:
 158 blocking, primary antibody, secondary antibody and wash steps. This system contain iBind
 159 device, iBind cards and iBind solution kits and rollers. Initially, we prepared primary (Rabbit
 160 sera, 1:2000 dilution, v/v; or Pooled human sera 1:8 dilution, v/v).and secondary (HRP-
 161 labelled goat anti-rabbit IgG, 1:2000 dilution, v/v; or Anti-human IgE, 1:200 dilution, v/v)
 162 antibodies with the ibind solution to a total volume of 2 mL. Afterward, the blotting
 163 membrane was immersed in 5 mL of iBind solution to prepare the run. Initially, the iBind card
 164 was placed in the device by handling it with the absorbance stacks. Next, pipetted 5 mL of
 165 iBind solution evenly across the flow region of the card followed by placing the membrane on
 166 top of the pooled solution with the protein side down and removed any air bubbles using the
 167 roller. Then closed the iBind device and opened the lid and added solution sequentially to the
 168 iBind valves (from 1-4): loaded diluted primary antibody (2 mL) into valve 1, loaded iBind
 169 solution (2 mL) into valve 2, loaded diluted secondary antibody (2 mL) into valve 3 and

170 finally loaded iBind solution (6 mL) into valve 4. Then closed the valve lid and placed it for
171 incubation at ambient temperature overnight. After incubation, rinse the membrane in water
172 and discarded the used iBind card. The immunoassay was then photographed by Tanon-
173 4200SF gel imaging system with ECL. The intensity of the bands indicated the amount of
174 specific IgG/IgE binding to TM.

175 2.6.2. *IgG/IgE binding capacity analysis*

176 The IgG/IgE binding potentials of treated and untreated samples were further analyzed by
177 indirect enzyme-linked immunosorbent assay (ELISA), using sera from shrimp allergic
178 patients (IgE) and rabbit polyclonal antibodies (IgG), as described earlier (Song, Li, Lin, Du,
179 Hao, Lin, & Zhu, 2015). At first, untreated and treated TM samples were diluted to 10 µg/ml
180 for IgG and 100 µg/ml for IgE in 50 mM sodium carbonate buffer (pH 9.6). A Microlon
181 ELISA plate was coated with 100 µl of diluted samples per well and incubated at a
182 refrigeration temperature overnight. Then, the plate was rinsed with PBST in triplicate,
183 followed by blocking using 1% BSA in PBST at 37 °C for 1.5 h to block the non-specific
184 binding. After blocking step, washing was repeated again in triplicate with PBST (phosphate-
185 buffered saline containing 0.1% Tween-20). The plate was either loaded with 100 µl of pooled
186 patient sera (1:50 dilution with blocking buffer, v/v) or rabbit polyclonal antibodies (1:2000
187 dilution with blocking buffer, v/v) and incubated at 37 °C for 1.5 h. Another 1.5 h incubation
188 at 37 °C with corresponding HRP-labeled goat anti-human IgE (1:1000 dilution with blocking
189 buffer, v/v) or HRP-labeled goat anti-rabbit IgG (1:5000 dilution with blocking buffer, v/v)
190 following the washing step. A substrate solution containing TMB was loaded (100 µl per well)
191 and incubated for 20 min at 37 °C followed by the addition of 2 M sulfuric acid to terminate
192 the reaction. The absorbance was recorded at 450 nm with a microplate reader (Multiscan
193 MK3, Thermo Fisher Scientific Inc., Waltham, MA, USA).

2.7. Structure analysis

2.7.1. UV absorption spectral analysis

The UV absorption spectra of native and Tyr-modified shrimp TM (0.5 mg/ml) were scanned (230-330 nm) at room temperature using UV-1101 system (Techcomp Co. Shanghai, China). Triplicate measurements were carried out in order to ensure reproductive results.

2.7.2. Intrinsic fluorescence analysis

The intrinsic fluorescence of all the samples (0.5 mg/ml) were scanned at a speed of 8 nm/s with a fluorescence spectrophotometer (F-4600, Hitachi, Japan), as described by Lv et al. (2017b). Data were acquired at the excitation and emission wavelengths of 280 nm and 300-400 nm, respectively, using 2.5 nm constant slit width. Rectangular quartz cuvette having 1 cm path-length were used in the experiment. Phosphate buffer (10 mM, pH 7.0) was used as a blank solution for all the samples. Each experiment was repeated three times.

2.7.3. Surface hydrophobicity analysis

The surface hydrophobicity of native and cross-linked TM samples were analyzed by a method adapted from Lin et al. (2015), with ANS used as a fluorescence probe. Samples were diluted in a sequence with phosphate buffer (10 mM, pH 7.0) to final concentrations of 0.01 to 0.5 mg/ml. Each protein samples were allowed to react for 3 min after the addition of 20 μ L of 8.0 mM ANS. Following reaction, F-4600 (Hitachi, Japan) was used to measure the relative fluorescence intensities of the solutions at an excitation wavelength of 370 nm and emission wavelength of 490 nm. The surface hydrophobicity of each sample was measured as the initial slope of the fluorescent curve plot versus protein concentration.

2.7.4. CD spectroscopy analysis

The alterations in secondary structure of shrimp TM were estimated at room temperature within a spectral range of 190-260 nm via a JASCO J-815 CD spectrometer (Japan Spectroscopic Co. Inc., Japan) (Zhong, Tu, Liu, Luo, & Liu, 2015). The samples were diluted to a concentration of 1 mg/ml with distilled water. The diluted samples were scanned at a speed of 100 nm/min using a 1 nm bandwidth at an interval of 2 s. Triplicate measurements were performed and the results were expressed as deg cm² dmol⁻¹ by analyzing with molar residue ellipticity.

2.8. Statistical analysis

One-way analysis of variance (ANOVA) with Tukey test honest significance differences 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.

3. Results and discussions

3.1. SDS-PAGE analysis of Tyr cross-linked TM

The SDS-PAGE profiles of untreated and Tyr/ Tyr + CA treated shrimp TM samples are shown in Fig. 1. The band of native TM (Fig. 1A, lane 1) aligned to the corresponding protein marker of 36 kDa (Lv et al., 2017b). Treatment of TM with various dosages of Tyr (Fig.1A) and Tyr + CA (Fig. 1B) led to diminution in the denseness of native TM bands. New bands with a molecular weight of approximately 72, 108, 140 kDa and above, were most obvious. The decrease in the densities, escorted by the appearance of high molecular weight smears were presumed to be the cross-links. The antibody-binding intensities showed considerable differences among treatments. These smears are supposed to be caused due to oxidation and crosslinking of TM. When oxidative enzymes oxidize the tyrosine residues of purified allergens, high molecular weight smears are formed and the bands of purified allergen

decreased in consequence (Chung et al., 2005; Fei et al., 2016). The association of tyrosine in the creation of new bands was justified using a monoclonal antibody against dityrosine (Chung et al., 2005). The intensity of the high molecular weight smears became denser, as the TM was treated with Tyr + CA (Fig. 1B). An intermediate high molecular mass smears were also noticed in Fig. 1B, lane 7 and 8, which were not obvious in Fig. 1A, lane 2. The possible reason is that CA might converted to quinone derivatives and led to cross-linking of purified TM, rather than only tyrosine residues. Chung et al. (2005) observed that treatments of peanut allergens (Ara h 1 and Ara h 2) by oxidative enzyme (PPO) and CA resulted in the formation of crosslinks, even if tyrosine residues were not available, confirming the formation of quinone derivatives. Fei et al. (2016) also observed that treating crab allergen, arginine kinase, with Tyr/CA increased the polymerization and caused extensive crosslinking with the dominant molecular weight above 116 kDa. Phenolic oxidation products could induce non-disulfide covalent interaction in protein, thereby creating new crosslinks (Ou, Wang, Tang, Huang, & Jackson, 2005; Vate & Benjakul, 2016a, 2016b).

