JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Article

pubs.acs.org/JAFC

Purification, Cloning, and Immunological Characterization of Arginine Kinase, a Novel Allergen of *Octopus fangsiao*

Hai-Wang Shen, Min-Jie Cao, Qiu-Feng Cai, Mi-Mi Ruan, Hai-Yan Mao, Wen-Jin Su, and Guang-Ming Liu*

College of Biological Engineering, The Key Laboratory of Science and Technology for Aquaculture and Food Safety, Jimei University, 43 Yindou Road, Xiamen, Fujian 361021, China

ABSTRACT: Arginine kinase (AK) is an important enzyme participating in energy metabolism in invertebrates, but, to date, there have been no reports that AK from octopus is an allergen. In this study, octopus AK was purified, and its molecular biological, immunological, and physicochemical characterizations were analyzed. The results showed that octopus AK was purified and confirmed by mass spectrometry for the first time, and its molecular mass was 38 kDa. The full-length gene sequence of octopus AK encompassed 1209 bp and was predicted to encode a protein with 348 amino acid residues. The homology of octopus AK and crustacean AK was about 54%, but the similarity between their three-dimensional structures was high. Octopus AK could react with mouse anti-shrimp AK and rabbit anti-crab AK polyclonal antibody singly. Octopus AK could also react with specific IgE of the sera from octopus-allergic patients effectively, whereas crab AK could inhibit the reaction between them. Finally, the IgE-binding activity of octopus AK could be reduced in the processes of thermal or acid−alkali treatment. In summary, AK was identified as a novel allergen in octopus, which had a sensitizing ability similar to that of crustacean AK. This is significant in allergy diagnosis and the treatment of octopus-allergic disorders.

KEYWORDS: *arginine kinase, allergen, octopus, purification, cDNA cloning*

INTRODUCTION

It has long been recognized that ingestion of seafood and working in fisheries can produce allergic symptoms in susceptible individuals, and seafood is one of the most frequently reported causes of allergic reactions.[1−3] Sensitized individuals can develop urticaria, angioedema, laryngospasm, asthma, and even life-threatening anaphylaxis.[4] With the consumption of seafood increasing worldwide, immediate hypersensitivity reactions to seafood have become an important issue. In the past decade, a number of allergens that stimulate IgE production and cause IgE-mediated disease have been identified. Parvalbumin, a 12 kDa protein, is the major allergen in fish.[5,6] Tropomyosin (TM), a thermally stable myofibrillar protein composed of two identical subunits, is the major allergen in molluscs and crustaceans.[7−10] In recent years, paramyosin in the disk abalone[11] and myosin light chain[12] and sarcoplasmic calcium-binding protein[13] in shrimps have been considered to be novel allergenic proteins. In addition, it was reported that arginine kinase (AK) is also an important allergen in crustaceans such as shrimps and crabs. However, whether AK is an allergen in other invertebrates has not been established.

AK (ATP:L-arginine phosphotransferase, EC 2.7.3.3) in invertebrates, similar to creatine kinase (ATP:creatine *N*-phosphotransferase, EC 2.7.3.2) in vertebrates, is a phosphagen kinase that participates 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.[14] AK has been investigated as an allergen mainly in crustaceans. Earlier, Binder et al. found that AK from the terricolous arthropod *Plodia interpunctella* could react with serum IgE from moth-allergic patients and is an invertebrate pan-allergen.[15] For aquatic crustaceans, Yu et al. purified, cloned, and expressed the novel allergen Pen m 2 with a molecular mass of 40 kDa from *Penaeus monodon*.[16] Krina et al. were the first to prove that AK is the allergen from the Pacific white shrimp species and found that AK has a 96% identity to Pen m 2.[17] Recently, Shen et al. in our laboratory studied and analyzed crab AK and showed that AK is an allergen of mud crab.[18] Thus far, there have been few studies investigating whether mollusc AK is an allergen or not.

As an important species among molluscs, the octopus has a high nutritional as well as economic value and was the subject of our research. In this study, octopus AK was purified for the first time, and its physicochemical, molecular biological, and immunological characteristics were analyzed.

MATERIALS AND METHODS

Octopus. Octopuses (*Octopus fangsiao*) were purchased live at Jimei market, Xiamen. They were immediately used or stored by freezing at −80 °C.

Chemicals. Q-Sepharose and Sephacryl S-200 were purchased from Amersham Bioscieces (Uppsala, Sweden). Protein standards for SDS-PAGE were from Fermentas (Lithuania) or New England BioLabs (Beverly, MA). Phenylmethanesulfonyl fluoride (PMSF) was purchased from Sigma-Aldrich (St. Louis, MO). Ethylenediaminetetraacetic acid (EDTA) was a product of Sinopharm (Shanghai, China). 2-Mercaptoethanol (2-Me) was from Biotech (Shanghai, China). Mouse anti-shrimp AK polyclonal antibodies, rabbit anti-crab AK polyclonal antibodies, and rabbit anti-octopus AK polyclonal antibodies were prepared in our laboratory. Horseradish peroxidase

Received: September 17, 2011
Revised: February 5, 2012
Accepted: February 6, 2012
Published: February 6, 2012

dx.doi.org/10.1021/jf203779w | J. Agric. Food Chem. 2012, 60, 2190−2199

(HRP)-conjugated rabbit anti-mouse IgG antibody and goat anti-rabbit IgG antibody were purchased from DAKO (Glostrup, Denmark). Peroxidase-conjugated goat anti-human IgE antibody was from Kirkegaard and Perry Laboratories (Gaithersburg, MD). The enhanced chemiluminescent (ECL) substrate for immunoblotting was from Pierce (Rockford, IL). 3,3′,5,5′-Tetramethylbenzidine (TMB) was purchased from Tiangen (Beijing, China). Other reagents were all of analytical grade.

Human Sera. Sera from patients were contributed by the Hospital of Jimei University. Twelve serum samples were contributed from octopus-allergic patients, and four sera contained specific IgE of octopus AK (the numbers of the four allergic patients' sera were 05979998, 04734483, 04366639, and 05981953). The number of the serum sample from a nonallergic individual used as a negative control was 05990077. Written informed consent was obtained from each patient or individual. All sera were stored at −80 °C until used.

Purification of AK. AK from octopus (*Octopus fangsiao*) was purified according to a modification of a previously described protocol.[16] All procedures were conducted at 0−4 °C. In brief, octopus muscles were minced and homogenized with 4 bed volumes of buffer A (0.1 M Tris-HCl, 10 mM 2-Me, 1 mM EDTA, 5 μM NaN_3, 25 μM PMSF, pH 8.0). The mixture was centrifuged at 12000*g* for 20 min; ammonium sulfate was added to the supernatant (myosinogen) to 70% saturation. After centrifugation at 15000*g* for 20 min, the resultant supernatant was adjusted to 90% saturation with ammonium sulfate and then centrifuged, and the precipitate was collected, dissolved in a small amount of buffer B (10 mM Tris-HCl, 10 mM 2-Me, 0.1 mM EDTA, pH 8.0), and dialyzed against the same buffer. The dialyzed solution was subsequently applied to a Q-Sepharose column (⌀ 1.2 cm × 5 cm) pre-equilibrated with buffer B. Bound proteins were eluted at 0.8 mL/min with a 0−0.2 M linear NaCl gradient in the buffer, and fractions of 3.2 mL per tube were collected. The fractions with AK were concentrated and then applied to a Sephacryl S-200 gel column (⌀ 1.0 cm × 100 cm) pre-equilibrated with buffer B with a flow rate of 0.4 mL/min. The fractions with purified AK were collected and identified.

