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**Purification, Characterization, and Analysis of the Allergenic
Properties of Myosin Light Chain in *Procambarus clarkii***

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ABSTRACT: Myosin light chain (MLC) plays a vital role in cell and muscle functions and has been identified as an allergen in shrimp. In this study, MLC with the molecular mass of 18 kDa was purified from crayfish (*Procambarus clarkii*) muscle. Its physicochemical characterization showed that the purified MLC is a glycoprotein with 4.3% carbohydrate, highly stable to heat, acid–alkali and digestion, and weakly retains IgE-binding activity when its secondary structure was altered. Serological assays suggested that conformational epitopes predominate over linear epitopes in the purified MLC. Two isoforms of the *MLC* gene (*MLC1* and *MLC2*) were cloned, and the purified MLC was identified as MLC1. Analysis of the secondary and tertiary structures of the MLCs indicated that MLC1 has four conformational epitopes and three linear epitopes, whereas MLC2 had a major conformational epitope and three linear epitopes. These results are significant for understanding hypersensitization of humans to crayfish.

KEYWORDS: *Procambarus clarkii*, myosin light chain, purification, physicochemical characterization, allergenicity, epitopes

INTRODUCTION

Seafood, including fish, crustaceans, and mollusks, is a widely consumed source of dietary protein because of its delicate flavor and high nutritive value. China, Japan, and Northern America are the three largest consumers of seafood, but the increasing consumption of seafood have been followed by increases in adverse reactions.¹ Most of these are mediated by immunoglobulin E (IgE) and generate mucocutaneous (95.7%), respiratory (23.9%), gastrointestinal (16.3%), anaphylactic (11.9%), and cardiovascular symptoms (3.3%).²

Procambarus clarkii, commonly known as the crayfish or freshwater lobster, is native to south-central United States and northeastern Mexico, and is one of the shellfish with the greatest commodity value worldwide. The crayfish is characterized by its high protein, low fat, low calorie, and high nutritional value, as well as its delicious taste and texture. Therefore, it is considered a popular food by consumers.^{3,4} The increased consumption of crayfish has been accompanied by more-frequent allergic reactions.² In China, *P. clarkii* is an important economic resource among freshwater shellfish, and the total production of crayfish in 2013 was about 603,520 tons, 8.78% higher than the 554,821 tons produced in 2012.⁵ A survey of 182 children suffering from urticaria in China reported that 58.79% of subjects had an allergic reaction to seafood, including crayfish, indicating that crayfish is an important allergenic food.⁶ Three well-characterized allergens in crayfish have been reported. The heat-stable myofibrillar protein tropomyosin (TM) is recognized as a major allergen of crayfish.⁷⁻⁹ Generally, TM is reported to be the pan-allergen of seafood, occurring in shrimp,^{10,11} lobster,⁷ crab,¹² and mollusks.¹³ Arginine kinase (AK), a myosinogen protein with a molecular mass of 40 kDa, is also a major allergen of crayfish,¹⁴ and has been identified as a major cross-reactive allergen in

58 mollusks and crustaceans.^{10,13} Another water-soluble allergic myosinogen protein discovered in
59 crayfish is sarcoplasmic calcium-binding protein (SCP), which consists of three subunits, and
60 occurs as three isoforms with molecular masses of 22 kDa, and all of its subunits and isoforms
61 are allergenic.⁶ SCP is also a shrimp allergen in the white leg Pacific shrimp (*Litopenaeus*
62 *vannamei*) and the black tiger prawn (*Penaeus monodon*).^{15,16} These three allergens share
63 similar physicochemical characteristics with most food allergens in that they are glycoproteins,
64 are relatively stable to heat and acid–alkali, and retain their immunological activity after
65 digestion.¹⁴ Several novel allergens of crustaceans have also been identified, such as
66 hemocyanin,¹⁷ triosephosphate isomerase,¹⁰ and troponin C.¹⁰

67 Myosin light chain (MLC) from *L. vannamei*, the North Sea shrimp (*Crangon crangon*),
68 and *P. monodon* is recognized as a novel shrimp allergen.^{10,18,19} Myosin, the most important
69 functional muscle protein, is a multimeric protein composed of two heavy chains of molecular
70 mass ~200 kDa and four light chains of molecular mass ~18–20 kDa.^{20,21} These proteins are
71 actin-binding molecular motors and play vital roles in a wide range of functions, including cell
72 adhesion, phagocytosis, muscle contraction, and cytokinesis. The light chains are categorized
73 into two classes: the essential light chain (MLC1) of molecular mass ~18 kDa and the
74 regulatory light chain (MLC2) of molecular mass ~20 kDa.^{20,22} It has been demonstrated that
75 MLC2 is phosphorylated by its kinase, which is regulated the expression of IgE, and is
76 reported to be increased in the smooth muscle of the allergen-sensitized human airway.²³ MLC
77 has been reported to have IgE-binding activity in *L. vannamei*, *C. crangon*, and *P. monodon*,
78 and the immunological effects of recombinant MLC have been analyzed in these organisms.
79 However, it is unclear whether MLC is the major allergen in *P. clarkii*; nor has native MLC

been characterized or the isoform identified.

The objectives of this study were to identify and purify MLC from *P. clarkii* and to analyze its physicochemical and immunological characteristics during changes in its secondary structure. We were able to clone the cDNA encoding the MLC proteins and to identify the allergenic MLC isoform. A homology model of the MLC structure was developed and used to predict its epitopes. This data will extend our understanding of MLC, and provide valuable references for further research into the molecular nature of hypersensitivity to crayfish.

MATERIALS AND METHODS

Crayfish. Crayfish (*P. clarkii*) was purchased from a local market in Xiamen, China. Crayfish was peeled, and the muscle samples were collected and immediately used in the experiments or frozen at -70°C for later analysis.

Chemicals. Diethylaminoethyl–Sephacel (DEAE–Sephacel) and Sephacryl S-200 HR were from Amersham Biosciences (Uppsala, Sweden). The protein standards for sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) were from Fermentas (Vilnius, Lithuania), and the prestained protein standards for Western blot analysis were from New England BioLabs (Beverly, Massachusetts, USA). Horseradish peroxidase (HRP)-conjugated goat anti-human IgE antibody was from Kirkegaard and Perry Laboratories (Gaithersburg, Maryland, USA), and the enhanced chemiluminescence (ECL) substrate for Western blot analysis was from Pierce (Rockford, Illinois, USA). 3,3',5,5'-Tetramethylbenzidine (TMB) was from Tiangen (Beijing, China). Ponceau S Concentrate and Porcine pancreatic enzymes were

102 purchased from Sigma–Aldrich (St. Louis, Missouri, USA). Porcine pepsin was prepared in
103 our laboratory. All reagents were of analytical grade.

104

105 **Human Sera.** Sera were collected from seven crustacean-allergic patients (No. 749, 514,
106 974, 067, 785, 980, and 791), who were referred by the Medical Center of Jimei University in
107 Xiamen, China. Serum samples from non-allergic (NA) individuals (No. 780 and 981) were
108 used as the negative controls. All patients and NA individuals voluntarily provided their sera
109 (No. U245749, U394514, T800974, U246067, U390785, U405980, U393791, U397780 and
110 V490981). Written informed consent was obtained from each patient. The IgE level in each
111 serum was measured with an ImmunoCAP assay (Phadia AB, Uppsala, Sweden), and the
112 results are shown in Table 1. An ImmunoCAP score > 0.35 kUA/L was considered positive.
113 All sera were stored at –30 °C until analysis.

114

115 **Purification of the Target Protein.** The preliminary treatment was carried out as described
116 previously,²⁴ with slight modifications. All procedures were performed at 0–4 °C unless
117 otherwise stated. The crayfish muscle tissue was minced and homogenized with 10 volumes
118 (v/w) of ice-cold 50 mM NaCl buffer containing 2 mM NaHCO₃ and 10 mM EDTA. The
119 homogenate was then centrifuged at 8,000 × g for 10 min at 4 °C. The precipitate was
120 resuspended in the same solution, and then homogenized and centrifuged again four more
121 times. The last precipitate, which contained the myofibrillar proteins, was washed three times
122 with cold absolute acetone, and dried completely. The dried powder was extracted overnight
123 with 10 volumes (v/w) of 20 mM Tris-HCl (pH 7.5) containing 1 M NaCl and 1 mM EDTA.

124 The mixture was then centrifuged at $12,000 \times g$ for 20 min at 4 °C. The supernatant was
125 subjected to isoelectric precipitation at pH 4.0 with 1 N HCl. After centrifugation at $12,000 \times g$
126 for 20 min at 4 °C, the precipitate was dissolved with 20 mM Tris-HCl (pH 7.5) and brought to
127 pH 7.6 with 1 N NaOH, and then subjected to ammonium sulfate fractionation with 41%–60%
128 saturated ammonium sulfate. Following centrifugation at $12,000 \times g$ for 20 min at 4 °C, the
129 precipitate was dissolved in 20 mM Tris-HCl (pH 7.5) containing 1 mM EDTA, and then
130 dialyzed in the same solution overnight. After centrifugation at $12,000 \times g$ for 20 min at 4 °C,
131 the supernatant was applied to a DEAE-Sephacel column (2.5×10 cm) and eluted with a linear
132 gradient of 0–0.5 M NaCl at a flow rate of 0.6 mL/min. The fractions containing the target
133 protein were concentrated by the ultrafiltration of a 3-kDa filtering membrane and then applied
134 to a Sephacryl S-200 HR gel column (1.5×100 cm) equilibrated with 20 mM Tris-HCl (pH 7.5)
135 containing 0.2 M NaCl. The column was eluted at a flow rate of 0.5 mL/min and the fractions
136 containing the target protein were collected and detected on a 12% SDS–PAGE gel stained
137 with Coomassie Brilliant Blue R-250 (CBB). The protein concentrations in the eluted solutions
138 were estimated by measuring the absorbance at 280 nm.

139

140 **Peptide Analysis by Matrix-assisted Laser Desorption/ionization Time-of-flight Mass**
141 **Spectrometry (MALDI – TOF – MS).** After the 18-kDa target protein had migrated on the
142 12% SDS–PAGE gel and was stained with CBB, the band was cut from the gel and sent to
143 Shanghai Applied Protein Technology Company Limited (Shanghai, China) to digest with
144 trypsin for analysis with a 4800 Plus MALDI TOF/TOF™ Analyzer from Applied Biosystems
145 (Foster City, California, USA), based on the method of Yan et al.²⁵. The MS conditions were as

146 follows: laser source, ND; wavelength, 355 nm; accelerating voltage, 2 kV; and substrate,
147 α -cyano-4-hydroxycinnamic acid. The positive ion mode was used and the data were obtained
148 automatically. The resulting peptide spectra were compared with those in the National Center
149 for Biotechnology Information (NCBI) database.

