



Mapping and IgE-binding capacity analysis of heat/digested stable epitopes of mud crab allergens

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ABSTRACT

Mud crab (*Scylla paramamosain*) is widely consumed after thermal processing. It is necessary to comprehensively evaluate the allergenic potential and epitopes of allergens in high temperature-pressure (HTP) treated *S. paramamosain*. Tropomyosin and arginine kinase presented higher prevalence (30.77% and 42.13%) than the other three important crab allergens by component-resolved diagnosis. The surface expression of basophils CD63 and CD203c were decreased in HTP treated crab, an effect that was even more evident after digestion and absorption by the intestinal Caco-2 cell model. Of the 35 stable epitope, six were for the first time identified in shellfish. Seven heat/digested stable peptides of tropomyosin retained IgE-binding capacity and were shown to interact with MHC-II. Five epitopes (amino acids 19–29, 99–109, 153–162, 170–188 and 211–221) were the first identified in crab. The study provides insight into prevention and therapy of crab allergy, as well as helps to reduce crab allergenicity during thermal processing.

1. Introduction

Food allergy is most common during the first few years of life, with an estimated prevalence of 6–8% in childhood (Nowak-Węgrzyn, Szajewska, & Lack, 2017). Allergy to shellfish is typically lifelong (Savage, Sicherer, & Wood, 2016). Crabs are widely bred and consumed in coastal areas and with rising consumption there is an accompanying increase in the rate of crab allergy (Liu et al., 2018; Lin, Lin, & Li, 2015). The prevalence of shellfish allergy in 4–6 years were 1.74% in Singapore and the prevalence is higher in Asian countries compared to western countries (Khora, 2016). While there is a lack of epidemiological data for childhood crab allergy in many countries, and this is particularly true in developing countries.

Component-resolved diagnosis (CRD), which is premised on IgE binding to a single allergic component of food, may provide more specific diagnostic information than diagnosis based on IgE binding to extracts comprised of a mixtures of food proteins (Sicherer, & Sampson, 2018). Studies suggest that the use of CRD is promising with improved

test sensitivity and analytic specificity (Lopata, Kleine-Tebbe, & Kamath, 2016). Though many CRD tests have become commercially available and are in widespread use (Klemans, van Os-Medendorp, Blankestijn, Bruijnzeel-Koomen, Knol, & Knulst, 2015; Beyer et al., 2015), questions continue to arise regarding their utility (Sicherer, & Sampson, 2018). Moreover, there are few CRD test reports for crab allergens.

Processing is the primary treatment for seafood before consumption (Costa et al., 2020), and food processing can potentially influence allergen structure, including the aggregation and the exposure of formerly hidden antigenic sites (Clare-Mills, Sancho, Rigby, Jenkins, & Mackie, 2009). Further, heating affects interactions within multi-component foods (Liu et al., 2019), which may affect allergic sensitization to food proteins and cause allergic reactions in sensitized individuals, further impacting the allergenic potential of food proteins (Vissers et al., 2011). In addition, processed crab should be subjected within the gastrointestinal tract prior to activation of the immune system (Costa et al., 2020; Li, & Damodaran, 2017). Digestibility and gut

Abbreviations: AK, arginine kinase; AP, apical; BAT, basophil activation test; BL, basolateral; CRD, component-resolved diagnosis; HTP, high temperature-pressure; iELISA, inhibitory ELISA; LAD-2, laboratory of allergic diseases 2; MLC, myosin light chain; SCP, sarcoplasmic calcium-binding protein; SGF, stimulated gastric fluid; SI, simulation index; SIF, simulated intestinal fluid; sIgE, specificity of IgE; TIM, triosephosphate isomerase; TM, tropomyosin.

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permeability are important factors that determine whether a food allergen invokes an allergic reaction in an individual (Costa et al., 2020; Moreno, 2007; Zheng, Lin, Pawar, Li, & Li, 2011). The complete digestion contains the continuous proteolytic action of pepsin and pancreatin in the whole food (Moreno, 2007). The comprehensive evaluation, which including the continuous digestion in the thermal processed whole food, and permeability of the gut mucosal barrier, is meaningful for the processed crab-contained food for allergic individuals. While the related reports, especially the allergens in processed crab, are still insufficient.

Food allergen epitopes are key to understanding the basis of food allergy (Matsuo, Yokooji, & Taogoshi, 2015). Epitopes recognized by specific antibodies can be either linear or conformational (Lin & Sampson, 2009). Linear epitopes are considered more important for food allergens, in that they are more stable in retain the integrity of epitopes than conformational, especially during thermal processing and gastrointestinal tract digestion (Liu & Sathe, 2018). The spatial structure of allergens are destroyed by thermal processing and digestive enzymes, which are common before food interaction with the immune system (Lin & Sampson, 2009; Zhang, Zhu, Zhang, Cai, & Chen, 2016). Extensive epitope mapping studies have been performed to identify and compare sequential epitopes for several shellfish allergens (Yang et al., 2019; Fu, Wang, Ni, Wang, & Wang, 2018). Wheat prolamins retain IgE reactivity after thermal processing in that the proteins possess thermo-stable IgE linear epitopes (Simonato, Pasini, Giannattasio, Peruffo, Lazzari, & Curioni, 2001). Further, allergens typically have structures that are preserved from degradation within the gastrointestinal tract (Moreno, 2007). However, analysis of heat/digested stable linear epitopes in food allergens is limited.

The aim of this study was, in a preliminarily manner, to assess five important crab allergens by CRD including the comprehensive evaluation of the allergenic potential of high temperature-pressure (HTP) treated *Scylla paramamosain*, as well as simulated gastrointestinal tract digestion and absorption of the allergens by use of the intestinal Caco-2 cell model. Further, the heat/digested stable epitopes of five important crab allergens, after thermal processing and digestion, were analyzed by bioinformatic and liquid chromatography-mass spectrometry. The IgE-binding capacity of stable mud crab epitope peptides was assessed by using sera from crab sensitized patients. As well, the peptides were assessed for their capacity to induce degranulation of human mast cells.

2. Material and methods

2.1. Materials

Live mud crabs (*S. paramamosain*) were purchased from RT-Mart in Xiamen, China, and immediately transported to laboratory within approximately 30 min. The obtained crabs were handled with care by placing in plastic bucket, which were filled with seawater.

Goat anti-human immunoglobulin E (IgE) horseradish antibody, pepsin (≥ 250 U mg⁻¹) and pancreatin (350 U mg⁻¹) were from Sigma Aldrich (Sigma Aldrich, St Louis, USA). Allergens, including tropomyosin (TM) (Liu et al., 2018), arginine kinase (AK) (Yang et al., 2019), sarcoplasmic calcium-binding protein (SCP) (Yang et al., 2019), triosephosphate isomerase (TIM) (Yang et al., 2017), and myosin light chain (MLC) (Li et al., 2019) were prepared according to the references. The detail information on the preparation of the allergens was in Supplementary material. Human Laboratory of Allergic Diseases 2 (LAD-2) mast cells and Caco-2 cells were purchased from ATCC by an agent (BeNa Culture Collection, Beijing, China). All other reagents were analytical grade.

2.2. Sera of crab-sensitized patients

Permission for sera collection was obtained from the Women and Children's Hospital Affiliated with Xiamen University (the human

ethical approval numbers are KY-2018-018 and KY-2019-014, Xiamen, China). There hundreds and three individuals were included in this study. Parents of participating children provided informed consent. The specificity of IgE (sIgE) levels for crab (f23) were assessed using ImmunoCap (Phadia AB, Uppsala, Sweden).

CRD test of mud crab allergens was based on the ELISA performed as described previously (Bublin et al., 2010) with some modification. The purified allergens were coated on 96-well plates in 0.05 M carbonate buffer (50 mM L⁻¹ Na₂CO₃, 130 mM L⁻¹ NaHCO₃, 3 mM L⁻¹ NaN₃, pH 9.6) overnight at 4 °C. Specific IgE value to crab allergens were measured by an Infinite® M200 PRO automatic microplate reader (Tecan, Männedorf, Switzerland). Absorbance > 0.1 were regarded as positive.

2.3. Thermal processing, continuous digestion, and absorption for mud crabs

Fresh raw crabs were cleaned with plenty of water to remove exterior contamination including sediment, then them was subjected to HTP treatment at 0.08 MPa, 115 °C for 15 min in an autoclave (ALP Co. Ltd., Ota-ku, Japan) to simulate home-cooking. Besides, parts of fresh raw crabs were cleaned and used as controls.

The *in vitro* continuous simulated gastrointestinal tract digestion procedure was conducted as previously described (Liu et al., 2019). In brief, the crab was spilt into aliquots of 20 mL (4 mg/mL) in preparation for the experiment. The pepsin was added (enzyme-to-substrate ratio, 1:50 w/w) at pH 2.0, and digestion was stopped after 60 min by raising the pH of the sample to 7.4 with NaOH. Then the gastric digestions samples were mixed with pancreatin (A broad-spectrum protease consisting of amylase, trypsin, lipase, ribonuclease, and protease in Sigma Aldrich) (enzyme-to-substrate ratio, 1:200 w/w) for 240 min. Digestion was stopped by boiling at 100 °C for 10 min, and the digestion samples were stored at 4 °C prior to analysis. The digestion samples were separated on 10-well 15% sodium dodecyl sulfate polyacrylamide gel electrophoresis gels.

Human intestinal Caco-2 cells were used for the *in vitro* absorption assay (Schulten, Lauer, Scheurer, Thalhammer, & Bohle, 2011). One of the important indicator is the dense monolayer, which was differentiated by cells. Meanwhile, the evaluation of monolayer including the integrity by *trans*-epithelial electrical resistance values. It was performed in duplicate when *trans*-epithelial electrical resistance measurements were >300 Ω ·cm² between the apical (AP) and the basolateral (BL) surfaces. After continuous digestion and dialysis, samples were added in triplicate to the AP side (0.5 mL/well) and incubated for 2 h.

