

Molecular Characterization and Cross-Allergenicity of Tropomyosin from Freshwater Crustaceans

Panyarat Laurchan, Teerasak E-kobon, Prapansak Srisapoome, Sasimanas Unajak, and Chomdao Sinthuvanich*



Cite This: <https://doi.org/10.1021/acs.jafc.1c00934>



Read Online

ACCESS |



Metrics & More



Article Recommendations

ABSTRACT: Tropomyosin is a major allergen responsible for cross-allergenicity in a number of shellfish species. Although extensively characterized in marine crustaceans, the information of tropomyosin is limited to a few freshwater crustacean species. As a result, more cross-reactivity evidence and information of tropomyosin at the molecular level are required for the detection of freshwater crustaceans in the food industry. In this study, we explored tropomyosin allergenicity in four freshwater crustacean species: prawn (*Macrobrachium rosenbergii* and *Macrobrachium lancesteri*) and crayfish (*Procambarus clarkii* and *Cherax quadricarinatus*). Immunoblotting, liquid chromatography–tandem mass spectrometry, and immunoprecipitation studies indicated that tropomyosin was recognized by the sera's IgE of crustacean-allergic volunteers. Cloning and characterization of nucleotide sequences of tropomyosin cDNA from *M. lancesteri* and *C. quadricarinatus* revealed highly conserved amino acid sequences with other crustaceans. This study emphasized the role of tropomyosin as a universal marker for the detection of both freshwater and marine crustaceans in the food industry.

KEYWORDS: allergen, tropomyosin, freshwater crustacean, crayfish, freshwater prawn

INTRODUCTION

Shellfish is one of the eight major food groups causing allergies in both children and adults.^{1–3} The study of shellfish allergies, particularly those mediated by crustaceans, has primarily focused on marine or estuarine crustaceans such as shrimp, prawn, lobster, and crab, as they are popularly cultivated, consumed, and distributed worldwide. As of 2021, allergens from at least 19 crustacean species have been reported and officially given the nomenclature according to WHO/IUIS Allergen Nomenclature (<http://allergen.org/>). However, information on the allergenicity of freshwater crustaceans is still limited as species are locally distinct to different parts of the world. Similarly, monoallergies to either freshwater or marine or estuarine crustaceans have been clinically described.^{3,4}

Currently, some freshwater crustacean species are reported to cause food allergies, including giant river prawn (*Macrobrachium rosenbergii*),^{5,6} riceland prawn (*Macrobrachium lancesteri*),^{3,7} oriental river prawn (*Macrobrachium nipponense*),⁸ Siberian prawn (*Exopalaemon modestus*),⁹ red swamp crayfish (*Procambarus clarkii*),^{10–13} and narrow-clawed crayfish (*Pontastacus leptodactylus*).¹⁴ In Thailand, four species of freshwater crustaceans are commonly consumed: giant river prawn (*M. rosenbergii*), riceland prawn (*M. lancesteri*), red swamp crayfish (*P. clarkii*), and red claw crayfish (*C. quadricarinatus*).

Giant river prawn (*M. rosenbergii*) and riceland prawn (*M. lancesteri*) are caridean groups in the family Palaemonidae. *M. rosenbergii* is native to the Indo-Pacific region and is commercially cultured and introduced worldwide. According to the Food and Agricultural Organization (FAO) of the

United Nations, in 2019, its global production reached 0.27 million tons with an economical value of more than 2.2 billion US dollars.¹⁵ The top producers of giant river prawn are China (51%), Bangladesh (19%), and Thailand (11%) which accounts for more than 80% of the world production.¹⁵ Giant river prawn has gained recognition in the global market as an alternative to marine shrimp due to its size, texture, and flavor. As a result, it has been used as a common representative of freshwater aquatic animals for shrimp allergy studies. *M. lancesteri* is a small translucent freshwater prawn commonly found in slowly flowing water such as rice paddle fields, swamp, ponds, and reservoirs in Southeast Asia.¹⁶ Due to its small size, riceland prawn is generally consumed as whole body or processed as paste or fermented food. Red swamp crayfish (*P. clarkii*) is a crayfish in the family Cambaridae. Although indigenous to northern Mexico and southern United States, the major sources of red swamp crayfish are from China and United States where it is distributed as fresh, frozen, or processed food.¹⁷ Its global production in 2019 reached 2.1 million tons with an economical value of more than 18 billion US dollars.¹⁵ Red claw crayfish (*C. quadricarinatus*), native to Australia, is a crayfish in the Parastacidae family. It is cultured mainly in Australia and Malaysia and exported worldwide

Received: February 13, 2021

Revised: June 18, 2021

Accepted: June 25, 2021



particularly to countries in Asia. It receives increased attention due to its relatively large size with an appearance of a lobster. In 2019, its production reached 0.17 million ton with an economical value of 2.1 million US dollars.¹⁵ Both red swamp crayfish and red claw crayfish are now cultivated for consumption in Thailand.

The major shellfish allergen is tropomyosin, which is responsible for cross-allergenicity in a number of shellfish species, due to its sequence similarity with conserved sequences of 88–100% among crustaceans.¹⁸ Accordingly, tropomyosin is used as a shellfish contamination marker in the food industry and for shellfish allergy diagnostics.^{19–23} Even though extensively studied in marine crustaceans, more allergenicity evidence and information at the molecular level of tropomyosin from freshwater crustaceans are still required. To date, tropomyosin in three freshwater crustacean species have been characterized: Mac r 1,⁵ Pro c 1,²⁴ and Exo m 1⁹ from *M. rosenbergii*, *P. clarkii*, and *E. modestus*, respectively.

The objective of this study was to explore the allergenicity of tropomyosin from four freshwater crustacean species: giant river prawn (*M. rosenbergii*), riceland prawn (*M. lanchesteri*), red swamp crayfish (*P. clarkii*), and red claw crayfish (*C. quadricarinatus*). The presence of tropomyosin was investigated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting. Their allergenicity was examined by immunoblotting, liquid chromatography–tandem mass spectrometry (LC–MS/MS), and immunoprecipitation studies. Tropomyosin cDNAs from *M. lanchesteri* and *C. quadricarinatus* were cloned and sequenced for comparison with other crustaceans. This work provides more insight into the molecular basis of allergenicity of tropomyosin from freshwater crustaceans.

MATERIALS AND METHODS

Crustacean Samples. The four freshwater crustacean species are shown in Table 1 and Figure 1. Live animals were purchased from

Table 1. Four Crustacean Species Used in This Study^a

no.	scientific name	common name	allergen	accession numbers (GenBank)
1	<i>Macrobrachium rosenbergii</i>	giant river prawn	Mac r 1	ADC55380.1
2	<i>Macrobrachium lanchesteri</i>	riceland prawn		MZ217127
3	<i>Procambarus clarkii</i>	red swamp crayfish	Pro c 1	ACN87223.1
4	<i>Cherax quadricarinatus</i>	red claw crayfish		MZ217128

^aAllergen nomenclature and tropomyosin accession number were retrieved from the allergen nomenclature database (www.allergen.org) and GenBank, respectively (<https://www.ncbi.nlm.nih.gov/genbank/>).

local markets, and each species was identified by morphological traits. On the day of purchase, fresh samples were extracted for crude proteins or stored in RNA-EZ Reagents RNA-Be-Locker A (Sangon Biotech, Shanghai, China) at −80 °C for RNA isolation.

Volunteer's Sera. This study was approved by the Kasetsart University Research Ethics Committee (COA no. COA63/004). Ten volunteers, with a history of crustacean allergies, and one healthy volunteer with no history of crustacean allergy and no allergic symptoms were recruited and provided written informed consent. Information on sex, age, and self-reported allergic symptoms are shown (Table 2). Sera from the volunteers were collected at the Infirmary of Kasetsart University and stored at −80 °C until required.

