



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



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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 D₃A₄K₄₃M₁A₅T₄₉T₄₄I₇, L₃₁K₃₃V₃₅T₃₂E₁₁E₁₈F₁₄S₃₄D₃₇, V₁₇₇G₁₇₂M₁₇₃D₁₇₆Q₁₇₈T₁₇₄L₁₈₁K₁₇₅L₁₈₇, and R₂₀₂L₁₇₀Y₂₀₃E₁₉₀P₂₀₅W₂₀₄L₁₈₇T₂₀₆Y₁₄₅ were identified with the program LocaPep, and mapped to *S. paramamosain* AK. The key amino acids of these conformational epitopes were D₃, K₃₃, T₁₇₄, and W₂₀₄, 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 Cys₂₀₁–Cys₂₇₁ is essential for its structural stability.

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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

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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 $\mu\text{g}/\text{mL}$ 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 $\mu\text{g}/\text{mL}$ and 25 $\mu\text{g}/\text{mL}$ 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 $\mu\text{g}/\text{mL}$ 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.

2.4.3. Phage DNA precipitation and sequencing

Single-stranded DNA was prepared from randomly selected clones with a standard protocol, according to the manufacturer's specifications. The primer-96gIII (5'-GCCCTCATAGTTAGCGTAACG-3') was used for automatic sequencing (Invitrogen Co., Shanghai, China). The sequences were then translated into peptide sequences and analyzed.

2.5. Locations of conformational epitopes of AK

The AK mimotope sequences obtained by competitively immunoscreening a phage display random peptide library were calculated, analyzed, and mapped onto the three-dimensional (3D) structure of AK using LocaPep (<http://atenea.montes.upm.es>), a dedicated epitope–protein surface software. The 3D structure of *L. vannamei* AK from the Protein Data Bank (<http://www.rcsb.org/pdb/>, PDB: 4bg4) was modeled with the Web service Swiss Model (<http://swissmodel.expasy.org/>) as the reference. The positions of the identified epitopes were assigned to the 3D structure of AK with the pyMOL software (DeLano Scientific, San Carlos, CA, USA).

2.6. Mapping the linear epitopes of AK

2.6.1. Sequence alignment

Selected epitope-mimicking sequences were aligned with one another or with the primary structure of AK using the DNAMAN program (Lynnon Inc., USA).

2.6.2. IgG/IgE-binding activity of overlapping synthetic peptides

Overlapping peptides (15-mers, overlapping by eight amino acids) corresponding to the primary sequence of *S. paramamosain* AK were designed. Nineteen peptides covered the mimotopes and the predicted linear epitopes (Mao et al., 2013) were commercially synthesized using organic solid-phase synthesis technology (Cellmano Biotech, Shanghai, China). The IgG/IgE-binding activity of the peptides was detected with dot blotting, based on a modification of a previously described protocol (Shen et al., 2012). Briefly, the peptides were dissolved in double-distilled water to a final concentration of 2 mg/mL. The samples were then directly spotted onto nitrocellulose membrane (2 μ L/dot) and allowed to dry. After the membranes were blocked with 5% skim milk, they were incubated with human serum (diluted 1:4). HRP-labeled goat anti-human IgG antibody (diluted 1:1000; Comwin Biotech Co., Beijing, China) or IgE antibody (diluted 1:20,000; Southern Biotech, Birmingham, USA) was used as the secondary antibody and the results were visualized by enhanced chemiluminescence (ECL) using the ECL substrate (Pierce, Rockford, Illinois, USA).

2.7. Confirmation of the linear epitopes with a degranulation assay in RBL-2H3 cells

2.7.1. Preparation of mouse anti-AK IgE

Female BALB/c mice, 5–6 weeks of age, were acquired from the Laboratory Animal Centre, Xiamen University. The mice were immunized twice, on days 0 and 14, with 100 μ g of purified *S. paramamosain* AK, together with an alum adjuvant, in a total volume of 200 μ L. The sera from individual mice were tested for AK-specific IgE with an ELISA.

2.7.2. Cell culture

The rat mast cell line RBL-2H3 was obtained from the American Type Culture Collection (Bethesda, MD, USA) and grown in essential medium with Eagle's salts supplemented with 10% fetal

bovine serum, 2 mM L-glutamine, and 100 U/mL penicillin at 37 °C in a humidified incubator under a 5% CO₂/95% air atmosphere.

2.7.3. Measurement of β -hexosaminidase

Degranulation was monitored by the release of β -hexosaminidase, as described by Han et al. (2011). RBL-2H3 cells were seeded at 1×10^5 cells per well in 48-well plates and incubated overnight with 1 μ g/mL mouse anti-AK IgE. The IgE-containing RBL-2H3 cells were treated with seropositive peptides for 6 h in Tyrode's buffer at 37 °C. AK was used as the positive control and a seronegative peptide as the negative control. The results are presented as the percentage β -hexosaminidase activity measured in the sample supernatants compared with the total activity in the corresponding cell lysates. The β -hexosaminidase activity of the RBL-2H3 cells was measured with the substrate 4-methylumbelliferyl-*N*-acetyl-*D*-glucosaminide (Sigma Aldrich Co. St. Louis, MO, USA). To measure the total activity, the cells were lysed with 0.1% Triton X-100 before the supernatant was removed. The β -hexosaminidase activity was quantified by measuring the fluorescence intensity of the hydrolyzed substrate with a fluorometer (Thermo Fisher Scientific, Waltham, MA, USA) using 360 nm excitation and 450 nm emission filters.

2.8. Denaturation of native AK

Native AK was diluted in 50 μ L of Tris–HCl (pH 8.0) containing different concentration of either dithiothreitol (DTT), guanidine hydrochloride (GdnHCl), SDS, urea, or β -mercaptoethanol (β -Me) (Biotech, Shanghai, China) to a final AK concentration of 0.25 mg/mL. All the mixtures were incubated at 4 °C overnight, and then analyzed by dotting them onto nitrocellulose membrane (3 μ L/dot) and probing them in an immunodot-blotting assay, as described in Section 2.6.2.

