



Effects of different processing methods on digestibility of *Scylla paramamosain* allergen (tropomyosin)

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ABSTRACT

Crustacean allergy is a significant health problem around the world, and tropomyosin (TM) represents the major allergen of crustaceans. The aim of this study was to evaluate three processing methods (boiling, CUB, HPS) and identify the one method that is most effective in the degradation of TM and reduction of its IgE-binding reactivity, and make it easier to be decomposed during gastrointestinal digestion. SDS-PAGE analysis indicated that boiling had little impact on the digestive stability of TM. In contrast, combined ultrasound and boiling (CUB), and high pressure steaming (HPS) both could accelerate the digestion of TM. Similarly, western blotting and inhibition ELISA also demonstrated that the reactivity of IgG/IgE-binding of TM that was extracted from processed crab was partially decreased after treating with CUB or HPS. Among the three processing methods, HPS was the most effective method to accelerate the digestion of TM in gastrointestinal digestion, and reduce the reactivity of IgG/IgE-binding of TM. These results suggest that proper processing of crab could promote the degradation of TM in simulated gastrointestinal digestion, reduce the reactivity of IgG/IgE-binding of TM, and decrease the incidence of crab hypersensitivity in humans.

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1. Introduction

There is growing concern about food allergy because it can lead to autoimmune diseases. Food allergy is representative of type I allergies that are mediated by IgE antibodies. The main food allergens are mostly proteins or glycoproteins with molecular masses of 10–60 kDa (Shainti et al., 1993). A report of the Centers for Disease Control and Prevention has indicated an 18% increase in childhood food allergy from 1997 to 2007, with an estimated 3.9% of children currently affected (Branum and Lukacs, 2008). In addition, according to an epidemiological survey, adverse immune responses to foods affect approximately 5% of young children and 3–4% of adults in westernized countries, and appear to have increased in prevalence (Scott et al., 2010).

Among a number of allergenic foods, crustaceans are considered to be one of the most common causes of food allergy due to their widespread consumption, especially in coastal countries (Lehrer et al., 2003; Wild and Lehrer, 2005). Crustaceans are one of the

eight major sources of food allergens proposed by the Food and Agriculture Organization (FAO) of the United Nations and World Health Organization (WHO) (FAO/WHO, 2001), and they are favored by people because of their delicacy and nutritious value. Recently, with the development of processed crab products, researchers around the world have paid much more attention to the study of crab allergens. It has been demonstrated that the major allergen of crab is tropomyosin (TM), a myofibrillar protein that is composed of two identical subunits with molecular masses of 35–38 kDa and an isoelectric point of 4.5. In contrast with other proteins, TM is stable and can tolerate heat, grinding and the normal processing methods (Leung et al., 1998; Motoyama et al., 2006, 2007).

According to the research of Lee and Park (2004), digestibility of common whelk allergens that were digested by simulated gastrointestinal fluid remained unchanged after thermal treatment, thus demonstrating that the main allergen of crustaceans is heat stable. Moreover, compare to the numerous food processing methods, ultrasound is an effective processing and preservation technology, on account of its good directivity and strong penetrability (Villamie and Jong, 2000). Currently, there have been few studies about the impact of ultrasound on food allergens. Li et al. (2006) have adopted ultrasonic treatment of shrimp protein extract and muscle. They have indicated that high-intensity ultrasound can clearly reduce the allergic properties of shrimp allergens, and this will be beneficial to allergic individuals. In addition, the effect of high

Abbreviations: CCE, crab crude extracts; CUB, combined ultrasound and boiling; DAB, 3,3'-diaminobenzidine tetrahydrochloride; HPS, high pressure steaming; HRP, horseradish peroxidase; SGF, simulated gastric fluid; SIF, simulated intestinal fluid; TBST, Tris-buffered saline with tween-20; TM, tropomyosin; TMB, 3,3',5,5'-tetramethylbenzidine.

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pressure treatment (300 MPa) on the hydrolysis of dairy whey proteins in simulated gastrointestinal digestion has been analyzed. After treatment with high pressure, dairy whey proteins and β -lactoglobulin were hydrolyzed easily by protease, and the IgE-binding was much lower (Izquierdo et al., 2005; Peñas et al., 2006). Therefore, it is meaningful to study the effect of different processing methods on crab allergens, and to establish proper processing methods to reduce or remove the stability and reactivity of crab TM.

In the present study, in order to identify the method that is most effective in the degradation of TM and reduction of its IgE-binding reactivity, *in vitro* simulated gastrointestinal digestion was adopted to investigate the digestive stability of TM extracted from crab treated by boiling, combined ultrasound and boiling (CUB), and high pressure steaming (HPS). Meanwhile, western blotting and inhibition ELISA were used to analyze the IgG/IgE-binding of TM from the digested crab crude extract (CCE) from processed crabs.

2. Materials and methods

2.1. Crab and chemicals

Crabs (*Scylla paramamosain*) were purchased alive at Jimei market, Xiamen. They were immediately used or stored by freezing at -80°C .

Protein standards for SDS-PAGE were purchased from Fermentas (Lithuania). Prestained protein standards for western blotting were purchased from New England Biolabs (Beverly, MA, USA). Horseradish peroxidase (HRP)-labeled goat anti-human IgE was from Kirkegaard & Perry Laboratories (Gaithersburg, MD, USA). HRP-labeled goat anti-rabbit IgG and 3,3'-diaminobenzindine tetrahydrochloride (DAB) were from Pierce (Rockford, IL, USA). Porcine trypsin and bovine α -chymotrypsin were purchased from Sigma (St. Louis, MO, USA) and Amresco (Cleveland, OH, USA). Rabbit anti-mud crab TM polyclonal antibodies were prepared in our laboratory (Liang et al., 2008). Porcine pepsin was prepared in our laboratory as previously described (Zhou et al., 2007).

