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A new chlorotoxin from *Mesobuthus eupeus* possessing insectotoxic property

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Abstract

The role of proteins and peptides in scorpion venoms has been established as one of the primary causes of their toxicity. Establishing their primary structures serves to extend fundamental knowledge. *Mesobuthus eupeus* is one of the widely spread scorpion species in the Central Asian region. In this work, the toxicities of various fractions from *M. eupeus* venom were compared to find the one with more significant potential. The toxicities of venom fractions on various insects were studied by administering in various doses to three insects – *Periplaneta americana*, *Tenebrio molitor*, and *Helicoverpa armigera*. We also isolated a pure peptide from one of those fractions. A new sequence of amino acids purified from *M. eupeus* was found to belong to chlorotoxins. The structure was established by mass spectral analysis. The peptide with a MW 3830,52 consisting of 36 amino acids and eight cysteine residues, was found to belong to chlorotoxins that inhibit Cl⁻ channels.

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Introduction

Scorpion venoms, as well as other venoms, can be a mixture of toxins that were used as treatments in low doses against various diseases, which are explained today with bioactive compounds, including proteins and peptides (Pennington *et al.* 2018). It contains antibacterial peptides and peptides affecting the neural and cardiac systems (Desales-Salazar *et al.* 2020). The scorpion venom is a cocktail of proteins and peptides with various functions, including blocking ion channels (Ali *et al.* 2016) which promotes their utilization as

insecticidal means to enhance plants insect resistance.

Among the toxic peptides in scorpion venom, chlorotoxins are a class of peptides that deserved attention. They are expected to be an inhibitor of ion channels. More than ten subfamilies of the toxins specific for K⁺ and Cl⁻ channels have been identified. They share sequence similarity (Tytgat *et al.* 1999). Chlorotoxins are among the factors that affect the normal functioning of the Cl⁻ channel (Dardevet *et al.* 2015). They are known to paralyze

insects (Kerkis *et al.* 2017). These peptides, consisting of 34-36 amino acid residues stabilized by four cystine residues, are expected to target chloride ion channels (DeBin *et al.* 1993; Tytgat *et al.* 1998). Chlorotoxins were established to paralyze cockroaches and bollworms; they are also known to kill some crustaceans like crayfish (Smith *et al.* 2013).

Identifying potential toxic peptides possessing insecticidal properties and establishing their primary structures can promote their utilization by introducing into plant or bacterial genomes either to breed resistant plant lines (Deng *et al.* 2019) or produce in bacteria (Amorim *et al.* 2018). In this work, we aimed to isolate and establish the structure of a peptide possessing insectotoxicity. As research object, we chose *Mesobuthus eupeus*, one of the scorpion species identified in the Asian region (Mohammadi *et al.* 2021). We intended to find an insectotoxic peptide with a potential to be used for plant resistance. By using several steps of purification of peptides, we planned to obtain several fractions from the venom of *M. eupeus*, widely spread in Uzbekistan, and compare their toxicities in three insects. Thus, we would select the most potential one for establishing its primary structure.

Experimental

Extraction/obtaining of scorpion

Approximately 10 % of scorpion venom was separated from non-toxic water-soluble mucoproteins by centrifugation of the venom solution. For this purpose, 90 mg of venom dissolved in 20 mM ammonium acetate buffer (pH 4.7) was centrifuged at 6000 rpm for 15 min. The precipitate was separated and dissolved again in this buffer. The solution was centrifuged. The separated supernatants were combined and used for chromatographic fractionation.

Gel chromatography

The gel filtration process was carried out in a 2.6 × 100 cm chromatographic column filled with ToyoPearl HW 55f gel. A 20 mM acetate buffer with pH 4.7 was used as the running buffer. The

flow rate was 1 ml/min. A 280 nm wavelength was used for protein detection.

Ion-exchange chromatography

At the next stage of our research, the ME-4 fraction, consisting of peptides obtained in gel filtration, was subjected to ion exchange chromatography. The ME-4 fraction was sent to a CM-TSK 650M sorbent column (1.5 × 25 cm) equilibrated with 20 mM ammonium acetate buffer at pH 4.7. The flow rate was 0.5 ml/min.

