

Identification of physicochemical properties of *Scylla paramamosain* allergen, arginin kinase

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Abstract

BACKGROUND: Arginine kinase (AK) is expressed in a wide variety of species, including human food sources (seafood) and pests (cockroaches and moths), and has been reported as a novel allergen. However, there has been little research on the allergenicity of AK in crustaceans. In this study the physicochemical properties of AK from mud crab (*Scylla paramamosain*) were investigated.

RESULTS: Analysis by sodium dodecyl sulfate polyacrylamide gel electrophoresis, immunoblotting and inhibition enzyme-linked immunosorbent assay revealed that purified AK was unstable in thermal processing and in acid buffer. Under simulated gastric fluid (SGF) and simulated intestinal fluid (SIF) conditions, purified AK was much more readily degraded by pepsin than by trypsin or chymotrypsin. The unpurified AK in crab myogen degraded more markedly than purified AK. In addition, in two-phase gastrointestinal digestion, AK was rapidly degraded by pepsin but resistant to trypsin and chymotrypsin digestion, while tropomyosin derived from mud crab was resistant to pepsin digestion but digested readily by trypsin or chymotrypsin. Further study of serum samples obtained from crab-allergic human patients indicated that the allergenicity of AK was markedly reduced by digestion with SGF but not SIF.

CONCLUSION: AK is an important food allergen despite its unstable physicochemical properties of digestibility.

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Keywords: *Scylla paramamosain*; allergen; arginine kinase; tropomyosin; stability; gastrointestinal digestion

INTRODUCTION

In recent years, food allergy has attracted much media and medical community attention. It has been reported that food-related type I hypersensitivity is mediated by immunoglobulin E (IgE) antibodies and manifested as urticaria, atopic dermatitis, bronchial asthma, rhinoconjunctivitis, Quincke's oedema, oral allergy syndrome and life-threatening anaphylaxis.^{1,2} Epidemiological survey has indicated that adverse immune responses to foods affect approximately 5% of young children and 3–4% of adults in western countries, and these rates represent a steady upward trend.³

Crustaceans are among the eight major human food sources associated with allergic response as classified by the FAO.⁴ Crustacean-based food products are widely consumed and have gained in popularity since the availability of processed crustacean products that possess a longer shelf life and are convenient to obtain and prepare. According to Sicherer *et al.*,⁵ the rate of food-allergic reactions (dyspnoea or throat tightness) to crustacean-based food products may be as high as 38%. Moreover, allergic cross-reactivity is common among crustacean allergies, so that patients who are allergic to one member of the crustacean family (e.g. shrimp) may also have an allergic reaction to another member (e.g. mud crab).⁵

A number of allergens have been identified that interact with serum IgE from atopic patients and can lead to IgE-mediated disease, but only a few of them have been described as major allergens of crustaceans.^{6,7} Tropomyosin (TM) has been shown to

be the major allergen of crab; it is heat-stable, with a molecular mass of 35–38 kDa and an isoelectric point of 4.5.^{8,9} TM has been recognised as a cross-reactive allergen and implicated in the cross-reactive allergies observed between various types of edible crustacean and mollusc.¹⁰ In addition, the phosphagen kinase arginine kinase (AK), which is normally involved in energy metabolism in invertebrates, has been confirmed as a novel crustacean allergen. Garcia-Orozco *et al.*¹¹ purified AK from white shrimp (*Litopenaeus vannamei*) and characterised its molecular mass as 40 kDa. It was also determined by enzyme-linked immunosorbent assay (ELISA) and immunoblotting analysis that AK was recognised by IgE in serum from shrimp-allergic individuals. AK was identified as the second most important allergen in snow crab using mass spectrometry, designated Chi o 3.¹² AK from mud crab has also been purified, cloned, expressed and analysed in our laboratory, and we have identified AK as a specific allergen of mud crab.¹³ Other studies have provided similar evidence to implicate AK from moths, dust mites and cockroaches as allergens.^{14–16}

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It has been asserted that the allergen is stable enough to retain its allergenicity during food-processing treatments or during *in vivo* digestion after consumption.¹⁷ However, according to Privalle *et al.*,¹⁸ thermal stability has no known predictive value in the allergenicity risk assessment of a novel food protein, but thermal deactivation may be related to the toxicological risk assessment for cooked or processed food. It is always important to study the physicochemical characterisation of food proteins, especially food allergens.

TM from mud crab has been experimentally demonstrated to be stable in normal food-processing treatments, except for high-pressure treatment, and to gastrointestinal digestion, although it may be degraded into smaller fragments with lower allergenicity.^{19,20} However, the physicochemical characterisation and digestive stability of AK from mud crab have yet not been established. Such characterisation of AK will help advance the development of accurate diagnostic and effective therapeutic tools for crab-allergic patients.

The focus of this study, therefore, was to investigate the digestibility of purified AK and myogen from mud crab by simulated gastrointestinal digestion and to determine the thermal and pH stability of AK. Moreover, the difference in digestibility between AK and TM in crab crude extract was investigated by two-phase simulated gastrointestinal digestion. Western blotting and inhibition ELISA were used to analyse the IgG/IgE-binding activity of AK and its digested products.

MATERIALS AND METHODS

Animals

Live mud crabs (*Scylla paramamosain*) were purchased from Jimei market, Xiamen, China and sacrificed in the laboratory for immediate use or storage at -80°C .

