



Influence of nonthermal extraction technique and allergenicity characteristics of tropomyosin from fish (*Larimichthys crocea*) in comparison with shrimp (*Litopenaeus vannamei*) and clam (*Ruditapes philippinarum*)

Xu Li Li^a, Lin Hong^a, Li Zhen Xing^{a,*}, Ahmed Ishfaq^a, Pramod S.N.^b, Lin Hang^c, Lv Liang Tao^a, Tian Sheng Lan^a, Yu Zhi Wen^a

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

^b Department of Studies in Biochemistry, Sahyadri Science College, Kuvempu University, Shimoga 577203, Karnataka 560037, India

^c The Affiliated Hospital of Medical College Qingdao University, Qingdao, Shandong Province 266071, PR China

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ABSTRACT

Recent reports showed that patients could be sensitized to fish tropomyosin (TM), who exhibited clinical symptoms. However, little information is available on differences in TM immune cross-reactivity among fish, shrimp and clam. Moreover, allergenicity might change during the food processing owing to the change of protein structure. In this study, we developed a nonthermal extraction technique to purified TM, IgG/IgE binding, cross-reactivity and structures were compared. Results showed that raw and boiled fish-TM were not cross reactive and had weak recognition of shrimp, while, shrimp-TM and clam-TM were cross reactive. The ELISA further confirmed that fish-TM was not able to trigger allergic immune response in shrimp sensitive subjects, while, surface hydrophobicity of fish-TM was higher. The study demonstrated that fish-TM, being with high sequence similarity, did not have cross-reactivity with shrimp and clam-TM. They could have a variable degree of IgE binding depending on subject sensitivity and allergenicity.

1. Introduction

Fish, belonging to vertebrate, is a popular dietary protein source with the delicate flavor and high nutritive value (Sicherer & Sampson, 2018). However, as one of the most frequent causes of food allergy, fish is responsible for triggering symptoms, such as urticaria, asthma, diarrhea, and even life-threatening anaphylaxis in sensitized individuals (Faber et al., 2017; Hu et al., 2017; Peixoto et al., 2017). Parvalbumin (10–12 kDa) identified as the major fish allergen is a heat-stable and intracellular EF-hand calcium-binding protein (Kalic et al., 2018). Meanwhile, tropomyosin (TM), generally refers as the major component of the myofibril is a heterodimer; composed of two identically coiled subunits with molecular masses of 35 to 38 kDa and has been known as the major allergens in crustacean and mollusk species (Alessandri et al., 2017; Peixoto et al., 2017; Rolland et al., 2018).

Typically, apart from invertebrate TM, vertebrate TM shares about 55% of sequence homology and have been considered non-allergenic protein except the fish species of *Oreochromis Mossambicus* (Cosme & Barbosa, 2016; Prester, 2016). However, recently in some cases, an immunoassay with patient serum showed cross-reactivity between 36

and 37 kDa TM from fish (hake and cod) and shrimp (Peixoto et al., 2017). Meanwhile, a patient with a history of house dust mite and shellfish allergy also showed sensitivity to fish-TM with clinical symptoms after fish ingestion (Verhoeckx et al., 2015).

Though TM is a type of heat-stable protein, food processing like boiling, cooking or grilling, may alter its allergenicity (Cai, Zhang, Zhu, & Chen, 2016; Fang, Li, Gu, Cai, & Lu, 2018; Kamath et al., 2016; Song, Li, Gao, Pavase, & Lin, 2016; Zhang, Zhang, Sheng, Wang, & Fu, 2016). It can either aggravate or mitigate allergic reactions among sensitized individuals (Liu et al., 2010; Mattison et al., 2014; Peyron, Mouecoucou, Fremont, Sanchez, & N., G, 2006). Different processing methods can lead to structural changes, mostly by altering the epitope structure, which might change the allergenic response to protein (Blanc et al., 2011; Broekman et al., 2015; Fei et al., 2016; Mattison et al., 2014; Zhang, Zhu, Zhang, Cai, & Chen, 2016). Earlier studies have shown that heating has a profound effect on the TM of crustaceans and mollusks by using specific monoclonal antibodies (Kamath, Abdel Rahman, Komoda, & Lopata, 2013). Another study identified that raw or boiled extracts of shrimps, American lobster, and spiny lobsters have shown significantly different responses by skin prick test reactions as

* Corresponding author.

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

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well as specific IgE levels (Carnes et al., 2007).

Currently, some studies provide evidence on the allergenicity of TM among the seafood, including vertebrate, crustacean and mollusk using different extraction methods (Carnes et al., 2007). However, it is essential to find the key structural information of TM, which is responsible for the huge difference in their allergenicity in different TM from different species of seafood. Besides, some people were interested in raw diets, such as *Oncorhynchus*, *Pleoticus muelleri*, *Octopodidae*, etc. The sensitization pattern and structural differences between raw and processed food should also be considered for evaluation to understand the extent of sensitization and cross-reactivity of TM in inducing allergenicity.

Thus, this study is aimed at investigating the structural differences of TM and their possible allergenic cross-reactivity among fish (*Larimichthys crocea*), shrimp (*Litopenaeus vannamei*) and clam (*Ruditapes philippinarum*) extracted from raw and boiled samples. In this paper, we compared the IgG/IgE binding and structures of raw and boiled fish-TM, shrimp-TM and clam-TM to understand immunogenic and allergic potential. The observations during this study will give an insight into the possible structural variations in raw and boiled TM and the ability to induce cross reactive allergic reactions among fish-TM, shrimp-TM and clam-TM. Therefore, this study will provide a new understanding of TM induced allergenic cross-reactivity among fish, shrimp and clam. It will also be helpful to delineate the implications TM in processed foods, to predict clinical sensitivity and allergenic reactivity among subjects with shrimp allergy.

2. Materials and methods

2.1. Materials

The anti-rabbit IgG obtained as purified TM specific IgG from shrimp-TM immunized rabbit sera using protein A sepharose column. Horseradish peroxidase (HRP) conjugated goat anti-rabbit IgG antibody or goat anti-human IgE antibody was purchased from Zhongshan Jinqiao Co. (Beijing, China). The enhanced chemiluminescent kit for Western blot analysis was obtained from Thermo Fisher Scientific Inc. (Waltham, USA). All other reagents used in this study were of analytical grade.

