Journal of Proteomics xxx (xxxx) xxx–xxx

Contents lists available at ScienceDirect

Journal of Proteomics

journal homepage: www.elsevier.com/locate/jprot

Proteomics, functional characterization and antivenom neutralization of the venom of Pakistani Russell's viper (*Daboia russelii*) from the wild

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ARTICLE INFO

Keywords:
Daboia russelii sochureki
Venomics
Procoagulant
Western Russell's viper
ED_{50}
Potency

ABSTRACT

The venom proteome of wild Pakistani Russell's viper (*Daboia russelii*) was investigated through nano-ESI-LCMS/MS of the reverse-phase HPLC fractions. A total of 54 venom proteins were identified and clustered into 11 protein families. Phospholipase A_2 (PLA_2, 63.8%) and Kunitz-type serine protease inhibitor (KSPI, 16.0%) were most abundant, followed by snake venom serine protease (SVSP, 5.5%, mainly Factor V activating enzyme), vascular endothelial growth factor (VEGF, 4.3%), snake venom metalloproteinase (SVMP, 2.5%, mainly Factor X activating enzyme) and phosphodiesterase (PDE, 2.5%). Other minor proteins include cysteine-rich secretory protein (CRiSP), snake venom C-type lectin/lectin-like protein (snaclec), nerve growth factor, L-amino acid oxidase and 5′-nucleotidase. PLA_2, KSPI, SVSP, snaclec and SVMP are hemotoxic proteins in the venom. The study indicated substantial venom variation in *D. russelii* venoms of different locales, including 3 Pakistani specimens kept in the USA. The venom exhibited potent procoagulant activity on human plasma (minimum clotting dose = 14.5 ng/ml) and high lethality (rodent LD_{50} = 0.19 μg/g) but lacked hemorrhagic effect locally. The Indian VINS Polyvalent Antivenom bound the venom immunologically in a concentration-dependent manner. It moderately neutralized the venom procoagulant and lethal effects (normalized potency against lethality = 2.7 mg venom neutralized per g antivenom).

Biological significance: Comprehensive venom proteomes of *D. russelii* from different locales will facilitate better understanding of the geographical variability of the venom in both qualitative and quantitative terms. This is essential to provide scientific basis for the interpretation of differences in the clinical presentation of Russell's viper envenomation. The study revealed a unique venom proteome of the Pakistani *D. russelii* from the wild (Indus Delta), in which PLA_2 predominated (~60% of total venom proteins). The finding unveiled remarkable differences in the venom compositions between the wild (present study) and the captive specimens reported previously. The integration of toxicity tests enabled the correlation of the venom proteome with the envenoming pathophysiology, where the venom showed potent lethality mediated through coagulopathic activity. The Indian VINS Polyvalent Antivenom (VPAV) showed binding activity toward the venom protein antigens; however the immunorecognition of small proteins and PLA_2-dominating fractions was low to moderate. Consistently, the antivenom neutralized the toxicity of the wild Pakistani Russell's viper venom at moderate efficacies. Our results suggest that it may be possible to enhance the Indian antivenom potency against the Pakistani viper venom by the inclusion of venoms from a wider geographical range including that from Pakistan into the immunogen formulation.

1. Introduction

The Russell's viper (family Viperidae, subfamily Viperinae, genus *Daboia*) is a medically important snake in South Asia and most parts of Southeast Asia [1,2]. The true viper, together with *Naja naja* (Indian cobra), *Bungarus caeruleus* (Indian krait) and *Echis carinatus* (saw-scaled viper) constitute 'The Big Four' or the four most medically important species of venomous snakes in the Indian subcontinent, including Pakistan. Snakebite cases in Pakistan are prevalent in the active agricultural areas as around the Indus Valley (Punjab) and the Indus Delta (Sindh) [3]. Of the Big Four in the region, the Russell's viper is considered as one of the most commonly encountered snakes. It is typically

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https://doi.org/10.1016/j.jprot.2018.05.003
Received 7 January 2018; Received in revised form 26 April 2018; Accepted 2 May 2018

found in the rice fields and villages and propagates very fast in the farming areas where rodent preys are abundant.

Envenomation by Russell's viper (*Daboia* sp.) can lead to diverse pathological events including systemic coagulopathy, pituitary infarct, neurotoxicity, acute kidney injury, hemorrhages and tissue necrosis [4–9]. The clinical presentations of Russell's viper envenomation are known to vary across geographical regions; for instance, besides coagulopathy and renal failure, envenoming cases in Sri Lanka were reported to show prominent neurotoxicity and myotoxicity [5,10], while envenoming cases from Myanmar were largely hemorrhagic with chronic complication of pituitary infarct [11]. Recent phylogenetic analysis indicated that the Russell's vipers constitute two distinct species, *i.e. Daboia russelii* in the South Asia (west to the Bay of Bengal) and *Daboia siamensis* in the Southeast Asia (east to the Bay of Bengal), however, the variation in the clinical effects of envenoming does not necessarily conform to the systematics [12]. Instead, the variation (clinical presentation) appears to reflect potential venom variability of Russell's vipers from different geographical locales [13–16]. The phenomenon highlights the importance of comprehensive knowledge on venom composition and pathophysiological action of the snake venom, as this has an important ramification on the production and the use of antivenom in the region. This is because antivenoms are typically raised against venom from snakes of a particular geographical origin, and very often, these snakes are kept under captivity or are of captive-bred strains, whereas snake envenomation is largely inflicted by wild snakes in various places. The variation in snake venom composition may be accompanied by different protein antigenicity that leads to suboptimal immunoreactivity and weak neutralization by clinically used antivenoms [17,18]. Currently, the treatment of *D. russelii* envenoming in Pakistan relies mainly on the polyvalent antivenom imported from India (*e.g.* products of VINS or Bharat) which are raised against the Big Four snake venoms of Indian origin [19]. A recent study revealed that the Indian antivenom was reactive and effective *in vitro* against the procoagulant effect of Pakistani Russell's viper venom (unspecified locale origin), but the *in vivo* neutralization of the venom remains to be further investigated. In addition, the venom tested in the previous study was sourced from 3 captive specimens housed in a facility in the USA (Kentucky Reptile Zoo) [20], hence it is relevant and important to also study the local specimens, in particular those from the wild, since long captivity may be a contributing factor to intraspecific snake venom variation [21–23].

