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Proteomic investigation of Sri Lankan hump-nosed pit viper (*Hypnale hypnale*) venom

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ABSTRACT

The hump-nosed pit viper, *Hypanle hypnale*, contributes to snakebite mortality and morbidity in Sri Lanka. Studies showed that the venom is hemotoxic and nephrotoxic, with some biochemical and antigenic properties similar to the venom of *Calloselasma rhodostoma* (Malayan pit viper). To further characterize the complexity composition of the venom, we investigated the proteome of a pooled venom sample from >10 Sri Lankan *H. hypnale* with reverse-phase high performance liquid chromatography (rp-HPLC), sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and peptide sequencing (tandem mass-spectrometry and/or N-terminal sequencing). The findings ascertained that two phospholipase A_2 subtypes (E6-PLA_2, W6-PLA_2) dominate the toxin composition by 40.1%, followed by snake venom metalloproteases (36.9%), L-amino acid oxidase (11.9%), C-type lectins (5.5%), serine proteases (3.3%) and others (2.3%). The presence of the major toxins correlates with the venom's major pathogenic effects, indicating these to be the principal target toxins for antivenom neutralization. This study supports the previous finding of PLA_2 dominance in the venom but diverges from the view that *H. hypnale* venom has low expression of large enzymatic toxins. The knowledge of the composition and abundance of toxins is essential to elucidate the pathophysiology of *H. hypnale* envenomation and to optimize antivenom formulation in the future.

1. Introduction

The advanced snakes, Caenophidians (ca. 2470 species), reached their considerably 'advanced' evolutionary state with the use of venom for prey capture (Vidal and Hedges, 2002). Genes for venom were first recruited from restricted sets of genes for physiological proteins. These genes evolved mainly through duplication which subsequently created molecular redundancy for protein neofunctionalization (Deshimaru et al., 1996; Ohno et al., 1998; Ogawa et al., 2005). Driven by changes in ecological niche, rapid amino acid sequence divergence within restricted protein families resulted in the selection and inheritance of 'toxin genes' with diverse functions adapted for prey immobilizing, killing and digestion. This leads to the great diversity of snake venom toxins; nonetheless, conserved sequence homology within restricted protein families of venom enable the current sequencing technologies and data mining to identify an unknown toxic gene/protein at high throughput scale (Angulo et al., 2008; Calvete et al., 2009; Fernández et al., 2010; Corrêa-Netto et al., 2011; Bernardes et al., 2013). The advancement as such in the field of toxinology is promising for a number of reasons: (1) the provision of deeper insights into envenomation pathophysiology; (2) the optimization of antivenom formulation; (3) the development of drug discovery from toxin; (4) the revision of an updated, robust snake systematics. Transcriptomic studies of snake venom glands virtually unmask the identities of most proteins/peptides at transcript level; however, the genetic findings may not necessarily correlate well with the relative abundances of post-translational proteins (Vogel and Marcotte, 2012). In this context, venom proteomic study, termed 'venomics' by Calvete et al. (2007) becomes invaluable as it is able to disclose the types and abundance of toxins in a snake venom.

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From the medical aspect, venomic studies are beneficial for improving the management of snake envenomation, a condition also known as a neglected tropical disease and a disease of poverty (Gutiérrez et al., 2006; Harrison et al., 2009; WHO, 2010). There are approximately 5.5 million snakebite cases yearly, of these at least 421,000 envenomings and 20,000 deaths occur worldwide, although the figures in reality may soar as high as 1,841,000 envenomings and 94,000 deaths (Kasturiratne et al., 2008). In view of the various financial and technical challenges faced confronting the production of good antivenom, the Global Snakebite Initiative (GSI) in collaboration with the International Society on Toxinology has proposed integrated strategies (Gutiérrez et al., 2010, 2014; Williams et al., 2011), one of which is the utilisation of venom proteomics as toxin profiling tool. The context is highly relevant for the envenomation problem caused by the endemic species *Hypnale hypnale* (hump-nosed pit viper) in Sri Lanka and Western Ghats of India (Joseph et al., 2007; Ariaratnam et al., 2008; Maduwage et al., 2013). In view of the lack of clinically indicated antivenom for *H. hypnale* envenomation, profiling of the venom composition hence becomes essential as the approach would contribute remarkably to the design, development and assessment of an effective antivenom.