Sodium Dodecyl Sulfate−Polyacrylamide Gel Electrophoresis (SDS-PAGE). SDS-PAGE was performed under reducing conditions according to the method of Laemmli.[19] Samples were separated in 12% polyacrylamide gels with 5% stacking gels. All gels were stained for protein with Coomassie brilliant blue R-250.

Peptide Analysis by Matrix-Assisted Laser Desorption Ionization Mass Spectrometry (MALDI MS). The target protein spot cut from the gel was sent to Shanghai Applied Protein Technology Co. Ltd. (Shanghai, China) and analyzed by MALDI MS. Mass spectrometric conditions were as follows: laser source, ND; wavelength, 355 nm; accelerating voltage, 2 kV; substrate, α-cyano-4-hydroxycinnamic acid (α-CHCA); the positive ion mode was adopted, and the data were obtained automatically. The peptide mass fingerprint was compared with sequences of octopus AK in the NCBI database and analyzed by DNAman software.

RNA Preparation and cDNA Synthesis. According to the method of Guo et al.,[20] total RNA was prepared from the muscle of octopus *O. fangsiao* using Tripure isolation reagent (Roche, USA). Single-stranded cDNA was synthesized with reverse transcriptase (Tiangen) and an oligo$(dT)_{15}$ primer according to the manufacturer's instructions.

Reverse Transcription Polymerase Chain Reaction (RT-PCR). On the basis of the DNA sequences of octopus AK (AB042331, arginine kinase, *Octopus vulgaris*)[21] in the NCBI database, two degenerate oligonucleotide primers, AKS and AKA, were designed as sense primers for PCR and are shown in Table 1. Using these primers and the cDNA synthesized, a fragment of the AK gene of approximately 300 bp was amplified by PCR in a thermal cycler, GeneAmp 9700 (Applied Biosystems, USA). The PCR program was performed as follows: 5 min at 94 °C followed by 30 cycles of 30 s at 94 °C, 45 s at 45 °C, 90 s at 72 °C, and a final extension of 10 min at 72 °C. The PCR product was purified by agarose gel electrophoresis and cloned into pGEM-T Easy vector (Promega) followed by DNA sequence analysis.

Table 1. Degenerate and Specific Primers Used in the Experiment

name	sequence (5′−3′)
AKS	AGGGTGGTAACTTGGGTGAAGTC
AKA	TTTCATTGACACCTCTCATCATTTC
AK5F1	TTCACCATGGATACCTCTGGCCTG
AK5F2	AGAAAGCTTAGGGATCTTAATATGCACA
AK3R1	AGGCCAGAGGTATCCATGGTGAAC
AK3R2	TATCCATGGTGAACATACAGAAAGTG

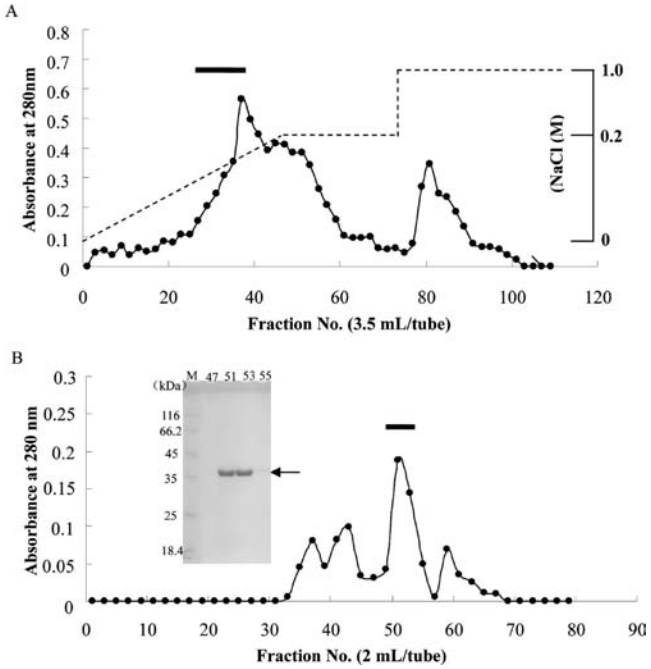


Figure 1. Chromatographic purification of octopus AK: (A) Q-Sepharose chromatography; (B) Sephacryl S-200 gel chromatography. The numbers on the tops of the lanes correspond to the fraction number. Target protein fractions under the bar were pooled.

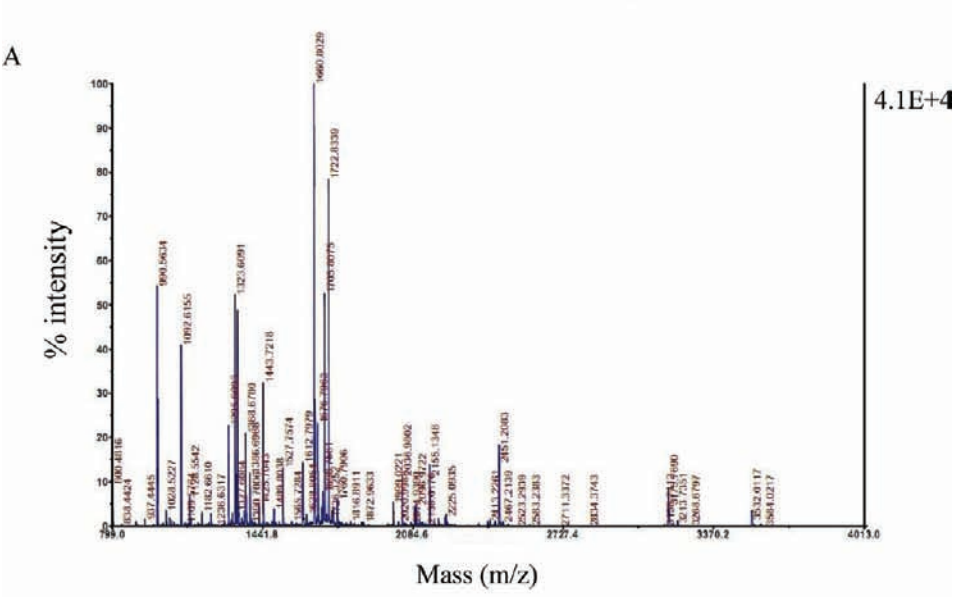
5′- and 3′-Rapid Amplification of cDNA Ends (5′- and 3′-RACE). On the basis of the sequence information from the RT-PCR product, gene-specific primers for 5′-RACE (AK5F1 and AK5F2) and 3′-RACE (AK3R1 and AK3R2) were designed and are shown in Table 1. RACE and RACE-PCR were conducted with the SMART RACE cDNA Amplification Kit and Advantage 2 PCR Kit (Clontech, USA). Touchdown PCR was adopted to improve the specificity of SMARTer RACE amplification. The program for 5′-RACE was performed as follows: 5 min at 94 °C followed by 30 cycles of 30 s at 94 °C, 45 s at 52 °C, 90 s at 72 °C, and a final extension of 10 min at 72 °C. The program for 3′-RACE was performed as follows: 5 min at 94 °C followed by 30 cycles of 30 s at 94 °C, 45 s at 50 °C, 60 s at 72 °C, and a final extension of 7 min at 72 °C. The PCR products, 5′-RACE (850 bp) and 3′-RACE (300 bp), were purified, cloned, and sequenced.

DNA sequencing was performed at Invitrogen Biotechnological Co. Ltd. (Guangzhou, China) using the DNA sequencer ABI Prism 3730 (CA). The sequencing was performed three times, and the sequences were confirmed. The full-length gene sequence of AK (1209 bp) was obtained by overlapping the RT-PCR fragment (300 bp), the 3′-RACE fragment (300 bp), and the 5′-RACE fragment (850 bp). The amino acid sequence of AK was deduced from the full-length gene sequence by DNAman software.

