



Thermal induced the structural alterations, increased IgG/IgE binding capacity and reduced immunodetection recovery of tropomyosin from shrimp (*Litopenaeus vannamei*)

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ABSTRACT

Shrimps were first subjected to various thermal processing, then tropomyosin (TM) was purified and their structure, IgG/IgE-binding ability and detectability were evaluated for elucidating the mechanisms responsible for thermal-induced TM immunodetection recovery alterations. According to CD and FT-IR analysis, heat-treated shrimp TM had significantly reduced α -helix and β -sheet contents with elevated random coil contents, contributing to an increase of 24.42%–62.22% in IgG/IgE reactivity as compared with raw shrimp TM. The exposure of hydrophobic residues and glycosylation occurred in various heated shrimps TM were confirmed by UV, intrinsic/extrinsic fluorescence spectrum and free amino group analysis, which caused some epitopes masking or modification, thereby inducing considerable TM recovery reduction (48.48%–90.44%). These results demonstrated that thermal-treated TMs with higher structural flexibility facilitated IgG/IgE recognition, however the lower number of epitopes within the thermal-treated TMs might cause considerable underestimation of recovery. The number of antigen binding sites might play a critical role in sandwich immunodetection.

1. Introduction

Crustacean shellfish play a crucial role in human nutrition and health, and are among the most popular foods consumed worldwide due to the trend towards healthier diets in the recent decades (Khan et al., 2019). Although enjoyed by most, allergic health problems have been reported more frequently in consumers due to the increasing production and consumption of shellfish, reported to affect up to 2.5% of the world's population (Fu et al., 2019; Kamath et al., 2013). Crustacean allergy reaction can be severe with life-threatening anaphylaxis or other harmful symptoms, and the adverse reactions typically persists throughout life in up to 90% of patients (Fu et al., 2019; Ruethers et al., 2018). Despite the adverse effects of crustacean allergy, there is still no specific therapy available. Strict dietary avoidance is the only way to combat the crustacean allergic reactions based on comprehensive and accurate information on food labels.

Currently, many highly sensitive methods such as polymerase chain reaction (PCR) assay and mass spectrometry (MS), have been developed

and employed to monitor the presence of allergens in finished foods, nevertheless, a protein-based immunodetection method, the sandwich enzyme-linked immunosorbent assay (sELISA) is most commonly used because of its high precision, ease of use, and potential for standardization (Fernandes et al., 2015; Sena-Torralba et al., 2020). The sELISA method employs antibodies that are specific with high affinity to detect protein/peptide biomarkers in finished foods that indicate the presence of offending ingredients. However, a major drawback of sELISA method is that detection of denatured or altered proteins tends to be difficult due to their structural alteration after processing compared to native proteins (Marsh et al., 2020; Sena-Torralba et al., 2020). Unlike other proteins in crustacean shellfish, tropomyosin (TM) is highly heat-stable due to its characteristic α -helical coiled coil, and is responsible for 72%–98% of shrimp allergic patients exhibiting a positive IgE-mediated allergic reaction (Khan et al., 2019; Ruethers et al., 2018), thus it is usually regarded as a representative crustacean allergen used in allergen detection to comply with food labeling regulations (Wang et al., 2021; Zhao et al., 2022a).

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Despite the fact that most crustacean foods are processed before consumption, few studies have examined the impact of processing on allergen detection. Thermal processing (e.g., boiling, baking, roasting, frying, grilling, pasteurizing, and sterilizing) is one of the most important and extensively used technique in the household and food industry, that can alter protein structure, reduce the solubility of allergenic proteins, and thus could alter the antigenicity of food allergens (Kamath et al., 2013; Marsh et al., 2020). It is often assumed that thermal processing could reduce allergens' IgG/IgE reactivity, since the structure of proteins could be catastrophically disrupted by various types of heating, such as thermal processing (Ekezie et al., 2018; Liu et al., 2021). Nevertheless, our recent studies suggested that although thermally treated shrimp extracts showed significantly reduced IgG/IgE binding ability, the TM from heat-treated shrimp extracts still possessed higher IgG/IgE activity (Zhao et al., 2022b; Zhao et al., 2022c). Similarly, some available studies indicated that TM from heat treated shrimps might display stronger IgG/IgE reactivity (Kamath et al., 2013; Liu et al., 2010).

Although previous studies have revealed that thermal processing can significantly affect protein quantitation by ELISA test, few have investigated the molecular basis for such an effect (Fu & Maks, 2013; Kamath et al., 2013; Marsh et al., 2020). TM was able to withstand heat and high-pressure processing and maintain its IgG reactivity (Kamath et al., 2013; Ruethers et al., 2018). Nevertheless, the available research implies that the greater underestimation of recovery might happen with TM-based immunochemical detection (Lin et al., 2018; Zhao et al., 2022b; Zhao et al., 2022c). Any changes in the target protein can interfere with its recognition by antibodies and significantly influence the detection results. A better understanding of the underlying causes of common thermal processing on the target protein is critical, both for establishing an appropriate allergen control protocol and mitigating risk to the allergic consumer.

Therefore, in the present study, the effect of common thermal processing, including boiling, baking, autoclaving, steaming, frying, and microwaving, on shrimp (*Litopenaeus vannamei*) structure, IgG/IgE binding capacity as well as immunodetection were investigated. The structural alterations and modifications were examined with circular dichroism (CD), fourier transform-infrared (FT-IR), ultraviolet (UV), intrinsic/extrinsic fluorescence spectra and free amino acid analysis, respectively. Western blotting, indirect ELISA, indirect competitive ELISA (ic-ELISA) and sELISA analysis were employed to evaluate the influence of thermal-processing on IgG/IgE recognition as well as immunodetection. These findings would provide a better understanding of the relationship between TM structural alterations, IgG/IgE binding capacity and its immunodetection, which have tremendous application potential in the developments of immunodetection methods for improved allergen detection in processed foods.

2. Materials and methods

2.1. Materials

Shrimps (*Litopenaeus vannamei*) were purchased from the local market of Qingdao. The preparation of mice and rabbit anti-shrimp TM polyclonal antisera were performed with the help of Hubei Proteingene Technology Co., Ltd. (Wuhan, China). Coomassie brilliant blue R-250, 4 × loading buffer, HRP-labelled rabbit anti-rat, HRP-labelled goat anti-rabbit IgG and 3,3',5,5'-Tetramethylbenzidine (TMB) were purchased from Beijing Solarbio Science & Technology Co., Ltd. (Beijing, China). Horseradish peroxidase (HRP) conjugated goat antihuman IgE antibody was obtained from Zhongshan Jinqiao Biotechnology Co., Ltd (Beijing, China). Ovalbumin (OVA), o-Phthalaldehyde (OPA), 1-anilino-8-naphthalene-sulfonate (ANS) were bought from Sigma Chemical Co. (St. Louis, MO, USA). All chemicals used in the study were of analytical grade.

