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J. Agric. Food Chem., **Just Accepted Manuscript** • DOI: 10.1021/acs.jafc.6b04587 • Publication Date (Web): 10 Jan 2017

Downloaded from <http://pubs.acs.org> on January 15, 2017

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**Triosephosphate isomerase and filamin C share common
epitopes as novel allergens of *Procambarus clarkii***

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24 **ABSTRACT:** Triosephosphate isomerase (TIM) is a key enzyme in glycolysis and
25 has been identified as an allergen in saltwater products. In this study, TIM with the
26 molecular mass of 28 kDa was purified from the freshwater crayfish (*Procambarus*
27 *clarkii*) muscle. A 90-kDa protein showed IgG/IgE cross-reactivity with TIM was
28 purified and identified as filamin C (FLN c), which is an actin-binding proteins. TIM
29 showed similar thermal and pH stability while better digestion resistant compared
30 with FLN c. Result of surface plasmon resonance (SPR) experiment demonstrated the
31 infinity of anti-TIM polyclonal antibody (pAb) to both TIM and FLN c. Five linear
32 and 3 conformational epitopes of TIM, and 9 linear and 10 conformational epitopes of
33 FLN c were mapped by phage display. Epitopes of TIM and FLN c showed the
34 sharing of certain residues, the occurrence of common epitopes in the two allergens
35 accounts for their cross-reactivity.

36 **KEYWORDS:** *Procambarus clarkii*, novel allergen, triosephosphate isomerase,
37 *filamin C*, common epitopes

INTRODUCTION

Aquatic products are important sources of dietary protein. Freshwater crayfish (*Procambarus clarkii*) have served as a local favorite in China, especially inland, and have played an important role in the history of human nutrition among indigenous people. Crayfish, for their characteristics of appealing taste, high protein, low fat and good balance of nutrition, have become the most important economic resource among freshwater shrimp, with a total production of 659,611 tones in 2014.¹ Meanwhile, the increased production and consumption have been accompanied by more frequent allergic reactions, involving diverse clinical symptoms that vary from local to life-threatening anaphylaxis.²⁻⁴

To date, numbers of allergens have been identified in crayfish, among those, tropomyosin (TM) is currently considered a most allergenic molecule in shellfish and mollusks,⁴ arginine kinase (AK), a 40 kDa protein, has also been recognized as a major allergen in crayfish.⁵ Crayfish also contains minor allergens, such as the 23 kDa sarcoplasmic calcium-binding protein (SCP), which has the polymorphic characterization,⁶ and myosin light chain (MLC), which is categorized into the essential light chain and the regulatory light chain.⁷ However, there is clear evidence from the report by the Xiamen Second Hospital that some patients with crayfish allergy exhibit strong clinical allergic symptoms have given negative results in the serologic tests against the known allergens (unpublished data), meaning that other potential allergenic proteins are yet to be examined.

Triosephosphate isomerase (TIM) has been identified by the International Union of

Immunological Societies as a minor allergen in seafood.⁸ TIM was initially reported as a food allergen in plant-derived foods, such as wheat flour, lychee, and watermelon.⁹⁻¹¹ In recent years, it was recognized as a novel allergen in saltwater products, such as amago salmon (*Oncorhynchus masou ishikawae*), mackerel (*Scomber japonicus*), North Sea shrimp (*Crangon crangon*), and black tiger prawn (*Penaeus monodon*).^{8,12-14} TIM appears to be a pan-allergen prevalent in saltwater fish and crustaceans, while it is still uncertain whether it is also an allergen in freshwater shellfish.

In addition, for food allergens, cross-reactivity has been observed not only between pan-allergens in different species but also between non-homologous allergens within a species,^{15,16} lead to more complex threaten in allergy suffers. Work focusing on novel allergens and their reacting patterns of specific epitopes is crucial but lacking at present, much research into novel allergens still needs to be done in developing a comprehensive understanding of crayfish allergy. The main objectives of this study were to purify and identify TIM from *P. clarkii*, to analyze its physicochemical and immunological characteristics, and further to determine the antigenic epitopes.

MATERIALS AND METHODS

Crayfish. Live crayfish (*P. clarkii*) was purchased from a local seafood market in Xiamen, China. Crayfish was peeled and deveined, and the muscle samples were collected immediately using or freezing at $-70\text{ }^{\circ}\text{C}$.

82 **Chemicals.** Q-Sepharose, Sephacryl S-200 HR, and Protein A Sepharose were
83 from GE Healthcare (Waukesha, Wisconsin, USA). The Ph.D.-12 Phage Display
84 Peptide Library for antigenic epitopes determination was from New England BioLabs
85 (Beverly, Massachusetts, USA). Freund's adjuvant was from Sigma (Aldrich, Saint
86 Louis, Missouri, USA). Goat anti-rabbit IgG-HRP antibody was from Abmart
87 (Berkeley Heights, New Jersey, USA). HRP-conjugated mouse anti-M13 phage
88 antibody was from Abcam (Cambridge, UK). Rabbit anti-crayfish TIM polyclonal
89 antibody was prepared in our laboratory. Others were the same as Zhang et al.⁷ used.
90 All reagents were of analytical grade.

92 **Patient Sera.** Thirteen subjects with a history of reaction to crayfish ingestion, a
93 positive ImmunoCAP score for shrimp extract (≥ 0.35 kUA/L; ImmunoCAP, Phadia
94 AB, Uppsala, Sweden) were recruited from Xiamen Second Hospital (China).
95 Additionally, Sera from normal individuals (No. 741 and 981) with no history of
96 crustacean sensitivity were collected as negative controls (Table 1). All subjects
97 voluntarily provided their sera (No. T802711, U399173, U393240, T796357,
98 U245749, U390894, T962520, U397454, T802846, T993931, U246002, U246007,
99 U245986, T982741 and V490981) with informed consent. Their use was approved by
100 the internal Ethical Committee of the hospital (XSH2012-EAN019).

102 **Identification of Proteins with IgE-binding Activity.** Crayfish muscle tissue was
103 minced and homogenized with 5 volumes (v/w) of 10 mM Tris-HCl buffer (pH 8.0)

104 containing 1 mM EDTA, 10 mM β -mercaptoethanol, and 25 mM PMSF. The sample
105 was separated on 12% SDS-PAGE, after which western blot was performed using sera
106 from crayfish-allergic patients.⁵ Patient sera recognize the target protein were used
107 separately or pooled as primary antibody in the following immunoblotting analysis.

108

109 **Purification and Identification of the Target Proteins.** The fresh muscle was
110 minced and homogenized before 40%-80% ammonium sulfate fractionation.⁶ The
111 extract was then dissolved in 10 mM Tris-HCl (pH 8.0) containing 0.1 mM EDTA
112 and 10 mM β -mercaptoethanol and dialyzed before applying to a Q-Sepharose
113 ion-exchange column (2.5 $\times$ 10 cm), bound proteins were eluted using a linear gradient
114 of 0-0.2 M NaCl at the flow rate of 0.6 mL/min. A 28-kDa protein-containing fraction
115 eluted was applied to a Sephacryl S-200 HR gel column (1.5 $\times$ 100 cm). The binding
116 proteins were eluted with 0.2 M NaCl at the flow rate of 0.5 mL/min. Obtained
117 28-kDa protein was analyzed by 12% SDS-PAGE, native PAGE,⁶ and dot blot.⁷ Pure
118 protein migrated on the SDS-PAGE was excised from the gel for MS/MS analysis
119 (Applied Protein Technology Inc, Shanghai, China).¹⁷ The resulting peptides spectra
120 were searched against the National Center for Biotechnology Information (NCBI,
121 <http://www.ncbi.nlm.nih.gov/protein/>) database using Matrix Science's Mascot 2.2
122 search engine (Matrix Science Inc, Boston, MA, USA).

123 The purified 28-kDa protein was adjusted to a concentration of 200 μ g/mL and then
124 emulsified with an equal volume of complete Freund's adjuvant as an antigen. A 100
125 μ g quantity of antigen was then subcutaneously injected into an adult female New

126 Zealand white rabbit. Further injections with the same amount of antigen were carried
127 out at 2, 4, and 5 weeks later. Five days after the final injection, the blood was
128 collected to obtain antiserum.⁶ Purification of specific polyclonal antibody (pAb) was
129 performed by using a Protein A Sepharose affinity column (1.5×5 cm), as previously
130 described.¹⁷ These experiments were performed in conformity with the laws and
131 regulations for treatment of live animals in Jimei University (Xiamen, China), SCXK
132 2012-0005.

133 During the study, a 90-kDa protein, which demonstrated immunoreactivity to the
134 specific pAb of the 28-kDa protein, was detected and further studied. The purification
135 of 90-kDa protein was carried out simultaneously with the purification of the 28-kDa
136 protein. In the first step of 28-kDa protein purification, the fraction eluted from the
137 Q-Sepharose column containing the 90-kDa protein was desalted before the
138 application to Q-Sepharose ion-exchange column (2.5×5 cm) again, binding proteins
139 were eluted with 0.05 M NaCl and subsequently eluted with a linear gradient of
140 0.05-0.2 M NaCl at the flow rate of 0.6 mL/min. Obtained 90-kDa protein was
141 analyzed by 12% SDS-PAGE, native PAGE, and dot blot as described previously.^{6,7}
142 The single band of 90-kDa protein was excised for MS/MS analysis.

