

Structural characterization and IgE epitope analysis of arginine kinase from *Scylla paramamosain*

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ARTICLE INFO

Article history:

Received 15 March 2013

Received in revised form 22 April 2013

Accepted 29 April 2013

Keywords:

Shellfish allergy

Arginine kinase

IgE epitope

Synthesized peptides

Protein homology modeling

Gene expression

ABSTRACT

Arginine kinase (AK) is reported to be the pan-allergen of shellfish. However, there is limited information on its IgE epitopes and structural characteristics. In this study, AK from *Scylla paramamosain* was purified and characterized. The purified AK is a glycoprotein with the molecular weight of 40 kDa and it demonstrates cross-reactivity with the related allergens present in other shellfish. The cDNA of *S. paramamosain* AK was cloned, which encodes 357 amino acid residues. Nine linear epitopes and seven conformational epitopes were predicted following bioinformatics analysis. In addition, the entire recombinant AK (rAK) and three partial recombinant AKs (rAK1, rAK2, and rAK3) were successfully expressed in *Escherichia coli* BL21 (DE3). The proteins of rAK1, rAK2 and rAK have strong IgE reactivity with the pooled sera from crab allergic patients, while rAK3 has significantly weaker IgE reactivity, which indicates that the IgE epitopes of AK are mainly distributed in the regions of rAK1 and rAK2. Furthermore, three experimental linear epitopes (epitope 1: AA 127–141, epitope 2: AA 141–155, and epitope 3: AA 211–225) were discovered in the region of rAK1 and rAK2 using synthesized overlapping peptides. The experimental linear epitopes were mapped onto the protein homology model of AK. Meanwhile, in the IgE-binding assays of the sera from nine crab allergic patients, only three sera reacted with the denatured, linear AK as shown by Western-blotting, eight sera reacted with the native, folded AK by both dot-blotting and ELISA, which indicates that the conformational IgE epitopes of *S. paramamosain* AK may be more predominant.

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1. Introduction

Shellfish is one of the most common causes of food-induced anaphylaxis in the Asia-Pacific region, especially in China (Lee et al., 2012). The production and consumption of shellfish have been accompanied by more frequent allergic diseases ranging from skin reactions, digestive disorders and respiratory symptoms to life-threatening reactions (Burks et al., 2012; Lopata et al., 2010). To date, several clinically relevant allergens of shellfish, such as tropomyosin, myosin light chain, sarcoplasmic calcium-binding protein, troponin C, triosephosphate isomerase, and hemocyanin (Bauermeister et al., 2011; Piboonpocanun et al., 2011), have been identified by proteomics, immunology and molecular biological approaches.

Arginine kinase (AK) is a pan-allergen in invertebrates, and plays an important role in inducing shellfish-derived allergic disorders (García-Orozco et al., 2007; Rahman et al., 2011; Yu et al., 2003). Our previous studies have confirmed that crab AK is bound by IgE from crab allergic patients' sera (Shen et al., 2012; Yu et al., 2013). Analysis of the IgE epitopes of AK is essential to the development of hypoallergenic food and safer immunotherapy in the future. Generally, there are two categories of antibody epitopes, linear and conformational, responsible for allergic reactions. Methods used to identify IgE epitopes included synthetic overlapping peptides, phage display, fingerprinting and micro-sequencing techniques (Ishikawa et al., 2001; Untersmayr et al., 2006). Recently, eight linear IgE epitopes (AA 1–18, 25–42, 64–96, 121–141, 142–159, 160–192, 232–255 and 319–342) of *Litopenaeus vannamei* AK has been obtained by overlapping peptides synthesis technique (Ayuso et al., 2012). However, the IgE epitopes and the related structural features of crab AK have not been reported.

Crab is well demanded in many countries due to its good taste and nutrition. China is the largest crab producer in the world and the production capability had exceeded 0.8 million tones in 2011 (Liu et al., 2012). It is worth noting that *Scylla paramamosain* is assumed to be the main species responsible for severe

Abbreviations: AA, amino acid number; ELISA, enzyme-linked immunosorbent assay; SDS-PAGE, sodium dodecylsulfate polyacrylamide gel electrophoresis; PDB, RCSB protein data bank; pI, isoelectric point; MW, molecular weight.

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hypersensitivity reactions, as it is widely distributed and diversified in China. Under these circumstances, the potential IgE epitopes of *S. paramamosain* AK were determined and the protein homology model was generated to observe the location of the IgE epitopes on the 3-dimensional structure.

2. Materials and methods

2.1. Human sera

The sera from nine crab allergic patients (No. 4917, 8557, 2452, 5240, 4922, 9527, 9985, 9815, and 2170) were obtained with the permission of the Hospital of Jimei University (Xiamen, China). These sera were used either individually or as a sera pool. All patients were thought to have crab allergy based on their clinical history. A sera pool collected from healthy donors (Nos. 0302, 8196, and 9655) was used as a negative control. All sera were stored in aliquots at -30°C until use.

2.2. Purification and identification

S. paramamosain AK was purified as previously described with minor modifications (Shen et al., 2011, 2012; Yu et al., 2013). The fresh muscle was minced and homogenized for ammonium sulfate fractionation. The extract was then dialyzed and applied to a HiTrapQ Sepharose Fast Flow column (Amersham Biosciences, Uppsala, Sweden). Fractions were eluted at 0.8 mL/min with a 0–0.15 M NaCl linear gradient. The bound protein was then collected and analyzed by 12% SDS-PAGE (Laemmli, 1970).