2.2. Shrimp patient sera

All clinical shrimp-allergic patient sera were obtained through The Affiliated Hospital of Qingdao University (Qingdao, China), which was approved by the Ethics Committee of the Affiliated Hospital of Qingdao University (Qingdao, Shandong, China), and written consent was obtained from all the participants (File No. QD-PF-20200216126). Allergic patient sera were documented by clinical history, and specific IgE levels > 0.35 kUA/L as assessed by the ImmunoCAP system (Phadia AB, Uppsala, Sweden), and the detailed information on serological characterization of shrimp-allergic patients were described in S Table 1.

2.3. Thermal processing of shrimp and purification of TM

Shrimps were first subjected to various thermal treatments including boiling (7 min), oven baking (180 °C for 10 min), autoclave steaming (0.104 MPa at 121 °C for 15 min), steaming (7 min), deep frying with soybean oil (>200 °C for 2 min), microwave heating using an MZ-2011 microwave oven (Haier Group, Qingdao, China) at 700 W for 4 min. Unheated shrimps were used as controls. All samples were treated in triplicate for each processing method.

Raw and heated shrimp TM purification were performed according to the methods described by Xu et al., 2020. All raw and heated shrimps were deshelled and deveined, and shrimp powder was prepared. The raw minced muscle was first homogenized in 10-fold Buffer A (50 mM KCl containing 2 mM NaHCO₃) for 4 h, followed by centrifugation at 4000 rpm for 40 min. The procedure was repeated three times under the same conditions. The shrimp residues were then washed with cold absolute acetone and dried completely at room temperature in a fume hood. For the preparation of heated shrimp powder, the shrimp sample was first crushed with a pulverizer, then the crushed shrimp muscle was washed with cold absolute acetone and dried completely to obtain heated shrimp powder. Then, the shrimp powder was suspended with Buffer B containing 0.02 M Tris-HCl, 1 M KCl and 0.1 mM DTT (1:15 w/v) adjusting the pH to 7.4. The extraction was carried out with stirring overnight. The mixture was then centrifuged at 9000 rpm for 30 min and the extract was fractionated with ammonium sulfate from 20% to 30% saturation. The precipitate was then dissolved in 0.01 M PBS and dialyzed in the same solution for 48 h to obtain raw and heated TM. It was then, freeze dried and stored at −80 °C until use.

2.4. CD spectra analysis

Raw and heated shrimp tropomyosins (TMs) were prepared with PBS to a concentration of 0.2 mg/mL and filtered through a 0.22 μm membrane. Then, CD spectral data from each sample between 190 and 260 nm were collected using a Jasco-810 spectropolarimeter (JASCO, Japan Spectroscopic Co. Inc., Tokyo, Japan) at 25 °C. All samples were scanned three times at a scanning speed of 1200 nm/min using a quartz cuvette with a 1 mm slice thickness.

2.5. FT-IR analysis

The freeze-dried raw and heated shrimp TMs (4.0 mg) were mixed with KBr (100 mg), and then pressed into a pellet. Subsequently, a Nicolet Is10 FTIR spectrometer (Thermo Nicolet Co., USA) was employed to collect the FT-IR spectral data ranging from 400 to 4000 cm^{−1} during 32 scans with 0.09 cm^{−1}. Finally, the data were further analyzed using Omnic 8.2 software (Thermo Fisher Scientific Inc., Madison, WI) and Peakfit 4.12 (Systat Software Inc., San Jose, CA).

2.6. UV absorption spectra measurements

The UV spectra data of raw and heated shrimp TMs (0.2 mg/mL) were measured from 200 to 320 nm at 1.0 nm wavelength interval by using a UV-vis spectrophotometer (UV-4802, Unico Instrument Co., Ltd.

USA) at 25 °C with PBS as the blank.

2.7. Intrinsic and extrinsic fluorescence measurements

The intrinsic and extrinsic fluorescence of raw and heated shrimp TMs (0.2 mg/mL) were determined using an F-4600 spectrofluorometer (Horiba Ltd., Kyoto, Japan). Each sample (2.0 mL) was mixed with 25 µL of 8.0 mM of ANS solution for a 5 min reaction using in extrinsic fluorescence measurements. The changes in tertiary structure were monitored by intrinsic fluorescence emission between 300 and 400 nm with a 280 nm excitation, and ANS fluorescence emission between 400 and 600 nm with a 380 nm excitation. The excitation and emission slit width were set to 5 nm.

2.8. Free amino group analysis

The free amino group contents of each sample were determined by the OPA method (Church et al., 1983). Briefly, 200 µL of each sample (0.2 mg/mL) was mixed with 4 mL of OPA solution and incubated at 25 °C for 2 min. The free amino group content was evaluated at a wavelength of 340 nm using a UV-1101 spectrometer. All the results were expressed as mol kg⁻¹ protein using L-serine as standard.

2.9. SDS-PAGE and western blotting analysis

SDS-PAGE and western blotting analysis were performed according to our recent methods (Zhao et al., 2022b). In SDS-PAGE analysis, protein samples were mixed and boiled with 4 × loading buffer at a 3:1 (v/v) ratio for 7 min. After that, samples (2 µg) were loaded onto SDS-PAGE gels prepared with the AnyKD PAGE kit (ATGene, Chongqing, China), and electrophoresed at 80 V for 30 min at 120 V for 1 h, and then the gels were stained with Coomassie brilliant blue R-250 (Solarbio Co.

mL) per well in a Corning-Costar ELISA microplate (Corning, NY, USA) at 4 °C overnight, and then being washed three times with PBST (10 mM PBS, 0.15 M NaCl, 0.05% Tween 20, pH 7.4). Then, the plate was blocked with 1% (w/v) BSA (200 µL/well) at 37 °C for 2 h. Then the plate was washed again three times with PBST and incubated with 100 µL of mice (diluted 200000) and rabbit antisera (diluted 320000) against TM, and shrimp-allergic patient sera (1:2 to 10 dilution) at 37 °C for 1 h. Next, the plate was washed and the HRP-labelled rabbit anti-mice IgG (100 µL, diluted 1:10000), HRP-labelled goat anti-rabbit IgG (100 µL, diluted to 1:20000) or HRP-labelled goat anti-human IgE (100 µL, diluted 1:3000) was added and incubated for 1 h at 37 °C. The plate was again washed three times with PBST before adding 100 µL/well of the tetramethyl benzidine solution (TMB) to develop the color in the dark for 10 min. Finally, the reaction was stopped by adding sulfuric acid (50 µL, 2.0 M), and the absorbance was evaluated at 450 nm using a MultiscanMK3 microplate reader (Thermo Fisher Scientific Inc., Waltham, MA, USA). Each sample was repeated three times.

