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PHYLOGENETIC ANALYSIS OF LYSOZYME C FROM THE SCORPION MESOBUTHUS EUPEUS VENOM GLAND

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Many studies have been carried out on peptides and genes encoding scorpion toxins from the venom of the scorpion *Mesobuthus eupeus*. The scorpion venom contains a diversity of bioactive peptides, which could cause toxic effects and can be candidates for drug design and development. The antimicrobial lysozymes among them are of great value. Lysozymes are hydrolytic enzymes characterized by the ability to cleave the β-(1,4)-glycosidic bond between N-acetylmuramic acid and N-acetyl-D-glucosamine in a peptidoglycan layer, the major bacterial cell wall polymer. The total RNA was extracted from venom glands of *Mesobuthus eupeus* species of Kuzestan, Iran. cDNA was synthesized with extracted total RNA as template and modified oligo-(dT) as primer. In order to amplify cDNA encoding a Lys-C peptide, semi-nested RT-PCR was performed with the specific primers followed by sequencing of the amplified fragment. The full-length cDNA sequence contains a 438 nucleotide open reading frame, which encodes a peptide of 144 amino acids with molecular weight of 16.702 kDa. A putative 22-residue signal peptide was identified. Based on the phylogenetic tree of MesoLys-C and C-type lysozyme of East Mediterranean *M. eupeus* it is concluded that *M. eupeus* of Khuzestan and East Mediterranean *M. eupeus* belong to different subspecies.

Phylogenetic analysis – antimicrobial protein – lysozyme C – scorpion venom

Բազմաթիվ հետազոտություններ են իրականացվել *Mesobuthus eupeus* կարիճի թունահեղուկի տոքսինները կոդավորող պեպտիդների և գեների վերաբերյալ: Կարիճի թունահեղուկը պարունակում է բազմազան կենսաակտիվ սպիտակուցներ, որոնք ունեն թունավոր ազդեցություն, ինչպես նաև լավ թեկնածուներ են դեղամիջոցների մշակման և զարգացման համար: Դրանց թվում մեծ արժեք ունեն հակամանրէային լիզոցիմները: Լիզոցիմները հիդրոլիտիկ ֆերմենտներ են, որոնք բնութագրվում են բակտերիալ պատի պեպտիդոգլիկանային շերտի N-ացետիլմուրամաթթվի և N-ացետիլ-D-գլյուկոզամինի միջև β-(1,4)-գլիկոզիդային կապը ճեղքելու ունակությամբ: Խուզեստանի (Իրան) տարածքում հավաքված *Mesobuthus eupeus* կարիճների թունագեղձերից անջատվել է ամբողջական ՌՆԹ-ն, որից սինթեզվել է cԴՆԹ, կիրառելով ամբողջական ՌՆԹ-ն որպես նմուշ և ձևափոխված օլիգո-(dT) որպես պրայմեր: Lys-C պեպտիդը կոդավորող cԴՆԹ-ի ամպլիֆիկացիան իրականացվել է semi-nested RT-PCR-ի միջոցով՝ կիրառելով սպեցիֆիկ պրայմերներ, որից հետո ամպլիֆիկացված հատվածը ենթարկվել է սեկվենավորման: Ամբողջական cԴՆԹ հատվածը կազմված է 438 նուկլեոտիդներից բաղկացած ORF, որը կոդավորում է 144 ամինաթթուներից կազմված 16.702 կԴա մոլեկուլային կշիռ ունեցող սպիտակուց: Որոշվել է 22 մնացորդ պարունակող ենթադրելի ազդանշանային պեպտիդը: Արևելամիջերկրական *M. eupeus* տեսակի MesoLys-C և C-տիպի լիզոցիմների ֆիլոգենետիկական ծառի հիման վրա կատարվել է եզրակացություն, որ Խուզեստանի *M. eupeus* և արևելա-միջերկրական *M. eupeus* տեսակները ֆիլոգենետիկորեն դասվում են տարբեր ենթատեսակների:

ֆիլոգենետիկական հետազոտություն - հակամանրէային սպիտակուց - լիզոցիմ C - կարիճի թույն

Проведены многочисленные исследования на пептидах и генах, кодирующих токсины яда скорпиона *Mesobuthus eupeus*. Яд скорпиона содержит разнообразные биоактивные пептиды, которые могут вызывать токсические эффекты и стать перспективными кандидатами для разработки лекарственных веществ. Среди этого ряда веществ антимикробиальные лизоцимы имеют наибольшую ценность. Лизоцимы – гидролитические энзимы, характеризующиеся способностью расщеплять β-(1,4)-гликозидную связь между N-ацетилмурамовой кислотой и N-ацетил-D-глюкозамина в пептидогликановом слое бактериальной клеточной стенки. Из ядовитых желез скорпионов *Mesobuthus eupeus*, отловленных в Хузестане (Иран), выделена тотальная РНК. кДНК синтезирована из тотальной РНК и модифицированного олиго-(dT)-праймера. С тем чтобы амплифицировать кДНК, кодирующую Lys-C пептид, был использован полугнездовой ОТ-ПЦР (semi-nested RT-PCR) со специфическими праймерами с последующим секвенированием амплифицированного фрагмента. Полноразмерный кДНК сиквенс содержит 438 нуклеотидный ORF, кодирующий пептид из 144 аминокислот с молекулярной массой 16.702 кДа. В итоге идентифицирован предполагаемый сигнальный пептид, состоящий из 22 аминокислотных остатков. На основе филогенетического анализа MesoLys-C и C-тип лизоцима восточносредиземноморского *M. eupeus* было сделано заключение, что *M. eupeus* из Хузестана и восточно-средиземноморский *M. eupeus* принадлежат к разным подвидам.

