



Purification, physicochemical and immunological characterization of arginine kinase, an allergen of crayfish (*Procambarus clarkii*)



Heng-Li Chen, Hai-Yan Mao, Min-Jie Cao, Qiu-Feng Cai, Wen-Jin Su, Yong-Xia Zhang, 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 361021, Fujian, China

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ABSTRACT

Arginine kinase (AK) has attracted considerable attention because it has been identified as a shellfish allergen. However, little information is available about AK in crayfish (*Procambarus clarkii*). In this study, crayfish AK was purified and cloned. Its physicochemical properties, processing stability, and immunological characteristics were analyzed. Crayfish AK was purified by column chromatography, which revealed a single band with molecular mass of 40 kDa; this result was further confirmed by mass spectrometry. The full-length gene sequence of crayfish AK was 1462 bp and encoded a protein of 357 amino acid residues. The results of this study revealed that crayfish AK is a glycoprotein with an isoelectric point of approximately 6.5. Thermal stability assays revealed that crayfish AK easily forms aggregates at temperatures >44 °C and was stable at pH 4.0–8.0. SDS–PAGE and dot blotting were used to assess processing stability of purified AK. The results revealed that the IgE-binding activity of crayfish AK is reduced after boiling.

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1. Introduction

Shellfish, which is an important source of dietary protein, is a common cause of food allergy in coastal countries. Shellfish is one of the eight major sources of food allergens. Unlike food allergy to milk and eggs which usually resolves by school age, seafood allergy is typically life-long and unsuccessful avoidance results in often severe allergic reactions (Lopata, 2012). The increasing production and consumption of shellfish have been accompanied by frequent reports of allergic reactions among consumers. Allergic reactions that are mediated by IgE antibodies may be triggered within minutes of shellfish consumption. Allergic reactions to shellfish, e.g., crustaceans and mollusks, can lead to diverse clinical symptoms ranging from mild urticaria to life-threatening anaphylactic reactions (Lopata et al., 2010).

Tropomyosin, a myofibrillar protein with a molecular mass of 34–38 kDa, is a major allergen in shellfish including shrimps (Shanti et al., 1993; Abdel Rahman et al., 2013), crabs (Leung et al., 1996; Rosmilah et al., 2012), and mollusks (Miyazawa et al., 1996; Nakamura et al., 2005). Other allergenic proteins in crustaceans include sarcoplasmic calcium-binding protein (Shiomi et al., 2008), myosin light chain (Ayuso et al., 2008), hemocyanin

(Piboonpocanun et al., 2011), troponin C (Bauermeister et al., 2011), triosephosphate isomerase (Bauermeister et al., 2011), and ferritin (González-de-olano et al., 2011).

Arginine kinase (AK) has also been identified as an important allergen in several shellfish species. AK, which is involved in cell metabolism of invertebrates, consists of a 40-kDa water-soluble protein present in myosinogen. Using immunological and molecular techniques, Yu et al. (2003) purified and identified AK as an allergen, designated Pen m 2, in black tiger shrimp (*Penaeus monodon*). García-Orozco et al. (2007) purified and characterized AK from white shrimp (*Litopenaeus vannamei*). Using mass spectrometry, Abdel Rahman et al. (2011) identified AK as the second most important allergen, designated Chi o 3, in snow crab (*Chionoecetes opilio*). In our laboratory, Yu et al. (2013) identified AK as a major allergen in mud crab (*Scylla paramamosain*), however, this allergen was not stable during digestion.

Red swamp crayfish (*Procambarus clarkii*), which is very popular worldwide, is native to south-central United States and northeastern Mexico (Huner, 1988). With increasing crayfish consumption, severe cases of allergic reactions among consumers have been reported. Crustacean-sensitive individuals in the United States have a high prevalence of positive skin tests (70%) when exposed to crayfish extract (Daul et al., 1993). In China, crayfish is an important economic species; in 2011, the total production of crayfish was approximately 486,319 tons (Anonymous, 2012). A study of 182 urticarial children in China reported that shellfish, including crayfish, is an important allergenic food. According to the authors

Abbreviations: AK, arginine kinase; ELISA, enzyme-linked immunosorbent assay; pI, isoelectric point; SDS–PAGE, SDS–polyacrylamide gel electrophoresis; SGF, simulated gastric fluid; SIF, simulated intestinal fluid.

* Corresponding author. Tel.: +86 592 6180378; fax: +86 592 6180470.

E-mail address: gmliu@jmu.edu.cn (G.-M. Liu).

Table 1The specific primers used in the cloning of *Procambarus clarkii* AK.

Name	Sequence (5'–3')
AK3R1	CACTTTCTGCCCCACCAACCTT
AK3R2	TGGAGGAAGTCGCCGCCAGGTA
AK5F1	ACGGGTGCCACGAACCTGGAGGC
AK5F2	GTGCCACGAACCTGGAGGCTGTA

of the study, approximately 58.79% of the children were sensitive to shellfish (Wang and Yu, 2009).

Until now, it is not known if AK is an allergen in red swamp crayfish. The aim of this study was to purify crayfish AK, clone the protein for B-cell epitopes prediction, and assess its physicochemical properties, processing stability, and immunological characteristics. The results can provide the valuable information for suitable food processing methods to reduce the sensitivity of shellfish.

2. Materials and methods

2.1. Crayfish and chemicals

Fresh red swamp crayfish (*P. clarkii*) was purchased at a local market in Xiamen, China. Crayfish muscle samples were either immediately used or stored at -80°C . Q-Sepharose was purchased from Amersham Biosciences (Uppsala, Sweden). Protein standards for SDS–PAGE were obtained from Fermentas (Vilnius, Lithuania); protein standards for western blotting were obtained from New England BioLabs (Beverly, USA). Periodic acid–Schiff (PAS) staining kit was from Genmed (Grand Rapids, USA); Horseradish peroxidase (HRP)-conjugated goat anti-human IgE antibody was from Kirkegaard and Perry Laboratories (Gaithersburg, USA). HRP-conjugated goat anti-rabbit IgG antibody and enhanced chemiluminescent (ECL) substrate for immunoblotting were from Pierce (Rockford, USA). 3,3',5,5'-Tetramethylbenzidine (TMB) was purchased from Tiangen (Beijing, China). Porcine pancreatic enzymes were obtained from Sigma–Aldrich (St. Louis, USA). Rabbit anti-crab (*S. paramamosain*) AK polyclonal antibody and porcine pepsin were prepared in our laboratory. All reagents were of analytical grade.

