



Crystal structure determination of *Scylla paramamosain* arginine kinase, an allergen that may cause cross-reactivity among invertebrates

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ABSTRACT

Shellfish are one of the most common causes of food allergy. Arginine kinase (AK) is known as an important allergen in shellfish. In the present study, AK from crab (*Scylla paramamosain*) was purified and its crystal structure was determined. A comparison of AK from *S. paramamosain* to AKs of other species showed high amino acid sequence and secondary structure identity, while the superposition of crystal structures of AKs from different species revealed only slight differences. Similarity of the linear epitope regions among species was observed in the epitope alignment of AKs; conformational epitopes were located in the regions where secondary structure was conserved. The structure of *S. paramamosain* AK is an accurate template for the analysis of the IgE binding pattern, and the structure conservation and epitope similarity of AKs among species could help to inform our understanding of the cross-reactivity and contribute to the prediction of cross-reactivity related epitopes.

1. Introduction

Immunoglobulin (Ig) E-mediated shellfish allergy constitutes an important health issue in both children and adults and its incidence is increasing (Shek et al., 2010). Unlike allergies caused by egg and milk, allergy to shellfish is typically a lifelong disorder that can significantly impair the quality of life (Jiménez-Saiz et al., 2017). Due to its nutritional value and delicious taste, crab is one of the most widely consumed shellfish around the world.

In recent decades, significant progress in biochemistry and molecular biology has enabled the identification and characterization of various crab allergens and their allergenic epitopes (Hu et al., 2017; Yang et al., 2015; Zhu et al., 2016). The myofibril protein tropomyosin (TM) (MW 34 kDa) was the earliest allergen identified in crab, and it is considered to be responsible for most of the allergenic activity in crustaceans and cross-reactivity among invertebrates (Leung et al., 1998; Purohit et al., 2007). At present, various IgE-binding B cells and putative T cell epitopes of TM have been described in detail (Faber et al., 2017). In addition to TM, Abdel Rahman et al identified arginine kinase (AK) (MW around 40 kDa) as an allergen in snow crab (*Chionoecetes opilio*) (Rahman, Kamath, Lopata, Robinson, & Helleur, 2011).

Similar to TM, AK has been demonstrated to be highly abundant in invertebrates, and it has been described in several shellfish species as well as invertebrates such as mites, cockroaches, spiders, and octopuses (García-Orozco, Aispuro-Hernández, Yepiz-Plascencia, Calderón-de-la-Barca, & Sotelo-Mundo, 2007; Laino et al., 2017; Shen et al., 2016; Teng, Yu, Sun, Wang, & Cui, 2018; Yang et al., 2016; Yu, Lin, Chiang, & Chow, 2003).

Our previous research identified AK in *Scylla paramamosain* as a major allergen with strong IgE-reactivity (Mao et al., 2013). AK displays high sequence identity among shellfish. Cross-reactivity of *S. paramamosain* AK with AKs from *Octopus fangsiao*, *Procambarus clarkia*, and *Litopenaeus vannamei* has been observed, although whether the cross-reactivity is due to high sequence identity or some structure related characteristic remains unknown (Chen et al., 2013; Shen et al., 2016). From immunological studies, epitopes are known to be responsible for the immune reaction so that the identification of epitopes is a necessary prerequisite to further understanding of the cross-reactivity. In subsequent research, the IgE-binding epitopes of *S. paramamosain* AK were identified, and these experimentally identified epitopes were found to be in accordance with the predicted epitopes of AKs from shrimp and octopus, which provides a molecular basis for the cross-reactivity

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(Ayuso et al., 2010; Shen et al., 2016; Yang et al., 2015). Previous studies established that AK is a panallergen in vertebrates (García-Orozco et al., 2007; Laino et al., 2017; Shen et al., 2016; Teng et al., 2018; Yang et al., 2016; Yu et al., 2003); nevertheless, whether AK is responsible for cross-reactivity between crustaceans and other arthropods is unclear.

Although AK contains both conformational and sequential epitopes, the importance of its conformational epitopes has been established, and the structural integrity of AK has proved to be essential for its allergenicity (Mao et al., 2013; Yang et al., 2015). Thus, determination of the three-dimensional structure of AK is essential. Though preliminary reports are available on *L. vannamei* AK crystallization (López-Zavala et al., 2013), the crystal structure of *S. paramamosain* AK has not been determined to date.

In the present study, AK from *S. paramamosain* myosinogen was purified to ensure optimum conditions for the formation of high quality crystals, which were prepared to acquire X-ray diffraction data. A comprehensive comparison of the primary, secondary, and crystal structures as well as of the allergenic epitopes of *S. paramamosain* AK with AKs from other species was performed to unravel AK-induced cross-reactivity among invertebrates.

2. Materials and methods

2.1. Materials

2.1.1. Crab

Live crab (*S. paramamosain*) was purchased from a local seafood market in Xiamen, China.

2.1.2. Chemicals

Q-Sepharose was from GE Healthcare (Waukesha, Wisconsin, USA). Goat anti-rabbit IgG-HRP antibody was from Abmart (Berkeley Heights, New Jersey, USA) and rabbit anti-crab AK polyclonal antibody was prepared in our laboratory (Yang et al., 2015). All reagents were of analytical grade.