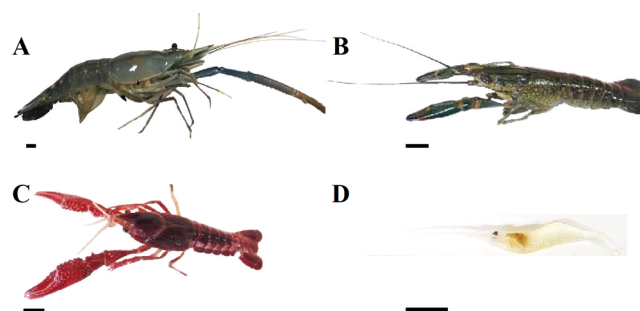


Figure 1. Four crustacean species used in this study. (A) Giant river prawn (*M. rosenbergii*), (B) red claw crayfish (*C. quadricarinatus*), (C) red swamp crayfish (*P. clarkii*), and (D) riceland prawn (*M. lanchesteri*), scale bar = 1 cm.

Table 2. Demographics and Sera's ImmunoCAP IgE Titer of Crustacean-Allergic Volunteers^a

subject (no.)	gender	age (years)	symptoms	ImmunoCAP (kU/L)
1	female	46	U	0.85
2	female	29	G, O, U	3.31
3	female	23	A, G	22.9
4	female	23	U	7.97
5	male	29	O, U	0.03
6	male	22	A, C, O, G, U	6.24
7	male	37	O	10.6
8	male	39	A, O, U	0.24
9	male	24	A, G	8.56
10	male	22	A, C, O	29.8

^aA: allergic rhinitis, C: conjunctivitis, G: gastrointestinal symptoms, O: oral symptoms, and U: urticaria.

ImmunoCAP assay (Thermo Fisher Scientific, Inc., Waltham, MA, USA) was performed for shrimp (f24) by GM Laboratory Co., Ltd. (Bangkok, Thailand).

Preparation of Crude Protein Extracts. Raw muscle was homogenized in 0.1 M ammonium bicarbonate buffer (pH 7.4) containing 0.2% phenol, followed by centrifugation at 12,000g for 15 min. The supernatant was collected and filtered through a 0.22 μm membrane (GE Healthcare, Chicago, Illinois, USA). Protein concentration was measured by Bio-Rad Protein assay according to the manufacturer's instructions (Bio-Rad Laboratories Ltd., Hercules, California, USA) using bovine serum albumin (BSA) as a standard. For boiled samples, fresh muscle was boiled in deionized water for 10 min before homogenization and crude proteins were extracted as described above.

Purification of Tropomyosin. Shrimp tropomyosin was purified as described by Lasekan and Nayak.²⁵ Briefly, fresh muscle of *Litopenaeus vannamei* was homogenized in 20 mM Tris-HCl buffer (pH 7.5) containing 50 mM KCl followed by centrifugation at 7000g for 15 min. The pellet was washed three times with cold acetone and dried overnight in a fume hood. Then, it was dissolved in 20 mM Tris-HCl buffer (pH 7.5) containing 1 M KCl and 10 mM 2-mercaptoethanol, followed by incubation at 4 °C overnight. The sample was then centrifuged at 7000g for 15 min to collect the supernatant, which was adjusted to a pH of 4.6. The pellet was collected and resuspended in 20 mM Tris-HCl buffer (pH 7.5). Protein was precipitated with 40–60% ammonium sulfate and centrifuged at 7000g for 15 min. After the pellet was redissolved in 20 mM Tris-HCl buffer (pH 7.5), soluble protein was passed through a 0.22 μm membrane and concentrated in a Vivaspin-20, with a 3 kDa cut-off (GE Healthcare, Chicago, Illinois, USA). The sample was loaded onto a 1-cm HiTrap DEAE-Sepharose fast flow column (GE Healthcare, Chicago, Illinois, USA) and eluted over a linear gradient

of 300–600 mM NaCl in 50 mM Tris-HCl buffer (pH 7.5). Purified tropomyosin was fraction-collected and stored at -20°C .

SDS-PAGE and Immunoblot Analysis. The protein extract was mixed in Laemmli sample buffer containing 2-mercaptoethanol and heated at 100°C for 10 min. The samples were then loaded onto a SDS-PAGE gel with 4% stacking gel and 12% separating gel and electrophoresed using a Mini-PROTEAN Tetra cell (Bio-Rad Laboratories Ltd., Hercules, California, USA) at 120 V for 1.50 h. Then, the gel was stained with InstantBlue (Expedeon Ltd., Cambridge, UK). The molecular weight marker was PageRuler Plus Prestained Protein Ladder (Thermo Fisher Scientific, Waltham, Massachusetts, USA).

For immunoblotting, proteins were transferred onto nitrocellulose membranes (NCs) using a Mini-Trans-Blot Cell (Bio-Rad Laboratories Ltd., Hercules, California, USA). After washing in Tris buffer saline with 0.1% Tween 20 (TBS-T), NCs were incubated at 4°C with volunteers' sera diluted to 1:2 (v/v) or rat monoclonal anti-tropomyosin antibody diluted to 1:1000 (v/v) (Cat. no. ab50567, Abcam, Cambridge, Massachusetts, USA) in TBS-T containing 0.1% BSA. After a 16 h incubation, NCs were washed and incubated with mouse monoclonal anti-human IgE-Fc antibody conjugated to horseradish peroxidase (Cat. no. ab99806, Abcam, Cambridge, Massachusetts, USA) or goat anti-rat IgG H&L antibody conjugated to horseradish peroxidase (Cat. no. ab205720, Abcam, Cambridge, Massachusetts, USA) for 1 h at room temperature. NCs were visualized using Western Lightning ECL Pro (PerkinElmer, Waltham, Massachusetts, USA) and analyzed on a GelDoc XR gel documentation system (Bio-Rad Laboratories Ltd., Hercules, California, USA).

Immunoprecipitation. 50 μL of Dynabeads M-280 sheep anti-mouse IgG (Thermo Fisher Scientific, Waltham, Massachusetts, USA) was washed twice in a wash buffer [0.01 M phosphate-buffered saline (pH 7.4) containing 0.1% BSA] for 5 min. After discarding the wash solution, the beads were incubated with 4 μg mouse monoclonal anti-IgE antibody (Cat. no. ab7295, Abcam, Cambridge, Massachusetts, USA) for 1 h at room temperature and washed twice. After discarding the wash solution, the anti-IgE conjugated beads were incubated at 4°C for 16–18 h with sera pooled from 10 crustacean-allergic volunteers diluted to 1:1 (v/v) in a wash buffer. After discarding the wash solution, the beads were further incubated with 20 μg crude protein extract for 3 h at room temperature. The beads were then washed twice. To analyze using SDS-PAGE and immunoblot, the beads were dissolved with 0.01 M phosphate-buffered saline (pH 7.4). Each sample was mixed in Laemmli sample buffer containing 2-mercaptoethanol and heated to 100°C for 10 min. The anti-IgE bound beads and the beads incubated with the pooled volunteers' sera were used as controls. The molecular weight marker was PageRuler Plus Prestained Protein Ladder (Fisher Scientific, Waltham, Massachusetts, USA). Gels were electrophoresed and processed as previously described.

Protein Identification Using Liquid Chromatography–Tandem Mass Spectrometry. After immunoblotting, NCs were cut, and on-membrane protein was digested by Trypsin/Lys-C mix (Promega Corporation, Madison, Wisconsin, USA).²⁶ Briefly, antibodies were removed by washing NCs three times with 20 mM sodium bicarbonate buffer (pH 7.4), followed by three washes in 100 mM glycine buffer (pH 2.4). Then, NCs were rewashed in 20 mM sodium bicarbonate buffer (pH 7.4). Next, NCs were blocked in 0.5% poly (vinylpyrrolidone) (PVP-40) in 0.5% acetic acid at 37°C for 30 min, before washing in ultrapure water. The on-membrane proteins were digested with 12.5 ng/ μL of Trypsin/Lys-C mix in 50 mM ammonium bicarbonate buffer (pH 8.0) and incubated at 37°C overnight. The reaction was stopped by adding 50% acetonitrile containing 0.1% formic acid. After digestion, the samples were submitted to the Research Instrument Center, Khon Kaen University for analysis by LC–MS/MS.