2.2. Human sera

Sera from 17 crustacean-sensitive patients (labeled No. 475, 362, 112, 252, 303, 555, 743, 468, 354, 336, 083, 300, 384, 282, 437, 221, and 347) were obtained from the Hospital of Jimei University in Xiamen, China. Crustacean allergy was confirmed based on allergic history, physicochemical examination, and clinical symptoms following the consumption of crustacean products. Sera from non-allergic individuals (labeled No. 307 and 776) without history of crustacean sensitivity were pooled and used as negative control. All patients and healthy individuals provided their sera voluntarily. Written informed consent was obtained from each patient. Ethics approval for this study was acquired at Jimei University. Sera were stored at -80°C until used.

2.3. Crayfish myosinogen preparation

Crayfish muscle tissue was minced and homogenized with 5 volumes of ice-cold 20 mM Tris–HCl buffer (pH 7.0). The homogenate was then centrifuged at 8000g at 4°C for 20 min. The resulting supernatant contained crayfish myosinogen.

2.4. Protein purification

A 40-kDa protein was purified according to the method of Yu et al. (2003) and Shen et al. (2012) with some modifications. All procedures were performed at 4°C . Crayfish muscle tissue was minced, homogenized, and centrifuged. The resulting supernatant was precipitated with 70–90% ammonium sulfate saturation. The resulting precipitate was dissolved and dialyzed before subjecting to a Q-Sepharose column (2.5 cm $\times$ 10 cm). Binding proteins were eluted using a linear gradient of 0–0.3 M NaCl at a flow rate of 1 mL/min. Protein concentration was estimated by the Lowry method (Lowry et al., 1951) with bovine serum albumin (BSA) as a standard. Fractions containing the target protein were detected by SDS–PAGE and collected.

2.5. Matrix-assisted laser desorption ionization mass spectrometry (MALDI-MS) analysis

The purified protein was run on a 12% SDS–PAGE gel and stained with Coomassie Brilliant Blue R-250 (CBB) for mass spectrometry. The target protein was destained with 100 mM NH_4HCO_3 /30% ACN. The disulfide bonds were reduced and methylated using DTT and IAA, respectively. Trypsin digestion (sequencing grade) was then performed at 37°C for 20 h in trypsin digestion solution (2.5–10 ng/ μL). The sample was desalted with ZipTip, lyophilized, and reconstituted for MALDI-MS analysis. The peptide fragments were analyzed using 4800 plus MALDI TOF/TOF analyser from ABI (Foster City, USA) by the Shanghai Applied Protein Technology Company Limited. The resulting peptides spectra were searched against the NCBI database using Matrix Science's Mascot 2.2 search engine.

2.6. Cloning and sequencing

Muscle samples from live crayfish were immediately frozen in liquid nitrogen. Total RNA was isolated from muscle samples using TRIzol reagent from Invitrogen (Carlsbad, USA). First-strand cDNA was synthesized with reverse transcriptase from Tiangen (Beijing, China); oligo(dT)₁₈ primers were designed according to the manufacturer's instructions. The primers (AK3R1, AK3R2, AK5F1, and AK5F2) for 5'-RACE and 3'-RACE are shown in Table 1. RACE and RACE-PCR were conducted using the SMART RACE Amplification Kit (Clontech, USA). The PCR amplification product was purified, cloned into pGEM-T Easy vector (Promega), and sequenced. DNA nucleotide sequences were analyzed using the ABI PRISM 3730 DNA sequencer (Foster City, USA), compared with the nucleotide sequences in the GenBank database and finally log in with the accession number JN828651 (gi|375298900) for *P. clarkii* AK cDNA.

2.7. Structure modeling and epitope prediction

Information on the simulated three-dimensional structure of AK can be obtained in the website <http://swissmodel.expasy.org/>. Linear (i.e., sequential) B-cell epitopes were predicted from hydropathic profiles as being the most hydrophilic, flexible, and surface-exposed regions according to the DNA Star Protean software. Conformational (i.e., non-sequential) B-cell epitopes were predicted using the Disco Tope software (<http://www.cbs.dtu.dk/services/DiscoTope/>) and displayed on the molecular surface of the protein with PyMOL.

2.8. Physicochemical characterization

2.8.1. PAS staining

To determine whether AK is a glycosylated protein, PAS staining was performed according to the manufacturer's instructions.

2.8.2. Two-dimensional electrophoresis (2-DE)

Purified crayfish AK was applied to a 7-cm immobilized pH gradient (IPG) strip (3.0–10.0). Isoelectric focusing was performed in an Ettan IPGphor 3 unit (GE Healthcare). After isoelectric focusing, the IPG strip was equilibrated and subjected to 12% gel SDS–PAGE and stained by CBB.

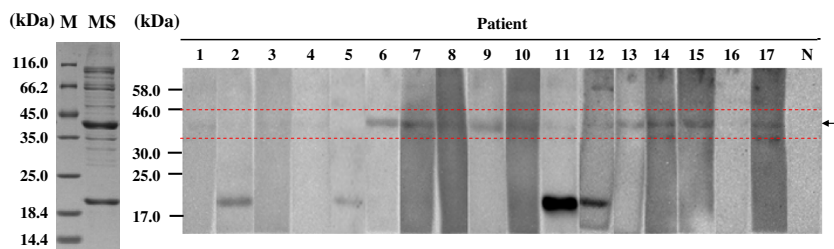


Fig. 1. SDS–PAGE and western blotting of crayfish myosinogen. M, protein marker; MS, SDS–PAGE analysis of myosinogen; 1–17, western blotting profiles of IgE-binding activity of myosinogen to patients' sera (1–17 correspond to sera No. 475, 362, 112, 252, 303, 555, 743, 468, 354, 336, 083, 300, 384, 282, 437, 221, and 347); N, western blotting profiles of pooled non-allergic sera (No. 307 and 776). The marker for western blotting consisted of pre-stained molecular mass standards.

2.8.3. Thermal and pH stabilities

For thermal stability, purified crayfish AK was incubated in the buffer (10 mM Tris–HCl, pH 7.0) at different temperatures for 30 min: 30 °C, 40 °C, 42 °C, 44 °C, 46 °C, 48 °C, 50 °C, 55 °C, 60 °C, 70 °C, 80 °C, 90 °C, or 100 °C. For pH stability, AK was incubated for 1.5 h at room temperature in buffers of different pH values: pH 1.0, 2.0, 3.0, 4.0, 5.0, 6.0, 7.0, 8.0, 9.0, 10.0, or 11.0. Buffers consisted of HAc–NaAc (pH 1.0–5.0), phosphate buffered saline (pH 7.0), and Na₂CO₃–NaHCO₃ (pH 9.0–11.0). Following the 1.5-h incubation, the samples were analyzed by SDS–PAGE and dot blotting using sera from the patients.

