

Identification of arginine kinase as an allergen of brown crab, *Callinectes bellicosus*, and *in silico* analysis of IgE-binding epitopes

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ABSTRACT

In recent years there has been an increase in the prevalence of allergic reactions to contact with/or consumption of crustaceans by immune responses mediated by IgE antibodies. Arginine kinase (AK) is considered one of the main allergens present in marine invertebrates. Currently, the allergenic potential of the brown crab (*Callinectes bellicosus*), which is a crustacean of great economic importance, has not been studied. Therefore, the aim of this work was to identify *C. bellicosus* AK as an allergen and to predict IgE-binding epitopes through immunobioinformatic analysis. AK was purified by precipitation with ammonium sulfate and ion-exchange chromatography. AK allergenicity was evaluated by IgE reactivity against sera from crustacean-allergic and non-allergic patients in both native and denaturing conditions. Additionally, a homology model was built based on the deduced amino acid sequence. A single band (~40 kDa) was found in SDS-PAGE, which was identified as an AK by mass spectrometry. AK showed immunoreactivity against crab-allergenic sera in both native and denaturing conditions with 70% and 80% positive reactions, respectively. Additionally, a 1073 bp ORF was obtained which codes for a deduced sequence of 357 amino acids corresponding to AK with > 90% identity with other AKs. Structural homology model of AK showed two main domains with conserved / folding of phospho-guanidine kinases. BediPred and Discotope were used for epitope prediction analysis, which suggests eight possible linear epitopes and seven conformational epitopes, respectively; and shows to be similar to other crustaceans AKs. *C. bellicosus* AK was identified as an allergenic protein by IgE reactivity and immunobioinformatic analysis indicates that both linear and conformational epitopes could be located in the surface of *C. bellicosus* AK structure.

1. Introduction

In recent years, there has been a high demand for the production of seafood worldwide since it is an important component of the human diet and represents an abundant source of nutrients. However, hypersensitivity due to consumption and inhalation of or contact with shellfish, as crustaceans, can result in angioedema, urticaria, and even anaphylactic reactions where IgE antibodies mediate immune responses (Munera et al., 2012; Sicherer et al., 2004). Several reports have identified shellfish immunoreactive proteins as tropomyosin, myosin, troponin C, hemocyanin, and triosephosphate isomerase, among others (Daul et al.,

1994; Rahman et al., 2011; Shiomi et al., 2008). Similarly, arginine kinase (AK) has been reported as one major allergen in shrimp and other crab species (L. Fu et al., 2018; García-Orozco et al., 2007; Mao et al., 2013; Yang et al., 2018). Recently, Mao et al. (2013) predicted eight linear (39–44, 87–103, 146–151, 174–181, 253–256, 294–298, 309–317, 325–330) and seven conformational (91–100, 106, 115–116, 172–183, 263, 295–299, 315, 320–321) epitopes of AK. They also experimentally identified *Scylla paramamosain* in mud crab, using different immunological and bioinformatics techniques (Mao et al., 2013; Yang et al., 2018).

The warrior swimming brown crab, *Callinectes bellicosus*, is one of the

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most important species captured in Mexico. In 2019, *C. bellicosus* represented about 95% of total crab hatcheries (46,702 tons approximately), which have great economic impact (Ramírez-Félix, E., Singh-Cabanillas, J., Gil-López, H., Sarmiento-Nafate, S., Salazar-Navarro, I., Montemayor-López, G., García-Borbón, J., Rodríguez-Domínguez, G., Castañeda-Lomas, N., 2003. La pesquería de Jaiba (*Callinectes spp.*) en el Pacífico Mexicano: diagnóstico y propuesta de regulación. Comisión Nacional de Acuacultura y Pesca (CONAPESCA)-Instituto Nacional de la Pesca y Agricultura. Mexico, 48p.). Despite the increase in crab allergy among the general population, there is a gap in the identification and standardization of substances, as major allergens, for an effective diagnostic of crab allergy. In this sense, epidemiological reports about brown crab allergenicity are scarce. In Mexico, shrimp and shellfish are related to most hypersensitivity reactions among school-children (Ontiveros et al., 2016). In Portugal, shellfish consumption triggered allergic reactions in 2.1% of 840 adults analyzed (Lozoya-Ibáñez et al., 2016). Meanwhile, shellfish allergy in the Asia-Pacific region is higher, with a prevalence of about 15% among children and adults (Hao et al., 2018). Additionally, in Taiwan, crab is the most common food allergen among infants with a prevalence of 88% (Wan et al., 2010). *C. bellicosus* is broadly commercialized in North America and could represent a well model to understand immune response mediated by IgE in crab allergenic population. Currently, the allergenic potential of *C. bellicosus* is yet to be studied and the proteins responsible for causing allergic reactions remain unknown.

There are several experimental techniques to identify and characterize allergenic proteins and both lineal and conformational epitopes from allergens. Among them are phage display, overlapping synthetic peptides, mass spectrometry, and surface plasmon resonance (Z. Fu and Lin, 2017; Lopata et al., 2016). However, these methods are costly, time-consuming, and demand the intervention of specialized personnel for their development in some cases. Immunobioinformatics methods are relatively low-cost, accessible, and useful for predicting potential epitopes from immunological and allergenic proteins. They have been reported as a first approach or complementary tool to identify conformational and lineal epitopes (L. Fu et al., 2018; Wai et al., 2020). Therefore, the objective of the present work was to characterize AK as an allergenic protein in the brown crab *C. bellicosus* and predict IgE-binding epitopes using immunobioinformatics tools.

2. Materials and methods

2.1. Human sera

Sera of ten people with allergies to crab and/or other crustaceans (No. 763, 974, 184, 447, 164, 330, 793, 365, 480, and 354) were obtained voluntarily with the permission of the Research Ethics Council of Universidad de Sonora (approval number: CEI-UNISON 007/2020); the participants signed an informed consent. Crab allergy was based on the subject's clinical history and their responses to a questionnaire related to crab and other crustacean allergies. In addition, sera were collected from two non-crustacean allergic individuals (No. 503 and 875). Each serum was used individually, aliquoted, and stored at -20°C until used. The total IgE binding potential was quantified in all sera using an ARCHITECT c8000® analyzer (Abbott Inc.). The clinical information obtained from questionnaires and total serum IgE binding potential of non- and crustacean-allergic patients are shown in Supplemental file 1 (S1).

