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ORIGINAL ARTICLE

Cloning and characterization of recombinant tropomyosin of giant freshwater shrimp *M. rosenbergii* to determine major allergens causing allergic reactions among shrimp-allergic children

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Abstract

Background: Seawater and freshwater shrimp are some of the most common causes of food allergy among children in Thailand. Tropomyosin has been reported as a major allergen for shrimp allergic populations around the world. Despite a high number of shrimp-allergic Thai children, however, it is unknown whether shrimp tropomyosin is a major cause of allergic reactions.

Objectives: To clone and characterize tropomyosin of giant freshwater shrimp *Macrobrachium rosenbergii* (*Mr*) and determine whether this tropomyosin is a major cross-reactive allergen for Thai children with shrimp allergy.

Methods: Recombinant shrimp *Mr* tropomyosin (Mac r1.0101) was expressed in yeast *Pichia pastoris*. Secondary structure composition of purified recombinant Mac r1.0101 was determined by Circular Dichroism. IgE reactivity was examined by immunoblot, direct binding ELISA and inhibition of IgE ELISA using serum from shrimp-allergic children.

Results and Conclusion: The amino acid sequence of Mac r1.0101 showed 2 polymorphic amino acids (F44 and S45) indicating a variant of tropomyosin. Purified recombinant Mac r1.0101 obtained a nature-like α-helix structure which can be bound by serum-specific IgE. The binding affinity of serum-specific IgE to Mac r1.0101 based on the IC50 value was ~1.8 ng/ml. Ten of 13 shrimp-allergic Thai children had serum-specific IgE against Mac r1.0101, but at different levels. Results of the inhibition of specific IgE using Mac r1.0101 showed that 7 of the tested serum samples also had specific IgE against other shrimp allergens in addition to IgE against Mac r1.0101. Tropomyosin therefore appears to be a major cross-reactive allergen for Thai children who are allergic to both seawater and giant freshwater shrimp.

Keywords: tropomyosin, cross-reactive allergen, shellfish allergy, shrimp allergy, food allergy

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Introduction

Shellfish, especially shrimp, is one of the eight groups of food that causes allergic reactions after ingestion by 10% of the world population.[1,2] Giant freshwater shrimp (*Macrobrachium rosenbergii, Mr*) is a river prawn that is popularly consumed and exported to countries around the world. Recently, allergic reactions after the ingestion of shrimp *Mr* among Thai children has been reported.[3,4]

To date, six shrimp allergens have been identified and characterized.[5] The major cross-reactive allergen of shrimp in many populations is tropomyosin, identified in many shrimp species such as *Macrobrachium rosenbergii, Penaeus (Farfantepenaeus) aztecus* (pen a 1), and *Penaeus monodon (Pm)* (pen m 1).[2,6,7] Arginine kinase, a phosphotransferase with a MW of 40 kDa, is the 2nd cross-reactive allergen of different shrimp species such as pen m 2 of shrimp *Pm*[8] and Lit v 2 of seawater pacific white leg shrimp *Litopenaeus vannamai (Lv)*.[9] The 3rd allergen, the 20-kDa myosin light-chain, is identified as Lit v 3 of shrimp *Lv*.[10] The 4th allergen, the 20-kDa sarcoplasmic calcium-binding protein, is identified in shrimp *Pm* and as Lit

v 4 in shrimp *Lv*.[11,12] Recently, the first novel non-cross-reactive allergen, hemocyanin, of shrimp *Mr*, was reported as a major non-cross-reactive allergen among Thai children with shrimp *Mr* allergy.[13]

Interestingly, besides hemocyanin, the other major shrimp allergens have never been determined, especially cross-reactive allergens among shrimp-allergic Thai patients. It is therefore necessary to identify and characterize major shrimp allergens sensitized among shrimp-allergic Thai children as shrimp is one of the most popularly consumed foods as well as the most sensitized food in Thailand.[4] In this study, we cloned and expressed tropomyosin from shrimp *Mr* and used recombinant allergens to determine whether it is a major cross-reactive allergen among Thai patients with shrimp allergy.

Methods

Shrimp allergic patients

Serum was prepared from blood samples of shrimp-allergic patients who had positive reactions to oral-challenge with both shrimp *Mr* and *Pm*, as fully described elsewhere.[4] The SPT, oral-food challenge protocols, and use of serum were approved by the Institutional Review Board at Siriraj Hospital (SiEc038/2005).[4]

