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Mapping and characterization of antigenic epitopes of arginine kinase of Scylla paramamosain

Yang Yang[a], Min-Jie Cao[a], Marcos Alcocer[b], Qing-Mei Liu[a], Dan-Xia Fei[a], Hai-Yan Mao[a], Guang-Ming Liu[a,*]

[a] College of Food and Biological Engineering, Fujian Collaborative Innovation Center for Exploitation and Utilization of Marine Biological Resources, Jimei University, 43 Yindou Road, Xiamen 361021, Fujian, China
[b] School of Biosciences, Sutton Bonington Campus, Loughborough LE12 5RD, UK

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ABSTRACT

Arginine kinase (AK) is a panallergen present in crustaceans, which can induce an immunoglobulin (Ig) E-mediated immune response in humans. The aim of this work was to map and characterize the antigenic epitopes of *Scylla paramamosain* AK. Specific-protein-A-enriched IgG raised in rabbits against purified *S. paramamosain* AK was used to screen a phage display random peptide library. Five AK mimotope clones were identified among 20 random clones after biopanning. Four conformational epitopes $D_3A_4K_{43}M_1A_5T_{49}T_{44}I_7$, $L_{31}K_{33}V_{35}T_{32}E_{11}E_{18}F_{14}S_{34}D_{37}$, $V_{177}G_{172}M_{173}D_{176}Q_{178}T_{174}L_{181}K_{175}L_{187}$, and $R_{202}L_{170}Y_{203}E_{190}P_{205}W_{204}L_{187}T_{206}Y_{145}$ were identified with the program LocaPep, and mapped to *S. paramamosain* AK. The key amino acids of these conformational epitopes were D_3, K_{33}, T_{174}, and W_{204}, respectively. On the basis of biopanning, six IgE-specific peptides were mapped with synthetic overlapping peptides using the sera from crab-allergic patients, and four seropositive peptides (amino acids 113–127, 127–141, 141–155, and 204–218) were confirmed as linear epitopes in a degranulation assay in RBL-2H3 cells. Stability experiments showed that the structural integrity of AK is essential for its allergenicity, and the intramolecular disulfide bond at Cys_{201}–Cys_{271} is essential for its structural stability.

1. Introduction

Seafood is a common dietary component and source of nutrition worldwide. However, seafood also constitutes one of the most important groups of foods inducing immediate (type I) food hypersensitivity, which can result in diverse clinical symptoms and diminish the sufferer's quality of life (Burks et al., 2012). In Asian countries, seafood allergy, especially shellfish allergy, is particularly common because its rate of consumption is high and individuals are exposed to seafood early in life (Leung et al., 2014). A survey of the prevalence of allergy in a population in China found that the most commonly identified allergens were fish, shrimp, crab, and seaweed, and a high incidence of crab allergy (60.8%) was detected (Hajeb and Selamat, 2012).

The muscle protein tropomyosin (MW 34 kDa) is considered responsible for most of the allergenic activity in shellfish and the cross-reactivity among different species (Lopata et al., 2010). In recent years, other proteins in shellfish, such as myosin light chain, sarcoplasmic calcium-binding protein, troponin C, and triosephosphate isomerase, have been identified as novel allergenic proteins (Bauermeister et al., 2011). The 40-kDa arginine kinase (AK) from the shrimps *Penaeus monodon* (Yu et al., 2003) and *Penaeus* (*Litopenaeus*) *vannamei* (Garcia-Orozco et al., 2007) and from the Indo-Pacific swamp crab *Scylla serrata* (Shen et al., 2011) has also been identified as an important allergen.

The antigenicity of an allergen depends on its epitopes. An epitope is a specific region recognized by a specific antibody, and is usually on the surface of the antigen. Allergen epitope characterization provides valuable information useful for the understanding of food allergens. The identification of epitopes in antibody–antigen interaction is a crucial step in the rational design of novel drugs and immunotherapeutic strategies. Epitopes are typically categorized as either sequential (linear and continuous) or conformational (discontinuous) (Willison et al., 2013). Different methods are used to identify epitopes. Linear epitopes can be identified by the fragmentation of the allergen, either enzymatically (Chen et al., 2012) or synthetically (Ayuso et al., 2012), whereas the identification of conformational epitopes requires more elaborate methods, such as

* Corresponding author. Tel.: +86 592 6180378; fax: +86 592 6180470.
E-mail addresses: gmliu@jmu.edu.cn, liugmo@sina.com (G.-M. Liu).

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Table 1
Specific IgE levels in patients in relation to the crab and shrimp.

ID	Sex	Age (y)	Specific IgE (kU/L)[a]	
			Crab	Shrimp
5991	M	1	0.86	0.65
7359	F	55	0.50	2.35
8517	F	28	0.40	0.01
0284	M	25	0.62	0.63
4583	M	35	0.78	0.19
0737	M	13	1.64	0.01
4723	M	1	0.63	0.52

[a] A serum with the specific IgE > 0.35 (kU/L) is defined as positive. M: male, F: female.

site-directed mutagenesis or probing with monoclonal antibodies (Miyaji et al., 2013). The phage display technology is an alternative to these methods, and is based on the affinity selection of antibodies of peptides containing the amino acids that describe the corresponding epitopes. A random nonapeptide library was constructed and used to mimic the discontinuous epitopes on human H-subunit ferritin (Luzzago et al., 1993) as early as in 1993, so the technique is considered a robust method for identifying the epitopes on allergens.