2.11. ic-ELISA analysis

For ic-ELISA, microtiter plates were coated with raw TM (5 µg/mL) and blocked, as indicated above. For the assay, appropriate dilutions of mice and rabbit anti-TM antisera were incubated with raw and various heated samples at final concentration of 0.5, 5.0, 50.0 and 500.0 µg/mL. After the mixtures (without positive control mixtures) were incubated for 20 min at 37 °C, they were put into the plates and incubated for 1 h at 37 °C. Plates were then washed with PBST and incubated with a 1:10000 dilution of HRP labelled rabbit anti-mice or goat anti-rabbit IgG for 60 min at 37 °C. The bound HRP was then determined as described for indirect ELISA and the percentage inhibition was calculated using the following formula:

$$\% \text{Inhibition} = 100 - \text{Inhibition} = 100 - \left(\frac{\text{OD}_{450} \text{ of antisera with inhibitor}}{\text{OD}_{450} \text{ of antisera without inhibitor}} \times 100 \right)$$

Beijing, China) or used for the following western blotting assay.

Western blotting was carried out after the above separating gel with resolved proteins was washed in 20% ethanol for 7 min before the proteins were transferred to a PVDF membrane by using the iBlot™ 2 Gel Transfer Device (Thermo Fisher Scientific, MA, USA). After blocking in 5% defat milk and 1% bovine serum albumin (BSA) (w/v) for 2 h at 25 °C, the membrane was incubated with mice antisera (diluted 1:10000 with PBST) and rabbit antisera (diluted 1:20000 with PBST) against TM in an incubator for 2 h. For IgE immunoblotting, different patient sera (diluted 1:10 with PBST) were reacted with the membrane overnight in a Surf-blot apparatus (Bio-Rad, CA, USA) at 4 °C. Then, the membrane was washed three times with PBST for 10 mins. Subsequently, the horseradish peroxidase (HRP)-labelled rabbit anti-mice IgG (1:10000 with PBST), HRP-labelled goat anti-rabbit IgG (1:10000 with PBST) and HRP-labelled goat anti-human IgE (1:2000 with PBST) for 2 h at room temperature. After washing with PBST three times for 10 mins, the results were obtained using multiple exposure time ECL solution to obtain an optimal signal from the BIO RAD Universal Hood II gel imaging system (Bio-Rad, CA, USA).

2.10. Indirect ELISA analysis

The IgG/IgE-binding ability of raw and various heated shrimp TMs were examined using indirect ELISA assay using mice and rabbit antisera against TM, and shrimp patients' sera. Briefly, standard ovalbumin (OVA), unheated and various heated TMs were coated at 100 µL (5 µg/

2.12. sELISA analysis

The immunodetection recovery was determined using sELISA following the recent report of Zhao et al. (2022a). Briefly, plates were coated with mice anti-TM antisera (diluted 1:100000 with CBS) and blocked, as described above. In the assay, TM standards (100–1 ng/mL, 7 concentration levels per curve) or different treated samples with equal concentration (100 µL/well) were added and incubated for 20 min at 37 °C. After washing three times with PBST, 100 µL/well of the rabbit anti-TM antisera (diluted into 1:10000 with PBST) were put for 20 min incubation at 37 °C. Plates were then were washed and incubated with a 1:10000 dilution of HRP-labelled goat anti-rabbit IgG in PBST for 20 min at 37 °C. Then the following determination was performed as described above.

2.13. Statistical analysis

All tests were performed in triplicate, and values were presented as mean ± standard deviation. Analyses of variance (ANOVA) and Duncan's means comparison test was applied with a significance level of 0.05 by using SPSS software (version 25, SPSS Inc. Chicago, IL, USA). Principal component analysis (PCA) was performed using SIMCA-P version 13.0 software package (Umetrics, Umeå, Sweden).

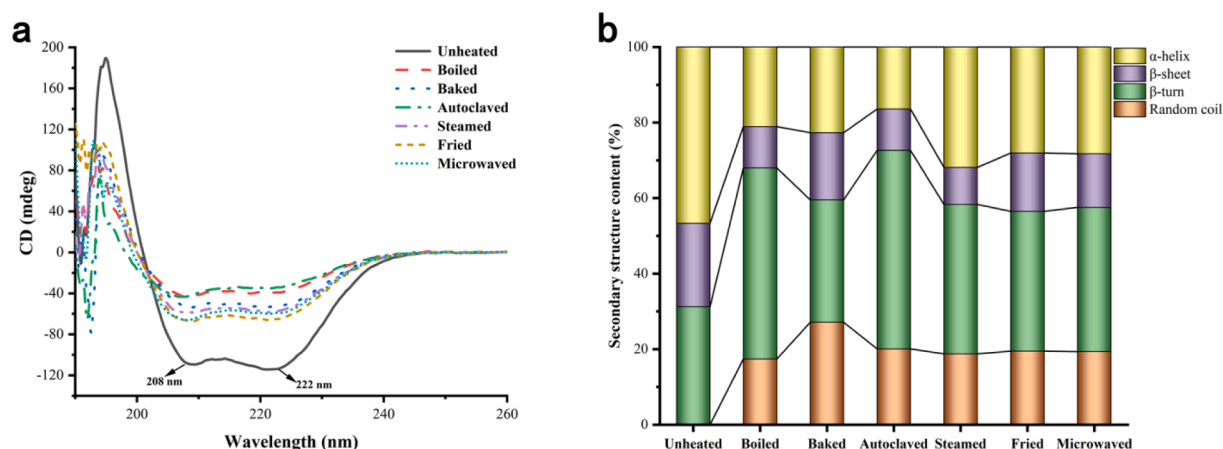


Fig. 1. The analysis of the secondary structure of TM purified from unheated or various heated shrimp. (a) Changes in CD spectra of raw or heated shrimp TM. (b) Secondary structural compositions of raw and unheated shrimp TM determined by FT-IR spectrum.

3. Results and discussions

3.1. Structural change analysis

3.1.1. CD spectra

The secondary structure alterations of raw and various heated

shrimp TMs were analyzed by CD spectrum. As shown in Fig. 1a, two apparent negative peaks at around 208 and 222 nm were observed in the CD curves, which are typical characteristic of the α -helix of protein secondary structure (He et al., 2021). Additionally, a minimum 222 nm is higher than that of at 208 nm, which indicated a coiled-coil structure again corroborating the structure of TMs (Liu et al., 2010). Upon various