2.2. Human sera

Clinically relevant sera from shrimp-allergic patients with a documented clinical history of shrimp allergy were obtained from the Affiliated Hospital of Medical College Qingdao University (Qingdao, China) (Table 1). The specific IgE levels of shrimp were assessed *in vivo* (skin prick test) or *in vitro* (serum immunoassay) using Phadia® 100 & IDM (Thermo Fisher Scientific, Waltham, MA, USA). The sera were kept at -80°C before analysis. The allergic sera were obtained after taking the consent signed by the subjects for the study. The study protocol was approved by the medical ethics committee.

2.3. Purification and identification of tropomyosin

TM from fish, shrimp and clam were purified by a combination of ammonium sulfate and isoelectric precipitation (Bailey, 1948; Lasekan & Nayak, 2016; Lin et al., 2015), and all procedures were carried out at $0-4^{\circ}\text{C}$ unless otherwise stated, as shown in Fig. 1A.

For fish and shrimp, the preliminary treatments were almost the same. The minced muscle (raw sample) was homogenized in 10-fold Buffer A (50 mM KCl containing 2 mM NaHCO_3) for at least 4 h, followed by centrifugation at 1300g for 20 min. The procedure was then suspended in Buffer A for about 2 h and centrifuged under the same conditions. The contents were washed by cold absolute acetone and dried completely under room temperature.

For clam, the edible part was minced and homogenized in 20 mM

Table 1

Characterization of sera from shrimp-allergic patients.

No.	Serum no.	Age (years)	Sex ^a	Symptoms ^a	Specific IgE (kUA/L)
1	3002	3	F	U	0.96
2	3004	6	M	Dr, P	2.17
3	3008	3	M	U, P	1.75
4	3009	4	F	A, Dr	1.08
5	3011	5	F	Dr, P	0.67
6	3014	57	F	A	0.56
7	3015	22	F	C	1.66
8	3016	62	M	Dr	2.71
9	4002	52	M	P, C	0.58
10	4005	27	M	A, C	1.46
11	4006	24	F	U, P	2.37
12	4009	35	M	Dr	0.78
13	4010	61	M	ND	0.01
14	4012	21	F	ND	0.01
15	4013	25	M	ND	0.00

^a F, female; M, male; A, asthma; C, cheilitis; Dr, dermatitis; P, pruritus; U, urticaria; ND, not determined. A serum with specific IgE > 0.35 (kUA/L) is defined as positive.

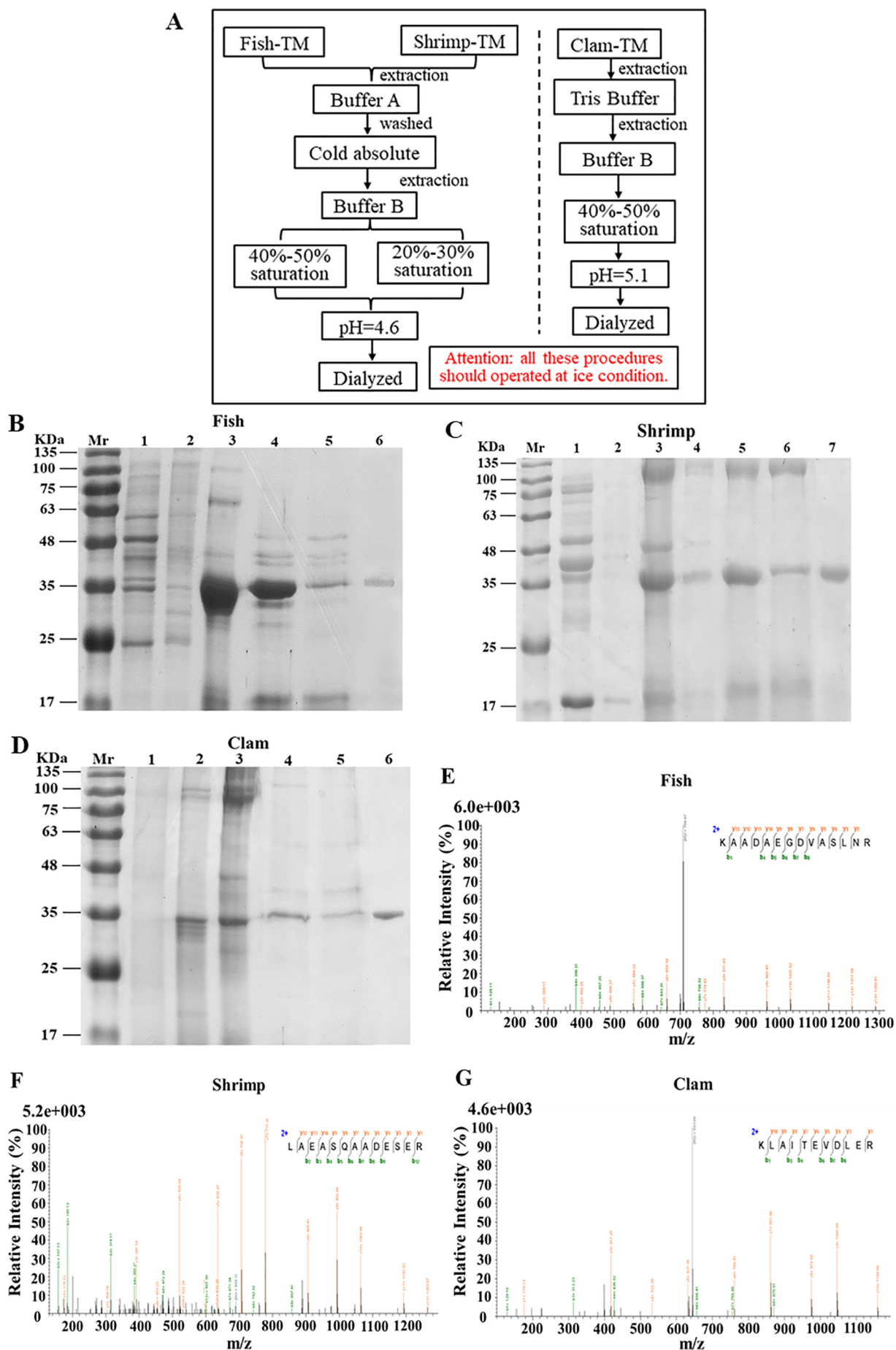
Tris-HCl buffer (1:2, w/v), pH 7.40 for more than 4 h. After centrifugation (1300g, 20 min), the content was suspended in Tris-HCl buffer for nearly 2 h and then centrifuged again, followed by the collection of the precipitates.