The proteolytic activity of pepsin was estimated by measuring trichloroacetic acid precipitable bovine hemoglobin after hydrolysis for 30 min. One unit of pepsin activity was defined as an increase in absorbance of 1.0 at 280 nm for 30 min incubation at 37°C . The proteolytic activity of trypsin was measured using Boc-Phe-Ser-Arg-MCA as substrate, whereas the activity of chymotrypsin was measured using Suc-Leu-Leu-Val-Tyr-MCA as substrate according to previously described methods (Cao et al., 2000; Yang et al., 2009). The fluorescence intensity of liberated 7-amino-4-methylcoumarin (AMC) was measured by a fluorescence spectrophotometer (FP-6200; Jasco, Tokyo, Japan) at an excitation wavelength of 380 nm and an emission wavelength of 450 nm. One unit of trypsin or chymotrypsin activity was defined as the amount of the enzyme required to release 1 nM/min of AMC.

2.2. Human sera

Sera from patients were obtained from the Hospital of Jimei University and the Second Hospital of Xiamen. Five tested patients were determined to have crab allergy. They were all confirmed as crustacean allergy on the basis of extensive history, physical examination and objective manifestations observed after ingestion of crustacean products. Written informed consent was obtained from each patient. In this study, five sera (No. 95762745, 95063666, 95063148, 95821251, and 95060123) were pooled and used for immunoassays, and sera from non-allergic individuals (No. 95948274, 95948357) were pooled and used as negative controls. All sera were stored at -80°C until used.

2.3. Different crab processing methods

Crab was treated with three different methods as follows: (a) boiling was done at 100°C for 20 min; (b) CUB was carried out in an ultrasonic cleaner (KQ5200DE; Shumei, China) at 200 W, 30°C for 60 min, followed by heating at 100°C for 20 min; or (c) HPS of 0.14 MPa, at 121°C in an autoclave (KT-30 L; ALP, Tokyo, Japan) for 20 min. Samples without treatment were used as controls.

2.4. Preparation of the CCE

Each processed sample was minced and homogenized with five volumes of ice-cold 20 mM Tris-HCl buffer (pH 7.5, containing 3% NaCl w/w) using a homogenizer (Kinematica, Lucerne, Switzerland). TM is heat stable, therefore, the homogenate was heated in boiling water for 15 min and centrifuged at 8000g for 20 min at

4°C , and the supernatant was regarded as the crude extract (Liang et al., 2008). Protein concentration of crude extracts was estimated by the Lowry method (Lowry et al., 1951) using bovine serum albumin as the standard.

2.5. SGF digestion stability assay

The digestibility of CCE in SGF was estimated as described by Takagi et al. (2003), with some modifications. SGF was prepared as described in the United States Pharmacopoeia (1995) and consisted of porcine pepsin (160 U/mg proteins) in 25 mM NaCl, pH 1.2. The total volume of reaction solution was 1 mL. For each sample, SGF (1:200 pepsin to protein, w:w) was preheated at 37°C for 15 min before adding the test protein. Digestion was performed at 37°C with continuous rocking. At each scheduled time point (0, 1, 2, 5, 10, 15, 30 and 60 min), 100 μL of the reaction solution was removed to a separate sampling tube, and the reaction was immediately terminated by adding 30 μL 200 mM Na_2CO_3 and 26 μL 4 $\times$ Laemmli buffer. The samples were heated at 95°C for 10 min and analyzed by SDS-PAGE or western blotting. For the 0 min samples, protein samples were added to SGF that was neutralized with Na_2CO_3 . In the control experiments, each protein sample was mixed with the reaction solution without pepsin and then reacted at 37°C for 60 min. All the experiments were repeated in triplicate.

2.6. SIF digestion stability assay

For intestinal digestion, SIF was also prepared as described in the United States Pharmacopoeia (1995), and consisted of trypsin or α -chymotrypsin in 0.05 M KH_2PO_4 , pH 7.5. The total volume of reaction solution was 1 mL. The enzyme activity of trypsin and α -chymotrypsin was 32 and 132 U/mg protein, respectively. The ratio of trypsin to protein was 1:2000 (w:w), and the ratio of chymotrypsin to protein was 1:5000 (w:w). Digestion was carried out at 37°C with continuous rocking. At each scheduled time point (0, 1, 15, 30, 60, 120, 180 and 240 min), 100 μL of the reaction solution was removed to a separate sampling tube, and the reaction was immediately terminated by heating at 95°C for 5 min. The samples were analyzed by SDS-PAGE or western blotting. In the 0 min samples, protein samples were added to SIF that was inactivated by heating at 95°C for 5 min. In the control experiments, each protein sample was mixed with the reaction solution without the corresponding enzyme and then reacted at 37°C for 4 h. All the experiments were repeated in triplicate.

2.7. SDS-PAGE and western blotting

For electrophoresis, samples (protein concentration, 2.25 mg/mL) were separated by 12% SDS-PAGE as described by Laemmli (1970), and stained with Coomassie Brilliant Blue R-250 (CBB).

