



Insight into IgG/IgE binding ability, *in vitro* digestibility and structural changes of shrimp (*Litopenaeus vannamei*) soluble extracts with thermal processing

Jinlong Zhao^a, Yonghong Li^{a,b}, Lili Xu^a, Yu Ji^a, Jianhua Zeng^a, Vaileth Timira^a, Ziye Zhang^a, Guanzhi Chen^c, Hong Lin^a, Zhenxing Li^{a,*}

^a College of Food Science and Engineering, Ocean University of China, No.5, Yushan Road, Qingdao, Shandong Province 266003, PR China

^b HOB Biotech Group Corp., Ltd., No. 218, Xinghu Road, Suzhou City, Jiangsu Province 215000, PR China

^c Department of Dermatology, The Affiliated Hospital of Qingdao University, Qingdao University, No.16, Jiangsu Road, Qingdao City, Shandong Province 266071, PR China

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ABSTRACT

The effects of six kinds of thermal processing on soluble protein recovery, potential allergenicity, *in vitro* digestibility and structural characteristics of shrimp soluble proteins were evaluated. Obtained results confirmed soluble protein recovery and IgG/IgE reactivity of shrimp soluble extracts were markedly suppressed by various thermal treatments with enhanced digestibility depended on the extent and type of heating applied, which correlated well with the structural alterations and modification. The maximum reduction of IgG/IgE-binding capacity and digestive stability were observed in the autoclaved shrimps because of unfolding of protein and hydrophobic residues exposed. Notably, tropomyosin (TM) and sarcoplasmic calcium-binding protein (SCP) were still IgG/IgE-reactive in various heat-processed shrimps, even higher IgG reactivity were found in heat-treated shrimps TM according to TM antiserum western-blotting and indirect ELISA results. Shrimp TM and SCP maintains its IgE/IgG-binding capacity after various cooking methods, thus most probably initiating allergic sensitization to both raw and cooked shrimps.

1. Introduction

Shrimp is an important primary protein source to feed the world's ever-increasing population due to the ample high-quality proteins and nutrients they contain (Fu et al., 2019; Liu et al., 2011). >900, 000 tons of shrimp have been consumed annually as a raw material in various processed foods, such as shrimp seasoning, shrimp paste, fried snack foods, etc. (Ayuso et al., 2008). However, a trace amount of shrimp component could induce severe allergic reactions, which persists throughout life. It has become a serious global public health problem with increasing prevalence because of increased consumption, resulting in up to 2.5% of the general population suffering worldwide (Fu et al., 2019; Ruethers et al., 2018).

Considering the hazard of shrimp allergies, numerous shrimp allergens have been identified and well-characterized, including TM (36 kDa), arginine kinase (40 kDa, AK), SCP (20 kDa), myosin light chain (17 kDa, MLC), troponin C (16.8 kDa), and triosephosphate isomerase

(27 kDa) (Fu et al., 2019; Ruethers et al., 2018). It can help to improve shrimp allergy diagnosis and management for ensuring consumer safety. Among these allergens, TM have been recognized as major panallergen in crustacean shellfish, which could elicit positive IgE binding in 72–98% of shrimp-allergic patients (Ayuso et al., 2010; Ruethers et al., 2018). Therefore, several processing technologies, such as enzyme-aided crosslinking (Ahmed et al., 2020; Wang et al., 2019), ultrasonic (Zhang et al., 2017), as well as irradiation treatments (Guan et al., 2018), have been explored as a means to modify shrimp allergen structure and reduce allergenicity. However, these methods maybe more suitable for industrial application, and only focused on purified allergens that exclude the potential impact of the food matrix. More importantly, not all allergens in shrimp are identified, so assays that are reliable and able to test various proteins from a complex food would be beneficial and reliable for evaluating possible allergic risk.

The effect of thermal processing on food allergens is of major interest for allergic patients who need to avoid all food containing active

* Corresponding author at: Food Safety Lab., College of Food Sci. & Engineering, Ocean University of China, PR China.

E-mail address: lizhenxing@ouc.edu.cn (Z. Li).

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allergens. Shrimps are usually consumed after being exposed to certain degrees of thermal treatments (such as boiling, baking, and frying) in our daily life. Besides, whole shrimp muscle is normally processed and consumed rather than a single purified protein, which might cause different structural modifications that affect interactions in the multi-component food (Liu et al., 2010; Liu et al., 2019; Zhao et al., 2022). Available research implied that food matrix could protect proteins against digestive enzyme degradation and processing denaturation leading to decreased probability of reaching the intestinal mucosa where absorption can occur (Martos, Lopez-Fandino, & Molina, 2013; Nowak-Węgrzyn et al., 2009). Consumption of allergens in a complex matrix containing different food components such as fats and carbohydrates may influence their digestibility and subsequently influence their ability to interact with the immune system (Jacob et al., 2020; Nowak-Węgrzyn & Fiocchi, 2009).

Setting up a study to investigate the alterations of IgE/IgG-binding properties and digestibility of shrimp extracts during real food processing conditions and to test the allergenic potential is more reliable and significant than using a purified single allergen. In the present study, whole shrimp instead of purified single allergen was first treated under common daily thermal processing, including boiling, baking, autoclaving, steaming, frying, and microwaving. Then, shrimp soluble proteins were extracted, and their IgG/IgE binding ability, digestibility, and structural alterations were investigated, respectively.

2. Materials and methods

2.1. Materials

White shrimps (*Litopenaeus vannamei*) were purchased from the local market of Qingdao city. TM from the white shrimp with at least 95% purity was obtained using a nonthermal extraction technique as described in our recent report (Xu et al., 2020). Sodium dodecyl sulfate (SDS), Coomassie Blue R-250, 8-Anilino-1-naphthalenesulfonate (ANS), and HRP-labelled goat anti-rabbit IgG were purchased from Beijing Solarbio Science & Technology Co., Ltd. (Beijing, China). Ovalbumin (OVA), porcine pepsin and trypsin were acquired from Sigma-Aldrich (St. Louis, MO, USA). Horseradish peroxidase (HRP) conjugated goat antihuman IgE antibody was obtained from Zhongshan Jinqiao Biotechnology Co., Ltd (Beijing, China). The iBindTM Solution Kit and enhanced chemiluminescent (ECL) kit for western blot analysis was purchased from Thermo Fisher Scientific Inc. (Waltham, MA, USA). All chemicals used in the study were of analytical grade.

2.2. Production of antisera against soluble raw shrimp extracts and TM

Polyclonal antisera against soluble raw shrimp extracts and TM were obtained from four New Zealand rabbits by injecting raw shrimp extracts and purified TM prepared by our recent methods (Yu et al., 2019). The specificity and titer of the antisera were monitored throughout the immunization period by an indirect ELISA, and when the antisera titer maintained a stable level, the rabbits were taken out to obtain the serum, which was centrifuged (5000 × g, 10 min). Finally, all antisera were divided into several 100 µL plastic tubes and kept at −80 °C for further analysis.

2.3. Shrimp allergic patient sera

All clinically shrimp-allergic patient sera were obtained from The Affiliated Hospital of Qingdao University (Qingdao, China), which was approved by the ethics committee of The Affiliated Hospital of Qingdao University, and the written informed consent was acquired from all participants (File No. QD-PF-20200216126). The allergic patient sera were documented by clinical history, and specific IgE levels ranged from 2.84 to 22.1 (>0.35 kU/L) assessed using the ImmunoCAP system (Phadia AB, Uppsala, Sweden) (S Table 1).