The nucleotide sequence data are available in the GenBank database under accession number JN127374 for the *O. fangsiao* AK cDNA.

Three-Dimensional Structure Analysis of AK. Information on the simulated three-dimensional structure of AK could be obtained when amino acid sequences of AK were sent to the Web site

A



B

Start-End	Calc. Mass	Obsrv. Mass	± da	Peptide Sequense
36-47	1338.6835	1338.7725	0.0890	TKFGGTLADCIR
87-116	3175.6018	3175.7683	0.1665	VQHPVPDFGDVNNLNIGDLDPSGSLIVSTR
122-133	1330.6791	1330.7236	0.0445	SHDSFGFPPVLK
172-185	1722.7719	1722.8340	0.0621	QLTDDHFLFNDSDR
209-227	2435.1846	2435.2258	0.0412	TFLCWVNEEDHLRLISMQK
301-319	1998.9517	1999.0178	0.0661	GIHGEHTESVGGVYDISNK
301-320	2155.0527	2155.1343	0.0816	GIHGEHTESVGGVYDISNKR
321-335	1816.8390	1816.8878	0.0488	RMGLTEYEAVTEMMR
322-335	1660.7379	1660.8042	0.0663	MGLTEYEAVTEMMR
322-335	1676.7329	1676.7943	0.0614	MGLTEYEAVTEMMR
322-335	1692.7278	1692.7955	0.0677	MGLTEYEAVTEMMR
336-342	800.4625	800.4818	0.0193	GVNEIIR

C

```
Purified protein                                          TKFGGTLADCIR
Octopus vulgaris  MAEELFKELQEAKECHSLLKKHLTKERVDKLKTLKTKFGGTLADCIRSGCKNPDSGVGIY  60

Purified protein                                  VQHPVPDFGDVNNLNIGDLDPSGSLIVSTR
Octopus vulgaris  ACDPDAYTVFADVLDAVIMDYHKIDKVQHPVPDFGDVNNLNIGDLDPSGSLIVSTRVRVG  120

Purified protein   SHDSFGFPPVLK                                        QLTDDHFLF
Octopus vulgaris  RSHDSFGFPPVLKKEDRVKMEQVSVEALKSLTGELAGNYYPLSTMTPDVQKQLTDDHFLF  180

Purified protein  NDSDR                          TFLCWVNEEDHLRLISMQ
Octopus vulgaris  NDSDRFLKAANGYDDWPTGRGIYFSANKTFLCWVNEEDHLRLISMQKGGNLGEVYRRLVE  240

Octopus vulgaris  AIHQMEKKLKFAKKDNMGYLTFCPSNLGTTMRASVHIKIPKLSQRADFKTICDKYHLQAR  300

Purified protein  GIHGEHTESVGGVYDISNKRRMGLTEYEAVTEMMRGVNEIIR
Octopus vulgaris  GIHGEHTESVGGVYDISNKRRMGLTEYEAVTEMMRGVNEIIREENNS              347
```

Figure 2. Mass spectrometric results for purified AK: (A) peptide mass fingerprinting (PMF) of purified protein from octopus; (B) effective peptides from MS compared with data in the NCBInr; (C) protein sequence alignment of AK with that from *Octopus vulgaris*.

http://www.cbs.dtu.dk/services/CPHmodels/. The three-dimensional structure of AK was analyzed using pyMOL software.

Immunoblotting, Dot Blotting, and IgE Inhibition Immunoblotting Assay. Immunoblotting was carried out as described by Towbin et al. with minor modification.[22] All procedures were conducted at 37 °C. Briefly, proteins on polyacrylamide gels were electrophoretically transferred onto nitrocellulose (NC) membranes. Then the NC membranes were blocked with 5% skim milk. After washing with TBST (20 mM Tris-HCl, 150 mM NaCl, 0.05% Tween-20, pH 8.0), the membranes were incubated with mouse anti-shrimp AK polyclonal antibodies (1:200 dilution), rabbit anti-crab AK polyclonal antibodies (1:2000 dilution), or rabbit anti-octopus AK polyclonal antibodies (1:20000 dilution) for 2 h and washed with TBST. After incubation with horseradish peroxidase (HRP)-labeled goat anti-mouse IgG or goat anti-rabbit IgG antibodies (1:2000 dilution), the membranes were washed extensively with TBST. Finally, the immunoassay was carried out using enhanced chemiluminescence (ECL).

The purified AK treated at different temperatures and different pH values was directly blotted on NC membrane instead of electrophoretically

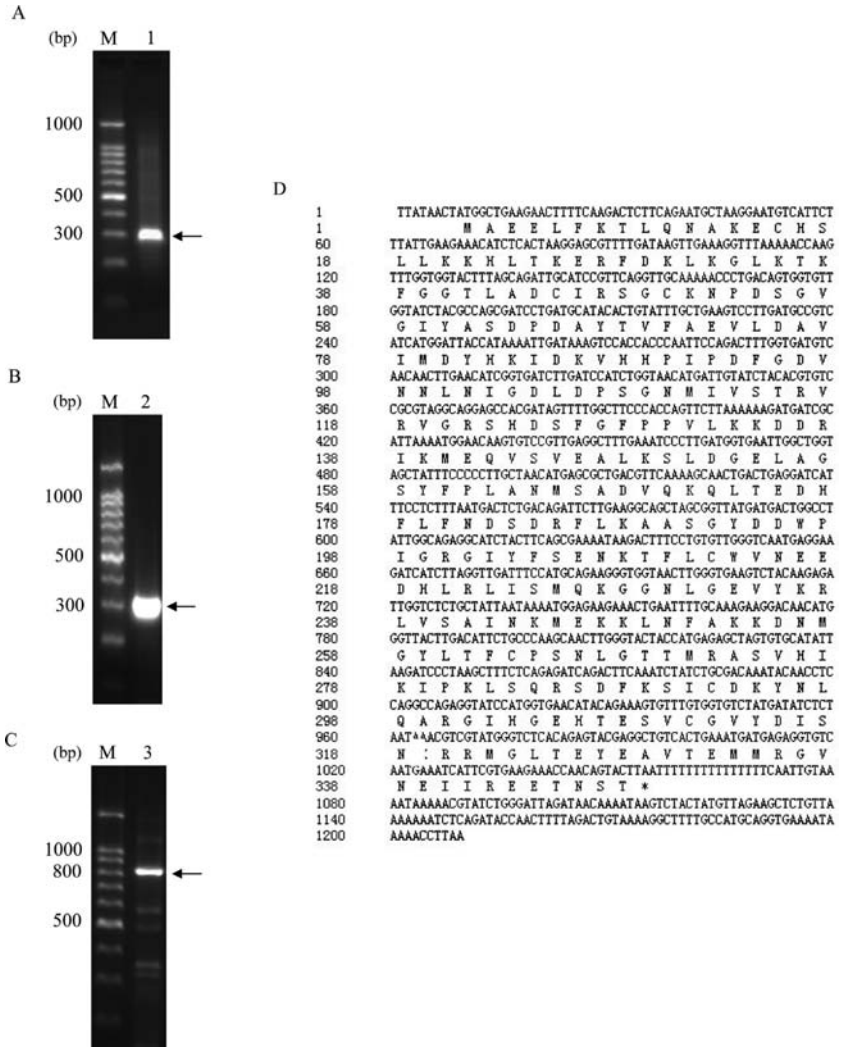


Figure 3. Amplification products and nucleotide and deduced amino acid sequences of octopus AK: (A) amplification product of RT-PCR; (B) amplification product of 3′-RACE; (C) amplification product of 5′-RACE (target amplification indicated by the arrows); (D) nucleotide and deduced amino acid sequences of octopus AK (residue numbers for both nucleotides and amino acids are indicated on the left).

transferred from the gel and incubated with mixed positive sera (1:7 dilution with 1% skim milk) for 4 h after being blocked with 5% skim milk for 1 h. After incubation with HRP-labeled goat anti-human IgE for 1.5 h, the immunoassay was carried out using ECL. The other procedures were identical with immunoblotting.