2.2. Purification and identification of AK

Purification of crab (*S. paramamosain*) AK was performed according to a previously published protocol (Mao et al., 2013). The crab muscle samples were minced and homogenized with 4 volumes (v/w) of 10 mM Tris-HCl buffer containing 1 mM ethylene diamine tetraacetic acid, 10 mM β -mercaptoethanol, and 25 μ M phenylmethanesulfonyl fluoride (pH 8.0). The homogenate was centrifuged at $12,000 \times g$ for 20 min and the supernatant, which contained crab myosinogen, was then collected and adjusted to 70%–90% ammonium sulfate for fractionation. The resulting precipitate was dissolved and dialyzed in 10 mM Tris-HCl containing 0.1 mM EDTA and 10 mM β -mercaptoethanol (pH 8.0) before being subjected to a Q-Sepharose column (2.5 cm $\times$ 10 cm). Bound proteins were eluted by a 0–0.15 M NaCl linear gradient at a flow rate of 1 mL/min.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed for the detection of the target protein. Samples were separated on 12% polyacrylamide gels with 5% stacking gels and stained with Coomassie brilliant blue. For the western blot, the samples separated by SDS-PAGE were electrotransferred to nitrocellulose membranes followed by blocking with 5% skim milk. Rabbit anti-crab AK IgG polyclonal antibody (1:10⁵ dilution) was the primary antibody, and HRP-labeled goat anti-rabbit IgG (1:20,000 dilution) was used for detection. Dot blot analysis was performed by directly spotting the protein (2 μ g/mL) on the nitrocellulose membranes instead of by electrotransfer. The primary antibody was sera from a crab allergic patient (sera No. Q648228, dilution 1:4). The results were visualized using enhanced chemiluminescence. The use of sera was approved by the internal Ethical Committee of the hospital (Xiamen second hospital,

Xiamen, China) (XSH2012-EAN019).

2.3. Crystallization of AK

Purified AK was concentrated to 40 mg/mL using Amicon centrifugal concentrators (Millipore, Billerica, MA, USA) before employing the hanging drop vapor diffusion method for crystallization. The drops were set up by mixing 0.2 μ L of concentrated AK and 0.2 μ L of crystallization solution. Multiple homemade as well as commercially available crystal screens were tested (MBP Screen, Crystal Screen™, Anion Suites™, Wizard I™, and Wizard III™, Hampton Research, Aliso Viejo, CA, USA). Crystals readily formed under conditions composed of ammonium sulfate and 2-(N-morpholino) ethanesulfonic acid (MES) at room temperature. Some additional amounts of 5% (V/V) ethylene glycol and 10% (V/V) glycerol were added to the crystallization solution to serve as a cryoprotectant solution when single crystals were flash cooled in liquid nitrogen.

2.4. X-ray diffraction, structure determination, and refinement of AK structure

X-ray diffraction data were collected at the Shanghai Synchrotron Radiation Facility beamline BL18U and BL19U1. Data sets of 360 images were collected at 12.680 keV with 0.5° rotation and 0.3 s exposure time.

Data were processed using the HKL2000 program suite and XDS (Otwinowski & Minor, 1997; Kabsch, 2010). The structure of *S. paramamosian* AK was determined by molecular replacement with Phaser (McCoy, Grosse-Kunstleve, Storoni, & Read, 2005) in the Collaborative Computational Project Number 4 program suite (Winn et al., 2011) using the published *Litopenaeus vannamei* AK structure as a search model (PDB: 4AM1). The final model was completed by alternative manual model building in the Coot and Phenix.refine refinement in Phenix GUI (Adams et al., 2010; Emsley, Lohkamp, Scott, & Cowtan, 2010). The crystal structures were validated by the Molprobiy server and RCSB ADIT validation serve (Chen et al., 2010; Yang et al., 2004). Molecular graphics were prepared using Pymol (Delano Scientific LLC, San Carlos, CA, USA) (Baker, Sept, Joseph, Holst, & McCammon, 2001).

2.5. Structure and epitope comparisons among allergenic AKs

Allergenic AKs from different species were identified in the Allergen Database for Food Safety (ADFS, <http://www.monika.org/node/231>). The structure of *S. paramamosian* AK was aligned with allergenic AKs from various species including: *Procambarus clarkii* (Chen et al., 2013), *Crangon crangon* (Bauermeister et al., 2011), *Penaeus chinensis* (Fu, Wang, Ni, Wang, & Wang, 2018), *Penaeus monodon* (Yu et al., 2003), *Litopenaeus vannamei* (Ayuso et al., 2010), *Periplaneta Americana* (Yang et al., 2016), *Blattella germanica* (Yan et al., 2011), *Dermatophagoides farinae* (Teng et al., 2018), *Octopus fangsiao* (Shen et al., 2016), and *Polybetes pythagoricus* (Laino et al., 2017). Amino acid sequence alignment was prepared using ClustalW (Thompson, Higgins, & Gibson, 1994). Structure-based sequence alignment of the allergenic AKs was completed by ESPrint 3.0 (Robert & Gouet, 2014). Available AK structures were downloaded from the Protein Data Bank (<http://www.rcsb.org/>), and the structures and epitopes were displayed with Pymol. The ConSurf server (<http://consurf.tau.ac.il/2016/>) was used to map sequence conservation on the structure of AK (Ashkenazy et al., 2016).

2.6. Protein data bank accession codes

The coordinates and structural factors of *S. paramamosian* AK have been deposited in the Protein Data Bank with accession code 5ZHQ.