3. Results

3.1. Purification and identification of AK

The purification steps were monitored with SDS–PAGE. After Q-Sepharose chromatography (Fig. 1A), one major band with a molecular mass of approximately 40 kDa was isolated on SDS–PAGE (Fig. 1B) and recognized by rabbit anti-AK IgG (Shen et al., 2011) (Fig. 1C), confirming the purification of AK from the muscle of *S. paramamosain*.

3.2. Phages isolated with biopanning with rabbit anti-AK IgG

After three rounds of biopanning, specific phages were isolated from the phage random library. A total of 20 random clones were identified with phage ELISA, and 19 of them bound to anti-AK IgG. The positive clones were sequenced. Of these 19 clones, 15 had identical sequences (ALRINTDPDFTE), so five peptides were assigned as mimotopes of *S. paramamosain* AK: mimotope 1 (DARMWGHMTTML), mimotope 2 (ALRINTDPDFTE), mimotope 3 (VWAMENSIRMTL), mimotope 4 (KLFDPLWLQSHF), and mimotope 5 (MHPNAGHGSLMR) (Table 2).

3.3. Determination of the conformational epitopes of *S. paramamosain* AK

The ELISA indicated that the five mimotopes (Table 2) bound *S. paramamosain* anti-AK IgG clones, and could therefore be considered candidate conformational epitopes. With the software LocaPep, four of the sequences were defined as the conformational epitopes: C-AK-1 (D₃A₄K₄₃M₁A₅T₄₉T₄₄I₇), C-AK-2 (L₃₁K₃₃V₃₅T₃₂E₁₁E₁₈F₁₄S₃₄D₃₇),

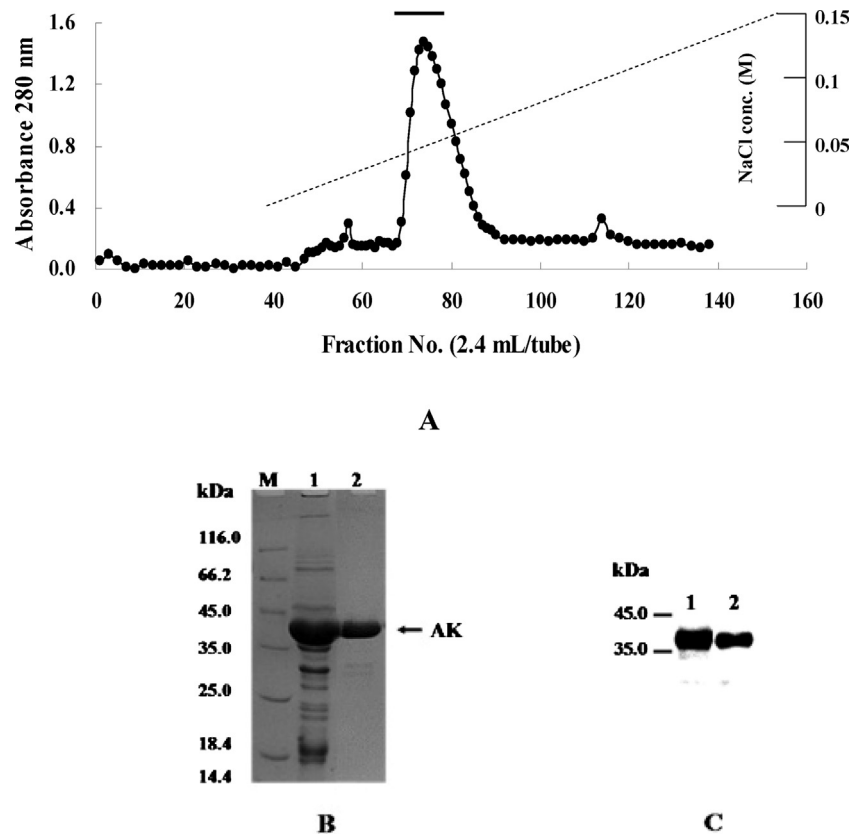


Fig. 1. Purification and identification of *S. paramamosain* AK. (A) Q-Sepharose chromatography profile. Target protein fractions under the bar were pooled (lane 2 in B and C). (B) SDS-PAGE of purified AK. M, protein marker; lane 1, crude extract from crab muscle; lane 2, purified AK from *S. paramamosain*. (C) Immunoblotting analysis using polyclonal rabbit anti-mud-crab (*Scylla serrata*) AK antibody. Lane 1, crude extract from crab muscle; lane 2, purified *S. paramamosain* AK.

Table 2

DNA sequences and deduced mimotope sequences of positive clones derived from biopanning against rabbit anti-AK IgG.

No.	Original nucleotide sequence (5'–3') ^a	Deduced mimotope sequence	Number of clone
1	GATGCGAGGATGTGGGGCATATGACGACGATGCTT	DARMWGHMTTML	1
2	GCTTTGAGGATTAATACTGATCCGATTTTACTGAG	ALRINTDPDFTE	15
3	GTTTGGGCTATGGAGAATTCTATTCGGATGACTCTG	VWAMENSIRMTL	1
4	AAGCTGTTTGATCCGTGGCTGGTTCAGTCTCATTTT	KLFDPWLVQSHF	1
5	ATGCATCCGAATGCGGGCATGGTTCGCTTATGCGG	MHPNAGHGSMLR	1

^a For deduce the amino acid sequences, the DNA sequences were read corresponds to the anticodon strand of the template.

C-AK-3 (V₁₇₇G₁₇₂M₁₇₃D₁₇₆Q₁₇₈T₁₇₄L₁₈₁K₁₇₅L₁₈₇), and C-AK-4 (R₂₀₂L₁₇₀Y₂₀₃E₁₉₀P₂₀₅W₂₀₄L₁₈₇T₂₀₆Y₁₄₅) (Table 3). Graphical molecular representations of these conformational epitopes on AK are shown in Fig. 2. The locations of these four conformational epitopes can be divided approximately into two areas at the antibody-binding interface: one includes C-AK-1 and C-AK-2, and the other includes C-AK-3 and C-AK-4. The two components in each area are close to one another and share the same amino acid residues to some extent.

