



Stability of major allergen tropomyosin and other food proteins of mud crab (*Scylla serrata*) by in vitro gastrointestinal digestion

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ABSTRACT

Stability in simulated gastric fluid is regarded as an important parameter for the estimation of food allergenicity. In this study, the digestive stability of allergenic protein tropomyosin (TM) and other food proteins from mud crab in simulated gastric fluid (SGF) and simulated intestinal fluid (SIF) digestion assay system was investigated and compared by SDS–PAGE and Western blot. In SGF system, proteins such as actin and the original band of myosin heavy chain (MHC) were rapidly degraded within a short period of time, while TM was relatively resistant to pepsin digestion. In SIF system, MHC was easily decomposed, while TM and actin were similarly resistant to digestion. Further study by IgE-immunoblotting and inhibition ELISA using sera from crab-allergic patients indicated that allergenicity of TM was partially decreased, suggesting proteinase digestion is effective in reducing the allergenicity of crab TM.

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1. Introduction

Scylla serrata, commonly known as mangrove crab or mud crab, is the only species of the Portunidae family that is closely associated with mangrove environments. It is widely distributed throughout the Indo-Pacific region and can also be found in countries with more temperate environments such as China and Japan (Macnae, 1968). There is a high consumer demand for mud crab, particularly in Southeast Asia because it is regarded as a delicacy for its large chelae and higher meat content. However, as one of the eight major sources of food allergens proposed by the Food and Agriculture Organization (FAO) of the United Nations and the World Health Organization (WHO), reports about allergic reactions induced by ingestion of shellfish such as crab and shrimp are frequent (FAO/WHO, 2001). Research has demonstrated that the major allergen of crab and shrimp is tropomyosin (TM), a myofibrillar protein composed of two identical subunits with molecular masses of 35–38 kDa, each in α -helix form coiled around one another in the coiled-coil formation (Shanti et al., 1993; Leung et al., 1998).

Abbreviations: DAB, 3,3'-diaminobenzindin tetrahydrochloride; GI, gastrointestinal; HRP, horseradish peroxidase; MHC, myosin heavy chain; SDS–PAGE, SDS–polyacrylamide gel electrophoresis; SIF, simulated intestinal fluid; SGF, simulated gastric fluid; TBST, Tris-buffered saline with Tween-20; TM, tropomyosin.

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Crustacean allergy is an IgE-mediated, type I allergy. A number of IgE-binding epitopes have been identified in the major allergen TM, though they may vary from one allergic individual to another (Leung et al., 1998; Motoyama et al., 2007). More recently, we have cloned the full-length amino acid sequences of TM from three species of crab (Chinese mitten crab, mud crab and swimming crab) and the amino acid sequence identity of TMs among these crabs reached as high as 99.2% (Liang et al., 2008). Thus, it is not surprising that crustacean TMs show high IgE cross-reactivity with one another (Leung et al., 1996). Furthermore, TM is extremely stable retaining its original allergenic activity after heat treatment (Leung et al., 1994; Shanti et al., 1993).

It is generally regarded that for a native or denatured food protein to retain its allergenicity, the specific structure or linear amino acid sequence of the protein to which human IgE antibody is directed (i.e., the structural and linear epitopes) must survive food-processing treatments and in vivo digestion. The latter property suggested that for an allergen sensitizing an individual via the gastrointestinal (GI) tract, it must endure degradation in the GI tract (such as resistance to low pH, bile salts and proteolysis), thus allowing allergen protein to reach the intestinal immune system (Taylor, 2002). Accordingly, food allergens are generally resistant to pepsin digestion. Digestibility test has been regarded as one of appropriate methods for evaluating the allergenicity of newly introduced proteins (FAO/WHO, 2001).

Known food allergens are more stable under simulated gastric fluid (SGF) conditions than non-allergens (Astwood et al., 1996). Allergens of peanut (Ara h 2) and milk (β -lactoglobulin) are more stable in SGF than non-allergenic proteins such as soybean seed lipoxygenase. Thus, it was proposed that the stability to digestion is a significant and valid parameter to distinguish food allergens from non-allergens (Astwood et al., 1996). Nevertheless, conflicting results have been obtained showing that food allergens are not necessarily more resistant to digestion than non-allergenic proteins (Fu et al., 2002; Yagami et al., 2000). Yagami et al. (2000) found that most fruit allergens and other non-allergenic proteins were decomposed by the SGF within 8 min while in the case of simulated intestinal fluid (SIF), allergens from avocado, banana, potato, melon and peach were not completely degraded even after digestion for 16 h.

