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Detecting Allergens From Black Tiger Shrimp *Penaeus monodon* That Can Bind and Cross-link IgE by ELISA, Western Blot, and a Humanized Rat Basophilic Leukemia Reporter Cell Line RS-ATL8

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Purpose: Black tiger shrimp *Penaeus monodon* is one of the common causes of shellfish allergy that is increasing worldwide. One of the important problems in the management of shellfish allergy is the lack of accurate diagnostic assay because the biological and immunological properties of allergens in black tiger shrimp have not been well characterized. This study aims to detect proteins with the ability to bind and cross-link immunoglobulin E (IgE) from black tiger shrimp by enzyme-linked immunosorbent assay (ELISA), Western blot, and a humanized rat basophilic leukemia reporter cell line RS-ATL8. **Methods:** Sera from shrimp allergic subjects were subjected to ELISA and Western blots using raw or cooked shrimp extract as antigens. Pooled sera were used to sensitize the RS-ATL8 reporter cell line and cells were activated by shrimp extract. Eluted protein extracts separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) were tested on the RS-ATL8 cell line and subjected to mass spectrometry to identify potential candidate allergens. **Results:** Allergic sera reacted stronger to raw shrimp extract than cooked shrimp extract (P=0.009). Western blot demonstrated that major IgE reactivity protein bands were at 32-39 kDa and 91-230 kDa in both raw and cooked shrimp extracts. The eluted protein bands at the molecular weight of 38 and 115 kDa from raw shrimp extract induced IgE cross-linking as assayed by the RS-ATL8 cell line. These protein bands were subjected to mass spectrometry for analysis. Ubiquitin-activating enzyme and crustacyanin were identified as potential candidate novel shrimp allergens. **Conclusions:** The RS-ATL8 reporter cell line can be used to identify potential new shrimp allergens that can functionally cross-link IgE and induce mast cell degranulation.

Key Words: *Penaeus monodon*; allergens; IgE; humanized basophilic leukaemia reporter cell

INTRODUCTION

The consumption of seafood increases worldwide and crustacean shellfish is one of the major causes of food allergy. In the United States, it has been reported that 1 in 50 individuals is diagnosed with prawn allergy.[1,2] A high prevalence of shellfish allergy is also found in Asia.[2] In Thailand, black tiger shrimp *Penaeus monodon* is the most frequently consumed shrimp species. The risk of shrimp allergy is triggered by various routes, such as inhaling of shrimp particles, and touching, working, and ingestion of meals containing shrimp. Allergic symptoms against shrimp involve several organs, such as the skin (52%-90%), respiratory tract (42%), gastrointestinal tract (35%), and cardiovascular system (anaphylaxis 10%).[3]

A correct diagnosis of food allergy is essential for proper treatment. At present, avoiding foods that are the causative of allergy is the best recommended treatment. Taking a clinical history is

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an important step in the diagnosis of food allergy, but it is often inaccurate. The skin prick test (SPT) is recommended as the primary method for the diagnosis of food allergy. SPT, however, has a low positive predictive value and oft en gives false positive results. Furthermore, the procedure can potentially induce systemic allergic reaction.[4] The gold standard for the diagnosis of food allergy is oral food challenge (OFC), but this test also has a great risk of severe reactions in allergic patients. Moreover, subsets of patients with positive SPT exhibit a negative OFC.[5]

Serum immunoglobulin E (IgE) from patients with shrimp allergy can be tested for its ability to bind with allergens by the ImmunoCAP test. This test is selected as the standard method for the quantification of IgE because of its accuracy and cost effectiveness. Nevertheless, false positivity can be obtained from this test due to the cross-reactivity.[6] As a result, allergen-specific IgE measurement by these methods can only be partially used for the diagnosis of food allergy.[6]

One of the most critical problems in the management of shellfish allergy is the lack of definite diagnostic assays because of the poor characterization of biological and immunological properties of the allergens. To date, some major allergens in shellfish have been identified, including tropomyosin, arginine kinase, myosin light chain, sarcoplasmic calcium-binding protein, hemocyanin, troponin-C, myosin heavy chain, glyceraldehyde phosphate dehydrogenase, and ubiquitin.[7] Tropomyosin, a major shellfish allergen, belongs to a group of muscle protein with a molecular weight of 34 to 39 kDa (Pen m 1 in *Penaeus monodon*).[8,9] Even though various allergens have been identified and characterized, a few can be used for diagnosis with no cross reactivity to other proteins from allergen-causing organisms, such as house dust mite (HDM).

The quantification of allergen-specific IgE is often used for the diagnosis of shellfish allergy, but it does not directly measure the capacity of an allergen to induce allergic reactions by IgE cross-linking. The basophil activation test (BAT) emerges as a diagnostic test for food allergy, but the procedure is quite complicated, involving whole blood stimulation and flow cytometry-based detection of activation markers.[10,11] Recently, a new detection assay for IgE cross-linking by specific allergens based on the nuclear factor of activated T-cells (NFAT) reporter gene in a humanized rat basophilic leukaemia line (RS-ATL8) was reported by Nakamura *et al.*[6,12] This assay, called the IgE cross-linking-induced luciferase expression (EXiLE) test can be used for the allergen evaluation of IgE cross-linking capacity with high sensitivity and great robustness.[13] Previous results showed that this method has a good correlation with the ImmunoCAP test and also OFC test in patients with egg allergy.[6,12]

This study aims to investigate the potential of using this reporter cell line for detecting shrimp allergenic proteins using allergic serum IgE and to correlate the results with enzyme-linked immunosorbent assay (ELISA) and Western blot. The results from this study may pave the way toward identification of novel shrimp allergen(s) for diagnostic and therapeutic purposes.

MATERIALS AND METHODS

Patient sera

Twenty-four sera were obtained from shrimp allergic patients from the Allergy Clinic of King Chulalongkorn Memorial Hospital. The inclusion criteria of this study were: 1) all patients had history of shrimp allergy; and 2) the allergic response was confirmed by the positive SPT (wheal ≥3 mm) and ImmunoCAP test. Serum from non-allergic healthy donor with no history of shrimp allergy and SPT-negative was used as a negative control. Detailed patient information is shown in Table 1. All procedures involving human subject were reviewed and approved by the Institutional Review Board of Chulalongkorn University (project review No. 469/58).

Preparation of shrimp extracts and HDM allergens

The shrimp extracts from a fresh black tiger shrimp *Penaeus monodon* were prepared as previously described.[14] Briefly, the outer shell of raw shrimp was removed, and the meat was cut into pieces and ground in a motar before resuspension in phosphate-buffered saline (PBS; pH 7.4). The homogenate was centrifuged at 8,600 ×g for 10 minutes at 4°C, and the supernatant was collected. The collected supernatant was sterilized by syringe filtration (0.22 μm) and stored at -80°C until used as raw shrimp extracts. For cooked shrimp extracts, shrimp was boiled in PBS at 100°C for 5 minutes before extraction using the same method described above. The bicinchoninic acid protein assay

Table 1. Information on patients in this study

Detailed information	No. (N=24)
Sex	Male (n=9) Female (n=15)
Age (year)	8-15 (n=8) 16-25 (n=4) 26-35 (n=5) 40-55 (n=3)
Symptoms	Anaphylaxis (n=4) Rash (n=4) Urticaria (n=4) Wheezing (n =7) Lip swell (n=1)
Diagnosis	SPT (n=15) ImmunoCAP test (n=15)
HDM SPT (*D. pteronyssinas* and *D. farinae*)	Positive (n=8) (S2, S8, S10, S18, S19, S20, S22, S24) Negative (n=1) (S7) Unknown (n=15) (S1, S3, S5, S6, S9, S11-S17, S21, S23)
Specific IgE to HDM by ImmunoCAP (kUA/L)	S2 (72.1), S3 (0.27), S19 (10.11), S20 (55.4)

SPT, skin prick test; HDM, house dust mite; IgE, immunoglobulin E.

(Pierce™ BCA Assay Protein Assay kit, ThermoFisher Scientific, Waltham, MS, USA) was used to determine total protein concentration, using bovine serum albumin (BSA) as a standard. HDM allergen, extract containing major dust mite allergens including and Der p, Der p2, Der f1, Der f2 (5,000 U/mL), was purchased from ALK Abello (ALK, Horsholm, Denmark).

The Der p10 cDNA was amplified by PCR from a whole *D. pteronyssinus* cDNA library. The amplified fragment was subsequently cloned into the pStaby1 expression vector (Delphi Genetics, Gosselies, Belgium) and upstream to a $(His)_6$ sequence tag. This DNA construct was transformed by electroporation into CYS21 *Escherichia coli* cells (Dephi Genetics). Finally, the recombinant plasmid purified from 1 CYS21 clone was transformed into the production SE1 *E. coli* cells (Delphi Genetics) according to the manual instructions. Recombinant Der p10 (rDer p10) was expressed at 37°C after addition of 1 mmol/L isopropyl-thiogalactoside (IPTG) for 2 hours. The induced bacteria were collected by centrifugation and lysed through a cell disrupter (Cell D; Constant, Low March, UK) at 1,800 bars. The cell lysate was centrifuged, and the supernatant was directly applied on a Ni^{2+} chelate high-performance column (GE Healthcare Bio-Sciences, Uppsala, Sweden) equilibrated with PBS buffer at pH 7.2. Protein elution proceeded by stepwise increasing imidazole concentration in the buffer (from 0 to 400 mmol/L). Fractions containing purified rDer p10 (elution with 200 mmol/L imidazole) were pooled and stored at -20°C.

ELISA

Indirect ELISA was performed as described previously with some modifications.[15] ELISA plates were coated with raw or cooked shrimp extract in PBS overnight at 4°C. After washing in PBS Tween-20 (PBS-T), the patient sera (dilution 1:50) were added to each well and incubated at room temperature for 1 hour. Horseradish peroxidase (HRP)-conjugated goat anti-human IgE (KPL; Seracare, Milford, MA, USA) was added after washing and the plates were incubated for 1 hour at room temperature. Then, 3, 3´, 5, 5´-tetramethylbezidine (TMB) was used as a substrate. The optical density (OD) was measured at 450 nm by a microplate reader (Anthos 2010; Biochrom, Holliston, MA, USA).

For competitive inhibition assay, the protocols described by Jeong *et al.*[16] was followed with some modifications. Similar procedures as indirect ELISA were performed, except that the patients' sera were incubated with various concentrations of competitors (shrimp extract, HDM extract or BSA for 1 hour before adding to the shrimp extract-coated ELISA plate). The %inhibition was calculated using the following formula:

$$\%\text{inhibition} = 100 - (\text{OD 450 of serum with inhibitor}/\text{OD 450 of serum without inhibitor}) \times 100$$

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot

SDS-PAGE was used to separate protein extracts from raw or cooked shrimp, and the gels were stained with Coomassie R-250 solution. For Western blot, membranes were incubated with patient sera at indicated dilutions and were further incubated with HRP-conjugated goat anti-human IgE (KPL, USA). After washing, blots were incubated with chemiluminescent substrates and the signals were detected by X-ray film (Amersham Biosciences, Amersham, UK).

Cell culture and reporter assay

The RS-ATL8 reporter cell line (a kind gift from Prof. Ryosuke Nakamura, Division of Novel Foods and Immunochemistry, National Institute of Health Science, Tokyo, Japan) was maintained in minimum essential medium (MEM) Gibco® (ThermoFisher Scientific) with supplement of 10% fetal bovine serum (FBS) (from Gibco®; ThermoFisher Scientific), penicillin (Hyclone®; GE Healthcare Bio-Sciences, Logan, UT, USA), geneticin® (0.5 mg/mL; ThermoFisher Scientific), hygromycin B (0.2 mg/mL Hygromix®; Elanco Animal Health, Greenfield, IN, USA), GlutaMAX™-I (Gibco®; ThermoFisher Scientific). To measure luciferase activity, the method described by Nakamura *et al.*[12] was followed. In brief, the RS-ATL8 cell line was cultured at 5×10^4 cells per well in 96 well-plate (50 μL per well). Cells were incubated for 3 hours before addition of diluted sera (1:100) in complete MEM for sensitization. After sensitization, cells were washed once gently with sterile PBS. The dissolved allergens in complete MEM were added (50 μL per well), and cells were incubated for another 3 hours for stimulation. After stimulation, luciferase activity was measured by the ONE-Glo™ Luciferase Assay System (Promega Corporation, Madison, WI, USA), and the assay were measured in a microplate reader (ThermoFisher Scientific). Measurements were done in triplicate, and the average was used for analysis. The positive control was stimulated with the affinity purified goat anti-human IgE (Bethyl Laboratories, Montgomery, TX, USA), and the negative control was medium alone or serum alone without shrimp extract.

