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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 A_2 (PLA_2s), Kunitz-type serine proteinase inhibitors (KSPIs), three- nger toxins (3FTxs), cysteine-rich secretory proteins (CRISPs), snaclecs, 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 signi cantly 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 g 0f ()-250(erized)82 0.7176%

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* identi ed 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	*Bungarus flaviceps*	6
3FTx	Non-conventional three nger toxin isoform 6	294961060	*Bungarus flaviceps*	5
3FTx	Short-chain three nger toxin isoform 4	294961042	*Bungarus flaviceps*	4
3FTx	Short-chain three nger toxin isoform 7	294961048	*Bungarus flaviceps*	9
3FTx	Short-chain three nger toxin isoform 6	294961046	*Bungarus flaviceps*	7
3FTx	Short-chain three nger toxin isoform 1	294961036	*Bungarus flaviceps*	2
3FTx	-bungarotoxin	809178	*Bungarus multicinctus*	1
3FTx	Short-chain three nger toxin isoform 3	294961040	*Bungarus flaviceps*	1
3FTx	- avitoxin	128938	*Bungarus flaviceps*	9

Table 1. *Cont*.

Protein Family	Protein	Accession No.	Species	Number of Peptides Matched
3FTx	Muscarinic toxin-like protein	294961066	*Bungarus flaviceps*	9
Serine protease inhibitor	-bungarotoxin B chain precursor	31745053	*Bungarus flaviceps*	7
Serine protease inhibitor	Kunitz-type serine proteinase inhibitor isoform 5	294961076	*Bungarus flaviceps*	8
Serine protease inhibitor	Kunitz-type serine proteinase inhibitor isoform 1	294961068	*Bungarus flaviceps*	4
Acetylcholinesterase	Acetylcholinesterase	1389604	*Bungarus flaviceps*	20
Acetylcholinesterase	Acetylcholinesterase DEN	476538388	*Denisoni adevisi*	4
PLA_2	-bungarotoxin A $_2$ chain precursor	31745049	*Bungarus flaviceps*	16
PLA_2	-bungarotoxin A $_1$ chain precursor	31745051	*Bungarus flaviceps*	3

Table 1. *Cont*.

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

Table 2. Snake venom protein families of *B. flaviceps* identi ed by de novo sequencing of tandem mass spectra followed by sequence-similarity analysis. The corresponding sequences are indicated.

Protein Family	Protein	Accession No.	Species	Database Sequence	Alignment Score	De Novo Sequence	ALC (%)	*mlz*	ppm to De Novo Derived Sequence
3FTx									

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. avicepsorigin 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 rst 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 Bungarusvenoms, as they were also reported in the venom of B. fasciatus [21].

Hyaluronidases from snake venoms are endo- -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 a hyaluronidase from the venom of the African Puff Adder, Bitis arietans, were identi ed in the venom of B. aviceps The low abundance (2.2%) of B. avicepshyaluronidase is consistent with observations in other venoms that this component is common but not abundant.

Nerve growth factors (NGFs) are relatively small proteins (up to 26 kDa) that induce growth and proliferation of certain neurons [45]. The NGFs encountered in snake venoms (vNGF) share signi cant sequence homology to their mammalian counterparts, but relatively little is known about their function and contribution to the envenomation process [46]. We detected three peptides related to vNGFs with homology to those from B. fasciatus and B. multicinctus, corroborating studies on the venoms of B. candidus [21

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

It is also interesting to compare the method of venom protein quanti cation 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 identi ed [

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

4.2. Tryptic Digestion of Crude Venom

Lyophilized venom (100 g) was dissolved in 40 L of 0.4 M ammonium bicarbonate and 8 M urea. After adding 10 L of 50 mM dithiothreitol (DTT), the solution was incubated for 3 h at 37 C. Additionally, 10 L 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 L) 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 L of water) and incubated with 10 L (2 g in 50 mM acetic acid) of trypsin (Promega, Madison, WI, USA) at 37 C overnight. Quenching of the reaction occurred by adding tri uoroacetic acid (40 L), and desalting of the sample was performed on Poros R2 microcolumns. As a nal step, the peptides were dried in a vacuum centrifuge and brought up in 1% formic acid solution (approx. 50 L).

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% -mercaptoethanol in Laemmli sample buffer (Bio-Rad, Hercules, CA, USA.) by incubation at 95 C for 10 min. Samples (20 g and 50 g 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% dd$H_2$0. 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/ L) 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 L of diluent (97.5% H_2O, 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 con guration. A total of 5 L of the sample was loaded onto a 200 m 0.5 mm trap column and eluted on an analytical 75 m 150 mm column. Trap and analytical columns were made of ChromXP C18-CL, 3 m (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 ow 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 identi cation 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 identi cation 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: Identi cation; Cys

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