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Purification and Antibacterial Activity of Indian Cobra and Viper Venoms

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Abstract

Snake venom contains a variety of chemicals including pharmacological and toxicological properties. Venom proteins that are enabling such pharmacological properties have not been understood in details yet. We extracted venoms from Indian cobra (*Naja naja*) and viper (*Vipera russelli*) and purified by ion-exchange chromatography with DEAE Sephadex A-25 column, followed by HPLC connected with GF-250 column. The highest purification peaks obtained for fraction numbers 3-6 of both snake venoms when elution buffer with 0.1-0.2M NaCl was used in ion-exchange chromatography. The purity and molecular mass of the eluted fractions have further analyzed and confirmed by HPLC and SDS-PAGE. These results reported that the purified proteins were low molecular masses ranged from 10-17kD, which resembled to phospholipase A2 of other snake venoms. The potential antibacterial activity of these purified proteins was found against gram negative bacteria like *E.coli* by agar diffusion assay using 20µl concentrations, suggested these venom proteins can be useful for some pharmacological applications. MALDI-TOF-MS studies will further be helpful to understand the molecular structure of these proteins in detail.

Keywords: *Naja naja*, *Vipera russelli*, Antibacterial activity, HPLC, Phospholipase A2

1. Introduction

Snake venom is a mixture of different enzymes includes phospholipase A2, L-amino acid oxidase, nucleosidase, ribonuclease and having toxic (cardiotoxin, myotoxin, neurotoxin, cytotoxin), and non-toxic activities (antimicrobial) produced by snake venom glands. Pharmacological and biochemical properties of those components in venoms are studied in many snakes, however, not yet every toxic ingredients in Indian snake venoms have been isolated and characterized for pharmacological perspectives. L-amino acid oxidase [1,2], lectin [3] and phospholipase A2 [4] have already investigated as antibacterial components in snake venoms.

Antibacterial proteins from snake venoms are purified by gel filtration [5], ion-exchange [6], reverse phase HPLC and FPLC [7,8] and further are characterized by SDS-PAGE [9], Iso-electric focusing and MALDI-TOF [10]. As most of the antibacterial proteins in snake venoms are belonged to small molecular weight, a multi-step purification strategy to obtain a high purity have been suggested [8,10]. Thus, this present work was aimed to purify small molecular weight proteins from venoms of *Naja naja* and *Vipera russelli* using ion-exchange chromatography followed by reverse phase HPLC with gel filtration column, and to evaluate the eluted fractions for antibacterial activities against gram negative bacteria, particularly *E.coli*. We hope that this study will useful to find some alternative for drug resistant microorganisms.

2. Methods

2.1 Venom collection

The venom was collected from adult snakes *Naja naja* and *Vipera russelli* captured in Western gates of India. A small sterilized plastic vial was covered with rubberized synthetic sheet, stretched and tied at the mouth. Then, the head of the snake was held and made to bite on the sheet. Simultaneously venom gland was pressed by hand to release venom into a plastic vial. It was mixed with sterile distilled water in 1:10 ratio and vacuum dried, and then kept at -20℃ in a deep freezer until purification.

2.2 Purification Ion-exchange chromatography

Lyophilized crude venom of snakes (1000mg) was dissolved in 5ml of 20mM phosphate buffer, pH 7.4, applied to a DEAE Sephadex A-25 column (1.5cm × 15cm, Pharmacia, USA), and then eluted with the same buffer with a linear gradient of 0-1.0mM NaCl [11]. The column was eluted at a flow rate of 20ml/h and collected in one micro-liter per tube using a fractional collector. Fractions with more protein concentration were pooled, desiccated, dialyzed

against 20mM phosphate buffer, pH 7.4. The purified enzyme solution was stored in the same buffer at 4℃ until use. The protein concentration in each fraction was tested by UV-spectrophotometer (UV-160A, Shimatzu, Japan) at 260 and 280nm.

2.3 High Performance Liquid Chromatography (HPLC)

The above purified fraction was again injected into a gel filtration column, GF-250 in HPLC. De-aerated phosphate buffer (20mM), pH 7.4 was used as a mobile phase. Flow rate (1ml/min), column temperature (25℃), column pressure (80psi) and wave length (280nm) were programmed by a system controller (SCL 6B). After equilibrating the column with the same buffer, the fraction was eluted at flow rate of 1ml/min. 100µl of the above sample and protein molecular weight marker were injected in reneodyne valve knob. The chromatographic run was conducted at constant flow rate of 1.0ml/min and monitored at 214nm by UV detector (SDD-6AV, 195-700nm). The retention time of individual fractions was assessed with standard proteins.