The fish or shrimp dried powder, as well as the clam precipitate, were all suspended in Buffer B (1:7 w/v) respectively, with pH adjusted to 7.40, containing 0.02 M Tris-HCl, 1 M KCl and 0.1 mM DTT. Extraction was carried out with stirring overnight. The mixture was then centrifuged at 2500g for 30 min and the extract was fractionated with ammonium sulfate from 40% to 50% saturation for fish, as for shrimp, the saturated ammonium sulfate was 20–30% and 40–50% ammonium sulfate saturation for clam. Then the precipitate was dissolved in 0.01 M PBS, while the supernatant was subjected to isoelectric precipitation at pH 4.6 (for fish or shrimp) or pH 5.1 (for clam) with 1 M HCl and then centrifuged at 2500g for 20 min. The precipitation was dissolved in 0.01 M PBS (pH 7.40) and then dialyzed in the same solution overnight to obtain raw TM. The dialyzed solution was heated in a water bath at boiling temperature for 10 min to obtain boiled TM. Both the raw and boiled TM were stored at -20°C until used. The target protein was migrated on the SDS-PAGE gel and followed by cutting the band from the gel and sent to Beijing Protein Institute (Beijing, China) and identification by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS), which was based on the method as described by Zhang et al. (2015). The resulted peptide spectra were searched by using the National Center for Biotechnology Information (NCBI).

2.4. SDS-PAGE and western blot analysis

Proteins were analyzed by SDS-PAGE using SurePAGE gradient gel (Genscript, New Jersey, U.S.A.) from 8% to 16% (1.0 mm) (Ahmed et al., 2018). Electrophoresis was performed at 120 V for 45 min under Tris-MOPS-SDS Running Buffer condition. The gel was placed into Coomassie Brilliant Blue R-250 (Solarbio, Beijing, China) stain solution for 1 h and then destained until the solution clear and then photographed.

Western blotting was used to evaluate the IgG binding, according to the method described as before (Lv et al., 2017). The proteins were transferred with iBlot™ gel transfer stacks (Thermo Fisher Scientific, Massachusetts, U.S.A.) to a PVDF membrane and then the membrane was incubated with IgG (rabbit serum, diluted 1:2000). While, HRP-labeled goat anti-rabbit IgG (diluted 1:2000) was used as the secondary antibodies and incubated using iBlot™ (Thermo Fisher Scientific, Massachusetts, U.S.A.) for 8 h at room temperature. Images were captured using Tanon-4200SF (Tanon Science & Technology Co., Ltd, Shanghai,



(caption on next page)

Fig. 1. Nonthermal extraction and purification analysis of fish-TM, shrimp-TM and clam-TM. (A) Nonthermal extraction and purification steps of fish-TM, shrimp-TM and clam-TM. (B) Raw fish-TM extraction. Line 1-the first extracts in Buffer A (50 mM KCl and 2 mM NaHCO₃), Line 2-the second extracts in Buffer A, Line 3-extracts in Buffer B (0.02 M Tris-HCl, 1 M KCl and 0.1 mM DTT), Line 4-40–50% ammonium sulfate cut, Line 5-isoelectric point cut (pH = 4.65), Line 6-the final purified raw fish-TM, Line Mr: protein marker. (C) Raw shrimp-TM extraction. Line 1-the first extracts in Buffer A (50 mM KCl and 2 mM NaHCO₃), Line 2-the second extracts in Buffer A, Line 3-the first extracts in Buffer B (0.02 M Tris-HCl, 1 M KCl and 0.1 mM DTT), Line 4-the second extracts in Buffer B, Line 5-20–30% ammonium sulfate cut, Line 6-isoelectric point cut (pH = 4.60), Line 7-the final purified raw shrimp-TM, Line Mr: protein marker. (D) Raw clam-TM extraction. Line 1-extracts in Tris Buffer (20 mM Tris-HCl), Line 2-extracts in Buffer B (0.02 M Tris-HCl, 1 M KCl and 0.1 mM DTT), Line 3-40–50% ammonium sulfate cut, Line 4-isoelectric point cut (pH = 5.10), Line 5-the final purified raw clam-TM, Line Mr: protein marker. (E) MS/MS of fish-TM. (F) MS/MS of shrimp-TM. (G) MS/MS of clam-TM.

China) using an ECL substrate.

2.5. ELISA and inhibition ELISA

ELISA and inhibition ELISA were carried out following the method described previously (Kamath et al., 2013; Lee, Lee, Han, & Oh, 2017). Briefly, for ELISA assay, 5 µg of raw and boiled fish-TM, shrimp-TM and clam-TM were placed overnight at 4 °C. For inhibition ELISA, 5 µg raw shrimp-TM was placed and the anti-rabbit IgG (diluted 1:2000) or human serum (diluted 1:4) was mixed with increasing concentration of inhibitors (raw and boiled fish-TM, shrimp-TM and clam-TM) for pre-incubation. Then, for both ELISA and inhibition ELISA, the anti-rabbit IgG (diluted 1:2000) or human serum (diluted 1:4) was used as the primary antibody after blocking the plate with BSA. Subsequently, the HRP-conjugated goat anti-rabbit IgG (diluted 1:2000) or HRP-conjugated goat anti-human IgE (diluted 1:2000) acted as the secondary antibody.

