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Full Length Research Paper

A new caerin-like antibacterial peptide from the venom gland of the Iranian scorpion *Mesobuthus eupeus*: cDNA amplification and sequence analysis

Masoumeh Baradaran[1], Amir Jalali[2], Abbas Jolodar[3] and Sepideh Ghasemian[3]*

[1]Toxicology Research Center, Ahvaz Jundishapur University of Medical Sciences, Ahvaz, Iran.
[2]Department of Pharmacology and Toxicology, School of Pharmacy and Toxicology Research Center, Ahvaz Jundishapur University of Medical Sciences, Ahvaz, Iran.
[3]Department of Biochemistry, School of Veterinary Medicine, Shaheed Chamran University, Ahvaz, Iran.

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Scorpion venom consists of different types of peptides and proteins which are encoded by individual genes. A full length cDNA consisting of 238 base pair nucleotides and encoding 74 amino acids peptide was isolated from the venom gland of the Iranian scorpion *Mesobuthus eupeus* (Buthidae family). This peptide named *M. eupeus* caerin-like antimicrobial peptide (Me-CLAP) belonging to the group of antibacterial peptide was previously described from scorpion. In this study, sequence of cDNA encoding Me-CLAP from the *M. eupeus* venom glands was amplified using reverse transcriptase polymerase chain reaction (RT-PCR) and was analyzed afterwards. Me-CLAP has similar molecular characteristics to antimicrobial peptides (AMPs) of same genus like *Mesobuthus martensii* and *M. eupeus* and more differences were seen with other genus.

Key words: Caerin-like antimicrobial peptide, *Mesobuthus eupeus*, semi-nested real-time polymerase chain reaction.

INTRODUCTION

Resistance to antibiotics is a rising concern among health care professionals, driving them to search peptides with antimicrobial activities in plants, bacteria and animals (Meylears et al., 2002; Shaini et al., 2010). Antimicrobial peptides (AMPs) are key effectors of the innate immune response of animals and show antimicrobial therapy potency (Bulet et al., 2004; Vancompernolle et al., 2005; Dhople et al., 2006; Yibao et al., 2009; Ramamoorthy, 2009). Most AMPs that display hydrophobic and cationic properties have a molecular mass below 25 to 30 kDa (Marsh et al., 2009; Reddy et al., 2004; Meylears et al., 2002). So far, more than 2000 natural AMPs have been isolated, sequenced and submitted until now. Some natural antimicrobial peptides are being developed for use either as antibiotics for topical use in healthcare or as preservatives in the food industry. Apart from the extreme diversity in their primary and secondary structures, AMPs have *in vitro* effects on a wide spectrum of bacteria (Gram-negative and -positive) and cells, including: parasites, tumor cells, fungi and viruses (Bulet et al., 2004). AMPs have non-specific processes involved in the antimicrobial action. The unique property of an antimicrobial peptide is its ability to interact selectively with bacterial lipid membrane and cause disruption. This property could decrease the likelihood of microbes acquiring resistance to AMPs. This could give AMPs a major advantage over conventional antibiotics, and in response, these peptides have been extensively investigated as potential antimicrobial agents (Harris et al., 2009; Thennarasu et al., 2010a,b; Ramamoorthy et al., 2010; Thennasu et al., 2005).

Scorpions are particularly resistant to bacterial aggressions, and it is of interest to analyze the molecules responsible for this resistance (Sabatier et al., 1996). The scorpion venoms have three different groups of ingredients and peptides, based on their molecular mass

*Corresponding author. E-mail: ghasemiansepideh@ymail.com.

(Yibao et al., 2009). The first group consists of proteins with enzyme activity such as hyaluronidases, phospholipases and sphyngomyelinases. The second group contains mainly a peptide fraction with molecular masses around and lower than 10 KDa such as toxins and cytolytic compounds. The third group is composed of components such as ions, free amino acids, biogenic amines, neurotransmitters, acylpolyamines, heterocyclic compounds and alkaloids (Ma et al., 2010; Kuhn-Nentwig., 2003). Different antimicrobial peptides such as imcroporin (He et al., 2008; Zhao et al., 2009), BmKb1 and BmKn2 (Zeng et al., 2004), Hadrurin (Torres-Larios et al., 2000) and Mucroporin (Chao et al., 2008) have been identified.