Western blotting was performed by the method of Towbin and Gordon (1984), with slight modifications. Protein samples were separated on gels and electrotransferred onto nitrocellulose membranes. To investigate IgG-binding activity of TM (from CCE of processed crabs), the membranes were incubated with rabbit anti-mud crab TM polyclonal antibodies (1:4000,000 dilution) at room temperature for 2 h, after blocking with 5% non-fat milk in Tris-buffered saline (TBS; 20 mM Tris-HCl, pH 7.5, 0.145 M NaCl), and the membranes were washed with TBST (TBS, 0.05% Tween-20). The HRP-labeled goat anti-rabbit IgG as secondary antibody (1:2000 dilution) was added and incubated for 1 h. The membranes were washed extensively with TBST. Finally, the color reaction was developed using DAB as a substrate.

To estimate IgE-binding activity of TM from CCE of processed crabs, and its digestive products, the pooled human serum (1:5 dilution) was used as first antibody, and peroxidase-conjugated goat anti-human IgE antibody (1:2000 dilution) was allowed to react with the membrane as second antibody at room temperature for 1 h, followed by extensive washing and detection by DAB.

2.8. Inhibition ELISA

Inhibition ELISA was carried out to determine the specificity of IgE-binding of TM (extracted from processed crab) that had been digested. Polystyrene 96-well ELISA plates (Nunc Maxisorb, Denmark) were coated overnight at 4°C using 0.16% (w/w) Na_2CO_3 and 0.29% (w/w) NaOH (pH 9.6) diluted in distilled water as the coating buffer, which contained about 200 ng TM. Coated plates were washed five times with TBST and the residue-free binding sites were blocked with 200 μL /well of blocking buffer (0.5% non-fat milk in TBST) at 37°C for 2 h. In another microtiter plate, 50 μL human sera (diluted 1/5 with 0.1% non-fat milk in TBS) were mixed with the same volumes of digested crude extracts as inhibitor (diluted 1/10 with 0.1% non-fat milk in TBS; the protein concentration was 0.3 mg/mL). After incubation at 37°C for 1 h, 50 μL of the mixture (serum-digested CCE) was transferred to the ELISA plate coated with TM and incubated at 37°C for 1 h. The plates were washed again and the HRP-conjugated goat anti-human IgE antibody (1:2000 dilution) was added. After incubation of the plates for 2 h at 37°C , the bound peroxidase activity was determined by reaction with TMB. Absorbance at 450 nm was determined on an automated ELISA plate reader (Benchmark

96; Bio-Rad Laboratories, Hercules, CA, USA). The hypersensitivity of the digested CCE and its digestive products were determined by calculating the inhibition rate using the following formula (Nakamura et al., 2006; Ma et al., 2008):

$$\text{Inhibition rate(\%)} = (X - Y)/(X - Z) \times 100$$

where *X* was the absorbance of patients' sera not incubated with inhibitors, and *Y* and *Z* were the absorbance of patients' and control sera reacted with inhibitors, respectively. All determinations were duplicated and mean values were used.

3. Results

3.1. Digestibility of processed CCE by SGF

In SGF, the digestibility of the CCE was analyzed by SDS–PAGE, and the original TM revealed a band with a molecular mass of 38 kDa (Fig. 1). Under digestion with pepsin, the original TM band and other proteins were gradually decreased with formation of digested fragments. On one hand, a main fragment of molecular mass 34 kDa was generated after 1 min, and the density of this fragment intensified during time extension and persisted until 1 h (Fig. 1A–C). On the other hand, compared with the untreated crab, the original TM bands with boiling and CUB treatments were digested rapidly, and produced another faint fragment of 32 kDa (Fig. 1B and C). After treatment with HPS, SDS–PAGE profiles indicated that the band of the original TM was a little different from those with the other treatments. The original TM band at about 38 kDa molecular mass was such a kind of broad and vague. In addition, it was difficult to identify the degraded band of the original TM (Fig. 1D).

The CCE contained not only the allergic protein (TM), but also the non-allergic proteins. So, it was difficult to distinguish the exactly degraded fragments of TM from the non-allergic proteins with low molecular weights just by SDS–PAGE. In order to make definitely understanding of the degradation of TM by digestive proteases, the western blotting experimentations were carried out with the polyclonal antibodies or human sera.

Western blotting using specific rabbit polyclonal antibodies against TM was performed to identify the degraded fragments of the original TM band. Fig. 2 shows the western blot figures that correspond to Fig. 1; the degradation pattern of TM was similar

to that with SDS–PAGE. In the SGF digestive assay, the original TM band was rapidly degraded to a 34-kDa fragment, and became undetectable within 30 min (A), 30 min (B), 30 min (C) and 15 min (D). However, the 34-kDa fragment still remained after 1 h (Fig. 2), which suggested that the 34-kDa fragment of TM was relatively resistant to SGF digestion. The density of the 34-kDa fragments was lower after treatment with HPS than with the other treatments (Fig. 2D). It is suggested that HPS accelerates the digestion of crab allergens in SGF.

3.2. Digestibility of processed CCE by SIF (trypsin)

In the digestion pattern of SIF, which consisted of trypsin, more degraded fragments were observed (Fig. 3A–C). As digestion time increased, the original TM band and other proteins were gradually degraded. Compared with the untreated crab, boiling had no effect on the digestibility of the original TM, but the proteolytic fragments treated by CUB were all degraded rapidly (Fig. 3A–C). However, for HPS treatment, the degradation bands were difficult to distinguish (Fig. 3D).