The UV absorption spectra of the absorption samples were scanned in the wavelength range of 200–300 nm at room temperature according to the previous methods (Liu et al., 2019). The presented results were deducted the value of background, and the background were detected with the same buffer in the reference cells.

2.3.1. Basophil activation test

After incubation, samples from the BL surface were used for the basophil activation test (BAT). The basophil source was whole blood of volunteers. (Nos. 2, 3, 6, and 18 comprised the sensitized group and Nos. 13 and 14 comprised the negative group). A cut-off value of f23 > 0.35 kU_A/L is indicative for allergy, as determined by ImmunoCap. BAT results are expressed as simulation index (SI) for CD63 and CD203c, as previously described (Yang et al., 2019; Li et al., 2019). According to the preliminary experiment, the protein volume was 50 μ L without changing the concentration to simulate the *in vivo* conditions.

2.4. LC-MS/MS

Analysis of peptides in absorption samples was by Wininnovate Bio. (Shenzhen, China) using Eksperit nanoLC 415 and AB Sciex Triple TOF® 6600 (AB Sciex Pte. Ltd., Redwood, USA). The trapping, desalting

procedure were carried out with a volume of 20 μ L 100% solvent A (0.1% formic acid). Then, an elution gradient of 8–38% solvent B (80% acetonitrile, 0.1% formic acid) in 30 min was used on an analytical column (Acclaim PepMap RSLC, 75 μ m $\times$ 25 cm, C18-2 μ m 100 Å). The chromatographic column used was Chrom XP Eksigent C18 (75 μ m $\times$ 15 cm, 3 μ m, 120 Å). Data was acquired using an ion spray voltage of 2.4 kV, and an interface heater temperature of 150°C. The analysis software used was PEAKS Studio 8.5 (version 8.5, Bioinformatics Solutions Inc., Waterloo, Canada). The local false discovery rate at PSM was 1.0% after searching against *S. paramamosain* database with a maximum of two missed cleavages.

2.5. Bioinformatic prediction and peptide synthesis

Allergen epitope sequences were submitted five bioinformatic tools; DNASTAR Protean, BepiPred 1.0, ABCpred, Immunomedicine Group, and NetMHC II 2.3, which are server-based computational approaches described previously (Li et al., 2019). The predicted epitopes were primarily based on hydrophilicity, flexibility, accessibility, and antigenicity of the amino acid sequences. The repeated epitopes predicted by more than three bioinformatic tools, were chosen for the candidate.

The epitopes, which was located simultaneously between the predicted linear epitopes by five immunoinformatic tools and the collected heat/digested stable peptides by LC-MS/MS, were synthesized. Synthetic TM epitope peptides were provided by Cellmano Biotech Limited Company (Hefei, China) and stored at -20°C until use.

2.6. IgE-binding capacity analysis of epitope peptides

An inhibitory ELISA (iELISA) was performed to analyze the IgE-binding capacity of epitope peptides using a sensitized serum pool (Supporting Information Table S1, No. 1–12 as the sensitized serum pool, of which the absorbance > 0.1 of TM by CRD). The procedure was as described previously by Li et al. (Li et al., 2019). The detail information of was in supplementary material. Peptide IgE-binding was assessed by pre-incubation with a sensitized serum pool (1:5 dilution).

The human LAD2 mast cell degranulation assay was performed to assess allergenicity of epitope peptides as previously described (Fu et al., 2018). Stimulation with PBS (pH 7.4) was used as negative control, and stimulation with native TM was used as positive control, which were purified from raw crab (Li et al., 2019). The percentage of β -hexosaminidase released was calculated: The absorbance of supernatant samples at 405 nm was compared to the total levels in the corresponding cell lysates.

2.7. Molecular docking

The crystal structure of MHC-II (HLA-DRB1*0401, PDB entry 1D5M) was used (Stern et al., 1994). Chain C and chain D were the ligands, which were removed prior to molecular modelling. Protein preparation was carried out using A and B chains by adding hydrogens, removing water molecules, and adding Kollman charges by standard molecular docking procedures (Prasanna, & Jing, 2018). The process of MHC-II docking with peptides was performed as previously described. The docking grid box size was $80 \times 126 \times 126$ points in the x, y, and z directions, respectively (Bello & Correa-Basurto, 2013).

2.8. Statistical analysis

Each experiment was repeated more than three times, with all results expressed as the mean $\pm$ standard deviation. Analysis of variance was used to assess differences between related groups using the Statistical Product and Service Solutions statistics software (SPSS for Windows, IBM Corp., New York, USA).

3. Results

3.1. Epidemiological investigation of mud crab

Sera were obtained from three hundred and three patients from the hospital clinical laboratory from January 3 to December 20, 2017. Of these patients, 19.47% (59/303) subjects were sIgE-positive for crab. Fig. 1A showed ratio between the positive and the whole number of people in each year group (7/73, 32/154, 14/49, 6/19 and 0/8, respectively). There was an increasing positive tendency with increased age for the first four groups, while the prevalence was zero in the group above 10 years of age. The crab positive patients typically showed early life cutaneous reactions (33.90%) in Fig. 1B. Respiratory reactions were found in 38.98% of patients. It is important to note that children's immune systems gradually develop with age as well as effector organ reactivity. Many children were found to be crab positive (18.64%) even though no obvious symptoms were apparent by health examination (the common physical examination). Malnutrition, hematuria of unknown origin, and other disorders were found in 3.39% of patients.

Fig. 1C showed the prevalence between the five mud crab allergens positive individuals and the total individuals in every year group by CRD, and the highest prevalence (73.08%) was found in patients 1–3 years of age. Fig. 1D was the ratio between the single allergen positive individuals and the total five allergens positive individuals by CRD. The percentage of patients positive for the five mud crab allergens were: TM, 30.77%; AK, 42.31%; MLC, 3.85%; SCP, 15.38%; and TIM, 19.23%.

3.2. Allergenic capacity analysis of digestion and absorption products

Raw crabs have a dense and compact monolithic structure, while HTP processed crabs (Fig. 2A) are loose, with more regular and slender fiber bundles. With simulated continuous digestion (Fig. 2B), HTP crabs exhibited fewer bands predominantly near 43 kDa and at less than 26 kDa. These bands were stable in stimulated gastric fluid (SGF) with digestion rate slower with simulated intestinal fluid (SIF). Further, the absorption peak of the BL side samples had a blue shift with raw or HTP crab digest samples compared to AP side samples from the Caco-2 cell monolayer (Fig. 2C). The absorption peak of HTP samples before digestion by SIF (204 nm) and SGF (203 nm) were significantly blue-shifted compared to raw crab, with decreased absorption maxima for HTP samples. The displacement of absorption peak which indicated that the structural modifications could be occurred or protein conformation were changed in processing.

The BAT was performed by assessing the ability of different processed samples to induce upregulation in the level of CD63 and CD203c expression on basophils from crab sensitised patients (Fig. 2D and Fig. 2E). The health individuals' sera (NA) showed lower SI value, while all the experiment group could activated basophils expression. Compared with the raw control, the surface expression of basophils CD63 and CD203c were decreased in HTP treated crab, an effect that was even more evident after digestion and absorption by the intestinal Caco-2 cell model.

3.3. Mapping of heat and digestion stable epitopes of allergens

Seventy-three linear epitopes of five crab allergens, recognized by CRD, were predicted by immunoinformatic analysis as shown in Supporting Information Table S2. The epitopes were contrasted with allergen peptides, which were collected by mass spectrometry. More amounts of peptides were collected from HTP crab than raw crab (the total peptides of HTP crab and raw crab were 1415/1145 and the total allergen peptides were 328/308), suggesting that thermal processing contributed to destruction of protein structure (Supporting Information Figure S1–S5).

There were 35 peptides located within predicted epitope regions of the five important mud crab allergens (Table 1). These 35 peptides were