Caerin is an antimicrobial peptide that was first found in amphibian, Australian green tree frog (Wong et al., 1997). Later different variants were isolated from a number of Australian frogs of Litoria genus (Brian et al., 2000; Steinborner et al., 1998) and African frog (Gottler and Ramamoorthy, 2009). In the recent years, caerin and caerin-like antimicrobial peptides were been found in scorpions such as *Mesobuthus eupeus* and *Mesobuthus martensii* (Luo et al., 2005). Antibacterial activity of caerin on Gram positive and negative bacteria has been shown with only small variation in their minimum inhibitory concentration (MIC) values (Boland and Separovic, 2006; Chia et al., 2011).

It is noteworthy that most of AMPs from scorpion's venom are obtained by either bioassay-guided fractionation or polymerase chain reaction (PCR)-based methods conducted with cDNA libraries. Most performed researches have focused on medically important family, Buthidae scorpions (Rodriguez and Possani, 2004; Rodriguez and Possani, 2005; Kozminsky-Atias et al., 2008). In this work, we isolated caerin-like antimicrobial peptide (CLAP) from the cDNA library of the venomous gland of the Iranian medically important scorpion *M. eupeus* which belong to Buthidae family.

MATERIALS AND METHODS

Sample collection and total ribonucleic acid (RNA) extraction

Scorpions were collected from the southwestern province of Iran, Khuzestan and identified by Razi reference scorpion laboratory of Ahvaz. The milking was carried out to allow the toxin - producing cells of the venom glands to enter the secretary phase. They were killed five days after milking 95% ethanol and freezing at -20°C. Six separated venom glands were used for total RNA extraction using RNA™ (Cinagene, Iran) according to the manufacturer's procedure. RNA pellet was dissolved in DEPC-ddH_2O and used for cDNA synthesis immediately.

cDNA library construction

First strand cDNA was synthesized using reverse transcriptase and amplified by polymerase chain reaction (RT-PCR). Extracted total RNA was used as template and modified oligodT (ModT) (5´-gggtctagagctcgagtcacttttttttttttttttt-3´) as primer for RT-PCR. ModT was added to extracted RNA and incubated at 70°C for 5 min and immediately was placed on ice for 2 min. Then, 5X buffer, dNTP, Ribolock, reverse transcriptase enzyme and ddH_2O were added to samples and was incubated for 60 min at 42°C. Samples were incubated for 10 min at 70°C and immediately placed on ice.

Semi-nested polymerase chain reaction (PCR)

Double strands cDNA was constructed and amplified using semi-nested RT-PCR. The first round of PCR was performed using ModT-R (5´-cccagatctcgagctcagtg-3´), MEC-F (5´-cgcGGATCCCGAAACTCTGCCAAGATGGA-3´) primers and first strand cDNA as template. Second round of PCR was performed using MEC-F, MEC-R (5′-cgcAAGCTTAGGAAACGACCGGAAGAGAG-3´) primers and PCR products of initial amplification as template. Both rounds of PCR was done in 35 cycles with denaturation at 94°C (40 s), anealing at 56°C (90 s) and extension at 72°C (1 min) with a initial denaturation at 95°C (5 min) and final extension at 72°C (10 min). Amplification products were separated by agarose gel electrophoresis and visualized by UV transilluminator.

Deoxyribonucleic acid (DNA) sequencing

PCR products were electrophoresed on 1% agarose gel and purified by gel extraction kit (Qiagen, Germany) according to the manufactures protocol, and stored at -20°C. Purified PCR products were sent to Kawsar Biotech Company for nucleotide sequencing.

