



Identification of the major allergenic epitopes of *Eriocheir sinensis* roe hemocyanin: A novel tool for food allergy diagnoses



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ABSTRACT

Crab meat and roe are highly nutritious delicacies in China. While extensive research has been conducted for allergens derived from crab-meat, data relevant to the allergenic potential of crab roe derived proteins, of which hemocyanin is a principal contender, are almost entirely absent. Using bioinformatics prediction and IgE-binding assays, the three principal immunodominant epitopes of hemocyanin were identified and then combined as a single recombinant fusion protein (rHc). This together with the full-length recombinant protein (Hc) were expressed in *Escherichia coli* and subsequently identified by SDS-PAGE and immunoblotting. Ninety-five percent of our patients were found to carry rHc-specific IgE antibodies by ELISA. Dot-blot inhibition, together with ELISA inhibition studies, showed that pre-incubation of patient sera with the recombinant epitope protein could inhibit 26% to 63% (mean: 50%) of IgE binding to immobilized, full-length Hc and the dose-response curve represents as a sigmoid shape. The recombinant protein (rHc) represents a versatile biologic tool with which to diagnose and investigate therapies for *E. sinensis* allergy.

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1. Introduction

Food allergy (FA) is a type I hypersensitivity disorder that affects up to 2–8% of the population (Munoz-Garcia et al., 2013), with clinical symptoms ranging from mild oral mucosa inflammation to life-threatening anaphylaxis. Shellfish allergy is relatively common, with a worldwide prevalence of 10% in food allergy (Nwaru et al., 2014; Wai et al., 2014), and a particularly high burden in Asian countries (Shek et al., 2010; Kim et al., 2008). Unlike egg and milk allergies, shellfish allergy is typically a life-long disorder, which can significantly impair the quality of life (Ayuso et al., 2010). Despite its prevalence, there are neither unified standardized test substances, nor an effective treatment for this allergic response. Among shellfish, *Eriocheir sinensis* is an economically important aquaculture species in China (Gong et al., 2010; He et al., 2015). Due to its nutritional value and delicious taste, crab roe, which includes the hepatopancreas and ovary, is the favored part of the crab for

consumption in China. Consequently, crab roe is, potentially, an important instigator of food allergy for which there is an urgent need to develop safe and effective diagnoses and therapies.

Over the past 25 years, a variety of allergens have been identified and their physicochemical properties analyzed (Lopes de Oliveira et al., 2013). As a result, there has been increased interest in the ability to measure component-specific immunoglobulins as a way to improve our diagnosis of food allergens (Hoffmann-Sommergruber et al., 2015; Sicherer and Sampson, 2014). While tropomyosin (TM) and arginine kinase (AK) are the major allergens of shellfish, others have shown that component sensitization can vary by region, and with the individual (Calderon et al., 2015; Salo et al., 2014). Consequently, interest has also been shown in the allergenic potential of other proteins. Additionally, research on allergens derived from crab-meat has almost entirely ignored the allergenic potential of crab roe.

In a previous study (Ying-Ying et al., 2015), we showed that hemocyanin bound IgE from the sera of 14 of 23 (61%) patients allergic to *E. sinensis*. Hemocyanin is an oxygen-transport protein found in crustacean hemolymph, which accounts for 75–95% of its total protein content (Pedrosa et al., 2015). We were interested in this protein because it is major allergen in *E. sinensis* roe (Cao et al., 2014), with demonstrably high IgE-binding. Hemocyanins

Abbreviations: FA, food allergy; MW, molecular weight; TM, tropomyosin; AK, arginine kinase.

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Table 1
Characterization of *E. sinensis* – allergic patients.

No.	Gender	Age(years)	other allergies	crab sIgE (kUA/L)
1	F	23	No	0.59
2	M	42	CN	0.93
3	F	1	milk	1.1
4	F	2	CN	1.8
5	M	19	No	0.73
6	F	36	EW	0.61
7	M	4	No	1.2
8	F	29	EW, milk	0.65
9	M	37	EW, shrimp	3.1
10	F	29	shrimp	10.5
11	F	24	beef, shrimp	1.9
12	F	4	CN, milk, shrimp	5.52
13	M	32	shrimp	14.7
14	M	36	EW, shrimp	5.6
15	F	21	No	0.49
16	M	24	CN, milk	2.90
17	F	60	shellfish, shrimp	12.3
18	F	2	milk, shrimp	8.7
19	F	45	CN, milk, shrimp, shellfish, PA	15.6
20	M	5	milk, shrimp	11.5
21	M	54	shrimp	8.7

M, male; F, female; CN, cashew nut; EW, egg white; PA, pineapple.

are large macromolecular assemblies relevant to molecular evolution, with potential biotechnological and therapeutic applications (Zhang et al., 2013; Glazer et al., 2013), as well as relevant to innate immune responses (Zhu et al., 2014). Therefore, research on *E. sinensis* roe hemocyanin is of particular significance for the Chinese population.

Some shellfish components, such as TM (Abramovitch et al., 2013) and AK (Mao et al., 2013), have made their availability in recombinant form a prerequisite for detailed immunologic characterization. This has substantially improved their use in diagnosing shellfish allergy by the measurement of IgE binding (Gamez et al., 2011). In the last several years, those epitopes to which IgE binds have also been identified in some food allergens (Perez-Gordo et al., 2013; Baar et al., 2014). Furthermore, for some foods such as peanut, milk, and egg, a positive relationship has been found between recognition of certain linear IgE-binding epitopes and the allergic reactivity of patients (Wang et al., 2010; Lin et al., 2012). The increasing availability of crab-rope derived recombinant allergens with known immunodominant epitopes will help to illuminate the underlying mechanisms of allergen-antibody interactions, and provide valuable data for diagnosis and, ultimately, allergen-specific immunotherapy (Gepp et al., 2014).

In this study, we identified ten epitopes of hemocyanin, including three immunodominant epitopes; and then design and express a tri-epitope fusion protein (rHc) in *Escherichia coli* (*E. coli*). The immunological characterization of rHc was then assessed using sera collected from patients allergic to *E. sinensis*.

2. Materials and methods

2.1. Patients' sera

Sera from 21 *E. sinensis* allergic patients were collected from the Academy of Traditional Chinese Medicine Affiliated Hospital (Tianjin, China), and then stored in aliquots at -20°C until use (Table 1). The diagnosis of *E. sinensis* allergy was based on symptoms observed following exposure to *E. sinensis*, a history of a typical case, and/or the presence of serum total IgE ($>100\text{ kUA/L}$), and crab-specific IgE ($>0.35\text{ kUA/L}$), as determined with the ImmunoCAP System (Thermo Fisher Scientific, Sweden). A sera pool was made by mixing equal volume of 20 sera for ELISA inhibition experiments. As negative controls, sera from 7 healthy

Table 2
Primer sequences used to clone Hc.

Primer Name	Primer Sequence	Restriction Site
H1for	ACATGGGGACCAACGAGTC	None
H1back	CGGCAGTCTGCTAGACTATGTG	None
H2for	AAGGAAACCATGGGGACCAACGAGTCCA	NcoI
H2back	AAGGAAACATATGGTGTCTCTCGATGTGGTAAACC	NdeI

volunteers, without history of type I allergy to common allergens, were included. Our study was approved by the Ethics Committee of the Tianjin Medical University. A written informed consent was obtained from all the allergic and normal subjects, including caretakers of children.

