

Physicochemical characterization and linear epitopes identification of arginine kinase allergen from *Crassostrea gigas*

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Abstract

BACKGROUND

Molluscan shellfish, including oysters, often cause allergic reactions in sensitive people throughout the world. It has been demonstrated that arginine kinase (AK) is one of the major allergens of oyster. The present study aimed to evaluate the immunoreactivity and structure of oyster AK as affected by heat treatment, pH change, and *in vitro* digestion. What is more, the immunoglobulin E-binding epitopes of this allergen were also predicted and validated.

RESULTS

Thermal and pH assays revealed that AK was unstable at temperature $>40^{\circ}\text{C}$ or $\text{pH} \leq 5.0$ by sodium dodecyl sulfate polyacrylamide gel electrophoresis and circular dichroism, and the digestibility assays suggested that AK was more easily digested by pepsin than by trypsin and chymotrypsin. The potential epitopes were predicted through immunoinformatics tools, and seven linear epitopes were identified by indirect competition enzyme-linked immunosorbent assay with pooled sera and individual serum from oyster-allergic patients. The critical amino acids in each epitope were also confirmed using mutant peptides. These linear epitopes and critical amino acids were apt to distribute on the outer surface of homology-based AK model. Moreover, the three denaturants (sodium dodecyl sulfate, β -mercaptoethanol, and urea) can destroy the spatial structure of AK and increase or reduce its allergenicity by denaturation treatments.

CONCLUSION

Processing conditions lay the foundation for the variation of allergenicity. Seven linear epitopes and their critical amino acids were identified by indirect competitive enzyme-

linked immunosorbent assay. These findings will be helpful in allergy diagnosis and development of hypoallergenic products in the near future. © 2021 Society of Chemical Industry.

INTRODUCTION

Food allergy, an abnormal immune response to certain food components, has appeared as a universal food safety issue because it can lead to a series of allergic symptoms.¹ Shellfish, including crustaceans and mollusks, is a common cause of food allergy in various countries with the acceleration of economic globalization.² Shellfish allergy could impact not only children but also the whole adult life.³ Nowadays, the studies of shellfish allergens mainly focus on the crustacean group designated as one of the eight commonly allergic foods,⁴ whereas relatively few mollusk allergens have been studied. The Pacific oyster *Crassostrea gigas*, which belongs to mollusks, makes up about half of the oysters consumed in the world.⁵ Though tropomyosin (TM) has been proved to be a major allergen of the oyster, we discovered that arginine kinase (AK) was also an allergen. The Pacific oyster AK, a myosinogen protein with a molecular mass of 40 kDa, plays an important role in cell energy metabolism of invertebrates.⁶

The majority of foods need to be cooked or processed before they are digested by the human body. Heating, pH, and digestion treatments enable the proteins to alter conformation and form new chemical bonds and intermolecular/intramolecular interactions. This may finally destroy certain epitopes in some allergens or produce and expose new ones to the molecular surface to influence sensitization.^{7, 8} However, the structures of allergic proteins are different, so they will show diverse allergenicity with different processing methods. Therefore, we first studied these physicochemical properties of AK so that we could figure out how the physicochemical properties can affect anaphylaxis during processing in future research.

The initiation of an allergic reaction is related to the binding of allergen epitopes with specific immunoglobulin E (IgE). Thus, it is important for us to understand these epitopes (linear and conformational epitopes) of allergens, which is useful to elucidate allergen–antibody interactions and to contribute to developing more effective treatments, diagnoses, and prognoses for food allergy, as well as for developing hypoallergenic food products.^{9–11} Although AK epitopes have been identified in *Litopenaeus vannamei*,¹² *Penaeus chinensis*,¹³ and *Scylla paramamosain*,¹⁴ the epitopes of oyster AK are still unknown. Epitope research has revealed that short linear epitopes bind specifically to IgE antibodies of allergic individuals, and the IgE-binding reactivity of the allergen may be altered by several amino acid mutations within each binding epitope.^{15, 16} In previous studies, the critical amino acids of epitopes were mostly summarized as charged or hydrophobic amino acids that influenced the structure and hydrophilicity of proteins or affinity to IgE.^{17, 18} Immunoinformatics, an interdisciplinary subject based on informatics and modern

immunology, has already become an efficient tool for predicting epitopes and further study of critical amino acids in linear epitopes.^{19, 20} In order to identify the predicted epitopes and critical amino acids, the indirect competitive enzyme-linked immunosorbent assay (icELISA) technique is also utilized.¹³

In this paper, we aimed to study the thermal stability, pH stability, and digestibility of AK from *Crassostrea gigas*. The potential allergenic epitopes were studied by three immunoinformatic analysis tools coupled with icELISA. Moreover, the critical amino acids based on the alanine substitution in the allergenic epitopes were verified, and the positions of linear epitopes and critical amino acids were labeled on the structure model of AK. Then, the effect of spatial structure changes on the reactivity of AK was explored by denaturing protein. These conclusions provide us with a deeper understanding of the allergic mechanism and will enable us to reduce the reactivity of oysters, which could be widely applied in other allergen research.

MATERIALS AND METHODS

Human sera

Serum samples from 12 oyster-allergic subjects (mean age 25 years), purchased from PlasmaLab International (Everett, WA, USA), were used to identify wild-type and mutant AK epitopes. All 12 subjects had a clinical history of oyster allergy (severe wheezing, urticaria, angioedema, vomiting, nausea, or diarrhea), oyster-specific IgE antibodies (from 25 to >100 kU L⁻¹, median >100 kU L⁻¹) measured using the CAP-system FEIA (Pharmacia Diagnostics, Uppsala, Sweden) and had strong IgE-binding capacity to purified oyster AK by immunoblot analysis. A serum pool was obtained by mixing equal aliquots of serum IgE. Sera from three normal volunteers without any previous history of shellfish allergies were used as a negative control. Sera were stored in aliquots at -80 °C until used. All assays have been approved by the medical ethics committee at the China National Research Institute of Food and Fermentation Industries (approval number: 2019-0001).