Elution of shrimp proteins from SDS-PAGE

After electrophoresis, gels were divided into 2 parts, one was used for Coomassie blue staining as a reference gel and the other was used for the elution of proteins. For the elution of proteins, the excised bands were minced into small pieces in the microcentrifuge tubes. The minced pieces were equilibrated in elution buffer and shaked at 25°C overnight. After equilibration, the supernatant was collected by centrifugation at $14,000\times g$ for 5 minutes. Aliquots of the supernatant were tested for the presence of proteins by SDS-PAGE. After the elution, the supernatant was dialyzed by dialysis cassette (Slide-A-Lyzer™ G2 Cassettes, 10K MWCO; ThermoFisher Scientific). The dialyzed samples were concentrated by the concentrator devices (3K MWCO; Pall Corporation, Cortland, NY, USA). The concentrated samples were used to test IgE cross-linking in the RS-ATL8

cell line and protein mass spectrometry.

Protein mass spectrometry

Protein samples were reduced with dithiothreitol (DTT), alkylated with iodoacetamide and digested with trypsin.[17] The digested peptide solutions were analyzed with Impact II UHR-TOF MS System (Bruker Daltonics Ltd., Billerica, MS, USA) coupled to a nanoLC system: UltiMate 3000 LC System (ThermoFisher Scientific). Peptides were separated on a nanocolumn (PepSwift monolithic column 100 μm i.d. ×50 mm). Eluent A was 0.1% formic acid and eluent B was 80% acetonitrile in water containing 0.1% formic acid. Peptide separation was achieved with a linear gradient from 10% to 45% B for 8.5 minutes at a flow rate of 1 μL/min, including a regeneration step at 90% B and an equilibration step at 1% B, one run took 20 minutes. Peptide fragment mass spectra were acquired in data-dependent AutoMS (2) mode with selecting most abundant precursor ions in 3-second cycle for fragmentation. The mass range of the mass spectrometry (MS) scan was set to extend from 150 to 2,200 m/z. The MS/MS data were submitted for a database search using the Mascot software (Matrix Science, London, UK).[18] The data was searched against the National Center for Biotechnology Information (NCBI) database for protein identification. Database interrogation was: taxonomy (*Other Metazoa*); enzyme (trypsin); variable modifications (carbamidomethyl, oxidation of methionine residues); mass values (monoisotopic); protein mass (unrestricted); peptide mass tolerance (1.2 Da); fragment mass tolerance (±0.6 Da), peptide charge state (1+, 2+, and 3+), and max missed cleavages.

To maximize the number of identified peptides and proteins at a given quality, we used our PeptideShaker version 1.16.5[19] for the interpretation of peptide and protein identifications. The MS/MS spectral files were again compared to the sequence database using multiple proteomics identification search engines, including Andromeda, Comet, MariMatch, MS Amanda, MS-GF+, OMSSA, Tide and X!Tandem with the help of SearchGUI version 3.2.14.[20] The decoy sequences were created by reversing the target sequences in SearchGUI. The identification settings were as follows. Trypsin with a maximum of 3 missed cleavages; 100 ppm as MS1 and 0.5 Da as MS2 tolerances; fixed

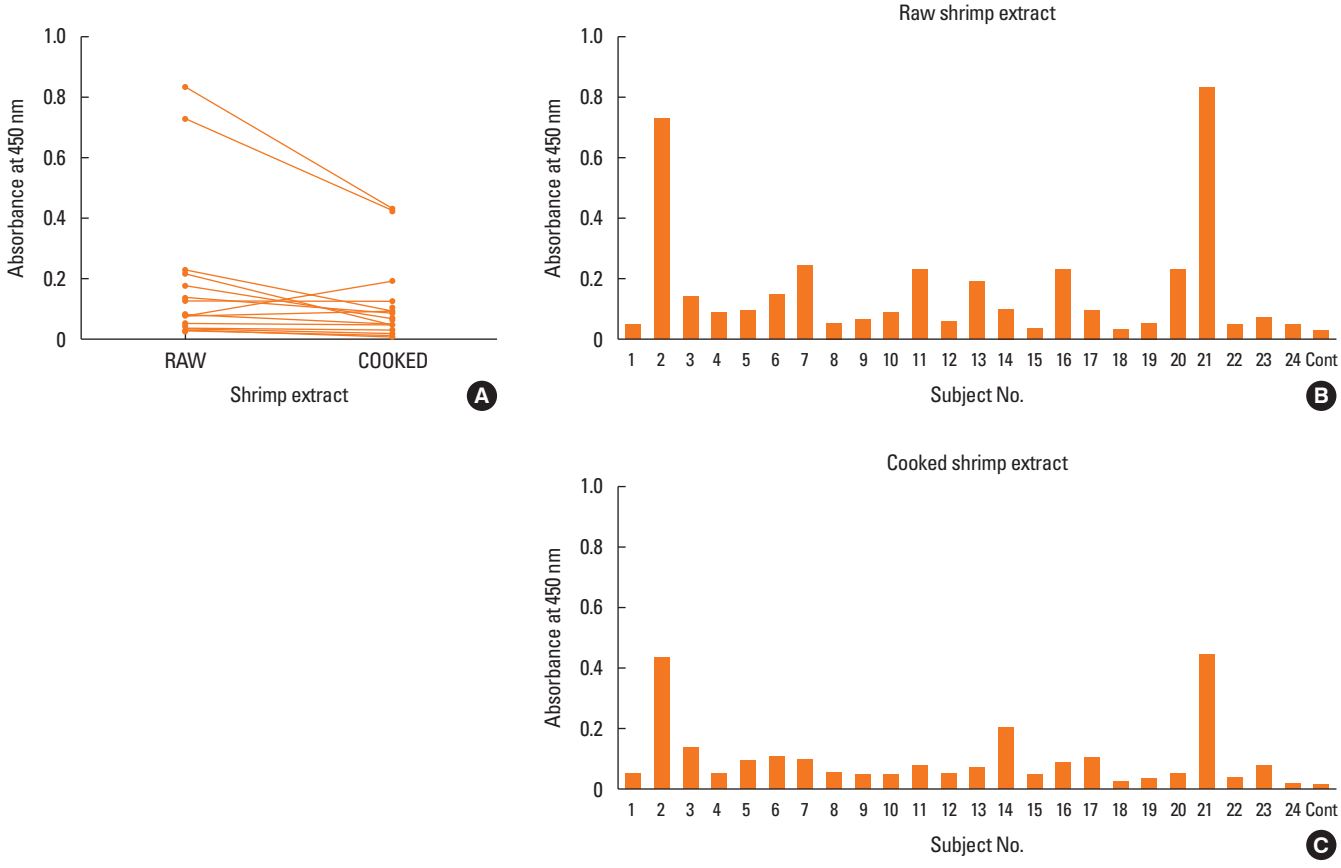


Fig. 1. Serum IgE reactivity to cooked and raw shrimp extracts by indirect ELISA. (A) Serum IgE reactivity to raw and cooked shrimp extracts of shrimp allergic patients were compared by indirect ELISA. The Wilcoxon test was used to compare serum IgE reactivity between the raw and cooked shrimp extracts (P=0.009). (B, C) Serum IgE reactivity to raw (B) or cooked (C) shrimp extracts was detected by indirect ELISA. The concentration of shrimp extracts was 250 μg/mL. Allergic sera were diluted at 1:50. Patient sera were labeled with arbitrary numbers and the control non-allergic serum was labeled as cont. IgE, immunoglobulin E; ELISA, enzyme-linked immunosorbent assay.

modifications: Carbamidomethylation of C (+57.021464 Da); and variable modifications: Oxidation of M (+15.994915 Da). Peptides and proteins were inferred from the spectrum identification results using PeptideShaker version 1.16.5.

Statistical analysis

The Wilcoxon signed rank test and the unpaired *t* test were used to compare between the groups. Differences between the groups were considered significant with a *P* value of less than 0.05. Analysis was performed using GraphPad Prism version 5.03 (GraphPad Software, Inc., La Jolla, CA, USA).

RESULTS

Specific serum IgE reactivity to raw and cooked shrimp extracts

The reactivity against raw and cooked shrimp extracts by the serum IgE of shrimp allergic patients by ELISA were performed. As shown in Fig. 1A, significantly higher reactivity to raw shrimp extract were observed compared to the cooked shimp extract,

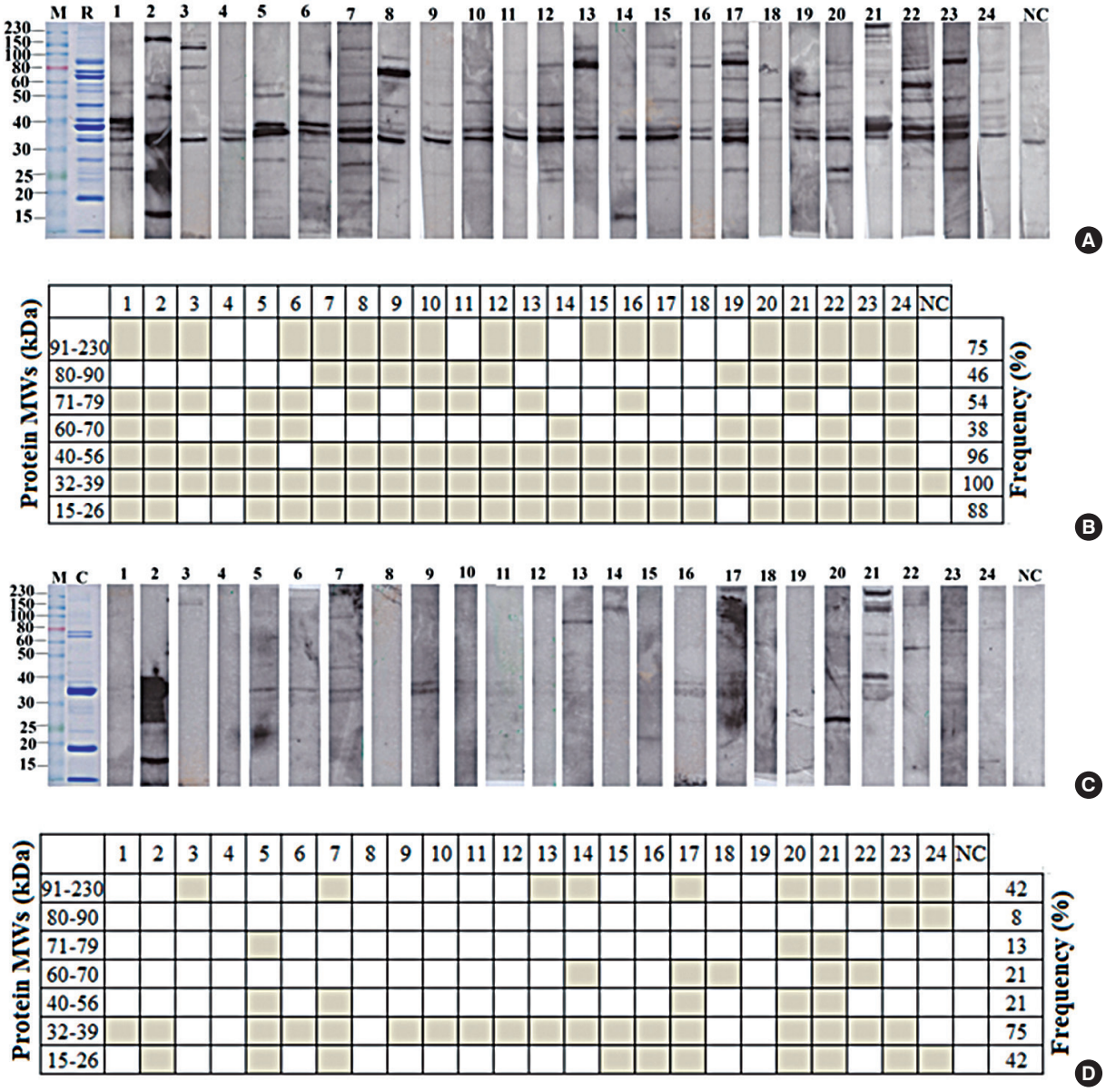


Fig. 2. Sera IgE reactivity patterns and the frequency of reactivity to raw and cooked shrimp extracts by Western blot. (A, C) All sera were diluted at 1:100. SDS-PAGE and Coomassie blue staining of raw and cooked extracts are shown as Lane R (raw shrimp extract) and Lane C (cooked shrimp extract). Serum from non-allergic healthy control was marked as NC. (B, D) The frequencies of specific sera IgE reactive to raw or cooked shrimp extract by Western blot in (A) and (C) are calculated and shown. The gray boxes indicate the patient sera reacting positively with the proteins with the indicated molecular weight range. IgE, immunoglobulin E; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; NC, control non-allergic serum.

(P=0.009) (Fig. 1A). When individual sera were directly compared, most samples yielded higher OD read than the healthy conrol subject. Futuremore, the sera from subject No. 2 and 21 showed the highest IgE reactivity to both extracts (Fig. 1B and C). Upon analysis of the raw and cooked shrimp extracts by SDS-PAGE, several protein bands that were found in the raw extracts were not visible in the cooked extracts (Fig. 2).