3.2. Antigenicity and allergenicity analyses of Tyr cross-linked TM

The effect of Tyr-catalyzed crosslinking, in the presence or absence of CA mediator, on antigenicity and potential allergenicity of shrimp TM was determined via western blotting. The IgG binding capacity was evaluated using rabbit polyclonal antibody against shrimp TM (Fig. 2A, B). The denseness of native TM bands declined, followed by the formation of new bands with molecular weight greater than untreated TM, thus indicating cross-links formation (Fig. 2A, B). While, the IgE binding potential of the treated TM was evaluated using pooled shrimp allergic patient's sera (Fig. 2C, D). Similar patterns were witnessed in the band width of native TM and high molecular weight new smears. Tyrosinase treatment resulted in the decrease of IgE-binding band at 36 kDa and crosslinks appeared at ~72 kDa, ~108 kDa and

~140 kDa. With the increase in the dosages of Tyr, the intensities of the high molecular weight bands markedly increased and the intensities of both IgG and IgE binding to native TM decreased. Lv et al. (2016) and Tong et al. (2012) also observed varying IgE binding patterns in pooled allergic patient's sera to shrimp TM and ovotransferrin, respectively. The addition of CA alone also led to the creation of new intermediate smears. However, TM samples treated with lower dosages of Tyr still bind to specific antibodies, exhibiting IgG/IgE binding potentials. These findings are in agreement with the previous results reported on cross-linked dietary proteins (Fei et al., 2016; Lv et al., 2017b; Yuan et al., 2017). Tyrosinase treatment might induced protein unfolding, which subsequently exposed several epitopes on the protein surface, and these epitopes further bound to IgE or IgG antibodies. In consequence of the unfolding of TM epitopes, the tertiary and quaternary structure denatured (Li, Lin, Pawar, Li, 2011; Lv et al., 2016; Williams, Badley, Davis, Puijk, & Meloen, 1998). This might explain the reason behind the reduction in the IgG/IgE binding capacity of Tyr-catalyzed crosslinked shrimp TM. However, western blotting could not exactly figure out whether there were an overall decrease in the IgE binding potentials of the treated samples. Nevertheless, despite the binding of cross-links to IgG and IgE antibodies, the crosslinks might not be as allergenic as the native allergens, because of the modifications of the IgE binding epitopes via enzymatic treatment.

Indirect ELISA was conducted using rabbit antibody and the same pooled serum as used in western blotting, to confirm whether there were indeed an overall reduction in the IgG and IgE binding activities of the cross-linked TM. A significant ($p < 0.05$) reduction in the antigenicity of TM was observed, when the protein was treated with different concentrations of Tyr and Tyr + CA, as shown in figure 3A and 3B, respectively. By treating shrimp TM with different dosages (20, 100, 400, 1000 and 2000 nkat/g) of Tyr, resulted in the attenuation of the antigenic activities by 0.95%, 3.05%, 10.17%, 20.76% and 33.83%, respectively. While,

treatment of TM with different doses of Tyr in conjunction with CA led to the reduction of
 1.68%, 4.21%, 10.80%, 25.94% and 37.19%, respectively. Likewise, the effect of Tyr
 treatment either alone or in conjunction with CA on the IgE binding capacity of shrimp TM
 are shown in figure 3C and 3D. The original IgE binding potentials of native TM was
 significantly ($p < 0.05$) greater than that of cross-linked TM. Following treatment with
 different doses of Tyr (20, 100, 400, 1000 and 2000 nkat/g), the IgE binding capacity of
 shrimp TM reduced by 5.70%, 22.57%, 25.50%, 33.25% and 43.90%, respectively. On the
 other hand, enzymatically treated TM along with CA diminished the IgE binding potential by
 9.96%, 22.39%, 37.12%, 41.24% and 49.41%, respectively. In the presence of 2000 nkat/g
 Tyr and CA, the maximum reduction (49.41%) in the IgE binding potential was observed,
 among all groups. As observed, the decline in the IgG and IgE binding capacities were more
 obvious when TM was treated with Tyr in the presence of CA mediator. Moreover, TM treated
 with lower dosages of either Tyr alone or Tyr and CA together, exhibited slightly lower
 reduction in IgG and IgE binding potentials. These results are correlated with the rate of
 crosslinking and oxidation of treated protein.

The results indicated that modification of shrimp TM via Tyr and CA treatments caused
 structural alterations, because of the modification of amino acids. These changes subsequently
 influenced the IgG and IgE binding potentials, which agreed to the previous studies on the
 modification of shrimp TM via various treatments (Lin et al., 2015; Song et al., 2015; Yuan et
 al., 2017). Treatment of TM with CA alone also induced very slight reduction in the IgG and
 IgE binding capacities. The efficiency of crosslinking was promoted by the addition of CA,
 which was similar to the previously reported work of Chung et al. (2005). The reduction in
 the immunoreactivity is probably due to cross-linking of the proteins and formation of active
 species, *o*-quinones, or other intermediates, altering the allergens tertiary structure (Garcia,
 Wichers, & Wichers, 2007). Additionally, this suggests that tyrosine residues had been buried

in the interior of Tyr-aided crosslinked TM molecule. Higher modifications induced by increasing the dosages of Tyr, further altered the amino acid side chains of TM, and binding epitopes were likely denatured, thereby reducing antigenicity and allergenicity. Recently, tyrosinase along with polyphenols were used to crosslink proteins for improving the properties of food products (Vate & Benjakul, 2016a, 2016b). Polyphenol oxidase/phenolic compounds treatment shown potential in mitigating allergenic properties of peanut allergens (Chung & Champagne, 2008). The potential allergenicity of crosslinked TM towards the serum IgE from pooled shrimp allergic patient showed changes similar to that observed in case of IgG-binding capacity. Other possible reason for the differences in the binding potentials might be due to the different binding sites of IgG and IgE or the differences in the binding mechanisms between the two assays or that the altered epitopes retain some IgG/IgE binding potentials. Nonetheless, Tyr treatment cannot entirely nullify the antibody recognition of shrimp TM, signifying the presence of some linear epitopes, which cannot possibly be diminished completely by enzyme and CA treatments.

3.3. Effect of Tyr aided crosslinking on conformational structure of shrimp TM

Various analyses are conducted to evaluate the changes in the conformational structure of shrimp TM. The UV absorption spectral analysis is one of the structural characteristic used to evaluate the conformational changes of proteins. The UV absorption spectra of native protein under normal condition are mainly dependent on tyrosine and tryptophan residues in the side chain groups (Tong et al., 2012). The maximum UV absorption spectrum of native TM appeared at 275 nm, specifying that the UV absorption peak of untreated TM predominantly based on tyrosine residues in the side-chain group, as tryptophan is absent in shrimp TM. Treatment of TM with Tyr and Tyr + CA induced reduction in the maximum absorption peaks, as shown in figure 4A and 4B, respectively, indicating alterations in tyrosine residues on the surface of TM. A blue shift was obvious in the UV absorbance peaks in samples treated with

Tyr (from 275 to 267 nm) and Tyr + CA (from 275 to 265 nm), respectively. Besides, the shift in the wavelengths of maximum absorbance peaks, the UV absorption peaks intensified in different samples. The modification of the polarities of amino acids microenvironment was responsible for the blue shift, possibly because of the broken peptide bonds. Changes in the micro-environmental polarities are caused by oxidation and crosslinking, and were responsible for the variation in the UV absorption peaks. The modification of the conformational structure of shrimp TM because of tyrosinase treatment might be the main cause of the reduction in IgG and IgE binding capacities.

