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Identification of triosephosphate isomerase as a novel allergen in *Octopus fangsiao*

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ABSTRACT

Octopus is an important mollusk in human dietary for its nutritional value, however it also causes allergic reactions in humans. Major allergens from octopus have been identified, while the knowledge of novel allergens remains poor. In the present study, a novel allergen with molecular weight of 28 kDa protein was purified from octopus (*Octopus fangsiao*) and identified as triosephosphate isomerase (TIM) by mass spectrometry. TIM aggregated beyond 45 °C, and its IgE-binding activity was affected under extreme pH conditions due to the altered secondary structure. In simulated gastric fluid digestion, TIM can be degraded into small fragments, while retaining over 80% of the IgE-binding activity. The full-length cDNA of *O. fangsiao* TIM (1140 bp) was cloned, which encodes 247 amino acid residues, and the entire recombinant TIM was successfully expressed in *Escherichia coli* BL21, which showed similar immunoreactivity to the native TIM. Different intensity of cross-reactivity among TIM from related species revealed the complexity of its epitopes. Eight linear epitopes of TIM were predicted following bioinformatic analysis. Furthermore, a conformational epitope ($A_{71}G_{74}S_{69}D_{75}T_{73}F_{72}V_{67}$) was confirmed by the phage display technology. The results revealed the physicochemical and immunological characteristics of TIM, which is significant in the development of hyposensitivity food and allergy diagnosis.

1. Introduction

Food allergy is an abnormal food-induced immune response encompassing several types of food hypersensitivity. According to the U.S. Food and Drug Administration, there are eight major allergenic foods, including peanuts, tree nuts, soy, wheat, fish, shellfish, milk and eggs. Exposure to these foods varies with dietary habits, living environment, as well as age. The symptoms of food hypersensitivity were grouped into three categories: oro-gastrointestinal symptoms, rhino-respiratory symptoms and dermatological symptoms (Wu et al., 2012). And the phenotypic heterogeneity of allergic disease indicates that their underlying mechanisms might change with various exotic foods (Peters et al., 2015).

Seafood plays an important role in worldwide diet due to its high nutritional value and delicious taste. The total Asian seafood production and supply is the highest in the world (FAO, 2014). The consumption of seafood products is growing rapidly, meanwhile, leading high frequency of adverse reactions. Seafood induced anaphylaxis is very common in Asian countries, such as Malaysia, Thailand, Singapore and China (Hajeb and Selamat, 2012). In the past decades, the major allergens in seafood have been identified and lots of researches focused on the allergen cross-reactivity among different species (Leung et al., 2014). Tropomysin (TM) is a major allergen in crustaceans and mollusks with a molecular weight of 34–39 kDa, Ishikawa et al. (2001) identified TM as an important allergen in *Octopus vulgaris* and named it Oct v 1. Arginine kinase (AK) with a molecular weight of about 40 kDa is a common allergen detected in several shellfish, such as *Penaeus monodon* and *Scylla paramamosain* (Shen et al., 2011; Yu et al., 2003). Besides, AK was also found to be a major allergen in *Octopus fangsiao*, with cross-reactivity among mollusk and crustaceans (Shen et al., 2012). In recent years, researches pay increasing attention to novel allergens, such as sarcoplasmic calcium-binding protein (SCP) and myosin light chain (MLC) in *Procambarus clarkia* and *Crangon crangon* (Bauermeister et al., 2011; Chen et al., 2013;

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Table 1
Clinical and laboratory characteristics of nine shellfish-allergic patients and two non-allergic individuals.

Serum No.	Age	Sex	Symptoms	sIgE octopus (f59) by ImmunoCAP (kUA/L)
099809	50	F	U	0.65
132965	30	M	U	0.54
152077	14	M	P	2.20
567055	6	M	Dr	5.62
645376	24	F	U	3.50
722655	29	M	U	0.63
722719	31	F	U	1.50
787956	12	M	Dr	1.15
833684	7	M	Dr	0.58
839758	25	M	ND	0.16
856386	38	F	ND	0.10

F: female, M: male, U: urticaria, P: pruritus, Dr: diarrhea, ND: not determined/unknown; Levels lower than 0.35 kU_A/L were considered negative.

Zhang et al., 2015). Besides, during our previous studies for the major *O. fangsiao* allergens, AK was identified as an allergen by immunoblot, accompanied with a 28-kDa protein that has significant IgE-binding activity with the sera of mollusk-allergic patients (Shen et al., 2012). The molecular weight of the IgE-reactive protein is similar to that of triosephosphate isomerase (TIM), a novel allergen reported in *P. monodon* and *Socomber scombrus* (Kamath et al., 2014; Pazos et al., 2014).

TIM is an enzyme (EC 5.3.1.1) that catalyzes the reversible interconversion of the triose phosphate isomers dihydroxyacetone phosphate and D-glyceraldehyde 3-phosphate (Wierenga et al., 2010). This protein is composed of 247 amino acids with a pI value of 6.2 (An et al., 2013). TIM is widely distributed among organisms, from bacteria and fungi, to plants and mammals, which has the potential to induce complex cross-reactive among species and lead to higher threaten in allergy suffers. Work focusing on the specific epitopes of TIM and its reactivity among species is crucial but lacking at present, much researches allergens are required for developing a comprehensive understanding of this allergen.