Desalination of fractions

The fractions were desalinated in LPLC (Bio-rad) provided with a C18 column (1 × 4 cm). Ions were washed out with water for the first 5 min. Further, proteins were eluted with 50 % acetonitrile for the next 7 min. The flow rate was 1 ml/min in both cases. Obtained fractions were freeze-dried and dissolved in water for the next experiments.

HPLC

HPLC fractionation of ME-4-2 fraction was carried out in a column Nucleosil 5C18 (4.6 mm × 250 mm) by using the following buffers and conditions. Buffer A – 0.1 % TFA, B – acetonitrile. Gradient – 35 min 5-60 % buffer B, 5 min – 60 % buffer B, 5 min – 60-5 % buffer B. Flow rate – 1 ml/min. 226 nm.

Insectotoxicity analysis

The insectotoxic activity of the fractions was determined by injecting them into the abdominal cavity of insects. For this purpose, cockroaches (*Periplaneta americana*) and mealworms (*Tenebrio molitor*) were injected with 16 dried fractions at 5 µg/g doses. The effect of fractions on insects was observed for 5 min, 3 hours, and 24 hours. The insectotoxicity of the isolated fractions by HPLC was determined by injecting them into the abdominal cavity of insects at a concentration of 1 µg/ml. During the research, the movement of insects was observed, and cases of paralysis and/or death were counted. Fractions that caused paralysis observed in insects were identified as insectotoxic

active. The effects of each protein fraction sample were studied in three replicates.

Mass spectral analyses of peptides

The peptide sequence was determined using Agilent Technologies CHIP-LC/MS/MS Q-TOF 6420B mass spectrometer: pump flow rate of 4 $\mu\text{L}/\text{min}$, Zorbax SB C18, 5 μm (150 $\mu\text{m} \times 43$ mm) CHIP -nano column. Mobile phase: A – 0.1 % formic acid, B – acetonitrile + 0.1 % formic acid. The adsorbed peptide was desorbed from the nano column using an Agilent Technologies 1260 Nano Pump at a flow rate of 0.6 $\mu\text{L}/\text{min}$ in the following gradient of the mobile phase: concentration of solution B: 5 % – 3 minutes, 80 % – 25-30 minutes, 5 % – 35 minutes.

Results and discussion

Insects are expected to be a part of a few scorpions' diet (Dos Santos *et al.* 2016), unless those insects do not hold defending compounds (Opitz and Müller 2009). Likewise, toads hold compounds called bufadienolides in their skin, used for their self-defence (Asrorov *et al.* 2023). Various mechanisms regulate the paralysis of insects used by scorpion for their diet (Wudayagiri *et al.* 2001; Mosbah *et al.* 2000). Cl^- channels are one of these mechanisms widely used by scorpions (Tytgat *et al.* 1998; Smith *et al.* 2013). In this work, we followed a classical approach to isolating insecticidal peptides: comparative analysis of fractions and selection of the one with the highest potential.

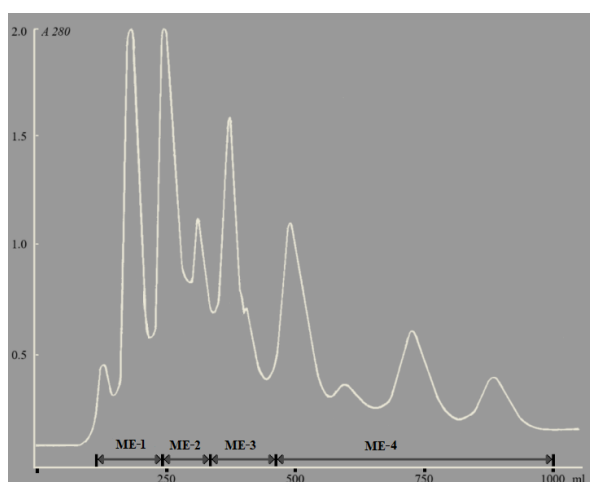


Figure 1: Gel filtration of *M. eupeus* scorpion venom.

Ten peaks were obtained by chromatographic separation (Fig. 1). Studying the molecular weight of all peaks showed a high similarity of the last four peaks to each other. Therefore, those four peaks were collected as one fraction and further used for detecting insectotoxic peptides by ion-exchange chromatography. ME-1, ME-2, and ME-3 fractions held higher molecular weight proteins and enzymes. Thus, they were not included in the insectotoxicity study.

