RESEARCH ARTICLE

Identification of hemocyanin as a novel non-cross-reactive allergen from the giant freshwater shrimp *Macrobrachium rosenbergii*

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Scope: Sensitization to giant freshwater shrimp *Macrobrachium rosenbergii* (*Mr*) was recently reported. However, the allergens have yet to be identified. This study aimed to identify and characterize a novel allergen of *Mr* shrimp.

Methods and results: Extracted proteins were separated and purified by anion and in some experiments, size-exclusion chromatography. Serum IgE from shrimp allergic donors identified a candidate protein, which was characterized by LC-MS/MS. The specificity of IgE binding was tested using immunoblotting and inhibition ELISA. The IgE-binding profiles from 12 of 13 *Mr* allergic subjects that were pre-incubated with an extract of *Penaeus monodon* showed residual binding to ~60–80 kDa proteins. The 60–80 kDa IgE-bound proteins were fractionated in the flow-through of anion chromatography showing a high IgE reactivity. Peptides identified by LC-MS/MS showed the proteins closely match subunits of hemocyanin (Hcs). Purified Hcs from hemolymph markedly inhibited binding of IgE from sera of *Mr* allergic subjects to solid-phased *Mr* proteins in inhibition ELISA.

Conclusion: Hcs were identified as heat-stable, non-cross-reactive, high-molecular-weight (MW) allergens from *Mr* shrimp. Since circulatory organs are not always removed during food preparation, high concentrations of Hcs may be present along with shrimp meat, which contains the known cross-reactive tropomyosin protein.

Keywords:
IgE reactivity / Seafood allergy / Shrimp allergen

1 Introduction

Seafood, including fish, crustacean and mollusks are commonly worldwide [1]. Both crustaceans and mollusks are referred to as shellfish and are recognized as one of the common causes of cross-reactive food allergy [1] in different countries such as Japan [2], Thailand [3] and the USA [4]. To date, four well-characterized allergens of crustaceans have been reported [1, 5]. Tropomyosin, a 35- to 38-kDa protein, was the first reported pan-allergen and has been thought to be the primary allergen in various species of crustaceans and mollusks [1, 4–6]. Recently, three other allergens of crustaceans were successfully identified using MS to identify the allergens [5–10]. A 40-kDa arginine kinase enzyme essential for energy metabolism was identified as a second pan-allergen in *Peneaus monodon* shrimp (Pen m 2) and the cross-reactive enzyme from *Litopenaeus vannamei* (Lit v 2) [5, 7]. Two 20-kDa proteins of shrimp *L. vannamei*, myosin light chain (Lit v 3) [8] and sarcoplasmic calcium-binding protein (SCP) (Lit v 4) [9] were identified by LC-MS/MS and characterized by recombinant protein as allergens. The

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Abbreviations: BCA, bicinchoninic acid; **DB,** dialyzed/boiled; **Hcs,** hemocyanin subunits; ***Mr,*** *Macrobrachium rosenbergii*; **MW,** molecular weight; ***Pm,*** *Penaeus monodon*; ***Ss,*** *Scyllaserrata*

sarcoplasmic calcium-binding protein of shrimp *P. monodon* has also been reported as an allergen [10].

Previously shellfish allergy reports have generally been attributed to salt water shrimp such as the black tiger shrimp *P. monodon* (*Pm*) or Pacific white leg shrimp *L. vannamei* (*Lv*). Recently, the giant freshwater shrimp *Macrobrachium rosenbergii* (*Mr*) was identified as a cause of shellfish allergy by oral-challenge tests [3]. Since freshwater shrimp *Mr* is exported from Thailand and Taiwan to many countries for human consumption, it is possible that new cases of hypersensitivity to *Mr* will arise in different populations in the near future. Thus, identification of the allergens of shrimp *Mr* would prove useful to ensure appropriate diagnosis of allergy to this freshwater shrimp.