2.4 SDS-PAGE and determination of molecular weight

The purified fractions of venom were incubated in 10% (w/v) trichloro acetic acid solution for 10min to precipitate the proteins and then centrifuged at 4℃ with 4000 rpm in a microfuge. After discarding the supernatant, pellet was dissolved in 0.5ml of 30% (w/v) NaOH solution. Electrophoresis was performed on a 15% polyacrylaminde gel following the methods of Laemmli [11]. Protein samples and molecular weight markers were separately mixed with an equal volume of sample buffer and loaded into the slots. The slots were filled with running gel buffer and casted in electrophoresis unit. The output voltage was adjusted to 80-100V. After the run was over, the gel was removed, washed with distilled water. The gel was stained with Coomassie Brilliant Blue R-250 (Sigma) and then detained. Molecular weight of the purified proteins was estimated by SDS-PAGE according to bands pattern obtained.

2.5 Antibacterial activity assay

A bacterium, *E.coli* (JM101) used in this study was obtained from Molecular Microbiology Laboratory, School of Biotechnology at Madurai Kamaraj University, Madurai, India. A disc diffusion assay was used with the following modifications [12]: bacteria (200 µl of a 0.1 A_{600} culture containing 1.75 × 10^9 colony forming units (CFU)/ml) were spread onto 15ml LB agar plates (90mm diameter). Sterile paper discs (7mm diameter) were then placed onto the agar surface and 20µl of sample (crude and purified fraction) was added per disc. Plates were incubated at 37℃. After 24h, the diameters of inhibition zones were recorded in micro-meter minus the disc diameter.

3. Results

Indian cobra and viper venoms contain about 70-80% protein components in general. We found 76.8% protein in cobra venom and 80.4% in viper venom, indicated that viper venom was slightly more concentrated than cobra venom. On SDS-PAGE gel, many clear protein bands observed for crude cobra venom (Figure 1) where as one thick band was for crude viper venom, which was distinctly ranged between 10-20kD.

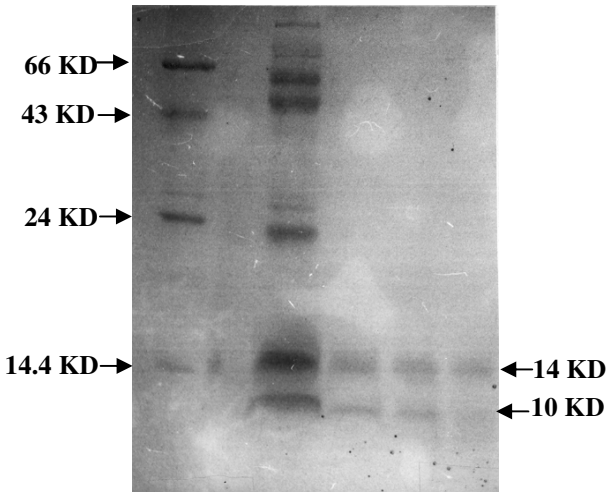

Figure 1. Molecular weight determination of purified venom proteins (Cobra) by SDS-PAGE. Lane 1. Markers; Lane 2. Crude venom proteins; Lane 3. Fraction 3; Lane 4. Fraction 4; Lane 5. Fraction 5. Protein standards and their molecular weights are: 1. γ-Globulin (160kD); 2. Bovine serum albumin (66kD); 3. Chicken egg ovalbumin (43kD); 4. Bovine trypsinogen (24kD); 5. Cytochrome-C oxidase (14.4kD).

The crude venom samples subjected to ion-exchange column on which 20 fractions with more protein concentration selected and the rest of the fractions ignored due to very low protein concentration. With 0.1-0.2M NaCl in phosphate buffer, the best elution of these fractions of both snake venoms was noted with high protein concentration and at this concentration it gave two high purity peaks as showed in Figure 2A & B. SDS-PAGE analysis showed that fraction No. 4 to 6 of cobra venom composed two proteins with 10 and 14kD, however, a distinct thick band seemed for fraction No. 4 and other fractions not showed good clarity. Similarly, in fraction No. 3 viper venom showed the highest peak and a dense band on SDS-PAGE gel corresponded with 17kD proteins. The results clearly revealed that the eluted fractions having small molecular proteins (10-17kD) with considerable protein concentration.

Figure 3A & C, the HPLC peaks indicated that crude venoms of both snakes having many proteins, however, most of the proteins belonged to small molecular masses correlated to proteins as in purified fractions of ion-exchange chromatography.

