Identification and Purification of two mammalian nourotoxins from Iranian Scorpion *(Buthotus schach)* venom

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ABSTRACT

Scorpion venoms contain of variety of peptides toxic to mammals، insects and crustaceans. Toxic peptides are the main factors in scorpion venom causing toxicity, (their amount being 1-3%of total venom.). Most of the scorpion toxins have been isolated from the venoms of scorpions in the family Buthidae. The scorpion *Buthotus Schach* belonging to the Buthidae family is widely found in the western region of Iran, but no published articles has been found to date on its venoms. Therefore in this study, we aimed to isolate and purify mammalian toxins from the venom of the scorpion *Buthotus Schach* present in Iran. For this study the crude venom was dialyzed against deionized water for 48 hrs., and centrifuged in order to separate soluble proteins from the insoluble mucoproteins and the soluble proteins was applied on a sephadex G-50 gel filtration. The toxicity of each fraction was determined by I.V injection to mice and toxic fractions were further purified by two steps ion-exchange (anion) and RP-HPLC chromatography. The purity of the final toxic protein fractions was checked and confirmed by RP-HPLC column & SDS-PAGE. Finally two neurotoxins, termed BS311 and BS313 were purified. Results in this study showed that the LD50 of crude venom on mice is 84µg/mice and contain at least 20 peptides from high molecular weight to low molecular weight out of which two of the peptides which showed toxicity to mice were isolated and purified. LD50 of these toxins were determined to be 3 and 2.17µg/mice respectively. The molecular weight of the purified toxins BS311 and BS313 were 7860and 7600 Da, respectively, as determined by SDS-PAGE. In conclusion this study showed that the main factor in the toxicity of scorpion (*Buthotus Schach*) venom is low molecular weight peptides.

Keywords: Scorpion, Venom, Purification, Isolation, Toxin, *Buthotus Schach*

INTRODUCTION

Scorpion venoms contain a variety of peptides toxic to mammals, insects and crustaceans (Loret & Hammock 2001, Zlotkin 2005). These toxic peptides can interact with ion channels with high affinity and selectivity. These biological features make scorpion toxins useful tools for probing the structures of different ion channels and evaluating their physiological contribution to cell and organ behavior (Massensini *et al* 2002, Wanke & Restano-Cassulini 2007). Scorpion venoms cause significant morbidity and mortality in many parts of the world

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(Rajarajesward *et al* 1979). Previously, the majority of chemical and pharmacological studies of scorpion venoms were performed primarily for medical importance as poisons (Lazarovici *et al* 1982). In addition, analyzing the relationships between the biological activity and three-dimensional structure of scorpion toxins may provide useful information for the design of novel insecticides and therapeutics (Menez, 1998; Zlotkin *et al* 2000). Scorpion toxins can be divided into two groups according to their molecular size. One is the long chain peptide group with 60–80 residues cross linked by three or four disulfide bridges (Rodriguez de la Vega & Possani, 2005), and the other is the short-chain peptide group with 20–40 residues cross-linked by two to four disulfide bridges(Rodriguez de la Vega & Possani, 2004). The majority of the long- and short-chain peptides are specific for voltage-dependent Na+ and K+ channels, respectively (Miyashita *et al* 2007). To date, about 400 toxic peptides have been identified in scorpion venoms, mainly from those of the Buthidae family (Tan *et al* 2006). Currently, the studies are motivated by the ability of scorpion venoms toserve as pharmacological tools for the excitation of biological systems (Catterall 1980). The main focus has been placed upon the Buthidae family because some species within this family possess particularly potent venoms that can be harmful to humans. The scorpion *Buthotus Schach* belonging to the Buthidae family is widely found in the western region of Iran, but no studies have been carried out to date on its venoms. Here, we report the purification and characterization of two mammalian toxins from the venom of the scorpion *Buthotus Schach.*

MATERIALS AND METHODS

Venom. Lyophilized crude venom was obtained from the Department of venomous animals and antivenom production, Razi Vaccine and Serum research Institute, Karaj, Iran. Crude venom (200 mg) was dissolved in 10 ml deionized water. The venom solution was dialyzed against deionized water for 48 hrs. Then the venom solution was centrifuged at 14000×g for 17 min to remove the insoluble material. The supernatant containing the solubilized venom was used for the study.