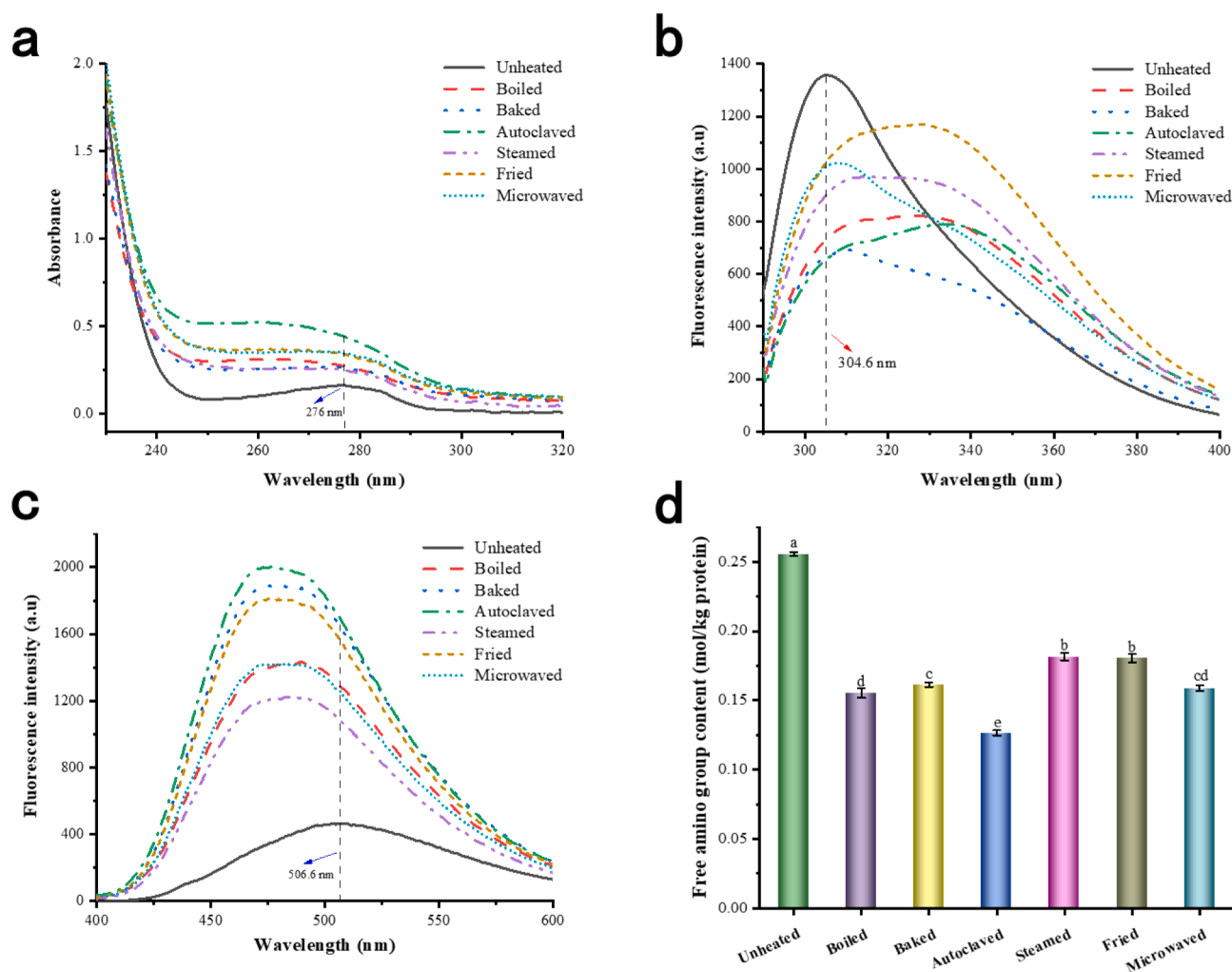


Fig. 2. UV spectra (a), intrinsic fluorescence spectra (b), extrinsic fluorescence spectra (c) and free amino group content (d) analysis of TM purified from raw and heated shrimps. Different lowercase letters in column mean significant differences ($P < 0.05$) among samples with different thermal processing types.

thermal treatments, 208 and 222 nm intensities decreased significantly in contrast to the raw TM, suggested the decreasing in α -helix content of structure induced by the thermal treatment, and the maximal decrease was observed in TM purified from autoclaved. Similar structural alterations were also observed in investigation of thermal-treated shrimp (*Litopenaeus vannamei*) TM at 95 °C (Liu et al., 2010) and purified TM from high pressure treated *Scylla paramamosain* (Liu et al., 2018).

3.1.2. FT-IR spectra

The amide I region (1700–1600 cm^{-1}) was analyzed using deconvolution combined with curve fitting of the amide I region for the calculation of secondary structure content of raw and various heated shrimp TMs (S Fig. 1). Significant differences in secondary structure content based on amide I region were found among the raw and various treated shrimp TMs. As shown in Fig. 1b, compared with the raw shrimp TM, the contents of the α -helix of different treated shrimp TM decreased from 46.68 % (raw), to 21.07% (boiled), 22.69% (boiled), 16.39% (autoclaved), 31.87% (steamed), 28.04% (fried), 28.25% (microwaved), respectively, while the β -turn and random coil contents increased significantly. The above data were in agreement with the CD spectra analysis, and suggested that increased unordered structure contents with unfolding of protein conformation might occur after various heating treatments. Previous research has shown that TM is a dimeric-helical coiled-coil protein along its entire length, and the interchain hydrophobic interactions in terms of the repeating pattern of nonpolar and polar residues in its amino acid sequence play a significant role in the stability of the coiled-coil structure (Hodges et al., 2009; Leesmiller & Helfman, 1991). Thus, the alteration and rearrangement of secondary structure caused by thermal processing, may signify the breakdown of hydrophobic interactions. Besides, the reduction of the helical structure with the increase of β -turn and random coil structure manifested the flexibility enhancement of TM purified from various heated shrimps. The above structural modifications of proteins to form new intra- or inter-molecular cross-linking might occur during thermal processing, which also are responsible for the secondary structure alteration (Fang et al., 2018; Liu et al., 2018). Such structural changes may incur a shift in immunological activity as a result of an altered hydrophobic surface that may carry IgG or IgE-binding epitopes.

3.1.3. UV absorption spectra

Structural changes of protein can be assessed by observing the direction and degree of UV spectra absorption peak, which is indicative of changes to the hydrophilic and hydrophobic environment. The maximum UV absorption peak of TM purified from unheated shrimp was observed at near 276 nm (Fig. 2a). Under various heating treatments, an evident blue shift of the maximum absorption peak from 270 nm (unheated) to approximately 258 ~ 265 nm depending on the degree of heating treatments were observed along with the increase absorption intensity, showing that the microenvironment of amino-acid residues exhibits significant changes as a result of the structural changes due to thermal processing.

3.1.4. Intrinsic fluorescence

Structural changes in the tertiary structure of TM purified from various heated shrimp can also be monitored by intrinsic fluorescence spectra (Fig. 2b). The fluorescence emission spectrum at 280 nm is mainly due to the presence of tyrosine (Tyr) residues in shrimp TM (Lv et al., 2017). The maximum fluorescence emission wavelength (λ_{max}) of raw shrimp TM was found to be about 304.6 nm, which is similar to that reported by Lv et al. (2017) in the study of the structure changes and IgE-binding capacity of shrimp (*Metapenaeus ensis*) TM following acrolein treatment. Additionally, there was a considerable decrease in fluorescence intensity in the maximum emission with an evident red shift in various heated shrimp TM, suggesting that the specific amino acids such as Trp and Tyr, which existed in the hydrophobic environment, might gradually move to the polar environments due to the tertiary structural

alterations during thermal processing (Zhao et al., 2022b).