Chemicals

HiTrapQ Sepharose for protein purification was purchased from Bio-Rad (San Francisco, CA, USA). Protein standards for sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting were obtained from Fermentas (Vilnius, Lithuania) and New England BioLabs (Beverly, MA, USA) respectively. Horseradish peroxidase (HRP)-labelled goat anti-human IgE was purchased from Kirkegaard & Perry Laboratories (Gaithersburg, MD, USA). HRP-labelled goat anti-rabbit IgG and 3,3'-diaminobenzidine tetrahydrochloride (DAB) were obtained from Pierce (Rockford, IL, USA). Porcine trypsin and bovine chymotrypsin were purchased from Sigma-Aldrich (St Louis, MO, USA) and Amresco (Cleveland, OH, USA) respectively. Purified rabbit anti-mud crab TM and rabbit anti-mud crab AK polyclonal antibodies were prepared in our laboratory.^{13,21} Porcine pepsin was prepared in our laboratory as described previously.²²

The activity of pepsin was estimated by measuring trichloroacetic acid-precipitable bovine haemoglobin after hydrolysis for 30 min. One unit of pepsin activity was defined as an increase in absorbance of 1.0 at 280 nm after 30 min of incubation at 37°C . The activity of trypsin was measured using Boc-Phe-Ser-Arg-4-methyl-coumaryl-7-amide (MCA) as substrate, while that of chymotrypsin was measured using Suc-Leu-Leu-Val-Tyr-MCA as substrate, both methods being applied as described previously.^{23,24} The fluorescence intensity of liberated 7-amino-4-methylcoumarin (AMC) was measured using a fluorescence spectrophotometer (FP-6200, Jasco, Tokyo, Japan) at an excitation

wavelength of 380 nm and an emission wavelength of 450 nm. One unit of trypsin or chymotrypsin activity was defined as the amount of enzyme required to release 1 nmol AMC $\text{L}^{-1} \text{min}^{-1}$.

Human serum samples

Serum samples were obtained from patients ($n = 6$) admitted to the Hospital of Jimei University and the Second Hospital of Xiamen. Crustacean allergy was confirmed on the basis of extensive history, physicochemical examination and objective manifestations observed after ingestion of crustacean products, usually crab. Written informed consent was obtained from each patient. In this study the six serum samples (05 990 605, 05 877 050, 05 886 969, 05 990 133, 05 991 423 and 04 366 639) were pooled for use in immunoassays. Negative controls were serum samples collected from healthy individuals without any history of crustacean allergy (05 981 132 and 05 980 025). All serum samples were stored at -80°C until use.

Purification of AK

AK was purified according to the method described by Yu *et al.*²⁵ with minor modifications. All procedures were performed either on ice or at 4°C . Crab muscle tissues were minced and homogenised in buffer A (0.1 mol L^{-1} Tris-HCl, 10 mmol L^{-1} β -mercaptoethanol (2-ME), 1 mmol L^{-1} ethylenediaminetetraacetic acid (EDTA), $5 \mu\text{mol L}^{-1}$ sodium azide (NaN_3), $25 \mu\text{mol L}^{-1}$ phenylmethanesulfonyl fluoride (PMSF), pH 8) using a homogeniser (Kinematica, Lucerne, Switzerland). The homogenate was then centrifuged at $12\,000 \times g$ for 20 min and the supernatant was adjusted to saturation by ammonium sulfate fractionation with 70–90% saturation. After centrifugation at $20\,000 \times g$ for 20 min the precipitate was collected, dissolved in buffer B (10 mmol L^{-1} Tris-HCl, 10 mmol L^{-1} 2-ME, 0.1 mmol L^{-1} EDTA, pH 8) and dialysed against the same buffer. After centrifugation at $12\,000 \times g$ for 20 min the clear supernatant was applied to a HiTrapQ Sepharose Fast Flow column pre-equilibrated with buffer B. Fractions with AK activity were eluted with a linear gradient of $0\text{--}1 \text{ mol L}^{-1}$ NaCl in buffer B. Protein concentration of the purified AK was estimated by the Lowry method²⁶ with bovine serum albumin (BSA) as standard.

Preparation of myogen and crude extract from mud crab

Mud crab muscle tissue was minced and homogenised with 5 volumes of ice-cold 10 mmol L^{-1} Tris-HCl buffer (pH 7 for myogen and pH 7 containing 0.25 mol L^{-1} NaCl for crude extract). The homogenate was then centrifuged at $8000 \times g$ for 20 min at 4°C and the supernatant was regarded as the crab myogen or crab crude extract.¹³ Protein concentration was estimated by the Lowry method²⁶ with BSA as standard.

Thermal and pH stability determination

To evaluate the thermal stability of AK, purified AK was diluted to a concentration of 0.15 mg mL^{-1} and incubated at pH 7 and different temperatures (30, 40, 42, 44, 46, 48, 50, 60, 70, 80, 90 and 100°C) for 30 min. Samples were then electrophoresed on SDS-PAGE gels, followed by Coomassie Brilliant Blue (CBB) R-250 staining, as described by Laemmli.²⁷ To investigate the effect of pH on the stability of AK, proteins were incubated at room temperature in buffers of different pH (1, 2, 3, 4, 5, 7, 9 and 11) for 1.5 h, followed by SDS-PAGE analysis. The buffers used were HAc/NaAc (pH 1–5), phosphate-buffered saline (PBS, pH 7) and $\text{Na}_2\text{CO}_3/\text{NaHCO}_3$ (pH 9 and 11).²⁸

Simulated gastric fluid (SGF) digestion stability assay

The digestibility of AK in SGF was estimated as described by Astwood *et al.*¹⁷ and Takagi *et al.*²⁹ with minor modifications. SGF was prepared as recommended by the USP³⁰ and consisted of porcine pepsin (109 U mg⁻¹ protein) in 25 mmol L⁻¹ NaCl (pH 1.2). The total volume of reaction solution was 1 mL. For each sample, SGF (1 : 5000 pepsin to AK (150 µg mL⁻¹)/crab myogen (3 mg mL⁻¹), w/w) was preheated at 37 °C for 15 min before adding the test protein. Digestion was performed at 37 °C with continuous rocking. At each scheduled time point (0, 1, 2, 5, 10, 15, 30 and 60 min), 100 µL of the reaction solution was removed to a separate sampling tube and the reaction was immediately terminated by adding 30 µL of 200 mmol L⁻¹ Na₂CO₃ and 26 µL of 4× Laemmli buffer. The samples were heated at 95 °C for 10 min and analysed by SDS-PAGE or western blotting. For the 0 min samples, protein samples were added to SGF that was neutralised with Na₂CO₃. In the control experiments, each protein sample was mixed with the reaction solution without pepsin and then reacted at 37 °C for 60 min. All experiments were performed in triplicate.