The raw samples were analyzed using a nano-liquid chromatography system, equipped with an ESI nano-sprayer (EASY-nLC II, Bruker, USA) and analyzed on an ion trap mass spectrometer (Amazon Speed ETD, Bruker, USA). The boiled samples were

analyzed using a nano-liquid chromatography system, equipped with an ESI nano-sprayer (EASY-nLC II, Bruker, USA) and analyzed by a quadrupole time of flight tandem mass spectrometer (micrOTOF-Q II, Bruker, USA). The samples were loaded onto an EASY-Column, C18-A2 (Thermo Scientific, Waltham, Massachusetts, USA). Mobile phase A comprised water with 0.1% formic acid and B was acetonitrile with 0.1% formic acid. The gradient was 5–35% B in 50 min followed by 10 min at 80% B. The tryptic digest of BSA was used as a standard. The LC–MS/MS spectra were analyzed using Compass data analysis v.4.0. Proteins were identified by searching against the protein database from Swiss-Prot [Metazoa (Animal)] using the MASCOT MS/MS Ion Search program (www.matrixscience.com). Search parameters were as follows: enzyme: trypsin; carbamidomethylation (C) as a fixed modification, and oxidation (HW) and oxidation (M) as variable modifications; peptide mass tolerance of 0.5 Da; a peptide charge state of +1, +2, +3; instrument type: ESI-TRAP for the raw samples and ESI-QUAD-TOF for the boiled samples; and report top: Auto.

Identification of cDNA Encoding Tropomyosin of *M. lanchesteri* and *C. quadricarinatus*. To identify tropomyosin cDNA, total RNA was isolated from fresh crustacean muscle using TRIzol reagent (Life Technologies, Carlsbad, California, USA). Total RNA was reverse-transcribed into first-strand cDNA, and full-length cDNA was amplified using a SMARTer RACE 5'/3' Kit (Takara Co., Ltd, Tokyo, Japan) as described in the manufacturer's instruction. The rapid amplification of cDNA ends (RACE) method was used to identify cDNA of tropomyosin.²⁷ The 3'RACE primer was designed using conserved tropomyosin sequences from 10 crustacean species including *Fenneropenaeus merguensis* (GU369817.6), *Metapenaeus ensis* (U08008.1), *Penaeus monodon* (AY827100.1), *M. rosenbergii* (KF571722.1), *Crangon crangon* (FJ457621.1), *Penaeus aztecus* (DQ151457.1), *Homarus americanus* (AF034954.1), *Jasus lalandii* (JX860677.1), *Cherax destructor* (KC291444.1), and *L. vannamei* (EU410072.1). The tropomyosin sequence amplified by the 3' RACE primer was used as a template to design the 5' RACE primer.

The primers were: 3'-RACEML (CTCAACACCGCCACCACC) and 5'-RACEML (GTCGGCCTCAACCATGG) for *M. lanchesteri* and 3'-RACECQ (ACGCCATCAAGAAGAAGATGCAGG) and 5'-RACECQ (ACAGTTCGCTGAATGTCTGGTCG) for *C. quadricarinatus*. After RACE-PCR, the full-length cDNAs were cloned into a pRACE vector using the In-Fusion HD Cloning kit (Takara Co., Ltd, Tokyo, Japan). Briefly, the PCR product was ligated into the linearized pRACE vector using the In-Fusion HD Enzyme Premix. The ligation reaction was incubated at 50°C for 15 min and placed on ice for 16 h before transformation into Stellar competent cells using the heat shock method. The cells were spread on an LB-plate containing 100 $\mu\text{g}/\text{mL}$ of ampicillin and incubated overnight at 37°C . Plasmid DNA from recombinant colonies was extracted using the TIANGEN Rapid Mini Plasmid kit (TIANGEN Biotech, Beijing, China) and sequenced by Macrogen Inc., South Korea. A phylogenetic tree was constructed from the tropomyosin amino acid sequence using MEGA-X 10.1 software and displayed using iTOL (<https://itol.embl.de>). The nucleotide sequences of tropomyosin from the following accession number (NCBI, GenBank Protein Database) were used for analysis: *Scylla serrata* (Scy s 1), ABS12233.1; *Charybdis feriata* (Cha f 1), AAF3543; *Portunus trituberculatus* (Por tr 1), ABS12234; *Portunus pelagicus* (Pro p 1), AGE44125; *P. aztecus* (Pen a 1), AAZ76743.1; *L. vannamei* (Lit v 1), ACB38288.1; *C. crangon* (Cra c 1), ACR43473.1; *H. americanus* (Hom a 1), O44119.1; *Panulirus stimpsoni* (Pan s 1), AAC38996.1; *Pandalus borealis* (Pan b 1), CBY17558.1; *P. monodon* (Pen m 1), AAX37288.1; *Melicertus latisulcatus* (Mel l 1), AGF86397.1; *M. ensis* (Met e 1), AAA60330.1; *E. modestus* (Exo m 1); *M. rosenbergii* (Mac r 1), ADC55380.1; *M. lanchesteri*, MZ217127; *P. clarkii* (Pro c 1), ACN87223.1; and *C. quadricarinatus*, MZ217128.

RESULTS AND DISCUSSION

Identification of Tropomyosin in Freshwater Crustaceans. To identify tropomyosin in four freshwater crustacean

species, crude protein was extracted from raw and boiled crustaceans. Different migratory protein patterns were observed by SDS-PAGE. In the raw samples, protein bands at 36–38 kDa, which reflected the molecular weight of tropomyosin, were visible for all species, although to a lesser extent in *M. lancesteri* (Figure 2A). Interestingly, in the boiled

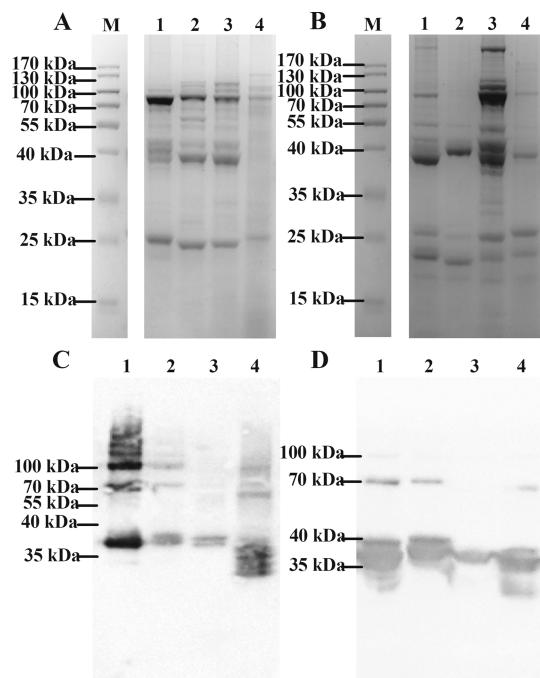


Figure 2. Presence of tropomyosin in (A,C) raw and (B,D) boiled samples. (A,B) SDS-PAGE of the protein extracts. (C,D) Immunoblot analysis of the protein extracts probed with an anti-tropomyosin antibody. M: molecular weight marker; lane 1: *M. rosenbergii*; lane 2: *C. quadricarinatus*; lane 3: *P. clarkii*; and lane 4: *M. lancesteri*.

samples, protein bands at 36–38 kDa were observed, regardless of the species (Figure 2B). This change in the protein migratory pattern was common as it was reported that after heat treatment, tropomyosin, a heat-stable protein, appeared with intense bands on SDS-PAGE.^{28–30}

To detect tropomyosin in crude extracts, a monoclonal anti-tropomyosin antibody was employed for western blot analysis. This antibody was produced using the troponin–tropomyosin complex purified from *Lethocerus indicus* (Waterbug) flight muscle as an immunogen. The antibody was reported to

recognize shellfish tropomyosin and has been used to identify tropomyosin in shellfish allergen extracts.^{28,30,31} In the raw samples, multiple protein bands were observed, with prominent protein bands at 36–38 kDa irrespective of the species. Interestingly, the bands at ~70 and >100 kDa were observed in all species, except *P. clarkii* (Figure 2C), while for *M. lancesteri*, a protein band recognized by anti-tropomyosin antibody showed a molecular weight lower than 37 kDa. These observed bands agreed with the boiled samples where high-intensity protein bands at 36–38 kDa were visible across all samples. In the boiled samples, the bands at ~70 kDa were observed only in *M. rosenbergii*, *C. quadricarinatus*, and *M. lancesteri* but not in *P. clarkii* (Figure 2D). These bands at molecular weights of ~70 and ~100 kDa could correspond to tropomyosin aggregates, which presented to a greater extent in the raw samples. In addition, heat processing has been reported to induce changes in the secondary structures, which can enhance antibody recognition of tropomyosin resulting in high-intensity protein bands at 36–38 kDa in the boiled samples.^{30,32} These data suggested the structural change of tropomyosin after heat treatment. Taken together, these data indicated that tropomyosin was present in these four freshwater crustacean species.