150

151 **Carbohydrate Content and Carbohydrate–peptide Linkage Characterization.** The total
152 carbohydrate content of 18-kDa target protein was determined with the phenol–sulfuric acid
153 method,²⁶ with some modifications. In brief, 0.3 mL of 6% phenol in water was added to the
154 sample solution (0.5 mL) in a test tube, and 1.5 mL of concentrated sulfuric acid was added
155 slowly down the side of the tube. The tube was then vortexed, covered, and incubated for 20
156 min at 100 °C. After all the tubes were cooled to room temperature, the absorbance of each was
157 read at 490 nm. All the experiments were performed in duplicate. The β -elimination was used
158 to determine the carbohydrate–peptide linkages in the target protein, according to a previously
159 described method.⁶ The target protein sample (0.3 mg/mL, 90 μ L) was mixed with 10 μ L of 0.5
160 M NaOH and incubated at 37 °C for 16 h, after which the absorbance at 220–300 nm was
161 measured. The target protein sample was replaced with the same volume of distilled water, as
162 the blank, and NaOH was replaced with the same volume of distilled water, as the negative
163 control. All experiments were performed in duplicate.

164

165 **Thermal Stability and pH Stability.** To determine the thermal stability of the 18-kDa
166 target protein, it was incubated in buffer (10 mM Tris-HCl, pH 7.5) at different temperatures
167 (30, 40, 50, 60, 70, 80, 90, and 100 °C) for 30 min. The protein was incubated at 0 °C as

168 control. To test its pH stability, the target protein was incubated for 1 h at room temperature in
169 buffer at different pH (1.0, 2.0, 3.0, 4.0, 5.0, 7.0, 9.0, and 11.0). The protein was incubated at
170 pH 7.5 as control. Then all the samples were analyzed with 15% SDS–PAGE and Dot blot.

171

172 **Stability during Simulated Digestion.** The stability of the target protein *in vitro* in
173 simulated gastric fluid (SGF, pepsin) and simulated intestinal fluid (SIF, pancreatin) was
174 assessed with the method of Chen et al.,¹⁴ with some modifications. The total volume of the
175 reaction solution was 1 mL. For gastric digestion, the ratio of pepsin to the target protein was
176 1:500 (w/w). An aliquot of 100 μ L of the reaction solution was removed after different time
177 intervals (0, 1, 2, 5, 10, 15, 30, or 60 min) and the reaction immediately terminated by the
178 addition of 30 μ L of 200 mM Na₂CO₃. For intestinal digestion, the ratio of pancreatin to the
179 target protein was 1:500 (w/w). The reactions were performed for 0, 1, 15, 30, 60, 120, 180, or
180 240 min, after which they were immediately terminated by heating at 95 °C for 5 min. A target
181 protein sample, dissolved in SGF/SIF with no corresponding enzyme, was used as the
182 undigested control. The final concentration of each sample was adjusted to 0.25 mg/mL. The
183 samples were analyzed with 15% SDS–PAGE and a competitive inhibition enzyme-linked
184 immunosorbent assay (ELISA).

185

186 **Western blot and Dot blot Analysis.** Western blot analysis was performed as described by
187 Chen et al.,¹⁴ with some modifications. Briefly, protein samples separated with SDS–PAGE
188 were electrophoretically transferred to nitrocellulose membrane and then the transfer efficiency
189 was determined by Ponceau S staining of the membrane. The membrane was washed with

190 TBST (20 mM Tris-HCl, pH 8.0, containing 0.15 M NaCl and 0.05% Tween-20), and blocked
191 with 5% skim milk in TBST. They were then incubated for 6 h at room temperature with
192 human serum (diluted 1:4) as the primary antibody and washed with TBST. The membrane was
193 then incubated for 1.5 h with HRP-labeled goat anti-human IgE antibody (diluted 1:20,000) as
194 the secondary antibody. After extensive washed with TBST, the proteins were visualized with
195 ECL. In the Dot blot analysis, the samples were blotted onto nitrocellulose membrane, blocked,
196 incubated with sera from patients (diluted 1:4) and HRP-labeled goat anti-human IgE antibody
197 (diluted 1:20,000) as described above. The signal was detected with ECL.

198

199 **Indirect and Competitive Inhibition ELISA (ciELISA).** Each well of a polystyrene
200 96-well ELISA plate (Nunc Maxisorb, Denmark) was coated with the 18-kDa target protein
201 (0.2 µg) and incubated at 4 °C overnight or at 37 °C for 2 h. The coated plates were washed
202 with TBST and blocked with 200 µL/well 5% skim milk at 37 °C for 2 h. After washing and
203 blocking, the coated plates were incubated at 37 °C for 2.5 h with 50 µL of patient serum
204 (diluted 1:5 with 1% skim milk in TBST) as the primary antibody, and then at 37 °C for 1.5 h
205 with HRP-conjugated goat anti-human IgE (diluted 1:2000) as the secondary antibody. TMB
206 (100 µL) was then added to each well and incubated at 37 °C for 20 min. After the reaction was
207 terminated by the addition of 50 µL of 2 M sulfuric acid, the absorbance was measured at 450
208 nm with an automated ELISA plate reader (Benchmark 96; Bio-Rad Laboratories, Hercules,
209 California, USA). The specific IgE-binding activity of the purified protein-derived digestion
210 fragments was confirmed with a ciELISA, based on the method of Li et al..²⁷ In contrast to
211 indirect ELISA, after the plates were blocked with 5% skim milk, 50 µL of human sera (diluted

1:2 with 1% skim milk in TBST) was mixed with the same volume of inhibitor sample (the purified protein-derived digestion fragments), in the concentration range of 0.025–50 µg/mL in another plate. After incubation at 37 °C for 1.5 h, 50 µL of the mixture (serum plus inhibitor) was transferred to the ELISA plate pre-coated with the purified protein as the allergen and incubated for 1.5 h at 37 °C. As above, after incubation with the secondary antibody and reaction with TMB, the absorbance at 450 nm was measured. The inhibition rate was calculated to evaluate the percentage of inhibition by each inhibitor sample, using the following formula:

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

Where X is the absorbance of the patient's serum not incubated with the inhibitor, and Y and Z are the absorbance of allergic patient's serum and control NA serum reacted with the inhibitor, respectively. All the samples were tested in duplicate and the mean values calculated.

Circular Dichroism (CD) Spectroscopy. Far-UV CD spectra were measured with a Chirascan™ circular dichroism spectrometer (Applied Photophysics Ltd, Surrey, UK). The measurements of purified protein in buffer (10 mM Tris-HCl, pH 7.5) were made at 25 °C using a rectangular quartz cuvette (Starna, Essex, UK) with a path length of 0.1 cm at a protein concentration of 0.12 mg/mL. The purified protein was treated at different temperatures (0 °C and 100 °C) for 30 min or at room temperature in different pH buffers (pH 1.0, 7.5, and 11.0) for 1 h. The samples were scanned three times at 1 nm/s, at a band width of 1 nm. The response time was 2 s, and the wavelength range was 190–260 nm. The data were averaged and the spectrum of the sample-free control was subtracted. The secondary structures of the proteins

234 were determined with the CDNN program (version 2.0) based on the variables of the amino
235 acid sequence and the molecular weight of the target protein.²⁸

236

237 **Cloning and Sequencing the MLC Genes.** Total RNA was prepared from crayfish (*P.*
238 *clarkii*) muscle based on the TRIzol method.²⁹ The first-strand cDNA was synthesized with the
239 TIANScript RT Kit (Tiangen, Beijing, China), according to the manufacturer's instructions,
240 and oligo(dT)₁₈ primers were designed according to the manufacturer's instructions. Based on
241 the MALDI-TOF-MS results and the alignment of the MLC cDNA sequences of *C. crangon*
242 and *P. monodon*, degenerate oligonucleotide primers were designed as the sense primers for
243 PCR. Using these primers and the synthesized cDNA, a 300-bp fragment of the *MLC* gene was
244 amplified by PCR in the GeneAmp 9700 thermal cycler (Applied Biosystems, Foster City,
245 California, USA). The PCR thermal cycling program was: 5 min at 94 °C, followed by 30
246 cycles of 45 s at 94 °C, 30 s at 57 °C, and 45 s at 72 °C, and a final extension at 72 °C for 7
247 min. The PCR product was purified by agarose gel electrophoresis and cloned into the
248 pGEM-T Easy vector (Promega), and the DNA sequence analyzed. 5'-RACE primers and
249 3'-RACE primers were designed based on the sequence information from the RT-PCR product.
250 RACE and RACE-PCR were performed with the SMART RACE Amplification Kit (Clontech,
251 Palo Alto, California, USA). The PCR amplification product was purified, cloned, and
252 sequenced. The full-length gene sequences of the *MLCs* (*MLC1*, 1096 bp; *MLC2*, 1362 bp)
253 were obtained by overlapping the RT-PCR fragment, the 3'-RACE fragment, and the 5'-RACE
254 fragment. DNA nucleotide sequencing was performed with the ABI PRISM 3730 DNA
255 Analyzer (Applied Biosystems, Foster City, California, USA), and the sequences were

256 compared with the nucleotide sequences in the GenBank database. The sequences were
257 submitted to GenBank under accession numbers JX173714 (gi|401606250) for *P. clarkii* *MLC1*
258 cDNA and JX013934 (gi|392499120) for *P. clarkii* *MLC2* cDNA.

259

260 **Structural Modeling and Epitope Prediction.** The three-dimensional structure of the target
261 protein was simulated at the website <http://swissmodel.expasy.org/> based on the homology
262 modeling by the amino acid sequence of the target protein to search the suitable template. The
263 continuous linear epitopes were predicted by the DNASTAR Protean software using the
264 method of Kyte-Doolittle for hydrophilicity plot prediction, the method of Karplus-Schulz for
265 flexible regions prediction, the method of Jameson-Wolf for antigenic index prediction, the
266 method of Emini for surface probability plot prediction. The peptide fragments were selected to
267 satisfy the conditions: i) highly hydrophilic; ii) appropriate antigenic index; and iii) situated in
268 a flexible and surface-exposed region. Discontinuous conformational epitopes were predicted
269 at the DiscoTope 2.0 Server (<http://www.cbs.dtu.dk/services/DiscoTope/>) by the calculation of
270 surface accessibility, a novel epitope propensity and the propensity of residues in spatial
271 proximity. The three-dimensional structure and epitopes were represented on the molecular
272 surface of the protein with PyMOL.