Western-blotting and two-dimensional electrophoretic (2-DE) analyses of the purified AK were performed as described in our previous study (Shen et al., 2011). Periodic acid-Schiff (PAS) staining was performed using a commercial kit (Genmed, Shanghai, China) to observe whether AK is glycosylated.

2.3. Cross-reactivity

The cross-reactivity between purified *S. paramamosain* AK and corresponding allergens in the myosinogen from crawfish (*Procambarus clarkii*), white shrimp (*L. vannamei*) and octopus (*Octopus fangsiao*) have been assayed by IgE immunoblotting inhibition experiments (Binder et al., 2001; Shen et al., 2012). Fresh muscle from the different species were minced and homogenized in ice-cold H_2O containing 25 μM PMSF, then centrifuged to obtain the supernatant as the myosinogen.

The extracts were subjected to a 12% SDS-PAGE (5 μg per lane) and were then electrophoretically transferred onto nitrocellulose (NC) membranes and blocked with 5% nonfat milk overnight at 4°C . Patient sera were mixed in an equal volume, diluted (1/10 in 20 mM PBS, pH 7.5) and pre-incubated with or without 100 $\mu\text{g}/\text{mL}$ *S. paramamosain* AK overnight at 4°C . The pre-incubated sera were then incubated with the NC membranes for 2 h at 37°C . After incubation with horseradish peroxidase (HRP)-conjugated mouse anti-human IgE secondary antibody (Southern Biotech, Birmingham, USA, 1/20,000 dilution), the immunoassay was carried out using enhanced chemiluminescence (ECL, Pierce, Rockford, IL, USA), according to manufacturer's instructions.

2.4. cDNA cloning

Total RNA was prepared from fresh *S. paramamosain* muscle using Trizol reagent (Invitrogen, Carlsbad, CA, USA). First stranded cDNA was synthesized with the TIANScript RT Kit (TIANGEN, Beijing, China).

Based on well-conserved regions of invertebrate AK sequences (GenBank: FJ457622, DQ975203, GQ246164, and HQ185192), a pair

Table 1

All the primers were used in cDNA amplification.

Primer	Sequence (5'–3')
AK-F	GAGGACTACCACAAGGGTTTCA
AK-R	ACCCTCAGCCTCAGTGTCTCGCC
AK-3R1	CAACGACAACAAGACCTTCCTGG
AK-3R2	ACCACTGTGCGTGCCTCCGTCCTCA
AK-5F1	CATCGCCGAAGTCCTTGTTAG
AK-5F2	TCAGTCTGCTTGAACCTTGTG

of primers (AK-F and AK-R) were designed (Table 1) and the PCR program was performed on GeneAmp 9700 (Applied Biosystems, Carlsbad, CA, USA). The PCR product was excised from the agarose gel and purified with the Universal DNA Purification Kit (TIANGEN, Beijing, China), then cloned into pGEM-T vector (Promega, Madison, WI, USA).

From the sequence information obtained above, gene specific primers (AK-3R1, AK-3R2, AK-5F1 and AK-5F2) were designed (Table 1) and used to selectively amplify the 5' and 3' portions of *S. paramamosain* AK with the SMART RACE Amplification Kit (Clontech, CA, USA). Nested-PCR was adopted to improve the specificity of SMARTer RACE amplification, and the PCR product was purified and cloned. All clones were sequenced by Invitrogen Corp. (Shanghai, China) with a DNA sequencer ABI PRISM 3730 (Applied Biosystems, Carlsbad, CA, USA).

2.5. Bioinformatics analysis

Protein sequence analysis was performed by InterPro Scan (Zdobnov and Apweiler, 2001), NetNGlyc (Gupta et al., 2005), SOPMA (Geourjon and Deléage, 1995), DNASTAR computer program (DNASTAR Inc., Madison, WI, USA) and DiscoTope 2.0 Server (Kringelum et al., 2012). Phylogenetic analysis of *S. paramamosain* AK was performed using MEGA 5.0 software (Tamura et al., 2007) with the intend to compare the amino acid residues with 17 other invertebrates.

The homology model of *S. paramamosain* AK was obtained by CPHmodels 3.2 Server (Nielsen et al., 2010) and analyzed using pyMOL software (DeLano Scientific, San Carlos, CA, USA).

2.6. Expression and purification of AK and partial AK fragments

In order to investigate the distribution of linear epitopes, three partial recombinant AKs were designed according to the distribution of the predicted linear epitopes, rAK1 have 3 predicted linear epitopes (AA 87–103, 146–151, and 174–181), while rAK2 and rAK3 have 2 predicted linear epitopes (rAK2: AA 174–181 and 253–256; rAK3: AA 294–298 and 309–319). The entire open reading frame of *S. paramamosain* AK and three partial recombinant AKs (rAK1: AA 87–186, rAK2: AA 172–265, and rAK3: AA 276–319) were cloned into the prokaryotic expression vector, pET 28 (Novagen, Madison, WI, USA), respectively. The constructs were then transformed into *Escherichia coli* BL21 (DE3) competent cells (Novagen, Madison, WI, USA). All clones were sequenced to make sure that they were correctly constructed.