The intrinsic fluorescence spectra, similar to UV absorption spectra, was carried out to assess the changes in the conformational structure of protein. Intrinsic fluorescence spectra of native and Tyr-treated TM samples, in the presence and absence of CA mediator, are presented in figure 5. The fluorophores of protein contain tyrosine, tryptophan and phenylalanine residues as a fluorescence emission spectrum and are responsible for the variation in the intrinsic fluorescence spectra (Li, Lin, Pawar, Li, & Li, 2011; Lv et al., 2017b; Lv et al., 2016). The maximum emission spectrum was observed at 316 nm upon excitation at 280 nm. Crosslinking of TM with increasing dosages of Tyr led to gradual reduction in the maximum fluorescence intensities. Furthermore, a very slight blue shift in the maximum emission wavelength (from 316 to 315 nm) was also noticed. The minimum intrinsic fluorescence intensities of shrimp TM reached to about 43.8% and 46.15% compared to control, when the TM was treated with 2000 nkat/g Tyr and 2000 nkat/g Tyr + CA, respectively. Treatment with CA alone also induced a 21.97% reduction in the fluorescence intensity. The decrease in the fluorescence intensities could be attributed to the changes in the microenvironment, in which tyrosine residues were located, due to the crosslinking and oxidation. These changes might also be due to the lower exposure of amino acids to the aqueous environment because of the coagulation of protein and modification of specific

amino acid residues (King & Li, 1999).

Surface hydrophobicity is an important structural characteristic for evaluating conformational changes. Significant ($p < 0.05$) decline in the surface hydrophobicity patterns of TM were observed with the increase of Tyr concentration, and were more intense when treated together with CA. Compared to control, surface hydrophobicities were declined by 3.05%, 11.26%, 21.16%, 35.11% and 49.14%, respectively, when treated with 20, 100, 400, 1000 and 2000 nkat/ g Tyr (Figure 6A). While, the addition of CA as a mediator along with Tyr, enhanced the reduction patterns (5.16%, 17.69%, 31.04%, 40.54%, 56.31% and 66.50%, respectively) of surface hydrophobicity values (Figure 6B). Shrimp TM was denatured as indicated by the changes in the fluorescence and UV absorption spectra. The unfolding of protein might resulted in the exposure of more hydrophobic regions on the protein surface and subsequently led to the modification of tertiary and quaternary structure (Li et al., 2011; Lv et al., 2016; Williams et al., 1998). The reduction in surface hydrophobicity might be attributed to the interaction of enzyme with the hydrophobic groups of the protein. The observed increase in surface hydrophilicity of Tyr-modified TM could be caused due to the formations of hydrophilic aggregates (containing $-\text{NH}_2$ and $-\text{OH}$) and partial unfolding of protein. Previous studies reported that phenolic oxidation products are electrophilic in nature and could induce non-disulfide covalent bonds in protein (Ou et al., 2005; Vate & Benjakul, 2016a, 2016b).

Far-ultraviolet CD spectra was employed to illustrate the influence of Tyr-mediated crosslinking on the secondary structure of shrimp TM. The secondary structure of protein comprises of α -helix, β -sheet, β -turn, and a random structure (Li et al., 2007). The negative peaks represent the presence of the α -helix and the positive peaks represents the β -sheet of protein. As given in Fig. 7, native TM spectra produced a positive peak at 193 nm and two negative peaks at 216 nm and 221 nm, thus specifying that α -helix is the prevailing secondary

structure of TM. These findings are in agreement with the previously reported works (Lv et al., 2017b; Lv et al., 2016; Yuan et al., 2017). By increasing the dosages of Tyr resulted in gradual lowering of the intensities of negative peaks, thereby indicating decrease in the proportions of α -helices in TM.

The changes in the positive and negative ellipticities in the far-UV CD spectra inferred considerable structural denaturation because of the alterations in the tertiary structures in the region of tyrosine residues. Tyrosinase is a bi-functional enzyme, which is capable of oxidizing the side-chains of tyrosine to quinone through L-DOPA intermediates. These quinones are very reactive and subsequently led to formation of melanin (Sánchez-Ferrer, Rodriguez-Lopez, García-Cánovas, & García-Carmona, 1995). Furthermore, treatment of protein with Tyr can polymerize peptides via covalent bond formation between aromatic side chains and cysteine, lysine or other tyrosine residues (Monogioudi, Faccio, Lille, Poutanen, Buchert, & Mattinen, 2011). The amino acid side chains of TM were oxidized and polymerized by treating with Tyr, thus led to the disruption of the original van der Waal bonds and altered the secondary structure. The unfolding of protein led to exposure of antigen epitopes (Williams et al., 1998). Addition of CA act as an enzyme substrate and intensified the crosslinking reaction. Fei et al. (2016) evaluated the influence of Tyr and CA, and found a reduction in the allergenicity of crab allergen because of the modification of the immunological and molecular properties. In conclusion, Tyr-aided crosslinking reduced the IgE binding potentials of TM by denaturing its conformational epitopes, which subsequently altered the tertiary epitopes of proteins, and might cause insolubility and random coiled aggregation.

4. Conclusions

Tyrosinase-aided crosslinking, either alone or in combination with CA, altered the structural features of shrimp TM that interfered with its potential to be recognized by IgG/IgE

antibodies. Such changes subsequently led to reduction of potential antigenicity and allergenicity, thus spotlighting the denaturation of IgE binding epitopes due to the alterations in linear and conformational structures. Tyr-aided crosslinking in conjunction with CA seems very promising in processing food allergens, as it induce site-specific modification under mild conditions, compared to chemical treatments. It is recommended to further elucidate the influence of Tyr and polyphenols on potential allergenicity *in vivo* and also clinical trials are needed for validation. Additionally, the results could possibly be exploited in mitigating other food allergies induced by TM via oxidative enzymes and is a novel approach in developing hypoallergenic food.

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References

- Ahmed, I., Lin, H., Zou, L., Brody, A. L., Li, Z., Qazi, I. M., Pavase, T. R., & Lv, L. (2017). A comprehensive review on the application of active packaging technologies to muscle foods. *Food Control*, 82, 163-178.
- Albrecht, M., Alessandri, S., Conti, A., Reuter, A., Lauer, I., Vieths, S., & Reese, G. (2008). High level expression, purification and physicochemical and immunochemical characterization of recombinant Pen a 1: A major allergen of shrimp. *Molecular Nutrition and Food Research*, 52(S2).
- Chu, K. H., Wong, S. H., & Leung, P. S. (2000). Tropomyosin is the major mollusk allergen: reverse transcriptase polymerase chain reaction, expression and IgE reactivity. *Marine Biotechnology*, 2(5), 499-509.
- Chung, S. Y., & Champagne, E. T. (2008). Using phenolic compounds to reduce the allergenic

properties of peanut extracts and peanut butter slurries. *Journal of Allergy and Clinical Immunology*, 121(2), pp. S249.

Chung, S. Y., Kato, Y., & Champagne, E. T. (2005). Polyphenol oxidase/caffeic acid may reduce the allergenic properties of peanut allergens. *Journal of the Science of Food and Agriculture*, 85(15), 2631-2637.

Clark, S., Espinola, J., Rudders, S. A., Banerji, A., & Camargo, C. A. (2011). Frequency of US emergency department visits for food-related acute allergic reactions. *Journal of Allergy and Clinical Immunology*, 127(3), 682-683.

Fei, D. X., Liu, Q. M., Chen, F., Yang, Y., Chen, Z. W., Cao, M. J., & Liu, G. M. (2016). Assessment of the sensitizing capacity and allergenicity of enzymatic cross-linked arginine kinase, the crab allergen. *Molecular Nutrition and Food Research*, 60(7), 1707-1718.

Feng, X., Fu, C., & Yang, H. (2017a). Gelatin addition improves the nutrient retention, texture and mass transfer of fish balls without altering their nanostructure during boiling. *LWT Food Science and Technology*, 77, 142-151.

Feng, X., Zhu, Y., Liu, Q., Lai, S., & Yang, H. (2017b). Effects of bromelain tenderisation on myofibrillar proteins, texture and flavour of fish balls prepared from golden pomfret. *Food and Bioprocess Technology*, 10(10), 1918-1930.

Garcia, A., Wichers, J. H., & Wichers, H. J. (2007). Decrease of the IgE-binding by Mal d 1, the major apple allergen, by means of polyphenol oxidase and peroxidase treatments. *Food Chemistry*, 103(1), 94-100.

King, A. J., & Li, S. J. (1999). Association of malonaldehyde with rabbit myosin sub fragment 1. *Quality Attributes of Muscle Foods*, 277-286.