Western blots that correspond to Fig. 3 showed that, in digestion with trypsin, the original TM band was separated into four main degraded fragments with molecular weights of approximately 37, 34, 28 and 22 kDa (Fig. 4A–C). For HPS, the main fragments were only 37 and 34 kDa (Fig. 4D). The original TM band and its degraded fragments disappeared within 4 h (A), 2 h (B), 1 h (C) and 1 h (D) (Fig. 4). After boiling, CUB and HPS treatments, all fragments of the original TM were digested more rapidly than those in the untreated crab. Of the three methods, the proteolytic fragments of TM treated with HPS were degraded much faster than with the other methods, and completely disappeared after 1 h (Fig. 4D).

3.3. Digestibility of crude extracts of TM by SIF (chymotrypsin)

In the presence of chymotrypsin, the original TM was susceptible to SIF digestion. The fragment of 37 kDa that was initially produced after 1 min, followed by the other three fragments which were 34, 28 and 22 kDa, and the 22-kDa band could not

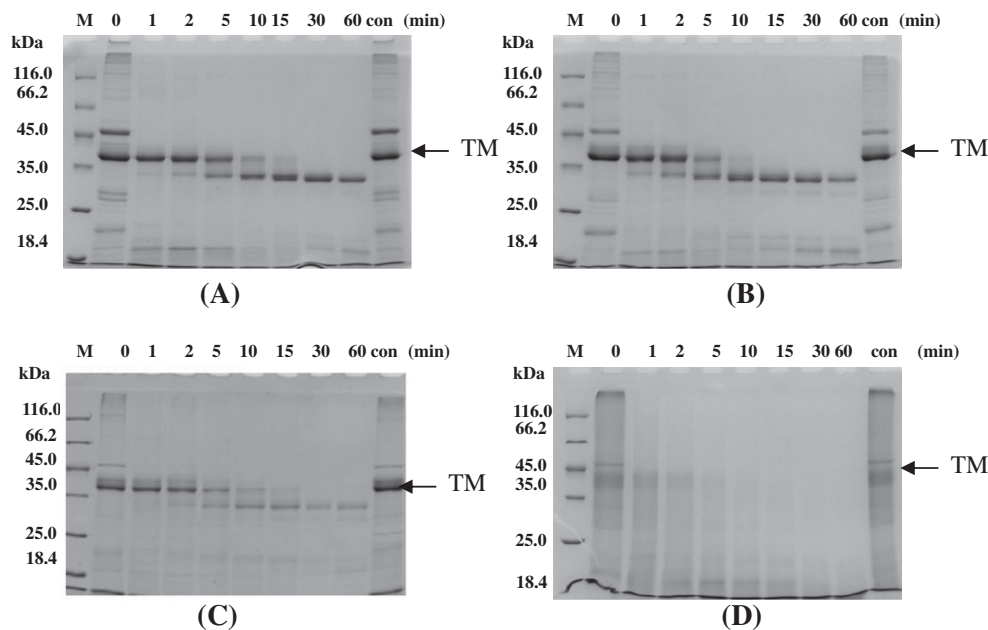


Fig. 1. Effect of SGF digestion (pepsin) on the crude extracts of processed crab. The crabs were untreated (A); treated with boiling (B); treated with CUB (C); or treated with HPS (D). Enzymatic digestion was performed, followed by SDS–PAGE. After electrophoresis, proteins were visualized by staining with CBB. In the control experiments (con), proteinase was replaced with 25 mM PBS (pH 7.5). Molecular masses of the protein markers (M) are shown on the left edges.

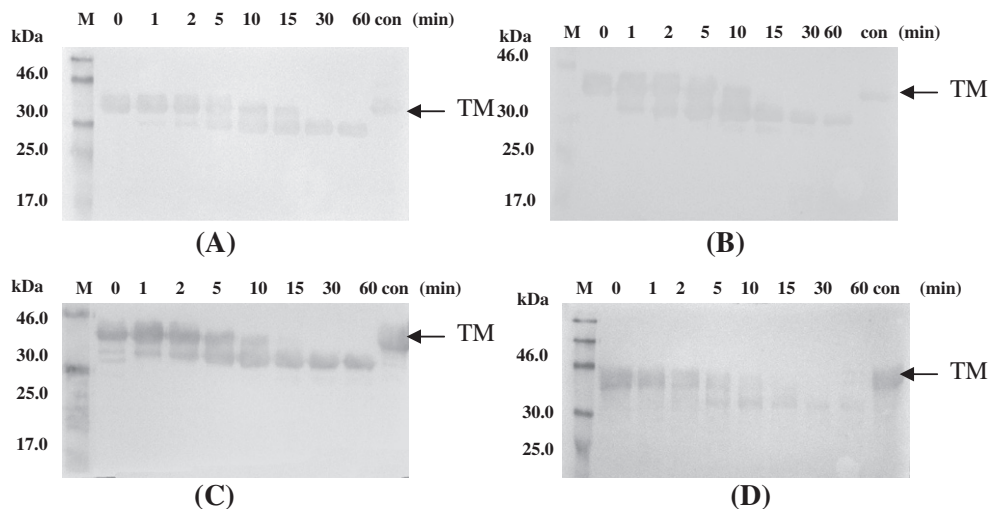


Fig. 2. Western blot detection of the degradation of the crude extracts of processed crab in SGF (pepsin). The crabs were untreated (A); treated with boiling (B); treated with CUB (C); or treated with HPS (D). Samples were separated by SDS–PAGE and electrophoretically transferred to nitrocellulose membranes. Immunological detection was performed. A rabbit anti-mud crab TM polyclonal antibody was used to detect the IgG-binding of digested samples. Positions of molecular mass standards (M) are labeled on the left.

