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Original Article:

Molecular Characterization of a cDNA Encoding of an Anionic Cysteine-Free Antimicrobial Peptide From the Iranian Scorpion Odontobuthus Doriae Venom Glands

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ABSTRACT

Background: The venom peptides from the scorpion fauna of Iran have been poorly characterized so far.

Objectives: In this study, we identified a cDNA encoding of an anionic cysteine-free antimicrobial peptide from the Iranian yellow scorpion *odontobuthus doriae (O.doriae)*.

Methods: The cDNA sequence of an anionic antimicrobial-peptide (AMP) was determined from the venom gland cDNA library of Iranian yellow scorpion *O.doriae* and was named ODAMP5. This sequence was characterized by a software. Then, the structure and function of its putative peptide were predicted in a bioinformatics manner. The library was constructed from 6 scorpion venom glands. The cDNA related to ODAMP5 was isolated from one positive clone of the library.

Results: The analysis of ODAMP5 reveals a 51-residue mature peptide with an anionic property that was stable in physiological states. ODAMP5 was similar to anionic peptide Aba-2 from Androctonus bicolor and according to its structure, it can be a member of helical structure AMPs with a new type of putative conserved domain. Putative ODAMP5 has a small size which makes it convenient for synthesis.

Conclusion: Furthermore, we created a framework to express the ODAMP5 peptide for future biomedical and pharmacological studies. ODAMP5 may be a new suitable therapeutic strategy for bacterial infection among a few recognized scorpion venom peptides without disulfide bridges.

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Introduction

Antimicrobial peptides (AMPs) play an effective role in assisting in predatory processes and in protecting them against infection [1]. AMPs are small peptides with 12 to 100 amino acids which are important components of the innate immune system in both vertebrates and invertebrates [2]. AMPs exhibit a broad spectrum of activities against gram-positive bacteria, gram-negative bacteria, and fungi, by causing membrane lyses [3]. Recently, cytotoxic effects of AMPs on cancerous cells and some viruses, such as HIV and HSV are reported [1, 2, 4]. The common feature of all targets of AMPs is to have a separate cell membrane [5, 6].

AMPs are divided into cationic and anionic groups [7]. AMPs commonly have positive charges that target negatively charged bacterial membranes. The anionic group commonly consists of 5 to 70 amino acids (rich in glutamic and aspartic acids) and often require cations (such as Zn^{2+} ions) as cofactors for biological activity [8]. The anionic AMPs inhibit ribonuclease activity by targeting ribosomes in microbial cells, thus resulting in cell death [9].

Resistance to antibiotics is a rising concern among health care professionals, driving them to search for peptides with antimicrobial activities in plants, bacteria, and animals, including scorpions [10, 11]. Scorpion venom offers a means of using specialist tools for searching for new antimicrobial that would otherwise prove prohibitively expensive. Researchers have the opportunity to use such tools for long periods as and when they need them. The use of scorpion venoms as a drug has been known since ancient times [12, 13]. Therefore, identifying and isolating AMPs from scorpions can be employed as new therapeutic strategies for bacterial infection [2, 5]. These peptides normally act specifically against targets.

Molecular identification of AMPs sequences can provide a framework to access large amounts of these proteins for more pharmaceutical, medical, and biological studies.

In this study, an antimicrobial cDNA sequence was isolated from the venom gland cDNA library of *Odontobuthus doriae (O.doriae)* scorpion. This Iranian yellow scorpion belongs to the Buthidae family of scorpions. This study is the first to characterize the venom gland AMPs of this native scorpion.

Materials and Methods

The cDNA library construction is done in the following procedure: total RNA was extracted from 6 telsons of Iranian yellow scorpion after 3 days of milking by the Qiagen total RNA extraction kit (Cat. No.74104).

