

Article

Proteomic Deep Mining the Venom of the Red-Headed Krait, *Bungarus flaviceps*

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Abstract: The use of -omics technologies allows for the characterization of snake venom composition at a fast rate and at high levels of detail. In the present study, we investigated the protein content of Red-headed Krait (*Bungarus flaviceps*) venom. This analysis revealed a high diversity of snake venom protein families, as evidenced by high-throughput mass spectrometric analysis. We found all six venom protein families previously reported in a transcriptome study of the venom gland of *B. flaviceps*, including phospholipases A₂ (PLA₂s), Kunitz-type serine proteinase inhibitors (KSPIs), three-nger toxins (3FTxs), cysteine-rich secretory proteins (CRISPs), snakelecs, and natriuretic peptides. A combined approach of automated database searches and de novo sequencing of tandem mass spectra, followed by sequence similarity searches, revealed the presence of 12 additional toxin families. De novo sequencing alone was able to identify 58 additional peptides, and this approach contributed significantly to the comprehensive description of the venom. Abundant protein families

1. Introduction

In the last decade, there has been a tremendous increase in the knowledge of snake venom composition and evolution, mainly because of the application of “omics” techniques, in particular, high-throughput transcriptomic investigations of venom gland tissue in combination with proteomic studies of venom [1–6]. The use of these highly sensitive technologies now makes it feasible to

2.1. Major Venom Components

The most abundant components of the venom of *B. aviceps* (22.3%; Figures 2 and 3) are represented by 3FTxs, similar to that observed for the related *B. fasciatus* in which 3FTxs make up 30% of the venom content [21]. As a non-enzymatic snake venom protein family, 3FTxs are structurally characterized by three-stranded loops protruding from a central core of the molecule. In spite of their similarity in structure and relatively small size (<10 kDa) of (~250 residues) 82.07176%

Figure 3. (A) Abundances of the venom protein families of *B. flaviceps* as evidenced by normalized mass spectrometric spectral count. (B) Comparison of abundances of venom protein families (*B. flaviceps*) by transcriptomic (red, adapted from [25], 2010, Springer Nature) and proteomic analysis. KSPI—Kunitz-type serine proteinase inhibitors; VEGF—vascular endothelial growth factor; VNGF—venom nerve growth factor; SVSP—snake venom serine proteinase; CVF—cobra venom factor.

Table 1. Snake venom protein families of *B. flaviceps* identified by automated database search. SVMP—snake venom metalloproteinase; SVSP—snake venom serine proteinase.

Protein Family	Protein	Accession No.	Species	Number of Peptides Matched
3FTx	Non-conventional three nger toxin isoform 1	294961050	<i>Bungarus flaviceps</i>	6
3FTx	Non-conventional three nger toxin isoform 6	294961060	<i>Bungarus flaviceps</i>	5
3FTx	Short-chain three nger toxin isoform 4	294961042	<i>Bungarus flaviceps</i>	4
3FTx	Short-chain three nger toxin isoform 7	294961048	<i>Bungarus flaviceps</i>	9
3FTx	Short-chain three nger toxin isoform 6	294961046	<i>Bungarus flaviceps</i>	7
3FTx	Short-chain three nger toxin isoform 1	294961036	<i>Bungarus flaviceps</i>	2
3FTx	-bungarotoxin	809178	<i>Bungarus multicinctus</i>	1
3FTx	Short-chain three nger toxin isoform 3	294961040	<i>Bungarus flaviceps</i>	1
3FTx	-avitoxin	128938	<i>Bungarus flaviceps</i>	9

Table 1. Cont.

Protein Family	Protein	Accession No.	Species	Number of Peptides Matched
3FTx	Muscarinic toxin-like protein	294961066	<i>Bungarus flaviceps</i>	9
Serine protease inhibitor	-bungarotoxin B chain precursor	31745053	<i>Bungarus flaviceps</i>	7
Serine protease inhibitor	Kunitz-type serine proteinase inhibitor isoform 5	294961076	<i>Bungarus flaviceps</i>	8
Serine protease inhibitor	Kunitz-type serine proteinase inhibitor isoform 1	294961068	<i>Bungarus flaviceps</i>	4
Acetylcholinesterase	Acetylcholinesterase	1389604	<i>Bungarus flaviceps</i>	20
Acetylcholinesterase	Acetylcholinesterase DEN	476538388	<i>Denisoni adevisi</i>	4
PLA ₂	-bungarotoxin A ₂ chain precursor	31745049	<i>Bungarus flaviceps</i>	16
PLA ₂	-bungarotoxin A ₁ chain precursor	31745051	<i>Bungarus flaviceps</i>	3

Table 1. Cont.