2.8.4. Digestion stability and inhibition ELISA

The digestibility of purified crayfish AK was assessed in a simulated gastric fluid (SGF, pH 1.2) and simulated intestinal fluid (SIF, pH 7.5) according to the method of Huang et al. (2010) and Yu et al. (2013) with some modifications. The pepsin to AK ratio was 1:4000 (w/w). The peptic digestion lasted 0, 1, 2, 5, 10, 15, 30, or 60 min. The pancreatic enzymes to AK ratio were 1:50 (w/w). The intestinal digestion lasted 0, 1, 15, 30, 60, 120, 180, or 240 min. AK dissolved in SGF/SIF, but with no digestive enzymes, was used as a control. Samples were analyzed by SDS–PAGE.

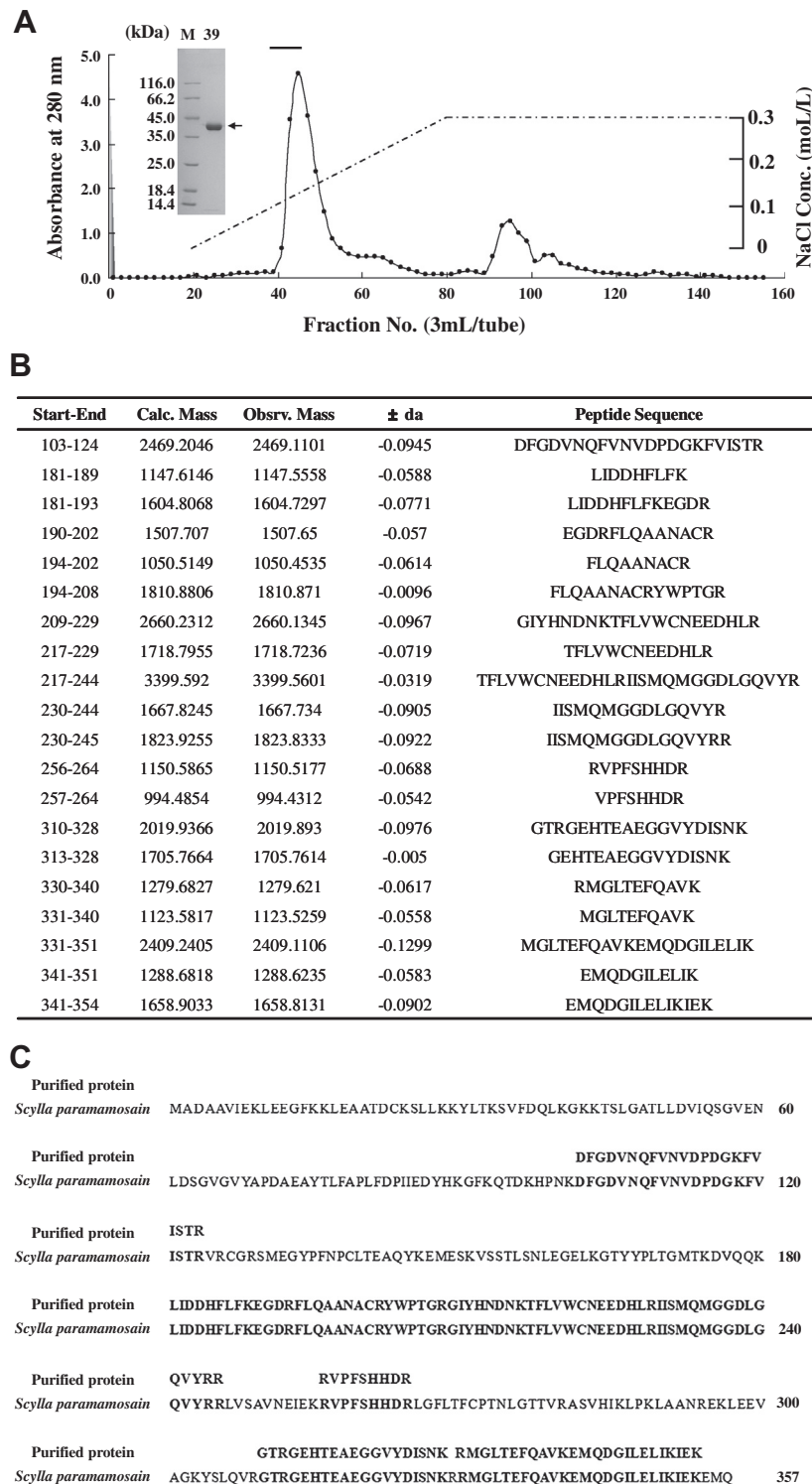


Fig. 2. Purification and identification of the 40-kDa crayfish protein. (A) Q-Sepharose chromatography. The numbers on the top of the lanes correspond to the fraction number. Target protein fractions under the bar were pooled. (B) Effective peptides from mass spectrometry (MS) compared with data in the NCBI. (C) Protein sequence alignment between the purified crayfish protein and *S. paramamosain* AK.

The specific IgE-binding activity of the digested AK was determined by inhibition ELISA according to the method of Yu et al. (2013). Digested AK in the concentration range of 0.02–40 µg/mL was used as an inhibitor. The IgE-binding activity of the digested AK was determined by calculating the inhibition rate.

2.9. Processing conditions

Purified crayfish AK underwent three different processing conditions: (1) ultrasonic treatment where purified AK was transferred to an ultrasonic cleaner (KQ5200DE; Kunshan, China) at 200 W and 30 °C for different times (10, 15, 30, 60, 90, 120, or 180 min); (2) microwave treatment where purified AK was exposed to microwave radiation in a commercial Galanz G8023CSL-K3 microwave (2450 MHz) at 800 W for different times (10, 15, 30, 60, 90, 120, or 180 s); or (3) boiling treatment where purified AK was boiled at 100 °C for different times (5, 10, 15, 20, 30, 40, or 60 min). After the different processing conditions, AK samples were analyzed by SDS–PAGE and dot blotting using sera from the patients.

2.10. SDS–PAGE, western blotting, and dot blotting

For SDS–PAGE, samples were separated on 12% polyacrylamide gels with 5% stacking gels and stained with CBB. For western blotting, samples separated by SDS–PAGE were transferred to nitrocellulose membranes, which were blocked with 5% skim milk. Human serum (1:4 dilution) or rabbit anti-crab AK polyclonal antibody (1:4000 dilution) was used as the primary antibody. HRP-labeled goat anti-human IgE antibody (1:20,000 dilution) or HRP-labeled goat anti-rabbit IgG antibody (1:20,000 dilution) was used. Western blotting was visualized using ECL.

Dot blotting was performed by first blotting the samples onto nitrocellulose membranes. The membranes were then blocked and incubated with sera from the patients (1:4 dilution). Subsequently, the membranes were incubated with HRP-labeled goat anti-human IgE antibody (1:20,000 dilution). The immunoassay was performed using ECL.

