

Purification, cloning, expression and immunological analysis of *Scylla serrata* arginine kinase, the crab allergen

Yuan Shen, Min-Jie Cao, Qiu-Feng Cai, Wen-Jin Su, Hui-Lin Yu, Wei-Wei Ruan and Guang-Ming Liu*

Abstract

BACKGROUND: Although crustaceans have been reported to be one of the most common causes of IgE-mediated allergic reactions, there are no reports about the characterization and identification of arginine kinase (AK) from the mud crab (*Scylla serrata*) as allergen. In the present study, the purification, molecular cloning, expression and immunological analyses of the IgE allergen AK from the mud crab were investigated.

RESULTS: The results showed that cloned DNA fragments of AK from the mud crab had open reading frames of 1021 bp, predicted to encode proteins with 356 amino acid residues. Sequence alignment revealed that mud crab AK shares high homology with other crustacean species. Mud crab AK gene was further recombined with the vector of pGEX-4T-3 and expressed in *Escherichia coli* BL 21. 2-D electrophoresis suggested that native AK (nAK) and recombinant AK (rAK) shared the same molecular weight of 40 kDa, and the pI is 6.5 and 6.3, respectively. The nAK and rAK were further confirmed by matrix-assisted laser desorption ionization time-of-flight mass spectrometry. Immunoblotting analysis and colloidal gold immunochromatographic assay (GICA) using sera from subjects with crustacean allergy confirmed that the nAK and rAK reacted positively with these sera, indicating AK is a specific allergen of mud crab.

CONCLUSION: Both of purified nAK and rAK reacted positively with sera from subjects with crustacean allergy in immunoblotting and GICA analysis, indicating AK is a common allergen of mud crab. *In vitro* expressed AK is proposed as a source of the protein for immunological or clinical studies.

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Keywords: *Scylla serrata*; arginine kinase; purification; cDNA cloning; expression; characterization; 2-D electrophoresis; MALDI-TOF MS; immunological analysis

INTRODUCTION

Consumption of seafood can produce allergic symptoms in susceptible individuals and crustacean allergies are the most frequently reported causes of allergic reactions.^{1,2} At the same time, crustacean allergies affect seafood workers who are occupationally exposed to the product. Sensitized individuals can develop urticaria, angioedema, asthma, and even life-threatening anaphylaxis.³ In the USA alone, more than 6 million individuals are at risk of having hypersensitive responses upon ingestion of shellfish.⁴ Since seafood trading increased worldwide, immediate hypersensitivity reactions to these products have become an important health issue and, in particular, IgE-mediated food allergies are the main goal in our seafood allergy research.

Arginine kinase (AK) (ATP:L-arginine phosphotransferase, EC 2.7.3.3), similar to creatine kinase (CK) (ATP: creatine N-phosphotransferase, EC 2.7.3.2) in vertebrates, is a phosphagen kinase participating in cell metabolism. It catalyzes the reversible transfer of a phosphoric group from Mg^{2+} ATP to arginine, leading to phosphoarginine and Mg^{2+} ADP, and plays an important role in cellular energy metabolism in invertebrates.⁵ Analysis of molecular information of crustacean AKs has revealed an evolutionary relationship between AK and vertebrate creatine kinase,

which has a similar metabolic role. In recent studies, it has also been demonstrated that AK caused allergic reaction in hypersensitive individuals.⁶ To date, only a few major IgE-binding proteins such as tropomyosin (36 kDa)^{7–9} and parvalbumin (12 kDa)^{10–12} in seafood have been identified as major allergens; more recently, a 40 kDa allergen from the shrimp *Penaeus monodon* was named Pen m 2,¹³ and an IgE allergen AK from the white shrimp *Litopenaeus vannamei* was reported.¹⁴

Among shellfish, crab is assumed to be mainly responsible for severe hypersensitive reactions in China, as there are more than 600 kinds of crab in China, including Chinese mitten crab (*Eriocheir sinensis*), mud crab and swimming crab, etc.¹⁵ Mud crab (*Scylla serrata*), which is widely distributed in China, is an economically important fished and farmed crab species in many areas of Asia.

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Although crustaceans have been reported to be one of the most common causes of IgE-mediated allergic reactions, there are no reports about the characterization and identification of AK from *S. serrata* as allergen. In this study, we report the purification, molecular cloning, expression and immunological analyses of the IgE allergen AK from *S. serrata*, which seems to play an important role as a specific crustacean allergen.

MATERIALS AND METHODS

Sera

The sera of patients were obtained from the Hospital of Jimei University and the Second Hospital of Xiamen. Patients were determined as crab allergic, and were all confirmed as having crustacean allergy on the basis of extensive history, physical examination and objective manifestations observed after crustacean product ingestion. Written informed consent was obtained from each patient. All patient sera containing AK-specific IgE antibodies were characterized using sandwich enzyme-linked immunosorbent assay (ELISA). Sera from non-allergic individuals were used as negative controls.

Crab

Mud crabs (*Scylla serrata*) were purchased live from the fresh market of Jimei, Xiamen, China. The muscle of crabs was obtained for immediate use or stored at -80°C .

Chemicals

HiTrapQ Sepharose was purchased from Bio-rad (San Francisco, CA, USA). Protein marker for sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) was from Fermentas, UAB (Vilnius, Lithuania). Pre-stained protein marker for western blot was from New England Biolabs (Richmond, VA, USA). Glutathione S-transferase (GST) affinity column was a product of Pharmacia Biotech (Uppsala, Sweden). Horseradish peroxidase (HRP)-conjugated goat anti-human IgE antibody was from Kirkegaard and Perry Laboratory (Gaithersburg, MD, USA). Rabbit anti-crab (*Scylla serrata*) AK polyclonal antibody was prepared in our own laboratory. Rabbit anti-shrimp (*Litopenaeus vannamei*) AK polyclonal antibody was kindly provided by Dr Cui-Luan Yao,¹⁶ Fisheries College, Jimei University (Xiamen, China). HRP-conjugated goat anti-rabbit IgG secondary antibody was from DAKO (Glostrup, Denmark). 3,3'-Diaminobenzidine tetrahydrochloride (DAB) was from Pierce (Rockford, IL, USA). All other chemicals used were of the highest reagent grade from commercial sources.

