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Research paper

2D gel electrophoresis and HPLC separation profile of *Echis carinatus sochureki* snake venom

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Abstract

Snakebite envenomation is a neglected tropical disease. In Pakistan, the venomous snakes responsible for high morbidity particularly in rural areas includes *Echis carinatus*, *Naja naja*, *Daboia russelii*, and *Bungarus* species. Our study presents HPLC and gel-based de-complexation of proteins and peptides from the venom of *Echis carinatus sochureki*, commonly called as saw-scaled viper. The combined analysis showed the presence of proteins in the range of 95-22 KDa as a major portion while the high and low molecular mass proteins were found to be in less abundance. The 2D gel showed the presence of acidic to basic range proteins. The 2D gel also reveals that the low molecular mass proteins are mostly at the acidic side.

Keywords: 2D gel, Echis, Venom, Venom proteins

Introduction

Envenomation by snakebite has a burden of about 2.7 million people around the world. The highest burden of bites exists in Asia, Africa, and the Middle East [1]. In Pakistan, there are 27 species of venomous snakes identified in different parts of the country including the coastal areas, few of which are native to the Pakistani region [2]. The snakebite envenomation (SBE) cases from in Pakistan exceeds 40,000 bites per year [3]. Snake venom is a modified saliva containing numerous proteins and peptides with fatal effects when injected into the prey. Phospholipases, Serine proteases, metalloproteases, hyaluronidases, and L-amino acid oxidases are main venom enzymes that induce cytotoxicity, neurotoxicity, hemorrhage, and other life-threatening symptoms in the victim. The process of recruitment involves tailoring by natural selection so that the modified end products (e.g. toxins) can target vital body systems of the prey [4-6]. Saw-scaled viper (*Echis carinatus sochureki*) belongs to the family *Viperidae*. The snake is commonly found in, Pakistan, northern India, Afghanistan, Iran, United Arab Emirates, and Oman [7].

Envenomation by *E. sochureki* results in severe bleeding, abdominal pain, headache, vomiting, and in some cases pancreatitis [8, 9]. The snake is considered to be very dangerous because of the aggressive and easily excitable nature. Studies conducted on the venom of *E. sochureki* reports the purification of phospholipases and toxins [10-11]. The assessment of the toxin composition of *E. sochureki* has been reported indirectly via venom gland transcriptomics [12]. The current study aims to explore venom proteome through proteomics centered approach to de-complex the venom proteins. Reversed phased HPLC (RP-HPLC) was performed utilizing the C18 column and fractions were collected which were subjected to 12.5% SDS PAGE analysis and stained with coomassie blue. Further analysis was conducted by 2D gel

electrophoresis of venom applied to isoelectric focusing followed by SDS-PAGE and staining procedure.

Method and Material

Sample collection

The snake specimens were collected from different districts of the province of Sindh, mainly from Thatta, Badin, Mirpur Khas, and Umerkot. The snakes were milked for venom followed by centrifugation for 30 minutes at 7,000 rpm at 4 °C and then by lyophilization. All samples were stored at -20 °C until further use.

HPLC and fractionation of proteins

The fractionation of the whole venom (1 mg) was carried out on a reverse-phase Nucleosil 300-5 C18 (250x4.6) column on HPLC. The RP-HPLC column was equilibrated with 0.1% TFA (tri-fluoro acetic acid) water and eluted with a linear gradient of acetonitrile containing 0.05% TFA from 5-70 % in 125 minutes. The flow rate was maintained at 1 mL/min and the effluent was monitored at 280 nm.

Characterization of fractionated proteins

The molecular mass pattern of proteins and peptides in each collected fraction was analyzed on SDS-PAGE with 12.5% gels. Samples under dissociating/reducing and denaturing conditions (with SDS, 2-mercaptoethanol/dithiothreitol, and 100 °C temperature) were loaded on the gels and run at a constant voltage of 70 volts. After the run, the gels were stained with coomassie blue for band visualization.