Sequence analysis

Sequence was compared with GenBank database using the BLAST software on the NCBI website (http://www.ncbi.nlm.nih.gov/BLAST). Nucleotide sequence was converted to amino acid with the software available at the Expasy website (http://ca.expasy.org/tools/pi_tool.html). The molecular weight and isoelectric point was estimated using ProtParam tool (http://www.expasy.org/tools/protparam.html). The signal peptide was predicted by SignalP (http://www.cbs.dtu.dk/services/SignalP/). Multiple sequence alignments were done using the CLUSTAL_W program and edited with the BOXSHADE software (http://www.ch.embnet.org/software/BOX_form.html). The SBASE online software (http://hydra.icgeb.trieste.it/sbase/) was used to determine the conserved domains.

RESULTS

Semi-nested RT-PCR amplification of *M. eupeus* caerin-like antimicrobial peptide (Me-CLAP) cDNA

First strand cDNA was constructed with RT-PCR. Semi-nested PCR amplification of Me-CLAP cDNA was carried out by specific primers. Figure 1 shows PCR amplification of the Me-CLAP c-DNA.

Characterization of the Me-CLAP gene

Sequence determination of amplified fragment revealed that Me-CLAP cDNA has 238 bp encoding for 74aa residue peptide. The 8541.33 Dalton molecular weight

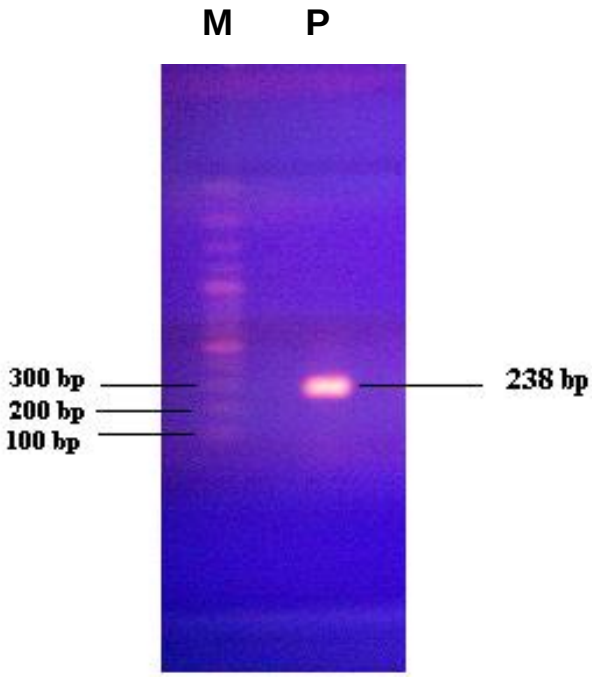


Figure 1. PCR amplification amplification of Me-CLAP from *Mesobuthus eupues*. Lane M, DNA marker; lane P, production of amplification of Me-CLAP. PCR, Polymerase chain reaction; Me-CLAP, *M. eupeus* caerin-like antimicrobial peptide.

and 8.89 theoretical pI were estimated. A 23-amino-acids signal peptide was identified. n- region, h- region and c-region of this signal peptide was predicted by SignalP software (Figure 2). The leucine at position 24 was assumed to represent the start of the mature protein. The size of mature protein is 51 amino acids and conserved domain of Me-CLAP was predicted using SBASE online software. As shown in Figure 3, Me-CLAP has one conserved domain.

Sequence alignment of *M. eupeus* Me-CLAP with similar sequences from other sources

In Figure 4, the amino acid sequences of Me-CLAP was aligned with similar molecules from five other scorpions including *M. eupeus*, *M. martensii* (20), *Tityus costatus* (Diego-Garcia et al., 2005), *Lychas mucronatus* (Dai et al., 2008), and *Isometrus maculates* (Zhao et al., 2009).