The cDNA library was constructed from 112 ng RNA using In-Fusion® SMARTer™ Directional cDNA Library Construction Kit (Cat. No.634933). In this way, the first-strand and second-strand cDNA were synthesized and adaptors were added by PCR along with the provided primers and linkers in the kit. The synthesized cDNA was cloned into pSMART2IFD linearized vectors of the kit. The cloned vectors were transformed into chemically competent cells of E. coli bacteria (DH5α strain). The transformed cells were grown on a Luria broth agar plate containing 100 µg/mL ampicillin, 1 mM IPTG, and 75 µg/mL X-Gal (for blue/white colony screening). Accordingly, positive colonies must be white given their non-functional β–galactosidase activities and consequences of the LacZ gene disruption by *O.doriae* sequences or transcripts. The colony PCR was done for each white colony by forward screening primer (TCACACAGGAAACAGCTATGA) and reverse screening primer (CCTCTTCGCTATTACGCCAGC) which were designed by In-Fusion® SMARTer®. Directional cDNA Library Construction Kit complemented the pSMART-2IFD linearized vector in the blunt ends flanking the insertion site. With this strategy, the presence of the insert would be checked.

cDNA library analysis

Plasmids were extracted from a positive colony by the QIAprep Spin Miniprep Kit (Qiagen company, Cat. No. 27104, Germany), and their inserted segment (cDNAs of venom peptides) sequenced by the Sanger method from both ends using specific forward (TCACACAGGAAACAGCTATGA) and reverse (CCTCTTCGCTATTACGCCAGC) primers by the ®Microgen laboratory sequencing service (South Korea).

The cDNA sequence of anionic AMPs was checked by VecScreen tools (http://www.ncbi.nlm.nih.gov/tools/vecscreen/) to trim from vector and primer sequence contaminations. The amino acid sequence of the obtained cDNA sequence was deduced using the open reading frame (ORF) finder program. The sequence of the ORF was confirmed via the protein BLAST founded on NCBI and the UniProt websites. The phylogenic tree and amino acid alignment were done by the online tool on the UniProt website. The signal peptide was predicted

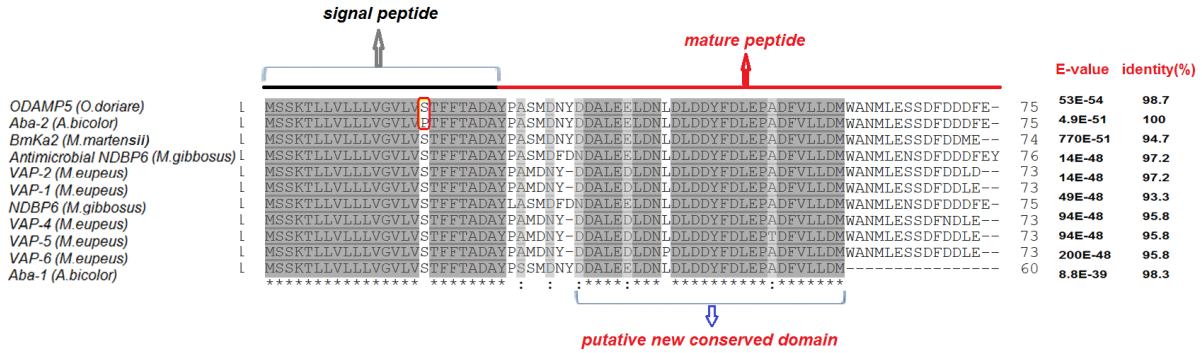

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Figure 1. Results From the Alignment of ODAMP5 Precursor Peptide With Homologue Peptides From Other Scorpion Species (Androctonus bicolor [A.bicolor], Mesobuthus martensii [M.martensii], Mesobuthus eupeus [M.eupeus], and Mesobuthus gibbosus [M.gibbosus])

Notes: The identity (%) and E-values are shown on the right sides that correspond to the precursor peptides. High and low similarities are shown by dark and light gray, respectively. Signal peptide and mature peptide sequences are marked on the top of the picture. A putative new type of conserved domain is marked at the bottom of the picture. Amino acid differences between ODAMP5 and Aba-2 are shown in the red box on their signal peptide sequences.

by the signalP4.1. Meanwhile, the presence or absence of disulfide bound was checked by the DISULFIND [14]. Physiochemical parameters were estimated by the ProtParam tool and molecular modeling, including the second and third structure of the putative mature peptide was done by the Phyre2 server [15].