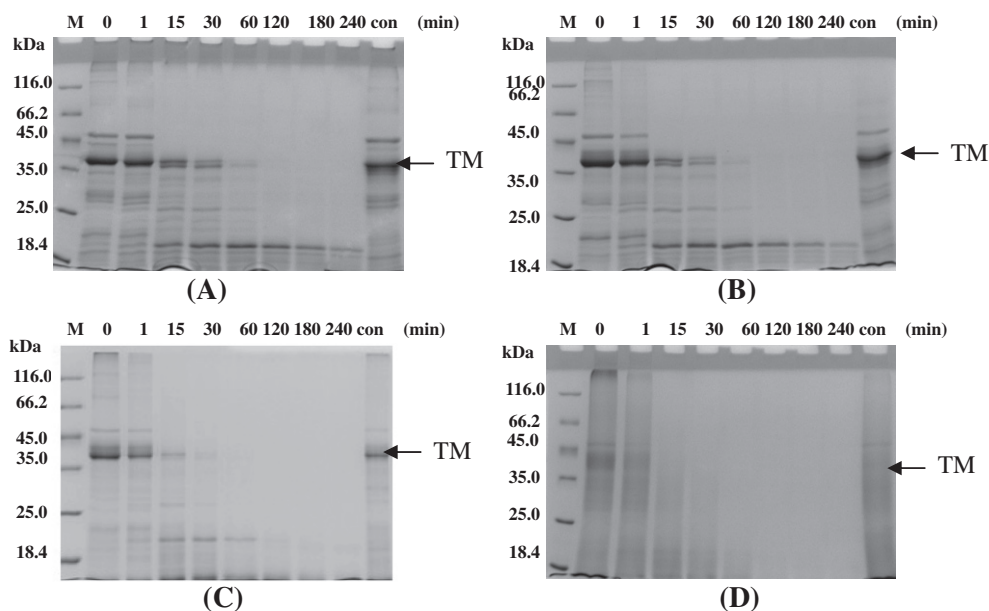


Fig. 3. Effect of SIF digestion (trypsin) on the crude extracts of processed crab. The crabs were untreated (A); treated with boiling (B); treated with CUB (C); or treated with HPS (D). Enzymatic digestion was performed, followed by SDS–PAGE as in Fig. 1. Positions of molecular mass standards (M) are labeled on the left.

be completely degraded within 4 h (Fig. 5A and B). On the contrary, with CUB treatment, the original TM band and its proteolytic fragments were undetectable when digested for 1 h (Fig. 5C). The protein bands of the original TM after HPS treatment were faint, therefore, it was hard to identify the degradation products of the original TM (Fig. 5D).

Western blot analysis of the digestion of TM by chymotrypsin showed that, similar to digestion by trypsin, the main degraded fragments were 37, 34, 28 and 22 kDa, and however, it is difficult to recognize 28 and 22 kDa fragments as to HPS treatment (Fig. 6 D). As whole, the original TM band were undetectable when digested for 4 h (A), 4 h (B), 2 h (C), 1 h (D) (Fig. 6). Whereas, 22 kDa proteolytic fragments still remained at 4 h (Fig. 6A and B). With regard to CUB and HPS, the proteolytic fragments degraded much more rapidly comparing with fragments of TM extracted from untreated and boiling treated crab (Fig. 6).

3.4. Immunoblotting and inhibition ELISA

The IgE-binding of TM obtained from CCE by proteinases (pepsin, trypsin and chymotrypsin) digestion was further analyzed by means of IgE immunoblotting. For TM extracted from boiled crab, western blotting with pool of serum samples from patients with crab allergy showed that their IgE antibodies positively reacted with the original TM band (38 kDa) and its degraded bands with sizes of 37, 34, 28 and 22 kDa (Fig. 7A and B). Clearly, IgE-binding epitopes in these fragments still existed, even when digested by pepsin, trypsin and chymotrypsin. However, for TM obtained by CUB or HPS, the color reactions of western blotting using the pooled sera were faint and hard to recognize (data not shown). The explanation might be that the IgE-binding sites of TM obtained with these two treatments were partly destroyed and were not able to react with IgE antibodies; as a result, the color