Tropomyosin as an Allergen in Freshwater Crustaceans. Pooled sera from 10 crustacean-allergic volunteers (Table 2) and one individual with no allergic history were used to identify IgE-reactive protein in the raw and boiled freshwater crustacean protein extracts. Regardless of the species, the pooled sera's IgE reacted with the protein bands at 36–38 kDa in all the extracts in both the raw (Figure 3A) and boiled samples (Figure 3B), while serum from the healthy volunteer showed no visible band. The pooled sera also recognized the purified tropomyosin isolated from *L. vannamei* which acted as a control (Figure 3C).

The protein bands at 36–38 kDa from each species were excised, on-membrane digested, and identified by LC–MS/MS. Table 3 shows the MASCOT search results for IgE-binding protein at 36–38 kDa. In the raw samples, tropomyosin was identified in *M. rosenbergii*, *M. lancesteri*, and *C. quadricarinatus*, whereas arginine kinase was identified in *M. rosenbergii* and *P. clarkii*. In the boiled samples, tropomyosin was identified in *M. rosenbergii*, *M. lancesteri*, and *C. quadricarinatus*. Similar to the raw samples, arginine kinase was also identified in *P. clarkii*. These results suggested that protein identification using on-membrane proteins from one-dimension electrophoresis could not discriminate proteins

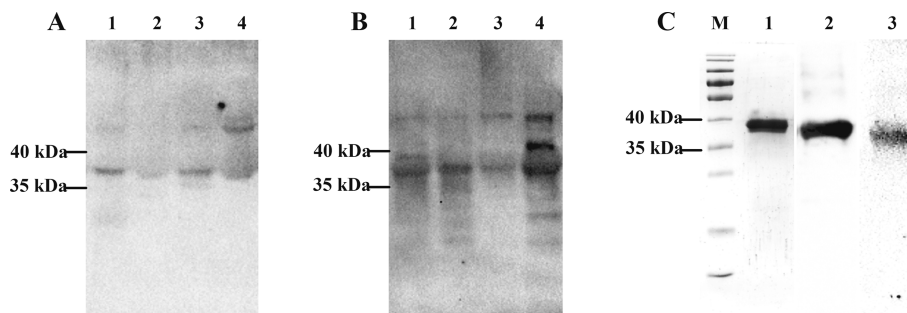


Figure 3. Immunoblot analysis of protein extracts from (A) raw and (B) boiled samples probed with the pooled sera from 10 crustacean-allergic volunteers; lane 1: *M. rosenbergii*; lane 2: *C. quadricarinatus*; lane 3: *P. clarkii*; and lane 4: *M. lancesteri*. (C) Purified tropomyosin; M: molecular weight marker; lane 1: purified tropomyosin from *L. vannamei* on 12% SDS-PAGE, lane 2: immunoblot with an anti-tropomyosin antibody; and lane 3: immunoblotting with pooled sera from 5 crustacean-allergic volunteers. Lane 2 and 3 were from separate immunoblots.

Table 3. MASCOT Search Results of IgE-Binding Protein at 36–38 kDa (Pooled Sera from 10 Crustacean-Allergic Volunteers) Using LC–MS/MS Analysis^a

samples	protein name	monoisotopic mass (Mr)	mascot score	coverage (%)	peptide sequences
Raw Samples					
<i>M. rosenbergii</i>	tropomyosin	32,830	70	4	LAEASQAADER
	arginine kinase	40,300	132	16	LVSANDIEK LIDHFLFK EMQDGILEIK VSSTLSGLELK IISMQMGDLGQVYR
<i>M. lanchesteri</i>	tropomyosin	32,655	163	20	FLAEADR MDALENLK LEDELVNEK LAMVEADLER IQLLEEDLER
					LAEASQAADER
					DIFDSLK
					LIDHFLFK
					VSSTLSGLELK IISMQMGDLGQVYR GEHTEAEGGIDISNK
<i>P. clarkii</i>	arginine kinase	40,300	82	16	FLAEADR IVEEELR LAMVEADLER IQLLEEDLER LAEASQAADER ALSNAEGEVALNR LAEASQAADERMR IQLLEEDLERSEER SLSDEERMDALENLK
<i>C. quadricarinatus</i>	tropomyosin	31,686	276	31	
Boiled Samples					
<i>M. rosenbergii</i>	tropomyosin	32,768	92	9	LAEASQAADER ADTLEQQNKEANNR
<i>M. lanchesteri</i>	tropomyosin	32,932	79	4	LAEASQAADER
<i>P. clarkii</i>	arginine kinase	40,420	29	3	EMQDGILEIK
<i>C. quadricarinatus</i>	tropomyosin	32,932	20	4	LAEASQAADER

^aSwiss-Prot was used for the database.

of similar molecular weight, that is, tropomyosin (37 kDa) and arginine kinase (40 kDa). Thus, to further confirm IgE reactivity of volunteer sera to tropomyosin, immunoprecipitation studies were performed.

Here, crude protein extracts were immunoprecipitated by the sera's IgE captured by an anti-human IgE antibody bound to Dynabeads. Immunoblot analysis of the immunoprecipitated protein using an anti-tropomyosin antibody revealed two protein bands at > 35 and ~55 kDa in the raw (Figure 4A) and boiled samples (Figure 4B). The upper band at 55 kDa was present in controls where Dynabeads M-280 sheep anti-mouse IgG was incubated with either an anti-human IgE antibody or the pooled sera from 10 crustacean-allergic volunteers (Figure 4C). This protein band was presumably the heavy chain of the mouse anti-human IgE antibody which cross-reacted with the goat anti-rat IgG H&L antibody which served as a secondary antibody. Thus, the lower band at ~36–38 kDa was expected to be tropomyosin from the protein extracts precipitated by sera's IgE. This band was not visible in the controls which confirmed no non-specific binding of the protein extracts to Dynabeads in the observed molecular weight range (Figure 4C). Thus, tropomyosin from freshwater crustacean was recognized by the pooled sera's IgE from individuals with crustacean allergies.

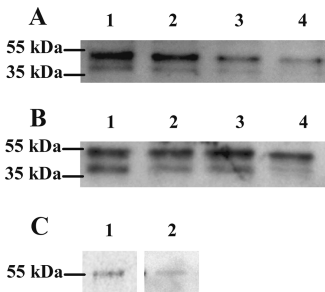


Figure 4. Immunoblot analysis using an anti-tropomyosin antibody. Protein extracts from (A) raw and (B) boiled samples were immunoprecipitated using the pooled sera from 10 crustacean-allergic volunteers and captured with mouse anti-human IgE on Dynabeads M-280 sheep anti-mouse IgG. Lane 1: *M. rosenbergii*; lane 2: *C. quadricarinatus*; lane 3: *P. clarkii*; and lane 4: *M. lanchesteri*. (C) Controls; lane 1: Dynabeads M-280 with an anti-human IgE antibody and lane 2: Dynabeads M-280 with pooled sera from 10 crustacean-allergic volunteers.