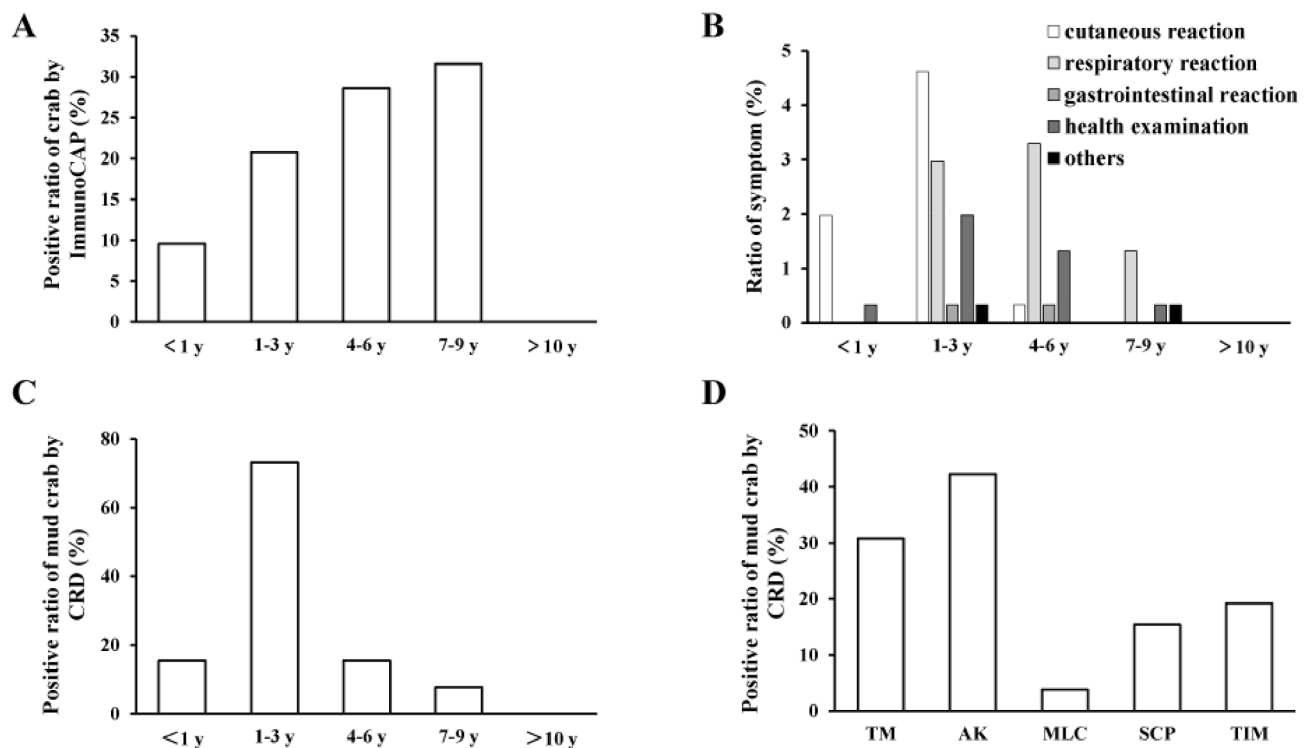


Fig. 1. Analysis of serum by ImmunoCAP and CRD. A) The positive individuals' age distribution by ImmunoCAP, with a cut-off value of sIgE (f23) > 0.35 kU_A/L. B) The positive individuals' symptom distribution ratio by ImmunoCAP. C) The positive individuals' age distribution of sIgE positive patients by CRD, with a cutoff value of absorbance > 0.15 at 450 nm. D) The positive ratio of five important allergens in crab by CRD.

mapped into modelled 3D structures (Fig. 3). For AK, 11 peptides mapped within the modelled 3D structure (template PDB: 5ZHQ) using Pymol. For SCP, four peptides were mapped (template PDB: 2SAS). For TIM, four peptides mapped to the modelled spatial structure (template PDB: 6JOX). For MLC, five peptides were mapped (template PDB: 5W1A.1.B). For TM, 11 peptides mapped to the modelled 3D structure (template PDB: 1C1G), evenly distributed throughout the protein. Of note, most epitopes were distributed within α helices of the proteins.

Compared with previously identified epitopes (Table 1), shellfish unique peptides were identified for the first time. Two such peptides (amino acids, AA294-304 and 340-349) were identified for AK, three peptides (AA39-51, 77-82, and 111-120) for SCP, one peptide (AA101-112) for TIM, and nine peptides (AA19-29, 77-82, 99-109, 134-139, 153-162, 170-188, 211-221, 250-254, and 267-274) for TM.

3.4. IgE-binding capacity analysis of heat and digestion stable epitope peptides of TM

In general, epitope peptides of 10–15 amino acids activate the immune system. Seven TM heat/digested stable peptides were identified as P1-P7 and synthesized for further analysis (Supporting Information Table S3).

Based on IgE competitive interaction between peptides and purified whole allergen protein (TM), a crab-allergenic serum pool was evaluated by iELISA. As shown in Fig. 4A, P1-P7 actively bound IgE. P3 had the greatest inhibition efficiency (90.07%) and P7 the least inhibition efficiency (70.59%) at 0.2 mg/mL. P4 displayed the highest (78.30%) and P7 the lowest ratio (50.59%) at 0.02 mg/mL. P4 had the highest ratio (96.21%) and P1 showed the lowest inhibition ratio (50.59%) at 0.002 mg/mL.

The release of β -hexosaminidase by human mast cells (LAD2 cells) is shown in Fig. 4B. Compared with the negative control-PBS (20%), positive control of purified native control-TM (32.80%) and all of the other peptides induce the degranulation of LAD2 cells. Compared with

TM, the degranulation capacity of TM or of its 7 linear peptides was decreased. While the seven peptides showed different β -hexosaminidase release rate. Among them, P4 had the highest degranulation efficiency (32.59%), and P7 showed the lowest ratio (24.55%).

3.5. Molecular docking analysis

Molecular docking was used to simulate peptide binding to MHC-II as shown in Fig. 5. There were four unfavorable bonds/forces for the complex of P7 and MHC-II, one unfavorable bond/force for the complex of P3 and MHC-II, one unfavorable bond/force for the complex of P4 and MHC-II, with no unfavorable bonds/forces in the complexes of other peptides and MHC-II.

MHC-II molecules have nine peptide binding sites, with pocket 1 the most predominant, and pocket 6 the next most predominant (Zaheer-ul-Haq, & Khan, 2011). For pocket 1; TM P1, P2, P4, P5, and P6 had one favorable amino acid. P3 had two amino acids, and P7 had no amino acids. For pocket 6; TM P1, P2, P3, and P7 had one favorable amino acid. P5 had two amino acids, P4 had six amino acids, and P6 had seven amino acids.

4. Discussion

Mud crabs accounted for more than half (53.68%) of all crabs consumed in 2018. The mud crab, *S. paramamosain*, is widely consumed in coastal regions during early childhood. Herein crab epidemiological data demonstrated 19.47% (59/303) of tested subjects to be sIgE-positive for crab allergens by ImmunoCAP; fewer positive subjects were detected by CRD. The likely difference is that f23 is a mixture of crab extracts from different crab species, while the reagents for CRD are purified allergens from *S. paramamosain*. CRD demonstrated specific food allergy phenotypes and with improved detection of possible cross-reactivities between different allergenic molecules (Sicherer, & Sampson, 2018). TM and AK are the major allergens of the mud crab (Yang