Simulated intestinal fluid (SIF) digestion stability assay

For intestinal digestion, SIF was also prepared as recommended by the USP³⁰ and consisted of trypsin or chymotrypsin in 0.05 mol L⁻¹ KH₂PO₄ (pH 7.5). The total volume of reaction solution was 1 mL. The enzyme activity of trypsin and chymotrypsin was 59 and 102 U mg⁻¹ protein respectively. The ratio of trypsin or chymotrypsin to purified AK (150 µg mL⁻¹) was 1 : 50 (w/w) and the ratio of trypsin or chymotrypsin to crab myogen (3 mg mL⁻¹) was 1 : 300 (w/w). Digestion was carried out at 37 °C with continuous rocking. At each scheduled time point (0, 1, 15, 30, 60, 120, 180 and 240 min), 100 µL of the reaction solution was removed to a separate sampling tube and the reaction was immediately terminated by adding the loading buffer and heating at 95 °C for 5 min. The samples were analysed by SDS-PAGE or western blotting. For the 0 min samples, protein samples were added to SIF that was inactivated by heating at 95 °C for 5 min. In the control experiments, each protein sample was mixed with the reaction solution without the corresponding enzyme and then reacted at 37 °C for 4 h. All experiments were performed in triplicate.

Two-phase digestion

The crab crude extracts were first subjected to SGF digestion, as described above (the ratio of pepsin to protein was 1 : 1000), and then switched to SIF digestion, as described above (the ratio of trypsin and chymotrypsin to protein was 1 : 1:50). The reactions were then followed by SDS-PAGE and western blotting analysis.³¹

Prediction of tertiary structures of mud crab TM and AK

The amino acid sequences of mud crab TM and AK were determined previously in our laboratory.^{13,21} Prediction of the tertiary structures of mud crab TM and AK was carried out using the CPH-models 3.0 Server (<http://www.cbs.dtu.dk/services/CPHmodels/>). The pyMol Molecular Graphics System (Version 1.3, Portland, USA) was employed to analyse the results.

SDS-PAGE analysis and immunoblotting

Proteins were separated by SDS-PAGE and the protein bands were visualised using CBB.

For detection of the IgG-binding activity of AK/TM, protein samples were subjected to SDS-PAGE followed by electrophoretic

transfer onto nitrocellulose membranes. The membranes were then blocked with skim milk in Tris-HCl-buffered saline (TBS; 20 mmol L⁻¹ Tris-HCl, pH 7.5, 0.145 mol L⁻¹ NaCl), after which they were incubated with rabbit anti-mud crab AK polyclonal antibodies (1 : 4000 dilution) or rabbit anti-mud crab TM polyclonal antibodies (1 : 4 000 000 dilution) at room temperature for 2 h and washed with TBST (TBS plus 0.05 mL L⁻¹ Tween-20). HRP-labelled goat anti-rabbit IgG secondary antibodies (1 : 2000 dilution) were added and incubation was carried out for 1 h, then the membranes were washed five times with TBST. The immunoassay detection was carried out using DAB as substrate.³²

To estimate the IgE-binding activity of AK and its digestion products, the pooled human serum (1 : 2 dilution) was used as primary antibody and peroxidase-conjugated goat anti-human IgE antibodies (1 : 2000 dilution) were used as secondary antibody. Incubations were carried out at room temperature for 2 h, followed by extensive washing and detection using DAB.

Inhibition ELISA

Inhibition ELISA was performed to determine the specificity of IgE binding of purified AK digestion fragments. First, 200 ng of AK was placed in each well of a polystyrene 96-well ELISA plate (Maxisorb, Nunc, Copenhagen, Denmark) and incubated overnight at 4 °C with 156.9 mmol L⁻¹ Na₂CO₃ and 72.5 mmol L⁻¹ NaOH (pH 9.6) diluted in distilled water as coating buffer. The next day, blocking was carried out by incubation with 5 g L⁻¹ skim milk in TBST for 2 h at 37 °C. In another microtitre plate, 50 µL aliquots of human serum (diluted 1 : 5 with 1 g L⁻¹ non-fat milk in TBS) were mixed with the same volumes of digested AK (in the concentration range 0.012–24 µg mL⁻¹) as inhibitor. After incubation at 37 °C for 1 h, 50 µL of the mixture (serum with digested AK) was transferred to the ELISA plate precoated with AK and incubation was carried out for 1 h at 37 °C. The plates were washed again and HRP-conjugated goat anti-human IgE antibodies (1 : 2000 dilution) were added. After incubation the bound peroxidase activity was determined by reaction with 3,3',5,5'-tetramethylbenzidine (TMB). The absorbance at 450 nm was determined using an automated ELISA plate reader (Benchmark 96, Bio-Rad Laboratories, Hercules, CA, USA). The hypersensitivity of the digested AK fragments was determined by calculating the inhibition rate using the formula³³

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

where X is the absorbance of patients' serum not incubated with inhibitors and Y and Z are the absorbances of patients' and control sera reacted with inhibitors respectively. All determinations were duplicated and mean values were used.