Purification of AK

AK of the Pacific oyster was purified as previously described with some modifications.²¹ The adductor muscle from *C. gigas* was minced and homogenized with 50 mL of buffer containing 50 mmol L⁻¹ sodium chloride (NaCl), 2 mmol L⁻¹ sodium bicarbonate (NaHCO₃), 10 mmol L⁻¹ ethylenediaminetetraacetic acid, and protease inhibitor cocktail (pH 8.0) and stirred for 16 h at 4 °C. The supernatant acquired by centrifugation was precipitated with ammonium sulfate (75–90% saturation). The sediment was then dialyzed and applied to a HiTrapQ Sepharose Fast Flow column (GE Healthcare, Pittsburgh, PA, USA). Target protein was eluted using a linear gradient from 0 to 0.5 mol L⁻¹ NaCl in buffer at a flow rate of 0.5 mL min⁻¹ and stored at -80 °C until used.

Thermal, pH, and digestion treatments of AK

For thermal stability, oyster AK ($0.3 \text{ mg} \cdot \text{L}^{-1}$) was incubated in the buffer (10 mmol L^{-1} Tris–hydrochloric acid (HCl), pH 8.0) at different temperatures (20, 30, 40, 50, 60, 70, 80, or 90°C) for 30 min. For pH stability, AK was incubated for 1.5 h at room temperature in buffer of different pH: pH 4.0, 5.0, 8.0, 9.0, or 11.0. Buffers consisted of HCl–potassium chloride (pH 4.0 and 5.0), Tris–HCl (pH 8.0), and sodium carbonate (Na_2CO_3)– NaHCO_3 (pH 9.0 and 11.0). They were analyzed using sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (PAGE) and circular dichroism (CD).

The digestibility of AK was assessed using *in vitro* simulated gastrointestinal digestion. The simulated gastric fluid (SGF) and simulated intestinal fluid (SIF) were prepared according to the method of Takagi *et al.*²² with a few modifications. SGF is composed of porcine pepsin (275 U mg^{-1}) and NaCl (25 mmol L^{-1}), pH 2.0. A centrifuge tube (10 mL) containing SGF was preheated at 37°C prior to addition of AK to adequately simulate human gastric fluid temperature. AK was then added to the tubes (1:1000 pepsin to AK, w/w), which was digested at 37°C . The reaction was terminated at seven different time points (0, 2, 5, 15, 30, 45, and 60 min) by adding 400 mmol L^{-1} Na_2CO_3 . SIF consists of trypsin, chymotrypsin, and 0.05 mol L^{-1} potassium dihydrogen phosphate (pH 7.5). The ratio of trypsin and chymotrypsin to AK was 1:1:50 (w/w). The reaction was terminated at seven different time points (0, 15, 30, 60, 120, 180, and 240 min) by heating at 100°C for 5 min. Other steps were the same as mentioned earlier, and AK without digestive enzymes was used as a control.

Linear epitope prediction

In the DNASTar Protean system, four properties of the amino acid sequence were regarded as the parameters for linear epitope prediction: hydrophilicity, flexibility, accessibility, and antigenicity. Furthermore, the Immunomedicine Group (<http://imed.med.ucm.es/Tools/antigenic.pl>) and the BepiPred 1.0 server (<http://www.cbs.dtu.dk/services/BepiPred/>) were also used to predict the epitopes of AK. These two online prediction servers only require the amino acid sequence of the AK to calculate and provide epitope results directly, which were based on the physicochemical character of amino acids, such as the exposed surface, polarity, and secondary structure. The BepiPred 1.0 server predicts the location of linear epitopes using a combination of a hidden Markov model and a propensity scale method, whereas the Immunomedicine Group was based on a table that reflected the occurrence of amino acid residues in experimentally known segmental epitopes. Segments are only reported if they have a minimum size of eight residues, and the reported accuracy of this method is about 75%.²³ The ultimate predicted epitopes were determined by combining the results of the three immunoinformatics tools.

Peptide synthesis

Peptides predicted as potential epitopes were synthesized using the standard Fmoc solid phase approach by GL Biochem (Shanghai, China). The purity was $>98\%$ and assessed using high-performance liquid chromatography. The molecular masses of the synthesized

peptides were measured using electrospray ionization mass spectrometry.

icELISA

icELISA was performed to analyze the IgE-binding ability of epitope peptides. AK ($10\text{ }\mu\text{g mL}^{-1}$), diluted with 0.05 mol L^{-1} carbonate buffer solution (CBS; pH 9.6), was placed in a polystyrene 96-well plate (Costar, USA) and incubated overnight at $4\text{ }^{\circ}\text{C}$. In the meantime, the pooled sera from 12 oyster-allergic subjects and five randomly selected individual sera from the same pool (diluted 1:10 with 2% non-fat milk in phosphate-buffered saline–Tween) together with an equal volume of wild-type or mutant peptides were also incubated overnight at $4\text{ }^{\circ}\text{C}$. After blocking with 5% non-fat milk for 3 h at $37\text{ }^{\circ}\text{C}$, the mixture was transferred to the ELISA plate precoated with AK at room temperature for 3 h. Horseradish peroxidase goat anti-human IgE serum (diluted 1:2000; KPL Inc., Gaithersburg, MD, USA) was added to the plate and incubated for 1 h at $37\text{ }^{\circ}\text{C}$. Subsequently, 3,3',5,5'-tetramethylbenzidine substrate was added to each well and incubated in a dark environment for 20 min; then, the reaction was terminated with 2 mol L^{-1} sulfuric acid. The absorbance was measured at 450 nm using a spectrophotometer (Dynex Technologies Inc., Chantilly, VA, USA). Meanwhile, uninhibited serum with CBS, uninhibited serum with AK, and serum only were used as a negative control, positive control, and blank control respectively. The plate was washed for 2 min with phosphate-buffered saline–Tween four times between each step. The ability of peptides to bind IgE antibody was expressed by the inhibition ratio. A high percentage of inhibition reflected a high IgE-binding ability of peptides.