In this study, we identified the subunits of hemocyanin (Hcs), an oxygen carrier protein in hemolymph, as the high-molecular-weight (MW) allergen from *Mr* shrimp visible in immunoblots with sera from *Mr* allergic subjects. The Hcs were purified by anion-exchange chromatography and identified by LC-MS/MS. We further characterized Hcs as a novel heat-stable and non-cross-reactive allergen based on inhibition studies using immunoblotting and ELISA.

2 Materials and methods

2.1 Shrimp allergic patients

Serum was prepared from blood samples of 13 shrimp allergic patients who had positive reactions to oral-challenge with the freshwater shrimp *Mr,* but negative reaction to the saltwater shrimp *Pm*. Food challenges to both shrimp *Pm* and *Mr* were performed in patients with positive skin tests to at least one species of shrimp. Food challenges were performed with one species at 2–4 wk before challenging with the second species [3]. Participants were asked to avoid consuming any shrimp for 2 wk before each challenge. The three-step food challenge was performed as described by Jirapongsananuruk et al [3]. Briefly, patients swallowed capsules containing increasing doses (500 mg, and 1, 2, 4 and 8 g) of raw lyophilized shrimp at a 15-min interval. The maximum cumulative dose was 15.5 g. Positive symptoms in some cases included airway or skin reactions, vomit or diarrhea or systemic reactions including anaphylaxis. The second step was to wipe a cooked shrimp on the inner lips of the subject and place it in the mouth without chewing for 5 min before it was removed. Positive oral-mucosal reactions were lip swelling/itching or throat itching. The third step was an open feeding challenge with increasing doses of cooked shrimp at 1, 2, 4, 8, 16 and 32 g at a 15-min interval. The cumulative maximum dose was 63 g. Positive responses to any three steps were considered as a positive challenge. Anaphylaxis was diagnosed by the recently published criteria [11]. Vital signs as well as patient's symptoms and signs were recorded every 15 min. Emergency resuscitation equipment and drugs were available in case of an emergency. The SPT, oral-food challenge protocols and the use of serum were approved by the Institutional Review Board at Siriraj Hospital (SiEc038/2005) [3].

Sera of five non-allergic subjects were also prepared and were used as a negative control. All sera were stored at −20°C until analysis.

2.2 Preparation of *Mr* shrimp extract

Live giant freshwater *Mr* shrimp and saltwater *Pm* shrimp were purchased from local markets in Bangkok, Thailand. The heads and shells were removed. The abdominal muscle was minced, homogenized and stirred in phosphate-buffered saline (pH 7.4) containing 1 mM PMSF at 4°C overnight before centrifugation at 17 210 × *g* for 30 min. The supernatant was filtered through 45-μM membranes and stored at −20°C. Total protein concentrations of *Mr* and *Pm* extracts were determined by the bicinchoninic acid (BCA) protein assay [12].

2.3 IgE-binding profile of patients allergic only to *Mr* shrimp

Fifty microgram of protein from each extract was loaded per lane in a 12% SDS-PAGE gel using Laemmli [13] buffer and reducing conditions. Separated proteins were electro-transferred onto nitrocellulose membranes. The membranes were blocked in 3% skimmed milk-blocking solution for 2 h. For inhibition assays, serum samples diluted one to four or one to eight sera were pre-incubated with extracts containing 500 μg of *Pm* for 2 h and centrifuged at 17 210 × *g* for 10 min before adding to the membranes. The pre-incubation was intended to block IgE binding to cross-reactive proteins. The sera were then incubated with membranes blotted from SDS-PAGE of *Mr* separated proteins for approximately 12 h at 4°C. Bound IgE was detected by incubating washed membranes with 1:50 000 diluted HRP-conjugated goat polyclonal IgG anti-human IgE antibody (cat no. 074–1004 from Kirkegaard & Perry Laboratories (KPL), MD, USA) followed by washing and addition of chemiluminescent substrate (Immobilon Western HRP substrate, Millipore, MA, USA). Light emissions were captured on X-ray film.

