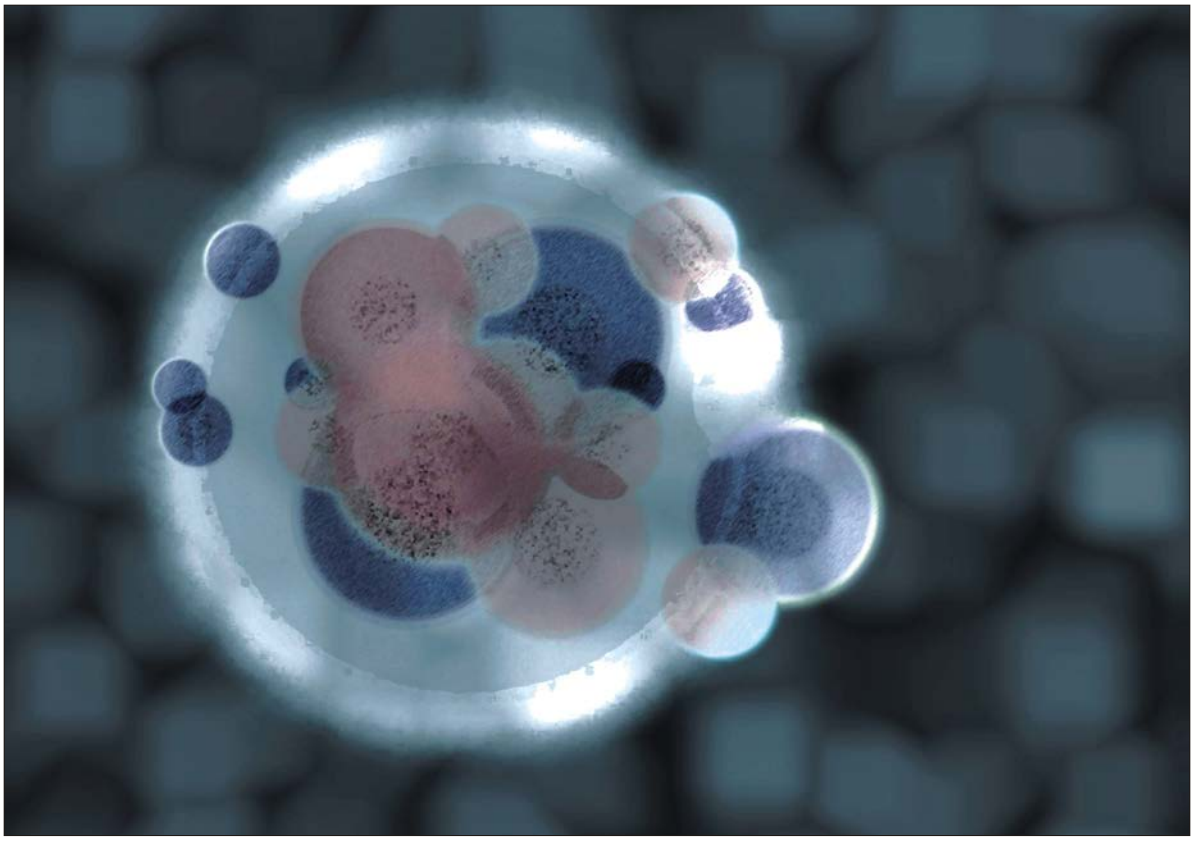

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Identification and Purification of the Three Toxins from *Odotobuthus bidentatus*, Lourenco and Pezier 2002, Venom (Scorpions, Buthidae)

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Abstract: *Odonthobuthus* belongs to Buthidae family from which 2 species *O.doriea* and *O. bidentatus* have been collected and reported from Iran. This study investigated the venom of *O. bidentatus* to identify mammal toxic fractions. After collecting scorpions, they were milked with electroshock technique and then the venoms was lyophilized. The LD_{50} of the venom was determined by injecting the venom to 18-20 g mice via., tail vein. In order to separate different fractions, the freeze dried venom was solubilized in distilled water and centrifuged at 15000 rpm for 20 min, to separate insoluble material and the clear supernatant containing soluble venom was loaded on a chromatography column packed with sephadex G50 gel and the fractions were collected according to UV absorption at 280 nm wavelength. Second fraction was toxic in Lab animal and in order to study sub fractions, fraction II (O_2) was loaded first on an anionic ion exchange resin (DEAE sephacel) out of which we obtained 2 toxic fractions (O_{21} and O_{23}). Then these 2 fraction were loaded on a cationic resin (CM sephadex C_{25}) out of which, from fraction 21, we got 2 toxic fractions (O_{211}, O_{213}) and from fraction O_{23} we got one toxic fraction (O_{232}). SDS PAGE electrophoretic studies on these toxins showed single band appearance that indicates purified toxins with molecular weight of O_{211} 9.2, O_{213} 8.6 and O_{233} 6.8 kD.