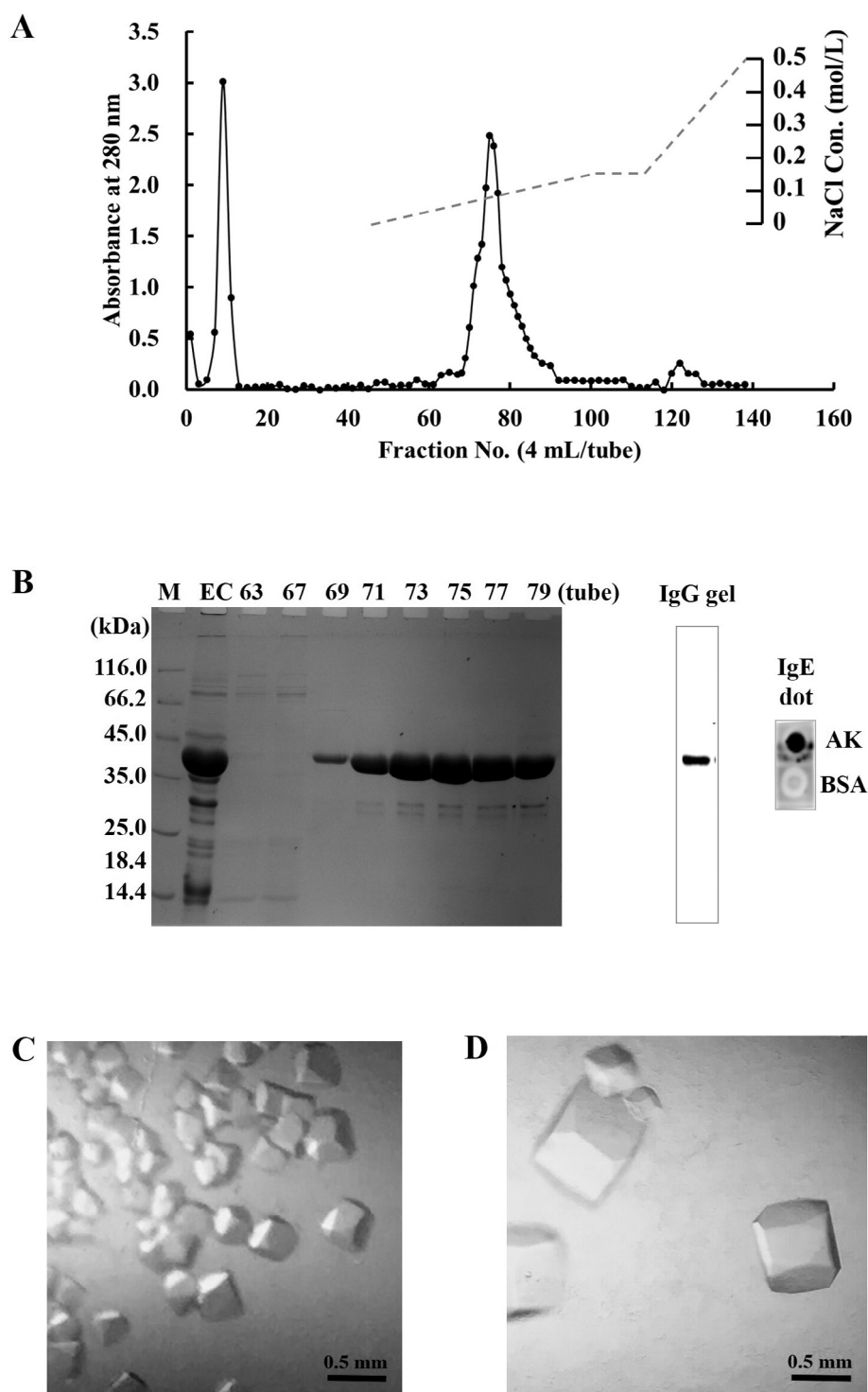


Fig. 1. Purification, identification and crystallization of *S. paramamosain* AK. A: Q-Sepharose ion exchange chromatography of AK. B: SDS-PAGE, IgG reactivity (primary antibody: rabbit anti-crab AK IgG pAb, dilution 1:10⁵), and IgE reactivity (primary antibody: sera from a crab allergic patient, sera No. Q648228, dilution 1:4) analysis of purified AK. M, protein marker; EC, protein precipitate at 70–90% saturation of ammonium sulfate. C: AK crystals obtained in the initial screen. D: AK crystals after optimization.

3. Results

3.1. Purification and identification of AK

AK from the muscle of *S. paramamosain* was highly purified by ammonium sulfate fractionation and Q-Sepharose ion exchange chromatography, and the purification steps were monitored with SDS-PAGE

(Fig. 1). After 90% ammonium sulfate precipitation, a protein band of approximately 40 kDa and of large quantity was detected (Fig. 1B, lane EC). In the Q-Sepharose anion exchange chromatography purification step, the major peak was eluted at 0.05–0.15 M NaCl (Fig. 1A). This fraction migrated as a single band with a molecular weight of approximately 40 kDa on an SDS-PAGE (Fig. 1B). These data agreed with results from previous studies (Mao et al., 2013; Yang et al., 2015). To

further confirm the identity of the eluted protein, western blot analysis was performed with rabbit anti-crab AK IgG polyclonal antibody (1:10⁵) (Yang et al., 2015). The strong positive reaction of the 40 kDa protein with antibody indicated the purified fraction was *S. paramamosain* AK. In addition, the purified AK showed strong reactivity to IgE from a crab allergic patient (Fig. 1B).

3.2. Optimization of the crystallization conditions of AK

The *S. paramamosain* AK was concentrated to 40 mg/mL and crystallized by the hanging-drop vapor-diffusion method at room temperature. In the crystallization screen carried out in the present study, the crystals appeared under the conditions of solution #54 of the MBP Screen™ kit (2.5 M ammonium sulfate, 0.1 M MES, pH 6.0) within a week. However, the obtained crystals were clusters of several individual crystals tightly joined together, which were not suitable for X-ray diffraction experiments (Fig. 1C). To optimize the crystallization conditions, a gradient of ammonium sulfate concentrations of 0–3 M was systematically screened, with an adjusted AK concentration of 30 and 60 mg/mL. Large crystals were obtained in the solution containing 2.5 M ammonium sulfate, at an AK concentration of 30 mg/mL, and the best crystals were grown to a size of 0.5 × 0.5 × 0.5 mm within 1 day (Fig. 1D). Single prismatic crystals that were suitable for data collection were picked up by nylon loops and cryoprotected with the cryoprotectant solution.

3.3. Data collection and structure determination of AK

The best AK crystal diffracted to 3.00 Å and its space group was determined to be P3₂21 with unit cell parameters of a = 136.72 Å, b = 136.72 Å, and c = 145.90 Å (Table 1). Matthew's coefficient analysis showed that, if there were two molecules in an asymmetric unit, the Matthew's coefficient was 4.89, which corresponded to a solvent content of 74.87%.

The structure was solved by a molecular replacement calculation using the *L. vannamei* AK (4AM1, 91.81% identity) as a search model.

