



Allergenicity of tropomyosin of shrimp (*Litopenaeus vannamei*) and clam (*Ruditapes philippinarum*) is higher than that of fish (*Larimichthys crocea*) via in vitro and in vivo assessment

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Abstract

Tropomyosin (TM), acting as heat-stable protein, has a high homology of amino acid sequences with different sensitizations. Fish belongs to vertebrate and its TM owns less allergenicity, whereas shrimp and clam belong to invertebrate and their TM are major allergens. Moreover, the allergenicity of TM might be altered during food processing. Therefore, shrimp-TM was administered intraperitoneally to BALB/c mice to establish an allergic mouse model, to evaluate the elicitation ability of these three proteins, while a mediator-releasing RBL cell line was used to estimate the variation in the stimulation abilities. The results showed that shrimp-TM owned stronger ability in the elicitation of the anaphylactic reactivity as well as in the IgE-mediated the RBL cell line. In comparison with shrimp-TM, clam-TM showed a similar allergic reaction and T-helper 2 (Th2)-based responses from spleen cells and mediums produced by RBL cell line, while fish-TM exhibited a weaker anaphylactic reactivity based on a mouse model and produced fewer active mediums based on cell model. Furthermore, raw and boiled TM did not exhibit a significant difference in allergic reactions and the capacity of degranulation. These results indicated that shrimp-TM owned stronger stimulation ability based on allergic mouse compared with fish-TM and clam-TM, which could supply information for clinical and anaphylaxis of TM in the stages of sensitization and simulation deserve further consideration.

Keywords Allergenicity · BALB/c · Tropomyosin · RBL · Shrimp

Introduction

With delicate flavor and high nutritive value, seafood is a kind of dietary protein source, which is very popular all over the world [1]. However, as one of the most frequent causes of food allergy, seafood is also responsible for many allergic diseases, leading to declining production [2, 3]. Generally speaking, tropomyosin (TM) is recognized as the major allergen in crustacean and mollusks [4]. TM has a molecular mass of 35–38 kDa, composed of two identical subunits and helix coiled-coil [5–7]. The amino acid sequence identity between crustacean and mollusk is about 65%, owing to the highly conserved amino acid sequences, and the allergic

reactions to TM are often cross reactive [8]. Typically, vertebrate TM shares about 55% of sequence homology with vertebrate TM. However, these have been considered as non-allergenic proteins except *Oreochromis mossambicus* [9].

Although TM is a type of thermo-stable protein, thermal treatment could alter the structure of a protein, by losing tertiary/secondary structure, exposing new B-cell epitopes or destroying old B-cell epitopes, the allergenicity of protein might be changed [10, 11]. Whereas, in some cases, food processing such as boiling, roasting, glycation, and frying, aggravated or mitigated the allergenicity [12–15]. However, some controversies have been found in the literature, the previous studies showed that thermal treatment could decrease allergen reactivity [16–18], while another study identified that purified TM from boiled shrimp owed enhanced IgE-binding ability than that of raw shrimp [10]. Meanwhile, an assay showed that there was a significant difference between raw or boiled extracts of shrimp and spiny lobster in skin prick test as well as the level of specific IgE [19].

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Nowadays, it has been reported that a patient serum showed cross reactivity between fish (hake and cod) and shrimp, and the molecular mass of the protein was around 36–37 kDa [7]. Correspondingly, it has been identified by many researchers that clam-TM has strong cross ability with shrimp-TM [5, 20]. As known, anaphylactic reaction mainly comprises the following three phases, sensitization, stimulation, and effector phase. When the human body is exposed to an allergen for the first time, it induces sensitization. Upon subsequent exposure, the body would be stimulated and cause serious problems such as urticarial, asthma, diarrhea, and even life-threatening anaphylaxis [20–22]. Thus, we postulated that shrimp-allergic patients would show clinical symptoms after eating other kinds of seafood, for the allergen shared similar epitopes with shrimp-TM that could be recognized by related cells, such as T lymphocyte cell or B lymphocyte cell, thereby causing T-helper 2 (Th2)-based responses, producing immune globulin (Ig), histamine, and tryptase, which could lead to allergic reaction.

In the previous work, we had developed a nonthermal extraction technique to purify TM from fish, shrimp, and clam. The IgG/IgE binding, cross reactivity, and structures of raw and boiled TM were compared. All those assays mainly focused on the relationship between antigen and antibody, as well as the structure change. More research about the allergic reaction among raw and boiled TM should be paying attention to. To figure out which phase of the anaphylactic reaction (sensitization or stimulation) works, we used raw shrimp-TM sensitized mice and a mediator-releasing RBL (rat basophil leukemia) cell line to assessment raw and boiled fish-TM, shrimp-TM, and clam-TM. The sera level of antibody and cytokine responses, as well as clinical symptoms in BALB/c mice were assessed. Meanwhile, Th2-type cytokines (IL-4 and IL-13) produced by RBL cell were also determined.

