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Research Article

Decomplexation of Venom Proteome of Pakistani Cobra (*Naja naja naja*)

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Abstract: The venom proteome of *Naja naja* from Sindh, Pakistan was decomplexed utilizing reverse phase HPLC and SDS PAGE. The results were compared with already reported *Naja naja* species of the region. The banding pattern represents all the major families of proteins including three-finger toxins, phospholipase A_2, snake venom metalloproteases, L-amino acid oxidases, phosphodiesterase, nucleotidases, cysteine-rich secretory proteins, serine proteases, nerve growth factor, cobra venom factor, acetylcholinesterases, Kunitz-type serine protease inhibitors and C-type lectin proteins. The decomplexation of the venom showed the best possible separation through RP-HPLC elution of venom components containing small peptides, small and large proteins based on hydrophobicity. The SDS PAGE under reducing and non-reducing conditions of HPLC fractions highlighted the presence of several proteins.

Keywords: *Naja naja*, Venom, Decomplexation, HPLC, SDS PAGE

1. INTRODUCTION

Snake venomics is a comparatively new field with tools and techniques related to understanding the mechanism of venom production, its composition and pathogenesis, and also because of its therapeutic value. Decomplexation of venom proteome is particularly important as venom is a mixture of numerous proteins and peptides or toxins with multiple functionalities [1].

Cobra (*Naja naja*) is one of the deadliest species of snake found in the Indian subcontinent. *Naja naja* (*N. naja*) belongs to the family Elapidae and is commonly known as Indian Cobra in this subcontinent. In Pakistan, the specie is distributed from Khyber Pakhtunkhwa, Sindh, and Punjab to a few areas of Baluchistan [2]. *N. naja* is part of the "Big four" group of snakes which include the four deadliest species of snakes responsible for high mortality and morbidity throughout the subcontinent [3]. Common symptoms followed by cobra bite are local swelling and tissue necrosis, difficulty in speaking and swallowing, paralysis, fixed dilated pupils and death due to respiratory failure [4].

The venom of *N. naja* has a variety of biologically active molecules divided into several classes and sub-classes. Enzymes, proteins, and small peptides are particularly important as these are the potential molecules responsible for venom pathophysiology. The main families of enzymes identified in the venom of *N. naja* are phospholipases A_2 (PLA_2), Snake venom metalloproteinases (SVMP), Snake venom serine proteases (SVSP), L-amino acid oxidases (LAAO), Endonucleases (END), Phosphodiesterases (PDEs) and Acetylcholinesterase (ACE). Three-finger peptides (3FTx), Cysteine-rich secretory proteins (CRISPs), Nerve growth factor (NGF), Cobra venom factor (CVF), and natriuretic peptides are non-enzymatic proteinous molecules with toxic effects. These have been found in the venom of *Naja* specie in several studies [5, 6]. Our study aimed to explore the venom proteome of the Pakistani cobra, *N. naja* from different geographical regions of Pakistan. Here the protein

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decomplexation technique including HPLC and polyacrylamide gel electrophoresis (SDS PAGE) was utilized to analyze the cobra venom collected from the province of Sindh. Separation profiles of the venom through HPLC and SDS-PAGE were compared based on respective estimated molecular masses of the protein bands and reported studies conducted on *N. naja* specie of the subcontinent. HPLC and acrylamide gel electrophoresis are the basic protein separation techniques that are most utilized and available resources in a basic protein separation facility. Our results demonstrate the complex nature of Pakistani cobra contributing towards understanding the genus *Naja* venom in South Asia specifically in the Indian subcontinent.

2. MATERIALS AND METHODS

2.1 Sample Collection

The venom was purchased from a snake charmer who collected snake specimens from different districts of the province of Sindh and brought them to the institute where snakes were milked for venom. The venom was centrifuged at 4 °C, 7,000 rpm for 30 minutes to remove any tissue material that may have come from the mouth of the snakes. The clear venom was lyophilized and kept at -20 °C until further use.