In this study, we focused on the digestive stability of the major crab allergen TM and unproven-allergenic proteins of crab muscle under SGF and SIF conditions. The pepsin/protein ratios were selected to represent the *in vivo* ratio in human gastrointestinal tract with a purpose to clarify the digestibility of TM. Furthermore, immunological assays were performed to make clear the effects of proteinase digestion on the IgE-binding of crab TM.

2. Materials and methods

2.1. Crab and chemicals

Mud crab (*S. serrata*) was purchased alive at Jimei market, Xiamen. After washing, crabs were sacrificed by freezing at -80°C . Both leg muscle and chest protection muscle were obtained and immediately used.

Protein standards for SDS–polyacrylamide gel electrophoresis (SDS–PAGE) were from Fermentas (Lithuania) or Bio-Rad (Richmond, CA, USA). Prestained protein standards for Western blot were from New England Biolabs (Beverly, MA, USA). Rat anti-common carp actin and rabbit anti-mud crab TM polyclonal antibodies were prepared in our laboratory (Liang et al., 2008). Horseradish peroxidase labeled rabbit anti-rat IgG was from DAKO (Glostrup, Denmark). Horseradish peroxidase labeled goat anti-human IgE was from Kirkegaard and Perry Laboratories (Maryland, USA). Horseradish peroxidase labeled goat anti-rabbit IgG and 3,3'-diaminobenzidine tetrahydrochloride (DAB) were from Pierce (Rockford, IL, USA). O-Phenyldiamine, porcine trypsin and bovine α -chymotrypsin were purchased from Sigma (St. Louis, MO). 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 of absorbance of 1.0 at 280 nm for 30 min incubation at 37°C . The activity of pepsin for hydrolyzing hemoglobin was 318.2 U and the specific activity of pepsin was 272 U/mg. Other reagents were all of analytical grade.

2.2. Human sera

Sera from five patients were obtained from the hospital of Jimei University. The patients with ages of 18–22 were all proposed of having crustacean allergy on the basis of their clinical history of immediate hypersensitivity reactions, such as urticaria and diarrhea, after ingestion of crustaceans. Written informed consent was obtained from each patient. These patient sera were used as a pooled positive sample or individually. All sera were stored at -80°C until used.

2.3. Preparation of myofibrillar proteins

Mud crab myofibrillar proteins were prepared as described (Cao et al., 2004). Briefly, crab muscle was minced and homogenized with five volumes of ice-cold 20 mM Tris–HCl buffer (pH 7.5) using a homogenizer (Kinematica, Lucerne, Switzerland). The homogenization process was performed using a polytron of PT-DA 2120 at speed indicator 15, and the operation was carried out three times for 30 s each with an interval of 1 min. The homogenate was centrifuged at 8000g, for 10 min at 4°C . The precipitate was collected and resuspended in five volumes of 20 mM Tris–HCl buffer. After four repeated cycles of homogenization and centrifugation, the resulting precipitate was dissolved in 100 mM Tris–HCl buffer (pH 7.5) containing 0.5 M NaCl and was regarded as the mud crab myofibril extract.

2.4. Purification of crab TM and protein content determination

The purification of crab TM was carried out as described (Liang et al., 2008). Briefly, an acetone powder of myofibrillar proteins was prepared from crab muscle as reported by Huang and Ochiai (2005) and extracted with 10 volumes of 20 mM Tris–HCl buffer (pH 7.5) containing 1 M KCl and 10 mM 2-mercaptoethanol. The homogenate was centrifuged at 20,000g for 20 min, the supernatant was isoelectric precipitated at pH 4.5 with HCl and the precipitate was dissolved in 20 mM Tris–HCl (pH 7.5) and successively salted out with 40–60% saturation of $(\text{NH}_4)_2\text{SO}_4$. After centrifugation at 20,000g for 20 min, the precipitate was collected and redissolved in 20 mM Tris–HCl (pH 7.5) followed by filtering off the residue. Finally, small amounts of impurities were removed by heating in a boiling water bath for 10 min followed by immediate cooling in ice water and centrifugation at 20,000g for 20 min. The supernatant containing TM with high purity was used for experiments or stored at -80°C until used.