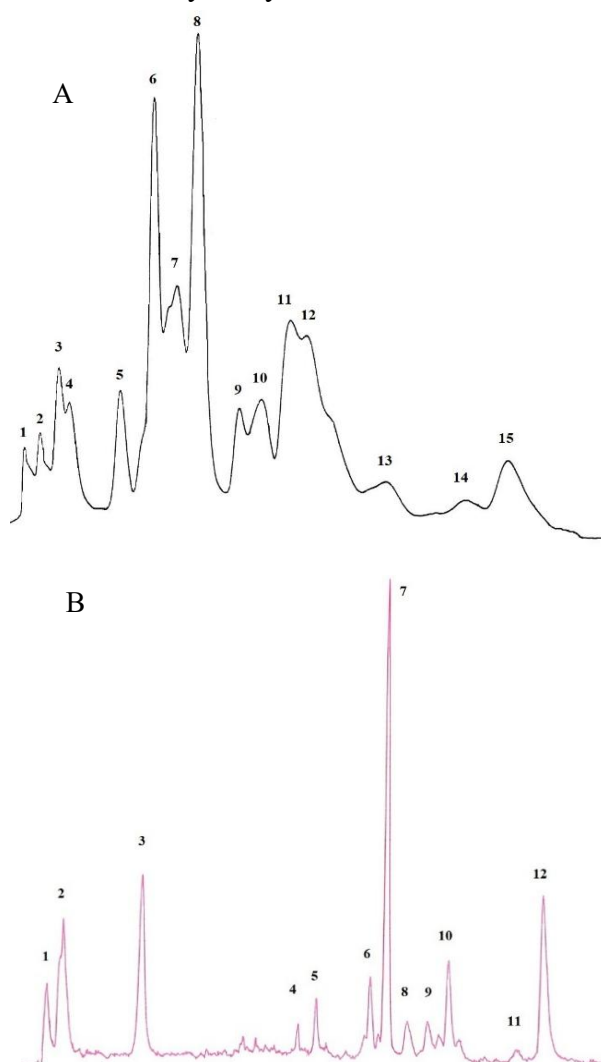


Figure 2: Fractionation of *M. eupeus* extraction by ion exchange chromatography (A) and HPLC (B).

Further fractionation of the ME-4 using CM-TSK enabled us to obtain 15 peaks from ME-4-1 to ME-4-15 (Fig. 2 A). Studying insectotoxicity by sending the fractions at 5 $\mu\text{g}/\text{g}$ doses clarified further selection of the fraction.

Table 1. Insectotoxicity of fractions obtained by ion-exchange chromatography (5 µg/g).

Fractions	Impact on <i>P. americana</i>			Impact on <i>T. molitor</i>		
	5 min	3 h	24 h	5 min	3 h	24 h
ME-4-1	+++	---	---	+++	---	---
ME-4-2	+++	+++	+++	+++	+++	+++
ME-4-3	+++	+++	---	+++	---	---
ME-4-4	+++	---	---	+++	++-	+--
ME-4-5	---	---	---	---	---	---
ME-4-6	+++	---	---	+++	---	---
ME-4-7	---	---	---	---	---	++-
ME-4-8	+--	---	---	---	+++	
ME-4-9	+++	---	---	---	+++	+++
ME-4-10	+++	+++	+++	+++	+++	++-
ME-4-11	---	---	---	---		
ME-4-12	+++	---	---	---	++-	++-
ME-4-13	---	---	---	---	---	
ME-4-14	---	---	++-	---	---	
ME-4-15	---	---	+++	---	---	+++

* The effects of the fractions were studied in three replicates: + stands for paralyzed insects at the given time; minus means either unaffected insects or the ones returned to active life form at the given time.

Table 2. Insectotoxic activity of the ME-4-2 fraction obtained using HPLC at 5 µg/g.