To analyze IgE reactive profiles, shrimp allergic patient IgE reactivity patterns against raw and cooked shrimp extracts were determined by Western blot. As shown in Fig. 2A and C, each se-

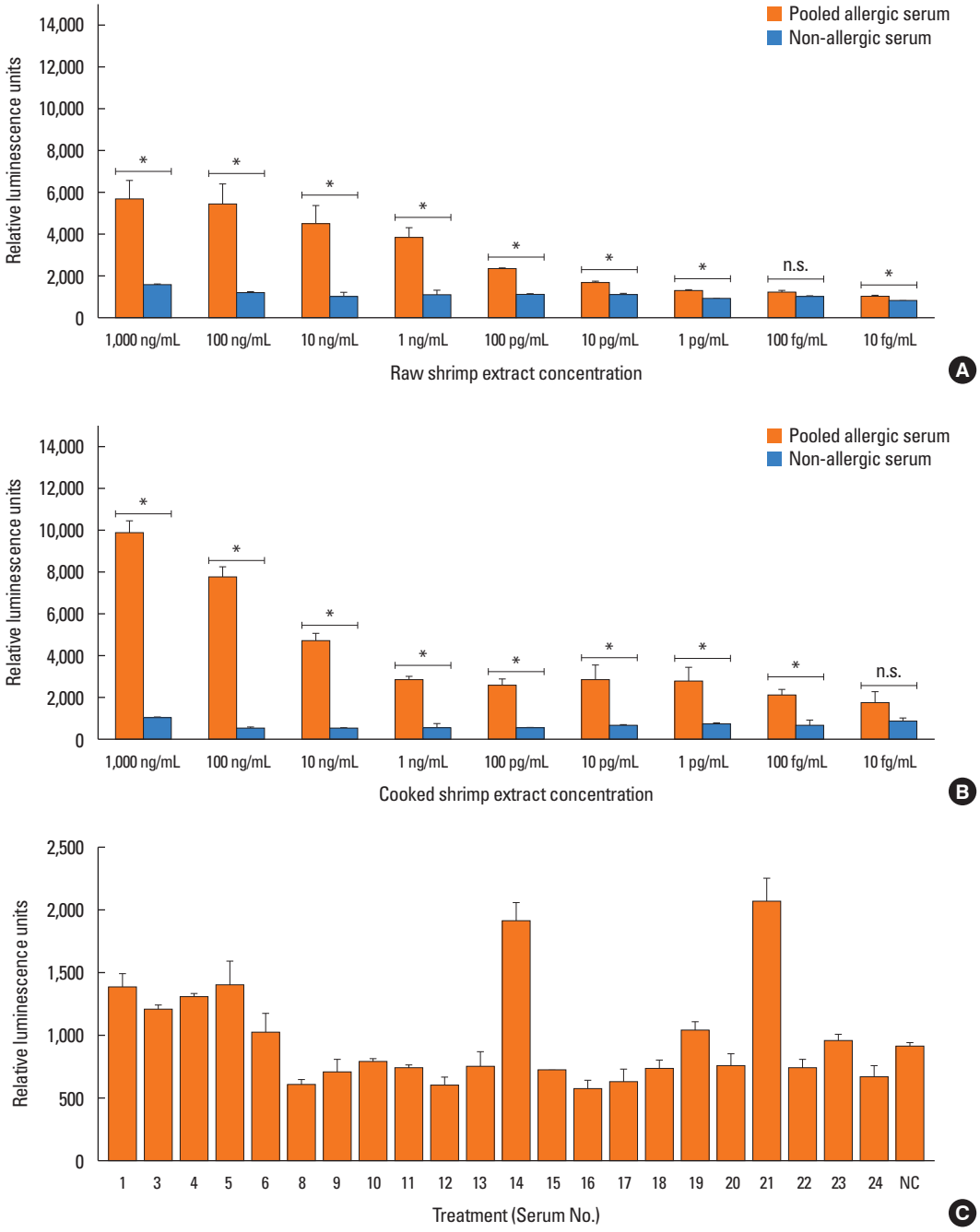


Fig. 3. Reporter assay for the RS-ATL8 reporter cell line against shrimp extracts (A, B) RS-ATL8 cells were sensitized overnight with diluted pooled sera (1:100) from shrimp allergic patients and non-allergic healthy control serum. Cells were stimulated with (A) raw or (B) cooked shrimp extracts. Pooled allergic sera are shown by the gray bars and non-allergic healthy control serum is shown by the black bars. Data are expressed as mean±SD of the readings of triplicates. (C) The RS-ATL8 cells were sensitized overnight with individual shrimp allergic serum and stimulated with 1 μg/mL raw shrimp extract. Luminescence was measured 3 hours after stimulation. Data are expressed as mean±SD of the readings of triplicates. n.s., not significant difference; SD, standard deviation; NC, control non-allergic serum. *P<0.05.

rum sample showed unique reactive pattern, and the allergic serum IgE showed stronger reactivity against raw over cooked shrimp extract, a result consistent with those of ELISA. The patterns of the reactive bands were summarized according to the ranges of molecular weight for cooked or raw shrimp extract in Fig. 2B and D, respectively. In most serum samples, 3 major groups of allergen bands around 91-230, 32-39, and 15-26 kDa in both raw and cooked shrimp extracts were observed. Eighteen out of 24 patients (75%) had specific IgE to the proteins of 32-39 kDa in cooked extracts, while all patients had specific IgE to the proteins of 32-39 kDa in raw extratcs (100%) (Fig. 2B and D). Two allergen bands around 15-26 and 91-230 kDa in the cooked shrimp extract were found in more than 40% of the samples.

Detection of IgE cross-linking shrimp extract using the RS-ATL8 reporter cell line

To detect whether shrimp extracts can induce IgE cross-linking, the RS-ATL8 cells were sensitized overnight with 1:100 of pooled sera from 5 shrimp allergic patients (patient No. 5, 8, 17, 21, and 23) that showed high IgE reactivity to the shrimp extracts on Western blot. Cells were stimulated with different protein concentrations of raw or cooked shrimp extracts. A dose response curve of the reporter activities against raw and cooked shrimp extracts are shown in Fig. 3A and B, respectively. The results showed that the raw or cooked shrimp extracts indeed cross-linked sera IgE and induced luciferase activity with wide a range of concentrations from 10 to 1,000 ng/mL. Therefore, the RS-ATL8 cell can be used to detect minute amounts of allergens that show IgE cross-linking ability in shellfish. To correlate this reporter assay with ELISA described above, the raw extracts were used to stimulate the RS-ALT8 cell line that were sensitized with individual serum. As shown in Fig. 3C, some samples, such as No. 21, showed a similar high response as ELISA but some samples, such as No. 14, showed the opposite re-

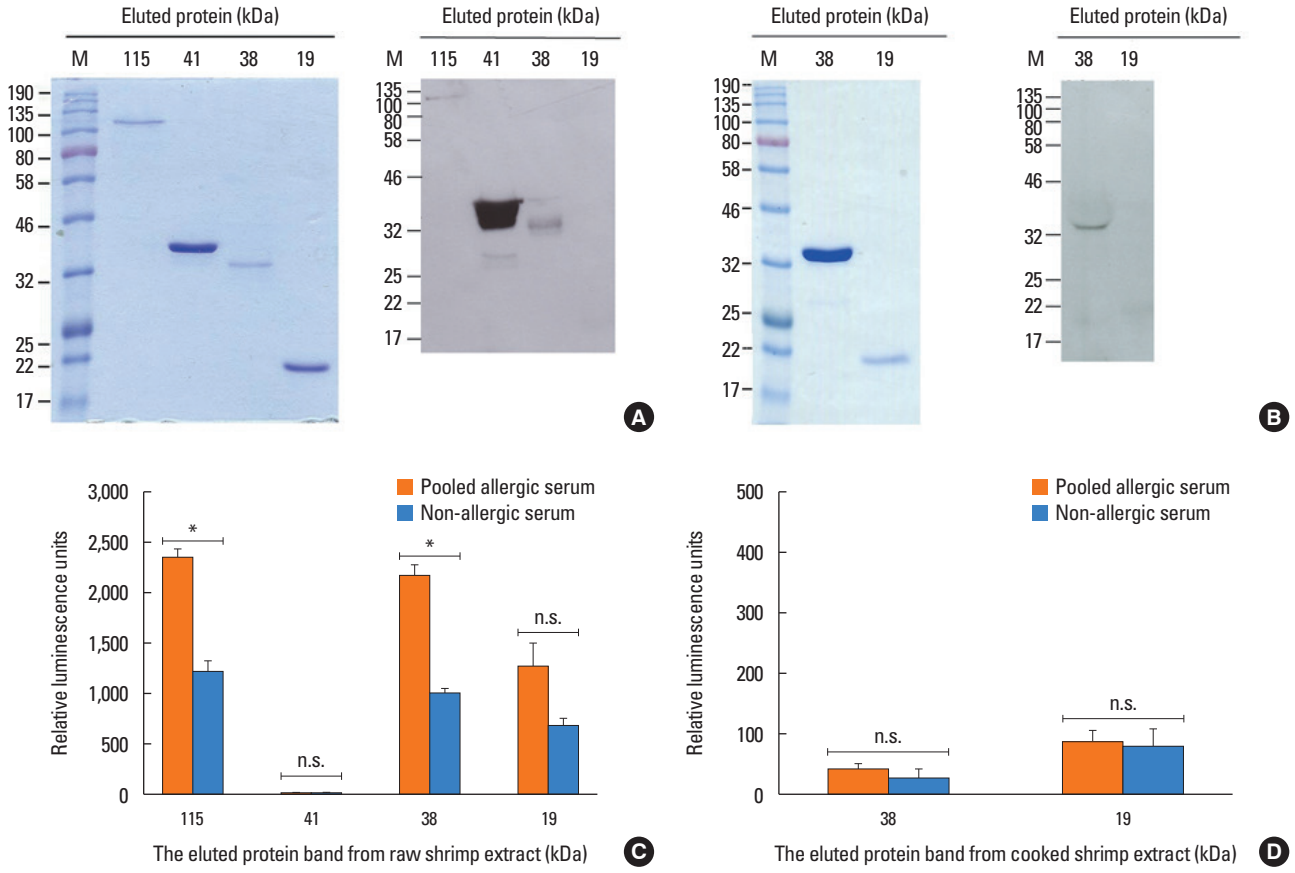


Fig. 4. Detection of the excised protein bands from raw and cooked shrimp extracts by Western blot and the RS-ATL8 reporter cell line. (A) The excised protein bands of raw shrimp extract (19, 38, 41, and 115 kDa) were analyzed by SDS-PAGE and Coomasie blue staining (left panel). The eluted proteins were detected by pooled allergic sera IgE by Western blot (right panel). (B) The excised protein band of cooked shrimp extract (19 and 38 kDa) were analyzed by SDS-PAGE and Coomasie blue staining (left panel). The eluted proteins were detected by pooled allergic sera IgE by Western blot (right panel). (C and D) RS-ATL 8 cells were sensitized overnight with diluted pooled sera (1:100) from shrimp allergic patient and non-allergic healthy control serum. Cells were stimulated with eluted protein bands of raw (C) and cooked shrimp extracts (D) as shown above. The results of pooled allergic serum are shown in the gray bars and non-allergic healthy control serum are shown in black bars. Data are expressed as mean±SD in triplicates. SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; IgE, immunoglobulin E; SD, standard deviation; n.s., no statistical significance. *$P<0.05$.

sponse strength.

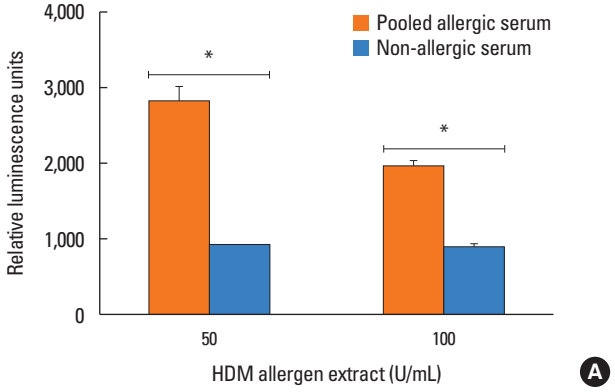
Detecting IgE cross-linking protein extracts eluted from SDS-PAGE using the RS-ATL8 cell line

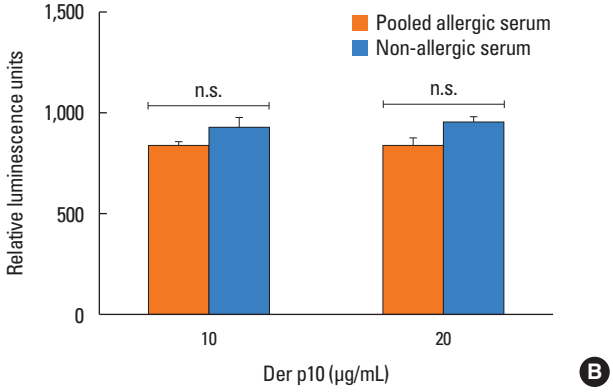
We next attempted to detect shrimp proteins after separation by SDS-PAGE that induce the cross-linking of IgE using the RS-ATL8 cell line. First, raw or cooked shrimp extracts were separated by SDS-PAGE, and 6 prominent protein bands were excised and eluted. Four protein bands from raw (19, 38, 41, and 115 kDa) and 2 protein bands from cooked (19 and 38 kDa) shrimp extracts were chosen based on the results obtained by Western blot described above. The eluted protein bands were subjected to SDS-PAGE for confirmation (Fig. 4A and B). In order to confirm that the eluted proteins still retained an IgE-binding ability, the eluted protein was subjected to Western blot using pooled allergic sera from 5 shrimp allergic patients as used above. The results showed that the eluted proteins at 38, 41, and 115 kDa from raw shrimp extract and 38 kDa from cooked shrimp extract still reacted positively to serum IgE. In contrast, the 19 kDa proteins from either extract did not show specific IgE binding.