To examine the IgE reactivity to tropomyosin, sera from six volunteers were individually incubated with the raw and boiled freshwater crustacean protein extracts as shown in Figure 5. Extracts from *L. vannamei* were used as a representative for a

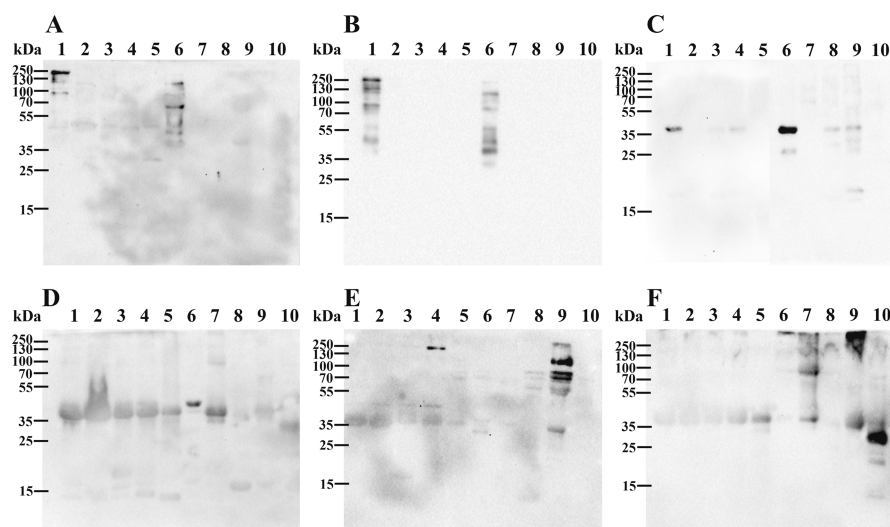


Figure 5. Immunoblot analysis of protein extracts from the boiled (lanes 1–5) and raw (lanes 6–10) samples probed with the sera's IgE from crustacean-allergic volunteers; (A) volunteer no. 1, (B) volunteer no. 2, (C) volunteer no. 3, (D) volunteer no. 7, (E) volunteer no. 9, and (F) volunteer no. 10. Lane 1,6: *L. vannamei*; lane 2, 7: *M. rosenbergii*; lane 3, 8: *C. quadricarinatus*; lane 4, 9: *P. clarkii*; and lane 5, 10: *M. lanchesteri*.

marine shrimp. IgE-immunoblotting revealed that the sera's IgE from each volunteer recognized the protein bands at ~36–38 kDa corresponding to the molecular weight of tropomyosin. These ~36–38 kDa bands were expected to be tropomyosin as confirmed by LC–MS/MS and immunoprecipitation experiments from the pooled sera as previously discussed. In volunteer number 1, tropomyosin bands were observed in raw *L. vannamei* extract and raw *P. clarkii* extract while protein bands at ~40 kDa, possibly arginine kinase, were observed in the boiled samples (Figure 5A). In volunteer number 2, no protein band was observed in the freshwater crustaceans but IgE-reactive tropomyosin was observed in *L. vannamei* extracts (Figure 5B). Interestingly, the sera's IgE from volunteer number 3 reacted to tropomyosin from *L. vannamei* and crayfishes, but not to the *M. rosenbergii* and *M. lanchesteri* (Figure 5C). In the case of volunteer number 7, 9, and 10, the sera's IgE recognized tropomyosin across all species used in this experiment (Figure 5D–F). The bands were more visible in the boiled samples compared to the raw samples which give a similar staining pattern to the protein extracts probed with anti-tropomyosin antibody (Figure 2C,D). From this experiment, the absence of the protein bands cannot rule out the fact that IgE-reactive tropomyosin may be present but not visible in Figure 5A–C because of the high background or the poor signal detection during imaging. However, it was reasonable to consider that, although not all volunteers' sera react to freshwater crustacean tropomyosin, cross-reactivity between tropomyosin from four freshwater crustaceans and *L. vannamei* was observed.

Molecular Cloning and Amino Acid Sequences of Tropomyosin in Freshwater Crustaceans. To gain more insight into the molecular level, we identified tropomyosin nucleotide sequences from *M. lanchesteri* and *C. quadricarinatus* using the RACE method. The determined nucleotide sequences of the full-length cDNAs were deposited in the GenBank databases and the accession numbers are shown in Table 1. Tropomyosin coding sequence from *M. lanchesteri* and *C. quadricarinatus* were 855 base-pair long. The open reading frames encoded a protein of 284 amino acids for each crustacean. When aligned, the amino acid sequences of

tropomyosin from *C. quadricarinatus* and *M. lanchesteri* were highly conserved, showing 95.4–99.3% homology between the four species of freshwater crustaceans and 81.7–99.6% identity between 18 crustacean species (Figure 6). Tropomyosin amino acid sequences were divided into a non-conserved region at location 1–79 amino acid residues and a conserved region at 80–284 amino acid residues.

The amino acid sequences were further analyzed and compared with previously reported IgE-binding epitopes of two crustacean tropomyosins. Figure 6 shows the IgE-binding epitopes of the following tropomyosins: Pen a 1 (43–55, 87–101, 137–141, 144–151, 187–197, 249–259, 266–273, and 273–281), a marine shrimp (*P. aztecus*) tropomyosin;³³ Lit v 1 (43–57, 85–105, 133–148, 187–202, and 247–284), a marine white shrimp (*L. vannamei*) tropomyosin;³⁴ Exo m 1 (43–59, 85–105, 131–164, 187–201, and 243–280), a freshwater Siberian prawn (*E. modestus*) tropomyosin;⁹ Scy s 1 (42–55, 90–100, 105–116, 133–142, 143–153, 196–206, 224–232, and 253–264), a mud crab (*S. serrata*) tropomyosin.³⁵ In comparison to Pen a 1 and Lit v 1, amino acid alignments revealed an alteration in the IgE-binding epitopes in the non-conserved region, with two alterations at the residues 44–45 in *M. lanchesteri* and one alteration at the residue 52 in *P. clarkii*. Similar alterations were observed when compared with Exo m 1, which were two alterations at the residues 44–45 in *M. lanchesteri*, two alterations at the residues 52 and 59 in *P. clarkii*, and one alteration at the residue 59 in *C. quadricarinatus*. When compared to the IgE-binding epitopes of tropomyosin from a mud crab, *S. serrata*, six alterations at the residues 43–46, 49, and 53 were observed in the non-conserved region of *M. rosenbergii*, *M. lanchesteri*, and *C. quadricarinatus*. In addition to the six alterations, an extra alteration at the residue 52 was observed in *P. clarkii*. Interestingly, no alteration in the conserved regions of the IgE-binding epitopes of Pen a 1 was observed in tropomyosin sequences from *S. serrata*, *L. vannamei*, *E. modestus*, *M. rosenbergii*, *M. lanchesteri*, *P. clarkii*, and *C. quadricarinatus*. This high sequence similarity to Pen a 1, Lit v 1, Exo m 1, and Scy s 1 suggested that the cross-reactivity of freshwater and marine crustaceans was likely based on the shared conserved