Fractions	Impact on <i>P. americana</i>			Impact on <i>T. molitor</i>			Impact on <i>H. armigera</i>		
	1 min	5 min	60 min	1 min	5 min	60 min	1 min	5 min	60 min
ME-4-2-1	---	---	---	---	---	---	---	---	---
ME-4-2-2	---	---	---	---	---	---	---	---	---
ME-4-2-3	---	---	---	---	---	---	---	---	---
ME-4-2-4	---	---	---	---	---	---	---	---	---
ME-4-2-5	+--	---	---	---	+--	---	---	+--	---
ME-4-2-6	++-	++-	+--	++-	++-	++-	++-	++-	++-
ME-4-2-7	+++	+++	+++	+++	+++	+++	+++	+++	+++
ME-4-2-8	++-	+--	+--	++-	++-	+--	++-	++-	---
ME-4-2-9	-+-	-+-	---	-+-	-+-	---	---	-+-	---
ME-4-2-10	---	---	---	---	---	---	---	---	---
ME-4-2-11	---	---	---	---	---	---	---	---	---
ME-4-2-12	---	---	---	---	---	---	---	---	---

* The effects of the fractions were studied in three replicates: + stands for paralyzed insects at the given time; minus means either unaffected insects or the ones returned to active life form at the given time.

Table 3. Insectotoxic activity of the ME-4-2 fraction obtained using HPLC (2.5 µg/g).

Fractions	Impact on <i>P. americana</i>			Impact on <i>T. molitor</i>			Impact on <i>H. armigera</i>		
	1 min	5 min	60 min	1 min	5 min	60 min	1 min	5 min	60 min
ME-4-2-1	---	---	---	---	---	---	---	---	---
ME-4-2-2	---	---	---	---	---	---	---	---	---
ME-4-2-3	---	---	---	---	---	---	---	---	---
ME-4-2-4	---	---	---	---	---	---	---	---	---
ME-4-2-5	---	---	---	---	---	---	---	---	---
ME-4-2-6	+ --	+ --	+ --	+ --	+ --	+ --	+ --	+ --	+ --
ME-4-2-7	+++	+++	+++	+++	+++	+++	+++	+++	+++
ME-4-2-8	++ -	---	---	+ --	+ --	+ --	---	---	---
ME-4-2-9	---	---	---	---	---	---	---	---	---
ME-4-2-10	---	---	---	---	---	---	---	---	---
ME-4-2-11	---	---	---	---	---	---	---	---	---
ME-4-2-12	---	---	---	---	---	---	---	---	---

* The effects of the fractions were studied in three replicates: + stands for paralyzed insects at the given time; minus means either unaffected insects or the ones returned to active life form at the given time.

The highest insectotoxic activity was determined in ME-4-2 and ME-4-10 fractions. These fractions showed their effects for 24 hours. Apart from them, ME-4-3 and ME-4-10 fractions had higher toxicity than the remaining others. In the ME-4-1 and ME-4-6 fractions, the experimental insects were paralyzed at the beginning of the injection, but they recovered from this state in 3 h. Reverse effects were observed in *T. molitor* cases. The fractions ME-4-7, ME-4-9, ME-4-12, and ME-4-15 caused the paralysis of *T. molitor* in 3 and/or 24 h, which means their slow impact.

Further, we divided the ME-4-2 fraction that showed the highest toxicity into fractions using HPLC. Thus, individual components were isolated because of fractionation. The resulting components were tentatively assigned with numbers as ME-4-2-n (1-12) (Fig. 2 B). All the fractions were dried in a lyophilizer, and their insectotoxicities were.

The obtained results showed the toxicity of only three HPLC fractions (Table 2). The ME-4-2-7 fraction at a concentration of 5 µg/ml caused paralysis in insects injected into the abdominal cavity of *P. americana*, *T. molitor*, and *H. armigera*. All the insects died in the first 5 minutes following the treatment with that fraction. Fractions ME-4-2-6 and ME-4-2-8 paralyzed them partially when

administered at 5 µg/ml (Table 2). Further, we studied the insectotoxic activity of the fractions at lower concentrations. Fractions were administered at a concentration of 2.5 µg/ml, and the behaviour of insects were observed over time (Table 3).

The ME-4-2-7 fraction at a concentration of 2.5 µg/ml caused paralysis in insects injected into the abdominal cavity of *P. americana*, *T. molitor*, and *H. armigera*. No recovery was observed among the insects treated with ME-4-2-7. The used dose of this fraction was enough for the complete death of all used insects for 1 minute. The fraction ME-4-2-6 caused the unrecovered paralysis of every third insect for 60 min. A similar effect of ME-4-2-8 was observed only in the case of mealworms. No effects of this fraction were observed on *P. americana* and *H. armigera* for 5 and 60 min. *P. americana* subjected to paralysis for a minute further recovered their movement. Thus, because of determining the insectotoxic activities of the components obtained from the fractionation of the ME-4-2 fraction using HPLC, ME-4-2-7 was found to be a strong insectotoxin.