Results

In this study, we found an anionic cysteine-free antimicrobial peptide in the cDNA library of the Iranian medically important scorpion *O.doriae* venom gland and named it "ODAMP5."

Non-disulfide-bridged peptides (NDBPs), belonging to the scorpion venom are a novel class of venom peptides that have special antimicrobial, immunological, or cellular signaling activities. Biological functions and potential applications of NDBPs scorpion venom peptides were reviewed [16]. Major information about NDBPs AMPs was elucidated using scorpion venom peptides. The study and discovery of new AMPs and the development of AMPs applications can solve the problem of antibiotic resistance. The natural source is frequently a straight point for searching for new antibiotics. The main approach which is called "genome mining" might code for new drugs, such as antimicrobials.

The ODAMP5 cDNA sequence was registered on NCBI Gene Bank by the following gene ID: KU212816.1 [17]. The sequence length of ODAMP5 cDNA was 369 nucleotides. The nucleotide sequence of ODAMP5 mRNA showed a 95% similarity to anionic peptide Aba-2 mRNA from Androctonus bicolor, one of the most poisonous scorpions worldwide [18].

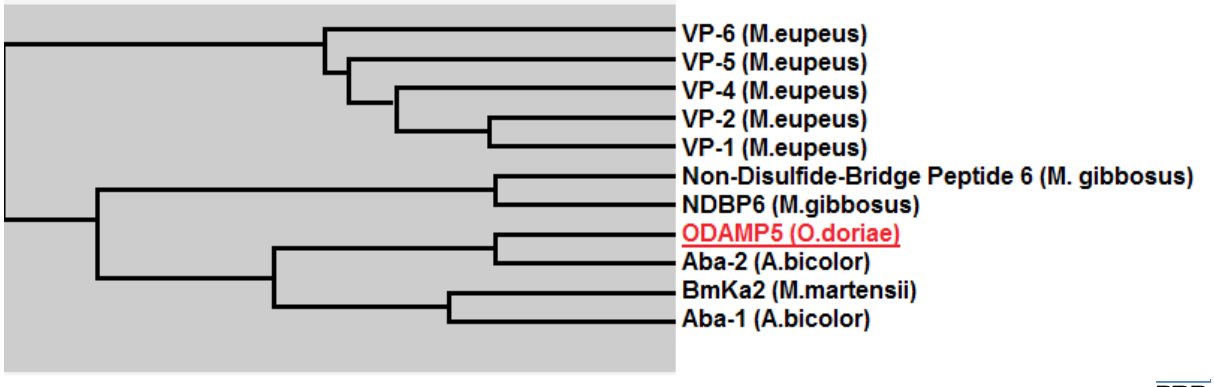

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Figure 2. Phylogenetic tree of ODAMP5 biodiversity with other similar anionic AMPs (10 peptides) from other species of the buthidae family