3.1.5. Extrinsic fluorescence

Extrinsic fluorescence analysis was further carried out to obtain a much deeper understanding of the thermally induced structural changes (Fig. 2c). TM purified from raw shrimp displayed the maximum emission wavelength (λ_{max} 506.6 nm) with minimal ANS fluorescence intensity. Nevertheless, a remarkable increase in the ANS fluorescence intensity with significant blue shift (λ_{max} from 506.6 nm to 479.6 nm) was observed in heated shrimp TM in contrast to raw shrimp TM. This result suggests that the internal hydrophobic residues of heated TM were evidently exposed to the molecular surface because of structural changes (Fang et al., 2018; Liu et al., 2018). Similar blue shifts in λ_{max} with increases in ANS fluorescence intensity was also observed in purified TM from high pressure treated *Scylla paramamosain* crude protein (Liu et al., 2018). Besides, *Cra g 1* (TM) from roasted (10 min, 180 °C) and boiled (100 °C, 10 min) oyster also showed similar increase in ANS fluorescence intensity compared to TM from native oyster (Fang et al., 2018). Combining the results of CD spectra, FT-IR spectra, UV absorption spectra, intrinsic and extrinsic fluorescence spectra, autoclaving treatment caused a considerable increase in hydrophobicity than that of boiling, baking, steaming, frying and microwaving, and the effect appears to be temperature-dependent. More exposure of hydrophobic regions occurred in autoclaved TM might be responsible for the maximal decrease or increase in IgG/IgE-binding capacity.

3.1.6. Free amino group

As compared to TM purified from raw shrimps, different degrees of reduction were observed in free amino group amounts of TM obtained from different treatments of shrimps (Fig. 2d). The maximum reduction was observed in the autoclaved shrimp TM, which was reduced by 50.48%. There are several amino acid residues in TM such as Asp, Gln, and Lys that contain free amino groups. The chemical modifications that occur in foods during processing are mostly caused by the reaction between free amino groups on TM and the oxidized lipids, aldehydes, or ketone groups in reducing sugars (Cabanillas & Novak, 2019; Mills, Sancho, Rigby, Jenkins, & Mackie, 2009). TM with rich lysine residues (29 aa/284 aa), may readily react with reducing sugars during heat-processing, resulting in the Maillard reaction. It might be responsible for the reduction of free amino group from treated shrimp TM. Additionally, according to previous studies (Ayuso et al., 2010; Zheng et al., 2011), seven IgE-binding epitopes on *Litopenaeus vannamei* shrimp TM contain Asp, Gln, and Lys. These amino group losses may affect the IgG/IgE-binding capacity and immunodetection recovery of TM in processed foods.

3.2. IgG binding capacity analysis

In consideration of potential food matrix-associated impacts on the outcome of allergen heating, shrimp were exposed to common thermal treatments, as well as the main allergen TM was isolated from raw and heated shrimp employing our previous methods (Xu et al., 2020). The SDS-PAGE patterns of TM purified from raw and various heated shrimps are shown in Fig. 3a. TM from raw, boiled, baked, autoclaved, steamed, fried and microwaved shrimps displayed a single band at approximately 36 kDa, and the purity was higher than 95% confirmed by Image Lab software. The TM from autoclaved shrimp (121 °C, 0.104 MPa, 15 min) showed a more diffused band in the gel with an increase of molecular weight, signifying the presence of structural modifications in the shrimp matrix during thermal processing. The modification was also confirmed by the reduced free amino group contents. Similar results were also observed in the investigation of TM obtained from *Scylla paramamosain* after high temperature-pressure (115 °C, 0.08 MPa, 15 min) (Liu et al., 2021).

Subsequently, WB analysis, indirect ELISA and ic-ELISA assays were performed to examine the alteration of IgG binding capacity of TM from

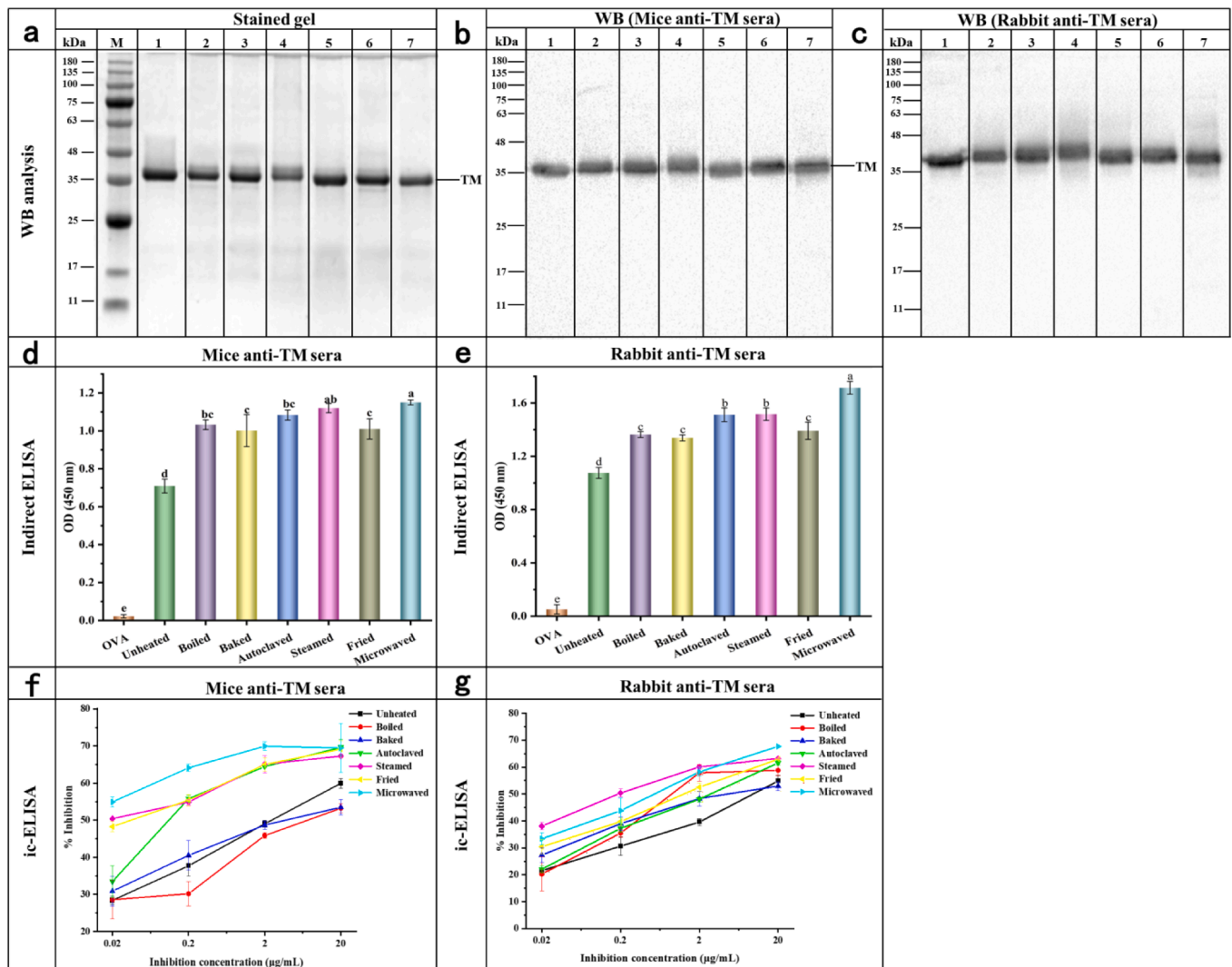


Fig. 3. IgG binding capacity analysis of raw and various heated shrimp TM using WB, indirect ELISA and ic-ELISA. SDS-PAGE analysis (a); IgG WB assays with mice antisera against TM (b) and rabbit antisera against TM (c); IgG indirect ELISA assays with mice antisera against TM (d) and rabbit antisera against TM (e); IgG ic-ELISA assays with mice antisera against TM (f) and rabbit antisera against TM (g). Lane 1–7, raw shrimp TM, boiled shrimp TM, baked shrimp TM, autoclaved shrimp TM, steamed shrimp TM, fried shrimp TM, and microwaved shrimp TM, respectively.