Cell strains containing the correct constructs were grown in Luria-Bertani (LB) medium at 37°C and induced with 1 mM isopropyl β -D-thiogalactoside (IPTG) at 28°C when the OD_{600} value had reached 0.6. The cells were then harvested by centrifugation and resuspended in pre-cooled lysis buffer (20 mM Tris-HCl, 50 mM NaCl, 10 mM imidazole, pH 8.0). The suspensions were subjected to freeze-thaw cycles, sonication, and then centrifuged for clarification. The supernatant was directly loaded onto a nickel-affinity chromatography column (His-Gravi TrapTM, GE Healthcare, Freiburg, Germany) to obtain the soluble proteins. The precipitate was dissolved in solution buffer (20 mM Tris-HCl, 500 mM NaCl,

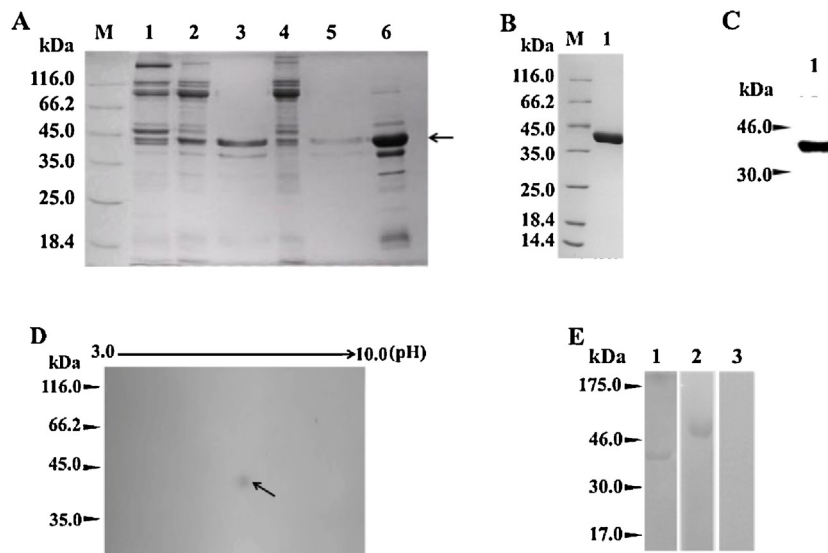


Fig. 1. Purification and characterization of *Scylla paramamosain* AK. (A) SDS-PAGE analysis of ammonium sulfate fractionation. M, protein marker; 1, muscle crude extracts; 2, supernatant after concentration of crude extracts; 3, supernatant following 70% ammonium sulfate; 4, 70% ammonium sulfate pellet; 5, supernatant following 90% ammonium sulfate; 6, 90% ammonium sulfate pellet. (B) SDS-PAGE analysis of the purified AK. M, protein marker; 1, the purified AK. (C) Western-blotting using rabbit anti-mud crab AK polyclonal antibody. (D) Two-dimensional electrophoresis map of the purified AK. (E) PAS staining analysis of glycoprotein. 1, the purified AK; 2, IgG heavy chain (the positive control); 3, BSA (the negative control).

10 mM imidazole, 6 M urea, pH 8.0), then refolded by gradient dialysis from 6 M urea to 0 M urea, change of buffer every 2 h and applied to nickel-affinity chromatography column. (His)₆-tagged proteins were eluted with 100 mM imidazole in lysis buffer. The eluted proteins were detected by SDS-PAGE and Western-blotting (Bauermeister et al., 2011).

2.7. IgE epitope mapping of AK

Overlapping peptides (15-mers, overlapping by 8 amino acids) corresponding to the primary sequence of *S. paramamosain* AK were designed (Lin et al., 2003). Twenty-six peptides covered the flexible sequences and predicted epitopes were commercially synthesized using organic solid phase synthesis technology (GL Biochem, Shanghai, China). Each peptide (2 µg) was spotted onto NC membranes for immunoassay with the sera from the nine crab allergic patients.

2.8. Western blot, dot blot and ELISA analysis

AK was denatured by treating with 2% SDS and 2% 2-Mercaptoethanol, contained in standard SDS-PAGE sample dye, and heated at 100 °C for 15 min. Following SDS-PAGE, the denatured (linear) AK (3 µg per lane) was electrophoretically transferred onto NC membranes for Western blot analysis. In the dot-blotting, 3 µg of the native (folded) AK was directly spotted onto the NC membrane and allowed to dry. The NC membranes were then blocked with 5% nonfat milk, and incubated with sera from nine crab allergic individuals or healthy donors (1/10 dilution). Then the NC membranes were then incubated with HRP-conjugated mouse anti-human IgE antibodies (1/20,000 dilution). The immunoassay was developed by incubation with ECL.

Enzyme-linked immunosorbent assay (ELISA) was performed as previously described (García-Orozco et al., 2007; Ishikawa et al., 1997). Briefly, 200 ng of native AK was immobilized on the microplates (Nunc Maxisorb, Denmark) in carbonate coating buffer (50 mM L⁻¹ Na₂CO₃, 130 mM L⁻¹ NaHCO₃, 3 mM L⁻¹ NaN₃, pH 9.6) and blocked with 5% nonfat milk. After washing 3 times with PBST (Phosphate-Buffered Saline with Tween-20, pH 8.5), sera from the

crab allergic patients or healthy donors were individually added (1/5 dilution in 1% nonfat milk) and incubated overnight at 4 °C. Plates were washed 5 times with PBST and then incubated with HRP-conjugated mouse anti-human IgE antibodies (1/2000 dilution in 1% nonfat milk) for 1.5 h at 37 °C. After washing, the plates were incubated with 100 µL of TMB (3,3',5,5'-tetramethylbenzidine) for 20 min. The reaction was terminated by adding 50 µL of 2 M H₂SO₄ and the optical density was measured at 450 nm (Bio-Rad, Philadelphia, PA, USA).