2.4. Thermal processing of shrimps

Shrimps were first subjected to various thermal treatments including boiling (7 min), baking in an oven (180 °C for 10 min), high pressure steaming in an autoclave (0.14 MPa at 121 °C for 15 min), steaming (7 min), frying with soybean oil (>200 °C for 2 min), microwaving using a MZ-2011 microwave oven (Haier Group, Qingdao, China) at 700 W (4 min). Unheated shrimps were used as a control. All the samples were treated in triplicates for each processing method. Afterward, all heated and unheated shrimps were deshelled and deveined. Then, shrimp powder from unheated and heated shrimp muscle was furthermore prepared according to our recent method (Guo et al., 2021). Briefly, shrimp muscle was crushed and suspended in ice-cold acetone (1:8, w/v) using a homogenizer for decoloring and degreasing, and destained until the supernatants was clear. Afterward, the shrimp residues were washed by cold absolute acetone and dried completely at room temperature. Then, the shrimp residues were milled to pass through 80-mesh sieves and stored at −80 °C until further use.

2.5. Preparation of raw and heat-treated shrimp soluble extracts

Shrimp soluble extracts were prepared according our optimized method (Guo et al., 2021). Briefly, the shrimp powder from raw and heat-treated shrimp muscle was suspended with 10-fold of 10 mM PBS (pH 7.4) containing 0.2 % SDS, and 1 mM DTT in a stirring state for 6 h at 4 °C. After centrifugation at 9000 × g for 30 min, the supernatants were dialyzed (3500 Da cutoff membrane) against 10 mM PBS for 36 h at 4 °C. Afterward, all the dialyzed supernatants from raw and heat-treated shrimp were filtered through 0.22 µm membrane, respectively, followed by freeze-drying and storage at −80 °C until use.

2.6. Indirect ELISA analysis

The IgG/IgE-binding abilities were assessed using indirect ELISA method with rabbit serum against shrimp soluble extracts and TM, and shrimp patients' sera. Briefly, the wells were coated with raw and heat-treated shrimp extracts (100 µL, 5 µg/mL) in a Corning-Costar ELISA microplate (Corning, NY, USA) at 4 °C overnight, followed by 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, and after that the plate was again washed with PBST three times and incubated with 100 µL of rabbit antisera against shrimp extracts with 1:320000 dilution, TM with 1:320,000 dilution, and shrimp-allergic patient sera with 1:2 to 10 dilution at 37 °C for 1 h. Next, the plate was washed and the HRP-labeled goat anti-rabbit IgG (100 µL, diluted to 1:20000) or HRP-labeled goat anti-human IgE (100 µL, diluted to 1:3000) was added and incubated for 1 h at 37 °C. The plate was washed again three times with PBST before adding 100 µL/well of the TMB solution for developing the color 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 via using a MultiscanMK3 microplate reader (Thermo Fisher Scientific Inc., Waltham, MA, USA). Each sample was repeated three times.

2.7. Western blotting analysis

Western blotting was performed to evaluate the IgG/IgE-binding abilities of different treated shrimp extracts according to our recent method (Zhao et al., 2021). Briefly, proteins in extracts were separated by SDS-PAGE and electrically transferred onto polyvinylidene fluoride (PVDF) membrane by iBlot® 2 Gel Transfer Device (Life Technologies Co., Israel). The membranes were then incubated with antisera against raw shrimp extracts and TM (diluted 1:5000 with iBind solution). Also, shrimp allergic patient sera (diluted 1:2 with iBind solution) and HRP-labeled goat anti-rabbit/human IgG/IgE (1:5000 and 1:2000 with iBind solution) at 25 °C for 6 h in iBindTM Western Device (Life Technologies

Co., Israel), respectively, followed by washing with PBST for 15 min. The results were obtained using multiple exposure time ECL solution to obtain an optimal signal by using the BIO RAD Universal Hood II gel imaging system (Bio-Rad Laboratories, Inc., CA, USA).

2.8. *In vitro* digestion assay

In vitro simulated gastrointestinal digestion of raw and heat-treated shrimp soluble extracts was performed as previously described by Guo et al. (2021). For the simulated gastric digestion process, different shrimp extracts were mixed with an equal volume of simulated gastric fluids (SGF) containing pepsin (2000 U/mL of digesta) and incubated in a water bath at 37 °C following shaking. Aliquots (100 µL) were taken at 0, 5, 10, 20, 30, 60, and 120 min, then the digestion solution was immediately stopped by adding 30 µL of 0.2 M Na₂CO₃ solution. For the simulated intestinal digestion process, equal simulated intestinal fluids (SIF) containing trypsin (100 U/mL of digesta) and bile (10 mM in the total digesta) were added at the 0 min point of the gastric phase and incubated for 0, 5, 10, 20, 30, 60 and 120 min, then digestion solution was taken out and terminated by the addition of Pefabloc® (5 mM final concentration) for further analysis.

2.9. SDS-PAGE analysis

Treated shrimp digestion products were separated via SDS-PAGE. Protein samples were mixed and boiled with 4 × loading buffer containing 0.4% bromophenol, 10% (w/v) SDS, and 400 mM DTT at a 3:1 (v/v) ratio for 7 min. Then the samples (10 µg) were loaded into SDS-PAGE gel prepared via using AnyKD PAGE kit (ATGene, Chongqing, China), and electrophoresed at 80 V for 30 min at 120 V for 1 h. Afterward, the gels were stained with Coomassie brilliant blue R-250 (Solarbio Co. Beijing, China) or used for the above western blotting assay.

2.10. CD spectra analysis

Raw and treated shrimp soluble extracts were prepared with PBS to a concentration of 0.2 mg/mL and filtered through a 0.22 µm membrane. Then, CD spectrum 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 the samples were scanned three times at a 1200 nm/min scanning speed with a 1 mm path length quartz cuvette.

2.11. FT-IR analysis

The lyophilized raw and treated shrimp extracts (4.0 mg) were mixed with KBr (200 mg), and then was pressed into a pellet. Subsequently, a Nicolet Is10 FTIR spectrometer (Thermo Nicolet Co., USA) was employed to collect the FT-IR spectrum data in the range from 400 to 4000 cm⁻¹ during 32 scans with 0.09 cm⁻¹. Finally, the data was further analyzed by Omnic 8.2 software (Thermo Fisher Scientific Inc., Madison, WI) and Peakfit 4.12 (Systat Software Inc., San Jose, CA).

2.12. UV absorption spectra measurements

The UV spectrum data of raw and treated shrimp soluble extracts (0.2 mg/mL) was recorded 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.

2.13. Intrinsic and extrinsic fluorescence measurements

Intrinsic and extrinsic fluorescence of raw and treated shrimp soluble extracts (0.2 mg/mL) were determined using F-4600 spectrofluorometer (Horiba Ltd., Kyoto, Japan). Before extrinsic fluorescence

measurements, each sample (2.0 mL) was mixed with 25 µL of 8.0 mM of ANS solution for a 5 min reaction. The alterations in the tertiary structure were monitored by intrinsic fluorescence emission between 300 and 400 nm at a 280 nm excitation, while ANS fluorescence emission between 400 and 600 nm at a 380 nm excitation. The excitation and emission slit width was set at 5 nm.

2.14. Statistical analysis

All assays were carried out in triplicates and the data was presented as mean ± standard deviations. Analyses of variance (ANOVA) and Duncan's means comparison test was applied with a significance level of 0.05 by using SPSS software (version 11.5, SPSS Inc. Chicago, IL, USA).

3. Results

3.1. SDS-PAGE analysis and total soluble protein recovery of raw and heat-treated shrimps

As shown in Fig. 1a, unheated shrimp extract bands presented a wider range of molecular weights between 11 kDa and 100 kDa, and the major shrimp allergen bands corresponding to AK (40 kDa), TM (36 kDa), SCP (20 kDa) and MLC (17.5 kDa) were visible. Nevertheless, protein bands found for unheated shrimp extracts were not visible in heated shrimp extracts. The heated shrimp soluble extracts only contain two major proteins (TM and SCP) with higher molecular weight and the more diffuse bands.

The results on the recovery of total soluble proteins from raw and thermally processed shrimps are shown in S Table 2. In contrast with raw shrimp soluble proteins, a significant loss of soluble shrimp proteins yields from the various processed shrimp. The maximal reduction was observed in autoclaved shrimp soluble proteins with 73.77% reduction compared to raw shrimp soluble proteins, followed by baked (71.15%), boiled (69.30%), steamed (66.50%), fried (64.53%), and microwaved (60.60%) shrimp soluble proteins.

