https://doi.org/10.18502/jad.v18i3.18575

Original Article

Purification and Molecular Characterization of a Mammalian Neurotoxin as a Pharmaceutical Tool from the Venom of Iranian Scorpion *Androctonus crassicauda*

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(Received 04 Sep 2022; accepted 04 Oct 2023)

Abstract

Background: Venom of scorpions are complex bioactive polypeptides. To gain greater insights into the structural and functional impacts of toxins from *Androctonus crassicauda* (Buthidae), a dangerously venomous scorpion species, its venom was isolated, purified, and characterized.

Methods: Long chain toxin with four disulfide bonds purified by size exclusion chromatography and reversed-phase HPLC and characterized by amino acid sequencing and molecular weight determination.

Results: The primary structure analysis exhibits a neurotoxin named AnCra2 with 7302.24 Da molecular weight and 64 amino acid residues that cause paralysis and lead to death in NIH mice. The LD_{50} of AnCra2 was determined to be 0.61±0.04 μg/mice. Phylogenetic analysis displays the toxin has 97% sequence similarity with alpha toxins reported from north African scorpions that affect voltage-gated sodium channels (VGSC), also proposed that differentiation among the scorpions of family Buthidae is affected by the geographical conditions and efficiency in evolutionary variations. AnCra2 exposed binding residues have a high affinity for receptor residues in site-3 (segment-3) of VGSC that are approved by three-dimensional structure and homology modeling.

Conclusion: Purified AnCra2 seems to be a new putative Alpha neurotoxin in homology with the structure of neurotoxins that act on VGSC as a pharmaceutical tool.

Keywords: *Androctonus crassicauda*; Phylogenetic analysis; Molecular modeling

Introduction

Venom of scorpions are complex bioactive polypeptides. The neurotoxins acting on different ion channels are the best-studied groups that impair their functional characteristics (1, 2). Studies on interactions between neurotoxic peptides and ion channels exhibit the molecular mechanism of ion channel action and show venom polypeptide structure had deferent binding properties to different receptors binding sites (3, 4). *Androctonus crassicauda* scorpion from the family Buthidae is considered a medically important species and vastly distributed in the Middle East (5–7). In Buthidae, scorpion venom contains a diversity of peptides, such as sodium, potassium, calcium, and chloride channel toxin family, cytolytic peptides, and enzymes (8, 9). The sodium channel toxins with four disulfide bonds are long-chain polypeptides containing 65–76 amino acid residues and, act as neurotoxins and influence the operation of the voltage-gated sodium channel (VGSC) (1, 6). The potassium channel toxins with three or four disulfide bonds and 45 amino acid residues are short peptides (10).

The main dangerous scorpion venom peptides to mammalian that affect sodium and potassium ion channel toxins have been widely studied as tools to analyze ion channels (11,

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Published Online: September 30, 2024

https://doi.org/10.18502/jad.v18i3.18575

12). *Androctonus crassicauda* venom contains complex toxic peptides that act on various ion channels causing severe autonomic and central nervous system abnormalities (2, 12). A previous report by Caliskan et al. reveals Acra 4 and Acra5 toxic peptides purified from the scorpion venom as specific VGSC α-type and beta-type toxins respectively (12). Our research group purified and characterized AnCra1 (*Androctonus crassicauda* neurotoxin 1) as a novel putative Alpha-mammal VGSC toxin with functional activity, from Iranian *A. crassicauda* (13).

In this study, we propose to purify the AnCra2 toxin from the Iranian scorpion *A. crassicauda*, to obtain amino acid sequencing and modeling of peptide, then compare with the VGSC blocker toxins that are certified in UniProt databanks and used for phylogenetic relationship analysis. The structural activity of toxins is understood by using molecular dynamic simulation software.

Materials and Methods

Sample preparation

Chemicals, solvents, and bovine serum albumin used in this study were purchased from Merck (Germany). Venom was filtered by a 0.2 µm filter (Sartorius, USA). The Pharmacia column (2.3×150 cm) and Sephadex G-50 were used for size exclusion chromatography and C18 analytical columns (4.6×150 mm) from Agilent were done on reversed-phase high-performance liquid chromatography (RP-HPLC) (Amersham Bioscience AKTA). Christ α1-2 (Germany) was used for lyophilization of the venom.

Live scorpions were captured from the southwestern region of Iran (Khuzestan Province, Baghmalek) from May to August 2018, and transferred to the Laboratory in Razi Vaccine and Serum Research Institute. Electrical stimulation was used to obtain venom from the telson of the scorpions and lyophilized (13). Venom (200 mg) dissolved in 10 ml distilled water. Mucoproteins were removed by centrifuging at 24100× g for 20 min. To determine protein concentration after the supernatant was filtered through a 0.45 µm filter (Sartorius USA), a Nanodrop spectrophotometer (Thermo Scientific, Wilmington, DE) was used (14).

Purification

The soluble *A. crassicauda* venom was initially separated by a sephadex G-50 size exclusion chromatography column. The column was eluted with 0.1M ammonium acetate, at a flow rate of 40 ml/h. Nine fractions (F1-F9) were collected by column elution profile separately and measured at 280 nm absorbance. The toxicity of each fraction was tested by injection of 0.5ml of the fraction intravenously in two mice and monitored for 24h. The linear acetonitrile gradient of 10 to 45% was used for elution and the measured at 215 nm and 280 nm. Fractions were collected based on the peaks absorbance and freeze-dried. Purification was repeated several times to obtain pure toxin. Fractions were freeze-dried by Christ α1-2 freeze drier and further purified by analytical RP-HPLC using a C-18 column (4.6×150 mm) with 0.1% trifluoroacetic acid (TFA) in water (15).