Cloning and expression of recombinant tropomyosin of Mr shrimp

Live giant freshwater shrimp *Mr* were purchased from local markets in Bangkok, Thailand. The heads and shells were removed before minced abdominal muscle was homogenized in TRIzol reagent (Invitrogen, USA) at a ratio of 0.1:1 (w/v) and incubated at room temperature (RT) for 5 min. Then, chloroform was added at a ratio of 0.2:1 (v/v), shaken for 15 sec and incubated at RT for 3 min. The reaction was centrifuged at 12,000 x g at 4°C for 15 min. Total RNA was precipitated from the upper aqueous phase by adding ice-cold isopropanol (1/2 volume of aqueous phase). After incubation at RT for 5-10 min, the mixture was centrifuged at 12,000 x g at 4°C for 15 min. The pellet was washed with ice-cold 70% ethanol, centrifuged and dried before being dissolved in DEPC-treated water. Tropomyosin cDNA was amplified by PCR using the forward primer 5'-GAATTC**ATG**GACGCCATCAAGAAGAAGATG-3', the reverse primer 5'-CATTCAGCGAACTGTCTGGC-TAC**TAA**GCGGCCGC 3' and *pfu* Taq polymerase (NEB, USA). Tropomyosin cDNA digested with EcoRI/NotI was ligated with pPICZαA. The DNA sequence of tropomyosin cDNA in pPICZαA was obtained by sequencing (Macrogen, Korea). The shrimp *Mr* tropomyosin (Mac r1.0101) cDNA sequence was deposited at GenBank (nucleotide accession# GU369816, protein accession# D3XNR9). Plasmid pPICZαA-Mac r1.0101 was electroporated into yeast *Pichia* strain KM71 (Invitrogen, USA). Yeast cells were induced to express Mac r1.0101 in media containing 1% (v/v) methanol for 4 days. The supernatant containing Mac r1.0101 was collected and stored at -30°C.

Purification of recombinant tropomyosin by anion exchange and size-exclusion columns

The supernatants of yeast cultures were dialyzed in 20 mM Tris-HCl pH 8 before being passed through a DEAE-Sephacel anion column (GE Healthcare, USA). Mac r1.0101 was eluted in 20 mM Tris-HCl pH 8 containing 200 and 300 mM NaCl before being purified through a Superdex 75 10/300 GL size-exclusion column (GE Healthcare, USA). Then, Mac r1.0101 was precipitated with ice-cold trichloroacetic acid (TCA) at a final concentration of 30% (v/v) for 1 h on ice before it was centrifuged at 17,210 x g for 15 min at RT and then air-dried and dissolved in 10 mM Tris-HCl pH 7.5, 500 mM $NaHCO_3$. The protein concentration was determined by Bicinchoninic acid (BCA) protein assay (Pierce, USA).

Circular dichroism

Purified Mac r1.0101 was dissolved in 10 mM Tris-HCl pH 7.5, 100 mM $NaHCO_3$ at a final concentration of 300 μg/ml. CD spectra were recorded from 260 to 190 nm at a path length of 0.02 cm and a resolution of 1 nm, with a scan speed of 50 nm/min on a Jasco J-700 spectropolarimeter. CD spectra of Mac r1.0101 were analyzed for secondary and tertiary structure using the program *CDPro*.

SDS-PAGE and Immunoblot analysis of recombinant tropomyosin

For SDS-PAGE analysis, 14% and 6% polyacrylamide gels were used for the resolving and stacking gels, respectively. Ten micrograms of recombinant Mac r1.0101 was mixed with 4x sample buffer (200 mM Tris HCl pH 6.8, 8% (w/v) SDS, 40% (v/v) glycerol, 10% (v/v) β-mercaptoethanol and 0.8% (w/v) bromphenol blue) in a 3:1 ratio. The polymerized gel was submerged in 1x running buffer (25 mM Tris-HCl, pH 8.2, 192 mM glycine and 0.1% (w/v) SDS) in electrophoresis apparatus. The samples were incubated at 95°C for 5 minutes and loaded into the wells. The molecular weight marker (Precision Plus Protein Dual Color, BioRad) was used. An electrical field was applied at 26 mA per gel. The SDS gel was stained overnight with gentle shaking in 50 ml of colloidal coomassie blue G-250 (0.08% (w/v) coomassie blue G250, 8% (w/v) ammonium sulfate, 1.6% (v/v) phosphoric acid and 20% methanol). Then, the SDS gel was de-stained in distilled water.

For the immunoblot assay, the process was the same as SDS-PAGE without staining. The molecular weight marker (Page Ruler Prestained Ladder, Thermo Scientific) was used in the immunoblot. Resolved Mac r1.0101 in 14% SDS-PAGE gels was electro-transferred onto a nitrocellulose membrane before being submerged in buffer A [PBS (137.9 mM NaCl, 2.7 mM KCl, 8 mM $Na_2HPO_4.2H_2O$, 1.76 mM KH_2PO_4, pH 7.4), 3% skim milk] for 1 hr. Serum of shrimp *Pm-Mr*-allergic patient ors non-allergic donors was diluted 1:8 in buffer A before being incubated with membranes at 4°C overnight. The membranes were washed with PBS buffer, 0.2% Tween-20 before being incubated with 1:10,000 HRP-labeled mouse IgG anti-human IgE (KPL, USA) at RT for 1 h. After washing, the membranes were incubated with a chemiluminescence HRP substrate (Millipore, USA) for 5 min. The emitted signals were captured by x-ray film.