3. Results

3.1. IgE-binding activity of patients' sera to crayfish myosinogen

Myosinogen, which contained several water-soluble proteins, was extracted from crayfish muscle tissue. Western blotting of crayfish myosinogen was performed using sera from 17 crustacean-sensitive patients to identify the specific IgE-binding allergens. As shown in Fig. 1 and 12 of 17 sera (with the exception of No. 362, 112, 252, 303, and 221) reacted to the 40-kDa protein. Four sera (No. 362, 303, 083, and 300) reacted to a 22-kDa protein. It should be noted that the 40-kDa protein had a higher IgE reactivity (70.59%) than the 22-kDa protein (23.53%). As expected, the control sera showed no reactivity.

3.2. Purification and identification of the 40-kDa protein

The 40-kDa protein was purified from crayfish muscle by ammonium sulfate precipitation and Q-Sepharose chromatography. In the Q-Sepharose column, a fraction of high protein content was eluted at 0–0.3 M NaCl and collected for subsequent assays. The collected fraction migrated as a single band with a molecular weight of approximately 40 kDa on SDS–PAGE, indicating its high purity (Fig. 2A). As a result, approximately 46.5 mg AK was obtained from 70 g of crayfish muscle.

To further confirm the purified protein, the protein band was excised, destained and digested in-gel with trypsin, and the resulting peptides were analyzed by MALDI-MS. The peptide mass fingerprinting (PMF) of the purified protein showed multiple peaks ranging from 800 to 4000 Da. The peaks that signal-to-noise ratios (SNR) of >50 were analyzed by mass spectrometry/mass spectrometry (MS/MS) (data not shown). The effective peaks were searched against the NCBI nr database using Mascot 2.2 search engine (Fig. 2B). Usually, protein scores are statistically significant when they are >75. After compared with the data in NCBI nr, there was one protein with a score >75, and the protein was AK (gi|375298903, *S. paramamosain*) with a score of 542. As shown in Fig. 2C, five peptide fragments with 140 amino acid residues were obtained and they were 100% identical to five peptide

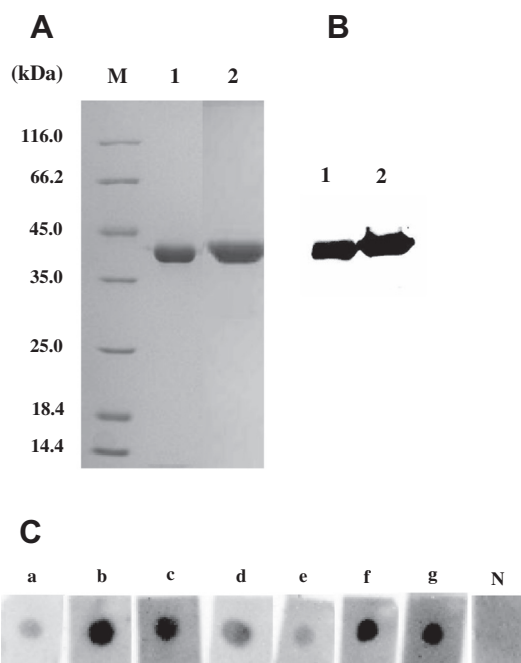


Fig. 3. (A) SDS–PAGE and (B) Western blotting of purified AK. M, protein marker; lane 1, purified crayfish AK; lane 2, purified crab AK (control). (C) IgE immunoblotting of purified AK from crayfish. a–g: sera of crustacean-allergic patients (correspond to sera No. 475, 555, 743, 354, 083, 384, and 282); N: pooled non-allergic sera (No. 307, 776).

fragments of crab (*S. paramamosain*) (gi|375298903) AK, suggesting that the 40-kDa purified protein was crayfish AK.

The molecular mass of crayfish AK was slightly smaller than the molecular mass of crab (*S. paramamosain*) AK on SDS–PAGE (Fig. 3A). The results of western blotting analysis of anti-crab AK polyclonal antibody revealed strong positive reactions with the purified crayfish AK and the purified crab AK (Fig. 3B), further supporting that the purified protein was crayfish AK. The purified crab AK was used as a positive control. To confirm the IgE-binding activity of the purified crayfish AK, AK was used as antigen and investigated by dot-blotting immunoassay with sera samples from crustacean-allergic patients. As shown in Fig. 3C, 7 dots showed different reactive intensity with patients' sera. No IgE-binding activity was detected with normal negative control sera. It revealed that AK was an allergen in crayfish.

3.3. Cloning and sequencing

According to the results obtained by MALDI-MS, the amino acid sequences of crayfish AK and crab AK were highly homologous. Therefore, the specific primers (AK3R1, AK3R2, AK5F1, and AK5F2; Table 1) used in RACE and RACE-PCR were designed on the basis of the DNA sequence of *S. paramamosain* (gi|375298902). The nucleic acid and the deduced amino acid sequence are shown in Fig. 4A. The full-length gene sequence of crayfish AK was 1462 bp including the ATG start codon in the 61–63 region, the TAA stop codon in the 1132–1134 region, and the poly-A tail. The open reading frame of crayfish AK was 1074 bp and the predicted protein consisted of 357 amino acid residues with a theoretical molecular weight of 40.12 kDa, which was equivalent to the 40 kDa molecular weight obtained by SDS–PAGE (Fig. 2A). In the amino acid sequence of crayfish AK, there were two N-glycosylated sites (Fig. 4A). The N-linked glycan is usually identified by its conserved sequence -Asn-X-Ser/Thr/Cys-. In addition, the results of motif analysis revealed that CPTNLGT (271–277 region) is the active site of crayfish AK. It was in accord with the