Experimental section

Mice

BALB/c mice (female, six-week-old) were purchased from Jinan Laboratory at the Animal Center (Jinan, China) and housed in a specific pathogen-free environment. No fish or shrimp protein was present in the diet (LAD3001M, Trophic Animal Feed High-tech Co., Ltd., Jiangsu, China). All experiments conformed to the established guidelines for the Ethical Committee of Experimental Animal Care at the Ocean University of China.

Materials and chemicals

Raw TM from fish, shrimp, and clam was purified using nonthermal extraction technique in our based on our lab's protocol [23], and then heated in a water bath at boiling temperature for 10 min to obtain boiled TM. Both raw and boiled TM were stored at -20°C until used. Alum adjuvant was obtained from Pierce (Rockford, IL, USA). An ELISA kit of IL-12 was acquired from ThermoFisher (Thermo Fisher Scientific, MA, USA). The ELISA kits of IgG1 and IgE were obtained from Bioss (Biosynthesis biotechnology Co., Ltd., Beijing, China). The ELISA kits of histamine, tryptase, mouse mast-cell protease-1 (MCP-1), IL-4, IL-10, IL-12, IL-13, and interferon (IFN)- γ were acquired from R&D systems (Minneapolis, MN, USA).

Experimental design in vivo

Sensitization testing was carried out according to the method previously described [24–26]. Mice experiment was established to investigate the differences in the reduced eliciting capacity of raw and boiled fish-TM, shrimp-TM, and clam-TM, based on the immunologic sensitized mouse model of raw shrimp-TM. For the sensitization, mice were sensitized with 200 μg raw shrimp-TM ($n=36$) with 2 mg/mL alum adjuvant by intraperitoneal injection at days 0, 7, 14, 21, and 25. After sensitization, mice were divided into six groups ($n=6$) that was raw fish-TM, boiled fish-TM, raw shrimp-TM, boiled shrimp-TM, raw clam-TM, and boiled clam-TM, respectively. Then, mice were challenged two times on days 29 and 33 by intraperitoneal injection with 100 μL (2 mg/mL) raw and boiled fish-TM, shrimp-TM, and clam-TM, respectively. Meanwhile, the mice of PBS groups were immunized with 100 μL phosphate-buffered saline (PBS) at days 29 and 33. The control group ($n=6$) received PBS together with alum adjuvant, injected the same volume of 200 μL at days 0, 7, 14, 21, and 25, and 100 μL at days 29 and 33, respectively. The anaphylactic responses were evaluated after the last injection. After overnight fasting, the mice were anesthetized and killed by cervical dislocation with the blood collected and spleen harvested under sterile conditions.

Anaphylactic responses

Body temperature and scoring clinical signs were assessed within 30 min after challenge, using a scoring system followed described previously [24]. The score was as follows: 0, no symptoms; 1, scratching and rubbing around the nose and mouth; 2, puffiness and scratching around eyes/nose and mouth; 3, wheezing, labored respiration,

cyanosis around the mouth and tail; 4, no activity after prodding; and 5, death.

Serum levels of IgE, IgG1, histamine, MCP-1, and tryptase

On day 34, blood samples from raw and boiled fish-TM, shrimp-TM, and clam-TM-treated mice were collected, respectively. After centrifugation, the sera were stored at -20°C until analyzed. The IgG1, IgE, histamine, MCP-1, and tryptase levels in sera were determined by sandwich ELISA using commercial kits according to the given instructions.

Histology

Samples of spleen and duodenum were collected, fixed with 4% paraformaldehyde, dehydrated using a graded series of ethanol, and embedded in paraffin wax. After that samples were cut, subjected to hematoxylin and eosin (H & E) staining and examined under a research inverted system microscope (Olympus Corporation, Tokyo, Japan).

Cytokine levels in culture supernatants

Cell cultures were performed as described previously [24, 27, 28]. Spleens were isolated immediately after sacrificed under aseptic conditions. After smashing the spleen gently on the cell strainer, the contents were then transferred into a centrifuge tube, added lymphocyte separation liquid, centrifuged at 500 g for about 20 min, and washed splenocytes twice with RPMI-1640 medium by centrifugation at 900 g. Following that, the splenocytes were resuspended and cultured in RPMI-1640 with 10% fetal bovine serum (FBS) and 1% Pen Strep. The cells were stimulated with raw and boiled fish-TM, shrimp-TM, and clam-TM for 3 days, respectively. After that, the supernatants were collected for the measurement of IL-4, IL-10, IL-12, IL-13, and IFN- γ using ELISA kits.

RBL mediator release assay

RBL cells (Chinese Academy of Sciences, Shanghai, China) were cultured at 37°C in a humidified atmosphere of 5% CO_2 as described previously [27, 29]. The grown RBL cells in DMEM media supplemented with 10% FBS and 1% Pen Strep were trypsinized and harvested to adjust the cell density at 2×10^5 cells/mL. Cells were seeded in a 96-well microtiter plate. Following that, shrimp-allergic serum was added for passive sensitization for 24 h. Then, the cells were stimulated with 0.1 mg/mL raw or boiled fish-TM, shrimp-TM, and clam-TM and Tyrode's buffer was used as a negative control for 1 h at 37°C . After incubation, the samples were

centrifuged and supernatants were collected. The cell supernatant was used to measure the mediators released such as β -hexosaminidase, histamine, tryptase, cysteinyl leukotriene, prostaglandin D2, IL-4, and IL-13 with standard ELISA kits, following the manufacturer's instructions.