Total shrimp extract

Minced shrimp abdominal muscle was stirred in PBS at 4°C for 1 h and centrifuged at 17,210 x g at 4°C for 30 min.

The supernatant was filtered through 0.45 µM and protease inhibitor (PMSF) was added at a final concentration of 1 mM. Total shrimp extract was stored at -80°C. Total protein concentration was determined by the BCA protein assay.

Direct binding ELISA

Total shrimp extract or Mac r1.0101 was diluted in PBS and added to each well at 500 ng/well in a 96-well plate and incubated at 4°C overnight. The plate was washed in buffer A (PBS, 0.05% Tween 20) and incubated with buffer B (PBS, 3% skim milk, 0.05% Tween 20) at RT for 1 h. Serum samples of 13 shrimp *Pm-Mr*-allergic donors and non-allergic donors were diluted 1:8 in buffer B and added to the plate and incubated at RT for 2 h. HRP-labeled mouse IgG anti-human IgE was diluted 1:10,000 in buffer B and added to the plate and incubated at RT for 1 h. The bound antibodies were detected using TMB substrate (3,3'5, 5-tetramethylbenzidine) (KPL, USA). The $OD_{650\ nm}$ was measured at 25 min.

Inhibition ELISA

Inhibition ELISA was carried out in this report with the 3 following purposes.

A) To determine the binding affinity of specific IgE against Mac r1.0101, sera of 6 shrimp *Pm-Mr* allergic patients who showed a high $OD_{650\ nm}$ value of specific IgE binding to coated Mac r1.0101 were used. The diluted native tropomyosin in PBS was coated at 500 ng/well in a 96-well plate and incubated at 4°C overnight. In a separate tube, serum diluted 1/8 was mixed with various amounts of either purified Mac r1.0101 (inhibitor) or buffer A (PBS, 3% skim milk, 0.05% Tween 20) (no inhibitor) and incubated at 4°C with rotating overnight. The serum-allergen (SA) mixture was centrifuged at 13,000 x g for 10 min and the SA supernatant was further used. The plate was washed with buffer B (PBS, 0.05% Tween 20) and incubated with buffer A at RT for 1 h. The washed plate was incubated with the SA supernatant at RT for 2 h. The plate was washed and incubated with diluted 1:10,000 HRP-labeled mouse IgG anti-human IgE at RT for 1 h. The washed plate was incubated with TMB substrate (3,3'5,5-tetramethylbenzidine). The $OD_{650\ nm}$ was measured at 25 min.

B) To estimate a level of specific IgE against Mac r1.0101 in individual serum, the sera of 13 shrimp *Pm-Mr*-allergic patients was used. The 96-well plate was coated with 500 ng/well of PBS-diluted total shrimp *Mr* extract and incubated at 4°C overnight. In a separate tube, serum diluted 1/8 was mixed with 2.4 µg/ml of purified Mac r1.0101 (inhibitor) or buffer A (no inhibitor) and incubated at 4°C with rotating overnight. The rest of the protocol was the same as that described in A.

C) To determine IgE cross-reactivity with *Pm* tropomyosin, serum samples of 6 tested patients were used. The 96-well plate was coated with 500 ng/well of PBS-diluted total shrimp *Mr* or *Pm* extract and incubated at 4°C overnight. In a separate tube, serum diluted 1/8 was mixed with 2.4 µg/ml of purified Mac r1.0101 (inhibitor) or buffer A (no inhibitor) and incubated at 4°C with rotating overnight. The rest of protocol was the same as that described in A.

The inhibition was calculated as the percent reduction in absorbance compared to the uninhibited value. The results were calculated as a mean average and standard error of the percent inhibition obtained with different sera. The IC50 was extrapo lated from a regression curve.

Results

This is the first report to successfully clone and express recombinant tropomyosin of *M. rosenbergii* (nucleic acid: GU369816; protein uniprot: D3XNR9) using the yeast *P. pastoris*. The results of DNA sequencing confirmed the identity of tropomyosin cDNA by comparison to that of tropomyosin of *M. rosenbergii* (accession number: ADC55380.1) from the NCBI databases. Interestingly, it appears that the amino acid sequence deduced from the sequence of Mac r1.0101 cDNA has 2 polymorphic amino acids at phenylalanine 44 (F44) and serine 45 (S45) (**Figure 1**). Secreted Mac r1.0101 in yeast media was purified through a weak-anion exchange column and a size exclusion column. Mac r1.0101 had a molecular weight (MW) of ~36 kDa compared with molecular weight markers, as shown in the results of SDS-PAGE gels (**Figure 2A**, lane M, 1) and immunoblots (**Figure 2A**, lane M1, 2, 3). The estimated yield of Mac r1.0101 was 0.4 g/L.