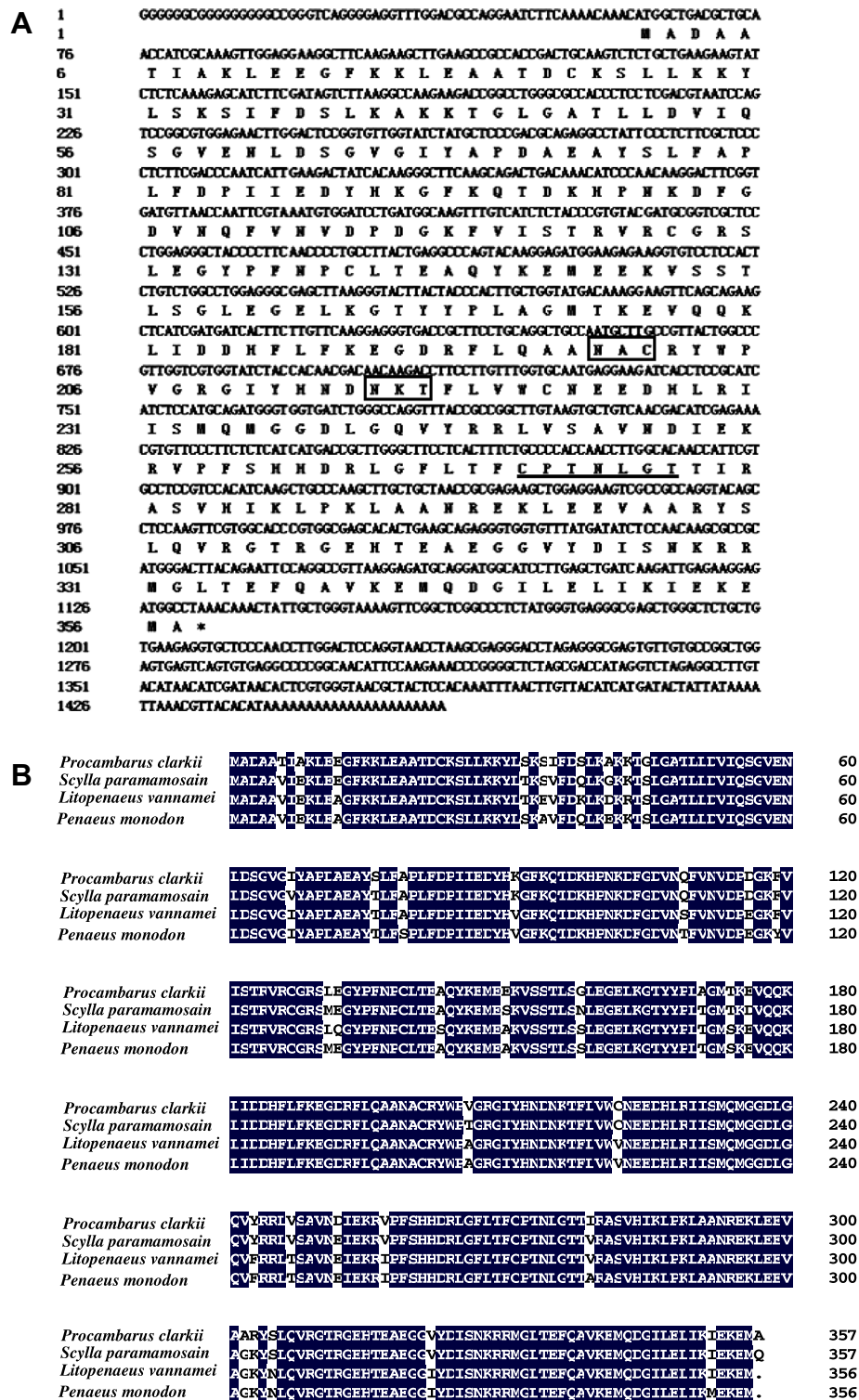


Fig. 4. Cloning and B-cell epitope prediction of purified crayfish AK. (A) The nucleotide and deduced amino acid sequence of crayfish AK (the N-glycosylation site is marked by a box; the active site is underlined). (B) Sequence alignment between crayfish AK and AK of other crustaceans (*S. paramamosain*, *L. vannamei*, and *P. monodon*). (C) Three-dimensional structure of crayfish AK. (D) Molecular mapping of the conformational B-cell epitopes of crayfish AK in the three-dimensional structure. N and C indicate the N- and C-termini, respectively. The areas occupied by the conformational B-epitopes are shown in cyan.

kinase active site of snow crab AK (Abdel Rahman et al., 2011). Protein sequence alignments showed that AK from different crustacean species had high homology (Fig. 4B). The amino acid sequence of crayfish AK was 94.40%, 91.04%, and 91.04% identical with the amino acid sequence of mud crab (*S. paramamosain*, gi|375298903) AK, white shrimp (*L. vannamei*, gi|115492980) AK, and black tiger shrimp (*P. monodon*, gi|27463265) AK, respectively.

3.4. Structure modeling and epitope prediction

On the basis of the amino acid sequence of crayfish AK, a molecular model of the three-dimensional structure of crayfish AK was constructed. Crayfish AK was composed of a small N-terminal domain and a large C-terminal domain (Fig. 4C). The N-terminal domain consisted of five α -helices, and the C-terminal domain

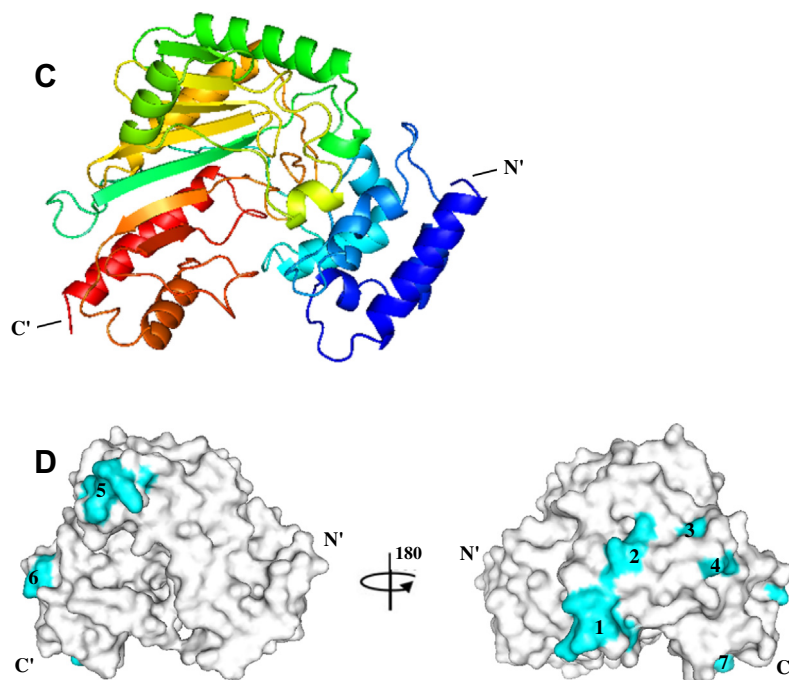


Fig. 4 (continued)

included eight-stranded antiparallel β -sheets flanked by seven α -helices. The linear (sequential) B-cell epitopes of crayfish AK were predicted based on secondary structures using DNA Star Protean software. Six sequential regions (39–43, 89–103, 146–152, 252–256, 311–317, and 325–330) of linear B-cell epitopes were the most hydrophilic, flexible, and surface-exposed regions. Twenty-four non-sequential residues (91–92, 94–98, 100–101, 106, 108, 115–116, 172, 174–176, 178–180, 183, 263, 295, and 299) of conformational B-cell epitopes were obtained by Disco Tope software. Molecular mapping of the 24 predicted non-sequential epitope residues on the three-dimensional structure of crayfish AK revealed seven conformational B-cell epitopes (labeled with cyan in Fig. 4D).