Purification of native AK

AK was purified according to Yu *et al.*¹³ and arginine kinase activity was assayed as in previous procedures.¹⁷ All procedures were conducted at $0-4^{\circ}\text{C}$ unless otherwise stated. Crab muscles were minced and homogenized with buffer A (0.1 mol L^{-1} Tris-HCl, 10 mmol L^{-1} 2-mercaptoethanol (2-ME), 1 mmol L^{-1} ethylenediaminetetraacetic acid (EDTA), $5\text{ }\mu\text{mol L}^{-1}$ Na_3P , and $25\text{ }\mu\text{mol L}^{-1}$ phenylmethanesulfonyl fluoride (PMSF), pH 8.0). The mixture was then centrifuged at $12\,000 \times g$ for 20 min; ammonium sulfate was added to the supernatant to 70% saturation and allowed to stand for 1 h. After centrifugation, the resultant supernatant was adjusted to 90% saturation with ammonium sulfate, then the precipitate was collected by centrifugation, dissolved in buffer B (10 mmol L^{-1} Tris-HCl, 10 mmol L^{-1} 2-ME, and 0.1 mmol L^{-1} EDTA, pH 8.0), and dialyzed against the same buffer.

After centrifugation at $12\,000 \times g$ for 20 min, the clear supernatant was then applied to a HiTrapQ Sepharose Fast Flow column pre-equilibrated with buffer B. Fractions with AK activity were eluted with a linear gradient from 0 to 1 mol L^{-1} NaCl in buffer B. Protein concentration of crude extracts was estimated by measuring by the Lowry method¹⁸ using bovine serum albumin as the standard.

Isolation of RNA, cDNA cloning, and sequencing

Isolation of RNA, cDNA cloning, and sequencing procedures were performed according to Liang *et al.*¹⁵ Total RNA was extracted from fresh muscle with Trizol reagent (Invitrogen, Carlsbad, CA, USA) following the manufacturer's protocol. Single-stranded cDNA was synthesized with reverse transcriptase (Invitrogen) and an oligo(dT)¹⁵ primer, according to the manufacturer's instructions and this cDNA was used as a template for reverse transcription polymerase chain reaction (RT-PCR). The AK cDNA was amplified using the RT-PCR system with one pair of primers (AK-F, AK-R). The primer sequences are listed as follows:

AK-F(57–74): 5'-CGGGATCC-ATGGCTGACGCTGCTGTT-3'
AK-R(1092–1110): 5'-GCGTCGAC-TTACATCTCCTCTCCATC-3'

PCR was performed with one cycle of denaturation at 94°C for 4 min; 35 cycles at 94°C for 40 s, annealing at 51°C for 45 s, and extension at 72°C for 1.5 min; finally terminal extension for 7 min at 72°C . All the PCR products were agarose gel purified, cloned, and sequenced by Invitrogen Corp. (Shanghai, China). The sequence homology and the deduced amino acid sequence comparisons were carried out using BLAST at NCBI (<http://www.ncbi.nlm.nih.gov/blast>).

In vitro expression and purification of GST-AK

In vitro expression and purification of GST-AK were performed according to Yao *et al.*¹⁶ The amplified full-length open reading frame, with *Bam* HI and *Sal* I sites of the AK gene, was purified with the DNA Purification System from Promega (Madison, WI, USA) and digested with *Bam* HI/*Sal* I, then subcloned into a prokaryotic expression vector, pGEX-4T-3 (GE Healthcare, Piscataway, NJ, USA), named pGEX-4T-3-AK. The recombinant plasmid was transformed into *E. coli* BL 21 for expression of the AK protein. Positive colonies were identified by PCR. A fresh, isolated colony was chosen and incubated overnight in liquid Luria-Bertani (LB) medium at 37°C . The overnight culture was diluted 1:10 in the same LB medium and grown at 37°C until A_{600} reached 0.6–0.8 absorbance units, at which point expression was induced by addition of 0.1 mmol L^{-1} isopropylthiogalactopyranoside (IPTG). After 4 h induction, bacterial cells were harvested by centrifuging at $4000 \times g$ for 5 min.

The pellet was resuspended with phosphate-buffered saline (PBS) (140 mmol L^{-1} NaCl, 2.70 mmol L^{-1} KCl, 1.8 mmol L^{-1} KH_2PO_4 , 10 mmol L^{-1} Na_2HPO_4). All subsequent procedures were carried out at 4°C . The supernatant was collected by centrifuging the lysate at $10\,000 \times g$ for 20 min and loaded into a GST Sepharose 4B affinity column previously equilibrated with PBS buffer according to the instructions of the manufacturer. The column was washed with elution buffer (50 mmol L^{-1} Tris-HCl, 10 mmol L^{-1} reduced glutathione, pH 8.0).

Fractions with the target protein were pooled and dialyzed against PBS containing 4 mol L^{-1} guanidine-HCl for 4 h at 4°C . Then, half the volume of the buffer was replaced by PBS without guanidine-HCl every 2 h, and the dialysis was continued overnight. The fusion protein was digested with thrombin (Sigma, Santa Clara,

CA, USA). Protein concentration of crude extracts was estimated by measuring with the Lowry method¹⁸ using bovine serum albumin as the standard.

The purified recombinant protein was quantified and characterized by SDS-PAGE and 2D-PAGE. IgG reactivity of the fusion protein was also detected by reacting with rabbit polyclonal antibody. Then the protein was stored at -18°C for further use.

SDS-PAGE and immunoblotting

nAK and GST-AK were analyzed by SDS-PAGE. For electrophoresis, samples (protein concentration 2.25 mg mL^{-1}) were separated by 12% SDS-PAGE as described by Laemmli,¹⁹ and stained with Coomassie Brilliant Blue R-250 (CBB). For immunoblot analyses, western blot was carried out as described by Towbin *et al.*²⁰ Briefly, proteins on polyacrylamide gel were electrophoretically transferred onto a nitrocellulose membrane, which was then blocked with 5% skimmed milk for 1 h and incubated with rabbit polyclonal antibody (1 : 200 000 dilution, overnight). Bound IgG were detected using HRP-conjugated goat anti-rabbit IgG (1 : 2000 dilution). Immunoassay was carried out using DAB as substrate for the development of color.