IgE inhibition immunoblotting assay was performed as described by Marina et al.[15] Briefly, myosinogen on polyacrylamide gels was electrophoretically transferred onto NC membranes. Then NC membranes were cut into strips and blocked with 5% skim milk. In the IgE inhibition immunoblotting assay, each of the positive sera (1:7 dilution) was divided into two parts, one part was preincubated with buffer for 1.5 h, and the another part was preincubated with purified octopus AK or crab AK (the final concentration was 100 μg/mL) for 1.5 h. Then preincubated sera were incubated with NC membranes for 4 h. After incubation with HRP-labeled goat anti-human IgE secondary antibodies (1:2000 dilution), the membranes were washed extensively with TBST. Finally, the immunoassay was also carried out using ECL.

Inhibition Enzyme-Linked Immunosorbent Assay (Inhibition ELISA). Inhibition ELISA was carried out as described by Yu et al. with minor modification.[23] Briefly, 96-well polystyrene ELISA plates (Nunc Maxisorb; Denmark) were coated with AK (200 ng well^{-1}) at 37 °C for 2 h using a coating buffer (0.16% Na_2CO_3 and 0.29% NaOH, w/w, diluted in distilled water, pH 9.5). After five washings with TBST, coated plates were blocked with 200 μL of 5% skim milk at 37 °C for 2 h. In another set of microtiter plates, 30 μL samples of human sera (diluted 1:5 with 1% skim milk) were mixed with the same volume of purified AK as inhibitor (diluted 1:10000, 1:1000, 1:100, 1:10, and 1:1 with 1% skim milk; the protein contents were 0.3, 3, 30, 300, and 3000 ng, respectively). After incubation at 37 °C for 2 h, 50 μL of the mixture (serum-AK) was transferred to the ELISA plates coated with AK and incubated at 37 °C for 2 h. The plates were washed five times again, and 100 μL of the HRP-conjugated goat anti-human IgE antibodies (diluted 1:2000 with 1% skim milk) was added. After incubation for 2 h, the plates were washed five times with TBST, and the bound horseradish peroxidase activity was determined by reaction with 100 μL of TMB at 37 °C for 20 min and terminated with 50 μL of 2 M H_2SO_4. The absorbance at 450 nm was determined on an automated ELISA plate reader (Benchmark 96; Bio-Rad Laboratories, Hercules, CA). The loss of specific IgE-binding activity of the patients' sera resulting from the treatment with human sera and purified AK as inhibitor was found by calculating the inhibition rate using the formula[24]

$$\text{inhibition rate } (\%) = (X - Y) \div (X - Z) \times 100\%$$

where X is the absorbance of the patients' sera without inhibitors and Y and Z are the absorbances of the patients' and control sera, respectively, treated with various amounts of AK as inhibitor. All determinations were duplicated, and variations between the two assays were always <10%. The mean values were used.

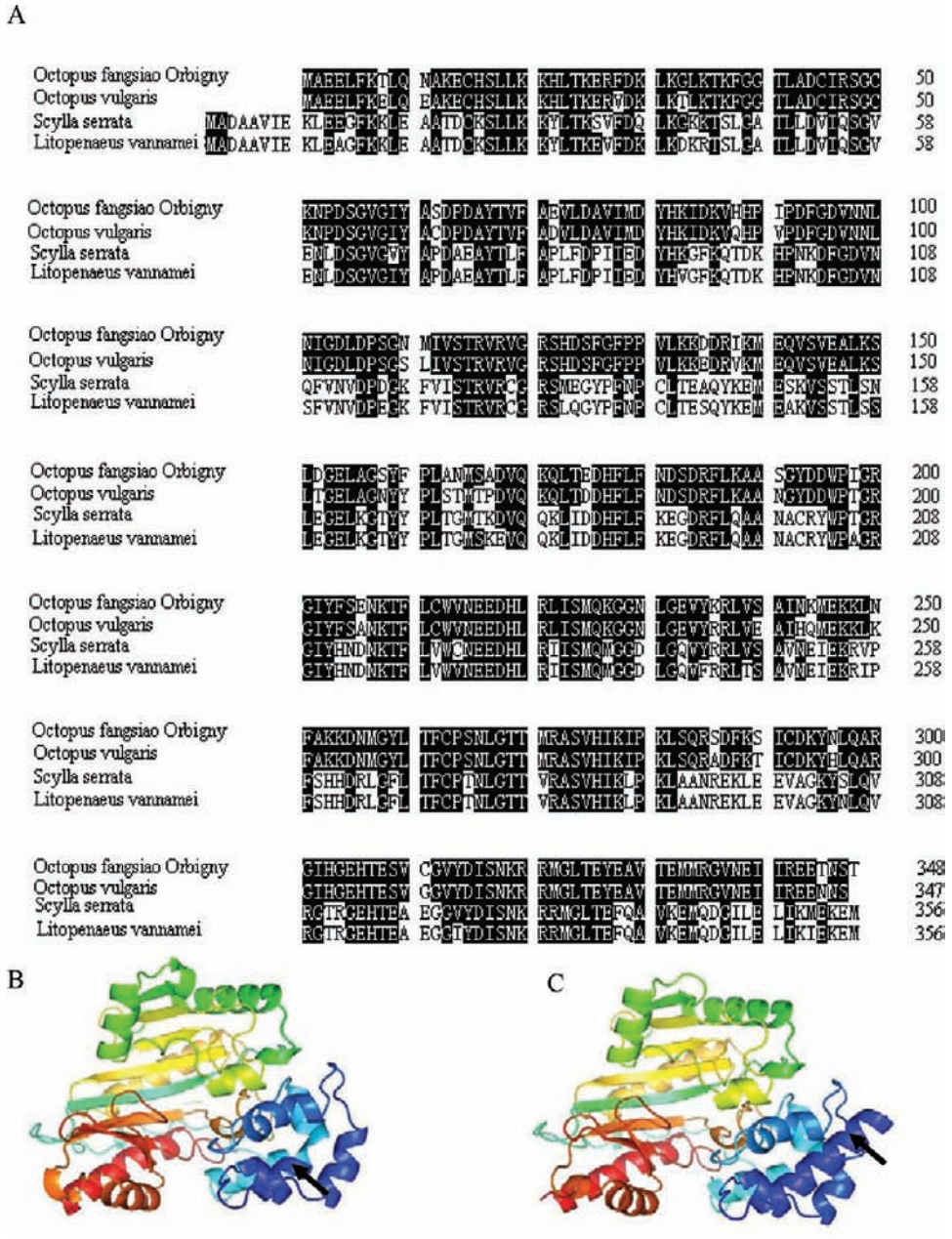


Figure 4. Alignment of primary structure and three-dimensional structure of AK: (A) amino acid sequence alignment of species of AK (species names and their corresponding GenBank accession numbers are as follows: octopus (*Octopus fangsiao*), JN127374; octopus (*Octopus vulgaris*), AB042331; crab (*Scylla serrata*), GQ851626; shrimp (*Litopenaeus vannamei*), DQ975203; amino acids marked with shadowing are the same in the four kinds of AK); (B) three-dimensional structure of octopus (*O. fangsiao*) AK; (C) three-dimensional structure of crab (*S. serrata*) AK. The main differences between octopus AK and crab AK are indicated by arrows.

