



# Identification of triosephosphate isomerase as a novel allergen in *Octopus fangsiao*



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## ABSTRACT

Octopus is an important mollusk in human dietary for its nutritional value, however it also causes allergic reactions in humans. Major allergens from octopus have been identified, while the knowledge of novel allergens remains poor. In the present study, a novel allergen with molecular weight of 28 kDa protein was purified from octopus (*Octopus fangsiao*) and identified as triosephosphate isomerase (TIM) by mass spectrometry. TIM aggregated beyond 45 °C, and its IgE-binding activity was affected under extreme pH conditions due to the altered secondary structure. In simulated gastric fluid digestion, TIM can be degraded into small fragments, while retaining over 80% of the IgE-binding activity. The full-length cDNA of *O. fangsiao* TIM (1140 bp) was cloned, which encodes 247 amino acid residues, and the entire recombinant TIM was successfully expressed in *Escherichia coli* BL21, which showed similar immunoreactivity to the native TIM. Different intensity of cross-reactivity among TIM from related species revealed the complexity of its epitopes. Eight linear epitopes of TIM were predicted following bioinformatic analysis. Furthermore, a conformational epitope (A<sub>71</sub>G<sub>74</sub>S<sub>69</sub>D<sub>75</sub>T<sub>73</sub>F<sub>72</sub>V<sub>67</sub>) was confirmed by the phage display technology. The results revealed the physicochemical and immunological characteristics of TIM, which is significant in the development of hyposensitivity food and allergy diagnosis.

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## 1. Introduction

Food allergy is an abnormal food-induced immune response encompassing several types of food hypersensitivity. According to the U.S. Food and Drug Administration, there are eight major allergenic foods, including peanuts, tree nuts, soy, wheat, fish, shellfish, milk and eggs. Exposure to these foods varies with dietary habits, living environment, as well as age. The symptoms of food hypersensitivity were grouped into three categories: oro-gastrointestinal symptoms, rhino-respiratory symptoms and dermatological symptoms (Wu et al., 2012). And the phenotypic heterogeneity of allergic disease indicates that their underlying mechanisms might change with various exotic foods (Peters et al., 2015).

Seafood plays an important role in worldwide diet due to its high nutritional value and delicious taste. The total Asian seafood

production and supply is the highest in the world (FAO, 2014). The consumption of seafood products is growing rapidly, meanwhile, leading high frequency of adverse reactions. Seafood induced anaphylaxis is very common in Asian countries, such as Malaysia, Thailand, Singapore and China (Hajeb and Selamat, 2012). In the past decades, the major allergens in seafood have been identified and lots of researches focused on the allergen cross-reactivity among different species (Leung et al., 2014). Tropomyosin (TM) is a major allergen in crustaceans and mollusks with a molecular weight of 34–39 kDa, Ishikawa et al. (2001) identified TM as an important allergen in *Octopus vulgaris* and named it Oct v 1. Arginine kinase (AK) with a molecular weight of about 40 kDa is a common allergen detected in several shellfish, such as *Penaeus monodon* and *Scylla paramamosain* (Shen et al., 2011; Yu et al., 2003). Besides, AK was also found to be a major allergen in *Octopus fangsiao*, with cross-reactivity among mollusk and crustaceans (Shen et al., 2012). In recent years, researches pay increasing attention to novel allergens, such as sarcoplasmic calcium-binding protein (SCP) and myosin light chain (MLC) in *Procambarus clarkia* and *Crangon crangon* (Bauermeister et al., 2011; Chen et al., 2013;

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**Table 1**

Clinical and laboratory characteristics of nine shellfish-allergic patients and two non-allergic individuals.

| Serum No. | Age | Sex | Symptoms | sIgE octopus (f59) by ImmunoCAP (kUA/L) |
|-----------|-----|-----|----------|-----------------------------------------|
| 099809    | 50  | F   | U        | 0.65                                    |
| 132965    | 30  | M   | U        | 0.54                                    |
| 152077    | 14  | M   | P        | 2.20                                    |
| 567055    | 6   | M   | Dr       | 5.62                                    |
| 645376    | 24  | F   | U        | 3.50                                    |
| 722655    | 29  | M   | U        | 0.63                                    |
| 722719    | 31  | F   | U        | 1.50                                    |
| 787956    | 12  | M   | Dr       | 1.15                                    |
| 833684    | 7   | M   | Dr       | 0.58                                    |
| 839758    | 25  | M   | ND       | 0.16                                    |
| 856386    | 38  | F   | ND       | 0.10                                    |

F: female, M: male, U: urticaria, P: pruritus, Dr: diarrhea, ND: not determined/unknown; Levels lower than 0.35 kUA/L were considered negative.

Zhang et al., 2015). Besides, during our previous studies for the major *O. fangsiao* allergens, AK was identified as an allergen by immunoblot, accompanied with a 28-kDa protein that has significant IgE-binding activity with the sera of mollusk-allergic patients (Shen et al., 2012). The molecular weight of the IgE-reactive protein is similar to that of triosephosphate isomerase (TIM), a novel allergen reported in *P. monodon* and *Scomber scombrus* (Kamath et al., 2014; Pazos et al., 2014).

TIM is an enzyme (EC 5.3.1.1) that catalyzes the reversible interconversion of the triose phosphate isomers dihydroxyacetone phosphate and D-glyceraldehyde 3-phosphate (Wierenga et al., 2010). This protein is composed of 247 amino acids with a pI value of 6.2 (An et al., 2013). TIM is widely distributed among organisms, from bacteria and fungi, to plants and mammals, which has the potential to induce complex cross-reactive among species and lead to higher threaten in allergy suffers. Work focusing on the specific epitopes of TIM and its reactivity among species is crucial but lacking at present, much researches allergens are required for developing a comprehensive understanding of this allergen.

*O. fangsiao* is a mollusk widely found in the southeast ocean of China, which is a kind of popular food and important commercial cephalopod, the novel IgE-binding protein should not be ignored for its high consumption. Under these circumstances, the 28-kDa protein was purified from *O. fangsiao*, and its physicochemical and immunological characteristics were determined. Sequence alignment and cross-reactivity of the protein among species were then investigated. Finally, the epitopes of 28-kDa protein were determined and the protein homology model was generated to observe the location of the IgE epitopes.

## 2. Materials and methods

### 2.1. Patient sera

Sera of 9 mollusk-allergic patients were collected by the Xiamen Second Hospital. Octopus-specific IgE level in sera were measured using the ImmunoCAP system (Phadia AB, Uppsala, Sweden) with octopus allergen (f59, Table 1). Levels lower than 0.35 kUA/L were considered negative. All subjects voluntarily provided their sera with informed consent. Their use was approved by the internal Ethical Committee of the hospital (XSH2012-EAN019).

### 2.2. Purification and IgE-immunoblot of the 28 kDa-protein

The target protein was purified from octopus as previously described (Shen et al., 2012) with modifications. In brief, ammonium sulfate was added to the crude extract of *O. fangsiao* with increasing saturation from 60% to 100% to precipitate the protein, which was then dialyzed using Tris-HCl buffer (20 mM, pH 8.0)

overnight before loading onto a Q-Sepharose column (2.5 × 10 cm, GE Healthcare, Waukesha, WI, USA). The elution was carried out with a linear gradient of 0–0.5 M NaCl at a flow rate of 0.8 mL/min. Fractions that contain the 28-kDa protein were collected and subjected to purification by a Sephacryl S-200 HR gel column (1.5 × 100 cm, GE Healthcare), which was equilibrated with Tris-HCl buffer (20 mM, pH 8.0) containing 0.2 M NaCl at a flow rate of 0.5 mL/min. Samples were separated in 12% SDS-PAGE, and the gels were stained for protein with Coomassie brilliant blue R-250. IgE-immunoblot was performed to validate the target protein. Briefly, protein samples were electrophoretically transferred to a nitrocellulose membrane, and blocked with 5% skim milk in TBST (20 mM Tris-HCl, pH 8.0, containing 0.15 M NaCl and 0.05% Tween-20). After washing with TBST, it was incubated with the allergy patients' serum pool (1:4 dilution) as the primary antibody, IgE antibody (diluted 1:20,000; Southern Biotech, Birmingham, UK) was used as the secondary antibody and the results were visualized by enhanced chemiluminescence (ECL) using the ECL substrate (Pierce, Rockford, Illinois, USA).