Identification of critical amino acids in allergenic epitopes

The alanine substitution is one of the frequent approaches to confirm the amino acids that are responsible for IgE binding. We selected the amino acids that appear more frequently in the epitopes than in AK. The amino acid sequences of AK from some allergenic foods in the Structural Database of Allergenic Proteins (<http://fermi.utmb.edu/SDAP/index.html>) were also searched, and the high conservative amino acids were screened out through multiple sequence comparison with DNAMAN software. The same amino acids in the results of both methods, together with the physicochemical properties, were regarded as the potential critical amino acids. The predicted critical amino acids in the epitopes were displaced by alanine, and icELISA was performed to discover the IgE-binding capacity of the mutations. The substituted amino acids of the mutant peptides whose IgE-binding capacity showed a sharp decline were identified as the critical amino acids.

Molecular modeling of AK

To further examine the relationship between the epitopes of AK and their structure, we generated a three-dimensional structure using the SWISS-MODEL server (<https://swissmodel.expasy.org/> interactive). AK was modeled based on the X-ray crystal structure of *Limulus polyphemus* AK (PDB code 4GVY), which is the most similar in the structure database. PyMOL software (<http://www.pymol.org/>) was utilized to mark the

positions of the verified linear epitopes and critical amino acids in the three-dimensional structure.

Denaturation of AK

The purified AK was mixed with denaturants SDS, β -mercaptoethanol (β -Me), or urea that diluted with 0.1 mol L⁻¹ Tris–HCl (pH 8.0) in different concentration gradients, and the total reaction system was 100 μ L. The concentrations of SDS were 0.3, 1.0, 3.0, 5.0, 7.0, 9.0, and 11.0, 15.0 mmol L⁻¹; the concentrations of urea were 1.0, 1.5, 2.0, 3.0, 4.0, 5.0, and 6.0 mol L⁻¹; and the concentrations of β -Me were 1.0, 1.5, 2.0, 2.5, and 3.0 mol L⁻¹. After reacting at 4 °C for 15 h, the change in IgE-binding activity was detected according to the ELISA method.²⁴

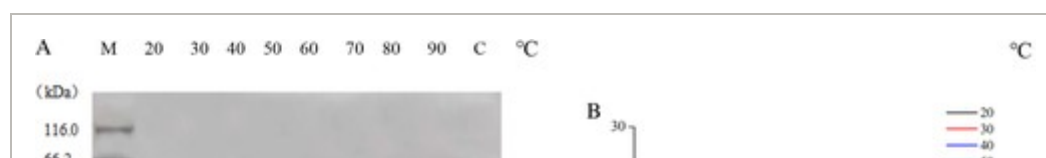
Statistical analysis

All data are exhibited as mean plus/minus standard deviation and were analyzed by a two-tailed Student's *t*-test using Originpro 8.0 (OriginLab Corp., Northampton, MA, USA). Significant difference in means between the samples was decided at $P < 0.05$ or $P < 0.01$.

RESULTS AND DISCUSSION

Thermal and pH processing of AK

AK was subjected to different temperature and pH treatments and analyzed by SDS-PAGE and CD. During thermal treatment, AK was relatively stable at 20–40 °C; however, an apparent protein band of approximately 35 kDa was observed when the temperature was increased. Degradation of AK led to lower molecular weight fragments and began to produce sediments at 80 °C (Fig. 1(A)). For pH processing, AK was easier to degrade in acidic conditions than in alkaline conditions (Fig. 1(C)). CD spectra displayed the secondary structure changes distinctly.²⁵ As shown in Fig. 1(B), the original AK has a positive peak at 191 nm, an obvious negative peak at 208 nm, and a gentle negative peak at 222 nm. This indicated that the relative content of AK secondary structure had 14.80% α -helix and 47.60% β -sheet. Compared with the original AK, the α -helix relative content of AK at pH 4.0 and pH 5.0 decreased 6.4% and 6.1% respectively, whereas the α -helix relative content of AK at pH 9.0 and pH 11.0 decreased 5.6%. When the temperature ranged from 40 to 90 °C, two negative peaks coupled with an increase in intensity became gentle gradually, revealing that α -helix content of AK decreased. The α -helix relative content at 40–90 °C decreased 4.6–6.2%. At the same time, acidic conditions (pH $\leq$ 5) resulted in more serious damage to the secondary structure (Fig. 1(D)). Yu *et al.*⁶ also found that *S. paramamosain* AK was unstable in thermal processing and acidic conditions, which was consistent with our results.



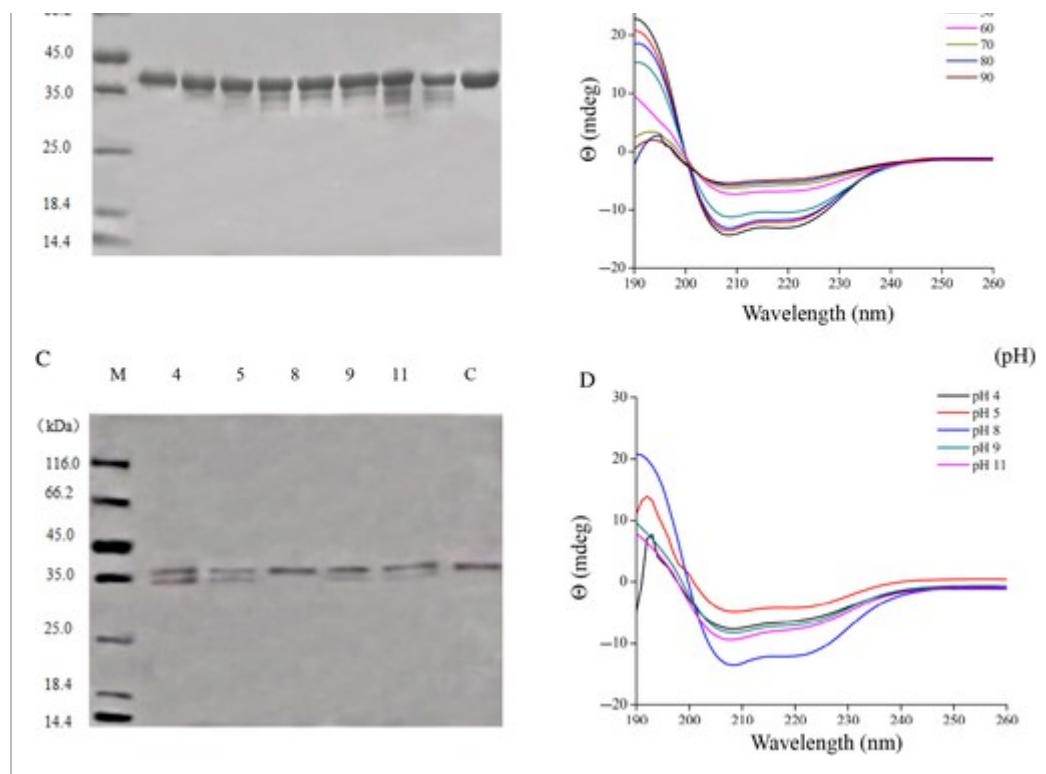


Figure 1

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Thermal and pH treatments of the oyster arginine kinase (AK). Detection of thermal stability of AK by (A) sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and (B) circular dichroism (CD). Detection of pH stability of AK by (C) SDS-PAGE and (D) CD. M, protein markers; C, control.