RESULTS AND DISCUSSION

Thermal and pH stability of AK

The thermal stability of AK was detected by SDS-PAGE. As shown in Fig. 1(a), AK was relatively stable at 30–40 °C. However, as the temperature increased, the protein structure began to change, accompanied by formation of precipitates in the reaction system, until an AK dimer was formed (apparent as a band at 80 kDa). When the temperature reached 90–100 °C, almost the entire band of AK appeared to be denatured. No band of AK or its dimer was observed from the analysis by SDS-PAGE, suggesting that AK became unstable and denatured with increasing temperature, especially at temperatures higher than 46 °C. Here we were focused mainly

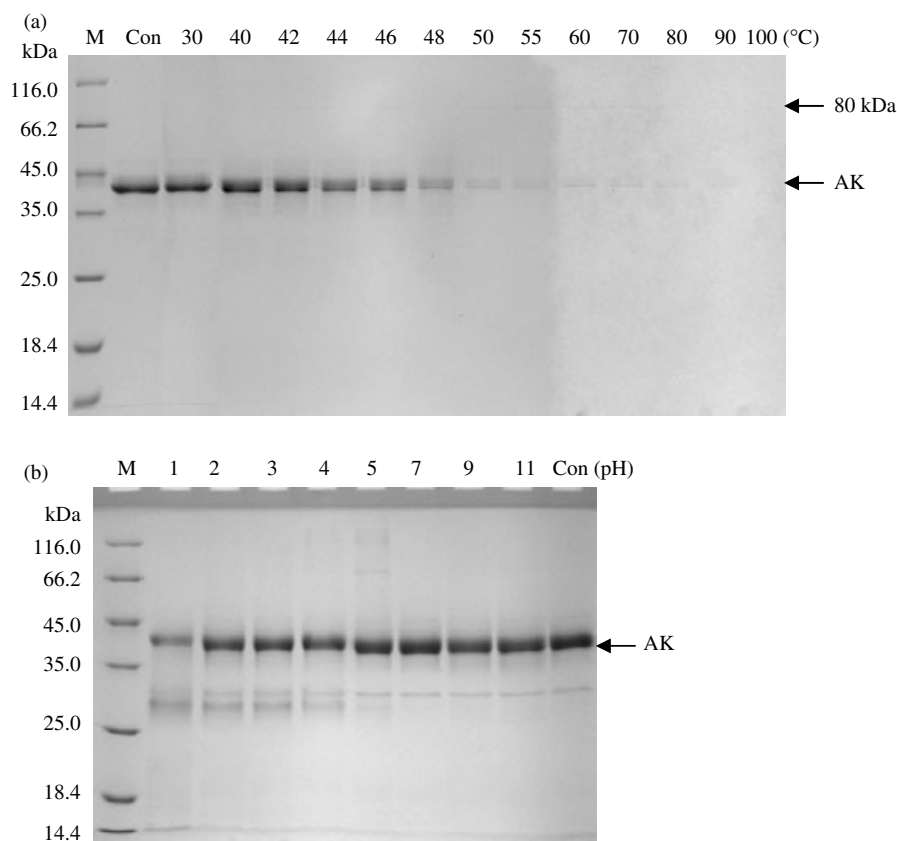


Figure 1. Physicochemical properties of purified AK. (a) Thermal stability: AK was incubated at different temperatures for 30 min. (b) pH stability: AK was incubated at 30 °C for 1.5 h in buffers of different pH. After incubation, samples were subjected to SDS-PAGE. Con, control; M, protein markers. Incubation temperature or pH is indicated above each lane.

on the denaturation temperature of AK, so the precipitates were not resolved for further analysis. It was previously reported that proteins exhibit a characteristic temperature-dependent pattern of loss of tertiary structure, followed by unfolding and loss of secondary structure (at 55–70 °C), after which cleavage of disulfide bonds occurs (at 70–80 °C), followed by new intra/intermolecular interactions, rearrangements of disulfide bonds (at 80–90 °C) and formation of aggregates (at 90–100 °C).³⁴ Thus it was indicated that AK possesses the characteristic of thermal instability.

The pH stability of AK was also examined by SDS-PAGE. AK appeared to be rather stable in the pH range 1–11 when it was incubated in buffers of different pH for 30 min (data not shown). However, when the incubation time was extended to 1.5 h, the stability of AK decreased (Fig. 1(b)). The results showed that the stability was particularly affected by acid buffers, and AK was somewhat degraded at pH 1. Whether the conformation and other optical properties of AK were altered or not needs to be further investigated.²⁸

Considering that crustacean allergens are generally characterised as heat- and acid-stable proteins, it was surprising to discover that AK was less stable to heat and acidity than expected. However, this finding is similar to that described for the allergens in milk (BSA) and egg (ovalbumin),²⁹ indicating that unstable allergens are still able to lead to allergic reactions.

Digestibility of purified AK by SGF and SIF

The digestibility of crab purified AK in SGF was analysed by SDS-PAGE. A band with a molecular mass of about 40 kDa was

observed (Fig. 2). *In vitro* digestion with pepsin led to AK being gradually digested over time. A major fragment of about 34 kDa was produced after 2 min of pepsin digestion, along with smaller amounts of approximately 25–28 kDa fragments. The density of the band at 34 kDa observed by SDS-PAGE gradually increased and then decreased with incubation time up to 1 h (Fig. 2(a)). In contrast, SIF digestion produced no detectable AK degradation by trypsin, regardless of self-degradation (Fig. 2(b)). In addition, the degradation during digestion with α -chymotrypsin was lower, and the fragments produced after 1 min were principally within the ranges 25–35 and 14.4–18.4 kDa. When the α -chymotrypsin digestion time was extended to 1 h, no degradation fragments appeared on SDS-PAGE. AK was determined to be incompletely degraded after 4 h of digestion with α -chymotrypsin (Fig. 2(c)).

Experimental conditions and enzyme ratios were optimised multiple times, and the ratios selected for the experiment were the most appropriate. The amount of enzymes might seem to be low, but their digesting capacity is much higher (109 U mg⁻¹ protein for pepsin) and is the most important value to measure. If we increased the amount of enzymes or reduced the amount of substrates, it might be difficult to observe and analyse the digestion of the target protein and its degraded products.