2.2 Chromatographic separation of venom

Reverse phase high-performance liquid chromatography (RP-HPLC) was utilized for protein separation of lyophilized venom. The venom sample (2 mg) was suspended in 200 uL of 0.1 % trifluoroacetic acid (TFA) water (solvent A) which is the initial buffer to start the sample run. The reverse phase column employed was a C-18 column with 250 x 10 mm column dimensions, 5 um particle size and 300 Å pore size. A linear gradient was run with 0.1 % TFA, acetonitrile (solvent B) 5 % to 70 % B in 120 minutes. Fractions were read at 280 nm and collected manually. After collection, the samples were desiccated and subjected to SDS PAGE analysis.

2.3 Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

Gel electrophoresis of crude venom was performed under reducing as well as non-reducing conditions SDS-PAGE gels of 12.5 % or 15 % were prepared using the standard protocol by Laemmli [7]. Samples were first dissolved in water and then diluted in 2x sample diluting buffer (SDB) which was modified (2 % SDS, 2 mM EDTA, 160 mM DTT, 20 % glycerol, 0.1mg/ml bromophenol blue in 20 mM tris HCl pH.6.8), and heated at 95 °C for 3 minutes. The sample diluting buffer for the loading of crude venom samples was modified as there were insolubility issues with the sample. Gels were run at constant a voltage of 65 or 70 volts for 2 hours. Gels were stained overnight using coomassie brilliant blue and de-stained with distilled water.

3. RESULTS AND DISCUSSION

3.1 Chromatographic separation of venom

The RP-HPLC program was run to fractionate the venom sample of *N. naja* and resolve the proteins and peptides into 22 fractions (Fig. 1). The fractions were eluted at different retention times with increasing concentrations of solvent B. The gels were run under reducing and non-reducing conditions of the collected fractions. The peaks collected until 25 minutes of the run did not show any bands on the gel (data not shown). It may be due to very small peptides or nucleosides [8]. However, the eluents after 30 minutes to 95 minutes of the run (peaks from 1 to 22) showed bands of proteins and peptides.

The chromatographic separation was followed with modifications as described by Tan *et al*. [1]. The HPLC program for the separation of venom proteins was scaled down from the original 180 minutes method to 120 minutes keeping the acetonitrile gradient in a similar shape with reading the eluted peaks at 280 nm. Lomonte and Calvete depicted a similar fractionation scheme of the venom [8]. Wong *et al*. have reported the decomplexation of *N. naja* venom of Pakistan and compared it with the Indian and Sri Lankan *N. naja* specie [9]. They have reported the variation in neurotoxin components which was found to be high in Pakistan *N. naja* as compared to other countries of the region. They performed decomplexation on HPLC with 180 minutes program and marked 33 peaks. The first ten peaks showed the presence of three-finger toxins or neurotoxins [9]. In our study, the HPLC run was

120 minutes and the number of peaks collected was 22 with high resolution. The collected peaks were analyzed in reducing as well as non-reducing SDS PAGE conditions. Another comparative study [10] conducted on the venom of *N. naja* and *Naja oxiana* from Pakistan utilizing mass spectrometric analysis also showed the presence of three-finger toxins (3FTx) but the percentage composition was found to be 21 % in contrast to the previous report of decomplexation where it was found to be more than 75 % [9]. This reflects the approach with which the venom analysis was performed and the region from where the snake specimen were collected. For example, the *N. naja* venom from South Punjab Pakistan showed the presence of three-finger toxins at 58 % [10] as compared to the northern side [5]. The decomplexation strategy however presents a much more detailed and comprehensive report of proteoforms [9]. Another study that compared the Indian and Sri Lankan *N. naja* applied the same decomplexation approach and identified the presence of three-finger toxins as 74 % and 80 % respectively. The specimen of *N. naja* included in this study were collected from the Rajasthan and Gujrat, regions of India. This area is close to the province of Sindh in Pakistan. The HPLC chromatogram of this study showed a different elution pattern with 160 minutes of program and 18 peaks [10]. However, the percentage composition of 3FTx in this study is similar to the *N. naja* venom content reported from Pakistan [9]. We report here the best possible HPLC separation of *N. naja* venom from the province of Sindh, Pakistan as compared to previous studies.