The secondary structure analysis of CD spectra, scanned from 190 to 260 nm, of the Mac r1.0101 suggested that the protein had a secondary structure of an α-helix structure, which showed minima at 222 and 208 nm (**Figure 2B**). Further analysis of the secondary structure compositions of Mac r1.0101 showed that it was composed of 68.2% helix, 2.4% strand, 9.5% turn and 20% un-ordered structure. To determine the binding affinity of specific IgE to Mac r1.0101, an inhibition ELISA was carried out. The results showed specific IgE bound to Mac r1.0101 with an IC50 of ~1.8 ng/ml (**Figure 2C**).

Individual patients may be sensitized to a number of allergens from one shrimp species. It is possible therefore that an individual who is allergic to two shrimp species would have several different specific IgE antibodies in their serum. As a result, to determine the distribution of these specific IgE antibodies, direct binding ELISA was performed using the sera of individual patients. The results of the direct binding of serum IgE to the total extract of both shrimp *Mr* and *Pm* showed that 3 patients (P1,P2,P8) had similar OD_{650} values of IgE-binding allergens in the total extract, while 4 patients (P3, P7,P9,P12) showed an OD_{650} value of IgE-binding allergens in the total extract of shrimp *Mr* (**Figure 3**, no-filled column) which was 15% higher than that of shrimp *Pm* (**Figure 3**, filled column). Moreover, 5 patients (P4-P6,P10,P11) showed an OD_{650} value of IgE-binding allergens in the total extract of shrimp *Mr* that was 25-35% higher (**Figure 3**, non-filled column) than that of shrimp *Pm* (**Figure 3**, filled column). Interestingly, one patient (P13) showed an OD_{650} value of IgE-binding allergens in the total extract of shrimp *Mr* which was 50% higher than that of shrimp *Pm*, while one patient showed an OD_{650} value of IgE-binding allergens that was less than 3 OD values above that of blank and non-atopic individuals (**Figure 3**).

To determine whether tropomyosin is one of the major allergens for shrimp-allergic Thai patients, the direct binding ELISA of serum IgE to Mac r1.0101 was carried out. When the direct binding of serum IgE to Mac r1.0101 was tested,

Figure 1. Amino acid sequence alignment of Mac r1.0101. Note: ** indicates polymorphic amino acids (H44F, N45S); nucleotide accession# GU369816, protein accession# D3XNR9.