The protein concentration of myofibrils and purified crab allergen TM was determined by measuring the absorbance at 280 nm or with the Lowry method as described (Lowry et al., 1951) using bovine serum albumin as the standard.

2.5. SGF digestion stability assay

The digestibility of purified TM and myofibrillar proteins in SGF was examined according to the method as described by Thomas et al. (2004) with some modifications. SGF was prepared as described in the United States Pharmacopoeia (1995) and consists of porcine pepsin A (272 U/mg) in 35 mM NaCl at pH 1.2. Volume of reaction solution was 1 mL. For each sample, a centrifuge tube (1.5 mL) containing SGF was preheated at 37°C prior to the addition of test protein. For the final digestion conditions, a ratio of 0.33 U of pepsin activity/ μg protein was selected for all tests (1:50 pepsin to protein, w:w). Digestion was performed at 37°C with continuous rocking. An aliquot (100 μL) of the digest was withdrawn at different time intervals (0.5, 1, 2, 5, 10, 15, 30 and 60 min) and was immediately terminated by addition of 30 μL of 200 mM Na_2CO_3 and 26 μL of $4 \times$ Laemmli buffer. Samples were heated at 95°C for 10 min and analyzed by SDS–PAGE or Western blot. For the 0 min sample preparation, protein samples were mixed with SGF that had been inactivated by neutralization with Na_2CO_3 . After adding Laemmli buffer, samples were then heated for SDS–PAGE. In the control experiments, each protein sample was dissolved in the reaction buffer without pepsin and then treated as described above. All the experiments were repeated three or more times and no obvious differences were identified.

2.6. SIF digestion stability assay

For duodenal digestion, porcine trypsin and bovine α -chymotrypsin were used. SIF was prepared as described in the United States Pharmacopoeia (1995) and consists of trypsin or α -chymotrypsin in 0.05 M KH_2PO_4 , pH 7.5. The final volume of reaction solution was 1 mL. For each sample, a centrifuge tube containing 1 mL of simulated intestinal fluid (SIF) was preheated at 37°C prior to the addition of test protein. The enzyme activity of trypsin and α -chymotrypsin was 325 U/mg protein and 62 U/mg protein, respectively.

Digestion conditions were as follows: a ratio of 0.20 U of trypsin activity/ μg protein was selected for all tests (1:50 trypsin to protein, w:w), and a ratio of 0.04 U of chymotrypsin activity/ μg protein was selected for all tests (1:50 chymotrypsin to protein, w:w). Digestions were performed at 37°C with continuous rocking. An aliquot (100 μL) of the digest was withdrawn at different time intervals (0, 1, 15, 30, 60, 120, 180 and 240 min), and 26 μL of the sample buffer was immediately added. The reaction was stopped by addition of serine proteinase inhibitor pefabloc SC to a final concentration of 5 mM and heating at 95°C for 10 min followed by SDS–PAGE. For 0 min sample preparation, each sample was mixed with SIF that had been inactivated by boiling for 5 min followed by addition of 26 μL of sample buffer and further heated at 95°C for 10 min for SDS–PAGE. In the control experiments, each protein sample was dissolved in the reaction buffer that did not contain corresponding enzyme and then treated as described above.

2.7. SDS–PAGE analysis and Western blot

SDS–polyacrylamide gel electrophoresis (SDS–PAGE) was performed under reducing conditions according to the method of Laemmli (1970). Samples were separated in 12% polyacrylamide gels with 5% stacking gel and the gels were stained for protein with Coomassie Brilliant Blue R-250 (CBB).

Western blot was carried out as described by Towbin et al. (1979). Briefly, protein samples were subjected to SDS–PAGE followed by electrophoretic transfer to nitrocellulose membranes. Non-specific protein sites were blocked with 5% non-fat milk in Tris–HCl buffered saline (TBS = 20 mM Tris–HCl, pH 7.5, containing 0.145 M NaCl). The membrane was incubated with rat anti-common carp actin or rabbit anti-mud crab TM polyclonal antibodies (1:2000 dilution) at room temperature for 2 h and washed with TBST (TBS, 0.05% Tween-20). After incubation for 1 h with horseradish peroxidase (HRP) labeled rabbit anti-rat IgG or goat anti-rabbit IgG secondary antibodies (1:2000 dilution), the membranes were washed extensively with TBST. Immunodetection was carried out using DAB as substrate.