Leung, N. Y. H., Wai, C. Y. Y., Shu, S., Wang, J., Kenny, T. P., Chu, K. H., Leung, P. S. C. (2014). Current immunological and molecular biological perspectives on seafood allergy:

a comprehensive review. *Clinical Reviews in Allergy and Immunology*, 46(3), 180-197.

Li, P., Yang, H., Zhu, Y., Wang, Y., Bai, D., Dai, R., Ren, X., Yang, H., & Ma, L. (2016).

Influence of washing and cold storage on lipid and protein oxidation in catfish (*Clarias lazera*) surimi. *Journal of Aquatic Food Product Technology*, 25(6), 790-801.

Li, Z., Lin, H., Limin, C., & Jamil, K. (2007). The influence of gamma irradiation on the allergenicity of shrimp (*Penaeus vannamei*). *Journal of Food Engineering*, 79(3), 945-949.

Li, Z., Lin, H., Pawar, R., Li, Z. X., & Li, M. H. (2011). Mapping IgE binding epitopes of major shrimp (*Penaeus monodon*) allergen with immunoinformatics tools. *Food and Chemical Toxicology*, 49(11), 2954-2960.

Lin, H., Li, Z., Lin, H., Song, Y., Lv, L., & Hao, Z. (2015). Effect of pH shifts on IgE-binding capacity and conformational structure of tropomyosin from short-neck clam (*Ruditapes philippinarum*). *Food Chemistry*, 188, 248-255.

Lv, L., Lin, H., Li, Z., Ahmed, I., & Chen, G. (2017a). Determining the effect of malondialdehyde on the IgE-binding capacity of shrimp tropomyosin upon in vitro digestion. *Journal of the Science of Food and Agriculture*.

Lv, L., Lin, H., Li, Z., Wang, J., Ahmed, I., & Chen, H. (2017b). Changes of structure and IgE binding capacity of shrimp (*Metapenaeus ensis*) tropomyosin followed by acrolein treatment. *Food and Function*, 8(3), 1028-1036.

Lv, L., Lin, H., Li, Z., Yuan, F., Gao, Q., & Ma, J. (2016). Effect of 4-hydroxy-2-nonenal treatment on the IgE binding capacity and structure of shrimp (*Metapenaeus ensis*) tropomyosin. *Food Chemistry*, 212, 313-322.

Monogioudi, E., Faccio, G., Lille, M., Poutanen, K., Buchert, J., & Mattinen, M. L. (2011). Effect of enzymatic cross-linking of β -casein on proteolysis by pepsin. *Food Hydrocolloids*, 25(1), 71-81.

- 488 Nayak, B., Li, Z., Ahmed, I., & Lin, H., (2017). Ultrasound: advances for food processing and
489 preservation. In D. Bermudez-Aguirre (Eds.), Removal of allergens in some food
490 products using ultrasound (pp. 267-292). USA: Academic Press, Elsevier.
- 491 Ou, S., Wang, Y., Tang, S., Huang, C., & Jackson, M. G. (2005). Role of ferulic acid in
492 preparing edible films from soy protein isolate. *Journal of Food Engineering*, 70(2), 205-
493 210.
- 494 Reese, G., Ayuso, R., & Lehrer, S. B. (1999). Tropomyosin: an invertebrate pan allergen.
495 *International Archives of Allergy and Immunology*, 119(4), 247-258.
- 496 Sánchez-Ferrer, Á., Rodríguez-López, J. N., García-Cánovas, F., & García-Carmona, F.
497 (1995). Tyrosinase: a comprehensive review of its mechanism. *Biochimica et Biophysica*
498 *Acta (BBA) Protein Structure and Molecular Enzymology*, 1247(1), 1-11.
- 499 Selinheimo, E., Lampila, P., Mattinen, M. L., & Buchert, J. (2008). Formation of protein
500 oligosaccharide conjugates by laccase and tyrosinase. *Journal of Agricultural and Food*
501 *Chemistry*, 56(9), 3118-3128.
- 502 Sicherer, S. H. (2011). Epidemiology of food allergy. *Journal of Allergy and Clinical*
503 *Immunology*, 127(3), 594-602.
- 504 Song, Y., Li, Z., Lin, H., Du, S., Hao, Z., Lin, H., & Zhu, Z. (2015). Effect of
505 malondialdehyde treatment on the IgE binding capacity and conformational structure of
506 shrimp tropomyosin. *Food Chemistry*, 175, 374-380.
- 507 Sow, L. C., Peh, Y. R., Pekerti, B. N., Fu, C., Bansal, N., & Yang, H. (2017). Nanostructural
508 analysis and textural modification of tilapia fish gelatin affected by gellan and calcium
509 chloride addition. *LWT-Food Science and Technology*, 85, 137-145.
- 510 Stanic, D., Monogioudi, E., Dilek, E., Radosavljevic, J., Atanaskovic-Markovic, M.,
511 Vuckovic, O., Raija, L., Mattinen, M., Buchert, J., & Cirkovic Velickovic, T. (2010).
512 Digestibility and allergenicity assessment of enzymatically crosslinked β -casein.

- 513 *Molecular Nutrition and Food Research*, 54(9), 1273-1284.
- 514 Takács, K., Guillamon, E., Pedrosa, M. M., Cuadrado, C., Burbano, C., Muzquiz, M., Haddad,
515 J., Allaf, K., Maczó, A., Polgár, M., & Gelencsér, É. (2014). Study of the effect of instant
516 controlled pressure drop (DIC) treatment on IgE-reactive legume-protein patterns by
517 electrophoresis and immunoblot. *Food and Agricultural Immunology*, 25(2), 173-185.
- 518 Tantoush, Z., Stanic, D., Stojadinovic, M., Ognjenovic, J., Mihajlovic, L., Atanaskovic
519 Markovic, M., & Velickovic, T. C. (2011). Digestibility and allergenicity of β
520 lactoglobulin following laccase-mediated cross-linking in the presence of sour cherry
521 phenolics. *Food Chemistry*, 125(1), 84-91.
- 522 Tong, P., Gao, J., Chen, H., Li, X., Zhang, Y., Jian, S., Wichers, H., Wu, Z., Yang, A., & Liu, F.
523 (2012). Effect of heat treatment on the potential allergenicity and conformational structure
524 of egg allergen ovotransferrin. *Food Chemistry*, 131(2), 603-610.
- 525 Vate, N. K., & Benjakul, S. (2016a). Combined effect of squid ink tyrosinase and tannic acid
526 on heat induced aggregation of natural actomyosin from sardine. *Food Hydrocolloids*, 56,
527 62-70.
- 528 Vate, N. K., & Benjakul, S. (2016b). Effect of the mixtures of squid ink tyrosinase and tannic
529 acid on properties of sardine surimi gel. *Journal of Food Science and Technology*, 53(1),
530 411-420.
- 531 Wai, C. Y., Leung, N. Y., Ho, M. H., Gershwin, L. J., Shu, S. A., Leung, P. S., & Chu, K. H.
532 (2014). Immunization with hypoallergens of shrimp allergen tropomyosin inhibits shrimp
533 tropomyosin specific IgE reactivity. *PloS one*, 9(11), e111649.
- 534 Wasserman, S., & Watson, W. (2011). Food allergy. *Allergy, Asthma and Clinical*
535 *Immunology*, 7(1), S7.
- 536 Williams, S. C., Badley, R. A., Davis, P. J., Puijk, W. C., & Meloen, R. H. (1998).
537 Identification of epitopes within beta lactoglobulin recognised by polyclonal antibodies