2.2. Preparation of hemocyanin cDNA and polymerase chain reaction (PCR)

E. sinensis was purchased from local suppliers (Tianjin, China), representing the type of crab consumed widely in China. Total RNA was isolated from 1 g *E. sinensis* roe using Trizol reagent (Invitrogen, USA). A detailed description of the primers is given in Table 2. The cDNA coding for hemocyanin was subsequently PCR amplified using the specific primers (detailed in Table 1) and Pfu DNA polymerase (Fermentas, Lithuania). The PCR parameters were as follows: 98°C for 30 s, 35 cycles of 98°C for 10 s, 68°C for 30 s, 72°C for 80 s, and finally 72°C for 10 min. The amplified PCR product was then excised from the agarose gel and purified using the Universal DNA Purification Kit (TIANGEN, China).

2.3. Epitope prediction and molecular modeling of hemocyanin protein

In order to improve the accuracy of antigen epitope prediction, we used three calculation softwares to predict antigenic hemocyanin epitopes, including DNASTAR Protean system (DNASTAR Inc., Madison, WI, USA), ABCpred (Saha and Raghava, 2006), and Bepipred Antibody Epitope Prediction (Larsen et al., 2006). The amino acid sequence coding for hemocyanin (GenBank database, Accession number AEG64817.1) was used as the input sequence for epitope prediction. The hemocyanin protein was analyzed for hydrophilicity, flexibility, accessibility and antigenic index. 10 peptides with high scores for each of these categories were selected as candidate antigenic epitopes. To further investigate epitope location, we then generated a three-dimensional (3D) model of hemocyanin using the SWISS-MODEL structure homology-modeling server (Arnold et al., 2006). Hemocyanin from *E. sinensis* was modeled based on the structure of the hemocyanin from *Panulirus Interruptus* (PDB ID: 1hcy), which is the mostly closely related to hemocyanin (highest sequence identity: 59%) in the structure database. The images were obtained and rendered using the PyMOL software (<http://www.pymol.org/>).

2.4. Epitope recognition by dot-blot assays

Dot-blot assay was performed as described (Chen et al., 2012). Our 10 candidate antigen epitopes were synthesized by Sangon Biotech Corporation (Shanghai, China) and solubilized in PBS buffer (27.4 mM NaCl, 0.54 mM KCl, 0.4 mM KH_2PO_4 , 2 mM Na_2HPO_4 , pH 7.4). 10 μg of each peptide were spotted onto Nitrocellulose membranes (Millipore, USA) and blocked with 5% skim milk in TBST (Tris-Buffered Saline with 0.05% Tween-20, pH 7.4), overnight at 4°C . The membranes were then washed three times with TBST for 10 min and incubated with a 1/10 dilution of the patient sera for 2 h at room temperature. Serum from a non-allergic patient was used as a negative control. After washing three times with

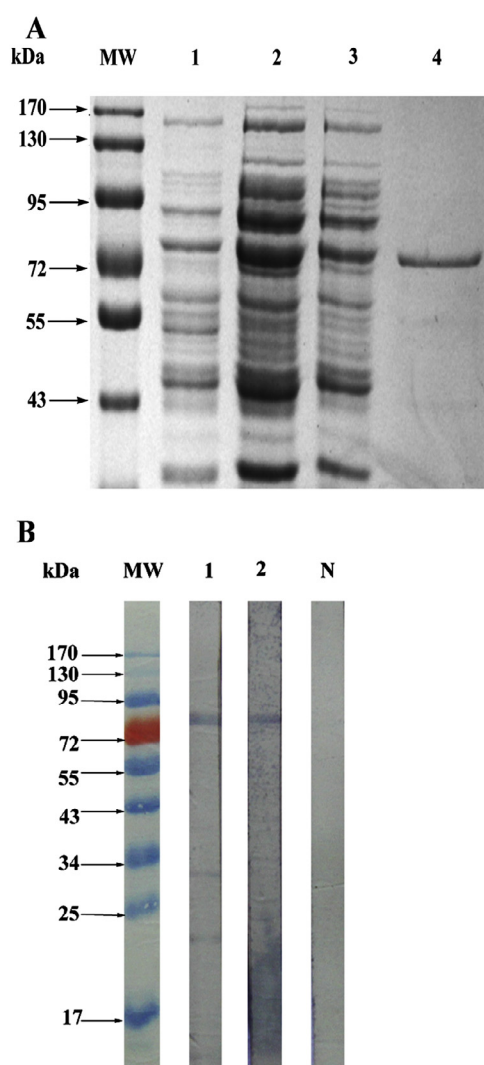


Fig. 1. SDS-PAGE and immunoblotting analysis of Hc. (A) SDS-PAGE: lane MW: molecular weight marker; lane 1: lysate from un-induced bacteria; lane 2: lysate from induced bacteria; lane 3: the wash fractions from nickel chelate columns; lane 4: the purified recombinant protein Hc. (B) Immunoblotting analysis: lane MW, pre-stained protein marker; lanes 1, immunoblotting using sera from a *E. sinensis* allergic patient; lanes 2, immunoblotting using mouse anti-His antibody; lane N, immunoblotting using control serum (serum from non-allergic subject).

TBST, the membranes were incubated with mouse anti-human IgE horseradish peroxidase-conjugate (Sigma, USA) at a dilution of 1:20000 in TBST for 2 h at room temperature. The membranes were washed three times with TBST for 10 min, before visualizing bound IgE antibodies using the ECL Plus Western blotting detection reagents (GE, USA). All incubation procedures were done with shaking.

2.5. Construction of recombinant plasmids

In order to investigate the immunogenicity of the predicted allergenic epitopes, we engineered a recombinant allergen, designated rHc, which was designed according to the reaction frequencies of the predicted linear epitopes. The three epitopes with the highest responder frequency (peptides 181–207, 331–350, and 360–378) were linearly combined in sequence, using two flexible intervening peptide sequences composed of 6 glycines respectively. The rHc sequence was synthesized at Sangon Biotech Corporation and then inserted into the pET-28a vector via XhoI and EcoRI. Additionally, the full-length cDNA of hemocyanin (Hc) was

PCR amplified, with restriction sites introduced by the primer set. The amplified product was purified and inserted into pET-28a as an NcoI/NdeI insert.

2.6. Expression and purification of the recombinant antigens

The plasmids pET28a-rHc and pET28a-Hc were transfected into *E. coli* Rosetta (DE3) competent cell (CWBIO, China); positive clones were then identified and sequenced. Protein induction in *E. coli* cultures were grown in Luria-Bertani (LB) medium at 37 °C and achieved with 1 mM isopropyl b-D-thiogalactoside (IPTG), for cultures grown to an optical density (OD) measured at 600 nm of 0.6–0.8. Induced bacteria were subsequently cultured overnight, at 16 °C. Bacteria were then harvested by centrifugation (10 min, 9000g, 4 °C) before being re-suspended in PBS (20 mM, pH 7.4). After ultrasonic disruption, proteins were collected by centrifugation (5 min, 12000g, 4 °C). Recombinant proteins were then purified under denaturing conditions using Ni²⁺-NTA affinity column chromatography (Invitrogen, USA) according to the manufacturer's instructions. Protein concentrations were measured using the PierceTM BCA Protein Assay Kit (Thermo Scientific, USA).