2.2. Purification and identification of AK

Male *C. bellicosus* specimens were obtained from the coastal area of Bahía de Kino, Sonora, Mexico. AK purification was performed using crab chela muscle, following the protocol described by García-Orozco et al. (2007) with certain modifications (García-Orozco et al., 2007). Briefly, 10 g crab muscle was homogenized with 50 mL lysis buffer containing 100 mM Tris-HCl, 25 μM PMSF, 10 mM 2-mercaptoethanol,

1 mM EDTA, and 1 mM benzamidine hydrochloride at pH 8.0, using a homogenizer (MRC®, model HOG-020) at medium speed for 10 s 3 times; there were 10-s intervals in an ice bath. The homogenate was incubated at 4°C overnight with constant stirring and clarified by centrifugation at $18,000 \times g$ at 4°C for 20 min. Afterwards, the supernatant was subjected to fractional precipitation with ammonium sulfate (60% and 90% saturation). The insoluble fraction at 90% of saturation was recovered and dissolved in buffer A (10 mM Tris-HCl, 10 mM 2-mercaptoethanol, and 0.1 mM EDTA; pH 8.0), and extensively dialyzed against buffer A using a 10–14 kDa cellulose membrane (Fisherbrand, USA). The dialysate was clarified as stated above, and protein concentration was measured by the Bradford assay (Bradford, 1976). This fraction was used in ion-exchange chromatography with a 5-mL Q Sepharose column (GE Healthcare, NY, USA). A linear gradient elution was performed from 0% to 100% with 1 M NaCl at a 3 mL/min flow. Fractions (3 mL each) were collected and analyzed by 12% SDS-PAGE (Laemmli, 1970). Fractions with a single band of ≈ 40 kDa were pooled and used for mass spectrometry analysis as follows: A single band was cut from the gel and reduced with dithiothreitol, alkylated with iodoacetamide, digested with trypsin, and analyzed by liquid chromatography with electrospray ionization and mass spectrometry (LC-ESI-MS/MS) at the Proteomics Laboratory, Universidad Nacional Autónoma de México. Partial sequence fragments were analyzed in Scaffold (Proteome Software), obtaining 25 unique peptides that were compared with the AK sequence reported for *C. sapidus* (UNIPROT: Q9NH49).

2.3. Immunoblotting and IgE reactivity

Fifteen micrograms of pure AK was loaded into both native-PAGE and SDS-PAGE and electrotransferred to a polyvinylidene fluoride (PVDF) membrane using a Trans-Blot Electrophoretic Transfer Cell (BioRad), as described by the manufacturer. Western blot was performed as follows: The membrane was washed with buffer (0.05% Tween 20 with TBS, TTBS) and incubated in blocking solution (5% non-fat dry milk in TTBS) at room temperature for 1 h. Then, the membrane was washed with TTBS, and several membrane strips were cut and incubated with serum (1: 250) from crab-allergic subjects as a primary antibody at room temperature for 24 h. The membranes were incubated with polyclonal rabbit anti-human IgE as secondary antibody (1:3000; Sigma Aldrich, USA) for 1.5 h. The secondary antibodies were previously biotinylated with 200 μL 0.1 M biotinyl-N-hydroxy-succinimide (BNHS) in dimethylformamide (DMF) and dialyzed against 0.1 M NaCl for 24 h (personal communication: PhD Ana Maria Calderon-De-la-Barca). Subsequently, the membrane was washed with TTBS buffer and incubated with peroxidase-conjugated streptavidin (HRP; EMD Millipore Corp, MA, USA) at a dilution of 1: 2000 with TTBS buffer for 1.5 h. Immunoreactivity was revealed using 3,3'-diaminobenzidine (DAB; Sigma Aldrich, USA) and hydrogen peroxide (30% w/v; H_2O_2) in TBS. The reaction was stopped once the presence of bands in the expected size was observed. Likewise, negative controls were carried out using the same described procedure with sera from non-crab allergic subjects. Finally, experimental controls for primary and secondary antibodies and HRP were assayed to evaluate signals or reactions or compare possible weak signals during immunoblotting.

2.4. cDNA and amino acid sequences of AK

Total RNA was extracted from brown crab muscle with TRIzol reagent (Invitrogen, CA, USA). The cDNA was synthesized using a SCRIPT cDNA Synthesis Kit (Jena Bioscience, Germany) and employed as a template to amplify AK gene by PCR. Two pairs of primers were designed based on the coding sequence of the Atlantic blue crab AK, *Callinectes sapidus* (GenBank: AF233355.1; Table 1). The PCR was performed in a C1000 Touch™ Thermal Cycler (Bio-Rad, USA) under the following conditions: 95°C for 5 min (one cycle), 95°C for 30 s, 60°C

Table 1
Primers used for cDNA amplification.

Primer	Sequence (5'–3')
AKFw1	ATGGCTGACGCTGCTACCATG
AKRv1	TTACTGCATTTCCTTCTCAATCTT
AKFw2	GACCAAGGAAGTGCAGCAAAAGC
AKRv2	GGAAGCGGTACCCCTCCTTGA

for 30 s, 72 °C for 1.5 min (35 cycles), and an extension of 72 °C for 10 min. PCR products were analyzed on 1% agarose gels and purified by GFX columns (General Electric, CA), following the manufacturer's instructions. They were sequenced at the Synthesis and Sequencing Unit, Universidad Nacional Autónoma de México. The cDNA sequence was analyzed by Chromas® (Technelysium, Australia) and BLAST algorithm. The deduced AK amino acid sequence of *C. bellicosus* was obtained in ExPASy (<https://web.expasy.org/translate/>). The AK from the *C. bellicosus* amino acid sequence was compared vs other crustacean AKs reported (GenBank: AF233355.1, AFK25805.1, Q004B5.1, and AAV83993.1), and an alignment was performed using the CLUSTALW algorithm.