### 2.3. Identification by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) and polyclonal antibody (pAb) preparation

The potential target protein bands were excised from the Coomassie Brilliant Blue stained SDS-PAGE gel and analyzed by Shanghai Applied Protein Technology Company Limited (Shanghai, China) using a 4800 Plus MALDI TOF/TOF Analyzer from Applied Biosystems (Foster City, CA, USA) based on the method of Yan et al. (2014). The purified protein was also used to generate a specific polyclonal antibody by subcutaneously injected of adult female New Zealand white rabbit, following the method of Yang et al. (2015). The specific pAb (1:40,000 dilution) was then recruited as primary antibody in the IgG-immunoblot performed below. HRP-labeled goat anti-rabbit IgG antibody (1:20,000 dilution, Abmart, Berkeley Heights, NJ, USA) was used as the secondary antibody and the results were also visualized by ECL.

### 2.4. Physicochemical characterization of the 28-kDa protein

#### 2.4.1. Two-dimensional (2-D) electrophoresis analysis

Two-dimensional (2-D) gel electrophoresis was carried out using a 7 cm immobilized dry strips (nonlinear, pH 3–10; BioRad, Hercules, CA, USA) with 12% SDS-polyacrylamide gel to determine the isoelectric point (pI) of target protein. Coomassie brilliant blue R-250 was used for the staining of protein.

#### 2.4.2. Thermal and pH stability determination

To determine the thermal stability, protein sample were adjusted to a final concentration of 0.15 mg/mL and incubated at different temperatures (−30, 0, 30, 35, 40, 45, 50, 55, 60, 70, 80, 90, 100 °C or 4 °C for the control) for 30 min. As to the pH stability, protein sample (0.15 mg/mL) was incubated for 30 min at 25 °C in buffer with different pH (1.0, 2.0, 3.0, 4.0, 5.0, 5.5, 6.0, 6.5, 7.0, 8.0, 9.0, 10.0, 11.0 or 7.5 for the control). All the samples were analyzed by 12% SDS-PAGE followed by western blot performed using specific pAb (1:40,000 dilution) as primary antibody. In the dot blot, the samples were immediately blotted onto the nitrocellulose membrane (2 µL/dot) and allowed to dry, and incubated with the serum pool (1:4 dilution) after blocked, as described in Section 2.2.

The protein (0.25 mg/mL) was treated at 4, 40, 50, 60, 90 or 100 °C, or incubated at 25 °C in different pH buffers (pH 2.0, 5.0, 7.5 or 11.0) for 1 h. The treated proteins were then scanned for Far-UV circular dichroism (CD) spectra using a Chirascan™ circular dichroism spectrometer (Applied Photophysics Ltd, Surrey, UK) at a concentration of 0.15 mg/mL in buffer (10 mM Tris-HCl, pH

8.0) at 25 °C with a 0.1 cm-path length rectangular quartz cuvette (Starna, Essex, UK). The secondary structures of target protein were analyzed by the CDNN program (version 2.0) (Bohm et al., 1992).

#### 2.4.3. In vitro digestion assay

The digestibility experiments were performed following the method of Huang et al. (2010) with some modifications. Pepsin (lab-produced) and pancreatin were used at a concentration of 1.6 µg/mL and 17.6 µg/mL, respectively. For gastric digestion, the ratio of pepsin/protein in the simulated gastric fluid (SGF) was 1:500 (w/w), aliquot of 100 µL was removed from the reaction solution over time intervals (0, 1, 5, 10, 15, 30, 60, 90 min) and stopped with 30 µL of 200 mM Na<sub>2</sub>CO<sub>3</sub>. For intestinal digestion, the ratio of pancreatin/protein in the simulated intestinal fluid (SIF) was 1:30 (w/w). Mixtures were removed at time intervals of 0, 1, 15, 30, 60, 120, 180 or 240 min and terminated by heating at 95 °C for 5 min. Samples were adjusted to final concentrations of 0.25 mg/mL and analyzed with 15% SDS-PAGE followed by western blot as described in Section 2.2.

An inhibition ELISA (i-ELISA) test was carried out to evaluate the IgE-binding activity of the digestion fragments (Zhang et al., 2015). Briefly, an 1:2 dilution of the patients' sera pool and the same volume of inhibitor (the digested allergens, in concentration range of 0.05–50 µg/mL) was preincubated for 1 h followed by transferred to the 96-well ELISA plate (Nunc Maxisorb, Denmark) coated with 20 µg purified allergen, and incubating for 1.5 h. Detection of the bound IgE antibodies and calculation of inhibition of IgE binding was done as described previously (Mao et al., 2013).

#### 2.5. Cloning, expression and sequence analysis of the 28-kDa protein

Total RNA was isolated from octopus muscle by the TRIZOL method (Guo et al., 2007) and single-stranded cDNA was generated by RT-PCR using the PrimeScript™ II kit (Takara, Japan) according to the manufacturer's instructions. On the basis of the result from MALDI-TOF-MS, three degenerated oligonucleotide primers, TIM-F1, TIM-R1 and TIM-R2, were designed as sense primers for PCR. Primers TIM-3F and TIM 5R were designed to complete the 5' and 3' ends respectively. The 5' and 3' ends were performed using the 5'-Full RACE Kit and TAP/3'-Full RACE Core Set with PrimeScript™ RTase (Takara, Japan) according to the manufacturer's instructions. The full length TIM fragment was amplified with primer set Oct-TIM-F and Oct-TIM-R, inserted in the *Nde*I/*Sall* sites of the pET-28a plasmid, and sequenced by Invitrogen Biotechnological Co. Ltd. (Guangzhou, China) using the ABI PRISM 3730 DNA Analyzer (Applied Biosystems, Foster City, CA, USA). All the gene-specific primers used are shown in Table 2.

The expression and purification of the TIM plasmid are following the method of Yan et al. (2014). The sequence of target protein was cloned into a bacterial plasmid pET-28a, the recombinant plasmid was expressed in *Escherichia coli* (*E. coli*) BL21 and induced with isopropyl β-D-1-thiogalactopyranoside (IPTG) at 37 °C for 4 h. The purification of recombinant protein was performed using a Ni-NTA agarose affinity column (His-Gravi Trap™, GE Healthcare) analyzed and inhibition immunoblotting was performed to compare the immune activity between recombinant and native proteins following the method of Binder et al. (2001). The IgE-binding activity was carried out with the allergy patients' serum pool (1:4 dilution) and the IgG-binding activity was with the specific pAb (1:40,000 dilution).

The gene nucleotide and its deduced amino acid sequences were analyzed using the DNASTar computer program (DNASTar Inc., Madison, WI, USA) and the ProtParam tool (<http://web.expasy.org/protparam/>). The analysis of protein N-glycosylation site and active site were performed by Motif scan ([http://myhits.isb-sib.ch/cgi-bin/motif\\_scan](http://myhits.isb-sib.ch/cgi-bin/motif_scan)).

Phylogenetic analysis was also carried out using the Molecular Evolutionary Genetics Analysis (MEGA) software (Ver. 5.2) (Li et al., 2014). All sequence databases were obtained from the National Centre for Biotechnology Information (NCBI).

#### 2.6. Immunologic cross-reactivity assay

The crude extracts of nine aquatic products including *O. fangsiao*, *Loligo chinensis*, *Crassostrea ariakensis*, *Ostrea plicatula*, *Ruditapes philippinarum*, *Scylla paramamosain*, *Fenneropenaeus chinensis*, *Procambarus clarkia*, and *Hypophthalmichthys molitrix* were prepared. All the samples were adjusted to a same concentrations (5 mg/mL), and the loading quantities was 20 µg in the SDS-PAGE analysis. The IgG-binding activity of those crude extracts was analyzed by western blot using the specific pAb and rabbit anti-crayfish TIM pAb (1:20,000 dilution) produced in our laboratory.