Digestibility of AK by SGF and SIF

The digestibility of oyster AK in SGF and SIF was analyzed using SDS-PAGE. Under SGF conditions, the original band (40 kDa) was gradually digested and accompanied by the production of small molecular mass protein bands (35, 33, 31 kDa) until 60 min (Fig. 2(A)). On the contrary, AK was degraded slowly during SIF digestion and only a fragment of ~38 kDa was observed. After 240 min digestion, the original band (40 kDa) still cannot be completely degraded (Fig. 2(B)). Compared with SGF digestion, AK was more stable to SIF digestion by trypsin and chymotrypsin. The results of *in vitro* simulated gastrointestinal digestion of AK from *S. paramamosain* and *Procambarus clarkia* were similar to our findings.^{6, 26}

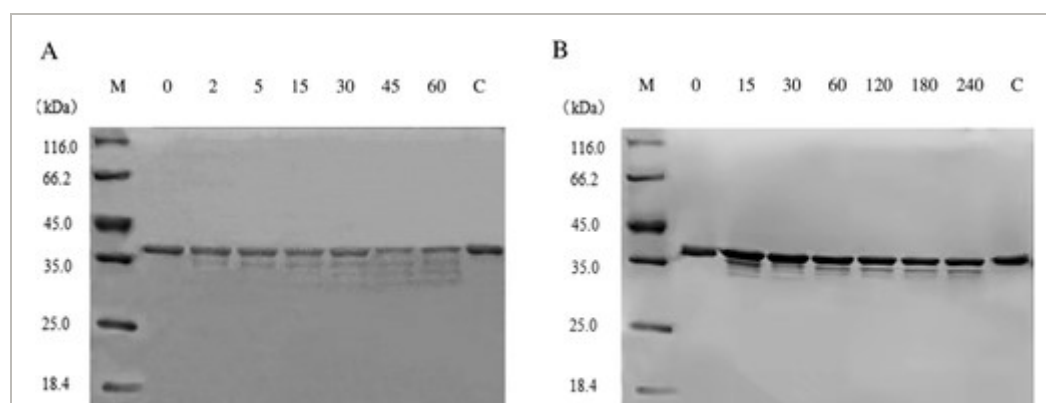




Figure 2

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Simulated gastric fluid (SGF) and simulated intestinal fluid (SIF) digestion of the oyster arginine kinase (AK). (A) Detection of SGF digestion on AK by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). (B) Detection of SIF digestion on AK by SDS-PAGE. M, protein markers; C, control.

Predicted linear epitopes

At present, there are many tools that have been applied to predict linear epitopes, such as DNASTar, BepiPred 1.0 Server, Immunomedicine Group, ABCpred, and AntheProt.²⁷ Using immunoinformatics methods, we could reduce the number of candidate epitopes that required synthesis and validation. The immunoinformatic tool of the DNASTar Protean system was useful for identifying AK epitopes according to the four properties of the amino acid sequence. As described in Fig. 3, the hydrophilicity regions (>0) were widely distributed in the AK sequence, accounting for 77.7% of the entire molecule. The frequent occurrence of flexibility (>0) and surface probability (>1) regions suggested they were likely to be exposed externally. The high antigenic index (>0) indicated that the regions have excellent epitope-forming capacity. For the aforementioned characteristics, 19 epitopes (3 to 13 residues in length) are displayed finally in Table 1. Moreover, eight epitopes from 8 to 40 residues and 12 antigenic regions between 3 and 23 residues in length were defined by the Immunomedicine Group and BepiPred 1.0 Server. The overlap regions predicted by at least two tools were regarded as the potential epitopes of AK for further investigation. Ultimately, the 13 predicted linear epitopes from 3 to 12 residues in length, named peptide 1 (P1) to peptide 13 (P13), were synthesized by the amino acid sequence (Table 2).

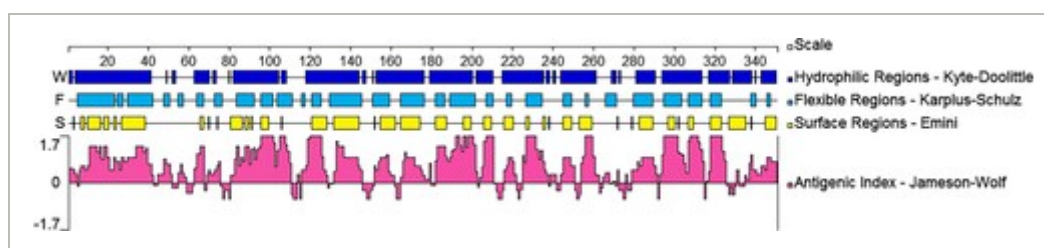


Figure 3

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Analysis results of the whole amino acid sequence of arginine kinase by the DNASTar Protean system.