Digestibility of crab myogen by SGF and SIF

Crab myogen is principally composed of AK along with other non-allergic proteins. In the SGF reaction system (Fig. 3) the protein with a band at 70 kDa and the intact band of AK were both gradually degraded within 1 h of incubation. The intact AK protein

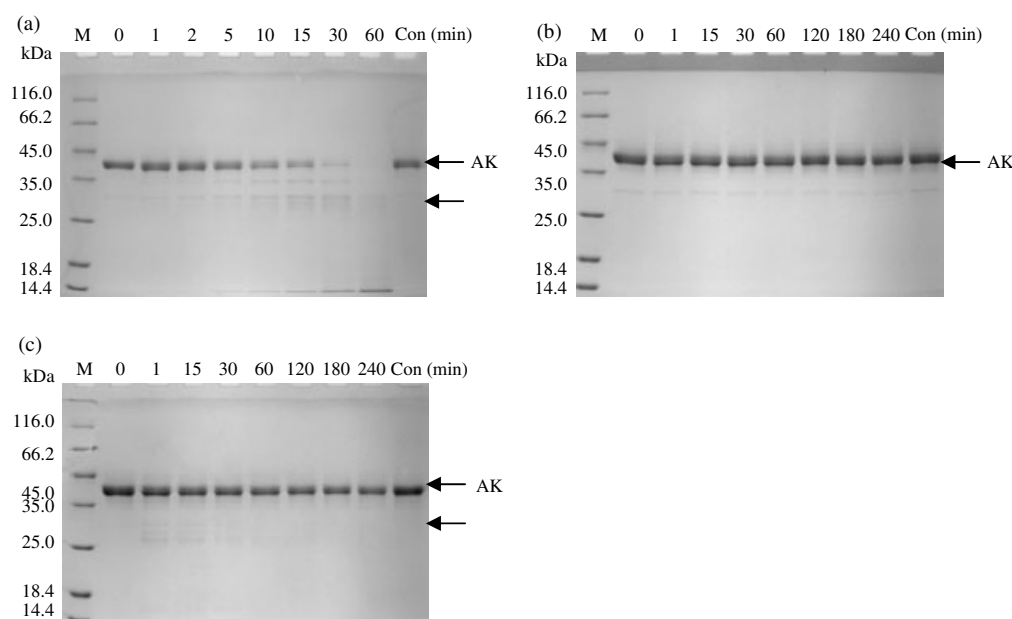


Figure 2. Effect of SGF and SIF digestion on crab purified AK. Proteinases used were (a) pepsin, (b) trypsin and (c) chymotrypsin. After electrophoresis, proteins were visualised by staining with CBB. In control (Con) experiments, proteinase was replaced with 25 mmol L⁻¹ PBS (pH 7.5). Molecular masses of protein markers (M) are shown on the left.

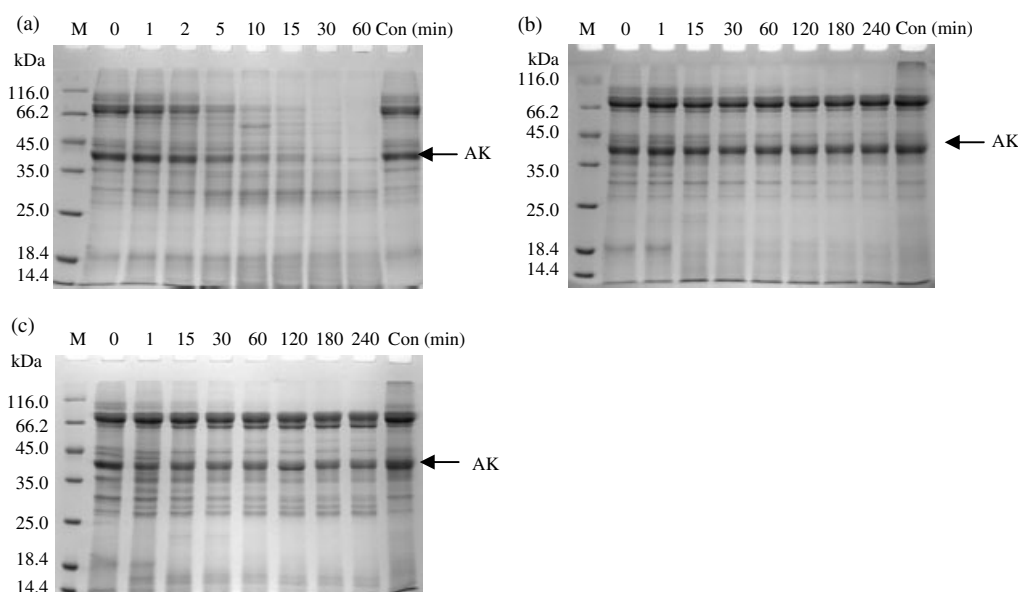


Figure 3. Effect of SGF and SIF digestion on crab myogen proteins. Myogen proteins were incubated with (a) pepsin, (b) trypsin and (c) chymotrypsin for the indicated times. Enzyme digestion was performed as described in 'Materials and methods', followed by SDS-PAGE.

was almost completely digested by the end of the SGF reaction (Fig. 3(a)). The digestibility of AK in myogen was almost the same as that of purified AK during the SIF reaction. AK was resistant to trypsin digestion (Fig. 3(b)) and was also hardly degraded by chymotrypsin (Fig. 3(c)).

Since the myogen contained non-allergic proteins in addition to the allergic protein (AK), it was difficult to distinguish between the degraded fragments originating from AK and those generated from non-allergic proteins with low molecular weights by SDS-PAGE. In order to attain a precise understanding of the degraded AK pattern from myogen, western blotting was used. Specific rabbit polyclonal antibodies against mud crab AK were applied

to identify the band of AK and its degraded fragments. Figure 4 shows the western blotting figures that correspond to Fig. 3. The degradation pattern of AK from myogen was found to be similar to that obtained with purified AK (Fig. 2). However, we noted that the band of AK of myogen was degraded slightly faster than that of purified AK. In the SGF digestion assay the band of AK was rapidly degraded to 34 kDa or smaller fragments; moreover, the band of AK and its degraded products was almost completely digested after 30 min, while the purified AK required 60 min of digestion (Figs 2(a) and 4(a)). On the other hand, digestion with the trypsin-based SIF reaction revealed that AK was rather stable to trypsin digestion and only produced a faint fragment at about