2-D electrophoresis

2-D electrophoresis was carried out according to the method of Chen *et al.*²¹ Isoelectric focusing (IEF) was performed on 7 cm Ready Strip IPG strips at pH 3–10. Purified native and recombinant AK (protein concentration 1.0 mg mL^{-1}) were resuspended in IEF buffer containing 2 mol L^{-1} thiourea, 7 mol L^{-1} urea, 2% (w/v) Chaps, 0.2% (v/v) pH 3–10 ampholites, 1 mol L^{-1} dithiothreitol (DTT), and bromophenol blue. IEF was performed in an Ettan IPGphor apparatus according to the instructions of GE Healthcare (San Francisco, USA) at 20°C as follows: the strips were rehydrated in IEF buffer at 50 V for 13 h, 30 min at 300 V, followed by 30 min at 1000 V; the voltage was then followed by 60 min at 5000 V, until 6000 Vh was reached, and thereafter the voltage was decreased to 500 V and held at that voltage for 3 h. After IEF, the strips were equilibrated with 50 mmol L^{-1} Tris-HCl (pH 8.8) containing 6 mol L^{-1} urea, 30% (v/v) glycerol, 2% (w/v) SDS, and 2% (w/v) DTT for 10 min, followed by another 10 min using the same buffer with 2.5% iodoacetamide instead of DTT.

2-D electrophoresis was performed on a MiniVECOMPLETE of Hoefer Inc. (San Francisco, USA). according to the method of Laemmli¹⁹ using 12% gels. After electrophoresis, the gels were stained with CBB.

Peptide analysis by matrix-assisted laser desorption ionization mass spectrometry (MALDI MS)

The target spot was sent to BGI LifeTech Co., Ltd (Beijing, China) and analyzed by MALDI MS. Mass spectrometric conditions were: laser source N_2 ; wavelength 337 nm; acceleration voltage 25 kV; substrate was 4-cyano-4-hydroxy cinnamic acid (a-CHCA); the positive ion mode was adopted and the data were obtained automatically. The peptide mass fingerprint data were compared with those in the NCBI database and analyzed by Mascot software according to the method of Bao *et al.*²²

IgE immunoassay with dot blot

Dot blot was performed applying $0.5\text{ }\mu\text{g}$ purified AK on nitrocellulose (NC) membranes, which were then blocked with 5% nonfat milk in Tris-HCl-buffered saline (TBS, 20 mmol L^{-1} Tris-HCl, pH 7.5,

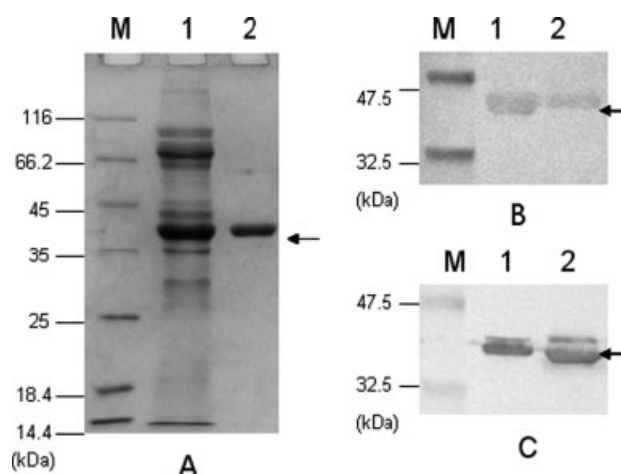


Figure 1. SDS-PAGE and immunoblotting of AK from mud crab muscle. (A) SDS-PAGE. M, protein marker; lane 1, crude extract of mud crab; lane 2, purified AK. (B) Immunoblotting using rabbit anti-shrimp (*Litopenaeus vannamei*) AK polyclonal antibody. M, pre-stained protein marker; lane 1, crude extract of mud crab; lane 2, purified AK. (C) Immunoblotting using rabbit anti-crab (*Scylla serrata*) AK polyclonal antibody. M, pre-stained protein marker; lane 1, crude extract of mud crab; lane 2, purified AK.

containing 0.145 mol L^{-1} NaCl) for 1 h, then the membranes were washed with TBST (TBS, 0.05% Tween-20). The NC membranes were incubated with a 1 : 5 dilution of allergic sera overnight at 4°C , and peroxidase-conjugated goat anti-human IgE antibody (1 : 2000 dilution) was then allowed to react with the membrane as second antibody at room temperature for 1 h, followed by extensive washing and detection by DAB.

Colloidal gold immunochromatographic assay (GICA)

GICA was performed on a prepared test kit. After assembling sample and adsorbent pads on laminated cards having an NC membrane, the cards were cut into strips. The lateral-flow-based dipstick kit was developed using an NC membrane on which a purified AK was bound to the test line and staphylococcal protein A (SPA) was bound to the control line as quality control.

The whole testing procedure was as follows. Test sera were first mixed with gold-labeled AK and applied to one end of the strip, after which the labeled immune complexes formed, driven by capillary forces, and migrated through the NC membrane to the line of immobilized capturing antigen (AK). Development of the reaction took 1–5 min, depending on the antibody (sera) concentration.

RESULTS

Protein extraction and purification

In the present study, AK was purified to homogeneity by 70–90% ammonium sulfate fractionation and column chromatography of HiTrap Q Sepharose. Fractions were washed using a linear gradient of 0–1 mol L^{-1} NaCl in buffer B. AK with enzymatic activity was finally washed out in fractions 7–14. Protein components of the crude extract as myosinogen from mud crab before HiTrap Q Sepharose were analyzed by SDS-PAGE and the results are shown in Fig. 1(A). After ammonium sulfate fractionation, the molecular masses of the remaining proteins were around 30, 40, and 80 kDa (Fig. 1(A), lane 1). After HiTrap Q Sepharose, there was one band on SDS-PAGE, and the molecular mass of the purified protein