We further studied the sequence of the ME-4-2-7 using mass spectrometry. The structure of the peptide was established as MCMPCFTTDANMARKCSDCCGGNGKCFGPQ

CLCNRA. The three-letter symbol style is Met-Cys-Met-Pro-Cys-Phe-Thr-Thr-Asp-Ala-Asn-Met-Ala-Arg-Lys-Cys-Ser-Asp-Cys-Cys-Gly-Gly-Asn-Gly-Lys-Cys-Phe-Gly-Pro-Gln-Cys-Leu-Cys-Asn-Arg-Ala consisted of 36 amino acid residues with 8 cysteines belonging to the chlorotoxin family. The MW of the identified peptide was 3830.52.

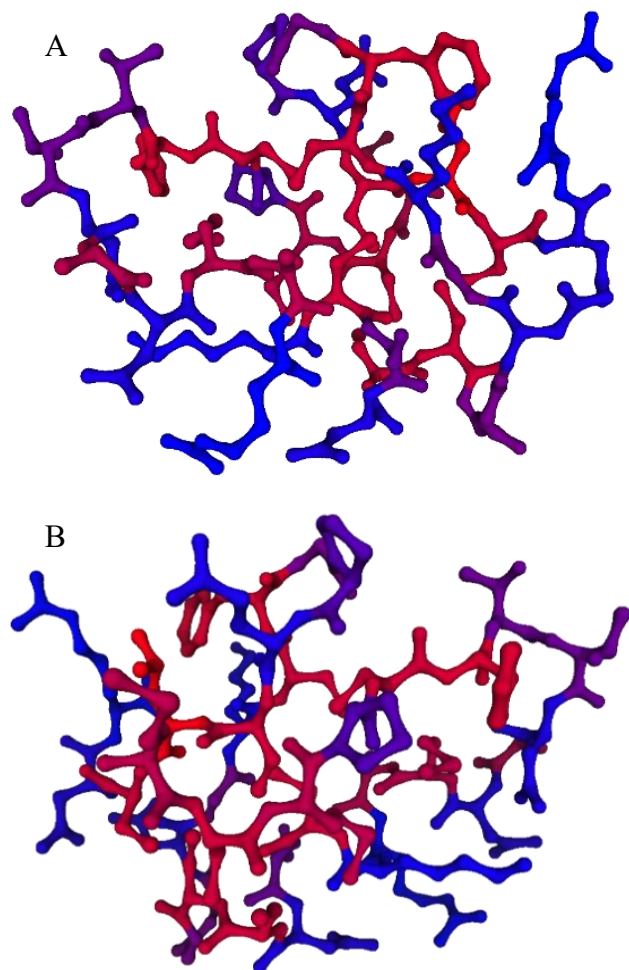


Figure 3: 3D model of the obtained chlorotoxin in this work: A and B are visions from different sides

Developing the peptide 3D model using Swiss-model revealed the location of hydrophobic amino acid residues in the middle of the peptide: red- and purple-coloured amino acid residues (Fig. 3). Polar amino acids, including Arg, Asp, Asn, and Gln, are located on the edge. Thus, we can suggest the charge-charge interactions with other charged groups. A higher ratio of hydrophobic amino acids than polar ones enable predicting its easy passage through biomembranes due to amphiphilic nature. A theoretical isoelectric point of the peptide

computed in https://web.expasy.org/compute_pi/ and equalled 8.17, might lead to normal distribution of the peptide in insects hemolymph that vary from pH 6.4 to 8 (Matthews 2017). In cockroaches its pH was found to be 7.3 (Matthews and White 2011).

Blue colour shows Asn, Asp, Gln, Lys, and Arg. Purple colour indicates Gly, Ser, Thr. Violet stands for Pro. Pink and red colours demonstrate Ala, Met, Cys, Leu, and Phe. The model was developed in the Swiss-model at <https://swissmodel.expasy.org/>.

Modelling suggested the location of disulfide bonds in the inner cavity of the peptide. Based on close locations of sulfhydryl groups of Cys16–Cys31, Cys5–Cys26, Cys20–Cys33, and Cys2–Cys19, we suggest high stability of the peptide resulting from four putative disulfide bonds (Fig. 4).