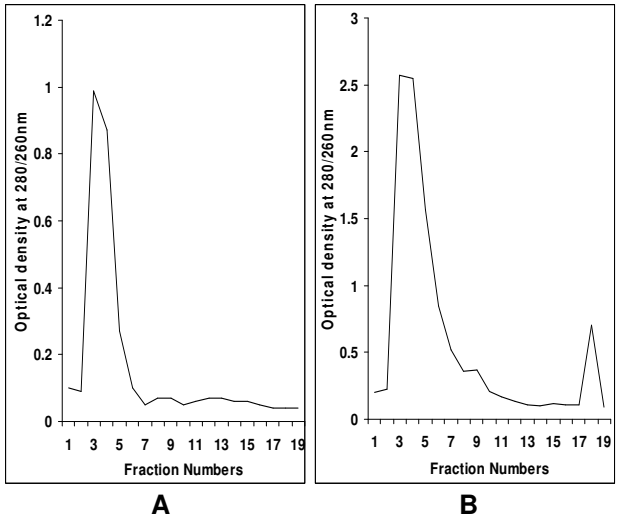

Figure 2. Chromatogram of fractions eluted from crude venom of snakes after DEAE Sephadex A-25 column chromatography using NaCl linear gradient (0-1.0 M).

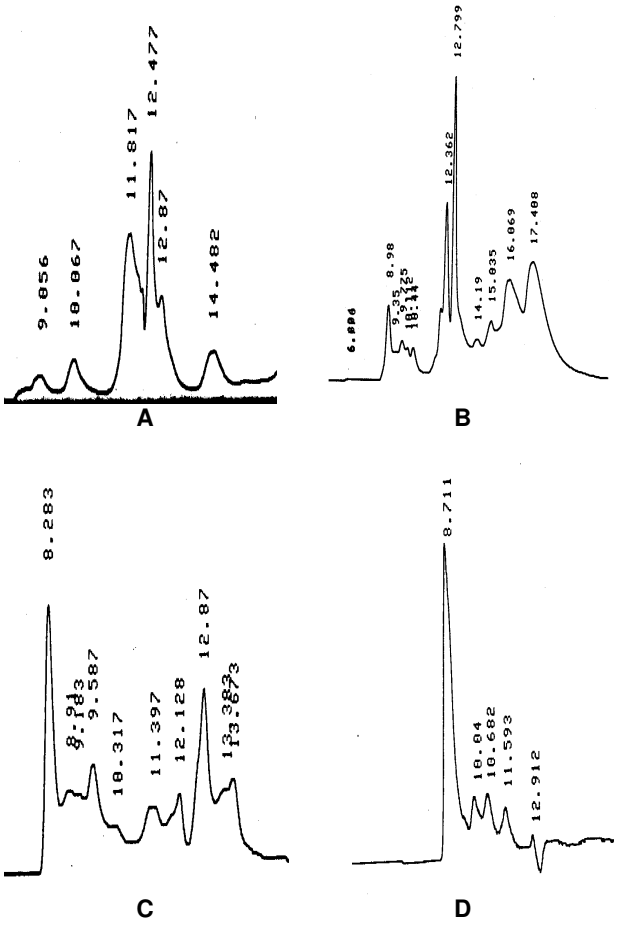

Figure 3. Chromatogram of fractions eluted after DEAE Sephadex A-25 column chromatography followed by HPLC connected with GF-250 column. (**A**) Crude venom of cobra; (**B**) Fraction No. 4 of cobra; (**C**) Crude venom of viper; (**D**) Fraction No. 3 of viper.

As shown in Figure 3B, the retention times obtained from HPLC study showed that fraction No.4 of cobra venom were 12.5 and 14.4 corresponded with the molecular weight of 14 and 10kD respectively. The retention times 8.9 and 11.2 reported for viper venom proteins (fraction No. 3) as in HPLC purification peaks (Figure 3D), which strongly coincided with the molecular weight of 66 and 17kD proteins, respectively. Nevertheless, a think band observed only for 17kD on gel and not for 66kD, suggesting a clear elution would be possible for purification of the proteins (17kD) from this fraction.

We carried out agar diffusion assay for examining antibacterial activity of purified fractions and crude venoms of both snakes (Figure 4A & B).