#### 2.7. Structural modeling and prediction of linear epitopes

The three-dimensional (3D) structure was modeled on the web-site (<http://swissmodel.expasy.org/>) by comparing the amino acid sequence and search the suitable template. The chosen model was then performed with PyMOL (DeLano Scientific, San Carlos, CA, USA) to represent its 3D structure. The linear epitopes were predicted using the DNASTar Protean software. The peptide fragments were selected to satisfy three conditions, highly hydrophilic, appropriate antigenic index, and situated in a flexible and surface-exposed region. The predicted epitopes were also represented on the molecular surface of the protein model with PyMOL. The characteristic pattern was checked in original sequence and further analysis was performed by the Prosite database (<http://prosite.expasy.org/>) to highlight the presence of functional motifs (Li et al., 2014).

#### 2.8. Conformational epitopes analysis with phage display

The Ph.D.-7 Phage Display Peptide Library (New England Bio-Labs, Beverly, MA, USA) was used for epitope the mapping according to the manufacturer's instructions. Upon automatic sequencing (Invitrogen Co., Shanghai, China), the selected peptides were analyzed for epitope locations based on protein surface properties using LocaPep (<http://atenea.montes.upm.es>). The output peptides were determined as the conformational epitopes and mapping to the 3D surface of the protein with the PyMOL software.

### 3. Results

#### 3.1. Purification and IgE-immunoblot analysis of the 28-kDa protein

Purification of the 28-kDa protein from *O. fangsiao* was achieved by salting-out with ammonium sulfate, followed by sequential column chromatography. The target proteins were present in the flow-through, revealed by SDS-PAGE (Fig. 1A). The fractions containing relatively high concentrations of the 28-kDa target protein were then gathered, concentrated, and loaded on a Sephacryl S-200 HR gel-filtration column. Following the SDS-PAGE of size-exclusion column chromatography fractions, a single band corresponding to the 28-kDa protein was observed (Fig. 1B).

With evidence of a strong reactivity with patient IgE (Fig. 1C), the target protein was confirmed as an allergen in the myosinogen of *O. fangsiao*. 2D-PAGE and western blot with the serum pool were carried out for the investigation of pI. Examination of the gel showed only one spot with obviously IgE-binding activity at a corresponding pI value of approximately 7.6 (Fig. 1D and E).

**Table 2**  
Specific primer sequences used for amplification of the TIM gene from *O. fangsiao*.

| Primers   | Orientation | Sequence (5'-3')                                               |
|-----------|-------------|----------------------------------------------------------------|
| TIM-F1    | Forward     | CTTCTGGTGCATTTACTGGAG                                          |
| TIM-R1    | Reverse     | GCCCACACTGGTTCATAAG                                            |
| TIM-R2    | Reverse     | CAGTTTACCAGCTTCTCTTTC                                          |
| TIM-3F    | Forward     | GTTGATTGGAGAGAAAATAGAGC                                        |
| TIM-5R    | Reverse     | CAAGATGACATAACGGAGACC                                          |
| Oct-TIM-F | Forward     | CATATGATGGGTCGAAATTTTCGT (containing the <i>Nde</i> I site)    |
| Oct-TIM-R | Reverse     | GTCGACTTAAGCTCGAGCATTGACAA (containing the <i>Sall</i> I site) |

### 3.2. Identification of the 28-kDa protein by MALDI-TOF-MS

The purified 28-kDa protein was subjected to MALDI-TOF-MS. Peptide mass fingerprinting of the purified protein showed multiple peaks ranging from 800 to 4000 Da, and the peaks with signal/noise ratio >50 were analyzed by MS/MS (Fig. 2A). A search for homologous proteins in the NCBI protein database showed that the ten peptides from the 28-kDa protein had over 50% identity with the sequences of *Octopus bimaculoides* (GenBank: AEJ33823.1) and *Enterctopus dofleini* (GenBank: AEJ33806.1) TIM proteins (Fig. 2B and C).

### 3.3. The stability of purified TIM under different thermal and pH conditions

The ability of native TIM to withstand the thermal and extreme pH condition were detected by SDS-PAGE and immunoblot. The purified rabbit anti-octopus IgG-pAb was used in immunoblot. As shown in Fig. 3A, TIM aggregated along with rising temperature, and started to form dimmers and polymers at 45 °C. As illustrated in Fig. 3C, result of western blot showed that the aggregates retained IgG-binding activity after heating for 60 min. The dot blot analysis showed that thermal treatment reduced the protein IgE-binding activity (Fig. 3E). The IgE-binding activity of TIM started to decrease after thermal treatment at 60 °C, and almost disappeared at 80 °C.

The stability of TIM exposed to different pH condition was also determined. As shown in Fig. 3B and D, the purified TIM was stable in solutions with different pH values, except for a little fainter in IgG-binding activity at pH 10.0 and 11.0. The analysis of dot blot using patient serum pool showed that IgE-binding activity of TIM had an obviously change when exposed in the extreme pH environment (pH 1.0–4.0 and pH 10.0–11.0) (Fig. 3F).

Folding properties of the native octopus TIM (control, 4 °C and pH 7.5) were analyzed by CD spectroscopy, and the effect of thermal and pH on its secondary structure were displayed. The Far-UV CD spectra displayed two negative peaks around 208 and 222 nm, along with a positive peak around 190 nm. At 4 °C, the secondary structure of TIM was 28.9%  $\alpha$ -helices, 23.7% extended strands, and 31.9% random coils. The structure had a change from 50 °C to 60 °C with a significantly reduced  $\alpha$ -helical content (19.5%), which remained at 18.5% at 100 °C (Fig. 3G). Acidic and alkaline solutions both affected the secondary structure of TIM. The content of  $\alpha$ -helical had a noticeable reduction from 28.9% to 16.9% and extended strands increased from 23.7% to 45.7%, upon pH decrease from 7.5 to 2.0 (Fig. 3H). Moreover, the strong alkaline conditions did not have such broad effect as the acidic ones.

### 3.4. The stability of TIM in simulated digestion in vitro

In addition to thermal and pH stability, digestibility of TIM in the *in vitro* systems using simulated fluids was compared. The intensity of the TIM protein band started to weaken from 5 min with pepsin digestion and decreased gradually along with the digestion time. After 60 min there are only visible fragments with low molecular weight (Fig. 4A), meanwhile few IgG-reactive fragment could

be observed (Fig. 4C). However, pancreatin digestion has effect on neither stability nor IgG-binding activity of TIM (Fig. 4B and D). The result of i-ELISA showed that at an inhibitor concentration of 0.05  $\mu$ g/mL, the IgE-binding activity of the pepsin-digested and the pancreatin-digested peptides were 43% and 45%, respectively, while the undigested control was about 50%. When the peptide inhibitor was increased to 5  $\mu$ g/mL, the pancreatin-digested peptides were able to bind 92% of IgE, which was comparable to the undigested TIM. While the pepsin-digestion fragments inhibited 83% of IgE-binding at the concentration of 5  $\mu$ g/mL and 89% at 50  $\mu$ g/mL (Fig. 4E). This result indicated that pepsin digestion had little effect on the immune characteristics of TIM, the peptides of which still had IgE-binding activity upon pepsin digestion for 90 min.

### 3.5. Cloning, expressing and characterization of the recombinant TIM

For further characterization, the gene of TIM was cloned. By amplification of cDNA from first strand synthesis products with degenerated oligonucleotide primers, the full-length cDNA of *O. fangsiao* TIM was obtained and submitted to the NCBI GenBank (accession number KT007222). As illustrated in Fig. 5A, the full-length cDNA sequence of *O. fangsiao* TIM is 1140 bp contains an open reading frame of 744 bp coding 247 amino acid residues. The 5'-end of the cDNA has a length of 101 bp and the length of 3'-untranslated region was 295 bp. Its theoretical molecular weight and pI was 26726.6 Da and 7.76, respectively. There is one potential N-glycosylation site (N<sub>194</sub>A<sub>195</sub>S<sub>196</sub>) (marked with a box), which was identified as the conserved motif Asn–X–Ser/Thr/Cys. The active site (<sup>162</sup>AYEPIWAIGTG<sup>172</sup>) of the motif was underlined.

Based on amino acid sequence alignment, the phylogenetic tree of TIM and other 10 invertebrate species including crustacean and mollusc were constructed by the Neighbor-Joining method. The result indicates that TIM from *O. fangsiao* is evolutionarily highly similar with *O. bimaculoides* based on the maximum probability of distances (Fig. 5B), which is consistent with the result of identification by MALDI-TOF-MS.