Statistical analysis

Data were expressed as mean $\pm$ SD and differences were analyzed by ANOVA of Duncan test. Significant differences between groups were considered statistically significant when p values were less than 0.05. Each experiment was repeated more than three times.

Results

Anaphylaxis score and body temperature after challenge with raw or boiled TM

After the last sensitization dose of 100 μg , mice sensitized to raw shrimp-TM were divided into six groups and challenged with raw or boiled fish-TM, shrimp-TM, and clam-TM, respectively (Fig. 1a). Clinical symptoms started to appear within 20 min and three mice developed puffiness around nose and mouth in the fish-TM group, while one or two mice belonging to the shrimp-TM group and clam-TM group showed wheezing, labored respiration, and cyanosis around the mouth. Moreover, one mouse of raw and boiled shrimp-TM groups, as well as the raw clam-TM group appeared no activity after prodding. However, no obvious anaphylaxis signs were observed in the control group (Fig. 1b). In groups of shrimp-TM and clam-TM, a significant drop of temperature was measured after being challenged with different raws or boiled TM, whereas no observed temperature drop was noticed in the control group (Fig. 1c).

Assessment of IgE, IgG1, histamine, MCP-1, and tryptase in serum

IgE and IgG1 were considered as important indicating allergy parameters when evaluating allergenic potential [30]. In some cases, the IgE levels of fish-TM and clam-TM samples were about 86% and 95% of that in raw shrimp-TM groups and 82% and 96% of that in boiled samples (Fig. 2a), while the IgG1 levels of fish-TM-treated mice were about 83% of that in raw shrimp-TM samples and 89% of that in boiled shrimp-TM. Clam-TM-treated mice showed 95% of that in raw shrimp-TM and 99% of that in boiled shrimp-TM samples. (Fig. 2b).

Histamine, MCP-1 and tryptase also play important roles in inflammatory responses, which has a direct correlation with the degree of allergic response. As shown in Fig. 2c–e,

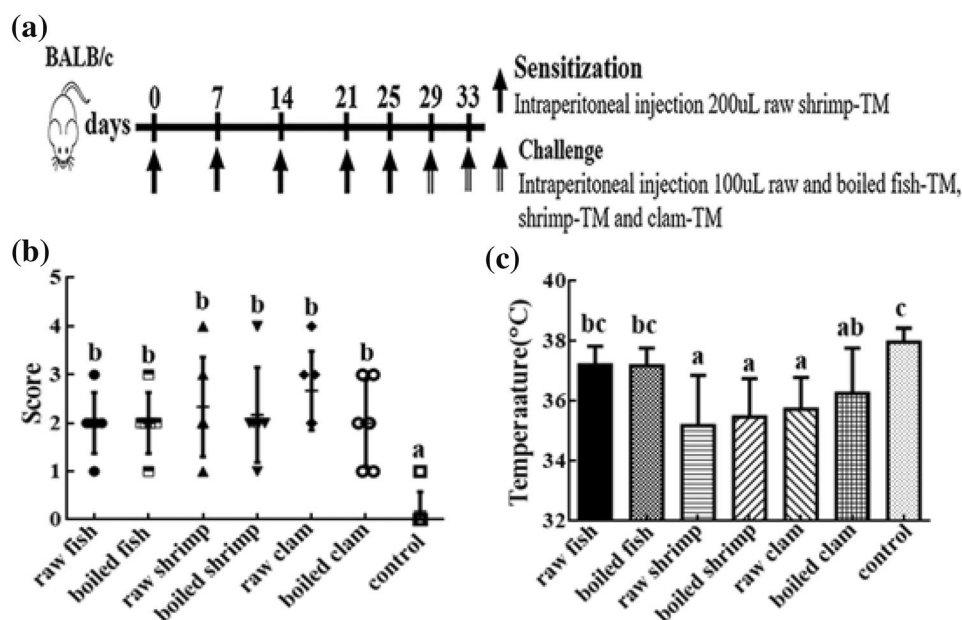


Fig. 1 Anaphylaxis in mice sensitized with raw shrimp-TM and challenged with raw and boiled fish-TM, shrimp-TM, and clam-TM. For the sensitization, mice were sensitized with raw shrimp-TM by intraperitoneal injection at days 0, 7, 14, 21, and 25. The mice were divided into six groups ($n=6$) that was raw fish-TM, boiled fish-TM, raw shrimp-TM, boiled shrimp-TM, raw clam-TM, and boiled clam-

TM, respectively. Then, mice were challenged two times on days 29 and 33 by intraperitoneal injection. Meanwhile, the mice of PBS groups were injected with same volume of PBS at the same day. PBS group was used as control. **a** Immunization procedure. **b** Clinical signs. **c** Temperature. Data are expressed as mean $\pm$ SD ($n=6$), and the different letter stands for significant difference ($p < 0.05$)

the concentration of histamine, MCP-1 and tryptase of fish-TM treated mice was about 88, 85, and 75% of that in the raw shrimp-TM group, and 86, 88, and 76% of that in the boiled shrimp-TM group. In another respect, the concentrations of histamine, MCP-1 and tryptase of the 5clam-TM group, were about 1.02 times, 98% and 98% of that in the raw shrimp-TM group, while 1.01 and 1.01 time, and 94% of that in boiled shrimp-TM sample. However, there was no significant difference between the raw and boiled group.