An epitope is assumed to form around a key residue, which is defined as a “seed” in the LocaPep software. To act as a seed, a residue must display a significantly large exposed surface area and have a small number of contacts compared with mean and extreme values for the corresponding amino acid in the protein (Pacios et al., 2011). The AK surface was scanned for seeds among the residues belonging to each cluster. The four key amino acids of the four conformational epitopes were identified as D₃, K₃₃, T₁₇₄, and W₂₀₄, which are marked in yellow on AK (Fig. 2). Mimotope

Table 3

Conformational epitopes of *S. paramamosain* AK analyzed by LocaPep based on the biopanning.

Name	Mimotopes	Amino acid residues and locations of epitope candidates	Score ^a
C-AK-1	DARMWGHMTTML	D ₃ A ₄ K ₄₃ M ₁ A ₅ T ₄₉ T ₄₄ I ₇	4.429
C-AK-2	ALRINTDPDFTE	L ₃₁ K ₃₃ V ₃₅ T ₃₂ E ₁₁ E ₁₈ F ₁₄ S ₃₄ D ₃₇	4.790
C-AK-3	VWAMENSIRMTL	V ₁₇₇ G ₁₇₂ M ₁₇₃ D ₁₇₆ Q ₁₇₈ T ₁₇₄ L ₁₈₁ K ₁₇₅ L ₁₈₇	5.007
C-AK-4	KLFDPWLVQSHF	R ₂₀₂ L ₁₇₀ Y ₂₀₃ E ₁₉₀ P ₂₀₅ W ₂₀₄ L ₁₈₇ T ₂₀₆ Y ₁₄₅	4.023

^a A cluster composed of n residues has a total score $T = \sum score_i, i = 1, n$. Total score for residue i is finally computed as: $score_i = 0.20scoA_i + 0.30scoE_i + 0.30scoC_i + 0.20scoD_i$. Area score, $scoA_i$; exposure score, $scoE_i$; contacts score, $scoC_i$; distance score, $scoD_i$. $0 \leq sco_i \leq 1.0$ ($i = A, E, C, D$). The bold font represents the key amino acid of a cluster.

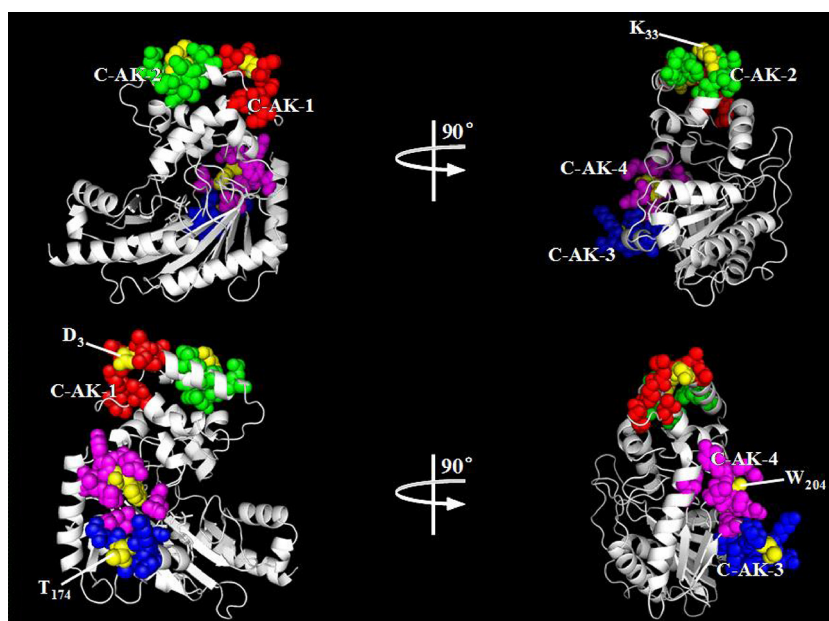


Fig. 2. Molecular graphics of the mimotopes recognized as conformational epitopes. The epitopes are highlighted in red, green, blue, and magenta. The yellow balls represent the key amino acid of each epitope. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

5 (MHPNAGHGSLMR) was excluded by the LocaPep program, because the software suggested that it is a linear epitope.

3.4. Determination of IgE-specific fragments of *S. paramamosain* AK

3.4.1. Alignment of mimotope sequences

Attempts by DNAMAN to find consensus sequences to the mimotopes were unsuccessful. Therefore, the mimotope sequences were aligned with the primary sequence of the AK molecule using DNAMAN, as shown in Fig. 3A. Mimotope 2, mimotope 5, and mimotope 1 corresponded to an area located at L₈₁–V₁₂₀, mimotope 4 to C₂₀₁–V₂₂₀, and mimotope 3 to V₃₂₂–L₃₃₃.

3.4.2. IgG/IgE-binding activity of synthetic peptides

To confirm the locations of the IgG/IgE-specific fragments of AK, the IgG/IgE-binding activities of the synthetic peptides were analyzed with dot blotting. The peptides used here are shown in Fig. 3B (peptides 8, 10, and 15 were confirmed as seropositive in our previous study). Among the 19 synthetic peptides, IgG-binding active peptides were peptides 6, 8, 9, 10, 14, 15, and 18 (Fig. 4A). Serum IgG of 7359 reacted with peptides 6, 10, 15, and 18; serum IgG of 4583 reacted with peptides 6 and 8; serum IgG of 8517 reacted with peptides 6, 8, 9, and 14. Six of the 19 peptides, including peptides 8, 10, and 15, were recognized by the sera of crab-allergic patients (Fig. 4B). Peptide 6 (113–VDPDGKFVISTRVRC–127) was recognized by the sera of patients 7359, 8517, 0284, and 0737; peptide 10 (141–TEAQYKEMESKVSST–155) was recognized by all the sera, except that of patient 5991; peptide 14 (204–WPTGRGIYHNDNKTF–218) was recognized by the sera of patients 0284 and 4723; peptide 15 (211–YHNDNKTLVWCNEE–225) was recognized by the sera of patients 7357, 0284, and 0737; peptide 18 (316–TEAEGGVYDISNKR–330) was recognized by the sera of patients 7359, 0284, and 4723. All the sera reacted strongly with peptide 6, suggesting that it might have a significant IgE-binding site. The locations of the experimental IgE-specific peptides were mapped on the model of AK (Fig. 4C).