143

144 **Thermal Stability and pH Stability.** To determine the thermal stabilities, proteins
145 at a concentration of 0.25 mg/mL were incubated at different temperatures for 30 min,
146 with 0 °C as the control. For the pH stability, proteins with concentration of 0.25
147 mg/mL were incubated for 1 h at 25 °C in buffers at different pH, with 8.0 as the

control. The treated samples were then analyzed by dot blot.⁷ Structure of proteins influenced by temperature and pH was observed by a ChirascanTM circular dichroism spectrometer (Applied Photophysics Ltd, Surrey, UK).⁷ The treated proteins were measured at a concentration of 0.15 mg/mL in buffer (10 mM Tris-HCl, pH 8.0) at 25 °C using a rectangular quartz cuvette (Starna, Essex, UK) with a path length of 0.1 cm. Samples were scanned three times at 1 nm/s at a bandwidth of 1 nm, with the wavelength range of 190-260 nm and then the data were calculated with the CDNN program (version 2.0).¹⁸

Digestion Stability. The digestibility of proteins in simulated gastric fluid (pepsin) and simulated intestinal fluid (pancreatin) was examined using the method of Zhang,⁷ with minor modifications. For gastric digestion, the ratios of pepsin (4534 nKat) to the 28-kDa and 90-kDa proteins were 1:50 and 1:1000 (w/w), respectively. For intestinal digestion, the ratios of pancreatin (18337 nKat) to the 28-kDa and 90-kDa proteins were 1:20 and 1:50 (w/w), respectively. Reactions were performed over time intervals, and the specific IgE-binding activity of digestion fragments was determined by competitive inhibition ELISA (ciELISA) as described, coated with 20 µg purified allergens, and an 1:2 dilution of the patients' sera and the same volume of inhibitor (the digested allergens, in concentration range of 0.025-50 µg/mL) was preincubated for 1 h before transferred to the allergen-coated 96-well ELISA plate (Nunc Maxisorb, Denmark) for incubating for 1.5 h. Detection of the bound IgE antibodies and calculation of inhibition of IgE binding was done as described previously.⁷

170

171 **Affinity Analysis by Surface Plasmon Resonance.** The affinities of 90-kDa
172 protein, 28-kDa protein, and the specific pAb were measured in surface plasmon
173 resonance (SPR) biacore experiments performed on a Biacore T200 instrument (GE
174 Healthcare, Uppsala, Sweden) as described by Wang,¹⁹ with some modification. The
175 kinetic analysis was performed in 10 mM Tris-HCl buffer (pH 8.0), 25 °C. The
176 90-kDa protein or the specific pAb was directly immobilized on a CM5 chip (GE
177 Healthcare, Uppsala, Sweden) by standard amine coupling according to the
178 manufacturer's instructions. The proteins or specific pAb was passed over the chip
179 surface separately at a flow rate of 30 μ L/min in 5 gradients of two-fold concentration
180 starting from 12.5 μ g/mL to 200 μ g/mL. The 12.5 μ g/mL concentration was run in
181 duplicate to confirm the reproducibility of the assay. The assay was performed using
182 the single-cycle kinetic method with two cycles of the buffer as a reference. For all
183 kinetic assays, the contact time and the dissociation time were set as 120 s and 900 s,
184 respectively, and the regeneration was processed with 60 s washing with 10 mM
185 glycine, pH 2.0. The kinetic constants for the 90-kDa protein and pAb of 28-kDa
186 protein were fitted with a 1:1 binding model.

187

188 **Epitopes Analysis with Phage Display.** The Ph.D.-12 Phage Display Peptide
189 Library was used for antigenic epitope mapping according to the method of Yang.²⁰
190 Purified pAb of 28-kDa protein was used as a target protein in biopanning. Single
191 strand phage DNA was prepared from the randomly biopanned clones and sequenced

(Invitrogen Co., Shanghai, China) based on the manufacturer's instructions. Phage clones with the highest reactivity to the specific pAb were selected by phage-capture ELISA, as described by Subbarayal,²¹ with slight modification. In short, ELISA plates were coated with 10 µg purified pAb. After blocking with BSA, the wells were incubated with the phage clones, which were serially diluted 10-fold, with a starting titer of 10¹² virions. All the steps described above were performed at room temperature for 1 h, and between each step, the plates were washed six times with 0.05% TBST. Detection of bound phages was done as previously described.²⁰

The obtained peptides were aligned with the proteins by DNAMAN program (Lynnon Biosoft, San Ramon, CA, USA) for the determination of linear epitopes. For conformational epitope mapping, the three-dimensional (3D) structures of the 28-kDa and 90-kDa proteins were modeled using the Web service Swiss Model (<http://swissmodel.expasy.org>) based on homology modeling, and the selected peptides were calculated, analyzed and mapped onto the 3D structures of the proteins by LocaPep (<http://atenea.montes.upm.es>) based on protein surface properties.²² The identified epitopes were localized on the 3D surfaces of the proteins using PyMOL software (DeLano Scientific, San Carlos, CA, USA).

IgG/IgE-binding Activity of Synthetic Peptides. Peptides corresponding to the identified epitopes of the two allergens were designed and commercially synthesized using organic solid-phase synthesis technology (Cell-mano Biotech, Shanghai, China). The IgG/IgE-binding activity of the peptides was detected with dot blot. Inhibition dot

214 blot was carried out by preincubated the pAb of 28-kDa protein with serial dilutions
215 of individual peptides before incubation with the 28-kDa and 90-kDa proteins.

216

217 **RESULTS**

218 **IgE-binding Activity of Patients' Sera to Crayfish Myosinogen.** Five of 13
219 patient sera (No. 711, 894, 454, 002, and 007) reacted to the 28-kDa protein, but the
220 reaction intensity of 28-kDa protein with patient sera was substantially weaker than
221 that of a band at approximately 40 kDa, which has been identified as AK, a major
222 allergen of crayfish, in our previous stud.⁵ As expected, the control sera did not show
223 any reactivity (Figure 1A). The 5 positive sera were used as primary antibody in the
224 following analysis.

225

226 **Purification and Identification of the 28-kDa Protein.** The 28-kDa protein
227 purified from the crayfish myosinogen by sequential column chromatography on
228 Q-Sepharose (Figure 1B) and Sephacryl S-200 HR, resolved by means of both
229 SDS-PAGE and native PAGE as a single band, with evidence of a strong reactivity
230 with patient IgE (Figure 1C), revealed that the purified 28-kDa protein is an allergen
231 in crayfish. The amino acid sequence of the distinct protein band on SDS-PAGE was
232 further analyzed, and Figure 2A summarizes the results of MS/MS. A search for
233 homologous proteins in the NCBI protein database showed that a total of sixteen
234 peptides had 100% identity with the amino acid sequences of TIM from *P. clarkii*
235 (gi|328900101), besides, the purified protein shared high identities with TIMs from

236 different species of crustacean (Figure 2B).

237 The anti-TIM pAb obtained by injecting rabbits with purified TIM showed a titer of
238 $1:10^5$ in dot blot. Meanwhile, during the specificity evaluation of pure anti-TIM pAb
239 (diluted $1:10^5$) by western blot, it was interesting to find that a protein with the
240 molecular mass of 90 kDa displayed specific activity toward the antibody (Supporting
241 Information Figure S1). Heating at 100 °C for 1 h or denaturated by 5% β -Me showed
242 that the 90-kDa protein was not a polymer of TIM, and it was speculated that
243 anti-TIM pAb might recognize some epitopes of the 90-kDa protein, which was worth
244 further investigation.

245

246 **Purification and Identification of the 90-kDa Protein.** The fractions eluted from
247 the Q-Sepharose column during TIM purification that contained the 90-kDa protein
248 were pooled and separated on another Q-Sepharose column (Fig. 1D). The eluted
249 90-kDa protein had migrated a single band on both SDS-PAGE and native PAGE,
250 with reactivity to patients' sera (Figure 1D), thus the protein was proved to be a
251 crayfish allergen. The 90-kDa protein was subjected to MS/MS, and the effective
252 peptides obtained were compared with the NCBI database. The obtained 11 peptides
253 with 144 amino acids was 100% identical to the sequences of filamin C (FLN c) of
254 *Pediculus humanus corporis* (gi|242004976), and the protein score confidence
255 interval (C. I.) output by Mascot was 99.4% (> 95% was judged a success), so the
256 90-kDa protein was identified as FLN c of *P. clarkii* (Figure 3).

257 Sera from crayfish-allergic patients were utilized as primary antibody for further

analysis of the immunoreactivity of TIM and FLN c by means of dot blot. The result of an IgE-binding activity determination of purified TIM was in accordance with that of the muscle tissue extract: 5 of the 13 patient sera (No. 711, 894, 454, 002, and 007) that reacted to the 28-kDa protein recognized purified TIM. What is more, FLN c showed positive activities only with the sera that reacted to TIM (Figure 1E).

Thermal Stability and pH Stability of TIM and FLN c. The thermal and pH stabilities of purified TIM and FLN c were determined using the far-Ultraviolet CD spectra and dot blot with patients' sera. The CDNN analysis showed that native TIM contains 31.7% α -helices, 12.4% antiparallel extended strands, and 7.4% parallel extended strands, which meets the structural standards of α + β proteins.²³ While heat treatment had an obvious effect on the secondary structure, especially after 100 °C treatment. Random coils were markedly reduced and β -turns partly increased (Figure 4A), the IgE-binding activity was reduced with the increasing temperature higher than 60 °C (Figure 4B). Extreme acidic conditions (pH 1.0) or alkaline conditions (pH 11.0) led to a reduction in α -helices in the structure (Figure 4C). The IgE-binding activity of TIM was relatively stable under acidic and alkaline conditions. However, it was interesting to find an increased IgE-binding activity at pH 2.0-3.0 (Figure 4D).

The CDNN analysis of native FLN c showed that it was an α / β protein, containing 25.6% α -helices, 8.1% antiparallel extended strands, and 15.2% parallel extended strand.²³ The secondary structure of FLN c was also affected by heat treatment: α -helices were markedly increased while random coils were significantly reduced

280 when the temperature increased to 60 °C, with a decreasing in IgE-binding activity.
281 (Figure 4E and F). When treated with extreme acid (pH 1.0), α -helices and random
282 coils in FLN c decreased while β -turns increased. Extreme alkaline treatment (pH
283 11.0) increased the α -helices and reduced the extended strands (Figure 4G). The
284 IgE-binding activity of FLN c was negligibly influenced by alkaline conditions, but
285 was slightly reduced under acidic conditions (pH 1.0-5.0) (Figure 4H).

286

287 **Digestibility of TIM and FLN c.** *In vitro* digestion tests were performed to
288 evaluate the stability of TIM and FLN c in simulated human gastrointestinal
289 conditions. As shown on the SDS-PAGE, original band of TIM gradually disappeared
290 upon treatment with pepsin, and two proteolytic fragments around 14.4 kDa appeared
291 after prolonged peptic digestion, with TIM digested completely after 1 h (Figure 4I).
292 However, TIM was stable during stimulated intestinal digestion even at higher
293 concentrations of pancreatic enzymes, and was only incompletely degraded after 4 h
294 (Figure 4J). When the digested TIM fragments were used as inhibitors in ciELISA,
295 the IgE-binding activity was not obviously reduced when the concentration of the
296 inhibitor was increased to 50 μ g/mL (Figure 4K).

297 In the simulated gastric fluid FLN c was gradually digested by pepsin, and three
298 proteolytic fragments around 60-80 kDa appeared with the increasing of time, and the
299 distinct band of FLN c was disappeared after 1 h (Figure 4L). Under the high ratio of
300 trypsin used in the digestion, an 80-kDa fragment appeared after 15 min, and was then
301 digested into smaller fragments in 4 h (Figure 4M). The results of ciELISA showed

302 that the IgE-binding activity was substantially reduced but 50% binding activity
303 remained when the concentration of the inhibitor was increased to 50 $\mu\text{g/mL}$ (Figure
304 4N).