Histopathological assessment of duodenum and spleen

Histopathological analysis by hematoxylin and eosin (HE) staining of spleen and duodenum is shown in Fig. 3a, b, respectively. Destruction of the mucosal lining and inflammatory cell infiltration was obtained in the duodenum of raw and boiled TM-treated mice. The pathological sections of the spleen showed lymphoid hyperplasia and macrophage in raw and boiled TM groups, while the normal structure of duodenum and spleen retained in the control group. Histopathological examination of shrimp-TM and clam-TM treated mice showed more severe allergic symptoms than the fish-TM group in duodenum and spleen. However, the fish-TM-treated mice group showed certain allergic symptoms. Moreover, boiled TM groups were found similar to raw TM-treated mice groups.

Th1/Th2 cytokines levels of spleen cell

Th1 cytokines (IL-10 and IFN- γ) and Th2 cytokines (IL-4, IL-5, and IL-13) were examined in splenocytes isolated from mice of raw and boiled TM-treated groups as well as the control group. These Th2 cytokines in shrimp-TM and clam-TM groups were markedly up-regulated, while fish-TM-treated mice owed lower contents. There was a significant decrease in IL-4, IL-5, and IL-13 levels in fish-TM-challenged groups in comparison with shrimp-TM and clam-TM-treated groups (Fig. 4a, b, d). The levels of IL-4, IL-5, and IL-13 in the groups of fish-TM and clam-TM were 67%/1.09 times, 67%/1.09 times and 62%/97% than those of raw shrimp-TM sample, and 68%/1.03 times, 60%/97% and 59%/99% than those of boiled shrimp-TM group. However, the levels of IL-10 and IFN- γ were significantly increased in fish-TM-injected groups, compared with shrimp-TM and clam-TM mice (Fig. 4c, e). IL-10 and IFN- γ concentrations of shrimp-TM and clam-TM-treated mice were 1.54/1.55 and 1.06/1.05 times higher than that of raw shrimp-TM groups and 1.67/1.70 and 1.13/1.10 times higher than that in boiled shrimp-TM samples. These findings indicate that shrimp-TM and clam-TM acted to promote food allergy symptoms through enhancing Th2 and inhibiting Th1 immune response.