2.6. CD spectroscopy

Purified raw and boiled TM samples (adjusted to 0.5 mg/mL with 0.01 M PBS) were analyzed by far-UV CD (190–260 nm) on a Jasco-810 spectropolarimeter (JASCO, Japan Spectroscopic Co. Inc., Tokyo, Japan) at room temperature according to a method described previously (Lv et al., 2016). The samples were scanned three times at a 50 nm/min at a bandwidth of 1 nm with 3 s response time. The data were averaged with the molar residue ellipticity, expressing as deg cm²/dmol.

2.7. UV absorption and intrinsic fluorescence spectral analyses

The UV absorption spectra of raw and boiled samples (0.5 mg/mL) were scanned from 230 to 330 nm with the UV-1101 system (Techcomp, Shanghai, China). The intrinsic fluorescence of all samples in 0.01 M PBS (pH 7.40) was measured using an RF-5301PC fluorescence spectrometer (Shimadzu, Kyoto, Japan), as reported previously (Wu, Wu, & Hu, 2010).

2.8. Surface hydrophobicity

The surface hydrophobicity of proteins was determined using *N*-Phenyl-8-naphthylamine-1-sulfonic acid (ANS) as described earlier (Lv et al., 2017). The samples were dissolved in PBS (0.01 M, pH 7.4) at different concentration from 0.01 to 0.5 mg/mL. Next, 5 µl of ANS was added to each sample (1 mL) and reacted for 15 min in dark. The operating parameters of an F-4600 fluorescence spectrophotometer

(Hitachi, Tokyo, Japan) were as follows: excitation wavelength/emission wavelength, 365 nm/484 nm; slit width, 5.0 nm; scanning speed, 1200 nm/s.

2.9. Statistical analysis

Statistical analyses and figures were performed using SPSS 22.0 (IBM Corporation, Armonk, New York, U.S.A.) and Origin 8 (Origin Lab, Northampton, MA, U.S.A.), respectively. Significant differences were calculated using a one-way analysis of variance (ANOVA) with a significance level of 5% ($p < 0.05$). The experiments were conducted for triplicate and reported as the means $\pm$ SD ($n = 3$).

3. Results

3.1. Establishment of a nonthermal extraction technique of fish-TM, shrimp-TM and clam-TM

TM proteins were purified by using ammonium sulfate precipitation and isoelectric precipitation with the nonthermal extraction technique. While, the protein components of the crude extracts from fish (Fig. 1B, lane 1), shrimp (Fig. 1C, lane 1) and clam (Fig. 1D, lane 1) were analyzed by SDS-PAGE, respectively. Then TM was further extracted with Buffer B (0.02 M Tris-HCl, 1 M KCl and 0.1 mM DTT) with the pH value 7.40 overnight at 4 °C, to obtain more protein content solution, and the process was repeated twice. Following that, the supernatant was deal with 40% (fish), 20% (shrimp) and 40% (clam) ammonium sulfate fractionation as well as one or two times of isoelectric precipitation to acquire purified TM (> 90%), which were expressed as single bands with a 35 kDa molecular weight, as observed in Fig. 1B, lane 6 (fish-TM), Fig. 1C, lane 7 (shrimp-TM) and Fig. 1D, lane 5 (clam-TM), respectively. All these procedures were operated at a low-temperature condition.

The purified 35 kDa proteins were excised from the gel, destained, digested with trypsin and then analyzed with MALDI-TOF-MS. Peptide mass fingerprinting of the purified proteins showed multiple peaks ranging from 100 to 1300 Da (Fig. 1E), 100 to 1280 Da (Fig. 1F) and 100 to 1180 Da (Fig. 1G), respectively and then the effective peaks were compared with the NCBI database. As shown in Table 2, the mascot search results showed that proteins listed with a high score, confirming the molecular weights of about 35 kDa purified proteins as fish-TM, shrimp-TM and clam-TM.

Table 2

Mascot search results of fish-TM, shrimp-TM and clam-TM.

GenBank	Protein description	Molecular weight/D	Score	Matches	Sequence
XP_019127831.1	<i>Larimichthys crocea</i>	32,694	3855	255 (131)	25 (22)
ACB38288.1	<i>Litopenaeus vannamei</i>	32,830	6372	255 (233)	26 (26)
BAH10157.1	<i>Ruditapes philippinarum</i>	32,690	6737	239 (221)	29 (28)

Matches stand for spectrogram number and the total number was outside the bracket, the match number was inside the bracket; Sequences mean sequence number and the total number was out the bracket, match number was in the bracket.

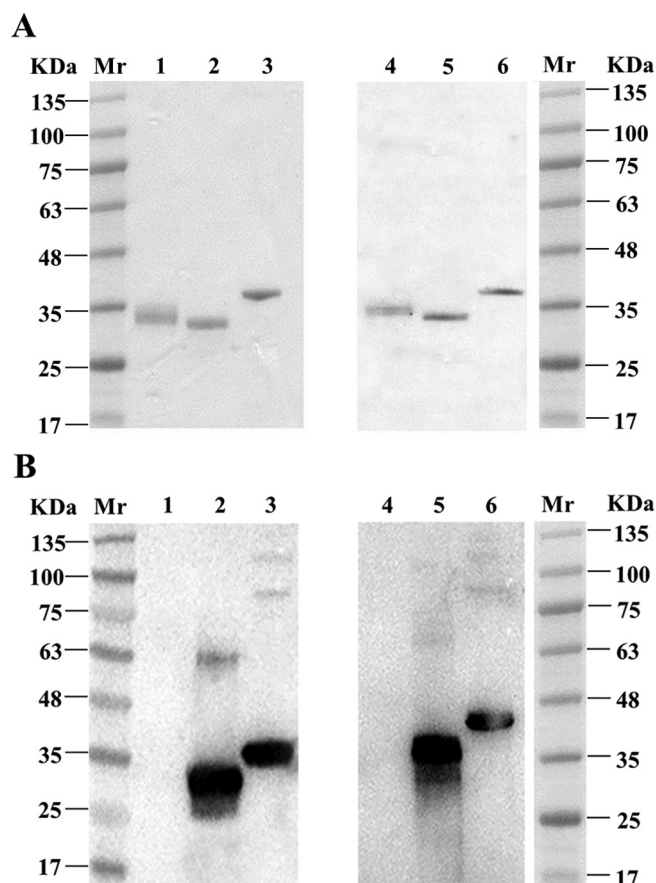


Fig. 2. SDS-PAGE & Western blot analysis of raw and boiled fish-TM, shrimp-TM and clam-TM. (A) SDS-PAGE. 1.5 μ g protein content for each band; (B) IgG-immunoblot. Lane1: raw fish, Line 2-raw shrimp, Line 3-raw clam, Line 4-boiled fish, Line 5-boiled shrimp, Line 6-boiled clam, Line Mr: protein marker.