3.2. IgG/IgE binding ability

As shown in Figs. 1 and 2, raw shrimp soluble extracts showed stronger binding to most stained shrimp extracts separated by SDS-PAGE (Fig. 1b). In contrast, the heat-treated shrimp soluble extracts displayed weaker binding capacity in AK and SCP compared to raw shrimp, especially autoclaved shrimp soluble extracts with negligible binding. Raw and treated TM clearly showed a single immunoblot band around 36 kDa in rabbit antisera against TM IgG immunoblotting. Besides, various treated TM with similar immunoblot intensity have higher immunoblotting intensity than the raw shrimp TM. (Fig. 1c). Fig. 1d shows that the raw and treated shrimp soluble extracts displayed an obvious IgE binding band in a pooled serum from 10 shrimp allergic patients' sera. Multiple bands were detected in the untreated shrimp extracts by the serum pool. The SCP, TM, and MLC bands in raw shrimp soluble extracts presented higher binding intensity. Although the allergen profile displayed an obvious alteration after thermal treatments, numerous IgE-reactive proteins were still recognized by the pooled sera with significantly reduced binding intensity. The negligible binding intensity was observed in autoclaved shrimp extracts.

Next, indirect ELISA was further performed to assess the IgG-binding ability of shrimp extracts to shrimp soluble extracts and TM antiserum, and shrimp allergic patients' sera (Fig. 2). Boiled, baked, autoclaved, steamed and fried shrimps showed significantly decreased ($P < 0.05$) IgG binding capacity in indirect ELISA with rabbit anti-shrimp extracts sera, which were reduced to 88.70%, 57.25%, 40.13%, 70.98%, and 66.88% of the unheated shrimp soluble extracts, respectively. While the microwaved shrimp presented an obvious increasing binding capacity (8.92%) (Fig. 2a). Interestingly, indirect ELISA with rabbit anti-TM sera indicated TM from all heated shrimp possessed higher IgG reactivity,

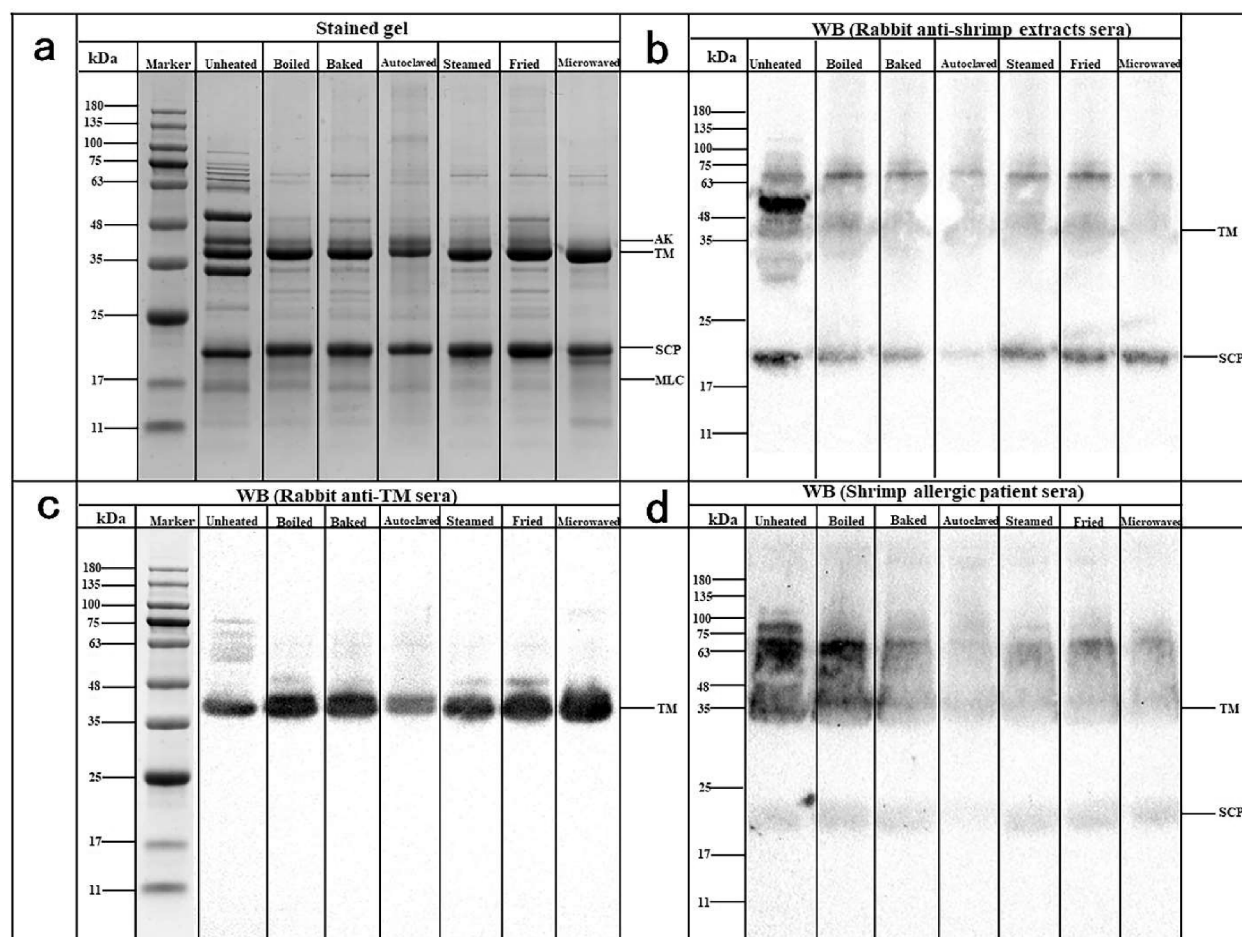


Fig. 1. SDS-PAGE reducing gel of raw and different heat-treated shrimp soluble extracts (a), and IgG immunoblots with pooled rabbit antisera against raw shrimp extracts (b) and TM (c) and IgE immunoblots with pooled shrimp allergic patients' sera (d).

and the microwaved shrimp TM showed the strongest IgG binding capacity, which was about 134.40% of the TM in raw shrimp extracts (Fig. 2b).

As illustrated in Fig. 2c, IgE reactivity of raw and different treated shrimp extracts with varying levels of IgE bindings were observed in all 10 shrimp allergic patients' sera with negligible IgE binding to OVA. Overall, heated shrimp extracts presented a similar decrease in IgE binding values with rabbit anti-shrimp extracts sera IgG ELISA results. It also showed that raw shrimp extracts had significantly higher IgE binding capacity ($P < 0.05$) than different heated shrimp extracts except for serum P5, and the lowest IgE binding to autoclaved shrimp extracts was observed in all 10 shrimp allergic patients.

3.3. *In vitro* digestion stability

The digestibility of raw and different treated shrimp extracts was assessed by SDS-PAGE, and the results were presented in Fig. 3. In the SGF digestion process, there was a significant change in band intensity of raw and treated shrimp samples with the expansion of digestion time (Fig. 3a). The raw shrimp soluble extracts began to be digested during the first 5 mins with many protein bands losing except for TM. With increasing digestion time, new fragments around 34, 19, and 17 kDa were observed in the gel, and the protein band around 34 kDa disappeared within 60 min digestion. While the digested fragments from raw shrimp sample were still detectable after 120 min SGF digestion. Heated shrimp extracts with only two main bands (TM and SCP) showed significantly decreased band intensity in the SGF digestion process with generations of new digested bands around 34, 19, and 17 kDa. The bands

from boiled, baked, steamed, fried and microwaved shrimps were undetectable at the end of 120 min SGF digestion. Significantly, the digested fractions would be almost invisible after 30 min digestion for the autoclaved shrimp extracts (Fig. 3a).