305

306 **Phages Isolated by Biopanning with Anti-TIM pAb.** TIM and FLN c displayed
307 cross-reactivity with the anti-TIM pAb and the same sera from crayfish-allergic
308 patients (Figure 1E). Interactions between proteins and antibody were measured by
309 the technique of SPR (Supporting Information Figure S2). As the kinetic constants
310 summarized in Table 2, binding ($K_D=1.275\times 10^{-8}$) between the anti-TIM pAb and TIM,
311 and binding ($K_D=4.210\times 10^{-6}$) between the anti-TIM pAb and FLN c were observed;
312 thus, purified anti-TIM pAb could be used as the target protein for biopanning of the
313 epitopes of TIM and FLN c. After three rounds of biopanning, 21 random clones were
314 identified with phage ELISA, and 17 of them bound to anti-TIM pAb (Table 3).

315

316 **Determination of the Epitopes of TIM.** The sequence alignment of the 17
317 mimotopes and TIM revealed a remarkable similarity, and the most frequent amino
318 acids were AA 17-30 (Figure 5A). Five peptides were defined as the linear epitopes,
319 named as linear epitope of TIM (L-TIM, Table 4). As the theory proposed by Pacios,²³
320 an epitope is assumed to form around a key residue. The TIM surface was scanned for
321 key residues belonging to clusters, 3 key residues were identified and then based on
322 the analysis of LocaPep program, and 17 peptides containing the residues of 3
323 conformational epitopes of TIM form around the key residues were output, named as

324 conformational epitopes of TIM (C-TIM, Table 4). Graphical molecular
325 representations of epitopes on TIM are shown in Figure 5B and C.

326

327 **Determination of the Epitopes of FLN c.** The sequence alignment between 17
328 mimotopes and FLN c indicated that the most frequent amino acids were AA 51-60
329 and 569-582, and 9 linear epitopes were defined (Figure 5D). Ten key residues and
330 conformational epitopes formed around the key residues were obtained from
331 calculations performed by LocaPep (Table 4). The 3D structure of FLN c was
332 modeled separately by five modeled structures, on which the linear and
333 conformational epitopes were displayed in Figure 5F and G.

334

335 **Identification of Common Epitopes shared by TIM and FLN c.** In addition to
336 the cross-reactivity results with the same sera from crayfish-allergic patients (Figure
337 1E) and K_D measurement by SPR (Table 2), the epitopes of TIM and FLN c showed
338 similarities, with the same residues in common to some extent, which are partly
339 marked in gray in Table 4. Peptides in agreement with the common epitopes of TIM
340 and FLN c were then synthesized (Table 5) and tested for the immunoreactivity. All
341 of the peptides showed binding activity to both anti-TIM pAb and crayfish-allergic
342 patients' sera (Figure 6A and B). In addition, dose-dependent inhibition was observed
343 when individual peptides were used to inhibit the binding between allergens and
344 anti-TIM pAb (Figure 6C), demonstrating the sharing of common epitopes by TIM
345 and FLN c, as expected.

346

347 **DISCUSSION**

348 Seafood is considered to be a common cause of IgE-mediated food allergy, and a
349 number of food allergens have been identified in various saltwater products over the
350 past few decades. However, freshwater products, which account for 48.37% of the
351 total fishery industry in China,¹ are important sources of nutrition in the Chinese diet.
352 As freshwater crayfish is the top-ranking aquaculture product, the frequency of
353 allergic reactions caused by crayfish has led to a recent focus on the diagnosis and
354 therapy of crayfish allergy. While a current limitation of crayfish allergen-based
355 diagnostics is that part of patients with crayfish allergy are overlooked as
356 false-negative responders (unpublished data). The situation has also been reported in
357 several of food, and turned out to be resulted from the novel allergens,²⁴⁻²⁶ prompted
358 the research for undescribed allergens in crayfish.

359 In the previous study of crayfish AK, a 28-kDa protein with IgE-binding activity
360 was discovered. In the current study, the IgE reactivity of sera from subjects sensitive
361 to crayfish was tested with the myosinogen of crayfish, the band volume of IgE
362 binding proteins (Figure 1A) suggested that AK is the major allergen, and the 28 kDa
363 band which was identified as TIM is a minor allergen in the myosinogen. With a low
364 concentration of approximately 0.071 mg/g crayfish muscle, the presence of TIM
365 could easily be undetected. TIM is a key enzyme in glycolysis that catalyzes the
366 interconversion of dihydroxyacetone phosphate and glyceraldehyde-3-phosphate.
367 There is a stable dimer of two 28-kDa monomer in its native state, and a typical

(β/α)₈ barrel fold in its spatial structure.²⁷ The current result revealed that TIM is also an allergen in freshwater products.

FLN c attracted our attention for its reactivity with anti-TIM pAb, positive reaction with crayfish-allergic patients' sera provided evidence that FLN c is a novel allergen in crayfish. Filamins are members of the superfamily of actin-binding proteins, which directly interact with many diverse cellular proteins and are involved in cell motility, cell signaling, transcription, and organ development.²⁸ FLN c, with a molecular weight of 90 kDa, is expressed only in skeletal muscle and cardiac tissues, and medaka FLN c was found to have an immunoglobulin-like domain;²⁹ however, there is a rare report about its role as an allergen in invertebrates, which might also result from the low content (approximately 0.083 mg/g crayfish muscle) in the tissue. It is the first time that FLN c has been recognized as an allergen, what's more, IgG and IgE cross-reactivity between TIM and FLN c were observed, taking the non-homology between TIM and FLN c into consideration, the cross-reactivity might be due to some primary structure unrelated similarities.

The immunoreactivity of allergens might change in harsh conditions, especially the thermal and extreme pH conditions that occur during food processing, due to structural variations. High temperature affects the secondary structure of TIM and FLN c, and led to a reduction of IgE-binding activity (Figure 4A, B, E, and F), which is similar to the thermal stabilities of AK and parvalbumin demonstrated in previous researches.^{5,30} As structure deformation during heating, the surface-exposed epitopes could become inaccessible to a certain extent, which is considered to be a key

influence for IgE-reactivity. The immunoreactivity of TIM was relatively stable in different pHs, except for an enhancement of IgE-binding activity at pH 3.0 (Figure 4C), a similar phenomenon has been observed in crayfish SCP, which might result from the exposure of hidden epitopes due to the unfolding of α -helices that contributed to the flexibility increasing of the structure.⁶ FLN c also demonstrated the obvious structure disorganized in acidic condition, with immunoreactivity changing. The results suggested similar physicochemical properties between TIM and FLN c, which were concert with the inference of some similarities existing in the two cross-reactive allergens.

A majority of food allergens remains immunoreactivity during gastrointestinal digestion.³¹⁻³⁴ Crayfish TIM appeared to be more resistant to gastrointestinal digestion than FLN c, the immunological activity of gastrointestinal digested TIM was influenced by the disorganization of structure, but the influence can be weakened by higher concentrations (Figure 4K). While the digested FLN c lost approximately 50% binding activity to the patients' sera (Figure 4N). TIM and FLN c were digestible by pepsin and trypsin, however, they may still cause allergic reactions, due to certain IgE-binding sites that remain unaffected by digestive enzymes. In addition to the thermal and pH stability, the digestibility assay further confirmed that the integrity, exposure, and masking of allergen epitopes are crucial for the immunological activity of allergens.

The immunoreactivity of an allergen depends on the number and characteristics of its epitopes, which are the specific regions recognized by the specific antibody.³⁵

412 Crayfish AK, SCP, MLC, TM, and the two novel allergens identified in the present
413 study, despite of their various degrees of proteolysis, were found to remain relatively
414 high immunoreactivity after gastrointestinal digestion,^{5-7,36} indicating that cleavage by
415 the digestive enzymes may disrupt their tertiary structures, but the separated minor
416 epitopes may retain immunoreactivity, or the major IgE-binding sites of the allergens
417 may be stable during gastrointestinal digestion. Importantly, a high degree of IgG/IgE
418 cross-reactivity between the two novel crayfish allergens were observed, in spite of
419 the low linear sequence identities and structural similarities. The reacting patterns of
420 epitopes seemed essential for explaining the relationship between the allergens and
421 the specific antibodies.

422 In the present study, epitopes of TIM and FLN c were determined by the technique
423 of phage display. Generally, biopanning for IgE-specific epitopes requires the
424 corresponding purified IgE from allergic suffers. Regrettably, a common limitation in
425 the IgE epitope mapping is that patient sera available were insufficient to purify IgE.
426 Researches on the IgE epitope mapping by Gieras et al. have recently found that IgG
427 reorganization of portions within the IgE epitopes,³⁷ specific IgG antibodies were
428 used for fast mapping of IgE epitopes of major allergens such as Bet v 1 from birch
429 pollen and Phl p 5 from grass pollen.^{38,39} Therefore, rabbit anti-TIM IgG pAb was
430 selected as the target protein for the panning procedure instead of IgE, the IgE
431 epitopes was further confirmed by the IgE-binding activity of synthetic peptides
432 derived from the identified common epitopes (Figure 6B). FLN c, in contrast with
433 TIM that tend to have mostly linear epitopes (approximately 63%) due to proteolytic

434 digestion of the antigen, might primarily have conformational epitopes displayed on
435 the surface of the protein (approximately 53%), which might explain the loss of 50%
436 immunoreactivity after digestion. Of note, some linear epitopes can also be
437 conformational epitopes if that sequence is surface-exposed,⁴⁰ and perhaps, this is the
438 case of the retained immunoreactivity in digested TIM and FLN c.

439 Data from the present study provide an experiment proved that the nonhomologous
440 allergens TIM, a key enzyme in glycolysis, and FLN c, an actin-binding protein, are
441 cross-reactive. Generally, cross-reactivity should be considered for proteins sharing
442 more than 35% identity in a window of 80 amino acids,⁴¹ nevertheless, TIM and FLN
443 c do not display obvious linear sequence identities and structural similarities. IgE
444 cross-reactivity among non-homologous peanut allergens, Ara h 1, a vicilin; Ara h 2,
445 a 2S albumin; and Ara h 3, a legumin has been reported by Bublin in the previous
446 study,¹⁶ which turned out to be a consequence of common epitopes shared among
447 allergens. The alignment performed between the epitopes of TIM and FLN c found
448 the presence of TIM IgE-binding epitopes with sequence similarities to several
449 peptides in FLN c (Table 4, marked in gray), which was likely to be the primary
450 region involved in the cross-reactivity. The dose-dependent cross-competitive dot blot
451 assays showed that the binding to specific antibody of TIM and FLN c were partially
452 inhibited by the synthetic peptides, this further confirms the existence of epitopes
453 cross-reactive between TIM and FLN c. Furthermore, the common epitopes of TIM
454 and FLN c showed low identify to the epitopes of other crayfish allergens SCP, MLC,
455 AK, and TM,^{6,7,20,42} the amino acid sequences of the 4 IgE-binding peptides

456 overlapped with the identified epitopes could thus be potential peptide biomarkers for
457 the diagnose of crayfish allergy instead of purified TIM and FLN c.