various heated shrimp using mice and rabbit antisera against TM (Fig. 3b–g). Both raw and cooked shrimp exhibited the similar single immunoblot band around 36 kDa with the similar immunoblotting intensities, confirming that conventional thermal processing did not eliminate or reduce TM's IgG reactivity (Fig. 3b and c).

Furthermore, the IgG binding ability of TM from raw and heated shrimp was assessed with indirect ELISA using mice and rabbit antisera against TM. The results are shown in Fig. 3d and e. Compared with raw shrimp TM, the IgG reactivity of various treated TM increased by 24.42%–59.34% and 41.28%–62.20% according to mice and rabbit antisera against TM IgG indirect ELISA, depending on the thermal processing types, suggesting increased IgG binding capacity (Fig. 3d, e). The increased IgG binding capacity also could be confirmed by higher inhibition of IgG binding than the corresponding TM obtained from raw shrimp (Fig. 3f and g). This suggested that TM is highly heat stable and common thermal processing changed the epitopes of TM by unmasking the epitopes with more surfaces accessible, resulting in a significantly increased IgG affinity (Cabanillas & Novak, 2019; Liu et al., 2010). Likewise, Kamath et al. (2013) observed that heating (100 °C, 20 min) boosted monoclonal antibody (mAb) reactivity for TM from crustaceans and several mollusk species. The ability of TM to tolerate common thermal processing methods can be attributed to its remarkably stable

linear alpha helical coiled-coil secondary structure along their entire length (Khan et al., 2019; Ruethers et al., 2018). The typical linear structure tends to have sequential IgG/IgE-binding epitopes, which can retain intact epitopes even under extreme thermal processing conditions. Despite the fact that the heating condition is intense enough to cause structural modification, the denatured forms of TM are easily accessible to bind IgG (Mills et al., 2009; Nowak-Wegrzyn & Flocchi, 2009). The possible reason is that the loss of ordered structure after thermal processing according to CD spectra and FT-IR results, make TM structure looser and higher accessibility. It can promote the antigen–antibody interaction and enhancing IgG binding, thus causing stronger IgG reactivity.

3.3. IgE binding capacity analysis

The IgE binding ability of raw and heated shrimp TM was evaluated with indirect ELISA using 10 shrimp allergic patient sera and 2 normal human sera. The results are shown in Fig. 4a. The raw and various heated shrimp TM presented varying levels IgE binding in all 10 shrimp allergic patients' sera with negligible IgE binding to OVA, and two normal sera displayed a negligible positive signal in the IgE ELISA assay. Overall, TM obtained from various heated shrimps showed significant

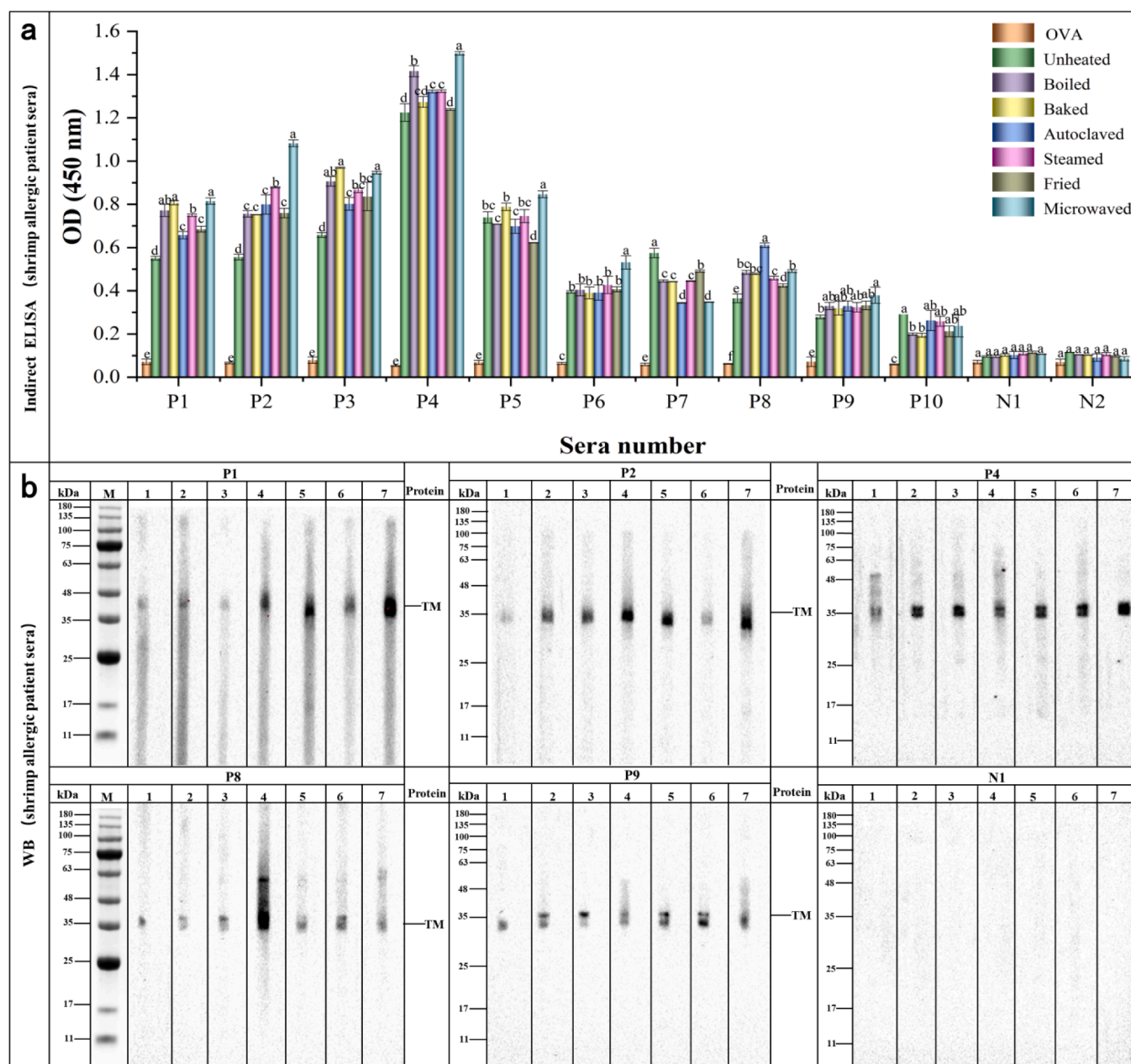


Fig. 4. IgE binding capacity analysis of raw and various heated shrimp TM using indirect ELISA (a) and WB (b) analysis with different shrimp patients' sera. P1-P10, ten different shrimp patients' sera. N1-N2, two non-allergic patients' sera. Different lowercase letters in column mean significant differences ($P < 0.05$) among samples with different thermal processing types.