For the detection of IgE-binding activity of TM and its digestive products, the membrane was incubated with human serum (1:7 dilution) at 4 °C for 16 h. Peroxidase-conjugated goat anti-human IgE antibody (1:2000 dilution) was then allowed to react with the membrane at room temperature for 1 h followed by extensive washing and detection by DAB.

2.8. Inhibition ELISA

Polystyrene 96-well ELISA plates (Nunc Maxisorb, Denmark) were coated with TM (200 ng/well) at 4 °C for 16 h using 15 mM sodium carbonate (pH 9.6) as the coating buffer. Coated plates were washed five times with TBST and then blocked with 200 µL blocking buffer (0.5% bovine serum albumin in TBST) at 37 °C for 2 h. In another microtiter plate, 100 µL human sera (diluted 1/20 with 0.1% bovine serum albumin in TBS) was mixed with equal volumes of digested TM (respectively with pepsin, trypsin and chymotrypsin) as inhibitor in the concentration range of 0.02–40 µg/mL. After incubation at 37 °C for 1 h, each 50 µL of the sera–TM mixture was transferred to the ELISA plate coated with TM and further incubated at 37 °C for 1 h. Plates were washed with TBST, followed by reaction with HRP-conjugated goat anti-human IgE antibody (1:2000 dilution) for 2 h at 37 °C. The color reaction was developed using 0.05 M phosphate-citrate buffer (pH 5.0) containing 0.1% *o*-phenylenediamine and 0.03% H₂O₂ and terminated by addition of 2 M sulfuric acid. The developed color was measured with a microplate reader (Benchmark 96, Bio-Rad Laboratories, Hercules, CA) at 490 nm. The loss of specific IgE-binding ability of patients' sera resulting from the treatment with human sera and digested TM as inhibitor was represented by calculating the inhibition rate using the following formula (Nakamura et al., 2006):

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

where *X* was the absorbance of patients' sera without inhibitors and *Y* and *Z* were the absorbance of patients' and control sera treated with various concentrations of inhibitors, respectively. All determinations were duplicated and variations between the two assays were always < 10%. The mean values were used.

3. Results

3.1. Digestibility of purified TM by SGF and SIF

The digestibility of the purified mud crab allergen TM was examined by SDS–PAGE. Purified TM revealed a band corresponding to the molecular mass of 38 kDa (Fig. 1). TM was relatively stable in SGF as the digestion of TM by pepsin was gradual and more proteolytic fragments appeared with increased digestion time while the largest fragment with size of approximately 34 kDa was resistant to digestion (Fig. 1A). On the other hand, in the SIF digestion assay, whether by trypsin or chymotrypsin, more degraded fragments appeared (Fig. 1B and C). Several proteolytic fragments (34, 28 and 22 kDa) could clearly be detected. As digestion time increased, larger molecular mass fragments (34 and 28 kDa) gradually disappeared, while the digestion-resistant 22 kDa fragment progressively intensified (Fig. 1B and C). Moreover, a faint original TM band was still detectable at 4 h by chymotrypsin (Fig. 1C).

3.2. Digestibility of crab myofibrillar proteins by SGF and SIF

Myofibrils mainly consist of myosin heavy chain (MHC, 200 kDa), actin (42 kDa) and other minor proteins such as α -actinin (100 kDa), TM, troponins and myosin light chain. In the SGF reaction system, the actin band disappeared within 1 min and MHC was undetected within 5 min while a fragment of approximately 60 kDa which survived digestion up to 60 min was observed (Fig. 2A). In contrast, intact TM was not completely digested under the same SGF conditions for 60 min.

The stability of myofibrillar proteins in SIF was different from proteins in SGF. MHC was rapidly digested within 1 h whether by trypsin or chymotrypsin. However, actin was more resistant to digestion by trypsin than TM (Fig. 2B). For the digestion by chymotrypsin, not much difference was found between TM and actin, both of their original bands were undetectable after 2 h (Fig. 2C).