273

274 RESULTS

275 **Purification of the 18-kDa Protein.** The 18-kDa protein was purified from crayfish muscle
276 with ammonium sulfate precipitation and sequential column chromatography on
277 DEAE-Sephacel and Sephacryl S-200 HR. The fractions from the DEAE-Sephacel column

278 were eluted with a linear gradient of 0–0.5 M NaCl (Fig. 1A). The fractions that contained
279 relatively high concentrations of the 18-kDa target protein were then pooled, concentrated, and
280 separated on a Sephacryl S-200 HR gel-filtration column, which removed the contaminating
281 proteins. After Sephacryl S-200 HR chromatography, the highly purified protein migrated on
282 SDS–PAGE as a single band with a molecular weight of about 18 kDa (Fig. 1B).
283 Approximately 5.6 mg of the purified protein was obtained from 140 g of crayfish muscle.

284

285 **Identification by MALDI–TOF–MS.** The purified 18-kDa protein band was excised from
286 the SDS–PAGE gel, destained, and digested in-gel with trypsin. The resulting peptides were
287 analyzed with MALDI–TOF–MS. Peptide mass fingerprinting of the purified protein showed
288 multiple peaks ranging from 800 to 4000 Da. The peaks with signal-to-noise ratios > 50 were
289 analyzed with MS/MS, as shown in Fig. 2A. The effective peaks were compared with the
290 NCBI database using the Mascot 2.2 search engine. One peptide fragment containing 13
291 amino acid residues was 100% identical to MLC of *Drosophila yakuba* (gi|195503715) and
292 92.3% identical to that of *C. crangon* (gi|238477331) and *Marsupenaeus japonicas*
293 (gi|290908988) (Fig. 2B). These results confirmed the purified 18-kDa protein as MLC.

294

295 **Glycoprotein Characterization.** The results of the phenol–sulfuric acid analysis indicated
296 that the total carbohydrates in MLC accounted for about $4.3 \pm 0.05\%$ (w/w) of the whole protein.
297 The β -elimination was used to determine the presence of O-linked glycans in the MLC
298 structure. As shown in Fig. 3A, when MLC was treated with alkali, the UV absorbance at 240
299 nm increased significantly compared with the absorbance before alkali treatment. This result

300 indicates that one or more O-glycans may be present in the structure of MLC.

301

302 **Thermal and pH Stability.** Thermal and pH stabilities are accepted characteristics of most
303 allergens. The thermal stability of purified MLC was analyzed with SDS–PAGE and Dot blot.
304 As illustrated in Fig. 3B, MLC was stable after exposure to different temperatures, even up to
305 100 °C. MLC IgE-binding activity at different temperatures was determined with Dot blot
306 using the patients' sera. The IgE-binding activity of crayfish MLC was detected in this assay,
307 and did not increase or decrease obviously in the temperature range of 30–100 °C (Fig. 3C).

308 The pH stability of purified crayfish MLC was also examined with SDS–PAGE and Dot blot.
309 As illustrated in Fig. 3D, MLC remained stable when exposed to acidic (pH 1.0–5.0) and
310 alkaline (pH 9.0, 11.0) conditions. Dot blot was also used to analyze the IgE-binding activity of
311 MLC. Compared with the control, no obvious change was detected in the IgE-binding activity
312 of MLC after exposure to different pHs (Fig. 3E).

313

314 **Stability during Digestion.** It was determined whether MLC was stable under *in vitro*
315 conditions simulating human gastrointestinal digestion. The stability of purified MLC in pepsin
316 digestion was analyzed with SDS–PAGE. The original band (18 kDa) was gradually digested
317 over time. Proteolytic fragments smaller than 14.4 kDa appeared after prolonged peptic
318 digestion. The original band was digested into fragments smaller than can be resolved by
319 SDS-PAGE by pepsin within 1 h (Fig. 3F). In contrast, a single proteolytic fragment smaller
320 than 14 kDa was observed after the pancreatin digestion of MLC. The ~14.4-kDa fragment
321 appeared after 15 min and remained undigested up to 240 minutes (Fig. 3G). Competitive

inhibition ELISA, was used to analyze the IgE-binding activity of the MLC-derived digestion fragments. As shown in Fig. 3H, the IgE-binding activity of the digested fragments was reduced by pancreatin digestion but not significantly with pepsin digestion. When the concentration of the inhibitor was 2.5 $\mu\text{g/mL}$, the inhibition rate of the pepsin digestion fragments was 80%, whereas that of the undigested MLC was 85%, and the inhibition rate of the pancreatin digestion fragments was only 57%. The pepsin digestion fragments at a concentration of 0.095 $\mu\text{g/mL}$ were capable of binding 50% of the IgE epitopes bound by undigested MLC at 0.075 $\mu\text{g/mL}$, whereas the pancreatic digestion fragments had a 50% inhibition rate at concentrations up to 0.224 $\mu\text{g/mL}$. This indicates that pepsin digestion had little effect on the IgE-binding activity of MLC. However, the IgE-binding activity of MLC that had been digested by pancreatin over a period of 4 h was reduced compared with that of the undigested MLC sample.

Secondary Structure of Purified MLC. Far-UV CD spectra were examined to confirm the predicted structure of purified MLC and to evaluate the effects of different physicochemical conditions on the protein's secondary structure. As shown in Fig. 4, the control curves (0 $^{\circ}\text{C}$ and pH 7.5) displayed two minima, around 222 and 208 nm, and one maximum around 190 nm, which are typical of α -helical secondary structures, although there was no fully-canonical shapes reflect the presence of other secondary structures, such as the extended strands, the β -turns and/or the random coils. At 0 $^{\circ}\text{C}$, the secondary structure was 33.5% α -helices, 16.2% extended strands, and 34.2% random coils. However, high temperature treatments had a noticeable effect on the secondary structure, significantly increasing the extended strands

(23.5%), which are characterized by a maximum at around 195 nm, and partly increasing the β -turns and/or the random coils (Fig. 4A). Extended strands (25.6%) and random coils (39.3%) were also markedly increased after strong acid treatment, whereas strong alkali did not significantly increase the extended strands (19.2%) (Fig. 4B). Among the different treatments tested, the secondary structure of purified crayfish MLC was most effectively altered by high temperature and strong acid.

350

Determination of the Predominant Epitope Type of Purified MLC. The IgE-binding activity of the purified crayfish MLC in folded form (with indirect ELISA and Dot blot analysis, Figs 5A and 5B) was compared with MLC in its denatured, linear form (Western blot analysis, Fig. 5C), with seven individual serum samples (No. 749, 514, 974, 067, 785, 980, and 791) from crustacean-allergic patients and two sera from NA individuals that were pooled (No. 780 and 981). With indirect ELISA (Fig. 5A), the IgE from each of the seven sera bound to the native folded MLC, at least two-fold higher than that of the negative control sera. In the Dot blot analysis, all seven sera reacted with the native folded MLC, but no IgE-binding activity was detected in the negative control sera (Fig. 5B). However, as shown in Fig. 5C, only three sera (No. 067, 785, and 791) reacted weakly to the denatured, linear MLC in the Western blot analysis. This indicates that the native folded MLC has higher IgE-binding activity than the denatured linear MLC.

363

Cloning and Sequencing MLC1. To identify the isoform of the purified MLC and to predict of the MLC structures, the *MLC1* gene was cloned. As shown in Fig. 6A, the

366 full-length gene sequence of crayfish *MLC1* is 1096 bp, including the ATG start codon at
367 nucleotides (nt) 63–65, the TAA stop codon at nt 523–525, and the poly-A tail. The open
368 reading frame of crayfish *MLC1* is 462 bp and the predicted protein consisted of 153 amino
369 acid residues, with a predicted molecular weight of 17.59 kDa by DNASTAR EditSeq software,
370 which is equivalent to the ~18-kDa molecular weight estimated with SDS–PAGE (Fig. 1B).
371 The protein has a predicted isoelectric point of 4.58 by DNASTAR EditSeq software. There are
372 two potential N-glycosylation sites in the amino acid sequence of crayfish MLC1 (marked with
373 a box in Fig. 6A), which were identified by the conserved motif Asn–X–Ser/Thr/Cys. A
374 protein sequence alignment showed that the MLC proteins from different crustacean species
375 share high homology (Fig. 6B). The amino acid sequence of crayfish MLC1 was 92.81%
376 identical to the amino acid sequence of *C. crangon* MLC (gi|238477331) and *M. japonicus*
377 MLC (gi|290908988), 84.97% to *Palaemon varians* MLC (gi|238683675), 64.05% to
378 *Periplaneta americana* MLC (gi|359326577), and 53.80% to *Artemia franciscana* MLC
379 (gi|152013723).

380

381 **Cloning and Sequencing MLC2.** The *MLC2* gene was also cloned to distinguish between
382 the isoforms of MLC. The full-length gene sequence of crayfish *MLC2* is 1362 bp, including
383 the ATG start codon at nt 63–65, the TAA stop codon at nt 589–591, and the poly-A tail (Fig.
384 6C). The open reading frame of crayfish *MLC2* is 528 bp and the predicted protein consists of
385 175 amino acid residues, with a predicted molecular weight of 19.07 kDa and a predicted
386 isoelectric point of 4.21 by DNASTAR EditSeq software. There is only one potential
387 N-glycosylation site in the amino acid sequence of crayfish MLC2 (marked with a box in Fig.

388 6C). A protein sequence alignment showed that the sequence homology of MLC1 and MLC2 is
389 only 14.2%, whereas the MLC2 proteins of different crustacean species share relatively high
390 homology (Fig. 6D). The amino acid sequence of crayfish MLC2 is 62.92% identical to the
391 amino acid sequence of *P. monodon* MLC2 (gi|357640499) and *L. vannamei* MLC
392 (gi|184198734).

393

394 **Structural Modeling and Epitope Prediction in Crayfish MLC.** The three-dimensional
395 structure of crayfish MLC1 was determined based on its amino acid sequence by a homology
396 model (Template: 4QBD, 56.95% identity, Fig. 7A). Crayfish MLC1 is mainly composed of
397 α -helices (54.25%) and random coils (22.22%), with a few β -turns or extended strands
398 analyzed by SOPMA secondary structure prediction. This is roughly consistent with the results
399 of the CD spectral analysis. The conformational epitopes were identified with the DiscoTope
400 2.0 Server, and the most likely regions included 10 discontinuous amino acid residues (25,
401 57–60, 96–99, and 136). The 10 predicted discontinuous epitope residues were molecularly
402 mapped onto the three-dimensional structure of crayfish MLC1, and formed four
403 conformational epitopes (labeled with slate in Fig. 7B). The linear (continuous) epitopes of
404 crayfish MLC1 were predicted with the DNASTAR Protean software based on its secondary
405 structure. Three continuous regions (amino acid residues 53–58, 76–83, and 95–99) of linear
406 epitopes occurred in the most hydrophilic, flexible, and surface-exposed regions, and were
407 shown in blue on the model of crayfish MLC1 (Fig. 7C). Crayfish MLC2 is also mainly
408 composed of α -helices (49.14%) and random coils (32.57%), with a few β -turns or extended
409 strands. The most likely regions of conformational epitopes were 47 discontinuous residues

(amino acid residues 31–32, 34–36, 38–39, 41–47, 49–71, 73–79, and 81–83), which composed a major conformational epitope. Three continuous regions (amino acid residues 5–11, 92–99, and 169–172) of linear epitopes were predicted (data not shown).