Thermal and pH Stabilities of AK. Thermal and pH stabilities of AK were tested as described by Liu et al.[25] with some modification. Purified AK (0.5 mg/mL) was stored for 30 min at different temperatures (0, 30, 40, 42, 44, 46, 48, 50, 60, 70, 80, 90, and 100 °C) and terminated in trash ice. For pH stability, purified AK (0.5 mg/mL) was incubated for 1 h at different pH values (1.0, 2.0, 3.0, 4.0, 5.0, 6.0, 7.0, 8.0, 9.0, 9.5, and 10.0) at 37 °C and terminated with isopyknic 1.5 M Tris-HCl (pH 8.0) in trash ice. After incubation, all of the samples were analyzed by SDS-PAGE and immunoblotting.

RESULTS

In this study, AK was highly purified from the muscle of octopus for the first time by ammonium sulfate fractionation and column chromatographies on Q-Sepharose (Figure 1A) and Sephacryl S-200 (Figure 1B). In the ionic Q-Sepharose column, AK fractions were eluted at a NaCl concentration of 0.1−0.2 M and pooled. The AK collected was applied to a gel filtration column (Sephacryl S-200), after enrichment, for further purification. As a result, highly purified AK was obtained to be used in subsequent assays. Purified octopus AK was separated in 12% polyacrylamide gels with 5% stacking gels, a single protein band appeared in the electrophoresis pattern, and the molecular mass was about 38 kDa (Figure 1B).

To identify the purified protein, it was excised from SDS-PAGE and digested in-gel with trypsin, and the resulting peptide mixture was analyzed by MALDI MS. The peptide mass fingerprinting (PMF) of the purified protein showed

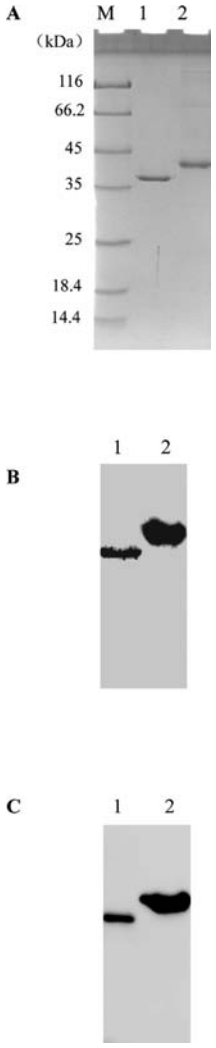


Figure 5. SDS-PAGE and immunoblotting of AK: (A) SDS-PAGE of AK (samples were separated in 12% polyacrylamide gels with 5% stacking gels); (B) immunoblotting (cross-reaction was accomplished using mouse anti-shrimp (*Litopenaeus vannamei*) AK polyclonal antibody); (C) immunoblotting (cross-reaction was accomplished using rabbit anti-crab (*Scylla serrata*) AK polyclonal antibody). Pierce ECL substrate was used for development, and all membranes were exposed to film for 30 s. Lanes: M, protein marker; 1, purified AK from octopus (*Octopus fangsiao*); 2, AK from mud crap (*S. serrata*), used as the positive control.

multiple peaks ranging from 800 to 4000 Da, and peaks having signal-to-noise ratios (SNR) of >50 were analyzed by mass spectrometry/mass spectrometry (MS/MS). The effective peaks were compared with data in the NCBInr database using the Mascot search tool (Figure 2A), and the Mowse value was observed. Usually, protein scores are statistically significant when they are >75, and a total of 12 effective peptides were obtained in the results (Figure 2B). As observed when the search results were compared with data in the NCBInr, there was only one protein with a score >75, and the protein was AK (AB042331, arginine kinase, *Octopus vulgaris*) with a score of 321. As shown in Figure 2C, six peptides with 128 amino acid residues in total were obtained, and they were 100% identical to six peptide fragments of AK from *O. vulgaris*. Therefore, we inferred that the purified protein was octopus AK.

According to the results of MALDI MS, the amino acid sequences of octopus AK from different species were highly homologous, so the degenerate primers used in RT-PCR were designed on the basis of the DNA sequences of octopus AK (AB042331) and generated a fragment of about 300 bp (Figure 3A). On the basis of the sequence information from this fragment, gene-specific primers were subsequently designed and used to generate 3′-RACE and 5′-RACE. The amplification products of 3′-RACE and 5′-RACE were a 300 bp fragment (Figure 3B) and an 850 bp fragment (Figure 3C), respectively. Determination of octopus AK was performed by comparing the overlapping fragment sequences with the full-length sequence, and the deduced amino acid sequence was shown in Figure 3D.

The full-length gene sequence of AK in octopus (*O. fangsiao*) contained 1209 bp including the start codon ATG at positions 9−11, the stop codon TAA at positions 1053−1055, and a polymeric nucleotide tail. The open reading frame of AK was 1047 bp, and the predicted protein consisted of 348 amino acid residues. In general, the average molecular mass of an amino acid was 128, and the molecular mass of AK was therefore approximated as 38 kDa, which was equivalent to the value of 38 kDa estimated by SDS-PAGE (Figure 1B).

A homology search in the GenBank databases revealed *O. fangsiao* AK shared a relatively high homology with AK in the octopus family. As shown in Figure 4A, it had 90.2% identity with AK from another octopus species (AB042331, *O. vulgaris*). Compared with crustaceans, octopus AK had about 54% identity with crab (GQ851626, *Scylla aerrata*) and 53.7% identity with shrimp (DQ975203, *Litopenaeus vannamei*), and the sequence of *O. fangsia* AK reduced eight amino acid residues from the N-terminus of the sequence of crustacean AK. However, it was worth mentioning that there were 13 peptides containing more than five consecutive amino acids that were identical in both octopus AK and crustacean AK. To understand their tertiary structure, their three-dimensional structures were modeled on the basis of the homology in the Web site and analyzed using pyMOL software. As can be seen in Figure 4B,C, the three-dimensional structure of octopus AK was highly similar to that of crab AK. They were both composed of a small N-terminal domain and a larger C-terminal domain. The N-terminal domain was made up of five α-helices, and the C-terminal domain includes an eight-stranded antiparallel β-sheet flanked by seven α-helices. Overall, the biggest difference between octopus AK and crab AK was in the N-terminal domain. The helices at the sequence end of crab AK were longer than in octopus AK (labeled with black arrow in Figure 4B,C).

Because octopus AK and crustacean AK had so many similarities, their immunoreactivity and cross-reactions were analyzed by immunoblotting. First, octopus AK and crab AK (positive control) were separated in SDS-PAGE. From the electrophoresis pattern (Figure 5A), we could see that the molecular mass of octopus AK was obviously smaller than that of crab AK (*S. serrata*), and the result was consistent with the results of amino acid sequence alignment (Figure 4). Immunoblotting analysis using anti-shrimp AK polyclonal antibodies and anti-crab AK polyclonal antibodies showed that octopus AK could undergo positive cross-reactions with crustacean AK, although the cross-reaction between octopus AK shrimp/crab AK was weaker than the reaction between the positive controls (Figure 5B,C). These results showed that some IgG binding activity of octopus AK was similar to that of crustacean AK.