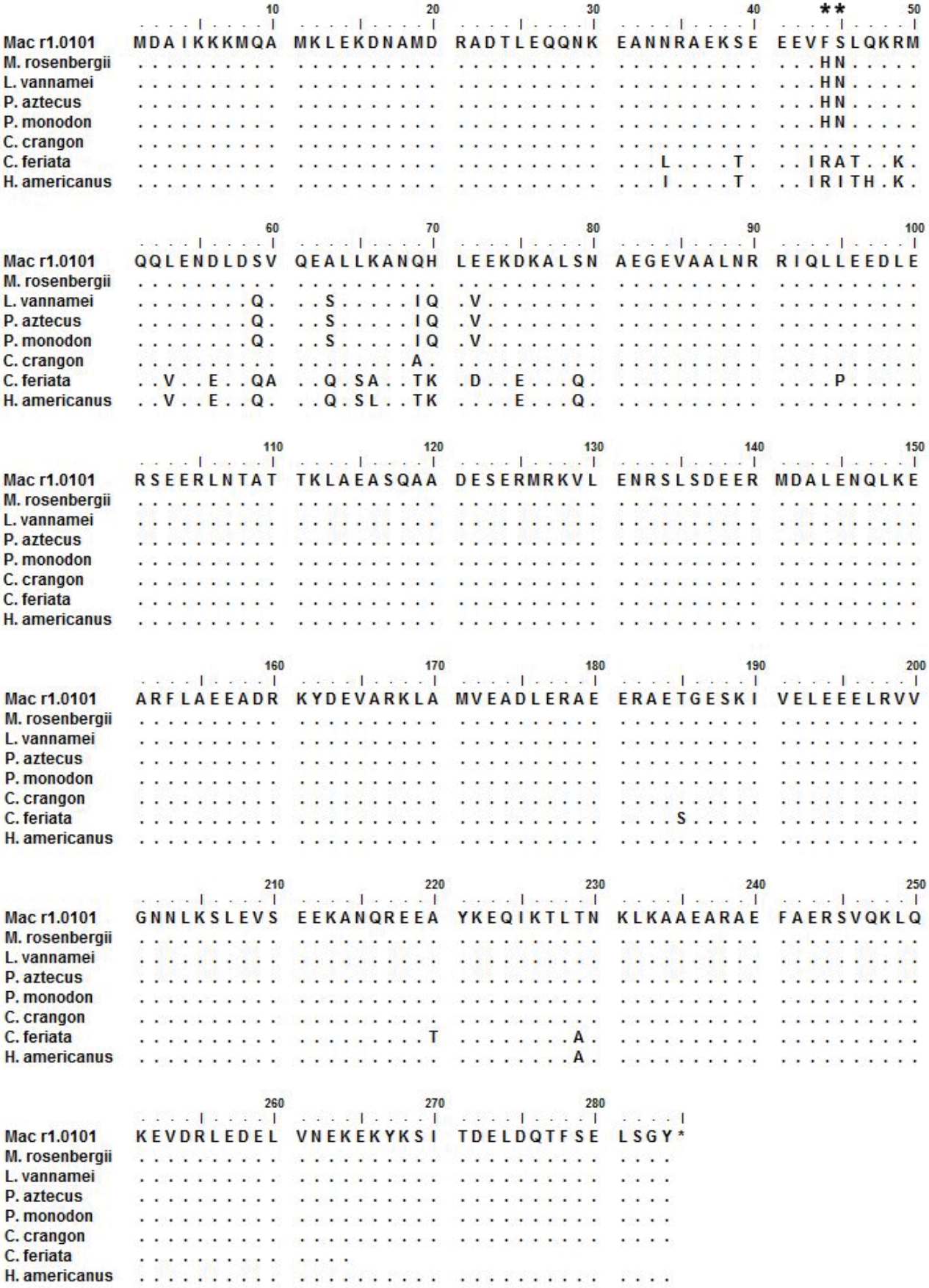
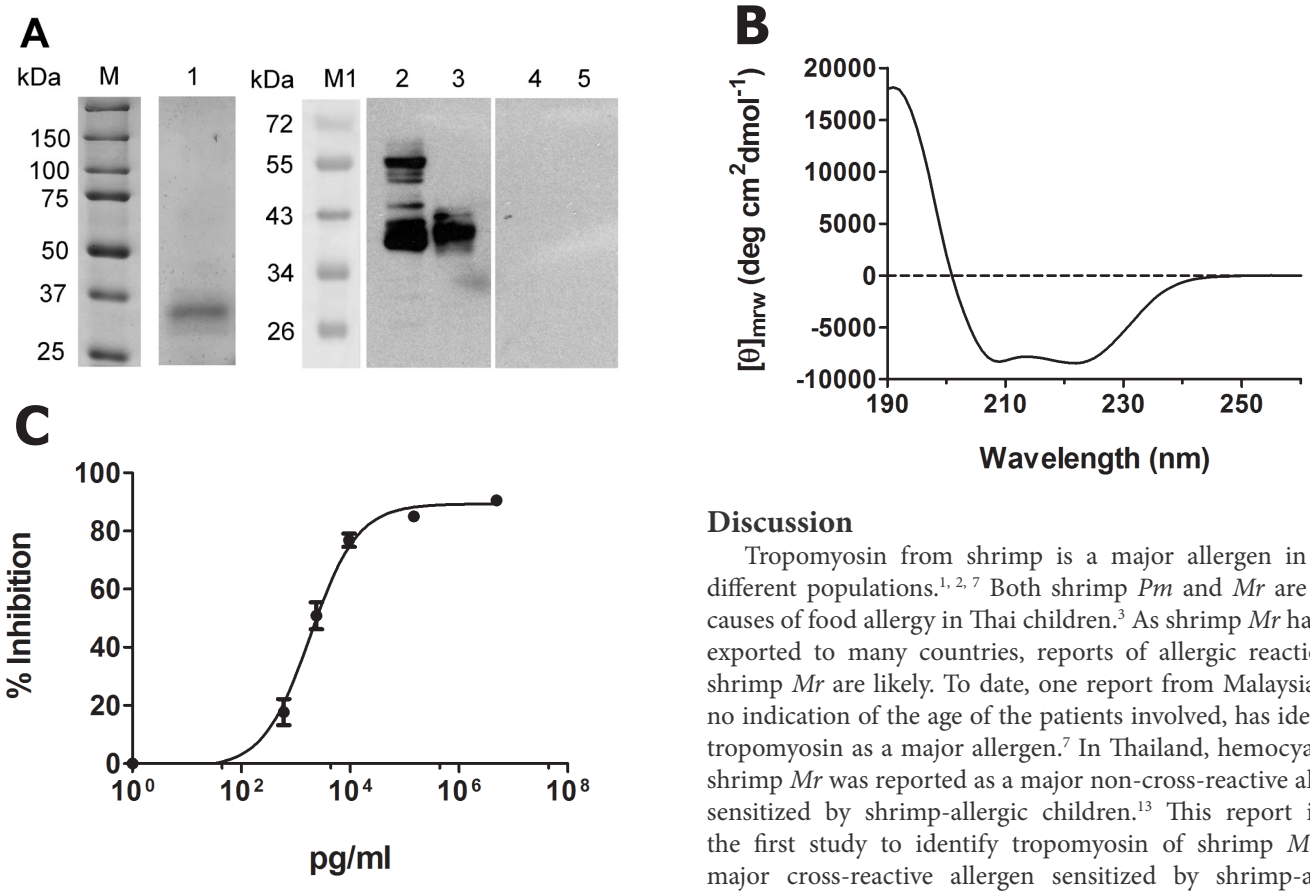