2.4 Isolation and characterization of *Mr* proteins by FPLC and LC-MS/MS

Proteins in *Mr* extracts were separated by an anion exchange (DEAE) column chromatography. Briefly, *Mr* extract was dialyzed in 20 mM Tris, pH 8 at 4°C with several buffer changes, then passed through a DEAE anion column.

Bound proteins were eluted using 20 mM Tris, pH 8, containing increasing concentrations of NaCl (100, 200, then 300 mM). Fractions were collected and dialyzed in 20 mM Tris, pH 8, 100 mM NaCl buffer. The protein concentration of each fraction was determined by the BCA method [12].

Identical protein samples (25 μg each) were separated in two 10% SDS-PAGE gels for immunoblotting and sequence analysis by liquid chromatography and tandem mass spectrometry (LC-MS/MS). Based on the results of immunoblotting, spots identified in the Coomassie G250-stained gel that matched the position of those bound by IgE were excised and submitted for analysis by LC-MS/MS at the proteomic service, Genome institute, National Center for Genetic Engineering and Biotechnology (BIOTEC) (Thailand). The LC-MS/MS spectral data were analyzed by SEQUEST (version 3.3, Thermo Fisher Scientific) using default analytical criteria recommended by the manufacturer. Sequences of the LC-MS/MS identified peptides were compared with Clustal W aligned with hemocyanin proteins of *L. vannamei*, *Pm* and *Cancer magister*.

2.5 Preparation of hemolymph

Hemolymph was collected from the arthrodial membrane of the walking legs of live shrimp using a 22-gauge needle and a syringe containing modified Alsever solution (AS) used as anti-coagulant (27 mM Na citrate, 34.22 mM NaCl, 104.5 mM glucose, 198.17 mM EDTA). In addition, hemolymph of the mud crab (*Scylla serrata* (*Ss*)) was collected from the arthrodial membrane of the last walking legs of the crab and mixed in the modified Alsever solution.

2.6 Partial purification of Hcs

Hemocyanin forms multimeric aggregates in hemolymph and the aggregates precipitate when boiled [14]. However, based on our results from preliminary experiments, dissociated or monomeric form of Hcs were not precipitated and not degraded when boiled. Thus, hemolymph was dialyzed in 50 mM Tris, pH 9.2, 10 mM EDTA at 4°C with several changes of buffer to obtain the monomeric Hcs. The dialyzed hemolymph was then boiled for 10 min and centrifuged at 12 000 × *g* 10 min. The supernatant (dialyzed/boiled or DB hemolymph) was used either in inhibition ELISA or for purification of Hcs. For purification, the DB hemolymph was loaded onto a DEAE anion column. Hcs were remained in the flow-through fraction in 50 mM Tris, pH 9.2, 10 mM EDTA and further purified through a Superdex 200 5/150 GL gel filtration column (GE Healthcare, USA) using 50 mM Tris, pH 9.2, 10 mM EDTA. Elution fractions containing Hcs were collected and tested by inhibition ELISA. Protein concentration was determined by the BCA method [12].

2.7 Immunoblot analysis of IgE binding to Hcs in DB hemolymph

Twenty-five microgram of DB *Mr* hemolymph was separated under reducing conditions in 10% SDS-PAGE gel for immunoblotting. Sera were diluted 1:4 or 1:6 for incubation with the membranes. Immunoblotting conditions were as described for those allergic to *Mr* shrimp.

