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Venomics of the beaked sea snake, *Hydrophis schistosus*: A minimalist toxin arsenal and its cross-neutralization by heterologous antivenoms

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ABSTRACT

The venom proteome of *Hydrophis schistosus* (syn: *Enhydrina schistosa*) captured in Malaysian waters was investigated using reverse-phase HPLC, SDS-PAGE and high-resolution liquid chromatography-tandem mass spectrometry. The findings revealed a minimalist profile with only 18 venom proteins. These proteins belong to 5 toxin families: three-finger toxin (3FTx), phospholipase A_2 (PLA_2), cysteine-rich secretory protein (CRISP), snake venom metalloprotease (SVMP) and L-amino acid oxidase (LAAO). The 3FTxs (3 short neurotoxins and 4 long neurotoxins) constitute 70.5% of total venom protein, 55.8% being short neurotoxins and 14.7% long neurotoxins. The PLA_2 family consists of four basic (21.4%) and three acidic (6.1%) isoforms. The minor proteins include one CRISP (1.3%), two SVMPs (0.5%) and one LAAO (0.2%). This is the first report of the presence of long neurotoxins, CRISP and LAAO in *H. schistosus* venom. The neurotoxins and the basic PLA_2 are highly lethal in mice with an intravenous median lethal dose of <0.2 μg/g. Cross-neutralization by heterologous elapid antivenoms (*Naja kaouthia* monovalent antivenom and Neuro polyvalent antivenom) was moderate against the long neurotoxin and basic PLA_2, but weak against the short neurotoxin, indicating that the latter is the limiting factor to be overcome for improving the antivenom cross-neutralization efficacy.

1. Introduction

Snakebite envenomation is a neglected tropical disease [1]. Persistent underestimation of its morbidity and mortality has made snakebite envenomation the most neglected of all "Neglected Tropical Diseases" listed by the World Health Organization [2]. Aptly described as a disease of poverty [3,4], it is primarily a problem of the poorer rural populations and affects mainly those involved in farming or fishing activities. Unfortunately, these snakebite victims are commonly the bread-winners in the family, and the young generation accountable for the country's economy [5]. This public health threat is shared among nations in the Southeast Asia, including Malaysia, the habitat native to a wide range of terrestrial and marine snakes. Where sea snake bites are concerned, the envenomation is a long recognized deadly occupational hazard for fishermen in Malaysian waters [6–8]. Globally, this is still a public health issue that remains relevant in the 21st century [4].

There are more than 20 species of sea snakes in Malaysian waters [9]; envenomation cases by sea snake have been reported sporadically but are not uncommon [8,10]. The beaked sea snake, *Hydrophis schistosus*, is one of the most commonly encountered species in this region. Formerly known as *Enhydrina schistosa*, this viviparous sea snake (Elapidae: Hydrophiinae) is now placed within the paraphyletic genus *Hydrophis* as indicated by molecular phylogenetic study [11]. The snake is often found entangled in fishing nets and were hauled in with the catch; mishaps happen as fishermen try removing them, or treading on them on the deck or in the murky water. The bite wound, in contrast to many terrestrial venomous snake bites, is relatively painless but systemic envenoming can ensue rapidly if victims are not treated promptly and correctly [4,12–14]. The incidence of fatalities from sea snake envenomation has been estimated to vary from 3.2% to 30% of the bites [15]. Clinically, *H. schistosus* envenomation is highly lethal; patients commonly develop neuromuscular paralysis that leads to respiratory failure, and rhabdomyolysis that can cause renal complication [13,14,16,17]. Earlier studies isolated two short neurotoxins [18] and a myotoxic, enzymatic phospholipase A_2 [19] from *H. schistosus* venom, supporting that these are the principal lethal toxins in the venom. Enzymatic activities of protease, alkaline phosphomonoesterase, acetylcholinesterase, 5′-nucleotidase and hyaluronidase have also been reported for the venom; the pathogenic effects of these enzymes in *H. schistosus* envenomation is however unclear [20]. To date, the only indicated antivenom for the treatment of *H. schistosus* envenomation is the CSL Sea Snake Antivenom manufactured by the Australian Commonwealth Serum Laboratory [4]; this product however is extremely costly in Southeast Asia (a retail price of close to RM 20,000