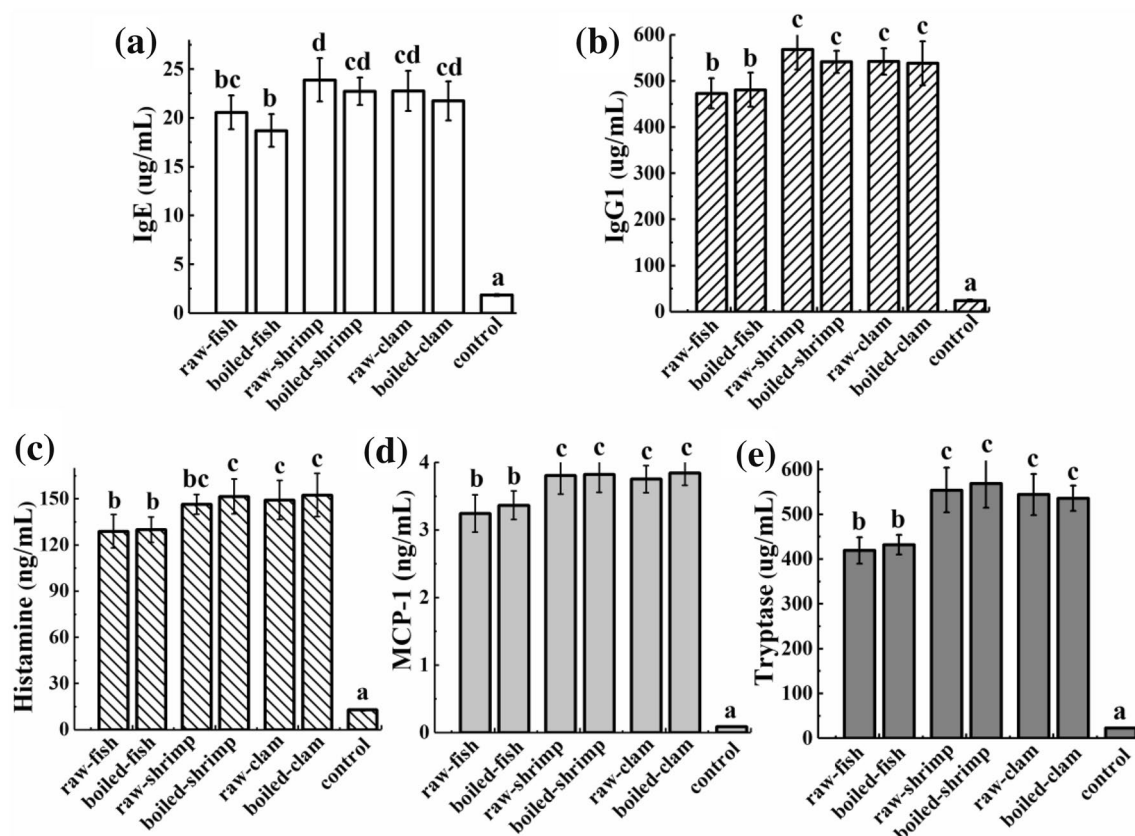


Fig. 2 Serum levels of immunoglobulin E, G1, and active mediators. The mice sensitized with raw shrimp-TM (1 mg per mouse) and challenged with raw and boiled fish-TM, shrimp-TM, and clam-TM (0.2 mg per mouse). PBS group was used as control. **a–b** Concentra-

tions of IgE and IgG1. **c–e** Concentrations of histamine, MCP-1 and tryptase. Data are expressed as mean $\pm$ SD ($n=3$), and the different letters stand for significant difference ($p < 0.05$)

Impact of raw/boiled TM on IgE-mediated RBL-cell degranulation assay

RBL cell is a type of IgE-mediated mucosal mast cells, which was used as a model of type I allergic reaction [31]. Therefore, RBL cells were employed to evaluate the allergic response to raw and boiled TM by monitoring the release of the active degranulation mediators. When compared with shrimp-TM, clam-TM showed a 20–30% reduction in histamine and tryptase release; however, no significant difference was observed in β -hexosaminidase release in both raw and boiled samples. During induced degranulation assay, fish-TM showed less than 30% release as compared to shrimp-TM for β -hexosaminidase, histamine, and tryptase and significantly higher (60%) with cysteinyl leukotriene. The percent release β -hexosaminidase by raw shrimp-TM and clam-TM was reduced by 21.1% and 11.3%, respectively (Fig. 5a). While histamine content reduced by 22.7% and 8.0% (Fig. 5b); tryptase content reduced by 7.9% and 13.0% (Fig. 5c); the contents of cysteinyl leukotriene reduced by 0.9% and 5.1% (Fig. 5d);

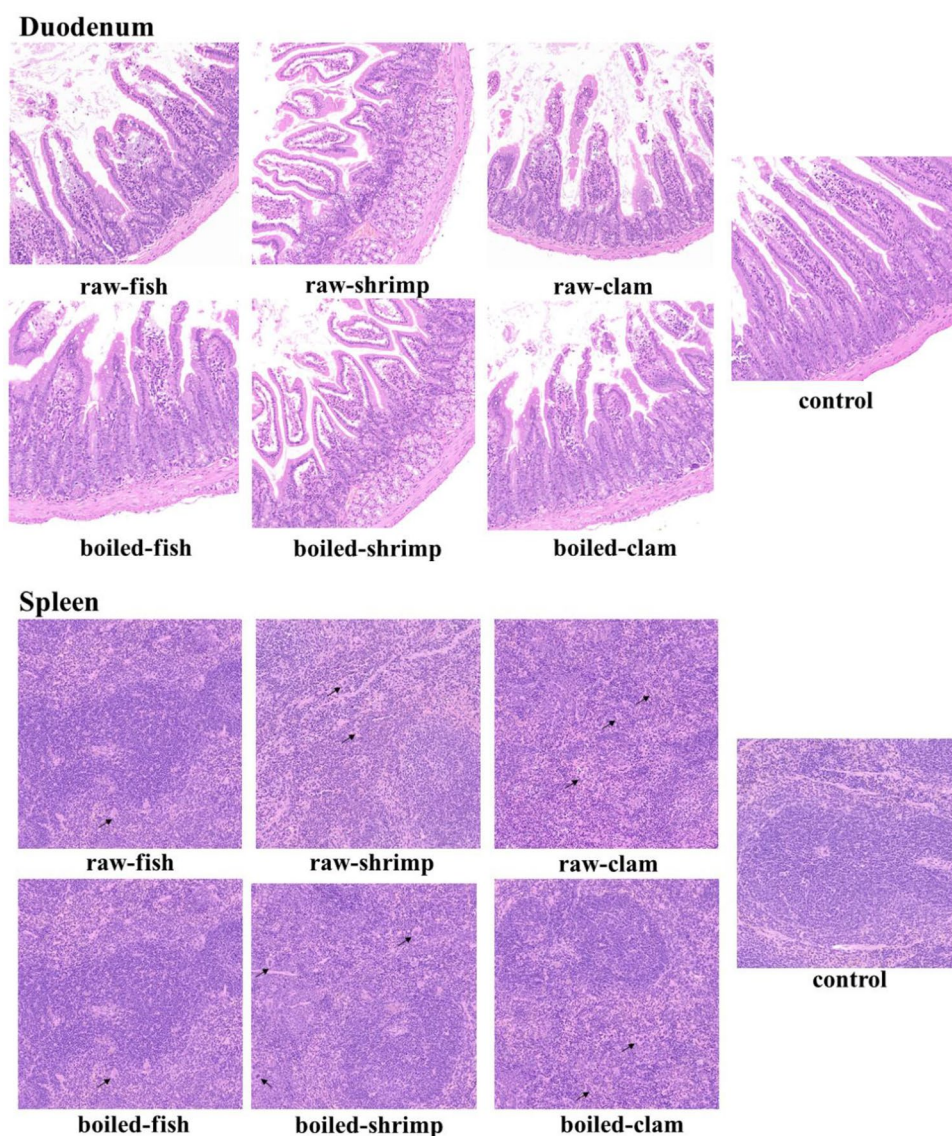
and the contents of prostaglandin D_2 reduced by 7.2% and 22.2% (Fig. 5e), respectively.