2.8 Inhibition ELISA

To confirm specific binding of IgE and Hcs in DB hemolymph, individual sera from nine *Mr* allergic patients who demonstrate specific IgE binding to Hcs by immunoblotting were diluted 1:2 to 1:4 in blocking buffer. Samples of diluted sera were incubated with 1, 100 or 500 μg/mL of DB *Mr* hemolymph in ELISA buffer (100mM $NaPO_4$/150mM NaCl, pH 7.0, 0.05% Tween 20) at 4°C overnight prior to addition to ELISA plates. In addition, the specificity of IgE against *Mr* Hcs was evaluated by preincubation with Hcs of other crustaceans (DB hemolymph from shrimp *Pm* and the mud crab *Ss*). The serum–DB hemolymph mixtures were clarified by centrifugation at 17 210 × *g* for 10 min. The supernatants added in duplicate to DB *Mr* hemolymph pre-coated wells (500 ng/well of DB *Mr* hemolymph overnight at 4°C per well) of 96-well Maxisorb ELISA plates (Nunc, Roskilde, Denmark) and incubated for 2 h at room temperature. After 2-h incubation, 1:1 000 diluted horseradish peroxidase (HRP)-labeled anti-human IgE antibodies (KPL) were added and incubated for 1 h at room temperature. Bound antibodies were detected with the chromogenic substrate (3,3′,5,5′-tetramethylbenzidine:TMB (KPL)). The color reaction was developed for 20 min; then, absorbance at 450 nm was recorded. The inhibition was calculated as the percent reduction in absorbance compared with the uninhibited value. The results were calculated as the mean and standard error of the percent inhibition obtained with the different sera. The IC_{50} was extrapolated from a regression curve.

For assays with purified Hcs, diluted sera of the same four patients as in inhibition with DB hemolymph were mixed and incubated with 100 μg/mL purified Hcs. Inhibition and calculations of percent inhibition were performed as described above.

3 Results

3.1 SDS-PAGE gel analysis of total proteins in *Mr* extract

One gram of abdominal muscle from shrimp yielded ~10 mg/mL of total proteins in the extract. The equivalent of 50 μg of protein in the extract was applied to a 12% SDS-PAGE gel and seven prominent protein bands were visible at ~40, ~50 and >70 kDa (Fig. 1A). Several faint bands of proteins were also visible at ~30, ~36 and ~60 kDa (Fig. 1A).

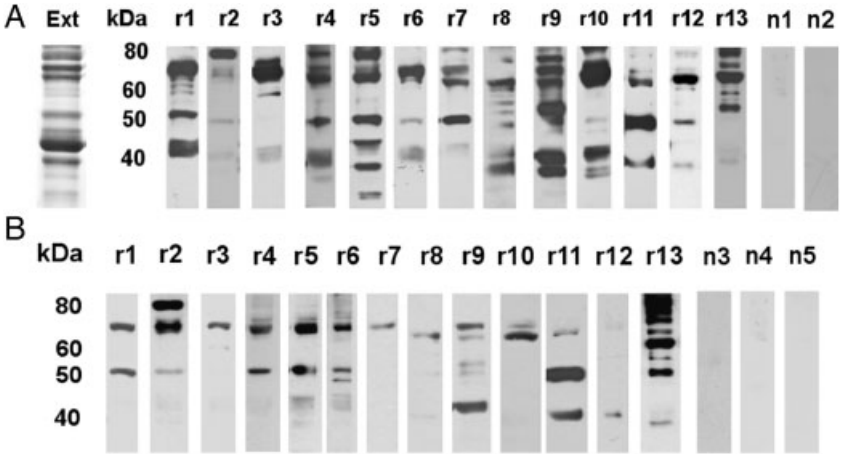


Figure 1. (A) Ext: SDS-PAGE gel analysis of proteins in *Mr* extract; kDa: molecular weight markers; r1–r13: immunoblot profiles of serum IgE-bound *Mr* proteins of 13 shrimp-allergic donors without pre-incubation with *Pm* extract; n1–2: immunoblot using normal sera. (B) r1–r13: Serum IgE-bound *Mr* proteins of the same 13 shrimp-allergic donors after pre-incubated with 500 μg *Pm* extract for 2 h before added to membranes. Note: n1–n5: immunoblot profiles of normal sera without pre-incubation with *Pm* extract.