These eluted proteins were used to test for IgE cross-linking in the RS-ATL8 cell line as described above. Cells were sensitized with 1:100 dilution of pooled shrimp allergic patient sera and non-allergic serum, and the eluted proteins were used to stimulate cells (Fig. 4 C and D). The results indicated that the eluted proteins at 38 and 115 kDa, but not those at 19 and 41 kDa, from raw shrimp extract induced significantly higher reporter activities in cells sensitized to allergic sera, compared to the healthy control serum. This result strongly indicated the ability of these proteins to cross-link IgE. In contrast, the eluted proteins at 19 and 38 kDa from cooked shrimp extract could not induce IgE cross-linking.

The protein bands from raw shrimp extract at 38 and 115 kDa that had ability to induce IgE cross-linking were excised from SDS-PAGE and were subjected to analysis by MS. Table 2 summarized the results of all identified proteins at 38 and 115 kDa. Three major proteins were identified in the 38 kDa band as glyceraldehyde-3-phosphate dehydrogenase, arginine kinase and crustacyanin C2 (Table 2). Five major proteins, including myosin heavy chain type 1, myosin heavy chain type 2, myosin heavy chain type 3, myosin heavy chain type 6A, and ubiquitin-activating enzyme (E1), were identified in the 115 kDa band (Table 2). Among these proteins, E1 and crustacyanin C2 have not been reported as shrimp allergens and are potential novel candidate shrimp allergens.

To test whether patient sera used in this study also cross react with the HDM allergens or tropomyosin, HDM allergen extract or recombinant Der p10 were tested in a pooled sera- sensitized RS-ATL8 cell line. As shown in Fig. 5A, patient sera induced significantly higher luciferase activity, suggesting that the sera contain HDM allergens-specific IgE. Interestingly,





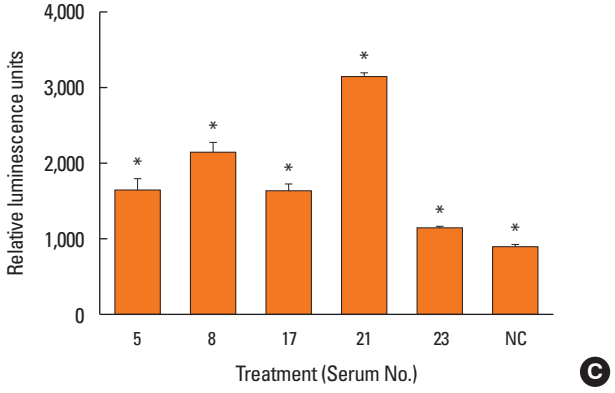


Fig. 5. Detection of dust mite allergens by the RS-ATL8 reporter cell line. (A, B) RS-ATL8 cells were sensitized overnight with diluted pooled sera (1:100) from shrimp allergic patient and non-allergic healthy control serum. Cells were stimulated with HDM allergen extract (A) or purified recombinant Der p10 (B). The results of pooled allergic serum are shown in the gray bars, and non-allergic healthy control serum are shown in black bars. Data are expressed as mean ± SD in triplicates. (C) RS-ATL8 cells were sensitized overnight with diluted individual sera (1:100) from shrimp allergic patients and non-allergic healthy control serum. Cells were stimulated with HDM allergen extract (100 U/mL). HDM, house dust mite; n.s., no statistical significance; SD, standard deviation. *$P<0.05$.

Table 2. Protein bands at 38 and 115 kDa identified by MS

Protein candidate	Nominal mass (Da)	Mascot protein score	Sequence coverage by Mascot (%)	Sequence coverage by PeptideShaker (%)	Peptide sequences from Mascot and PeptideShaker
38 kDa protein					
Glyceraldehyde-3-phosphate dehydrogenase	13,922	1,426	78.3	67.4	PMFVCGVNMEKYTK KITVFNEMKPENIPWSKA AGAEYIVESTGVFTTIEK KVIISAPSADAPMFVCGVN KVVSNASCTTNCLAPVAKV DMKVVSNASCTTNCLAPVAK KAGAEYIVESTGVFTTIEKA ADAPMFVCGVNMEKYTKDMK VIISAPSADAPMFVCGVNMEK KVIISAPSADAPMFVCGVNMEKY VLHENFEILEGLMTTVHAVTATQK DMKVVSNASCTTNCLAPVAKVLHEN KVLHENFEILEGLMTTVHAVTATQKT KITVFNEMKPENIPWSKAGAEYIVESTGVFTTIEKA
Arginine kinase	40,427	108	31.7	36.8	KTFLVWVNEEDHLRI EMQDGILELIKMEKEM RLGFLTFCPTNLGTTVRA EGDRFLQAANACRYWPTGR RMGLTEFQAVKEMQDGILELIKM FLQAANACRYWPTGRGIYHNDNK IISMQMGGDLGQVFRRLTSAVNEIEK KTSLGATLLDVIQSGVENLDSGVGIYAPDA EAYTLFSPLFDPIIEDYHVGFKQ
Crustacyanin C2	19,816	18	22.5	78.1	RCEAAFMNIGIDPSRFTKT KFNVRSTGTDANGNAITRR SPSLADRYHRRCEAAFMNIG MLYDQQKPNHQRYAGTWYEI RCEAAFMNIGIDPSRFTKTT MLYDQQKPNHQRYAGTWYEIA YHRRCEAAFMNIGIDPSRFTK VVDERMLYDQQKPNHQRYAGTW QCVRNEYTFDGIKFNVRSTGTDA FGYYSDFAFIFSRSPSLADRYHR CEAAFMNIGIDPSRFTKTTQGGSC GQALPNPFGEPHLSVDYEASWIAPY
115 kDa protein					
Myosin heavy chain type 1	220,933	561	15.6	42.3	RVFSWLVKK RTQLELSQVRQ KIEDEQALVYRD ECRNYSTEHFRLK VHDEIKDAETRVK KLSLTNQLDDTRK RRVHDEIKDAETRV QIEEAEEIAALNLAK EGESKQNLEDQIIQT GELEESRTLLEQSDRGR

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Table 2. Continued

Protein candidate	Nominal mass (Da)	Mascot protein score	Sequence coverage by Mascot (%)	Sequence coverage by PeptideShaker (%)	Peptide sequences from Mascot and PeptideShaker
					RQIEEAEEIAALNLAKF KAANDENIEQLDSIRRE AIDSMQASLEVEAKGKAE KSTGPDPDPTEYLFISRE REQINNFTNQNTALTASKR RDIEESNLQHEAALATLRK RDDCYKVTASVMHHGNMKF ELEELELSVQKGEQDKQTK RKLEGEMSTLQADLEEMLN LEHDIQALRDQLDEESDAK KCHQRAIDSMQASLEVEAK KKHNDVVAEMSEQVDYLNK KDLQTRLEESESAAMKAGKK LNDTVVDQLKKASNALTVEI LIYTYSGLFCIVINPYKRYP ELSELGERLDEAGGATAAQI EEELEKLEATAVKAEEEFGK VFFRAGVLGTLEEVRDDRIMK KKHNDVVAEMSEQVDYLNKMK RNEVPPHLFAICDGAYQNMNQ AVSNMEARIRDLESALDDETRR CIVPNEFKKPGEVDSGLIMHQL ALEITAKDLQTRLEESESAAMK KMCLLSDDIYDYYYEAQGKVTV KKMGLLSILEEESMFPKATDKT RKLEGEMSTLQADLEEMLNEAKN KIKTYKRQIEEAEEIAALNLAKF MLSAANAEALMWRSKYESEGVAR QTKDQQLTNLNEEISHQEELITK DQRQVKELQARLEELEEDVEHER VTVPSIDDKEDMQFTHDAFDVLN QEIDKRIQEKEEEFDATRKCHQR KPGEVDSGLIMHQLTCNGVLEGH EELQAALEEAEAALEQEENKVLR DKTTAEKTTKQLQHQYGELCALAK VGAEFVTKGMNVDQVNYNIGAMAK AMFIGVLDIAGFEIFDFNGFEQIC EGIVWQFVDFGMDLQACIELFEKK ELSELGERLDEAGGATAAQIEINK TEKDKEAMKRDADDAKASMDSLAR SKYESEGVARAEELEAARMKLAAR CNMTLETGQTRAMFIGVLDIAGFEI MRAAAELDDLHASAERAQALASAAE MPGHVKKSTGPDPDPTEYLFISREQR RELSELGERLDEAGGATAAQIEINKK KKHNDVVAEMSEQVDYLNKMKARTEK ARVISQSPAERGYHIFYQLMCDQIDYVK AKKAMLDAARLADELRSEQEHAQSQEKMR KDPLNDTVVDQLKKASNALTVEIFADHPGQSGDGGGKGKG

(Continued to the next page)

Table 2. Continued

Protein candidate	Nominal mass (Da)	Mascot protein score	Sequence coverage by Mascot (%)	Sequence coverage by PeptideShaker (%)	Peptide sequences from Mascot and PeptideShaker
Myosin heavy chain type 2	220,231	118	11.6	19.9	KLSVENGDLLRQ ILAADVMATEKDDK KTIQQQINEVNVKL IEELETEAEHERQAR ICQKGFPNRMQYPDFK RNVDQVYYSVSAMAKG RLEEAELQIEQLNVKN KMRKGLDLSVKDLQARL LDEAGGATGAQMELNKK GLAEEIKDLMEQISEGGR AVYEENLEHLDSVRRENK QLNSLMTTLHSTHPHFVR NIRKFMEMSNWSWFIFWQK RDLEEANIQHESALANLRK LIYTYSGLFCIVINPYKRYP QVKKAQAEMKDMQARVEEEQR SKRKVEGDLKMTQETVADIER MQYPDFKHRYKILAADVMATEK EGFAEGLIQGAKGDKLVSVQLK KQNLEDQIVQTNPPLEAYGNAKT KRKLEGEIQTLHAELDDMLNEAKN QNLEDQIVQTNPPLEAYGNAKTTR LGLLAILEEESMFPKATDKSFEEK AIESMQASLEAEAKSKAEALRMKKK AMVDAARLADELRAEQEHAQTQEKMR KLEGEIQTLHAELDDMLNEAKNSEDKAKK KRRNEVPPHLFAISDGAYMDMLQSQQNQSMLITGESGAGK-TENTKK
Myosin heavy chain type 3	34,519	511	15.2	74.8	DELRSEQEHAQVQEK QIEEAEEIAALNLAK GELEESRTLLEQSDRGR RQIEEAEEIAALNLAKF FRKAQQELEETEMTVTVSH AISNLEGRIRDLESALDDETR VKEEQRLASEYREQYGIAERR ANSDLHKHYRKIQDDVKDMEAR RKLEGEMQTLQADLEEMLSEAK KLEGEMQTLQADLEEMLSEAKN ELAFQTDEDKKNHDRMQDLVDK KIKTYKRQIEEAEEIAALNLAKF TELNDARDQINNFNSQNGALAATKR
Myosin heavy chain type 6a	33,075	511	16.7	76.4	QIEEAEEIAALNLAK RQIEEAEEIAALNLAKY VNGELEESRTLLEQSDR KAQAEMKDMQARLEEEQR LEEEQRLASEYREQCSASER IRELETQLDDEARRHADAQKNL

(Continued to the next page)

Table 2. Continued

Protein candidate	Nominal mass (Da)	Mascot protein score	Sequence coverage by Mascot (%)	Sequence coverage by PeptideShaker (%)	Peptide sequences from Mascot and PeptideShaker
					ELSFQSDEDKKNHERMQDLVDK KIKTYKRQIEEAEEIAALNLAKY ELADANESLSHLTAQHGSLSMAKR KRKLEGEIQTLHAELDDMLNEAKN AMVDAARLADELRAEQDHAQNSEKMR KLEGEIQTLHAELDDMLNEAKNSEDKAK
Ubiquitin-activating enzyme E1	116,441	68	0.1	7.51	KIEIPEFVPRS KFLHAFKEMNEGHPAKV RIANLQSAIPQATSFGALKL RDAVADIVNKIEIPEFVPRS SFKALEESIKEPEFIMTDFAKFDR NFAMLGIGAGEGGKLVVTDMDLIEK HNLEITMLSQGVSMLYSFFMPPAKRNER RLEPAVNGSTNMAANGSQQDIDESLYSRQLYVLGHDAMRRM

MS, mass spectrometry.

