

# Comparative study of *in vitro* digestibility of major allergen tropomyosin and other food proteins of Chinese mitten crab (*Eriocheir sinensis*)

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## Abstract

**BACKGROUND:** China is the largest producer and consumer of aquatic products in the world; however, many people in China suffer from allergies upon consuming crab. Stability in simulated gastric fluid is regarded as an important parameter for the estimation of food allergenicity.

**RESULTS:** The digestive stability of allergenic protein tropomyosin (TM) and other food proteins from Chinese mitten crab in simulated gastric fluid (SGF) and simulated intestinal fluid (SIF) digestion assay systems was investigated and compared by SDS-PAGE, western blot and inhibition ELISA. In the SGF system, proteins such as the original band of myosin heavy chain (MHC) and actin were rapidly degraded within a short period of time, while TM was relatively resistant to pepsin digestion. In the 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.

**CONCLUSION:** Chinese mitten crab major allergen 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 revealed that proteinase digestion is effective in reducing the allergenicity of crab TM.

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**Keywords:** Chinese mitten crab; allergen; tropomyosin; digestibility; simulated gastric fluid; simulated intestinal fluid; western blot; inhibition ELISA

## INTRODUCTION

*Eriocheir sinensis*, commonly known as Chinese mitten crab, is a native of east Asia and predominantly lives in freshwater but migrates seawards to breed. Chinese mitten crab has long been a fashionable delicacy in China. This kind of crab constitutes a promising freshwater fishery industry in China with a production of 420 000 tons in 2004.<sup>1</sup> 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.<sup>2</sup> 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  $\alpha$ -helix form coiled around one another in the coiled-coil formation.<sup>3,4</sup> 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.<sup>4,5</sup> More recently, we have reported the isolation, identification and primary structure determination of

crab allergen and confirmed that tropomyosin is the major allergen of *Eriocheir sinensis*, and 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 TM among these crabs reached as high as 99.2%.<sup>6</sup> Thus, it is not surprising that crustacean TMs show high IgE cross-reactivity with one another.<sup>7</sup> Furthermore, TM is extremely stable retaining its original allergenic activity after heat treatment.<sup>3,8</sup>

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

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food-processing treatments and *in vivo* digestion. The latter property suggested that, for an allergen sensitising 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.<sup>9</sup> Accordingly, food allergens are generally resistant to pepsin digestion. The digestibility test has been regarded as one of the appropriate methods for evaluating the allergenicity of newly introduced proteins.<sup>2</sup>

Known food allergens are more stable under simulated gastric fluid (SGF) conditions than non-allergens.<sup>10</sup> Allergens of peanut (Ara h 2) and milk ( $\beta$ -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.<sup>10</sup> Nevertheless, conflicting results have been obtained showing that food allergens are not necessarily more resistant to digestion than non-allergenic proteins.<sup>11,12</sup> Yagami *et al.*<sup>12</sup> 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 the present 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 digestion on the IgE-binding of crab TM.

## MATERIALS AND METHODS

### Crab

Chinese mitten crab (*Eriocheir sinensis*) was purchased alive at Jimei market, Xiamen. After washing, crabs were killed by freezing at  $-80^{\circ}\text{C}$ . Both leg muscle and chest protection muscle were obtained and immediately used.

### Chemicals

Protein standards for sodium dodecyl sulfate–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-mud crab TM polyclonal antibodies were prepared in our laboratory.<sup>6</sup> Horseradish peroxidase-labelled rabbit anti-rat IgG was from DAKO (Glostrup, Denmark). Horseradish peroxidase-labelled goat anti-human IgE was from Kirkegaard & Perry Laboratories (Gaithersburg, MD, USA). Horseradish peroxidase-labelled goat anti-rabbit IgG and 3,3'-diaminobenzidine tetrahydrochloride (DAB) were from Pierce (Rockford, IL, USA). *o*-Phenyldiamine, porcine trypsin and bovine  $\alpha$ -chymotrypsin were purchased from Sigma (St Louis, MO, USA). Porcine pepsin was prepared in our laboratory as previously described.<sup>13</sup> The proteolytic activity of pepsin was estimated by measuring trichloroacetic acid precipitable bovine haemoglobin 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  $37^{\circ}\text{C}$ . The activity of pepsin for hydrolysing haemoglobin was 318.2 U and the specific activity of pepsin was  $272\text{ U mg}^{-1}$ . Other reagents were all of analytical grade.