458 The identification of epitopes, on another hand, is crucial for the development of
459 hypoallergenic food.⁴³ On the basis of epitope describing, the IgE-binding activity and
460 allergenicity of AK was mitigated by heating treatment and cross-link processing
461 catalyzed by tyrosinase, an oxidase that acts mostly on tyrosyl residues leading to
462 cross-linking of proteins or proteins with polysaccharides.⁴⁴ As a certain number of
463 tyrosyl residues distributed in the epitopes of TIM and FLN c, cross-linking
464 processing with tyrosyl might also appropriate for the reduction of immunoreactivity
465 of TIM and FLN c. The result of thermal stability suggested the TIM and FLN c
466 showed similar characters to AK, indicating that heating treatment might also be
467 conducive to the hypoallergenic processing of the currently identified novel allergens.
468 What's more, based on the similarities among AK and the two novel allergens, the
469 method of enzymatic cross-link processing combined with heating treatment
470 established previously⁴⁴ is expected to eliminate the allergenicity of these proteins
471 simultaneously. Likewise, the searching for other effective processing methods (e.g.
472 maillard reaction and galactosylated modification) could be more founded according
473 to the characters and reaction patterns of epitopes.

474 In summary, two novel allergens of crayfish, TIM and FLN c were identified, and
475 cross-reactivity between the novel allergens was observed. The epitopes mapped by
476 phage display and the common epitopes discovered between the allergens offer the
477 basis for their cross-reactivity. The identification of novel allergens might contribute

478 to an increase in diagnostic sensitivity, and their epitopes and structural features might
479 add information for the development of low allergenic products. As the mechanism by
480 which TIM and FLN c act during anaphylaxis is not completely understood, more *in*
481 *vitro* and *in vivo* experiments should be incorporated in further research.

482

483 **CONFLICT OF INTEREST**

484 The authors declare no competing financial interest.

485

486 **ABBREVIATIONS USED**

487 **AA**: amino acid; **AK**: arginine kinase; **CD**: circular dichroism; **ciELISA**:
488 competitive inhibition enzyme linked immunosorbent assay; **FLN c**: filamin C; **MLC**:
489 myosin light chain; **NCBI**: national center for biotechnology information; **pAb**:
490 polyclonal antibody; **SCP**: sarcoplasmic calcium-binding protein; **SPR**: surface
491 plasmon resonance; **TIM**: triosephosphate isomerase; **TM**: tropomyosin

492

493 **ACKNOWLEDGEMENT**

494 This work was supported by the Grant from the National Natural Scientific
495 Foundation of China (31171660, U1405214), the Foundation for Innovative Research
496 Team of Jimei University (2010A005).

497

498 **SUPPORTING INFORMATION**

499 For additional experimental details, see Supporting information.

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- 630

631 **Figure Legends**

632 **Table 1.** Clinical and laboratory characterizations of thirteen crustacean-allergic
633 patients and two non-allergic individuals.

634

635 **Table 2.** Kinetic constants for FLN c and anti-TIM pAb and TIM determined by
636 Biacore T200.

637

638 **Table 3.** Mimotope sequences of positive clones derived from biopanning against
639 rabbit anti-TIM pAb.

640

641 **Table 4.** Epitopes of *P. clarkii* TIM and FLN c mapped by the bio-panning.

642

643 **Table 5** Sequences of synthetic peptides.

644

645 **Figure 1.** Purification and dot blot analysis of the IgE-binding proteins. (A) Analysis
646 of specific IgE reactivity to crayfish muscle protein with sera from the patients with
647 crayfish allergy. M, protein marker; MS, SDS-PAGE analysis of myosinogen. Lanes
648 1-13, representatives of sera from crayfish-allergic subjects; N, pooled non-allergic
649 sera from healthy individuals (negative control). (B) Q-Sepharose chromatography
650 elution profile of the crayfish muscle protein after the ammonium sulfate precipitated.
651 Target protein fractions under the bar were pooled. (C) Sephacryl S-200 HR gel
652 chromatography elution profile of fraction contained the 28-kDa from (B). (D)

Q-Sepharose chromatography elution profile of the 90-kDa protein containing fraction from (B). The protein concentrations were estimated by measuring the absorbance at 280 nm. The fractions containing target proteins in (C) and (D) were subjected to SDS-PAGE and native PAGE analysis (gel concentration of 12%). IgE-binding activity of the purified proteins was then analyzed by dot blot using crayfish-allergic patients' serum pool (positive sera of (A)) (dot 1) and non-allergic serum pool (dot 2) (No. 741 and 981). (E) IgE-binding activity of TIM and FLN c was analyzed by dot blot. 1-13, dot blot profiles of IgE-binding activity of purified TIM and FLN c to patients' sera (1-13 correspond to sera from crayfish-allergic subjects); N, pooled non-allergic sera.

663

Figure 2. Mass spectrometric analysis of the 28-kDa protein in crayfish. (A) Map of MS/MS. (B) Protein sequence alignments of TIMs from *P. clarkii* (gi|328900101), *Archaeopotamobius sibiricus* (gi|19848023), *Cherax quadricarinatus* (gi|342326238), *Eriocheir sinensis* (gi|307564155), and *C. crangon* (gi|238477329). The identical residues are marked with grey.

669

Figure 3. Mass spectrometric analysis of the 90-kDa protein in crayfish. (A) Map of MS/MS. (B) Protein sequence alignments of FLN c from *Pediculus humanus corporis* (gi|242004976). The identical residues are marked with grey.

673

Figure 4. Thermal, pH, and digestion stability analysis of the purified allergens. (A-D)

675 Thermal and pH stability of TIM. CD spectral analysis of TIM subjected to different
676 temperatures (A) and pHs (C). Dot blot of TIM treated at different temperatures (B)
677 and pHs (D) with patients' sera pool (No. 711, 894, 454, 002, and 007). (E-H)
678 Thermal and pH stability of FLN c. CD spectral analysis of FLN c subjected to
679 different temperatures (E) and pHs (G). Dot blot of FLN c treated at different
680 temperatures (F) and pHs (H) with patients' sera pool (No. 711, 894, 454, 002, and
681 007).. (I-K) Digest stability of TIM. SDS-PAGE analysis of TIM stability to pepsin
682 digestion (I) and pancreatin digestion (J); M, protein marker; con, control. (K)
683 ciELISA of the digested fragments of TIM using patients' sera pool as primary
684 antibody; TIM digested by pepsin (●, 1 h), pancreatin (△, 4 h), and undigested (○).
685 (L-N) Digest stability of FLN c. SDS-PAGE analysis of FLN c stability to pepsin
686 digestion (L) and pancreatin digestion (M); M, protein marker; con, control. (N)
687 ciELISA of the digested fragments of FLN c using patients' sera pool as primary
688 antibody; FLN c digested by pepsin (●, 1 h), pancreatin (△, 4 h), and undigested (○).
689
690 **Figure 5.** Sequences alignment and molecular graphics of the epitopes of TIM (A-C)
691 and FLN c (D-F). (A) The amino acid sequence alignment of *P. clarkii* TIM and 17
692 mimotopes derived with biopanning. (B) The 5 linear epitopes of TIM on the 3D
693 structure. (C) The 3 conformational epitopes of TIM on the 3D structure (Template:
694 2I9E, 71.3% identity). (D) The amino acid sequence alignment of FLN c and 17
695 mimotopes derived with biopanning. (E) The 9 linear epitopes of FLN c on the 3D
696 structure. (F) The 10 conformational epitopes of FLN c on the 3D structure. FLN c

697 was modeled separately by 5 modeled structures: I (Template: 2K7P, 57.93% identity),
698 II (Template: 2DMC, 51.82% identity), III (Template: 2BRQ, 68.13% identity), IV
699 (Template: 2D7P, 45.36% identity), V (Template: 2EEC, 50.89% identity).

700

701 **Figure 6.** Determination of the IgG/IgE-specific peptides of *P. clarkii* TIM and FLN c.

702 (A) Dot blot analysis of IgG binding-activities of the 4 synthetic peptides using
703 anti-crayfish TIM pAb as primary antibody. (B) Dot blot profiles of IgE-binding
704 activities of the 4 synthetic peptides to patients' sera pool (sera No. 0735, 8551, 4780,
705 3498, 3764, and 5603). (C) IgG inhibition immunoblotting of TIM and FLN c with
706 the synthetic peptides as the inhibitor, using anti-TIM pAb (diluted $1:10^5$) as the
707 primary antibody, synthetic peptides were serial diluted by 1:5, 1:10, and 1:50 as
708 inhibitors.

Table 1

Serum No.	Age	Sex	Symptoms	sIgE shrimp (f24) by ImmunoCAP (kUA/L)
711	8	F	U, P	9.68
173	14	M	N, P	0.65
240	18	M	A, D	1.28
357	22	F	U, P, E	0.63
749	13	M	E, Dr	1.64
894	19	F	U, D, P	1.93
313	34	M	A, D	0.62
454	17	F	E, N	11.1
846	21	M	A, D, R	0.78
931	28	F	E, Dr	0.86
002	18	F	A, R	2.35
007	15	F	N, Dr	61.7
986	10	M	U, E	45.1
741	19	F	ND	0.15
981	23	M	ND	0.06

F: female, M: male, U: urticaria, P: pruritus, E: emesis, N: nausea, A: asthma, D: dyspnea, R:

rhinitis, Dr: diarrhea, ND: not determined / unknown.