Table 1

X-ray data collection and refinement table of arginine kinase.

<i>Data collection</i>	
Space group	P3 ₂ 21
Unit cell (a, b, c) (Å)	136.72, 136.72, 145.90
(α, β, γ) (°)	90, 90, 120
Resolution (Å) (Last shell)	50–3.00 (3.08–3.00) [*]
No. of reflections (total/unique)	153,749/30,704
Redundancy (Last shell)	5.0 (5.0) [*]
Completeness (%)	96.0 (97.1)
I/σ (I) (Last shell)	18.35 (1.28) [*]
R _{meas} (Last shell) (%) [*]	7.3 (148.9) [*]
<i>Refinement</i>	
Resolution (Å)	50–3.00
Number of protein atoms	5696
No. of solvent/hetero-atoms	40
Rmsd bond length (Å)	0.006
Rmsd bond angles (°)	0.839
R _{work} (%)	17.84
R _{free} (%)	22.79
Ramachandran plot favored/disallowed ^{**}	97.2/0
PDB code	5ZHQ

^{*}R_{work} = $\sum_h ||F_{obs}(h)| - |F_{calc}(h)|| / \sum_h |F_{obs}(h)|$, where $F_{obs}(h)$ and $F_{calc}(h)$ are the observed and calculated structure factors, respectively. No I/σ cutoff was applied.

^{*}R_{free} is the R value obtained for a test set of reflections consisting of a randomly selected 5% subset of the data set excluded from refinement.

^{*} Asterisk numbers correspond to the last resolution shell.

^{*} $R_{meas} = \sum_h (n/n - 1)^{1/2} \sum_i |I_i(h) - \langle I(h) \rangle| / \sum_h \sum_i I_i(h)$, where $I_i(h)$ and $\langle I(h) \rangle$ are the i th and mean measurement of the intensity of reflection h .

^{**} Values from Molprobity server (<http://molprobity.biochem.duke.edu/>).

The refinement was terminated when there were no significant changes in R_{work} and R_{free} and inspection of the difference density map suggested that no further corrections or additions were justified. All of the 3.0 Å resolution data were used in the final refinement, with R_{work} = 17.84% and R_{free} = 22.79%. Molprobity validation showed 97.2% of all protein residues were in favored regions and there were no outliers in the Ramachandran plot. There were no C-beta outliers. The geometry of the model is acceptable (Table 1) with a root mean square deviation (RMSD) bond of 0.008 Å and an RMSD angle of 0.94°.

3.4. Overall structure of AK

The full-length *S. paramamosain* AK molecule is composed of 357 residues. Overall, the AK crystal structure shows a canonical AK fold with an α-helical N-terminal domain and an α-β C-terminal domain. The N-terminal domain contains five α-helices, and the C-terminal domain contains seven α-helices and eight β-sheets (Fig. 2A). The epitopes of *S. paramamosain* AK reported previously (Yang et al., 2015) were mapped on the crystal structure. All of the linear epitopes: amino acids (AA) 113–127, 127–141, 141–155, and 204–218, as well as two conformational epitopes: V₁₇₇G₁₇₂M₁₇₃D₁₇₆Q₁₇₈T₁₇₄L₁₈₁K₁₇₅L₁₈₇ and R₂₀₂L₁₇₀Y₂₀₃E₁₉₀P₂₀₅W₂₀₄L₁₈₇T₂₀₆Y₁₄₅, were distributed in the α-β C-terminal domain (Fig. 2B and C). Another two conformational epitopes (D₃A₄K₄₃M₁A₅T₄₉T₄₄I₇ and L₃₁K₃₃V₃₅T₃₂E₁₁E₁₈F₁₄S₃₄D₃₇) were distributed in the α-helical N-terminal domain (Fig. 2C).

3.5. Comparison of *S. paramamosain* AK with AKs from other species

Fig. 3A shows a ribbon representation of the AK molecule, indicating residue conservation among 150 AK-related protein sequences selected by ConSurf. The most viable residues are in the regions of AA 3–19, AA 33–40, AA 176–184, AA 294–304 in the α-helices, and AA 110–120 in a loop. The regions AA 3–19, AA 33–40, and AA 176–184 are located within the conformational epitopes, while AA 110–120 is one of the linear epitopes of *S. paramamosain* AK. AK has been identified as an allergen in many species of invertebrates; allergenic AKs from different species searched for with the ADFS are summarized in Table 2, showing relatively high primary and secondary structure identity (Fig. 3B). Predicted or experimental linear epitopes of these allergens are labeled under the aligned sequences, and all of the allergens reported more than one linear epitope in AA 130–155, which is an α-helix region. Besides, two other high frequency linear epitope regions are AA 25–44 in an α-helix region and AA 308–321 in a loop (Fig. 3B).

Structural alignment of *S. paramamosain* AK with the available allergenic AKs, *L. vannamei* AK without a ligand (apo-AK, PDB: 4AM1), AK in a ternary transition state analog complex with ADP-Mg²⁺ and arginine (AK-TSAC, PDB: 4BG4), and *P. pythagoricus* AK in a binary complex with arginine (AK-Arg, PDB: 5U8E) are shown in Fig. 4. The structures of apo-AK, AK-Arg, and *S. paramamosain* AK are highly conserved. The RMSD value is 0.750 between *S. paramamosain* AK and apo-AK and 0.469 is between *S. paramamosain* AK and AK-Arg. In comparison with the other three AK structures, AK-TSAC displays significant differences, with an RMSD value of 1.704 relative to apo-AK and of 1.568 relative to *S. paramamosain* AK. The variable residue regions AA 3–19, AA 33–40, AA 110–120, and AA 294–304 are conserved in the spatial structure; differences were observed mainly in the two α-helix regions: region-1 (AA 175–184) and region-2 (AA 288–304, 335–355), containing two α-helices in the C-terminus. The helix AA 288–304 in region-1 is located in the variable residue region (Fig. 3A), indicating the structure variation might be due to the difference in primary structure.