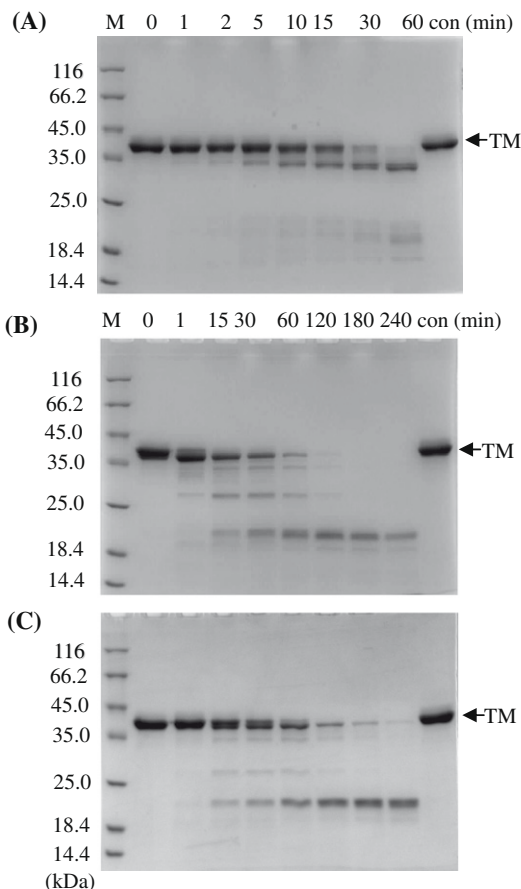


Fig. 1. Effect of SGF and SIF digestion on mud crab TM. Proteinases used were pepsin (A), trypsin (B) and α -chymotrypsin (C). Enzyme digestion was performed as described in Section 2 followed by SDS–PAGE. After electrophoresis, proteins were visualized by staining with CBB. In the control experiments (con) proteinase was excluded. Molecular masses of the protein markers (M) were shown at the left edges.

3.3. Western blot analysis of individual proteins

Myofibrillar proteins with larger molecular mass such as MHC, nebulin and titin present sources of fragment products that are difficult to ascertain by SDS–PAGE. To clarify the identity of the degraded myofibrillar protein fragments, Western blot using specific polyclonal antibodies against actin and TM were carried out. In the SGF digestion assay, actin was rapidly degraded to a fragment (30 kDa) that was undetectable after 10 min (Fig. 3A), suggesting SGF is quite effective in the degradation of actin. On the other hand, in the SIF digestion assay, actin was relatively resistant to trypsin, its original band could still be observed even after 4 h and some faint fragments were detected (Fig. 3B). Interestingly, actin was susceptible to chymotrypsin digestion and a fragment of 32.5 kDa was detectable after 1 min while all the bands disappeared after 1 h (Fig. 3C), so it is reasonable to propose that the fragment in Fig. 2B at approximately 32.5 kDa is also a digested product of actin.

The degradation of allergen TM in the myofibril extract by SGF and SIF was also investigated by Western blot. In contrast to actin, TM was relatively resistant to SGF digestion. Although a new degradation band (34 kDa) appeared within 1 min, both the original protein band and the 34 kDa fragment remained after 60 min (Fig. 4A), suggesting its stability against SGF digestion. Compared with SGF, under SIF conditions, TM was digested into fragments by trypsin and produced three main fragments with sizes of