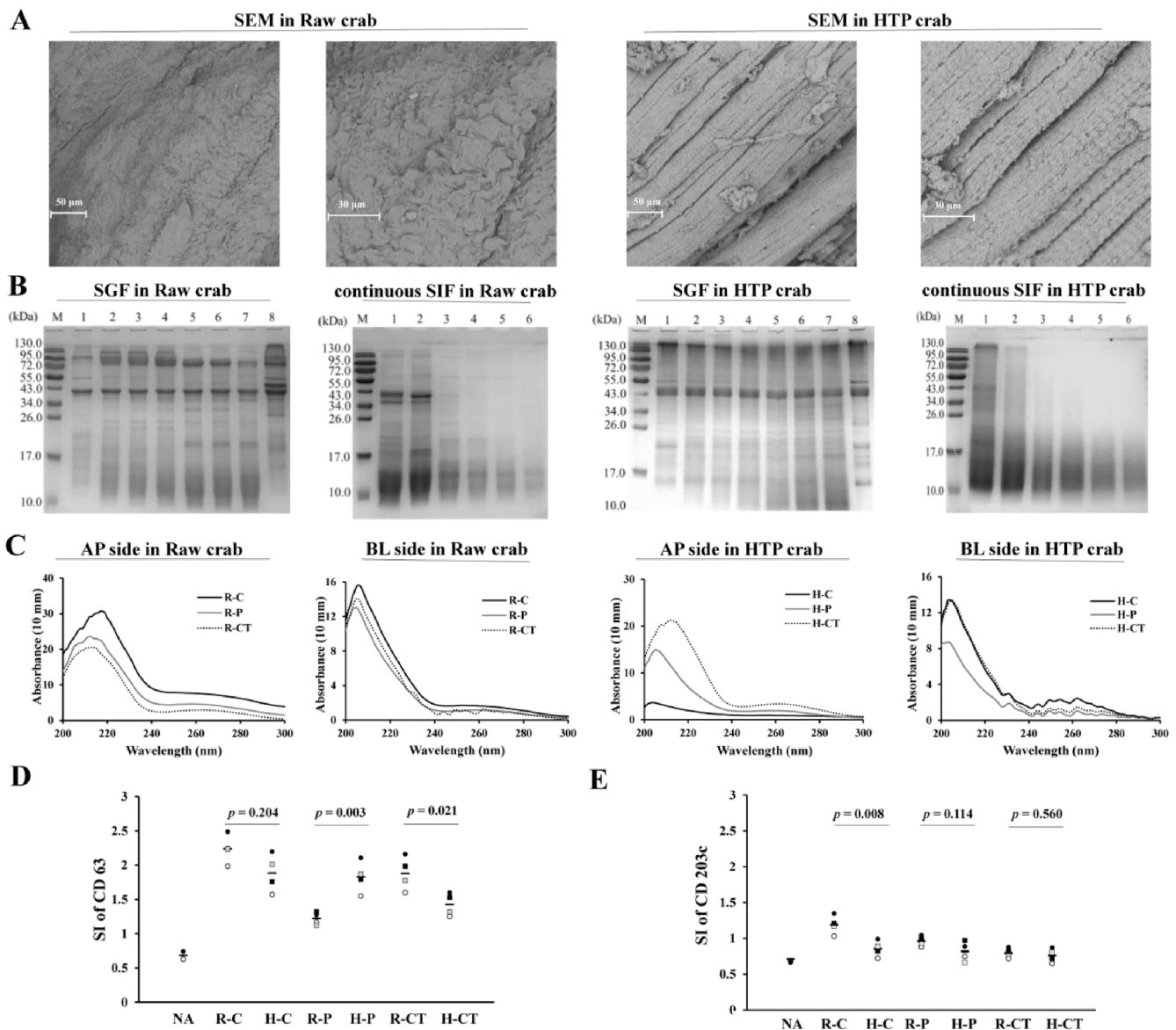


Fig. 2. Analysis of allergenic potential in crab muscle after digestion and absorption. A) SEM of crab muscle tissue at different magnifications. B) SDS-PAGE of simulated continuous gastrointestinal tract digestion of crab. Lanes in SGF: Lane M, protein marker. lane 1, protein digested in gastric phase for 0 min; lane 2, protein digested for 1 min; lane 3, protein digested for 2 min; lane 4, protein digested for 5 min; lane 5, protein digested for 15 min; lane 6, protein digested for 30 min; lane 7, protein digested for 60 min; lane 8, protein after cooking and before gastric digestion. Lanes in continuous SIF: Lane M, protein marker; lane 1, protein in duodenal digestion for 0 min after gastric digestion; lane 2, protein digested for 10 min; lane 3, protein digested for 60 min; lane 4, protein digested for 120 min; lane 5, protein digested for 180 min; lane 6, protein digested for 240 min. C) UV absorbance scanning of absorption samples by Caco-2 cell. R-C, control samples before digestion of raw crab. R-P, products after simulated gastric fluid digestion for 60 min. R-CT, products after simulated gastric fluid digestion for 60 min and continuous intestinal digestion for 240 min. H-C, control samples before digestion of HTP crab. H-P, products after simulated gastric fluid digestion for 60 min. H-CT, products after simulated gastric fluid digestion for 60 min and continuous intestinal digestion for 240 min. D) The surface expression of basophils CD63 evaluated as SI in BAT by crab sensitised sera. E) The surface expression of basophils CD203c evaluated as SI in BAT by crab sensitised sera. BAT of patients with crab sensitised serum ($n = 4$) or nonallergenic individuals' serum ($n = 2$). Data shown (individual values [circles and box] and mean values [lines]) are combined results. NA, the sera from nonallergenic individuals as the negative control. R-C, control samples before digestion of raw crab. R-P, the products after simulated gastric fluids digestion for 60 min. R-CT, the products after simulated gastric fluid digestion for 60 min and continuous intestinal digestion for 240 min. H-C, control sample before digestion of HTP crab. H-P, the products after simulated gastric fluid digestion for 60 min. H-CT, the products after simulated gastric fluid digestion for 60 min and continuous intestinal digestion for 240 min. The p value refers to comparisons of the stimulation index mean values of CD63 or CD203 of basophils between individuals with raw samples and HTP samples.

et al., 2019), with higher positive CRD prevalence than other allergens. For CRD positive individuals, the frequency of myosinogen allergens (AK, SCP, and TIM) were above 60%, consistent with a previous report of 65.14% (Yang et al., 2019).

Mud crab are widely prepared for consumption by thermal treatment. Further, gastrointestinal tract digestion and intestinal cell absorption contribute to induce mud crab allergic reactions. Analysis of

such is under reported. Herein, HTP crab was shown to have an orderly arrangement of fiber bundles, potentially conducive to digestion, consistent with shrimp muscle (Liu et al., 2019), and likely the major proteins digested. Protein transferred after HTP treatment resulted in fewer protein bands before digestion by SGF, with proteins likely denatured by thermal processing (Cabanillas, & Novak, 2017). Absorption properties of proteins are identified by UV absorption spectra (Prasanna,

Table 1
Comparison of allergen epitopes and peptides in different species.

Allergen	Species	Genebank accession No.	Identified linear epitope regions	Stable peptides collected by LC-MS in mud crab
AK	<i>Scylla paramamosain</i>	AFA45340.1 ²²	30–53,100–109,127–143, 257–284,307–314	34–47,61–68,91–97, 101–117,159–166,179–195,230–243,246–256, 294–304,310–323,340–349
	<i>Penaeus chinensis</i>	AAV83993.1 ²³	19–33,55–72,123–135,140–154,250–266,286–299,320–333	
	<i>Litopenaeus vannamei</i>	ABI98020.1 ³⁹	1–18,25–42,64–96,121–141,142–159,160–192,232–255,319–342	
SCP	<i>Litopenaeus vannamei</i>	ACM89179.1 ³⁹	10–36,49–72,130–147	39–51,77–82,111–120, 139–149
TIM	<i>Scylla paramamosain</i>	APP94292.1 ²²	45–56,79–110,183–188, 198–235	15–28,101–112,149–157, 230–240
	<i>Procambarus clarkii</i>	AEB54655.1 ²⁵	18–27,67–70,78–91, 151–158,208–215	
MLC	<i>Scylla paramamosain</i>	MK749844.1 ²⁶	7–19,35–47,51–61, 66–80,96–106, 109–120,135–147	39–48,66–73,77–87, 109–126,136–145
TM	<i>Penaeus chinensis</i>	ADA70137.1 ²³	12–25,47–60,87–105, 112–128,131–147,155–167,187–203,221–239, 243–259,263–280	19–29,39–48,77–82, 99–109,110–123,134–139, 153–162,170–188, 211–221,250–254,267–274
	<i>Penaeus monodon</i>	AAX37288.1 ¹⁷	89–105,115–128,131–142, 145–164,177–190,210–224,243–259,263–280	
	<i>Penaeus aztecus</i>	AAZ76743.1 ⁴⁰	43–55,87–101,137–141, 144–151,187–197,249–259,266–273,273–281	
	<i>Litopenaeus vannamei</i>	ACB38288 ³⁹	1–36,37–63,61–81,82–105,115–150,142–162, 157–183,190–210,246–284	
	<i>Metapenaeus ensis</i>	AAA60330 ⁴¹	25–30,43–60,87–103,146–154,161–165,191–211,236–241,247–255,269–281	
	<i>Scylla serrata</i>	— ^{a3}	44–55,90–100,105–116, 133–142,143–154,196–206,224–232,253–264	19–29,39–48,77–82,99–109,110–123,134–139, 153–162,170–188,211–221,250–254,267–274

Note: a-means no shown in the publish paper. The light gray marks the coincident regions between reported epitopes and stable peptides.

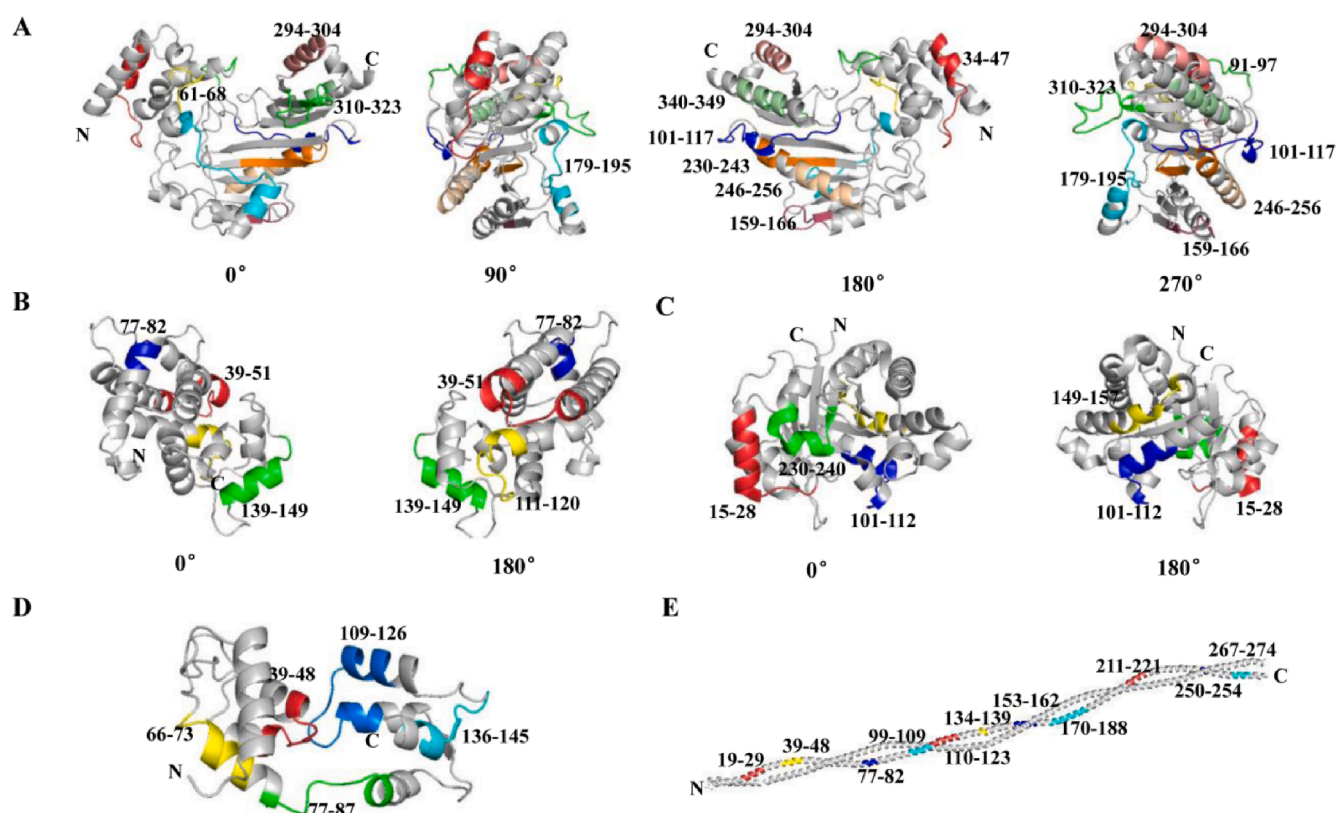


Fig. 3. Mapping of heat/digested stable peptides of five mud crab allergens. A) Eleven heat/digested stable peptides of AK. B) Four heat/digested stable peptides of SCP. C) Four heat/digested stable peptides of TIM. D) Five heat/digested stable peptides of MLC. E) Eleven heat/digested stable peptides of TM. Gray color, the 3D structure of allergens based on Pymol software. The other color are the stable peptides of allergens.