DISCUSSION

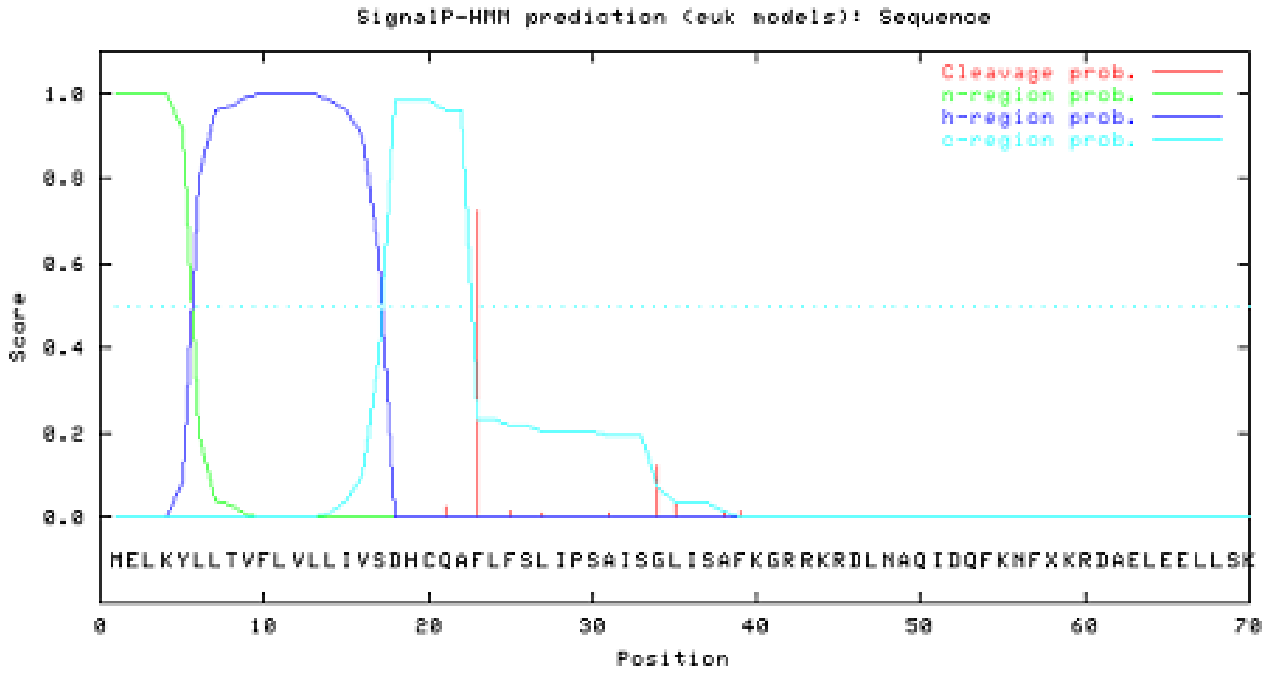
So far, a few antimicrobial peptide including Meucin-24, Meucin-25 (Gao et al., 2010), Meucin-13 and Meucin-18 (Gao et al., 2009) have been identified from *M. eupeus* venom gland. In this study Me-CLAP was amplified and characterized from the Iranian scorpion gland *M. eupeus*. The molecular weight and theoretical pI of Me-CLAP were calculated to be 8541.33 Daltons and 8.89 respectively. Comparison of the cDNA fragment and amino acid sequence of Me-CLAP with the GenBank database revealed that the DNA and amino acid sequence is highly homologous with scorpion antimicrobial peptides suggesting that it belongs to the antmicrobial peptides.

Since mature antimicrobial peptides generally contain 12 to 100 amino acid residues (Ping et al., 2011) with molecular mass lower than 10 KDa (Matsuzaki, 1999) therefore Me-CLAP could be an antimicrobial peptide. All of the previous studies identify *M. eupeus* as antimicrobial peptides which belong to Muecin family, but in this study we have identified and characterized an antimicrobial from caerin family.