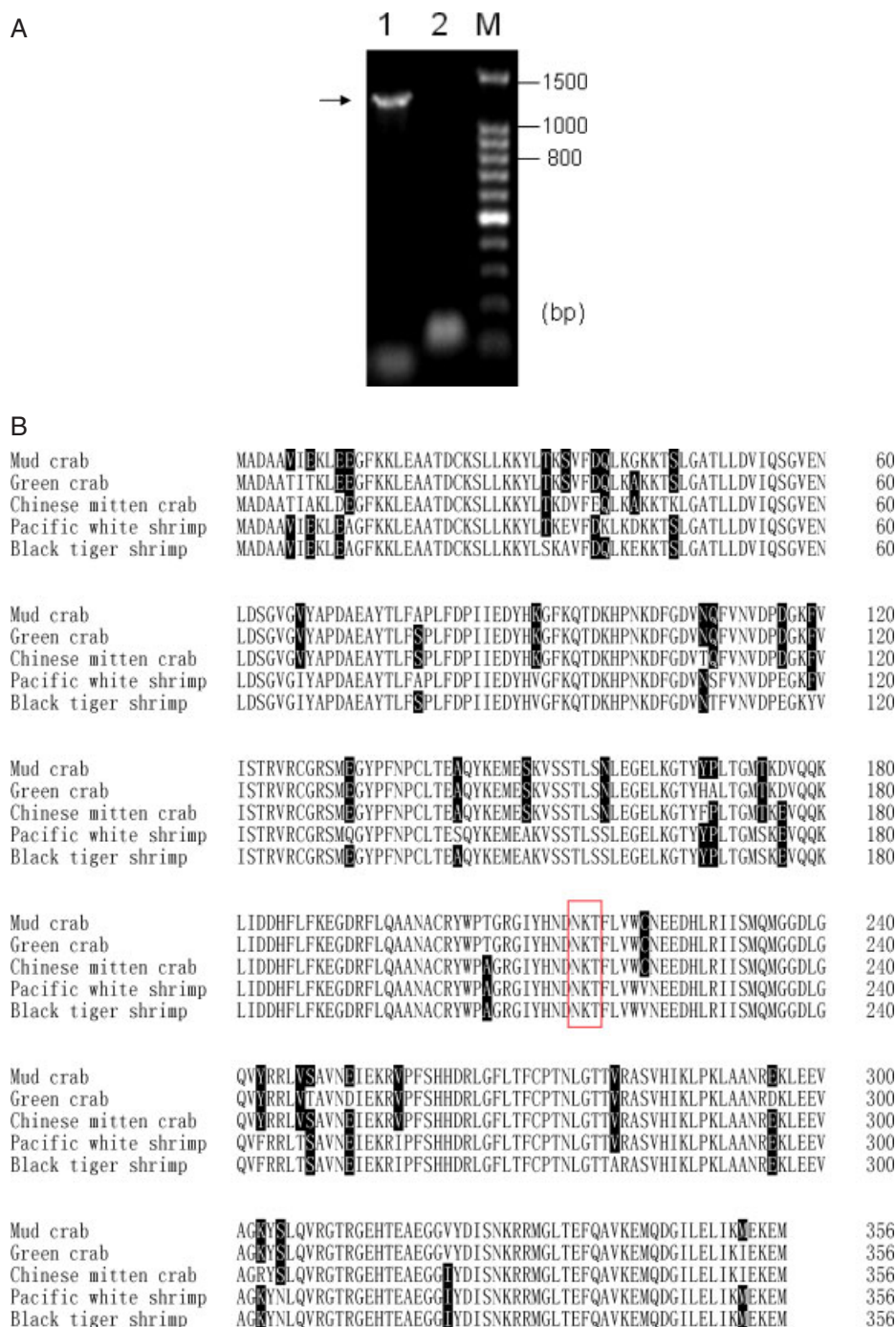


Figure 2. PCR amplification of AK gene from mud crab (*Scylla serrata*) and amino acid sequence alignment of species of AK. (A) PCR amplification of AK gene from mud crab. M, DNA marker; lane 1, PCR amplification products from mud crab; lane 2, negative control. (B) Amino acid sequence alignment of species of AK. The listed species' names and their corresponding GenBank accession numbers are as follows: green crab (*Carcinus maenas*), AAD48470; mud crab (*Scylla serrata*), GQ851626; Chinese mitten crab (*Eriocheir sinensis*), AAF43437; Pacific white shrimp (*Litopenaeus vannamei*), ABI98020; black tiger shrimp (*Penaeus monodon*), ACT34086; *Trypanosoma brucei*, AAF23164. amino acid sequence residues of No. 215–218 was one N-glycosylation site (window rectangle).

was approximately 40 kDa (Fig. 1(A), lane 2). Kinase activity and relative yields in the various purification steps are summarized as follows. About 30.4 mg of 40 kDa protein was purified from the muscle of mud crab, the final purification being ~5.24-fold, with a specific activity of 498 U mg⁻¹ (1 unit of AK activity is defined as the amount of enzyme that catalyzes the formation of

1 mol L⁻¹ min⁻¹ of NADH (reduced form of nicotinamide adenine dinucleotide) under the assay conditions).

The purified 40 kDa native protein was confirmed by immunoblotting with rabbit anti-shrimp (*Litopenaeus vannamei*) AK polyclonal antibody (Fig. 1(B)) and rabbit anti-crab (*Scylla serrata*) AK polyclonal antibody (Fig. 1(C)). From the western blot results

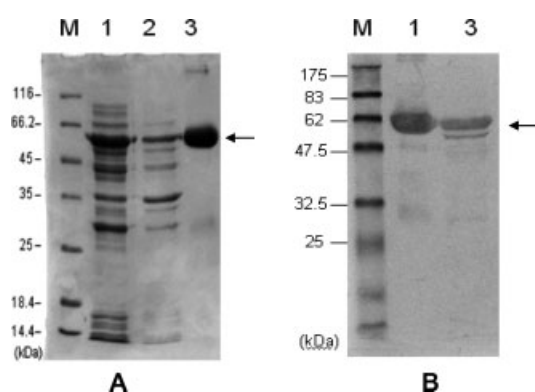


Figure 3. SDS-PAGE and immunoblotting of GST-AK of mud crab. (A) SDS-PAGE. M, protein marker; lane 1, crude extract from BL 21 GST induced with IPTG; lane 2, crude extract from recombinant BL 21 GST-AK before induction with IPTG; lane 3, purified GST-AK. (B) Immunoblotting using rabbit anti-crab (*Scylla serrata*) AK polyclonal antibody. M, pre-stained protein marker; lane 1, purified GST-AK; lane 3, crude GST-AK.

that both polyclonal antibodies positively reacted with 40 kDa protein, we could deduce that the purified 40 kDa protein obtained with antigenic activity is probably arginine kinase from mud crab.