& Jing, 2018), with characteristic peaks for different amino acids. The absorption peaks of the BL side shifted blue indicating that protein structure was changed and amino acids were exposed or marked after

thermal processing. The digestion and absorption samples reduced SI for BAT, and it is likely that digestive enzymes further influenced allergen structure (Pali-Schöll, Untermayr, Klems, & Jensen-Jarolim, 2018). The

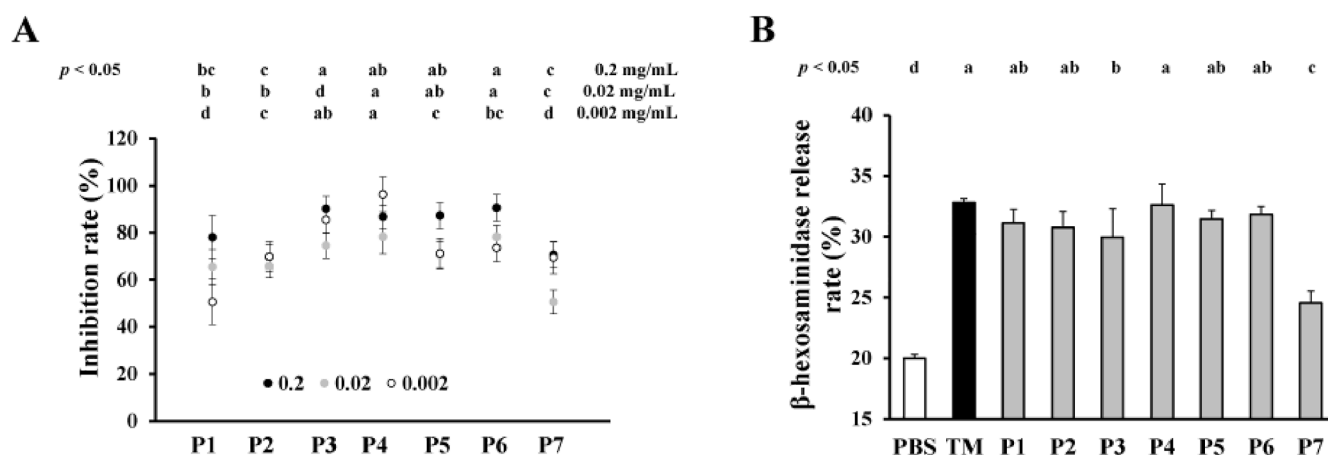


Fig. 4. IgE-binding capacity analysis of heat/digestion stable peptides of TM. A) IgE-binding capacity of seven peptides by iELISA with the sensitized serum pool. B) Validation of seven peptides by LAD2 cell degranulation assay. PBS was used as a negative control and TM was used as a positive control. All data are represented as the mean $\pm$ SD (n = 3).

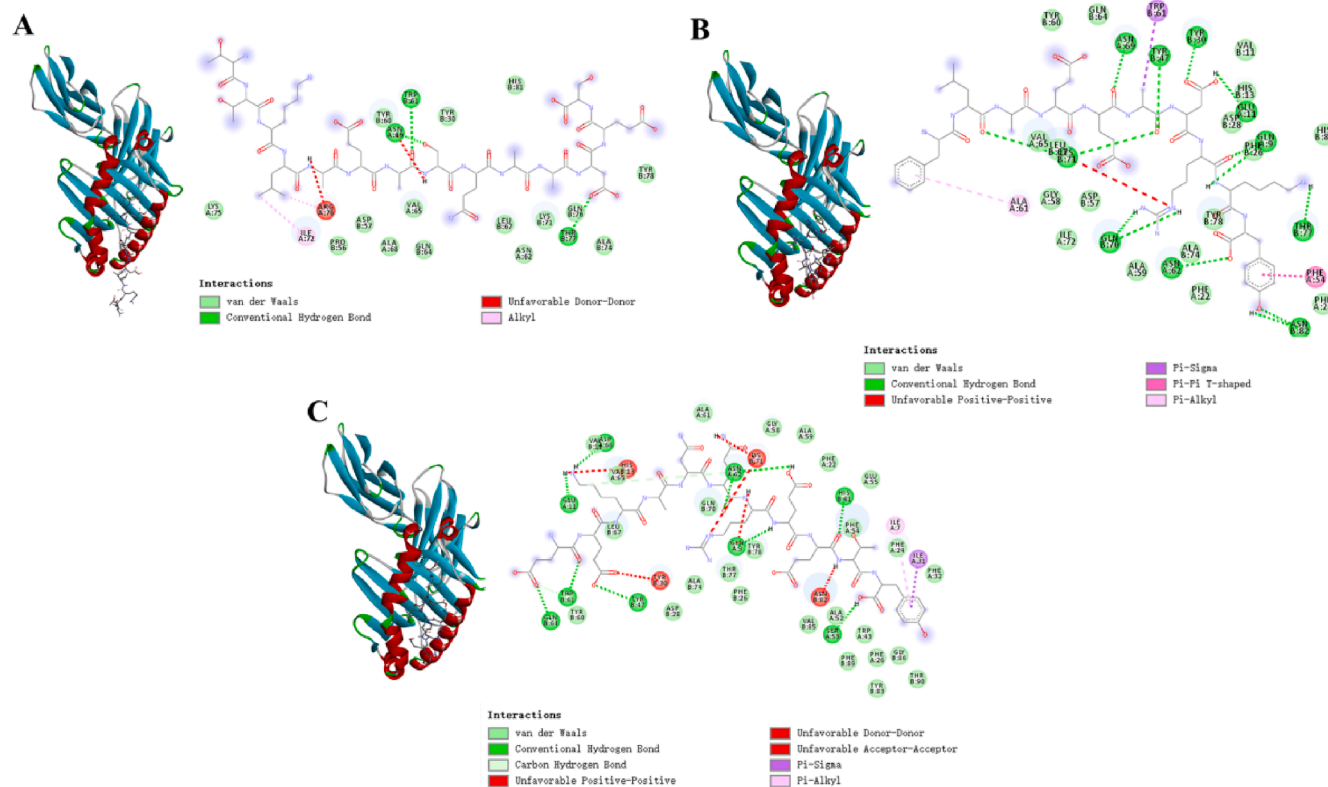


Fig. 5. Molecular docking results for MHC-II with TM stable epitopes. A) 3D and 2D structures of MHC-II-P4. B) 3D and 2D structures of MHC-II-P5. C) 3D and 2D structures of MHC-II-P7. Images obtained with Accelrys DS Visualizer software.

downregulation of CD63 and CD203c for HTP crab was likely due to reduce the allergenic potential of crab allergens (Pali-Schöll et al., 2018). Therefore, HTP likely changed the structure of fiber bundles within the food matrix, influencing protein structure and improving the digestibility of mud crab.

It is well known that allergen is the main reason for causing the allergic reactions. We found digestion and absorption samples to have basophil-activation activity, which is consistent with large allergen digestion-resistant fragments, with stable IgE epitopes, that stimulate the immune system (Moreno, 2007; Breiteneder, 2018). The present study focused on the heat and digestion stable linear epitopes of five mud crab allergens. These allergens were identified by CRD,

immunoinformatic software, and mass spectrometry of the peptides after digestion and absorption. There were 35 satisfactory peptides located within the predicted linear epitope regions of these five important mud crab allergens. Not all of the epitopes were degraded at predicted digestive enzyme sites, based on described methodology (Erickson & Kim, 1990). Meanwhile, there were large numbers peptides collected from HTP, which was similar with the opinion of thermal processing contribute to destroy the protein structure (Cabanillas & Novak, 2019). It means that the interaction between food matrixes greatly affects the digestion and absorption of allergens, and analysis of digestion and absorption of allergens is essential to an understanding of the allergenic nature of food.

Of the 35 stable peptides, two of 11 peptides (AA294-304 and 340–349) were not reported in shellfish AK (Yang et al., 2019; Fu et al., 2018; Ayuso et al., 2010), and three of four peptides (AA39-51, 77–82 and 111–120) were not reported in shellfish SCP (Ayuso et al., 2010). The peptide AA101-112 was not reported in shellfish TIM (Yang et al., 2019, 2017). MLC of *S. paramamosain* was reported to have seven linear epitopes (Li et al., 2019), and those were within the same linear region as the MLC peptides described herein. However, the linear epitope (AA7-19) was not a heat or digestion stable epitope of crab MLC. The stable peptides of TM in *S. paramamosain* shared a common sequence with the reported shrimp TM, including *P. chinensis* (Fu et al., 2018), *P. monodon* (Zheng et al., 2011), *P. aztecus* (Ayuso, Reese, Leong-Kee, Plante, & Lehrer, 2002), *L. vannamei* (Ayuso et al., 2010), and *M. ensis* (Wai et al., 2014). Compared with the other TM sequences, *S. paramamosain* TM shared >89% identity. There are few reports that focus on crab TM epitopes, even though TM is an atypical coiled-coil, heat stable protein that is highly conserved (Klueber et al., 2020). Liu verified eight linear epitopes of *S. serrata* TM, which were located at amino acids; 44–55, 90–100, 105–116, 133–142, 143–154, 196–206, 224–232, and 253–264 (Liu et al., 2018). Of 11 stable peptides, AA39-48 and 110–123 were partly in superposition with *S. serrata* TM. The other nine stable peptides are first reported for crab TM. Compared with other reported shellfish allergen linear epitopes, the epitopes identified in this study were of a shorter sequence. The previously reported epitopes were synthetic overlapping peptides (Ayuso et al., 2002) or identified by phage display (Yang et al., 2019). The present peptides were based on simulated gastrointestinal digestion and immunoinformatic prediction. Further, the heat and digestion stable epitope peptides were found in α helix of the allergens, which would be available for reaction with antibodies.