In the SIF digestion process, an evidently decreased band from raw shrimp soluble extracts were found during the first 10 min SIF digestion, and bands corresponding to AK (40 kDa), TM, 37 kDa, and SCP (20 kDa) were almost invisible after 30 min digestion with massive digested fragments around 19 and 11 kDa. Nonetheless, the bands around 49 kDa could retain visibility until 120 min digestion (Fig. 3b). In contrast, soluble extracts from heated shrimps displayed apparent reduced band density with the expansion of SIF digestion time. TM and SCP bands in various heated shrimp soluble extracts were no longer visible at 20 min during the SIF digestion with many new digested fragments around 19 and 11 kDa. In addition, the band of autoclaved shrimp extracts disappeared at the end of 10 min digestion.

3.4. CD spectra analysis

CD spectra analysis showed a typical characteristic α -helical structure peaks, with one clear positive peak near 196 nm and two negative bands around 208 and 222 nm, while a negative peak around 216 nm attributed to β -sheet contents (Fig. 4a) (He et al., 2020). Following thermal treatment, an obvious decrease in negative CD signal values near 208 and 222 nm were observed in soluble extracts from various heated shrimp with a more prominent negative peak.

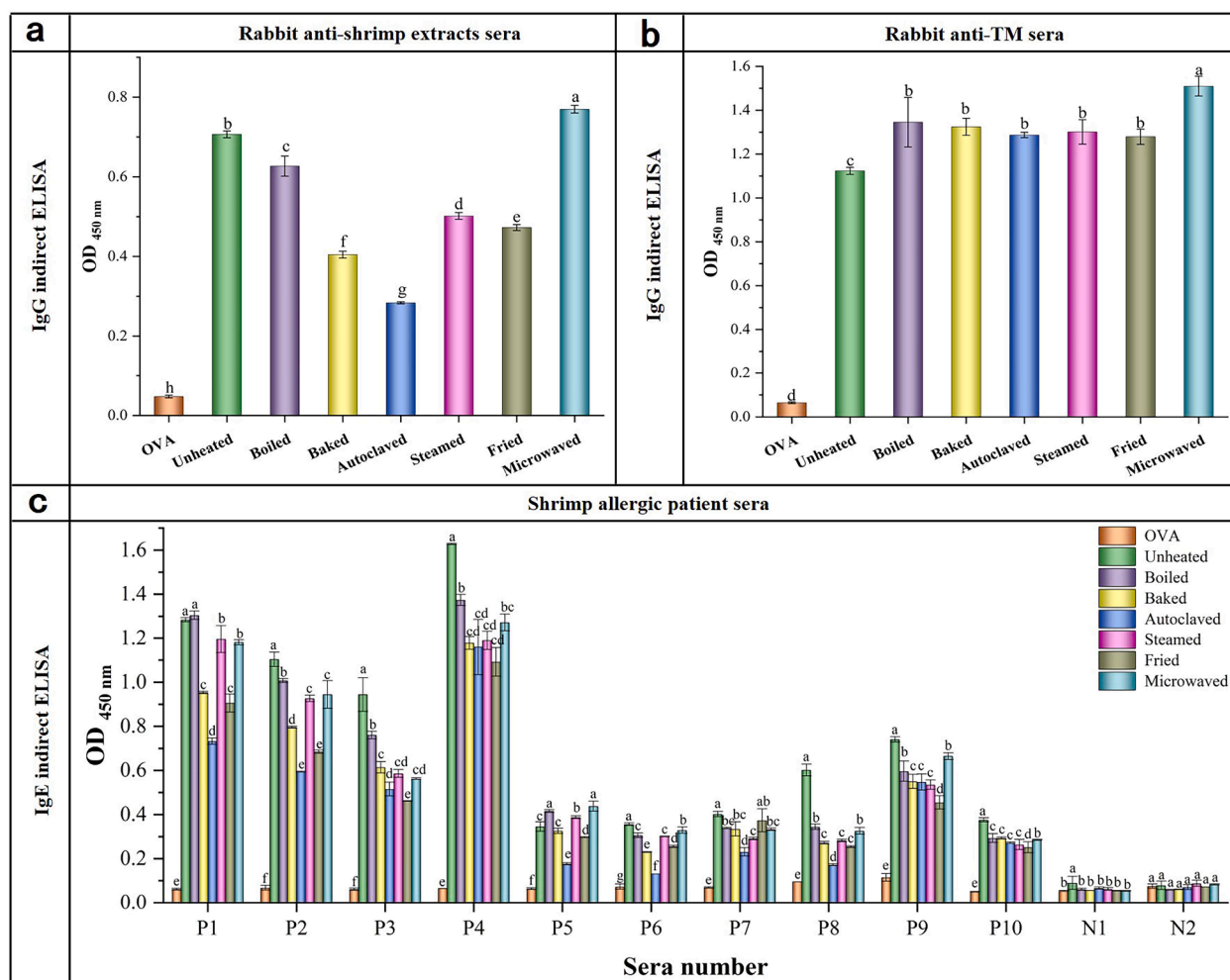


Fig. 2. Indirect ELISA for pooled rabbit antisera against raw shrimp soluble extracts (b) and TM (c) IgG binding, and shrimp patient serum IgE binding to raw and different heat-treated shrimp extracts. The data is presented as the mean values for triplicates and the standard deviation is indicated by error bars. Different lowercase letters in column mean significant differences ($P < 0.05$) among raw and different heat-treated shrimp extracts.

3.5. FT-IR spectra analysis

As shown in Fig. 4b, two characteristic bands around 3303 and 2960 cm^{-1} , which corresponded to amide A and amide B regions arising from N—H and C—N stretching vibrations, respectively, could be found in FT-IR spectra (Han et al., 2015). Compared with soluble extracts from raw shrimp, significant movement of the amide A absorption peak of treated shrimp extracts to a higher wavenumber, suggesting that the stretching vibration of N—H was strengthened, and no significant change was observed in the amide B band. Additionally, amides I, II, and III were applied to monitor structural changes of protein in FT-IR spectra, and the three regions in the FT-IR spectra of raw shrimp soluble extracts were found near 1653.72, 1544.68, and 1238 cm^{-1} , respectively. The absorption peak of heated shrimp soluble extracts showed a mild blue shift in the amide I, a red shift in amide II, and an insignificant shift in amide III compared with raw shrimp samples. Additionally, a significant increase in absorption peaks near 1157, 1080, 976, and 860 cm^{-1} was observed in FT-IR spectra of heated shrimp extracts. In Fig. 5, the amide I region of raw and treated shrimp soluble extracts were analyzed using deconvolution combined with curve fitting of the amide I region to calculate the content of each secondary structure. Differences in secondary structure content based on the amide I region were observed among the raw and treated shrimp soluble extracts. Compared to the raw shrimp extracts, the contents of α -helix of treated shrimp soluble extracts presented a significant increase, and β -sheet decreased from

26.06% (unheated) to 14.38% (boiled), 15.83% (baked), 11.62% (autoclaved), 11.43% (steamed), 11.80% (fried), 12.08% (microwaved), respectively, while the β -turn and random coil content showed insignificant alteration.

3.6. UV absorption spectra analysis

The maximum UV absorbance peak of raw shrimp soluble extracts was found around 256 nm (Fig. 6a). A significant red shift of the absorbance peak with the reduction in absorbance intensity was observed in boiled, baked, steamed, fried, and microwaved shrimp soluble extracts, while the UV absorption intensity from autoclaved shrimp extracts decreased with similar red shift.

3.7. Intrinsic and extrinsic fluorescence analysis

As shown in Fig. 6b, the untreated shrimp soluble extracts exhibited a maximum fluorescence intensity (1524 a.u) at 332.8 nm. Heated shrimp soluble extracts presented an apparent decrease in the maximum fluorescence intensity along with mild red shift (2–4 nm) in the λ_{max} . The lowest fluorescence intensity was found in autoclaved shrimp extracts.