### Human sera

Sera from five patients were obtained from the Hospital of Jimei University. The patients, aged 18–22 years, were all proposed to have crustacean allergy on the basis of their clinical history of immediate hypersensitivity reactions, such as urticaria and diarrhoea, 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 aliquot stored at  $-80^{\circ}\text{C}$  until used.

### Preparation of myofibrillar proteins

Chinese mitten crab myofibrillar proteins were prepared as described.<sup>14</sup> Briefly, crab muscle was minced and homogenised with five volumes of ice-cold  $20\text{ mmol L}^{-1}$  Tris-HCl buffer (pH 7.5) using a homogeniser (Kinematica, Lucerne, Switzerland). The homogenisation 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  $8000 \times g$  for 10 min at  $4^{\circ}\text{C}$ . The precipitate was collected and resuspended in five volumes of  $20\text{ mmol L}^{-1}$  Tris-HCl buffer. After four repeated cycles of homogenisation and centrifugation, the resulting precipitate was dissolved in  $100\text{ mmol L}^{-1}$  Tris-HCl buffer (pH 7.5) containing  $0.5\text{ mol L}^{-1}$  NaCl and was regarded as the Chinese mitten crab myofibril extract.

### Purification of crab tropomyosin

The purification of crab TM was carried out as described.<sup>6</sup> Briefly, an acetone powder of myofibrillar proteins was prepared from crab muscle as reported by Huang and Ochial,<sup>15</sup> and extracted with 10 volumes of  $20\text{ mmol L}^{-1}$  Tris-HCl buffer (pH 7.5) containing  $1\text{ mol L}^{-1}$  KCl and  $10\text{ mmol L}^{-1}$  2-mercaptoethanol. The homogenate was centrifuged at  $2000 \times g$  for 20 min, the supernatant was isoelectrically precipitated at pH 4.5 with HCl and the precipitate was dissolved in  $20\text{ mmol L}^{-1}$  Tris-HCl (pH 7.5) and successively salted out with 40–60% saturation of  $(\text{NH}_4)_2\text{SO}_4$ . After centrifugation at  $20\,000 \times g$  for 20 min, the precipitate was collected and redissolved in  $20\text{ mmol L}^{-1}$  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  $20\,000 \times g$  for 20 min. The supernatant containing TM with high purity was used for experiments or stored at  $-80^{\circ}\text{C}$ .

### Determination of protein content

The protein concentration of myofibrils and purified crab allergen TM was determined by measuring the absorbance at 280 nm or with the method of Lowry *et al.*,<sup>16</sup> as described, using bovine serum albumin as the standard.

### Simulated gastric fluid digestion stability assay

The digestibility of purified TM and myofibrillar proteins in SGF was examined according to the method described by Thomas *et al.*<sup>17</sup> with some modifications. SGF was prepared as described in the United States Pharmacopoeia<sup>18</sup> and consists of porcine pepsin A ( $272\text{ U mg}^{-1}$ ) in  $35\text{ mmol L}^{-1}$  NaCl at pH 1.2. The volume of reaction solution was 1 mL. For each sample, a centrifuge tube (1.5 mL) containing SGF was preheated at  $37^{\circ}\text{C}$  prior to the addition of test protein. For the final digestion conditions, a ratio of 0.33 U of pepsin activity  $\mu\text{g}^{-1}$  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 mmol L<sup>-1</sup> Na<sub>2</sub>CO<sub>3</sub> and 26 µL of 4× Laemmli buffer. Samples were heated at 95 °C for 10 min and analysed by SDS-PAGE or western blot. For the 0 min sample preparation, protein samples were mixed with SGF that had been inactivated by neutralisation with Na<sub>2</sub>CO<sub>3</sub>. 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.

#### Simulated intestinal fluid digestion stability assay

For duodenal digestion, porcine trypsin and bovine α-chymotrypsin were used. SIF was prepared as described in the United States Pharmacopoeia<sup>18</sup> and consists of trypsin or α-chymotrypsin in 0.05 mol L<sup>-1</sup> KH<sub>2</sub>PO<sub>4</sub>, 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<sup>-1</sup> proteins and 62 U mg<sup>-1</sup> proteins, respectively.