Figure 2. Characterization of Mac r1.0101. A) SDS-PAGE and Immunoblot, B) Analysis of CD spectra and C) Determination of IgE binding affinity. Note: A) lane M = MW marker (Precision Plus Protein Dual Color), M1= MW marker (Page Ruler Prestained Ladder), lane 1,3,4 = purified Mac r1.0101, lane 2,4 = total shrimp *Mr* extract. Serum of patient (P1) was incubated with lane 2 and 3, serum of non-allergic donor was incubated with lane 4 and 5. B) CD spectra were scanned from 190 to 260 nm showing minima at 222 and 208 nm. C) a value of binding affinity of serum specific IgE against Mac r1.0101. Each point represents a mean average with standard error of 6 individual serum selected from 13 patients included in this study.



9 patients (P1-6,P8,P10-12) (**Figure 4**) showed an IgE binding value of OD_{650} which was equal to or higher than that of serum IgE-bound allergens in the total extract of shrimp *Mr* (**Figure 3**, no-filled column). Interestingly, 3 patients (P7,P9,P13) showed no serum IgE bound to the Mac r1.0101 (**Figure 4**).

For the further estimation of a level of specific IgE against the Mac r1.0101, serum IgE was mixed with the Mac r1.0101 overnight before unbound IgE was incubated with coated allergens in the total extract of shrimp *Mr*. The results showed that 2 patients (P1,3) had <15% IgE-bound coated allergens, while 7 patients (P5,6,7-9,11-13) had >70% IgE-bound coated allergens (**Figure 5A**). Four other patients (P2,4,6,10) had 30-60% IgE-bound coated allergens (**Figure 5A**).

To determine IgE cross-reactivity with *Pm* tropomyosin, the serum of 6 patients (P1,4-6,11,12) was mixed with the Mac r1.0101 overnight before unbound IgE was incubated with coated allergens in total extract of shrimp *Pm*. The results showed 3 patients (P1,4,6) with <35% IgE-bound coated *Pm* allergens (**Figure 5B**). One patient (P5) had ~55% IgE-bound *Pm* allergens, while 2 patients (P11,12) had >-67% IgE-bound *Pm* allergens (**Figure 5B**).

Discussion

Tropomyosin from shrimp is a major allergen in many different populations.[1, 2, 7] Both shrimp *Pm* and *Mr* are major causes of food allergy in Thai children.[3] As shrimp *Mr* has been exported to many countries, reports of allergic reactions to shrimp *Mr* are likely. To date, one report from Malaysia, with no indication of the age of the patients involved, has identified tropomyosin as a major allergen.[7] In Thailand, hemocyanin of shrimp *Mr* was reported as a major non-cross-reactive allergen sensitized by shrimp-allergic children.[13] This report is also the first study to identify tropomyosin of shrimp *Mr* as a major cross-reactive allergen sensitized by shrimp-allergic Thai children. Recombinant tropomyosin of shrimp *Mr* (Mac r1.0101) was cloned and expressed, and purified Mac r1.0101 was used to determine whether tropomyosin is one of the major allergens causing allergic reactions among Thai children. After being refolded and purified, the Mac r1.0101 obtained secondary structure compositions of α-helix proteins similar to recombinant tropomyosin of shrimp Penaeus aztecus (rPen a 1).[14] Moreover, the results of the immunoblot indicated that the Mac r1.0101 had an estimated MW of 36 kDa when compared to the molecular weight markers. The alignment of the amino acid sequence of Mac r1.0101 from 10 cDNA clones showed 2 altered amino acids at phenylalanine 44 (F44) and serine 45 (S45). Interestingly, when compared with the amino acid sequence of tropomyosin from different shrimp species, the region spanning amino acid residues K30 to N80 was shown to not be conserved and several polymorphic residues were observed in tropomyosin of other shrimp species such as *L. vannamai, P. monodon and P. aztecus*, which are in different groups in the phylogenetic tree.[2] However, based on the phylogenetic tree, the shrimp *M. rosenbergii* and *C. crangon* are in the same group;[2] thus, the amino acid sequence of Mac r1.0101 is identical to that of *C. crangon*, except at alanine 69

Figure 3. Serum IgE binding to allergens in total extract of shrimp Mr compared to allergens in total extract of shrimp Pm. A mean average of duplicated experiments was showed. Note: individual serum of 13 shrimp *Pm-Mr* allergic patients; dot-line = 3 OD above OD value of blank and non-atopic individuals; □ = total extract of shrimp *Mr*; ■ = total extract of shrimp *Pm*, C1 = blank, C2 = non-atopic individuals.