With the advent of proteomics, the venom compositions of Russell's vipers from several locales have been profiled, including that of *D. russelii siamensis* (proper nomenclature: *D. siamensis*) from Myanmar and *D. russelii* from India, Pakistan and Sri Lanka [20,24,25]. The reported venom profiles varied to some extent among the studies, with notable differences in terms of the protein type/family detected and the protein abundance quantitated. The observation was likely reflective of true geographical venom variability, although it could also be partly confounded by the proteomic or analytical method. Knowledge on the venom composition of Russell's viper venom especially that of authenticated specimen from known locality, is very crucial to improve our understanding of the venom complexity and variability. Furthermore, when coupled with functional studies, the knowledge will contribute toward elucidating the clinical pathophysiology of Russell's viper envenoming and how antivenom production and use can be improved.

In this study, we aimed to investigate the venom proteome of wild Pakistani Russell's vipers through a protein decomplexation approach using reverse-phase high performance liquid chromatography (RP-HPLC) followed by nano-electrospray ionization (ESI) liquid chromatography tandem mass spectrometry (nano-ESI-LCMS/MS) of the digested peptides. The specimens were wild adult snakes collected in Pakistan (specifically the Indus Delta). The preliminary findings indicated substantial variations from the venom proteome reported recently for the Pakistani *D. russelii* of a different source, which was a venom sample of 3 captive snakes from a serpentarium in the USA [20]. In addition, we also examined the effectiveness of the commonly used India-manufactured polyvalent antivenom in neutralizing the procoagulant and lethal effects of the venom. It is hoped that the findings will shed light on the venom variability of Russell's viper, thus propelling the improvement of antivenom production and use in the region. The proteomic results will also enrich the database for comparison to venom proteomes of Russell's vipers of various geographical regions.

2. Material and methods

2.1. Venoms and antivenom

Daboia russelii (Indus Delta, Pakistan) venom was milked and pooled from > 10 adult snakes authenticated by author NQ. The venom was lyophilized and stored at −20 °C prior to use. The antivenom used was Indian VINS Polyvalent Antivenom (VPAV, Batch no:01AS12041, expiry date: March 2016, used before expiry) reconstituted in 10 ml of sterile water prior to use. This antivenom was obtained from the sera of horses hyperimmunized against venoms of the Indian “Big Four”: *Naja naja* (Indian cobra), *Bungarus caerelus* (common krait), *Daboia russelii* (Russell's viper), and *Echis carinatus* (saw-scaled viper).

2.2. Mice and ethics clearance

The albino ICR strain (20–25 g) mice were supplied by the Animal Experimental Unit, University of Malaya. Animal studies protocol was designed based on the Council for International Organizations of Medical Sciences (CIOMS) guidelines on animal experimentation [26]. The protocol was approved by the Faculty of Medicine Institutional Animal Care and Use Committee of the University of Malaya (Ref: 2014–09-11/PHAR/R/TCH).

2.3. Materials

Ammonium bicarbonate, dithiothreitol (DTT) and iodoacetamide were purchased from Sigma-Aldrich (USA). Mass-spectrometry grade trypsin protease, Spectra™ Multicolor Broad Range Protein Ladder (10 to 260 kDa), and HPLC grade solvents used in the studies were purchased from Thermo Scientific™ Pierce™ (USA). Millipore ZipTip® C_{18} Pipette Tips were obtained from Merck (USA). Other chemicals and reagents of analytical grade were purchased from Sigma-Aldrich (USA).

2.4. C_{18} reverse-phase HPLC of the Pakistani D. russelii venom

The lyophilized venom (3 mg) was reconstituted in 0.1% trifluoroacetic acid (TFA) and centrifuged at 10,000*g* for 12 min at 4 °C. The supernatant was then fractionated by a LiChrospher® WP 300 C_{18} reverse-phase column (250 mm × 4.6 mm, 100 Å) using a Shimadzu LC-20AD HPLC system. Elution was carried out at a flow rate of 1 ml/min [27], using a linear gradient of 0.1% TFA in water (Solvent A) and 0.1% TFA in 100% acetonitrile (Solvent B) (0–5% B for 10 min, followed by 5–30% B over 30 min, 30–55% B over 150 min and 50–70% B over 170 min). Protein elution was monitored at 215 nm. The eluted fractions were subjected to SDS-PAGE and separately analyzed by nano-ESI-LCMS/MS shotgun proteomic analysis after in-solution tryptic digestion.

2.5. SDS PAGE of venom and HPLC fractions

SDS-PAGE was conducted according to Laemmli [28]. The Prestained Protein Ladder (10–260 kDa) was used for calibration. The crude venom (50 µg) and HPLC-eluted protein fractions were individually loaded onto a 15% gel and electrophoresis was performed under reducing condition at 90 V for 2 h. Proteins were visualized using Coomassie Brilliant Blue R-250. The relative density of protein band