Key words: Scorpion, venom, fraction, *Odontobuthus bidentatus*, gel

INTRODUCTION

Scorpion venom is a rich source of various polypeptides, which are proved to be high affinity ligands of ion channels making them useful as pharmacological probes. Among the well characterized peptide toxins are those from scorpions belonging to Buthidae family (Chirstian *et al*., 1996; Nastain, 2002; Rajendra, 2001). The lyophilized milked venom was a matrix containing glycoprotein, cellular debris and mucus material (Gawade, 2003) and various concentration of soluble material like neurotoxin, cardiotoxin, nephrotoxin, hyoluronidases, glycosaminoglacans, histamine (Chirstian, 1996; David, 2005). Also, source of highly concentrated active enzymes and cytotoxine (Badhe, 2006). The classification of scorpion toxins have been made on various criteria such as ion channel specificity, number of amino acid residues or target specificity (Cook *et al*., 2002; Srinivasan, 2002). Odontobuthus is one of the medically important buthid scorpion in Iran (Zare *et al*., 2007). Navidpour *et al*. (2008) showed that in South and Southwestern provinces of Iran there are 2 species of Odontobuthus: *O. doriae* and *O. bidentatus*. Biochemical and clinical manifestations as well as toxins identification and purification from the venom of *O. doriae* was previously reported by Zare *et al*. (2006). However, no report exist about the toxins present in *O. bidentatus*. Hence, in this study we attempt to identify and purify the mammalian toxins present in *O. bidentatus*.

MATERIALS AND METHODS

The scorpions were collected with UV light at night (Lowe *et al*., 2003) from different parts of Khoozestan Province and they were milked by electric shock at the end of tail. The venom was water dialyzed and freeze dried at -20°C until use (Debont, 1998). The LD_{50} was determined by sperman and carber method on albino mice weighting 18- 20 g (Masihipour, 2005; Kalapothakis and Chavez, 1997).

Preparation of the sample: The freeze dried venom (812 mg) was dissolved in distilled water and placed in dialysis bag and dialyzed against distilled water at 4°C for 48 h (Masihipour, 2005) after dialysis the venom solution was centrifuged at 15000 rpm for 15 min and supernatant was collected (Kalapothakis and Chavez, 1997).

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Sephadex gel filtration: Crude venom loaded over a sephadex G50 (fine) (More *et al*., 2005) in a 100×50 cm column equilibrated with ammonium acetate buffer (0.1 mol and pH = 7) and flow rate was adjusted to 60 mL h^{-1} (Davila, 1996). Sample collection was carried out in 10 mL tubes and protein content was determined by UV spectrometer at 280 nm wave length. Each fractions toxicity was tested on mice by intravenous injection of 0.5 mL of each fraction to albino mice weighting 18-20 g.

Ion exchange chromatography: Toxic fraction derived from gel filtration was load on DEAE sephacel an anionic ion exchanger (0.1 mL, pH = 8.9) and flow rate was adjusted to 30 mL h^{-1}. The elusion was carried out using gradient of NaCl (0.0 -0.05 mL). The eluted fractions from this column after testing on albino mice and finding the toxic fractions as previously mentioned were load on a CM sephadex C_{25} an cation exchanger and toxic fractions were collected and checked for toxicity.

SDS PAGE electrophoresis: Crude venom as well as each toxic fraction and finally the purified toxins were loaded on SDS PAGE electrophoresis to determines the trend of purification. The concentration percentage of SDS-PAGE was 10% for crude venom and 12% for pure toxins.

RESULTS AND DISCUSSION

The toxicity of venom was tested by determination of LD_{50} of crude venom in mice for 24 h. the average LD_{50} of crude venom was found to be 10.35 μg.

Total protein of the crude venom was found to be 353 mg after removal of mucoproteins. That means percentage of mucoprotein in the crude venom using electroshock method for venom milking in 47.3% (Table 1). Electrophoretic pattern of electrophoresis showed the presence of at least 14 separated bands (Fig. 1). Total amount of protein loaded on the gel filtration column was 459 mg. After elution from gel filtration column we got 4 peaks (Fig. 2) out of which the peaks No O_2 was found

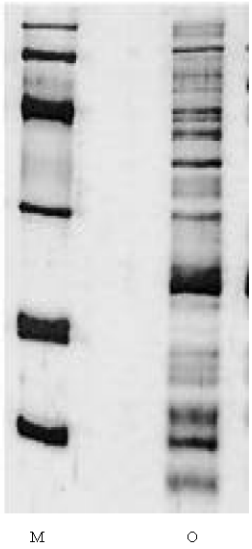

Fig. 1: Comparison of electrophoretic bands of *O. bidentatus* with control marker