Mass spectroscopy and Amino acid sequence

Matrix-assisted laser desorption ionization-time of a fight (MALDI-TOF/TOF) mass spectrometer analysis performed on a Centre of Excellence in Mass Spectrometry (University of York, Heslington, York, YO10 5DD, UK), was used for venom fraction analysis in the positive linear mode. The AnCra2 was reduced by dithiothreitol and the cysteines were *S*-alkylated with 4-vinyl-pyridine as described previously (13). The alkylated peptide was subsequently desalted by C8-reversed-phase HPLC. Determination of amino acid sequences performed by Edman degradation on a PPSQ-23 peptide sequencer (Shimadzu Co., Japan) based on recommendations. Phenylthiohydantoin derivatives were characterized as described previously (16). Position of disulfide bound and

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Published Online: September 30, 2024

https://doi.org/10.18502/jad.v18i3.18575

amino acids similarity in sequence were performed using BLAST (ncbi.nlm.nih.gov/blast) and Cysteines Disulfide Bonding State and Connectivity Predictor (disulfind.dsi.unifi.it).

Lethality test

The lethality of crud venom (whole venom) was tested in NIH mice (weighting 18–20 g) following injections of various concentrations of the venom to determine LD_{50} by the Spearman-Kärber method (۱۷, ۱۸). However, partially purified fractions as well as purified toxins were also tested for toxicity by injection into the tail vein of the mice just to determine the presence of toxin in fraction. In the control group, 0.5 ml of normal saline was injected into the mice. The mice were observed for 24 h, post injection. Approval for this study was obtained from the ethical committee of the Razi Vaccine and Serum Research Institute according to the National Institutes of Health guide for the care and use of laboratory animals (NIH Publications no. 8023, revised 1978).

Homology modeling

The multiple sequence alignment analysis was done by Bioedit 7.2.6 (Clustal W) and BLASTP algorithm (blast.ncbi.nlm.nih.gov/Blast.cgi) methods. The Basic Local Alignment Search Tool (uniprot.org/blast) was used by BLOSUM80 matrices for getting scores. The molecular modelling of the peptide was generated by Swiss Modeler (19) and RaptorX web server (20). To obtain the final model structure by Molegro Virtual Docker 6.0 software, then the toxin was subjected to refinement by energy minimization in Swiss Modeler using a GROMOS96 force field. Based on the Uniprot database, the homology model for the pore domain of human VGSC (NCBI entry Q99250.3) was simulated using the Cryo-EM structure of human VGSC (PDB ID: 6NT4). The VGSC –toxin complex is generated by docking the toxin structure to the VGSC model using the program HDOCK (hdock.phys.hust.edu.cn) (21) and further analysis accomplished by Molegro Virtual Docker 6.0. Molecular dynamics simulations exhibit channel-toxin complexes to identify the residues of toxin interacting with VGSC channel.

Alignment and phylogenetic analysis

The phylogenetic analysis of the purified toxin of this study with sodium channel toxins of the family Buthidae reported in the UniProt database (uniprot.org) involved 21 amino acid sequences. All the sequences used in the phylogenetic tree drawing had 64 amino acids in length. Multiple sequence alignments were performed by Bioedit software (22) to draw the phylogenetic tree and consensus amino acids. The phylogenetic tree was determined by MEGA 10.0.5 software with 1000 Bootstrap replications, the Neighbor-Joining method, and the evolutionary distances were computed using the p-distance method (23).

Results

Toxin Purification

Size exclusion column chromatography was used to fractionate 100 mg crude venom. In total, nine fractions (F1 to F9) were obtained (Fig. 1A). NIH mice were used to evaluate the biological activity of all fractions. Fractions F5 and F6 were identified as toxic fractions, and the other fractions were nontoxic. We selected fraction 6 for this study however fraction 5 was previously published (13). Afterward F6 was loaded on the C18 RP-HPLC column for further purification and eluted by acetonitrile gradient (Fig. 1B). Two separate zones of peaks were isolated, and the 15.48 min peak was explored as a toxic fraction (F61). The purified toxin was eluted at 15.48 min (Fig. 1C) with extra purification by RP-HPLC on a C18 column and named AnCra2.

Bioassay

The purified toxin from C18 RP-HPLC column (AnCra2) was injected into NIH mice, the LD_{50} was 0.61±0.04 µg/mice (18–20 g) or 30.5±2 µg/kg mice based on Spearman-Kärber

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Published Online: September 30, 2024

https://doi.org/10.18502/jad.v18i3.18575

method (17, 18). *Androctonus crassicauda* whole venom in different concentrations was injected intravenously into NIH mice weighing 18–20 g and its LD_{50} was found to be 4.06±0.3 µg/mice. Signs and symptoms that appeared following the whole venom injection were found to be severe paralysis, leading to death. As Fig. 2 shows the AnCra2 LD_{50} was calculated and found to be 0.61±0.04 µg/mice (18–20 g). The symptom that appeared following toxin injection in mice was muscle contraction, leading to severe paralysis and animal death indicating that, AnCra2 is a mammalian lethal toxin.

Structural analysis

Purified toxin AnCra2 fully sequenced, at the first step, 36 amino acid residues from the N-terminus of toxin, revealed by reduced and alkylated in Direct Edman degradation. Three additional RP-HPLC fragments lead to a full sequence of 64 amino acid residues obtained. The experimental molecular mass of AnCra2 was determined about 7302.24 Da, which corresponds to the MALDI TOF/TOF mass spectrometer (Fig. 3). The AnCra2 sequence analysis shows, toxin with four disulfide bonds (eight cysteines) has a long chain peptide (Fig. 3).