2.7. SDS-PAGE and immunoblotting

SDS-PAGE under reducing conditions was carried out following the Laemmli method (Laemmli, 1970). Purified rHc and Hc proteins were treated in a denaturing buffer and heated at 100 °C for 5 min. Protein extracts, together with purified proteins and pre-stained molecular weight markers (Biorad, USA) were then resolved using a 12% separating gel with a 5% stacking gel (Mini Protean 3 System, BioRad, USA). To ensure proper protein visualization, gels were stained with Coomassie Brilliant Blue R-250.

To assess their IgE-binding properties, our recombinant epitope construct (rHc), and full-length (Hc) proteins were transferred onto polyvinylidene fluoride membranes (0.22 μm, Millipore, USA) for immunoblotting analysis (Hilger et al., 2011). And then the membranes were blocked with 5% skimmed milk in TBST for 2 h at 37 °C. Sera from 1 *E. sinensis* –allergic patient and a non-allergic control were diluted 1:10 in TBST and mouse anti-His antibody was diluted 1:1000 in TBST. Then, the membranes were incubated with antibodies overnight. For sera incubated membranes, alkaline phosphatase conjugated mouse anti-human IgE (Sigma) was used as the secondary antibody at a dilution of 1:1000. For anti-His antibody incubated membranes, alkaline phosphatase conjugated rabbit anti-mouse IgE (Sigma) was used as the secondary antibody at a dilution of 1:5000. Visualization was with the NBT/BCIP substrate (BioRad, USA). All incubation procedures were done with shaking.

2.8. IgE-Binding capacity of rHc in comparison with Hc by dot-blot and dot-blot inhibition assay

For dot-blot assay, blocking and incubation were performed, as described above. 5 μg of Hc and rHc were spotted onto Nitrocellulose membranes and blocked with 5% skimmed milk in TBST, overnight at 4 °C. Membranes were then washed three times with TBST for 10 min and incubated with a 1/10 dilution of the patient sera for 2 h at room temperature. Serum from a non-allergic patient was used as negative control. For dot inhibition assays, the membranes were incubated with patient sera, following its pre-incubation with 5 μg of inhibitor (rHc) per milliliter of serum for 2 h at room temperature. After washing three times with TBST, the membranes were incubated with alkaline phosphatase conjugated mouse anti-human IgE (Sigma) at a dilution of 1:1000 in TBST for 2 h at room temperature. The membranes were washed three times with TBST for 10 min, and visualization of bound membrane IgE was

performed using the NBT/BCIP substrate. All incubation procedures were done with shaking.

2.9. ELISA for rHc-specific IgE

For IgE ELISA (Hilger et al., 2014), a 96-well plate (Thermo, USA) was coated with 100 ng/well of rHc, diluted in coating buffer (0.1 M sodium carbonate, pH 9.6), overnight at 4 °C. The plate was then blocked with 200 µl PBS containing 0.05% Tween-20 and 5% skimmed milk for 1 h at 37 °C. After washing with PBS containing 0.05% Tween-20 three times, the plate was incubated with 200 µl/well patient sera diluted 1:20 in blocking buffer. The plate was then washed, and incubated with 150 µl/well horseradish peroxidase conjugated mouse anti-human IgE (Sigma), at a dilution of 1:20000, for 1 h at 37 °C. After termination of the reaction using 2 M H₂SO₄, IgE reactivity was detected by the addition of 3,3',5,5'-tetramethylbenzidine substrate solution (Thermo, USA), with colorimetric measurements taken at 450 nm. Sera from five non-allergic patients were used as negative controls. All tests were performed in duplicate, with the average OD value used.

2.10. ELISA Inhibition and Sequential ELISA Inhibition

For ELISA inhibition, blocking and detection were performed, as described above. In inhibition assays, the plates were coated with 100 ng purified Hc protein overnight at 4 °C. Sera from 20 allergic patients were diluted 1:20 in blocking buffer and pre-incubated for 2 h at room temperature with 5 µg of inhibitor (rHc) per milliliter of serum. Serum from a non-allergic patient was used as negative control. After blocking with 5% skimmed milk, the plates were incubated with patient sera-rHc mix. Bound human IgE antibody was then detected with a 1:20000 dilution of a HRP-mouse anti-human IgE antibody conjugate.

Then, we performed a sequential ELISA inhibition assay to illustrate the dose-dependency of rHc mediated inhibition. A sera pool from 20 allergic patients was diluted 1:20 in blocking buffer and pre-incubated for 2 h at room temperature with dilutions of rHc ranging from 0.5 µg to 500 µg per milliliter of serum. Serum from a non-allergic individual was again used as a negative control. The percentage inhibition of IgE binding was then calculated as follows: Percentage inhibition (%) = 100 – (ODp/ODs) × 100, where ODp and ODs represent the optical densities after pre-incubation with the pre-immune and immune serum, respectively.

2.11. Statistical analysis

Statistical evaluation of our ELISA experiment was performed using an unpaired-sample *t*-test; data from the ELISA inhibition assay with human IgE were examined using a paired-sample *t*-test. All statistical analyses were carried out using GraphPad Prism 5 software. A *p*-value < 0.05 was considered as statistically significant.

3. Results

3.1. Expression and identification of full-length recombinant Hc

Recombinant Hc was expressed in *E. coli* as a (C-terminal) hexahistidine fusion protein. A 2067 bp open reading frame for the *E. sinensis* hemocyanin cDNA (GenBank accession AEG64817.1) was identified and cloned following PCR amplification. Sequence alignment analyses confirmed 100% sequence identity with the known hemocyanin gene sequence. The mature hemocyanin protein comprises 688 amino acids, with a theoretical molecular mass of 79 kDa. The purification steps were monitored by SDS-PAGE (Fig. 1A). Resolution by SDS-PAGE resulted in a correct size band (lane 4) at the

Table 3

Statistical analysis of epitopes recognized by patient sera.

Hc peptides	Sera with reaction
E1	5, 10, 13–16, 19–21
E2	2, 11, 14–21
E3	13
E4	1–7, 9–21
E5	14, 17
E6	19
E7	4, 20
E8	1, 8, 11
E9	8, 9
E10	6, 16

expected molecular weight. The recombinant protein Hc was highly purified by Ni²⁺-NTA affinity column chromatography. Given the predicted immunogenicity of Hc, the purified protein was then subjected to immunoblotting analysis using sera obtained from patient allergic to *E. sinensis* and anti-His antibody (Fig. 1B). In contrast to a non-allergic control serum, both of the “allergic” sera and anti-His antibody showed reactivity to the 79 kDa protein. So we confirmed that the purified protein Hc have IgE-binding activity.

3.2. Identification of IgE-binding epitopes of hemocyanin

Firstly, we used three calculation softwares to predict antigenic hemocyanin epitopes, including DNASTAR Protean system, ABCpred, and Bepipred Antibody Epitope Prediction. The hydrophilicity plot, flexibility plot, surface probability plot, and the antigenic index of hemocyanin were obtained by DNASTAR Protean system (Fig. 2A). Most amino acid sequence of hemocyanin had high hydrophilicity, better flexibility and surface probability, and the regions which antigenic index more than 1.0 were distributed. The IgE-binding epitopes of hemocyanin were further analyzed by ABCpred, and Bepipred Antibody Epitope Prediction (Data not show). Based on the results from these three tools, ten candidate linear epitopes (Fig. 2B) of hemocyanin were synthesized. For further analysis of the interaction of IgE with hemocyanin, we predicted its tertiary structure by SWISS-MODEL structure homology-modeling server (Fig. 2C).