increase in IgE binding ability in seven out of ten shrimp allergic patient sera test, with the exception of sera P5, P7, and P10. This suggests an increase in IgE reactivity of TM after thermal treatment, and the enhanced antibody binding reactivity are good agreement with above IgG binding capacity analysis results. Varied degrees of increase in IgE binding with different sera might be attributed to the difference of patients' sera, because each patient serum has its own specific IgE binding proteins or epitopes (Lin et al., 2015). Surprisingly, the autoclaved shrimp TM had higher IgE binding compared to raw shrimp TM, especially in serum P8.

Subsequently, six serum samples from P1, P2, P4, P8, P9 (Fig. 4b) and N1 were then further used in IgE WB assays, and the results are illustrated in Fig. 4b. A single immunoblot band around 36 kDa was observed at TM from raw and different heated shrimp with 5 shrimp allergic patient sera, and no specific IgE binding band were found in the

normal human serum for raw and heated shrimp TM. The increased IgE binding from different heated shrimp TM was also confirmed by clearly enhanced immunoblot intensity. The obtained results demonstrated that shrimp TM is difficult to be suppressed by common thermal processing methods, and the extent of antigen-antibody binding required to elicit a reaction is significantly enhanced. Although the extracts from raw shrimp (*Litopenaeus vannamei*) had higher immunoreactivity than the extracts from boiled shrimp, dot-blot assay results showed a higher IgE binding to purified TM from boiled shrimp than raw shrimp (Liu et al., 2010). Abramovitch et al. (2013) also found boiled blue swimmer crab (*Portunus pelagicus*) TM have higher reactivity than the raw crab and prawn TM by immunoblotting, and they demonstrated this may be due to the more common ingestion of cooked crab and prawn. Our results indicated the elevated IgE reactivity to heated shrimp TM might be attributed to: (i) unmasking of IgE epitopes to have more accessible

surfaces as a result of secondary structure changes and more hydrophobic residues exposed (Fang et al., 2018; Liu et al., 2010); (ii) conformational alteration of the epitopes that increased antibody binding inducing changes to the conformational epitopes, thus promoting the antigen–antibody interaction and enhancing IgE reactivity (Albrecht et al., 2009; Kamath et al., 2013); (iii) enhancing and activating protein modification including through the Maillard reaction between free amino acids, and aldehyde or ketone groups of sugars (Ilchmann et al., 2010; Nowak-Węgrzyn & Flocchi, 2009).

3.4. Immunodetection recovery analysis

Reliable immunodetection is critical in appropriate labeling guidelines for consumer strict dietary avoidance, and the sELISA are the most commonly employed to detect allergenic foods (Sena-Torralba et al., 2020). As a result, the heated shrimp TM was tested further using sELISA, and the results are shown in Fig. 5a. In general, heat treatment resulted in a significant lower level of TM detected by the sELISA, but the extent of decrease differed. The maximal underestimation was found in autoclaved shrimp TM with 90.44% reduction in recovery when compared to raw shrimp TM, followed by microwaved (83.96%), steamed (80.60%), boiled (63.45%), baked (53.98%) and fried (48.48%) shrimp TM. These results are of high concern as the widespread usage of shrimp component in processed foods can cause the potential hazard of hidden allergens for allergic consumers, especially the higher IgE reactivity in heat-processed shrimp TMs (Abramovitch et al., 2013; Khan et al., 2019). The depressed recoveries of each allergen following thermal processing also were found in peanut allergen with dry-heat treatment for 10 min at different temperatures (Fu & Maks, 2013), milk allergens in a pastry dough food matrix subjected to boiling (100 °C for 2 min), baking (190 °C for 30 min), frying (190 °C for 2 min), and retorting (121 °C for 20 min with 17 psi overpressure) (Downs & Taylor, 2010), and egg allergens in six different heat processed food model (Faeste et al., 2007).

We attempted to explain the greater underestimation in recovery of various heat-processed shrimp TM by the sELISA using a schematic diagram (Fig. 5b). The test format of sELISA consists of a pair of antibodies: one is a captured IgG coupled to the inner surface of microtiter plate wells, and the other is an HRP-linked detection IgG. To construct the antibody–antigen–antibody complex, sELISA requires at least two or more binding sites in the target allergen (Fernandes et al., 2015; Sena-Torralba et al., 2020). According to above structural analysis results, despite TM with high thermostability, the tertiary structure alteration with more hydrophobic amino acid exposed and some epitopes

modification occurred in heat-processed shrimp TM, which might mask allergenic epitopes, and reduce allergen IgG binding sites (Ekezie et al., 2018; Marsh et al., 2020). Moreover, due to the fact that a certain amount of IgG is first coated in the sELISA microtiter plate wells, a lower number of IgG-binding sites of allergen would result in significantly reduced antibody–antigen–antibody production, and will ultimately lead to an underestimation of TM recovery. Nevertheless, in the indirect ELISA format, excess antigens are first coated onto the microtiter plate, then added IgG and HRP-labeled IgG, followed by color development using TMB. The transition from α -helix to random coil happened in TM from heated shrimps, which might contribute to elevated flexibility of TM structure, and facilitate epitope recognition. Thus, the higher content of antigen–antibody complex would be obtained in thermal-treated shrimp TM with indirect ELISA analysis.

3.5. Relationship among immunodetection recovery, IgG binding ability, and structural properties of TM

PCA analysis was performed to correlate the variance in IgG binding ability and immunodetection recovery observed due to the difference of structural properties in the raw and heat-processed shrimp TM (Fig. 6). With the prediction goodness parameter $Q^2 = 0.9564$, the PCA model could explain 99.05 percent of the total variance ($R^2 = 0.9905$). The IgG binding ability of TM purified from raw and various heated shrimp is clustered with β -T, coil content, UV, and ANS in the loading plot, whereas TM recovery of raw and various heated shrimp TM is clustered together with α -H, β -S, FAA, and IF (Fig. 6a). The above results indicated that unordered structure (β -turn and random coil) and surface hydrophobicity are positively correlated with IgG binding ability, inversely correlated with TM recovery, nevertheless, intrinsic fluorescence intensity, ordered structure, free amino group content make a positively contribution to the TM recovery. As shown in Fig. 6b, the coefficients are calculated to describe the positive or negative attribute of each variable to the TM recovery. The importance of a given X-variable is proportional to its distance (coefficient value) from the origin (zero). In general, higher β -sheet, free amino group and α -helix content would improve TM detection by ELISA. In contrast, higher IgG binding ability, β -turn, random coil content and UV absorbance on the other hand would result in lower TM recovery. Taken together, the ordered structure (α -helix and β -sheet) possess higher chemical bonds energy, which can be more solid to retain the structure of protein molecules, and was resistant to specific interactions with IgG/IgE antibodies. After thermal treatments, transition from α -helix to random coil happened in TM from heated shrimp with unordered structure and elevated