3.2 Specific IgE-allergen profiles

Immunoblotting of *Mr* proteins was performed using sera from *Mr* allergic patients either with or without pre-incubation of sera with *Pm* extract, to identify non-cross-reactive allergens (Fig. 1A and B). The results of immunoblots from 13 *Mr* allergic subjects without preincubation with *Pm* extract showed all patients had IgE-bound proteins at ~60–80 kDa (Fig. 1A). Ten of 13 patients also showed IgE binding to proteins between 40 and ~50 kDa (Fig. 1A). Interestingly, the results following pre-incubation with *Pm* extract showed that 12 of 13 had IgE binding to proteins at ~60–80 kDa (Fig. 1A). Eight of 13 had IgE binding to proteins at ~50 kDa (Fig. 1B) and only 5 of 13 had IgE binding to proteins at 40 kDa (Fig. 1B).

3.3 Fractionation of *Mr* proteins and immunoblot analysis of fractionated proteins

Many proteins migrate at approximately the same apparent molecular mass in a 1-D SDS-PAGE, thus complicating identification of individual allergenic proteins. Therefore, the proteins in the extract were fractionated by anion-exchange chromatography prior to separation by immunoblotting. A total of four fractions were obtained. Two anion fractions flow-through and bound proteins that eluted in 100 mM NaCl were analyzed by both SDS-PAGE gel and immunoblotting (Fig. 2). The results of immunoblots with eight representative sera showed that one or two bands of high MW 70–100 kDa were bound by IgE in the flow-through (f0) fractions (Fig. 2B and C). These two 70–100 kDa bands were excised (box in Fig. 2A), trypsin-digested and analyzed by LC-MS/MS.

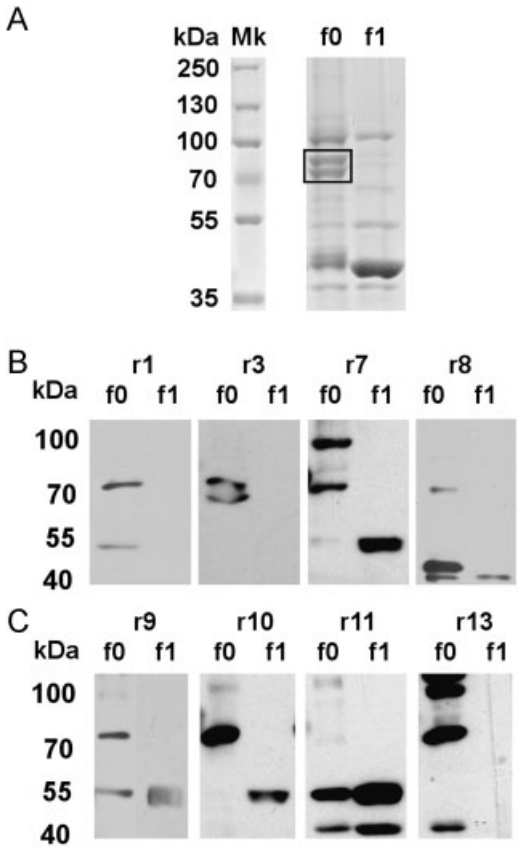


Figure 2. (A) SDS-PAGE gel analysis of anion fractions. f0: flow-through, f1: proteins eluted with 100 mM NaCl. Box around bands in f0 indicates excised protein bands analyzed by LC-MS/MS. (B and C) Immunoblot profiles of serum IgE of eight *Mr* allergic patients: r1, r3, r7, r8, r9, r10, r11 and r13, bound proteins in f0 (flow-through) and f1: (proteins eluted with 100 mM NaCl).