Epitopes for hemocyanin were chosen based on the intensity and frequency of their recognition by dot-blot assays (Fig. 3). In agreement with our *in-silico* epitope mapping exercise for hemocyanin, we then showed that sera from twenty-one patients with allergy to *E. sinensis* reacted to at least one of the synthesized peptides (Fig. 3A). Given that all ten of the predicted linear epitopes were recognized by “allergic” sera, it would appear that allergenic epitopes are distributed over the full-length allergen. The percentage of patients that react to each epitope varied from 5% to 95% (Fig. 3B), although only three peptides generated a frequency of response in excess of 40%. The statistical analysis of epitopes recognized by patient sera was shown in Table 3. IgE reactivity of epitopes is depicted by colors based on their response frequency as follows: strong (red) and weak (blue) (Fig. 2C). Based on these criteria, three peptides (1, 2, and 4) were identified as the immunodominant hemocyanin –specific IgE binding sequences. A recombinant allergen combining these sequences (rHc) was then designed.

3.3. Expression, purification and identification of rHc

The recombinant allergen rHc was expressed as a fusion protein and assayed for IgE reactivity. SDS-PAGE of the purified protein showed a single major band migrating at about 12 kDa (Fig. 4A), which corresponded to the predicted molecular mass. Immunoblotting was then performed using serum IgE from 1 allergic patient to determine the reactivity of IgE to this protein species.

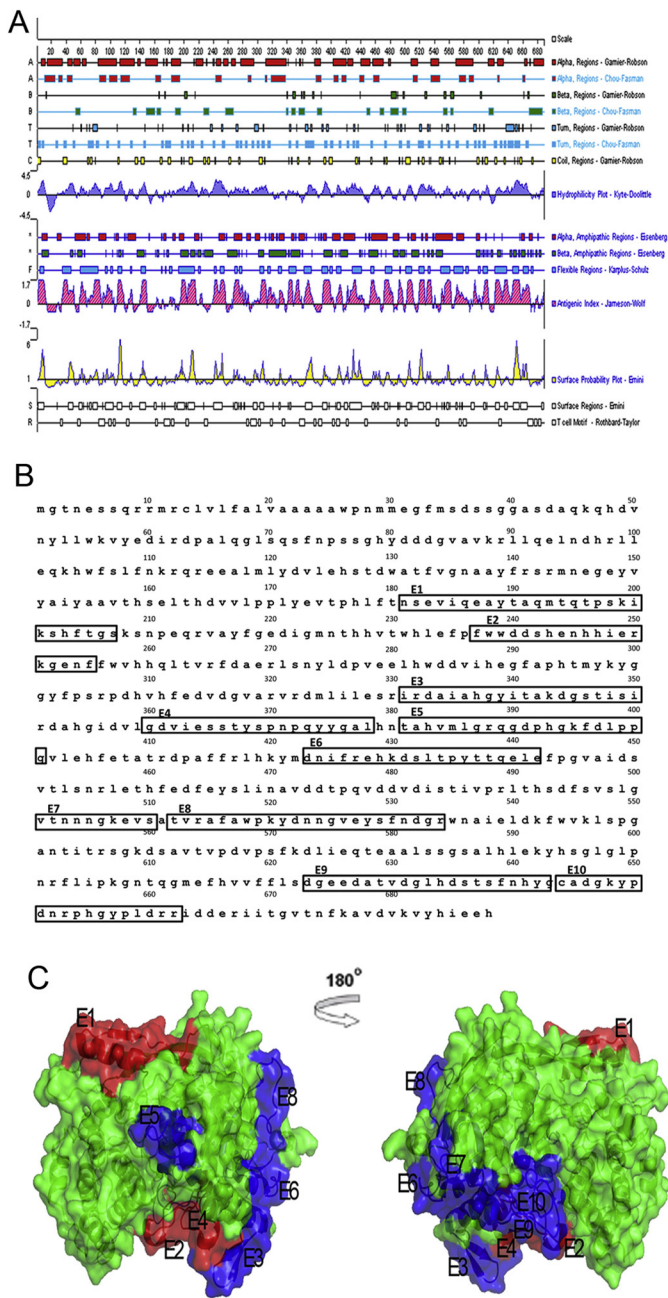


Fig. 2. The cDNA cloning and bioinformatics analysis of hemocyanin. (A) Linear epitopes of hemocyanin predicted by the DNASTAR Protean system. (B) Location of the IgE-binding epitopes in hemocyanin. The IgE epitopes designated as E1–E10 are shown in boxes. (C) Molecular model and the predicted IgE-binding epitopes of hemocyanin. The different strengths of IgE-binding epitopes are colored blue (weak), and red (strong). Different levels of IgE binding intensity are defined as: weak (0–20%), and strong (40–100%). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

As shown in Fig. 4B, immunoblotting revealed a 12 kDa band, indicating the rHc protein; both patient sera and anti-His antibody can react to the 12 kDa species.

3.4. Immunologic characterization of rHc

Sera from 21 allergic patients and 5 non-allergic subjects were then used to analyze sIgE binding activities to rHc by direct ELISA (Fig. 5). The results indicated that the serum of twenty (95%) subjects demonstrated IgE binding to the purified 12 kDa protein, with an increase in ELISA unit (over control serum) of 1.3- to 2.0-fold.

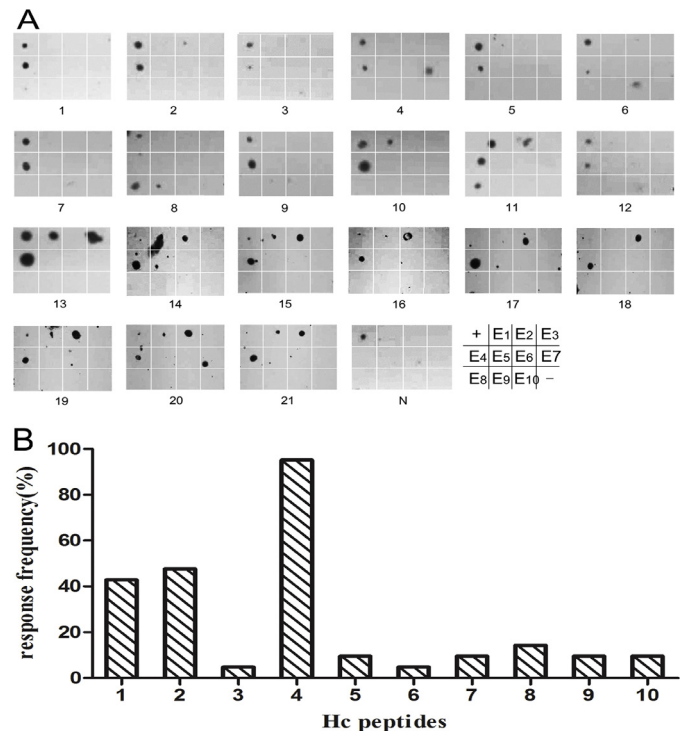


Fig. 3. Mapping of IgE-binding epitopes (E1–E10) by dot-blotting. (A) Ten peptides spanning almost the entire sequence of hemocyanin were tested for reactivity toward serum IgE from 21 *E. sinensis*-allergic patients and a non-allergic control (N). +: goat anti-mouse IgG as positive control; -: PBS buffer as negative control. The diagram on the bottom-right shows the pattern of peptides on the blots. (B) Donor response frequency profile for the 10 hemocyanin peptides.