2.5. Epitope prediction and molecular modeling

We predicted AK linear (i.e., sequential) B-cell epitopes using BepiPred 1.0 (Larsen et al., 2006) while conformational ones (i.e., non-sequential) were predicted using DiscoTope 2.0 (Kringelum et al., 2012). All predicted epitopes were compared against experimental and theoretical reported AK epitopes from other crustacean species like crabs and shrimps. The AK homology model was built using Phyre2 in intensive mode (Kelley et al., 2015). The best model obtained (100% confidence and > 80% coverage) was based on the crystal structure of *Limulus polyphemus* AK (PDB: 1RL9), which shares 75% amino acid identity to *C. bellicosus* AK. The AK homology model was used to locate predicted linear and conformational epitopes. Structural analysis and figures were performed using UCSF Chimera software (Pettersen et al., 2004).

3. Results

3.1. Purification and identification of *C. bellicosus* AK

The AK was purified from brown crab (*C. bellicosus*) muscle by fractional precipitation with 60% and 90% ammonium sulfate followed by ionic exchange chromatography. The SDS-PAGE analysis showed an abundant band corresponding to the expected molecular weight (~40 kDa) of AK in fractionation with ammonium sulfate at 90% saturation (Fig. 1, lane 3). This fraction was applied to ion-exchange chromatography. The elution at 40 mM NaCl showed a single band around 40 kDa (Fig. 1 lanes 6–10), corresponding to the molecular weight of AK. These fractions were pooled, extensively dialyzed to eliminate NaCl, and used to identify the protein by LC-MS chromatography. The partial sequences obtained were analyzed in Scaffold (Proteome Software) and compared against the AK sequence of *C. sapidus* (GenBank: AF233355.1). We obtained 25 unique peptides that correspond to 60% coverage of *C. sapidus* AK sequence and 100% of similarity; therefore the purified protein from *C. bellicosus* was identified as an AK (peptide sequence and alignment are showed in Supplemental file: S2).

3.2. Detection of AK as an allergen by IgE-binding

The immunoreactivity of purified AK against sera from crab/crustacean non-allergic and allergic patients was evaluated by western blot. Ten sera from allergic subjects (763, 974, 184, 447, 164, 330, 793, 365, 480, and 354) and two from non-allergic subjects (503 and 875) were used for immunoreactivity assays. Denatured AK reactivity was detected in 8 allergic sera from subjects 184, 447, 164, 330, 793, 365, 480, and 354 (shown in Fig. 2a, lanes 3–10). Only two allergic sera showed no reactivity with AK (Fig. 2a, lanes 1–2). After the immunoassay with native AK, 70% of the crab allergic patients showed reactivity against purified AK (Fig. 2b, lanes 4–10). Sera from non-allergic patients and experimental controls showed no reactivity against AK. These results demonstrated that purified AK of *C. bellicosus* is an IgE-reactive protein under denaturing and native conditions.

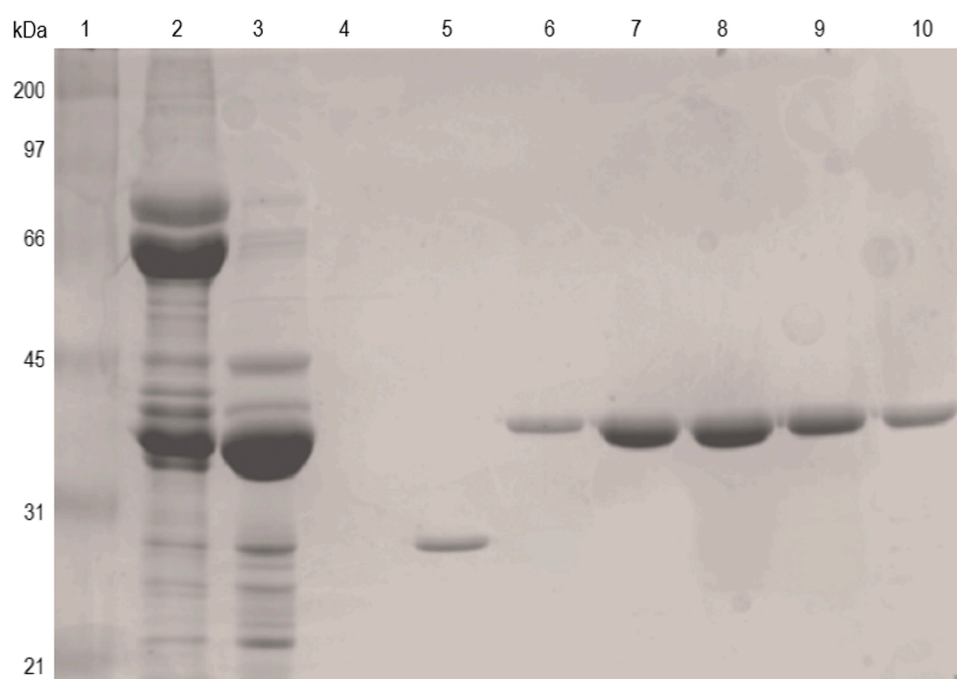


Fig. 1. Electrophoretic analysis of AK purification. Lane 1: molecular weight marker (MW), lane 2: clarified extract, lane 3: precipitated with 90% of ammonium sulfate, lane 4: empty, lane 5: non-bound protein to column and lane 6–10: eluted (0.04 M NaCl) purified AK from Q-sepharose column.