3.4 LC-MS/MS analysis of the high MW allergens

The results from LC-MS/MS analysis of two IgE-bound proteins migrating at 70–80 kDa identified six peptides from the 72-kDa spot and five peptides from the 75-kDa spot, which matched amino acid sequences of different Hcs from different crustaceans with a significant SEQUEST scores (Table 1). The results for the 72-kDa spot showed six

peptides that matched Hcs from different crustacean species in the top 10 ranking based on MASCOT (Table 1). For the 75-kDa spot, results showed six peptides matched Hcs from different crustacean species in the top 20 ranking based on MASCOT (Table 1). All peptides matched amino acid sequences of Hcs from different crustacean species with a significant p value as well as X_{corr} value above cut-off values, which are 1.5 for $Z = 1$ and at 2.2 for $Z = 2$. Moreover, the DelCN value of all peptides was above the cut-off value at 0.1. The matched peptides had 62.5–100% identity with aligned amino acid sequence of Hcs from different crustaceans, except Hcs of shrimp *Pm* (Supporting Information Table 1). When aligned with the amino acid sequences of Hcs of shrimp *Pm,* two of six peptides of the 72-kDa spot and two of five peptides of the 75-kDa spot had 18.8–27.3% identity (Supporting Information Table 1). Thus, based on the results of LC-MS/MS analysis, two high MW IgE-bound proteins are likely Hcs from *Mr*.

3.5 SDS-PAGE and immunoblot analysis of Hcs

An extract of ~6 g of shrimp *Mr* yielded a volume of 1.5 mL with ~13 mg/mL of total proteins. Samples of dialyzed *Mr* hemolymph were separated in 8% SDS-PAGE and showed broad bands of Hcs ranging from 70 to 250 kDa (Fig. 3A; lane D). These appear to be multimeric forms of Hcs from various protein mixtures, resulting in ill-defined, smeared bands. However, electrophoresis of DB *Mr* hemolymph resulted in the appearance of monomeric Hcs, seen as distinct bands at MW ~75 kDa (Fig. 3A; lane DB). Elution of Hcs from the gel filtration column occurred at a similar elution volume as conalbumin, a 75-kD standard protein marker (Fig. 3B; lanes 2 and 3). Immunoblot analysis qof DB *Mr* hemolymph using sera from seven *Mr* allergic patients showed specific IgE bound to the 75 kDa *Mr* Hcs

Table 1. LC-MS/MS analysis of 2 high-molecular-mass spots

72 kDa protein spot	Matched Hcs of crustacean species[a)]
R.IRDAIAHGYIIK.E	7
R.QREEALMLFTVLNQCK.D	1, 2, 3, 4
K.HWFSLFNER.Q[b)]	9, 10
K.YMDNIFK.K[c)]	1, 2, 3, 4, 8
K.FNMPPGVMEHFETATR.D[b)]	1, 2, 3, 4, 5, 9, 10
R.KGENFFWVHHQLTVR.F[b)]	8
75 kDa protein spot	
R.EEALMLFDVLMHCK.S	5, 6
K.HWFSLFNER.Q[b)]	11
K.YMDNIFR.E[c)]	12
K.FNMPPGVMEHFETATR.D[b)]	5, 11
R.KGENFFWVHHQLTVR.F[b)]	12

Identified peptides of the two spots matched with published protein sequence of hemocyanin from crustacean species are shown.

a) Crustacean species: 1: hemocyanin subunit 1 (*Palinurus vulgaris*); 2: hemocyanin subunit 2 (*P. vulgaris*); 3: hemocyanin subunit 3 (*P. vulgaris*); 4: hemocyanin subunit 4 (*Palinurus elephas*); 5: hemocyanin α-subunit (*Homarus americanus*); 6: hemocyanin β subunit (*H. americanus*); 7: hemocyanin (*Palaemonetes pugio*); 8: hemocyanin (*P. monodon*); 9: hemocyanin subunit 1 (*C. magister*); 10: hemocyanin subunit 2 (*C. magister*); 11: hemocyanin subunit 2 (*C. magister*); 12: hemocyanin subunit 3 (*C. magister*).

b) Four peptides found in the two spots.

c) Residue no. 8 of this peptide is different.