Digestion conditions were as follows: a ratio of 0.20 U of trypsin activity µg<sup>-1</sup> protein was selected for all tests (1:50 trypsin to protein, w/w), and a ratio of 0.04 U of chymotrypsin activity µg<sup>-1</sup> 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 the serine proteinase inhibitor pefabloc SC to a final concentration of 5 mmol L<sup>-1</sup> 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.

#### SDS-PAGE analysis

SDS-PAGE was performed under reducing conditions according to the method of Laemmli.<sup>19</sup> 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 analysis

Western blot was carried out as described by Towbin *et al.*<sup>20</sup> 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 mmol L<sup>-1</sup> Tris-HCl, pH 7.5, containing 0.145 mol L<sup>-1</sup> 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)-labelled 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.

#### Inhibition ELISA

Polystyrene 96-well ELISA plates (Nunc Maxisorb; Nunc Maxisorp, Roskilde, Denmark) were coated with TM (200 ng well<sup>-1</sup>) at 4 °C for 16 h using 15 mmol L<sup>-1</sup> 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 microtitre 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 µL mL<sup>-1</sup>. 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 colour reaction was developed using 0.05 mol L<sup>-1</sup> phosphate-citrate buffer (pH 5.0) containing 0.1% o-phenyldiamine and 0.03% H<sub>2</sub>O<sub>2</sub> and terminated by addition of 2 mol L<sup>-1</sup> sulfuric acid. The developed colour was measured with a microplate reader (Benchmark 96; Bio-Rad Laboratories, Hercules, CA, USA) 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 formula:<sup>21</sup> inhibition rate (%) = [(X - Y) ÷ (X - Z)] × 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.

## RESULTS AND DISCUSSION

#### Digestibility of crab myofibrillar proteins by simulated gastric fluid and simulated intestinal fluid

Myofibrils mainly consist of TM, myosin heavy chain (MHC, 200 kDa), actin (42 kDa) and other minor proteins such as α-actinin (100 kDa), troponins and myosin light chain. It is necessary to compare the digestive stability of TM with non-allergenic proteins or proteins with unproven allergenicity in myofibrils. Considering the time that foods stay in the human digestive system, the digestion time in stomach and intestine was designated as 1 h and 4 h, respectively. 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. 1A). In contrast, intact TM was not completely digested under the same SGF conditions for 60 min. As shown in Fig. 1A, degraded protein fragments gradually increased with the prolongation of digestion time. Interestingly, most of these fragments were in the molecular masses range between 200 kDa and 66 kDa which are quite possibly degraded products of MHC, although the possibility that some of them

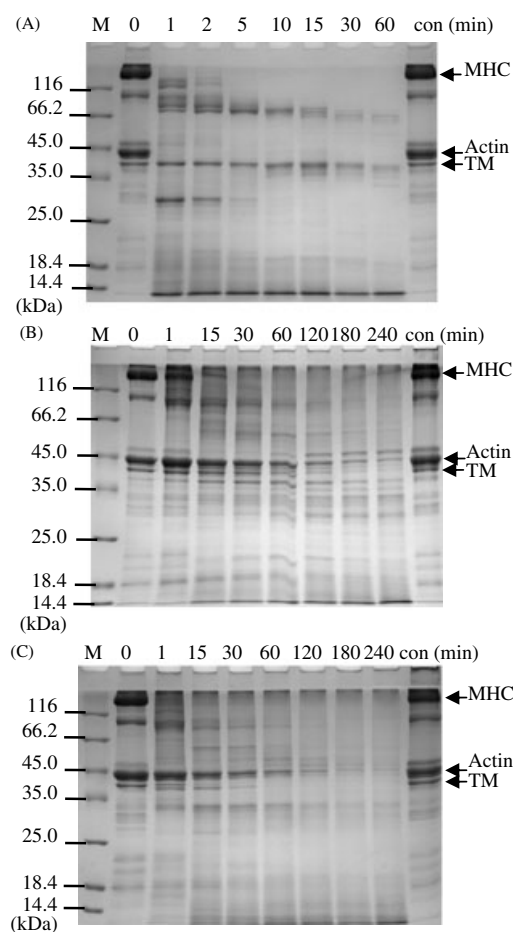


**Figure 1.** Effect of SGF and SIF digestion on Chinese mitten crab myofibrillar proteins. Proteinases used were pepsin (A), trypsin (B) and chymotrypsin (C). Enzyme digestion was performed as described in 'Materials and Methods' followed by SDS-PAGE. After electrophoresis, proteins were visualised 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.