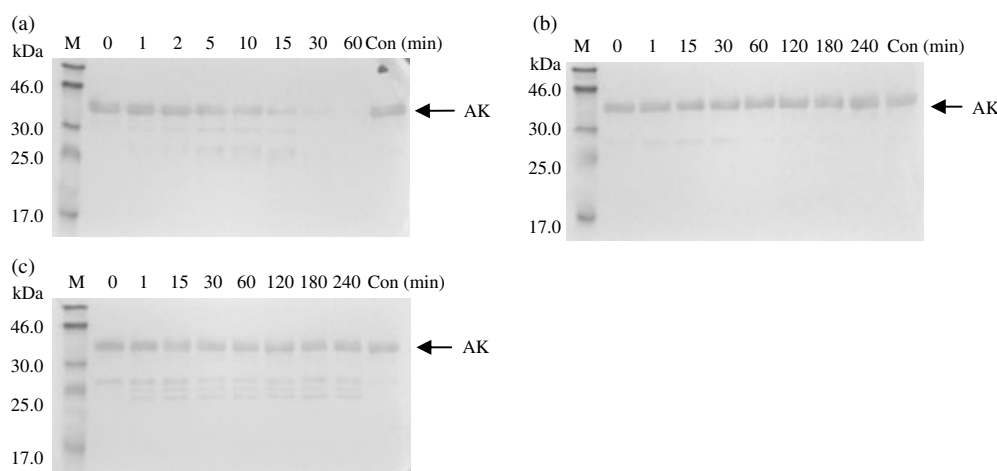


Figure 4. Differential degradation properties of AK and myogen in SGF and SIF. Proteinases used were (a) pepsin, (b) trypsin and (c) chymotrypsin. Western blotting detection was performed using a polyclonal antibody against mud crab AK. Positions of prestained molecular mass standards (M) are shown on the left.

28 kDa. This fragment quickly disappeared after 1 h of digestion, but the band of AK remained even after 4 h of digestion (Fig. 4(b)). Chymotrypsin digestion produced a distinct degradation pattern that was different from that of purified AK (Figs 2(c) and 4(c)). In this reaction the band of AK was robustly digested to give digested products in the range 25–28 kDa. AK was not completely digested by the end of the reaction (4 h).

Our results from *in vitro* simulated gastrointestinal digestion of purified AK and crab myogen demonstrated that the digestive patterns of the three proteases (pepsin, trypsin and chymotrypsin) were more robustly different because of their different cleavage specificities for peptide bonds. In SGF digestion the band of AK (as visualised by SDS-PAGE) mainly produced a proteolytic fragment at 34 kDa and faint fragments between 25 and 28 kDa (Figs 2(a) and 4(a)). In contrast, in SIF digestion the main proteolytic fragments were of 25–28 kDa and the band of AK could not be digested even after 4 h of reaction (Figs 2(c), 4(b) and 4(c)).

Trypsin and chymotrypsin both belong to the family of serine proteinases. Serine proteinase inhibitors have been identified in marine species, including fish, where they have been demonstrated to modulate proteinase activities and regulate a variety of crucial proteinase-mediated processes.^{23,35} So far, all marine species' serine proteinase inhibitors have been purified from myogen. Therefore the crab myogen may contain an inhibitor of trypsin and chymotrypsin. The purified AK may also contain a trypsin and chymotrypsin inhibitor. Unfortunately, if such inhibitors were present in very low concentrations, they would be undetectable by CBB staining. Their presence, however, would explain why the enzymatic activity of trypsin and chymotrypsin appeared to be ineffective against AK (Figs 2(b) and 2(c)).

It was intriguing that the band of AK in crab myogen was digested more rapidly than that of purified AK in both SGF and SIF reactions. It is possible that this kind of inhibitor in crab myogen may react with other proteins, leaving the enzymatic activity of trypsin/chymotrypsin unaffected, so that the AK in myogen may be digested more obviously (Figs 4(b) and 4(c)) than the purified AK (Figs 2(b) and 2(c)). In addition, other kinds of enzyme present in myogen may affect the digestion of AK. We observed that the digestibility of AK in crab myogen was obviously affected by the existence of other proteins. However, in order to gain a detailed

understanding of the effect of trypsin/chymotrypsin inhibitor(s) on the digestion of AK, further studies need to be performed.

In the experimental system used in this study, we set up control ('Con') and '0 min' samples for the digestion experiment. The 'Con' samples would identify the stability of the target protein AK during the reaction in SGF for 1 h or in SIF for 4 h without digestive enzymes, while the '0 min' samples would not. As shown in Figs 3(b) and 3(c), there were slight differences between the protein bands detected from the 'Con' and '0 min' reactions in SIF digestion, while in SGF digestion the 'Con' reaction produced nearly identical protein bands to the '0 min' reaction (Fig. 3(a)). The reason for the difference may be that the reaction time for SIF was as long as 4 h, causing some unstable proteins of the crab myogen to display faint degradation.

Immunoblotting and inhibition ELISA

The IgE-binding capabilities of AK fragments produced by digestion with proteinases (pepsin, trypsin and chymotrypsin) were analysed by SDS-PAGE (Fig. 5(a)) and IgE immunoblotting (Fig. 5(b)). Western blotting using pooled serum samples from patients with crab AK allergy showed that their IgE antibodies reacted positively with the band of AK (40 kDa) (Fig. 5(b)). Clearly, IgE-binding epitopes in the original AK still existed, even after digestion by pepsin, trypsin or chymotrypsin. However, the degraded bands of AK with sizes of 34 and 25–28 kDa reacted only slightly with the pooled serum, as evidenced by their very faint detection by western blotting (Fig. 5(b)). These findings suggested that the IgE-binding sites corresponding to AK digested products may have been partly (or completely) destroyed during the digestion process and were unable to react with IgE antibodies.