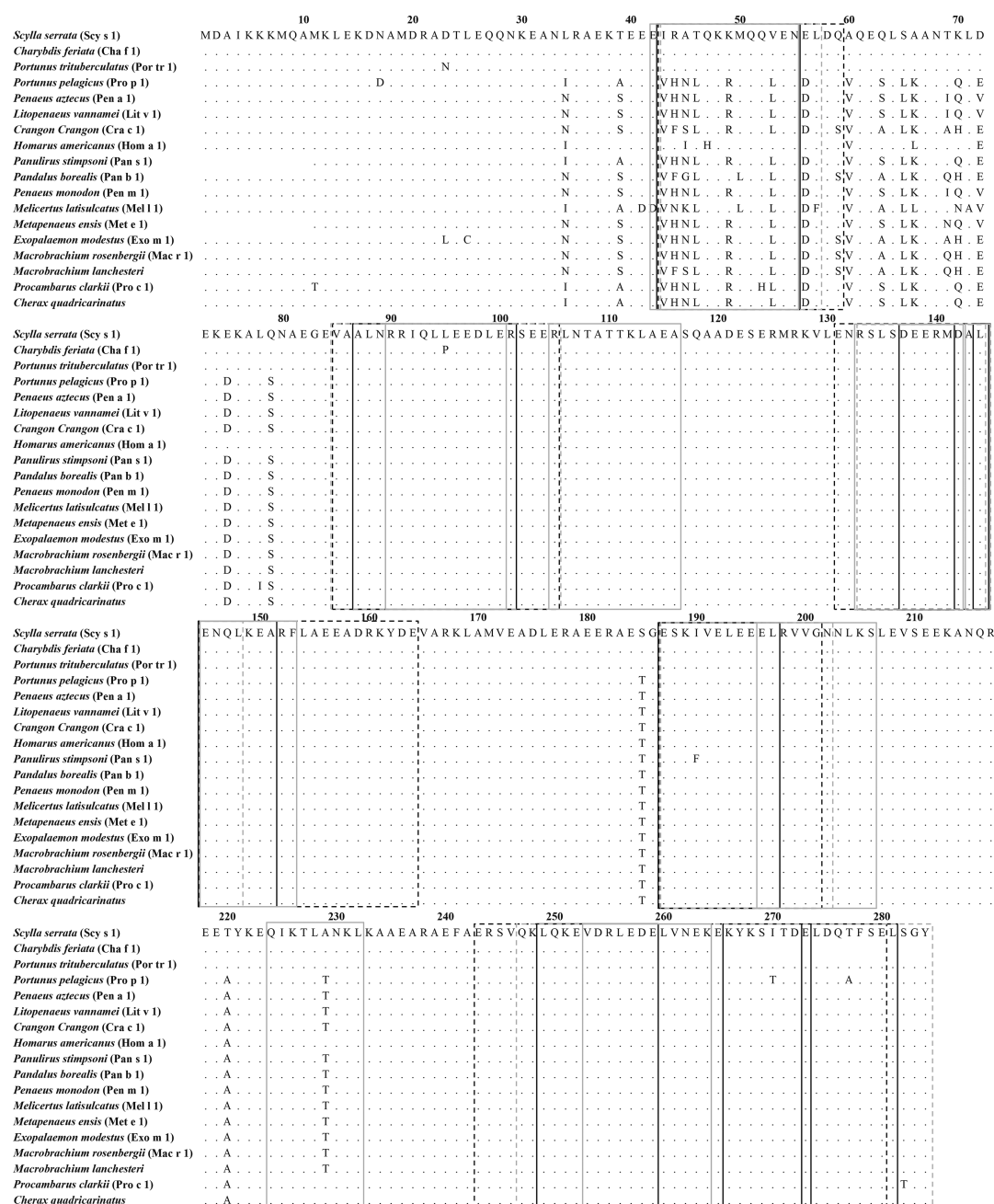


Figure 6. Amino acid sequence alignment of crustacean tropomyosin. Accession numbers (NCBI, GenBank Protein Database): *S. serrata* (Scy s 1), ABS12233.1; *C. feriata* (Cha f 1), AAF3543; *P. trituberculatus* (Por tr 1), ABS12234; *P. pelagicus* (Pro p 1), AGE44125; *P. aztecus* (Pen a 1), AAZ76743.1; *L. vannamei* (Lit v 1), ACB38288.1; *C. crangon* (Cra c 1), ACR43473.1; *H. americanus* (Hom a 1), O44119.1; *P. stimpsoni* (Pan s 1), AAC38996.1; *P. borealis* (Pan b 1), CBY17558.1; *P. monodon* (Pen m 1), AAX37288.1; *M. latisulcatus* (Mel l 1), AGF86397.1; *M. ensis* (Met e 1), AAA60330.1; *E. modestus* (Exo m 1); *M. rosenbergii* (Mac r 1), ADC55380.1; *M. lanchesteri*, MZ217127; *P. clarkii* (Pro c 1), ACN87223.1; and *C. quadricarinatus*, MZ217128. Dots represent identical amino acid residues. The IgE-binding epitopes of Scy s 1 are shown in the gray-line boxes. The IgE-binding epitopes of Pen a 1 are shown in the black-lined boxes. The IgE-binding epitopes of Lit v 1 are shown in the broken gray-lined boxes. The IgE-binding epitopes of Exo m 1 are shown in the broken black-lined boxes.

IgE-binding epitopes. The phylogenetic tree of tropomyosin from 18 crustaceans based on amino acid sequence comparisons, constructed using the neighbor-joining method, confirmed our finding (Figure 7). The analyses suggested that crustacean species in the tree evolved from a series of common ancestors. *C. quadricarinatus* was the most recent common ancestor with *P. clarkii* (Pro c 1). *M. lanchesteri* was the most recent common ancestor with *C. crangon* (Cra c 1), which was

in the same clade as *M. rosenbergii* (Mac r 1) and *E. modestus* (Exo m 1).

Tropomyosin is a major allergen in crustaceans, with 50–98.0% of shrimp-allergic patients showing IgE binding to tropomyosin.^{21–23} Interestingly, the incidents of allergy specific to either freshwater or marine crustacean have been reported.^{3,36} Our findings suggested that irrelevant to the natural habitat, species-specific allergies were not caused by the tropomyosin, but possibly by other minor shellfish allergens.

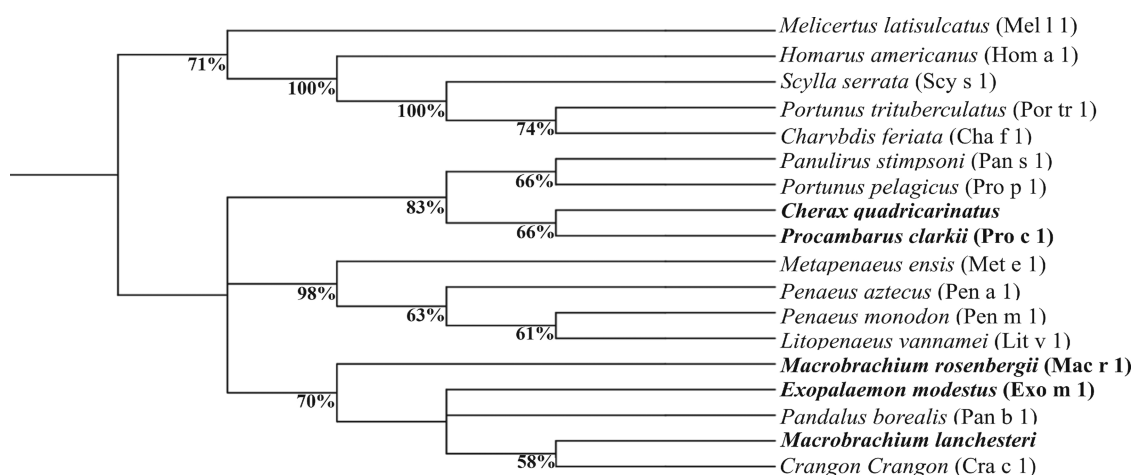


Figure 7. Phylogenetic tree of tropomyosin from *C. quadricarinatus*, *M. lancesteri*, and other crustaceans, based on amino acid sequence comparisons using the neighbor-joining method. The numbers at each branch represent the percentage of bootstrap support from 1000 replicates. Bold text represents freshwater crustaceans.