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. 1A). This result was further confirmed in Fig. 2A which agreed well with that using purified TM as shown in Fig. 3A, suggesting the presence of other proteins did not interfere with the digestion of TM by SGF.

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 (Fig. 1B and C). However, actin was more resistant to digestion in SIF than TM. For the digestion by trypsin, it was found the original band of TM to be undetectable in 3 h, while that of actin still to be visible after 4 h (Fig. 1B). For the digestion by chymotrypsin, it was found the original band of TM to be undetectable in 1 h, while that of actin still to be visible after 3 h (Fig. 1C).

#### Digestibility of purified tropomyosin by simulated gastric fluid and simulated intestinal fluid

The digestibility of the purified Chinese mitten crab allergen TM was examined by SDS-PAGE. Purified TM revealed a band

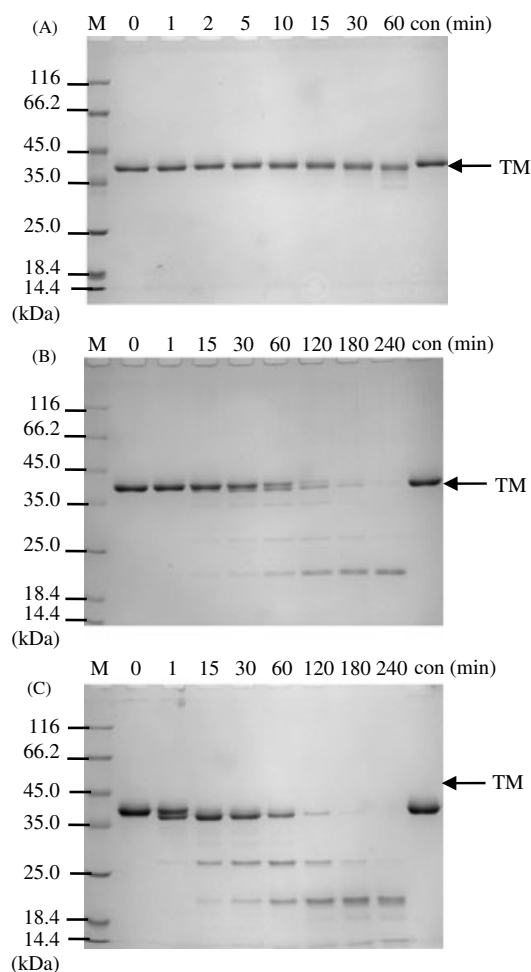


**Figure 2.** Effect of SGF and SIF digestion on Chinese mitten crab TM. Proteinases used were pepsin (A), trypsin (B) and  $\alpha$ -chymotrypsin (C). Enzyme digestion was performed as described in 'Materials and Methods' followed by SDS-PAGE as in Fig. 1.

corresponding to the molecular mass of 38 kDa (Fig. 2). Digestive patterns of TM by pepsin, trypsin and chymotrypsin were quite different. TM was relatively stable in SGF, it was hardly digested by pepsin, only a bit of fragments (34–38 kDa) could be detected after 60 min (Fig. 2A). On the other hand, in the SIF digestion assay, whether by trypsin or chymotrypsin, more degraded fragments appeared (Fig. 2B and C). Several proteolytic fragments (34, 28, 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. 2B and C). Moreover, two faint fragments (14 and 18 kDa) appeared after being digested for 3–4 h by chymotrypsin (Fig. 2C).

The different TM digestion patterns *in vitro* by pepsin, trypsin and chymotrypsin 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.<sup>22</sup> Trypsin reveals a well-defined specificity, cleaving next to the hydrophilic amino acid residues Lys and Arg at the P<sub>1</sub> position.<sup>23</sup> 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 P<sub>1</sub> position, and to a much lower extent next to a few other amino acids.<sup>23</sup>