In order to further analyse the IgE-binding capabilities of AK-derived digestion fragments, the inhibition ELISA technique was used based on its sensitivity in detecting proteolytic fragments with low IgE reactivity. The degree of IgE binding was indirectly measured by determining the extent of inhibition of IgE reactivity as observed by the digested fragment pattern visualised by SDS-PAGE: the greater the inhibition, the more allergenic were the digested proteins.³⁶ As shown in Fig. 6, AK digested by pepsin for 1 h displayed less inhibition than undigested AK. However, trypsin and chymotrypsin digestion carried out for 4 h had no effect on

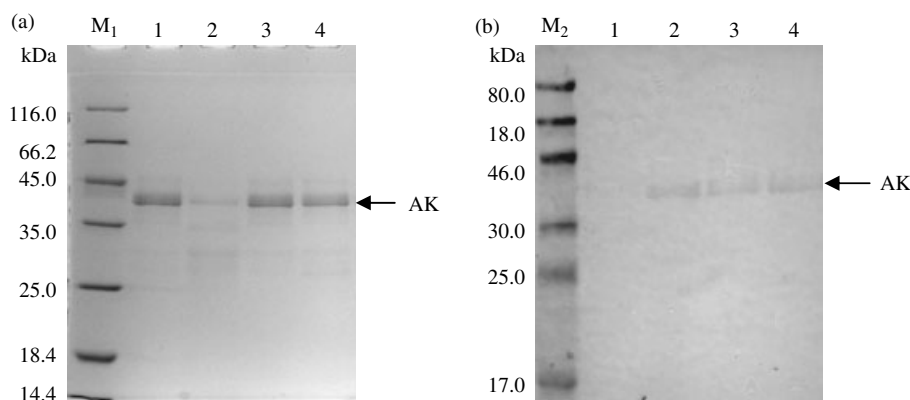


Figure 5. IgE binding of purified AK after digestion by SGF and SIF. (a) SDS-PAGE: lane M₁, protein marker; lane 1, purified AK; lane 2, digested with pepsin; lane 3, digested with trypsin; lane 4, digested with chymotrypsin. (b) IgE western blotting of human serum: lane M₂, prestained molecular mass standards; lane 1, digested with pepsin; lane 2, digested with trypsin; lane 3, digestion time of 15 min; 4, purified AK.

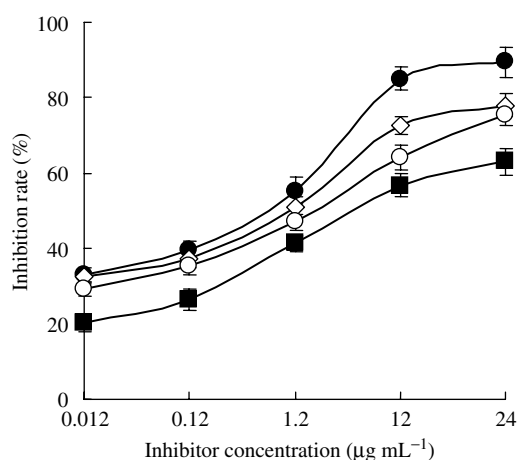


Figure 6. Inhibition ELISA analysis of mud crab AK digested fragments. AK samples digested by pepsin (■, 1 h), trypsin (◇, 4 h) and chymotrypsin (○, 4 h) and undigested AK (●) were diluted 1:5, 1:10, 1:100, 1:1000 and 1:10 000 and used as inhibitor solutions. Pooled patients' serum (1:2 dilution) was used. The initial protein concentration of each sample was 150 μg mL⁻¹.

the IgE-binding activity of AK, indicating that there might be an inhibitory effect.

Importantly, the data from immunoblotting and inhibition ELISA were concordant and supported the conclusion that IgE binding of AK remained intact under gastrointestinal-like digestion conditions. To further evaluate the IgE-binding alterations in AK-derived proteinase-digested fragments, which may be useful in developing novel therapeutic tools for crab-allergic patients, experiments such as the release of histamine from basophils and the skin prick test should be carried out.

***In vitro* gastrointestinal digestion of crab crude extract (two-phase digestion)**

During phase 1 digestion in SGF, TM was found to be very resistant to pepsin. There was only one degraded fragment (~34 kDa) produced by digestion for 30 min, and the original band of TM was insufficient to completely degrade by digestion for 1 h (Figs 7(a) and 8(a)). In contrast, AK was readily digested by pepsin after 1 min of incubation, and the degraded fragments were of 25–36 kDa. Digestion for 10 min led to nearly complete

degradation of the original AK, highlighting the differences in physiochemical properties between AK and TM (Fig. 8(c)). The difference in pepsin sensitivity might reflect the differences in primary and secondary structures of TM and AK. Analysis of the amino acid sequences of mud crab TM and AK¹³ indicated that both contain the enzyme-cutting sites for pepsin (at amino acid 14 in TM and at amino acid 30 in AK), suggesting further analysis of the tertiary structure in order to determine why AK was much more readily digested by pepsin than TM (discussed below).

During phase 2 digestion in SIF, the original band of TM and its derived fragments were further degraded by trypsin and chymotrypsin, producing another 36 kDa fragment and leading to complete degradation after 4 h (Figs 7(b) and 8(b)). When compared with the digestion patterns obtained with purified AK and myogen, TM was considered to be relatively sensitive to trypsin and chymotrypsin digestion.

Since orally ingested foods travel to the stomach, SGF digestion plays an important role in the overall digestion process. According to our findings from the *in vitro* two-phase simulated digestion (SGF and SIF), AK was more readily digested by pepsin than TM and was a more unstable allergen.

Prediction of tertiary structures of mud crab TM and AK

We only cloned the DNA encoding for a single strand of the TM protein, so our prediction of the TM tertiary structure was based on that strand alone. The structure of TM was linear and composed almost completely of an α -helix (Fig. 9(a)). The structure of AK, on the other hand, was much more complex and composed of eight antiparallel β -sheets surrounded by nine α -helices (Fig. 9(b)). In addition, analysis of the two tertiary structures by the pyMol system indicated that the trypsin and chymotrypsin enzyme-cutting sites were almost exposed on the TM surface but mostly embedded inside the AK protein. Therefore AK would be expected to be much more resistant to trypsin and chymotrypsin digestion, as observed in the SIF assay.