The difference in absorbance between the positive (i.e. allergic), and negative serum samples, were significantly different ($p < 0.001$). No antibody binding was detected to non-allergic controls.

To determine the relative IgE-binding capacity of rHc in comparison with Hc, non-denaturing dot-blot and dot-blot inhibition assays were then performed using sera from 21 allergic patients (Fig. 6); each showed strong binding to Hc (row a) and weaker binding to rHc (row b) which was absent from a control “non-allergic” serum. Pre-incubation of patient sera with 5 $\mu\text{g}/\text{ml}$ rHc affected IgE binding to Hc (row c). The binding of serum IgE from 48% patients were completely inhibited by pre-incubation with rHc, with partial inhibition achieved for serum IgE from other patients. These inhibition experiments suggested that rHc and Hc shared IgE-binding immunodominant epitopes.

Using an IgE-binding inhibition ELISA assays, we confirmed the reduction of IgE reactivity with 5 $\mu\text{g}/\text{ml}$ inhibitor rHc. For this purpose, sera from 20 allergic patients with rHc-specific IgE were pre-incubated with 5 $\mu\text{g}/\text{ml}$ rHc. As shown in Fig. 7A, compared with the IgE reactions from a control array (Bar N), which was incubated with serum from a non-allergic patient, rHc strongly inhibited IgE binding to Hc for 20 patient sera. In the absence of the inhibitory protein, the absorbance attributable to IgE binding, measured at 450 nm was 0.292 ± 0.023 (Black bars). With inhibition, absorbance was reduced to 0.146 ± 0.006 (Grey bars). Clearly, pre-incubation with rHc could greatly attenuate the binding of IgE in “allergic” sera to Hc ($p < 0.001$), with a percent inhibition of between 26% and 63% (mean: 50%).

We then investigated the dose-dependency of this inhibition using a sequential ELISA inhibition assay. A serum pool from 20 allergic patients was pre-incubated with different concentrations of soluble inhibitor (rHc) ranging from 0.5 $\mu\text{g}/\text{ml}$ to 500 $\mu\text{g}/\text{ml}$. Then, soluble, unbound Hc-specific antibodies were allowed to react to the coated Hc. The sigmoidal shape of the dose-response

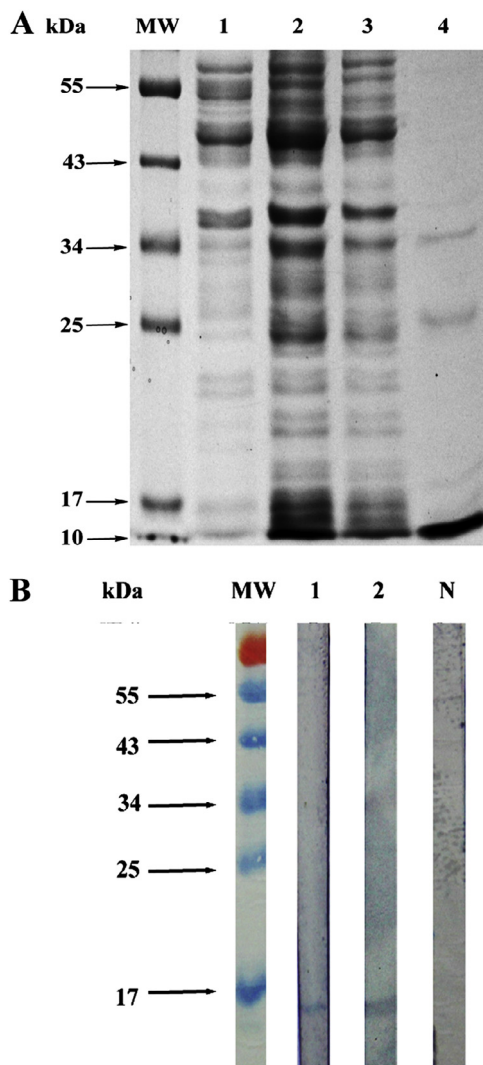


Fig. 4. SDS-PAGE and immunoblotting analysis of rHc. (A) SDS-PAGE: lane MW: molecular weight markers; lane 1: lysate from un-induced bacteria; lane 2: lysate from induced bacteria; lane 3: the wash fractions from nickel chelate columns; lane 4: the purified recombinant protein rHc. (B) Immunoblotting analysis: lane MW, pre-stained protein marker; lanes 1, immunoblotting using sera from a *E. sinensis* allergic patient; lanes 2, immunoblotting using anti-His antibody; lane N, immunoblotting using control serum (serum from non-allergic subject).

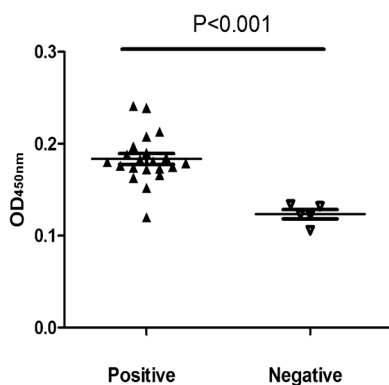


Fig. 5. IgE-reactivity of human sera against rHc. The binding of rHc to serum IgE from 21 patients allergic to *E. sinensis*, and 5 control individuals (negative) were tested by ELISA.

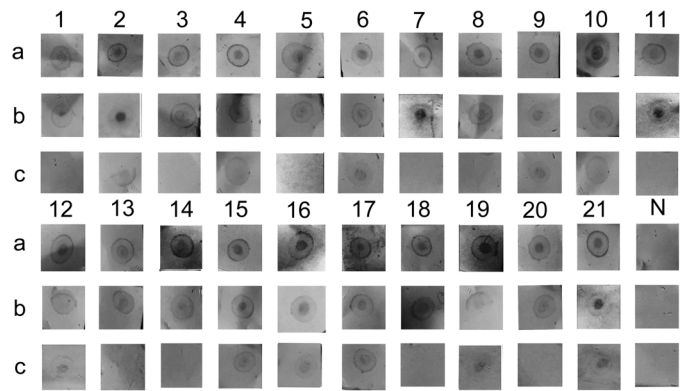


Fig. 6. Dot-blot immunoassay of Hc and rHc. (a) Dot-blot immunoassay of Hc binding to serum without pre-incubation. (b) Dot-blot immunoassay of rHc binding to serum without pre-incubation. (c) Dot-blot immunoassay of Hc binding to serum with pre-incubation. Lanes 1–21: dot-blot immunoassay using sera from 21 patients allergic to *E. sinensis*; lane N: dot-blot immunoassay using serum from a non-allergic control.