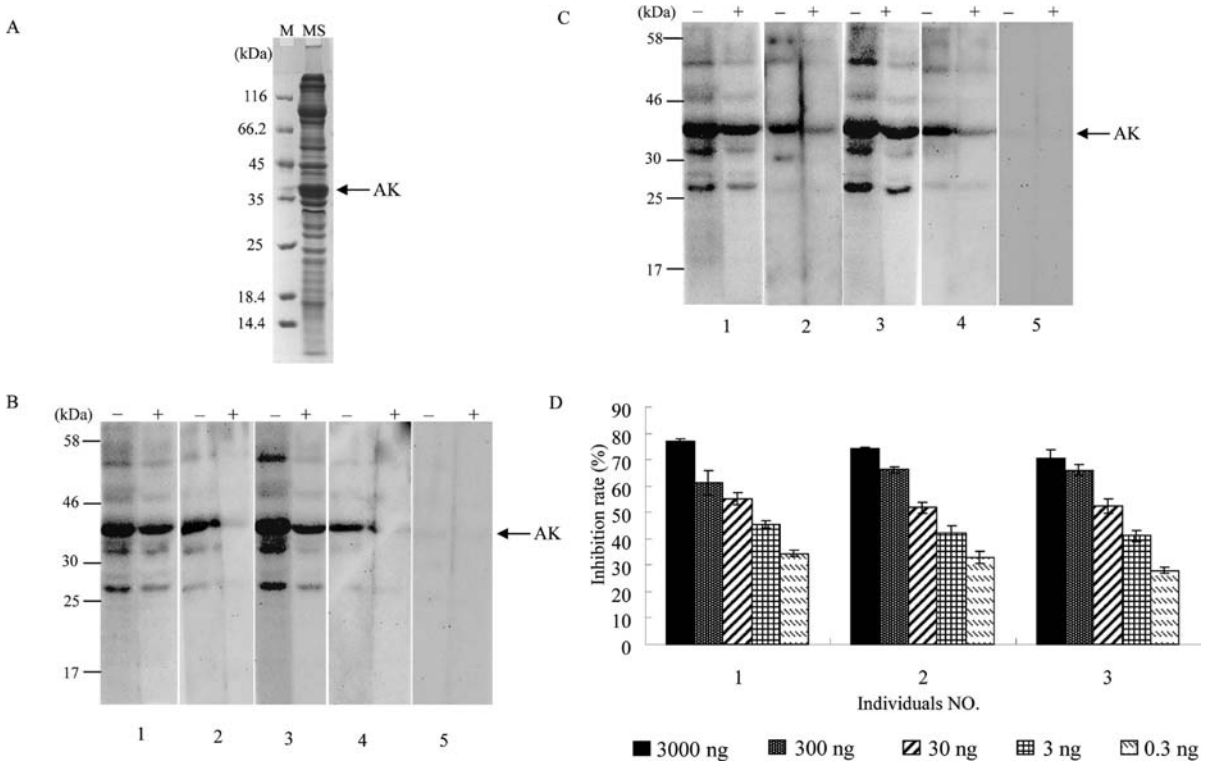


Figure 6. SDS-PAGE and IgE inhibition immunoblotting assay of octopus myosinogen and inhibition ELISA of purified octopus AK: (A) SDS-PAGE of octopus myosinogen (lanes: M, marker; MS, myosinogen); (B) IgE inhibition immunoblotting of myosinogen with octopus AK as the inhibitor; (C) IgE inhibition immunoblotting of myosinogen with crab AK as the inhibitor (NC membrane-blotted myosinogen from octopus muscle was incubated with AK-positive sera, preincubated with purified AK (+) or with buffer only (−); Pierce ECL substrate was used for development, and the membrane was exposed to film for 2 min; sera from octopus-allergic patients were diluted 1:7 with 1% skim milk); (D) inhibition of the IgE reactivity to purified AK (sera diluted 1:5 with 1% skim milk were incubated with an equal volume of inhibitor solution at 37 °C for 2 h, respectively, and then transferred into plates). All data are expressed as the mean ± SD, $n = 2$. Values for the different requirements of AK were significantly different from those of the control ($P < 0.05$). Numerals 1−5 correspond to sera numbers 05979998, 04734483, 04366639, 05981953, and 05990077.

To identify the allergenicity of octopus AK, four sera (05979998, 04734483, 04366639, and 05981953) containing specific IgE of octopus AK were selected from 12 octopus-allergic patients and used to perform the IgE inhibition immunoblotting assays. Myosinogen was extracted from octopus muscle and contained AK and various water-soluble proteins (Figure 6A). As shown in Figure 6B, purified octopus AK (the final incubating concentration was 100 μg/mL) could effectively inhibit the reaction of a 38 kDa protein in the myosinogen with the specific IgE in the four sera. These showed that the 38 kDa protein might be octopus AK. The hybridized bands of octopus AK with sera IgE also became faint obviously when the sera were preincubated with the same concentration of purified crab AK (Figure 6C). The results showed that octopus AK could cross-react with crab AK, and some of their IgE-binding sites were similar to each other. It had been proved that AK was the allergen of shrimps and crabs, so we inferred that AK also was a novel allergen of octopus. To understand the efficiency of raw octopus AK reacted with sera IgE, inhibition ELISA also was carried out. As shown in the Figure 6D, the inhibition rate increased as the dose of AK increased. When the inhibition rates were 50%, the requirement of AK incubated with different sera samples was about 30 ng. In addition, the inhibition rates of all three sera were >70% when 3000 ng of AK was added to the incubated sera. Those demonstrated that there were IgE-binding sites on the surface of octopus AK so that it could combine effectively with IgE to cause an allergic reaction.

Thermal and pH stabilities are very important characteristics of an allergen. Therefore, purified AK was treated at different temperatures and different pH values and detected by SDS-PAGE and immunoblotting. In the process of thermal treatment, the solution began to become turbid at 46 °C after 30 min, and some flocculent precipitate appeared at 70 °C for 30 min. As seen from the SDS-PAGE pattern (Figure 7A), AK was relatively stable below 30 °C, but the original bands of AK became faint and the bands of aggregated AK (>70 kDa) appeared when the temperature was above 40 °C. With detection by rabbit anti-octopus AK polyclonal antibodies, the immunoblotting pattern was very coincident with the SDS-PAGE pattern, and these showed that the IgG-binding activity was retained after thermal treatment (Figure 7B). However, with detection by the patients' sera, the IgE-binding activity of octopus AK was decreased as the temperature increased and could not be detected when the temperature was 48 °C (Figure 7C).

Purified AK was incubated in solutions with different pH values, and the samples were analyzed by SDS-PAGE and immunoblotting. As shown in Figure 8A, AK had no obvious change when the pH value was below 9.0, but the original band of AK was faint and the degraded fragments of AK were observed when AK was incubated at pH 9.5 and 10.0 for 1 h. In the corresponding immunoblotting pattern (Figure 8B), the

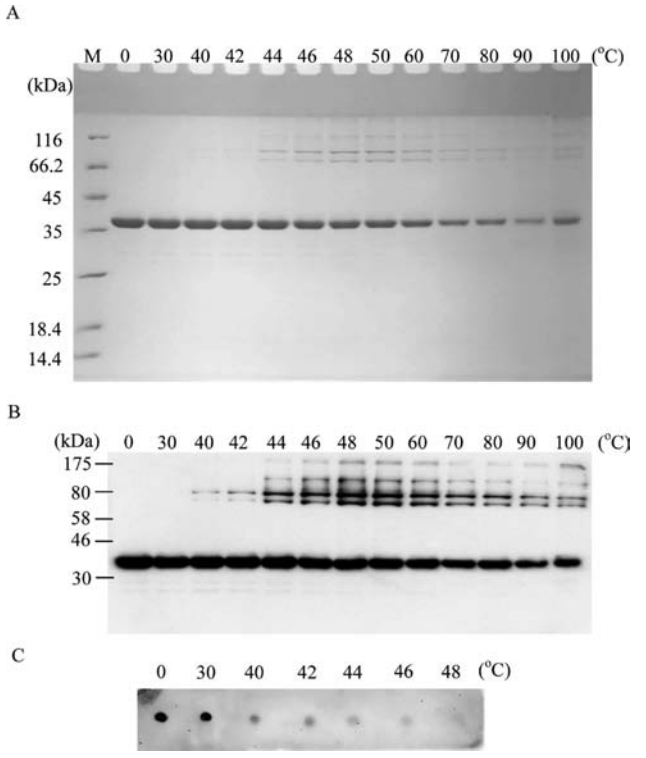


Figure 7. Thermal stabilities of purified AK: (A) SDS-PAGE of thermal stability of octopus AK (lanes: M, protein marker); (B) immunoblotting of thermal stability of octopus AK by polyclonal antibody; (C) IgE-binding activity of thermal treated octopus AK. Pierce ECL substrate was used for development, and the membranes were exposed to film for 30 s (B) and 2 min (C), respectively.