3.2. SDS-PAGE and western blot analysis of raw/boiled fish-TM, shrimp-TM and clam-TM

Raw and boiled TM were extracted and purified from fish, shrimp and clam using ammonium sulfate and isoelectric precipitation. They were found homogenous with high purity (> 95%) and were run as single bands around 35 kDa molecular weight when observed by SDS-PAGE (Fig. 2A). Moreover, no noticeable variations were observed between raw and boiled TM. The fish-TM, shrimp-TM and clam-TM were subsequently evaluated for IgG immunoblot (Fig. 2B). The clam-TM was recognized by the sera of shrimp anti-IgG with > 60% binding intensity, where the fish-TM was not, indicating that clam-TM has observable cross-reactivity and shared common epitopes with shrimp-TM. The results of IgG immunoblot showed no variation between raw and boiled TM.

3.3. Shrimp allergic subjects and IgE binding to purified TM

Shrimp allergic sera with described allergic symptoms after the ingestion of shrimp were collected. The sera include 8 male and 7 female subjects, aged between 3 and 62 years with the levels of shrimp TM specific IgE ranged from 0.58 to 2.71 kUA/L (Table 1). These sera showed high IgE binding to shrimp-TM and clam-TM and all other sera were observed to have marginal specific IgE to shrimp, clam and fish TM (Fig. 3A). Compared to shrimp-TM, clam-TM showed 50–70% binding, where the shrimp-TM had only 20–30% variation in IgE binding between raw and boiled TM.

In addition, The shrimp-TM and clam-TM had strong binding to the

sera from subjects 1–12, much weaker binding to serum 13, 14 and 15, where the levels of shrimp-TM specific IgE were from 0.58 to 2.71, and 0.00–0.01 kUA/L, respectively, and specific IgE > 0.35 kUA/L defined as a positive subject. The absorbances of fish-TM, shrimp-TM and clam-TM were between 0.21–0.49, 0.21–1.43 and 0.21–1.37, respectively, indicated that fish-TM had weak IgE binding compared with that of shrimp-TM and clam-TM. While, shrimp-TM and clam-TM retained higher cross-activity, appearing to be similar results as western blot. Meanwhile, there was no overall difference between raw and boiled TM. The results of different sera showed the difference between raw and boiled TM. Therefore, much more individual serum should be collected for the evaluated of the IgE binding ability.

3.4. Analysis of the IgG/IgE binding of raw/boiled fish-TM, shrimp-TM and clam-TM

The IgG/IgE binding of raw/boiled TM was determined by ELISA and inhibition ELISA with rabbit antibodies and pooled human sera. IgG binding to raw and boiled shrimp-TM was 1.43 and 1.40, respectively, with no significant difference. However, the clam-TM showed absorbance of 0.8 (raw) and 1.16 (boiled), indicating a high recognition of shrimp-TM specific IgG on boiling compared to raw TM (Fig. 3B). The fish-TM had significantly 70% less recognition of IgG and demonstrated no difference between raw and boiled TM. In the case of binding to human IgE, shrimp showed high specificity with no difference between raw and boiled TM. The clam-TM had almost equal IgE recognition as compared to shrimp-TM and the fish-TM has very marginal and was equal to negative control BSA (Fig. 3C). This indicated the shrimp-TM and clam-TM share IgE epitopes and had a high chance of cross-reactivity as compared to fish-TM.

Additionally, during the inhibition ELISA, the IgG binding was decreased markedly when the concentration of shrimp-TM and clam-TM were increased from 0.001 μ g/mL to 1 μ g/mL, whereas no significant increase was found with fish-TM. The percent maximum inhibition of shrimp-TM and clam-TM were 76.3% (raw shrimp), 72.2% (boiled shrimp), 74.1% (raw clam) and 71.3% (boiled clam), respectively (Fig. 3D). The observed IgG inhibition implied that there was high cross-reactivity between shrimp-TM and clam-TM for IgG binding and exhibit no differences whether the TM were boiled or not.

Moreover, the same trend for IgE binding was seen as that of IgG for both raw and boiled samples, that could speculate the same conclusions. The maximum percent inhibition of fish-TM, shrimp-TM and clam-TM for shrimp allergic sera were 24.4% (raw fish), 21.9% (boiled fish), 65.5% (raw shrimp), 66.1% (boiled shrimp), 63.9% (raw clam) and 61.4% (boiled clam), respectively. The cross-reactivity of the shrimp-TM to clam-TM was 97.6% (for raw TM) and 92.9% (for boiled TM), respectively (Fig. 3E). The fish-TM was not cross-reactivity and had shown no inhibition to shrimp-TM IgE binding. The results of IgG/IgE indicate the high possible cross-reactivity with shrimp-TM and clam-TM and no observed IgE recognition was seen with fish-TM. It also appeared that a small degree of difference in IgE binding was observed between raw and boiled TM of clam without any significant difference in the IgG binding between raw and boiled TM.