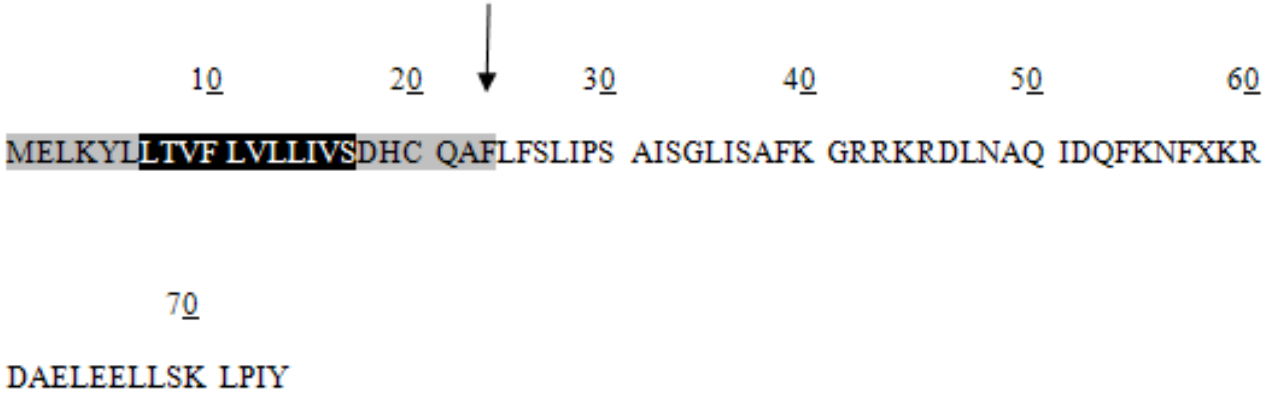
A newly synthesized secretary protein in cells bears a special sequence, called signal peptide which plays the role of “address tag” in guiding the protein to wherever it is needed (Liu et al., 2007). Me-CLAP has signal peptide and is suggested as a secretory protein (Bals, 2000). Signal sequences have a tripartite structure, consisting of a hydrophobic core region (H-region) flanked by an N- and C- region. The latter contains the consensus cleavage site that signals peptidase enzyme and act at this position (Nielsen and Krogh, 1998). N-, H- and C-regions of Me-CLAP are shown in Figure 2. It was assumed that cleavage site of Me-CLAP is between 23 and 24, and leucine at position 24 is the first amino acid of mature protein.

N-terminal signal sequences mediate targeting of nascent secretory and membrane proteins to the endoplasmic reticulum (ER) in signal recognition particle (SRP) dependent manner. Signal sequences are extremely variable; both in their length and the steps beyond are affected by the signal sequence. This variability may account for additional functions, that is, post-targeting functions (Nielsen and Krogh, 1998). Although, Me-CLAP show high level of similarity if compared with other identified scorpion AMPs, but Me-CLAP signal peptide is different from signal peptide of similar AMPs of other genus (Figure 4). These similarities and differences suggest that ER targeting and the steps beyond post-targeting functions of caerin that is translocation, signal peptidase cleavage site etc. are similar in different species of genus Mesobuthus and is different with caerin from other genus.

The 74 amino acids residues Me-CLAP represents one of the longest described antimicrobial peptides if compared with scorpion AMPs such as defensin from the scorpion *Leirus quin questriatus* hebraeus (Cociancich et al., 1993) and *Androctonus australis* (Ehret-Sabatier et al., 1996), buthinin and androctinin from the venom of *A. australis* (Ehret-Sabatier et al., 1996) and scorpine isolated from *Pandinus imperator* venom (Conde et al., 2000). Scorpine from the west and central African scorpion (*Pandinus imperator*) has 75 amino acid



A



B

Figure 2. Signal peptide analysis of Me-CLAP. A, Prediction of N-region, C-region and H-region. Cleavage site, between position 23 and 24 is shown with red line; B, amino acid sequence of Me-CLAP. Amino acid sequence related to N-region, C-region and H-region of signal peptide are shown on N-terminal in gray, black and gray respectively. Site effect of signal peptidase enzyme is indicated by arrow. Me-CLAP, *M. eupeus* caerin-like antimicrobial peptide.

residues, 8449.8 Dalton molecular weight and 8.8 pI shows a similar molecular characteristics with Me-CLAP (Conde et al., 2000). So the identification of Me-CLAP peptide in the Iranian scorpion (*M. eupeus*) confirms wide spread occurrence and significant biological function of antimicrobial peptides in scorpion venoms.