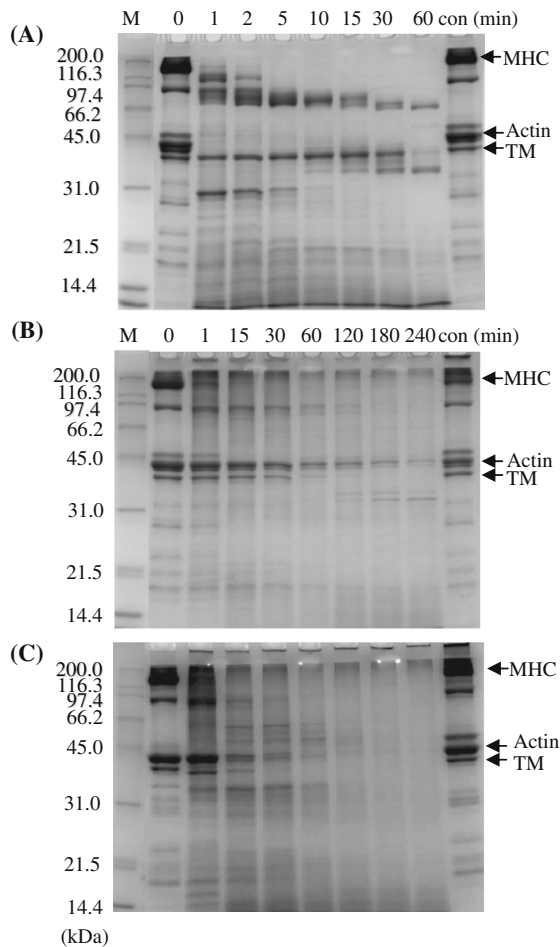


Fig. 2. Effect of SGF and SIF digestion on mud crab myofibrillar proteins. Proteinases used were pepsin (A), trypsin (B) and chymotrypsin (C). Enzyme digestion was performed as described in Section 2 followed by SDS-PAGE as in Fig. 1.

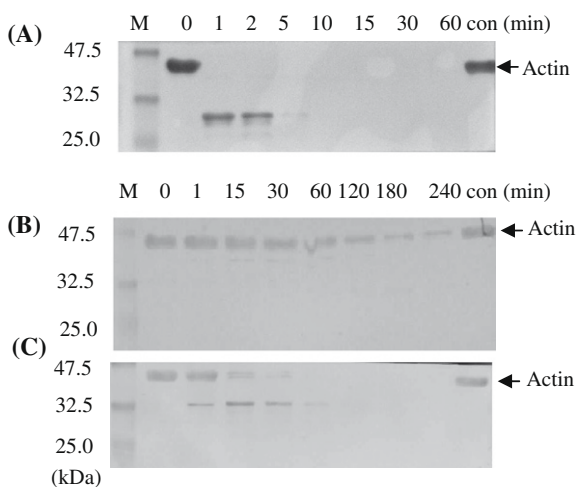


Fig. 3. Western blot detection of the degradation of actin in SGF and SIF. Proteinases used were pepsin (A), trypsin (B) and chymotrypsin (C). Samples were separated by SDS-PAGE, electrophoretically transferred to nitrocellulose membranes. Immunological detection was performed using a polyclonal antisera against common carp actin. Positions of molecular mass standards (M) were labeled on the left.

approximately 34, 28 and 22 kDa (Fig. 4B). All the bands disappeared completely after incubation for 3 h. In the presence of chy-

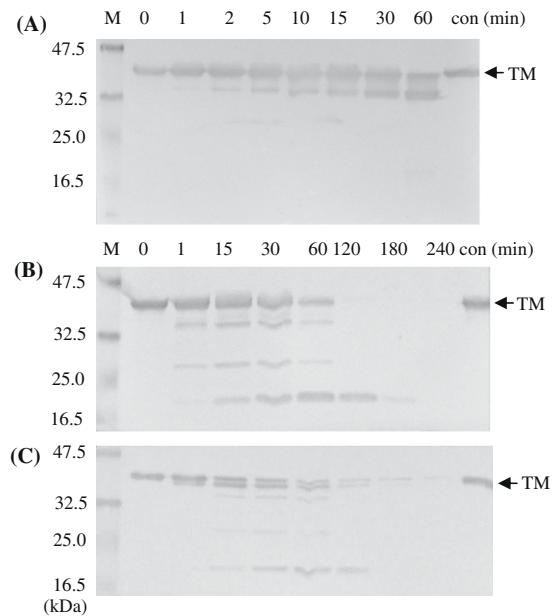


Fig. 4. Western blot detection of the degradation of TM in SGF and SIF. Proteinases used were pepsin (A), trypsin (B) and chymotrypsin (C). Samples were separated by SDS-PAGE followed by immunological detection using a polyclonal antisera against mud crab TM.

motrypsin, TM was also digested and produced four main fragments (37, 34, 28 and 22 kDa) while a faint original protein band still existed even after 4 h (Fig. 4C).

3.4. IgE-binding of TM by proteinase digestion

The IgE-binding of TM by proteinases (pepsin, trypsin and chymotrypsin) digestion was further analyzed by means of SDS-PAGE (Fig. 5A) and IgE-immunoblotting (Fig. 5B). Western blot with a serum pool of patients showed that their IgE antibodies positively reacted with the original TM band (38 kDa) and protein bands with size of 37 and 34 kDa. However, no IgE reactivity was observed against smaller fragments (28, 22 kDa). Possibly, IgE-binding epitopes in these fragments were destroyed by trypsin and chymotrypsin digestion. However, it should be reminded that unlike proteins, small peptides are less well immobilized by immunoblots. Therefore, inhibition ELISA which is more sensitive in detecting proteolytic fragments with IgE reactivity was conducted. As shown in Fig. 6, the effectiveness of proteinase digestion to the IgE-binding of mud crab TM was evaluated. Compared with