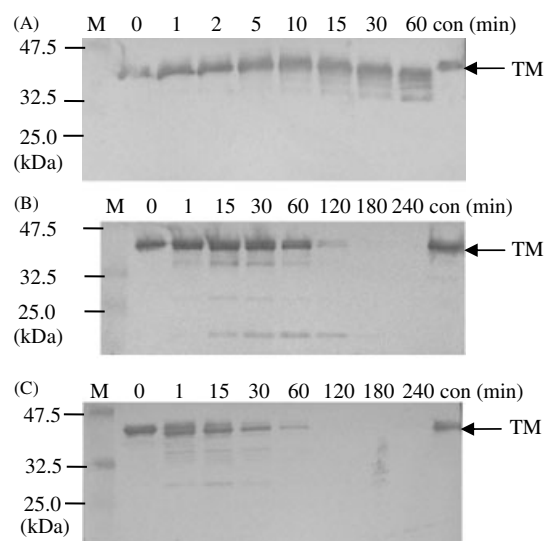


**Figure 3.** Western blot using polyclonal antibody to detect the degradation of TM in SGF and SIF. Proteinases used were pepsin (A), trypsin (B) and chymotrypsin (C). Samples were separated by SDS-PAGE followed by immunological detection using a polyclonal antiserum against mud crab TM. Positions of molecular mass standards (M) were labelled on the left.

### 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 antibody against TM was carried out. TM was relatively resistant to SGF digestion. Although two new degradation bands (34 and 32 kDa) appeared within 10 min, both the original TM band and the 34 and 32 kDa fragments remained after 60 min (Fig. 3A), 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 approximately 34 kDa, 28 kDa and 22 kDa. All the bands disappeared completely after incubation for 3 h (Fig. 3B). In the presence of chymotrypsin, TM was also digested and produced two main fragments (34 kDa and 28 kDa), all the bands disappeared completely after 4 h (Fig. 3C). Though from the primary structure of Chinese mitten crab TM there are more cleavage sites for trypsin than for chymotrypsin,<sup>6</sup> our present result revealed that chymotrypsin cleaved TM more randomly. It is noteworthy that the results using purified TM by means of SDS-PAGE (Fig. 2) were in accordance with those by western blot, which is more specific and sensitive (Fig. 3), suggesting the degradation pattern of TM by SGF and SIF was not affected by the presence of other myofibrillar proteins. In the present study, rabbit anti-mud crab TM polyclonal antibody was used to evaluate the digestion of TM in the presence of other myofibrillar proteins. Though the antibody against TM was prepared using mud crab (*Scylla serrata*), this protein is well-conserved in evolution, and actually the polyclonal antibody was effective in detecting Chinese mitten crab TM 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  $\beta$ -lactoglobulin B,<sup>10,24</sup> the degradation rate of other allergens such as ovalbumin and non-allergenic enzymes was affected by the ratio.<sup>11</sup> 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,<sup>25,26</sup> 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 the present study, porcine pepsin was prepared and its specific activity was 272 U mg<sup>-1</sup> according to our definition and it reached 8185 U mg<sup>-1</sup> according to the method of Sigma Chemical Co. As the pepsin we used was of higher activity than commercial pepsin (800–4500 U mg<sup>-1</sup>), 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. 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, such as hypoacidity as a physiological feature in newborns, atrophic gastritis or partial gastrectomy, might critically affect the digestive capacity of the stomach.<sup>27</sup> 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 sensitisation. 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 to proteins is a relative and comparative result.

### IgE-binding of tropomyosin by proteinase digestion

The IgE-binding of TM by proteinases (pepsin, trypsin and chymotrypsin) digestion was further analysed by means of IgE immunoblotting (Fig. 4). Western blot with a serum pool of patients showed that their IgE antibodies positively reacted with the original TM band (38 kDa) and degraded band with size of 34 kDa both in digestion of pepsin and trypsin. It was faint for the IgE antibodies reacted with the digestion product

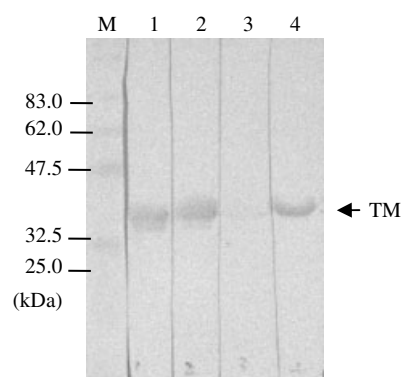


**Figure 4.** Western blot using human serum to detect the IgE binding of TM after digestion by SGF and SIF. Lanes: M, Prestained molecular mass standards; 1, purified TM; 2, digested with pepsin; 3, digested with trypsin; 4, digested with chymotrypsin. Digestion time was 60 min.