As described previously, antimicrobial peptide mucroporin from scorpion *L. mucronatus* is an important defensive molecules of the ancient innate immunity (Bhattacharjva and Ramamoorthy, 2009; Ruiming et al.,

LFSLIPSAISGLISAFKGRRKRDLNAQIDQFKNFXKRDAELEELLS

KLPIY

Figure 3. Mature protein of Me-CLAP. Conserved domain of Me-CLAP is indicated in black color. Me-CLAP, *M. eupeus* caerin-like antimicrobial peptide

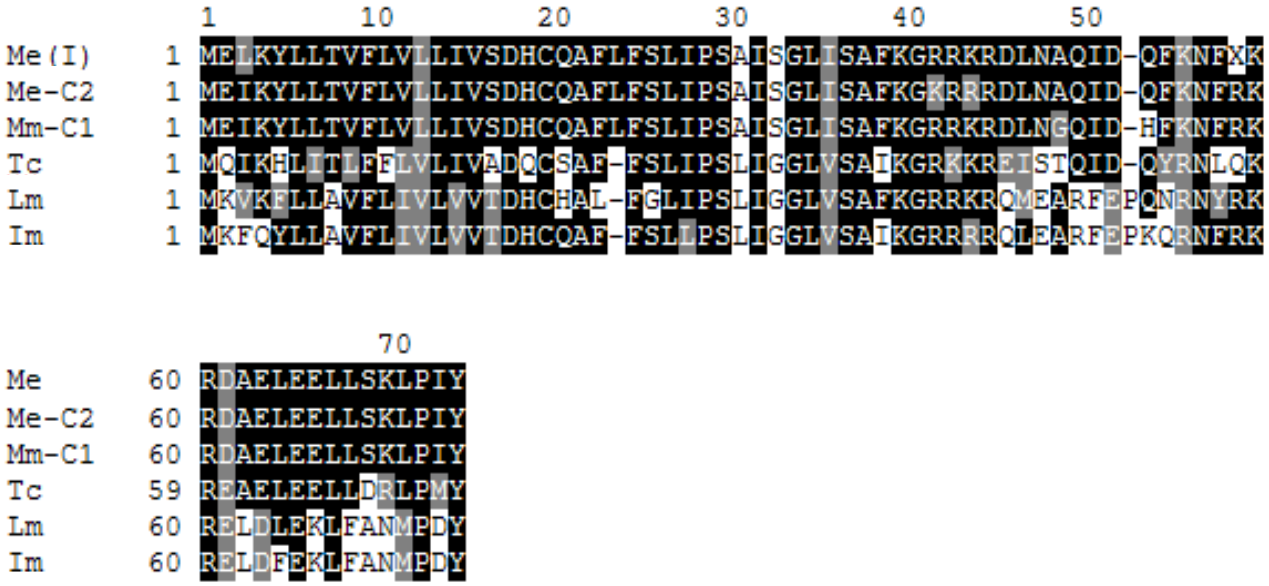


Figure 4. Sequence alignment of Me-CLAP with the similar proteins from five different scorpions. Dashes represent gaps introduced to optimize the alignment. Shaded areas indicate matching residues. Me (I), *M. eupeus* (Iran); Me-C2, *M. eupeus* (lesser Asian scorpion) (ABL68083); Mm-C1, *M. martensii* (AAW23032); Tc, *T. costatus* (AAW72458); Lm, *L. mucronatus* (ACF93401); Im, *I. maculates* (C7B247).

2010). So we can conclude that Me-CLAP could be a defensive molecule with significant biological function of innate immunity.

As showed in Figure 4, four amino acids differences is seen between Me-CLAP and other isolated antimicrobial peptide from *M. eupeus* (lesser Asian scorpion), Me-C_2. If noticed well, only three amino acids difference is seen between Me-CLAP and Mm-C_1 that belongs to other *Mesobuthus* sp. (*martensii*). So we can propose that Me-CLAP belongs to C_1 group.

Me-CLAP has similar molecular characteristics to AMPs of the same genus like *M. martensii* and *M. eupeus*. More differences in Me-CLAP sequence are seen with other genus.

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