3.5. Structural analysis of raw/boiled fish-TM, shrimp-TM and clam-TM

The protein structures were analyzed by CD spectra, UV spectra, intrinsic fluorescence spectra and surface hydrophobicity assay. TM, which are typical α -helical secondary structures, expressed no significant difference in α -helices, β -sheet, β -turn and random coil after boiling (Fig. 4A). Then the structure was observed to show alterations in UV absorption and second-derivative UV absorption. The maximum UV absorbance peaks of raw/boiled fish-TM, raw/boiled shrimp-TM and raw/boiled clam-TM were 216.5 nm/215.5 nm, 207.0 nm/206.5 nm and 214.5 nm/214.5 nm, respectively, showing no significant difference between raw and boiled samples (Fig. 4B, C). An intrinsic

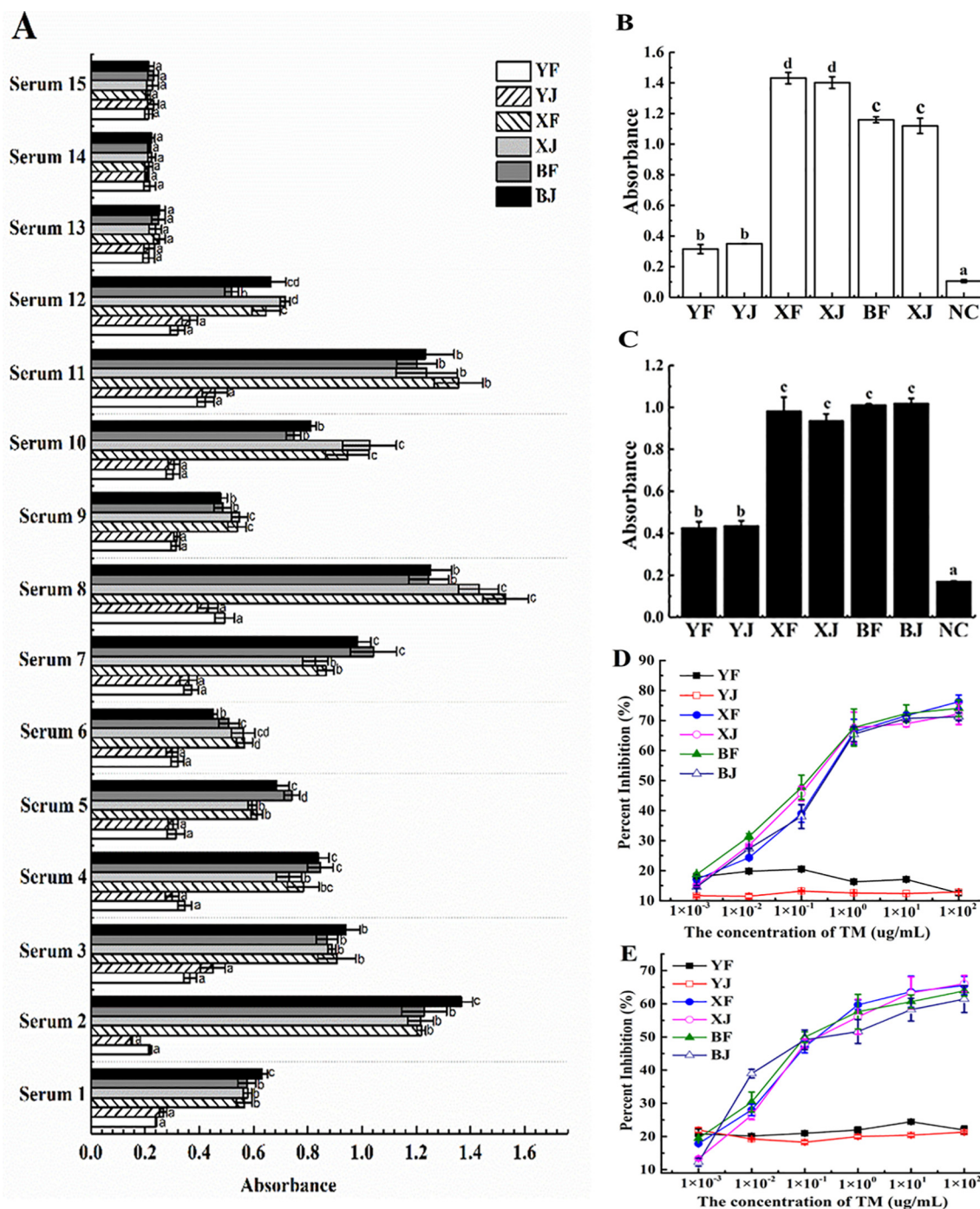


Fig. 3. ELISA and inhibition-ELISA for IgG/IgE binding raw and boiled fish-TM, shrimp-TM and clam-TM. (A) ELISA assay of individual serum. Serum Nos. 1–12 and 13–15 were used as the negative control. (B, C) IgG-ELISA and IgE-ELISA. 5 μ g raw and boiled fish-TM, shrimp-TM and clam-TM were placed. Pooled sera were used for IgE-ELISA assay. (D, E) IgG-inhibition ELISA and IgE-inhibition ELISA. 5 μ g raw shrimp-TM was placed. Pooled sera were used for IgE-inhibition ELISA assay. YF: raw fish-TM group; YJ: boiled fish-TM group; XF: raw shrimp-TM group; XJ: boiled shrimp-TM group; BF: raw clam-TM group; BJ: boiled clam-TM group; NC: negative control. The data represent the mean $\pm$ SD ($n = 3$), while the different letter stands for significant difference ($p < 0.05$).

fluorescence spectrum was used to assess the structural changes. The wavelength of the maximum absorption peaks for raw and boiled fish-TM, shrimp-TM and clam-TM were 341.6 nm/332.2 nm, 334.2 nm/330.6 nm and 342.0 nm/340.8 nm, respectively (Fig. 4D). Thus, only a slight blue shift in the maximum emission wavelength was noticed between the TM. According to the results of surface hydrophobicity (Fig. 4E), there was a significant difference among vertebrate-fish, crustacean-shrimp and mollusk-clam. The surface hydrophobicity of fish-TM was 2123.1, which was 2.63 times and 1.71 times higher than

that of shrimp-TM (806.8) and clam-TM (124.0). Additionally, raw TM showed significantly higher levels of hydrophobicity compared to boiled TM with a marked decline of 80.0, 21.4 and 59.2% for fish-TM, shrimp-TM and clam-TM, respectively.