The pET28-derived expression plasmid pET-TIM was constructed, expressed, and highly purified, as shown in Fig. 5C, the highly purified rTIM showed slightly larger molecular mass than that of native TIM. This difference may result from the mobility change caused by His-tag. The purified rTIM also illustrated IgG-binding activity with rabbit anti-octopus TIM pAb (Fig. 5C). The IgE immunoblot inhibition experiment was then carried out, preincubation with allergic patients' serum pool against rTIM inhibited IgE-binding to TIM and rTIM on nitrocellulose membrane. The IgG/IgE inhibition ELISA assay were also conducted and rTIM has the same inhibition ability as the TIM control (data not shown). These results revealed that the IgE and IgG binding activity of rTIM was comparable to that of native TIM, suggesting that native TIM can be replaced by rTIM in allergy diagnosis and further studies.

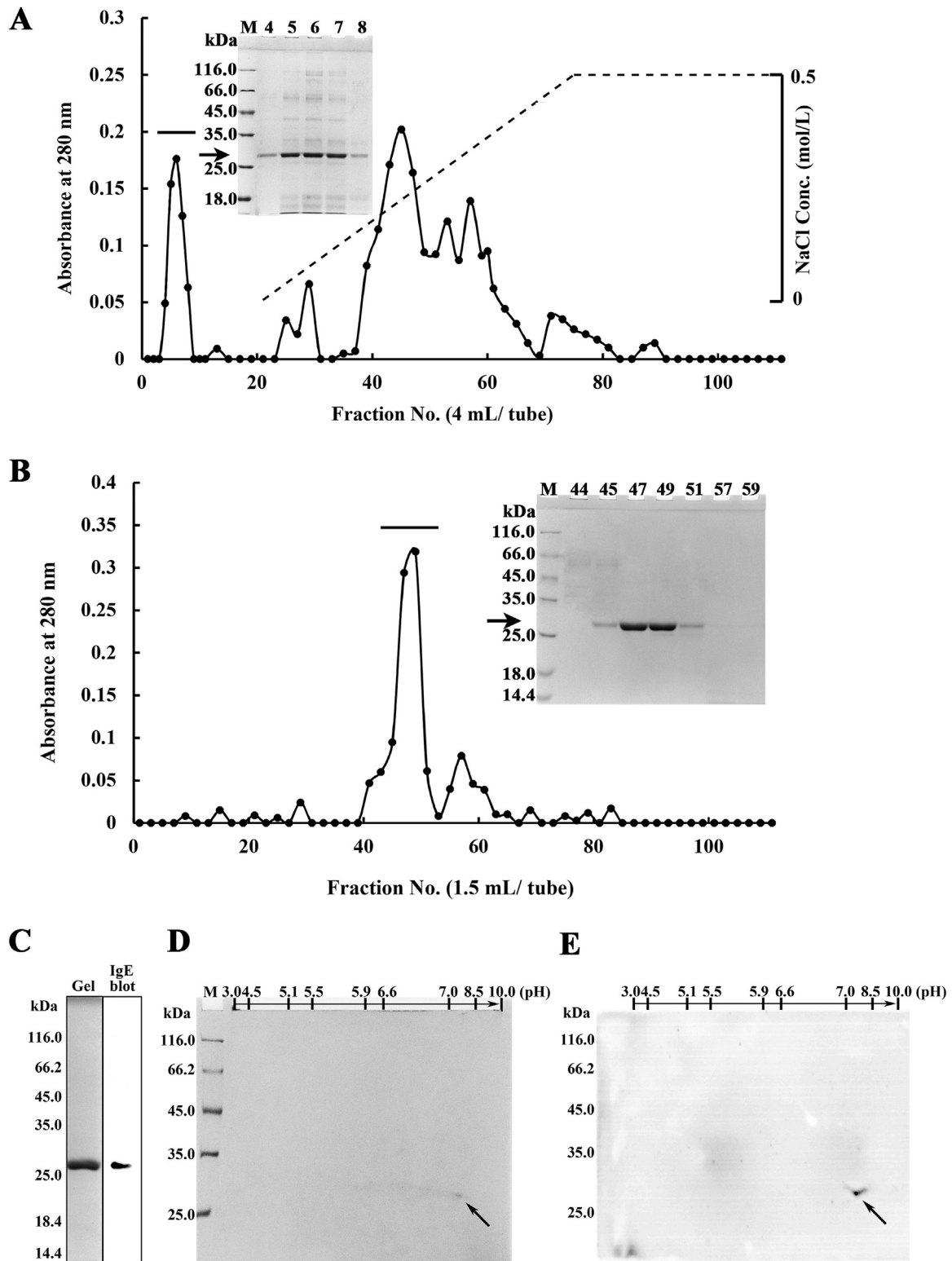


**Fig. 1.** Chromatographic purification of the 28 kDa-protein from *O. fingsiao*. (A) and (B) Representative SDS-PAGE image of the collected fractions was shown above the absorption curve. The numbers on the top correspond to the fraction numbers. The target protein fractions under the bar were pooled. M: protein marker. (A) Q-Sepharose chromatography. (B) Sephacryl S-200 HR gel chromatography. (C) SDS-PAGE, IgE immunoblot analysis of the purified protein using octopus allergic patients' serum pool (dilution 1:4) as primary antibody. (D) and (E) 2-D PAGE of the purified protein (D) and immunoblot analysis (E) using the patient serum pool (dilution 1:4) as primary antibody.

### 3.6. Cross-reactivity analysis of TIM among species

According to the result of MALDI-TOF-MS and phylogenetic tree of TIM, there is an evolutionarily highly similarity of TIMs in crus-

tacean and mollusk. In order to analyze the cross-reactivity of TIM from different species, an immunologic assay was performed using rabbit anti-octopus TIM pAb and rabbit anti-crayfish TIM pAb prepared in our laboratory previously. The crude extracts of nine