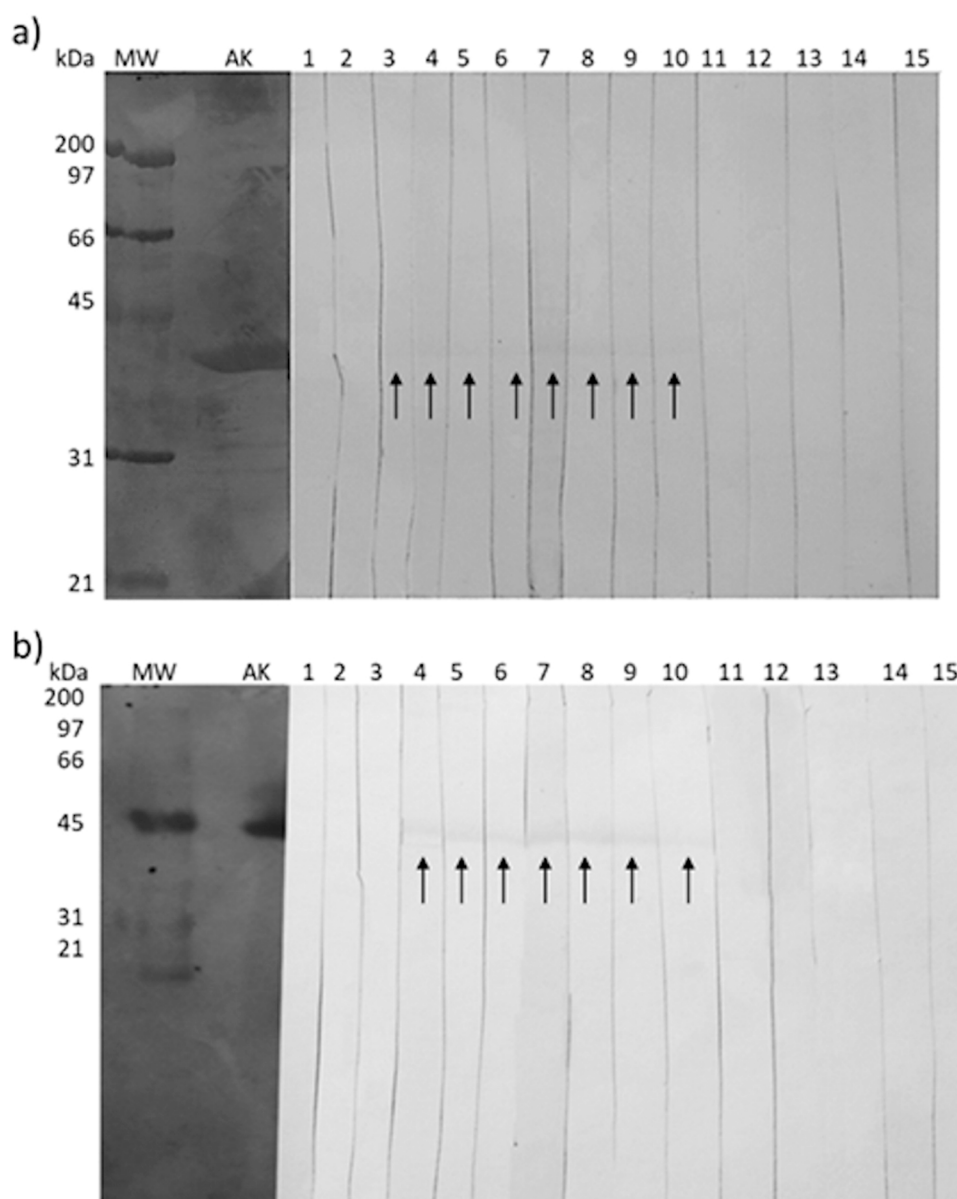


Fig. 2. Western blot analysis of *C. bellicosus* AK against both crab-allergic and non-allergic subjects. a) IgE-binding to denatured AK. b) IgE-binding to native AK. Sera numbers from crab-allergic patients 763, 974, 184, 447, 164, 330, 793, 365, 480, and 354 are indicated from lane 1–10, respectively. The non-allergic sera 503 and 875 are 11–12. The experimental controls without primary or secondary antibodies or HRP control are 13–15, respectively. Arrows indicated the detected bands. MW: molecular weight marker.

3.3. Sequence analysis and theoretical 3D structure of AK

The obtained coding sequence of brown crab AK showed an open reading frame of 1075 bp, start codon (ATG) at position 2, and stop codon (TAA) at 1073 bp. The nucleotide sequence was compared Atlantic blue crab (*C. sapidus*) AK (GenBank: AF233355.1), obtaining an identity of 99%. Therefore, the obtained sequence corresponds to an AK transcript. Subsequently, the deduced amino acid sequence (357 residues) was obtained using ExPASy (Fig. 3). An alignment of the deduced amino acid sequence was made with the AK from other crab and shrimp species (*C. sapidus*, Q9NH49; *S. paramamosain*, K7PD48; *L. vannamei*, Q004B5; and *P. chinensis*, Q4KY22), achieving > 92% similarity (Supplemental file S3). The cDNA and deduced amino acid sequences were deposited in GenBank under the accession number MW602301.

The deduced amino acid sequence of AK was used to build a structural homology model in Phyre2. The crystallographic structure of Atlantic horseshoe crab (*Limulus polyphemus*, PDB: 1RL9) AK was used as template; since shares 75% amino acid identity to *C. bellicosus* AK. The final model of *C. bellicosus* AK was obtained with a confidence level of 100% and a sequence coverage > 95%, indicating that the predicted

model is accurate under Phyre2 parameters (Fig. 4). The overall-theoretical structure of *C. bellicosus* AK consists of eight antiparallel β -sheets surrounded by seven α -helices, organized in a small N-terminal α -helical domain (residues 1–101) that interacts with a guanidine substrate. The large C-terminal domain (residues 102–357) contains an ATP-binding site, as shown in Fig. 4 (Suzuki et al., 2000). In addition, a well-conserved putative *C. bellicosus* AK active site was located in the vicinity of both domains; comprising Glu225, Cys271, and Glu314 which are implicated in an acid-base catalytic mechanism described in AK, as well as a guanidine/arginine binding-site, including Gly64, Val65, and Gly64 (Zhou et al., 1998).