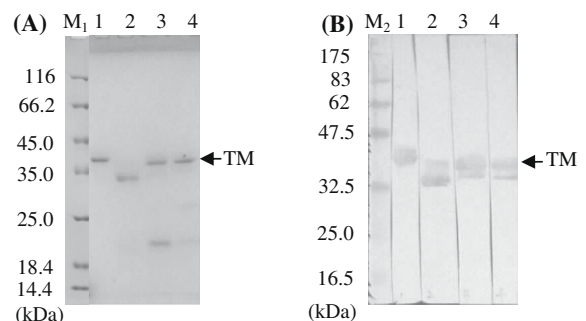


Fig. 5. SDS-PAGE (A) and IgE-Western blot (B) using human serum to detect the IgE-binding of TM after digestion by SGF and SIF. Lanes: M₁, protein marker; M₂, prestained molecular mass standards; 1, purified TM; 2, digested with pepsin; 3, digested with trypsin; 4, digested with chymotrypsin. Digestion time was 60 min.

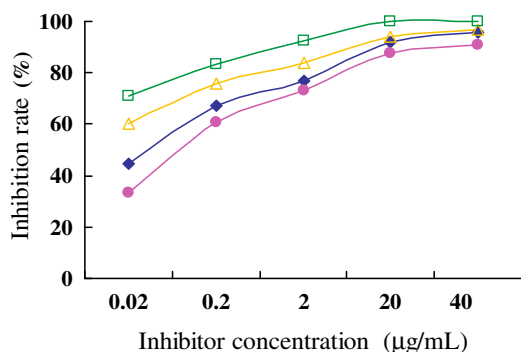


Fig. 6. Inhibition-ELISA of TM digestive samples of mud crab. The digestive samples by pepsin (Δ), trypsin (●), chymotrypsin (◆) for 1, 4 and 4 h, respectively and undigested TM (□) were diluted 1:5, 1:10, 1:100, 1:1000, 1:10,000 and used as inhibitor solutions. Pooled patient serum (1:20 dilution) was incubated with an equal volume of inhibitor solution at 37 °C for 1 h followed by ELISA. The initial protein concentration of each sample was 200 μg/mL.

undigested sample, the IgE-binding of all digested samples was reduced by proteinases treatment, especially by trypsin, suggesting proteinase treatment is an appropriate way in reducing TM IgE-binding.

4. Discussion

Recently, much attention has been paid on allergens from aquatic products, especially crustaceans and fish. Although characteristics of the major allergen TM of crustaceans have been widely studied (Leung et al., 1996, 1998; Shanti et al., 1993; Motoyama et al., 2007), detailed information concerning simulated digestion of TM is still not available. In this study, the digestibility of mud crab major allergen TM and other myofibrillar proteins by SGF and SIF was investigated. Considering the time that foods stay in the human digestive system, the digestion time by SGF and SIF was designated as 1 and 4 h, respectively. Our results showed that digestive patterns of TM by pepsin, trypsin and chymotrypsin were quite different (Fig. 1). The differences are the result of different cleavage specificities for peptide bonds of these three different proteinases. Pepsin A prefers to cleave peptide bonds following Phe or Tyr residues (Mikita and Padlan, 2007). Trypsin reveals a well-defined specificity, cleaving next to the hydrophilic amino acid residues Lys and Arg at the P₁ position (Barrett et al., 2004). Chymotrypsin, on the other hand, has a broader specificity, strongly preferring cleavage next to aromatic amino acids (Tyr, Trp and Phe) and Leu in the P₁ position, and to a much lower extent next to a few other amino acids (Barrett et al., 2004).

As TM represents only a small portion of crab muscular proteins, it is necessary to compare the digestive stability of TM with non-allergenic proteins or proteins with unproven-allergenicity in myofibrils. During the digestion of mud crab myofibrillar proteins by pepsin, degraded protein fragments gradually increased with the prolongation of digestion time (Fig. 2A). Interestingly, most of these fragments were in the molecular masses range between 200 and 66 kDa which are quite possibly degraded products of MHC, although the possibility that some of them were from much larger sized proteins such as titin (3000 kDa) and nebulin (600–800 kDa) should not be excluded. On the other hand, a new protein band (34 kDa) which is quite possibly the proteolytic fragment of TM could also be observed (Fig. 2A). This result was further confirmed in Fig. 4A by Western blot.