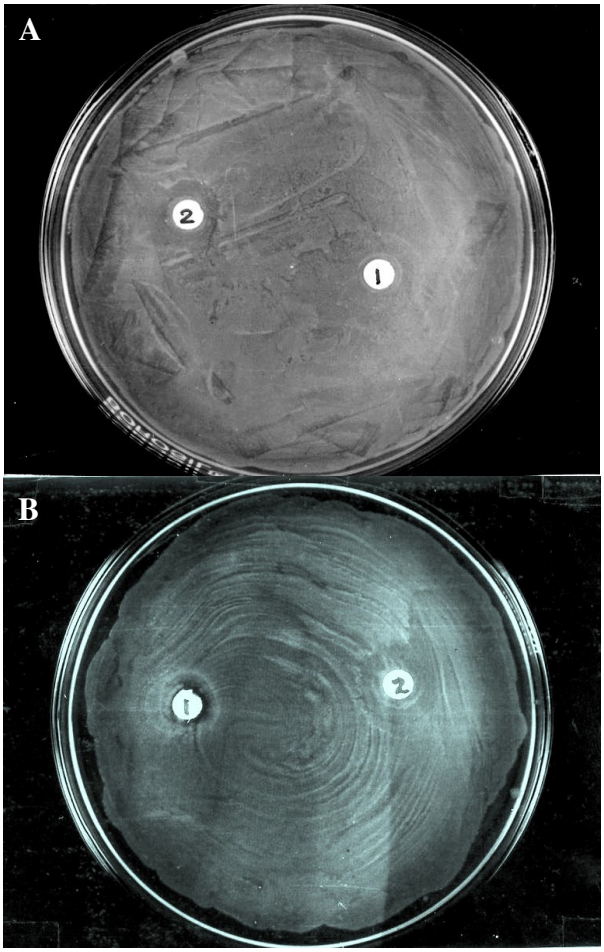

Figure 4. Antibacterial activity of crude and purified venoms of Indian cobra and viper against *E.coli*. The values represent an inhibition zone in mm, minus the 7mm diameter of the disc, after 18h incubation. Bacteria inoculums per plate contained 1.75 × 10^9 colony-forming units, which were spread onto agar plates. Sterile paper discs (7mm diameter) were then placed onto the agar surface and 15µl of sample was added per disc. 1. Crude; 2. Purified. (**A**) Cobra venom; (**B**) Viper venom.

It was observed that cobra venom showed the antibacterial effect against *E.coli*, a gram-negative bacteria on LB agar plate and the purified fraction and crude venom gave a clear zone diameter 1.8cm and 0.8cm, respectively. When crude venom and purified fraction of viper venom tested, the purified

fraction showed wider clear zone (1.6cm) than crude venom (0.7cm). On comparison to crude venoms of both snakes, the venoms proteins purified by ion-exchange chromatography with DEAE Sephadex A-25 was the best choice to bring better antibacterial effect of these venoms against gram-negative bacteria. On the other hand, purified venom from *Naja naja* was much better than purified *Vipera russelli* venom for such antibacterial properties.

4. Discussion

DEAE Sephadex A-25 column connected with HPLC was used for purification of small molecular weight proteins, myotoxin I (14kD) from *Naja naja*, *Bothraps jararacussu* [7] and *Trimeresurus jerdonii* [13]. The highest purification peaks have reported in this study as similar to the above strategies and the purified proteins are also small molecular weight proteins such as 10 and 14kD from cobra venom and 17kD from viper venom. Wu et al. [14] reported that crotalin (von Willebrand Factor, vWF) from *Crotalus atrox* auto-proteolytically degraded to about 14 and 10kD fragments in the presence of SDS and reduce crotalin able to bind vWF is conformation-independent. This literature evidenced to explain the possibility of forming two auto-proteolytic fragments such as 10 and 14kD in purified fractions of cobra venom. Furthermore, the molecular weight of L-amino acid oxidase from the same specie but distributed in Taiwan was reported to show 140kD by analytical gel filtration and 70kD by SDS-PAGE [15].

This variation may be due to different composition or differed glycosylation. In fact, variation in snake venom composition is a ubiquitous phenomenon at all taxonomic level [16]. Recent studies showed that the composition and some main components of phospholipase A2 [17] are different between the *Trimeresurus mucrosquamatus* venom from Hunan Province (in Chinese mainland) and Taiwan Province. Daltry et al. [18] also demonstrated a significant relationship between geographic variation in diet and geographic variation in venom composition might reflect natural selection for the greater efficiency in killing and/or digesting different prey types in different regions. The intra-specific venom variation in *T. mucrosquamatus* may be explained by this hypothesis. Consequently, the molecular weights of these proteins are showed such variation in this study.