- 538 using phage display and PEPSCAN. *Journal of Immunological Methods*, 213(1), 1–17.
- 539 Yang, D. M., & Ouyang, M. A. (2012). Antioxidant and anti-tyrosinase activity from olea leaf
540 extract depended on seasonal variations and chromatography treatment. *International*
541 *Journal of Organic Chemistry*, 2(04), 391-397.
- 542 Yuan, F., Lv, L., Li, Z., Mi, N., Chen, H., & Lin, H. (2017). Effect of transglutaminase
543 catalyzed glycosylation on the allergenicity and conformational structure of shrimp
544 (*Metapenaeus ensis*) tropomyosin. *Food Chemistry*, 219, 215-222.
- 545 Zhong, J., Tu, Y., Liu, W., Luo, S., & Liu, C. (2015). Comparative study on the effects of
546 nystose and fructofuranosyl nystose in the glycation reaction on the antigenicity and
547 conformation of β -lactoglobulin. *Food Chemistry*, 188, 658-663.
- 548

549 **FIGURE CAPTIONS**

550 **Figure 1.** Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis
551 of tyrosinase (Tyr) and caffeic acid (CA) treated shrimp tropomyosin (TM). The molecular
552 mass of the standard protein marker ranged from 14.4 to 116 kDa. (A) Lane M: Protein
553 marker; Lane 1: shrimp TM (Control); Lane 2: 20 nkat/g Tyr; Lane 3: 100 nkat/g Tyr; Lane 4:
554 400 nkat/g Tyr; Lane 5: 1000 nkat/g Tyr; Lane 6: 2000 nkat/g Tyr. (B) Lane 7: 0.1M CA; Lane
555 8: 20 nkat/g Tyr + 0.1M CA; Lane 9: 100 nkat/g Tyr + 0.1M CA; Lane 10: 400 nkat/g Tyr +
556 0.1M CA; Lane 11: 1000 nkat/g Tyr + 0.1M CA; Lane 12: 2000 nkat/g Tyr + 0.1M CA.

557 **Figure 2.** Western blot analysis of the tyrosinase (Tyr) and caffeic acid (CA) treated shrimp
558 tropomyosin (TM). (A and B) IgG binding potential for detecting changes in the antigenic
559 activity of treated shrimp TM using rabbit polyclonal antibodies. (C and D) IgE binding
560 potential for detecting changes in the antigenic activity of treated shrimp TM using pooled
561 sera from patients allergic to shrimp. Lane M: Protein marker; Lane 1: shrimp TM (Control);
562 Lane 2: 20 nkat/g Tyr; Lane 3: 100 nkat/g Tyr; Lane 4: 400 nkat/g Tyr; Lane 5: 1000 nkat/g
563 Tyr; Lane 6: 2000 nkat/g Tyr. (B) Lane 7: 0.1M CA; Lane 8: 20 nkat/g Tyr + 0.1M CA; Lane
564 9: 100 nkat/g Tyr + 0.1M CA; Lane 10: 400 nkat/g Tyr + 0.1M CA; Lane 11: 1000 nkat/g Tyr
565 + 0.1M CA; Lane 12: 2000 nkat/g Tyr + 0.1M CA.

566 **Figure 3.** Indirect enzyme-linked immunosorbent assay (ELISA) analysis of the tyrosinase
567 (Tyr) and caffeic acid (CA) treated shrimp tropomyosin (TM). (A and B) Investigate specific
568 IgG-binding activity of treated shrimp TM using rabbit polyclonal antibodies. (C and D)
569 Investigate IgE-binding activity of treated shrimp TM using pooled sera from patients allergic
570 to shrimp. (A and C) Cross-linked with different levels (0, 20, 100, 400, 1000 and 2000 nkat/g,
571 respectively) of Tyr. (B and D) Cross-linked with different levels of Tyr and 0.1M CA.
572 Different letters above the columns indicate significant differences ($p < 0.05$) among

treatments, based on $n = 3$ replica each. Error bars represent standard error of the mean.

Figure 4. Structural changes of treated tropomyosin (TM) measured by ultraviolet (UV) absorption spectroscopy. (A) Cross-linked with different levels (0, 20, 100, 400, 1000 and 2000 nkat/g, respectively) of tyrosinase (Tyr). (B) Cross-linked with different levels of Tyr and 0.1M caffeic acid (CA).

Figure 5. Structural changes of treated tropomyosin (TM) measured by intrinsic fluorescence. (A) Cross-linked with different levels (0, 20, 100, 400, 1000 and 2000 nkat/g, respectively) of tyrosinase (Tyr). (B) Cross-linked with different levels of Tyr and 0.1M caffeic acid (CA).

Figure 6. Surface hydrophobicity analyses of treated tropomyosin (TM). (A) Cross-linked with different levels (0, 20, 100, 400, 1000 and 2000 nkat/g, respectively) of tyrosinase (Tyr). (B) Cross-linked with different levels of Tyr and 0.1M caffeic acid (CA). Different letters above the columns indicate significant differences ($p < 0.05$) among treatments, based on $n = 3$ replica each. Error bars represent standard error of the mean.