Table 2

Ligand	analyte	K_a ($M^{-1}s^{-1}$)	k_d (s^{-1})	K_D (M)
FLN c	Anti-TIM pAb	17.66	7.437×10^{-5}	4.210×10^{-6}
	TIM	N/A	N/A	1.084×10^{-5}

Table 3

Mimotope Sequence	Clone No.	Mimotope Sequence	Clone No.
KFPDIDSISLW	2, 21, 30	NFSPSNVPGDKF	14
FEDSIVSTRETF	4	HVDTGSDKKLDH	15
YYDDTASIRSSR	5	SDSDSIRTYMNI	17
VNLSDSLHYP	6	RSEGEVLSPETL	19
SGVYKVAYDWQH	9	DSISSIVTSQAF	23
HTEDDTASITTS	10	MDGLDSIYTSSR	24
DRSSDSIVSWRG	11	GQIIQDFDWVQN	25
STDSIITNSQRL	12, 20	SSTD SIMSSYIG	28
AYAGSTGLFERG	13	ACCDIDSIKSSV	29

Table 4

TIM		FLN c	
Linear epitopes	1	D ₁₈ R ₁₉ A ₂₀ G ₂₁ I ₂₂ D ₂₃ S ₂₄ I ₂₅ I ₂₆ S ₂₇	1 G ₄₃ S ₄₄ P ₄₅ F ₄₆
			2 D ₅₁ S ₅₂ I ₅₃ A ₅₄ S ₅₅ G ₅₆ Y ₅₇ V ₅₈ T ₅₉
			3 D ₁₀₇ D ₁₀₈ T ₁₀₉ V ₁₁₀ S ₁₁₁
			4 I ₂₂₁ T ₂₂₂ N ₂₂₃ S ₂₂₄
	2	Y ₆₇ K ₆₈ V ₆₉ A ₇₀	5 D ₄₇₉ R ₄₈₀ K ₄₈₁ D ₄₈₂ G ₄₈₃ S ₄₈₄ C ₄₈₅ Y ₄₈₆ V ₄₈₇ S ₄₈₈
	3	I ₇₈ S ₇₉ P ₈₀ S ₈₁ M ₈₂ I ₈₃ K ₈₄ D ₈₅ C ₈₆ G ₈₇ C ₈₈ E ₈₉ W ₉₀ V ₉₁	6 G ₄₉₅ E ₄₉₆ Y ₄₉₇ R ₄₉₈ V ₄₉₉
	4	V ₁₅₁ P ₁₅₂ N ₁₅₃ I ₁₅₄ S ₁₅₅ D ₁₅₆ W ₁₅₇ S ₁₅₈	7 E ₅₆₇ D ₅₆₈ D ₅₆₉ C ₅₇₀ F ₅₇₁ I ₅₇₂ Q ₅₇₃ S ₅₇₄ I ₅₇₅ D ₅₇₆ S ₅₇₇ D ₅₇₈ T ₅₇₉ Y ₅₈₀ S ₅₈₁ V ₅₈₂ R ₅₈₃ F ₅₈₄ M ₅₈₅ P ₅₈₆ R ₅₈₇ E ₅₈₈
			8 G ₆₂₈ L ₆₂₉ T ₆₃₀ K ₆₃₁ I ₆₃₂ K ₆₃₃ T ₆₃₄
	5	Y ₂₀₈ G ₂₀₉ G ₂₁₀ S ₂₁₁ V ₂₁₂ T ₂₁₃ P ₂₁₄ G ₂₁₅ L ₁₃₁ E ₁₃₂ R ₁₃₄ E ₁₃₅ P ₁₆₆ V ₁₆₇ W ₁₆₈ A ₁₆₉	9 T ₇₀₆ G ₇₀₇ E ₇₀₈ D ₇₀₉ L ₇₁₀ A ₇₁₁ E ₇₁₂ R ₇₁₃ G ₇₁₄
	1	I ₁₇₀ G ₁₇₁ T ₁₇₂ G ₁₇₃ K ₁₇₄ T ₁₇₅ A ₁₇₆ T ₁₇₇ E ₁₇₉ Q ₁₈₀ E ₁₈₃ V ₁₈₄ G ₂₁₀ S ₂₁₁ T ₂₁₃	1 V ₁₀ I ₁₁ E ₁₂ D ₁₃ N ₁₄ D ₁₆ G ₁₇ T ₁₈ V ₁₉ S ₂₀ I ₂₁ N ₂₂ L ₃₃ I ₃₅ S ₄₅ P ₄₆ F ₄₇ F ₄₉ S ₆₇ S ₁₄₀ K ₁₄₂
Conf. epitopes	2	N ₆₅ F ₇₄ G ₇₆ E ₇₇ N ₁₀₀ V ₁₀₁ F ₁₀₂ N ₁₀₃ E ₁₀₄ L ₁₀₈	2 S ₃ G ₄ K ₅ V ₆ D ₇ K ₂₆ E ₂₇ E ₂₈ D ₅₂ S ₅₃ I ₅₄ A ₅₅ S ₅₆ G ₅₇ Y ₅₈ Y ₈₀ G ₈₃ A ₈₄
	3	S ₁₅₈ R ₁₈₉ R ₁₉₃ V ₁₉₆ A ₁₉₈ E ₁₉₉ V ₂₀₀ D ₂₀₂ S ₂₀₃	3 S ₇₅ T ₇₈ L ₈₉ A ₉₀ M ₉₁ S ₉₂ I ₁₀₁ S ₁₀₂ C ₁₀₃ H ₁₀₄ D ₁₀₅ T ₁₁₀ V ₁₁₁ S ₁₁₂ S ₁₁₄ S ₁₂₆ K ₁₂₈ S ₁₃₇
			4 Q ₁₈₀ A ₁₈₁ P ₁₈₂ E ₂₁₀ Q ₂₁₂ S ₂₁₄ S ₂₂₅ P ₂₂₆ K ₂₂₈
			5 T ₁₄₄ G ₁₄₇ R ₁₄₈ N ₁₅₁ Q ₁₅₂ I ₁₅₃ S ₁₅₄ V ₁₅₅ S ₁₅₇ S ₁₅₉ I ₂₂₉ V ₂₃₁
			6 T ₁₆₈ D ₁₆₉ S ₁₇₀ E ₁₇₁ L ₁₇₂ R ₁₇₃ T ₁₇₄ L ₁₇₅ H ₄₃₅ G ₄₃₇ E ₄₅₁ N ₄₅₃ W ₄₅₅ T ₄₅₆ R ₄₅₇ E ₄₅₈ A ₄₅₉ G ₄₆₀ A ₄₆₁ G ₄₆₂
			7 S ₄₆₃ L ₄₆₄ A ₄₆₅ I ₄₆₆ E ₄₇₅ I ₄₇₆ D ₄₇₇ F ₄₇₈ K ₄₇₉ D ₄₈₀ R ₄₈₁ K ₄₈₂ D ₄₈₃ G ₄₈₄ S ₄₈₅ C ₄₈₆ Y ₄₈₇ S ₄₈₉ F ₅₀₄ D ₅₀₆
			8 E ₄₄₅ Q ₄₄₆ P ₄₇₁ S ₄₇₂ V ₄₉₂ G ₄₉₃ T ₄₉₄ P ₄₉₅ I ₅₁₈ S ₅₁₉ A ₅₂₁ G ₅₂₃ D ₅₂₄ K ₅₂₇ L ₅₂₈ V ₅₃₀ A ₅₃₁ Q ₅₃₂ F ₅₃₃ P ₅₃₄ N ₅₄₅ F ₅₄₆
			9 L ₅₄₇ V ₅₄₈ R ₅₄₉ K ₅₅₀ G ₅₅₂ A ₅₅₃ K ₅₅₄ G ₅₅₅ E ₅₅₆ E ₅₆₈ D ₅₇₀ F ₅₇₂ I ₅₇₃ Q ₅₇₄ S ₅₇₅ I ₅₇₆ D ₅₇₇ S ₅₇₈ D ₅₇₉ T ₅₈₀ Y ₅₈₁ S ₅₈₂ V ₅₈₃ R ₅₈₄ F ₅₈₅
			G ₆₂₇ N ₆₂₈ L ₆₃₀ T ₆₃₁ K ₆₃₂ K ₆₃₄ T ₆₃₇ T ₆₃₉ G ₆₄₉ A ₆₅₀ G ₆₅₁ T ₆₅₂
	10	L ₆₅₃ A ₆₅₄ V ₆₅₅ Q ₆₅₆ I ₆₅₇ D ₆₅₈ V ₆₆₃ M ₆₆₅ D ₆₆₆ C ₆₆₇ T ₆₆₈ E ₆₆₉ Y ₆₇₄ T ₆₇₉ F ₆₈₇ V ₆₈₈ S ₆₈₉ K ₆₉₁ Y ₆₉₂ G ₆₉₄ S ₇₀₀ V ₇₀₄	

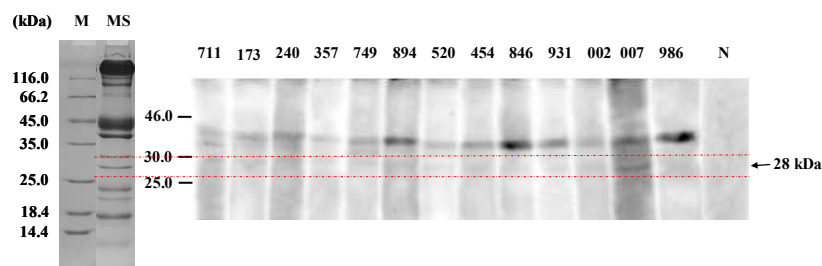
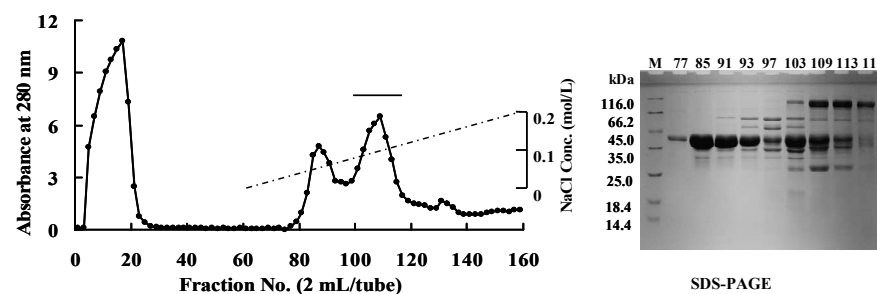
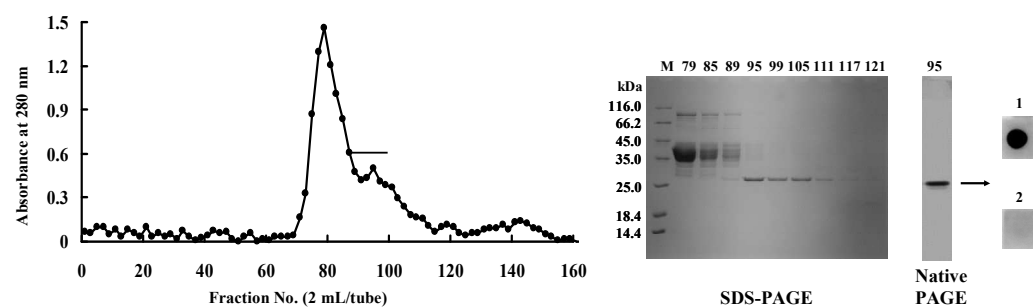
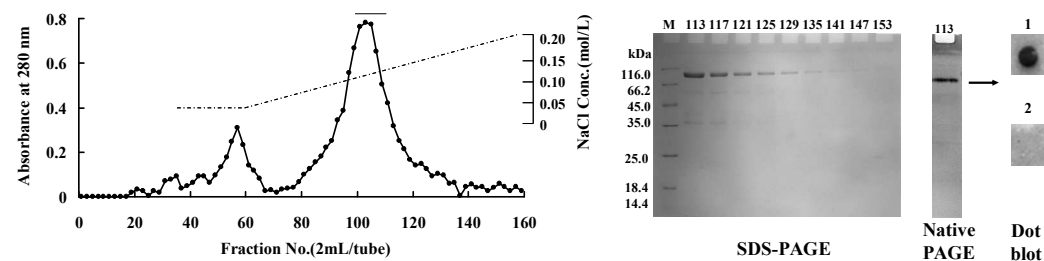
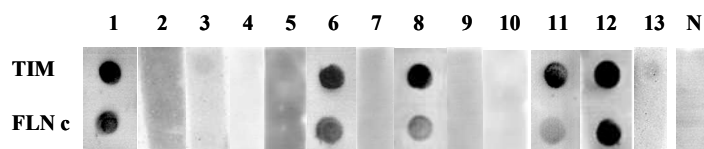
The boldface represents the key residue of a cluster. The light grey marked the co-epitopes

between TIM and FLN c. Conf. epitope, Conformational epitope.