DISCUSSION

The growing international trade in crayfish has increased its popularity and the frequency of its consumption, which has been accompanied by an increase in the incidence of allergic diseases.^{1,2} MLC was identified as a novel allergen in the white leg Pacific shrimp (*L. vannamei*),¹⁸ the North Sea Shrimp (*C. crangon*),¹⁰ and the black tiger prawn (*P. monodon*).¹⁹ A previous study of crayfish TM demonstrated that an undefined myofibrillar protein, with a molecular mass about 18 kDa, had IgE-binding activity in the sera from crustacean-allergic patients.⁹ It was inferred to be a new allergen in *P. clarkii*, although it is unclear whether this *P. clarkii* protein was MLC, and if so, which isoform it might be. This study was undertaken to determine whether this 18-kDa protein was MLC.

Few studies have purified native MLC for further analysis. In this study, the 18-kDa protein was purified by a combination of isoelectric precipitation, ammonium sulfate precipitation, ion exchange and size exclusion chromatography, and identified as MLC with MALDI–TOF–MS. The MLC content in muscle tissue is very low compared with the content of crayfish AK.¹⁴ Only 11 sera from 137 sera collected from crayfish-allergic patients (which also had IgE-binding activity to other crustaceans) reacted with *P. clarkii* MLC in a serological assay. This indicates that MLC is a minor allergen in *P. clarkii*, and may cross-react with the myofibrillar proteins of other crustaceans.

432 The molecular mass of crayfish MLC is 18 kDa, which is similar to that of *C. crangon*
433 MLC, smaller than that of *L. vannamei* MLC (20 kDa), and larger than that of *P. monodon*
434 MLC (16 kDa). It is interesting that the cloned sequence of the purified MLC was highly
435 homologous to *C. crangon* MLC (Fig. 6B), whereas *L. vannamei* MLC (gi|184198734) was
436 only 13.11% identical and *P. monodon* MLC (gi|357640499) only 13.97% identical to the
437 purified MLC, whereas the *L. vannamei* and *P. monodon* MLCs were 62.92% identical to
438 MLC2 from *P. clarkii* (gi|392499121). This indicates that the purified MLC from *P. clarkii* and
439 MLC from *C. crangon* are MLC1, and that the MLCs from *L. vannamei* and *P. monodon* are
440 MLC2. Because MLC1 is the essential light chain and MLC2 is the regulatory light chain,
441 further studies should perform to determine whether MLC2 in *P. clarkii* is also allergenic and
442 to compare the allergenic properties of MLC1 and MLC2.

443 Most food allergens share similar physicochemical characteristics in that they are
444 glycoproteins, relatively stable to heat and acid-alkali, and may remain allergenic after
445 digestion.¹⁴ In this study, the total carbohydrate content of MLC1 was about 4.3±0.05% (w/w)
446 of the whole protein. Two identical potential N-glycosylated sites (Asn-Gly-Thr) were
447 identified by analyzing the primary structure and mass spectra of the protein. The
448 carbohydrates in crayfish SCP are reported to constitute about 4.9% of the protein, with one
449 N-glycosylation site. MLC and SCP have reportedly similar characteristics, and data in this
450 report support this conclusion.^{6,18} An O-linked glycan in the MLC1 structure was detected with
451 β -elimination. The O-glycan, which occurs in clusters along short regions of the peptide chain
452 that contain repeating units of Ser and Thr, is unstable under alkaline conditions at low
453 temperature. After NaOH treatment, Ser and Thr are transformed into α -amino acids. Both of

454 these unsaturated amino acids display characteristic UV absorption at 240 nm.⁶ According to
455 the typical characterization of glycoproteins, MLC1 is a glycoprotein. N- and O-linked
456 allergenic glycoproteins, which have common core structures, are associated with the induction
457 of an IgE response in allergic patients.³⁰ The N-glycosylated sites of MLC1 were partly at the
458 predicted epitopes regions (amino acid residues 25 and 99). Therefore, the relationship between
459 the allergenicity and glycosylation of crayfish MLC1 warrants further research.

460 In this study, MLC1 was stable at 30–100 °C and under strongly acidic and alkaline
461 conditions, and retained its IgE-binding activity at different temperatures and pHs. In contrast,
462 a study by Kamath et al.¹⁹ demonstrated that IgE binding to MLC of *P. monodon* was only
463 detected in heated prawn extract and not in raw prawn extract. It was inferred that the thermal
464 stability of MLC1 differs from that of MLC2. The thermal stability and pH stability of crayfish
465 MLC1 were similar to those of TM, another myofibrillar protein and the major allergen of
466 shellfish, which is stable under acidic and alkaline conditions and retains its IgE-binding
467 activity after high-temperature and high-pressure processing.³¹ However, the IgE-binding
468 activities of crayfish myosinogens AK and SCP are reduced at high temperatures or under
469 different acidic or alkaline conditions.^{6,14} It has been reported that, under different
470 physiological and experimental conditions, some allergens may undergo conformational
471 changes that cause the exposure of different combinations of epitopes recognized by the IgE
472 antibodies of allergic subjects.¹⁸ In this study, the changes in the secondary structure of
473 crayfish MLC1 under severe conditions, including high temperature (100 °C), strong acid (pH
474 1.0), and strong alkali (pH 11.0), were assessed to observe the changes in its secondary
475 structure. High temperature and strong acid treatments had significant effects on the secondary

476 structure of MLC1, but its IgE-binding activity remained intact despite the changes in its
477 structure. A future study should focus on effective processing methods to mask or disrupt the
478 IgE epitopes. Food allergens generally remain intact during gastrointestinal digestion. Crayfish
479 MLC1 appeared to be more resistant to gastric digestion than to intestinal digestion, and
480 retained its immunological activity (detected with ciELISA). Interestingly, MLC1 was
481 degraded after gastrointestinal digestion, but the degraded fragments had IgE-binding activity
482 almost equivalent to that of the undigested MLC1 sample. In a previous study, crayfish AK and
483 SCP were also degraded to varying degrees by gastrointestinal digestion, but remained
484 immunologically active.^{6,14} Certain IgE-binding sites on crayfish MLC1 are thought to be left
485 intact by digestive enzymes, and the major IgE-binding sites of allergens in crayfish are stable
486 after digestion. It was reported that Ca^{2+} binding was essential for the formation of the
487 conformation structure of calcium binding proteins like parvalbumin, and the depletion of Ca^{2+}
488 resulted in the reduction of IgE-binding activity.^{32,33} MLC belongs to EF-hand superfamily, the
489 influences of Ca^{2+} to the structure of MLC and its allergenicity need further research.

490 The epitopes can be classified as continuous or discontinuous. Continuous epitopes can be
491 identified in denatured proteins and peptide fragments, whereas discontinuous epitopes depend
492 on the higher-order structures of the native protein.³⁴ The study of epitopes is critical to
493 understanding the nature of allergens and the improvement of diagnosis, detection and
494 therapies. To determine the predominant IgE epitope type in crayfish MLC1, indirect ELISA
495 and Dot blot analysis were performed on folded MLC1 and Western blot analysis was
496 performed on the unfolded MLC1 with sera from allergic individuals. In both the indirect
497 ELISA and the Dot blot analysis, the native MLC1 was immobilized on the plate or a

498 nitrocellulose membrane, respectively. In both of these assays, the folded MLC1 was found to
499 maintain IgE binding under high heat and different pH conditions for all allergic sera tested. In
500 the Western blot, where MLC1 was in denatured or linear form only the serum IgE from three
501 allergic individuals displayed weakly positive binding. These results imply that the
502 conformational epitopes of MLC1 are predominantly recognized by IgE, rather than the linear
503 epitopes. Considering that the IgE-binding by unfolded MLC1 is significantly reduced
504 compared to the folded MLC1, it is possible that the digested fragments retain some structural
505 features that are able to compete with intact MLC1 for IgE binding.

506 The conformational and linear epitopes of the crayfish MLCs were also predicted *in silico*.
507 MLC1 was predicted to contain four conformation epitopes and three linear epitopes, and the
508 regions formed by amino acids 57–60 and 96–99 contained both conformational and linear
509 epitopes. MLC2 also showed a major predicted conformational epitope and three linear
510 epitopes. MLC2 clearly was predicted to have more epitopes than MLC1, but it was not
511 recognized by the IgE in sera from crustacean-allergic patients in a previous study. However,
512 in future studies, it will be important to investigate IgE binding to MLC2 with the same patient
513 sera used in this study for MLC1. Fundamental studies of allergens focus on IgE-binding
514 epitopes, which are used as biomarkers to predict the clinical reactivity of subjects who are
515 sensitized to a specific food, and provide alternative immunotherapeutic strategies.³⁵ If some
516 parallels can be found between predicted and the experimental IgE binding patterns, perhaps
517 efforts to reduce the allergenicity of food and diagnostic tools may be improved via epitope
518 prediction tools. Also, recombinant MLC1 and MLC 2 can be used to aid in clinical diagnoses

519 and in further research to provide basic information for the hypersensitization to crayfish and
520 the evaluation of food allergies.

521

522 **CONFLICT OF INTEREST**

523 The authors declare no competing financial interest.

524

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529

530 **SUPPORTING INFORMATION**

531 Supporting Information Available: Description. This material is available free of charge
532 via the Internet at <http://pubs.acs.org>.

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- 619

620 **Figure Legends**

621 **Table 1** Clinical and laboratory characterizations of seven crustacean-allergic patients and two
622 non-allergic individuals.

623

624 **Fig. 1.** Chromatographic purification of the 18-kDa protein in crayfish. (A) DEAE – Sephacel
625 chromatography. (B) Sephacryl S-200 HR gel chromatography. The numbers above the lanes
626 correspond to the fraction numbers. Target protein fractions under the bar were pooled.

627

628 **Fig. 2.** Mass spectrometric analysis of the 18-kDa protein in crayfish. (A) Map of MS/MS. (B)
629 Protein sequence alignments of MLCs from *Procambarus clarkii*, *Drosophila yakuba*, *Crangon*
630 *crangon*, and *Marsupenaeus japonicus*.