Fig. 1

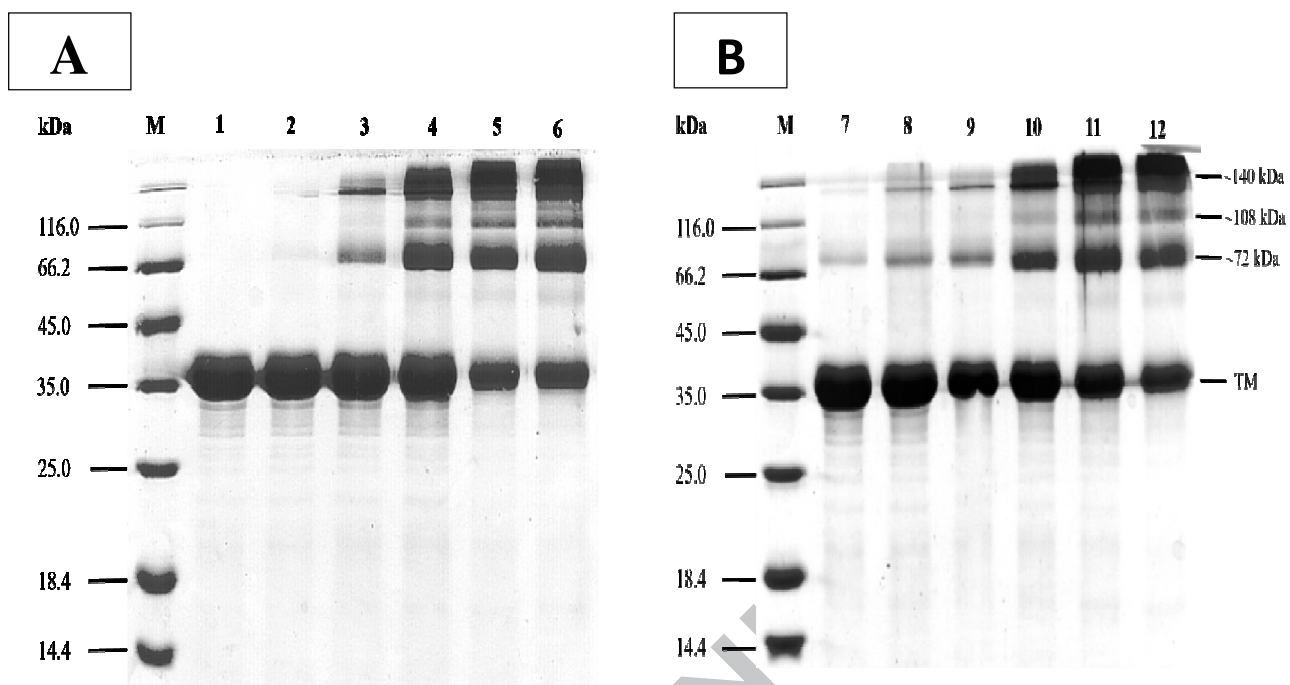


Fig. 2

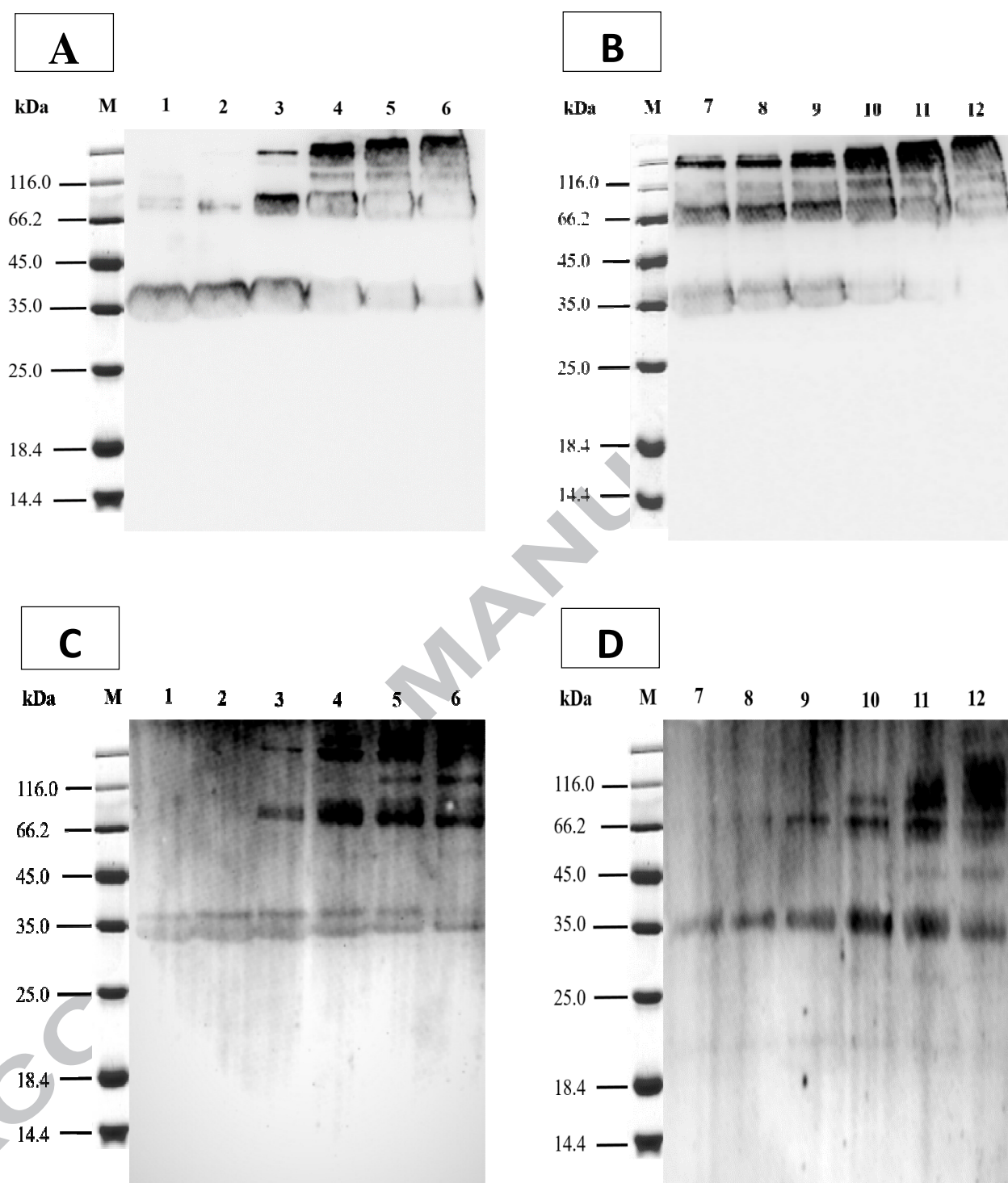


Fig. 3

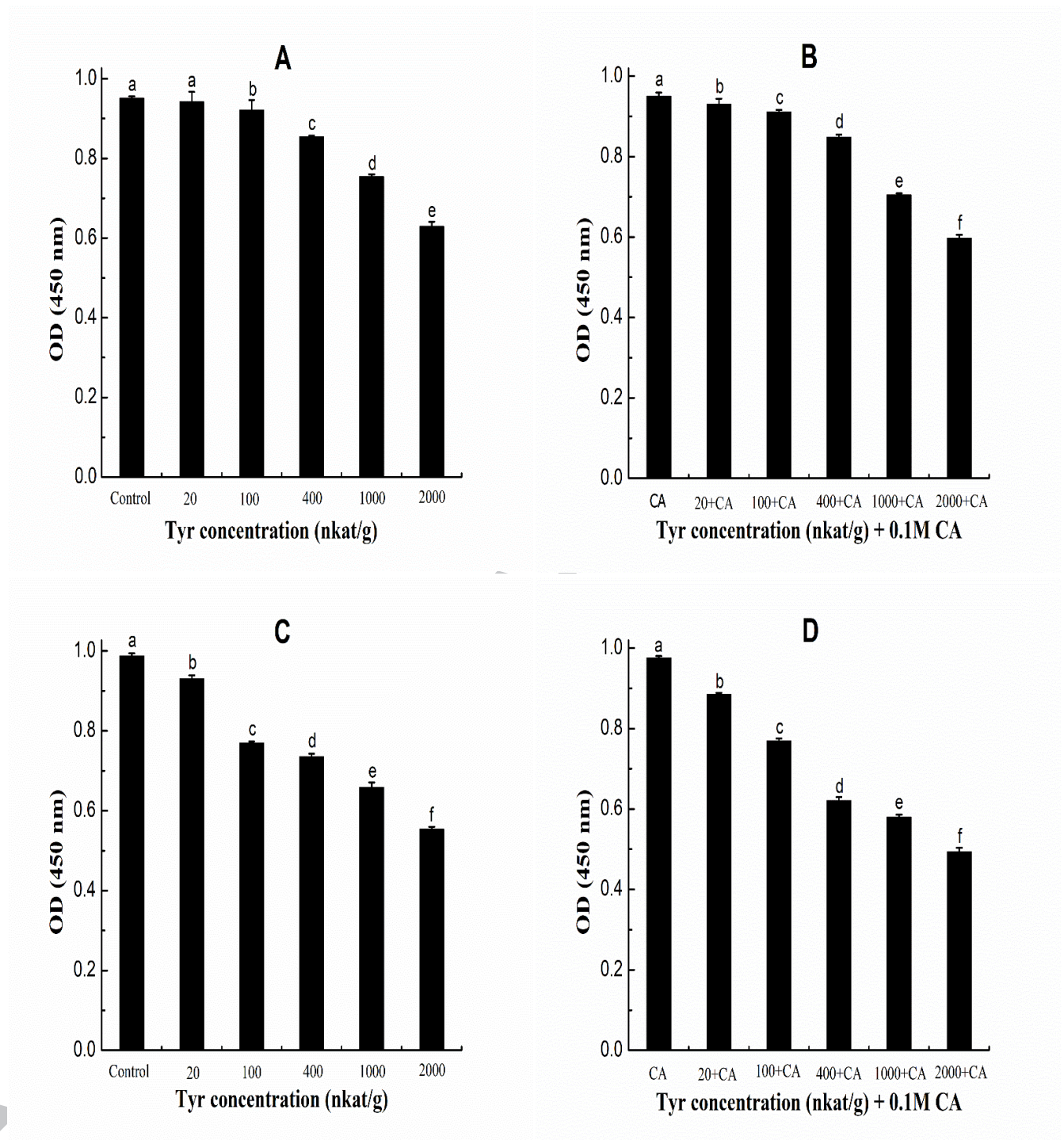