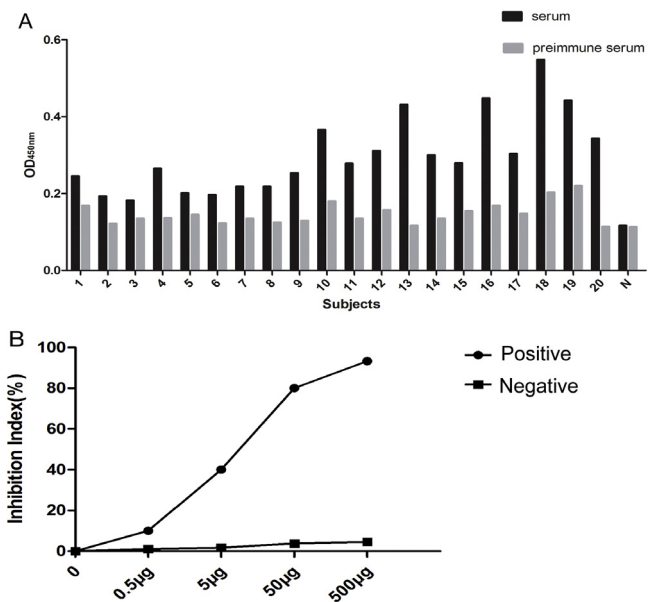


Fig. 7. Purified rHc inhibited patient derived IgE from binding Hc. (A) 5 μ g/ml of inhibitor (rHc) inhibited 20 patients allergic to *E. sinensis* derived IgE from binding Hc. Bar N, control serum (serum from a non-allergic subject). Black bars are average OD₄₅₀ value of Hc binding to serum without pre-incubation, grey bars are average OD₄₅₀ value of Hc binding to serum with pre-incubation. (B) Hc (100 ng) was used as solid phase, with a positive serum pool pre-incubated with increasing concentrations of purified rHc (circles). Serum from a non-allergic subject pre-incubated with increasing concentrations of purified rHc was our negative control (squares). All tests were performed in duplicate, with the average OD value used.

curve confirmed the specificity of Ag-Ab binding (Fig. 7B). The binding of serum IgE to immobilized Hc was inhibited by 93% when using 500 μ g of soluble rHc per milliliter of serum; a 50% inhibition (IC₅₀) was achieved using 6 μ g rHc per milliliter of serum. Taken together, these results demonstrate that the rHc construct carried the major allergenic epitopes of hemocyanin.

4. Discussion

In this study, the IgE binding epitopes and diagnostic utility of a recombinant allergen comprising the immunodominant epitopes of hemocyanin derived from *E. sinensis* roe are described for the first time. Investigation of the allergenic properties of rHc included an evaluation of IgE reactivity by immunoblotting, ELISA, dot-blot

inhibition, and ELISA inhibition assay. These findings are significant given that this recombinant allergen, carrying immunodominant epitopes (rHc), can be used to standardize testing and, potentially, provide a therapeutic tool against *E. sinensis* allergy.

Ten IgE-binding epitopes, including three immunodominant epitopes of hemocyanin, were first identified by bioinformatics prediction and then confirmed by IgE-binding assays. Hemocyanin undergoes heat processing and enzymatic digestion before its induction of an anaphylactic reaction. Hemocyanin is a heat-stable protein due to its copper active site and disulphide bridges (Piboonpocanun et al., 2011; Zanjani et al., 2014; Yesilyurt et al., 2008). The feature of heat-stable allows hemocyanin to retain the ability to elicit an immune reaction under conditions such as heating and enzymatic degradation as tropomyosin (Yadzir et al., 2015). But in these proteins, conformational changes and linear epitopes exposure will increase antibody binding after heating (Kamath et al., 2014). Therefore the immune response to linear epitopes was considered to be relevant (Mao et al., 2013). As seen in Fig. 3, different epitopes of hemocyanin were recognized by IgE derived from different allergic patients, in agreement with previous findings suggesting the existence of various epitopes that could be differentially recognized (Wang et al., 2010; Lin et al., 2012; Martinez-Botas et al., 2013). All of the candidate epitopes could be bound by sera from some allergic patients, suggesting that they were genuine, linear, IgE-binding epitopes of hemocyanin. These data indicated that our epitope prediction using the bioinformatics approach correlated well with subsequent experimental data (Chen et al., 2014). The IgE reactivity to the tested epitopes varied from 5% to 95%, with the response frequency to epitopes 1, 2, and 4 predominating (all >40%); these data lead to our categorization of these epitopes as immunodominant. The determination of allergen-specific, IgE-binding epitopes, is an important first step in understanding the complexity of hypersensitivity reactions and provides a useful tool for testing and, potentially, therapy.

Generally, the low performance of commercially available extracts can complicate the diagnosis of food allergy (Aalberse et al., 2013; Palomares et al., 2014). Confounding factors includes extraction procedure, batch-to-batch variation, protease content, and storage conditions (Palomares et al., 2014). Therefore, recombinant allergens offer a more reliable alternative for the in-vitro diagnosis of food allergy (Mao et al., 2013; Cramer, 2013). Exploitation of molecular cloning allowed us to generate a recombinant protein containing the three immune-dominant epitopes, between flexible spacer peptides. Our SDS-PAGE analyses indicated the expected 12 kDa protein that bound IgE derived from allergic sera, indicating preservation of the IgE epitopes.

In this study, the cDNA encoding hemocyanin was isolated from a cDNA expression library constructed from *E. sinensis* roe. We chose to clone and express the full-length cDNA of hemocyanin (Hc) in order to evaluate the immunogenicity of this construct relative to a recombinant allergen containing the three primary immunodominant epitopes alone (rHc). The results showed that hemocyanin genes are 2067 bp in size, encoding 688 amino acid residues. The amino acid sequence deduced from the hemocyanin encoding cDNA was identical with those described in the earlier researches (Sun et al., 2012).

To assess whether rHc could serve as a candidate recombinant allergen for the diagnosis and treatment of patients allergic to *E. sinensis*, we conducted a series of inhibition and ELISA assays. Firstly, we detected sIgE reactivity in allergic and non-allergic patients using the recombinant rHc allergen by ELISA. Ninety-five percent of these patients had generated rHc-specific IgE. The results showed that rHc reactivity could be used to discriminate between positive and negative sera, suggesting suitability for clinical diagnostic applications. Dot-blot and inhibition assays confirmed the allergenicity of rHc relative to Hc. All 21 of a cohort of *E. sinensis*

– sensitized patients showed IgE reactivity to Hc and rHc, confirming its relevance as an important *E. sinensis* allergen, while pre-incubation with rHc abolishing IgE binding to Hc (Fig. 6), suggesting their shared expression of allergenic epitopes. An ELISA competition experiment then allowed us to quantitate the inhibition achieved using the rHc construct as between 26% and 63% (mean: 50%). Plausible explanations for these data (i.e. incomplete inhibition) include of the rHc protein construct to express all of the relevant allergenic epitopes in hemocyanin and/or an insufficient concentration of inhibiting rHc. Subsequent investigations of the specificity of the antigen-antibody interaction (using a sequential competitive ELISA) indicated a sigmoidal dose-response curve, confirming the specificity of Ag-Ab binding (Fig. 7B). 500 µg of soluble rHc was required to compete out 93% of serum IgE binding to immobilized Hc. This indicated expression of the major immunodominant epitopes of hemocyanin within the rHc fusion protein. An increasing knowledge of allergen content and the characteristics of individual food allergens can facilitate the replacement of unstandardized food protein extracts by a component-resolved strategy (Aleksic et al., 2012). Such an approach applied for *Eriocheir sinensis* allergy diagnosis and treatment increased the sensitivity of the diagnostic tests and individual therapy.