Table 1: Percentage recovery and LD_{50} of extracted toxins

Steps of purification	Fractions (mg)	Recovery in relation 812 mg crude dry venom (%)	Recovery (%)	LD_{50} (μg $mice^{-1}$)
Extraction and dialysis	459 mg	56.30		10.35
Sephadex G-50	33.6 = O1	4.13	7.32	7.20*
	183.3 = O2*	22.57	39.93	
	153.4 = O3	18.89	33.42	
	43.3 = O4	5.33	9.43	
DEAE sephacel	424.6 = SUM	52.29	90.10	3.78*
	183.3 mg			
	43.4 = O21*	5.34	23.63	
	37.8 = O22	4.65	20.62	
	5.8 = O23*	0.71	3.16	1.15*
	3.6 = O24	0.44	1.96	
	90.9 = SUM	11.14	49.37	
CM sephadex C25	31.3 mg			
	5.4 = O211*	0.665	17.25	
	2.6 = O212	0.320	8.30	
	1.5 = O213*	0.184	4.79	
	8.7 = O214	1.070	27.79	
	18.7 = SUM	2.300	58.13	
	3.48 mg			
	0.29 = O231	0.035	8.33	
	o.36 = O232	0.044	10.34	
	0.46 = O233*	0.056	13.21	
	1.11 = SUM	0.136	31.89	

*Fractions which killed mice

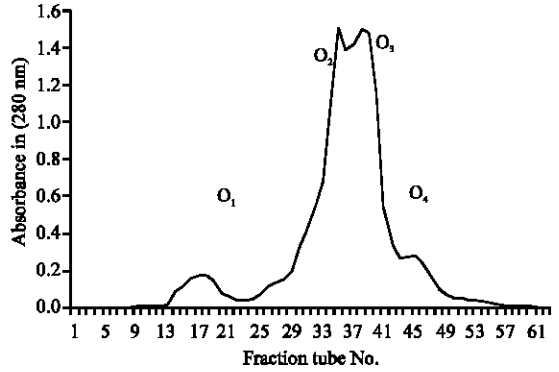

Fig. 2: Sephadex G-50 gel filtration crude venom on a 2.5×125 cm column with 0.1 M ammonium acetate buffer, pH = 8.6 and 60-70 mL h^{-1} elution rate

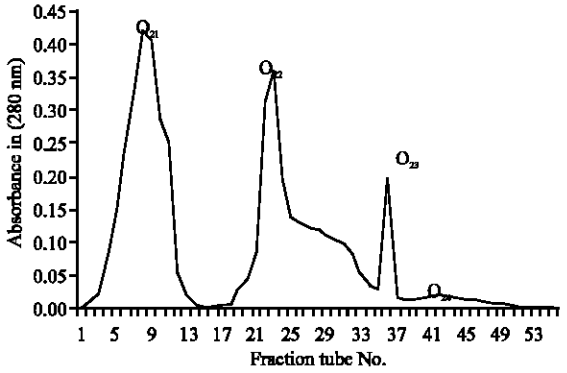

Fig. 3: DEAE sephacel ion exchange chromatography of O_2 fraction on a 1.3×50 cm column with 0.5 mol ammonium acetate buffer with 0.5 mol salt gradient, pH = 8.9 and 30-35 mL h^{-1} elution rate

Table 2: Results obtained from IV injection of 0.5 mL of each fractions to mice after 24 h

Fraction	Before dialysis and concentration	After dialysis and concentration
O_{21}	+	+
O_{22}	-	-
O_{23}	+	+
O_{24}	-	-
O_{211}	+	+
O_{212}	-	-
O_{213}	-	+
O_{214}	-	-
O_{231}	-	-
O_{232}	-	-
O_{233}	-	+

+: Kill the mice -: Non kill the mice

to be toxic on mice. The total protein of O_2 were determined and found to be 183 mg. The LD_{50} for O_2 were found to be 7.2 μg, respectively (Table 1). Yield of mammalian toxins for O_2 were, DEAE sephacel amonic chromatography of fraction O_2 from previous step, four

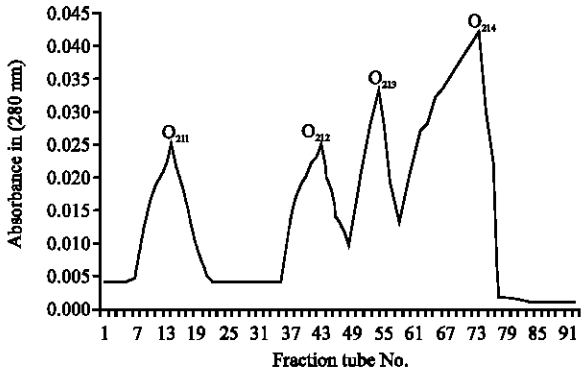

Fig. 4: CM sephadex O_{25} ion exchange chromatography of $O_{2.1}$ fraction on a 1.5×50 cm column with 0.1 Tri sodium citrate buffer and 0-0.5 mol salt gradient and 30-35 mL h^{-1} elution rate