The 7302.24 Da purified AnCra2 is the alpha-toxin active polypeptide. The AnCra2 multiple sequence alignment, based on cysteine, and other conserved N and C-terminal residues exhibits highly considerable homology of AnCra2 and mammalian neurotoxins with long-chain polypeptides belonging to VGSC blockers (Fig. 4). The sequence identity matrix exhibits the AnCra2 has significant homology with the Alpha-toxins (BioEdit 7), including α- toxin Amm5 (97%) from *Androctonus mauritanicus mauritanicus*, α- toxin Lqq5 (95%) from *Leiurus quinquestriatus quinquestriatus*, α- toxin Acra8 (93%) from *A. crassicauda* and α- toxin AahP1005 (88%) from *A. australis*.

Figure 6 exhibits the molecular docking for the VGSC (6NT4)/AnCra2 complex (Fig. 6). Toxin and channel make contacts together with two and four amino acid residues respectively. The C-terminal coil of AnCra2 toxin residues consists of Arg and Lys. The exclusively exposed and accessible segments in peptides with hydrophilic amino acids are shown as antigenic sites. Polypeptide residues of VGSC in domain IV voltage sensor, segment site-3/ site-4, consisting of Glu, Phe, and Leu are functional residues present in protein-protein interaction between VGSC and AnCra2.

Phylogenetic analysis

AnCra2 toxin from Iranian scorpion *A. crassicauda* with 21 known specific peptide sequences of VGSC toxins from other Buthidae family scorpions was used to construct a molecular phylogenetic tree by the Neighbor-Joining, using the p- distance method and 1000 replicates bootstrap as the evolutionary history of taxa in consensus tree (Fig. 7).

The phylogenetic tree in Figure 7, confirms that VGSC toxins formed the consensus sequences from the family Buthidae are divided into two main groups with four branches. The relationship between the scorpion toxins in cluster (II), group (A), exhibits: AahP1005 from *A. australis*, alpha-toxin Acra8 from *A. crassicauda*, Alpha-mammal toxin Lqq5 from scorpion (*L. quinquestriatus quinquestriatus*), Amm5 toxin from *A. mauritanicus mauritanicus* and AnCra2 from *A. crassicauda*. The cluster (II) presents high similarity with Alpha VGSC toxin sequences. The sequence similarity shows that AnCra2 (Iran) and Amm5 toxin from Morocco have a close relationship together. Based on the phylogenetic relationship of consensus toxin sequences, all of the toxins belonging to the family Buthidae species with highly conserved domains are from geographical areas of North Africa. All VGSC sequences in group one have high sequence similarities, high affinity to VGSC (receptor site 3), disulfide bond significant homology, and impairing of VGSC (inhibit inactivation or slow).

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Published Online: September 30, 2024