Gel filtration, ion exchange and reversed phase HPLC chromatography. Solubilized crude venom was applied to a 1.6 × 150 cm column containing sephadex G-50 equilibrated with a 0.1 M ammonium acetate buffer (pH 7.4). The flow rate was held at 60 ml/hr. Eight milliliter of solubilized venom (135 mg) was applied to the column and the eluted material was collected in 10 ml fractions. The optical absorbance of the eluent was measured at 280 nm. Fractions were separated and toxicity of each fraction was determined by I.V injection to mice. Fraction displaying biological activity was pooled and dialyzed against deionized water after concentrated using poly ethylen glycol (PEG).Concentrated biologically active fractions was loaded on to a 2.5 × 50 cm column containing DEAE-Sepharose (Pharmacia Biotech, Piscataway, NJ) anion exchange resin column equilibrated with 20 mM Tris buffer (pH 8.2) at. Fractions were eluted and collected with a linear gradient of NaCl from 0.0 to 0.5 M. The flow rate was 30ml/h and 5 ml fractions were collected. Four absorbance peaks were observed and toxicity was determined as mentioned earlier. Fractions displaying biological activity were pooled and dialyzed against deionized water and concentrated using poly ethylen glycol (PEG). Fractions showing toxicity were applied on a C18 RP-HPLC column which was equilibrated with solvent A (H2O, 0.1% trifluoroacetic acid), and eluted with a concentration gradient of solvent B (acetonitrile, 0.1% trifluoroacetic acid) from 0 to 100%, at a flow rate of 0.5 mL/min during 60 min. The peaks were monitored through the A280. Each RP- HPLC peak was collected individually and

lyophilized and each individual fractions were tested for toxic activity against mice.

SDS-Polyacrylamide gel electrophoresis (SDS-PAGE). SDS-PAGE was performed using the following protocol: acrylamide (30%) and Bis acrylamide (0.8%) and potassium acetate were polymerized by ammonium sulfate and N, N, N', N'-tetra methyl ethylene diamine (TEMED). A 5% stacking gel was applied above a 15% acrylamide resolving gel. The electrode buffer was potassium acetate (pH 4.3). Slab gels were stained in 0.25% Coomasie blue for 40 min and destained in 10% acetic acid and 30% methanol (Laemmli 1970, Schagger, von Jagow 1987).

Determination of molecular mass. 15% SDS-PAGE was performed according to Laemmli (1970) and using molecular mass standard of low molecular mass ranging from 6.5 KDa to 45 KDa (Laemmli 1970, Schagger, von Jagow, 1987).

Measurement of lethal toxicity (LD50). The toxicity of crude venom and Fractions were assayed using albino mice (20.0 ± 2.0 g). Toxins were administered to mice by intra ventricular (i.v) injection. Method used for LD50 was based on Sperman & Karber method (1948).

Purity analysis. Purity analysis carried out with HPLC on C18 column as previously described and SDS-PAGE.

Protein determination. Protein concentration was measured by the method of Bradford *et al* (1976) using Bovine Serum Albumin (BSA) as a standard.

RESULTS

Fractionation of scorpion venom. Venom from the scorpion *Buthotus schach* was 200 mg before removing the mucoproteins. However after dialysis and centrifugation of sample, the amount of protein was reduced to 135mg. Figure 1 shows the results of adding 8 ml of solubilized venom (135 mg) to the column sephadex G-50. Six absorbance peaks were obtained (BS1 to BS6). When all the fractions were

tested on mice for Toxicity, it was found that fraction BS3 showing toxicity to mice. The LD50 of crude venom was 84µg/mice and the yield after dialysis was 135mg that is 67.5% of crude venom (Table1).