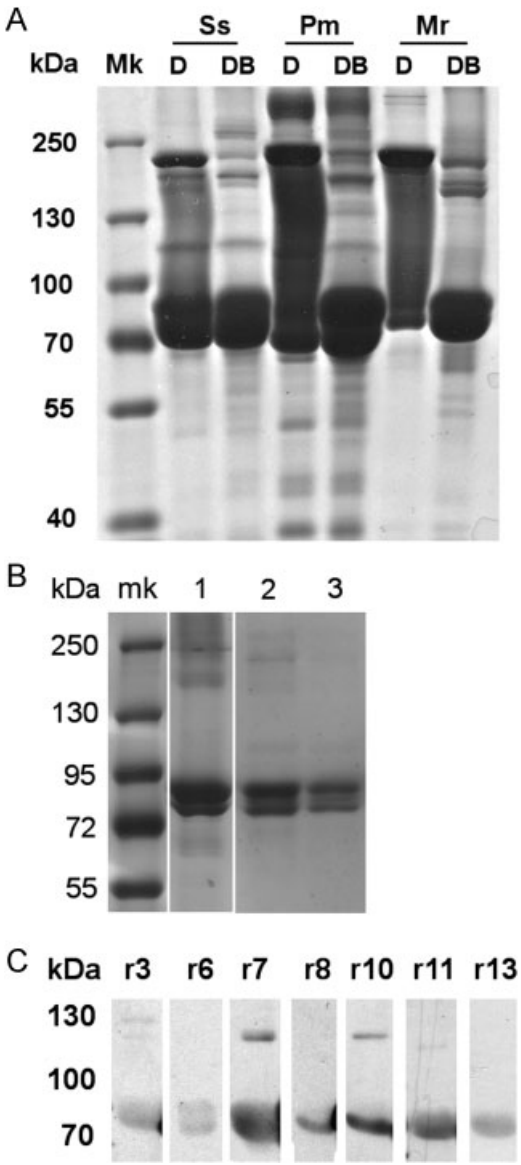


Figure 3. (A) SDS-PAGE analysis of Hc in hemolymph of three crustacean species: shrimp *Pm*: *P. monodon*, shrimp *Mr*: *M. rosenbergii* and mud crab Ss: *S. serrata,* after dialysis (D) in Tris, pH 9.2, 10 mM EDTA and after both dialysis and boiling (DB) for 10 min. (B) SDS-PAGE analysis of purified *Mr* Hcs. 1: DB Hcs; 2 and 3: eluted Hcs from size-exclusion column. (C) Immunoblot of serum IgE of seven *Mr* allergic patients: r3, r6, r7, r8, r10, r11 and r13, bound to DB Hcs (as shown in B, lane 1).

with different intensities (Fig. 3C). Moreover, results from peptide mass fingerprinting also confirmed the identity as Hcs.

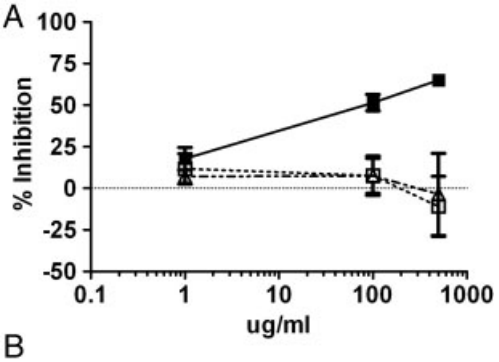
3.6 Inhibition ELISA of Hcs

IgE reactivity against monomeric Hcs in DB hemolymph was determined by inhibition ELISA. The inhibition results showed only DB of *Mr* hemolymph inhibited IgE from sera of nine patients to bind to coated DB *Mr* hemolymph with the IC_{50} of ~100 ug/mL whereas neither Hcs in DB *Pm* nor *Ss* hemolymph could inhibit IgE binding (Fig. 4A).