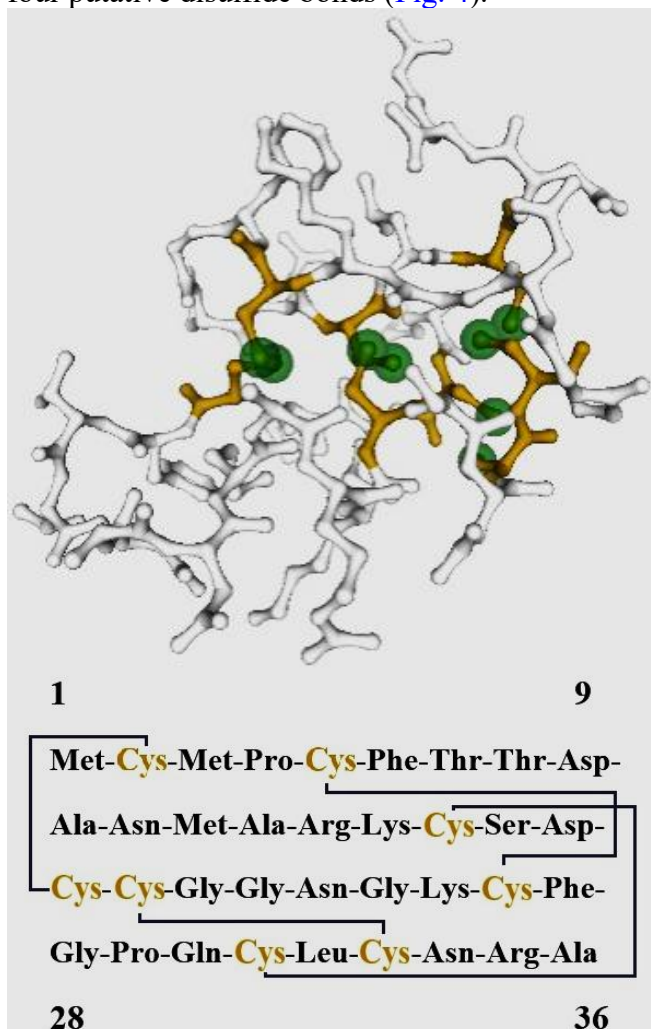


Figure 4: Locations of putative disulfide bonds in the chlorotoxin molecule identified in this work. The model was developed in the Swiss-model platform at <https://swissmodel.expasy.org/>.

24746279.1	MKFLYGIVFITLFLTVMIATHTEAAMCMPCFTTNLMNEQECRDCCGGT--GRCFGPQCLC GYD	61
24746279.2	MKFLYGIVFIALFLTVMIATHTE-AMCMPCFTTRPNMAQQCRDCCRGR--GKCFGPQCLC GYD	60
24746279.3	MKFLYGIVFITLFLTVMIATHTE-AMCMPCFTTRPNMAQQCRDCCRGR--GKCFGPQCLC GYD	60
29483648	-----GSMCMPCFTTDHQMARKCDDCCGGKGRGKCYGPQCLC R--	38
8383429	-----MCMPCFTTDHQMARKCDDCCGGKGRGKCYGPQCLC R--	36
22887697	MKFLYGIVFIALFLTVMFATQ--TDGCGPCFTTDANMARKCRECCGGN--GKCFGPQCLC NRE	59
10673158	MKFLYGIVFIALFLTVMFATQ--TDGCGPCFTTDANMARKCRECCGGI--GKCFGPQCLC NRI	59
35524923	-----MCMPCFTTDHNMAKKCNDCCGGY--GKCFGPQCLC R--	34
9891977.2	-----MCMPCFTTDPNMANKCRDCCGGG--KKCFGPQCLC NR-	35
Ch1T.Bio.	-----MCMPCFTTDANMARKCSDDCCGGN--GKCFGPQCLC NRA	36
9891977.1	-----MCMPCFTTDHNMAKKCRDCCGGN--GKCFGPQCLC NR-	35
6679783	-----MCMPCFTTDPNMAKKCRDCCGGN--GKCFGPQCLC NR-	35
2085324	-----MCMPCFTTDPNMAKKCRDCCGGN--GKCFGPQCLC NRX	36

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Figure 5: Comparison of amino acid sequences of the peptide identified in this work with others found in the NCBI database. The numbers showing the belongings of the sequences indicate their PubMed ID. The right-side numbers show the amino acid numbers in the peptides. The peptide identified in this work is shown in the rectangle.