We found a reasonable antibacterial activity of these snake venoms against gram negative bacteria in which purified protein fractions are reported here for more suitability. There are many proteins have been reported as showing antibacterial activity on wide range of microorganisms so far. L-amino acid oxidase is a dimeric flavoprotein containing non-covalently bound FAD or FMN as cofactor, and is present in various resources including snake venoms. It catalyzes the oxidative deamination of L-amino acids to produce the corresponding α-keto acids along with the production of ammonia and hydrogen peroxide via an imino acid intermediate [19]. Two antibacterial components from the venom of an Australian elapid, *Pseudechis australis* (Australian king brown or mulga snake) are purified to homogeneity, which had potent antibacterial properties associated with L-amino acid oxidase activity [20]. Although L-amino acid oxidase in cobra and viper venoms determined antibacterial activity against gram negative and gram positive bacteria [2], it is a large molecular weight protein with 128kD [1]. Therefore, we assumed that our purified fractions could not be belonged to L-amino acid oxidase as reported earlier. In contrast, galactose binding lectin [3], phospholipase A2 [2,4,21], acuthrobin-C [5], onwaprin, 50-amino acid cationic protein [22] purified from snake venoms have already found to be small molecular weight proteins (10-17kD) with a potent antibacterial activity. Since omwaprin lost its antibacterial activity upon reduction and alkylation of its cysteine residues, or upon deletion of six N-terminal amino acid residues, four of which are positively charged, the three-dimensional structure constrained by four disulfide bonds and the N-terminal residues are essential for its activity [20].

Naturally occurring venom peptides and phospholipase A2 proved to possess highly potent antimicrobial activity against *Burkholderia pseudomallei* [2]. Phospholipase cleaves glycophospho lipids at the Sn-2 position. The reaction products are lysophospholipid and arachidonic acid. The metabolites derived from arachidonic acid control a wide variety of cellular functions including inflammation [23]. There is a family of secreted phospholipase A2 comprising low molecular weight (13-15kD) disulfide linked proteins [4,9,10] that depend on Ca^{2+} ion for enzyme activity that are important defensive mechanism of innate immunity capable of killing bacteria [2,24]. Thirty-four crude venoms, nine purified phospholipase A2s and two L-amino acid oxidases were studied by Perumal samy et al. [25] for antibacterial activity by disc-diffusion assay. Several snake venoms (*Daboia russelli russelli, Crotalus adamanteus, Naja sumatrana, Pseudechis guttata, Agkistrodon halys, Acanthophis praelongus* and *Daboia russelli siamensis*) showed activity against two to four different pathogenic bacteria [25]. L-Amino acid oxidase inhibited the growth of *E.coli, Staphylococcus aurues* and *Bacillus dysenteriae*. Among the bacteria tested, *S.aurues* was most sensitive to L-amino acid oxidase. In addition, the antibacterial effect was neutralized by catalase, suggesting H_2O_2 produced by L-amino acid oxidation is involved in the antibacterial effect. These results are in accordance with the effects of two L-amino acid oxidases from the venom of *Pseudechis austrlis* [22]. No one has been reported

galactose binding lectin in venoms of Indian cobra and viper as our hearable knowledge yet. Therefore, it suggests that our purified proteins are not considered as L-amino acid oxidase and they could be phospholipase A2 because of resemblance in molecular weight as well as potent antibacterial properties. Manjunatha [26] proposed that their unique ability to 'target' themselves to a specific organ or tissue is due to their high affinity binding to specific proteins which act as receptors (more precisely, acceptors). This specific binding of phospholipase A2 is conferred by the presence of a 'pharmacological site' on its surface which is independent of the catalytic site. The high affinity interaction of phospholipase A2 with its acceptor (or target protein) is probably due to the complementarities, in terms of charges, hydrophobicity and van der Waal's contact surfaces, between the pharmacological site and the binding site on the surface of the acceptor protein. Upon binding to the target, the phospholipase A2 can induce its pharmacological effects by mechanisms either dependent on or independent of its catalytic activity.

5. Conclusions

Overall, we conclude that DEAE Sephadex column chromatography A-25 followed by gel filtration (GF-250) HPLC used to purify small molecular weight proteins (10-17kD) from crude venoms of Indian cobra (*Naja naja*) and viper (*Vipera russeli*). These purified fractions showed the potential antibacterial activities against gram negative bacteria in which cobra venom was more effective than viper venom. According to earlier investigations, it is thought to be having similar functions as phospholipase A2 family. Moreover, this is a first report on finding small molecular weight proteins with antibacterial principles from venoms of Indian cobra and viper. The recent development of mass spectrometry and structural bioinformatics will, perhaps, be helpful to elucidate the chemical structure and peptide families of these purified proteins. Thus, the antibacterial profile of snake venoms reported herein will hopefully be useful for developing a potential antibacterial candidate against drug resistant gram negative bacteria.

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