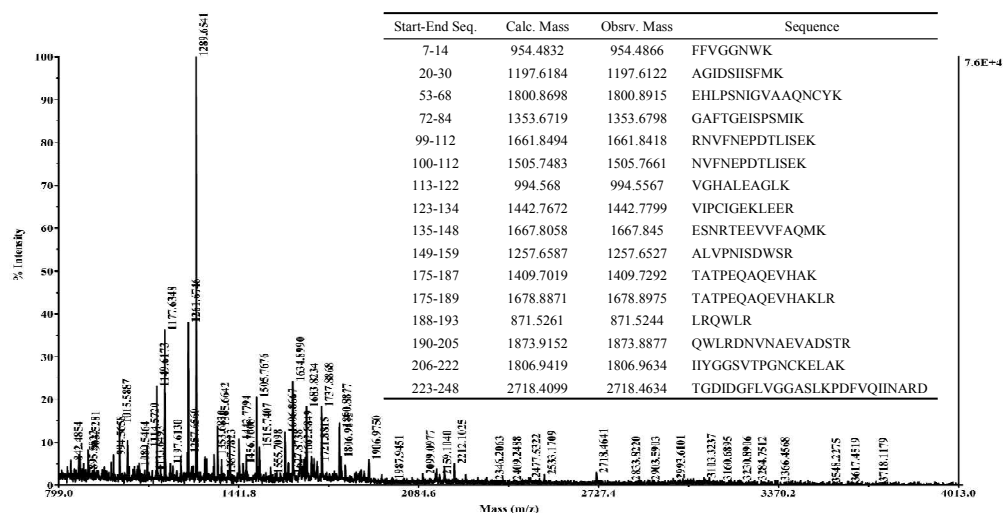
Table 5

Peptide No.	Amino acid residues and locations of epitope candidates	Sequence of synthesized peptides
1	L-TIM-1: D ₁₈ R ₁₉ A ₂₀ G ₂₁ I ₂₂ D ₂₃ S ₂₄ I ₂₅ I ₂₆ S ₂₇	NGDRAGIDSIISFM K
	L-TIM-4: V ₁₅₁ P ₁₅₂ N ₁₅₃ I ₁₅₄ S ₁₅₅ D ₁₅₆ W ₁₅₇ S ₁₅₈	
	L-FLN c-2: D ₅₁ S ₅₂ I ₅₃ A ₅₄ S ₅₅ G ₅₆ Y ₅₇ V ₅₈ T ₅₉	
2	L-FLN c-7: E ₅₆₇ D ₅₆₈ D ₅₆₉ C ₅₇₀ F ₅₇₁ I ₅₇₂ Q ₅₇₃ S ₅₇₄ I ₅₇₅ D ₅₇₆ S ₅₇₇ D ₅₇₈	FKDRKDGSCYVSY KV
	L-TIM-2: Y ₆₇ K ₆₈ V ₆₉ A ₇₀	
	L-FLN c-5: D ₄₇₉ R ₄₈₀ K ₄₈₁ D ₄₈₂ G ₄₈₃ S ₄₈₄ C ₄₈₅ Y ₄₈₆ V ₄₈₇ S ₄₈₈	
3	L-TIM-5: Y ₂₀₈ G ₂₀₉ G ₂₁₀ S ₂₁₁ V ₂₁₂ T ₂₁₃ P ₂₁₄ G ₂₁₅	RIIYGGSVTPGNCK E
	C-FLN c-1:	
	V ₁₀ I ₁₁ E ₁₂ D ₁₃ N ₁₄ D ₁₆ G ₁₇ T ₁₈ V ₁₉ S ₂₀ I ₂₁ N ₂₂ L ₃₃ I ₃₅ S ₄₅ P ₄₆ F ₄₇ F ₄₉ S ₆₇	
4	C-TIM-1:	PVWAIGTGKTATP EQ
	L ₁₃₁ E ₁₃₂ R ₁₃₄ E ₁₃₅ P ₁₆₆ V ₁₆₇ W ₁₆₈ A ₁₆₉ I ₁₇₀ G ₁₇₁ T ₁₇₂ G ₁₇₃ K ₁₇₄ T ₁₇₅ A ₁₇₆ T ₁₇₇ E ₁₇₉ Q ₁₈₀ E ₁₈₃ V ₁₈₄ G ₂₁₀ S ₂₁₁ T ₂₁₃	
	C-FLN c-8:	
	G ₆₂₇ N ₆₂₈ L ₆₃₀ T ₆₃₁ K ₆₃₂ K ₆₃₄ T ₆₃₇ T ₆₃₉ G ₆₄₉ A ₆₅₀ G ₆₅₁ T ₆₅₂ L ₆₅₃ A ₆₅₄ V ₆₅₅ Q ₆₅₆ I ₆₅₇ D ₆₅₈ V ₆₆₃ M ₆₆₅ D ₆₆₆ C ₆₆₇ T ₆₆₈ E ₆₆₉ Y ₆₇₄ T ₆₇₉ F ₆₈₇ V ₆₈₈ S ₆₈₉	

The boldface represents the key residue of a cluster. The light grey marked the co-epitopes between TIM and FLN c, and the dark grey marked the residues contribute to co-epitopes.