Extrinsic fluorescence spectra showed that raw shrimp extracts had maximum emission wavelength with minimum ANS fluorescence intensity. As well as treated shrimp extracts showed a significant increase in the ANS fluorescence intensity without obvious red or blue shift

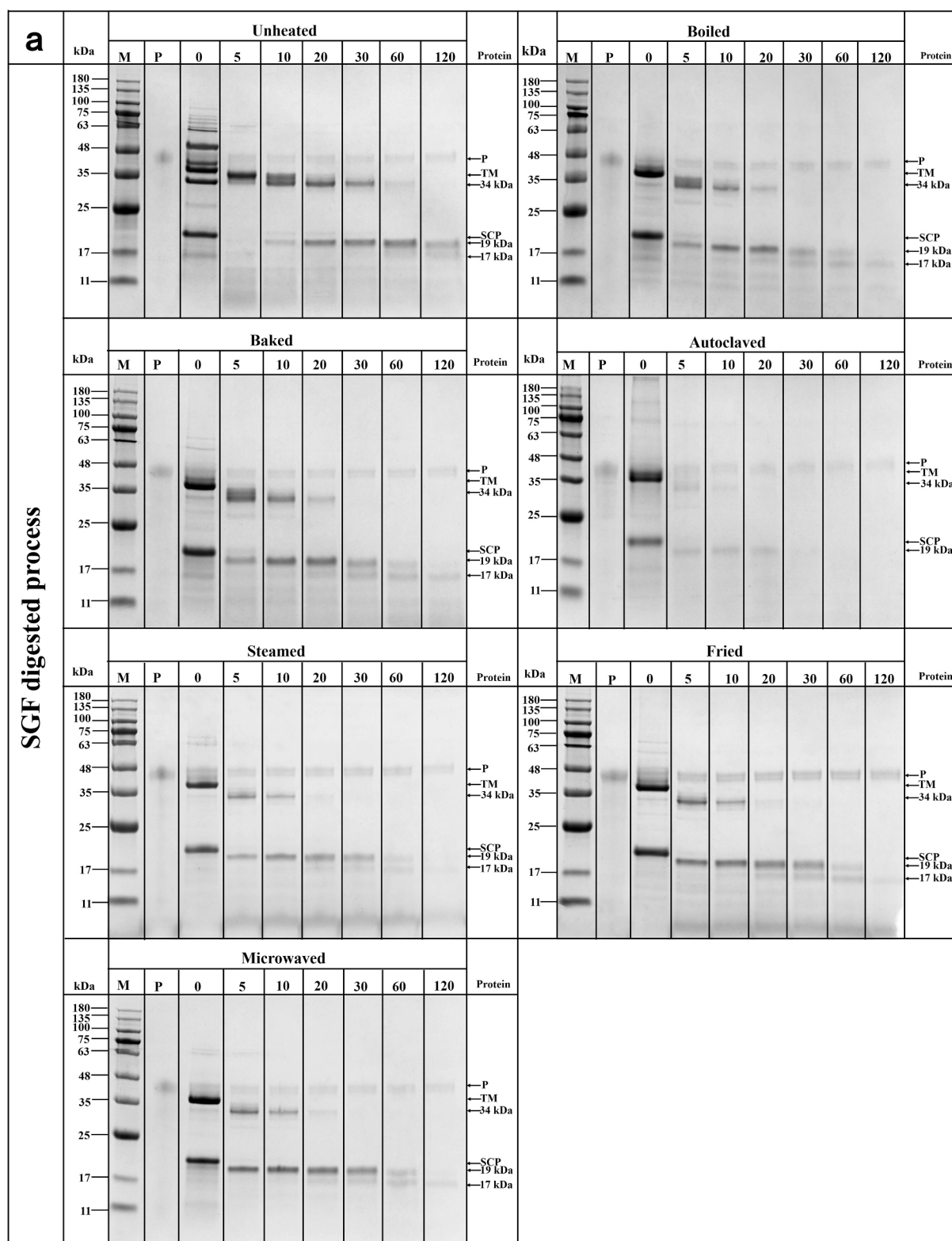


Fig. 3. The digestibility of unheated and different heat-treated shrimp soluble extracts in simulated gastric fluids (SGF) digestion (a) and intestinal fluids (SGF) digestion (a), respectively, assessed by SDS-PAGE. Lane M, protein marker; Lane P, pepsin; Lane T, trypsin; Lane 0, 5, 10, 20, 30, 60 and 120, SGF or SIF digestion for 0, 10, 20, 30, 60 and 120 min, respectively.

compared to the unheated shrimp soluble extracts (Fig. 6c). The microwaved shrimp extracts displayed the maximal fluorescence intensity.

4. Discussion

Almost all conventional processing may modify the structure and biochemical characteristics of food proteins. It is often assumed that

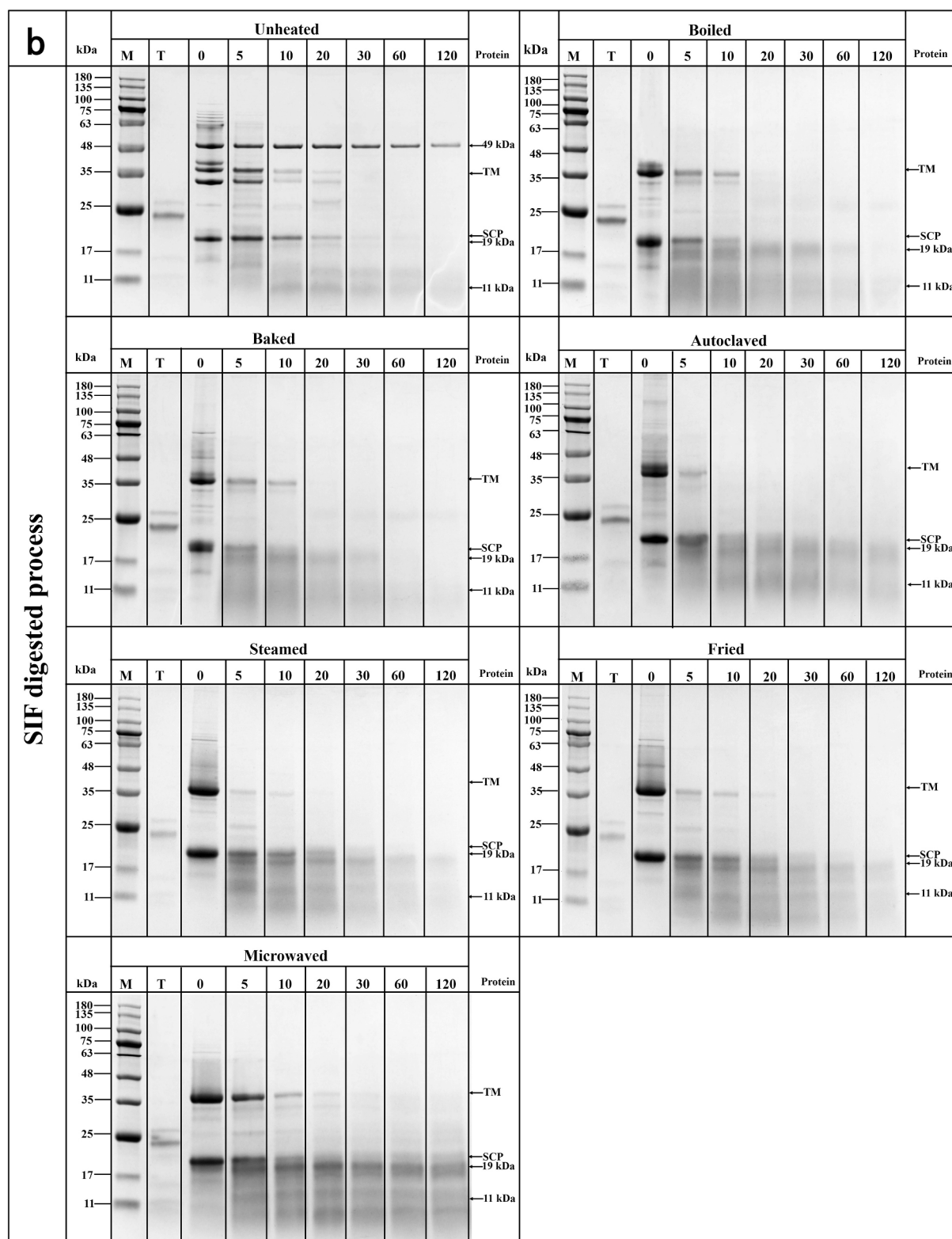


Fig. 3. (continued).

thermal processing could cause significant decrease in the IgG/IgE binding reactivity to allergen, since a catastrophic disruption of protein structure might occur with exposure of various heating types (Cabanillas & Novak, 2017; Liu et al., 2019). Nevertheless, thermal processed shrimps still could elicit allergic reaction, and few researchers focus on the potential allergenicity alteration of shrimp with various cooking treatments and its underlying causes.