Филогенетический анализ – антимикробиальный протеин – лизоцим С – яд скорпиона

All known scorpion species possess a venom apparatus, which has been an important determinant in contributing to the successful survival of these animals for more than 400 million years. Scorpion venom is a combinatorial library of peptides and proteins that could cause toxicological responses and can be candidates for drug design and development [1]. Several recent studies have demonstrated that scorpion-like peptides isolated from the venomous gland of some scorpion species have anti-bacterial and anti-malaria effects [2]. These and other antimicrobial peptides found in scorpions may serve as a promising lead candidate in the development of novel antibiotic molecules. In this context lysozymes are of great importance. Lysozymes are muramidases that damage the peptidoglycan layer of the bacterial cell wall by hydrolysing β-(1,4)-glycosidic linkages between N-acetylmuramic acid and N-acetyl-D-glucosamine residues [3]. The known lysozymes within the animal phyla are generally classified into 3 main types: chicken-type (c-type), invertebrate-type (i-type), and goose-type (g-type) [4]. The c-type lysozyme has been found in many organisms including vira, bacteria, plants, insects, reptiles, birds, and mammals [5], including scorpions [6]. Generally, lysozymes play an important defense role in the innate immunity. However, the exact biological role of lysozymes from scorpion venoms remains to be explored, as they have a relatively high expression level.

In this work, we report the characterization and phylogenetic analysis of c-type lysozyme from the venom glands of *Mesobuthus eupeus* scorpions of *Buthidae* family, which are widespread in Iran, especially in Khuzestan province.

Materials and methods. *Scorpion samples.* The specimens of *M. eupeus* were collected in Khuzestan province (Iran) and transported to the reference laboratory of the Razi Institute. They were killed two days after manual extraction of the venom to allow the toxin producing cells of the venom glands to enter into a secretory phase. Twenty separated venom glands were used for total RNA extraction.

Total RNA extraction. Four μg of total RNA was extracted from the venom glands of scorpions (0.5 g of tissue material) using RNA™ (Cinagene, Iran), according to the manufacture procedure. The RNA pellets were dissolved in DEPC-ddH2O and used for cDNA synthesis immediately.

cDNA library Synthesis. cDNA was synthesized from the extracted total RNA as template and modT (modified oligo-dT) (5′-gggtctagagctcgagtcacttttttttttttttt-3′) as primer. ModT was added to the extracted RNA and incubated at 70ºC for 5 min and immediately transferred into ice for 2 min. The mixture of 5Ч buffer, dNTPs, Ribolock, Reverse transcriptase and ddH2O was added to the samples followed by incubation at 42ºC for 60 min, after which the samples were incubated at 70ºC for 10 min and immediately transferred into ice.

Semi-nested RT-PCR amplification. For the cDNA amplification semi-nested RT-PCR technique was used. The first round of PCR was performed using modT-R (5'- cccagatctcgagctcagtg-3'), lys-F 5'- gcgcggatccaagatggctttcaagttttcatt-3' primers, and synthesized cDNA as template. The second round of PCR was performed using lys-F and lys-R 5'-gcgcaagctttacagttgttatcattgataaatt-3') primers, and the PCR products of the initial amplification as templates. The PCR conditions for both rounds were as follows: initial denaturation at 95ºC (5 min), followed by 35 cycles of denaturation at 94ºC (40 sec), annealing at 56ºC (90 sec) and extension at 72ºC (1 min), with a final extension at 72ºC (10 min). Amplicons were separated by 1% agarose gel electrophoresis and visualized by UV transilluminator.

DNA sequencing and Bioinformatics analysis. The amplified cDNA fragments were purified from the gel by QIAquick Agarose Gel Extraction kit (www.fermentas.com) and sent to Kawsar Biotech Company for nucleotide sequencing. Sequence similarity analysis against GenBank database entries was performed using BLAST at the NCBI website (http://www.ncbi.nlm.nih.gov). The nucleotide sequences were translated into the corresponding amino acids, and the predicted signal peptide sequence was identified using online tool software at the Expasy website (http://expasy.org/tools). The sequences used for alignment and phylogenetic analysis were retrieved from SWISS-PROT database (http://www.expasy.org/sprot). The alignment was performed using the multiple sequence alignment program ClustalW 2.03 followed by manual adjustment [7], and viewed by the Jalview software [8]. Phylogenetic analysis was carried out with Neighbor-Joining method implemented in MEGA 3.1 [5].