For instance, Ayuso et al. reported that 2 of 38 sera from subjects with shrimp allergies recognized the myosin light chain, but not tropomyosin.³⁷ Giuffrida et al. reported that 8 of 40 shrimp-allergic patient sera recognized arginine kinase (rPen m 2) and/or sarcoplasmic calcium-binding protein (rPen m 4) but not tropomyosin (rPen m 1).³⁸ Other minor allergens with low sequence similarity may also cause species-specific allergy; for example, hemocyanin which has 54% sequence identity to that of *P. monodon* was reported as a non-cross-reactive allergen in *M. rosenbergii*.⁴ In addition, species-specific allergies may involve the recognition of non-conserved species-specific IgE-binding epitopes. In the case of tropomyosin, our analysis showed that one to seven alterations of amino acid can be observed in the non-conserved IgE-binding epitopes of the freshwater crustaceans, which may contribute to the different IgE reactivity observed in each volunteer. Another example includes profilin, a cross-reactive plant allergen, predicted to contain nine variable epitopes and two conserved epitopes.³⁹ Patients with IgE that reacted to the non-conserved species-specific epitopes displayed an allergic reaction to a lower number of plant foods than patients with IgE reacted to the cross-reactive epitopes of profilin.³⁵ Thus, to better understand species-specific shellfish allergies, more studies must elucidate IgE-binding epitopes and sequence homology of minor allergens in crustaceans.

This work emphasized the role of tropomyosin as a major allergen in crustaceans that is responsible for IgE cross-reactivity among crustacean-allergic patients. Evidence at the primary structure of tropomyosin from freshwater crustacean provided a greater understanding of crustacean allergy and highlights the use of tropomyosin as a universal marker to detect freshwater and marine crustaceans for the purpose of food allergen labeling. These data may be used to predict cross-species allergies, to develop component-resolved diagnostic platform, and to develop a test kit to detect allergen contamination in the food industry.

AUTHOR INFORMATION

Corresponding Author

Chomdao Sinthuvanich – Department of Biochemistry,
Faculty of Science, Kasetsart University, Bangkok 19000,

Thailand; orcid.org/0000-0002-2432-2122;
Email: chomdao.si@ku.th

Authors

Panyarat Laurchan – Department of Biochemistry, Faculty of Science, Kasetsart University, Bangkok 19000, Thailand
Teerasak E-kobon – Department of Genetics, Faculty of Science, Kasetsart University, Bangkok 19000, Thailand
Prapansak Srisapoom – Department of Aquaculture, Faculty of Fisheries, Kasetsart University, Bangkok 19000, Thailand
Sasimanas Unajak – Department of Biochemistry, Faculty of Science, Kasetsart University, Bangkok 19000, Thailand

Complete contact information is available at:
<https://pubs.acs.org/10.1021/acs.jafc.1c00934>

Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

This research is funded by Kasetsart University through the Graduate School Fellowship Program. We thank Tonkla Insawang for advice on LC–MS/MS studies. P.L. acknowledges Narita Thungsatianpun, Duangdow Seachou, Pakawat Jarupund, Chatchai Purananunak, Jarawee Saibua, and Thanakrit Khammeetong for scientific advices. P.L. also thanks Chawang Laurchan, Auchara Laurchan, Kittapart Laurchan, Anchasa Laurchan, and Roongroj Rojvisutthinun for support, advice, and encouragement during this work.

REFERENCES

- (1) Sripramong, C.; Visitsunthorn, K.; Srisuwatthari, W.; Pacharn, P.; Jirapongsananuruk, O.; Visitsunthorn, N. Food sensitization and food allergy in allergic Thai patients from a tertiary care center in Thailand. *Asian Pac. J. Allergy Immunol.* **2019**, DOI: [10.12932/AP-210119-0475](https://doi.org/10.12932/AP-210119-0475).
- (2) Moonesinghe, H.; Mackenzie, H.; Venter, C.; Kilburn, S.; Turner, P.; Weir, K.; Dean, T. Prevalence of fish and shellfish allergy: a systematic review. *Ann. Allergy. Asthma. Immunol.* **2016**, *117*, 264–272.
- (3) Lao-araya, M.; Trakultivakorn, M. Prevalence of food allergy among preschool children in northern Thailand. *Pediatr. Int.* **2012**, *54*, 238–243.
- (4) Piboonpocanun, S.; Jirapongsananuruk, O.; Tipayanon, T.; Boonchoo, S.; Goodman, R. E. Identification of hemocyanin as a

novel non-cross-reactive allergen from the giant freshwater shrimp *Macrobrachium rosenbergii*. *Mol. Nutr. Food Res.* **2011**, *55*, 1492–1498.

(5) Kumjim, S.; Jirapongsananuruk, O.; Piboonpocanun, S. Cloning and characterization of recombinant tropomyosin of giant freshwater shrimp *M. rosenbergii* to determine major allergens causing allergic reactions among shrimp-allergic children. *Asian Pac. J. Allergy Immunol.* **2016**, *34*, 229–235.

(6) Ittiporn, S.; Piboonpocanun, S.; Pacharn, P.; Visitsunthorn, N.; Thongngarm, T.; Jirapongsananuruk, O. Natural resolution of non-anaphylactic shrimp allergy in patients diagnosed 10 years earlier by oral food challenge. *Asian Pac. J. Allergy Immunol.* **2019**, DOI: 10.12932/AP-080119-0470.

(7) Srinroch, C.; Srisomsap, C.; Chokchaichamnankit, D.; Punyarit, P.; Phiriyaikul, P. Identification of novel allergen in edible insect, *Gryllus bimaculatus* and its cross-reactivity with *Macrobrachium* spp. allergens. *Food Chem.* **2015**, *184*, 160–166.

(8) Xie, Y.; Shao, H.; Hu, X.; Hua, X.; Meng, X.; Chen, H. Characterization of systemic allergenicity of tropomyosin from shrimp (*Macrobrachium nipponense*) and anaphylactic reactions in digestive tract. *J. Sci. Food Agric.* **2021**, *101*, 2940–2949.

(9) Zhang, Z.; Li, X.-M.; Xiao, H.; Nowak-Węgrzyn, A.; Zhou, P. IgE-binding epitope mapping of tropomyosin allergen (Exo m 1) from *Exopalaemon modestus*, the freshwater Siberian prawn. *Food Chem.* **2020**, *309*, 125603.

(10) Chen, H.-L.; Mao, H.-Y.; Cao, M.-J.; Cai, Q.-F.; Su, W.-J.; Zhang, Y.-X.; Liu, G.-M. Purification, physicochemical and immunological characterization of arginine kinase, an allergen of crayfish (*Procambarus clarkii*). *Food Chem. Toxicol.* **2013**, *62*, 475–484.

(11) Chen, H. L.; Cao, M. J.; Cai, Q. F.; Su, W. J.; Mao, H. Y.; Liu, G. M. Purification and characterisation of sarcoplasmic calcium-binding protein, a novel allergen of red swamp crayfish (*Procambarus clarkii*). *Food Chem.* **2013**, *139*, 213–223.

(12) Zhang, Y.-X.; Chen, H.-L.; Maleki, S. J.; Cao, M.-J.; Zhang, L.-J.; Su, W.-J.; Liu, G.-M. Purification, characterization, and analysis of the allergenic properties of myosin light chain in *Procambarus clarkii*. *J. Agric. Food Chem.* **2015**, *63*, 6271–6282.

(13) Yang, Y.; Zhang, Y.-X.; Liu, M.; Maleki, S. J.; Zhang, M.-L.; Liu, Q.-M.; Cao, M.-J.; Su, W.-J.; Liu, G.-M. Triosephosphate isomerase and filamin C share common epitopes as novel allergens of *Procambarus clarkii*. *J. Agric. Food Chem.* **2017**, *65*, 950–963.

(14) Jauregui-Adell, J.; Wnuk, W.; Cox, J. A. Complete amino acid sequence of the sarcoplasmic calcium-binding protein (SCP-I) from crayfish (*Astacus leptodactylus*). *FEBS Lett.* **1989**, *243*, 209–212.

(15) Food and Agricultural Organization (FAO) of the United Nations, FishStatJ, a tool for fishery statistics analysis. <http://www.fao.org/fishery/statistics/global-aquaculture-production/query/zh>, 2021.

(16) Khanarnpai, R.; Thaewnon-ngwi, B.; Kongim, B. Genetic variation of *Macrobrachium lanchesteri* (De Man, 1911) in Northeastern Thailand. *Cogent Biol.* **2019**, *5*, 1677126.