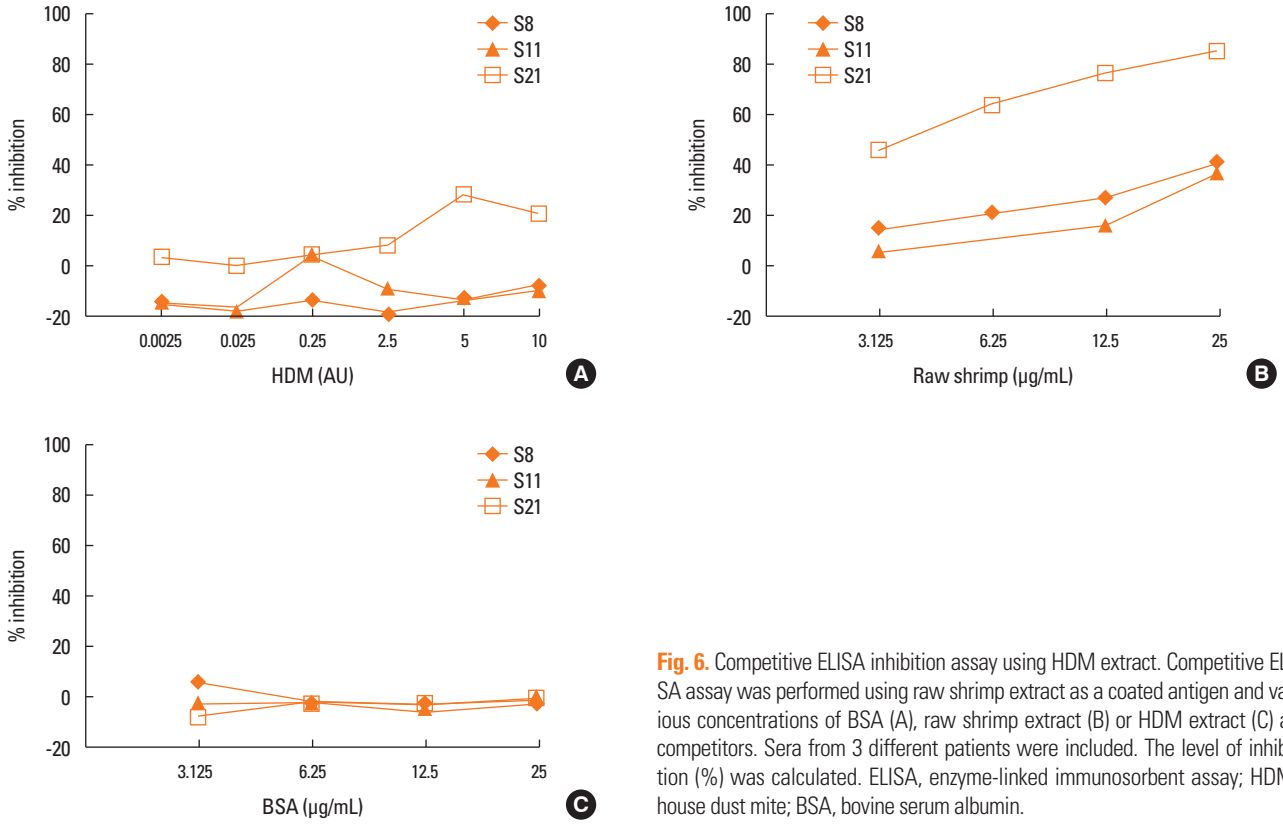

Fig. 6. Competitive ELISA inhibition assay using HDM extract. Competitive ELISA assay was performed using raw shrimp extract as a coated antigen and various concentrations of BSA (A), raw shrimp extract (B) or HDM extract (C) as competitors. Sera from 3 different patients were included. The level of inhibition (%) was calculated. ELISA, enzyme-linked immunosorbent assay; HDM, house dust mite; BSA, bovine serum albumin.

when recombinant Der p10 was used, no significant increase in luciferase activity was observed (Fig. 5B). When individual serum was separately tested, all sera showed significantly increased luciferase activity upon stimulation with HDM extract. Furthermore, competitive inhibition assay was performed using raw shrimp extract as a coated antigen. As shown in Fig. 6A and B, competition with BSA showed no cross reactivity with raw shrimp extract, while competition with raw shrimp extract showed the range of inhibition between 18%-85%. In contrast, competition with HDM extract only showed competitive re-

sponse up to 28% by serum No. 21, while no inhibition was found in other sera (Fig. 6C). These results suggest that at least sera from 5 patients tested here contained IgE specific for HDM allergens, but competition with HDM extract yielded only partial inhibition.

DISCUSSION

An accurate diagnosis of food allergy is essential for proper treatment of allergic symptoms. Lack of definite diagnostic tests is one of the obstacles to shellfish allergy management. Currently, there are 5 allergens listed by the World Health Organization/International Union of Immunological Societies (WHO/IUIS) Allergen Nomenclature Sub-Committee, *i.e.*, Pen m 1-m 6, and it is likely that other shrimp allergens remain uncharacterized. The primary objective of this study is to compare the IgE reactivity of allergic subjects by traditional methods of ELISA and Western blot using a humanized rat basophilic leukaemia line, RS-ATL8. Our ELISA results showed in shrimp patients, that sera IgE reactivity was higher to raw extract than to cooked extracts and that more reactive bands were detected on Western blot using raw shrimp extract (Figs. 1 and 2). Similar results were reported by other groups on shrimp and other food allergens.[21-23] A study by Paschke and Besler[24] showed that heating of cow's milk for 10 minutes decreased the allergenecity of bovine allergens. This may be due to epitope structures modification from cooking process; likewise, Nakamura *et al.*[25] found that the Maillard reaction could cause reduction in the allergenicity of squid tropomyosin in some epitopes. Our results are consistent with this hypothesis that reduction in shrimp allergenicity is caused by thermal treatment.[26] In contrast, several reports suggested that heat treatment resulted in increased IgE binding for shrimp allergens.[27,28] The differences may be due to the temperature used for treatment and the composition of the extracts subjected for heat treatment as purified proteins and crude extracts yield different sensitivities to heat treatment. Interestingly, Liu *et al.*[26] reported that patient IgE reacted stronger to the raw shrimp extract, than to the boiled shrimp extract, but when purified tropomyosin was used, it gave the opposite results. From our ELISA and Western blot, stronger IgE reactivity was observed against raw shrimp extract but the cooked shrimp extract gave higher relative luciferase activity when compared to the raw extract (Fig. 3A and B). This result indicated that the cooked shrimp extract may contain allergens that can crosslink IgE more than the raw shrimp extract or the heat treatment may confer IgE cross-linking ability to neoepitope. Therefore, IgE binding using ELISA/Western blot may yield contrasting results to the IgE cross-linking assay as was found in this study.

When HDM allergen extract was tested for IgE cross-linking in some patient sera used in this study, all sera indeed induced higher luciferase activity than the non-allergic control. This result indicated that some patients may contain HDM allergen-specific IgE. The purified recombinant Der p10 did not yield positive reporter assay, suggesting that the Der p10 is not the cross-reactive allergens in the sera used in this study or recombinant Der p10 is not capable of cross-linking IgE. Interestingly, at least 8 patients included in this study are positive to SPT using HDM extract as summarized in Table 1. When competitive inhibition assay was performed using HDM extract as competitor against shrimp extract, only 1 serum showed inhibition (Fig. 6). This result implied that some sera IgE cross-reacted with HDM and raw shrimp extract, but some reacted more specifically to shrimp extract. Tropomyosin is considered to be the link between shrimp allergy and dust mite allergy where dust mite tropomyosin may function as a primary sensitizer to shrimp allergy.[9]

By Western blot, sera IgE from all patients reacted positively to the raw shrimp extract at the region around 32-39 kDa and also 75% reacted to the same region in the cooked shrimp extract. In previous studies, proteins in these areas were identified to include a major shellfish allergen tropomyosin that is heat-stable and water-soluble.[22,23,26,29-33] In the sera of 3 patients (No. 4, 8, and 19), there were no detectable IgE reactivity by Western blot (Fig. 2A and C). These patients may have specific IgE against proteins that were lost during preparation or the IgE may recognize non-linear epitopes.

ELISA and Western blot are immunological methods that depend on the binding of specific IgE in sera of allergic patients to the coated allergens. The results obtained using these methods often do not correlate with data obtained from clinical history and the *in vivo* test (SPT).[6,10] Furthermore, they do not provide any information on the capacity of proteins to promote IgE-mediated hypersensitivity reaction.[10,34] In contrast, activation of mast cell requires 2 or more epitopes on the allergen to cross-link specific IgE that bound to its receptor (FcεRI) on the surface of mast cells.[34] In recent years, BAT has been used for the *in vitro* diagnosis of IgE-mediated hypersensitivity reactions based on the phenotypic changes induced by histamine release.[35-37] Recently, it was reported that the rat basophillic leukemia (RBL)-derived mast cell line was developed for the assessment of the cross-linking capacity of allergens in the measurement of allergen-specific IgE.[6,10,38] The reporter cell line RS-ATL8, was used in several studies to detect allergens in *Schistosoma mansoni* and egg white.[12,37,39] However, the assay of the crude raw and cooked shrimp extracts that induce IgE cross-linking using this cell line has not been reported. The results showed in Fig. 3 clearly demonstrated that the RS-ATL8 reporter cell line has high sensitivity for the detection of both crude raw and cooked shrimp allergens even at the ng/mL level. Therefore, using this reporter system, it is possible to detect allergens inducing IgE cross-linking with high sensitivity that may be used as diagnostic tools in the future.[13] Different responses obtained by Western blot and EXiLE assay may reflect structural differences in allergens based on different methods

of sample preparation. While Western blot mainly detects denatured proteins, EXiLE can detect native form of proteins.

In recent years, identification of the food allergens by proteomic analysis becomes one of the major approaches to discover novel allergens.[40] In various studies, allergens in black tiger shrimp were identified using this approach.[21,22,41] However, it does not often accompany with the test of IgE cross-linking ability. In this study, using SDS-PAGE and Western blot, we selected 4 single bands from raw shrimp extract and 2 single bands from cooked shrimp extract that clearly bind to IgE from patient sera and used them for screening in the reporter cell line. Proteins at 38 kDa from raw shrimp extract have the ability to induce an IgE cross-linking (Fig. 4). In contrast, the proteins at 115 kDa from raw shrimp extract exhibited only weak reactivity on Western blot, but it is sufficient to induce IgE cross-linking (Fig. 4). Interestingly, the 41 kDa band from the raw shrimp extract clearly reacted with IgE on Western blot, but the eluted fraction failed to induce IgE cross-linking (Fig. 4A and C), suggesting that they are not able to crosslink IgE and induce degranulation. The 2 protein bands from the cooked shrimp extract failed to induce reporter gene activity, implying that heat treatment may destroy epitopes necessary for IgE cross-linking.

From the results obtained using the reporter cell line, we identified proteins in the 2 protein bands (38 and 115 kDa) from raw black tiger shrimp extract by MS (Table 2). The results indicated that the 115 kDa had 5 major proteins, including known shrimp allergens, such as myosin heavy chain types 1, 2, 3, 6A, and a potential novel candidate allergen, ubiquitin-activating enzyme. The band at 38 kDa contained 3 major proteins, including known allergens like glyceraldehyde-3-phosphate dehydrogenase, arginine kinase, and a novel candidate allergen crustacyanin C2.[7] Arginine kinase or Pen m 2 is reported to be heat stable, but the cooked proteins at 38 kDa which may contain this protein lost the ability of IgE crosslinking, implying that heat treatment may affect IgE cross-linking.[41] Ubiquitin-activating enzyme E1 and crustacyanin C2 have not been reported to be shrimp allergens. Myosin heavy chain and glyceraldehyde-3-phosphate dehydrogenase were reported as an allergen in banana shrimp (*Fenneropenaeus merguiensis*).[31] The study by Gámez *et al.*[32] reported the protein allergens in *Solenocera melantho* shrimp by using MS as α-actinin, β-actin, arginine kinase, biphosphate aldolase, fructose, sarcoplasmic calcium-binding protein, and ubiquitin. Ubiquitin-activating enzyme E1 activates ubiquitin,[42] and crustacyanin is a carotenoprotein pigment that is involved in the shell color.[43] In this study, we could detect only 1 black tiger shrimp allergen recognized by WHO/IUIS Allergen Nomenclature Sub-Committee. Other allergens, such as Pen m 1, were not detected in our MS analysis. This may be due to the size of protein bands excised from gel for analysis. In this study, we excised only 4 bands corresponding to molecular weights of approximately 19, 38, 41, and 115 kDa. Other proteins with differing size may not have been included in our analysis. Further studies are needed to directly test whether these newly identified proteins are true shrimp allergens by using either recombinant or native proteins. Taken together, using the RS-ATL8 cell line together with the proteomics approach may detect novel shrimp allergens with the biological function of cross-linking IgE upon binding.