Table 1. Predicted results of DNASTar Protean, Immunomedicine, and BepiPred 1.0 server

Tool	Amino acid regions
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Tool	Amino acid regions
DNAstar Protean	10–16, 18–20, 30–38, 84–86, 88–91, 96–99, 121–125, 132–144, 155–159, 165–174, 182–186, 196–199, 207–209, 217–219, 245–249, 283–289, 297–300, 307–309, 318–323
Immunomedicine	16–30, 40–54, 56–95, 110–120, 125–133, 231–266, 272–282, 310–317
BepiPred 1.0 server	12–17, 32–39, 61–69, 86–108, 121–143, 154–157, 165–174, 186–199, 228–234, 283–285, 298–315, 347–350

Table 2. Final predicted immunoglobulin E-binding epitopes of combined immunoinformatics methods

Peptide no.	Amino acid sequence	Position	Length of sequence
1	SLL	18–20	3
2	LKDKKTK	32–38	7
3	VEHP	88–91	4
4	GPQD	96–99	4
5	GRSHE	121–125	5
6	VSTDEQRKDMEN	132–143	12
7	ELK	155–157	3
8	TMTPEENQQL	165–174	10
9	QLEKK	245–249	5
10	LAK	283–285	3
11	KLQ	298–300	3
12	EHT	307–309	3
13	ESVGGV	310–315	6

Validation of allergenic epitopes by icELISA

To validate allergenicity of the potential epitopes, icELISA was performed based on the competitive interaction between potential epitope peptides and oyster AK with oyster-allergic human sera. Compared with CBS (negative control), seven epitopes (P2, P3, P5, P6,

P7, P9, and P12) of AK showed stronger inhibition of IgE-binding than that of the other peptides ($P < 0.05$ or $P < 0.01$). They were identified as the IgE epitopes with positions at 32–38, 88–91, 121–125, 132–143, 155–157, 245–249, and 307–309, because they can be recognized by more than half of sera (Fig. 4), whereas the other peptides (P1, P4, P8, P10, P11, and P13) were only identified by less than three human sera (data not shown), which presented a variable degree depending on individual sensitivity. These seven epitopes from AK (3 to 12 amino acids in length) were shorter than the epitopes of shrimp *L. vannamei*¹² and crab *S. paramamosain*²⁸ that were determined by the overlapping peptides scan. Except for P12, P2, P3, P5, P6, P7, and P9 partly overlapping with the epitope regions of *L. vannamei* AK,¹² P5 and P6 completely overlap with the IgE-binding regions of *S. paramamosain*,²⁸ which may be on account of similarities and homology among species. We have known that amino acid sequence of AK shared 55–60% similarity in the mollusks and crustaceans; hence, the oyster allergenic epitopes would offer further molecular basis for immunologic cross-reactivity.