3. Results

3.1. Purification and characterization of *S. paramamosain* AK

The purification steps were monitored by SDS-PAGE (Fig. 1). Originally, multiple proteins were present in the water-soluble extracts from the muscle of *S. paramamosain*. After ammonium sulfate fractionation, most of the unwanted proteins were removed (Fig. 1A). The target protein was highly purified by ion exchange chromatography (Fig. 1B). The purified protein was then identified as AK by Western-blot analysis with rabbit anti-mud crab AK polyclonal antibody (Fig. 1C). In the 2-DE map of AK, there was a single spot with MW of 40 kDa and pI value of 6.5 (Fig. 1D). In addition, the results of PAS staining to determine if AK is glycosylated revealed a magenta staining band of AK (lane 2) as that of the heavy chain of IgG (the positive control, lane 1), by contrast, no such band was observed for BSA (the negative control, lane 3) (Fig. 1E).

3.2. Cross-reactivity of *S. paramamosain* AK

To investigate whether *S. paramamosain* AK is cross-reactive with related allergens in other shellfish, the pooled sera from crab allergic patients was used to perform the IgE inhibition immunoblotting. IgE reactivity to the protein with MW of 40 kDa in *P. clarkii*, *L. vannamei* and the protein with MW of 38 kDa in *O. fangsiao* was blocked after pre-incubation of the pooled patient sera with the purified AK from *S. paramamosain* (Fig. 2).

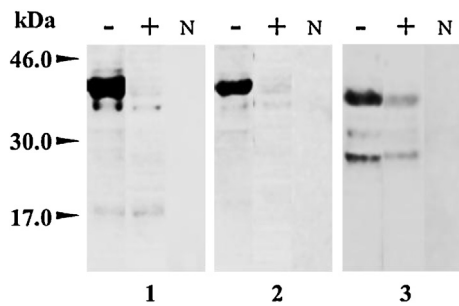


Fig. 2. Competitive inhibition immunoblotting assay. 1–3, nitrocellulose-blotted extracts from *Procambarus clarkii*, *Litopenaeus vannamei*, *Octopus fangsiao* were incubated with the pooled sera of crab allergic patients which were preincubated with *Scylla paramamosain* AK (+) or with buffer only (–). The positions of protein marker are indicated at the left side. N, the pooled sera of healthy donors.

3.3. Bioinformatics and structural analysis of *S. paramamosain* AK

The full-length cDNA of *S. paramamosain* AK was obtained, which was 1457 bp with an open reading frame encoding 357 amino acid residues. The predicted MW was 40.29 kDa and the theoretical pI value was 6.44, which were consistent with the results of 2-DE analysis. The nucleotide sequences were submitted to the GenBank/EBI databases (JN828652). *S. paramamosain* AK belongs to phosphagen kinase super-family and its putative enzymatic active site is located at Cys²⁷¹–Thr²⁷⁷, and an N-glycosylation site at the position of Asn²¹⁵–Thr²¹⁷ (Fig. 3A).

An amino acid sequence comparison between AK and the other 17 invertebrate species was also carried out, which suggested that they are relatively conserved. The phylogenetic tree was then constructed by the Neighbor-joining method, which indicates that *Scylla olivacea* and *Scylla serrata* are evolutionarily highly similar with *S. paramamosain* based on the maximum probability of distances (Fig. 3B).

As the molecular structure of natural *S. paramamosain* AK was not available in the RCSB Protein Data Bank, a homology model was constructed and analyzed. The results exhibited that the molecular model of *S. paramamosain* AK was highly similar to *L. vannamei* AK (PDB: 4AM1, 89% identity) and comparatively similar to *Limulus polyphemus* AK (PDB: 1RL9, 77% identity). There were two globular domains in the structure; one of it is composed of five α -helices and about a 30 amino acid residue N-terminus region. The other domain (C-terminal) included an eight-stranded antiparallel β -sheet flanked by seven α -helices (Fig. 3C). SOPMA analysis showed that there were 43.42% α -helices, 33.89% random coil, 15.69% extended strand and 7% beta turn in its secondary structure (Azzi et al., 2004; Genfa et al., 1998; Lopez-Zavala et al., 2012).

On the basis of the hydrophilicity, surface probability, flexibility and the antigenic index, nine linear epitopes of *S. paramamosain* AK were predicted by the DNA STAR computer program and then located on its molecular model (Fig. 4A). In addition, according to the amino acid composition, spatial neighborhood and surface measure of *S. paramamosain* AK, seven conformational epitopes were predicted by the DiscoTope 2.0 Server, then mapped onto its solvent-exposed surface (Fig. 4B) and highlighted as green sticks (Fig. 4C).

3.4. Expression, partial expression and purification of recombinant proteins

The pET28-derived expression plasmids pET-AK, pET-AK1, pET-AK2, and pET-AK3 were constructed, expressed, and highly purified (Fig. 5A). The full length AK was about 2 kDa larger than the native *S. paramamosain* AK, presumably because it carried six His-tag and



Fig. 3. The cDNA cloning and bioinformatics analysis of AK. (A) Nucleic acid and deduced amino acid sequences of AK from *Scylla paramamosain*. Residue numbers for nucleic acid and amino acid are indicated in the left of each row. The putatively ATP: guanine phosphor-transferase, active site is underlined. The N-glycosylation site is shown in a rectangular box. (B) Phylogenetic tree based on the amino acid sequences of *Scylla paramamosain* AK in comparison with other invertebrate. (C) Cartoon diagram of the molecular model of *Scylla paramamosain* AK structure.