https://doi.org/10.18502/jad.v18i3.18575

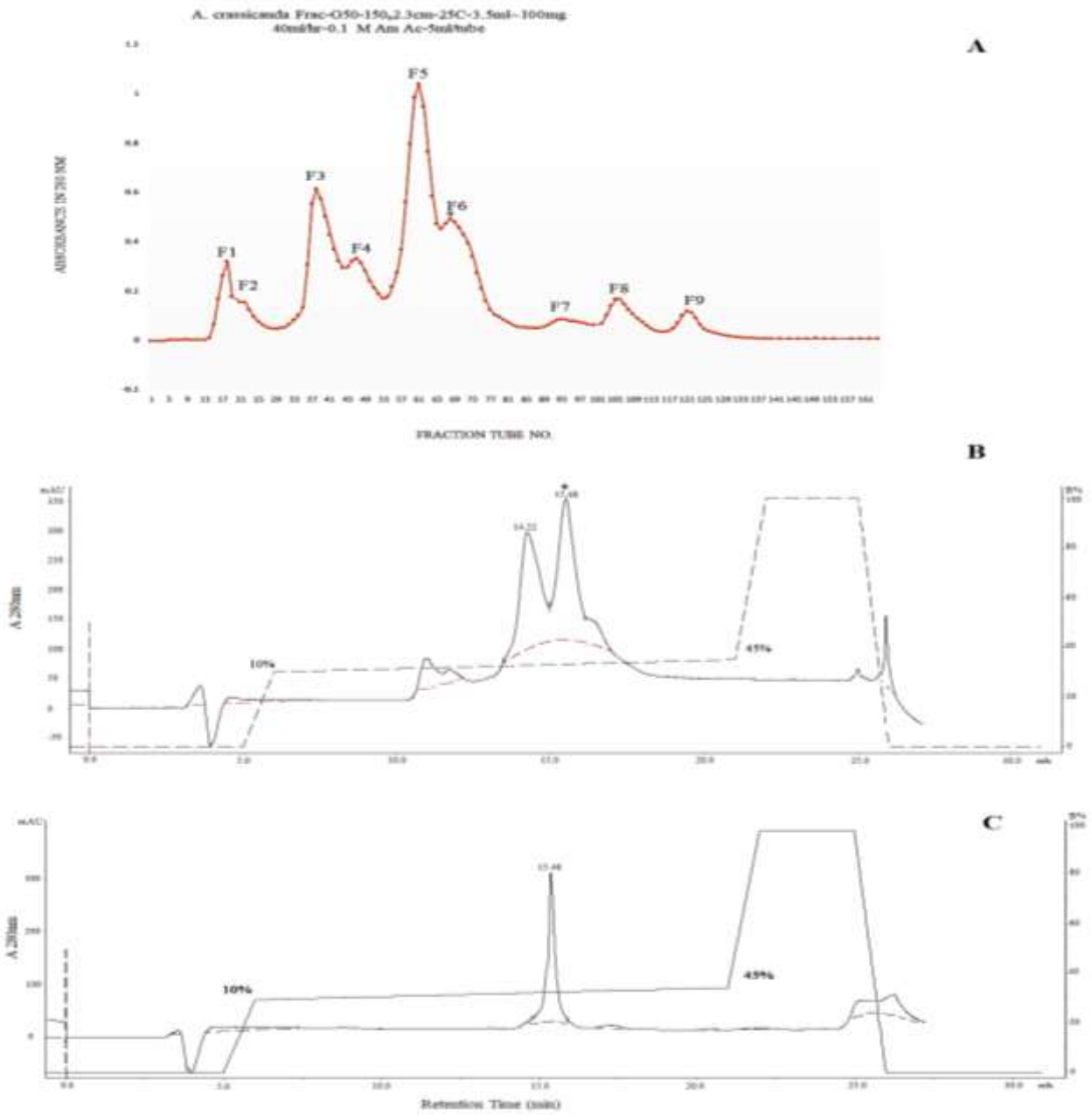

Fig. 1. A. Sephadex G- 50 gel chromatography profile of the *Androctonus crassicauda* venom. Elution was carried out using 0.1M ammonium acetate, at a flow rate of 40 ml/h. The eluents profile exhibited nine peaks (F1-F9). The toxic F6 fraction was lyophilized. B. Purification of AnCra2. RP-HPLC profile of the F6 fraction. Further purification of the fraction was carried out on C18 Pharmacia analytical RP-HPLC column, equilibrated with 0.1% of TFA in water. Elution was carried out using linear gradient of acetonitrile from 10 to 45%. The AnCra2 (F61 fraction) retention time was at 15.48 min, labeled with (*) was shown to be lethal to mice. C. Purification of AnCra2. RP-HPLC profile of extra purified toxic sub-fraction on a C18 column. The final purified toxin eluted at 15.48 min which is named as AnCra2

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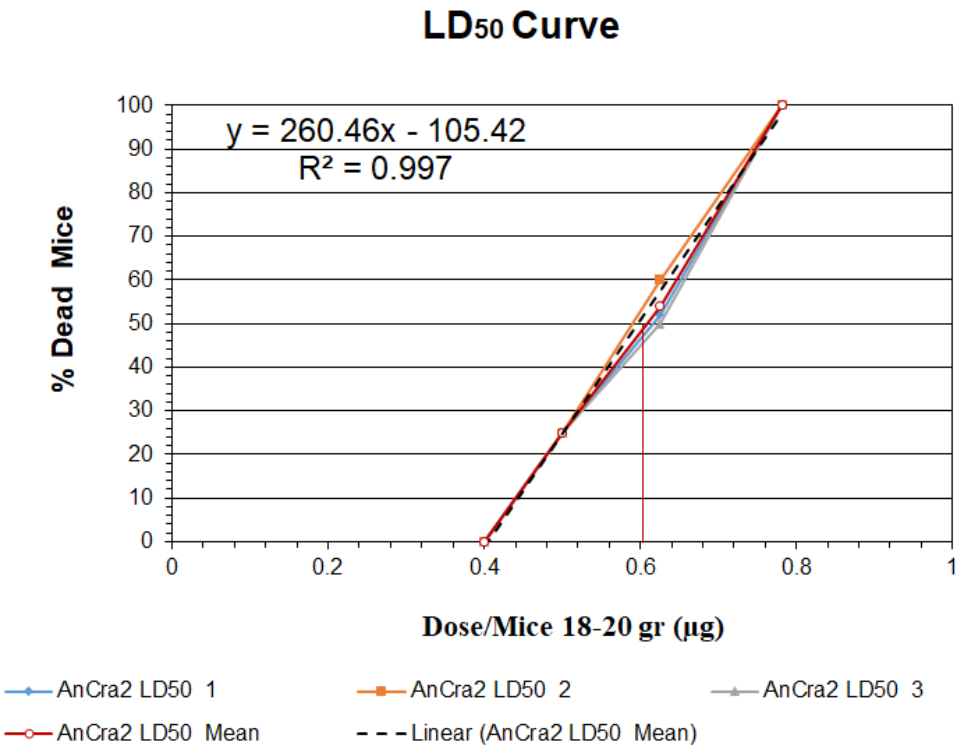

Fig. 2. The LD_{50} curve (graph) of AnCra2 purified toxin originated from *Androctonus crassicauda* venom from Khuzestan Province, southwest Iran, purified and injected intravenously to NIH mice weighing 18–20 gr. The LD_{50} was calculated and found to be 0.61±0.04 µg/mice. The graph was drawn by GraphPad Prism 8.0 software