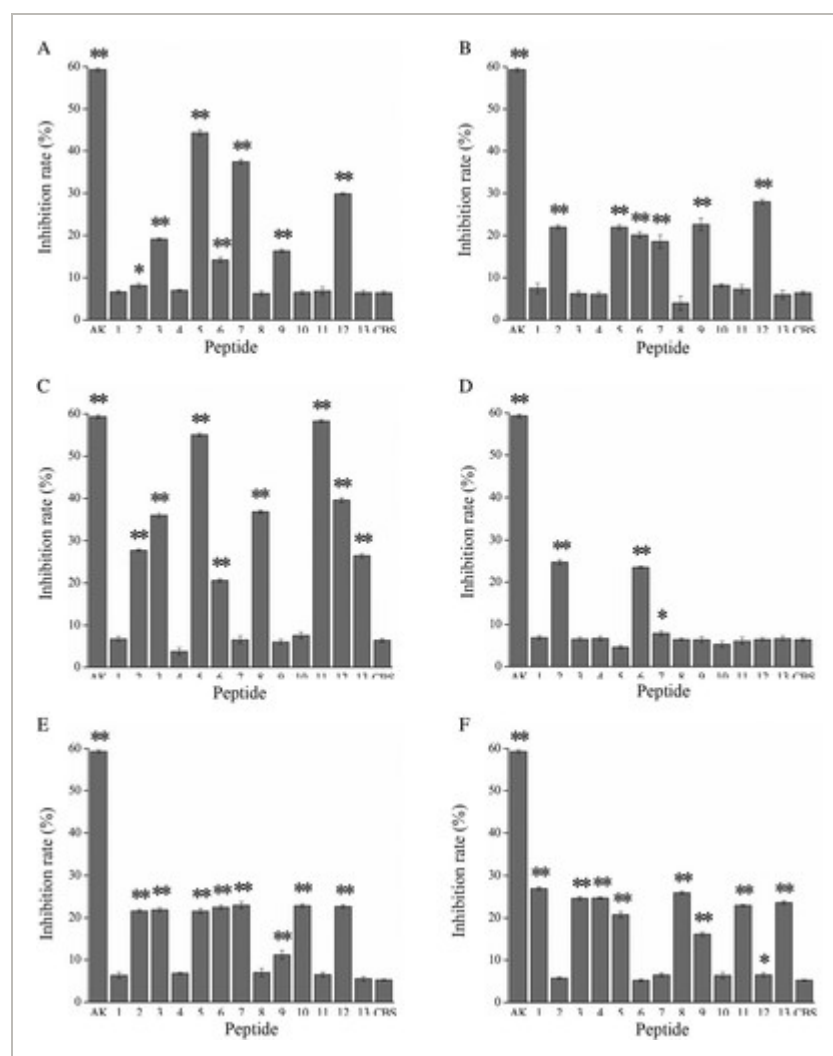


Figure 4

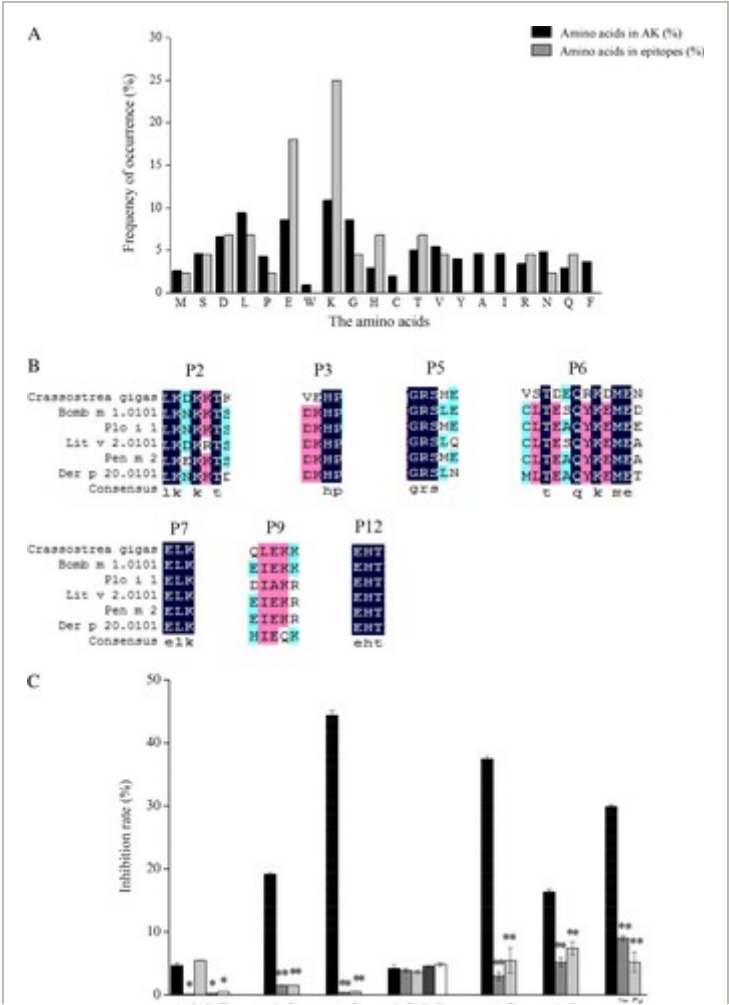
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Determination of the major arginine kinase (AK) epitopes. (A) Histogram of the immunoglobulin E (IgE)-binding reactivity against the AK peptides as determined by indirect competitive enzyme-linked immunosorbent assay (icELISA) using the

pooled sera from 12 oyster-allergic subjects. (B–F) Histogram of the IgE-binding reactivity against the AK peptides as determined by icELISA using individual serum from five randomly selected subjects whose serum was included in the pool. All data are presented as the mean plus/minus standard deviation ($n = 3$). $*P < 0.05$ or $**P < 0.01$ versus carbonate buffer solution (CBS).

Identification of critical amino acids in allergenic epitopes

Amino acids (E, K, D, H, R, T, Q) appear more frequently in AK linear epitopes than in the whole molecule (Fig. 5(A)). On the other hand, multiple sequence alignment of AK from allergenic species in the Structural Database of Allergenic Proteins displayed that L, K, T, H, P, G, R, S, Q, M, and E were conserved in all the aligned sequences (Fig. 5(B)). Considering the aforementioned two methods comprehensively, the final candidates were E, K, T, Q, H, and R. To verify whether the predicted critical amino acids were accurate, we turned these amino acids into alanine in the epitopes identified. The 18 mutant peptides were designed (Table 3) and their IgE-binding capacity was assessed by icELISA with specific human sera. Compared with wild-type peptides, P2-1, P2-3, P2-4, P3-1, P3-2, P5-1, P5-2, P7-1, P7-2, P9-1, P9-2, P12-1, and P12-2 remarkably decreased allergenicity (Fig. 5(C)) ($P < 0.05$ or $P < 0.01$). Altogether, the experimental results implied that K-35, T-37 in P2, E-89, H-90 in P3, R-122, H-124, E-125 in P5, E-155, K-157 in P7, Q-245, E-247, K-248, K-249 in P9, and E-307, H-308 in P12 were critical amino acids for the reactivity of oyster AK.



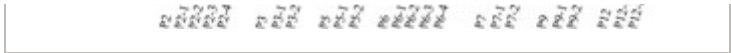


Figure 5

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Demonstration of critical amino acids for allergenic epitopes by immunoinformatics tools and indirect competitive enzyme-linked immunosorbent assay. (A) Amino acids frequency of the whole arginine kinase (AK) and allergenic epitopes were measured by BioEdit. (B) Conservative property of amino acids in AK allergenic epitopes across different species obtained from the Structural Database of Allergenic Proteins. Alignment of different AK sequences was from five species (National Center for Biotechnology Information accession numbers, allergen): *Bombyx mori* (ABB88514, Bomb m 1.0101), *Plodia interpunctella* (Q95PM9, Plo i 1), *Litopenaeus vannamei* (Q004B5, Lit v 2.0101), *Penaeus monodon* (AAO15713, Pen m 2), *Dermatophagoides pteronyssinus* (ACD50950, Der p 20.0101). (C) Identification of critical amino acids within the major AK epitopes. Wild-type and mutant peptides were synthesized and probed with the pooled sera from 12 oyster-allergic patients. **P* < 0.05 or ***P* < 0.01 versus corresponding wild-type peptide. Data represent the mean plus/minus standard deviation (*n* = 3).

Table 3. Mutagenesis of arginine kinase linear epitopes

Original sequence	Mutant point	Mutant sequence	Name
LKDKKTK (32–38)	K-35, T-37	LK DA KAK	P2-1
	K-33, 35, 36, 38	L DA A TA	P2-2
	K-35	LK DA TK	P2-3
	T-37	LKDK K AK	P2-4
VEHP (88–91)	E-89	VA HP	P3-1
	H-90	VE AP	P3-2
GRSHE (121–125)	E-125	GR SH A	P5-1
	R-122, H-124	GAS AE	P5-2
VSTDEQRKDMEN (132–143)	E-136, K-139	VST DA QRAD MEN	P6-1
	Q-137, R-138	VSTDE AA KDMEN	P6-2
	E-136, R-138, K-139	VST DAQA AD MEN	P6-3
	T-134, Q-137	VS AD E ARKDMEN	P6-4
ELK (155–157)	E-155, K-157	ALA	P7-1
	K-157	EL A	P7-2
QLEKK (245–249)	Q-245, E-247	ALAK K	P9-1

The mutant amino acids are shown as the bold residues.