4 restriction enzyme cut sites. The recombinant, full length and native proteins were both recognized by the rabbit anti-mud crab AK polyclonal antibody (Fig. 5B). Both the rAK and the native AK show IgE-binding activity with the pooled sera from the crab allergic patients (Fig. 5C).

In addition, rAK1, rAK2 and rAK3 were also expressed and purified (Fig. 5D). rAK1 and rAK2 have strong IgE-binding activity with the pooled sera from crab allergic patients, while rAK3 has significantly weaker IgE-binding activity (Fig. 5E).

3.5. Linear epitope determination in *S. paramamosain* AK

The synthesized peptides utilized here are shown below (Fig. 6A). Three linear IgE epitopes were recognized by the sera from crab allergic patients. Epitope 1 (127-CGRSMGYFPNPLCT-141) was recognized by the sera of Nos. 9985 and 9815. Epitope 2 (141-TEAQYKEMESKVSST-155) was recognized by the serum of No. 9815. Epitope 3 (211-YHNDNKTFLVWCNEE-225) was recognized by the serum of No. 2170 (Fig. 6B). The locations of the experimental

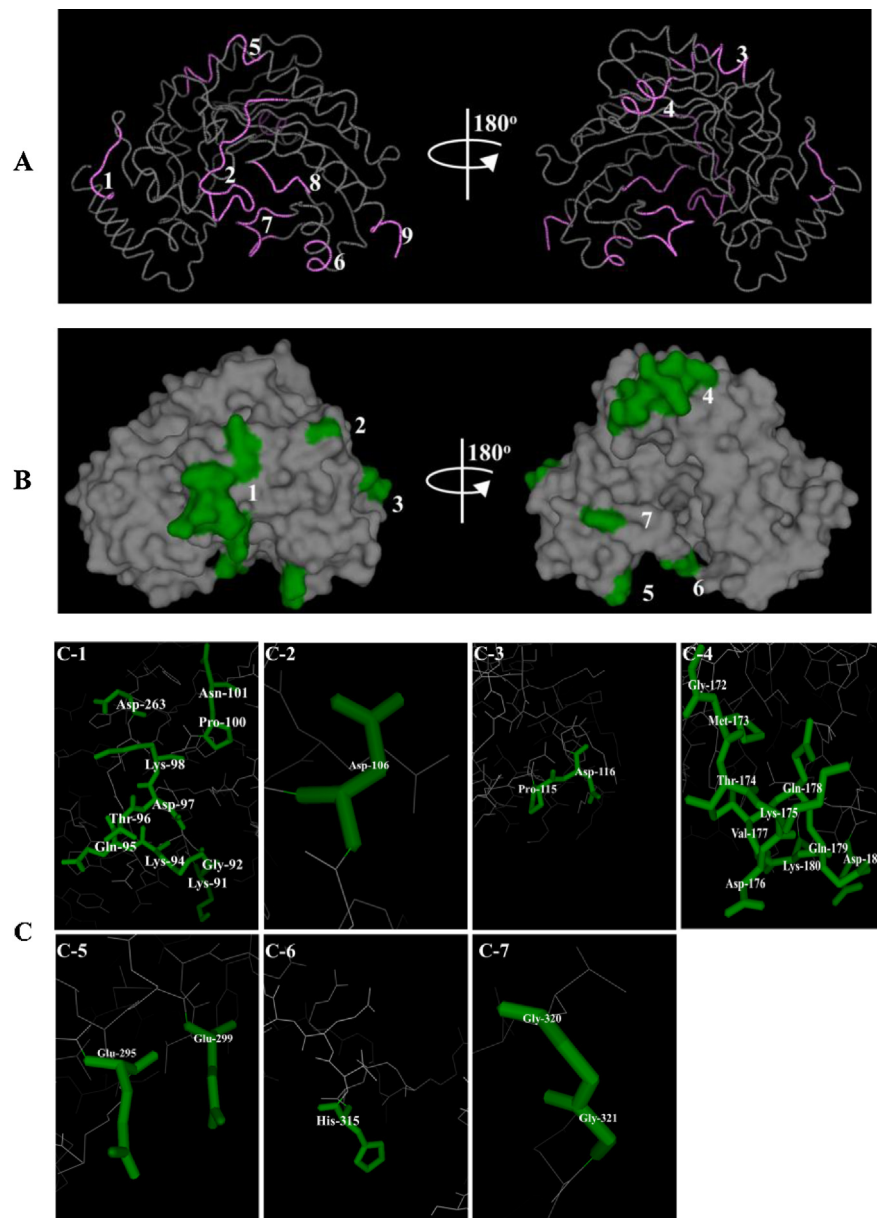


Fig. 4. Computational predicted epitopes of *Scylla paramamosain* AK. (A) The nine predicted linear epitopes, which are displayed in violet on the model of *Scylla paramamosain* AK (ribbon). The epitopes 1–9 are Leu³⁹–Thr⁴⁴, Glu⁸⁷–Asp¹⁰³, Lys¹⁴⁶–Lys¹⁵¹, Thr¹⁷⁴–Leu¹⁸¹, Ile²⁵³–Arg²⁵⁶, Arg²⁹⁴–Glu²⁹⁸, Arg³⁰⁹–Glu³¹⁷, Ile³²⁵–Arg³³⁰, and Ile³⁵²–Gln³⁵⁷, respectively. (B) The seven predicted conformational epitopes modeled onto the solvent-exposed surface of AK structure. (C) The seven predicted conformational epitopes separately shown by green sticks. The epitope numbers correspond to the ones indicated in 4A. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

linear epitopes are mapped onto the surface of protein homology model of *S. paramamosain* AK molecular model (four orientations, Fig. 6C).