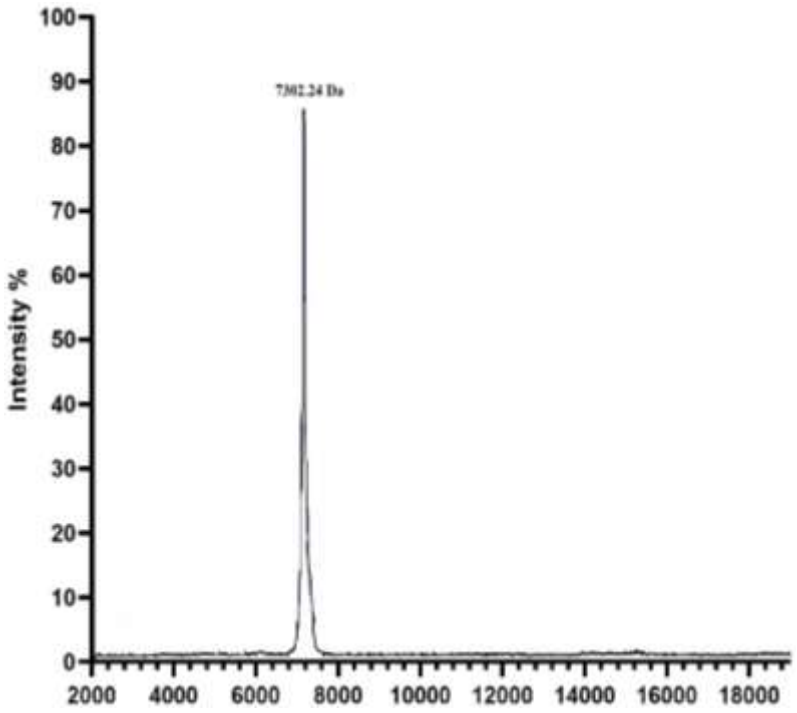

Fig. 3. Determination of AnCra2 molecular mass originated from *Androctonus crassicauda* venom from Khuzestan Province, southwest Iran. The 7302.24 Da of 64 amino acid residues with four disulfide bridges for AnCra2 as a long chain scorpion toxin

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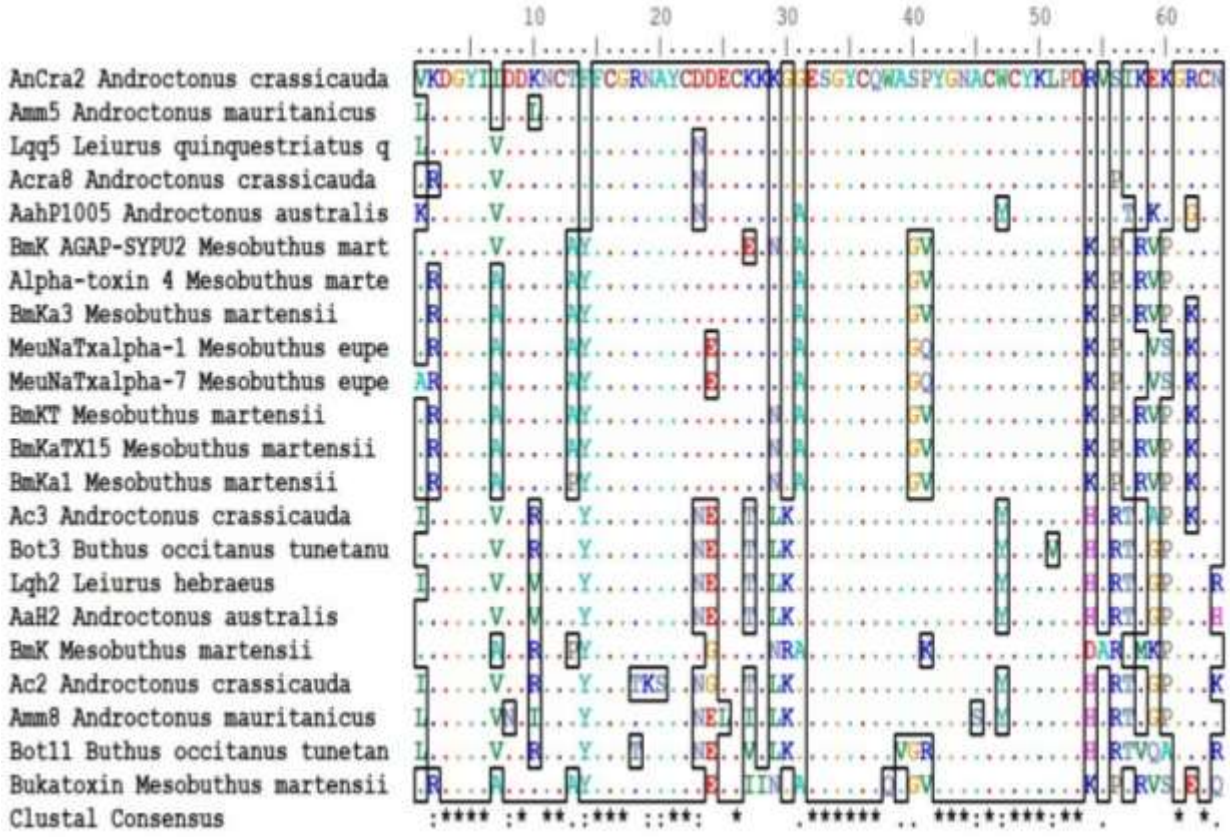

Fig. 4. The toxin, AnCra2 Sequence analyses of amino acid residue sequence alignment of AnCra2 originated from *Androctonus crassicauda* venom from Khuzestan Province, southwest Iran with the known scorpion alpha-neurotoxins belong to VGSC blockers. Multiple alignment analysis of AnCra2 and other VGSC blocker amino acid sequence from UniProt data bank exhibits that conserved residues existed in βαββ structure. The conservation score below that position is an asterisk (*), indicating that the amino acid at that position is conserved. (:) conservative mutation, (.) semi conservative mutation. The alignment was done by Bioedit 7

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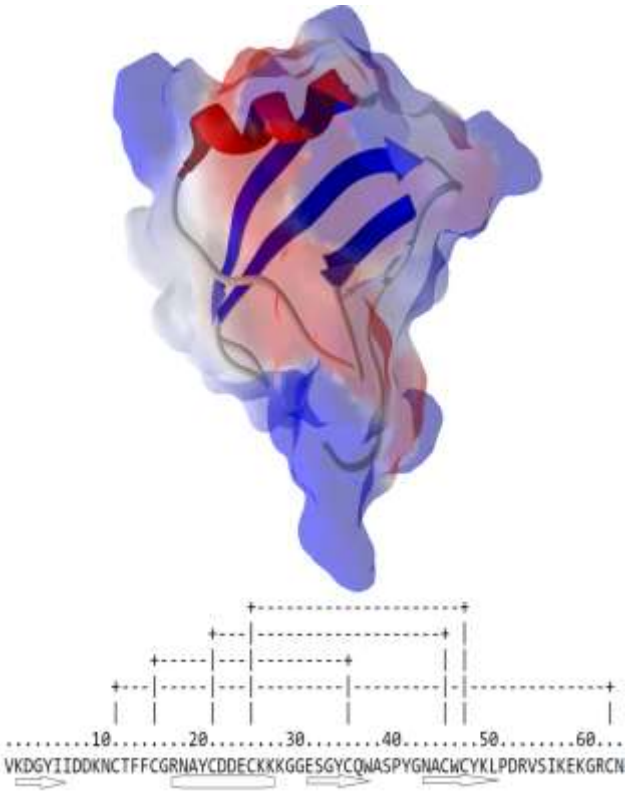