Table 1. Protein content, LD50 and yield of toxic fractions of scorpion (*B. Schach*) *venom.*

Step	Protein(mg)	LD50(µg/mice)	Yield (%)
Venom	135	84	67.5
BS3	47.6	49.2	35.3
BS31	23	19.8	17.1
BS311	0.72	3	0.53
BS313	1.52	2.17	1.12

The amount of protein in toxic fraction BS3 was estimated to be 47.6 mg which the yield was calculated to be 35.3% of 135 mg protein before fractionation. The LD50 of this fraction was estimated to be 49.2 µg/mice. Further purification was carried out by ion exchange chromatography on DEAE-Sepharose resin. Figure 2 shows the results of adding concentrated toxic fraction BS3 from the Sephadex G-50 column to an anion exchange column (DEAE sepharos). Four absorbance peaks were observed (BS31 to BS34). The fraction BS31 showed toxic activity to mice. The LD50 of BS31 fraction was found to be 19.8 µg/mice and the yield was calculated and found to be 17.1% which account for 23mg protein. The toxic fraction BS31 was pooled, dialyzed and applied to a reverse-phase HPLC C18 column. Five peaks (BS311 to BS315) were obtained (Figure 3), Fractions BS311 and BS313 were Toxic to mice. The amount of protein of purified toxins BS311 and BS313 were estimated to be 0.72 and 1.52 mg respectively with the yields of 0.53% and 1.12% of 135 mg protein respectively. The LD50 of these toxins were estimated to be 3 and 2.17µg/mice respectively (table 1).

Purity and Determination of molecular mass.
The homogeneity of purified toxins was confirmed by SDS-PAGE and HPLC, as shown in Figure 4, 5 and 6 respectively. Isolated BS311 and BS313 showed high purity as analyzed by C18 reverse phase HPLC (Figures 4 & 5). The chromatographic profile of BS311 and BS313 samples at 280nm does not show the contaminating peptide. This toxins showed a single band in SDS–PAGE too (Figure 6). The molecular mass of this enzyme was estimated to be about 7860and 7600 Da respectively under reduced condition.

DISCUSSION

Venomous animals produce a variety of toxins of which only a tiny minority has been characterized to date. Because of generally high activity and high specificity of animal toxins, they are useful for study of highly complex biological mechanisms. Scorpion venoms are indispensable for studying the ion-channel functions. Over 400 scorpion toxins have been reported to date, but most of the insecticidal toxins isolated from scorpion venoms are long chain toxins with 6–8 kDa molecular masses and act on Na+ channels (Zlotkin *et al* 2005). The venoms from scorpions belonging to Buthidae family are well characterized as peptide toxins (Zlotkin *et al* 2005, Wudayagiri *et al* 2001, Inceoglu *et al* 2001, 2002, 2005, 2003). Buthoid venoms have been reported to affect a wide variety of vertebrate and invertebrate organisms (Zlotkin *et al* 2005) and their toxicity is attributed to the presence of a variety of polypeptides cross-linked by three to four disulfide bridges (Zlotkin *et al* 1978, Rochat *et al* 2004). In this study, the yield of solubilized venom was 67.5% of crude venom. One the important part of scorpion venom is mucoprotein which is non toxic to mammalians. It seems that scorpion (*B. Schach*) venom extraction by electric shock causing the release of mucoproteins in the venom and the amount of mucoproteins was about 32.5%. The amount of mucoproteins in the venom of different scorpions varied by different methods of venom milking.
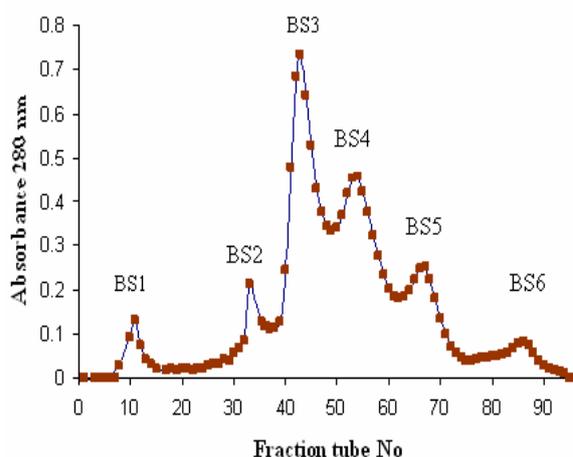
Figure 1. Sephadex G-50 chromatography of Iranian *Buthotus Schach* scorpion. Crude venom (135 mg) was applied to Sephadex G-50 column (3 × 150 cm) using buffer ammonium acetate 0.1 M and PH 7.5. Flow rate was 60 ml/hr and 9 ml fractions per tube were collected.