4. Discussion

Shellfish, particularly crabs, are known to cause severe type I respiratory allergies with a growing prevalence of IgE sensitization

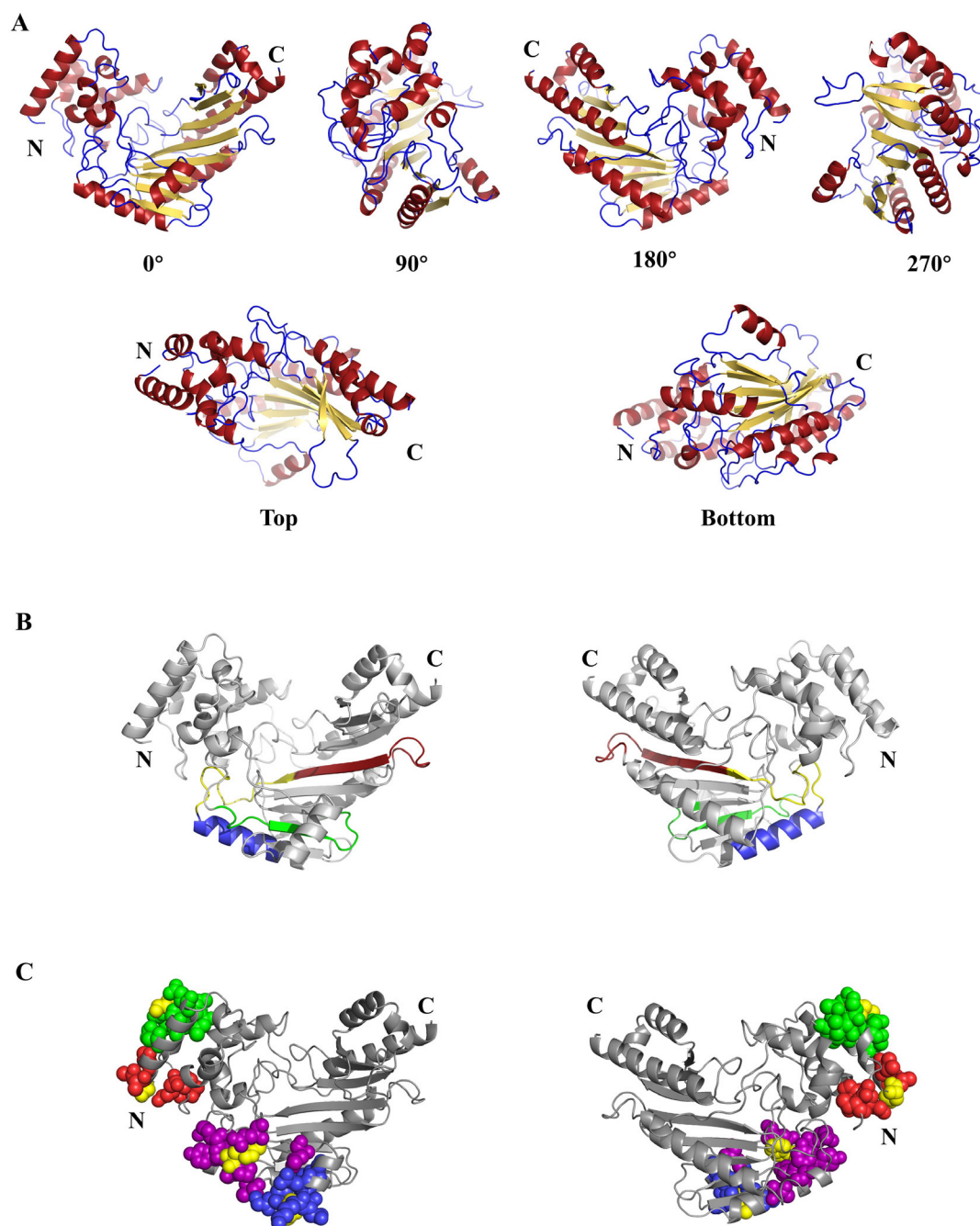


Fig. 2. Crystal structure and epitope mapping of AK. A: structure of AK shown in the diagram, with the α -helices colored red, β -sheets colored yellow, and random coils colored blue. B: reported linear epitopes were mapped on the structure of AK shown in the diagram. Red: 113–127, yellow: 127–141, blue: 141–155, green: 204–218. C: reported conformational epitopes were mapped on the structure of AK shown in the diagram. Red: D₃ A₄ K₄₃ M₁ A₅ T₄₉ T₄₄ I₇; green: L₃₁ K₃₃ V₃₅ T₃₂ E₁₁ E₁₈ F₁₄ S₃₄ D₃₇; blue: V₁₇₇ G₁₇₂ M₁₇₃ D₁₇₆ Q₁₇₈ T₁₇₄ L₁₈₁ K₁₇₅ L₁₈₇; purple: R₂₀₂ L₁₇₀ Y₂₀₃ E₁₉₀ P₂₀₅ W₂₀₄ L₁₈₇ T₂₀₆ Y₁₄₅. The key amino acids are colored yellow. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

among Asians (Shek et al., 2010). A number of shellfish allergens have been reported thus far, and among those molecules, TM in the myofibrils and AK in the myosinogen are considered to be major allergens (Ayuso et al., 2010; Chen et al., 2013; Mao et al., 2013; Shen et al., 2016), and TM and AK were reported as panallergens in invertebrates (Faber et al., 2017). The cross-reactivity induced by TM has been extensively characterized in recent decades, while the structure and cross-reactivity characteristics of AK have not yet been fully documented.