In our present study, whole shrimps were treated with different common food processing, then the soluble proteins were extracted using our recent optimized method (Guo et al., 2021). SDS-PAGE results showed that various heat-treated shrimp soluble proteins displayed a more uniform protein-banding pattern with two major protein bands around 18 and 37 kD, which suggested that TM and SCP have high thermostability (Fig. 1a). This makes its role in shrimp-induced allergic

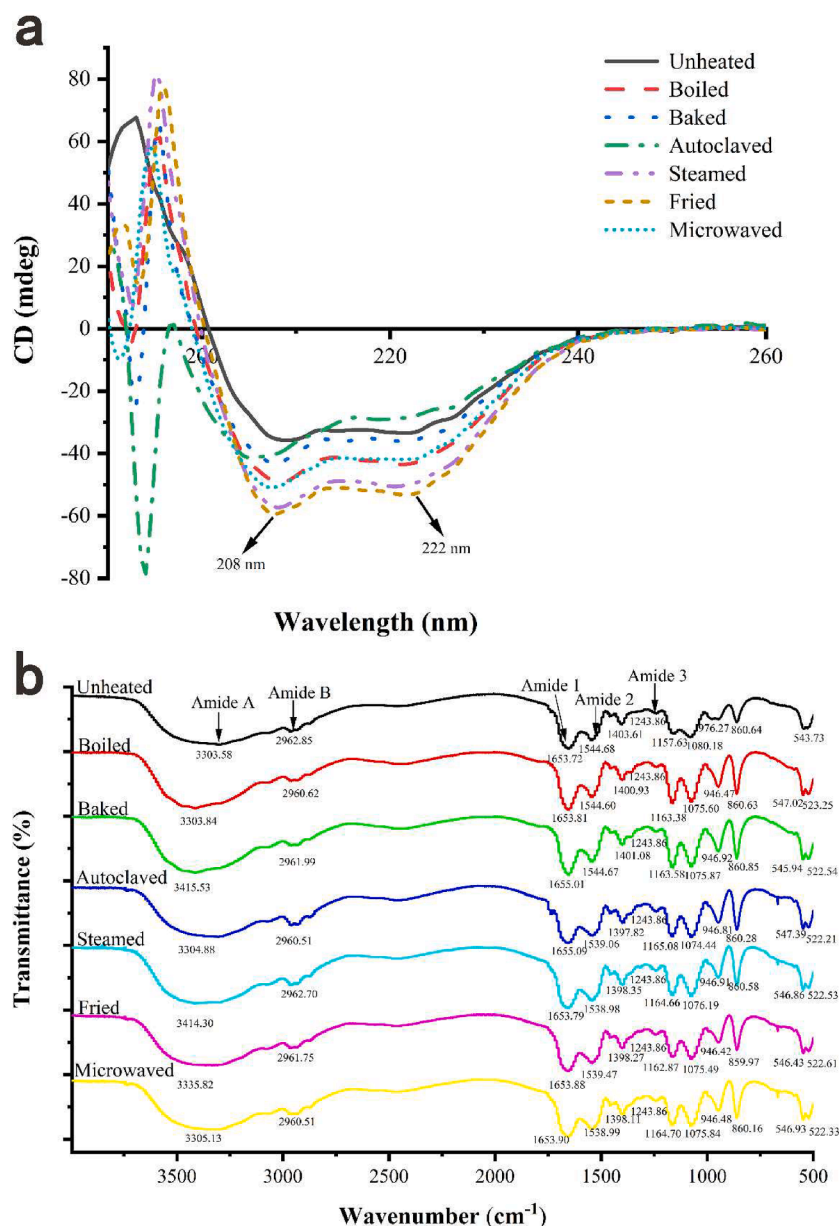


Fig. 4. The analysis of the secondary structure of raw or heat-treated shrimp soluble extracts. (a) Changes in CD spectra of raw or heat-treated shrimp extracts. (b) FTIR spectra of raw or heat-treated shrimp soluble extracts. Numbers indicate the positions of main peaks.

reactions even more important because shrimps usually are consumed cooked. Besides, increased molecular weight, more diffused bands, and newly formed bands were also found, signifying the presence of protein modifications in the shrimp matrix. The modification was also confirmed by the following FT-IR results. Similar protein profiles were also observed in other various heated crustacean extracts (Kamath et al., 2013; Liu et al., 2018; Zhao et al., 2022). Meanwhile, a significant reduction (60.60–73.77%) in total soluble protein yield was found in differentially processed shrimp samples. These might be attributed to the denaturation and aggregation of shrimp muscle proteins due to intra- and inter-molecular interactions of the proteins in the complex food matrix during the thermal treatments (Liu et al., 2018; Liu et al., 2019).

The above extraction to obtain the shrimp soluble protein including the major allergens, has a huge advantage in combining several protein fractions into a single test using western blotting, indirect ELISA analysis, and the assays that are robust and able to evaluate a number of shrimp allergens characteristics, and would be useful for identifying

possible allergy risk (Zhao et al., 2021). Subsequently, the treated shrimp extracts were analyzed by western blotting and indirect ELISA using rabbit antisera against raw shrimp extracts and TM, and 10 shrimp allergic patients' sera. The obtained results demonstrated that heat-treated shrimp extracts except for TM showed weaker immunoblot band intensity in contrast to unheated shrimp, implying lower IgG/IgE binding to heated shrimp extracts (Fig. 1b and d). Besides, the autoclaved shrimp extracts presented a negligible binding in shrimp soluble extracts rabbit antisera IgG immunoblot and IgE immunoblot assays. The above results were also confirmed by indirect ELISA with anti-shrimp extracts sera. As shown in Fig. 2a, heat-treated shrimp soluble extracts showed varying degrees of reduction in IgG binding as compared to raw shrimp extracts depending on the extent and type of heating applied. The maximum reduction of IgG-binding reactivity was observed in autoclaved shrimp extracts, with reduced values up to 40.13% of raw shrimp extracts. Moreover, the reduction of 25.96 to 71.34 % varied with different shrimp allergic patient sera was found in IgE binding capacity compared to raw shrimp extracts (Fig. 2c).