3.5. The effect of seropositive peptides on the degranulation of antigen-stimulated RBL-2H3 cells

To determine the allergic activity of the seropositive peptides, we investigated their effects on the degranulation of antigen-stimulated RBL-2H3 cells. The β -hexosaminidase release was measured as an index of degranulation. Purified AK and seronegative peptide 7 were used as the positive and negative controls, respectively. A methyl thiazolyl tetrazolium (MTT) assay showed that none of the fractions (10–100 μ g/mL) affected cell viability (data not shown). As shown in Fig. 5, AK induced obvious degranulation in IgE-sensitized RBL-2H3 cells at a concentration of 200 ng/mL. Four of the six seropositive peptides also induced degranulation. Peptide 15 and peptide 18 seemed to have little effect on the degranulation of cells. Among the effective fractions, β -hexosaminidase release was induced in the order: peptide 14 > peptide 6 > peptide 10 > peptide 8. However, the effective concentrations of these peptides were orders of magnitude higher than that of the AK. Linear epitope mapping has been done by Ayuso et al. (2012) for Lit v 2, considering high conservation of arginine kinase among crustacean species, an amino acid sequence alignment of linear epitopes of prawn and crab arginine kinase was present to describe the similarities and differences between the two species (Fig. 6).

3.6. Effect of AK denaturation on its IgE reactivity

To investigate the IgE-binding activity of purified AK under different denaturation conditions, the denatured proteins were dotted blotted and probed with the patients' sera. The denaturants used were SDS, β -Me, DTT, SDS, urea, and GdnHCl. The result of dot-blotting experiment (Fig. 7A) revealed that all five denaturants influenced the IgE-binding activity of AK to different degrees. SDS reduced AK reactivity at low concentrations (0.3 mM), and the reactivity of AK was almost completely abolished when the SDS concentration reached 15 mM. In the urea-treated samples, the reactivity of AK was enhanced at urea concentrations of 2.0–3.0 M, whereas at concentrations higher than 4.0 M, the samples had lower IgE-binding activity than nondenatured AK. There was a

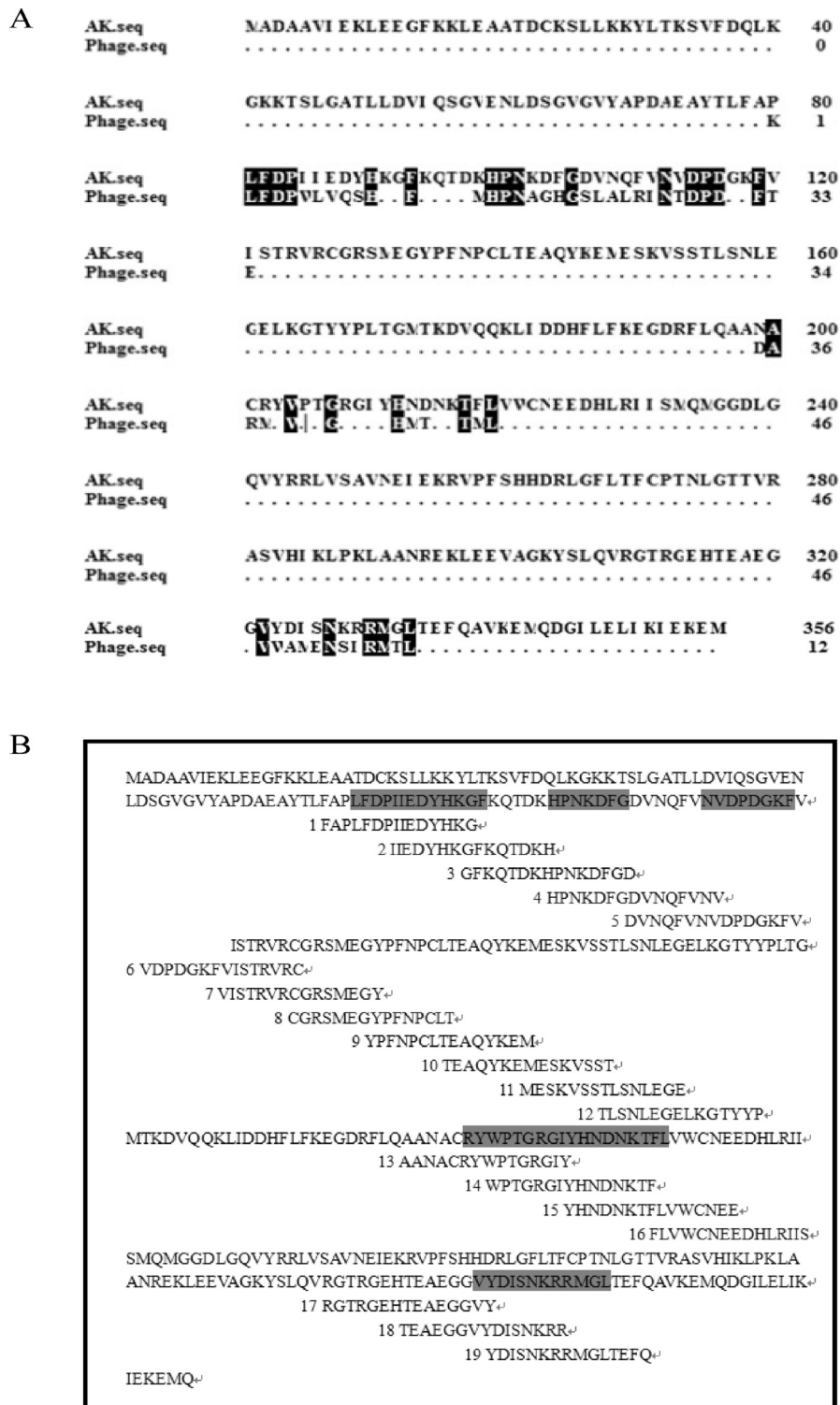


Fig. 3. Amino acid sequences of *S. paramamosain* AK. (A) The amino acid sequence alignment of *S. paramamosain* AK and five mimotopes derived with biopanning. The common sequences of AK and mimotopes were marked in black. (B) Amino acid sequences of *S. paramamosain* AK and 19 synthetic peptides; the peptides derived with biopanning are highlighted (peptide 8, peptide 10, and peptide 15 were defined as IgE-specific fragments of *S. paramamosain* AK in our previous study (Mao et al., 2013)).

slight change in the IgE-binding activity of AK after GdnHCl treatment, but when the concentration of GdnHCl reached 1.5 M, the binding capacity of the samples was unchanged. DTT, at a concentration of 10 mM, caused a slight reduction in AK reactivity, and the IgE-binding activity of AK decreased in a dose-dependent manner at concentrations of 10–250 mM, but remained steady at higher concentrations. The IgE-binding activity of AK treated with 2.5 M

β -Me was lower than that of nondenatured AK. Among the five treatments, the IgE-binding activity of purified AK was effectively reduced by SDS, β -Me, DTT, and high-concentration urea. Based on these results and previous studies of the structure–activity relationships of AK, the AK structure was further analyzed by a bioinformatics method, and the inferred key chemical bonds that are crucial for the allergenicity of AK are shown in Fig. 7B.