We isolated, for the first time, toxins from the venom of the Iranian scorpion *B. schach*, termed BS311 and BS313. These neurotoxins were isolated and purified by a combination of gel filtration on Sephadex G-50 (Figure 1), ion-exchange chromatography on DEAE-Sepharose (Figure 2) and HPLC on C18 column (Figure 3). As toxins BS311 & BS313 have a net positive charge, it was submitted to anion exchange purification. The toxin did not bind onto the column and got out on flow-through (Figure 2), while contaminant proteins did bind onto the column. This indicated that isoelectric point of these toxins, were low. Retention time of toxins BS311 and BS313 in HPLC were 8 and 16 min respectively. The yield of purified toxin BS311 and BS313 were found to be 0.53% and 1.12% which is in accordance with other studies (Darbon, *et al* 1983).
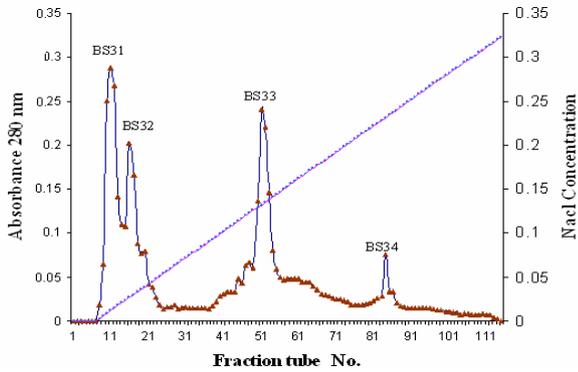
Figure2. DEAE-Sepharose chromatography of BS3 obtained from Sephadex G-50. The pooled fractions from Figure 1 were applied to DEAE-Sepharose column (2.5 × 20 cm) equilibrated with 20 mM Tris±HCl buffer (pH 8.2). Proteins were eluted with a linear concentration gradient of NaCl from 0 to 0.5 M, and 5 ml fractions per tube were collected.
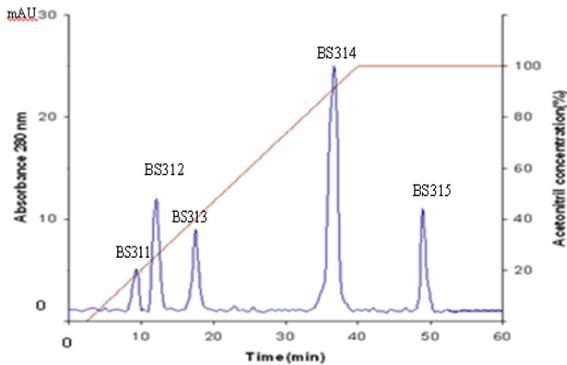
Figure 3. HPLC chromatography of BS31 obtained from DEAE-Sepharose chromatography. BS31 was applied on an HPLC C18 column which was equilibrated with solvent A (H2O, 0.1% TFA), and eluted with a concentration gradient of solvent B (acetonitrile, 0.1% TFA) from 0 to 100%, at a flow rate of 0.5 ml/min during 60 min.