3.2 Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS PAGE) of venom

Crude venom was subjected to SDS PAGE under reducing and non-reducing conditions. Both gels showed protein bands ranging from as high as ~120 kDa to as low as ~6.5 kDa of molecular mass. Three individual venom samples collected from three specimens were run side by side, presenting a similar pattern of bands in both conditions (Fig. 2). The gel under reducing conditions of whole venom was compared with gels reported from India and Sri Lanka under the same condition of SDS PAGE.

The SDS PAGE results of whole *N. naja* venom was compared to the results reported by Vanuopadath *et al*. 2022 [11]. They described the proteome of *N. naja* species from Kerala, India [11]. They have identified the bands obtained from

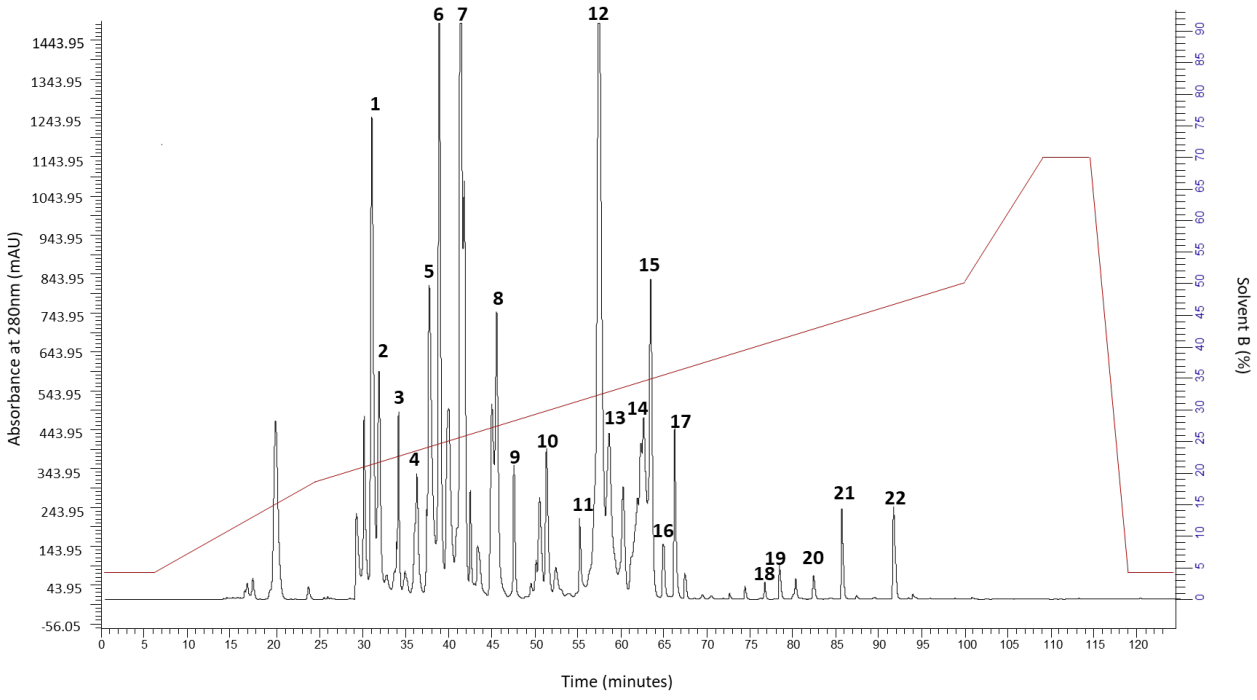

Fig. 1. HPLC chromatogram of cobra (*Naja naja*) venom on C18 column. The numbers represent the collected peaks.

similar SDS PAGE procedures performed under reducing conditions. Our gel results represent an almost similar band pattern (Fig. 3).