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or USD 5000 per vial), demanding stringent cold-chain transport and storage, and is not widely available or adequately stocked in local hospitals [21]. Nonetheless, cross-neutralization of this venom in mice lethality study by other elapid antivenoms such as Thai cobra antivenoms had been reported, presumably due to the presence of common epitopes between the venoms [21–23]. A detailed profiling of *H. schistosus* venom and assessment of antivenom cross-neutralization of individual toxins remain to be explored.

Indeed, the comprehensive knowledge of venom composition is essential to elucidate the pathophysiology and immunoneutralization profile of snake venom, a complex mixture of diverse toxins. The approach established by the community of international toxinologists through the Global Snakebite Initiative (GSI) aims to improve the management of snakebite, through an integrated strategy of coupling modern proteomic, immunological, pharmacological and molecular biological techniques to improve the therapeutics and the understanding of the underlying pathophysiology [2,24,25]. The meticulous global profiling of venom toxins was once a challenging and difficult task; nonetheless recent advances in proteomic technology have made it possible to reliably identify and quantitate toxin proteins in snake venom, including the minor components. Our knowledge on the proteomes of venom has advanced remarkably since then, shedding light on the improvement of snakebite management as well as drug discovery [26–29]. To date, the proteomes of a considerable number of medically important venomous snakes have been reported (for examples: [30–36]), however, there were limited studies published on the proteome of sea snake venoms [37,38]. In this study, we investigated the venom proteome of *H. schistosus* captured in the northwestern coastal area of Peninsular Malaysia through sequential fractionation of the venom, followed by high-resolution mass spectrometry analysis for protein identification. The subtypes and relative abundances of toxins were analyzed for a deeper insight into the compositional diversity of *H. schistosus* venom. We also hypothesized that the major lethal toxins of *H. schistosus* venom can be cross-neutralized by the heterologous Thai cobra antivenoms (*Naja kaouthia* monovalent antivenom, NKMAV, and Neuro polyvalent snake antivenom, NPAV), and this has been examined in an in vivo rodent model which provided comparative data on the effectiveness of the antivenoms against individual toxins.

2. Materials and methods

2.1. Venoms and antivenoms

Venoms were milked from 10 adult *H. schistosus* from the northwestern waters of Peninsular Malaya (State of Penang) by one of the authors (CHT) into sterile plastic containers. The venom samples were subsequently lyophilized and pooled in this study. The snakes (identified by CHT) were then released back into the sea. Antivenoms used were *N. kaouthia* Monovalent Snake Antivenom (NKMAV, Lyophilised; Batch no. NK00310; Exp. Date 9th August, 2015, a purified $F(ab')_2$ obtained from serum of equines hyperimmunized against Thai *N. kaouthia*) and Neuro Polyvalent Snake Antivenom (NPAV, Lyophilized, Batch no. NP00109, Exp. Date 5th October, 2014; a purified $F(ab')_2$ obtained from serum of equines hyperimmunized against venoms of Thai *N. kaouthia*, *Ophiophagus hannah*, *Bungarus candidus* and *Bungarus fasciatus*). Both antivenoms were manufactured by Queen Saovabha Memorial Institute, Bangkok, Thailand.

2.2. Animals and ethics clearance

Mice used in this study were of albino ICR strain, 4–5 week old male weighing 20–25 g supplied by the Animal Experimental Unit, University of Malaya. The protocol of experimental animal use in this study was based on the guidelines given by CIOMS [39] and the use of animals was approved by the Institutional Animal Care and Use Committee of the University of Malaya (Ref: 2014-09-11/PHAR/R/TCH).