3.5. Physicochemical characterization of AK

3.5.1. Glycoprotein characterization

PAS staining was used to determine whether crayfish AK is a glycoprotein. In the positive control, an IgG heavy chain band (magenta) was observed (Fig. 5A, lane 2); in the BSA negative control, no such band was observed (Fig. 5A, lane 1). On the other hand, the crayfish AK band revealed a slight magenta staining (Fig. 5A, lane 3), suggesting that AK is a glycoprotein.

3.5.2. Isoelectric point determination

To further characterize the protein, crayfish AK was subjected to 2-DE for separation by isoelectric point in the horizontal direction and by molecular mass in the vertical direction. Crayfish AK produced a single spot (Fig. 5B) with a molecular mass consistent with the SDS-PAGE results (Fig. 2A) and a pI value of approximately 6.5.

3.5.3. Thermal stability and pH stability

Thermal and pH stabilities are major characteristics of an allergen. Purified crayfish AK was subjected to different temperature treatments and analyzed by SDS-PAGE and dot blotting. The results revealed that during thermal treatment, AK was stable at 30–44 °C (Fig. 6A). However, as temperature increased (>44 °C), AK aggregates and precipitates formed in the reaction systems.

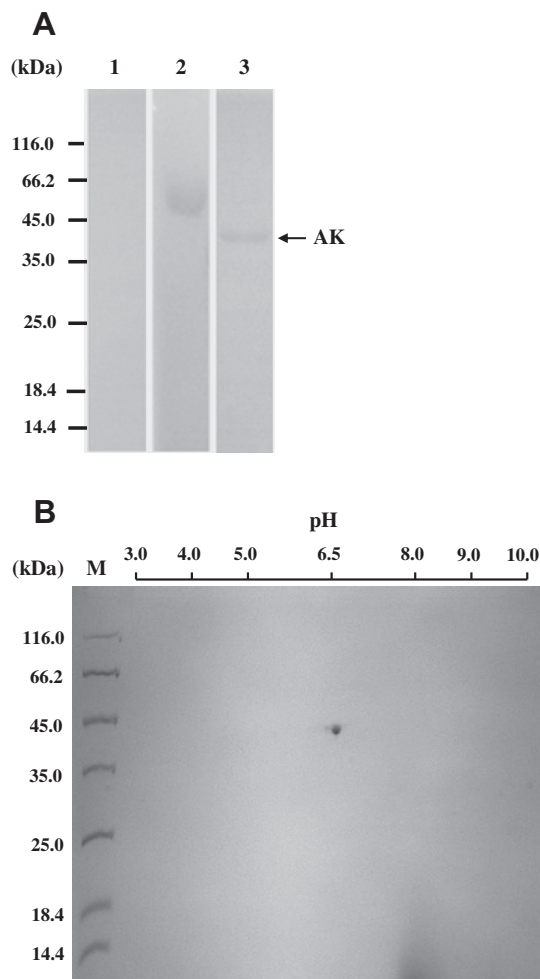


Fig. 5. Glycosylation and isoelectric point of purified crayfish AK. (A) PAS staining: lane 1, BSA (negative control); lane 2, IgG heavy chain (positive control); lane 3, purified crayfish AK. (B) 2-DE map. M, protein marker.

With increasing temperatures, more AK aggregates were formed. The IgE-binding activity of crayfish AK increased in 44–70 °C; however, IgE-binding activity of crayfish AK could not be detected when temperatures were 80 °C or higher (Fig. 6B).

The pH stability of the purified crayfish AK was also assessed by SDS–PAGE and dot blotting. As shown in Fig. 6C, AK was affected by buffers at pH 1.0–4.0. As shown in Fig. 6D, IgE-binding activity of AK was reduced under alkaline conditions (pH 9.0–11.0); however, the IgE-binding activity of AK increased when AK was exposed to acidic conditions (pH 1.0–3.0).

3.5.4. Digestion stability

Most food allergens remain intact during digestion, thereby causing anaphylaxis in consumers. An in vitro digestion model was performed to determine whether AK is stable to digestion. The digestibility of the purified crayfish AK in SGF was analyzed by SDS–PAGE. The original band (40 kDa) was gradually digested over time. Peptide fragments (25–35 kDa) appeared after a prolonged peptic digestion. AK was almost completely digested by the end of the SGF digestion (Fig. 6E). AK was stable during the SIF digestion and it was hardly degraded by pancreatic enzymes even after 4 h of intestinal digestion (Fig. 6F). The digestion-resistant products were further analyzed by inhibition ELISA, which is quite sensitive for detecting proteolytic fragments with IgE-binding activity. As shown in Fig. 6G, the IgE-binding activity of AK that had been digested by pepsin for 1 h was reduced in comparison with the undigested AK sample. A 4-h intestinal digestion had little effect on the IgE-binding activity of AK. Thus crayfish AK still had immunological activity following the in vitro gastrointestinal digestion.

3.6. Effects of different processing conditions on IgE-binding activity of purified AK

To investigate the changes in IgE-binding activity of purified crayfish AK under different processing conditions, SDS–PAGE and dot blotting using patients' sera were performed. The processing conditions consisted of ultrasound, microwave, and boiling treatments. The SDS–PAGE profile indicated that the band of the original AK had no obvious change after ultrasound treatment (Fig. 7A). Similarly, the dot blotting assay revealed no changes in IgE-binding activity of AK after ultrasound treatment (Fig. 7B). When subjected to microwave treatment, the original AK appeared to be intact, but some AK aggregates were produced after 120 s (Fig. 7C). The dot blotting assay revealed that the IgE-binding activity of AK subjected to 180 s of microwave treatment was reduced compared with that of the untreated sample (Fig. 7D). In the boiled samples, AK aggregates were observed when AK was boiled at 100 °C for 60 min (Fig. 7E). The formation of AK aggregates can occur even after boiling the sample for 5 min; the number of AK aggregates increased over time. The boiled AK had a lower IgE-binding activity than the untreated sample (Fig. 7F). The reduction in IgE-binding activity of AK was correlated with the treatment duration. Among the three treatments, IgE-binding activity of purified crayfish AK was effectively reduced after the boiling and microwave treatments. Boiling was the most effective treatment for reducing IgE-binding activity of AK.

4. Discussion

With increasing crayfish consumption, several cases of IgE-mediated allergies have been reported (Daul et al., 1993; González-de-olano et al., 2011). Therefore, we performed a thorough investigation of crayfish allergens. Serological assays are crucial for the identification of allergens. The 40-kDa protein from crayfish myosinogen had higher IgE reactivity to crustacean-sensitive

patients' sera than the 22-kDa protein, which was identified as sarcoplasmic calcium-binding protein (Chen et al., 2013).