Results and Discussion. In this study we identified and compared the MesoLys-C amino acid sequence with the representative C-type lysozymes of three major phyla: scorpion, invertebrate, and vertebrate. In order to characterize and assay the mRNAs, single strand cDNAs were synthesized and the cDNA fragments were amplified by RT-PCR technique. The length of the coding region was 438 bp, encoding a polypeptide of 144 amino acid residues with a calculated molecular weight of 16.702 kDa and theoretical isoelectric point of 7.54. To obtain a cDNA probe for the screening of the cDNA library we performed PCR, which yielded a predominant 450 bp product (Fig. 1). From this fragment a cDNA probe sequence was determined.

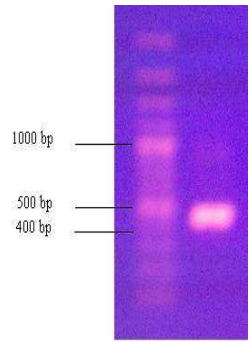

Fig. 1. PCR amplification of Lys-C cDNA from *M. eupeus* venom gland.

The precursor sequence of a 438 nucleotide open reading frame consisted of a putative 22-amino-acids length signal peptide, and lysine at the position 23 was assumed to represent the start of the mature protein (Fig. 2).

M A F K F S F F T V L C L C V F I E N L D G K R F G R C E L A K L L V F N G I P Y K D P
D W V C L A Y Y Q S R L E S S F M S P V S N G H R E Y G I F Q I S S T D D N L D D D I K
C A K L I H R R H K F D A W Y A W K A H V K D K E L S Q F I N D N N C M A F K F

Fig. 2. The full-length cDNA sequence of Lys-C.
The signal peptide is highlighted; the mature peptide is underlined.

The MesoLys-C (*Mesobuthus* Lys-C) amino acid sequence comparison performed against GenBank NCBI database revealed that the amino acid sequence of MesoLys-C is highly homologous to C-type lysozymes from other scorpions and arthropods.

To analyze the evolutionary aspects of the MesoLys-C peptide we generated a phylogenetic tree encompassing known c-type lysozymes within the animal phyla: scorpion, invertebrate, and vertebrate (Fig. 3).

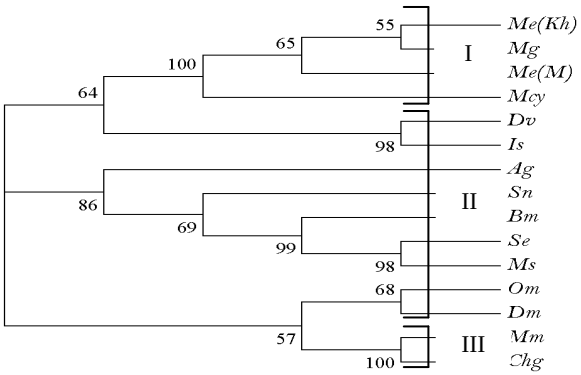

Fig. 3. Phylogenetic tree of MesoLys-C and lysozymes C from other species.

Me(Kh): *Mesobuthus eupeus* of Khuzestan, Mg: *Mesobuthus gibbosus*, Me(M): East Mediterranean *Mesobuthus eupeus*, Mcy: *Mesobuthus cyprius*, Dv: *Dermacentor variabilis*, Is: *Ixodes scapularis*, Ag: *Anopheles gambiae*, Sn: *Simulium nigrimanum*, Bm: *Bombyx mori*, Se: *Spodoptera exigua*, Ms: *Manduca sexta*, Om: *Ornithodoros moubata*, Dm: *Drosophila melanogaster*, Mm: *Mus musculus*, Chg: *Chicken Gallus*. Groups: I – scorpion; II – invertebrate; III – vertebrate. Numbers indicate bootstrap support based on 100 replicates.

According to the dendrogram, all scorpion samples analyzed (*M. eupeus* of Khuzestan, *M. gibbosus*, *M. cyprius*, and East Mediterranean *M. eupeus*) could be considered phylogenetically different subspecies by possessing the lysozyme C peptide in their venom. Moreover, MesoLys-C isolated from *M. eupeus* of Khuzestan displayed the highest and the lowest sequence similarities with *M. gibbosus* and *M. cyprius*, respectively. This result is in accordance with other similar studies [9,10]. Further comparison of MesoLys-C with those from the Groups II and III species showed marked difference between the lysozyme C amino acid sequences displaying the lowest homology with the vertebtrate Group. The study also revealed that the residues of catalytic site in C-type lysozyme of scorpions are different from those of chicken and other organisms. Based on the phylogenetic tree of MesoLys-C and C-type lysozyme of East Mediterranean *M. eupeus* it is concluded that *M. eupeus* of Khuzestan and East Mediterranean *M. eupeus* belong to different subspecies.

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