Purification of Scorpion Venom Protein Components by Reverse Phase High Performance Liquid Chromatography.

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Summary: Reverse phase high performance liquid chromatography has been employed for the separation of proteins from the venom of scorpion Buthus sindicus. The separated proteins consist of three neurotoxins, a toxin and a 14 kDa cysteine/½cystine rich protein. These proteins have been characterized by SDS-PAGE, amino acid composition and N-terminal amino acid sequence analysis.

Introduction

Buthus sindicus is a pale yellow scorpion, belongs to the group Buthoids [1] and is commonly found in the Sind Province of Pakistan.

Scorpion venom, particularly from the Buthoids, has been the subject of extensive chemical and pharmacological investigations in recent years [2]. The venom is a mixture of polypeptides, each having 36-70 amino acid residues with cross linked disulphide bridges [3-6]. These toxic polypeptides have been demonstrated to interfere with the basic events in biological membranes [7-10].

Several methods have been employed for the purification of these proteins for the structural studies. These include combination of conventional gel filtration and ion exchange chromatography [11].

In our present study on venom proteins from Buthus sindicus, we have employed reverse phase HPLC (RP-HPLC) using Vydac C-18 column for separation. The proteins were checked for their purity using gel electrophoresis, N-terminal sequence analysis and amino acid composition. This separation has resulted in the isolation and partial characterization of three neurotoxins, a toxin and a 14 kDa cysteine/½cystine rich protein not yet reported from scorpion venom proteins.

Materials and Methods

Scorpions were collected from the Karachi region and venom was obtained by electric stimulation [12] in deionized water and centrifuged at 6000 rpm for 30 minutes, at 10°C. The supernatant was lyophilized and stored at -20°C for further use.

RP-HPLC of Scorpion Venom Proteins:

The lyophilized venom was separated on a Vydac C-18 column (Phenomenex, New York, U.S.A.), crude venom (5 mg) was dissolved in 0.1% TFA and eluted with a linear gradient of 0.1% TFA (A) and acetonitrile containing 0.1% TFA (B).

Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE):

The fractions from RP-HPLC were checked for purity by SDS (PAGE) in 15 % gels according to the described

procedure [13]. The standards used for molecular mass determination were obtained from FLUKA.

Amino Acid Composition:

The proteins were hydrolyzed under vacuum with 5.7N HCl 0.5% phenol for 20 h. Samples were analyzed on Biotronik LC 6001 amino acid analyzer (Biotronik GmbH, West Germany).

N-terminal Sequence Analysis:

The N-terminal sequence of the proteins/peptides isolated by RP-HPLC was determined by 4-(dimethylamino) azobenzene 4'-isothiocyanate) DABITC method [14].

Results

The separation profile of scorpion venom by RP-HPLC is shown in Fig.1. Five major and several minor peaks were observed. At present only the major peaks have been studied.

A single band on SDS-PAGE was observed for peak 2, 3, 4, 5 and 6 (Fig.1). A band corresponding to 3.5 kDa was obtained for peak 2, peaks 4, 5, 6 gave single bands in the molecular range of 6-7 kDa whereas peak 3 gave a single band in the range of 14 kDa (Fig.2). The amino acid compositions of some peaks from RP-HPLC are shown in table 1.

The N-terminal sequence of proteins analyzed are shown in table-2.

Discussion

The peaks obtained from RP-Vydac C-18 column were analyzed by SDS-PAGE, amino acid composition and N-terminal sequence analysis. The SDS-PAGE of various peaks gave bands in the range of 3.5-6.6 kDa which is typical for scorpion neurotoxins.

The amino acid composition of peaks 5 & 6 (Table 1) resembles with that of neurotoxins characterized from other scorpion species [15-17]. Their N-terminal sequence also show identities to characterized scorpion venom proteins (Table 2). (These two

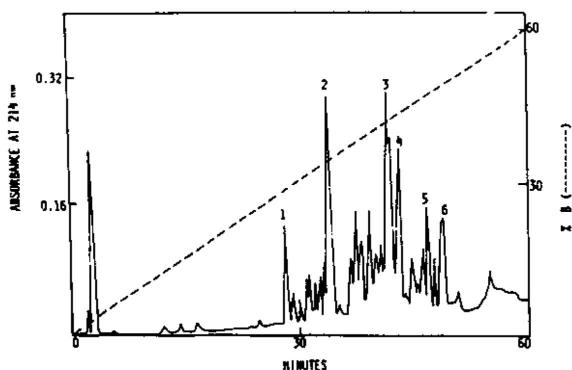

Fig-1: Separation of scorpion venom proteins on a reverse phase Vydac C-18 column. The sample was eluted in 0.1% TFA with a linear gradient of 0.1% TFA in acetonitrile. Fractions were pooled as indicated by numbers and are mentioned as such in the text.