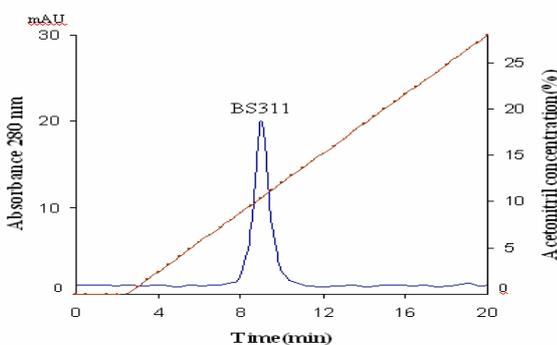
Figure 4. Purity assay of BS311 by HPLC
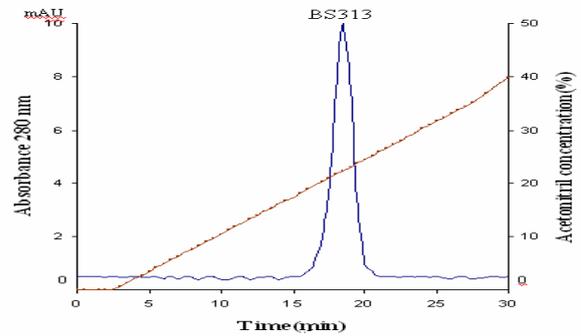
Figure 5. Purity assay of BS313 by HPLC
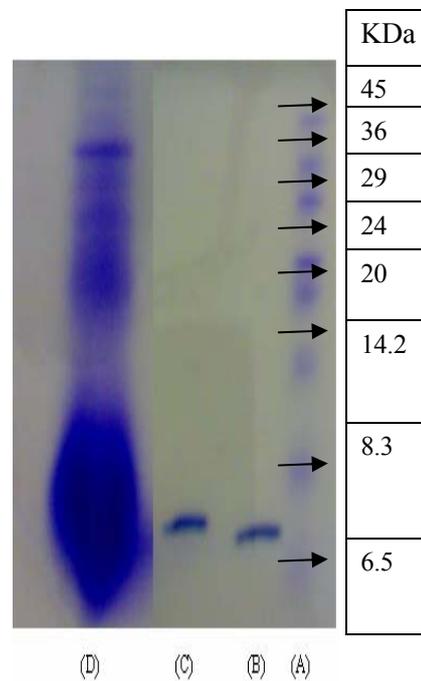
Figure 5. SDS-PAGE of M.W (A), BS311 (B), BS313 (C) and crude venom(D) was analyzed on SDS-polyacrylamide gel in the presence of 1% 2-mercaptoethanol

The homogeneity of purified toxins can be determined by HPLC and SDS-PAGE which showed almost homogeneity. However to confirm the 100% homogeneity of purified toxins, it requires the method of mass spectroscopy. A few number of studies reported that the difficulties of standardizing the venom quality and LD50 determination are depend on various factors (Krifi *et al* 2006, Theakston *et al* 2003). In our study we used LD50 mouse lethality assay. LD50 of crude venom and toxins (BS311 & BS313) of *B.Schach* scorpion were

found to be 84, 3 and 2.17µg/20 g mouse respectively. This indicates about 39 folds purification of toxins. Since scorpion's toxins are resistant to denaturizing at room temperature, we carried out the whole process at room temperature (Jover *et al* 1980). Molecular mass ranging toxins of Buthidae family scorpions were 5000 to 10,000 Da (Pimenta *et al* 2001, Batista *et al* 2006, Caliskan *et al* 2006, Favreau *et al* 2006). Molecular mass determination of these neurotoxins performed by SDS-PAGE was about 7860and 7600 Da. for toxins BS311 and BS313 respectively.

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