Cloning and sequencing of Mud crab AK

The cDNA fragment of 1071 bp (open reading frame of mud crab AK gene) was finally amplified by PCR with primers of AK-F (57–74) and AK-R (1092–1110) (Fig. 2(A)). The cDNA sequence of AK from *Scylla serrata* was deposited in GenBank under accession number GQ: 851 626. The deduced amino acid sequences of AK from mud crab and other species are shown in Fig. 2(B). An open reading frame of AK encoded 356 amino acid residues, which contained one N-glycosylation site (No. 215–218, window rectangle).

Database sequence homology searches of the predicted AK amino acid sequence were performed using the BLASTP program. Multiple sequence alignment showed that the deduced amino acid sequence of AK from *Scylla serrata* shared high identity with those from other crustaceans such as *Carcinus maenas*, *Eriocheir sinensis*, *Litopenaeus vannamei*, and *Penaeus monodon* (the score was 97%, 95%, 94%, and 94%).

Expression of recombinant AK in *E. coli*

The AK of mud crab was recombined with the expression vector pGEX-4T-3, and expressed in *E. coli* BL 21 after IPTG induction. Then the collection of recombinant bacteria and the ultrasonic extraction of GST-AK fusion protein were performed. Finally the soluble recombinant protein was purified by GST Sepharose 4B affinity chromatography. Figure 3(A) shows the SDS-PAGE result of the GST-AK fusion protein. As shown in lane 3, the expressed GST-tagged fusion protein exhibited a major single band of molecular mass around 65 kDa (25 kDa for GST and 40 kDa for AK), suggesting that the molecular mass of the expressed target protein is in accordance with its native form.

The identity of the crude (lane 3) and purified (lane 1) expressed fusion AK was further confirmed by immunoblotting with rabbit anti-crab (*Scylla serrata*) AK polyclonal antibody (Fig. 3(B)). From the western blot results that 65 kDa protein reacted with polyclonal antibody positively, we could deduce that the purified 65 kDa protein obtained with antigenic activity is probably recombinant arginine kinase.

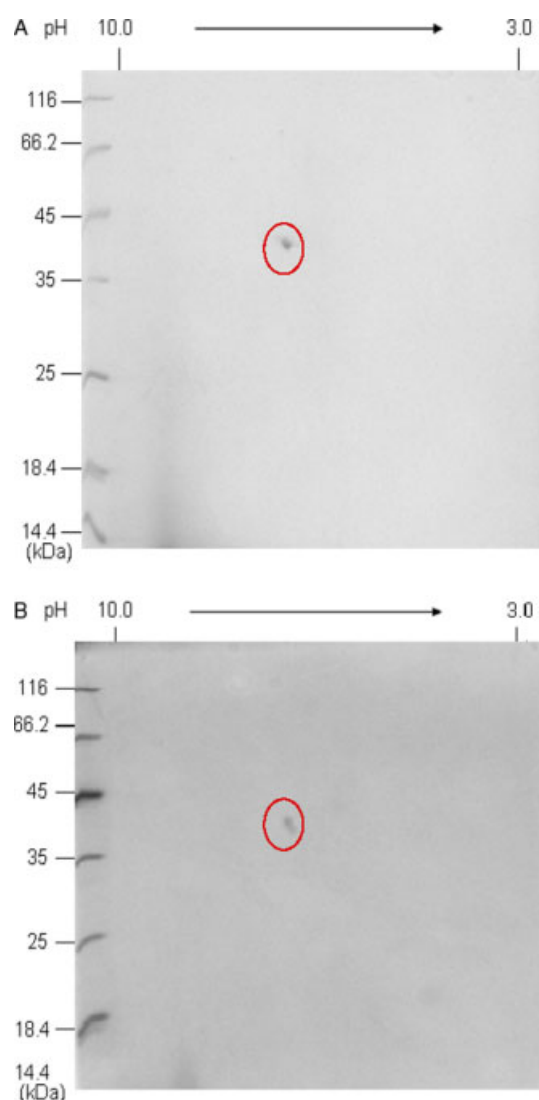


Figure 4. Two-dimensional gel electrophoresis of purified AK. (A) The molecular mass of native AK is 40 kDa and pI is 6.5. (B) The molecular mass of recombinant AK is 40 kDa and pI is 6.3. The gel was stained with CBB.

2-D PAGE of purified AK

The purified 40 kDa native protein was further confirmed by 2-D PAGE, which produced a single spot (Fig. 4(A)); the estimated molecular masses of approximately 40 kDa and pI value of approximately 6.5, which is in accordance with other related research,¹³ provided further evidence of the validity of AK.

To make clear the pI value of recombinant AK, the rAK digested by thrombin was further confirmed by 2-D PAGE, which produced a single spot; the estimated molecular masses were approximately 40 kDa, and pI values were approximately 6.3 (Fig. 4(B)), which showed little difference from the native form. This may be due to a different type of protein folding, or the recombinant protein might have been affected by the protease used to release the GST protein.

Peptide analysis by matrix-assisted laser desorption/ionization (MALDI) MS

To identify the spot, it was excised and digested in-gel with trypsin. The resulting peptide mixtures were analyzed by MALDI-TOF MS.

As shown in Fig. 5(A), the mass spectral profile of the peptides of the spot – the 40 kDa protein with a pI of 6.5 – showed multiple peaks ranging from 750 to 2750 Da. The signal at 1718.8 Da, as the prominent peak, was compared with data in the NCBI database using the Mascot search tool, and the Mowse value was observed. As observed from the result, 41 proteins scored greater than 75 and all of them were AK. Figure 5(C) represents the relation between number of hits and probability-based Mowse score. The probability that the observed random match was 5% was the significance threshold. The hatched area represents scores below the significant threshold; protein scores greater than 75 are significant ($P < 0.05$). Figure 5(D) lists some information on the top seven peptides, including score, accession number, molecular

mass and queries matched with these peptides. The protein with the highest score was found to be AK from *Scylla olivacea*, which had a score of 138, and 17 queries matched the peptide. Based on the results, we infer that the target protein was AK of mud crab (*Scylla serrata*).