Cytokines as IL-4 and IL-13 were also produced by RBL cells, which played key pathologic roles in eliciting IgE response and trigger allergic symptoms in sensitive individuals. As shown in Fig. 5f, g, the levels of IL-4 and IL-13 were found to have a similar trend as that of inflammatory mediators released during degranulation of RBL cells in response to shrimp-TM, clam-TM, and fish-TM. Both IL-4 and IL-13 production were high in shrimp-TM and clam-TM stimulated cells as compared to fish-TM and the raw TM were more effective compared to boiled TM.

Discussion

In this paper, we aimed to assess the elicitation abilities of shrimp-TM and clam-TM (regarded as major allergens), as well as fish-TM (considered as no allergic properties) using in vitro and in vivo assays. As a consequence of this, the BALB/c mouse model was used to compare the stimulation

Fig. 3 Histopathological analysis. Samples were sectioned at 5 μ m thickness, stained with hematoxylin and eosin (HE), photographed at $\times 20$ magnification using 3D HISTECH Panoramic 250. The black arrow stands for macrophage



capacity of these three proteins based on shrimp-TM-treated mice. Meanwhile, RBL cells sensitized with sera from shrimp-allergic patients were applied to compare the elicitation abilities of fish-TM, shrimp-TM, and clam-TM by measuring the active mediators and Th2-type cytokines. In addition, though TM is a kind of heat-stable allergen in crustaceans and mollusks, their allergenicity may be altered during food processing. When the temperature was above 70 °C, the tertiary structures of protein could be destroyed, followed by the loss of secondary structure and formation of intra/intermolecular interactions and the rearrangement of disulfide bonds. These might also lead to the destruction or/and exposure of epitopes and cause a different degree of anaphylactic reactivity in BALB/c mouse [17, 32]. In addition, we had developed a nonthermal extraction technique to obtain purified fish-TM, shrimp-TM, and clam-TM and compared the IgG/IgE binding, cross reactivity, and protein

structures between raw and boiled TM. Those experiments were mainly focused on the relationship between antigen and antibody. Here, we shifted to the attention of antigen and anaphylactic reaction. Thus, the allergenicity between raw and boiled TM was evaluated in this study.

While shrimp-TM and clam-TM are known as the major allergens in crustaceans and mollusks, fish-TM is not identified as an allergen. However, in this study, fish-TM-treated BALB/c mice experienced temperature drops and clinical scores compared with the control group. Shrimp-TM and clam-TM-treated BALB/c mice exhibited higher drops of temperature and more severe anaphylactic sensitivities. The similar results with serum levels of specific IgE, IgG1, histamine, MCP-1, and tryptase were found and our measurements corresponded to shrimp-TM-sensitized mice previously reported in studies in BALB/c mice [25, 27, 33, 34]. However, a few kinds of literature researched anaphylactic