The present study describes the first crystal structures ever reported for *S. paramamosain* AK. With few exceptions, most published crystal structures of AK were obtained from recombinant protein or protein

with ligands (e.g. arginine, ATP) (Laino et al., 2017; López-Zavala et al., 2013). However, AK has been proven to contain predominantly conformational epitopes; thus, in this study, we determined the crystal structure of purified natural AK with a preserved active site, confirming an overall fold of AK. Purified AK crystallized in a form with a high solvent content of 74.87% and a Matthew's coefficient of 4.89, which may have contributed to how well the crystal diffracted, as crystals with lower solvent content tend to diffract better (Kantardjieff & Rupp, 2003). Notably, the structure of AK from *S. paramamosain* was determined for the first time, and the model obtained provides an accurate template for analysis of the allergenic epitopes.

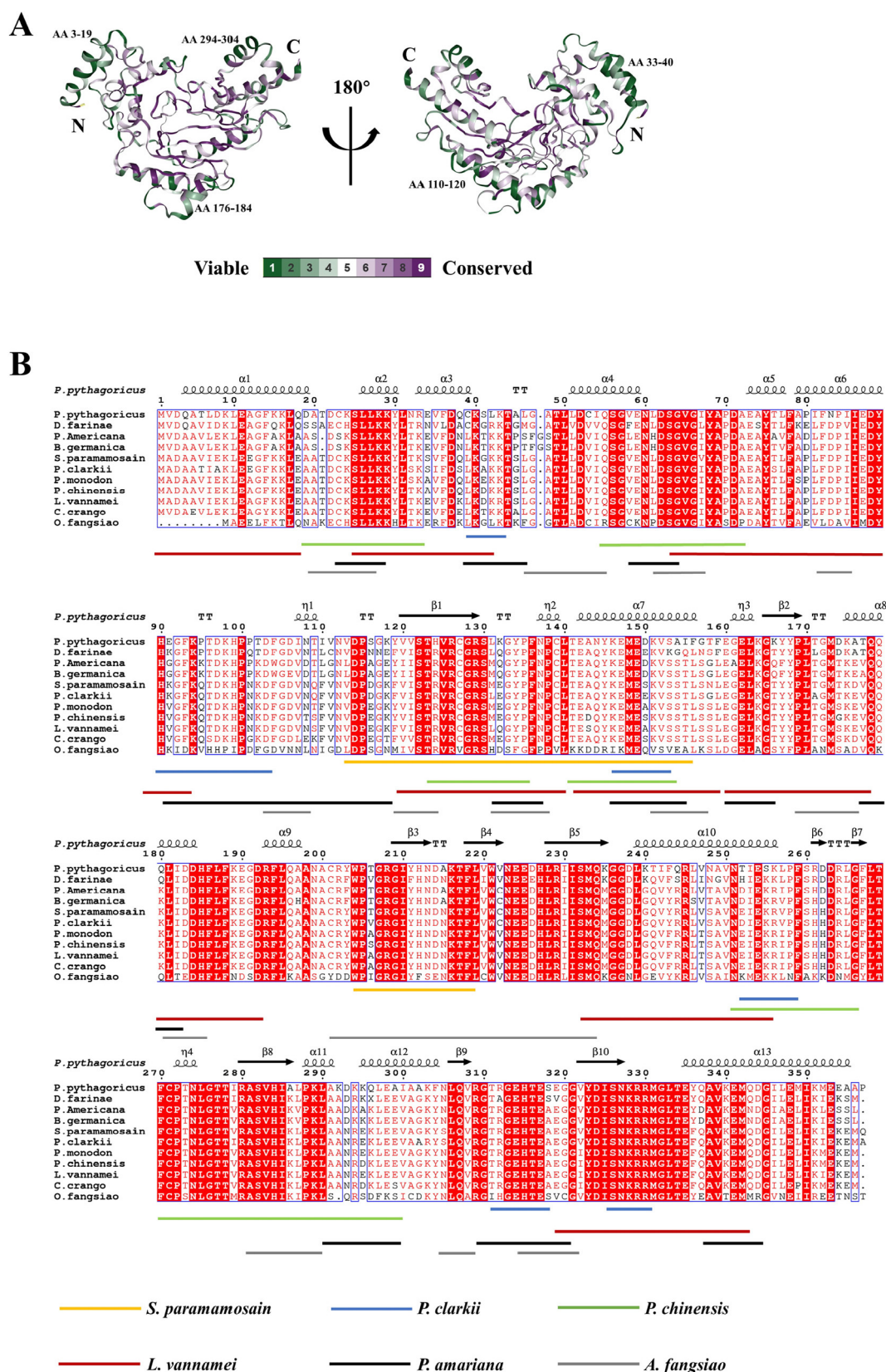


Fig. 3. Structural comparison of *S. paramamosain* AK to AKs from other species. A: ribbon representation of AK showing conserved residues derived from an alignment of 150 AK-related protein sequences selected by the ConSurf server. The most conserved residues are shown in purple, whereas the most variable residues are shown in green. B: sequence, epitopes and secondary structure alignment of AK from *S. paramamosain* with other species. The secondary structure elements of AKs are shown on the top of the aligned sequences. Identical regions are colored red, and conserved regions (identity > 70%) are shown in boxes. Epitopes are designated by colored lines below the AK sequence, red: experimental epitopes of *L. vannamei* AK; yellow: experimental epitopes of *S. paramamosain*; blue: predicted epitopes of *P. clarkii* AK; black: predicted epitopes of *P. amariana* AK; grey: predicted epitopes of *A. fangsiiao* AK; green: experimental epitopes of *P. chinensis* AK. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 2
Comparison of allergic arginine kinase in different species.