Fig. 5. Structural analyses of AnCra2 originated from *Androctonus crassicauda* venom from Khuzestan Province, southwest Iran. The alpha-toxin structure was predicted by Molegro Virtual Docker 6.0. AnCra2 scaffold as VGSC toxin, consist of two disulfide bridges that linked a β-sheet to α-helix, while third Disulfide Bridge between β-strand and coil and fourth bridge linked coil to turn. (+----+) means disulfide bridges sites, 3 arrows stand for β-sheets and rectangular box refers to α-helix

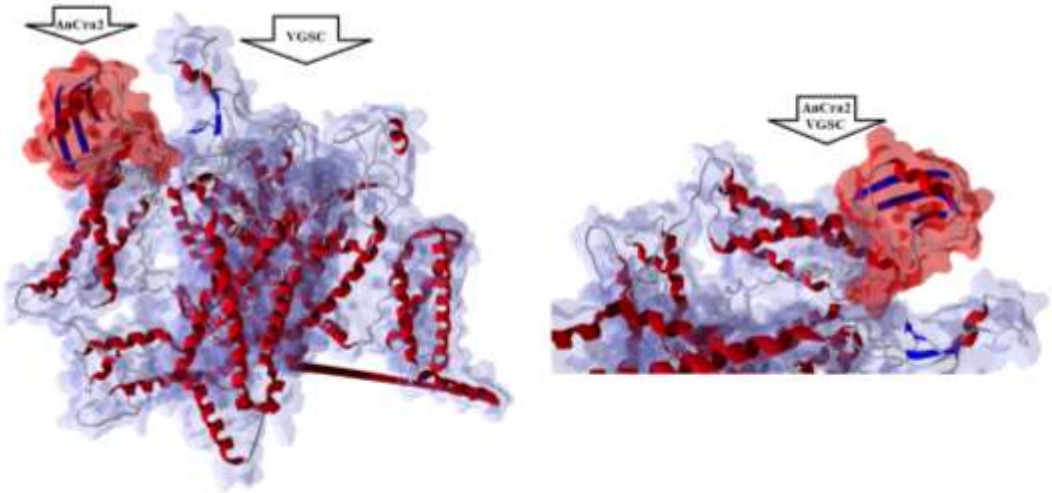

Fig. 6. The molecular dynamic simulations of voltage-gated sodium channel –AnCra2 complex originated from *Androctonus crassicauda* venom from Khuzestan Province, southwest Iran. Important interactions indicated explicitly. Two toxin residues that contact four amino acids on the channel. Residues on exposed surface part of the AnCra2 exhibit the β-sheet and C-Terminal coil that have significant function for binding to the site-3 segment of the domain IV voltage sensor at helical region of receptor in VGSC. (⏢) contain the name of peptides. Predicted by Molegro Virtual Docker 6.0

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Published Online: September 30, 2024