(17) Yi, S.; Li, Y.; Shi, L.; Zhang, L.; Li, Q.; Chen, J. Characterization of population genetic structure of red swamp crayfish, *Procambarus clarkii*, in China. *Sci. Rep.* **2018**, *8*, 5586.

(18) Ruethers, T.; Taki, A. C.; Johnston, E. B.; Nugraha, R.; Le, T. T. K.; Kalic, T.; McLean, T. R.; Kamath, S. D.; Lopata, A. L. Seafood allergy: a comprehensive review of fish and shellfish allergens. *Mol. Immunol.* **2018**, *100*, 28–57.

(19) Yu, Z.; Wang, Y.; Li, Z.; Pramod, S. N.; Zhang, L.; Lin, H. Development of ELISA method for detecting crustacean major allergen tropomyosin in processed food samples. *Food. Anal. Method* **2019**, *12*, 2719–2729.

(20) Sakai, S.; Matsuda, R.; Adachi, R.; Akiyama, H.; Maitani, T.; Ohno, Y.; Oka, M.; Abe, A.; Seiki, K.; Oda, H.; Shiomi, K.; Urisu, A. Interlaboratory evaluation of two enzyme-linked immunosorbent assay kits for the determination of crustacean protein in processed foods. *J. AOAC Int.* **2008**, *91*, 123–129.

(21) Pascal, M.; Grishina, G.; Yang, A. C.; Sánchez-García, S.; Lin, J.; Towle, D.; Ibáñez, M. D.; Sastre, J.; Sampson, H. A.; Ayuso, R. Molecular diagnosis of shrimp allergy: efficiency of several allergens to

predict clinical reactivity. *J. Allergy Clin. Immunol. Pract.* **2015**, *3*, 521–529.

(22) Yang, A. C.; Arruda, L. K.; Santos, A. B. R.; Barbosa, M. C. R.; Chapman, M. D.; Galvão, C. E. S.; Kalil, J.; Morato-Castro, F. F. Measurement of IgE antibodies to shrimp tropomyosin is superior to skin prick testing with commercial extract and measurement of IgE to shrimp for predicting clinically relevant allergic reactions after shrimp ingestion. *J. Allergy Clin. Immunol.* **2010**, *125*, 872–878.

(23) Gámez, C.; Sánchez-García, S.; Ibáñez, M. D.; López, R.; Aguado, E.; López, E.; Sastre, B.; Sastre, J.; del Pozo, V. Tropomyosin IgE-positive results are a good predictor of shrimp allergy. *Allergy* **2011**, *66*, 1375–1383.

(24) Zhigang, L.; Xia, L. X.; Liu, F.; Yan, H.; Huang, Z.; Tang, M. J.; Chen, D. W.; Liu, Z. G. The cloning expression, purification and characterization of a fragment of red swamp crayfish major allergen tropomyosin. *J. Jiangxi Norm. Univ.* **2011**, *3*, 280–285.

(25) Lasekan, A. O.; Nayak, B. Effects of buffer additives and thermal processing methods on the solubility of shrimp (*Penaeus monodon*) proteins and the immunoreactivity of its major allergen. *Food Chem.* **2016**, *200*, 146–153.

(26) Luque-García, J. L.; Neubert, T. A. On-membrane tryptic digestion of proteins for mass spectrometry analysis. *Methods Mol. Biol.* **2009**, *536*, 331–341.

(27) Frohman, M. A. Rapid amplification of complementary DNA ends for generation of full-length complementary DNAs: Thermal race. *Methods Enzymol.* **1993**, *218*, 340–356.

(28) Faisal, M.; Vasiljevic, T.; Donkor, O. N. Effects of selected processing treatments on antigenicity of banana prawn (*Fenneropenaeus merguensis*) tropomyosin. *Int. J. Food Sci. Technol.* **2019**, *54*, 183–193.

(29) Kamath, S. D.; Rahman, A. M. A.; Voskamp, A.; Komoda, T.; Rolland, J. M.; O'Hehir, R. E.; Lopata, A. L. Effect of heat processing on antibody reactivity to allergen variants and fragments of black tiger prawn: a comprehensive allergenomic approach. *Mol. Nutr. Food Res.* **2014**, *58*, 1144–1155.

(30) Kamath, S. D.; Abdel Rahman, A. M.; Komoda, T.; Lopata, A. L. Impact of heat processing on the detection of the major shellfish allergen tropomyosin in crustaceans and molluscs using specific monoclonal antibodies. *Food Chem.* **2013**, *141*, 4031–4039.

(31) Faisal, M.; Buckow, R.; Vasiljevic, T.; Donkor, O. N. Effect of simulated digestion on antigenicity of banana prawn (*Fenneropenaeus merguensis*) after high pressure processing at different temperatures. *Food Control* **2019**, *104*, 187–192.

(32) Costa, J.; Villa, C.; Verhoeckx, K.; Cirkovic-Velickovic, T.; Schrama, D.; Roncada, P.; Rodrigues, P. M.; Piras, C.; Martin-Pedraza, L.; Monaci, L.; Molina, E.; Mazzucchi, G.; Mafrá, I.; Lupi, R.; Lozano-Ojalvo, D.; Larre, C.; Klueber, J.; Gelencser, E.; Bueno-Díaz, C.; Díaz-Perales, A.; Benede, S.; Bavaro, S. L.; Kuehn, A.; Hoffmann-Sommergruber, K.; Holzhauser, T. Are Physicochemical properties shaping the allergenic potency of animal allergens. *Clin. Rev. Allergy Immunol.* **2021**, DOI: 10.1007/s12016-020-08826-1.

(33) Ayuso, R.; Lehrer, S. B.; Reese, G. Identification of continuous, allergenic regions of the major shrimp allergen Pen a 1 (tropomyosin). *Int. Arch. Allergy Immunol.* **2002**, *127*, 27–37.

(34) Matsuo, H.; Yokooji, T.; Taogoshi, T. Common food allergens and their IgE-binding epitopes. *Allergol. Int.* **2015**, *64*, 332–343.

(35) Liu, G.-Y.; Mei, X.-J.; Hu, M.-J.; Yang, Y.; Liu, M.; Li, M.-S.; Zhang, M.-L.; Cao, M.-J.; Liu, G.-M. Analysis of the allergenic epitopes of tropomyosin from mud crab using phage display and site-directed mutagenesis. *J. Agric. Food Chem.* **2018**, *66*, 9127–9137.

(36) Jirapongsananuruk, O.; Sripamong, C.; Pacharn, P.; Udompunturak, S.; Chinratanaipit, S.; Piboonpocanun, S.; Visitsunthorn, N.; Vichyanond, P. Specific allergy to *Penaeus monodon* (seawater shrimp) or *Macrobrachium rosenbergii* (freshwater shrimp) in shrimp-allergic children. *Clin. Exp. Allergy* **2008**, *38*, 1038–1047.

(37) Ayuso, R.; Grishina, G.; Bardina, L.; Carrillo, T.; Blanco, C.; Ibáñez, M. D.; Sampson, H. A.; Beyer, K. Myosin light chain is a novel shrimp allergen, Lit v 3. *J. Allergy Clin. Immunol.* **2008**, *122*, 795–802.

(38) Giuffrida, M. G.; Villalta, D.; Mistrello, G.; Amato, S.; Asero, R. Shrimp allergy beyond tropomyosin in Italy: clinical relevance of arginine kinase, sarcoplasmic calcium binding protein and hemocyanin. *Eur. Ann. Allergy Clin. Immunol.* **2014**, *46*, 172–177.

(39) Radauer, C.; Willeroider, M.; Fuchs, H.; Hoffmann-Sommergruber, K.; Thalhamer, J.; Ferreira, F.; Scheiner, O.; Breiteneder, H. Cross-reactive and species-specific immunoglobulin E epitopes of plant profilins: an experimental and structure-based analysis. *Clin. Exp. Allergy* **2006**, *36*, 920–929.