To identify the fusion protein, the spot after SDS-PAGE was also excised, digested in-gel with trypsin and then analyzed by MALDI-TOF MS. Parameters of database searching were the same as that for the native protein except that number of queries was 63. As a result, the mass spectral profile of the peptides of the spot – the 65 kDa protein – showed multiple peaks ranging from 750 to 2750 Da. The signals at approximately 1660.8 Da and 2360.2 Da as the prominent peaks were compared with those in the NCBI

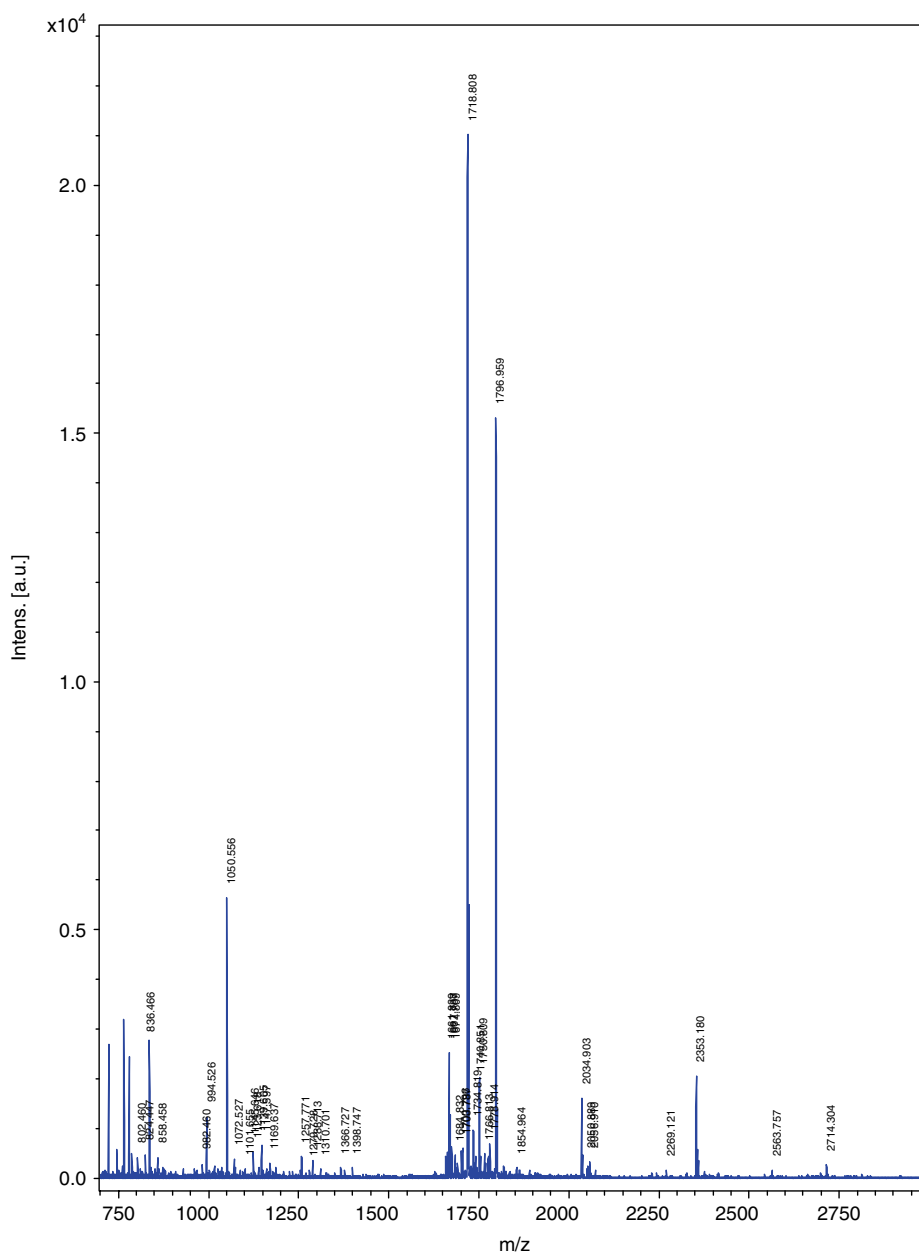


Figure 5. Mass spectrometric results of native and expressed AK. (A) PMF of mud crab (*Scylla serrata*) native AK. (B) PMF of fusion protein. (C) Number of hits versus probability-based Mowse score (nAK). Protein score is $-10 \times \log(P)$, where P is the probability that the observed match is a random event. Protein scores greater than 75 are significant ($P < 0.05$). (D) Summary report of the top seven peptides (nAK). (E) Summary report of the protein which had the highest score (GST-AK).