Figure 1**A****B****C****D****E**

A

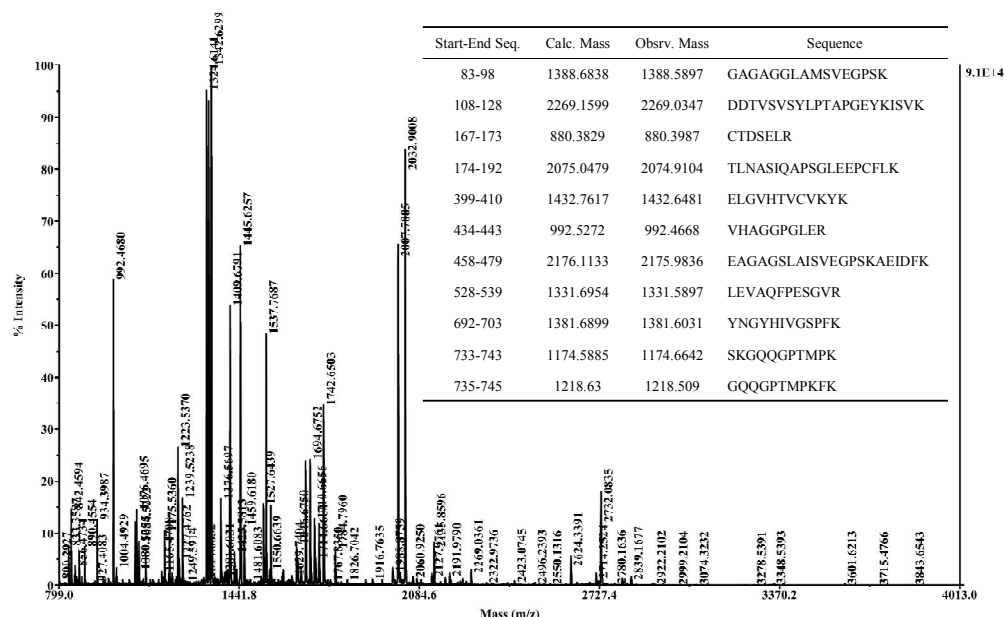


B

<i>Procambarus clarkii</i>	MANQRKFVGGNWKMMNGDKAAGIDSI	ISFMKGLPSADTEVVVGGPCQCYLMY	50		
<i>Archaeopotamobius sibiriensis</i>	GGNWKMMNGDRASIDSI	ISFMKGLPSGDEVVVVGGPCQCYLMY	41		
<i>Cherax quadricarinatus</i>	MSDHRKFFVGGNWKMMNGDKAJAIDA	VSFMKGLSPNTEVVVGGAPQCYLSY	50		
<i>Eriocheir sinensis</i>	MANQRKFFVGGNWKMMNGDKAJAIDGI	ITFMKGLSPDEVVVVGGPCQCYLMY	50		
<i>Crangon crangon</i>	MSGSRKFFVGGNWKMMNGDKAJAIDG	VFDMKGLPNPTEVVVGGPCQCYLSY	50		
purified protein	FFVGGNWK.....AGIDSI	ISFMK.....	19		
<i>Procambarus clarkii</i>	TREHLP	SNIGVAAQNCYKVKAGGAGTGEISPSMI	KDCGGEWVILGHSERRN	100	
<i>Archaeopotamobius sibiriensis</i>	TREHLP	SNIGVAAQNCYKVKAGGAGTGEISPSMI	KDCGGEWVILGHSERRN	91	
<i>Cherax quadricarinatus</i>	TREHIP	ANIGVAAQNCYKVKAGGAGTGEISPA	MIKDCGGEWVILGHSERRN	100	
<i>Eriocheir sinensis</i>	TREHP	ANIGVAAQNCYKVKAGGAGTGEISPA	MIKDCGGEWVILGHSERRN	100	
<i>Crangon crangon</i>	TREKLP	AEIGVAAQNCYKVKAGGAGTGEISPA	MVKDCGGEWVILGHSERRN	100	
purified protein	..EHL	PSNIGVAAQNCYK...GAGTGEISPSMI	K.....RN	50	
<i>Procambarus clarkii</i>	VFNEP	DTLISEKVGHAEAGLKVIPICIGEK	LEERESNRTTEVVFAQMKA	150	
<i>Archaeopotamobius sibiriensis</i>	VFNEP	DTLISEKVGHAEAGLKVIPICIGEK	LEERESNRTTEVVFAQMKA	141	
<i>Cherax quadricarinatus</i>	VFNEP	DQLISEKVGHAEAGLKVIPICIGEK	LEERESNRTTEVVFAQMKA	150	
<i>Eriocheir sinensis</i>	VFDEP	DQLISEKVGHAEAGLKVIPICIGEK	LDERESNRTTEVVFAQMKA	150	
<i>Crangon crangon</i>	VFNEP	DQLISEKVGHAEAGLKVIPICIGEK	LDERESNRTTEVVFAQMKA	150	
purified protein	VFNEP	DTLISEKVGHAEAGLKVIPICIGEK	LEERESNRTTEVVFAQMKA	100	
<i>Procambarus clarkii</i>	VPNIS	SDWSRVVIAIEPVWAI	GTGKTATPEQAQEVHAKLRQWL	RDNVNAEV	200
<i>Archaeopotamobius sibiriensis</i>	VPNIS	SDWSRVVIAIEPVWAI	GTGKTATPEQAQEVHAKLRQWL	RDNVNAEV	191
<i>Cherax quadricarinatus</i>	VPNIS	SDWSRVVIAIEPVWAI	GTGKTATPEQAQEVHAKLRQWL	RDNVNAEV	200
<i>Eriocheir sinensis</i>	VPNIS	SDWSRVVIAIEPVWAI	GTGKTASPEQAQDVHAKLRQWL	RDNVSPQV	200
<i>Crangon crangon</i>	VPNIS	SDWSRVVIAIEPVWAI	GTGKTASPEQAQDVHAKLRQWL	TENVSAEV	200
purified protein	VPNIS	SDWSR.....	TATPEQAQEVHAKLRQWL	RDNVNAEV	135
<i>Procambarus clarkii</i>	ADSTR	IYGGSVTPGNCKELAKTG	IDGFLVGGASLKP	DFVQIINAR	247
<i>Archaeopotamobius sibiriensis</i>	ADSTR	IYGGSVTPGNCKELAKTG	IDGFLVGG.....		224
<i>Cherax quadricarinatus</i>	ADSTR	IYGGSVTPGNQDLAKKG	IDGFLVGGASLKP	DFV.....	241
<i>Eriocheir sinensis</i>	ADPTR	IYGGSVSAGNCKELAKTG	IDGFLVGGASLKP	DFVQIINAR	247
<i>Crangon crangon</i>	AESCR	IYGGSVSPSNGAELAKMG	IDGFLVGGASLKP	DFVTIINAR	247
purified protein	ADSTR	IYGGSVTPGNCKELAKTG	IDGFLVGGASLKP	DFVQIINAR	182

Figure 3

A



B

<i>Pediculus humanus corporis</i> purified protein	MPSGKVDKPVIEDNRDGTVSINYPKKEEGLHELHIKYNGEHVQGSPPKFHVDSIASGYVT	60
<i>Pediculus humanus corporis</i> purified protein	AYGPGLSHGVCGEPSNFTIYTKGAGAGGLAMSVEGPSKAEISCHDNKDDTVSVSYLPTAP	120
<i>Pediculus humanus corporis</i> purified protein	GEYKISVKFGDKHIGSPYSAKITGEGRKRNIQSVGSCSEFSIPGKCTDSELRITLNASIQ	180
<i>Pediculus humanus corporis</i> purified protein	APSGLEPCFLKMPNGTLGISFTPREVGEHQVSVKRMGNHITNSPFKISVGAKEVGDAK	240
<i>Pediculus humanus corporis</i> purified protein	KVKVTGSLKEGVTHKDNVFTVDTKNAGYGLSLISIEGPSKAEISCKDNEDGTLKIAKYPT	300
<i>Pediculus humanus corporis</i> purified protein	EPGYYIVNLKFADHHVDGSPFTVKVSGEGSNRVREKIQRRQREAVPIIEVGSCKCLTFKMP	360
<i>Pediculus humanus corporis</i> purified protein	GITAFDLSSSVISPGGVIEDAEINEVEDGLYAVHFVPKELGVHTVCVKYKDIHIPGSPFQ	420
<i>Pediculus humanus corporis</i> purified protein	FTVGPLKDGGAHRVHAGGPGLERGEQGEACEFNWNTREAGAGSLAISVEGPSKAEIDFKD	480
<i>Pediculus humanus corporis</i> purified protein	RKDGSCTVSYKVGTPGEYRVGKFNDDQHIPDSPYKVIISPAMGDAHKLEVAQFPESGVRA	540
<i>Pediculus humanus corporis</i> purified protein	NHPSNFLVRKNGAKGELDCKIISPSGIEDDCFIQSIDSDTYSVRFMPRENGIHNIHCKFN	600
<i>Pediculus humanus corporis</i> purified protein	GVHIPGSPFRIKVGKEDADPAAVRATGNGLTKIKTGKTDFIIDTCNAGATLAVQIDGP	660
<i>Pediculus humanus corporis</i> purified protein	SKVMDCTEAEEGYKVRYTPLMHGDFVSIKYNHYHIVGSPFKVSCTGEDLAERGPQETS	720
<i>Pediculus humanus corporis</i> purified protein	SVVVETVHKVAKSKGQQGPTMPKFKSDASKVTSKGMGLKAYLGKQNNFTVNCGDAGNNI	780
<i>Pediculus humanus corporis</i> purified protein	LYVGVYGPKGPCEEVFIKHTGRNNYQVGYLVKERGDYLVIVKMGDDHIRGSPFKVD	836