At present, clinical diagnostic kits mostly used crab muscle extracts for detecting serum sIgE. The addition of allergens from crab roe to clinical diagnostic kits would improve the sensitivity of the crab allergy diagnosis. In the case of hemocyanin, we identified three immunodominant epitopes and confirmed their utility as allergenic epitopes. The recombinant tri-epitope allergen will, compared to crude *E. sinensis* extracts, enable standardization and adequate quality control of allergenic biologics, with similar IgE binding reactivity sensitivity for *in vitro* and *in vivo* diagnostic procedures. Compared to the full length Hc, the recombinant tri-epitope allergen rHc may further increase the positive predictive value of laboratory tests, provide information on the natural history of *E. sinensis* allergy, that is whether the patients may outgrow their allergy, and perhaps provide information on the potential severity of the allergic reaction to *E. sinensis* (Caubet et al., 2011). Further research is now needed to fully understand the relationship between linear IgE-binding epitopes and the allergic reactivity of patients. Any clinical role for rHc remains to be determined in future trial studies with patients allergic to *E. sinensis*. This study increases our understanding of relevant Hc epitopes, and provides much needed data for diagnosis and immunotherapeutic approaches to allergic disease.

In conclusion, our combination of the three major immunodominant epitopes of *E. sinensis* hemocyanin as a fusion protein will allow us to develop more specific diagnostic and therapeutic approaches with which to diagnose *E. sinensis* allergy.

Conflict of interest

The authors have declared no conflict of interest.

Author contributions

YZ participated in all stages of the project, prepared and performed the experiments, provided figures and data for the paper, and was the main author of the manuscript. LZ carried out ELISA analysis and performed the statistical analysis. JZ revised the manuscript. SL collected serum and patients information. JY performed SDS-PAGE analysis. YB performed isolation of total mRNA. HL conceived and designed the project. All authors provided critical review of the manuscript.