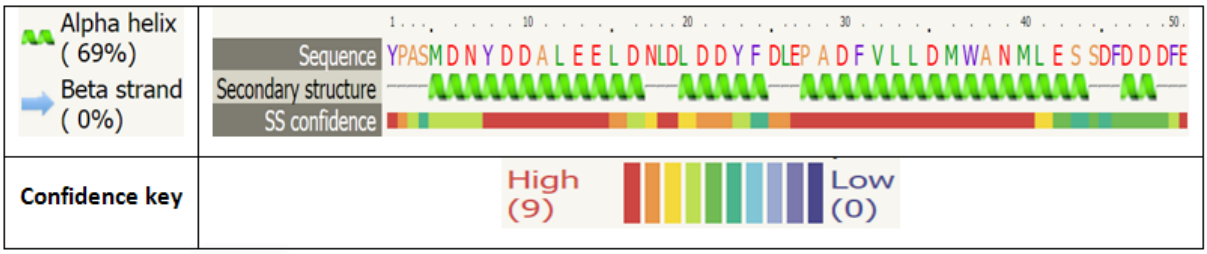

Figure 3. Secondary structure of putative mature ODAMP5

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The results of the ORF finding tool revealed that ODAMP5 has a putative 75 amino acid residues precursor peptide. A total of 24 amino acid residues signal peptide was predicted in ODAMP5 precursor peptide which proves the secretory nature of its function. Similarly, searching for ODAMP5 precursor peptide with other proteins or peptides in databases showed that this peptide is akin to some anionic AMPs in other scorpions. The most similar peptide to ODAMP5 is the anionic peptide Aba-2 with 98.7% identity. The results from the alignment of ODAMP5 with 9 of the most similar peptides from Mesobuthus martenssi (M.martenssi) and Mesobuthus eupus (M.eupeus) are shown in Figure 1.

The results of the phylogenic assessment of ODAMP5 precursor with similar peptides are demonstrated in Figure 2. In this assessment, ODAMP5 is close to the Aba-2 venom peptide of Androctonus bicolor (A.bicolor) because of its sequence similarity.

The results of the secondary structure and 3D model prediction of ODAMP5 designed by the Phyre2 software are shown in Figure 3 and Figure 4, respectively.

Discussion

Given the alignment of ODAMP5 with similar peptides in the databases, there is only one amino acid difference between ODAMP5 and Aba-2 on position 17 of the signal peptide. In this position, hydrophobic amino acid "proline" of Aba-2 is replaced by hydrophilic tiny amino acid "serine." The presence of serine in this position of ODAMP5 was similar to other homolog AMPs. Both serine and proline amino acids are small; however, serine is a polar amino acid compared to proline. Instead of this difference between ODAMP5 and Aba-2, a signal peptide of aligned homolog AMPs in other positions was conserved (Figure 1). Because of the alignment of ODAMP5 with homolog AMPs, the sequence of ODAMP5 was conserved in the signal peptide position and the middle part of the mature peptide. Based on the conservation of the mature peptide middle part in all of the homologous AMPs which are shown in Figure 1, it seems to be a new type of conserved domain in this position; however, further structural studies are needed.

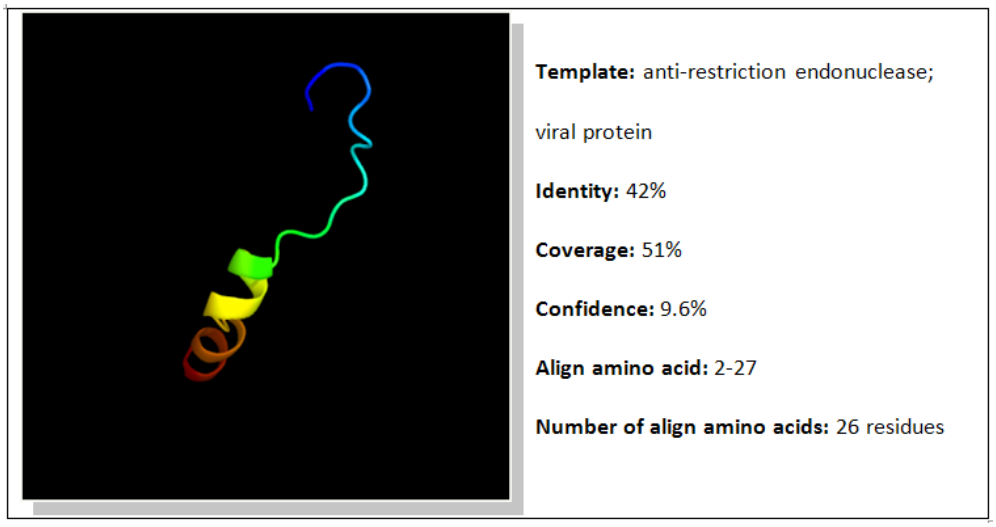

Figure 4. Molecular modeling of putative mature ODAMP5

Notes: the image is colored by rainbow N → C terminus.