Based on the band comparison the expected identification of proteins could provide an idea of the groups of proteins present in each band marked in figure 3 (A). To elaborate on such a scenario, band 1 might represent snake venom metalloprotease (SVMP), cobra venom factor (CVF), and phosphodiesterases (PDE). Band 2-4 represents acetylcholinesterase (ACE), 5'-nucleotidase (5-ND), snake venom serine protease (SVSP), Phospholipase B (PLB) and L-amino acid oxidase (LAAO) in addition to SVMP and CVF. Band 5 may include endonuclease (END) in addition to SVMP, CVF and LAAO. Similarly bands 7 and 8 might comprise of Ohanin/vespryn family proteins (OLP), cysteine-rich secretory protein (CRISP), glutathione peroxidase (GPrx), phospholipase A_2 (PLA_2), C type lectin (CTL) and nerve growth factor (NGF). Band 9 symbolizes three-finger toxins (3FTx) in addition to OLP and PLA_2 whereas, band 10 could include Kunitz-type serine protease inhibitor (KSPI) with 3FTx, PLA_2, and OLP.

Fractions collected from HPLC were subjected to SDS PAGE to determine the protein band pattern. Multiple bands were found in each collected fraction on gel under both reducing and non-reducing conditions. The banding pattern obtained under reducing SDS PAGE conditions were compared with the already reported gels of *N. naja* venom [9-11]. Fractions 3, 4 and 5 mainly showed low molecular mass proteins (~7 kDa). Chromatographic peaks from 6 to 13 presented bands in the range of molecular masses of ~85 to 6.5 kDa. The rest of the peaks showed the presence of high molecular weight proteins (~140 to 55 kDa). Over all more than 40 bands were observed in reducing SDS PAGE gels of the HPLC collected fractions. Hashmi *et al*. reported the *N. naja* venom decomplexation utilizing the 180 minutes' acetonitrile gradient program and analyzed the collected fractions on 12.5 % SDS PAGE gel [12]. In our study, we analyzed the first 9 fractions on 15 % gels and the rest of the fractions on 12.5 % SDS PAGE. We adopted the strategy based on the literature review where it was observed that the 3FTx are the main eluent in the initial phase of the HPLC program. The analysis of fractions under non-reducing conditions was performed to have an idea about the fractions with proteins consisting of more than one subunit. Fractions 6 and 10 showed the presence of such proteins (Fig. 4). Most of the literature report only reducing SDS PAGE gels of the collected venom fractions. However non-reducing SDS PAGE could provide functional

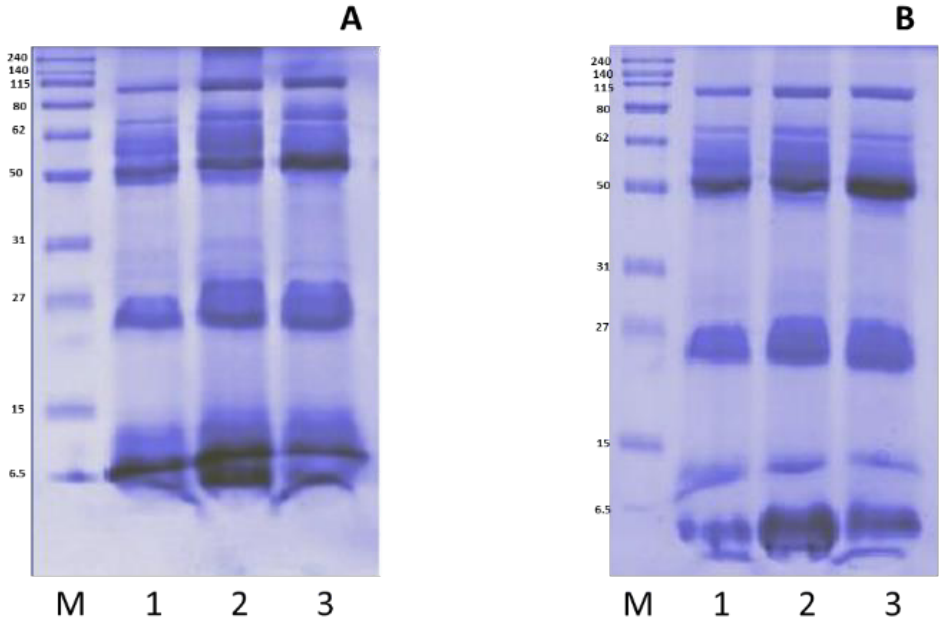

Fig. 2. 12 % SDS PAGE under non-reducing (A) and reducing (B) conditions. 'M' represents the molecular weight marker with respective bands in kDa in lane 1. From lane 2-4 the number represent venom samples of three individual specimens of *Naja naja*.