https://doi.org/10.18502/jad.v18i3.18575

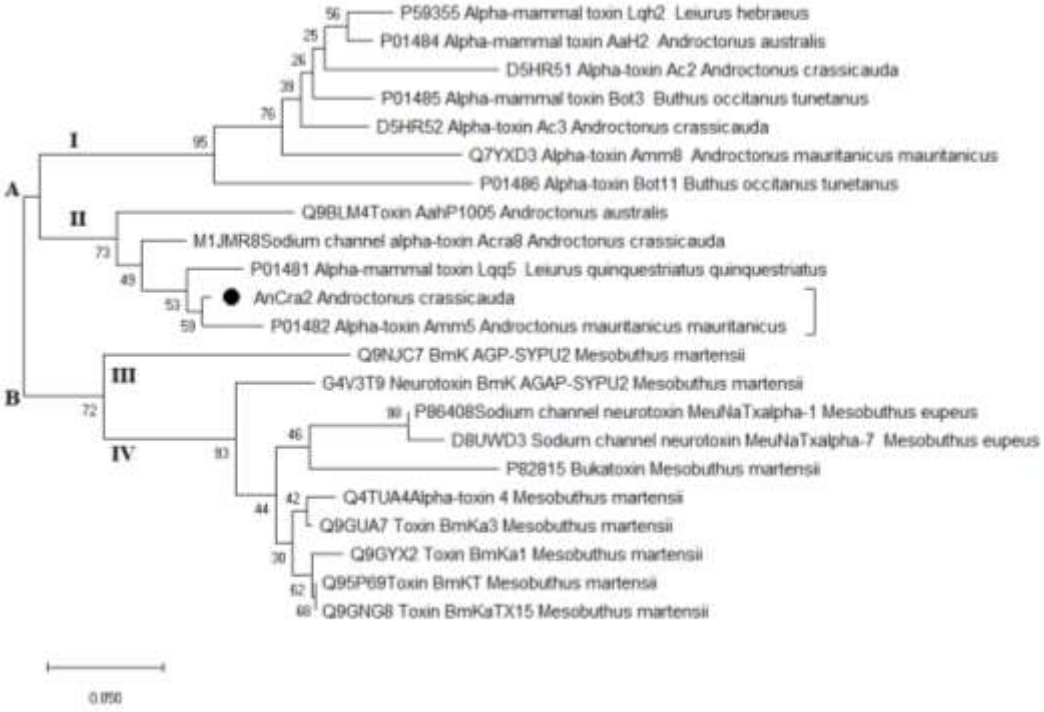

Fig. 7. The phylogenetic tree of AnCra2 originated from *Androctonus crassicauda* venom from Khuzestan Province, southwest Iran together with 21 sequences specific for VGSC toxins from other Buthidae species. All the sequences used in the phylogenetic tree drawing had 64 amino acids length. AnCra2 toxin (•) has close relationship with alpha-toxin Amm5 (Morocco) that labeled with bracket. A and B are first and I, II, III, IV are second branch types, the numbers on the nodes are bootstrap values. The Neighbor-Joining method embed in MEGA X was used to draw the tree

Discussion

This research characterizes AnCra2 as a new purified long-chain α-toxin from *A. crassicauda* venom and illustrates its structural features by MALDI TOF/TOF mass spectrometry, Direct Edman degradation amino acid sequencing, modelling, and phylogenetic analysis. AnCra2 has biological activity, low molecular mass, and four disulfide bridges, therefore is more stable during RP-HPLC so separated by RP-HPLC with structural protein folding (24). Figure 3 shows the 7302.24 Da 64 amino acid residues with four disulfide bridges for AnCra2 as a long-chain scorpion toxin.

The VGSC α-toxins have a positive surface potential binding affinity (25) that represents the physical linkage between positively charged toxin surfaces with negatively charged receptor site residues. The net charge of the AnCra2 neurotoxin peptide is 2, indicating the pI: 8.15. The presence of eleven Arg and Lys as positively charged residues, and nine Asp and Glu as negatively charged residues may determine the positive charge (web.expasy.org/cgi-bin/protparam/protparam and pepcalc.com). Molecular alignment showed this peptide has considerable homology with neurotoxins; alpha- toxin Amm5 (97%) from *A. mauritanicus*, alpha-toxin Lqq5 (95%) from *L. quinquestriatus*, alpha-toxin Acra8 (93%) from *A. crassicauda*. These VGSC neurotoxins are subjected to modelling and explaining the interaction between AnCra2/VGSC. The structure of the AaH2 toxin (4aei.1) (26) with a P-value of 1.08e.6, uGDT 66, and GDT 67 were taken as templates, to determine the AnCra2 structure. The AaH2- Fab4C1 complex crystal structure was previously determined by X-RAY. AnCra2 alpha-toxin structure de-

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Published Online: September 30, 2024

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termined by the SWISS-MODEL server and for final modeling, used Molegro Virtual Docker 6.0. AnCra2 peptide has α- helix from Arg/R 18 to Lys/K 28 and three β -strands, one from Lys/K 2 to Ile/I 7, the second from Glu/E 32 to Trp/W 38, and the third from Asn/N 44 to Leu/L 51 (Fig. 5).

The evaluation of the AnCra2 model quality was shown by the WHAT IF program and based on stereochemical quality implication, the AaH2 (SCX2_ANDAU) toxin structure confirmed the AnCra2 model as a homologous toxin. The PROCHECK v.3.5.4 (27, 28) reported that the toxin model has a Ramachandran score of 78 and the AnCra2 structure has a similar scaffold with neurotoxins that act on the VGSC. Because of the high relation and great molecular homology between AnCra2 and AaH2 and recent molecular structure of interaction between AaH2 and VGSC (6NT4) reported (29), therefore in our study we used VGSC (6NT4) structure for molecular dynamic simulations analysis.

The explanation of the interaction between AnCra2 and VGSC was obtained by molecular modelling and not confirmed experimentally. The core βαββ scaffold in VGSC channel scorpion toxins contains an α-helix and three antiparallel β-sheet, intermediate variable loops, and exclusive homology with particular functional properties (30, 31). Four disulfide bonds in VGSC toxins lead to folded active peptides that consist of three conserved disulfide bridges in the core structure and a fourth disulfide bond can be adjusted in diverse arrangements (6). Toxin peptide secondary structure impressed the constitution of toxins epitopes intensively. AnCra2 scaffold as VGSC toxin, consists of two disulfide bridges that link a β-sheet to α-helix, while a third Disulfide Bridge between β-strand and coil and a fourth bridge links coil to turn (Fig. 5). This study modelling shows the pattern of disulfide bonds of AnCra2 toxin is similar to the VGSC blocker toxins. The biologically active conformation of small molecular size toxin peptides that are stabilized by a high number of disulfide bonds, are stable in tropical environments and are highly resistant to denaturation (2). Comparison between scorpions VGSC neurotoxins in the Buthidae family by multiple sequence alignment exhibit consensus sequence, for instance, a special motif Cys-X-X- X-Cys exists in the core of α-helix in potassium channel blocker toxins (32). The primary structure of AnCra2 shows an α-helix with Cys-D-D-E-Cys (Cys-X-X-X- Cys) pattern and a β-sheet with Cys-W-Cys (Cys-X-Cys) pattern similar to VGSC blockers that organized two disulfide bonds between α-helix and third β-sheet. In AnCra2, folding the α-helix and three β-sheets accomplished by three disulfide bonds and the core of the toxins stabilized through, α –helix link to β3-sheet by residue Cys3-Cys6 and Cys4-Cys7, creating two disulfide bridges (conserved from N- to C-terminal). The third conserved structurally disulfide bond crate between Cys5-Cys2. The fourth disulfide bond, links the N-terminal (Cys1) to the C-terminal (Cys8) varies in position but is exposed on the molecular surface (Fig. 5). The core of the toxins in the βαββ scaffold, three conserved disulfide bonds have existed and only in excitatory toxins the fourth bond is conserved (10).