Considering the length of epitope peptides, seven TM stable peptides (AA19-29, 39–48, 99–109, 110–123, 153–162, 170–188, and 211–221) were synthesized for the detection of IgE-binding capacity by iELISA and LAD2 mast cell degranulation assays. P1-P7 of TM had obvious IgE-binding activity and induced degranulation of mast cells, suggested that the seven peptides retained IgE-binding capacity and the potential to induce allergic reactions. These results are consistent with digested allergens retaining interaction with IgE (Moreno, 2007). Meanwhile, the degranulation capacity of TM or of its 7 linear peptides was very similar and it was consistent with the previous reported (Li & Damodaran, 2017), which means that the allergenicity of this protein is probably more related with linear epitopes than with conformational ones. Actually, crab TM are highly homologous alpha-helical protein sharing characteristic rod-shaped structures of two parallel helices (Klueber et al., 2020), linear epitopes are more important than conformational epitopes. Most of the TM stable peptides examined in this study, except P2, were located in conserved segments in Supporting Information Figure S6. Of the seven epitopes, five TM epitopes (AA19-29, 99–109, 153–162, 170–188, and 211–221) are the first identified in crab.

MHC-II molecules are surface proteins of antigen presenting cells, the binding of peptide to MHC-II molecules is considered to be a fundamental and pre-requisite step to the initiation of adaptive immunity (Degroot, Chirove, & Ndifon, 2018). Pocket 1 of MHC-II molecules is the most predominant exhibiting a strong preference for large hydrophobic side chains (Trp, Tyr, Phe, Leu, and Ile). Pocket 6 is the next most with a strong preference for smaller residues (Gly, Ala, Ser, and Pro) (Zaheer-ul-Haq & Khan, 2011). Molecular docking is a widely used method to simulate the binding between ligand (epitope) and receptor (MHC-II). Then, P1-P7 of TM interact with MHC-II by molecular docking, suggesting that the seven peptides may play an important role in crab allergy. Of the seven epitope peptides, P7 had the least IgE-binding and degranulation activity, while P4 had the greatest. This may be due unfavorable bonds/forces in the complex of P7 and MHC-II or the less favorable amino acids within pockets 1 and 6 of MHC-II (Zaheer-ul-Haq & Khan, 2011).

5. Conclusion

In the present study, TM and AK had higher positive recognition ratios (30.77% and 42.13%, respectively) than the other three allergens by CRD. Basophil surface expression of CD63 and CD203c was lower in HTP digested samples likely due to a change in the structure of fiber bundles, and improved digestibility of the food matrix. There remain 35 heat/digested stable peptides of the five allergens located in predicted epitope regions of digestion samples, with six epitope peptides (two peptides in AK, three peptides in SCP, and one peptide in TIM) were the first reported in shellfish. Furthermore, seven epitope peptides of TM were identified that interact with MHC-II, of which five epitopes (AA 19–29, 99–109, 153–162, 170–188, and 211–221) were the first identified in mud crab. This study enriches our knowledge of allergens within the food matrix by in-depth analysis of the heat and digestion stable epitopes of *S. paramamosain*. Results of this study provide insight into prevention and therapy of crab allergy as well as the elimination of allergenicity during thermal processing of mud crab.

CRedit authorship contribution statement

Meng Liu: Conceptualization, Methodology, Validation, Investigation, Formal analysis, Writing - original draft, Visualization. **Fei Huan:** Investigation, Data curation. **Mengsi Li:** Conceptualization, Methodology. **Tianjiao Han:** Methodology. **Fei Xia:** Methodology. **Yang Yang:** Investigation. **Qingmei Liu:** Investigation. **Guixia Chen:** Resources. **Minjie Cao:** Supervision, Writing - review & editing. **Guangming Liu:** Writing - review & editing, Supervision, Project administration, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.foodchem.2020.128735>.

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1 **Supporting Information**

2 **Mapping and IgE-binding capacity analysis of heat and digestion stable epitopes**
3 **of mud crab allergens**

4 **Running title:** Analysis of stable crab allergen epitopes

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Methods

The preparation of the allergens

Raw crab muscles were minced and homogenised and the pellet was resuspended for five times to remove sarcoplasmic proteins effectively. The last obtained precipitation was dissolved and the solution was termed myofibrillar protein. The myofibrillar protein was treated with absolute acetone, and dried overnight. The completely dried powder was extracted with 10-fold (v/w) of 20 mM Tris-HCl (pH 7.5), containing 1 M KCl and 10 mM 2-mercaptoethanol, overnight. After centrifuged, the resulting supernatant was subjected to isoelectric precipitation and ammonium sulfate fractionation from 40% to 60%. The bound protein ~38 kDa was then collected and identified as TM (Liu, Mei, Hu et al., 2018). The myofibrillar protein was eluted (flow rate of 1 mL/min) with three linear gradients (0–0.15, 0.15–0.3, and 0.3–0.5 M NaCl) on a Q-Sepharose column. The bound protein ~18 kDa was then collected and identified as MLC (Li, Xia, Liu, He, Chen, Bai et al., 2019).

The fresh muscle was minced and homogenized for ammonium sulfate fractionation. The extract was then dialyzed and applied to a HiTrapQ Sepharose Fast Flow column. Fractions were eluted at 0.8 mL/min with a 0–0.15 M NaCl linear gradient. The bound protein ~40 kDa was then collected and identified as AK (Yang et al., 2019). Fractions was carried out with three linear gradients of 0–0.15, 0.15–0.3, and 0.3–0.5 M NaCl at a flow rate of 0.8 mL/min. The elution contain ~21 kDa protein were collected and subjected to a Sephacryl S-200 HR gel column, which was pre-equilibrated with Tris-HCl buffer, at a flow rate of 0.4 mL/min. The bound protein ~21 kDa was then collected

and identified as SCP (Yang et al., 2019). The fresh muscle was minced and homogenized before 40%– 80% ammonium sulfate fractionation. The extract was then dialyzed and applied to a Q-Sepharose ion-exchange column (2.5×10 cm). Fractions were eluted at 0.6 mL/min with a 0–0.2 M NaCl linear gradient. The bound protein ~28 kDa was then collected and eluted with 0.2 M NaCl at a flow rate of 0.5 mL/min on a Sephacryl S-200 HR gel column (1.5×100 cm). The bound protein ~28 kDa was then collected and identified as TIM (Yang, et al., 2017).

49

Table S1 Specific IgE levels and symptoms of the crab sensitive individuals.

Number	Sex ^a	Symptom	Specific IgE (f23, kU _A /L)
1	M	urticaria	≥100.0
2	F	allergic dermatitis	97.3
3	F	dermatitis	78.6
4	M	anaphylactic rhinitis	32.1
5	F	gastrointestinal disorders	17.6
6	F	eczema	23.6
7	F	pruritus	14.3
8	M	acute bronchitis	9.3
9	M	urticaria	7.4
10	M	rhinitis	6.6
11	F	allergic dermatitis	34.7
12	M	pruritus and nuasea	9.2
13	F	- ^c	0.3
14	M	- ^c	0.2

Note: ^a- M, male; F, female. ^b- means food allergy-free.

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52 **Table S2 The predicted linear epitopes of mud crab allergens by immunoinformatic tools**

Allergen	Genebank	
	accession No.	Peptides location in the allergen sequence
TM	QHW05413.1	19-29, 39-48, 67-82, 99-109, 110-123, 126-139, 153-162, 164-175, 176-188, 211-221, 222-231, 243-254, 267-274
AK	AFA45340.1	10-16, 18-47, 61-87, 91-117, 135-142, 150-155, 160-166, 172-187, 189-210, 216-222, 226-227, 229-232, 236-266, 282-290, 294-298, 301-306, 309-322, 341-346
MLC	NK749844.1	7-10, 35-47, 53-61, 66-75, 76-85, 89-94, 96-100, 103-106, 176-188, 109-126, 131-132, 135-144
SCP	AFJ80778.1	21-37, 39-55, 72-78, 80-88, 91-93, 95-114, 116-126, 129-132, 139-145, 149-159, 164-165, 170-172, 174-186
TIM	APP94292.1	14-19, 23-26, 30-39, 44-46, 57-79, 82-94, 96-99, 101-108, 110-128, 129-141, 143-159, 163-183, 194-206, 209-212, 217-219, 223-225, 228-238

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Table S3 The information of synthesis TM linear epitope peptides

Peptides	Position	Sequence	Molecular weight
P1	19-29	MDRADTLEQQN	1320.38
P2	39-48	TEEEIRATQK	1204.28
P3	99-109	LERSEERLNTA	1317.40
P4	110-123	TTKLAEASQAADES	1421.46
P5	153-162	FLAEEADRKY	1241.35
P6	170-188	AMVEADLERAEERAESGES	2079.15
P7	211-221	EEKANQREETY	1396.41

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Figure S1. Analysis of crab muscle after HTP by scanning electron microscopy (SEM), sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), and particle size

A) SEM of crab muscle tissue at different magnifications. SEM of raw crab. SEM of HTP crab.
B) SDS-PAGE of simulated continuous gastrointestinal tract digestion of crab. Lane M, protein marker. Con, control sample before digestion.
C) Particle size in Caco-2 transwell filter by laser light scattering of crab. R-C, control samples before digestion of raw crab. R-P, products after simulated gastric fluid digestion for 60 min. R-CT, products after simulated gastric fluid digestion for 60 min and continuous intestinal digestion for 240 min. H-C, control samples before digestion of HTP crab. H-P, products after simulated gastric fluid digestion for 60 min. H-CT, products after simulated gastric fluid digestion for 60 min and continuous intestinal digestion for 240 min.

Figure S2. Blast of collected peptides by LC-MS/MS and linear epitopes of TM

A) Collected peptides after digestion and transportation of raw crab.
B) Collected peptides after digestion and transportation of HTP crab.
Red lines, the predicted epitopes of TM from *S. paramamosain*. Blue lines, the peptides collected by LC-MS/MS.