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References

- Aalberse, J.A., Meijer, Y., Derksen, N., van der Palen-Merkus, T., Knol, E., Aalberse, R.C., 2013. Moving from peanut extract to peanut components: towards validation of component-resolved IgE tests. *Allergy* 68, 748–756.
- Abramovitch, J.B., Kamath, S., Varese, N., Zubrinich, C., Lopata, A.L., O'Hehir, R.E., et al., 2013. 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* 8, e67487.
- Aleksic, I., Popovic, M., Dimitrijevic, R., Andjelkovic, U., Vassilopoulou, E., Sinaniotis, A., et al., 2012. Molecular and immunological characterization of Mus a 5 allergen from banana fruit. *Mol. Nutr. Food Res.* 56, 446–453.
- Arnold, K., Bordoli, L., Kopp, J., Schwede, T., 2006. The SWISS-MODEL workspace: a web-based environment for protein structure homology modelling. *Bioinformatics* 22, 195–201.
- Ayuso, R., Sanchez-Garcia, S., Lin, J., Fu, Z., Ibanez, M.D., Carrillo, T., et al., 2010. Greater epitope recognition of shrimp allergens by children than by adults suggests that shrimp sensitization decreases with age. *J. Allergy Clin. Immunol.* 125, 1286–1293, e1283.
- Baar, A., Pahr, S., Constantin, C., Giavi, S., Papadopoulos, N.G., Pelkonen, A.S., et al., 2014. The high molecular weight glutenin subunit Bx7 allergen from wheat contains repetitive IgE epitopes. *Allergy* 69, 1316–1323.
- Calderon, M.A., Linneberg, A., Kleine-Tebbe, J., De Blay, F., Hernandez Fernandez de Rojas, D., Virchow, J.C., et al., 2015. Respiratory allergy caused by house dust mites: what do we really know? *J. Allergy Clin. Immunol.* 136, 38–48.
- Cao, J., Wang, Z., Zhang, Y., Qu, F., Guo, L., Zhong, M., et al., 2014. Identification and characterization of the related immune-enhancing proteins in crab *Scylla paramamosain* stimulated with rhubarb polysaccharides. *Mol. Immunol.* 57, 263–273.
- Caubet, J.C., Kondo, Y., Urisu, A., Nowak-Wegrzyn, A., 2011. Molecular diagnosis of egg allergy. *Curr. Opin. Allergy Clin. Immunol.* 11, 210–215.
- Chen, J.C., Chiu, L.L., Lee, K.L., Huang, W.N., Chuang, J.G., Liao, H.K., et al., 2012. Identification of critical amino acids in an immunodominant IgE epitope of Pen c 13, a major allergen from *Penicillium citrinum*. *PLoS One* 7, e34627.
- Chen, H., Yang, H.W., Wei, J.F., Tao, A.L., 2014. In silico prediction of the T-cell and IgE-binding epitopes of Per a 6 and Bla g 6 allergens in cockroaches. *Mol. Med. Rep.* 10, 2130–2136.
- Cramer, R., 2013. The crux with a reliable in vitro and in vivo diagnosis of allergy. *Allergy* 68, 693–694.
- Gamez, C., Sanchez-Garcia, S., Ibanez, M.D., Lopez, R., Aguado, E., Lopez, E., et al., 2011. Tropomyosin IgE-positive results are a good predictor of shrimp allergy. *Allergy* 66, 1375–1383.
- Gepp, B., Lengger, N., Bublin, M., Hemmer, W., Breiteneder, H., Radauer, C., 2014. Chimeras of Bet v 1 and Api g 1 reveal heterogeneous IgE responses in patients with birch pollen allergy. *J. Allergy Clin. Immunol.* 134, 188–194.
- Glazer, L., Tom, M., Weil, S., Roth, Z., Khalaila, I., Mittelman, B., et al., 2013. Hemocyanin with phenoloxidase activity in the chitin matrix of the crayfish gastrolith. *J. Exp. Biol.* 216, 1898–1904.
- Gong, Y.N., Li, W.W., Sun, J.L., Ren, F., He, L., Jiang, H., et al., 2010. Molecular cloning and tissue expression of the fatty acid-binding protein (Es-FABP) gene in female Chinese mitten crab (*Eriocheir sinensis*). *BMC Mol. Biol.* 11, 71.
- He, L., Li, Q., Liu, L.H., Wang, Y.L., Xie, J., Yang, H.D., et al., 2015. A catalog of proteins expressed in the AG secreted fluid during the mature phase of the chinese mitten crabs (*Eriocheir sinensis*). *PLoS One* 10.
- Hilger, C., Swiontek, K., Kler, S., Diederich, C., Lehnert, C., Vogel, L., et al., 2011. Evaluation of two new recombinant guinea-pig lipocalins, Cav p 2 and Cav p 3: in the diagnosis of guinea-pig allergy. *Clin. Exp. Allergy: J. Br. Soc. Allergy Clin. Immunol.* 41, 899–908.
- Hilger, C., Kler, S., Arumugam, K., Revets, D., Muller, C.P., Charpentier, C., et al., 2014. Identification and isolation of a Fel d 1-like molecule as a major rabbit allergen. *J. Allergy Clin. Immunol.* 133, 759–766.
- Hoffmann-Sommergruber, K., Pfeifer, S., Bublin, M., 2015. Applications of molecular diagnostic testing in food allergy. *Curr. Allergy Asthma Rep.* 15, 56.
- Kamath, S.D., Rahman, A.M., Voskamp, A., Komoda, T., Rolland, J.M., O'Hehir, R.E., et al., 2014. Effect of heat processing on antibody reactivity to allergen variants and fragments of black tiger prawn: a comprehensive allergenomic approach. *Mol. Nutr. Food Res.* 58, 1144–1155.
- Kim, J.S., Ouyang, F., Pongracic, J.A., Fang, Y., Wang, B., Liu, X., et al., 2008. Dissociation between the prevalence of atopy and allergic disease in rural China among children and adults. *J. Allergy Clin. Immunol.* 122, 929–935.
- Laemmli, U.K., 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227, 680–685.
- Larsen, J.E., Lund, O., Nielsen, M., 2006. Improved method for predicting linear B-cell epitopes. *Immun. Res.* 2, 2.
- Lin, J., Bruni, F.M., Fu, Z., Maloney, J., Bardina, L., Boner, A.L., et al., 2012. A bioinformatics approach to identify patients with symptomatic peanut allergy using peptide microarray immunoassay. *J. Allergy Clin. Immunol.* 129, 1321–1328.
- Lopes de Oliveira, L.C., Aderhold, M., Brill, M., Schulz, G., Rolinck-Werninghaus, C., Clare Mills, E.N., et al., 2013. The value of specific IgE to peanut and its component Ara h 2 in the diagnosis of peanut allergy. *J. Allergy Clin. Immunol. Pract.* 1, 394–398.
- Mao, H.Y., Cao, M.J., Maleki, S.J., Cai, Q.F., Su, W.J., Yang, Y., et al., 2013. Structural characterization and IgE epitope analysis of arginine kinase from *Scylla paramamosain*. *Mol. Immunol.* 56, 463–470.
- Martinez-Botas, J., Cerecedo, I., Zamora, J., Vlaicu, C., Dieguez, M.C., Gomez-Coronado, D., et al., 2013. Mapping of the IgE and IgG4 sequential epitopes of ovomucoid with a peptide microarray immunoassay. *Int. Arch. Allergy Immunol.* 161, 11–20.
- Munoz-Garcia, E., Luengo-Sanchez, O., Haroun-Diaz, E., Maroto, A.S., Palacin, A., Diaz-Perales, A., et al., 2013. Identification of thaumatin-like protein and aspartyl protease as new major allergens in lettuce (*Lactuca sativa*). *Mol. Nutr. Food Res.* 57, 2245–2252.
- Nwaru, B.I., Hickstein, L., Panesar, S.S., Roberts, G., Muraro, A., Sheikh, A., et al., 2014. Prevalence of common food allergies in Europe: a systematic review and meta-analysis. *Allergy* 69, 992–1007.
- Palomares, O., Cramer, R., Rhyner, C., 2014. The contribution of biotechnology toward progress in diagnosis, management, and treatment of allergic diseases. *Allergy* 69, 1588–1601.
- Pedrosa, M., Boyano-Martinez, T., Garcia-Ara, C., Quirce, S., 2015. Shellfish allergy: a comprehensive review. *Clin. Rev. Allergy Immunol.* 49, 203–216.
- Perez-Gordo, M., Pastor-Vargas, C., Lin, J., Bardina, L., Cases, B., Ibanez, M.D., et al., 2013. Epitope mapping of the major allergen from Atlantic cod in Spanish population reveals different IgE-binding patterns. *Mol. Nutr. Food Res.* 57, 1283–1290.
- Piboonpocanun, S., Jirapongsananuruk, O., Tipayanon, T., Boonchoo, S., Goodman, R.E., 2011. Identification of hemocyanin as a novel non-cross-reactive allergen from the giant freshwater shrimp *Macrobrachium rosenbergii*. *Mol. Nutr. Food Res.* 55, 1492–1498.
- Saha, S., Raghava, G.P., 2006. Prediction of continuous B-cell epitopes in an antigen using recurrent neural network. *Proteins* 65, 40–48.
- Salo, P.M., Arbes Jr., S.J., Jaramillo, R., Calatroni, A., Weir, C.H., Sever, M.L., et al., 2014. Prevalence of allergic sensitization in the United States: results from the national health and nutrition examination survey (NHANES) 2005–2006. *J. Allergy Clin. Immunol.* 134, 350–359.
- Shek, L.P., Cabrera-Morales, E.A., Soh, S.E., Gerez, I., Ng, P.Z., Yi, F.C., et al., 2010. A population-based questionnaire survey on the prevalence of peanut, tree nut, and shellfish allergy in 2 Asian populations. *J. Allergy Clin. Immunol.* 126, 324–331.
- Sicherer, S.H., Sampson, H.A., 2014. Food allergy: epidemiology, pathogenesis, diagnosis, and treatment. *J. Allergy Clin. Immunol.* 133, 291–307.
- Sun, S., Chen, L., Qin, J., Ye, J., Qin, C., Jiang, H., et al., 2012. Molecular cloning, characterization and mRNA expression of copper-binding protein hemocyanin subunit in Chinese mitten crab, *Eriocheir sinensis*. *Fish Shellfish Immunol.* 33, 1222–1228.
- Wai, C.Y., Leung, N.Y., Ho, M.H., Gershwin, L.J., Shu, S.A., Leung, P.S., et al., 2014. Immunization with Hypoallergens of shrimp allergen tropomyosin inhibits shrimp tropomyosin specific IgE reactivity. *PLoS One* 9, e111649.
- Wang, J., Lin, J., Bardina, L., Goldis, M., Nowak-Wegrzyn, A., Shreffler, W.G., et al., 2010. Correlation of IgE/IgG4 milk epitopes and affinity of milk-specific IgE antibodies with different phenotypes of clinical milk allergy. *J. Allergy Clin. Immunol.* 125, 695–702.
- Yadzir, Z.H., Misnan, R., Bakhtiar, F., Abdullah, N., Murad, S., 2015. Tropomyosin, the major tropical oyster *Crassostrea belcheri* allergen and effect of cooking on its allergenicity. *Allergy Asthma Clin. Immunol.* 11 (30).
- Yesilyurt, B.T., Gielens, C., Meersman, F., 2008. Thermal stability of homologous functional units of Helix pomatia hemocyanin does not correlate with carbohydrate content. *FEBS J.* 275, 3625–3632.
- Ying-Ying, Z., Li-Na, Z., Shao-Shen, L., Li-Ying, H., Hui-Qiang, L., 2015. Hemocyanin is an important high-molecular-weight allergen of *Eriocheir sinensis*. *Chin. J. Immunol.* 31, 1375–1379.
- Zanjani, N.T., Sairi, F., Marshall, G., Saksena, M.M., Valtchev, P., Gomes, V.G., et al., 2014. Formulation of abalone hemocyanin with high antiviral activity and stability. *Eur. J. Pharm. Sci.* 53, 77–85.
- Zhang, Q., Dai, X., Cong, Y., Zhang, J., Chen, D.H., Dougherty, M.T., et al., 2013. Cryo-EM structure of a molluscan hemocyanin suggests its allosteric mechanism. *Structure* 21, 604–613.
- Zhu, H., Zhuang, J., Feng, H., Liang, R., Wang, J., Xie, L., et al., 2014. Cryo-EM structure of isomeric molluscan hemocyanin triggered by viral infection. *PLoS One* 9, e98766.