3.6. Determination of the predominant epitope type in *S. paramamosain* AK

In order to investigate whether conformational IgE epitopes existed in *S. paramamosain* AK, the comparison of IgE-binding activity of AK between the native folded and the denatured linear forms of the protein.

The results showed that the sera from the nine crab allergic patients, eight (Nos. 4917, 8557, 2452, 5240, 4922, 9527, 9985, and 9815) of them are capable of reacting with the native folded AK as shown in dot-blotting (Fig. 7A), and the AK-specific IgE titers were

2.5 times higher than those in the healthy donor sera shown in ELISA analysis (Fig. 7B). However, only three of the patients (Nos. 9985, 9815, and 2170) reacted with the denatured linear AK in Western blot analysis (Fig. 7C) indicating that the conformational epitopes were recognized by more patient sera than the linear epitopes.

4. Discussion

In this study, AK from *S. paramamosain* was cloned, expressed, purified and characterized. The results indicated that *S. paramamosain* AK was a glycoprotein, with a MW of 40 kDa and pI value of 6.5. The IgE inhibition immunoblotting experiments confirmed that *S. paramamosain* AK is cross-reactive with AKs from other shellfish. This is not unexpected based on our characterization of

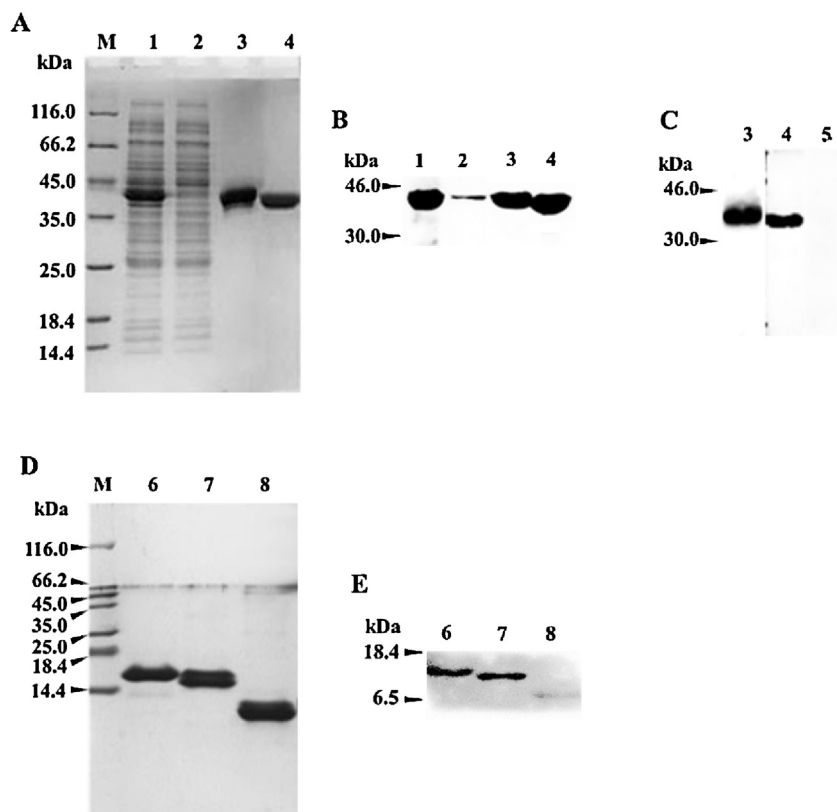


Fig. 5. Purification and identification of the recombinant proteins. (A) SDS-PAGE analysis of the purification of rAK. M, protein marker; 1, the lysate from induced cells; 2, the wash fractions from nickel chelate columns; 3, the purified rAK; 4, the native AK. (B) Western-blot analysis with rabbit anti-mud crab AK polyclonal antibody. 1, the lysate from the induced cells; 2, the wash fractions from nickel chelate column; 3, the purified rAK; 4, the native AK. (C) Western-blot analysis with the pooled sera of crab allergic patients. 3, the purified rAK; 4, the native AK; 5, the expression product of pET-28a in *E. coli* (the negative control). (D) Tricine-SDS-PAGE. M, protein marker; 6, the purified rAK1; 7, the purified rAK2; 8, the purified rAK3. (E) Western-blot analysis with crab allergic patient sera pool. 6, rAK1; 7, rAK2; 8, rAK3.

S. paramamosain AK which was shown to have a relatively high sequence and structural similarity with other invertebrate AKs. Research has shown that pan-allergens are cross-reactive structures involving phylogenetically conserved proteins, such as profilins, lipid transfer proteins, and cyclophilins (Limacher et al., 2007). All the information here indicates that *S. paramamosain* AK exhibits the typical characteristics of a pan-allergen.