2D-gel electrophoresis and image analysis

For two-dimensional (2-D) gel electrophoresis, 80µg of venom was dissolved in rehydration buffer (8 M urea, 0.2 M EDTA, 0.5 M Dithiothreitol (DTT), glycerol, NP-40, ampholyte solution pH 3–10 in 0.5 M Tris–HCl) for passive rehydration of 7 cm IPG strip 3–10 overnight at room temperature. Proteins were focused on the IEF Multiphor II system (GE Healthcare) at 20 ˚C using a total of 7000 V/h. After IEF, IPG strips were equilibrated using equilibration buffer I (6 M Urea, 30 % Glycerol, 2 % SDS, 50 mM Tris–HCl pH 8.8, and 1 % DTT) and equilibration buffer II (6 M Urea, 30 % Glycerol, 2% SDS, 50 mM Tris–HCl pH 8.8, and 2.5 % Iodoacetamide). Following equilibration, the focused proteins were separated on 12.5 % SDS-PAGE. Gels were visualized after silver staining to detect proteins after electrophoretic separation on 12.5% polyacrylamide gels.

Results and Discussion

The crude venom of *E. sochureki* snake was subjected to reverse phase HPLC analysis. The venom was fractionated into more than 40 small to high-intensity peaks (Fig. 1). The fractions were collected and dried in a speed vac. The chromatogram showed well-separated peaks although the sample amount injected was approximately 1 mg. The peaks were collected manually, therefore, showing traces of bands from the consecutive previous peak. The dried fractions were re-dissolved in sample diluting buffer for SDS PAGE analysis (Fig. 1). Most of the collected fractions showed protein bands ranging from high to low molecular masses. Fractions 9-12 showed less number of bands with fraction 10 showing an intense band of more than 30 kDa relative to the molecular mass marker. Fractions from 23-35 showed the presence of most of the protein components eluting from the column. The rest of the fractions (36-44) showed bands in the range of 175-42 kDa with very low-intensity other bands.

The 2D gel also showed the presence of acidic to basic proteins and peptides focused and resolved on the IPG strip for pH gradient of 3 to 10. The gel showed the presence of more than 70 protein spots the majority of which corresponds to the same molecular mass range of 95-22 kDa as was observed in the SDS PAGE of HPLC fractions. The

presence of high to medium molecular mass polypeptide components is the characteristic of viper snake venoms and has been reported from other species [13, 14]. Overall gel-based and liquid chromatography based techniques were utilized to de-complex the snake venom components and it was observed that combining the techniques would reveal more data about the proteins and peptides present in the venom. Both the strategies are well suited for the separation of venom from *E. sochureki* and can be adopted for protein decomplexation.