3.4. Prediction of AK IgE-binding epitopes

Eight possible allergenic linear epitopes were predicted from the deduced AK amino acid sequence: LI: 40–45, LII: 58–74, LIII: 91–104, LIV: 112–117, LV: 130–136, LVI: 145–151, LVII: 203–213, and LVIII: 312–325 (Figs. 5a and 6). The spatial position of the predicted linear epitopes was identified using the 3D-theoretical model of *C. bellicosus* AK, (Fig. 5b). Only LII epitope was located in non-exposed regions while

1	TATGGCTGAAGCTGCTACCATGCCAAGCTGGAGGAGGGCTTCAAGAAGCTGGAGGCTGCC	61
1	M A E A A T I A K L E E G F K K L E A A	20
62	ACTGACTGCAAGTCTCTCCTCAAGAAGTACCTCACCAAGTCTGTGTTGACCAGCTCAAG	121
21	T D C K S L L K K Y L T K S V F D Q L K	40
122	GGCAAGAAGACAAGCCTTGGCGCCACCCCTCCTCGATGTGATCCAGTCTGGTGTGAGAAC	181
41	G K K T S L G A T L L D V I Q S G V E N	60
182	CTGGACTCTGGCGTTGGTGTGTATGCCCTGACGCCGAGGCCTACACCCCTCTTCGCCCT	241
61	L D S G V G V Y A P D A E A Y T L F A P	80
242	CTGTTGACCCCATCATCGAGGACTACCACAAGGGTTTCAAGCAGACTGACAAGCACCCCT	301
81	L F D P I I E D Y H K G F K Q T D K H P	100
302	AACAAGGACTTCGGCGATGTCAACCAGTTCGTCAACGTGGACCCCGATGGCAAGTTCGTG	361
101	N K D F G D V N Q F V N V D P D G K F V	120
362	ATCTCCACTCGCGTTCGTTGCGGTGCTCCATGGAGGGCTACCCCTTCAACCCCTGTCTC	421
121	I S T R V R C G R S M E G Y P F N P C L	140
422	ACCGAGGCCAGTACAAGGAGATGGAGTCCAAGGTTTCCTCCACCCTGTCCAACCTCGAG	481
141	T E A Q Y K E M E S K V S S T L S N L E	160
482	GGCGAGCTCAAGGGCACTTACTTCCCCCTCACCGGCATGACCAAGGATGTGCAGCAGAAG	541
161	G E L K G T Y F P L T G M T K D V Q Q K	180
542	CTGATCGACGACCACTTCTCTTCAAGGAGGGTGACCGCTTCTGTCAGGCCGCTAATGCC	601
181	L I D D H F L F K E G D R F L Q A A N A	200
602	TGCCGCTACTGGCCCACTGGCCGTGGCATCTACCACAACGACAACAAGACCTTCTGGTG	661
201	C R Y W P T G R G I Y H N D N K T F L V	220
662	TGGTGCAACGAAGAGGATCACCTCCGCATCATCTCCATGCAGATGGGTGGTGACCTGGGC	721
221	W C N E E D H L R I I S M Q M G G D L G	240
722	CAGGTCTACCGCCGCTTGTGTCAGCGCTGTTAACGAGATTGAGAAGCGCGTGCCCTTCTCC	781
241	Q V Y R R L V S A V N E I E K R V P F S	260
782	CACCATGACCGCCTGGGCTTCTCACCTTCTGCCCCACCAACCTTGGCACCACCGTGCGT	841
261	H H D R L G F L T F C P T N L G T T V R	280
842	GCCTCCGTCCACATCAAGCTCCCCAAGCTGGCCGCCAACCGCGAAAAGCTTGAGGAAGTC	901
281	A S V H I K L P K L A A N R E K L E E V	300
902	GCTGGCAAGTACAGTCTCCAGGTTTCGCGGCACCCGCGGCGAGCACACCGAGGCTGAGGGC	961
301	A G K Y S L Q V R G T R G E H T E A E G	320
962	GGCGTGACGACATCTCCAACAAGCGCCGCATGGGTCTTACTGAGTACCAGGCTGTTAAG	1021
321	G V Y D I S N K R R M G L T E Y Q A V K	340
1022	GAGATGCAGGACGGTATCCTTGAGCTCATCAAGATTGAGAAGGAAATGCAGTAA	1075
341	E M Q D G I L E L I K I E K E M Q -	357

Fig. 3. cDNA and amino acid sequence deduced of *C. bellicosus* AK. Alignment of the nucleotide sequence obtained and its translation into amino acids. The start codon (ATG) and the stop codon (TAA) are shown in yellow.

the rest were located near the surface of the AK structure. This suggests that IgE might bind to the enzyme through this type of epitopes. The LII, LIII, LV, LVI, and LVIII epitopes of the brown crab AK were the most conserved when compared to other AKs (similarity of > 50%) (Fig. 6).

DiscoTope 2.0 predicted 28 discontinuous residues in the 3D-homology model: 92, 94–98, 100–101, 103, 106, 108, 115–117, 172, 174–180, 183, 263, 293, 295, 317, 319–320, and 334 (Fig. 7a). Seven possible conformational epitopes were found as follows: CI 92, 94–98, 263; CII