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REFERENCES

1. Sicherer SH. Food allergy. Lancet 2002;360:701-10.
2. Lee AJ, Thalayasingam M, Lee BW. Food allergy in Asia: how does it compare? Asia Pac Allergy 2013;3:3-14.
3. Daul CB, Morgan JE, Hughes J, Lehrer SB. Provocation-challenge studies in shrimp-sensitive individuals. J Allergy Clin Immunol 1988;81:1180-6.
4. Bernstein IL, Li JT, Bernstein DI, Hamilton R, Spector SL, Tan R, et al. Allergy diagnostic testing: an updated practice parameter. Ann Allergy Asthma Immunol 2008;100:S1-148.
5. Musmand JJ, Daul CB, Lehrer SB. Crustacea allergy. Clin Exp Allergy 1993;23:722-32.
6. Nakamura R, Uchida Y, Higuchi M, Nakamura R, Tsuge I, Urisu A, et al. A convenient and sensitive allergy test: IgE crosslinking-induced luciferase expression in cultured mast cells. Allergy 2010;65:1266-73.
7. Faber MA, Pascal M, El Kharbouchi O, Sabato V, Hagendorens MM, Decuyper II, et al. Shellfish allergens: tropomyosin and beyond. Allergy 2017;72:842-8.
8. Leung PS, Chu KH, Chow WK, Ansari A, Bandea CI, Kwan HS, et al. Cloning, expression, and primary structure of *Metapenaeus ensis* tropomyosin, the major heat-stable shrimp allergen. J Allergy Clin Immunol 1994;94:882-90.
9. Wong L, Huang CH, Lee BW. Shellfish and house dust mite allergies: is the link tropomyosin? Allergy Asthma Immunol Res 2016;8:101-6.
10. Vogel L, Lüttkopf D, Hatahet L, Haustein D, Vieths S. Development of a functional *in vitro* assay as a novel tool for the standardization of allergen extracts in the human system. Allergy 2005;60:1021-8.
11. Pedrosa M, Boyano-Martínez T, García-Ara C, Quirce S. Shellfish allergy: a comprehensive review. Clin Rev Allergy Immunol 2015;49:203-16.
12. Nakamura R, Ishiwatari A, Higuchi M, Uchida Y, Nakamura R, Kawakami H, et al. Evaluation of the luciferase assay-based *in vitro* elicitation test for serum IgE. Allergol Int 2012;61:431-7.
13. Falcone FH, Alcocer MJ, Okamoto-Uchida Y, Nakamura R. Use of humanized rat basophilic leukemia reporter cell lines as a diagnostic tool for detection of allergen-specific IgE in allergic patients: time for a reappraisal? Curr Allergy Asthma Rep 2015;15:67.
14. Abramovitch JB, Kamath S, Varese N, Zubrinich C, Lopata AL, O'Hehir RE, et al. IgE reactivity of blue swimmer crab (*Portunus pelagicus*) tropomyosin, Por p 1, and other allergens; cross-reactivity with black tiger prawn and effects of heating. PLoS One 2013;

8:e67487.
15. Abugoch L, Tapia C, Plasencia D, Pastor A, Castro-Mandujano O, López L, et al. Shelf-life of fresh blueberries coated with quinoa protein/chitosan/sunflower oil edible film. J Sci Food Agric 2016; 96:619-26.
16. Jeong KY, Hwang H, Lee J, Lee IY, Kim DS, Hong CS, et al. Allergenic characterization of tropomyosin from the dusky brown cockroach, Periplaneta fuliginosa. Clin Diagn Lab Immunol 2004;11: 680-5.
17. Paemanee A, Wikan N, Roytrakul S, Smith DR. Application of gelC-MS/MS to proteomic profiling of chikungunya virus infection: preparation of peptides for analysis. Methods Mol Biol 2016;1426: 179-93.
18. Perkins DN, Pappin DJ, Creasy DM, Cottrell JS. Probability-based protein identification by searching sequence databases using mass spectrometry data. Electrophoresis 1999;20:3551-67.
19. Vaudel M, Burkhart JM, Zahedi RP, Oveland E, Berven FS, Sickmann A, et al. PeptideShaker enables reanalysis of MS-derived proteomics data sets. Nat Biotechnol 2015;33:22-4.
20. Vaudel M, Barsnes H, Berven FS, Sickmann A, Martens L. SearchGUI: an open-source graphical user interface for simultaneous OMSSA and X!Tandem searches. Proteomics 2011;11:996-9.
21. Sahabudin S, Misnan R, Yadzir ZH, Mohamad J, Abdullah N, Bakhtiar F, et al. Identification of major and minor allergens of black tiger prawn (*Penaeus monodon*) and king prawn (*Penaeus latisulcatus*). Malays J Med Sci 2011;18:27-32.
22. Abdel Rahman AM, Kamath S, Lopata AL, Helleur RJ. Analysis of the allergenic proteins in black tiger prawn (*Penaeus monodon*) and characterization of the major allergen tropomyosin using mass spectrometry. Rapid Commun Mass Spectrom 2010;24:2462-70.
23. Samson KT, Chen FH, Miura K, Odajima Y, Iikura Y, Naval Rivas M, et al. IgE binding to raw and boiled shrimp proteins in atopic and nonatopic patients with adverse reactions to shrimp. Int Arch Allergy Immunol 2004;133:225-32.
24. Paschke A, Besler M. Stability of bovine allergens during food processing. Ann Allergy Asthma Immunol 2002;89:16-20.
25. Nakamura A, Sasaki F, Watanabe K, Ojima T, Ahn DH, Saeki H. Changes in allergenicity and digestibility of squid tropomyosin during the Maillard reaction with ribose. J Agric Food Chem 2006; 54:9529-34.
26. Liu GM, Cheng H, Nesbit JB, Su WJ, Cao MJ, Maleki SJ. Effects of boiling on the IgE-binding properties of tropomyosin of shrimp (*Litopenaeus vannamei*). J Food Sci 2010;75:T1-5.
27. Carnés J, Ferrer A, Huertas AJ, Andreu C, Larramendi CH, Fernández-Caldas E. The use of raw or boiled crustacean extracts for the diagnosis of seafood allergic individuals. Ann Allergy Asthma Immunol 2007;98:349-54.
28. Gámez C, Zafra MP, Sanz V, Mazzeo C, Ibáñez MD, Sastre J, et al. Simulated gastrointestinal digestion reduces the allergic reactivity of shrimp extract proteins and tropomyosin. Food Chem 2015;173: 475-81.
29. Usui M, Harada A, Ishimaru T, Sakumichi E, Saratani F, Sato-Minami C, et al. Contribution of structural reversibility to the heat stability of the tropomyosin shrimp allergen. Biosci Biotechnol Biochem 2013;77:948-53.
30. Daul CB, Slattery M, Reese G, Lehrer SB. Identification of the major brown shrimp (*Penaeus aztecus*) allergen as the muscle protein tropomyosin. Int Arch Allergy Immunol 1994;105:49-55.
31. Khanaruksombat S, Srisomsap C, Chokchaichamnankit D, Punyarit P, Phiriyangkul P. Identification of a novel allergen from muscle and various organs in banana shrimp (*Fenneropenaeus merguiensis*). Ann Allergy Asthma Immunol 2014;113:301-6.
32. Gámez C, Zafra M, Boquete M, Sanz V, Mazzeo C, Ibáñez MD, et al. New shrimp IgE-binding proteins involved in mite-seafood cross-reactivity. Mol Nutr Food Res 2014;58:1915-25.
33. Yu CJ, Lin YF, Chiang BL, Chow LP. Proteomics and immunological analysis of a novel shrimp allergen, Pen m 2. J Immunol 2003;170: 445-53.
34. Bodinier M, Brossard C, Triballeau S, Morisset M, Guérin-Marchand C, Pineau F, et al. Evaluation of an *in vitro* mast cell degranulation test in the context of food allergy to wheat. Int Arch Allergy Immunol 2008;146:307-20.
35. Eberlein-König B, Varga R, Mempel M, Darsow U, Behrendt H, Ring J. Comparison of basophil activation tests using CD63 or CD203c expression in patients with insect venom allergy. Allergy 2006;61:1084-5.
36. Bridts CH, Sabato V, Mertens C, Hagendorens MM, De Clerck LS, Ebo DG. Flow cytometric allergy diagnosis: basophil activation techniques. Methods Mol Biol 2014;1192:147-59.
37. Wan D, Wang X, Nakamura R, Alcocer MJ, Falcone FH. Use of humanized rat basophil leukemia (RBL) reporter systems for detection of allergen-specific IgE sensitization in human serum. Methods Mol Biol 2014;1192:177-84.
38. Dibbern DA Jr, Palmer GW, Williams PB, Bock SA, Dreskin SC. RBL cells expressing human Fc epsilon RI are a sensitive tool for exploring functional IgE-allergen interactions: studies with sera from peanut-sensitive patients. J Immunol Methods 2003;274:37-45.
39. Wan D, Ludolf F, Alanine DG, Stretton O, Ali Ali E, Al-Barwary N, et al. Use of humanised rat basophilic leukaemia cell line RS-ATL8 for the assessment of allergenicity of *Schistosoma mansoni* proteins. PLoS Negl Trop Dis 2014;8:e3124.
40. Sotkovský P, Hubálek M, Hernychová L, Novák P, Havranová M, Setinová I, et al. Proteomic analysis of wheat proteins recognized by IgE antibodies of allergic patients. Proteomics 2008;8:1677-91.
41. Kamath SD, Rahman AM, Voskamp A, Komoda T, Rolland JM, O'Hehir RE, et al. 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;58:1144-55.
42. Nagai Y, Kaneda S, Nomura K, Yasuda H, Seno T, Yamao F. Ubiquitin-activating enzyme, E1, is phosphorylated in mammalian cells by the protein kinase Cdc2. J Cell Sci 1995;108:2145-52.
43. Ferrari M, Folli C, Pincolini E, McClintock TS, Rössle M, Berni R, et al. Structural characterization of recombinant crustacyanin subunits from the lobster *Homarus americanus*. Acta Crystallogr Sect F Struct Biol Cryst Commun 2012;68:846-53.

SUPPLEMENTARY INFORMATION

Amino acid sequence of peptides of each protein are shown. Amino acids in regular letters belong to the candidate proteins while those in yellow highlight corresponding to the peptide analyzed by Mascot. Peptide sequences analyzed by PeptideShaker were highlighted in red.

Result identified by Mascot

>gi|902943505|gb|AKS26013.1| crustacyanin C2

VVDERMLYDQQKPNHQRYAGTWYEIALTNNPYQLIKQCVRNEYTFDGIKFNVRSTGTDANGNAITRRGQALPNPFGEPHLSVDYEASWIAPYVILDTDYENFSCIYSCSGYNFGYYSDFAFIFSRSPSLADRYHRRCEAAFMNIGIDPSRFTKTTQGGSCSYNTNYRSW

>gi|543335549|arginine kinase

MADAAVIEKLEAGFKKLEAATDCKSLLKKYLSKAVFDQLKEKKTSLGATLLDVIQSGVENLDSGVGIYAPDAEAYTLFSPLFDPIIEDYHVGFKQTDKHPNKDFGDVNTFVNVDPEGKYVISTRVRRGRSMEGYPFNPCLTEAQYKEMEAKVSSTLSSLEGELKGTYYPLTGMSKEVQQKLIDDHFLFKEGDRFLQAANACRYWPTGRGIYHNDNKTFLVWVNEEDHLRIISMQMGGDLGQVFRRLTSAVNEIEKRIPFSHHDRLGFLTFCPTNLGTTVRASVHIKLPKLAANREKLEEVAGKYNLQVRGTRGEHTEAEGGIYDISNKRRMGLTEFQAVKEMQDGILELIKMEKEM

>>gi|694174556|Glyceraldehyde-3-phosphate dehydrogenase

GCLVVDGHKITVFNEMKPENIPWSKAGAEYIVESTGVFTTIEKASAHFQGGAKKVIISAPSADAPMFVCGVNMEKYTKDMKVVSNASCTTNCLAPVAKVLHENFEILEGLMTTVHAVTATQKTVDGPSA