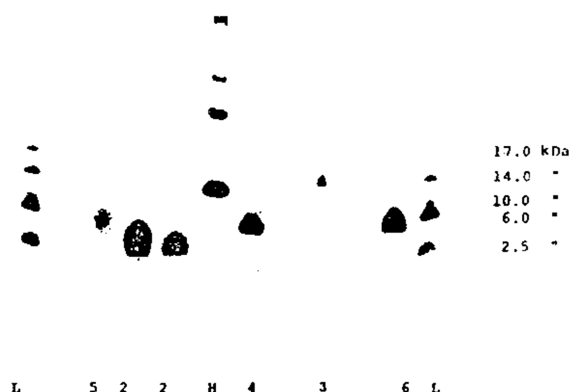

Fig-2: SDS-PAGE of peaks from reverse-phase HPLC of scorpion venom on 15% gels. (L) represents low molecular mass standard, (H) represents high molecular mass standard and numbers represent peptides from Fig.1. The position for each standard is mentioned in the Figure.

Table-1

Amino Acid	Peak 2	Peak 3	Peak 5	Peak 6	Neurotoxin of Be [17]
Asp	7.80	10.54	13.36	12.46	13.8
Thr	7.71	7.48	4.71	5.51	-
Ser	4.17	3.14	5.27	4.07	3.07
Glu	8.42	8.52	5.03	4.75	7.69
Pro	9.35	4.97	4.38	3.78	3.07
Gly	17.78	14.37	11.01	17.5	10.7
Ala	4.87	4.39	3.94	3.52	9.23
Cys	11.98	10.43	n.d(8)	n.d(8)	12.3
Val	-	2.52	3.89	-	4.61
Met	2.49	0.22	-	-	-
Ile	-	4.0	4.51	3.71	3.07
Leu	3.46	3.62	5.49	5.56	1.53
Tyr	3.56	4.21	6.72	7.57	9.23
Phe	3.21	4.25	2.80	2.11	1.53
His	4.33	3.41	9.65	9.37	-
Trp	n.d	n.d	n.d(1)	n.d(1)	4.61
Lys	6.17	8.58	8.99	6.43	12.3
Arg	3.56	5.35	1.18	4.61	3.0
Molecular mass (kDa)					
On SDS PAGE	3.5	14.0	6.6	6.6	7.3

Be = Buthus eupeus.

Table-1: Amino acid composition of various proteins of scorpion venom after reverse phase HPLC purification. Values are given in mole % after hydrolysis. The values for tryptophan could not be determined due to destruction after acid hydrolysis, values for cysteine were determined without modification. Values for cysteine and tryptophan in peak 5 and 6 mentioned in parentheses are based on comparison with characterized neurotoxin from Buthus eupeus [17].

proteins are identified as neurotoxins). The amino acid analyses show certain differences in their composition, which provide an explanation to the difference in their elution time on reverse phase column.

Characterization of major peaks also resulted in identification of a short peptide having molecular mass of about 3.5 kDa, (peak 2 fig.2). This protein is identified to be related to a neurotoxin on the basis of its N-terminal sequence analysis [18]. The complete structural elucidation of this protein is underway, which would explain the species variation in this protein.

Peak.4 (fig.1) was identified as toxin on N-terminal sequence comparison (table 2) with a reported toxin sequence [19]. The molecular mass is also similar to short neurotoxins (Fig.2), therefore this protein is characterized as neurotoxin.

Table-2

Peak in Fig.1	N-terminal sequence									
	1	2	3	4	5	6	7	8	9	10
Peak 2	Arg	Arg	Lys	Pro	Cys	Phe	Thr	Asp	Pro	
Peak 4	Glu	Lys	Asp	Gly	Tyr	Ala	Val	Val	Asp	Ser
Peak 5	Asp	Gly	Tyr	Ser	His	Asp				
Peak 6	Asp	Gly	Tyr	Ser	Asn	Asp				
Buthus eupeus [17]	Asp	Gly	Tyr	Ile	Ala	Asp	Asp	Lys	Asp	Cys

Table-2: N-terminal amino acid sequences of peaks after reverse-phase HPLC. The peaks 5 and 6 were identified by comparison of their N-terminal amino acid sequences with sequence of known toxin [17]. Peak numbers as in Fig.1.

A 14 kDa protein (peak.3, fig.2) was also isolated and found to be rich in cysteine/½ cystine residues, which is characteristic of neurotoxins. The protein however has different amino acid composition, which does not correspond to toxins of this molecular mass or any other characterized protein from venom. Structural characterization of this protein is in progress and would explain the nature of this protein and its relationship to characterized venom proteins.

In conclusion the separation of various protein components of scorpion venom has been achieved by RP-HPLC. The separation of venom protein by this method is helpful in purification and identification of almost all the major venom proteins which have previously been separated by lengthy chromatographic procedures.

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