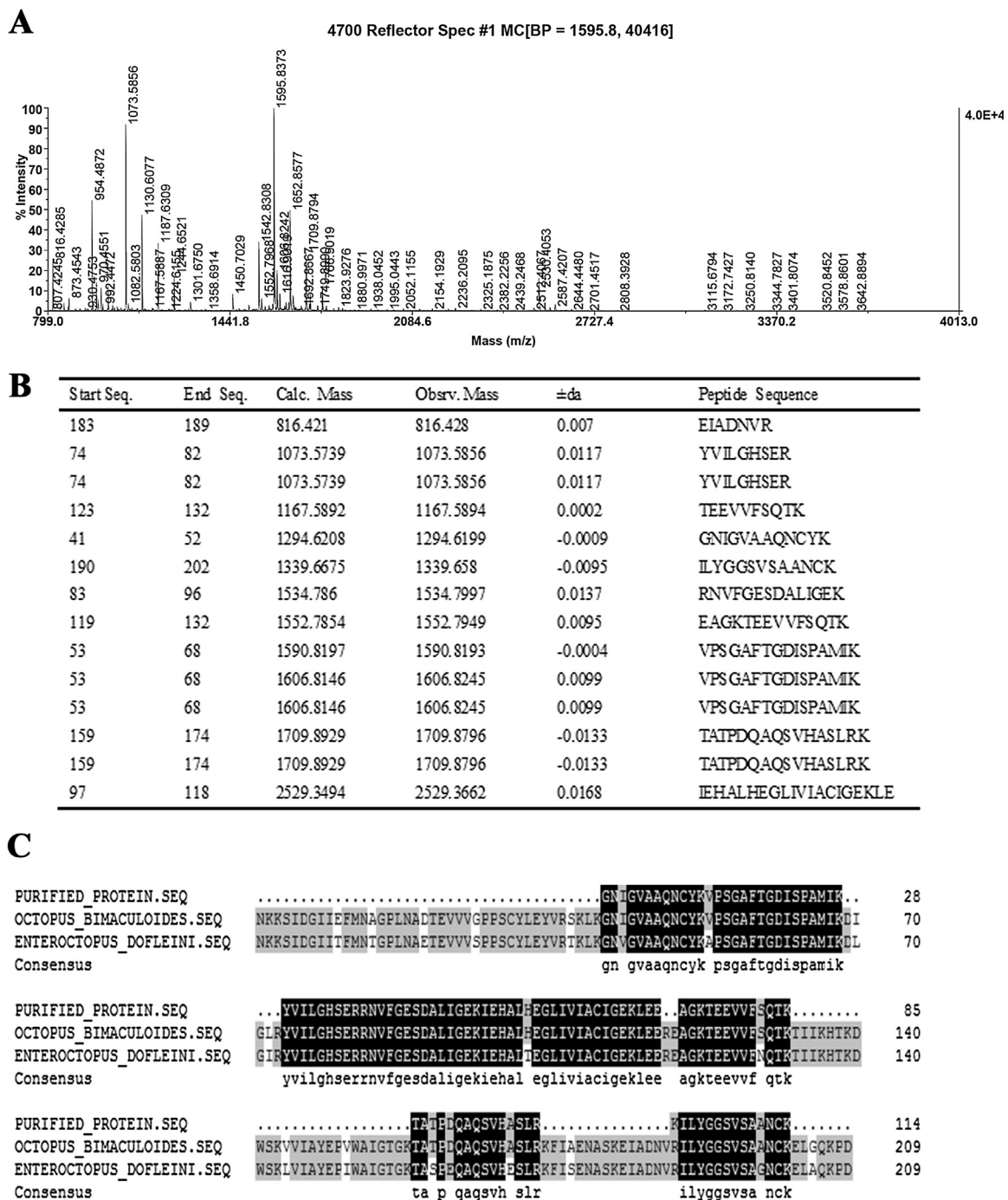


**Fig. 2.** Mass spectrometry (MS) of the 28 kDa-protein in *O. fangsiao*. (A) Peptide mass fingerprinting (PMF) analysis of the purified protein from octopus. (B) Peptides identified by MS, mapped to the NCBI peptide database. (C) Protein sequence alignments of TIM from *O. bimaculoides* and *E. dofleini*.

seafood products were subjected to SDS-PAGE, and as showed in Fig. 5D, the TIM corresponding band can be detected clearly in all the samples. However, it is not the major protein in myosinogen. The different contents of TIM in the crude extract of nine species (Fig. 5D) may correlate with energy metabolism of the species, for its important role in glycolysis and energy production.

For cross-reactivity with anti-octopus TIM pAb, the TIM binding activity of *L. chinensis*, *S. paramamosain* and *H. molitrix* was relative higher than that of other five species, and TIM of *F. chinensis* and *P. clarkia* did not show clear result (Fig. 5E). The western blot analysis of cross-reaction with anti-crashfish TIM pAb was quite different, TIM of *S. paramamosain* and *F. chinensis* had a strong binding to

anti-crashfish TIM pAb, while TIM of *O. fangsiao*, *L. chinensis* and *H. molitrix* showed weaker binding to anti-octopus TIM pAb (Fig. 5F).

### 3.7. Structural modeling and epitope analysis of TIM

The 3D structure of octopus TIM was determined based on a homology model (Template: *Tenebrio molitor* TIM, 2i9e.1.A, 70.73% identity, Fig. 6A). As predicted by self-optimized prediction method with alignment (SOPMA), octopus TIM contained 38.06%  $\alpha$ -helices, 23.08% extended strand, 12.15%  $\beta$ -turn and 26.72% random coils. The results were similar to that of the CD spectral analysis, indicating the validity of the model.

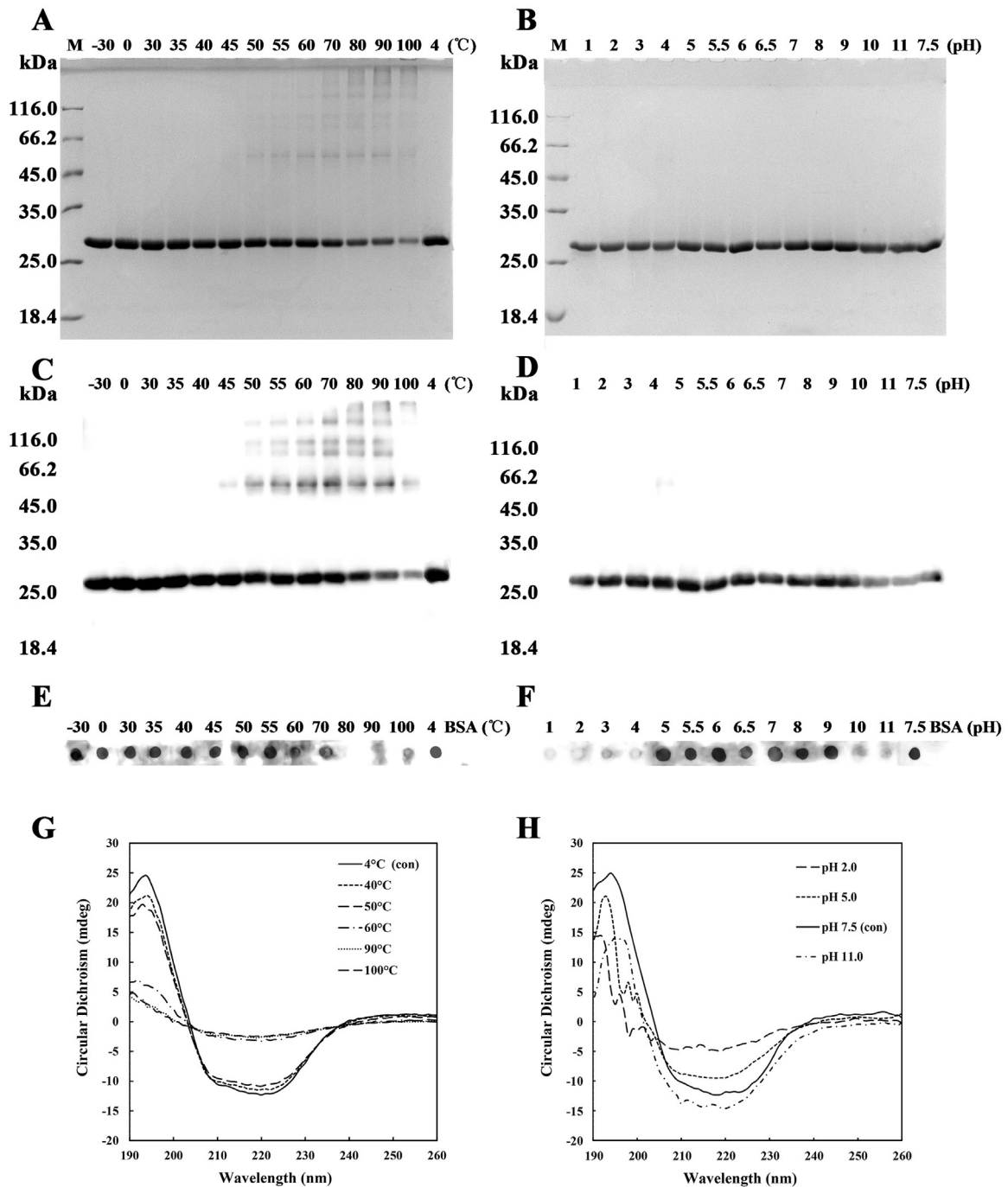


**Fig. 3.** Physicochemical characterization of the purified TIM protein, detected by immunoblot. (A), (C), and (E) Different temperatures. (B), (D), and (F) Different pH values. (A) and (B) SDS-PAGE of the thermal (A) and pH (B) stability of TIM. M: Marker. (C) and (D) Western blot analysis of thermally treated (C) and different pH treated (D) TIM using rabbit anti-TIM pAb (dilution 1:40,000) as the primary antibody. (E) and (F) Dot blot analysis of heat treated (E) and different pH treated (F) TIM, using octopus allergic patients' serum pool (dilution 1:4) as primary antibody. BSA: bovine serum albumin, the negative control. (G) and (H) CD spectral analysis of TIM subjected to different temperatures (G) and pH values (H).