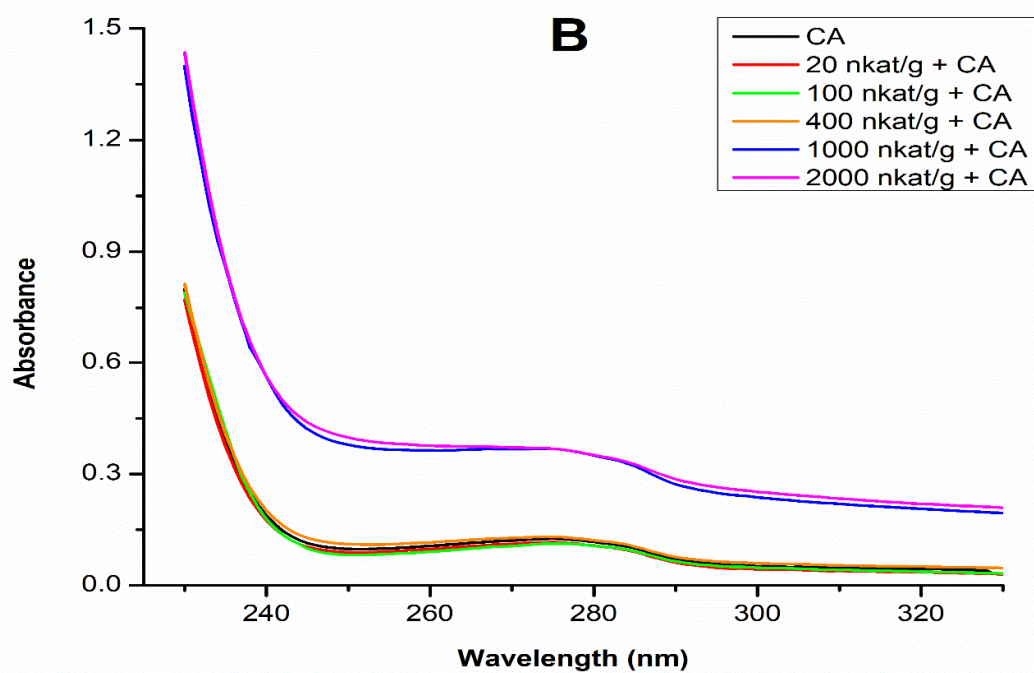
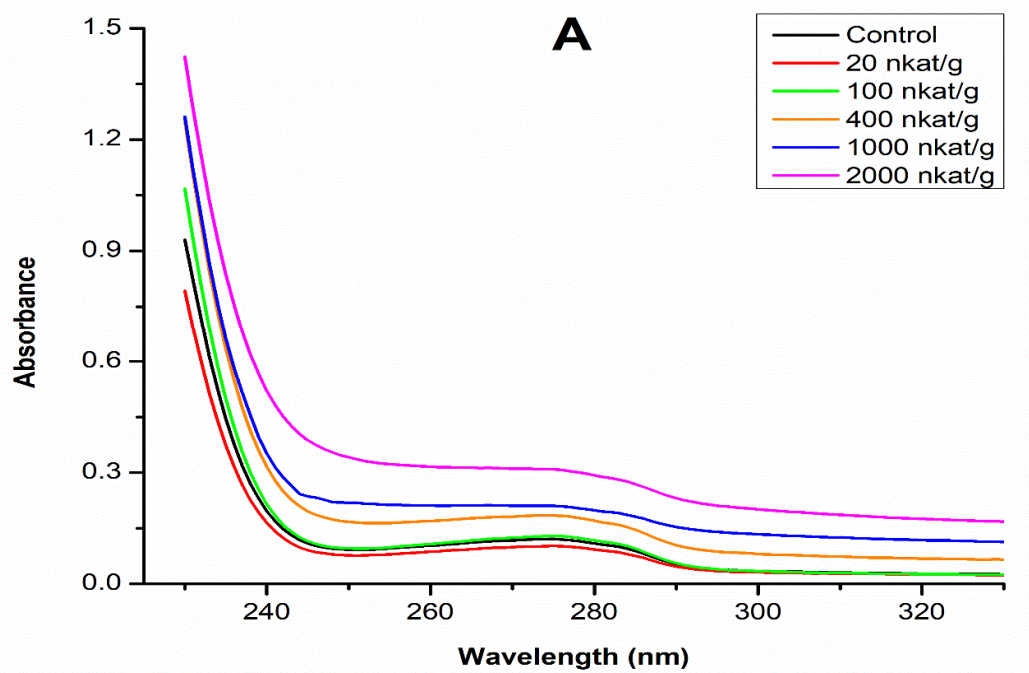
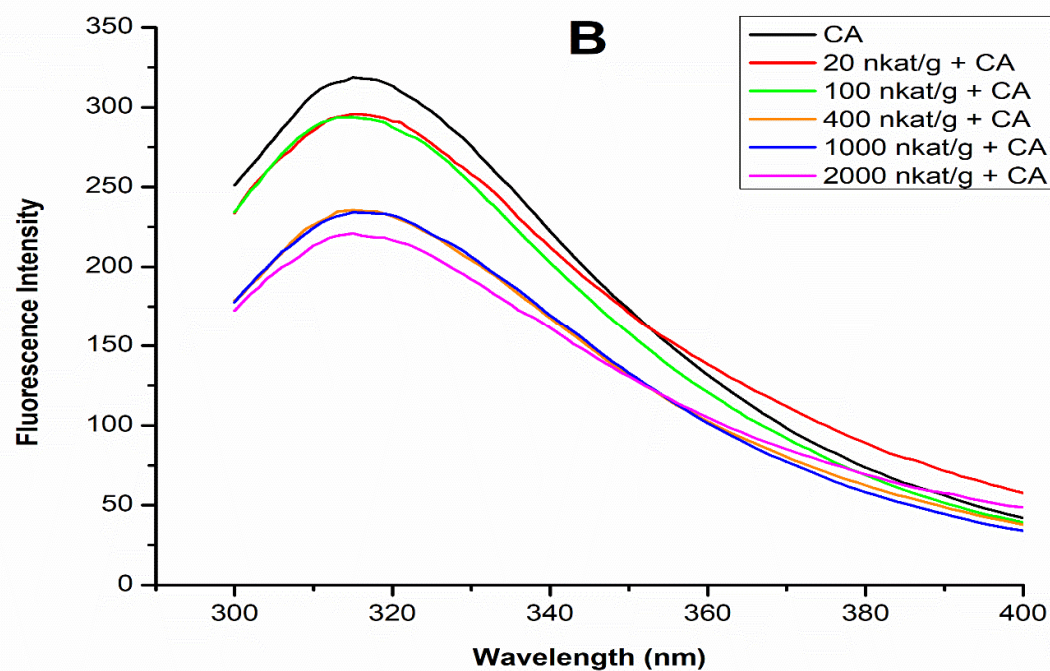
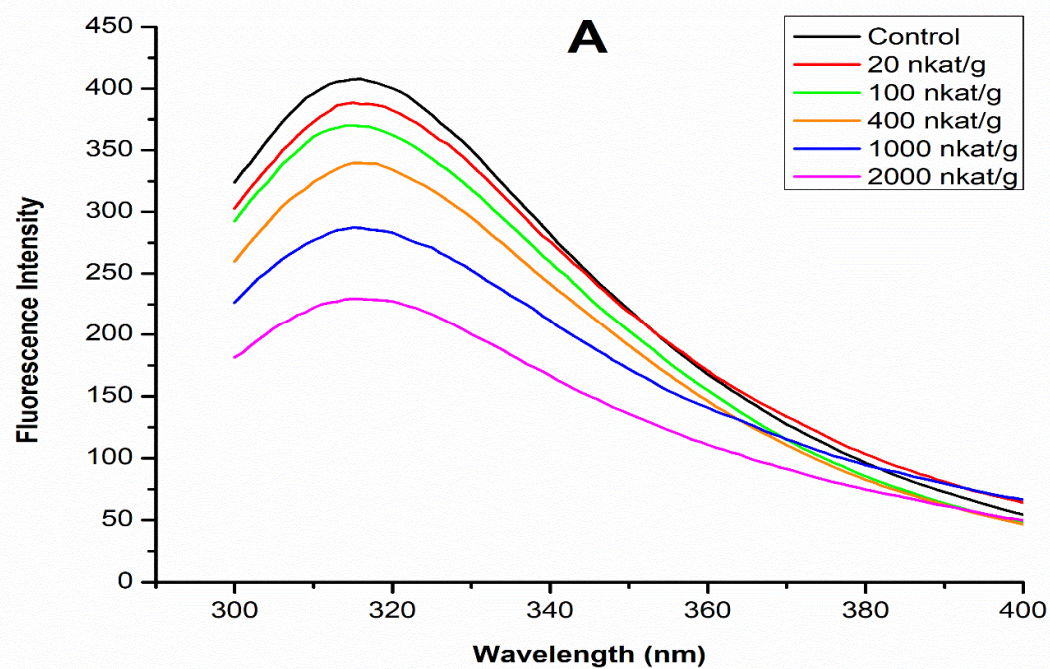