631

632 **Fig. 3.** Physicochemical characterization of purified crayfish MLC. (A) UV spectral analysis of
633 the purified MLC. Line 1: untreated MLC; line 2: MLC treated with low-concentration alkali.
634 The change at 240 nm is marked by an arrow. (B) SDS–PAGE analysis of the thermal stability
635 of MLC. con: MLC was incubated at 0 °C. (C) Dot blot of thermally treated MLC. con: MLC
636 was incubated at 0 °C. (D) SDS–PAGE analysis of the pH stability of MLC. con: MLC was
637 incubated at pH 7.5. (E) Dot blot of MLC treated with different pHs. con: MLC was incubated
638 at pH 7.5. (F) SDS–PAGE analysis of MLC stability to pepsin digestion. con: MLC was
639 untreated with pepsin. (G) SDS–PAGE analysis of MLC stability to pancreatin digestion. con:
640 MLC was untreated with pancreatin. M: protein marker; con: control. (H) ciELISA of the
641 digested fragments of MLC. MLC digested by pepsin (○, 1 h); pancreatin (▲, 4 h); and

undigested (●).

Fig. 4. Effects of different experimental conditions on the stability of purified crayfish MLC1.

(A) CD spectral analysis of MLC1 subjected to high temperature (100 °C, - - -) or 0 °C

(—) as the control. (B) CD spectral analysis of MLC1 subjected to strong acid (pH

1.0, - · - ·); strong alkali (pH 11.0, - - -); or pH 7.5 (—) as the control.

Fig. 5. Comparison of IgE reactivity to native and denatured MLC from crayfish. 1–7: sera of

seven crustacean-allergic patients (corresponding to serum No. 749, 514, 974, 067, 785, 980,

and 791); N: pooled sera from non-allergic individuals (No. 780 and 981) as the negative

control. (A) ELISA. (B) Dot blot. (C) Western blot. M: protein marker; MLC: SDS–PAGE

analysis of the purified MLC.

Fig. 6. Cloning *MLC* genes and sequence alignment of crayfish MLC proteins. (A) The

nucleotide and deduced amino acid sequences of crayfish MLC1 (the N-glycosylation site is

marked with a box). (B) Sequence alignment of crayfish MLC1 and the MLC proteins of other

crustaceans. (C) The nucleotide and deduced amino acid sequences of crayfish MLC2 (the

N-glycosylation site is marked with a box). (D) Sequence alignment of crayfish MLC2 and the

MLC proteins of other crustaceans.

Fig. 7. Structural modeling and epitope prediction of crayfish MLC1. (A) Three-dimensional

structure of crayfish MLC1. N and C indicate the N- and C-termini, respectively. (B)

664 Molecular mapping of the conformational epitopes of crayfish MLC1 onto its
665 three-dimensional structure. The areas occupied by the conformational epitopes are shown in
666 slate. Epitopes 1–4 are Asn²⁵, Lys⁵⁷–Glu⁶⁰, Lys⁹⁶–Asn⁹⁹, and Glu¹³⁶, respectively. (C) Molecular
667 mapping of the linear epitopes of crayfish MLC1 onto its three-dimensional structure. The
668 areas occupied by the linear epitopes are shown in blue. Epitopes I–III, Gly⁵³–Arg⁵⁸,
669 Lys⁷⁶–Ser⁸³, and Asp⁹⁵–Asn⁹⁹, respectively.

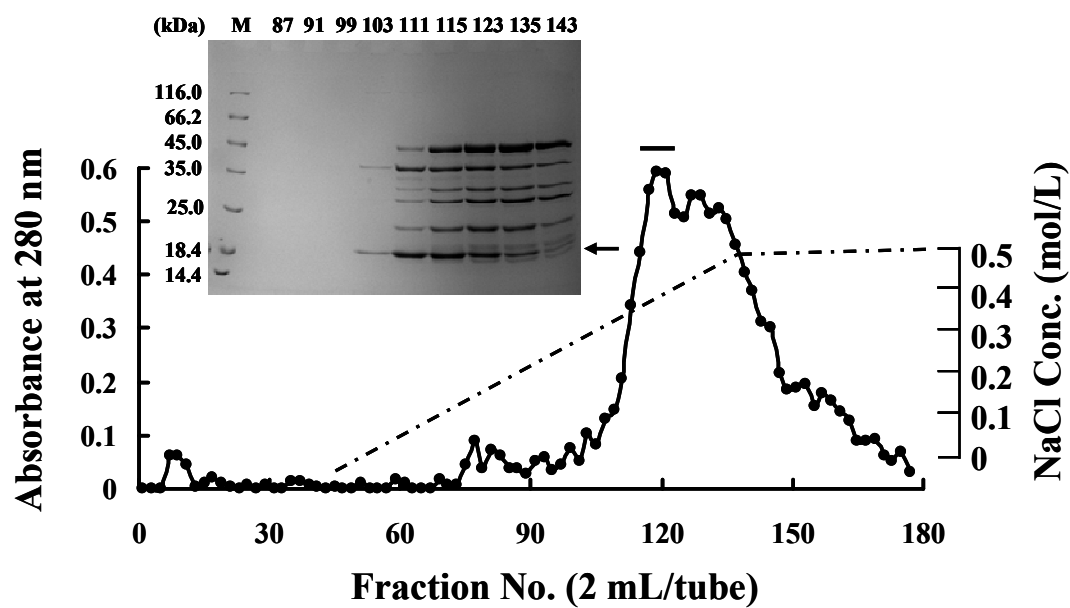
Table 1

Serum No.	Age	Sex	Symptoms	sIgE shrimp (f24) by ImmunoCAP (kUA/L)
749	23	F	U, P	11.1
514	19	M	E, N	1.28
974	18	M	N, P	45.1
067	22	F	A, D, R	9.68
785	19	F	GIT, Dr	61.7
980	19	F	U, D, P	1.93
791	24	M	A, D	2.35
780	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, GIT: gastrointestinal problems, Dr: diarrhea, ND: not determined / unknown.

Fig. 1

A



B

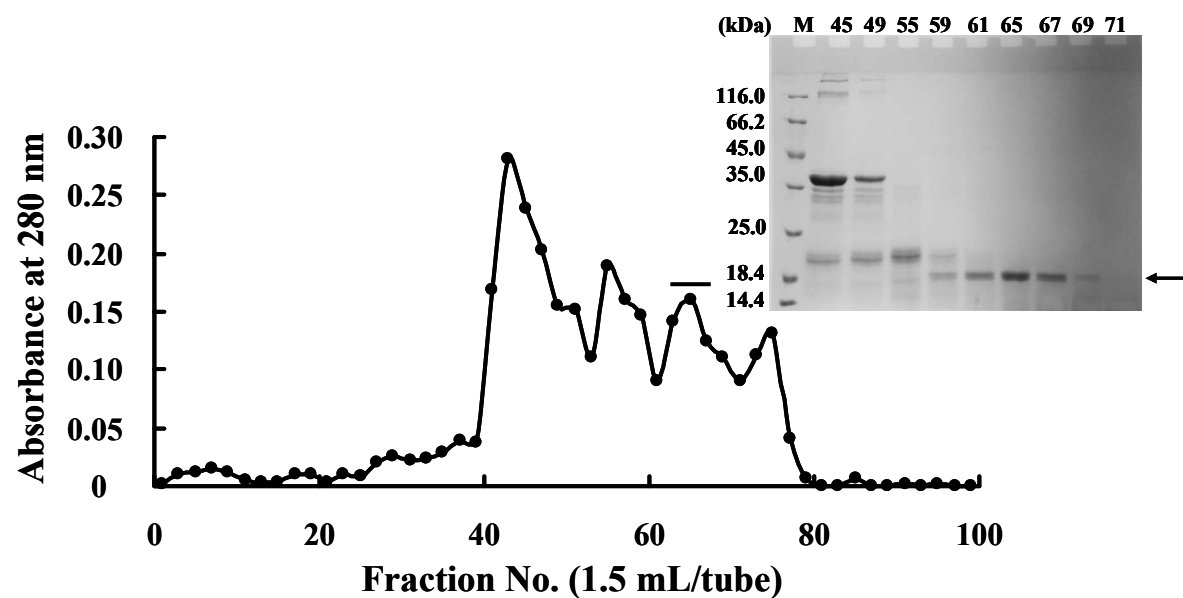


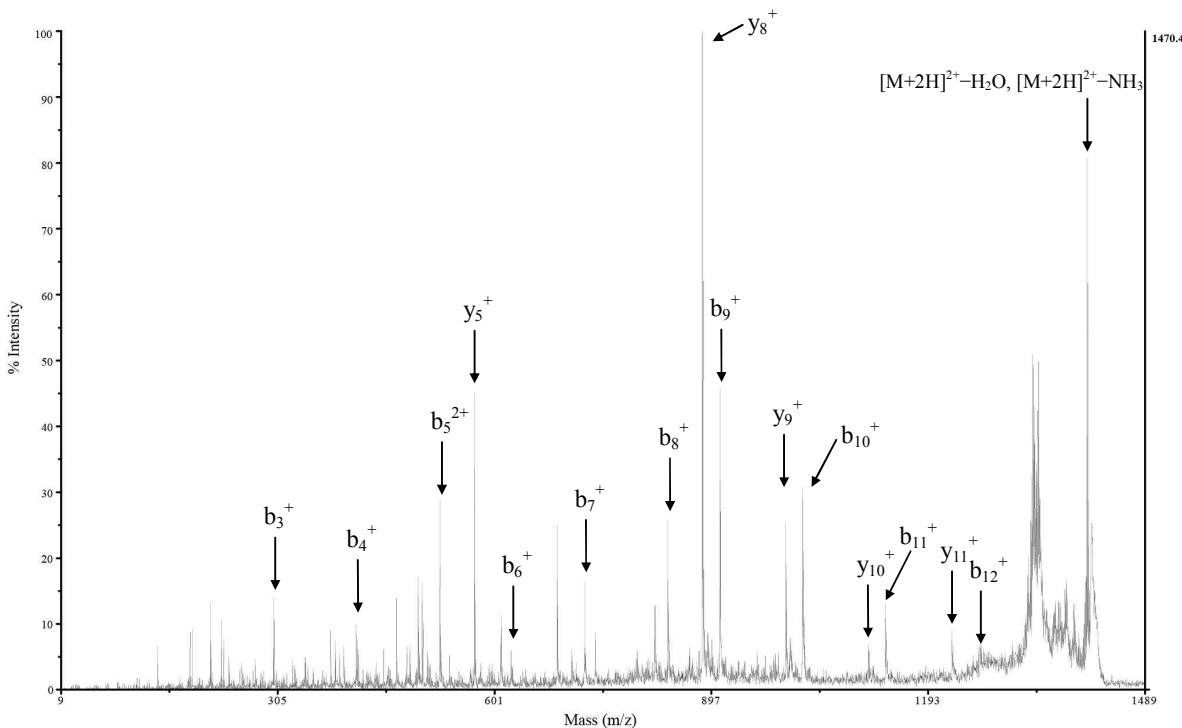
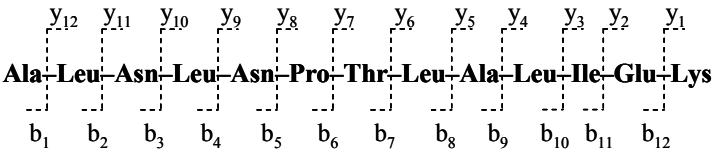
Fig.2