Given all this, a total of 39 amino acids presented in the seven allergenic epitopes, of which 23 were charged (59%), 8 were hydrophobic residues (21%), and 31 were polar (79%) (Fig. 5(A)). Except for Q and T (polar neutral amino acids), the other four verified critical amino acids (E, K, H and R) belonged to charged amino acids that were closely relevant to allergenicity. Furthermore, researchers discovered that the substitution of a single amino acid from peanut allergen Ara h 1 could significantly reduce the IgE-binding ability of epitopes.²⁹ Reese *et al.*³⁰ suggested that the allergenic potency of shrimp allergen Pen a 1 was alleviated by replacing more than two amino acids, and the most critical amino acids lay in the center of the epitopes. Our data demonstrated that a combination of two amino acid substitutions may reduce IgE-binding ability more strikingly than a single substitution. Interestingly, P2-2 (four amino acid substitutions) led to an increase of IgE reactivity, implying that the more amino acid substitutions were not necessary to decrease IgE binding. Meanwhile, the impact of alanine substitutions in the center of AK epitopes was roughly the same as those found in the peripheral parts, which was in accordance with the conclusions of the peanut allergen Ara h 2 and oyster allergen Cra g 1.^{31, 32} In addition, the critical amino acids of P6 need further confirmation with new allergic sera because there was no significant consequence. These phenomena might allow us to design hypoallergenic products that would be beneficial to clinical diagnosis and therapeutic measures.

Location of the IgE-binding epitopes on the three-dimensional structure of AK

The crystal structure of AK from *L. polyphemus* (PDB code 4GVY) was used as the template for homology-based modeling. The oyster AK was modeled through SWISS-MODEL (Fig. 6(A)), and the quality of this model was evaluated by the Ramachandran diagram to reflect the conformational reasonability. Most backbone torsion angles for non-glycine residues were located within the allowed regions of the Ramachandran plot; only 1.16% of the amino acids were disallowed (Fig. 6(B)), so the homology-based AK tertiary structure was reasonable. From the AK model, we found that it had a regularly advanced fold, containing an α -helical domain of N-terminus and an α - β domain of C-terminus. The N-terminal domain consisted of three α -helixes, and the C-terminal domain was composed of five α -helixes and eight β -sheets. Additionally, the authenticated linear epitopes and critical amino acids were labeled on the AK tertiary structure, which provided more detailed information of IgE-binding. The seven epitopes were mainly composed of α -helix or random coil and distributed on the outer surface of the allergenic molecule, which was in accordance with the reported results of other allergens (Fig. 6(A)).^{33, 34} As for critical amino acids, we inferred that they might interact with serum IgE through electrostatic attraction, but the alanine substitutions changed charges (E-89/125/155/247/307, K-35/157/248/249,

H-90/124/308, R-122, Q-245, T-37) of critical residues, leading to damage of the interactions between IgE and AK. At the same time, a number of studies have shown that ~90% of the epitopes in globular proteins are discontinuous.^{13, 35} We speculated that most of the oyster AK epitopes were conformational. Thus, detailed studies of conformational epitopes in the future are expected to shed light on the sensitization of the globular protein AK.

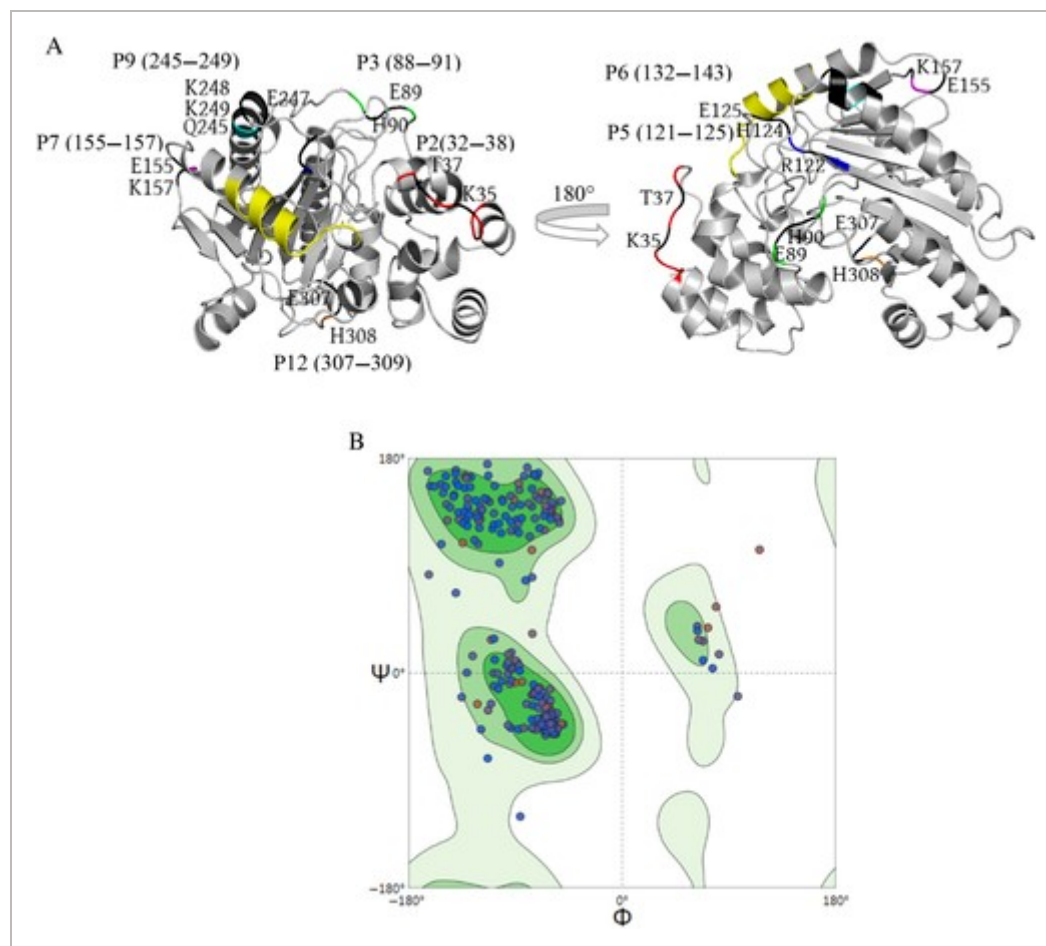


Figure 6

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Location of the linear epitopes on the arginine kinase (AK) three-dimensional structure. (A) The major epitope regions and critical amino acids are labeled on the ribbon model. (B) The Ramachandran diagram of AK model. A plot of the Ψ and Φ angles for the amino acids in the AK three-dimensional structure model is shown. Each dot within the quadrants represents an amino acid.