4. Discussion

In the present work, most researchers extracted and purified protein via extracting crude protein solution coupled with chromatographic

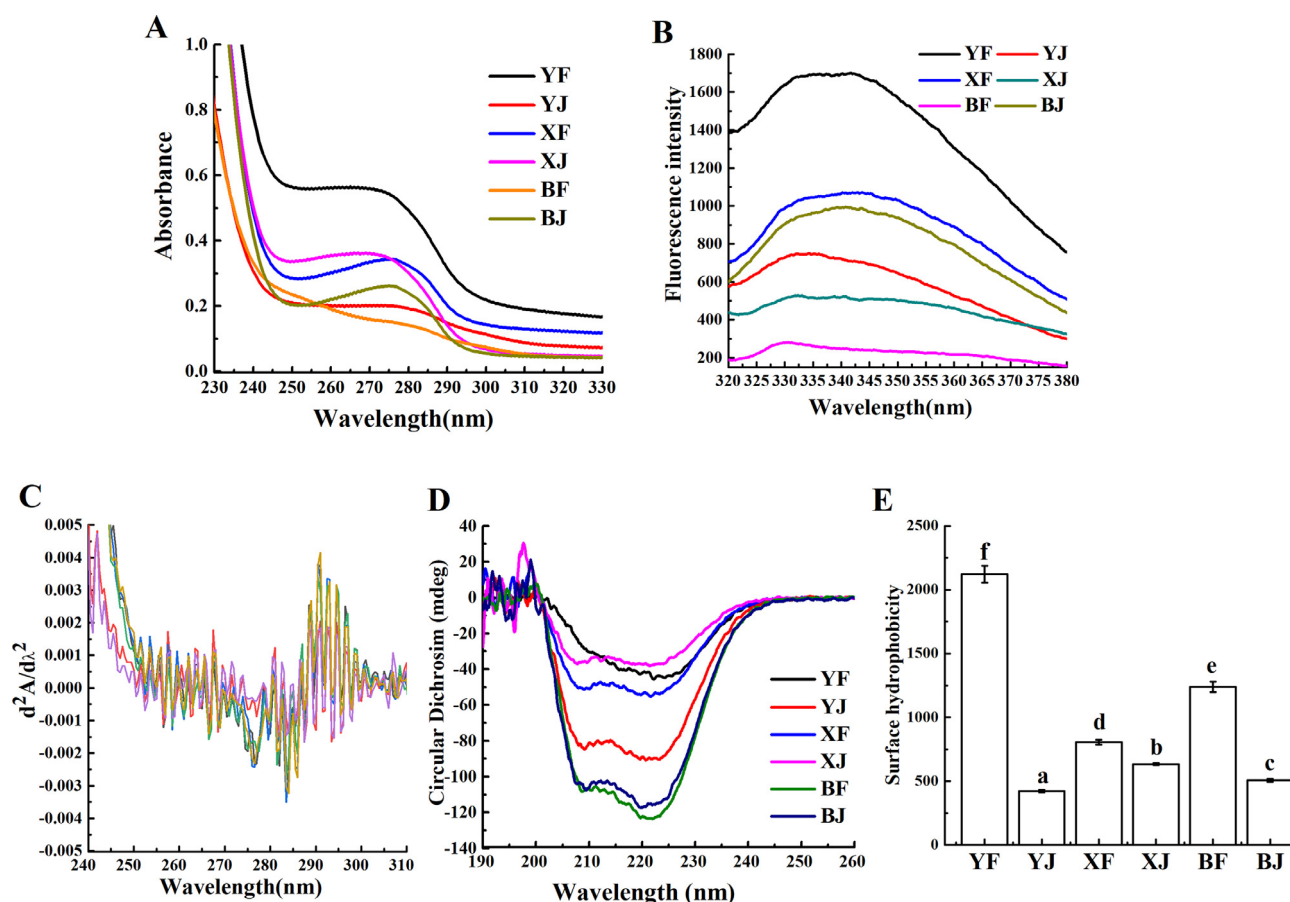


Fig. 4. Structure of raw and boiled fish-TM, shrimp-TM and clam-TM. (A) CD spectra. The measurements of raw and boiled fish-TM, shrimp-TM and clam-TM in 0.01 M PBS (pH 7.40) with the concentration of 0.5 mg/mL for CD spectra. (B, C) UV spectra and second-derivative UV spectra. (B) UV spectra; (C) Second-derivative UV spectra. The measurements of raw and boiled fish-TM, shrimp-TM and clam-TM in 0.01 M PBS (pH 7.40) with the concentration of 0.5 mg/mL for UV spectra. (D) Intrinsic fluorescence spectra. The measurements of raw and boiled fish-TM, shrimp-TM and clam-TM in 0.01 M PBS (pH 7.40) with the concentration of 0.5 mg/mL for intrinsic fluorescence spectra. (E) surface hydrophobicity analysis. The samples were dissolved in PBS (0.01 M, pH 7.40) at different concentration from 0.01 to 0.5 mg/mL. YF: raw fish-TM group; YJ: boiled fish-TM group; XF: raw shrimp-TM group; XJ: boiled shrimp-TM group; BF: raw clam-TM group; BJ: boiled clam-TM group. The data represent the mean $\pm$ SD ($n = 3$), while the different letter stands for significant difference ($p < 0.05$).

technique or boiling the crude protein solution binding ammonium sulfate methods to obtain purified protein (Ahmed et al., 2018; Hu et al., 2017; Kamath et al., 2013). Previous allergen sensitization assays have mainly focused on using raw/unprocessed allergen as an antigen to produce monoclonal/multi-clone antibody or sensitize cells, whereas, in most cases, processed proteins were used in antigen-antibody binding assays or allergen stimulating tests (Lin et al., 2015; Liu et al., 2017). Owing to the allergen, conducting with different treatment methods, was used in the whole allergic reaction, including the sensitization stage and excitation stage. Additionally, considering that the structure of protein could be changed in different processing methods, which induced changes in the binding or stimulating ability. Though this variation might make no sense of its sensitization, resulting in a decrease in the credibility of the evaluation results. Nevertheless, TM is a type of thermostable protein, the protein structure would be changed during processing, like boiling, cooking, etc., which could cause the destruction of some epitopes, or the exposure of new epitopes and consequently, affect the allergenicity of protein. Hence, we established a nonthermal extraction technique to obtain native fish-TM, shrimp-TM and clam-TM. The method mainly included ammonium sulfate precipitation and isoelectric precipitation and all these steps should be operated at low-temperature condition (4 °C) to prevent the degeneration of albuminous (Bailey, 1948; Lv et al., 2017). Without using an ion-exchange column, much more protein could be collected. Approximately, 20 mg TM was obtained from 100 g of fish, shrimp,

clam muscle, respectively.