Protein Family	Protein	Accession No.	Species	Number of Peptides Matched
CRISP	Opharin precursor	225547744	<i>Ophiophagus hannah</i>	2
SVMP	Scutase-1 (PIII)	145982766	<i>Notechis scutatus</i>	8
SVMP	Metalloproteinase (PIII)	126035640	<i>Bungarus multicinctus</i>	6
SVMP	Metalloproteinase MTP9 (PIII)	336042214	<i>Drysdalia coronoides</i>	4
SVMP	Metalloproteinase (PIII)	126035635	<i>Bungarus fasciatus</i>	4
SVMP	P-III	633276509	<i>Micropechis ikaheka</i>	4
SVMP	MTP4 (PIII)	537463069	<i>Micrurus fulvius</i>	3
SVMP	Atragin precursor(PIII)	224482347	<i>Naja atra</i>	3
SVMP	Metalloproteinase isoform 3 (PIII)	109254964	<i>Sistrurus catenatus edwardsi</i>	2
SVMP	SVMP-Hop-14, partial (PIII)	476539284	<i>Hoplocephalus bungaroides</i>	2
SVMP	SVMP-Hop-46, partial(PIII)	476539268	<i>Hoplocephalus bungaroides</i>	2
SVMP	SVMP 1	537444726	<i>Micrurus fulvius</i>	2
SVMP	Metalloproteinase (PII)	82466485	<i>Bothrops asper</i>	1
SVMP	Fur-1, partial (PI)	476538467	<i>Furinaor nata</i>	1
SVMP	jararhagin (PIII)	62468	<i>Bothrops jararaca</i>	1
SVMP	Metalloproteinase (PIII)	241995585	<i>Philodrya solfersii</i>	1
SVMP	Leucurolysin-B (PIII)	223635807	<i>Bothrops leucurus</i>	1
SVMP	Ech-32 (PIII)	476538400	<i>Echiopsis curta</i>	1
SVMP	Cobrin precursor(PIII)	6006966	<i>Naja naja</i>	1
SVMP	Metalloproteinase (PII)	297594122	<i>Echis pyramidum leakeyi</i>	1
SVMP	CohPH-3 (PII)	522802426	<i>Crotalus oreganus helleri</i>	1
SVSP	Serine proteinase isoform 2	109254940	<i>Sistrurus catenatus edwardsi</i>	2
SVSP	SVSP 11	387014258	<i>Crotalus adamanteus</i>	1
Natriuretic peptide	Natriuretic peptide	294961100	<i>Bungarus flaviceps</i>	1
Complement-depleting factor	Complement-depleting factor	126035660	<i>Bungarus fasciatus</i>	1

Serine proteinase inhibitors were the second most abundant toxin family in the venom of *B. flaviceps* and accounted for 19% of the total spectral count (Figure 3

Acetylcholinesterases (AChEs) are important regulators of neurotransmission at the neuromuscular junction, and they rapidly hydrolyze acetylcholine. Acetylcholinesterases were the

and hyperalgesia in mice [42]. Vespryns matching those from several species, including *O. hannah*, *Pseudechis australis* and *Drysdalia coronoides* were encountered in the present study. Recently, vespryns were also found in the venom of *B. candidus* [21], but tryptic peptides of *B. aviceps* origin did not match these proteins.

Phosphodiesterases (PDE) are basic enzymes with molecular masses in the range of 98 to 140 kDa [43], and they catalyze the hydrolysis of phosphodiester bonds from the 3' terminus of polynucleotides. Though PDEs have long been known to be present in many venoms, the first complete primary structure of a PDE from snake venom was only recently published [44]. We encountered sequences similar to PDEs of the elapid *Micrurus fulvius* and of several different pitvipers (Tables 1 and 2). These results further corroborate the presence of PDE in *Bungarus* venoms, as they were also reported in the venom of *B. fasciatus* [21].

Hyaluronidases from snake venoms are endo- α -N-acetyl-hexosaminidases with molecular masses in the range of 30 to 110 kDa [43]. These enzymes cleave hyaluronan, a major glycosaminoglycan constituent of the extracellular matrix, into N-acetylglucosamine and oligocarbohydrates. Therefore, hyaluronidases have been implicated as important factors for the distribution and dissemination of the venom in tissues and are often called "spreading factors" [43]. Peptides with pronounced homology to-acetyl

of the total spectral count (Figure 3). While most of the identified sequences were related to SVMPs of congeneric *B. multicinctus* and the elapid *Notechis scutatus*, we also identified peptides matching to SVMPs of viperid (e.g., *Bothrops asper*

It is also interesting to compare the method of venom protein quantification used in the present study to other quantitative snake venom investigations. Quantitative analysis of the venoms of *B. candidus* and *B. fasciatus* for example, was based on the number of the corresponding proteins of the different protein venom families identified [

extracted four times each and the average yield was 24.7 L. Range = 5–75 L. No animals were sacrificed during this study.

4.2. Tryptic Digestion of Crude Venom

Lyophilized venom (100 µg) was dissolved in 40 µL of 0.4 M ammonium bicarbonate and 8 M urea. After adding 10 µL of 50 mM dithiothreitol (DTT), the solution was incubated for 3 h at 37 °C. Additionally, 10 µL of iodoacetamide (150 mM) was added at room temperature and the reaction was allowed to proceed for 15 min. The reaction was quenched with 50 mM of DTT (6 µL) for 15 min. The last two steps were performed in the dark. For digestion, the sample solution was diluted to 1 M urea (by adding 254 µL of water) and incubated with 10 µL (2 µg in 50 mM acetic acid) of trypsin (Promega, Madison, WI, USA) at 37 °C overnight. Quenching of the reaction occurred by adding tri-n-octanoic acid (40 µL), and desalting of the sample was performed on Poros R2 microcolumns. As a final step, the peptides were dried in a vacuum centrifuge and brought up in 1% formic acid solution (approx. 50 µL).