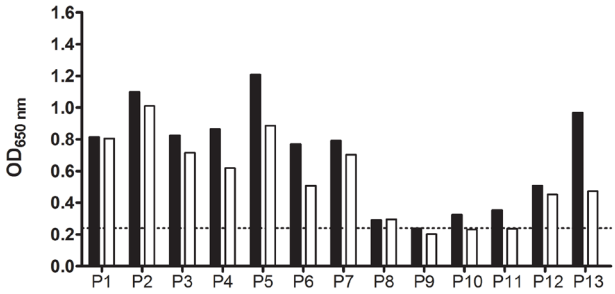


Figure 4. Serum IgE binding to Mac r1.0101. A mean average of duplicate experiments was showed. Note: individual serum of 13 shrimp *Pm-Mr* allergic patients; dot-line = 3 OD above OD value of blank and non-atopic individuals; ■ = Mac r1.0101.

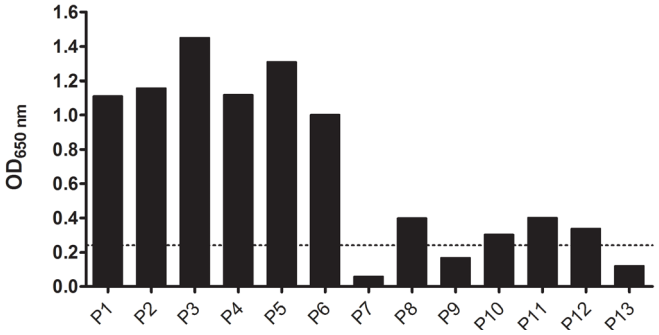


Figure 5. Binding of serum IgE to coated allergens in total extract of shrimp A) Mr and B) Pm after preincubating with 2.4 µg/ml of Mac r1.0101. A mean average of duplicated experiments was showed. Note: individual serum of 6 shrimp *Pm-Mr* allergic patients.

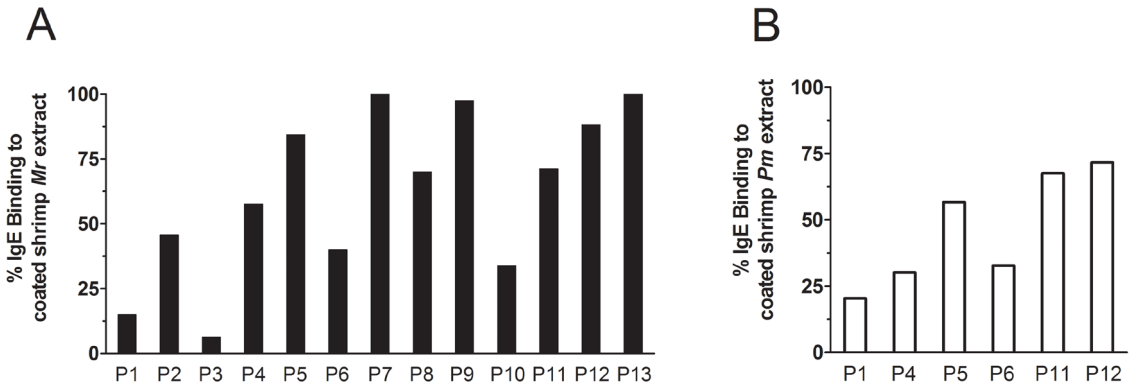


Table 1. Demographic data and symptoms upon challenges to both M. rosenbergii (Mr) and P. monodon (Pm).

Patient number	Sex	Age	Symptom after challenge (Mr)	Symptom after challenge (Pm)	Anaphylaxis
1	M	13	LSI, AP, A	LI, U, R, EI	to Mr
2	F	8	LSI, NV, AP, A	LSI, U	to Mr
3	M	5	LSI, U	LSI, U	-
4	M	7	LSI, U	LSI, U	-
5	M	8	LSI, U, R	LSI, U	-
6	M	13	LSI, U	LSI, U	-
7	M	10	LSI, U, NV, R, A	LSI, U, R	to Mr
8	M	5	LSI	U, AG	-
9	M	11	LSI, U	U	-
10	M	9	LSI	LSI	-
11	M	6	LSI, U	LSI, U	-
12	M	10	LSI, U	LSI	-
13	F	8	LSI, AG, R, EI	LSI, U, R, EI	-

A, anaphylaxis; AG, angioedema; AP, abdominal pain; EI, eye itching; LI, lip itching; LSI, lip swelling/itching; NV, nausea/vomiting; R, rhinitis; U, urticaria.

(A69) (**Table 1**). Because our cDNA was synthesized with *pfu* Taq polymerase, which has a proof reading property, any single point mutation would be rare. It is likely that variants with F44 and S45 could be found in nature. As shown in the results, these 2 polymorphic residues did not reduce the IgE affinity of Mac r1.0101 (IC_{50} of ~1.8 ng/ml) indicating that both polymorphic residues may not be a region of the IgE epitope. Moreover, the IC_{50} value of Mac r1.0101 is ~50-fold better than that of rPen a 1.[14] Thus, the prepared Mac r1.0101 could be used as a diagnostic material to measure serum-specific IgE in shellfish allergy.