Purified Hcs from DB *Mr* hemolymph was retested for IgE reactivity. Owing to a limitation of sera, only four of nine sera, previously used in DB hemolymph inhibition ELISA, were used. The results of inhibition using 100 ug/mL purified Hcs also showed ~50% binding of IgE from tested sera (Fig. 4B).

4 Discussion

This is the first study to identify high MW Hcs proteins as novel non-cross-reactive allergens from fresh water *Mr* shrimp. In the hemolymph of crustaceans, hemocyanin is composed of hexamers or multi-hexamers of Hc with individual subunits having MW ~75 kDa. These complexes are oxygen transport proteins [14, 15]. Hcs of crustaceans reportedly contain multiple subunits that are species-specific [15–18]. The heterogeneity of Hcs from three tested crustaceans could be observed in SDS-PAGE (Fig. 3A; lane D) showing different mobilities across the species. The amino acid sequence alignment results suggest that amino acid sequence of Hc subunits of shrimp *Mr* differs from those of shrimp *Pm* Hcs. The results of immunoblotting using sera pre-incubated with shrimp *Pm* extract to occupy cross-reactive IgE still demonstrated IgE in 13 of the subjects that retained residual binding to the two high MW *Mr* proteins. These results suggested that *Mr* Hc subunits are unique allergens. Interestingly, the results of the same immunoblotting also showed reduced signal of IgE bound to proteins for some patients. These results suggest that some other IgE cross-reactive *Pm* proteins with approximate MW of 75 kDa may exist. However, the results of inhibition ELISA demonstrated that only *Mr* hemolymph could inhibit serum IgE binding for *Mr* allergic patients. This confirms that the *Mr* Hcs act as unique allergens. Although Hcs appear to be unique allergens, ~100 μg/mL of hemolymph or of purified Hcs protein was required to achieve 50% inhibition, suggesting the affinity of binding may be low. One possible explanation is that a dominant epitope might be a complex carbohydrate. A second possible explanation would be that the structure of the epitope, as presented in this study, is not native. Although one glycosylation site has been reported for Hcs of some crustaceans such spiny lobster (*Panulirus spp.*) and the shame-faced crab *Calappa granulata* [17–19], the full sequence of the *Mr* shrimp is still unknown and we did not attempt to determine the glycosylation status of the proteins. Structural masking or unfolding of the Hcs proteins used in our tests may also contribute to a weak affinity suggested by the high amount of Hcs needed to achieve more than 50% inhibition. Structural hindrance of high MW allergens was previously observed with peanut allergens Ara h1 and Ara h3 [20].



B

Patient	% Inhibition
r1	49
r2	64
r7	63
r14	66

Figure 4. (A) Inhibition of IgE binding using 1, 100 and 500 μg/mL of DB hemolymph containing Hcs subunits from shrimp *Mr* (■), *Pm* (□) and mud crab *Ss* (△). Plotted values are a mean with SE of nine individual patients in duplicate wells. (B) Percent inhibition of IgE by 100 μg/mL of size-exclusion column eluted *Mr* Hcs incubated with sera from four individual patients in duplicate wells.

In crustaceans, 75–95% of total proteins in hemolymph are composed of Hc. Hemolymph circulates through the abdominal segment via the posterior aorta and ventral artery [21]. During the preparation of samples used in this study the aorta and ventral artery was not removed. As these structures are often not removed in the preparation of shrimp used for food, it is likely that Hcs in hemolymph would be present in shrimp that are consumed in a typical meal. Also cooking by boiling should not cause Hcs to degrade or denature as shown by results from SDS gel and immunoblot analysis of DB hemolymph. Thus, it is likely that allergic reactions could be initiated by *Mr* Hcs as well as by other allergen such as tropomyosin when *Mr* allergic subjects consume cooked shrimp *Mr*.

In conclusion, Hcs of freshwater shrimp *Mr* should be regarded as a novel and unique heat-stable allergen.

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The authors have declared no conflict of interest.

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