Species	IUIS name	MW (kDa)	AA length	Genbank accession No.	Linear IgE epitope regions
<i>Scylla paramamosain</i> (Yang et al., 2015; Mao et al., 2013)	–	40	357	AFA45340.1	113–127, 127–141, 141–155, 204–218,
<i>Procambarus clarkii</i> (Chen et al., 2013)	–	40	357	AFA45339.1	*39–43, 89–103, 146–152, 252–256, 311–317, 325–330
<i>Crangon crangon</i> (Bauermeister et al., 2011)	Cra c 2	40	356	ACR43474.1	–
<i>Penaeus chinensis</i> (Fu et al., 2018)	–	40	356	AAV83993	19–33, 55–72, 123–135, 140–154, 250–266, 286–299, 269–281
<i>Penaeus monodon</i> (Yu et al., 2003)	Pen m 2	40	356	ACT34086.1	–
<i>Litopenaeus vannamei</i> (Ayuso et al., 2010)	Lit v 2	40	356	ABI98020.1	1–18, 25–42, 64–96, 121–141, 142–159, 160–192, 232–255, 319–342
<i>Periplaneta americana</i> (Yang et al., 2016)	Per a 9	43	356	AAT77152.1	*23–28, 39–46, 58–64, 91–118, 131–136, 145–154, 159–165, 176–183, 290–299, 309–320, 338–344
<i>Blattella germanica</i> (Yan et al., 2011)	Bla g 9	40	356	AEV23883.1	–
<i>Dermatophagoides farinae</i> (Teng et al., 2018)	Der f 20	42	356	AAP57094.1	–
<i>Octopus fangsiao</i> (Shen et al., 2016)	–	38	348	AEK65120.1	*20–28, 46–54, 61–66, 81–85, 103–109, 119–124, 131–137, 150–157, 168–176, 181–185, 221–233, 281–289, 304–308, 314–321
<i>Polybetes pythagoricus</i> (Laino et al., 2017)	–	43	357	ASV64865.1	–

* The asterisk marked results represents the predicted epitopes.

Panallergens are thought to be involved in vital cellular processes and, accordingly, have similar structures although they come from species that may be quite distant evolutionarily. The primary structure of AK is highly conserved. Among 150 AK-related protein sequences selected by ConSurf, variable residue regions are mainly distributed in AA 3–19, AA 33–40, AA 110–120, AA 176–184, and AA 288–304 (Fig. 3A), which are lacking in linear epitopes of AK (Mao et al., 2013; Yang et al., 2015). In parallel, the secondary structures of allergenic AKs display relatively high identity. According to the allergenic epitopes labeled in Fig. 3B, three hotspot epitope regions were observed. The high identities in amino acid sequences and allergenic epitopes among AKs from crustaceans (Ayuso et al., 2010; Bauermeister et al., 2011; Chen et al., 2013; Fu et al., 2018; Yu et al., 2003), insects (Yan et al., 2011; Yang et al., 2016), arachnids (Teng et al., 2018), and mollusks (Shen et al., 2016) support the possibility of cross-reactivity among invertebrates. Furthermore, judging from the crystal structure, the three hotspot epitope regions are located on the surface of the protein, where they are likely to be accessible for direct IgE binding to the intact protein. These results provide more accurate information for the *in silico* prediction of IgE cross-reactivity among AKs.

Spatial structural similarity is a prerequisite for cross-reactivity of conformational epitopes (Stemeseder et al., 2017). To get a better understanding of the structural determinants relevant for cross-reactivity, the obtained three-dimensional structure of AK was compared with known structures of *L. vannamei* AK and *P. pythagoricus* AK. The *S. paramamosain* AK displayed a well-conserved structure to apo-AK and AK-Arg, while AK-TSAC had a significant difference. The superposition of the structures reveals structural differences at AA 175–184 and the C-terminus (AA 288–304, AA 335–355) according to *S. paramamosain* AK numbering. This is in accordance with the previous research of López-Zavala et al. and Laino et al., which demonstrated that the binary sequence of arginine to apo-AK did not cause structural changes, while significant differences could be observed in AK-TSAC (Laino et al., 2017; López-Zavala et al., 2013). That means that the structural differences were resulting from the formation of a ternary transition state analog complex rather than a species gap. Moreover, none of the previously reported conformational epitopes was found to be located in the structurally variable regions in the C-terminus (AA 288–304, AA 335–355). In other words, the spatial structural variations in these regions would not result in a significant reactivity difference.

Another inconsistent region is AA 175–184, which is also a variable sequence area. A conformational epitope of *S. paramamosain* AK (V₁₇₇G₁₇₂M₁₇₃D₁₇₆Q₁₇₈T₁₇₄L₁₈₁K₁₇₅L₁₈₇) distributes in this area (Yang et al., 2015). However, as a food allergen, AK is more likely to be in a ligand-free state rather than an AK-TSAC with enzymatic activity. Hence, the differences between *S. paramamosain* AK and AK-TSAC

might not impact on the conservation of conformational epitopes and the cross-reactivity among species. In addition, the reported conformational epitope of AK (L₃₁K₃₃V₃₅T₃₂E₁E₁₈F₁₄S₃₄D₃₇) is in the variable residue region AA 33–40 (Yang et al., 2015), while this region displays high spatial structural identity. In fact, except for AA 175–184, the other sequence variable regions show high identity in spatial structure, which might ensure the common properties of the conformational epitopes of AKs.

Reports on IgE from patients allergic to crustaceans that binds to proteins from different insects or arachnids are not rare, and homology among the proteins of crustaceans, insects, and arachnids has been previously documented (Yang et al., 2018). That is not surprising, for these species belong to the Arthropoda phylum and, as a result of this phylogenetic relationship, the homology is to be expected (Srinroch, Srisomsap, Chokchaichamnankit, Punyarit, & Phiriyangkul, 2015). In previous research it has been shown that there is cross-reactivity between crustacean and insect AKs, while the reason for this cross-reactivity is still unclear (Srinroch et al., 2015). The present research comprehensively compared allergenic AKs from various species. High sequence identity, linear epitope similarity, and conservation of spatial structure in the conformational epitope regions were observed not only among crustaceans and mollusks but also among shellfish and arachnids, revealing the foundation of the cross-reactivity of AK among species. To date, except for *S. paramamosain*, reports on conformational epitopes of AK have been few. The resolution of the crystal structure and the structural and epitope comparisons completed in the present work add information that will assist conformational epitope prediction and the identification of AKs.