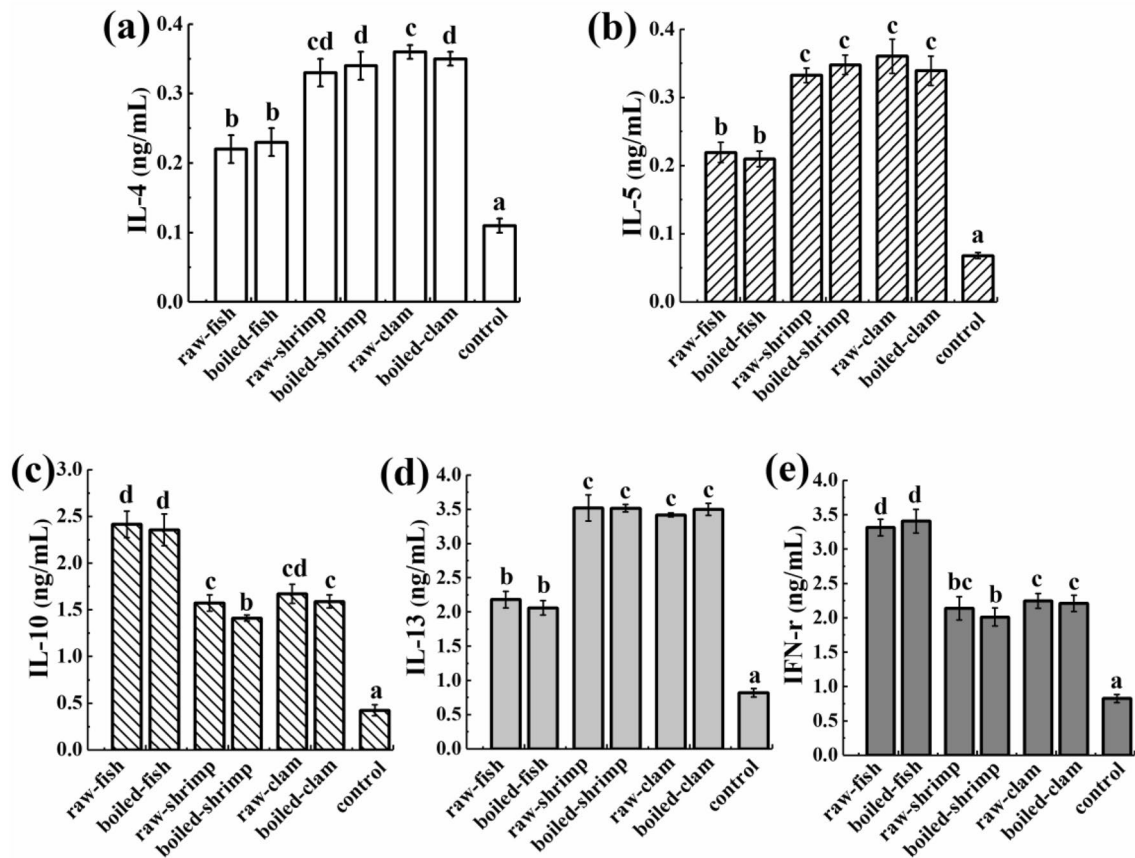


Fig. 4 Cytokine production by spleen cells. The mice sensitized with raw shrimp-TM and challenged with raw and boiled fish-TM, shrimp-TM and clam-TM. PBS group was used as control. Data are

expressed as mean ± SD (pooled spleen cells from six mice stimulated in duplicate), and the different letters stand for significant difference ($p < 0.05$)

reactions about clam-TM using a mouse model experiment. Only two papers reported a phenomenon with respect to fish-TM anaphylactic characteristics. In addition, all shrimp-TM sensitized mice were divided into groups and finally challenged with fish-TM, shrimp-TM, and clam-TM, respectively. The histopathological analysis also confirmed that mice with finally clam-TM challenge could found biological symptoms, such as inflammatory cell infiltration in the duodenum as well as lymphoid hyperplasia and macrophage in the spleen. In comparison with fish-TM fed mice, the groups of shrimp-TM and clam-TM mice showed significantly higher levels of early immunoglobulin E, G1, and active mediums (histamine, MCP-1, and tryptase) and more severe allergic reactions in the sections of duodenum and spleen.

Alternatively, ex vivo spleen cell production of Th1-type cytokines (IL-10 and IFN- γ) and Th2-type cytokines (IL-4, IL-5, and IL-13) were observed in this study. In contrast to the control group, the Th2-type cytokines were up-regulated, whereas the Th1-type cytokines were down-regulated with treated fish-TM, shrimp-TM, and clam-TM. Compared with fish-TM, shrimp-TM, and clam-TM shared higher levels of

Th2-type cytokines, which implies increased allergenicity. Th2-type cytokines could cause the production of IgE antibody, which would combine with mast cell and prompt mastocyte degranulation, produce histamine, leukotriene, etc. These factors could trigger allergic symptoms, such as urticaria, asthma, diarrhea, and even life-threatening anaphylaxis in sensitized patients [22, 35, 36]. Altogether, it is documented that fish-TM could exhibit allergic features based on shrimp-TM-sensitized mice, which might be associated with the decreased immunity of sensitization mice and the high homology of tropomyosin. Furthermore, in the mouse model, we found no major differences between raw and boiled TM in clinical symptoms such as sera levels of IgE, IgG1 and active mediums, histology, and cytokines of spleen cells. These findings did not correlate well with the previous study of thermal-processing effects on peanut allergen Ara h 2 [17], the boiling induced the down-regulation of the allergenicity of peanut, and the reason could be the different disposes of protein.

To address the IgE-binding variations and their relativity in clinical sensitization, the cellular degranulation and