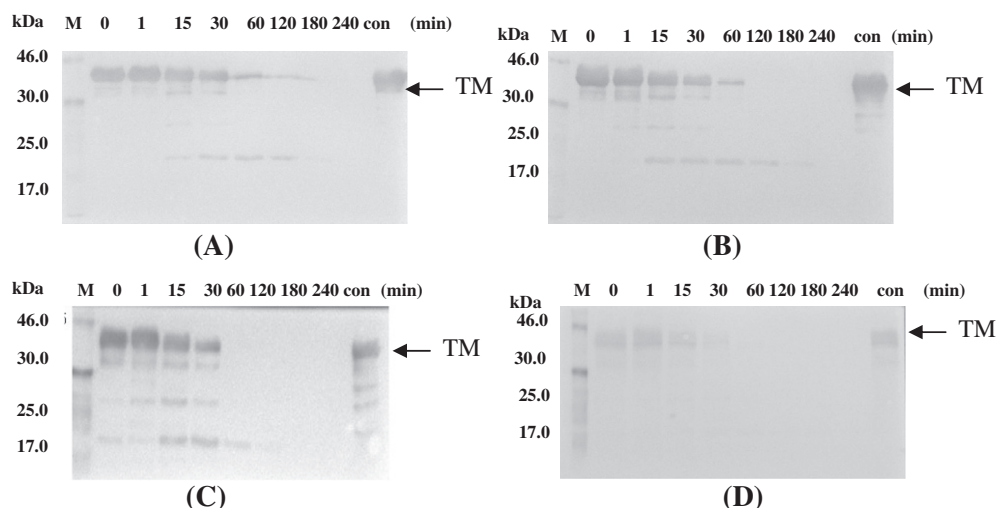


Fig. 4. Western blot detection of the degradation of TM from the crude extracts of processed crab in SIF (trypsin). The crabs were untreated (A); treated with boiling (B); treated with CUB (C); or treated with HPS treated (D). The proteins which were separated by SDS–PAGE were blotted onto nitrocellulose, followed by an immunological detection with rabbit anti-mud crab TM polyclonal antibodies, to detect the IgG-binding of digested samples. Positions of molecular mass standards (M) are labeled on the left.

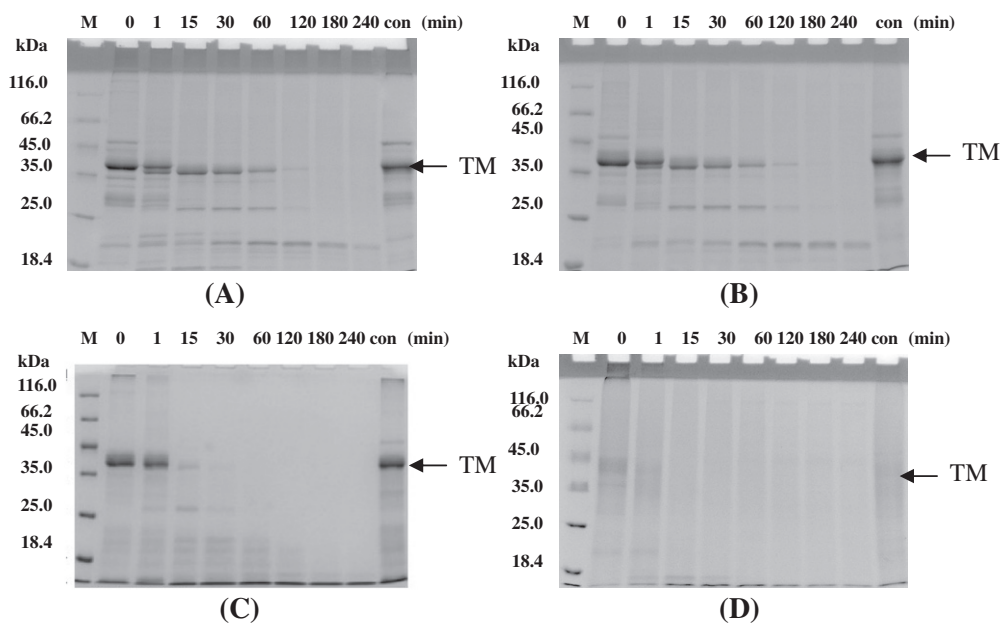


Fig. 5. Effect of SIF digestion (chymotrypsin) on the crude extracts of processed crab. The crabs were untreated (A); treated with boiling (B); treated with CUB (C); or treated with HPS (D). Enzymatic digestion was performed, followed by SDS–PAGE as in Fig. 1. Positions of molecular mass standards (M) are labeled on the left.

reaction with DAB was hard to recognize. In the other way, it could be due to the smears from protein aggregation.

To analyze further the IgE-binding of TM digested by proteinases (pepsin, trypsin and chymotrypsin) and its degraded fragments, we used inhibition ELISA because it is more sensitive for detecting proteolytic fragments with IgE reactivity. The degree of IgE-binding was measured on the basis of inhibition of IgE reactivity according to treatment methods and digestion pattern; the greater the inhibition, the more allergenic were the digested proteins (Chart et al., 1984). As shown in Fig. 8, TM from the crab treated with boiling, CUB or HPS displayed less inhibition than that from untreated crab. Among these treatments, HPS greatly affected

the IgE-binding of TM, which was clearly reduced after gastrointestinal digestion.

4. Discussion

Recently, studies on crab allergens have focused on their purification and characterization, and details about the effects of different processing methods on the digestive stability of TM are still not available. Crabs are always consumed after processing, followed by gastrointestinal digestion with proteases; the proteins are digested through the gastric and then the intestinal tracts. Since the human