IgE reactivity of AK after denaturation treatment

The alteration of spatial structure would destroy conformational epitopes and affect the sensitization. Typical chemical reagents (SDS, β -Me, and urea) act on intermolecular/intramolecular interactions, disulfide bonds, and hydrophobic interactions that maintain the spatial structure of proteins. Different concentrations of denaturants have various influences on the affinity between IgE and AK. When SDS was at a concentration of 1 mmol L⁻¹, the binding capacity of AK began to increase and then decreased with increasing

concentration. When the concentration reached 3 mmol L^{-1} , the IgE-binding ability was almost lost (Fig. 7(A)). SDS could enter the interior of the protein and break the hydrophobic interactions, so that the hydrophobic area of the molecule was exposed and lost most of the IgE-binding capacity.³⁶ Compared with the control, AK treated with urea enhanced IgE reactivity when the urea concentrations were $1\text{--}6 \text{ mol L}^{-1}$ (Fig. 7(B)). This trend might be attributed to the increase in solubility of non-polar side chains.³⁷ After treatment of β -Me, the anaphylaxis increased gradually when the concentration was less than 2 mol L^{-1} , because the lower concentration of β -Me caused hidden epitopes to be exposed on the molecular surface. However, when β -Me was $>2.5 \text{ mol L}^{-1}$, intramolecular disulfide bonds that maintained the spatial conformation were broken, resulting in the reduction of IgE-binding (Fig. 7(C)).³⁸ As a result, the spatial structure (conformational epitopes) of oyster AK is one of the important factors for its allergenicity.

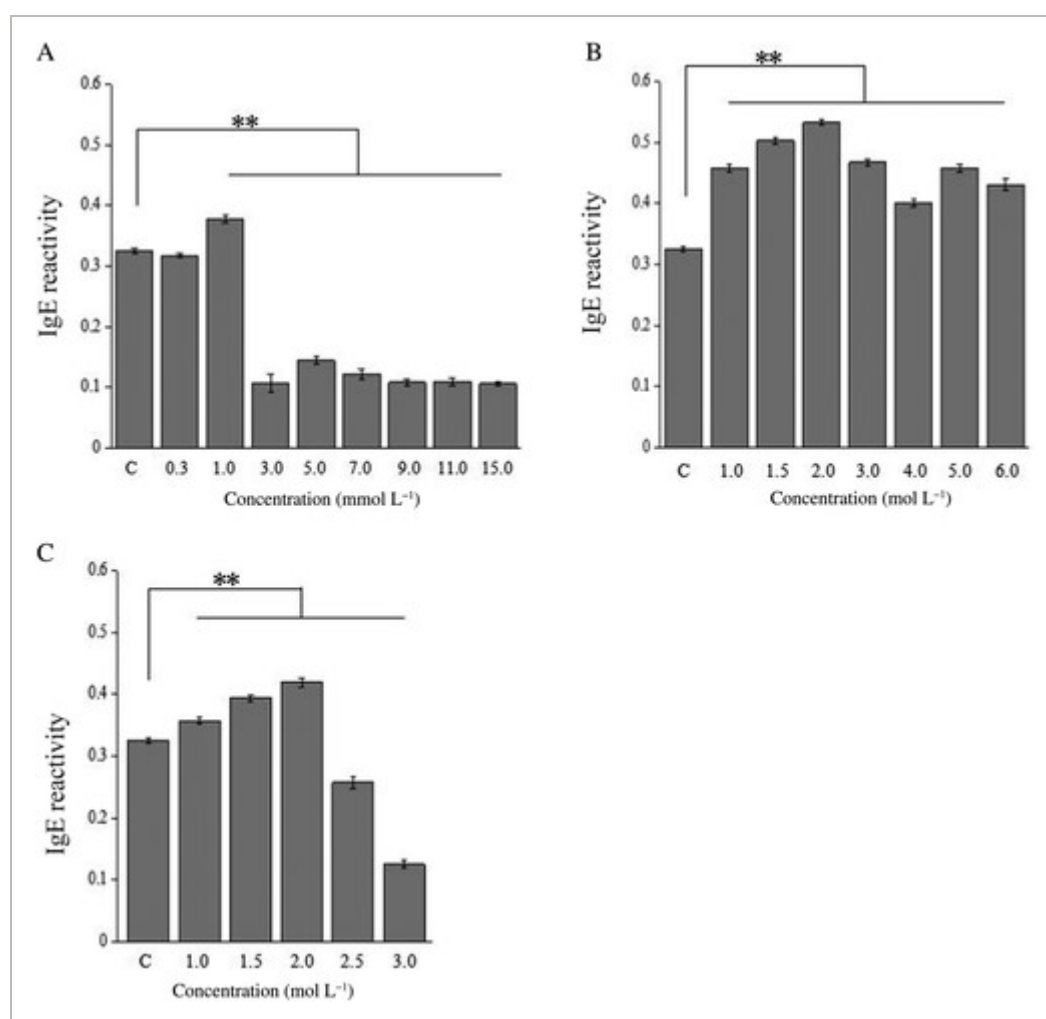


Figure 7

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Immunoglobulin E (IgE) reactivity of the denatured arginine kinase (AK) using enzyme-linked immunosorbent assay. Effects of (A) sodium dodecyl sulfate, (B) urea, and (C) β -mercaptoethanol on the IgE reactivity of AK. C, control. $**P < 0.01$ versus control. Data represent the mean plus/minus standard deviation ($n = 3$).

CONCLUSIONS

Till now, some AKs from crustaceans have been identified, such as Cra c 2 (*Crangon crangon*),³⁹ Lit v 2 (*L. vannamei*)⁴⁰ and Pen m 2 (*Penaeus monodon*),⁴¹ but detailed verification of their characterization and IgE-binding epitopes are lacking. Therefore, the further investigation of AK allergens from the Pacific oyster was carried out. We illustrated the physicochemical properties of AK, identified its allergenic epitopes and the critical amino acids, and explored the effect of spatial structure changes on the IgE reactivity. Immunoinformatics methods and icELISA improved the accuracy of epitopes prediction and the efficiency of epitopes detection. These results will have great implications in clinical diagnosis and immunotherapy. Future work will be oriented towards analyzing conformational epitopes of oyster AK and the IgE-mediated allergic signaling pathway through animal and cell models.

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CONFLICT OF INTERESTS

The authors declare that there are no conflicts of interest.

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