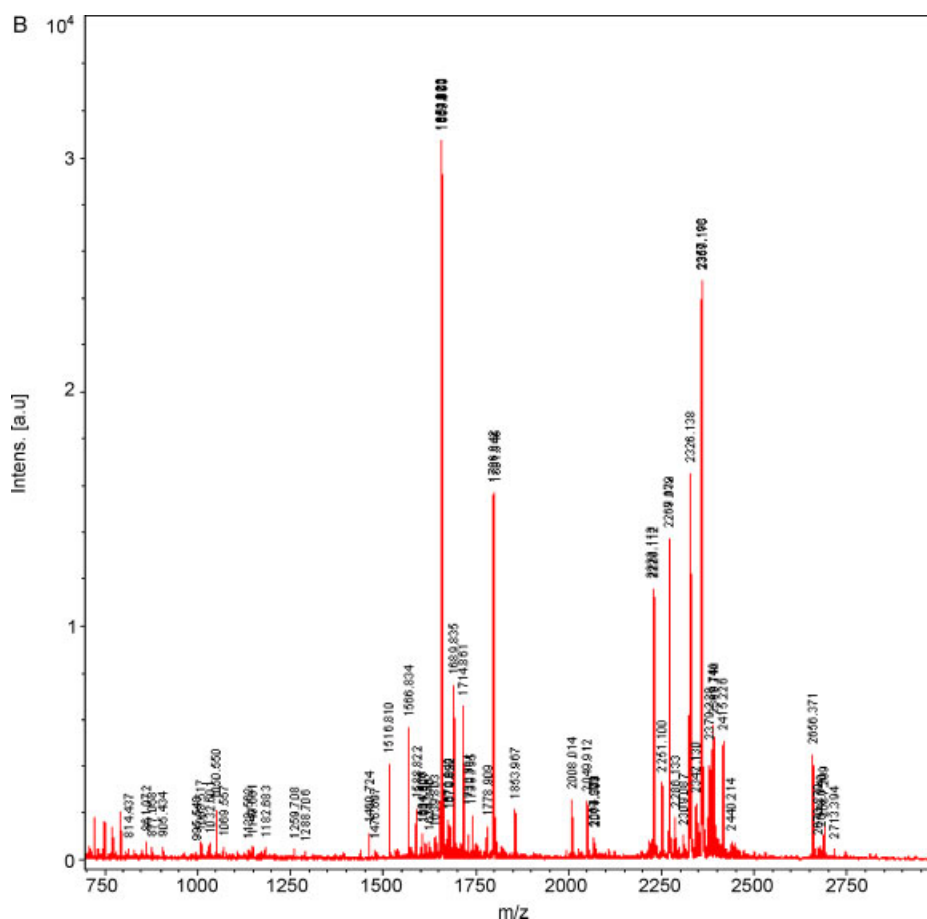


Figure 5. (Continued).

database using the Mascot search tool (Fig. 5(B)) and the Mowse values were observed. The protein with the highest score (171) was found to be a mixture composed of chain A, orthorhombic glutathione *S*-transferase of *Schistosoma japonicum* and AK from *Fenneropenaeus merguensis* (Fig. 5(E)). Consequently, we infer that the fusion protein of AK of mud crab (*Scylla serrata*) was expressed successfully.

Dot blot immunoassay with IgE

To identify the IgE reactivity of the native and recombinant fusion protein, nAK and GAT-AK were used as antigens and investigated by dot blot immunoassay with sera samples from four crustacean-allergic patients. As shown in Fig. 6(Aa), dots 1–4 (native protein), all four sera showed a positive result. No IgE-binding protein was detected with normal negative control sera (dot 5). On the other hand, in Fig. 6(Ab), dots 1–4 (expressed protein), which used the same sera, showed a different result: dots 2 and 3 could be identified as reacting with the crustacean-allergic patients' sera strongly, but dots 1 and 4 showed an uncertain result. The weaker reaction of expressed protein may be due to the presence of GST, which affects the recombinant protein in the structural epitopes.

GICA result

Diluted human sera from subjects with crustacean allergy were used as samples, then added to the sample well and the response was recorded using a digital camera after 3 min of sample addition. As shown in Figs 6(Ba, b), the control line and test line of these

two strips both showed a strong red color and we can predicate that both give a positive result, which was attributed to strong dative bonds between the gold and the protein molecules. On the other hand, the two strips were observed with not much difference in color intensity, and revealed nAK and GST-AK with no significant difference in immunity character (Fig. 6(B)). In Fig. 6(Bc), when the normal negative sera were added to the sample well, only the control line showed a red color, suggesting that there was no reaction between negative sera and AK.

DISCUSSION

Consumption of seafood can produce allergic symptoms in susceptible individuals and crustacean allergies are one of the most frequently reported causes of allergic reactions. To date, only a few kinds of allergens, such as tropomyosin¹⁵ and parvalbumins,^{10,11} have been identified in seafood as a major IgE-reactive component, and AK in the shrimp as an allergen has been identified by a few researchers,^{13,14,23} but only few reports on the identification of crab AK as another kind of allergen. This is the first report of allergenicity of AK identified from *Scylla serrata*.

In this study, nAK from mud crab was purified to homogeneity and then checked by SDS-PAGE and 2-D PAGE. The single band of 40 kDa protein suggested the procedures of ammonium sulfate fractionation and HiTrap Q Sepharose were sufficient and effective

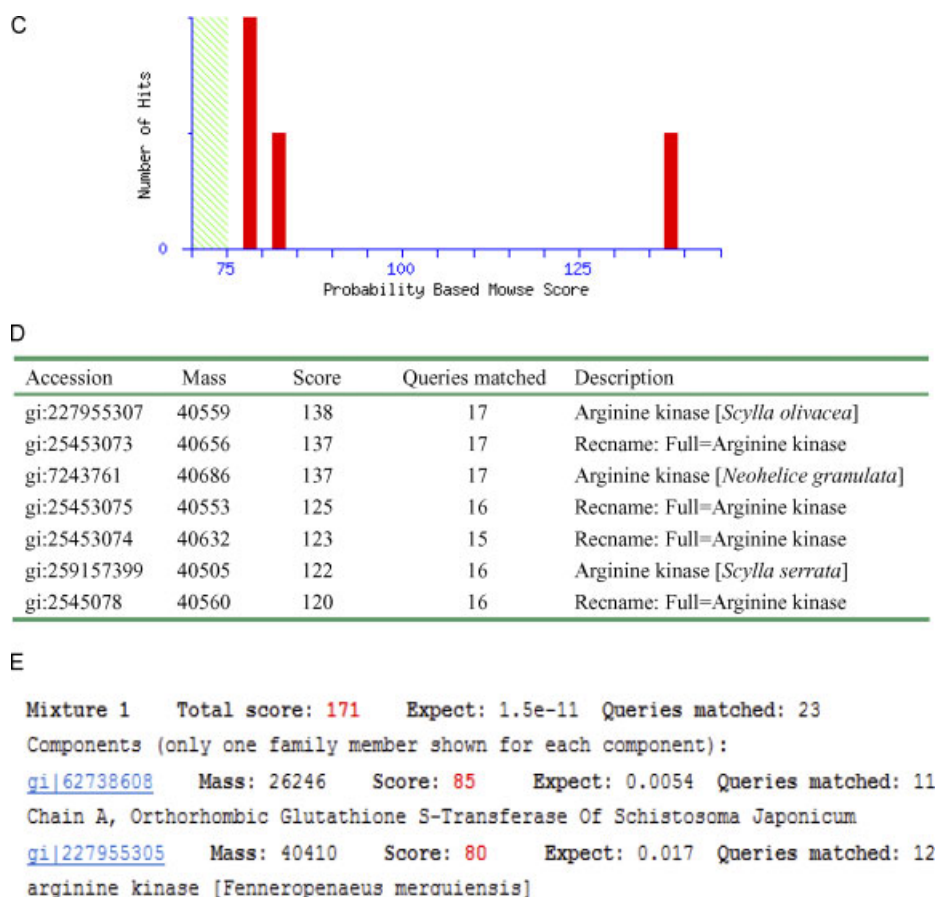


Figure 5. (Continued).

enough for the purification of nAK. Western blot with rabbit anti-shrimp AK antibody confirmed that the purified protein was AK. In addition, the estimated molecular masses and *pI* value of the protein on 2-D PAGE provided evidence for AK but not tropomyosin, with similar molecular masses.