>BAM65719.1 myosin heavy chain type 1

MPGHVKKSTGPDPDPTEYLFISREQRMKDQTKPYDPKKSFWCPDPNEGFVECELQGAKGDKHVTVKLPSGETKDFKKEQVGQVNPPKYEKCEDVSNLTFLNDPSVFYVLKSRYQAKLIYTYSGLFCIAVNPYKRYPIYTNRAVKIYIGKRRNEVPPHLFAICDGAYQNMNQERQNQSMLITGESGAGKTENTKKVLSYFANVGASEKKEGESKQNLEDQIIQTNPILEAYGNAKTTRNDNSSRFGKFIRVHFAPNGKLSGADIEVYLLEKARVISQSPAERGYHIFYQLMCDQIDYVKKMCLLSDDIYDYYYEAQGKVTVPSIDDKEDMQFTHDAFDVLNFSHEERDDCYKVTASVMHHGNMKFKQRGREEQAEADGTEAGEIVAKLLGVDAEELYRNFCKPKIKVGAEFVTKGMNVDQVNYNIGAMAKGLFSRVFSWLVKKCNMTLETGQTRAMFIGVLDIAGFEIFDFNGFEQICINFCNEKLQQFFNHHMFVLEQEEYAKEGIVWQFVDFGMDLQACIELFEKKMGLLSILEEESMFPKATDKTFEEKLNNNHLGKSRCFIKPKPPKAGQPENHFAIVHYAGTVSYNLTGWLEKNKDPLNDTVVDQLKKASNALTVEIFADHPGQSGDGGGKGKGGKQQTGFKTVSSGYKDQLANLMKTLNATHPHFIRCIVPNEFKKPGEVDSGLIMHQLTCNGVLEGHPLFARRASPTGMPYKDFKLRYNILAAKEMLEAKDDKKAATACFERAGLNPELYRTGNTKVFFRAGVLGTLEEVRDDRIMKLVSWLQAWVRGWASRKYYSKMQKQRTALIVMQRNIRKFKIMRSWLWYELWIMLKPRLKATRGEEELEKLEATAVKAEEEFGKVVKVREELEAQNALLMQERNELLAAVDSTKGGMSEYLDKQAKLLAQKAELEGQLNDTLERLRKEEDARNQIANGKKKCEQEVTNLKKELEELELSVQKGEQDKQTKDQQLTNLNEEISHQEELITKVNKEKKHLQECNQKTAEDLQGVEDKCNNLNKVKTKLESSLDELEDTLEREKKLRAEVEKSKRKVEGDLRLTQEAVSDLERNLKELEVAAERKEKEISAITAKIEDEQALVYRDQRQVKELQARLEELEEDVEHERQARGKAEKAKNVLSRELSELGERLDEAGGATAAQIEINKKREGELGKVRRDIEESNLQHEAALATLRKKHNDVVAEMSEQVDYLNKMKARTEKDKEAMKRDADDAKASMDSLARDKTTAEKTTKQLQHQYGELCAKLDEVNRTLSDFDATKKKLACENTDLVRQLEEAENQVSQLSRVKLSLTNQLDDTRKMCDEESRGRATLLGKFRNLEHDIQALRDQLDEESDAKGDVLRMLSAANAEALMWRSKYESEGVARAEELEAARMKLAARLEEAEMQIESLNVRNLHLEKTKMRAAAELDDLHASAERAQALASAAEKKQKNFDKIISEWKLKVDDLAAEVDASQKECRNYSTEHFRLKAANDENIEQLDSIRRENKNLSDEIRDLMDQLGEGGRAYHEVQKNARRLELEKEELQAALEEAEAALEQEENKVLRTQLELSQVRQEIDRRVQEKEEEFDNTRKCHQRAIDSMQASLEVEAKGKAEALRMKKKLESDINELEIALDHANKANSDLHKHLRRVHDEIKDAETRVKEEQRVASEFREQYGIAERRFNALHGELEESRTLLEQSDRGRRHAETELNDAREQINNFTNQNTALTASKRKLEGEMSTLQADLEEMLNEAKNSEEKAKKAMLDAARLADELRSEQEHAQSQEKMRKALEITAKDLQTRLEESESAAMKAGKKAVSNMEARIRDLESALDDETRRHADSQKNLRKCERRIKELAFQTEEDKKNHDRMQDLVDKLQQKIKTYKRQIEEAEEIAALNLAKFRKTQQELEESETVTVVHY

>BAM65720.1 myosin heavy chain type 2

MPGHVVKSTGPDPDPTEFLFISAEQRMLDQTKPYDPKKSCWVPDDKEGFAEGLIQGAKGDKLVSVQLKSGEVKDFKKDLVVQVNPPKYEKCEDVSNLTFLNDASVLYNLKSRYQAKLIYTYSGLFCIVINPYKRYPIYTNRTVKIYQGKRRNEVPPHLFAISDGAYMDMLQSQQNQSMLITGESGAGKTENTKKVLSYFANVGATSKKKEDENKQNLEDQIVQTNPPLEAYGNAKTTRNDNSSRFGKFIRIHFAPNGKLSGADIEVYLLEKARVVSQAPAERGYHIFYQMMSDQVDYLKKLCHLSDDIYDYRYECQGKVTVPSIDDKEDMEFTHNAFTILNFTNEERDSCYKITAAVMHHGNMKFKQRGREEQAEPDGTDAGDVIADLMGVETEELYKNFCKPKIKVGAEFVTQGRNVDQVYYSVSAMAKGLFDRLFKWIVKKCNQTLETGMKRAMFIGVLDIAGFEIFDFNGFEQICINFCNEKLQQFFNHHMFVLEQEEYKAEGIDWVFVDFGMDLQACIELFEKKLGLLAILEEESMFPKATDKSFEEKLKANHLGKSPCFIKPKPPKAGQSEGHFAIVHYAGTVTYNLSGWLEKNKDPLNDTVVDQLKKSKLPLVVELFADHPGQSAPAEAKGGKKKKTGGFKTVSSGYREQLNSLMTTLHSTHPHFVRCIVPNETKSPGVVEAGLIMHQLTCNGVLEGIRICQKGFPNRMQYPDFKHRYKILAADVMATEKDDKKAAEMTFQKAGLDKELYRCGKTKVFFRAGVLGTMEELRDDRLAKIITWMQSWIRGLIGRKEYTRLQEQRVSLVVLQRNIRKFMEMSNWSWFIFWQKVKPLINQPRIEDEINKLKDRAEKAVADLDRETTRRKELEDSNLSLAEELNTLKETLESTKGNVSKFIEEQAKISAAKADLEAQLSDASARLQKEEESRTEMFQLKRKAEQDVNAMRKDLEDFELNVQKTNQDKATKDHQIRNINDEIAHQDEIINKVNKEKKLLQEMNQKTAEDLQAVEEKASHLNKIKSKLEQTLDELESSVGREKKLRAEIERSKRKVEGDLKMTQETVADIERQHKDLEQTIQRKDKEIGNLANKLEEEQGVVSKVQKTIKEMQARIEELETEAEHERQARAKAEKGKGNMSRELNDLNERLDEAGGATGAQMELNKKREAELAKLRRDLEEANIQHESALANLRKKHNDAVAEMTEQIDHLNKMKAKTEKEKEMMKYQADEAKSAMDSLARDKALAEKANKTIQQQINEVNVKLDEANRTLRDFDAQKKLSVENGDLLRQLEEADNQINQLNNLKVSLTTQLEDTKKMVDDESRERSVLLGKFRNLEHDLDGLREQLDEECEAKADANRQLSKAHAEAQMWRSKYESEGVARAEEIEAARLKLAARLEEAELQIEQLNVKNMQLEKAKGRIISEIDEMQMQVERAQGMAIAAERRQKDFDRVVNEWKIKVDQLATELDASQKECRQYSTEHFRIKAVYEENLEHLDSVRRENKGLAEEIKDLMEQISEGGRSLHEIEKNAKRFEIEKEELQAALEEAEAALEQEENKVLRGQLELSQVRQEIDKRIQEKEEEFDATRKCHQRAIESMQASLEAEAKSKAEALRMKKKLESDIGELEIALDHANKANADIQKQVKKAQAEMKDMQARVEEEQRLASEYREQCSASERKANAVNGELEESRTLLEQSDRGRRQAESELADANESLSHLTAQHGSLSMAKRKLEGEIQTLHAELDDMLNEAKNSEDKAKKAMVDAARLADELRAEQEHAQTQEKMRKGLDLSVKDLQARLDEFESSAHKTGKKALAKLEARIRELENQLDDESRRHADAQKNLRKCERRIKELSFQSEEDKKNHERMQDLVDKLQQKIKTYKRQIEEAEEIAALDLAKYRKAQQELETVQRS

>BAN17317.1 myosin heavy chain type 3, partial [Penaeus monodon] 45/297 15.2%

ESDINELEIALDHANKANSDLHKHYRKIQDDVKDMEARVKEEQRLASEYREQYGIAERRFNALHGELEESRTLLEQSDRGRRHAETELNDARDQINNFNSQNGALAAT-
KRKLEGEMQTLQADLEEMLSEAKNSEEKAKKAMLDAARLADELRSEQEHAQVQEKMRKALEGTVKDLQTRLEESEAAALKTGKKAISNLEGRIRDLESALDDETRRHADSQKNLRK-
CERRIKELAFQTDEDKKNHDRMQDLVDKLQQKIKTYKRQIEEAEEIAALNLAKFRKAQQELEETEMTVTVSHY

>BAN17320.1 myosin heavy chain type 6a, partial [Penaeus monodon] 48/288 16.7%

ESDIGELEIALDHANKANGDIQKQVKKAQAEMKDMQARLEEEQRLASEYREQCSASERKANAVNGELEESRTLLEQSDRGRRQAESELADANESLSHLTAQHGSLS-
MAKRKLEGEIQTLHAELDDMLNEAKNSEDKAKKAMVDAARLADELRAEQDHAQNSEKMRKGLEVSVKDLHARLDDFESSGHKTGKKALAKLEVRIRELETQLDDEARRHA-
DAQKNLRKCERRIKELSFQSDEDKKNHERMQDLVDKLQQKIKTYKRQIEEAEEIAALNLAKYRK
AQQELESK

>ANZ80593.1 ubiquitin-activating enzyme E1 [Penaeus monodon]

MSELQAANSQPPCKRPRLEPAVNGSTNMAANGSQQDIDESLYSRQLYVLGHDAMRRMASSDVLISGLGGLGVEIAKNVILGGVKSVTLHDTKNTSVRDLSTQFFLTSDDVGTN-
RATACHSRLAELNTYVPISASISPLTEEFLKKFRVVVLTDSSLEEQMQVSSFCHSNNIAVIVAATKGLFGQIFCDFGEEFEVVDTNGESPVSAMIAGVTKEENSVVTCLDESRHGIEDG-
DYVTFSEVQGMTELNGCKPVKIKVLGPFTFSIGDTAQFSDYVRGGIVTQVKVPVKKSFKALEESIKEPEFIMTDFAKFDRPGILHIAFQTLHKYEKENGSAPRPWNEEDAEKFLHAFKEM-
NEGHPAKVEEFDENLVKQFAKIASGGVAPLDAVIGGIVAQEVMKACSGKFTPIYQWLYFDALECLPDDLSTITEDLCTPAGDRYDSQVAVFGRDFQKKLGSQKYFIVGAGAIG-
CELLKNFAMLGIGAGEGGKLVVTDMDLIEKSNLNRQFLFRPWDVQKAKSDTAAAAVKKMNPQVNIIAHQNRVGPETERVYDDSFFEPLDGVANALDNVEARLYMDRRCVYYRK-
PLLESGTLGTKGNVQVVVPHLTESYGSSQDPPEKSIPICTLKNFPNAIEHTLQWARDMFEGEFRQAAENAAQYLVDSRFMERTLKLPGSQPMEVLESVKRALVEDRPQSFEDCVL-
WARKHFAEQFHNQIVQLLHNFPKDQTTSSGEPFWSGPKRCPHPLKFDANNQLHLDYILSAANLKAEVYGITQVRDRDAVADIVNKIEIPEFVPRSGVKIAVTDAEAEAQSNQVDSERI-
ANLQSAIPQATSFGALKLTPLDFEKDDDTNFHMDYIVAASNLRAENYDIPPADRHKSKLIAGKIIPAIATTTALISGLVSLELLKLVQGHNSIEKFKNGFVNLALPFFGFSEPISAPKNKYYD-
KEWTLWDRFEVDGEMTLEEFINYFEEKHNLEITMLSQGVSMLYSFFMPPAKRNERMSLPMSEVVKKVSKKKIEDHVRALVLELCANDKDGEDVEVPYVKYNLP-
TQOOOOOOOOOOOOOOOOOOOOOOOOOO

Result identified by PeptideShaker

>gi|902943505|gb|AKS26013.1| crustacyanin C2

VVDERMLYDQQKPNHQRYAGTWYEIALTNNPYQLIKQCVRNEYTFDGIKFNVRSTGTDANGNAITRRGQALPNPFGEPHLSVDYEASWIAPYVILDTDYENFSCIYSCSGYNFGYYS-
DFAFIFSRSPSLADRYHRRCEAAFMNIGIDPSRFTKTTQGGSCSYNTNYRSW

>gi|543335549|arginine kinase

MADAAVIEKLEAGFKKLEAATDCKSLLKKYLSKAVFDQLKEKKTSLGATLLDVIQSGVENLDSGVGIYAPDAEAYTLFSPLFDPIIEDYHVGFKQTDKHPNKDFGDVNTFVNVDPEG-
KYVISTRVRRGRSMEGYPFNPCLTEAQYKEMEAKVSSTLSSLEGELKGTYYPLTGMSKEVQQKLIDDHFLFKEGDRFLQAANACRYWPTGRGIYHNDNKTFLVWVNEEDHLRI-
ISMQMGGDLGQVFRRLTSAVNEIEKRIPFSHHDRLGFLTFCPTNLGTTVRASVHIKLPKLAANREKLEEVAGKYNLQVRGTRGEHTEAEGGIYDISNKRRMGLTEFQAVKEMQDGILE-
LIKMEKEM