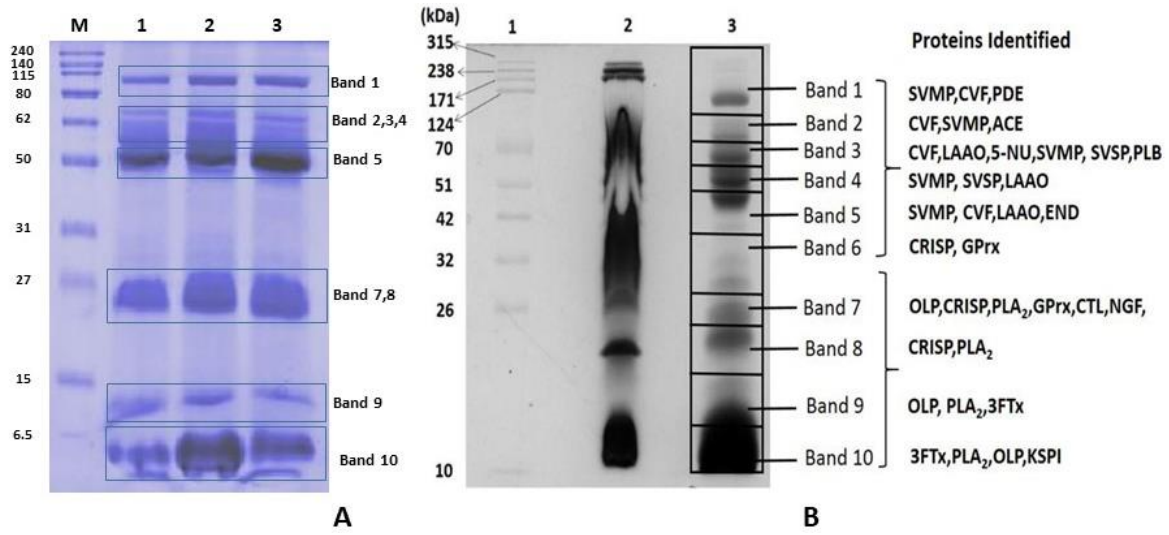

Fig. 3. 12.5 % SDS PAGE under reducing conditions (A) where bands are marked in boxes in comparison to the Vanuopadath *et. al*. 2022 [11]. 'M' represents the molecular weight marker with respective molecular mass (kDa) in lane 1. From lane 2-4 the number represent venom samples of three individual specimens of *Naja naja* venom. Gel image opted from Vanuopadath *et al*. 2022 [11] (B) representing molecular weight marker in lane 1, whole venom of N. naja under non-reducing conditions in lane 2 and in reducing conditions in lane 3. The proteins identified in each band are mentioned on the right side of the image.