The 40-kDa IgE-binding protein was purified by ammonium sulfate precipitation and a Q-Sepharose chromatography and identified as AK by MALDI-MS. The molecular mass of crayfish AK was 40 kDa, which is slightly smaller than the molecular mass of crab (*S. paramamosain*) AK (Fig. 3A). It has been reported that the molecular masses of AK from shrimp (*P. monodon*) (Yu et al., 2003), crab (*Scylla serrata*) (Shen et al., 2011), and lobster (*Homarus americanus*) (Blethen and Kaplan, 1967) are also 40 kDa. This result was attributed to the high homology of AK among different crustacean species (Fig. 4B).

A molecular model of the three-dimensional structure of crayfish AK was constructed to predict the conformational B-cell epitopes (Fig. 4C and D). Both linear and conformational B-cell epitopes were predicted in crayfish AK. The 91–92, 94–98, and 100–101 regions comprised both the linear and conformational B-cell epitopes. Ayuso et al. (2012) reported that IgE antibodies to certain epitopes of shrimp AK could be used as biomarkers for the prediction of clinical reactivity in subjects with sensitization to shrimp. Therefore, future studies should assess the clinical reactivity in subjects with sensitization to crayfish using the predicted epitopes of this study.

Most food allergens share similar physicochemical characteristics, e.g., food allergens are glycoproteins with low isoelectric points, stable at different temperatures or pH values, and may still possess immunological activity following digestion (Chandra, 1997; Lopata et al., 2010). According to the results obtained in this study, the isoelectric point of the crayfish AK was about 6.5 and crayfish AK was a glycoprotein identified by PAS staining (Fig. 5A and B). Two potential N-glycosylated sites (NAC and NKT) were identified by analyzing the primary structure and mass spectrometry, but it indicated no presence of the N-glycans. In order to clarify the conflicting, the determination of the molecular mass of AK by MALDI-TOF and comparison of the theoretically calculated molecular mass with the experimentally determined molecular mass will be done in our future research. On the other hand, the thermal stability assays revealed that crayfish AK was unstable and formed aggregates at temperatures >44 °C. The IgE-binding activity increased at 44–70 °C, but decreased at temperatures >80 °C (Fig. 6A and B). It is possible that, with temperatures increasing, AK unfolds and hidden AK epitopes are unveiled, thereby exposing more sequential epitopes that can bind to IgE antibodies (Ayuso et al., 2012). Temperatures >80 °C might result in a potentially allergenic structure that is created by surface-exposed epitopes and AK aggregate formation. A certain amount of AK aggregates may have a masking effect on the recognition of sequential (i.e., linear) epitopes (Taheri-Kafrani et al., 2009). Therefore, high temperature treatments may be effective in reducing IgE-binding activity of AK. According to the pH stability results, crayfish AK was degraded in strongly acidic conditions; however, the IgE-binding activity of AK was decreased in alkaline conditions (Fig. 6C and D). These results may be attributed to changes in the conformational structure of AK by acid and alkali treatments, thereby resulting in the exposure of hidden epitopes on the surface of AK. However, alkaline conditions can result in a reduction in IgE binding capacity of proteins (Baur et al., 1997; Bernardi et al., 2010); to test this, circular dichroism (CD) will be performed in our future studies. Similar to crab AK (Yu et al., 2013), crayfish AK was degraded by pepsin but was resistant to pancreatic enzymes (Fig. 6E and F). Furthermore, after the in vitro digestion, crayfish AK still had immunological activity (Fig. 6G). Although AK is structurally unstable, it may still cause an allergic reaction, probably due to certain IgE-binding sites of AK that remain unaffected by digestive enzymes.

Few studies have been conducted on the processing conditions of AK. In this study, the changes of IgE-binding activity of crayfish AK were assessed under different processing conditions. The