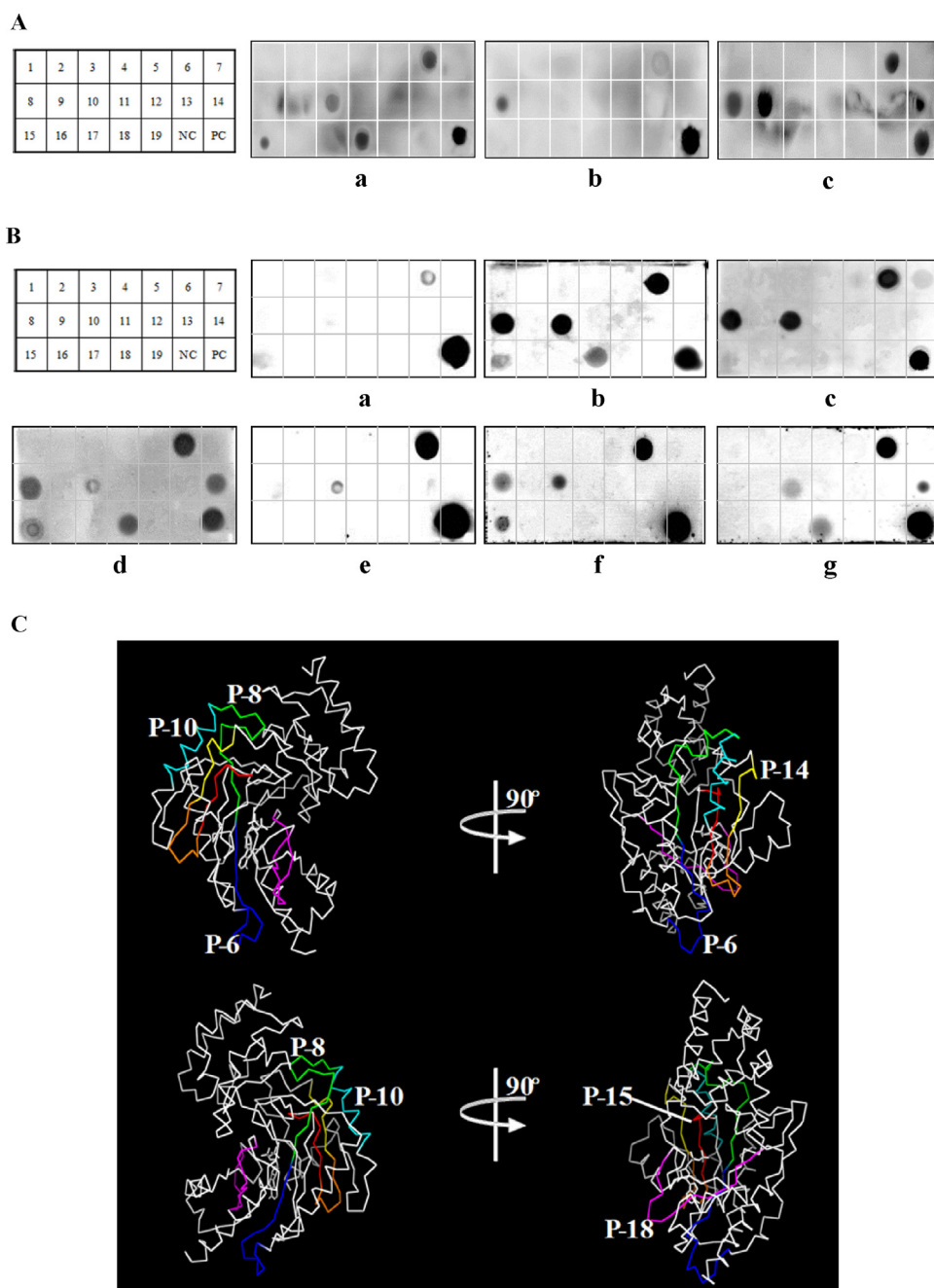


Fig. 4. Determination of the IgG/IgE-specific fragments of *S. paramamosain* AK. (A) Dot-blot analysis of IgG binding activity of the 19 synthetic peptides. (a–c) Sera of crab-allergic patients (corresponding to sera no. 7359, 4583, and 8517); PC: native AK; NC: BSA. (B) Dot-blot analysis of IgE-binding activity of the 19 synthetic peptides. a–g: sera of crab-allergic patients (corresponding to sera nos. 5991, 7359, 8517, 0284, 4583, 0737, and 4723); PC: native AK; NC: BSA. (C) Mapping the IgE-specific peptides onto a molecular model of *S. paramamosain* AK. P, peptide.

4. Discussion

A better understanding of the issues related to allergen-specific immune reactions are essential if we are to establish new and better diagnostic and therapeutic tools for food allergies. Work focusing on the patterns of specific epitope reactions in patients suffering from allergies has contributed to our knowledge of the general mechanisms of allergy. The identification of the IgE-binding epitopes might provide a better understanding of the functional role that allergens play in the disease and could have implications for immunodiagnosis and immunotherapy. To map the conformational epitopes of proteins, some authors have suggested that the technique of phage display

peptide libraries is a rapid and convenient method for identifying both linear and conformational epitopes (Zeng et al., 2012).