The cDNA of *S. paramamosain* AK was successfully cloned and its immunological properties could be studied. Nine linear and seven conformational epitopes of *S. paramamosain* AK were predicted. Generally, many food allergens undergo heat processing and enzymatic digestion before inducing anaphylactic reaction. Thus food allergens are not always in their native conformation, the immune response to linear epitope may be favored (Ayuso et al., 2012). Three of the nine predicted linear epitopes (AA 146–151, 174–181, 325–330) have much the same amino acid residues with the linear IgE epitopes of *L. vannamei* AK (Lit v 2), while the other three (AA 39–44, 87–103, 253–256) were partly overlapped with the linear IgE epitopes of Lit v 2 (Ayuso et al., 2012).

Furthermore, the general distribution of IgE epitopes in *S. paramamosain* AK was determined by analysis of the IgE-binding reactivity of three partial recombinant AKs (rAK1, rAK2 and rAK3). Three partial recombinant AKs was designed according to the distribution of the predicted linear epitopes, rAK1 have 3 predicted linear epitopes (AA 87–103, 146–151, and 174–181), while rAK2 and rAK3 have 2 predicted linear epitopes (rAK2: AA 174–181 and 253–256; rAK3: AA 294–298 and 309–319). The results showed that the epitopes of *S. paramamosain* AK exist in the regions encompassed by rAK1 and rAK2. Five epitopes of Lit v 2 (AA 64–96, 121–141,

142–159, 160–192 and 232–255) were included in the region of rAK1 and rAK2, however, neither one was found in the region of rAK3.

Three linear epitopes of *S. paramamosain* AK were identified by synthetic overlapping peptide technology. As the random coils and turns in the secondary structure of the protein were flexible and available as potential linear epitopes (Berger et al., 1999), the peptides covering the flexible regions, and predicted linear epitopes of *S. paramamosain* AK were synthesized (AA 15–29, 29–43, 36–50, 57–71, 78–92, 85–99, 92–106, 106–120, 127–141, 141–155, 148–162, 155–169, 169–183, 183–197, 197–211, 211–225, 218–232, 255–269, 253–267, 267–281, 281–295, 288–302, 295–309, 303–316, 309–323, 323–337). The linear epitopes identified were mapped onto the protein homology model. Their distribution revealed that epitope-bearing peptides are likely to have a substantial number of surface exposed residues. All of the three linear IgE epitopes were located on rAK1 and rAK2, and the Epitope 2 shared eleven identical amino acids (AA 141–151) with the predicted linear epitope (AA 146–151). This result indicated that the method of predicting epitope by bioinformatics technique had limitations.

Furthermore, three experimental IgE epitopes (AA 127–141, 141–155, 211–225) of *S. paramamosain* AK were individually compared with the epitopes of Lit v 2. Epitope 1 (AA 127–141) was included in the IgE epitope (AA 121–141) of Lit v 2 with two alterations at the positions of 131 and 132 (ME to LQ). Epitope 2 (AA 141–155) corresponded to the IgE epitope (AA 142–159) of Lit v 2 and had two alterations at positions 143 (A to S) and 150 (S to A). These alterations did not seem to affect its binding