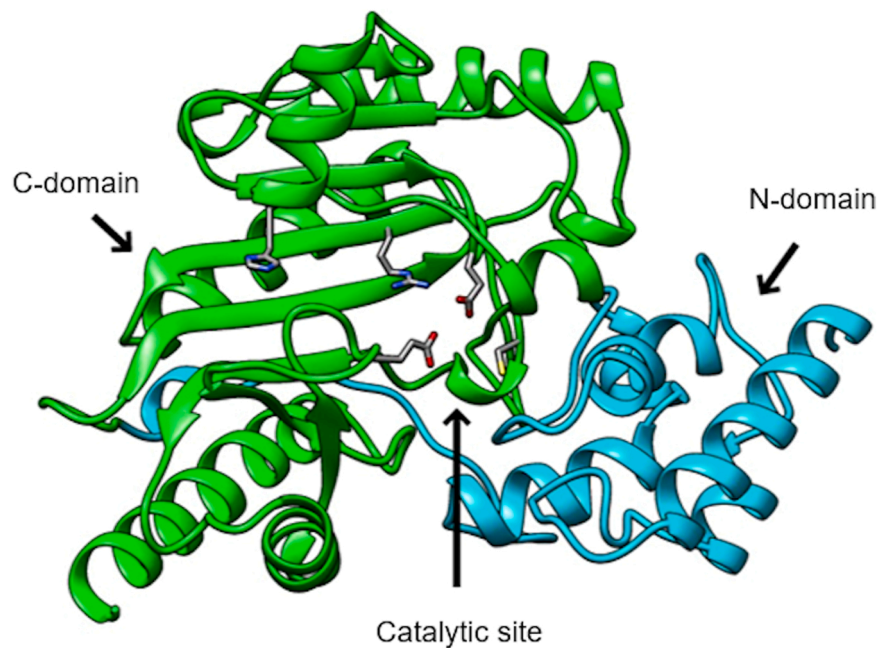


Fig. 4. 3D-homology model of *C. bellicosus* AK. C-domain is represented in green and N-domain in blue. Active site amino acids are shown in sticks and colored by atom type.

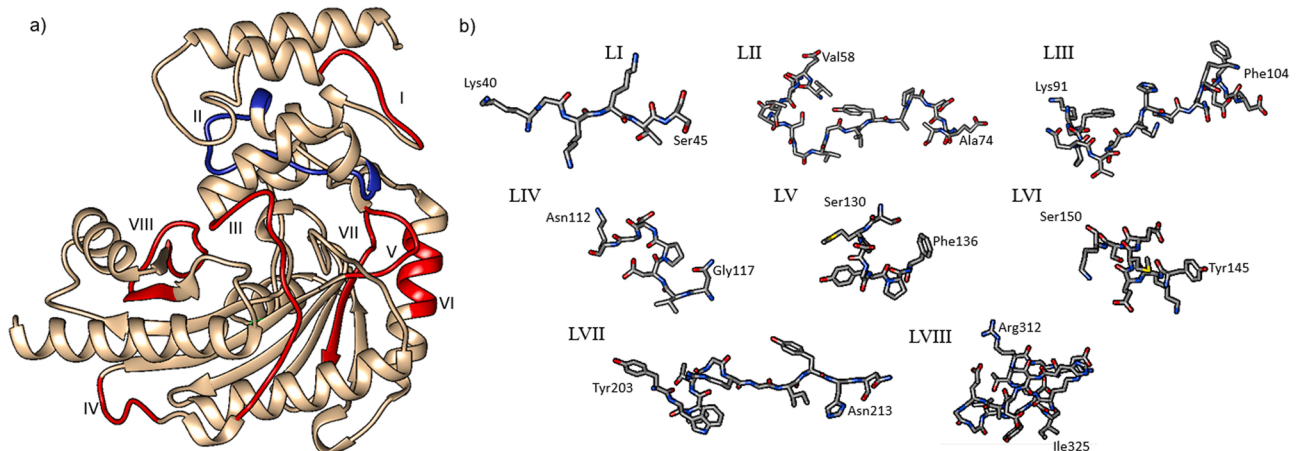


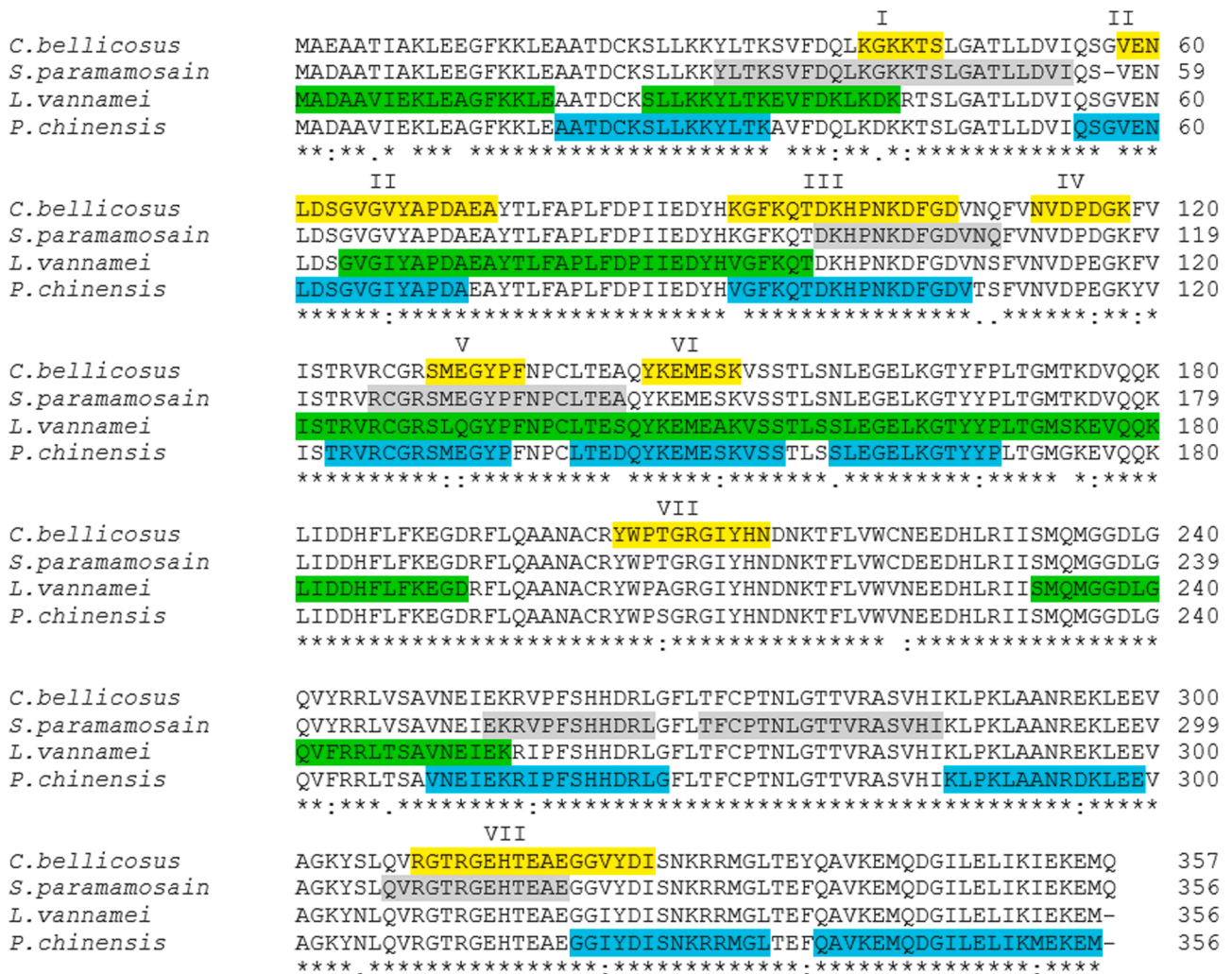
Fig. 5. Predicted linear epitopes of *C. bellicosus* AK. a) 3D-localization of predicted linear epitopes in the AK homology model. Exposed antigenic regions (I, III, IV, V, VI, VII, and VIII) are colored in red; while internal epitope (II) is shown in blue. b) Stick representation of the eight linear epitopes, which are colored by atom type.