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Table 1. Some physicochemical properties measured for mature ODAMP5

Amino Acid Residue	Molecular Weight	Isoelectric PH (pI)	Formula	Total Number of Atoms	Instability Index (II)	N-Terminal Residue
51	6019.3	2.80	C266H371N55099S3	794	37.93/stable	y (Tyr)

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According to the dendrogram of the phylogenic tree (Figure 2), the ODAMP5 isolated from Iranian *O.doriae* displayed the highest similarity with the anionic peptide Aba-2 of A.bicolor. Odontobuthus and Androctonus both belong to the Buthidae family [19].

O. doriae is one of the most medically important scorpions and has a wide dispersion in the south and central areas of Iran [20]. We suggest that the Iranian *O.doriae* scorpion is a homolog to the Chinese A.bicolor, according to the anionic AMPs biodiversity studies.

According to the secondary structure (Figure 3), 69% of mature ODAMP5 in the secondary structure view is composed of α-helix and does not have any β strand on the predicted structure. This lack of β strand in the secondary structure of ODAMP5 was similar to α-helical AMPs [21], such as Magainin antimicrobial peptide from Xenopus laevis. Magainin is one of the pore-forming toxins that fought off the microbes. Magainin showed a broad spectrum of antimicrobial activities against gram-negative and gram-positive bacteria, fungi, and protozoa. This family of linear cationic AMPs is cytotoxic for cancer lines. Similar findings in reports about Aba-2 and BmKa-2, two other homolog anionic AMPs, were obtained [16, 22].

The new studies support the concept that molecular and structural characteristics of the primary structure, rather than the 3-dimensional folding, are important determinants for the recognition of biological functions. The structure of ODAMP5 in a 3-dimension state (Figure 4) was predicted by the single highest scoring template which is an anti-restriction endonuclease in viruses [23]. In this model, 26 residues (51% of the sequence) have been modeled with 9.6% confidence. Although this model has a very low confidence level, other laboratory studies, such as nuclear magnetic resonance spectroscopy and mass spectrometry will be required.

Some other properties of mature ODAMP5, such as physicochemical parameters are shown in Table 1. The N-terminal amino acid residue of ODAMP5 is tyrosine (Tyr). Given its anionic nature, the measured isoelectric pH (pI) is 2.8 units. ODAMP5 is a stable molecule that can be a suitable candidate for any pharmaceutical purpose.

Conclusion

We found a cDNA that belongs to an anionic AMP and molecularly characterized its putative peptide. Molecular characterization and homology search for cDNA sequence and putative peptide of ODAMP5 revealed that this sequence has a high homology rate in some scorpion species of the Butidae family, such as Odontobuthus, Androctonus, and Mesobuthus.

High conservation of these homolog peptides indicates that these peptides have a critical role in the life and survival of these scorpions.

Since the ODAMP5 was similar to the Aba-2 anionic peptide from A.bicolor, it possibly acts similarly. No investigation into the antimicrobial activity of these peptides was conducted; however, based on the sequence similarities to other AMPs, it was suggested that ODAMP5 may classify these new peptides as a part of novel NDBPs.

ODAMP5 has a small size with only 51 amino acid residues which makes it convenient for synthesis. Furthermore, we created a framework to express the ODAMP5 peptide for future studies. NDBPs from scorpion venom may be the target for novel drugs against antibiotic-resistant pathogens.

Ethical Considerations

Compliance with ethical guidelines

There were no ethical considerations to be considered in this research.

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Authors' contributions

All authors equally contributed to preparing this article.

Conflict of interest

The authors declared no conflict of interest.

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