Figure 4

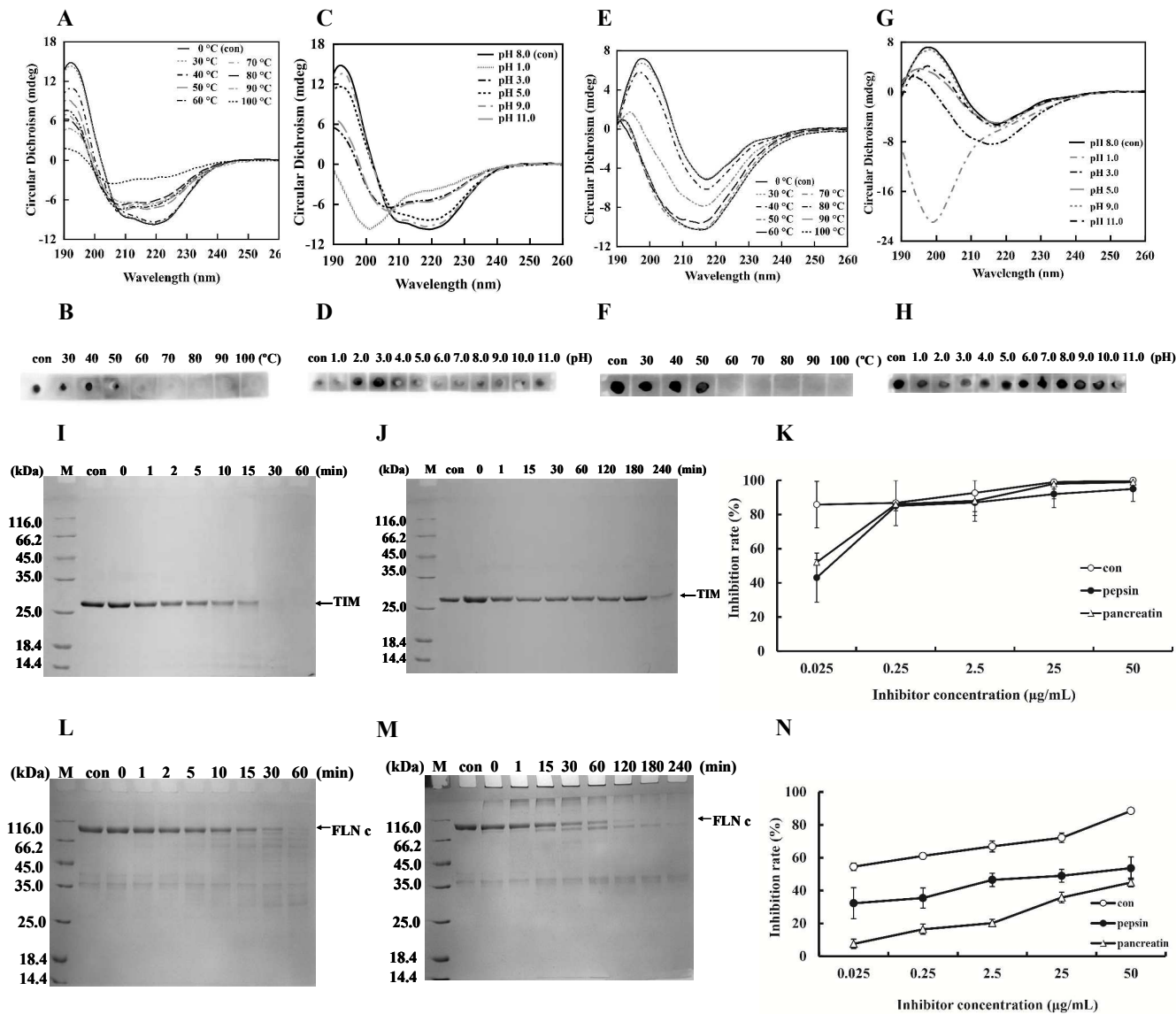


Figure 5

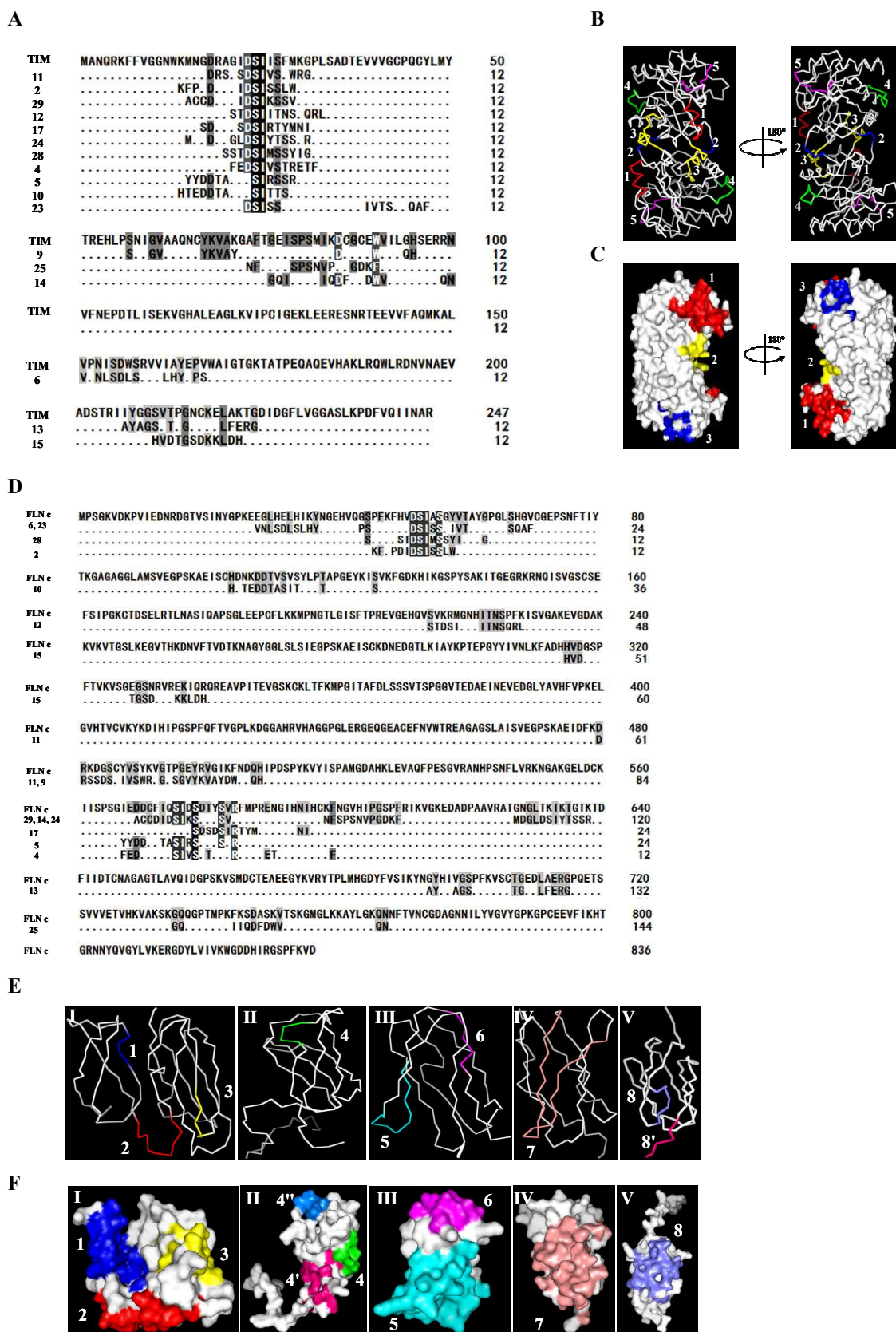


Figure 6

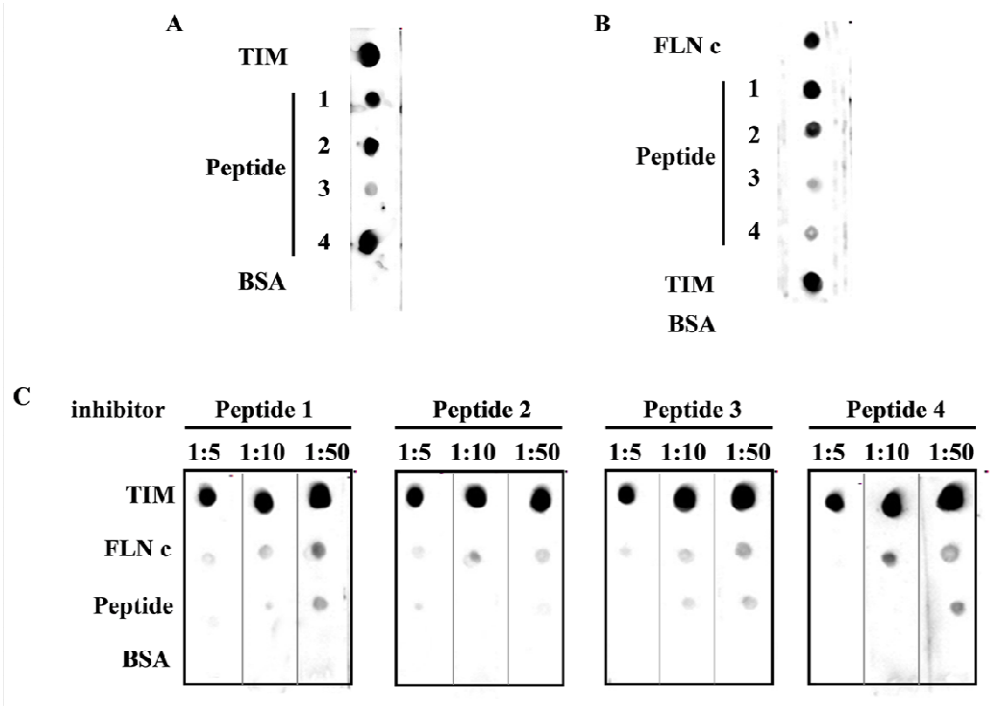
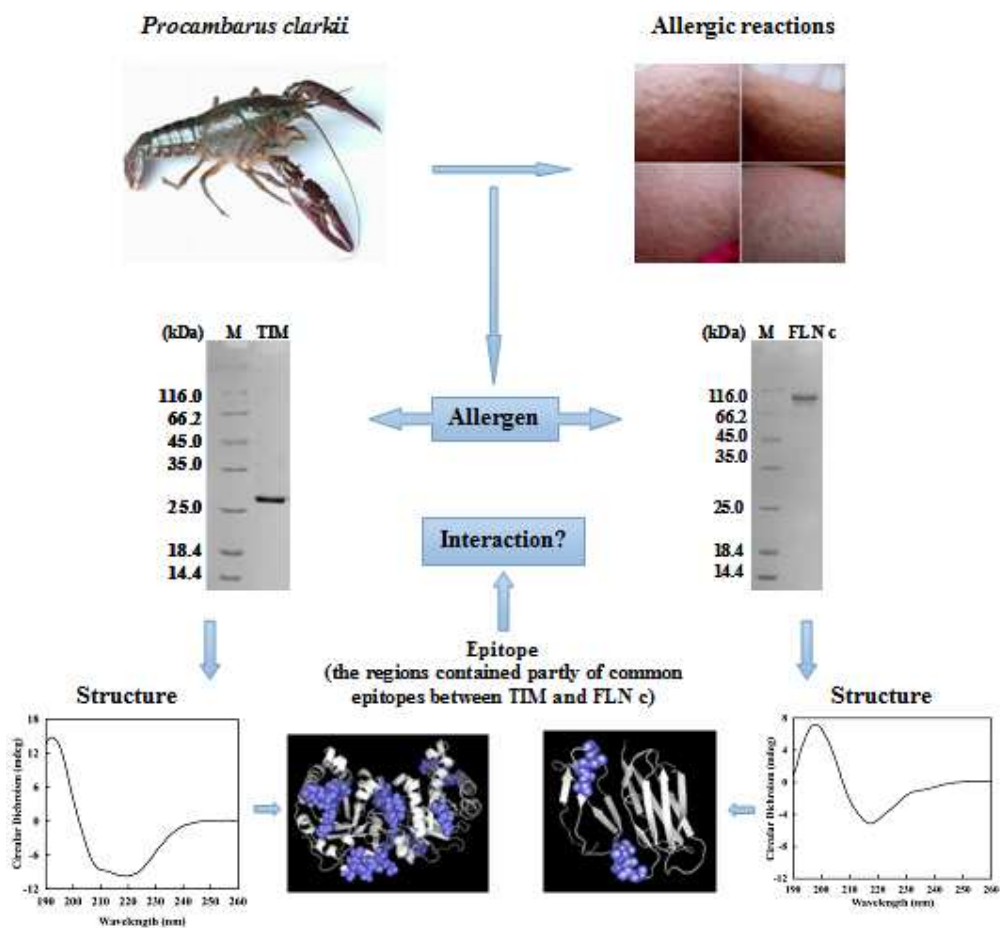
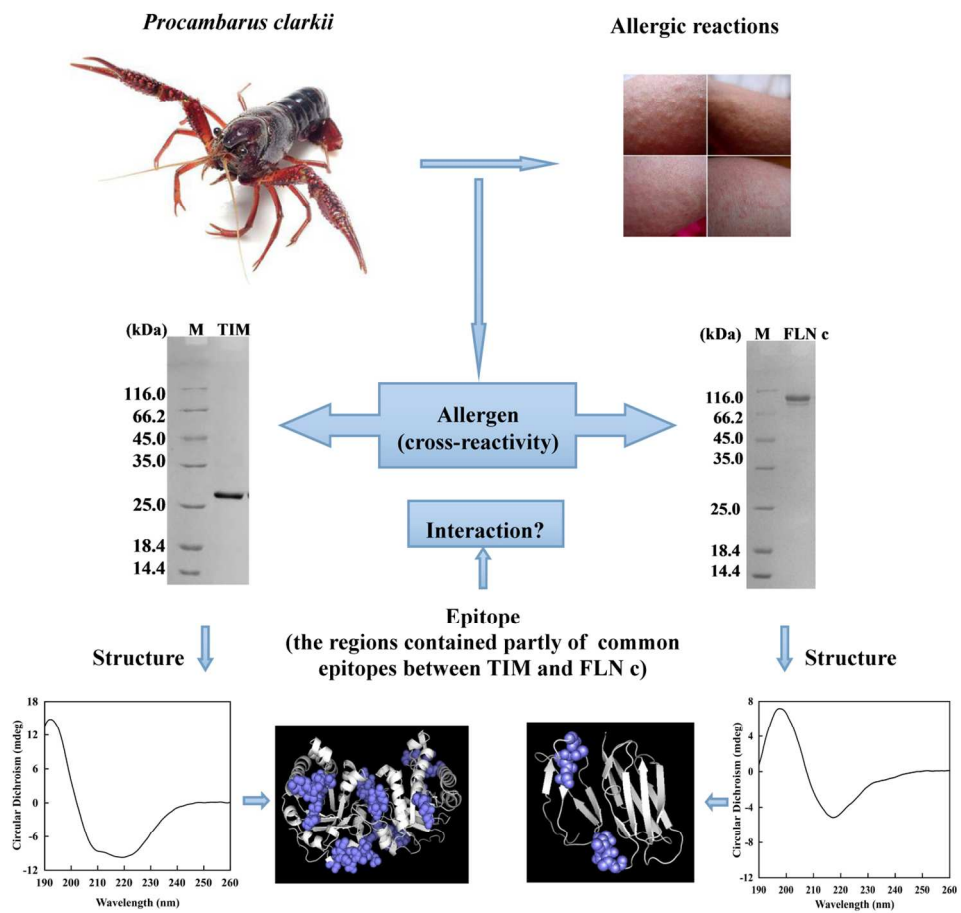


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188x176mm (200 x 200 DPI)