Hypnale hypnale envenomation usually has low fatality, but associates with severe complications e.g. extensive tissue destruction, venom-induced coagulopathy and acute kidney injury that is often the cause of death (Alirol et al., 2010). Unfortunately, specific antivenom against this species is not available, while the heterologous Indian polyvalent antivenoms (raised against the venoms of Indian Big Four: Indian cobra, Indian krait, saw-scaled viper, Russell's viper) were proven ineffective but risking hypersensitive reactions in the patients (Sellahewa and Kumararatne, 1994; Premawardena et al., 1996, 1998; Joseph et al., 2007; Ariaratnam et al., 2008). Tan et al. (2011a, 2012a) demonstrated that Thai Hemato polyvalent antivenom (raised against Malaysian pit viper, white-lipped green pit viper and Siamese Russell's viper) conferred paraspecific protection against *H. hypnale* venom's various toxicities in mice; the findings of efficacy however still await clinical validation. Laboratory studies thus far confirmed the activities of enzymatic toxins like phospholipases A_2 (PLA_2), thrombin-like enzymes (TLE), proteolytic enzymes (snake venom metalloproteinases, SVMP) and L-amino acid oxidases (LAAO) in the venom (Wang et al., 1999a; Tan et al., 2011b). Recently, Ali et al. (2013) reported the identities of major proteins from the venom of a single *H. hypnale* specimen using 1-dimensional (1D) and a 2-dimensional (2D) gel electrophoresis. In brief, it was concluded that PLA_2 dominated among other toxin classes, and that the venom lacks large enzymatic toxins (higher molecular weight proteins above 15 kDa). However, the quantitative abundance of toxin composition of *H. hypnale* venom toxins was not determined, possibly limited by the discrepancies of protein spots/bands between the two electrophoretic methods. Moreover, as the venom was sourced from a single snake, the choice of a more representative sample pooled from multiple snakes in the region remains to be explored. With an additional fractionation step using a reverse-phase high performance liquid chromatography, the current study examined the proteome of *H. hypnale* venom pooled from >10 Sri Lankan specimens for a better coverage of inter-individual and geographical variations. The characterisation focused specifically on the toxin components and relative composition in order to gain better insights into the venom's toxic actions and suggestions for antivenom formulation.

2. Materials and methods

2.1. Venom sample

H. hypnale venom was a pooled sample obtained from the milking of >10 adult snakes of both sexes captured in Sri Lanka (Gamapha, Kelaniya, Avissawela, Colombo regions). The snakes were kept at the Snake Venom Research Laboratory and Serpentarium at the University of Colombo, Sri Lanka and were identified by Anslem de Silva, an expert herpetologist. Reagents and equipments used in this study are stated in the description of methodology.

2.2. Reverse-phase HPLC separation of H. hypnale venom proteins

Two milligrams of *H. hypnale* venom dissolved in 0.1% trifluoroacetic acid (TFA) was fractionated on a LiChrospher® RP100C18 reverse-phase column (Merck, 250 × 4 mm, 5 μm particle size). The sample was eluted at 1 mL/min with a linear gradient of 0–66% acetonitrile (ACN) containing 0.1% TFA over 60 min. The solvent delivery and gradient formation over 60 min was achieved using the Shimadzu LC-20AD HPLC system. Eluted fractions were collected manually according to 215 nm absorbance, and lyophilized in a Speed-Vac system.

2.3. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) for reverse-phase HPLC fractions

Isolated protein fractions were further assayed by SDS-PAGE on 15% polyacrylamide gels under reducing condition with beta-mercaptoethanol as the reducing agent. The assay was conducted in an electrophoresis (slab) system (Enduro PAGE System; Labnet International, USA) according to the principle of Laemmli (1970) and method as modified by Studier (1973). The vacuum-dried protein fractions (from reverse-phase HPLC) were first reconstituted in 100 μL deionized water each. Ten μL of each reconstituted fraction were mixed with sample incubation buffer containing mercaptoethanol in 1:1 volume ration, and heated in a boiling water bath for 15 min. The samples along with standard protein markers (Thermo Scientific, USA; Bio-Rad, USA) were then loaded on 15% polyacrylamide gels and subjected to electrophoresis at a constant voltage of 80 V for sample stacking (14 min), followed by a constant voltage of 100 V for sample separation (2 h). The gels upon completion of SDS-PAGE were destained overnight and the protein bands were checked for their relative intensities using MYImageAnalysis software (Thermo Scientific). Intensities of the protein bands were integrated with chromatographic peak areas to estimate the relative abundance of proteins (subsequently grouped by toxin classes) expressed in percentage of total protein.

2.4. Electro-transference of SDS-PAGE proteins for N-terminal sequencing

Upon completion of SDS-PAGE, gels were swiftly removed and proteins were transferred onto polyvinylidene fluoride (PVDF) membranes by using Invitrogen iBlot™ System (Program P2, 8–10 min). Protein bands on the PVDF membranes were made visible by Coomassie Blue R-250 stain. The protein samples were then subjected to peptide N-terminal sequencing by Edman degradation method using the Procise Sequencer (Applied Biosystems Sciex, USA). The N-terminal amino acid sequences determined were subsequently subjected to similarity searches against the available databases using the BLAST search program (Basic Local Alignment Search Tool) search in the *blastp* suite at www.ncbi.nlm.nih.gov/BLAST, using the following settings for the non-redundant

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