Though from the primary structure of mud crab TM there are more cleavage sites for trypsin than for chymotrypsin (Liang et al., 2008), our results revealed that chymotrypsin cleaved TM

more randomly. Quite possibly, the steric structure of TM is more available for chymotrypsin attack than that by trypsin. It is noteworthy that the degradation results using purified TM by means of SDS-PAGE (Fig. 1) were in accordance with that by Western blot which is more specific and sensitive (Fig. 4), suggesting the degradation pattern of TM by SGF and SIF was not affected by the presence of other myofibrillar proteins. In this study, rat anti-common carp actin and rabbit anti-mud crab TM polyclonal antibodies were used to evaluate the digestion of actin and TM in the presence of other myofibrillar proteins. Though the antibody against actin was prepared using common carp actin, this protein is well-conserved in evolution, and actually the polyclonal antibody was effective in detecting mud crab actin as well as certain of its proteolytic fragments.

It should be noticed that though the pepsin to allergen ratio is not a determinant factor for the degradation of extremely stable allergenic proteins such as β-lactoglobulin B (Astwood et al., 1996; Fu, 2002), the degradation rate of other allergens such as ovalbumin and non-allergenic enzymes was affected by the ratio (Fu et al., 2002). Our present study also indicated that enzyme-to-substrate ratio was influential in the stability of crab TM. When the pepsin to TM ratio (weight to weight) increased from 0.02 to 1, the protein was highly unstable. When the pepsin/test protein ratio increased to 5, neither the original protein nor degradation fragments were detectable after 10 min (data not shown). It is widely acknowledged that human digestion is variable from person to person (Moreno, 2007; Mills et al., 2004), so it is not easy to choose the optimal pepsin to substrate ratio which reflects the physiological situation. Choosing a ratio to compare the differences in the rate of digestibility in the presence of the same amount of enzyme allows us to determine if one protein is more resistant to digestion than another. In this study, porcine pepsin was prepared and its specific activity was 272 U/mg according to our definition and it reached 8185 U/mg according to the method of Sigma Chemical Co. As the pepsin we used was of higher activity than commercial pepsin (800–4500 U/mg), a lesser amount of pepsin was used for experiment and this may be the main reason why the pepsin band was not discernible on SDS-PAGE. In fact, for protein digestion analysis, the amount of proteinases used should be based on proteolytic activity but not on weight.

Gastric acidity also plays an important role in digestion because pepsin shows optimal proteolytic activity at low pH. Conditions with low net acid output, like, for example, hypoacidity as a physiologic feature in newborns, atrophic gastritis, or partial gastrectomy, might critically affect the digestive capacity of the stomach (Untersmayr et al., 2005). Under these circumstances, the digestion of allergenic proteins and some other food proteins might be suppressed, and then allergenic proteins might enter the gut with a subsequent absorption and sensitization. Except for the pepsin to protein ratio and pH, some other factors (purity of enzyme, methods of detection, food matrix and so on) will also have influence to the digestion. Therefore, the effect of simulated gastrointestinal digestion on proteins is a relative and comparative result.

In addition, it is noteworthy that our inhibition ELISA data only measured the IgE-binding which may not completely equate to allergenicity. This is because allergenicity determination requires divalent reactivity of the allergenic protein to cross-link IgE antibodies on the surface of mast cell or basophil membranes. For partially digested proteins, some fragments may just contain one IgE-binding site which may be able to bind IgE but not able to lead mediators release from mast cells or basophils. Thus, to further evaluate the allergenicity alteration of TM after proteinase treatment, experiments such as the release of histamine from basophils and skin prick test are required.

5. Conclusions

The digestibility of mud crab major allergen TM by SGF and SIF revealed that TM was resistant to pepsin while relatively susceptible to trypsin and chymotrypsin digestion. Both SDS–PAGE using purified TM and Western blot using myofibrillar proteins indicated that the degradation pattern of TM by SGF and SIF was not affected by the presence of other myofibrillar proteins. Inhibition ELISA results showed that proteinase treatment is a relatively effective way for reducing the IgE-binding of TM.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

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