100–101, 103, 334; CIII 106, 108; CIV 115–117; CV 172, 174–180, 183; CVI 293, 295; CVII 317, 319–320 (Fig. 7b). Predicted CI, CII, CIII, CIV, and CV epitopes were the most conserved (similarity > 81%) when compared to other crustacean AKs (Fig. 8).

4. Discussion

In recent years, cases of food allergies have been on the rise, becoming a major health problem, with shellfish allergies being one of the most common (Matricardi, 2014; Ruethers et al., 2018). In the last two decades, several allergens have been identified in crustaceans such as *Penaeus monodon*, *Crangon crangon*, *P. clarkii*, *S. paramamosain*, and *Portunus pelagicus* (Bauermeister et al., 2011; Chen et al., 2013; Mao et al., 2013; Rosmilah et al., 2012; Shiomi et al., 2008). This is the first report of an allergenic protein mediated by IgE immunoreactivity in the brown crab *C. bellicosus*. AK was purified and identified as arginine kinase with > 90% identity compared to *C. sapidus* sequence and > 70% to other crustaceans AKs. AK was identified as an allergenic protein in the

brown crab by IgE reactivity against crab-allergic subjects serum. Western blotting experiments showed that 80% of the allergic sera were positive for denatured AK, while native enzyme was assayed, 70% of allergic subjects sera showed IgE reactivity. Serum from subject 184 (Fig. 2, lane 3) showed IgE reactivity only against denatured AK. IgE antibodies from serum 184 could be sensitive to non-exposed linear epitopes of the enzyme, since this interaction may be favored when are AK was denatured. Mao et al. (2013) evaluated native and denatured AK of *S. paramamosain* against sera from allergic patients. IgE immunoreactivity was most predominant (nine subjects) to native AK; while under denaturing conditions, only three sera showed reactivity, concluding that AK from *S. paramamosain* has predominance for conformational epitopes (Mao et al., 2013). Our experimental results (Fig. 2) and complemented with bioinformatics analysis (Figs. 5–8), suggest that the antibodies in seven of ten serum from allergic patients could have allergenicity to both linear and conformational epitopes. While, one patient did not show IgE-reactivity only against denatured AK (Fig. 2, lane 3), which indicates that allergenicity is related to a conformational



The coding sequence of the AK was obtained and deduced amino acid sequence is well-conserved (>90% identity) when compared with other crustaceans AKs as crabs (*C. sapidus* and *S. paramamosain*) and > 70% identity to shrimps (*L. vannamei* and *P. chinensis*). *C. bellicosus* AK active site is also invariant such as guanidine binding amino acids (Gly64, Val65, Gly66, and Tyr68) and catalytic residues as Cys271 and Glu225; this last is related to an acid-base mechanism which is involved Glu314 (López-Zavala et al., 2013; Yousef et al., 2003). Bioinformatics tools allow obtaining accurate protein structural homology models when an experimental structure is not available as *C. bellicosus* AK. Homology model of *C. bellicosus* AK was built with 100% of coverage and confidence factors based on Phyre2 algorithm; and shows typical folding of monomeric AK as reported in horseshoe *Limulus polyphemus* AK (PDB entry 1BG0) and the white shrimp *L. vannamei* AK (PDB entry: 4AM1). *C. bellicosus* AK is composed of two domains: the small N-terminal is comprised mainly by α -helices from residues 1–100, while the large C-terminal domain shows an α - β folding; within the active site located in the vicinity of both domains (Fig. 4) (Kenyon et al., 2012; Suzuki et al., 2000; Uda and Suzuki, 2004; Wu et al., 2012; Yousef et al., 2003; Zhou

In recent years, the amino acid composition in allergenic proteins has been studied to understand the interaction with antibodies. It has been found that epitopes are mainly composed of polar and charged amino acids, which are frequently located on the surface of the protein (Dal-l'Antonia et al., 2011; Minkiewicz et al., 2015; Pomés et al., 2020). This promotes the antigen-antibody formation complex since the amino acid side chains are exposed, thus favoring the union with antibodies (Dal-l'Antonia et al., 2011; Zhao et al., 2012). Though, less frequently hydrophobic aliphatic amino acids in allergenic epitopes are also found as tyrosine (Rubinstein et al., 2008). In our study, eight linear and seven conformational epitopes were predicted; containing mostly charged amino acids as lysine, glutamine, aspartic acid, and glutamic acid residues (Figs. 6 and 8). Aromatic amino acids (except for tyrosine) and aliphatic appear less frequently. Likewise, several authors have reported that epitopes are constantly found in the loops of the secondary structure of proteins since these are flexible regions and are exposed on the surface of the molecule (Janin and Chothia, 1990; Reimer, 2009; Zhao et al., 2012). The LVI and LVII linear epitopes are located in α -helix and β -sheet, respectively, while the remaining epitopes were found in loops (Fig. 5). While, all conformational epitopes were located on the surface of *C. bellicosus* AK homology model as noted in Fig. 7b. The linear epitopes predicted were compared with those reported in *S. paramamosain* (Mao et al., 2013), *L. vannamei* (Ayuso et al., 2010) and *P. chinensis* (L. Fu