Figure S3. Blast of collected peptides by LC-MS/MS and linear epitopes of AK

A) Collected peptides after digestion and transportation of raw crab.
B) Collected peptides after digestion and transportation of HTP crab.
Red lines, the predicted epitopes of AK from *S. paramamosain*. Blue lines, the peptides collected by LC-MS/MS.

Figure S4. Blast of collected peptides by LC-MS/MS and linear epitopes of MLC

A) Collected peptides after digestion and transportation of raw crab.
B) Collected peptides after digestion and transportation of HTP crab.
Red lines, the predicted epitopes of MLC from *S. paramamosain*. Blue lines, the peptides collected by LC-MS/MS.

Figure S5. Blast of collected peptides by LC-MS/MS and linear epitopes of SCP

A) Collected peptides after digestion and transportation of raw crab.
B) Collected peptides after digestion and transportation of HTP crab.

87 Red lines, the predicted epitopes of SCP in *S. paramamosain*. Blue lines, the peptides collected by
88 LC-MS/MS.

89 **Figure S6 Blast of collected peptides by LC-MS/MS and linear epitopes of TIM**

90 A) Collected peptides after digestion and transportation of raw crab.

91 B) Collected peptides after digestion and transportation of HTP crab.

92 Red lines, the predicted epitopes of TIM from *S. paramamosain*. Blue lines, the peptides collected
93 by LC-MS/MS.

94 **Figure S7. Conservation of allergenic TM sequences across different shellfish species**

95 Red lines, the heat and digestion epitopes of TM from *S. paramamosain*. Yellow lines, the identified
96 linear epitopes of TM from *S. serrata*. Light blue lines, the identified linear epitopes of TM (Pen c
97 1) from *P. chinensis*. Middle blue lines, the identified linear epitopes of TM (Pen m 1) from *P.*
98 *monodon*. Dark blue lines, the identified linear epitopes of TM (Pen a 1) from *P. aztecus*. Purple
99 lines, the identified linear epitopes of TM (Lit v 1) from *L. vannamei*. Green lines, the identified
100 linear epitopes of TM (Met e 1) from *M. ensis*.

101

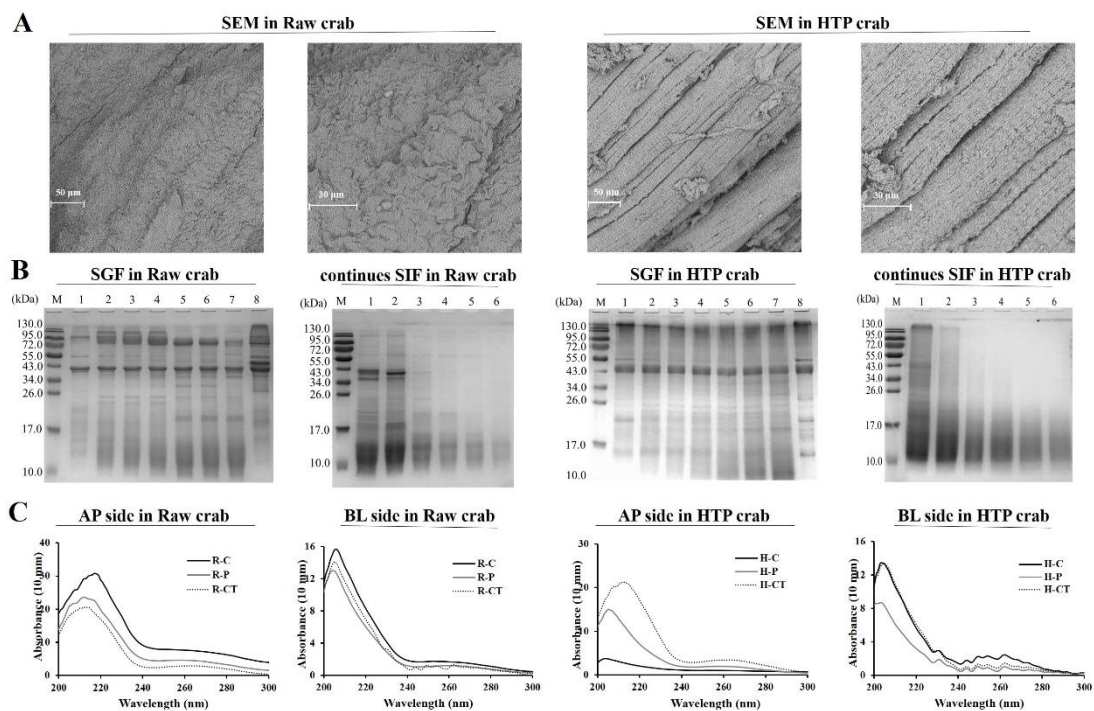
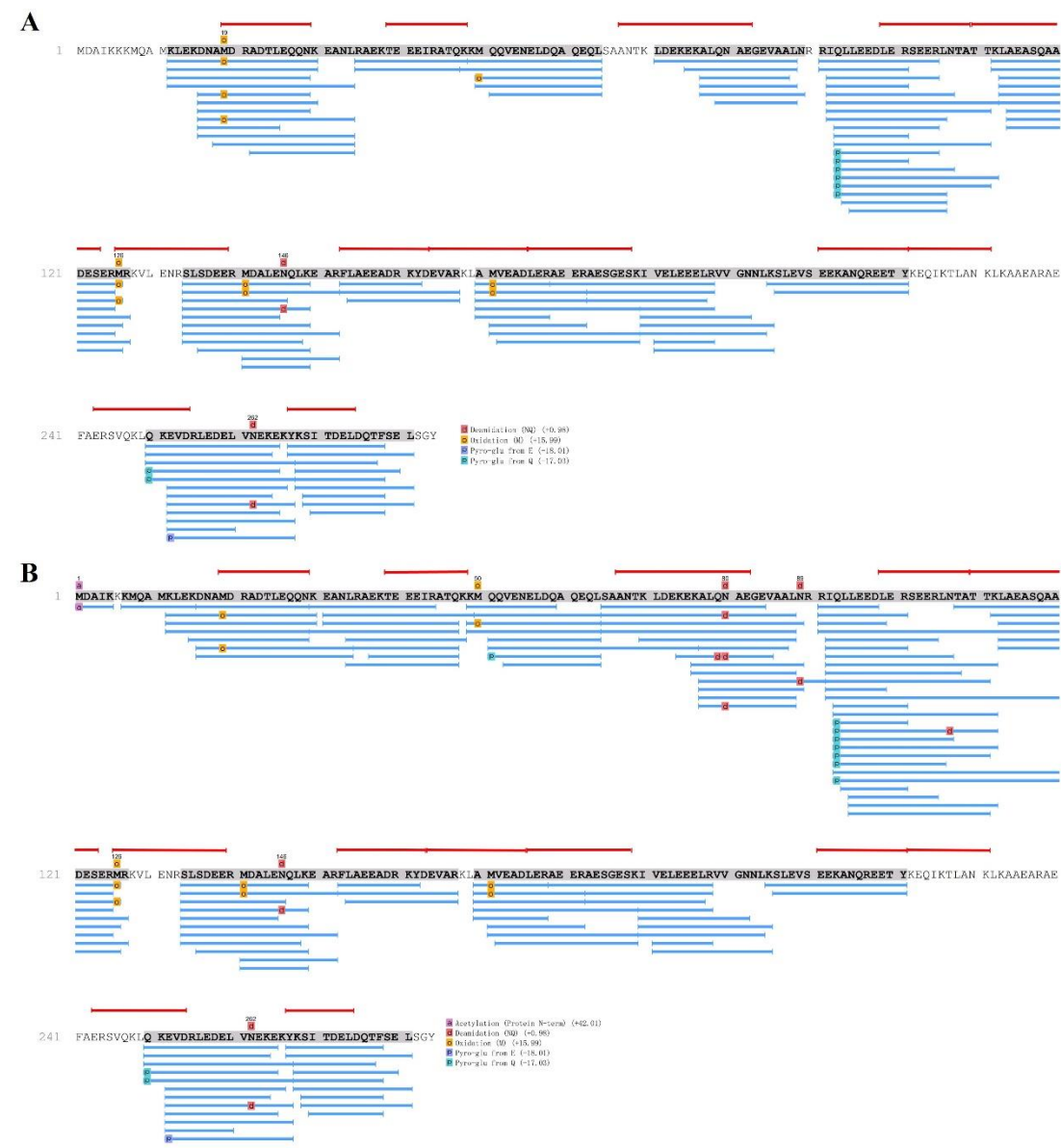
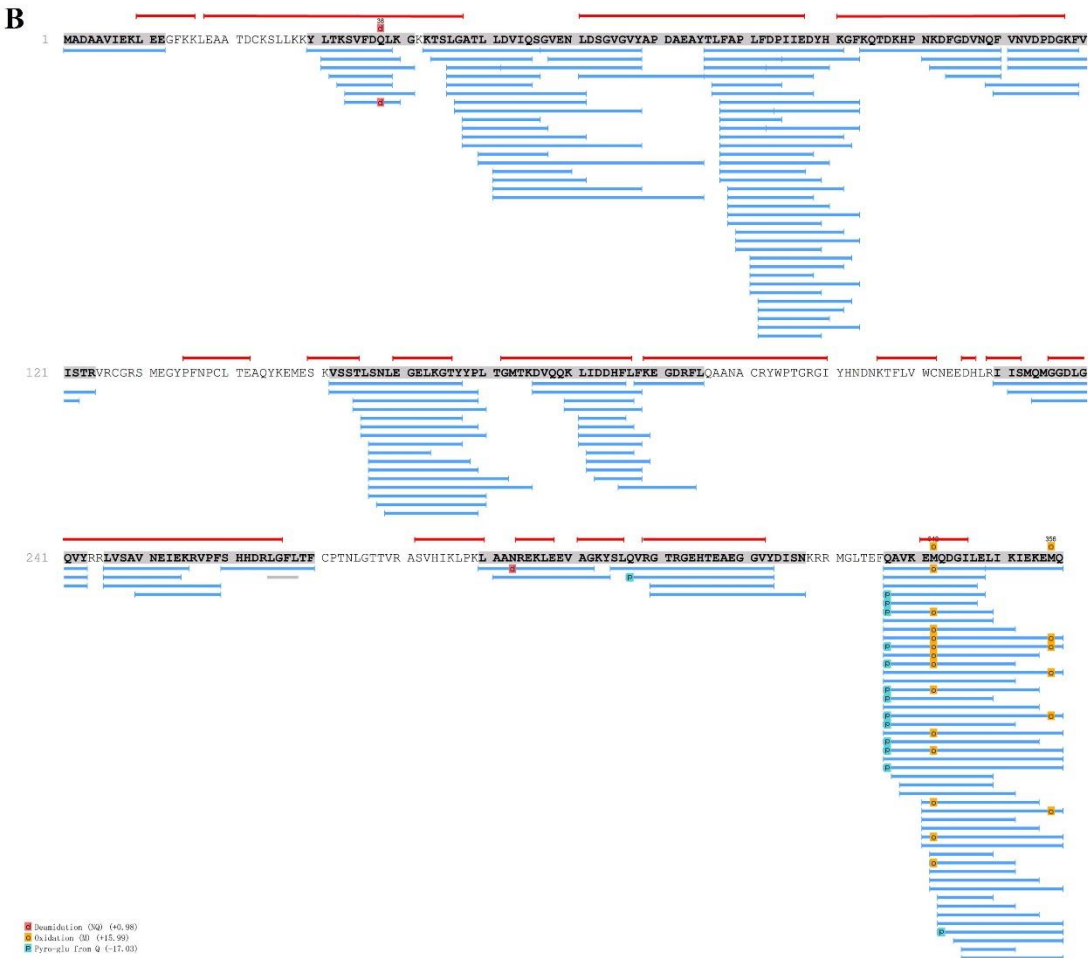
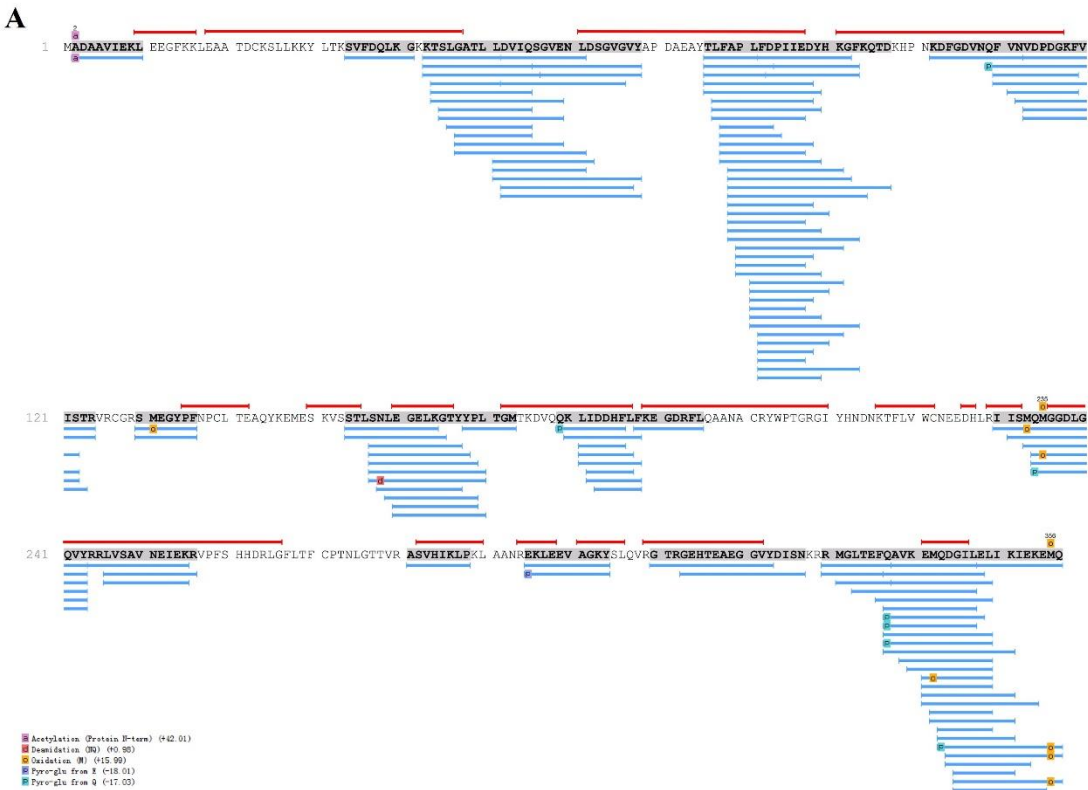