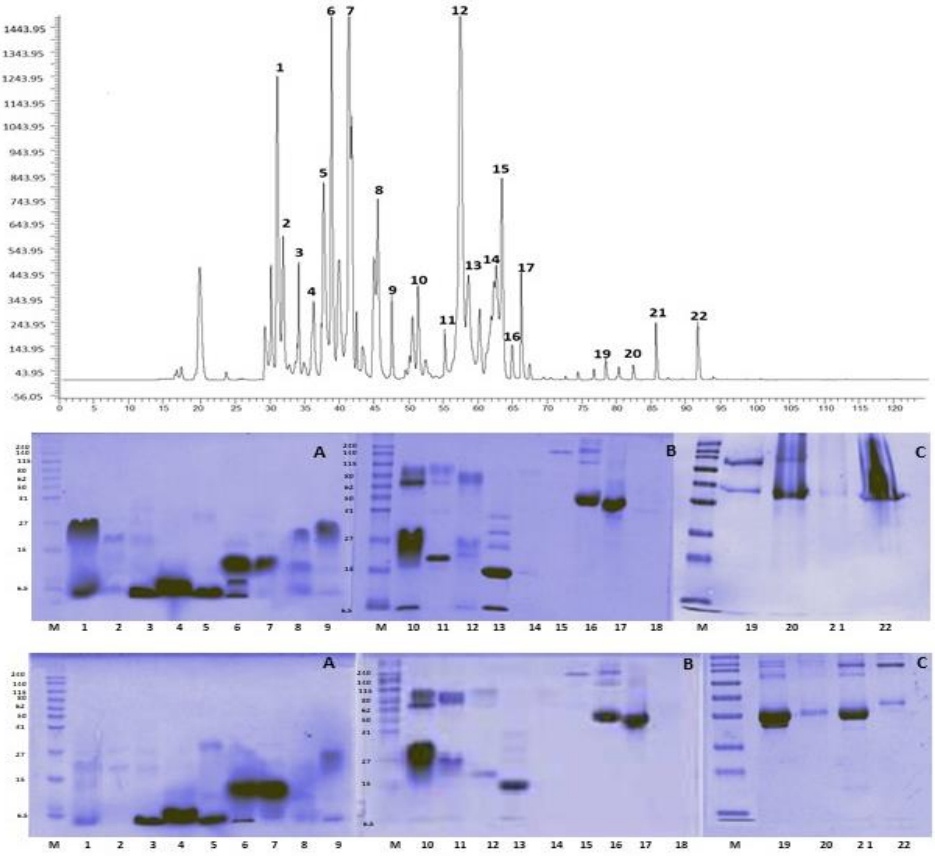

Fig. 4. Biochemical characterization of *N. naja* venom. Upper panel RP-HPLC chromatogram of the venom with collected fractions (1-22). Middle panel, reducing SDS PAGE gels and lower panel non-reducing SDS PAGE of the fractions. 15 % gel (A), and 12% gels (B and C) marked by fraction or peak numbers at the bottom. 'M' represents the molecular weight marker with respective molecular weights (kDa).

information as the non-denaturing conditions signify the intact interactions of proteins that are necessary for biological activities [12].

The separation pattern of the *N. naja* venom showed the generalized elution of proteins and peptides. A linear acetonitrile gradient starting from 5 % and reaching up to 50 % elutes small peptides, followed by small proteins, medium-sized proteins and large or most hydrophobic proteins in the sequence of the HPLC program [8]. The pattern of HPLC separation on reversed phase C18 column is almost similar in most studies reporting the decomplexation of cobra venom even with unique findings. For example, venom from a cobra species *Naja senegalensis* showed an absence of the Phospholipase A_2 component and high levels of 3FTx (~76 %). The specie belongs to the Western Africa subgenus Uraeus of genus *Naja* highlighting insights that could be used for the availability of region-specific antivenom and reducing envenomation-related deaths [13]. The decomplexation studies of snake venom target the ultimate goal of anti-venom development. The region-specific antivenom against a specific specie of snake, such as cobra should include all the members of that genus present in a particular region. This strategy would support the development of a remedy or anti-venom that would provide an effective treatment against envenomation. Our data and similar work find its contribution attention to understanding the complexity of *N. naja* venom in South Asia specifically in the Indian subcontinent.

4. CONCLUSION

The decomplexation of *Naja naja* venom presented all the major proteins in the venom reported from the *N. naja* species in the Indian subcontinent including different regions of Pakistan, North India, South India and Sri Lanka. We report the best possible HPLC separation of *Naja naja* venom from the province of Sindh, Pakistan as compared to previous studies. Fractions 6 and 10 showed the presence of proteins with more than one subunit as demonstrated by SDS PAGE results under non-reducing conditions. Our data highlights the complexity of the Pakistani cobra species *Naja naja naja*.

5. CONFLICT OF INTEREST

The authors declared no conflict of interest.

6. DECLARATION

The author declared no conflict of interest.

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