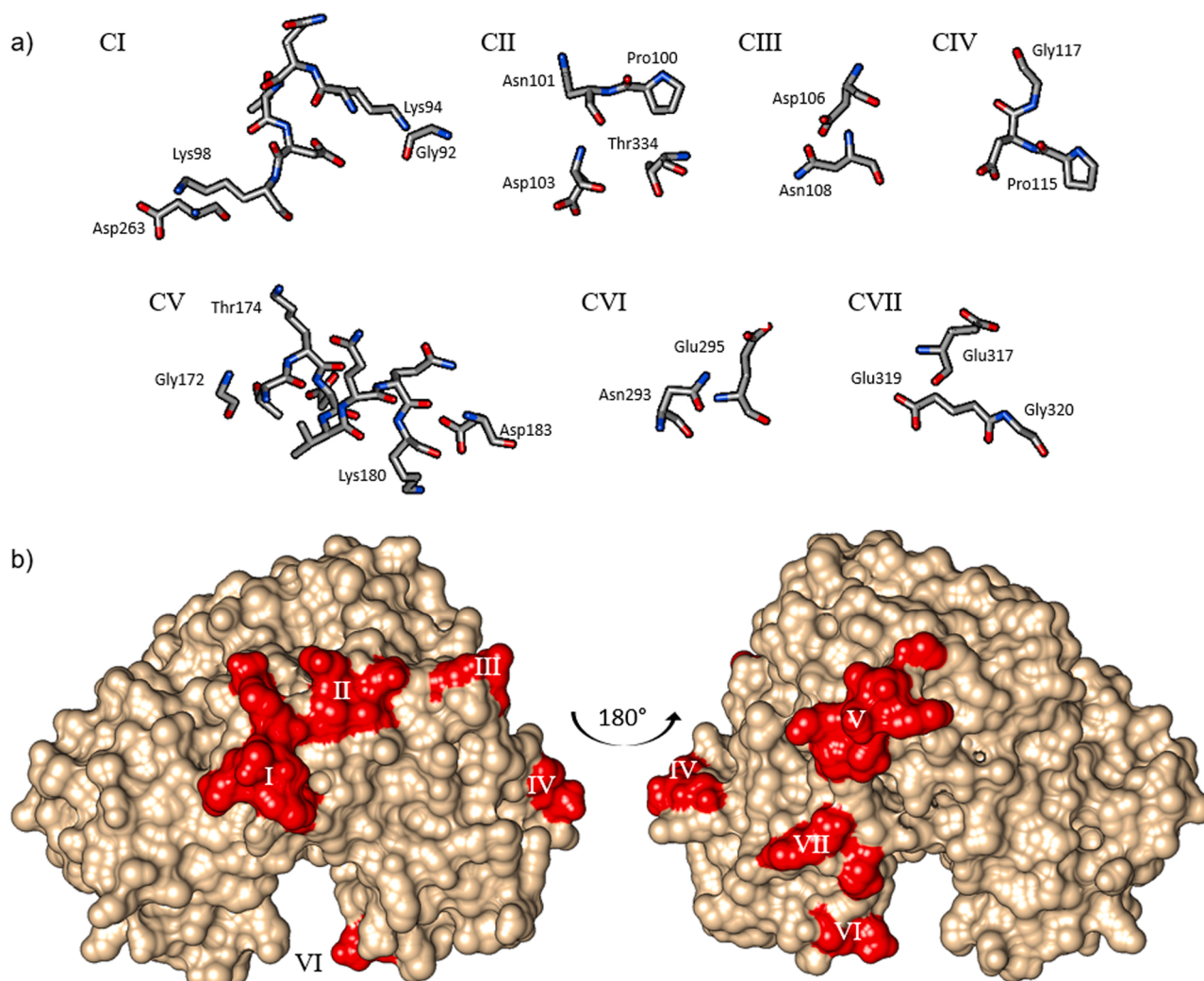


Fig. 7. Spatial localization of *C. bellicosus* AK predicted conformational epitopes. a) Structure of the eight conformational epitopes is shown in sticks. b) 3D-structural surface representation of predicted conformational epitopes are colored in red. The model was rotated 180 degrees on axis X to facilitate visualization of predicted epitopes.

et al., 2018) AK with > 50% of identity (Fig. 6). Whereas, conformational epitopes are also well conserved (> 81% identity) with the conformational epitopes reported in *P. clarkii* (Chen et al., 2013) and *S. paramamosain* AK (Mao et al., 2013) (Fig. 8). Additional experimental studies such as synthetic overlapping peptides or phage techniques are necessary to confirm and characterize both linear and/or conformational of epitopes predicted *C. bellicosus* AK.

In conclusion, our results suggest that *C. bellicosus* AK is related to crab-allergenicity mediated by IgE response against sera from crab-allergy patients. Also, *in silico* analysis indicates that both linear and conformational epitopes are found in *C. bellicosus* AK and may be related to cross-reactivity with others crustaceans AKs. Further immunological, biochemical and structural studies will help to elucidate the cellular response of *C. bellicosus* AK and to confirm predicted epitopes. This information will help in the development of more accurate allergy-crab diagnostic tools.

Ethics approval and consent to participate

This research was approved by the Research Ethical Committee from Universidad de Sonora (CEI-UNISON 007/2020). All patients/subjects that participates in this study signed written informed consent.

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