The linear epitopes of octopus TIM were predicted by the DNASTAR Protean software based on its hydrophilicity, surface probability, flexibility and the antigenic index. Eight linear epitopes were selected: L-TIM-1 (N<sub>14</sub>G<sub>15</sub>N<sub>16</sub>K<sub>17</sub>K<sub>18</sub>S<sub>19</sub>), L-TIM-2 (N<sub>33</sub>A<sub>34</sub>D<sub>35</sub>), L-TIM-3 (R<sub>51</sub>S<sub>52</sub>K<sub>53</sub>L<sub>54</sub>), L-TIM-4 (S<sub>95</sub>E<sub>96</sub>R<sub>97</sub>R<sub>98</sub>), L-TIM-5 (K<sub>129</sub>L<sub>130</sub>E<sub>131</sub>E<sub>132</sub>R<sub>133</sub>E<sub>134</sub>A<sub>135</sub>G<sub>136</sub>K<sub>137</sub>T<sub>138</sub>), L-TIM-6 (H<sub>152</sub>T<sub>153</sub>K<sub>154</sub>D<sub>155</sub>W<sub>156</sub>), L-TIM-7 (N<sub>194</sub>A<sub>195</sub>S<sub>196</sub>), L-TIM-8 (L<sub>219</sub>G<sub>220</sub>Q<sub>221</sub>K<sub>222</sub>P<sub>223</sub>D<sub>224</sub>), and shown in color on the model (Fig. 6B).

After biopanning with rabbit anti-TIM pAb, one positive clone was selected and sequenced (GASESYL) as a mimotope. Analyzed by LocaPep, the sequence was defined as a conformational epitope C-TIM (A<sub>71</sub>G<sub>74</sub>S<sub>69</sub>D<sub>75</sub>T<sub>73</sub>F<sub>72</sub>V<sub>67</sub>). An epitope is assumed to form around a key residue, which is defined as a “seed” in the LocaPep software. To act as a seed, the residue must display a significantly large exposure surface area and have a small number of contacts in the protein (Pacios et al., 2011). The key amino acid of the epitope was identified as F<sub>72</sub>, which was marked in red on TIM (Fig. 6C).

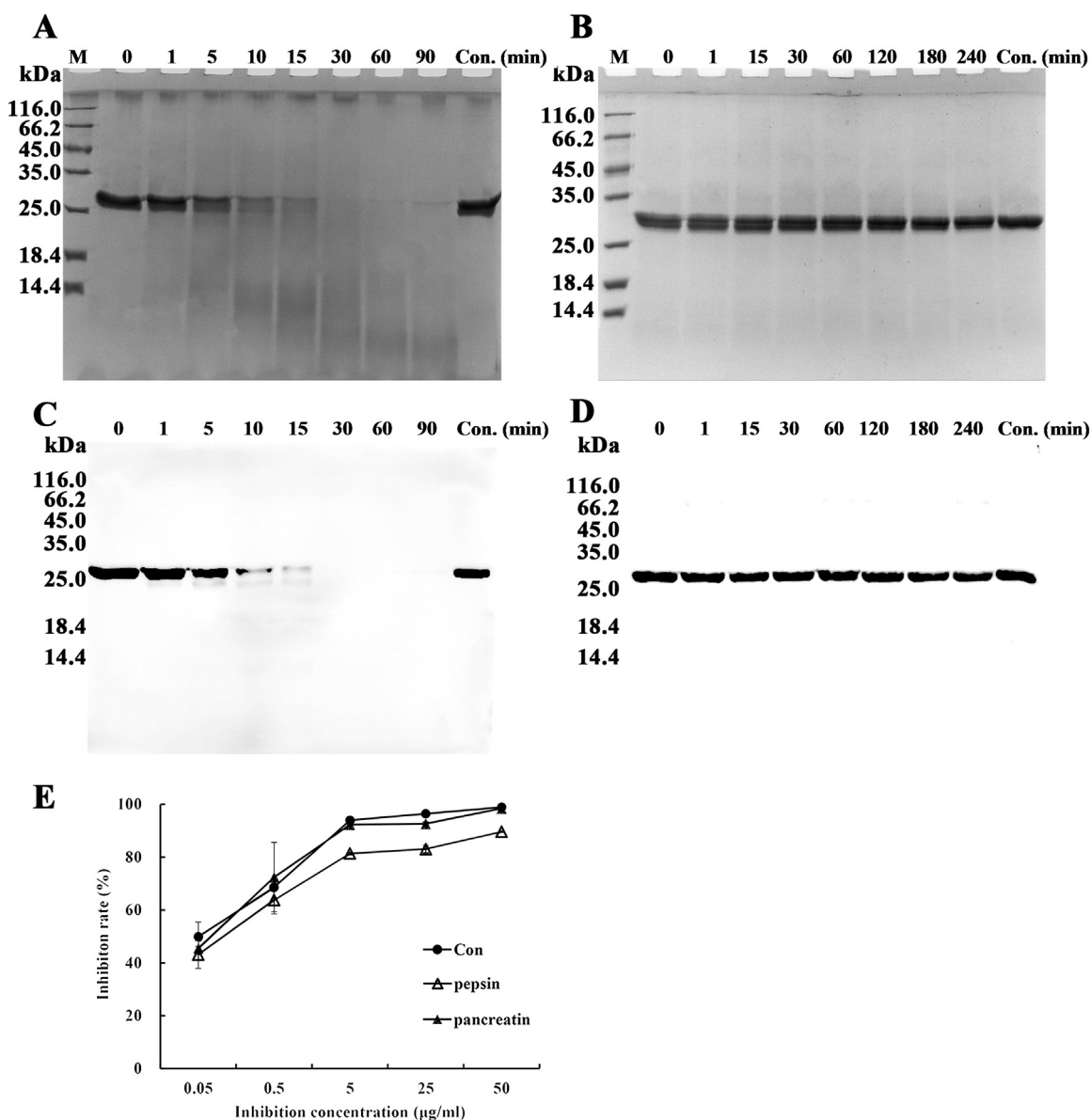


**Fig. 4.** The stabilities of TIM to simulated digestion. (A) and (B) SDS-PAGE of TIM upon pepsin (A) and pancreatin (B) digestion. M: protein marker; Con.: untreated TIM. (C) and (D) Western blot of pepsin-digested (C) and pancreatin-digested (D) TIM using rabbit anti-TIM pAb (dilution 1:40,000) as the primary antibody. (E) i-ELISA of the digested TIM fragments. TIM digested by pepsin (△, 1 h); pancreatin (▲, 4 h); and undigested (●) using octopus allergic patients' serum pool (dilution 1:4) as primary antibody.

To examine if the IgE-reactive octopus TIM shares the homology sequence with known allergens, the database of WHO/IUIS Allergen Nomenclature Subcommittee website was searched for TIMs that were reported as allergens in other species. An alignment of these allergens with TIM from *Octopus f.* was performed (Fig. 6D). Five candidates including plant, insect, and crustaceans share over 60% sequence homology were obtained. The amino acid sequence of octopus TIM was 66.0% identical to that of *Archaeopota-mobius sibiriensis* (Arc s 8, emb|CAD29196.1), 66.27% to *C. crangon* TIM (Cra c 8, gb|ACR43476.1), 67.45% to *Dermatophagoides farinae* TIM (Der f 25, gb|AIO08860.1) and *Triticum aestivum* TIM (Tri a 31, emb|CAC14917.1).

Earlier studies showed that the amino acids Ala, Ser, Asn, Gly and particularly Lys play a key role in IgE-binding to antigenic epitopes (Oezguen et al., 2008). In the present study, all of the 8 predicted epitopes were found to have one or more special five-amino acid sequences and the common residue was Lys (Fig. 6D). In addition, the majority of predicted epitopes are located in the loops

between  $\alpha$ -helices and extended strands (Fig. 6E). The potential N-glycosylation site N-A-S was also a predicted epitope.

#### 4. Discussion

Seafood is considered to be a common cause of IgE-mediated food allergy, and a number of food allergens have been identified in various saltwater products over the past few decades. However, previous studies on the allergens of crustaceans and molluscs mainly focused on the major allergens like TM and AK, while in fact, novel protein with IgE-binding activity in the IgE-immunoblot has been observed in our previous research (Shen et al., 2012). A comprehensive understanding of the allergens of crustaceans and molluscs is still limited.