Few recent articles reported that a patient serum showed IgE cross-reactivity for 36–37 kDa protein from fish and shrimp, which appear to be TM (Alessandri et al., 2017; Cosme & Barbosa, 2016; Kalic et al., 2018; Rolland et al., 2018). Another patient who had a history of house dust mite and shellfish allergy was also identified to have IgE sensitization to fish-TM (Peixoto et al., 2017). Based on these observations made in the literature, we decided to reassess the allergenic cross-reactivity of fish-TM, with shrimp-TM and clam-TM, since vertebrate fish-TM was considered non-allergic as compared to allergic invertebrate shrimp-TM and clam-TM (Ahmed et al., 2018). In this paper, the results of both western blot, ELISA and inhibition ELISA indicated that shrimp-TM and clam-TM shared high cross-reactivity, while, fish-TM had weak IgG-/IgE-binding compared with that of shrimp-TM. Our study reveals that based on IgE binding the clam-TM and shrimp-TM were found cross-reactivity with a similarity of more than 80% in IgE recognition and the fish-TM was very weak and could not bind shrimp specific IgE. The same observations were documented with human IgE obtained from shrimp allergic subjects, which clearly demonstrate that fish-TM was not cross-reactivity and clam-TM and shrimp-TM with high IgE binding share the epitopes with a high degree of possible cross-reactivity. Meanwhile, there was no obvious difference between raw and boiled TM both in SDS-PAGE and western blot, as well as in ELISA and inhibition ELISA assay. Though it appeared to be a small degree of difference in the IgE binding between raw and boiled clam-TM, there

was still no significant difference in the IgG binding between raw and boiled TM. Furthermore, TM was generally recognized as the major allergen obtained from invertebrates (i.e. crustacean and mollusk) and regarded as non-allergenic proteins for vertebrates, such as fish and rabbit (Ahmed et al., 2018; Sicherer & Sampson, 2018). It is indicated that, during the evolution of vertebrates from invertebrates, the conserved gene sequence of TM appeared to be altered, which changed the amino acid sequence with some subtle differences that may lead to the change in protein structure, making difference in antigenic determining epitopes. This might have changed in the courses that result in the great distinction of hyperacuity of TM between invertebrate and vertebrate animals.

Subsequently, we continued to study the difference in protein structure between raw and boiled fish-TM and shrimp-TM or clam-TM. As earlier reported, the stability of allergens and their allergenicity may be altered after different food processing methods may destroy, like heating, changed pH, simulated digestion or enzymatic hydrolysis (Liu et al., 2017). All these processing methods would destroy the original epitopes or expose fresh epitopes via the change of protein structure. Although TM is a type of thermostable protein, the protein structure would be changed during processing, which could cause the destruction of some epitopes or the exposure of new ones, consequently affecting the allergenicity of protein. Our results indicate that, compared with the raw TM, boiled TM had no remarkable changes in protein structure, except a minute blue-shift in UV and intrinsic fluorescence spectra. Therefore, slightly altered sensitization ability was encountered to raw TM compare to boiled TM, which was well correlated with the heat-stability of TM (Faber et al., 2017; Santiago, Bennuru, Boyd, Eberhard, & Nutman, 2011). In addition, in comparison with boiled TM, raw TM showed significantly higher levels of hydrophobicity with a marked decline of fish-TM, shrimp-TM and clam-TM, respectively, indicating the loss or gain of epitopes and structural variation during boiling. Therefore, the structure of protein could be changed with different processing methods, which might cause the change in binding or stimulating ability resulting in a decrease in the credibility of the evaluation result of sensitization ability (Lee et al., 2017; Liu et al., 2016).

All these findings observed in our study might be associated with the source and quality of human serum and the nature of allergenic proteins and their interaction in sensitized subjects. Our findings were not in agreement with a previously reported study that states boiled extracts seems to be more effective in diagnosing of shrimp (*Penaeus* sp.), American lobster (*Homarus* sp.) and spiny lobster (*Palinurus* sp.) and the reasons might be the species variation and extraction methods that induce a difference in IgE recognition (Fei et al., 2016). Different extraction methods of allergic protein may cause different allergenicity features. There existed two methods of extracting the boiled protein sample at present, one was extracted from the raw sample and then boiled the raw protein without heating during the whole extraction processes, while, another method was extracted from the boiled sample. The former method mainly focused on the properties of the protein itself, while, the other one was more focus on the food processing method. However, this study had a few limitations as the IgE binding assays were done completely based on shrimp-allergic sera and didn't have any fish or clam specific IgE. It mainly focused on understanding the cross-reactivity of shrimp-TM with clam-TM and fish-TM. As different sources of sera might lead to various consequences. Therefore, more experiments relied on clam-allergic serum and even fish-allergic serum would provide more information and should be studied in the future. In addition, more experiments about *in vitro* and *in vivo* should be taken to assessment of allergenicity characteristic between raw and boiled TM.

5. Conclusions

The study followed a systematic approach towards the prediction of allergenicity to fish-TM and compared that of shrimp-TM and clam-TM

and its variance during processing. In addition, it observed that fish-TM could have IgE binding, about 30–35% compared to shrimp-TM and clam-TM, depending on the source and quality of human serum to some degree and was not cross-reactivity. High cross-reactivity resulted between shrimp-TM and clam-TM with high IgE binding and epitope sharing. Moreover, we further confirmed that there was no significant difference between raw TM and boiled TM, though the allergenic potential of raw TM was slightly higher than that of boiled TM. The observations in this paper would provide more significance for further study on the allergenic nature of TM during different food processing methods. It also provided information on the possible cross-reactivity of TM and could be used during clinical interpretation on predicting cross-reactivity between fish, clam and shrimp sensitivity among seafood allergic subjects.

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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