Considering the results reported by Huang *et al.*,¹⁹ the simulated two-phase digestion study revealed that crab AK displayed different digestion fragment patterns from crab TM and TM of other crustaceans. Our immunoassay study also revealed that AK is an allergic protein of crab, which can bind to IgE and may lead to anaphylactic reactions.

AKs from other invertebrate species such as migratory locust,³⁷ beetle³⁸ and greasyback shrimp³⁹ have been investigated previ-

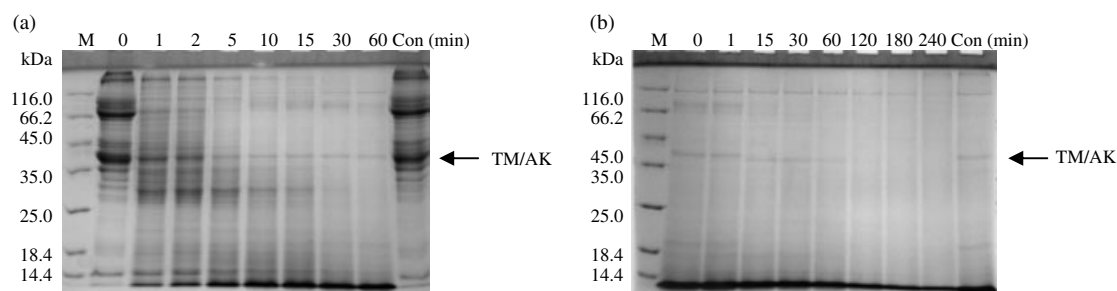


Figure 7. Effect of SGF and SIF digestion on crab crude extract. Pepsin was used in phase 1 of enzymatic digestion (a), followed by trypsin and chymotrypsin in phase 2 (b). Enzymatic digestion was performed as described in 'Materials and methods', followed by SDS-PAGE. In control (Con) reactions, proteinase was replaced with 25 mmol L⁻¹ PBS (pH 7.5). Molecular masses of protein markers (M) are shown on the left.

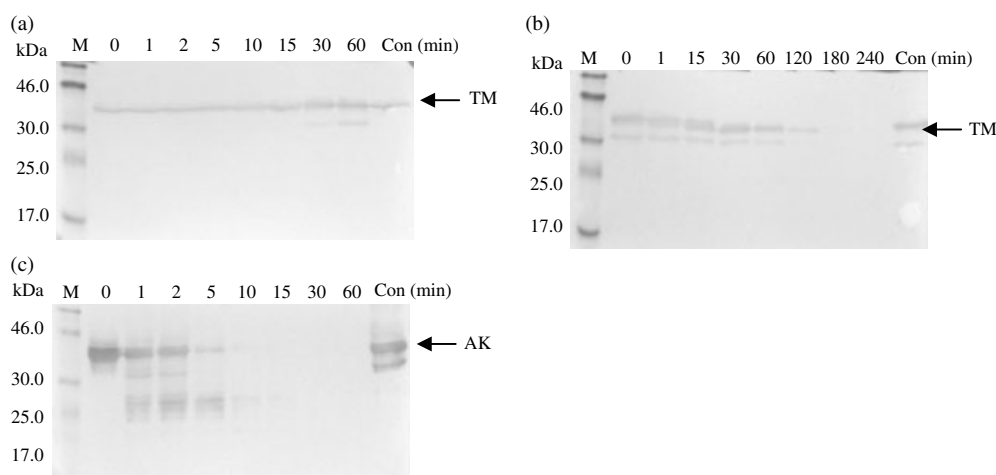


Figure 8. Western blotting detection of degradation of TM and AK from crab crude extract in SGF and SIF. Proteinases used were (a, c) pepsin and (b) trypsin/chymotrypsin. Immunological detection of electrophoretically separated proteins on membranes was performed using a polyclonal antibody against (a, b) mud crab TM or (c) mud crab AK. Positions of molecular mass standards (M) are shown on the left.

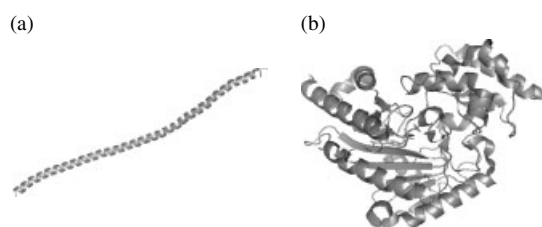


Figure 9. Tertiary structures of (a) mud crab TM (single strand) and (b) mud crab AK.

ously and may provide further insights into the as yet uncharacterised activities of mud crab AK. Since the enzymatic properties of AK were not the key point of the present study, we did not focus on them. However, future investigations on the functional relationship between mud crab AK activity and allergenicity will advance our overall understanding of crab-specific allergies.

CONCLUSIONS

We have provided the first characterisation of AK properties related to thermal and pH conditions and determined the digestibility of AK mediated by enzymes encountered in the human gastric system. In particular, AK was found to be vulnerable to higher temperatures (>46 °C) and high acidity (pH 1). During simulated gastrointestinal digestion, AK was more readily digested by pepsin

than by trypsin or chymotrypsin. Moreover, the digestibility of AK was partly affected by the other protein components of myogen. AK from crab myogen was more robustly degraded by pepsin, trypsin or chymotrypsin than purified AK. In addition, simulated two-phase digestion of crab crude extract revealed that AK was readily degraded by pepsin, while TM was sensitive to trypsin and chymotrypsin. We confirmed that AK has characteristics distinct from those of TM and likely represents a unique mud crab allergen.

The physicochemical properties of mud crab AK make it a much more unstable protein than TM. Nonetheless, AK could also elicit an allergenic reaction based upon its interaction with IgE. Thus alterations in amino acid sequence, IgE epitopes and protein structure that may be generated by food processing and/or *in vivo* digestion need to be further studied to fully elucidate the allergenic mechanism(s) of crab AK.

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