A

b ions: 89.08, 185.13, 299.18, 412.24, 526.31, 623.35, 724.43, 837.52, 908.56, 1021.63, 1134.69, 1263.73

y ions: 141.11, 274.17, 389.24, 502.33, 573.37, 686.47, 784.48, 884.57, 998.61, 1111.71, 1225.70, 1337.86

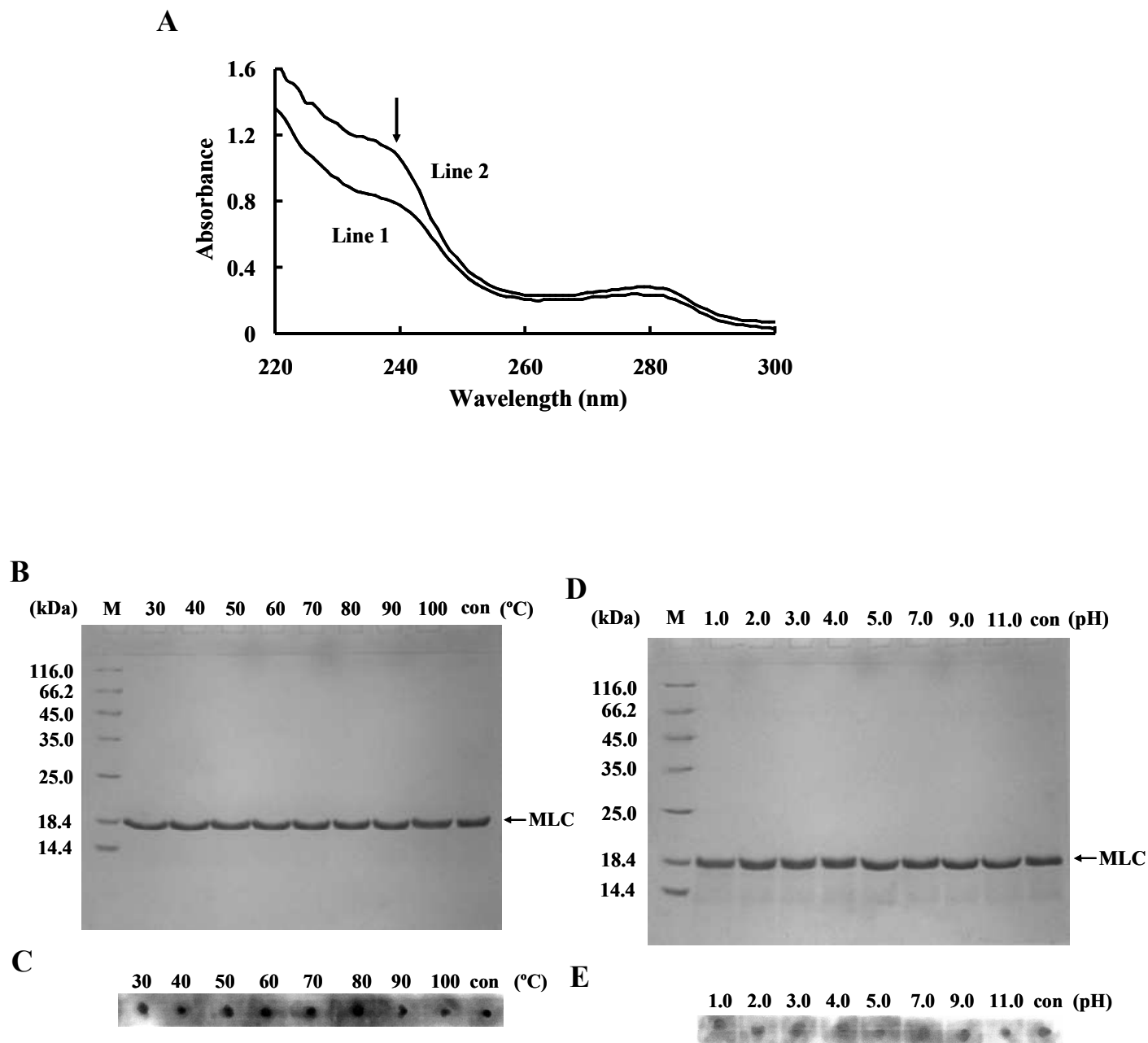
m/z: 1409.82, Charge: +1



B

Purified protein	ALNLTPTLALTEK.....	13
<i>Drosophila yakuba</i>	.MADVPKREVENVEFVFVEMGSPGEG.IDAVDLGDALRALNLTPTLALIEKLGGTKKRNE	58
<i>Crangon crangon</i>	MAADLSARDVERVKFAFSIYDFEGNGQIDAFYIGDCLRALNLTPTLALIAKLGGEKTRKE	60
<i>Marsupenaeus japonicus</i>	MAADLSARDVERVKFAFSIYDFEGNGTMDAYYIGDCLRALNLTPTLAVIEKVGGEKTRKE	60
Purified protein	KKIKLDEFLLPISQVKKKEQGCYEDFIECLKLYDKEENGTMLLAELQHALLALGENLDD	13
<i>Drosophila yakuba</i>	KKIKLDEFLLPISQVKKKEQGCYEDFIECLKLYDKEENGTMLLAELQHALLALGENLDD	118
<i>Crangon crangon</i>	KM IKLDDFMPLFAQVKKDKDAGSYEDFIEVLKLYDKAENGTM MYAELEHILLSLGERLDK	120
<i>Marsupenaeus japonicus</i>	KM IKLDEFMP IFAQVKKDKDAGSFEDFMEVLKLYDKAENGTM MYAELEHILLSLGERLEK	120
Purified protein	EQVETLFADGMDPEDDEGFIPYSQFVQRLMSDPVVF	13
<i>Drosophila yakuba</i>	EQVETLFADGMDPEDDEGFIPYSQFVQRLMSDPVVF	154
<i>Crangon crangon</i>	AELEPILRECCPPEDDEGLIPFEPFVKKL TQLL...	153
<i>Marsupenaeus japonicus</i>	AELEPVLKECCPEEDEEGCIPYEPFLKKITQLL...	153

Fig. 3



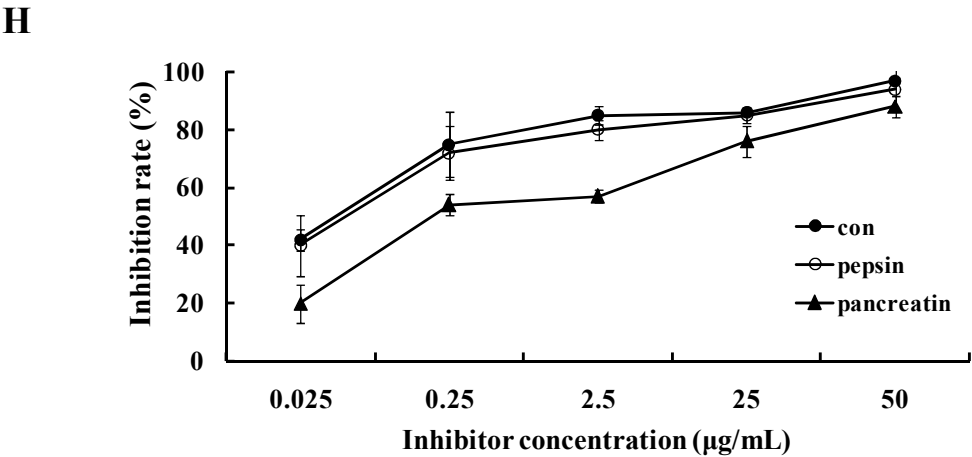
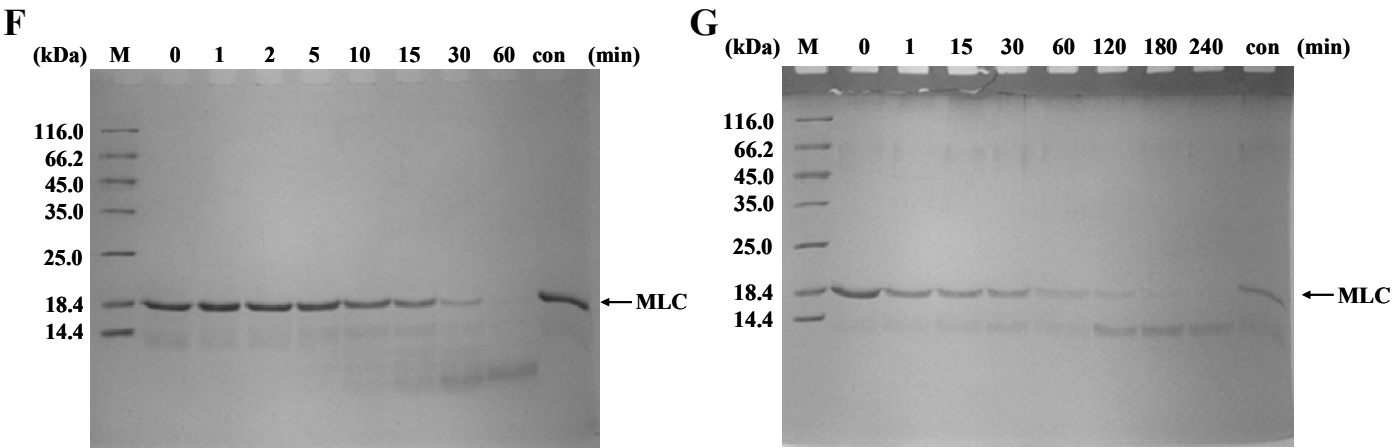
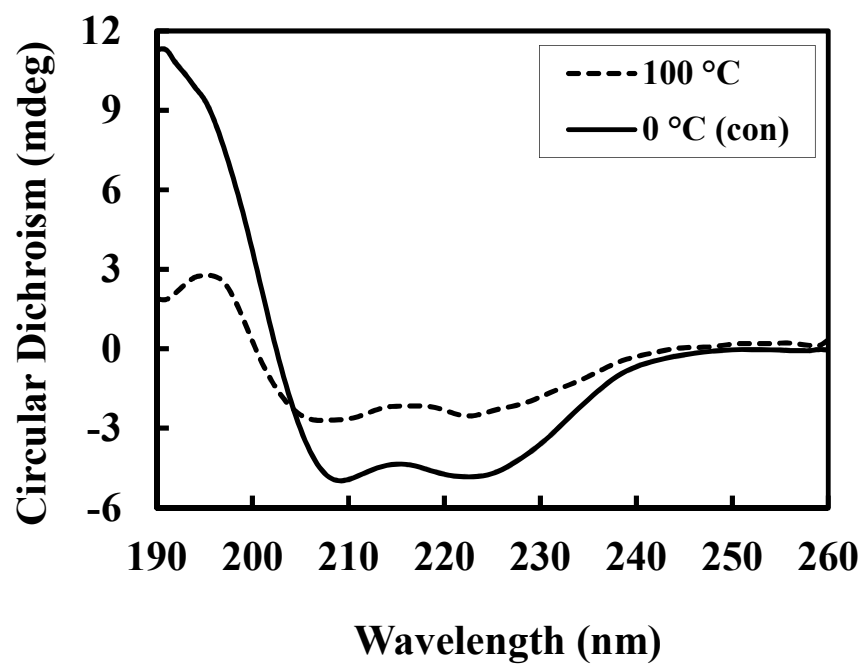