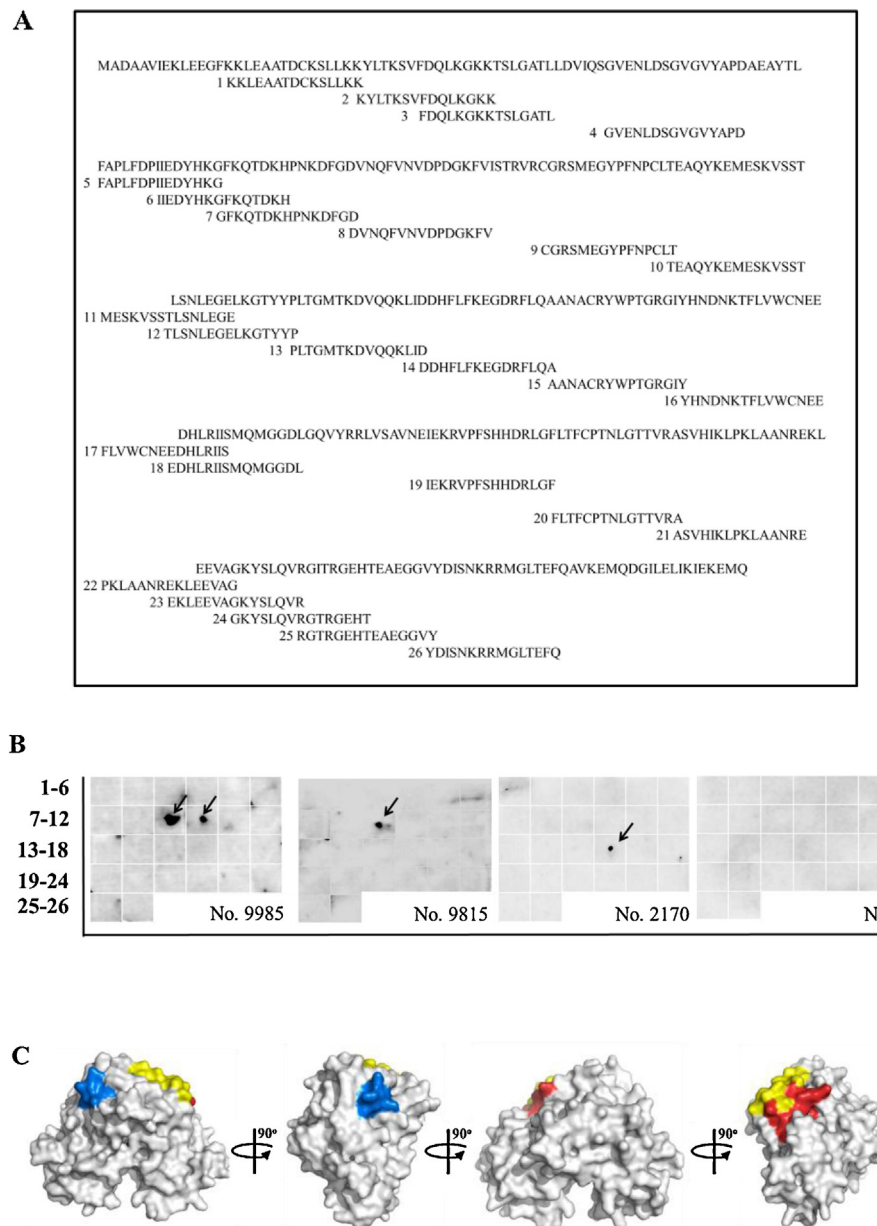


Fig. 6. Linear epitope determination of *Scylla paramamosain* AK. (A) Amino acid of *Scylla paramamosain* AK and 26 synthetic peptides. (B) IgE Dot-blot analysis of the 26 synthetic peptides. Serum 998 reacted with peptides 9 and 10; serum 981 reacted with peptide 9; serum 2170 reacted with peptide 16; N, the pooled sera of healthy donors. (C) Mapping of the linear epitopes on the molecular model of AK. The epitopes are highlighted in yellow, red and blue. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

ability to specific IgE antibodies in the sensitized sera. Epitope 3 (AA 211–225) did not correspond with the IgE epitope of Lit v 2. It can be indicated that the differences stated above are non-conservative amino acid changes. The dot-blotting results also indicate that the different IgE epitopes of *S. paramamosain* AK were present in the sera from different crab allergic patients.

To confirm the predominant epitope type in *S. paramamosain* AK, the allergenicity of the denatured, linear, AK was compared with the native, folded, AK using the serological assay. In dot-blotting, the native AK was immobilized on the NC membranes directly, thus its spatial structure could be maintained. ELISA auxiliary illustrated that the result of dot-blotting were credible. Western-blotting was used to analysis the IgE-binding activity of the denatured (linear) AK. The number of specific sera which reacted with the native AK was significantly higher than that

which reacted with the denatured AK. To some extent, our findings indicate that, in this population of patients, the conformational epitopes of *S. paramamosain* AK were preferentially recognized. These results also explain why the IgE-binding activity of AK was reduced during the processes of thermal treatment (Yu et al., 2013). Many studies have indicated that approximately 90% of epitopes in globular proteins are discontinuous (Kringelum et al., 2012).

The identification of the epitopes and structural features of AK might provide basis information for the development of low allergic products and the immunodiagnostic of food allergy. However, certain regions (AA 1–108, 234–300 and 320–357) of *S. paramamosain* AK have not been studied, the accurate distribution of the linear and conformational IgE epitopes of *S. paramamosain* AK are still incomplete. More sera from crab allergic patients with AK-specific IgE are

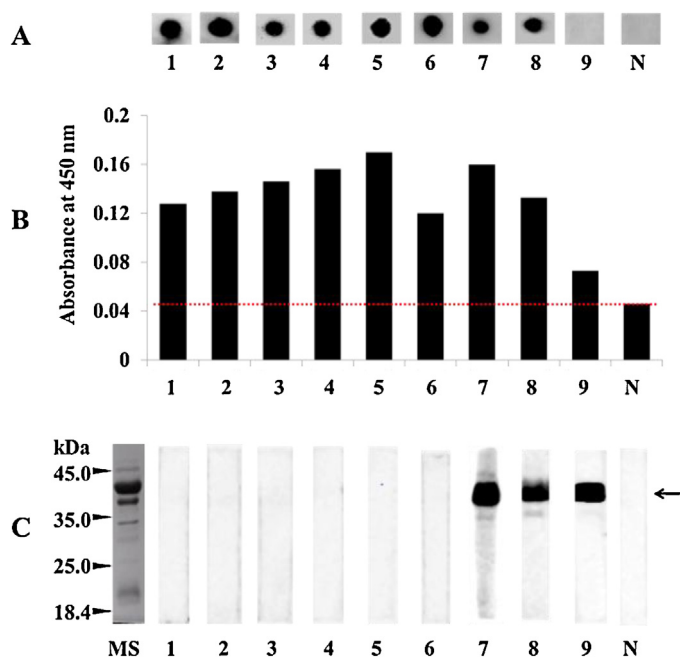


Fig. 7. Comparison of serum IgE-binding to native (folded) versus linear (unfolded) *Scylla paramamosain* AK. (A) Dot-blot and (B) ELISA analysis of the native, folded AK. (C) Western-blotting analysis of the denatured linear AK. Sera from crab allergic patients' number 4917, 8557, 2452, 5240, 4922, 9527, 9985, 9815, and 2170, are indicated as numbers 1–9 respectively. N, represents the pooled sera of healthy donors.

needed to understand the detailed information about IgE epitopes of *S. paramamosain* AK.

Acknowledgements

This work was supported by grants from the National Natural Scientific Foundation of China (31171660), the Natural Scientific Foundation of Fujian Province (2010J06012), the Program for New Century Excellent Talents in Fujian Province University (NCETFJ-2007), the Foundation for Innovative Research Team of Jimei University (2010A005).

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