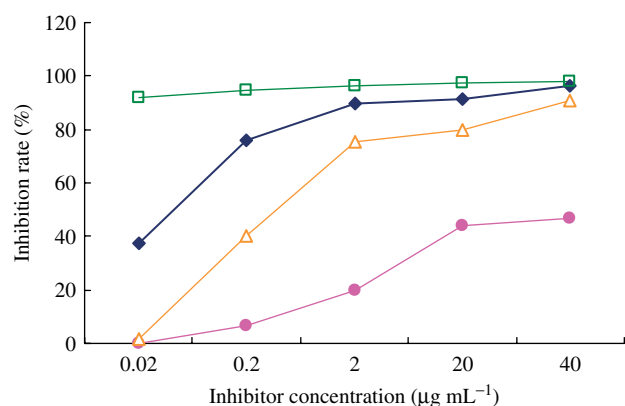


**Figure 5.** Inhibition ELISA of TM digestive samples of Chinese mitten crab. The digestive samples by pepsin ( $\Delta$ ), trypsin ( $\bullet$ ), chymotrypsin ( $\blacklozenge$ ) for 1 h, 4 h, 4 h, respectively and undigested TM ( $\square$ ) were diluted 1:5, 1:10, 1:100, 1:1000, 1:10 000 and used 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  $\mu\text{g mL}^{-1}$ .

of chymotrypsin (Fig. 4, lane 3). However, no IgE reactivity was observed against smaller fragments (28 kDa and 22 kDa). Possibly, IgE-binding epitopes in these fragments were destroyed by trypsin and chymotrypsin digestion. However, it should be remembered that, unlike proteins, small peptides are less well immobilised by immunoblot. Therefore, inhibition ELISA which is more sensitive in detecting proteolytic fragments with IgE reactivity was conducted. As shown in Fig. 5, the effectiveness of proteinase digestion to the allergenicity of Chinese mitten crab TM was evaluated. Compared with undigested sample, the IgE binding of all digested samples was obviously reduced by proteinases treatment, especially by trypsin, suggesting proteinase treatment is an appropriate way in reducing TM IgE binding.

It is noteworthy that our present study 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.

## CONCLUSIONS

The digestibility of Chinese mitten crab major allergen TM by SGF and SIF was studied. 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 of ELISA results revealed that proteinase treatment is a relatively effective way for reducing the IgE binding of TM.