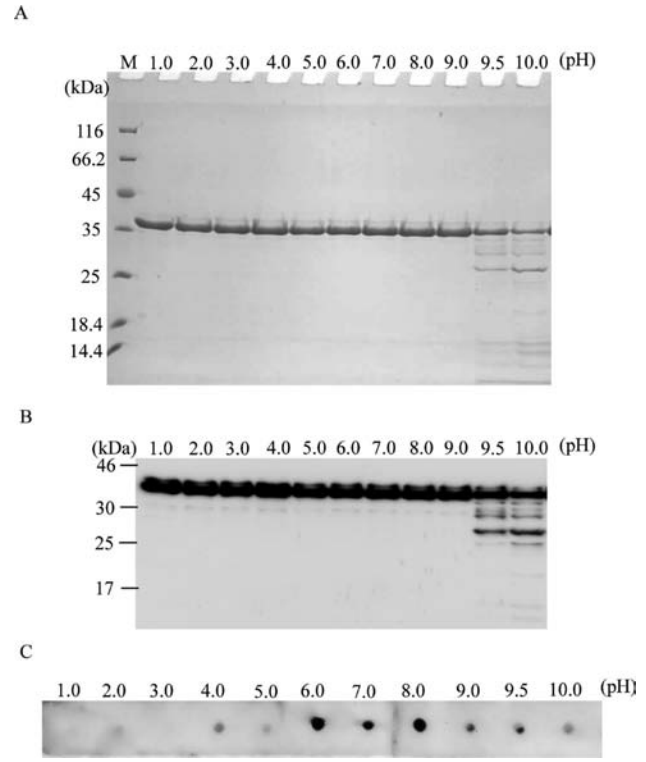


Figure 8. pH stabilities of purified AK: (A) SDS-PAGE of pH stability of octopus AK (lanes: M, protein marker); (B) immunoblotting of pH stability of octopus AK by polyclonal antibody; (C) IgE-binding activity of octopus treated at different pH values. Pierce ECL substrate was used for development, and the membranes were exposed to film for 30 s (B) and 2 min (C), respectively.

change trend of AK was similar to the SDS-PAGE pattern, and the degraded fragments could also be observed clearly. By analysis by positive sera, the results showed that IgE-binding activity could be reduced when AK was treated in acidic or alkali condition (Figure 8C).

DISCUSSION

This is the first study to identify AK as a novel allergen in octopus. Like the major allergen TM of molluscs and crustaceans, AK is also a highly conserved protein as there is a relatively high homology among different species.[26] AK has also been shown to be an important allergen in crustaceans.[16−18] Therefore, AK might be considered a good candidate as an allergenic protein in octopus from a theoretical point of view.

Ammonium sulfate precipitation, anion column chromatography, and gel column chromatography were employed to isolate AK from the muscle of octopus for the first time. It was reported that the molecular masses of shrimp AK,[15] crab AK,[18,27] and lobster AK[28] are 40 kDa, and that of cockroach AK[29] is about 43 kDa, whereas the molecular mass of octopus AK is about 38 kDa, which is obviously smaller than AK from arthropod animals (Figure 5A). This is because *O. fangsiao* AK is made up of 348 amino acids, whereas shrimp and crab AK are made up of no less than 356 amino acids (Figure 4A). Interestingly, although there is a larger gap between the molecular masses of octopus AK and crustacean AK, and the homology between them is about 54%, their simulated three-dimensional structures are highly similar: a small α-helical N-terminal domain is followed by a larger C-terminal domain that is similar to the C-terminal domain of glutamine synthetase.[30,31] On the basis of the amino acid sequence of octopus AK and crustaceans AK, there are 13 identical peptides, and 11 of them are in the random coils of the three-dimensional structure of AK, 1 in the α-helices, and 1 in the β-sheet. Immunoblotting analysis using polyclonal antibody shows that all of these AK samples from crustaceans and molluscs appear to display immunological cross-reactions (Figure 5B,C). All of these show that octopus AK has the similar immunoreactivity to crab AK, and some of the 13 identical peptides might be their mutual epitopes. For these reasons, octopus AK may be an allergen.

In the process of identifying the allergen, a serological assay is crucial and essential. The major allergen of octopus is tropomyosin, which belongs to salt-soluble protein in the myofibril. AK is one kind of water-soluble protein in the myosinogen, so octopus myosinogen is chosen to prevent reaction of tropomyosin with the positive sera in the immunblotting. In the inhibition assays, both octopus AK and crab AK could inhibit the reaction of octopus AK with sera IgE; these show that octopus AK could cross-react with crab AK and some of their IgE-binding sites might be similar. So far, shrimp AK and crab AK have been proved to be the allergen in the crustaceans;[16−18] therefore, octopus AK has been identified as a novel allergenic protein in octopus distinct from TM. In addition, there is an interesting phenomenon in Figure 6B,C that other proteins (26 and 50 kDa) in the myosinogen also appear to be reactive with positive sera, and these proteins should be researched further.

Most food allergens share the characteristic that they are stable at different temperatures or pH values and may still have immunological activity after digestion.[2] For example, tropomyosin,

the major allergen of seafood, is stable in acid and alkali conditions and still has immunological reactivity after high-temperature and high-pressure processing, even after being digested by trypsin or chymotrypsin.[23] However, octopus AK is unstable and can degrade in strongly alkaline conditions, and the IgE-binding activity of AK is decreased significantly. In addition, AK is unstable in the process of thermal treatment, and the aggregated protein and the precipitate begin to appear, which results in IgE-binding activity of AK decreasing significantly while the temperature is increased. It can be speculated that octopus AK is denatured and aggregated in the process of thermal treatment, and its solubility is sharply lower. Although AK is structurally unstable, it may still cause an allergic reaction. The reason may be that some of the IgE-binding sites of AK are stable and unaffected by the processes of protein denaturation, such as high-temperature treatment. From these we could also infer that the linear epitopes of octopus AK might play a more important role of causing allergic reaction.

In summary, this is the first reported purification of AK from octopus, and the purified AK was confirmed through MALDI MS. The physicochemical and molecular biological characteristics of AK were analyzed. AK was identified as an allergen in octopus through immunoblotting, IgE inhibition immumnobloting assay, and inhibition ELISA. However, the functional relationship between octopus AK activity and allergenicity has been not elucidated. Knowledge of the enzymatic properties of AK may advance our overall understanding of this novel allergen. In the future, it is worth discussing the relationship of the structural properties and the allergenicity of AK and optimizing the processing methods to reduce the allergenicity of AK.

AUTHOR INFORMATION

Corresponding Author

*Phone: +86-592-6180378. Fax: +86-592-6180470. E-mail: gmliu@jmu.edu.cn.

Funding

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), and the Foundation for Innovative Research Team of Jimei University (2010A005).

Notes

The authors declare no competing financial interest.

REFERENCES

(1) Daul, C. B.; Morgan, J. E.; Lehrer, S. B. Hypersensitivity reactions to crustacea and mollusks. *Clin. Rev. Allergy* **1993**, *11*, 201−222.

(2) Lopata, A. L.; O'Hehir, R. E.; Lehrer, S. B. Shell allergy. *Clin. Exp. Allergy* **2010**, *40*, 850−858.