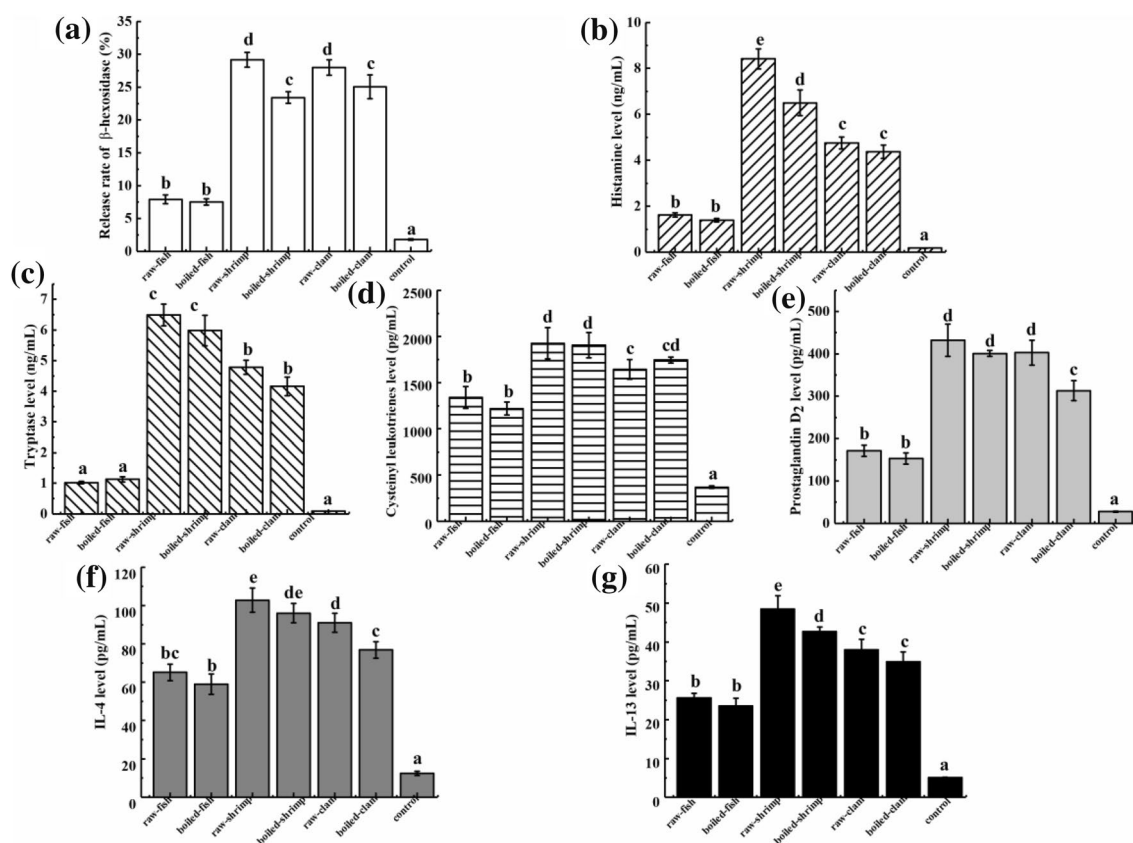


Fig. 5 RBL-cell assay for raw and boiled fish-TM, shrimp-TM, and clam-TM. **a** Release of β -hexosaminidase; **b** level of histamine; **c** level of tryptase; **d** level of cysteinyl leukotriene; **e** level of prostaglandin D₂; **f** level of IL-4; **g** level of IL-13. The content of protein

used in cell assay was 0.1 mg/mL. Tyrode's buffer was used as a negative control. The data represent the mean $\pm$ SD ($n=3$), while the different letters stand for significant difference ($p < 0.05$)

mediator release assays are commonly employed. These are more predictive of possible allergenicity, as they directly indicate the ability to trigger clinical symptoms in sensitized subjects as compared to in vitro IgE immune assays. Moreover, histamine and prostaglandin play important roles in inflammatory responses, which have a direct correlation with the degree of allergic responses. RBL cells were used as a model for type I anaphylactic reaction and to establish allergen-specific standards. In this study, we studied the effect of raw and boiled fish-TM and shrimp-TM or clam-TM on potential sensitization by determining the release of active mediators (β -hexosaminidase, histamine, tryptase, cysteinyl leukotriene, and prostaglandin D₂) and cytokines (IL-4 and IL-13). Our study showed that shrimp-TM and clam-TM showed higher mediator release compared to that of fish-TM, whereas clam-TM and shrimp-TM were highly cross reactive and owned the same strength of the stimulatory effect and induced mediator release. Interestingly, the raw TM showed a little higher percent release of mediators and cytokine compared to

boiled TM. That may be due to the change in some epitope structure during the boiling process.

In conclusion, this study showed that shrimp-TM owed stronger ability in the elicitation of the anaphylactic reactivity in a mouse model of allergy as well as in IgE-mediated RBL cell line. Moreover, with respect to allergic action of shrimp-TM, clam-TM could share a similar elicitation with shrimp-TM, whereas fish-TM could exhibit lower stimulation ability based on sensitized mice. However, there was no significant difference between raw and boiled TM in the phase of excitation during the process of food allergy using in vitro and in vivo assays. Therefore, shrimp-allergic patients could take relatively high risks of eating mollusk and lower risk of eating fish in comparison with shrimp. This study will advance our knowledge on TM from fish, shrimp and clam, and shed light on the sensitization and elicitation of anaphylactic sensitivity and the prevention and therapy of allergy.

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Compliance with ethical standards

Conflict of interest The authors declare that there is no conflict of interest related to this paper.

Compliance with ethics requirements All experiments conformed to the established guidelines for the Ethical Committee of Experimental Animal Care at the Ocean University of China.

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