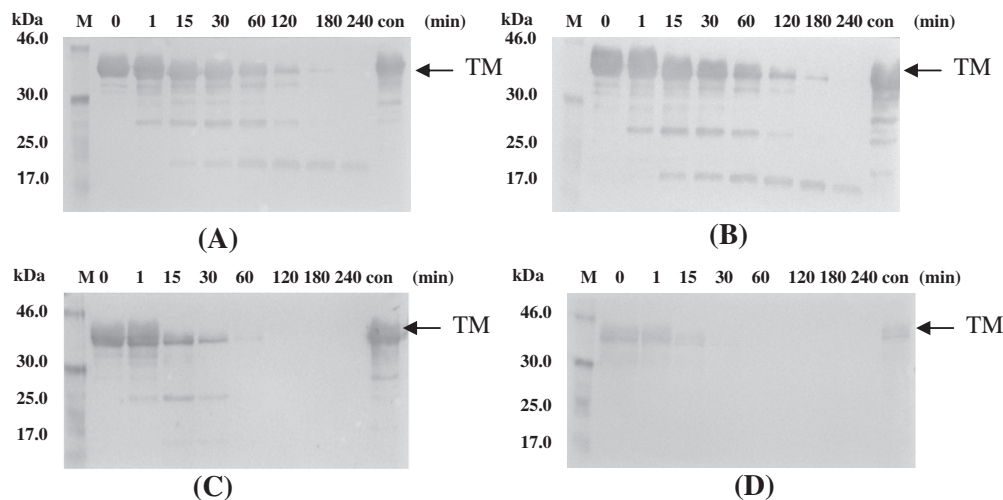


Fig. 6. Western blot detection of the degradation of TM from the crude extracts of processed crab in SIF (chymotrypsin). The crabs were untreated (A); treated with boiling (B); treated with CUB (C); or treated with HPS (D). The proteins which were separated by SDS–PAGE were blotted onto nitrocellulose, followed by an immunological detection with rabbit anti-mud crab TM polyclonal antibodies, to detect the IgG-binding of digested samples. Positions of molecular mass standards (M) are labeled on the left.

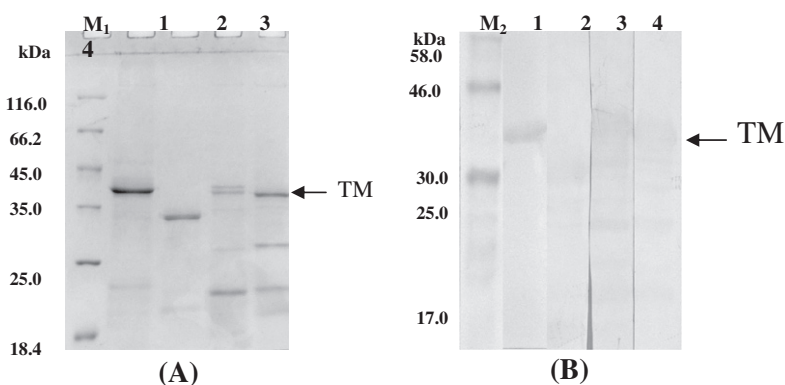


Fig. 7. Western blotting using the pooled sera to detect the IgE-binding of TM (from the crude extract of boiled crab) digested by SGF and SIF. Lanes: M₁, protein marker; M₂, prestained molecular mass standards; 1, CCE from crab treated with boiling (control); 2, CCE (boiling-treated crab) digested with pepsin; 3, CCE (boiling-treated crab) digested with trypsin; 4, CCE (boiling-treated crab) digested with chymotrypsin. The digestion time was 30 min. Samples were separated by SDS–PAGE (A), and then followed by immunological detection of the IgE-binding of TM with the pooled sera (B).

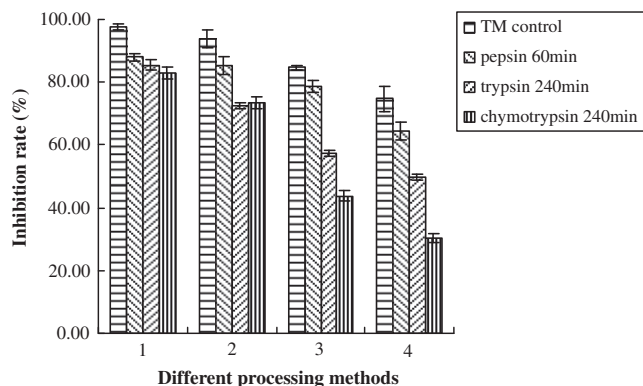


Fig. 8. Inhibition ELISA analysis of digestive samples of treated crab. 1, untreated crab; 2, crab treated with boiling; 3, crab treated with CUB; 4, crab treated with HPS. Samples digested by pepsin, trypsin or chymotrypsin for 1, 4 or 4 h, respectively. Undigested samples (controls) were diluted 1:10, and used as inhibitor solutions. Pooled patients' serum (1:5 dilution) and peroxidase-conjugated goat anti-human IgE antibody (1:2000 dilution) were used. All data expressed as the mean $\pm$ SD, $n = 2$. Values of the three treatments were significantly different from those of the control ($P < 0.05$).

gastrointestinal digestive system was very complicated correlated to kinds of enzymes, pepsin, trypsin and chymotrypsin were the main digestive enzymes, so they were chosen to act in the digestive pattern separately in this study and the digestive time for SGF and SIF was selected as 1 and 4 h, respectively. On that basis, it was considerable to be conducted for the sequential gastrointestinal digestive pattern for the further study.

Our results with *in vitro* simulated gastrointestinal digestion demonstrated that the digestive patterns of the three proteases were different because of their different cleavage specificities for peptide bonds, and the digestive stability of TM was higher than that of the other proteins in CCE. In SGF digestion, the original TM bands mainly produced a proteolytic fragment at 34 kDa, which had strong resistance to pepsin. Otherwise, in the case of SIF digestion, the main proteolytic fragments were bands of 37, 34, 28 and 22 kDa.

Research on the structure of the crab allergen TM has revealed that it is rather stable even when treated by normal processing methods (Leung et al., 1998; Motoyama et al., 2006, 2007). Besler et al. (2001) have researched the allergenicity of processed food (including crustaceans), and have shown that processing methods can lessen food allergenicity. Allergic proteins normally contain

numerous and structurally identical or different IgE-binding sites. After food has been processed, it might induce changes in the structure and quantity of those sites, which alters IgE binding. Normally, food processing is followed by repression of food allergenicity (Sathe et al., 2005). We used boiling, CUB and HPS treatment of crab, and showed that the three methods did influence the digestive stability of TM from CCE.