O. fangsiao is a mollusk widely found in the southeast ocean of China, which is a kind of popular food and important commercial cephalopod, the novel IgE-binding protein should not be ignored for its high consumption. Under these circumstances, the 28-kDa protein was purified from *O. fangsiao*, and its physicochemical and immunological characteristics were determined. Sequence alignment and cross-reactivity of the protein among species were then investigated. Finally, the epitopes of 28-kDa protein were determined and the protein homology model was generated to observe the location of the IgE epitopes.

2. Materials and methods

2.1. Patient sera

Sera of 9 mollusk-allergic patients were collected by the Xiamen Second Hospital. Octopus-specific IgE level in sera were measured using the ImmunoCAP system (Phadia AB, Uppsala, Sweden) with octopus allergen (f59, Table 1). Levels lower than 0.35 kU_A/L were considered negative. All subjects voluntarily provided their sera with informed consent. Their use was approved by the internal Ethical Committee of the hospital (XSH2012-EAN019).

2.2. Purification and IgE-immunoblot of the 28 kDa-protein

The target protein was purified from octopus as previously described (Shen et al., 2012) with modifications. In brief, ammonium sulfate was added to the crude extract of *O. fangsiao* with increasing saturation from 60% to 100% to precipitate the protein, which was then dialyzed using Tris-HCl buffer (20 mM, pH 8.0) overnight before loading onto a Q-Sepharose column (2.5 × 10 cm, GE Healthcare, Waukesha, WI, USA). The elution was carried out with a linear gradient of 0–0.5 M NaCl at a flow rate of 0.8 mL/min. Fractions that contain the 28-kDa protein were collected and subjected to purification by a Sephacryl S-200 HR gel column (1.5 × 100 cm, GE Healthcare), which was equilibrated with Tris-HCl buffer (20 mM, pH 8.0) containing 0.2 M NaCl at a flow rate of 0.5 mL/min. Samples were separated in 12% SDS-PAGE, and the gels were stained for protein with Coomassie brilliant blue R-250. IgE-immunoblot was performed to validate the target protein. Briefly, protein samples were electrophoretically transferred to a nitrocellulose membrane, and blocked with 5% skim milk in TBST (20 mM Tris-HCl, pH 8.0, containing 0.15 M NaCl and 0.05% Tween-20). After washing with TBST, it was incubated with the allergy patients' serum pool (1:4 dilution) as the primary antibody, IgE antibody (diluted 1:20,000; Southern Biotech, Birmingham, UK) was used as the secondary antibody and the results were visualized by enhanced chemiluminescence (ECL) using the ECL substrate (Pierce, Rockford, Illinois, USA).

2.3. Identification by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) and polyclonal antibody (pAb) preparation

The potential target protein bands were excised from the Coomassie Brilliant Blue stained SDS-PAGE gel and analyzed by Shanghai Applied Protein Technology Company Limited (Shanghai, China) using a 4800 Plus MALDI TOF/TOF Analyzer from Applied Biosystems (Foster City, CA, USA) based on the method of Yan et al. (2014). The purified protein was also used to generate a specific polyclonal antibody by subcutaneously injected of adult female New Zealand white rabbit, following the method of Yang et al. (2015). The specific pAb (1:40,000 dilution) was then recruited as primary antibody in the IgG-immunoblot performed below. HRP-labeled goat anti-rabbit IgG antibody (1:20,000 dilution, Abmart, Berkeley Heights, NJ, USA) was used as the secondary antibody and the results were also visualized by ECL.

2.4. Physicochemical characterization of the 28-kDa protein

2.4.1. Two-dimensional (2-D) electrophoresis analysis

Two-dimensional (2-D) gel electrophoresis was carried out using a 7 cm immobilized dry strips (nonlinear, pH 3–10; BioRad, Hercules, CA, USA) with 12% SDS-polyacrylamide gel to determine the isoelectric point (pI) of target protein. Coomassie brilliant blue R-250 was used for the staining of protein.

2.4.2. Thermal and pH stability determination

To determine the thermal stability, protein sample were adjusted to a final concentration of 0.15 mg/mL and incubated at different temperatures (-30, 0, 30, 35, 40, 45, 50, 55, 60, 70, 80, 90, 100 °C or 4 °C for the control) for 30 min. As to the pH stability, protein sample (0.15 mg/mL) was incubated for 30 min at 25 °C in buffer with different pH (1.0, 2.0, 3.0, 4.0, 5.0, 5.5, 6.0, 6.5, 7.0, 8.0, 9.0, 10.0, 11.0 or 7.5 for the control). All the samples were analyzed by 12% SDS-PAGE followed by western blot performed using specific pAb (1:40,000 dilution) as primary antibody. In the dot blot, the samples were immediately blotted onto the nitrocellulose membrane (2 μL/dot) and allowed to dry, and incubated with the serum pool (1:4 dilution) after blocked, as described in Section 2.2.

The protein (0.25 mg/mL) was treated at 4, 40, 50, 60, 90 or 100 °C, or incubated at 25 °C in different pH buffers (pH 2.0, 5.0, 7.5 or 11.0) for 1 h. The treated proteins were then scanned for Far-UV circular dichroism (CD) spectra using a Chirascan™ circular dichroism spectrometer (Applied Photophysics Ltd, Surrey, UK) at a concentration of 0.15 mg/mL in buffer (10 mM Tris-HCl, pH