4.3. Chromatography and Mass Spectrometry

4.3.1. One-Dimensional Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Crude venom was reduced with 2.5% β-mercaptoethanol in Laemmli sample buffer (Bio-Rad, Hercules, CA, USA.) by incubation at 95 °C for 10 min. Samples (20 µg and 50 µg amounts) were run on a 12% Bis-Tris acrylamide gel, stained with 0.1% Coomassie Brilliant Blue R-250 overnight, and destained in 10% acetic acid: 40% methanol: 50% ddH₂O. A Novex Mark 12 unstained mass standard (Life Technologies, Grand Island, NY, USA) was also run for band mass estimation. Protein bands were excised and submitted to the Protein and Proteomics Centre in the Department of Biological Sciences, National University of Singapore, for LC-MS/MS analysis.

4.3.2. Liquid Chromatography-Tandem Mass Spectrometry (LC-MS/MS) Analysis of Excised Gel Bands

Bands were reduced with 10 mM DTT (dithiothreitol) and alkylated with 55 mM IAA (iodoacetamide), then digested with trypsin (13 ng/µL) overnight in 25 mM ammonium bicarbonate, 10% ACN (acetonitrile). Samples were desalted using a Sep-Pak tC18 Elution Plate (Waters, Milford, MA, USA), and reconstituted with 20 µL of diluent (97.5% H₂O, 2% ACN, 0.05% formic acid). Peptide separation was carried out on an Eksigent nanoLC Ultra and ChiPLC-nano exLC-MS (Eksigent, Dublin, CA, USA) in Trap Elute configuration. A total of 5 µL of the sample was loaded onto a 200 µm × 0.5 mm trap column and eluted on an analytical 75 µm × 150 mm column. Trap and analytical columns were made of ChromXP C18-CL, 3 µm (Eksigent, Dublin, CA, USA). Peptides were separated by a gradient formed by 2% ACN, 0.1% FA (mobile phase A) and 98% ACN, 0.1% FA (mobile phase B); 5 to 7% of mobile phase B in 0.1 min, 7 to 30% of mobile phase B in 10 min, 30 to 60% of mobile phase B in 4 min, 60 to 90% of mobile phase B in 1 min, 90 to 90% of mobile phase B in 5 min, 90 to 5% of mobile phase B in 1 min and 5% of mobile phase B for 10 min, at a flow rate of 300 nL/min. The MS analysis was performed on a TripleTOF 5600 system (AB SCIEX, Redwood City, CA, USA) in Information Dependent Mode. MS spectra were acquired across the mass range of 400–1250 m/z in high resolution mode (>30,000) using 250 ms accumulation time per spectrum. A maximum of 10 precursors per cycle were chosen for fragmentation from each MS spectrum with 100 ms minimum accumulation time for each precursor and dynamic exclusion for 8 s. Tandem mass spectra were recorded in high sensitivity mode (resolution > 15,000) with rolling collision energy on adjustment. Survey-IDA experiment with charge states 2 to 4, which exceeds 125 cps, was selected. Peptide identification was achieved with ProteinPilot 5.0 software Revision 4769 (AB SCIEX, Redwood City, CA, USA) using the Paragon database search algorithm (5.0.0.0.4767) for peptide identification and the integrated false discovery rate (FDR) analysis function. The data were searched against a database consisting of SerpentesDB

database (total 345,092 entries). The search parameters are as follows: Sample Type: Identification; Cys

applied: no restrictions on species of origin or protein molecular weight, two tryptic missed cleavages allowed, variable modifications of cysteine (carbamidomethylation) and methionine (oxidation), and pyro-glutamate formation at N-terminal glutamine of peptides. The enzyme conditions were set to “semi-tryptic” including the option to search for non-tryptic cleavage sites either at the N-terminus or C-terminus of the corresponding peptide. The database search of electrospray data (Orbitrap Q Exactive™, ThermoFisher Scientific, Waltham, MA, USA) applying a p -value of 0.05% showed a false discovery rate (FDR) of 1.82%, while the database search of spectra acquired with MALDI mass spectrometry revealed a FDR of 0.49% (p -value 0.05%).

4.5. De Novo Sequencing and Similarity-Driven Analysis of Digested Venom

In addition to automated database searches, all tandem mass spectra were sequenced by de novo analysis using the program PEAKS version 6 (Bioinformatics Solutions Inc., Waterloo, KW, Canada). The following search parameters were applied: cysteine modification (carbamidomethylation of cysteine), deamidation of asparagine (N), oxidation of methionine, and pyroglutamate modification at N-terminal glutamate; 20 ppm precursor mass tolerance and 0.1 Da fragment mass tolerance. High quality de novo sequences (Peaks ALC score higher than 50 and sequences with six or more amino acid residues) were submitted to the algorithm PepExplorer [[64oxins](#)

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