Fig. 4



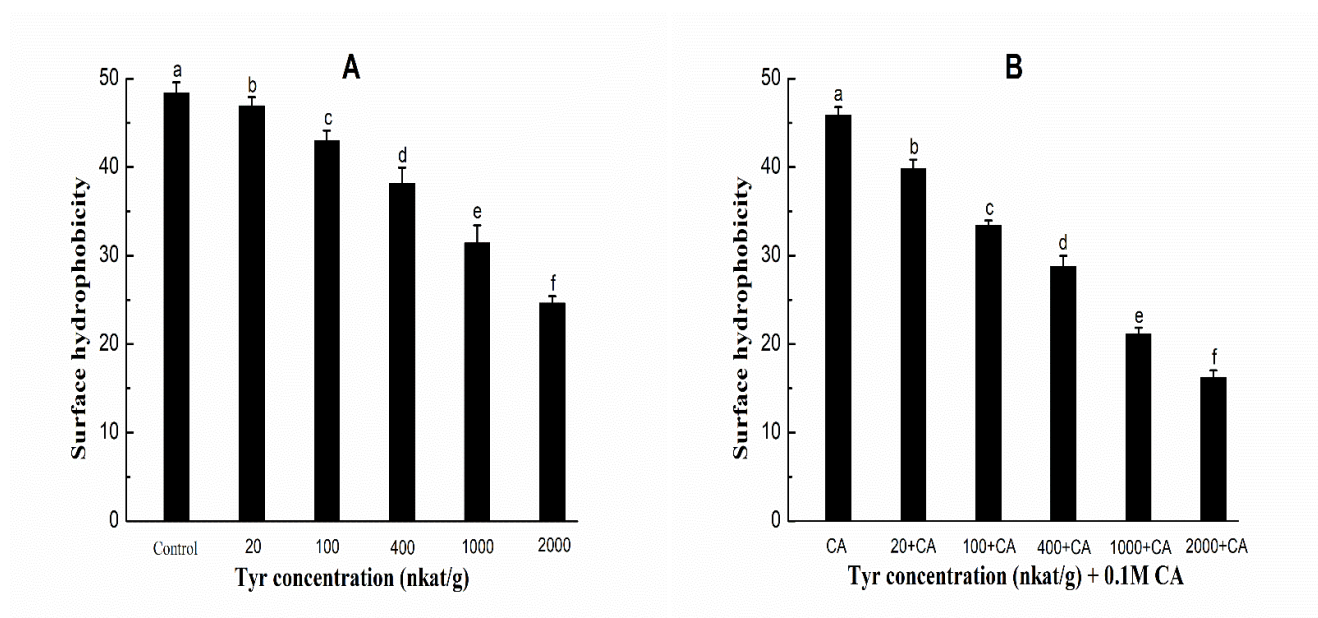
615

616 **Fig. 5**



617

618 **Fig. 6**

**Fig. 7**

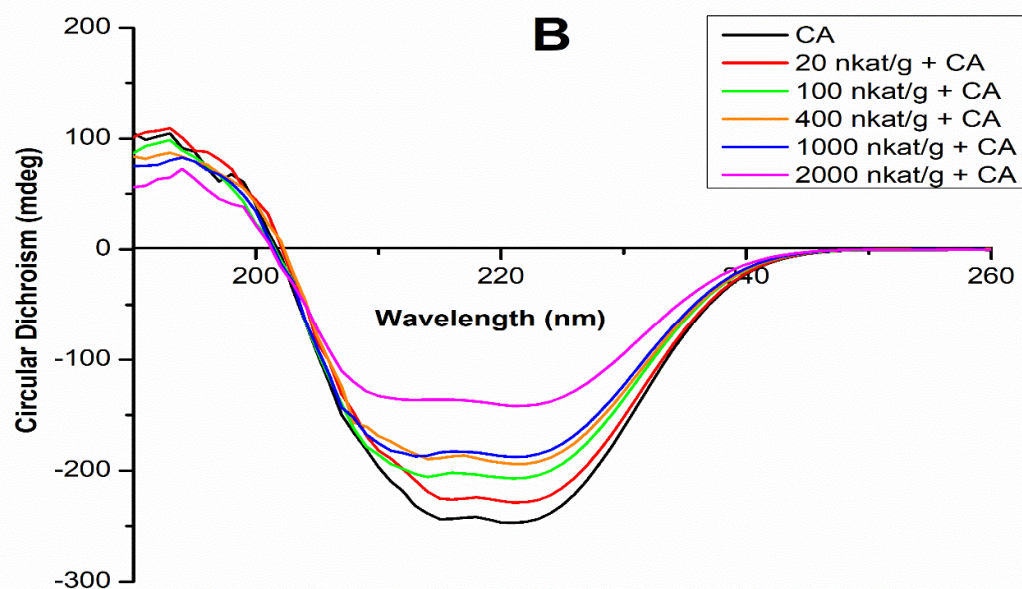
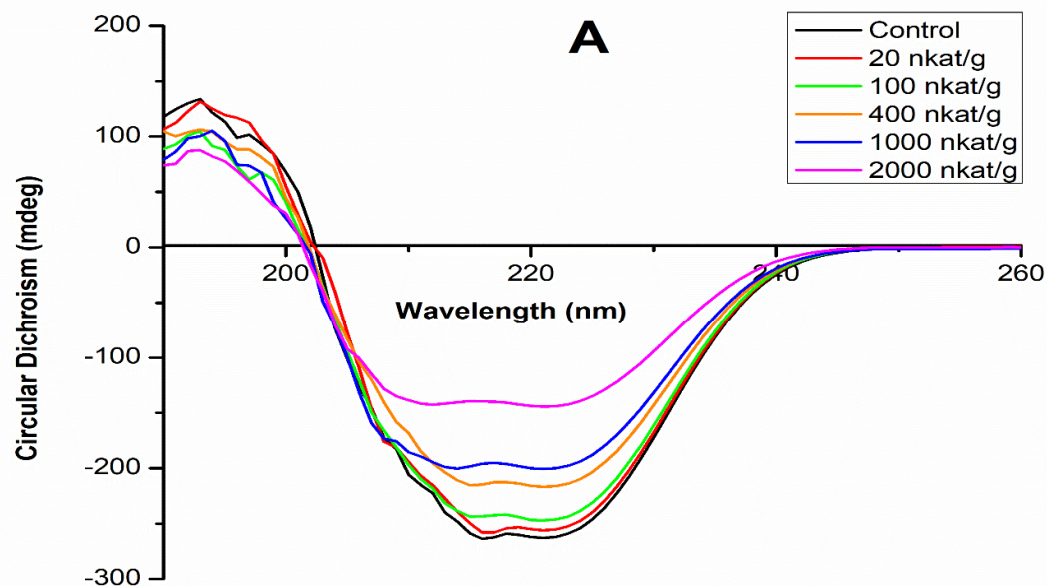


Figure 7. Structural changes of treated tropomyosin (TM), measured by Far-ultraviolet circular dichroism (CD) spectroscopy. (A) Cross-linked with different levels (0, 20, 100, 400,

624 1000 and 2000 nkat/g, respectively) of tyrosinase (Tyr). (B) Cross-linked with different levels
625 of Tyr and 0.1M caffeic acid (CA).

626

627

628 **Highlights:**

- 629 • Treatment of shrimp TM with Tyr and CA led to the formation of crosslinks.
- 630 • Crosslinking and oxidation altered the structure and the IgG/IgE binding levels.
- 631 • Tyr and CA showed effective in mitigating allergenicity caused by shrimp TM.
- 632 • Hypoallergenic food could possibly be tailored via Tyr-catalyzed crosslinking.
- 633