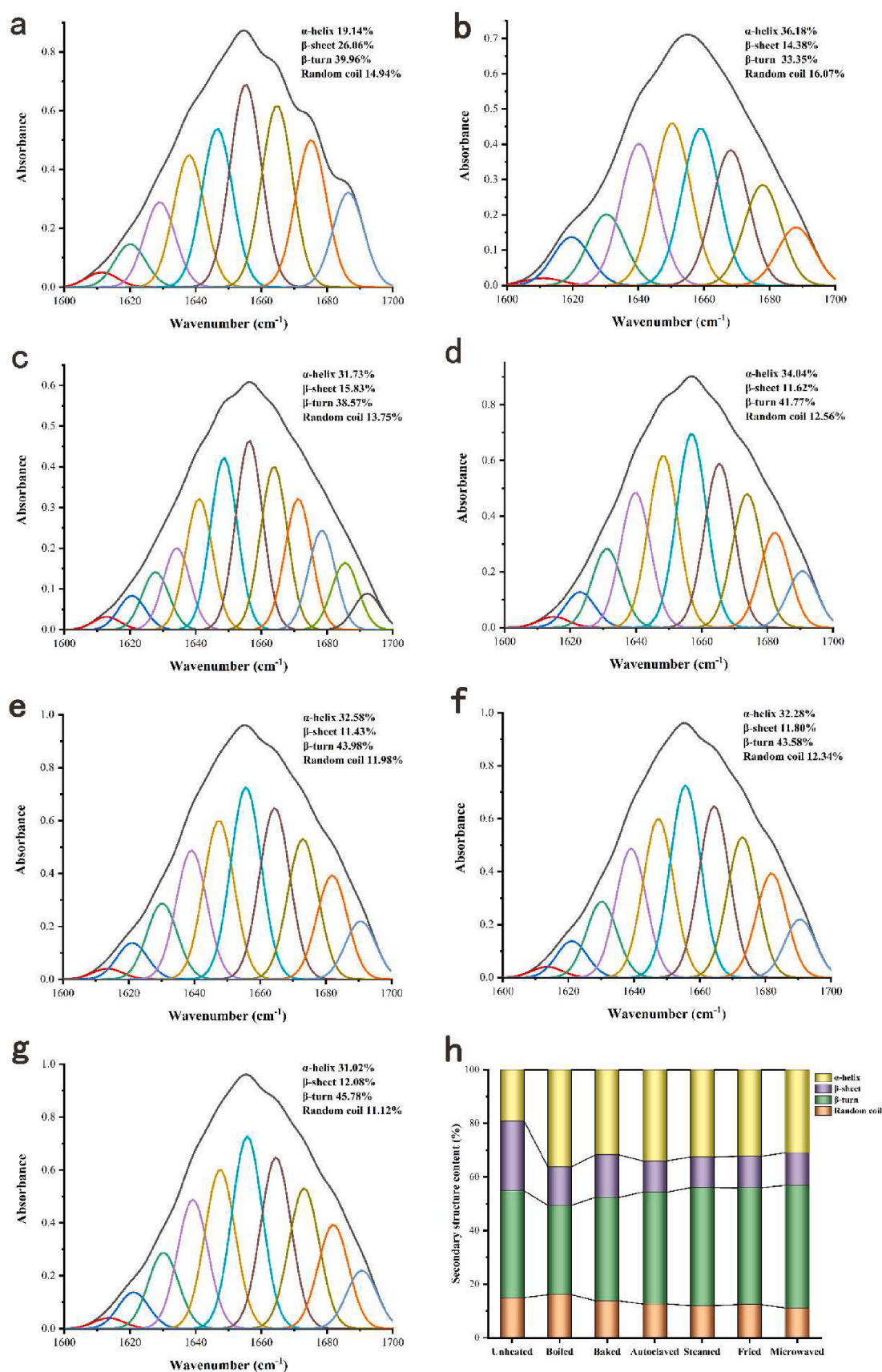


Fig. 5. Self-deconvoluted FT-IR spectrum and their curve fitting results of amide I (1600–1700 cm^{-1}) of unheated (c) or boiled (d), baked (e), autoclaved (f), steamed (g), fried (h) and microwaved (i) shrimp soluble extracts.

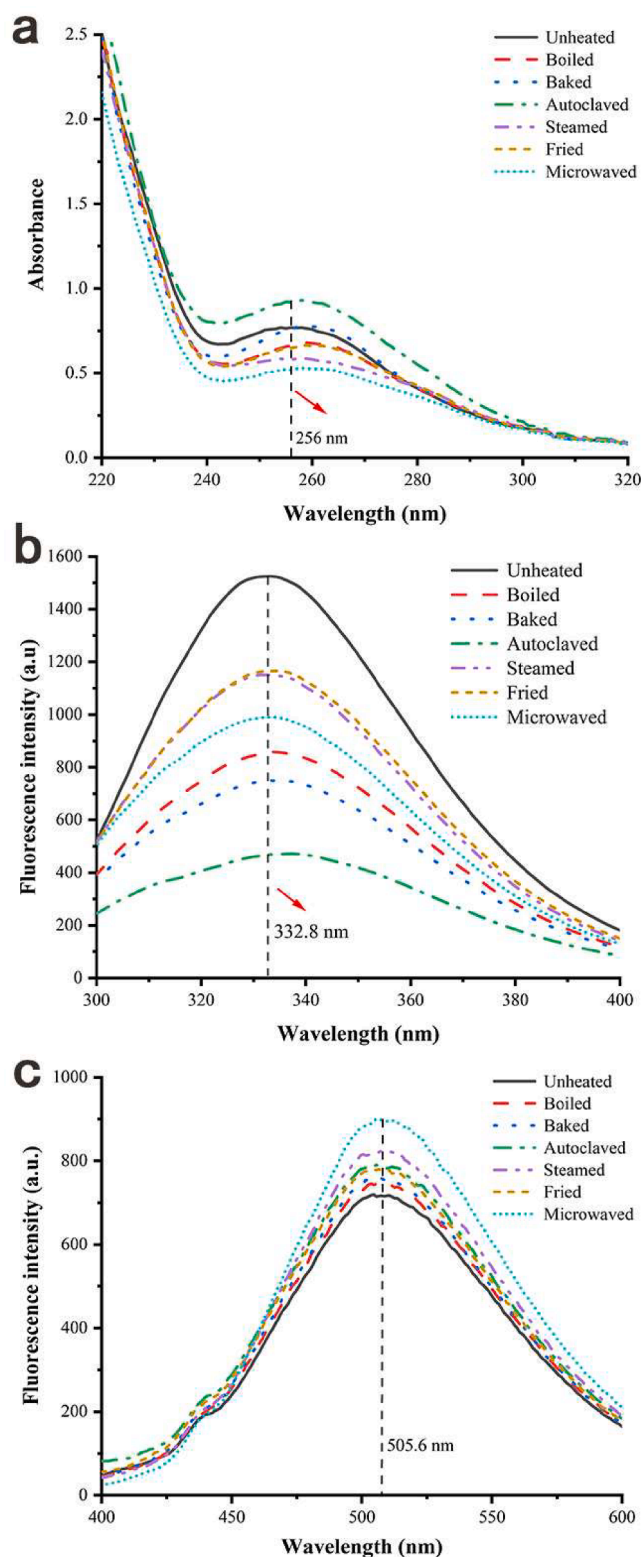


Fig. 6. UV spectra (a), intrinsic fluorescence spectra (b), and extrinsic fluorescence spectra (c) of shrimp soluble extracts with different thermal treatment.

Similarly reduced IgE binding was observed in the common household processed *Scylla paramamosain* muscle protein (Liu et al., 2018). Thermal processing may cause lower IgG/IgE binding because certain epitopes were disrupted and masked as a result of structural alterations and protein-chemical crosslinking in the shrimp matrix (Cabanillas & Novak, 2017). Microwaved shrimp soluble extracts with higher IgG binding

capacity might be due to increased IgG activity of TM and relatively stable IgG activity of SCP during microwaved treatment according to western blotting and indirect ELISA using rabbit antisera against raw shrimp extracts and TM.

Although the thermal treatments reduced the allergenic potential of shrimp, but the allergenicity cannot be completely eliminated. Significantly, TM from different heat-treated shrimp soluble extracts even posed higher IgG/IgE-binding ability, which increased by 13.86–34.39% of TM from raw shrimp according to anti-TM IgG WB and indirect ELISA, and the maximum enhancement of IgG-binding reactivity was found in microwaved shrimp extracts (Fig. 1c, Fig. 2b). Given that IgG/IgE binding to TM in the heated shrimps is even stronger than raw, we can argue that the working epitopes on TM are linear instead of conformational because the primary structure can tolerate with common thermal treatments. Additionally, TM is highly heat-stable due to α -helical coiled-coil structure. Structure alterations would cause enhanced structural flexibility and higher epitope accessibility after certain thermal treatment. It can promote the antigen-antibody interaction and enhancing IgG/IgE binding, thus led to stronger IgG/IgE reactivity (Gobl et al., 2017). Some available studies also indicated that TM from heat-treated shrimp might display stronger antibody binding reactivity. Liu et al. (2010) found that boiled shrimp extracts showed lower IgE binding than raw shrimp extracts, yet the TM purified from boiled shrimp displayed a stronger IgE-binding activity. Besides, heating (100 °C, 20 min) might elicit higher monoclonal antibodies (mAb) binding reactivity to TM from crustacean and some mollusk species was reported by Kamath et al. (2013).