We have previously shown that AK is specifically recognized by IgE collected from crab-sensitized allergic patients (Shen et al., 2011), and have defined two areas of *S. paramamosain* AK (amino acids 174–181 and 253–256) as the main epitope regions of the protein. Further serological assays of synthetic peptides defined three experimental linear epitopes (amino acids 127–141, 141–155, and 211–225) (Mao et al., 2013). Ayuso et al. (2012) characterized eight linear epitopes of AK (amino acids 1–18, 25–42, 64–96, 121–141, 142–159, 160–192, 232–255, and 319–342) from *L. vannamei* using synthetic peptides. However, information about the conformational epitopes of AK is still limited. Chen et al. (2013) predicted 24 discontinuous residues of *Procambarus clarkii* AK, Mao et al. (2013) predicted seven conformational epitopes of *S. paramamosain* AK, and the differences in IgE-binding activities of the linear and native AK molecules indicate that conformational epitopes play a key role in its antigenicity. However, there is no experimental evidence for the predicted conformational epitopes, and it is unclear how the structure of AK affects its allergenicity. Thus, the research of conformational epitopes of AK is still incomplete.

In this study, the conformational and linear epitopes of AK were mapped with competitive immunoscreening of a phage display random peptide library using a rabbit anti-AK immunoglobulin G (IgG), and the epitopes were confirmed with *in silico* analysis, synthetic peptides, and a degranulation assay in RBL-2H3 cells. The effects of its conformation on the allergenicity of AK were investigated.

2. Materials and methods

2.1. Patient sera

Sera from crab-allergic patients (no. 5991, 7359, 8517, 0284, 4583, 0737, and 4723) were used, with the informed consent of the subjects and with the approval of the local Ethics Committee of the First Affiliated Hospital of Xiamen University (Xiamen, Fujian, China). The patients had convincing histories of crab anaphylaxis with clear crab-exposure-related symptoms. Their crab-specific IgE antibodies were quantified *in vitro* with ImmunoCAP (Phadia AB, Uppsala, Sweden). Table 1 shows the data of the patients who were allergic to crab. All sera were stored at −30 °C until analysis.

2.2. Purification and identification of S. paramamosain AK

Crabs (*S. paramamosain*) were purchased live at Jimei Market, Xiamen. The AK fractions were isolated with Q-Sepharose and characterized with sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS–PAGE)/immunoblotting using a polyclonal rabbit anti-mud-crab (*S. serrata*) AK antibody, as previously described (Mao et al., 2013).

2.3. Preparation of rabbit anti-AK IgG

Polyclonal AK-specific antiserum was generated at Xiamen University in the Laboratory Animal Centre, as described by Qian et al. (2012). The polyclonal rabbit anti-AK antiserum was purified by affinity chromatography on Protein A Sepharose (GE Healthcare, New York, NY, USA). The fractions containing the purified IgG were collected and stored in −20 °C until analysis.

2.4. Biopanning a phage display peptide library

2.4.1. Panning procedure

The Ph.D.-12 Phage Display Peptide Library (New England BioLabs, Beverly, MA, USA) was used in this study for epitope mapping, essentially as described by Bøgh et al. (2014), with some modification. Briefly, the wells of a microtiter plate (Nunc Maxisorb, Copenhagen, Denmark) were coated overnight at 4 °C with 150 μL of purified rabbit anti-AK IgG (100 μg/mL in 0.1 M $NaHCO_3$, pH 8.6), and then blocked with bovine serum albumin (BSA; 5 mg/mL in 0.1 M $NaHCO_3$, pH 8.6) for 2 h at 4 °C. After the samples were washed six times with TBST (TBS containing 0.1% Tween 20; TBS: 50 mM Tris–HCl, 150 mM NaCl, pH 7.5), the wells were incubated for 45 min with 1 μL ($\sim 2 \times 10^{10}$) phage from the library diluted in 100 μL of TBST. The wells were then washed extensively to remove any unbound phage. *Escherichia coli* ER2738 (New England BioLabs, Beverly, MA, USA) was infected with the bound phage to amplify the phage, which were then concentrated with PEG-8000/NaCl precipitation.

In the second and third round of biopanning, 50 μg/mL and 25 μg/mL IgG were incubated with $\sim 10^9$–10^{10} amplified phage selected in the previous biopanning round, to enrich those phage displaying high-affinity binding. The Tween 20 concentrations in the TBST were increased to 0.5% (v/v) in the washing step. In the third round, the bound phages were not amplified but titered, and single colonies were picked, isolated, and amplified as described by the library manufacturer.

2.4.2. Analysis of phage clones with an enzyme-linked immunosorbent assay (ELISA)

The phage clones with the highest reactivity to purified anti-AK IgG were selected with a phage capture ELISA, performed as described by Bøgh et al. (2014), with slight modification. In short, ELISA plates were coated overnight with 100 μL of 100 μg/mL purified rabbit anti-AK IgG at 4 °C, and then blocked with BSA at 4 °C for 2 h. After washing, the wells were incubated with the picked clones, which were serially diluted 10-fold in TBST, with a starting titer of 10^{12} virions. To detect positive reactions, horseradish peroxidase (HRP)-conjugated mouse anti-M13 phage antibody (Abcam, Cambridge, UK), diluted 1:20, was used as the secondary antibody. The samples were then incubated with 3,3′,5,5′-tetramethylbenzidine (Tiangen, Beijing, China) at 37 °C for 20 min and the reaction terminated with 2 M H_2SO_4. The absorbance at 450 nm was measured on an automated ELISA plate reader (Bio-Rad, Philadelphia, PA, USA). All the steps described above were performed at room temperature for 1 h, and between each step, the plates were washed six times with 0.05% TBST.