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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 inhibit26% 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.

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-roe 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 kUA/L), and crab-specific IgE (>0.35 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 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.

Table 2
Primer sequences used to clone Hc.

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

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