Shrimp or shrimp-derived foods are usually consumed after processing, followed by gastrointestinal digestion with proteases, and the proteins are digested through the gastric and then the intestinal tracts (Yu et al., 2011). Resistance to gastrointestinal digestion is one of the major characteristics that allows food proteins to retain intact epitopes that have the potential to bind IgE and play a role in the sensitization (Liu et al., 2019; Moreno, 2007). Our results indicated that the digestive patterns of the SGF and SIF were different due to their different protease cleavage sites, and the digestive stability of TM was much higher than that of the other proteins in raw and treated shrimp soluble extracts. The results are in good agreement with some previous investigations of the *in vitro* digestibility of raw or proceed mud crab (*Scylla serrata*) crude extracts (Huang et al., 2010; Yu et al., 2011). The processed shrimp extracts showed a faster degradation both in SGF and SIF digestion process compared to raw shrimp soluble extracts (Fig. 3). Significantly, autoclaved shrimp extracts showed the fastest degradation, and the digested fraction was invisible within 30 min of SGF digestion and 20 min SIF digestion, respectively. Heat treatments could significantly enhance the digestibility of shrimp proteins, which might be due to the heat-induced structural alterations with exposure of more cleavage sites to make the protein more susceptible to proteolytic attack (Liu et al., 2019; Zhang et al., 2017).

Herein, structural analyses were further applied to explore the structural changes responsible for the IgG/IgE binding ability and *in vitro* digestibility. Raw and heated shrimp soluble extracts displayed two obvious negative bands around 208 and 222 nm in CD spectra (Fig. 4a), which were the main characteristics of the α -helix of protein, and a negative peak at around 218 nm was attributed to β -sheet contents (Greenfield, 2004; He et al., 2020). Compared to unheated shrimp extracts, a negative CD signal value near 208 and 222 nm was more obvious after various thermal treatments, suggesting that it might induce an increase in α -helix content of shrimp soluble extracts. The secondary structural alterations of shrimp subjected to heat treatments was further monitored by FT-IR (Fig. 4b), the left shift of the amide A band occurred in heated shrimp, suggesting reduced hydrophilicity of shrimp soluble extracts (Guan et al., 2018), which could be attributed to the protein structural alteration. Amides I, II, and III were applied to analyze structural changes of proteins in FT-IR spectra, which resulted from C—O stretching vibration (1700 and 1600 cm^{-1}), N—H bending

vibration (1600 and 1500 cm^{-1}), and C—N and N—H stretching vibration (1360 and 1200 cm^{-1}), respectively (Guan et al., 2018; Han et al., 2015). A mild blue shift in the amide I and red shift in amide II were found in the absorption peak of heated shrimp soluble extracts, which indicated the protein secondary structure alteration and rearrangement because of the damage and formation of hydrogen bonds during the thermal processing (Secundo & Guerrieri, 2005). The significantly increased absorption peak near 1157, 1080, 976, and 860 cm^{-1} were found, which might be attributed to the structural modification due to the numerous chemical interactions, such as other proteins, carbohydrates and lipid ingredients, that may be induced during processing in the complex matrices (Han et al., 2015; Liu et al., 2019). Among all the amides regions, the infrared amide I band ranging between 1700 and 1600 cm^{-1} was highly sensitive to the alterations of secondary structure elements in the protein. Whichever it has been applied in analyzing the protein secondary structural changes, and 1610–1638 cm^{-1} attributed to β -sheet, 1638–1648 cm^{-1} attributed to the random coil, 1649–1660 cm^{-1} belonged to α -helix, and 1660–1692 cm^{-1} belonged to β -turn structures (Guan et al., 2018; Han et al., 2015; He et al., 2021). The curve-fitted spectra of the infrared amide I band suggested that thermal treatments could cause an obvious increase in the α -helix content. Of which the value was increased to 36.18% in boiled shrimp extracts, 31.73% in baked shrimp extracts, 34.04% in autoclaved shrimp extracts, 32.58% in steamed shrimp extracts, 32.28% in fried shrimp extracts, and 31.02% microwaved shrimp extracts, while the β -sheet content was decreased significantly, and insignificant alteration in β -sheet and random coil contents were observed (Fig. 5). The obtained results were also consistent with the data of CD. Native TM showed a characteristic α -helical coiled coil structure, with nearly 100% α -helix along entire sequence. Heat-induced structural alterations and higher TM content in heated shrimp soluble extracts in relative to unheated shrimp soluble extracts, which may together contribute to increased α -helix content. In combination with the experimental results obtained, these structural alterations might cause the unfolding of proteins, and then IgG/IgE epitopes might be easier to be destroyed and modified with the exposure of more proteolytic cleavage sites, resulting in the reduction of IgG/IgE binding ability and *in vitro* digestibility (Guan et al., 2018; Liu et al., 2019; Zhao et al., 2019).

Furthermore, structural changes of heated shrimp extract were also determined by alterations in UV absorption spectra, intrinsic and extrinsic fluorescence spectra (Fig. 6). The maximum UV absorption peak of raw shrimp extracts was found at around 256 nm (Fig. 6a). In addition, a slight red shift of the absorption peak with decreased UV absorption intensity was found in heated shrimp extracts, suggesting that more hydrophobic amino acid exposed on the surface of protein resulting in the unfolding of the proteins, especially in autoclaved shrimp extracts (Lin et al., 2015). There was a mild red shift in the maximum emission with a considerable decrease in intrinsic fluorescence intensity of heat-treated shrimp extracts (Fig. 6b). This indicated that the hydrophobic amino acids located in the protein interior might gradually move to the polar regions of the protein surface due to thermal treatments (Zhao et al., 2019), which also could be confirmed by the increase of extrinsic fluorescence intensity (Fig. 6c). These hydrophobic regions seemed unlikely to be epitopes of allergens based on the basic role of epitopes forming (Zheng et al., 2011). Additionally, the exposure of more hydrophobic amino acid residues depending on the extent and type of heating applied may mask the epitopes due to the unfolding of proteins, which might cause shrimp allergens unrecognized by IgG/IgE antibodies. The above structure alterations can reflect reduced IgG/IgE-binding capacity, which also correlated well with structural changes, as well as *in vitro* digestibility.

5. Conclusions

Taken together, the effects of common thermal processing methods on the potential allergenicity and *in vitro* digestion stability of shrimp

edible portions were characterized in the study. Thermal processing could significantly reduce the protein recovery and IgG/IgE binding and improved the digestibility of shrimp muscle extracts. The degree of reduced protein amounts and IgG/IgE activity differed, depending on the extent and type of heating applied, and the maximum reduction of IgG/IgE binding was observed in the autoclaved treatments. However, thermal processed shrimp extracts still posed IgG/IgE binding, which is mainly because of heat resistance of TM and SCP, especially TM. Notably, the TM from heat-processed shrimp soluble extracts displayed higher IgG binding. Since some protein fractions became insolubilized during heating, future studies should focus on examining the effects of heat processing methods on the structure and allergenicity of insoluble protein and promote shrimp thermal processing.

CRediT authorship contribution statement

Jinlong Zhao: Investigation, Methodology, Writing – original draft. **Yonghong Li:** Validation, Resources. **Lili Xu:** Validation, Resources. **Yu Ji:** Validation, Resources. **Jianhua Zeng:** Validation, Resources. **Vai-leth Timira:** Writing – review & editing, Formal analysis, Validation, Resources. **Ziye Zhang:** Validation, Resources. **Guanzhi Chen:** Methodology. **Hong Lin:** Data curation, Supervision. **Zhenxing Li:** Conceptualization, Methodology, Writing – review & editing, Supervision, Funding acquisition, Data curation, Supervision.

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.2022.132177>.

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