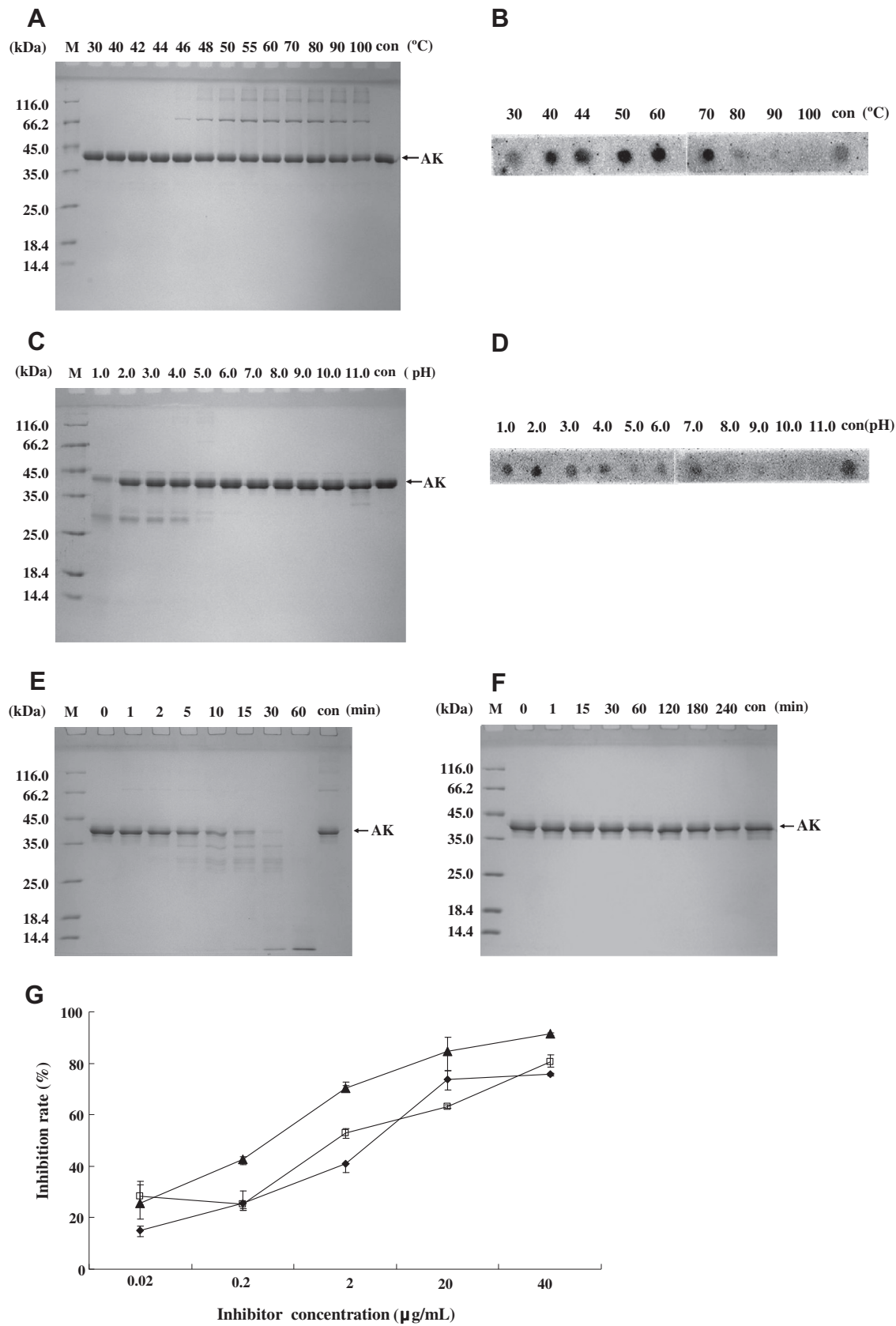


Fig. 6. Stability of the purified crayfish AK. (A) SDS–PAGE of the thermal stability of AK. (B) Dot blotting of thermal-treated AK. (C) SDS–PAGE of the pH stability of AK. (D) Dot blotting of AK treated at different pH values. (E) SDS–PAGE analysis of the AK stability to SGF digestion. (F) SDS–PAGE analysis of the AK stability to SIF digestion. M, protein marker, con, control. (G) Inhibition-ELISA of the digested fragments of AK. AK digested by pepsin (◆, 1 h); pancreatic enzymes (□, 4 h); and undigested (▲).

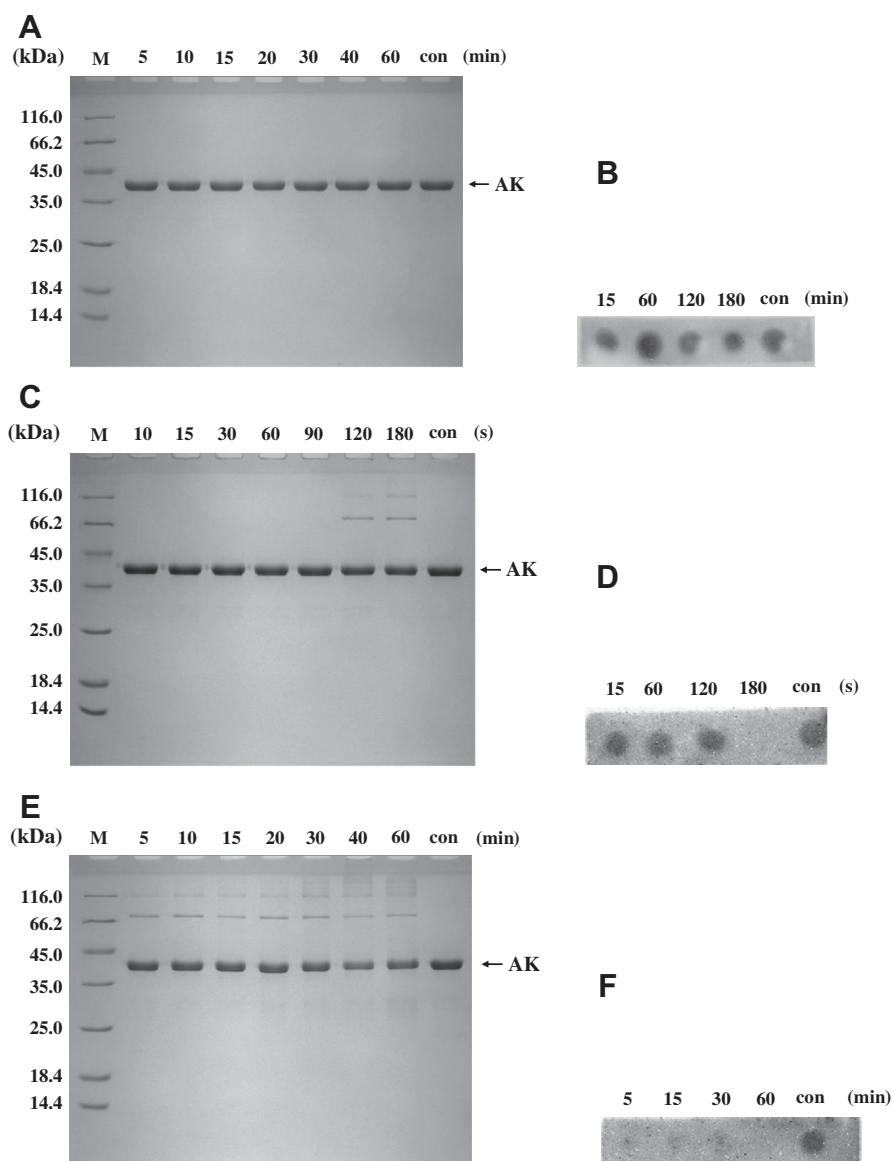


Fig. 7. Effects of different processing conditions on the purified crayfish AK. (A) SDS–PAGE of AK subjected to ultrasonic treatment. (B) Dot blotting of AK subjected to ultrasonic treatment. (C) SDS–PAGE of AK subjected to microwave treatment. (D) Dot blotting of AK subjected to microwave treatment. (E) SDS–PAGE of AK subjected to boiling treatment. (F) Dot blotting of AK subjected to boiling treatment. M, protein marker, con, control.

application of high-intensity ultrasound results in chemical and physical changes (Gunasekaran, 1994). Li et al. (2006) reduced the allergic properties of shrimp allergens using high-intensity ultrasound. However, in our study, ultrasound treatment did not degrade crayfish AK or denature the IgE-binding sites of the protein (Fig. 7A and B). Food processing conditions that rely on severe heating methods are known to induce physical and chemical changes in protein molecules. The allergenicity of bovine allergens can be reduced by heating cow's milk for 10 min (Paschke and Besler, 2002). Our results revealed that the microwave and boiling treatments resulted in the formation of AK aggregates (Fig. 7C and E). The IgE-binding activity of AK was significantly reduced after boiling (Fig. 7F). The purified protein is not a food source; therefore, future research should process the original crayfish and then extract and characterize crayfish AK.

5. Conclusions

AK with a molecular mass of 40 kDa was extracted from freshwater crayfish (*P. clarkii*) and confirmed by MALDI-MS. AK was

identified as a crayfish allergen by its IgE-binding activity. The gene that codes for crayfish AK, which consists of 357 amino acid residues, was cloned for B-cell epitope prediction. The physicochemical characteristics of crayfish AK, including glycosylation, isoelectric point, and thermal, pH and digestion stabilities, were assessed. The IgE-binding activity of AK is reduced by boiling. Future research should focus on effective processing methods that simultaneously reduce the allergenicity of AK, tropomyosin, and other allergens.

Conflict of Interest

The authors declare that there are no conflicts of interest.

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