The core secondary structure of α-toxins like, Aah2 and Aah3 and Bot3 consists of βαββ scaffold including an α-helix and three antiparallel β-sheets with some diversity in sequence homology (1, 4, 6, 33). The specificity of toxic peptide residues to bind to ion channel receptors is different in diverse ion channels. In potassium channel toxins the important residues are located in the β –sheet at the solvent-exposed part of the toxin that functionally interacts with the outer cavity and blocks the channel (34). In scorpion VGSC toxins, important residues interact with two distinct pharmacologically receptor binding sites. Scorpion VGSC toxins bind at receptor site-3 (α-toxins) and site-4 (β-toxins) and prevent channel inactivation, instead shift the channel voltage-dependent activation (35, 36). Residues on

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Published Online: September 30, 2024

https://doi.org/10.18502/jad.v18i3.18575

the exposed surface part of the AnCra2 exhibit the existence of Lys located on the β-sheet and Arg and Glu on the C-Terminal coil, which have significant functions for binding to the site-3 segment of the domain IV voltage sensor residues, Phe, Glu, and Leu at the helical region of receptor in VGSC (Fig. 6). The suggested interaction site was based on VGSC (PDB ID: 6NT4) due to previous studies and availability of the structure (13). Beta scorpion VGSC toxins interact with an extracellular side at exposing site-3/site-4 loop of domain- II of VGSC receptor (37, 38). The active site of AnCra2 with flexible hydrophilic amino acids, indicates that the exposed antigenic sites are located in the same molecular surface region as other α-toxins. Linker-domain of AnCra2 as a potentially alpha VGSC toxin docked in the extracellular side of VGSC at the voltage-sensing domain, accordingly, the model demonstrated that functional surface of toxin located in the middle of linker domain.

Based on structural analysis AnCra2 can be defective VGSC and molecular modeling shows that scorpion alpha mammalian toxins together with AnCra2 as a potentially alpha VGSC toxin, bind to site-3/site-4 extracellular loop in domain IV and corroborate previous reports (36, 37). Molecular docking and binding pattern of alpha toxins with the extracellular side of the VGSC site-3/site-4 linkers, demonstrate the considerable function of various regions of VGSC for defective in gating process. Previous physiological studies show that, alpha toxins like AaH2 and Amm8, raid the extracellular loop in domain IV of VGSC, receptor site 3 positions and cause to inhibit the fast inactivation procedure of the channel (4, 39). Multiple alignment analysis of AnCra2 and other VGSC blocker amino acid sequence from UniProt data bank exhibits that 17 conserved residues have existed in βαββ structure (Fig. 4). Previous studies showed that the presence of a second β-sheet with fully conserved residues (ESGYCQW), is homologous in alpha-mammal VGSC toxins subfamilies (13) and AnCra2 has correlation with this structure, furthermore highly positive charged in C-terminal and 12 similar amino acid residues with high homology pattern to VGSC blocker neurotoxins and its binding affinity emphasis that AnCra2 is potentially alpha VGSC toxin.

The alpha toxin, AnCra2 similar to AaH2 and Amm8 can induce inhibition fast inactivation of the VGSC Based on binding to the site-3 of the channel (36–40). The pharmacological characteristics of VGSC toxins due to contact surface situations and their surface charges during the interaction between toxin and targeted channels demonstrate α- or/and β-toxins properties (2, 13, 41, 42).

Different geographical conditions might be influencing the efficacy of scorpion anti-venoms. In tropical regions like the Middle East, the south region of Iran, especially the rural and countryside areas, scorpion envenomation is a considerable public health subject (7, 43). Toxicological properties and dispersion of the family Buthidae are impressionable by biogeographic situations (44, 45). Phylogenetic relationship analysis represented the specific anti-venoms against the Buthidae species haven't the equivalent outcome in the neutralization of envenomation by other species of scorpion in this family (21, 45). A previous study showed the cross reactivity with no significant neutralization effect of anti-venom against *A. crassicauda* and venom of *Hottentotta zagrosensis* (46). Therefore, the preparation of anti-venoms is influenced by the differences in biogeographical conditions of countries. The family Buthidae toxins from North Africa, structurally and pharmacologically a correlation. Based on the phylogenetic analysis of this study AnCra2 has high similarity and adjustment with North African scorpions and showed that the VGSC α-toxins present in the same climate conditions have the same structural and functional activity and vide diversity in scorpion species venom.

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https://doi.org/10.18502/jad.v18i3.18575

Conclusion

In conclusion, AnCra2 as a potentially Alpha-mammal VGSC toxin from Iranian scorpion *A. crassicauda* has similar structure, binding activity, and functional properties with VGSC neurotoxins. Our phylogenetic analysis and molecular modelling exhibit that AnCra2 has the highest homology with VGSC alpha toxins particularly toxin peptides that are isolated from North African scorpions. This neurotoxin can be used as a pharmaceutical tool to investigate the physiological checkup of VGSC targets.

Acknowledgements

This work was supported by grants from the Ministry of Agriculture, Agricultural Research- Education and Extension Organization (AREEO), Razi Vaccine and Serum Institute of Iran (Grant number: 96043-100-18-18-01).

Ethical considerations

All experiments have adhered to the National Institutes of Health guide for the care and use of laboratory animals (NIH Publications No. 8023, revised 1978) in addition All experimental animals were confirmed by the ethics committee of the Razi Vaccine and Serum Research Institute (No. RVSRI.REC.98. 016).

Conflict of interest statement

The authors declare there is no conflict of interest.

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