>>gi|694174556|Glyceraldehyde-3-phosphate dehydrogenase

GCLVVDGHKITVFNEMKPENIPWSKAGAEYIVESTGVFTTIEKASAHFQGGAKKVIISAPSADAPMFVCGVNMEKYTKDMKVVSNASCTTNCLAPVAKVLHENFEILEGLMTT-
VHAVTATQKTVDGPSA

>BAM65719.1 myosin heavy chain type 1

MPGHVKKSTGPDPDPTEYLFISREQRMKDQTKPYDPKKSFWCPDPNEGFVECELQGAKGDKHVTVKLPSGETKDFKKEQVGQVNPPKYEKCEDVSNLTFLNDPSVFYVLKSRYQAK-
LIYTYSGLFCIAVNPYKRYPIYTNRAVKIYIGKRRNEVPPHLFAICDGAYQNMNQERQNQSMLITGESGAGKTENTKKVLSYFANVGASEKKEGESKQNLEDQIIQTNPILEAYGNAKT-
TRNDNSSRFGKFIRVHFAPNGKLSGADIEVYLLEKARVISQSPAERGYHIFYQLMCDQIDYVKKMCLLSDDIYDYYYEAQGKVTVPSIDDKEDMQFTHDAFDVLNFSHEERD-
DCYKVTASVMHHGNMKFKQRGREEQAEADGTEAGEIVAKLLGVDAEELYRNFCKPKIKVGAEFVTKGMNVDQVNYNIGAMAKGLFSRVFSWLVKKCNMTLETGQTRAMFIGVLDI-
AGFEIFDFNGFEQICINFCNEKLQQFFNHHMFVLEQEEYAKEGIVWQFVDFGMDLQACIELFEKKMGLLSILEEESMFPKATDKTFEEKLNNNHLGKSRCFIKPKPPKAGQPENHFAIVHY-
AGTVSYNLTGWLEKNKDPLNDTVVDQLKKASNALTVEIFADHPGQSGDGGGKGKGGKQQTGFKTVSSGYKDQLANLMKTLNATHPHFIRCIVPNEFKKPGEVDSGLIMHQLTCNGV-
LEGHPLFARRASPTGMPYKDFKLRYNILAAKEMLEAKDDKKAATACFERAGLNPELYRTGNTKVFFRAGVLGTLEEVRDDRIMKLVSWLQAWVRGWASRKYYSKMQKQR-
TALIVMQRNIRKFKIMRSWLWYELWIMLKPRLKATRGEEELEKLEATAVKAEEEFGKVVKVREELEAQNALLMQERNELLAAVDSTKGGMSEYLDKQAK-
LLAQKAELEGQLNDTLERLRKEEDARNQIANGKKKCEQEVTNLKKELEELELSVQKGEQDKQTKDQQLTNLNEEISHQEELITKVNKEKKHLQECNQKTAEDLQGVEDKCNNLNKVKT-
KLESSLDELEDTLEREKKLRAEVEKSKRKVEGDLRLTQEAVSDLERNLKELEVAAERKEKEISAITAKIEDEQALVYRDQRQVKELQARLEELEEDVEHERQARGKAEKAKNVLSRELSEL-
GERLDEAGGATAAQIEINKKREGELGKVRRDIEESNLQHEAALATLRKKHNDVVAEMSEQVDYLNKMKARTEKDKEAMKRDADDAKASMDSLARDKTTAEKTTKQLQHQYGEL-
CAKLDEVNRTLSDFDATKKKLACENTDLVRQLEEAENQVSQLSRVKLSLTNQLDDTRKMCDEESRGRATLLGKFRNLEHDIQALRDQLDEESDAKGDVLRMLSAANAEALM-
WRSKYESEGVARAEELEAARMKLAARLEEAEMQIESLNVRNLHLEKTKMRAAAELDDLHASAERAQALASAAEKKQKNFDKIISEWKLKVDDLAAEVDASQKECRNYSTEH-
FRLKAANDENIEQLDSIRRENKNLSDEIRDLMDQLGEGGRAYHEVQKNARRLELEKEELQAALEEAEAALEQEENKVLRTQLELSQVRQEIDRRVQEKEEEFDNTRKCHQRAIDSMQA-
SLEVEAKGKAEALRMKKKLESDINELEIALDHANKANSDLHKHLRRVHDEIKDAETRVKEEQRVASEFREQYGIAERRFNALHGELEESRTLLEQSDRGRRHAETELNDAREQINNFTN-
QNTALTASKRKLEGEMSTLQADLEEMLNEAKNSEEKAKKAMLDAARLADELRSEQEHAQSQEKMRKALEITAKDLQTRLEESESAAMKAGKKAVSNMEARIRDLESALDDE-
TRRHADSQKNLRKCERRIKELAFQTEEDKKNHDRMQDLVDKLQQKIKTYKRQIEEAEEIAALNLAKFRKTQQELEESETVTVVHY

>BAM65720.1 myosin heavy chain type 2 [Penaeus monodon]

MPGHVVKSTGPDPDPTEFLFISAEQRMLDQTKPYDPKKSCWVPDDKEGFAEGLIQGAKGDKLVSVQLKSGEVKDFKKDLVVQVNPPKYEKCEDVSNLTFLNDASVLYNLKSRYQAKLI-
YTYSGLFCIVINPYKRYPIYTNRTVKIYQGKRRNEVPPHLFAISDGAYMDMLQSQQNQSMLITGESGAGKTENTKKVLSYFANVGATSKKKEDENKQNLEDQIVQTNPPLEAYGNAKT-
TRNDNSSRFGKFIRIHFAPNGKLSGADIEVYLLEKARVVSQAPAERGYHIFYQMMSDQVDYLKKLCHLSDDIYDYRYECQGKVTVPSIDDKEDMEFTHNAFTILNFTNEERDSCYKITA-
AVMHHGNMKFKQRGREEQAEPDGTDAGDVIADLMGVETEELYKNFCKPKIKVGAEFVTQGRNVDQVYYSVSAMAKGLFDRLFKWIVKKCNQTLETGMKRAMFIGVLDIAGFEIFD-
FNGFEQICINFCNEKLQQFFNHHMFVLEQEEYKAEGIDWVFVDFGMDLQACIELFEKKLGLLAILEEESMFPKATDKSFEEKLKANHLGKSPCFIKPKPPKAGQSEGHFAIVHYAGTV-
TYNLSGWLEKNKDPLNDTVVDQLKKSKLPLVVELFADHPGQSAPAEAKGGKKKKTGGFKTVSSGYREQLNSLMTTLHSTHPHFVRCIVPNETKSPGVVEAGLIMHQLTCNGVLE-
GIRICQKGFPNRMQYPDFKHRYKILAADVMATEKDDKKAAEMTFQKAGLDKELYRCGKTKVFFRAGVLGTMEELRDDRLAKIITWMQSWIRGLIGRKEYTRLQEQRVSLVVLQRNIRK-
FMEMSNWSWFIFWQKVKPLINQPRIEDEINKLKDRAEKAVADLDRETTRRKELEDSNLSLAEELNTLKETLESTKGNVSKFIEEQAKISAAKADLEAQLSDASARLQKEEESRTEM-
FQLKRKAEQDVNAMRKDLEDFELNVQKTNQDKATKDHQIRNINDEIAHQDEIINKVNKEKKLLQEMNQKTAEDLQAVEEKASHLNKIKSKLEQTLDELESSVGREKKLRAEIER-
SKRKVEGDLKMTQETVADIERQHKDLEQTIQRKDKEIGNLANKLEEEQGVVSKVQKTIKEMQARIEELETEAEHERQARAKAEKGKGNMSRELNDLNERLDEAGGATGAQMELNK-
KREAELAKLRRDLEEANIQHESALANLRKKHNDAVAEMTEQIDHLNKMKAKTEKEKEMMKYQADEAKSAMDSLARDKALAEKANKTIQQQINEVNVKLDEANRTLRDFDAQK-
KKLSVENGDLLRQLEEADNQINQLNNLKVSLTTQLEDTKKMVDDESRERSVLLGKFRNLEHDLDGLREQLDEECEAKADANRQLSKAHAEAQMWRSKYESEGVARAEEIEAARLK-
LAARLEEAELQIEQLNVKNMQLEKAKGRIISEIDEMQMQVERAQGMAIAAERRQKDFDRVVNEWKIKVDQLATELDASQKECRQYSTEHFRIKAVYEENLEHLDSVRRENKGLAEEIK-
DLMEQISEGGRSLHEIEKNAKRFEIEKEELQAALEEAEAALEQEENKVLRGQLELSQVRQEIDKRIQEKEEEFDATRKCHQRAIESMQASLEAEAKSKAEALRMKKKLESDIGELEIALD-
HANKANADIQKQVKKAQAEMKDMQARVEEEQRLASEYREQCSASERKANAVNGELEESRTLLEQSDRGRRQAESELADANESLSHLTAQHGSLSMAKRKLEGEIQTLHAELD-
DMLNEAKNSEDKAKKAMVDAARLADELRAEQEHAQTQEKMRKGLDLSVKDLQARLDEFESSAHKTGKKALAKLEARIRELENQLDDESRRHADAQKNLRKCERRIKELSFQSEED-
KKNHERMQDLVDKLQQKIKTYKRQIEEAEEIAALDLAKYRKAQQELETVQRS

>BAN17317.1 myosin heavy chain type 3, partial [Penaeus monodon]

ESDINELEIALDHANKANSDLHKHYRKIQDDVKDMEARVKEEQRLASEYREQYGIAERRFNALHGELEESRTLLEQSDRGRRHAETELNDARDQINNFNSQNGALAAT-
KRKLEGEMQTLQADLEEMLSEAKNSEEKAKKAMLDAARLADELRSEQEHAQVQEKMRKALEGTVKDLQTRLEESEAAALKTGKKAISNLEGRIRDLESALDDETRRHADSQKNLRK-
CERRIKELAFQTDEDKKNHDRMQDLVDKLQQKIKTYKRQIEEAEEIAALNLAKFRKAQQELEETEMTVTVSHY

>BAN17320.1 myosin heavy chain type 6a, partial [Penaeus monodon]

ESDIGELEIALDHANKANGDIQKQVKKAQAEMKDMQARLEEEQRLASEYREQCSASERKANAVNGELEESRTLLEQSDRGRRQAESELADANESLSHLTAQHGSLS-
MAKRKLEGEIQTLHAELDDMLNEAKNSEDKAKKAMVDAARLADELRAEQDHAQNSEKMRKGLEVSVKDLHARLDDFESSGHKTGKKALAKLEVRIRELETQLDDEARRHA-
DAQKNLRKCERRIKELSFQSDEDKKNHERMQDLVDKLQQKIKTYKRQIEEAEEIAALNLAKYRKAQQELESK

>ANZ80593.1 ubiquitin-activating enzyme E1 [Penaeus monodon]

MSELQAANSQPPCKRPRLEPAVNGSTNMAANGSQQDIDESLYSRQLYVLGHDAMRRMASSDVLISGLGGLGVEIAKNVILGGVKSVTLHDTKNTSVRDLSTQFFLTSDDVGTN-
RATACHSRLAELNTYVPISASISPLTEEFLKKFRVVVLTDSSLEEQMQVSSFCHSNNIAVIVAATKGLFGQIFCDFGEEFEVVDTNGESPVSAMIAGVTKEENSVVTCLDESRHGIEDG-
DYVTFSEVQGMTELNGCKPVKIKVLGPFTFSIGDTAQFSDYVRGGIVTQVKVPVKKSFKALEESIKEPEFIMTDFAKFDRPGILHIAFQTLHKYEKENGSAPRPWNEEDAEKFLHAFKEM-
NEGHPAKVEEFDENLVKQFAKIASGGVAPLDAVIGGIVAQEVMKACSGKFTPIYQWLYFDALECLPDDLSTITEDLCTPAGDRYDSQVAVFGRDFQKKLGSQKYFIVGAGAIG-
CELLKNFAMLGIGAGEGGKLVVTDMDLIEKSNLNRQFLFRPWDVQKAKSDTAAAAVKKMNPQVNIIAHQNRVGPETERVYDDSFFEPLDGVANALDNVEARLYMDRRCVYYRK-
PLLESGTLGTKGNVQVVVPHLTESYGSSQDPPEKSIPICTLKNFPNAIEHTLQWARDMFEGEFRQAAENAAQYLVDSRFMERTLKLPGSQPMEVLESVKRALVEDRPQSFEDCVL-
WARKHFAEQFHNQIVQLLHNFPKDQTTSSGEPFWSGPKRCPHPLKFDANNQLHLDYILSAANLKAEVYGITQVRDRDAVADIVNKIEIPEFVPRSGVKIAVTDAEAEAQSNQVDSERI-
ANLQSAIPQATSFGALKLTPLDFEKDDDTNFHMDYIVAASNLRAENYDIPPADRHKSKLIAGKIIPAIATTTALISGLVSLELLKLVQGHNSIEKFKNGFVNLALPFFGFSEPISAPKNKYYD-
KEWTLWDRFEVDGEMTLEEFINYFEEKHNLEITMLSQGVSMLYSFFMPPAKRNERMSLPMSEVVKKVSKKKIEDHVRALVLELCANDKDGEDVEVPYVKYNLPTQ