Figure S2





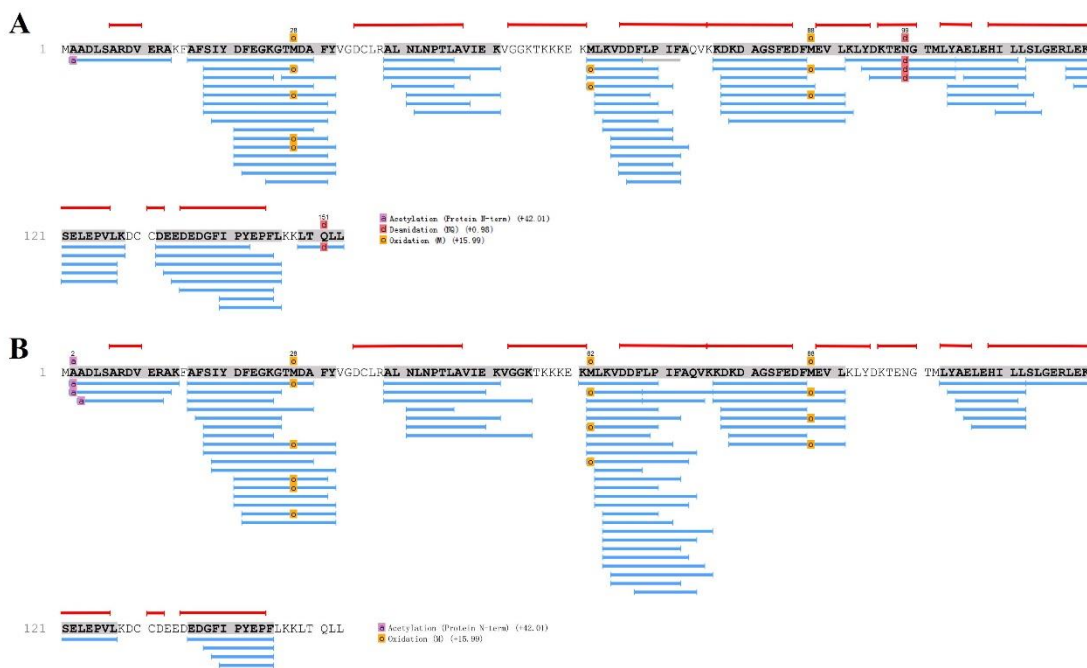
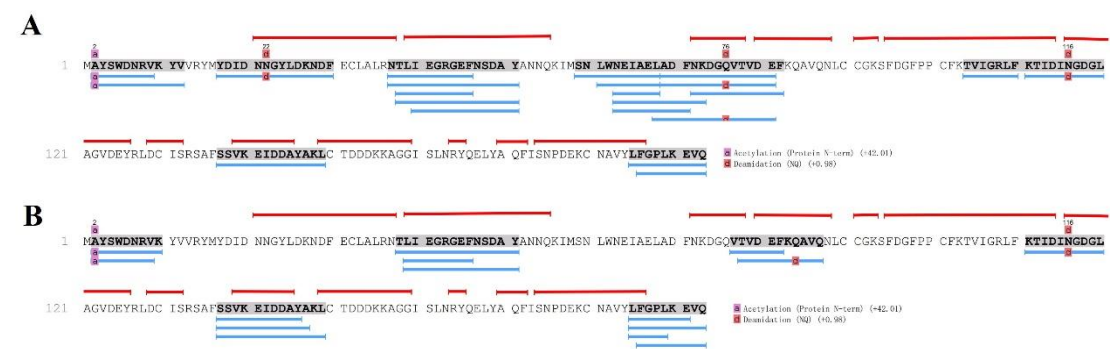
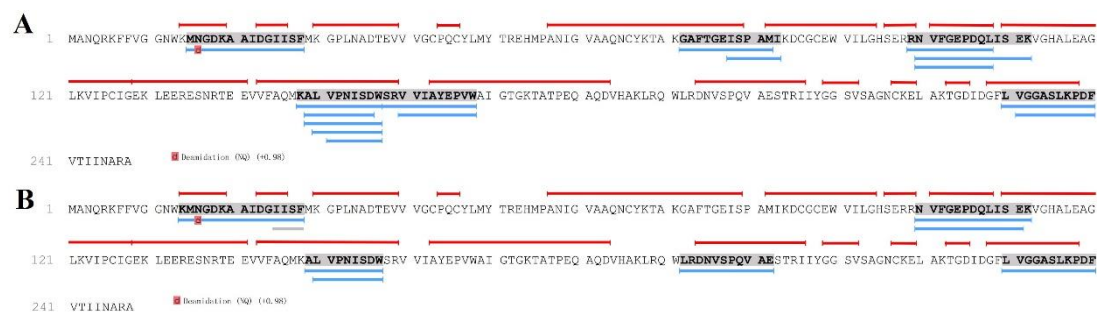


Figure S5



117 **Figure S6**



118

119

Figure S7