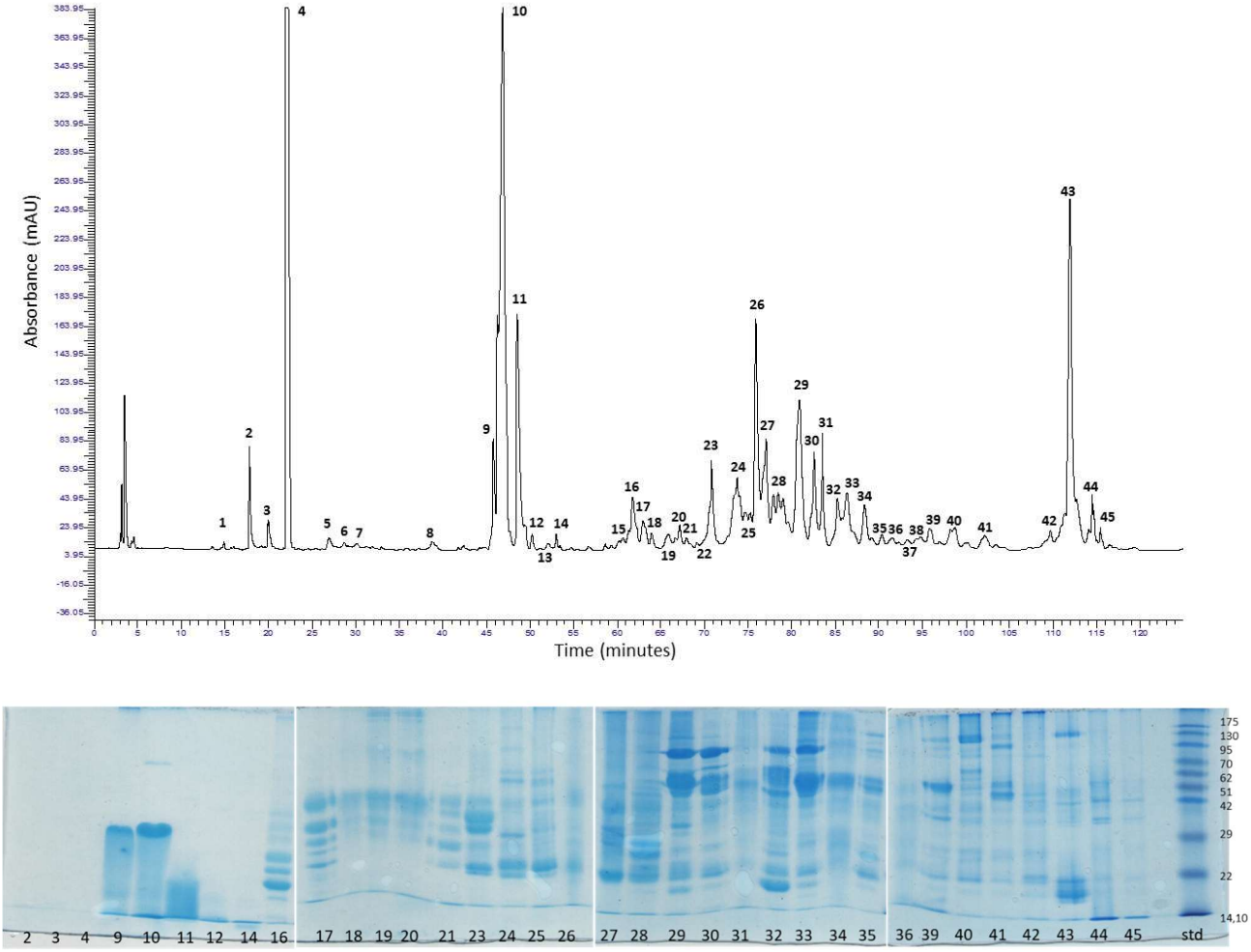

Figure 1. HPLC separation of venom of *E. sochureki*: Crude venom (1.0 mg) was loaded on to Nucleosil 300-5 C18 (250x4.6) RP-HPLC column equilibrated with 0.1% TFA water and eluted with a linear gradient of acetonitrile (containing 0.05% TFA) from 5-70 % in 125 minutes. The flow rate was maintained at 60 mL/hr and the effluent was monitored at 280 nm. Numbers indicate the collected fractions. 12.5% SDS PAGE gels were run at a constant voltage of 70 volts. Gels were visualized after coomassie staining to detect protein bands. The standard molecular weight ladder (std) was run as a reference with numbers indicating the fraction number of HPLC analysis.

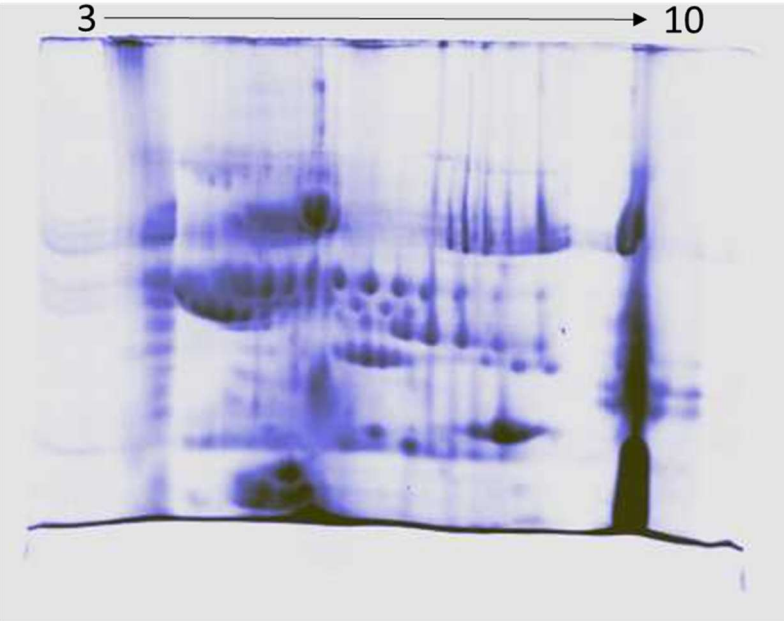

Figure 2. 2D gel of venom of *E. sochureki*: Crude venom (80 µg) was applied to an IPG strip (Bio-Rad) of pH 3-10 and iso-electric focusing was performed on IEF Multiphor II system (GE Healthcare) at 20 ˚C using a total of 7000 V/h. Following equilibration, the strip was run on 12.5 % SDS-PAGE at a constant voltage of 70 volts. Gels were visualized after coomassie staining to detect protein spots. Arrow indicates the IPG strip pH gradient.

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