Fig. 4

A



B

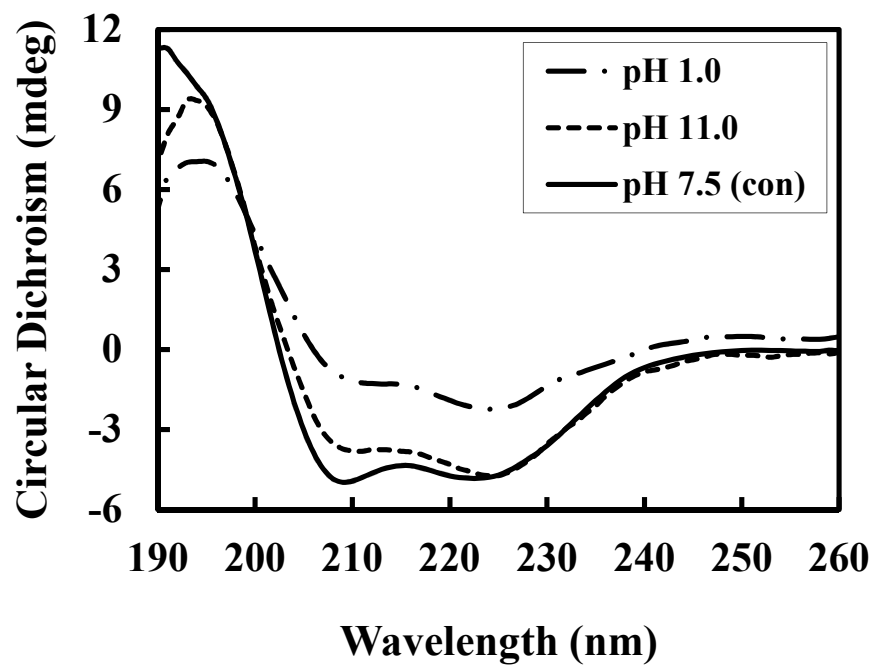


Fig. 5

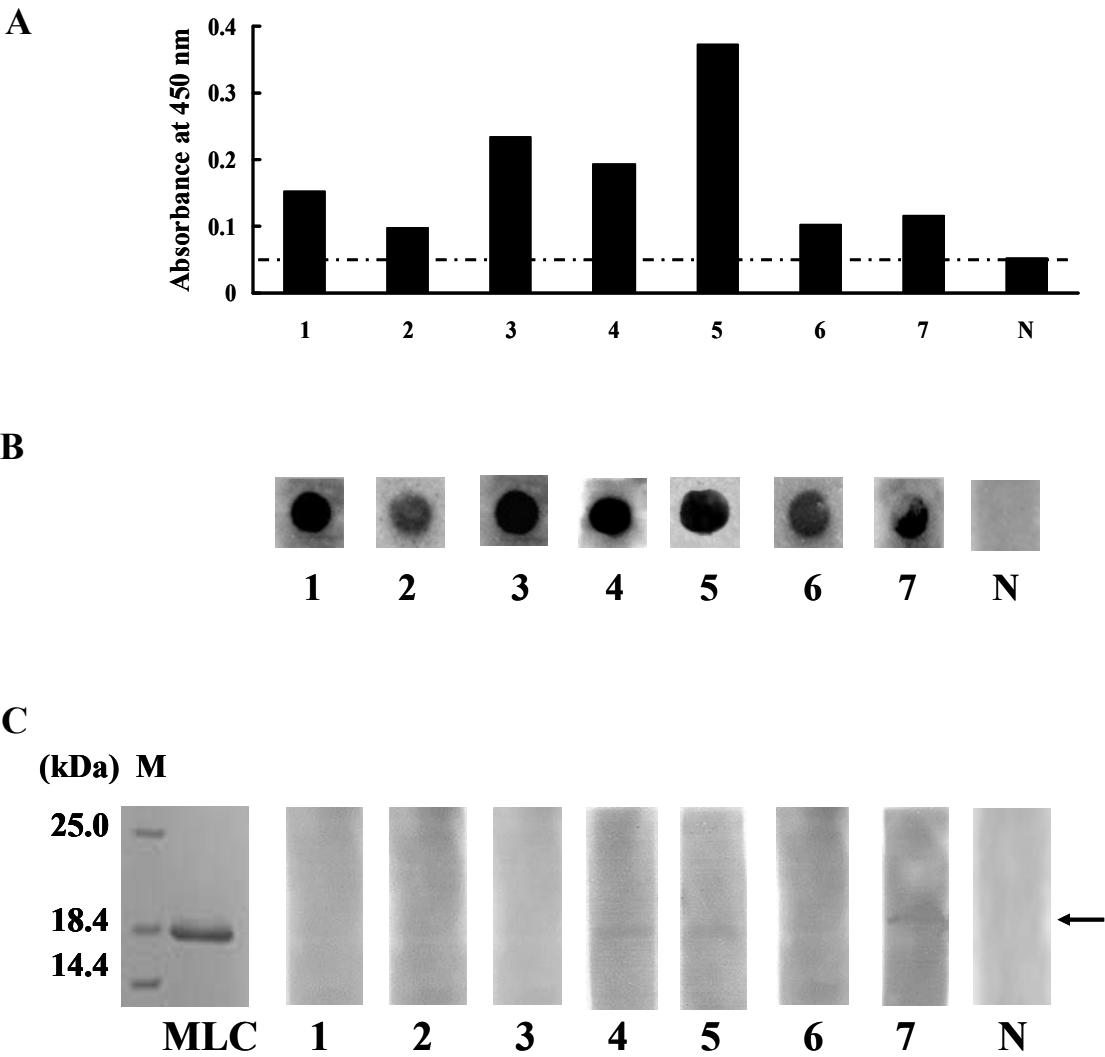


Fig. 6

A

```

1      ACGCGGGGACTTCAGGTCTGCCTTTTGCCCCGTAAACGTGGTGTGCTAGAGGCTAAGTCA
61     AGATGGCCGCGGATCTCAGTGCTCGTGATGTAGAGAGGGTGAAATTCGCCTTCTCTATCT
1      M A A D L S A R D V E R V K F A F S I
121    ATGACTTCGAAGGCAACGGCACCATGGATGCCTTCTACATTGGCGATTGCCTTCGCGCCC
20     Y D F E G N G T M D A F Y I G D C L R A
181    TGAACCTCAACCCACCTTAGCTATCAATTGAGAAGGTCGGAGGCAAGGAGAAGAGGAAGG
40     L N L N P T L A I I E K V G G K E K R K
241    AGAAGATTATTAAGCTCGAGGAATTCATACCCATCTTCGCTCAGGTTAAGAAGGACAAGG
60     E K I I K L E E F I P I F A Q V K K D K
301    ACGCCGGCTCCTACGAGGACTTCATGGAAGTGCTGAAGCTCTACGACAAA TCTGAGAAATG
80     D A G S Y E D F M E V L K L Y D K S E N
361    GCACTATGATGTACGCCGAGCTTGAACACATCCTCCTGTCCCTTGGTGAGCGCCTCGAAA
100    G T M M Y A E L E H I L L S L G E R L E
421    AGTCCGAATTGGAGCCAGTGCTGAAGGAGTGCTGCAACGAAGAGGATGAAGACGGCTTCA
120    K S E L E P V L K E C C N E E D E D G F
481    TTCCTACGAGCCTTTCCTTAAGAAGATGACTCAACTACTCTAAATACGTCCGCCATCTT
140    I P Y E P F L K K M T Q L L *
541    CTTTTGTGACGTCATCACAGAGTGCGCATGCGTGCAACACCACGCCATTCGGAGTGTC
601    GTGCGCAGTGTACAATCATTCTGTGGGCTCGACCAACCACAGACATTTATAGCTAAAT
661    TATACACATACACCAAAAGTATATAAAGATTACCAA GATGAAAGTGAATAAAGTGAA
721    AATGTTATAATTGACAA TGTGAGCCACCCGTCACA TGCTCA TCACTTCCTGTCCGTATG
781    TGTCTTGTGTCCGTGTAGCGGTGAAGGTCCCATGGATCTGTGGAACGTCTCCTTTTCTG
841    TCTCA TCCACGCGGGGTGAAGGAGGGGTCAAGGCAGGCGCGACCCGTGTGACTTGTA
901    CCTGAAGTGACCACTGAATTGATCCGCACATAGTTGACCACCCGACATCACCTAGAGGTG
961    GGAGACTATAGACGCCATTATCAAGACTCCCTCCCCGCAATAGCCAGCCGGCCAGCTGA
1021   CCATCGCCGCCATCTTGCGGGCCCCGCGCGCGCGGGAGGGACAAGAAAACCAAAAA
1081   AAAAAAAAAAAAAAAAAA