In the present study, an IgE-binding, 28-kDa protein was purified from *O. fangsiao* for the first time. The purified 28-kDa protein was identified as TIM with MALDI-TOF-MS. The theoretical molecular weight of *O. fangsiao* TIM is 26.73 kDa, which is similar to that

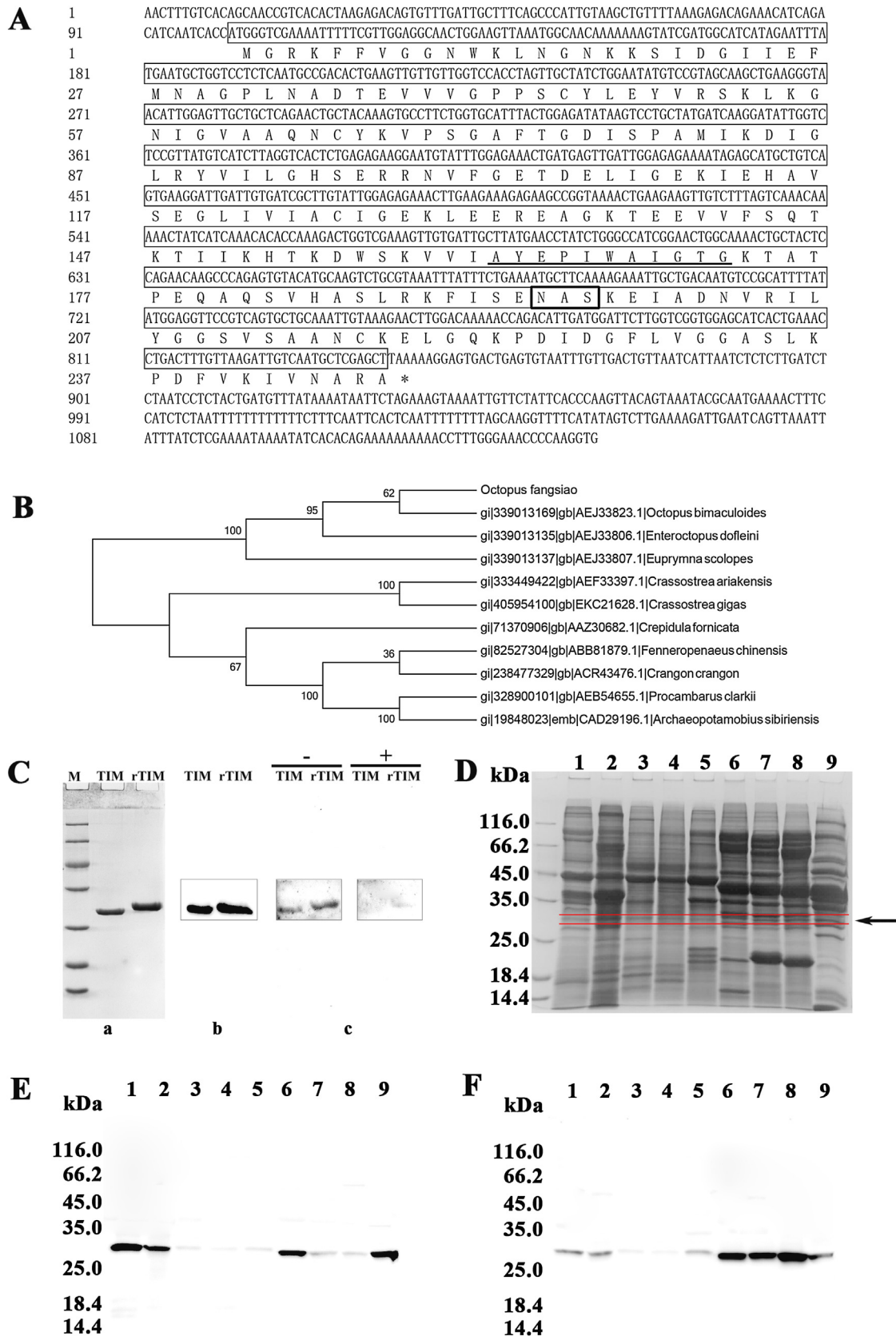


**Fig. 5.** The cDNA cloning, bioinformatics analysis and structural modeling of TIM. (A) Nucleic acid and deduced amino acid sequences of TIM from *O. fangsiao*. Residue numbers for nucleic acids and amino acids are indicated on the left of each row. The N-glycosylation site is shown in a rectangular box. The active site of TIM is underlined. (B) Phylogenetic tree based on the amino acid sequences of *O. fangsiao* TIM in comparison with other invertebrate. (C) Comparison of native TIM and the recombinant TIM (rTIM). a: SDS-PAGE; b: IgG western blot; c: IgE inhibition western blot, preincubated with either rTIM (+) or buffer only (-). (D) SDS-PAGE of the crude extracts from different aquatic products. 1–9, *O. fangsiao*, *L. chinensis*, *C. ariakensis*, *O. plicatula*, *R. philippinarum*, *S. paramamosain*, *F. chinensis*, *P. clarkii* and *H. molitrix*. (E) Western blot analysis of the crude extracts as (D) with a rabbit anti-octopus TIM pAb (1:20,000 dilution). (F) Western blot analysis of the crude extracts as (D) with a rabbit anti-crayfish TIM pAb (1:40,000 dilution).

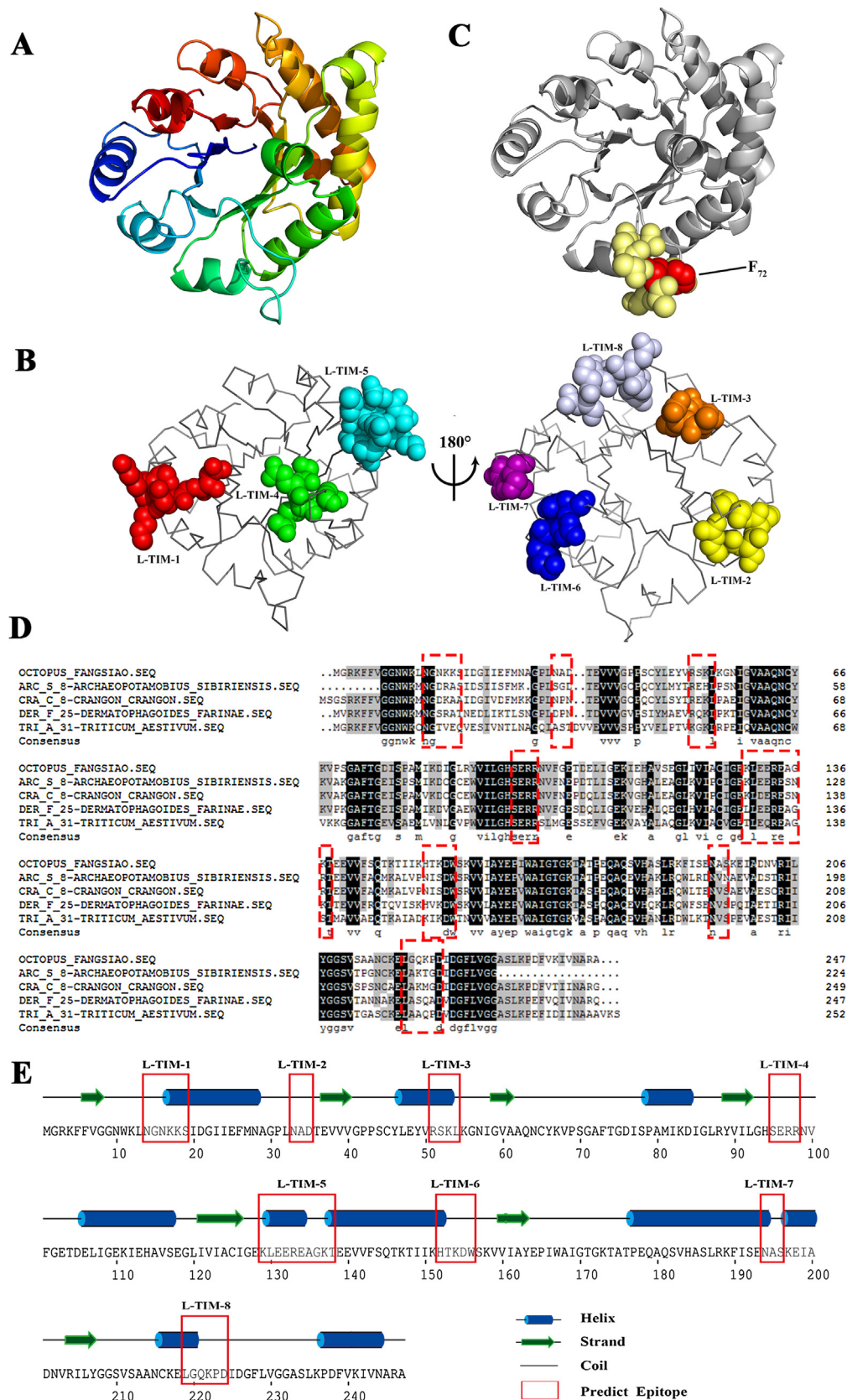


**Fig. 6.** Epitope analysis of octopus TIM. (A) Model of 3D structure of octopus TIM. (B) Molecular mapping of the predicted linear epitopes of TIM onto its 3D structure. The areas occupied by the linear epitopes are shown in color and named L-TIM-1 to L-TIM-8. (C) Molecular mapping of the conformational epitope of TIM onto its 3D structure. The areas occupied by the conformational epitopes are shown in red and the key amino acid is shown in yellow. (D) Protein sequence alignments of TIM from *O. fangsiao* and other four TIM proteins that have been confirmed as an allergen. Predicted linear epitopes were boxed. (E) The location sites of linear epitopes on TIM secondary structure. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