The high identity of the structures and epitopes of AK results in cross-reactivity that might induce more serious anaphylaxis in patients. On the other hand, the processing methods for reducing the allergenicity of AK could be applicable across species. In a previous study, enzymatic cross-linking by tyrosinase and caffeic acid combined with thermal polymerization of AK effectively mitigated the allergenicity by modifying the tyrosines in the epitopes or structurally related sites (Fei et al., 2016). Based on the structure identity and epitope similarity, this method has the potential to reduce the IgE binding activity of AKs in other species. Besides, AK modified by the Maillard reaction with arabinose at residues K₃₃, K₁₁₈, and R₂₀₂ had the ability to induce oral tolerance in AK-sensitized mice (Han et al., 2018). Tolerance to crab AK induced by the modified allergen might also promote tolerance to AKs from shellfish, mollusks, and insects.

5. Conclusion

The natural folded structure of a protein offers information about

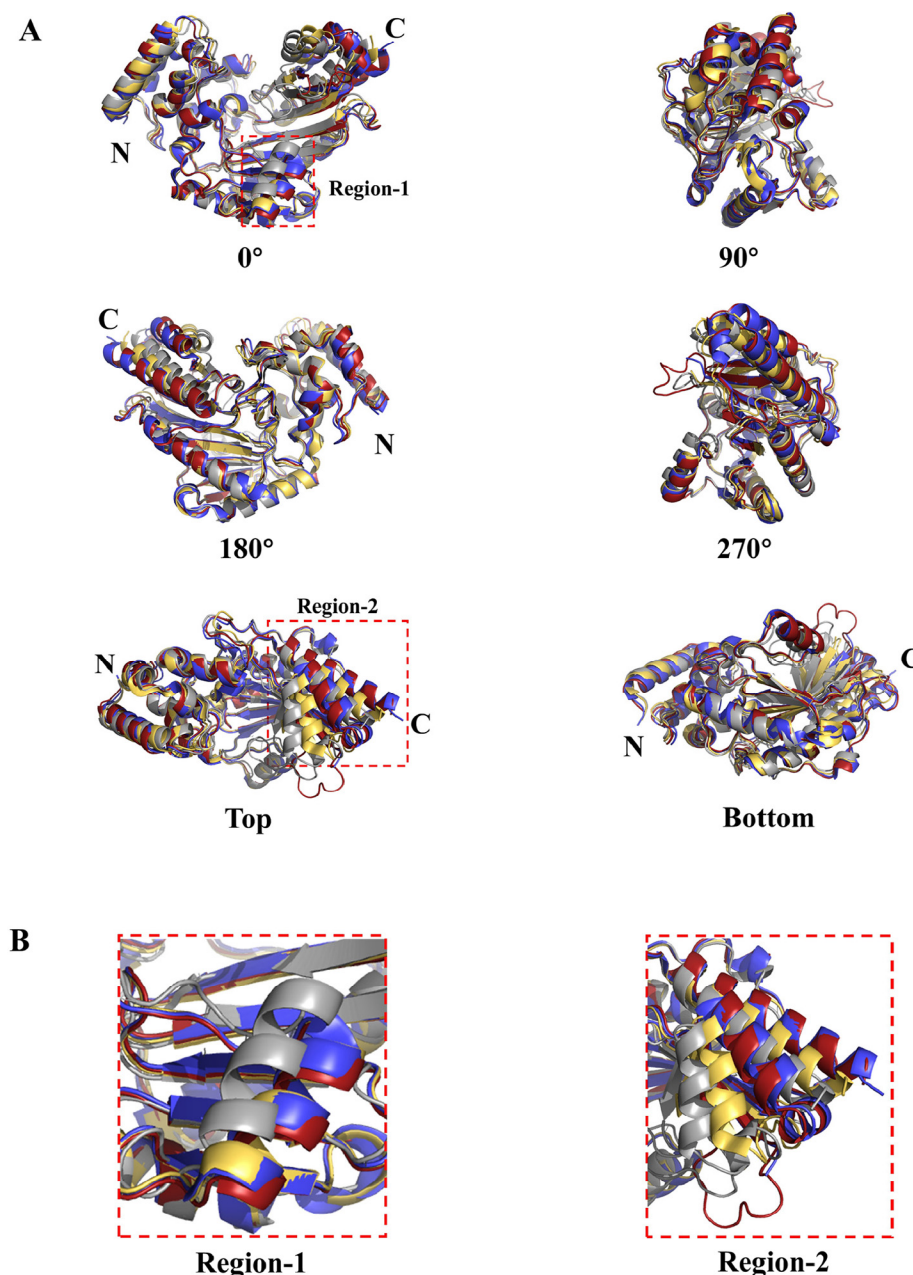


Fig. 4. Structural comparison of *S. paramamosain* AK with AKs from *L. vannamei* and *P. pythagoricus*. A: structure superposition of crab AK (5ZHQ, red) on the available AKs, yellow: shrimp AK with no ligand (apo-AK, PDB: 4AM1); grey: shrimp AK in a ternary transition state analog complex (AK-TSAC, PDB: 4BG4); blue: spider AK in a binary complex with arginine (AK-Arg, PDB: 5U8E). Two regions displaying structure differences are highlighted in boxes. B: detailed view of the two structurally divergent regions. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

the identities of relevant surface-exposed areas that are important for antibody binding. Hitherto, only a small fraction of food allergens have had their structures determined experimentally. The crystal structure of natural purified AK obtained in this study provides an accurate template for epitope evaluation and mapping. On the other hand, comparing structural data from distantly related species will allow for a more reliable basis for predictions of allergenic epitopes, especially those involved in cross-reactivities. The newly identified *S. paramamosain* AK structure will help to unravel the structural and immunological basis for cross-reactivity among species.

Conflict of interest

The authors declare no competing financial interest.

Protein data bank accession code

The coordinates and structural factors of *S. paramamosain* arginine kinase have been deposited in the Protein Data Bank with accession code 5ZHQ.

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