(3) Wild, L. G.; Lehre, S. B. Fish and shellfish allergy. *Curr. Allergy Asthma Rep.* **2005**, *5*, 74−79.

(4) Davis, C. M. Food allergies: clinical manifestations, diagnosis, and management. *Curr. Probl. Pediatr. Adolesc. Health Care* **2009**, *39*, 236−254.

(5) Elsayed, S.; Aas, K. Isolation of purified allergens (cod) by isoelectric focusing. *Int. Arch. Allergy Appl. Immunol.* **1971**, *40*, 428−438.

(6) Cai, Q. F.; Liu, G. M.; Li, T.; Hara, K.; Wang, X. C.; Su, W. J.; Cao, M. J. Purification and caracterization of parvalbumins, the major allergens in red stingray (*Dasyatis akajei*). *J. Agric. Food Chem.* **2010**, *58*, 12964−12969.

(7) Ishikawa, M.; Suzuki, F.; Ishida, M.; Nagashima, Y. J.; Shiomi, K. Identification of tromomyosin as a major allergen in the octopus *Octopus vulgaris* and elucidation of its IgE-binding epitopes. *Fish. Sci.* **2001**, *67*, 934−942.

(8) Nakamura, A.; Watanabe, K.; Ojima, T.; Ahn, D. H.; Saeki, H. Effect of Maillard reaction on allergenicity of scallop tropomyosin. *J. Agric. Food Chem.* **2005**, *53*, 7559−7564.

(9) Shanti, K. N.; Martin, B. M.; Nagpal, S.; Metcalfe, D. D.; Rao, P. V. Identification of tropomyosin as the major shrimp allergen and characterization of its IgE-binding epitopes. *J. Immunol.* **1993**, *151*, 5354−5363.

(10) Liang, Y. L.; Cao, M. J.; Su, W. J.; Zhang, L. J.; Huang, Y. Y.; Liu, G. M. Identification and characterization of the major allergen of Chinese mitten crab (*Eriocheir sinensis*). *Food Chem.* **2008**, *111*, 998−1003.

(11) Suzuki, M.; Kobayashi, Y.; Hiraki, Y. M.; Nakata, H.; Shiomi, K. Paramyosin of the disc abalone *Haliotis discus discus*: Identification as a new allergen and cross-reactivity with tropomyosin. *Food Chem.* **2011**, *124*, 921−926.

(12) Ayuso, R.; Grishina, G.; Bardina, L.; Carrillo, T.; Blanco, C.; Ibáñez, M. D.; Sampson, H. A.; Beyer, K. Myosin light chain is a novel shrimp allergen, Lit v 3. *J. Allergy Clin. Immunol.* **2008**, *122*, 795−802.

(13) Shiomi, K.; Sato, Y.; Hamamoto, S.; Mita, H.; Shimakura, K. Sarcoplasmic calcium-binding protein: identification as a new allergen of the black tiger shrimp *Penaeus monodon*. *Int. Arch. Allergy Immunol.* **2008**, *146*, 91−98.

(14) Mühlebach, S. M.; Gross, M. W. T.; Wallimann, T.; Perriard, J. C.; Wyss, M. Sequence homology and structure predictions of the creatine kinase isoenzymes. *Mol. Cell. Biochem.* **1994**, *133*−*134*, 245−262.

(15) Marina, B.; Vera, M.; Brigitte, H.; Wolfgang, R. S.; Matthias, S.; Sabin, P.; Gerhard, W.; Peter, V.; Rudolf, V.; Michael, D. Molecular and immunological characterization of arginine kinase from the indianmeal moth, *Plodia interpunctella*, a novel cross-reactive invertebrate pan-allergen. *J. Immunol.* **2001**, *167*, 5470−5477.

(16) Yu, C. J.; Lin, Y. F.; Chiang, B.; Chow, L. P. Proteomics and immunological analysis of a novel shrimp allergen, Pen m 2. *J. Immunol.* **2003**, *170*, 445−453.

(17) Karina, D.; Orozco, G.; Emmanuel, A. H.; Gloria, Y. P.; Ana, M. C.; Rogerio, R. S.; Sotelo, M. Molecular characterization of arginine kinase, an allergen from the shrimp *Litopenaeus vannamei*. *Int. Arch. Allergy Immunol.* **2007**, *144*, 23−28.

(18) Shen, Y.; Cao, M. J.; Cai, Q. F.; Su, W. J.; Yu, H. L.; Ruan, W. W.; Liu, G. M. Purification, cloning, expression and immunological analysis of *Scylla serrata* arginine kinase, the crab allergen. *J. Sci. Food Agric.* **2011**, *91*, 1326−1335.

(19) Laemmli, U. K. Cleavage of structural proteins during the assembly of the head of the bacteriophage T4. *Nature* **1970**, *227*, 680−685.

(20) Guo, C.; Cao, M. J; Liu, G. M.; Lin, X. S.; Hara, K.; Su, W. J. Purification, characterzation, and cDNA cloning of a myofibril-bound serine proteinase from the skeletal muscle of crucian carp (*Carassius auratus*). *J. Agric. Food Chem.* **2007**, *55*, 1510−1516.

(21) Suzuki, T.; Fukuta, H.; Nagato, H.; Umekawa, M. Arginine kinase from *Nautilus pomplilius*, a living fossil. *J. Biol. Chem.* **2000**, *275*, 23884−23890.

(22) Towbin, H.; Staehelin, T.; Gordon, J. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc. Natl. Acad. Sci. U.S.A.* **1979**, *76*, 4350−4354.

(23) Yu, H. L.; Cao, M. J.; Cai, Q. F.; Weng, W. Y.; Su, W. J.; Liu, G. M. Effects of different processing methods on digestibility of *Scylla paramamosain* allergen (tropomyosin). *Food Chem. Toxicol.* **2011**, *49*, 791−798.

(24) Nakamura, A.; Sasaki, F.; Watanabe, K.; Ojima, T.; Ahn, D. H.; Saeki, H. Changes in allergenicity and digestibility of squid tropomyosin during the Maillard reaction with ribose. *J. Agric. Food Chem.* **2006**, *54*, 9529−9534.

(25) Liu, G. M.; Cao, M. J.; Yu, H. L.; Hu, Y. H.; Zhang, L. J.; Su, W. J. Optimisation of enzymatic hydrolysis of the by-products of marine crab processing using mixed enzymes. *Int. J. Food Sci. Technol.* **2010**, *45*, 1198−1204.
(26) Reddy, S. R.; Watts, D. C. Hybridization of matrix-bound MM-creatine kinase with BB-creatine kinase and arginine kinase. *Comp. Biochem. Mol. Biol.* **1994**, *108*, 73−78.
(27) France, R. M.; Sellers, D. S.; Grossman, S. H. Purification, characterization, and hydrodynamic properties of arginine kinase from gulf shrimp (*Penaeus aztecus*). *Arch. Biochem. Biophys.* **1997**, *345*, 73−78.
(28) Blethen, S. L.; Kaplan, N. O. Purification of arginine kinase from lobster and a study of some factors affecting its reactivation. *Biochemisty* **1967**, *6*, 1413−1421.
(29) Brown, A. E.; France, R. M.; Grossman, S. H. Purification and characterization of arginine kinase from the American cockroach (*Periplaneta americana*). *Arch. Insect Biochem.* **2004**, *56*, 51−60.
(30) Almassy, R. J.; Janson, C. A.; Hamlin, R.; Xuong, N. H.; Eisenberg, D. S. Novel subunit—subunit interactions in the structure of glutamine synthetase. *Nature* **1986**, *323*, 304−309.
(31) Karin, F. W.; Thomas, S.; Theo, W.; Kabsch, W. F. Structure of mitochondrial creatine kinase. *Nature* **1996**, *381*, 341−345.