2.3. Materials

All chemicals and reagents used in the studies were of analytical grade. Ammonium bicarbonate, dithiothreitol (DTT) and iodoacetamide were purchased from Sigma-Aldrich (USA). Trypsin protease of mass spectrometry sequencing grade, Spectra™ Multicolor Broad Range Protein Ladder (10–260 kDa), and HPLC grade solvents used in the studies were purchased from Thermo Scientific™ Pierce™ (USA). LiChroCART® 250-4 LiChrospher® WP 300 RP-18 (5 μm) HPLC cartridge and Millipore ZipTip® C_{18} Pipette Tips were purchased from Merck (USA).

2.4. C_{18} reverse-phase HPLC fractionation

Crude venom (2 mg) was reconstituted in ultrapure water and centrifuged at 10,000 *g* for 5 min. The supernatant was subjected to LiChrospher® WP 300 C_{18} reverse-phase column using a Shimadzu LC-20AD HPLC system (Japan). The venom components were eluted at 1 mL/min with a linear gradient of 0.1% TFA (trifluoroacetic acid) in water (solvent A) and 0.1% TFA in 100% ACN (acetonitrile) (solvent B) (0–5% B for 10 min, followed by 5–15% B over 20 min, 15–45% B over 120 min and 45–70% B over 20 min). Protein was detected by measurement of absorbance at 215 nm and the peaks were collected manually and lyophilized.

2.5. SDS-PAGE and protein tryptic digestion

The lyophilized protein fractions obtained from reverse-phase HPLC were reconstituted in ultrapure water, subsequently subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE, 15% gel, reducing condition) according to the procedure of Laemmli [40]. Spectra™ Multicolor Broad Range Protein Ladder (10 to 260 kDa) was used for molecular mass calibration. Protein bands were excised from the Coomassie Brilliant Blue-stained electrophoretic gel and subjected to the standard procedure of reduction with DTT, alkylation with iodoacetamide, and in-gel digestion with MS grade trypsin protease according to the manufacturer's protocol (Thermo Scientific™ Pierce™, Rockford, IL, USA). The tryptic digested peptides were desalted using standard bed Millipore ZipTip® C_{18} Pipette Tips (Merck, USA) according to the manufacturer's protocol to enhance the performance of mass spectrometry.

2.6. High resolution nano-HPLC tandem mass spectrometry analysis

Following in-gel trypsin digestion, the digested peptide samples TOF were subjected to Thermo Scientific™ Pierce™ Orbitrap Fusion™ Tribrid™ with an Easy-nLC™ 1000 ultra-high pressure LC on a Thermo Scientific™ Pierce™ EASY-Spray™ PepMap C_{18} column (2 μm, 75 μm × 25 cm). The peptides were separated over 44 min gradient eluted at 300 nL/min with 0.1% formic acid in water (Solvent A) and 0.1% FA in 100% ACN (solvent B) (0–5% B in 5 min, followed by 5–50% B over 30 min and 50–95% B over 2 min). The run was completed by holding a 95% B for 7 min. MS1 data was acquired on an Orbitrap Fusion mass spectrometry using a full scan method according to the following parameters: scan range 300–2000 m/z, orbitrap resolution 240,000; AGC target 200,000; and maximum injection time of 50 ms. MS2 data were collected using the following parameters: rapid scan rate, CID collision energy 30%, 2 m/z isolation window, AGC 10,000 and maximum injection time of 35 ms. MS2 precursors were selected for a 3 s cycle. The precursors with an assigned monoisotopic m/z and a charge state of 2–6 were interrogated. The precursors were filtered using a 70 s dynamic exclusion window. MS/MS spectra were searched using Thermo Scientific™ Pierce™ Proteome Discoverer™ Software Version 1.4 with SEQUEST® against the non-redundant NCBI Serpentes database (taxid: 8570) (updated August, 2014) downloaded from NCBI database (http://www.ncbi.nlm.nih.gov/protein/?term=Serpentes).