In this study, rabbit anti-AK immunoglobulin was used to pan a phage display random peptide library to determine the mimotopes of *S. paramamosain* AK. In general, biopanning for IgE-specific epitopes requires the corresponding purified IgE from allergic suffers. Regrettably, the limited patient sera available were far from sufficient to purify IgE. However, IgG is reported to recognize the same epitopes as IgE, at least to some degree (Subbarayal et al., 2013; Wachholz et al., 2003), so rabbit anti-AK IgG enriched with protein A was selected as the target protein for the panning procedure instead of IgE.

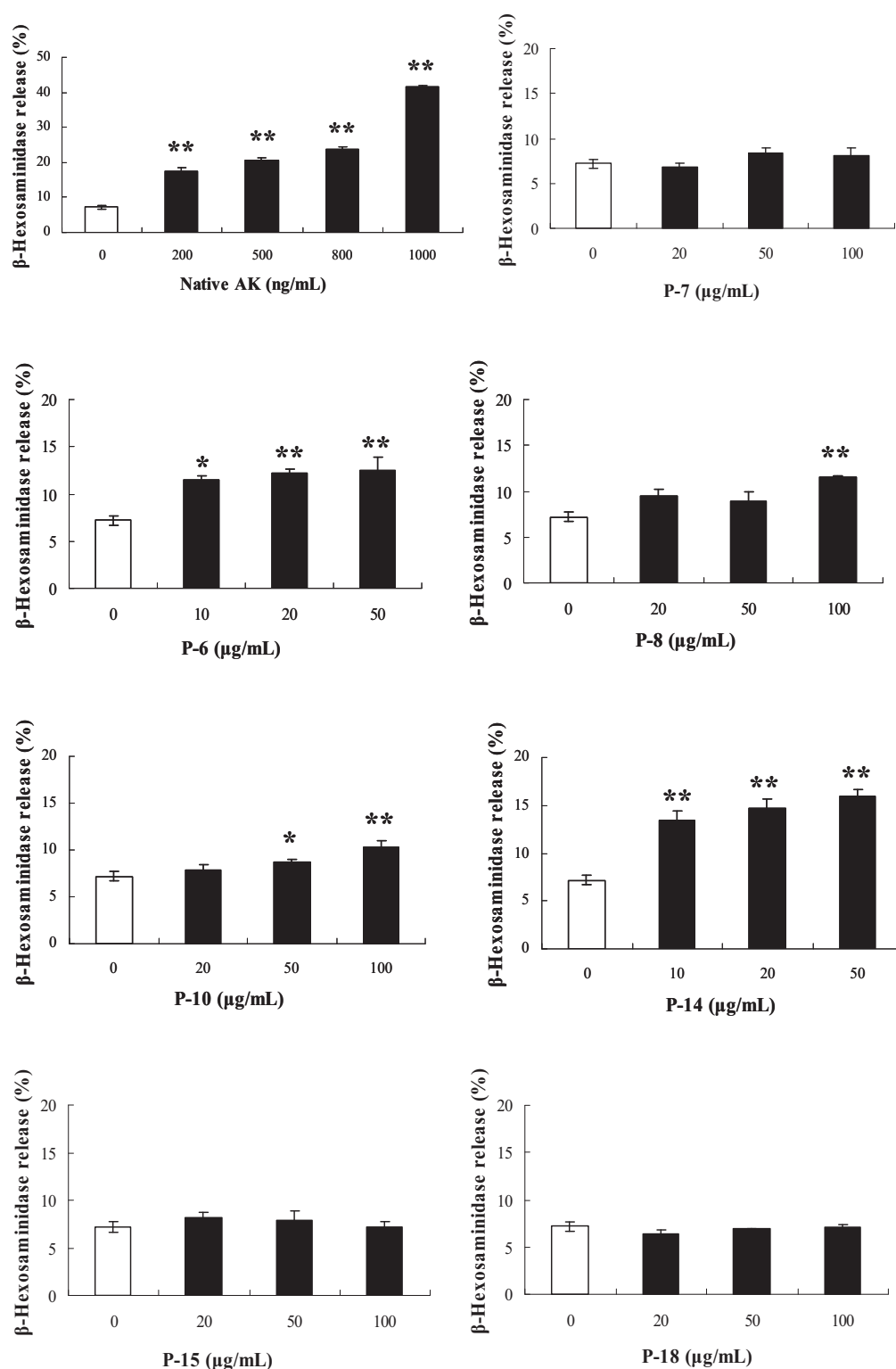


Fig. 5. Effects of seropositive peptides on degranulation. The degranulation was determined by measurement of β -hexosaminidase release in RBL-2H3 cells. The AK specific-IgE sensitized RBL-2H3 cells were stimulated with seropositive peptides for 6 h at 37 °C. PC: native AK; NC: seronegative peptide (P-7); P, peptide. Each value represents the mean $\pm$ SE of three experiments. * Indicates significant differences from the control at $p < 0.05$, ** indicates significant differences from the control at $p < 0.01$.

Based on the results of biopanning, four conformational epitopes of *S. paramamosain* AK (C-AKs) were mapped onto the 3D model of the AK molecule with the LocaPep program. C-AK-1 and C-AK-2 partly overlap the predicted conformational epitope (amino acids 39–44) of *S. paramamosain* AK, whereas C-AK-3 has amino acid residues very similar to those of the predicted conformational

epitope of *S. paramamosain* AK (amino acids 172–180, 183) and *P. clarkii* AK (Mao et al., 2013; Chen et al., 2013). There was no predicted conformational epitope corresponding to C-AK-4. However, this epitope coincides with the main sensitization areas (amino acids 142–159 and 160–192) of *L. vannamei* AK (Lit v 2), as defined by Ayuso et al. (2012). However, the main sensitization areas of