```

B

<i>Procambarus clarkii</i>	MAADLSARDVERVKFAFSIYDFEGNGTMDAFYIGDCLRALNLP TLAIIEKVGGKEKRKE	60
<i>Crangon crangon</i>	MAADLSARDVERVKFAFSIYDFEGNGQIDAFYIGDCLRALNLP TLALIAKLGSTKRKE	60
<i>Marsupinaeus japonicus</i>	MAADLSARDVERVKFAFSIYDFEGNGTMDAYYIGDCLRALNLP TLAVIEKVGGKEKRKE	60
<i>Palaemon varians</i>	MAADLSARDVERVKFAFSIYDFEGNGTMDAFYIGDCLRALNLP TVVTIEKLGGAEKRKE	60
<i>Periplaneta americana</i>	MAGDLSARDIERANFAFSIYDFEGTGTVDAYLGLDILRGLNLP TQATVEKMGSTKKKNE	60
<i>Artemia franciscana</i>	MA.....FSFTILMETRQLMAFYLGDC LRALNLP TLAQIEKLGGSTKKKNE	46
<i>Procambarus clarkii</i>	KIIEKLEEFIPFAQVKKDKDAGSYEDFMEVLKLYDKSENGTMMYAELEHILLSLGERLEK	120
<i>Crangon crangon</i>	KMIKLDDFMPLFAQVKKDKDAGSYEDFIEVLKLYDKAENG TMMYAELEHILLSLGERLDK	120
<i>Marsupinaeus japonicus</i>	KMIKLDEFMPLFAQVKKDKDAGSFEDFMEVLKLYDKAENG TMMYAELEHILLSLGERLEK	120
<i>Palaemon varians</i>	KMIKLDDFMPLFAQVKKDKDAGGYEDFIEVLKLYGKSENGTMMYAELEHILPSLGERLEK	120
<i>Periplaneta americana</i>	KKLKVEEFLPIFGQVKKEKDVGCYEDFLECLKLYDKAEDGKMLLAELHTLLSLGERLSD	120
<i>Artemia franciscana</i>	KKLKVEEFLPIFSQVKKEKDHGTLEDFIECMKLYDKSENGTMM LAELNHILLALGEKLED	106
<i>Procambarus clarkii</i>	SELEPVLKECCNEEDEDGFIPEYEPFLKKMTQLL...	153
<i>Crangon crangon</i>	AELEPILREGCPPEDEEGLIPFEPFVKKL TQLL...	153
<i>Marsupinaeus japonicus</i>	AELEPVLKECCPEEDEDGCIPEYEPFLKKITQLL...	153
<i>Palaemon varians</i>	AELEPILKEGCPPEDEEGCIPEYEPFVKKL TLL...	153
<i>Periplaneta americana</i>	REVDTIVKDCAEPEDEDGFIPIAQLKNVMS.....	151
<i>Artemia franciscana</i>	SQVDSIFKEGMDPEDEENIPYTPFLKRLLAGPYPDA	143

C

1 ACGCGGGAGAGAGGAGGTCGAGGAACCTGACTATGGGAGTGATAAGGTTGATGTATCGCG
61 ATCATGCCTGCCTCAAAGGGTTCCCGTTCTTCTCCAAGAAGGCGAAGAAGGGAGGAAGC
1 M P A S K G S R S S S K K A K K G G S
121 AATGTCTTCGATATGTTACCCAGAAGCAGGTGGCGGAGTTCAAGGAGGGCTTCCAGGTG
20 N V F D M F T Q K Q V A E F K E G F Q V
181 ATGGACCGTGATCGTGATGGCATCATCAGCAAGGACGACCTCCGTGGTGTC TACGACGAA
40 M D R D R D G I I S K D D L R G V Y D E
241 ATCGGCCGTATTGTCACCGACAAGGACCTGGACGACA TGATTGCTGACGCACCTGGCCCA
60 I G R I V T D K D L D D M I A D A P G P
301 ATTA ACTTCACCACCCTCCCGCACA TGTTCGCCTCAGCTCCTCTGGAGAGTCCGACGAC
80 I N F T T L P H M F A S R S S G E S D D
361 GACGATGTTGTTGCCAAGTCTTTCCTG GCCTTCGAGAAGGAAGCCGGTCAGATCGACTCC
100 D D V V A K S F R A F E K E A G Q I D S
421 GAGCAGTTCCGTGCAATGCTTATGGCTTTCGGTGACAAGTTCACGCCCCGCGGAAGTCGAC
120 E Q F R A M L M A F G D K F T P A E V D
481 GATGCC TTTGAGCAGATGGAATCGACGAGGACACCGGCA TCATCGACTCCAACGCCCTC
140 D A F E Q M E I D E D T G I I D S N A L
541 ATCGCCAT GTTGTGCGCCGGTGGAGCAGAAAAGGGTGAGGGTGAGGCTTAAATACCCAAA
160 I A M L C A G G A E K G E G E A *
601 TTCCCGTCCATCGTCCGTGCCCCGAAGGAAATGTGGGTGAGCAGTAGCGGAAGCCATCTGC
661 AGGGGTTGCTACCTGTAGCTGT GACACTCAGGCGCTTC TTGGCTAT CTTCT GGGCCCTGC
721 TGCTGCTACTTCCTCTCTCA TCAATGCTCGCCACT TCTT CCA TACG TCACCACCACCA T
781 ATT TCTTACGGGCGAAGTCACAAGCAGGATAGACAATTGAGGCTGGACCCTTCGCCC TCT
841 CCCTCTTC TGT AAT CCTGCCGTCA TTATC TCC ATCTACGATA GACCCTACAAGCGGCGGC
901 TTTCACCAGACCTCAAGAT CGGCGTCCGCGAACTCGCAGTGTCTTTGTGAAAAGAAAAA
961 GCCAAGCCGGGAGCGGAGTGACAGTGACAGTGAATATGAATGTAAACATTGCCGCCGTGA
1021 TCCACAACCAGAGGGCCTACCGCTCCC TTGCTAGTGTTACAT CCGAACAGAACT AAAACG
1081 AGACAT CTGTGAC TAAAGTAAAGTACGAGGTCGTAGTGTGAGGGAGCTCAGTGTCTCTG
1141 CCTCAT CTTTAAACCACTGTAGTGACGACTTTTACGAGCT GGAGAAGCCACCGCGGTGAC
1201 CGCTACGCAGTTGTCGTCACCTGATGCTGGACCAGAGTCGGGTGCCGGTGACTGCAGCTC
1261 TAGCACTTGT AAATGTTT CAGTTGTGAAGCA TTGTAT T AACA TCGGCAAGATA TA TTCAA
1321 TAAAT GTTTT AAAAAAAAAAAAAAAAAAAAAAAAAAAAAA

D

<i>Procambarus clarkii</i>	MPASKGSRSSSKKAKK. . GGSNVFDMFTQKQVAEFKEGFQVMDRDRDGIISKDDL RGVYD	58
<i>Litopenaeus vannamei</i>	MSRKSGSRSSSKRSKSGGGSNVFDMFTQROVAEFKEGFQLMDRDKDGVIGKTDLRGTFD	60
<i>Penaeus monodon</i>	MSRKSGSRSSSKRSKSGGGSNVFDMFTQROVAEFKEGFQLMDRDKDGVIGKTDLRGTFD	60
<i>Procambarus clarkii</i>	EIGRIVTDKDLDDMIADAPGPINFTTLPHMFASRSSGESDDDDVVAKSFRAFEKEAGQID	118
<i>Litopenaeus vannamei</i>	EIGRIATDQELDEMLADAPAPINFTMLLNMFARQTGESDDDDVVAKAF LAFADEEGNID	120
<i>Penaeus monodon</i>	EIGRIATDQELDEMLADAPAPINFTMLLNMFARQTGESDDDDVVAKAF LAFADEEGNID	120
<i>Procambarus clarkii</i>	SEQFRAMLMAFGDKFTPAEVDDAFEQMEIDEDTGII DSNALIAMLCAGGAEKGE. EA	175
<i>Litopenaeus vannamei</i>	CDTFRHALMTWGDKFSSQEADDALDQMDIDDG. GKIDVQGV IQMLTAGGGDDAAAEAA	177
<i>Penaeus monodon</i>	CDTFRHALMTWGDKFSSQEADDALDQMDIDDA. GKIDVQSV IQMLTAGGGDDAAAEAA	177

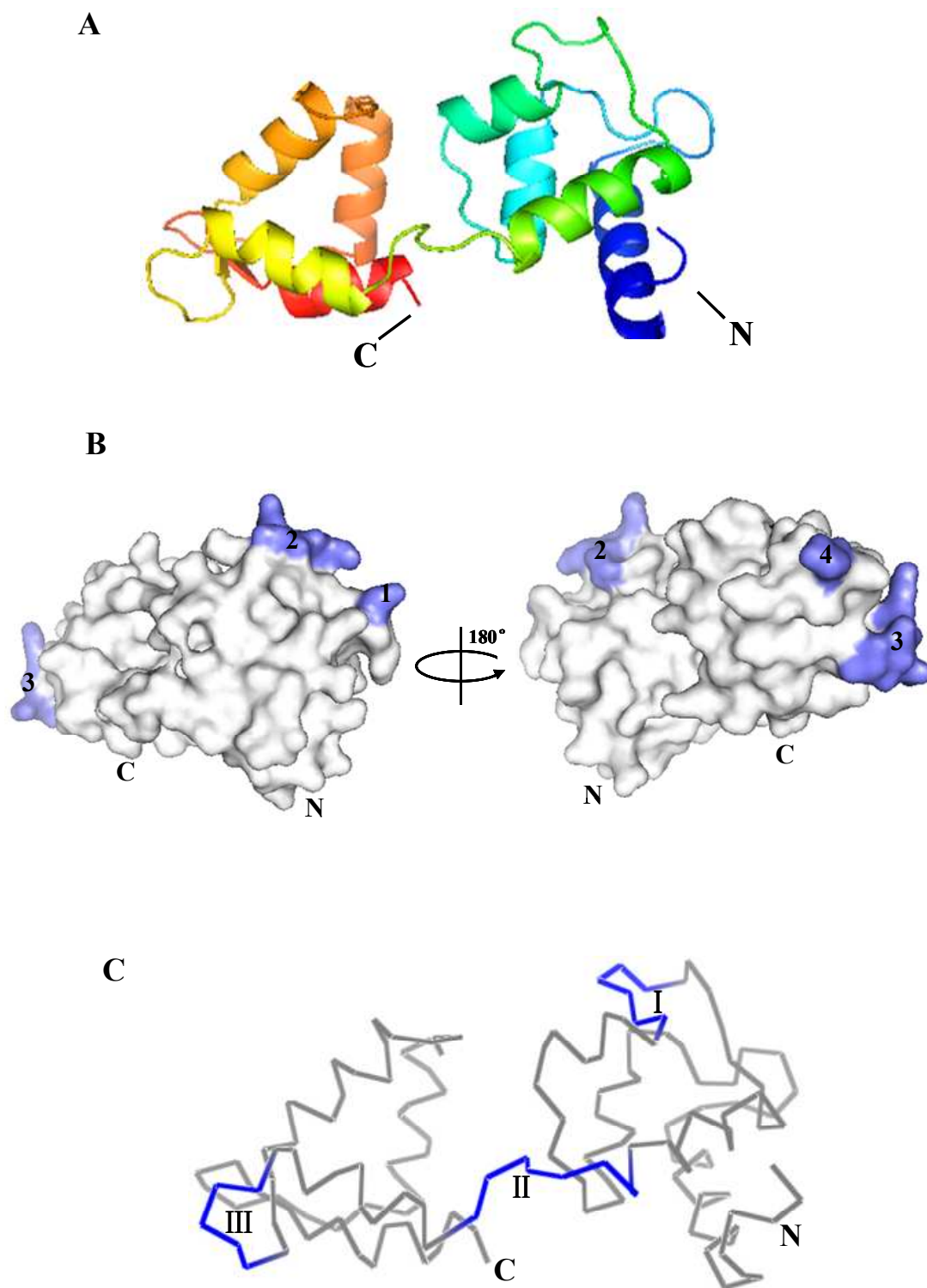
Fig. 7

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