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## REFERENCES

- 1 Zhao QH, Wang XZ, Chen LJ, Li W and Liu YP, Purification, identification and characterization of the major allergen from *Linnaeus*. *Chin J Immunol* **23**:256–259 (2007).
- 2 FAO/WHO, Evaluation of allergenicity of genetically modified foods, in *Report of a Joint FAO/WHO Expert Consultation of Allergenicity of Foods Derived from Biotechnology*. WHO, Geneva, pp. 22–25 (2001).
- 3 Shanti KN, Martin BM, Nagpal S, Metcalfe DD and Rao PV, Identification of tropomyosin as the major shrimp allergen and characterization of its IgE-binding epitopes. *J Immunol* **151**:5354–5363 (1993).
- 4 Leung PSC, Chen YS, Gershwin ME, Wong SH, Kwan HS and Chu KH, Identification and molecular characterization of *Charybdis feriatu* tropomyosin, the major crab allergen. *J Allergy Clin Immunol* **102**:847–852 (1998).
- 5 Motoyama K, Suma Y, Ishizaki S, Nagashima Y and Shiomi K, Molecular cloning of tropomyosins identified as allergens in six species of crustaceans. *J Agric Food Chem* **55**:985–991 (2007).
- 6 Liang YL, Cao MJ, Su WJ, Zhang LJ, Huang YY and Liu GM, Identification and characterization of the major allergen of Chinese mitten crab (*Eriocheir sinensis*). *Food Chem* **111**:998–1003 (2008).
- 7 Leung PSC, Chow WK, Duffey S, Kwan HS, Gershwin ME and Chu KH, IgE reactivity against a cross-reactive allergen in crustacea and mollusca: Evidence for tropomyosin as the common allergen. *J Allergy Clin Immunol* **98**:954–961 (1996).
- 8 Shimakura K, Tonomura Y, Hamada Y, Nagashima Y and Shiomi K, Allergenicity of crustacean extractives and its reduction by protease digestion. *Food Chem* **91**:247–253 (2005).
- 9 Taylor SL, Protein allergenicity assessment of foods produced through agricultural biotechnology. *Annu Rev Pharmacol Toxicol* **42**:99–112 (2002).
- 10 Astwood JD, Leach JN and Fuchs RL, Stability of food allergens to digestion in vitro. *Nat Biotechnol* **14**:1269–1273 (1996).
- 11 Fu TJ, Abbott UR and Hatzos C, Digestibility of food allergens and nonallergenic proteins in simulated gastric fluid and simulated intestinal fluid – A comparative study. *J Agric Food Chem* **50**:7154–7160 (2002).
- 12 Yagami T, Haishima Y, Nakamura A, Osuna H and Ikezawa Z, Digestibility of allergens extracted from natural rubber latex and vegetable foods. *J Allergy Clin Immunol* **106**:752–762 (2000).
- 13 Zhou Q, Fu XP, Zhang LJ, Su WJ and Cao MJ, Purification and characterization of sea bream (*Sparus latus* Houttuyn) pepsinogens and pepsins. *Food Chem* **103**:795–801 (2007).
- 14 Cao MJ, Shao W, Li Y, Hara K, Wang XC and Su WJ, Identification of a myofibril-bound serine proteinase in the skeletal muscle of silver carp. *J Food Biochem* **28**:373–386 (2004).
- 15 Huang MC and Ochiai Y, Fish fast skeletal muscle tropomyosins show species-specific thermal stability. *Comp Biochem Physiol B* **141**:461–471 (2005).
- 16 Lowry OH, Rosebrough NJ, Farr AL and Randall RJ, Protein measurement with the Folin phenol reagent. *J Biol Chem* **193**:265–275 (1951).
- 17 Thomas K, Aalbers M, Bannon GA, Bartels M, Dearman RJ, Esdaile DJ, *et al*, A multi-laboratory evaluation of a common in vitro pepsin digestion assay protocol used in assessing the safety of novel proteins. *Regul Toxicol Pharmacol* **39**:87–98 (2004).
- 18 US Pharmacopoeia, the National Formulary. Simulated gastric fluid and simulated intestinal fluid, TS, in *The United States Pharmacopoeia* 23, *The National Formulary* 18. The United States Pharmacopoeial Convention Inc., Rockville, MD, p. 2053 (1995).
- 19 Laemmli UK, Cleavage of structural proteins during the assembly of the head of the bacteriophage T4. *Nature*, **227**:680–685 (1970).
- 20 Towbin H, Staehelin T and Gordon J, Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: Procedure and some applications. *Proc Natl Acad Sci U S A* **76**:4350–4354 (1979).
- 21 Nakamura A, Sasaki F, Watanabe K, Ojima T, Ahn DH and Saeki H, Changes in allergenicity and digestibility of squid tropomyosin

- during the maillard reaction with ribose. *J Agric Food Chem* **54**:9529–9534 (2006).
- 22 Mikita CP and Padlan EA, Why is there a greater incidence of allergy to the tropomyosin of certain animals than to that of others? *Med Hypotheses* **69**:1070–1073 (2007).
- 23 Barrett AJ, Rawlings ND and Woessner JF, *Handbook of Proteolytic Enzymes*, 2nd edition. Elsevier, London (2004).
- 24 Fu TJ, Digestion stability as a criterion for protein allergenicity assessment. *Ann NY Acad Sci* **964**:99–110 (2002).
- 25 Moreno FJ, Gastrointestinal digestion of food allergens: effect on their allergenicity. *Biomed Pharmacother* **61**:50–60 (2007).
- 26 Mills ENC, Jenkins JA, Robertson JA, Griffiths-Jones S and Shewry PR, Identifying allergenic proteins in food, in *Pesticides, Veterinary and Other Residues in Food*, ed. by Watson DH. Woodhead Publishing, Cambridge, pp. 577–597 (2004).
- 27 Untersmayr E, Poulsen LK, Platzer MH, Pedersen MH, Boltz-Nitulescu G, Skov PS, et al, The effects of gastric digestion on codfish allergenicity. *J Allergy Clin Immunol* **115**:377–382 (2005).