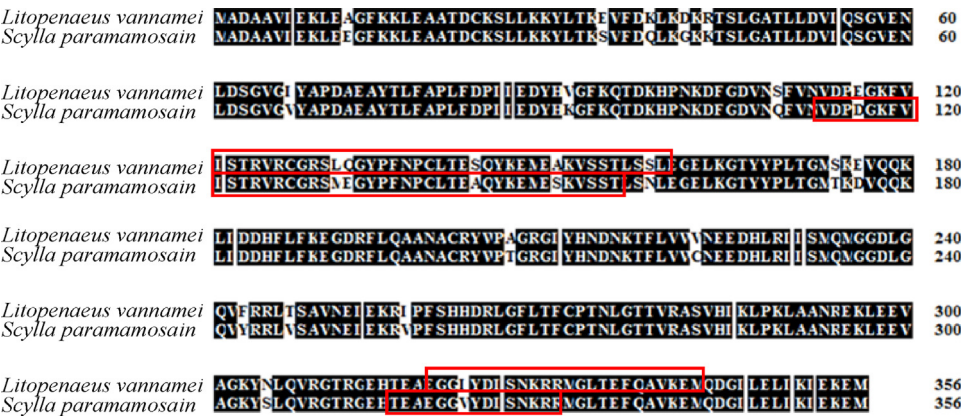


Fig. 6. Amino acid sequence alignment of *L. vannamei* and *S. paramamosain* AK (corresponding GenBank accession numbers are DQ975203 and JN828652). Amino acids marked with shadowing are the same in the two kinds of AK, the sequences marked in red frames are the coincident seropositive peptides between prawn AK and crab AK.

Lit v 2 were defined with synthetic peptides, indicating that the sensitization areas contain linear epitopes. However, our study suggests that C-AK-4 is a conformational epitope, so this area might contribute to both conformational and linear epitopes.

The four clusters were approximately divided into two domains of AK based on their positions: one domain including C-AK-1 and C-AK-2 and the other including C-AK-3 and C-AK-4. Among the predicted AK conformational epitopes in *P. clarkii* and *S. paramamosain*, only two clusters (39–44 and 172–180, 183, respectively) are located in the domains described above. This indicates that the prediction of epitopes with bioinformatics techniques is still with some discrepancy, and should be backed by experimental results.

It is generally believed that most of the IgE epitopes are conformational, nevertheless, food allergens might first suffer the gastrointestinal tract in the body, and linear epitopes have been suggested to be important as well. To confirm these six IgE-specific peptides, they were identified with synthetic overlapping peptides. As described by Mao et al. (2013), the IgE epitopes in *S. paramamosain* AK are mainly distributed in two regions: amino acids 87–186 and amino acids 172–265. In the present case, peptide 6 (amino acids 113–127), peptide 8 (amino acids 127–141), and peptide 10 (amino acids 141–155) were located within amino acids 87–186, whereas peptide 14 (amino acids 204–218) and peptide 15 (amino acids 211–225) were located within amino acids 172–265. Peptides

8 and 10 share some amino acid residues with the predicted linear epitopes (amino acids 146–152) of *P. clarkii* AK and *S. paramamosain* AK (Chen et al., 2013; Mao et al., 2013). Besides, it is worth mentioning that peptides recognized by IgG from crab-allergic patient sera are peptides 6, 8, 9, 10, 14, 15, and 18; peptides recognized by the specific IgE are peptides 6, 8, 10, 14, 15, and 18. In other words, the sequences recognized by IgG and IgE from crab-allergic patient sera are in line with each other, although the reactivity intensity of IgG and IgE was not so consistent for each peptide, indicating that the epitopes conducted by IgG in the present study can extrapolate to the IgE epitopes.

Ayuso et al. (2012) has mapped the linear epitopes of *Litopenaeus vannamei* AK by peptide microarray analysis, an amino acid sequence alignment of prawn and crab arginine kinase, as shown in Fig. 6 has drawn a comparison between previous and present studies, which demonstrated that the IgE epitopes in prawns and crabs are very similar, but not identical. Compared with the experimental seropositive peptides of *S. paramamosain* AK, epitopes 4a and 4b (amino acids 121–141 and 142–159) of *L. vannamei* AK were similar to the seropositive peptides 6, 8, and 10, which were included in the predominant epitope region amino acids 87–186 defined by Mao et al. (2013), and epitope 7 (amino acids 319–342) of *L. vannamei* AK was corresponded to peptide 18 (amino acids 316–330). However, peptides 14 and 15 did not correspond with the IgE epitope of *L. vannamei* AK, this might result from the non-conservation

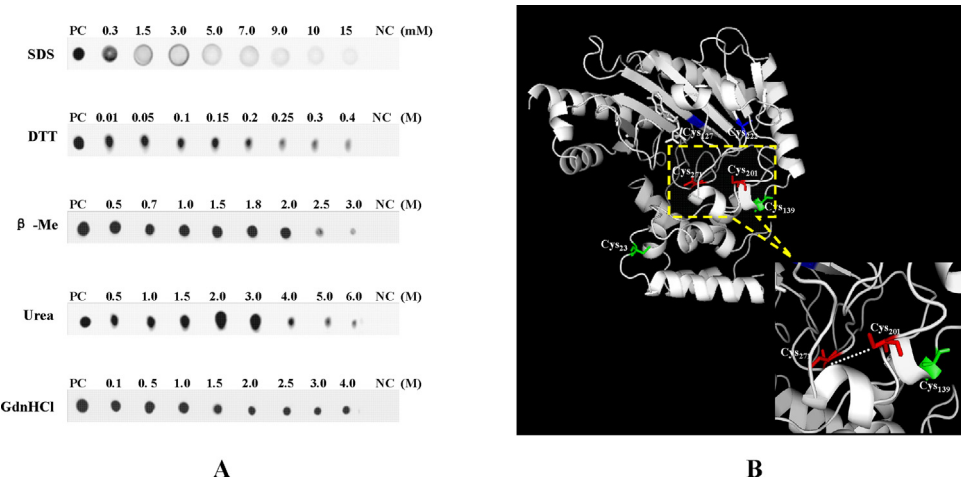


Fig. 7. Effects of denaturants on the allergenicity of AK. (A) Dot-blotting analysis of the IgE-binding activity of denaturant-treated *S. paramamosain* AK. An ECL substrate was used for visualization, and the membranes were exposed to film for 5 min. Sera pool (nos. 7359, 8517, 0284, and 0737); PC: native AK; NC: BSA. (B) Structural model of AK and its cysteine residues. Cysteine residues are shown in the stick models. The disulfide bond formed between Cys₂₀₁ and Cys₂₇₁ is marked with a line.

between species, in addition, and the individual difference of sera from different patients may also lead to this non-corresponding.