AK gene from mud crab was amplified and sequenced, and the results showed that AK genes are 1071 bp in size, encoding 356 amino acid residues. Nucleotide sequence analysis revealed that the comparison of *S. serrata* AK amino acid sequence with other crustacean AKs resulted in 97% similarity with *Carcinus maenas* AK and 94% similarity with *Penaeus monodon* AK and *Litopenaeus vannamei* AK. The high homology of these AK and this high sequence identity established the molecular basis for immunological cross-reactivity among crustacean AK; the cross-reaction of AK from mud crab and Pacific white shrimp (Fig. 1(c, d) provides evidence of this.

The AK of mud crab was recombined with the vector pGEX-4T-3 and expressed in *E. coli* BL 21. The fusion protein was soluble and of about 65 kDa in size on SDS-PAGE, which was identical to the predicted molecular mass of GST-AK. Also we have confirmed rAK by four methods: molecular mass in SDS-PAGE, *pI* in 2-D PAGE, immunoblotting and mass spectrometry, and comparison with literature reports.^{13,14}

IgE binding of nAK and GST-AK was recognized with sera from crustacean-allergic individuals by immunoblotting, providing evidence of the protein as an allergen. The proposed dipstick GICA assay seems to be a fast and reliable field test for preliminary qualitative screening of sera from humans allergic to AK. The

human samples can be analyzed without pre-treatment and provide a result in 5 min. For further research, we may study the developed assay for semi-quantitative screening of allergic samples with higher sensitivity and accuracy.

CONCLUSIONS

The purification, cloning, expression and immunological analysis of arginine kinase of mud crab (*Scylla serrata*) was studied. SDS-PAGE, 2-D PAGE and MALDI-TOF MS confirmed the purified 40 kDa native and expressed protein as AK; the *pI* of the two were 6.5 and 6.3, respectively. This recombinant protein was compared with native arginine kinase and characterized as an IgE allergen. Both purified arginine kinases reacted positively with sera from subjects with crustacean allergy in immunoblotting and GICA analysis, indicating that AK is a common allergen of mud crab. *In vitro* expressed arginine kinase is proposed as a source of the protein for immunological or clinical studies.

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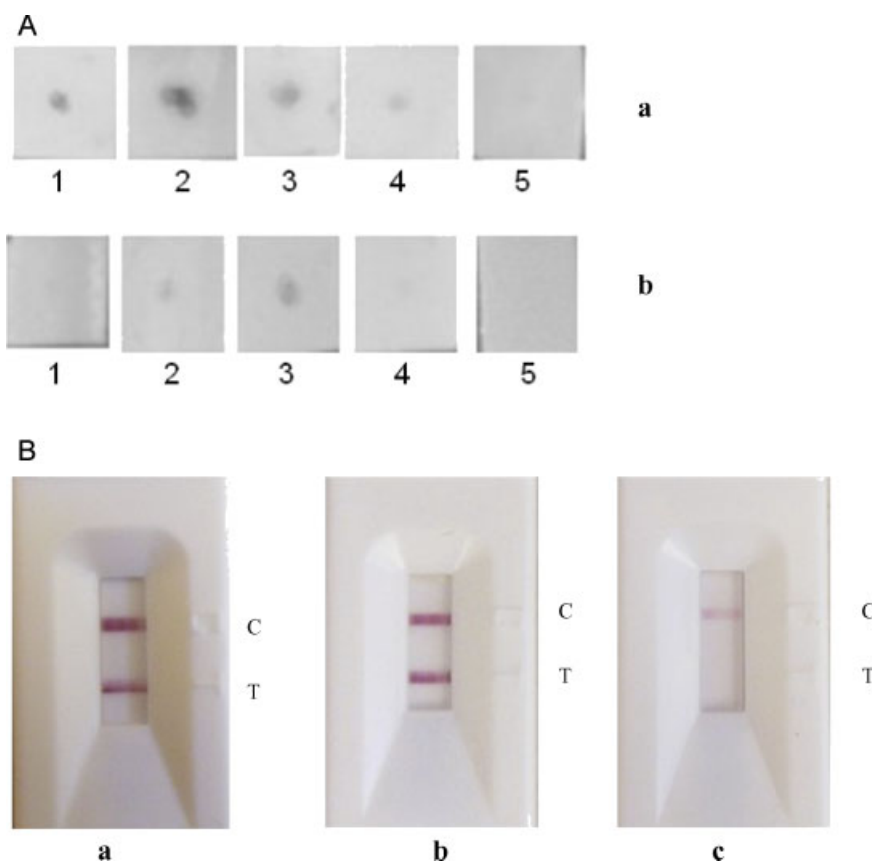


Figure 6. Reactivity of IgE in patients' sera with purified native and recombinant AK of mud crab by dot blot (A) and GICA assay (B). (A) Dot-blot assay: (a) native AK; dots 1–4, sera from subjects with crustacean allergy; dot 5, normal negative control sera; (b) expressed AK; dots 1–4, the same sera from subjects with crustacean allergy; dot 5, normal negative control sera. (B) GICA assay: (a) sera from allergic individual, SPA coated on the C line, nAK coated on the T line; (b) sera from allergic individual, SPA coated on the C line, GST-AK coated on the T line; (c) sera from non-allergic individual as negative control, SPA coated on the C line, GST-AK coated on the T line.

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