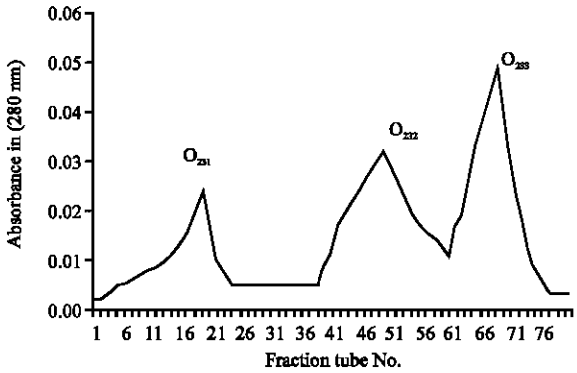

Fig. 5: CM sephadex O_{25} ion exchange chromatography of $O_{2.3}$ fraction on a 1.5×50 cm column with 0.1 mol Tri sodium citrate buffer and 0-0.5 mol salt gradient and 30-35 mL h^{-1} elution rate

fractions O_{21}, O_{22}, O_{23} and O_{24} were obtained (Fig. 3) after injection of these 4 fractions to animals it was found that fractions O_{21} and O_{23} are toxic (Table 1). After that, the 2 toxic fraction from above step, were loaded on CM sephadex C_{25} column, from O_{21} fraction 4 sub fractions O_{2I1},O_{2I2}, O_{2I3}, O_{2I4} (Fig. 4) were eluted containing 5.4, 2.6, 1.5 and 8.7 mg protein, respectively (Table 1). After injection of these fractions to mice, it was clear that, 2 fractions O_{2I1} and O_{2I3} were toxic (Table 2). Fraction O_{23} after elution on the CM sephadex O_{25} gave three sub fractions O_{23I}, O_{23II}, O_{23III} (Fig. 5) with protein content of 0.29, 0.36 and 0.46 mg, respectively (Table 1), out of which only fraction O_{23III} was toxic (Table 1). Electrophoretic pattern of purified toxins showed a single band which was an indicator for purity of toxins.

The present study examined, the toxic fractions and subfractions present in the venom of *O. bidentatus* which

has a wide distribution in South Provinces of Iran and is one of the medically important scorpion in the region. The envenomation by this scorpion cause local pain accompanied with extreme edema in severe cases it manifests dizziness, delirium vomiting nausea and finally sleepiness. The primary action of venom is through both sympathetic and parasympathic postganglionic stimulation (Dittrich *et al*., 2002). Four different fractions were obtained by sephadex G_{50} (fine) chromatography, out of which, second fraction O_2 was toxic for lab animal and other fraction were not toxic for mammals even though they can be toxic for insects. In a research Zare *et al*. (2006) separated three hommogen toxic fraction from the venom of scorpion (*Odonthobuthus doriea*) the O_2 fraction from previous step was loaded on a DEAE sephacel amion exchange column. Four Sub fractions were eluted, out of which O_{21} and O_{23} were toxic. Then sub fractions toxic were loaded on a CM sephadex O_{25} cation exchange column from O_{21} fraction 2 toxic peptide i.e., 211 and 213 were obtained and from O_{23} one toxic peptide was elutedie 233. The omittion of higher molecular bands from O_2 fraction after G50 sephdex gel chromatography, was due to lack of toxicity but O_2 fraction which was toxic, had low molecular weight. Electrophoretic results showed that O_{211}, O_{213} and O_{233} are homogen. It is necessary to notify that presence of single proteinic band on gel is necessary to proof homogenecity but is not enough. Because neurotoxins purified from a venom may have similar appearance locations and approval of final purity of toxins and the determination of their exact molecular weight needs amino acid analysis in further researches. Usually, toxins with high activities are found in the venoms of Buthidae family which are effective on ionic channels ie Na, K, Cl channels in different tissues. This study showed different toxic fractions of *O. bidentatus* venom which are specifically active on mammals, thus serving as important pharmaceutical tools for excitability and sodium channel structure and serve as specific targets for many neurotoxins. These toxins occupy different receptor sites and have been used as tools for functional mapping and characterization of the channels. Scorpion toxins affecting inactivation of sodium current may be divided on different groups according to their mammal or insect activities. The first group comprise highest activity on mammals sodium channels and lowest affinity for insect neuronal membrane. Second group are very weakly active on insects but express its toxic activity mainly on mammals. Third group are active on both insects and mammals, fourth group which oppose low affinity to mammals and high affinity for insects. Clarification of these different selectively in action of toxins requires three dimensional structural knowledge of the toxins coupled with molecular localization of amino acids directly interacting with recognition points which is suggested to be important areas of future studies.

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