Human type I immediate allergic response symptoms are caused by the release of mediators, such as β -hexosaminidase and histamine, from basophils and mast cells. This event is triggered by allergens aggregating preformed IgE bound to the high-affinity receptor on these cells (Holm et al., 2004). Therefore, two factors are crucial for the cascade leading to the allergic response: the allergen/IgE interaction and the release of the mediator from the immune cells. In other words, the IgE reactivity of a protein or peptide alone is insufficient to induce the symptoms of an allergic response. Mast cells express high-affinity receptors for IgE on their surfaces and play a critical role in inducing allergic inflammation (Kasakura et al., 2014). In a study of the major shrimp allergen Pen a 1 (tropomyosin) by Reese et al. (2005), the mediator released from rat basophilic leukemia (RBL-2H3) cells was one of the indices used to measure allergenicity. Consequently, in our study, mast cells were used to identify the linear epitopes among the six IgE-specific peptides of AK. Only four of the IgE-specific peptides (peptide 6, peptide 8, peptide 10, and peptide 14) induced the degranulation of mast cells, and peptides 6 and 14 induced degranulation at lower concentrations than peptides 8 and 10. Although peptides 15 and peptide 18 were recognized by the patients' sera, they did not induce a subsequent allergic reaction, confirming that the linear epitopes of AK (L-AKs) are L-AK-1 (amino acids 113–127), L-AK-2 (amino acids 127–141), L-AK-3 (amino acids 141–155) and L-AK-4 (amino acids 204–218). Of these, L-AK-1 and L-AK-4 may be major linear epitopes. Sequences of L-AK-1 and L-AK-4 are also similar to that of mimotope 1 (ALRINTDPDFTE) and mimotope 4 (DARMWGHMTTML), suggesting that the IgG-specific epitopes in AK overlap the IgE-specific epitopes to a large extent, so biopanning against IgG rather than IgE was appropriate. From the prediction results, only L-AK-3 shared some amino acid residues with the predicted linear epitope at amino acids 146–152 (Mao et al., 2013; Chen et al., 2013), confirming that the epitopes predicted with bioinformatics techniques must be confirmed with experimental evidence. Furthermore, IgE-binding activity is not enough for a peptide to be defined as an epitope.

A number of studies have shown that in globular proteins, approximately 90% of the epitopes are discontinuous (Kringelum et al., 2012). The results of Mao et al. (2013) are consistent with previous suggestions that the predominant epitope type in *S. paramamosain* AK is the conformational epitope. However, how the structure of AK affects its antigenicity is still unclear, although a denaturation study provided some insight into the stability of the protein structure during sensitization. In order to investigate the allergenicity changes during different structure variations, denaturants including SDS, β -Me, DTT, urea, and GdnHCl, which were typical and frequently used in the studies on structure-activity relationship were used, these denaturants act on different chemical bonds such as intermolecular forces, disulfide linkages, and hydrophobic forces, which are important in maintaining structure of proteins. A dot-blotting assay showed that SDS was an efficient denaturant, and that AK was completely inactivated in 15 mM SDS. With its hydrophobic tail, SDS can penetrate the interior of proteins and disrupt the hydrophobic interactions therein, changing the overall charge on the protein (Sudhakar and Chin, 2006). The low IgE-binding activities of SDS-treated AK suggest that most IgE-binding epitopes in AK are conformational.

Urea and GdnHCl presumably exert their effects by interacting with peptide bonds (Gianni et al., 2001). Treatment with 2–3 M urea resulted in the exposure to the solvent of buried residues present in the native protein, increasing the antigenicity of AK, whereas at higher concentrations, urea acted as a classical denaturant, unfolding the protein chain. Urea has only chaotropic effects, whereas GdnHCl is a monovalent salt with ionic effects, rather than

chaotropic effects. GdnHCl is an electrolyte with a pKa of 11 (Rashid et al., 2005). In the buffer used here (pH 8.0), GdnHCl is fully protonated to Gdn⁺, and Gdn⁺ can affinity bind to one or more sites on proteins, possibly stabilizing the molecular structures of the proteins (Ahmad et al., 2001). In the present case, AK might have been stabilized by the bound Gdn⁺ on its molecular surface. Consequently, slight variations in the IgE-binding activity of AK were detected in the GdnHCl-treated samples.

Disulfide bonds in proteins are essential for maintaining their native conformations (Choi et al., 2005). As described by Wang et al. (2013), AK contains six free cysteine thiols in its molecule, and the intramolecular disulfide bond that forms between Cys₂₀₁ and Cys₂₇₁ is essential for the structural stability of AK. Cys₂₇₁ is reported to be located in the hinge region between the two domains of AK and causes conformational changes in the enzyme when the substrate is added (Liu et al., 2011). DTT and β -Me are typical disulfide-bond-reducing agents, often used to investigate the relationships between bioactivity and the disulfide bonds in proteins. β -Me can penetrate a protein and break the disulfide bonds inside it, whereas in contrast, DTT can only affect disulfide linkages on the molecular surface (Terence, 1990; Zhang et al., 2000). In this study, both of these reagents reduced the IgE-binding activity of AK, suggesting again that conformational epitopes are important for its allergenicity. At lower concentrations, the effective concentration of DTT was much lower than the effective concentration of β -Me, but as the concentration increased, the effect of β -Me was stronger than that of DTT, whose full denaturant effect cannot be reached at the maximum concentration used. These findings lend further support to the notion that the intramolecular disulfide bond at Cys₂₀₁–Cys₂₇₁ is a stability relative bond that plays an important role in maintaining the AK structure and therefore its allergenicity (Fig. 7B).

5. Conclusions

Four conformational epitopes, and their key amino acid residues, were defined by biopanning a phage random peptide library with the LocaPep program. Four IgE-specific peptides were derived with biopanning, and the synthesized overlapping peptides were identified as linear epitopes in a mediator release assay with RBL-2H3 cells. Stability experiments showed that the intramolecular disulfide bond at Cys₂₀₁–Cys₂₇₁ is essential for the allergenicity of AK. However, more sera containing crab-AK-specific IgE from allergic patients is required to fully understand the IgE epitopes of *S. paramamosain* AK.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

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