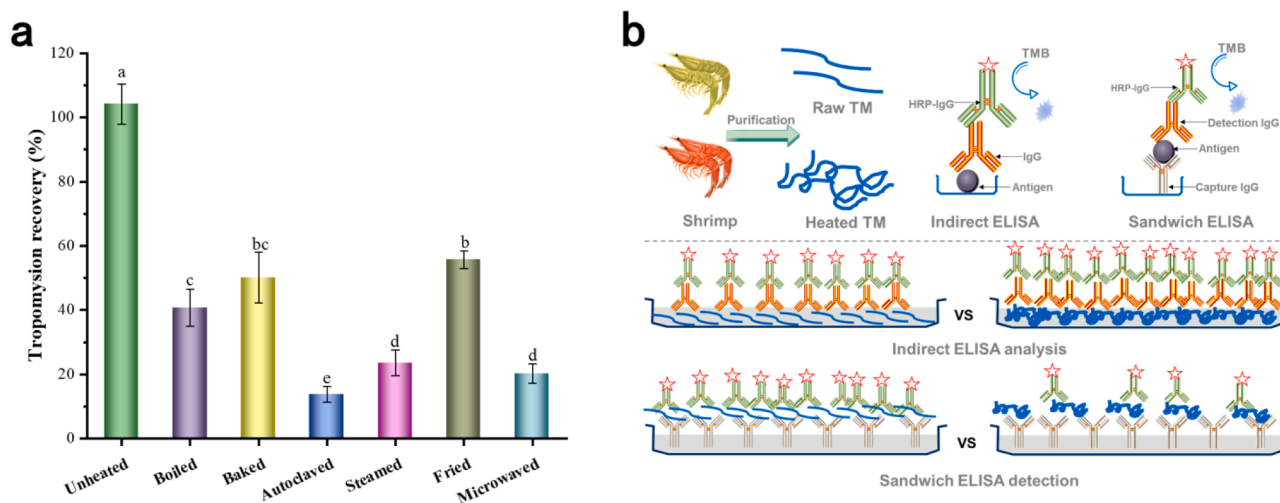


Fig. 5. Percentage recoveries of TM purified from raw and various heated shrimps as detected by ELISA assays (a) and its schematic diagram (b). Different lowercase letters in column mean significant differences ($P < 0.05$) among samples with different thermal processing types.

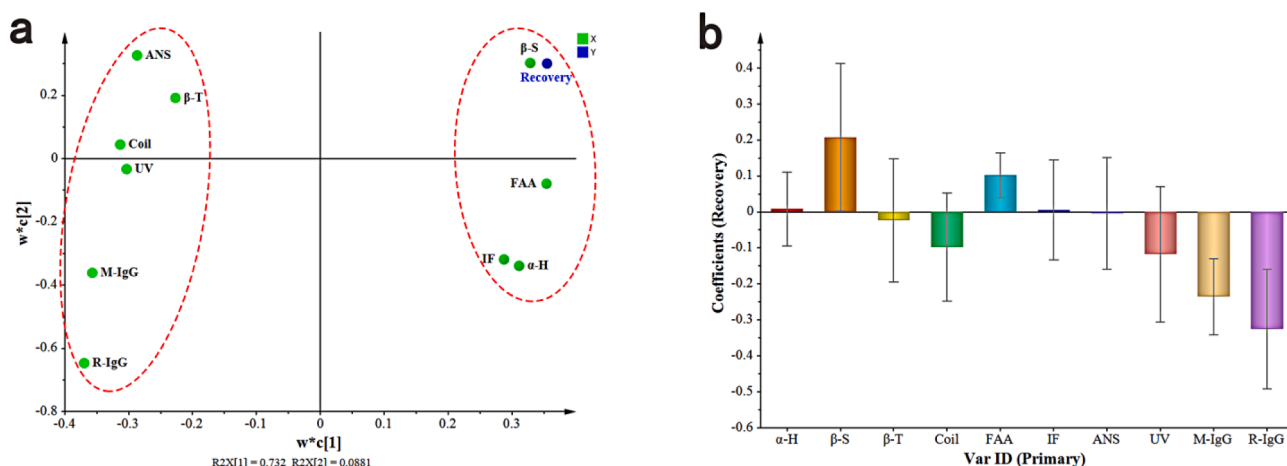


Fig. 6. Loading score plot (a) and coefficients plot (b) of the PCA model analysis. M–IgG and R–IgG, IgG binding ability to mice and rabbit anti-sera against TM; Recovery, immunodetection recovery using sELISA; α -H, α -helix; β -S, β -sheet; β -T, β -turn; Coil, random coil; UV, UV absorbance at 276 nm; IF, intrinsic fluorescence intensity at 304.6 nm; ANS, extrinsic fluorescence intensity at 506.6 nm; FAA, free amino acid content.

flexibility, which might bring about better satirical epitope recognition, especially in continuous epitope (James et al, 2018; Kim et al., 2021). Meanwhile, increased unordered structure with reduced free amino group content during thermal treatments can result in some epitopes masking and modification due to glycation and structural alteration. Less antibody binding sites in heat-treated shrimp TM are primarily responsible for the greater underestimation in recovery (Fu & Maks, 2013; Marsh et al., 2020).

4. Conclusions

In summary, our findings indicated that heat processed shrimp TM would undergo structural alteration, including the loss of ordered structure (transition from α -helix and β -sheet to β -turn and random coil), increased surface hydrophobicity, and decreased free amino group contents. Despite some epitopes masking and modification of shrimp TM occurred after thermal treatments, the above structural changes caused TM structure to become looser and more accessible, allowing TM to bind IgG/IgE more easily. Furthermore, the loss of IgG binding sites as a result of structural modification brought about 48.48%–90.44% reduction in immunodetection recovery as compared to raw shrimp TM. Our findings could provide some valuable information that help to better understand how IgG/IgE binding capacity and immunodetection recovery relates to structure characteristics of shrimp TM, and also add to new evidence on the raised concerns about possible risk from processed foods and its reliable detection of allergens. Structural crystallography techniques should be used to further characterize the molecular level changes in the structure of TM caused by thermal processing.

CRediT authorship contribution statement

Jinlong Zhao: Investigation, Methodology, Writing – original draft. **Yonghong Li:** Validation, Resources. **Lili Xu:** Validation, Resources. **Jianhua Zeng:** Validation, Resources. **Yuhai Liu:** Resources, Methodology. **Vaileth Timira:** Writing – review & editing, Formal analysis, Validation, Resources. **Ziye Zhang:** Validation, Resources. **Hong Lin:** Data curation, Supervision. **Zhenxing Li:** Conceptualization, Methodology, Writing – review & editing, Supervision, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.foodchem.2022.133215>.

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