Sera IgE used in this study were from 13 pediatric patients allergic to both shrimp *Mr* and *Pm*. When a level of specific IgE to allergens of shrimp *Mr* was compared with that to allergens of shrimp *Pm*, 11 of the 13 patients had a level of specific IgE to allergens of shrimp *Mr* which was higher than to allergens of shrimp *Pm* (**Figure 3**). When determining whether IgE against tropomyosin is a predominant antibody in a repertoire of IgE against shrimp allergens in 13 patients, the results (**Figure 4**) suggested that a repertoire of IgE against shrimp allergens in 9 patients would be IgE against tropomyosin. Moreover, 6 of the 9 patients (P1-6) had a high level of specific IgE against tropomyosin, as indicated by a high value of OD_{650}, indicating that IgE against tropomyosin would be the predominant antibody. However, 4 other patients (P8-12) had a low level of specific IgE against tropomyosin (**Figure 4**),

suggesting that specific IgE against tropomyosin may not be the predominant IgE in the serum of P8-12. Interestingly, when the results of direct binding ELISA were compared (**Figure 3, 4**), we also observed an OD_{650} value of IgE-bound Mac r1.0101 which was higher than that of IgE-bound coated allergens of total shrimp *Mr* extract in 5 patients (P1-5). This may be due to that amount of coated Mac r1.0101 (500 ng/well) being likely to be several folds higher than the amount of tropomyosin in 500 ng of total extract.

To further confirm the predominant IgE against tropomyosin in a repertoire of IgE against shrimp allergens in patients P1-6, inhibition ELISA was performed (**Figure 5A**). The results showed that 2 patients (P1,P3) had <15% IgE-bound coated allergens, while 2 patients (P2,P6) had 40% IgE-bound coated allergens. The results suggest that >60% of serum-specific IgE is against tropomyosin. However, 2 patients (P4,P5) had >60% IgE-bound coated allergens. This suggests that the level of specific IgE against other allergens may be higher than specific IgE against tropomyosin in these 2 patients. Interestingly, we observed that another 6 patients (P7-9,11-13) had <0.4 OD_{650} of IgE-bound Mac r1.0101 (**Figure 4**) and >70% of IgE-bound coated allergens (**Figure 5A**). This suggests the predominance of IgE specific to other shrimp allergens such as arginine kinase[8] and hemocyanin[13] in these patients. These findings indicate that allergic individuals may have a diverse repertoire of specific IgE against shrimp allergens.

To determine the cross-reactivity of serum-specific IgE against *Mr* tropomyosin with *Pm* tropomyosin, selected sera (P1,P4,P5,P6,P11,P12) containing specific IgE against tropomyosin were mixed and incubated overnight with 2.4 µg/ml of Mac r1. Unbound IgE were incubated with coated allergens in the total extract of shrimp *Pm* (**Figure 5B**). Because specific IgE bound Mac r1.0101 with a very high affinity, in individual serum containing a high percentage of IgE against tropomyosin, the results of inhibition would show a small percentage of IgE bound to coated allergens. As the results showed, in the same patients, the pattern of IgE-bound coated allergens extracted from shrimp *Pm* was similar to that of IgE-bound coated allergens extracted from shrimp *Mr* (**Figure 5A, B**). However, the percent of IgE-bound coated *Pm* allergens was lower than that of IgE-bound coated *Mr* allergens (**Figure 5A, B**). The results indicate that of the 3 patients of interest (P1, P4, P6), specific IgE against tropomyosin would be the predominant IgE antibody since overnight incubation could deplete specific IgE against tropomyosin by >50%. Moreover, in another 3 patients (P5,P11,P12), specific IgE to other cross-reactive shrimp allergens would be the predominant IgE antibody.

In this study, 10 of the 13 patients had specific IgE against tropomyosin but at different levels. However, as demonstrated, the IgE affinity of Mac r1.0101 was very strong, thus even with low level of IgE, Mac r1.0101 could likely cause clinical symptoms.[15] Also allergic reactions of these patients may occur via specific IgE against different shrimp allergens. Based on these findings, diagnosis with tropomyosin could identify 76% of shrimp-allergic patients. Therefore, it may be more prudent to include other shrimp allergens in diagnosis, especially, if information is needed for allergen-specific immunotherapy.[16]

In conclusion, recombinant tropomyosin Mac r1.0101 of shrimp *Mr* has been cloned and expressed. The Mac r1.0101 had a nature-like structure and interacted with serum-specific IgE with high affinity. Tropomyosin is a major cross-reactive allergen among the tested shrimp-allergic Thai children.

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