BLAST search in the NCBI database showed tens of structures that share highly homologous sequences to the one identified in this work. We found similar structures from those consisting of 35-36 amino acids to others with 60 amino acid residues (Fig. 5).

The peptide identified in this work was found a new sequence compared to others found in NCBI database. Similar structures were identified in scorpion so far (Fig. 5). BLAST-ing revealed 3 or 5 amino acid differences in the sequences of peptides that share the closest homology with the peptide found in this work.

Insecticidal transgenes have been incorporated with several biomolecules such as protease inhibitors, lectins, amylase inhibitors and others (Rakesh *et al.* 2023). The usage of scorpion toxins in genetic engineering has deserved attention. The review on the insecticidal potential of scorpion toxins highlights their involvement in the voltage-gated sodium channels. A few works utilized scorpion genes to improve the insect resistance of plants (Gurevitz *et al.* 2007). In one work, the antineuroexcitation peptide III from scorpion was expressed in tomato and tobacco plants. The work proved the effective expression of the peptide in two plants. The protein expression of the protein in tomato made greater quality (Song *et al.* 2011). The expression of scorpion insect toxin AaHIT in cotton plants led to enhanced insect resistance of

the plant in another work. The plants expressing scorpion toxin showed high resistance to cotton bollworms compared to those that do not express the toxin. After infestation, high insect mortality was observed in plants expressing the scorpion toxin (Wu *et al.* 2008).

The expression of chlorotoxins in recombinant organisms explains the possibility of their production as recombinant products (Quintero-Hernández *et al.* 2011). A few research works report their expression bacteria. The expression of insectotoxin I5A from *B. eupeus* in *E. coli* was found only in trace amounts. The authors detected a high level of expression in bacterium; the low amount was explained with probable instability (Pang *et al.* 1992). The yield of the chlorotoxin-like peptide from *B. martensii* Karsch in the model bacterium made 7.8 mg/L, which can consider as satisfying result (Fu *et al.* 2005). In another work, scorpion beta neurotoxin CsxII was obtained recombinantly at a dose of 24.6 mg/L in *E. coli*. The proper folding structure was revealed *in vitro* (Estrada *et al.* 2007). These results explain the possibility of obtaining chlorotoxins recombinantly in bacteria. Greater yields of chlorotoxin were detected in yeasts. The expression of *B. eupeus* insectotoxin I5A in *Saccharomyces cerevisiae* equalled 50-100 mg/L (Pang *et al.* 1992). The expression of ButaIT from *Mesobuthus tamulus* in *Pichia pastoris* made 25 mg/L. The authors suggested its usage as recombinant fusion (Fitches

et al. 2010). The expression of insectotoxin I5A in tobacco plants was proved by Southern hybridization analysis. The mRNA was detected in gel electrophoresis that was different from non-transformed plants. The protein expression was proven by purifying the toxin from the protein extract (Pang *et al.* 1992). The expression of another scorpion toxin, termed neurotoxin AaIT, was expressed in tobacco. Southern blotting analysis proved its integration into the plant genome. Even though the protein was not isolated after expression, transformed plants showed notable insect resistance compared to the non-transformed ones (Yao *et al.* 1996).

Conclusions

We isolated and purified a peptide from scorpion venom with insectotoxic activity. It showed high homology to toxins, termed chlorotoxins, that inhibit Cl⁻ channels. The peptide consisted of 36 amino acids and eight putative cysteine residues reveals amphiphilic property.

The established amino acid sequence in this work can be efficiently used to obtain genetically modified organisms with enhanced resistance against *H. armigera*, *P. americana*, *T. molitor*, economically important insects. The established sequence expands the knowledge on amino acid sequence-related primary structures of peptides that could be used to identify potential insectotoxic molecules.

The peptide with 36 amino acids was found to have high insectotoxicity on cockroaches. Introducing the gene into the bacterial genome will enable obtaining the recombinant peptide that could be purified in preparative amounts and utilized to produce various forms of insecticides.

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