in *P. monodon* of 26 kDa and *C. crangon* of 28 kDa (Bauermeister et al., 2011; Kamath et al., 2014). But it was different from that of *D. farinae* with 34 kDa. As shown in 2D-PAGE (Fig. 1D), the experimentally-determined pI was calculated to be 7.6 which matched the theoretical pI of 7.76. This is quite different from those of shrimp which are classified into acidic proteins with a pI about 5.5–6.4. After comparison with other TIM amino acid sequences by ProtParam, the positively charged arginine residues in the N-terminal and C-terminal regions are likely responsible for the alkaline pI of *O. fangsiao* TIM, while these two arginines are not located in the functional regions (Wierenga et al., 2010).

The stabilities with thermal and acid to alkaline treatment can make food allergens retain allergenic capacity after food processing. The stability of TIM to harsh thermal and extreme pH conditions were measured in this study. TIM from *O. fangsiao* was relatively stable in mild temperature conditions while aggregates started to form at 45 °C. The dimer and aggregates retained IgG-binding activity after high-temperature treatment, while the IgE-binding activities were decreased. The stability characteristics of TIM are similar with that of AK (Shen et al., 2012; Yu et al., 2003; Chen et al., 2013). Thermal treatment caused denaturation and aggregation of *O. fangsiao* AK and its solubility was also sharply lower (Shen et al., 2012). Kamath et al. (2014) also reported that TIM from *P. monodon* is a heat-labile protein. The formation of oligomeric protein aggregates seems to have considerable influence on the allergenic reactivity as discussed by Rosenberg (2006), thermal treatment caused aggregation of the monomer might lead a more complex structure of allergen protein but retain the sites for IgE binding. As demonstrated in the present research, heated TIM maintained IgE-binding activity to a certain level, indicating that food processing like steaming, boiling, or frying wouldn't completely eliminate the immunoreactivity of this allergen. The IgE-binding activity at extreme pH condition was also reduced while that of IgG-binding was relatively stable. Conformational and secondary structure change of TIM after treated by heating and different pH values can also be seen in the CD spectrum. The conformational instability followed by reduction of  $\alpha$ -helical and increasing of extended strands indicated an unfolded state of protein, which results in epitopes having either reduced affinity and/or accessibility for specific IgE (Bublin et al., 2008; Lin et al., 2015; Vissers et al., 2011).

Considering the importance of the resistance of food allergen to gastrointestinal digestion in their activity to modulate the immune response, digestibility of TIM was evaluated. TIM from octopus could be quickly degraded in the early stages of digestion, while reactivity against IgE evaluated by inhibition ELISA revealed high inhibition rate of gastric digestion products, indicating that the major IgE epitopes were maintained. Similarly, it was shown that digested  $\beta$ -casein, the major milk allergen, retained IgE-binding properties of the intact protein (Benede et al., 2014). Therefore, the assessment of TIM potential IgE epitopes seemed to be crucial for the resistant to digestion enzymes.

The reported partial amino acid sequences of *O. fangsiao*, *O. bimaculoides* and *E. dofleini* TIM need further analysis to verify the common characteristic of TIM from octopus. The phylogenetic tree of TIM revealed evolutionarily highly similarity between TIM from *O. fangsiao* and *O. bimaculoides* (Fig. 5B). Follow-up studies were performed to compare the allergenic properties of TIM of different species. difference in binding activity between *O. fangsiao* and *P. clarkia* TIM pAb was observed, suggesting that some of the immune epitope might also vary. TIM has been found in nearly every organism, and for its epitope diversity, research on the epitopes of TIM is of good importance in setting basis for the allergy diagnosis and allergenic reduction.

The basic sequence properties and the 2D and 3D structures of TIM from *O. fangsiao* were analyzed, for better knowledge of the structure and position of predicted epitopes in TIM. With the

development of immunoinformatics, a growing number of computational analysis methods are available publicly. Based on the physicochemical properties analysis of the amino acids with DNASTar, three types of peptide fragments were taken into consideration: i) highly hydrophilic; ii) appropriate antigenic index; and iii) situated in a flexible and surface-exposed region. All the 8 predicted epitopes contained special 5 amino acids (Ala, Ser, Asn, Gly and Lys) with Lys as the common residue. The linear epitopes in AK from *S. paramamosain* and MLC 1 from *P. clarkia* also indicated that Lys was the major amino acid residue in antigenic epitopes (Yang et al., 2015; Zhang et al., 2015). An earlier study on TIM showed that the TIM active site is comprised of flexible loops (Wierenga et al., 2010). In this work, the majority of the predicted epitopes are located in the loops between  $\alpha$ -helices and extended strands (Fig. 6D). However, these predicted linear epitopes required experimental confirmation, such as the method of synthetic peptides used in the conformation of linear epitopes in the major *Penaeus vannamei* allergen Pen a 1 and AK from *S. paramamosain* (Ayuso et al., 2002; Mao et al., 2013; Yang et al., 2015).

In the last several years, some methods have substantially improved their accuracy to identify conformational epitopes. Epitope mimics using page display technique have been widely used in defining the conformational epitopes. Four conformational epitopes of *S. paramamosain* AK were identified by Yang using specific IgG with the phage display method (Yang et al., 2015). In general, biopanning for IgE-specific epitopes requires the corresponding purified IgE from allergic suffers. Regrettably, the limited patient sera available were far from sufficient to purify IgE. However, IgG is reported to recognize the same epitopes as IgE, at least to some degree (Subbarayal et al., 2013; Wachholz et al., 2003), so rabbit anti-octopus TIM IgG enriched with protein A was selected as the target protein for the panning procedure instead of IgE. One conformation epitope located in the longest flexible loop of TIM was identified in the present study. The sequence of the conformational epitope candidates was composed of three hydrophobic amino acids and four hydrophilic amino acids, corresponding to nonpolar and polar.

The high sequence homology may lead to cross-reactivity between allergens. For example, AK as a major allergen in shellfish and mollusks caused wide cross-reactivity due to its conservative characteristics in different species (Mao et al., 2013). Several shrimp allergens have also been identified to be responsible for the cross-reactivity between mite and seafood for their high extent of sequence homology (Gamez et al., 2014). The epitopes of TIM in *O. fangsiao* defined in the present research, including a defined conformational epitope and 8 linear epitopes, were overlapping with those of Der f 25 except for L-TIM-4 and L-TIM-6 (Li et al., 2014). The identification of epitopes and IgE-reactive TIM among species highlights its importance as a possible invertebrate pan-allergen, more works focus on the IgE-binding epitopes and the cross-reactivity among species are needed in the future.

## 5. Conclusion

In summary, this is the first time the 28-kDa protein from *O. fangsiao* has been purified. The protein was identified to be TIM and the results of immunological assays showed that it is a novel allergen in octopus. The physicochemical and immunology characteristics were also investigated. Cross-reactivity of TIM among species suggested its possibility as a pan-allergen and revealed the complexity of its epitopes. Furthermore, 8 linear epitopes were predicted and one conformational epitope was identified. These studies will allow us to give a further understanding of seafood allergens, especially mollusk allergens, and lay foundation for the

development of diagnostic and therapeutic approaches to seafood allergy.

## Conflict of interest

The authors declare that there are no conflicts of interest.

## Acknowledgement

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