Takagi et al. (2003) have found that heating can enhance the digestion of parvalbumin in simulated gastrointestinal digestion, and affect the digestive pattern and formation of the degraded products. Other studies have shown that the main allergen of crustaceans, TM, is heat-stable. However, our study showed that boiling accelerated TM digestion in SGF (Fig. 1B), which suggested that the whole crab was processed, and there might have been other crab proteins that could have affected the structure and characterization of TM. But for the SIF digestion, boiling treatment did not accelerate TM digestion comparing with the untreated crab, the explanation for this phenomenon need to be further studied. For CUB or HPS treatment, the allergic protein TM was degraded much more quickly than the untreated TM in SGF and SIF. The original bands were undetectable after 15 min of digestion by pepsin, whereas the untreated TM needed 30 min (Fig. 2A, C and D). In contrast, during digestion by trypsin and chymotrypsin, the time needed for complete degradation of TM was shorter than that for untreated TM, especially for HPS treatment. The reasons for the rapid digestion of TM that was extracted using CUB or HPS treatment might be as follows. During CUB, proteins were connected by S–S bonds to form polymers, which resulted in changes in the surface properties and molecular dimensions of the proteins (Sancho et al., 2005). Ultrasound treatment could have resulted in chemical and physical changes in a viscous medium by cyclic generation and collapse of cavities, and the increased pressure and temperature in the vicinity of these cavities could have led to changes in the native protein structure (Li et al., 2006). In addition, the temperature and pressure of HPS could have destroyed the structure of TM significantly, loosened the protein formation and weakened the interaction between the bonds, and led to easier degradation by protease.

In addition, the control samples and the “0 min” samples in the digestion experiment were set separately, because the control samples could be used to identify the stability of the target protein during the reaction in SGF for 1 h or in SIF for 4 h without digestive enzymes, while the “0 min” samples can not. As shown in Fig. 3 and Fig. 5, there is a little difference between the protein bands of the “Con” and “0 min” in SIF digestion, while the “Con” remained almost the same as the “0 min” in SGF digestion (Fig. 1). The reason for the difference may be that the reacting time for SIF was as long as 4 h, some unstable proteins of the CCEs would display faint degradation.

With regard to the allergenicity of TM, it is noteworthy that our immunoblotting and inhibition ELISA data similarly proved that the IgE-binding of TM from CCE was greatly decreased when treated by CUB or HPS. These results confirmed those of Li et al. (2006) and Peñas et al. (2006). Different treatment might change the IgE-binding activity of degraded fragments of TM. As we know that, after treatment and digestion, TM would produce much more degraded fragments than we can see on the SDS–PAGE. It is possible that the IgE-reactive substances are low molecular weight peptide fragments with IgE-binding epitopes derived from TM. Small fragments or peptides may be not detected by SDS–PAGE or immobilized by immunoblots. So, inhibition ELISA is more sensitive in detecting all the proteolytic fragments with IgE reactivity which can not be shown just from SDS–PAGE or immunoblots, so the results of SDS–PAGE or immunoblots might different from inhibition ELISA. Inhibition ELISA showed the difference of IgE reactivity of TM treated by three methods in much further details. In the study of Shimakura et al. (2005), proteins were undetectable in crab

extractive 2 as analyzed by SDS–PAGE and immunoblots but the presence of IgE-reactive substances was confirmed by inhibition ELISA. It is ascertained from HPLC that the majority of IgE-reactive substances in crab extractive 2 appeared in low molecular weight fractions, indicating the presence of IgE-reactive peptide fragments. Nevertheless, IgE-binding might not equate completely to allergenicity. To evaluate further the IgE-binding alterations of TM after processing and proteinase digestion, experiments such as the release of histamine from basophils and skin prick test are required, so that we can establish the processing methods that can reduce allergenicity.

Huang et al. (2010) only used purified TM from untreated crab. In contrast, we processed the whole crab and crude extracts were subjected to gastrointestinal digestion. This enabled us to establish whether the processing methods, proteins other than TM, and proteases could affect the digestibility and allergenicity of crab TM. Our results showed that TM from treated crab was more easily digested in SGF and SIF than was purified TM from untreated crab. This suggests that processing methods, proteins other than TM, or proteases can affect digestibility of TM.

With regard to canned crab meat, the results of our study indicated that CUB with certain modifications can be used in the canned crab industry, because it can accelerate the digestion of TM and reduce its allergenicity. For the traditional family cooking of crab, a pressure cooker can be used, because HPS clearly changes the structure of TM and makes it easy to digest, with a concomitant reduction in hypersensitivity.

5. Conclusions

The digestive stability and reactivity of TM from *S. paramamosain* treated with different processing methods were studied by *in vitro* simulated gastrointestinal digestion and immunoassay. In conclusion, boiling was found to accelerate the digestion of TM in simulated gastric fluid. CUB could also increase the digestion of TM in SGF and SIF. Comparing the three processing methods, HPS was found the most effective method to promote the degradation of TM by digestive protease, and to decrease the IgG/IgE-binding of TM in the simulated gastrointestinal system. To some extent, the results provide a reference value for the achievement of lower allergenicity of processed crab products.

Conflict of Interest

The authors declare that there are no conflicts of interest.

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