Toxicon 107 (2015) 197–209

Contents lists available at ScienceDirect

Toxicon

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

Quantitative proteomic analysis of Vietnamese krait venoms: Neurotoxins are the major components in *Bungarus multicinctus* and phospholipases A_2 in *Bungarus fasciatus*

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ARTICLE INFO

Article history:
Received 23 May 2015
Received in revised form
21 August 2015
Accepted 27 August 2015
Available online 2 September 2015

Keywords:
Snake venom
Krait
Bungarus multicinctus
Bungarus fasciatus
Proteome
Mass-spectrometry

ABSTRACT

Kraits are venomous snakes of genus Bungarus from family Elapidae. Krait venoms are generally neurotoxic, but toxicity strongly depends on the particular species and regional origin of snakes. We analyzed the proteomes of Vietnamese *Bungarus multicinctus* and *Bungarus fasciatus* venoms both qualitatively and quantitatively. It should be noted that no proteomic data for *B. multicinctus* venom existed so far. We have found that in this venom, almost half (45%) of the proteins by weight was represented by β-bungarotoxins, followed by three finger toxins (28%) and phospholipases A_2 (16%), other proteins being present at the level of 1–3%. In *B. fasciatus* venom, phospholipase A_2 was the main component (71%), followed by oxidase of L-amino acids (8%), acetylcholinesterase (5%) and metalloproteinases (4%). Unexpectedly, extremely low amount of three finger toxins (1%) was found in this venom. Interestingly, the presence of complement depleting factor was observed in both venoms. Although our data showed the presence of the same toxin families in Vietnamese krait venoms as those found earlier in the venoms of kraits from other geographic regions, their relative ratio is completely different. This concerns especially *B. fasciatus* venom with predominant content of phospholipases A_2 and very low amount of three finger toxins.

1. Introduction

Genus Bungarus (kraits) belongs to the family Elapidae and includes 14 species (http://reptile-database.reptarium.cz/advanced_search?taxon=Elapidae&submit=Search) according to the latest data. Venoms of kraits have neurotoxic effects and contain a variety of toxins that block the nerve impulse transmission. Among all the krait venoms that of many-banded krait *B. multicinctus* is the best studied. The first snake α-neurotoxin α-bungarotoxin (α-Bgt),[2] which is still used as an effective tool for investigating certain subtypes of nicotinic cholinergic receptors, was isolated from this venom over 50 years ago (Chang and Lee, 1963). In addition to the three-finger α- and κ-neurotoxins, phospholipases A2 (PLA2), neurotoxic β-bungarotoxins (β-Bgt), Kunitz type proteinase inhibitors, in the venom are present some enzymes and other proteins, which do not possess known enzymatic activity. The banded krait *Bungarus fasciatus* venom is also fairly well studied. However, the present data indicate that different toxins were found in *B. fasciatus* venoms from different geographic regions (Tsai et al., 2007). The proteomic analysis of Malaysian *B. fasciatus* venom revealed the presence of several new peptides and proteins, which were not found earlier in this venom (Rusmili et al., 2014a). Surprisingly, no proteomic analysis of *B. multicinctus* venom has been performed so far and only the transcriptomic analysis of Chinese *B. multicinctus* has been done (Jiang et al., 2011). More than one

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[2] Ammonium bicarbonate buffer, ABC; α-bungartoxin, α-Bgt; β-bungarotoxin, β-Bgt; dithiothreitol, DTT; exponentially modified protein abundance index, emPAI; expressed sequences tags, ESTs; extracted ion chromatograms, XIC; natriuretic peptide, NP; iodoacetamide, IAA; local false discovery rate, LFDR; phospholipases A2, PLA2; three-finger toxin, 3Ftx; trifluoroacetic acid, TFA.

http://dx.doi.org/10.1016/j.toxicon.2015.08.026
0041-0101/

thousand of valid expressed sequences tags (ESTs) for *B. multicinctus* were analyzed. Three-finger toxins (3Ftx, 64.5%) and β-Bgt (25.1%) comprised the main toxin classes, and a cluster of natriuretic peptide (NP) precursors was found. However, it is not obvious how this cDNA composition is translated into distinct proteins in vivo.

In this paper, we have analyzed the proteomes of Vietnamese *B. multicinctus* and *B. fasciatus* venoms. The data obtained indicated that venoms of Vietnamese snakes differ substantially from those of kraits of other geographical origin.

2. Material and methods

The venoms of *B. multicinctus* and *B. fasciatus* kraits were collected from snakes obtained from the authorized, licensed local establishment for snake breeding and venom production with permission of its owner Mr. Ha Van Tien (Vinh Shon, Vinh Tuong, Vinh Phuc province, Vietnam). The venoms obtained were dried over anhydrous $CaCl_2$ and stored at −20 °C.

2.1. In-solution trypsin digestion of venom samples

Lyophilized venom sample (100 μg each) was dissolved in 20 μl of 50 mM ammonium bicarbonate buffer (ABC), containing 8 M urea and 5 mM dithiothreitol (DTT), and incubated for 40 min at 54 °C to reduce disulphide bonds. Then 5 μl of 50 mM iodoacetamide (IAA) in water was added and the mixture was incubated in the dark for 30 min at room temperature. Residual IAA was blocked by 5 μl of 50 mM DTT. One tenth (3 μl) of the mixture obtained (equivalent to 10 μg of original venom material) was added to 20 μl 3% acetonitrile, 0.1% trifluoroacetic acid (TFA) in water and used for further isolation of endogenous venom peptides. The remaining main portion was diluted with 50 μl 50 mM ABC and trypsin solution was added at 1:100 enzyme/protein ratio to the final volume of 100 μl resulting in venom concentration of ~1 mg/ml. Samples were incubated overnight at 37 °C, trypsin was inactivated by addition of 5 μl 10% TFA. Tryptic peptides were extracted and desalted using reverse-phase solid extraction cartridges Discovery DSC-18 (100 mg) (Supelco) according to the manufacturer protocol. Final peptide solution was dried in vacuum and stored at −80 °C prior to LC–MS/MS analysis.

2.2. Endogenous venom peptides isolation

Endogenous peptides from venom samples were isolated using C_{18} StageTips (Rappsilber et al., 2007). To make StageTips, two pieces of C18 Empore extraction disk (Sigma–Aldrich Co. LLC) were cut using blunt-point 16-gauge needle and packed into a P200 pipette tip (Gilson S.A.S., France). The filling was conditioned with 20 μl of methanol and equilibrated with 20 μl of 0.1% aqueous TFA. Venom solutions were applied onto the conditioned tips, followed by washing with 20 μl of 0.1% aqueous TFA. Peptides were eluted with 20 μl of 80% acetonitrile, 0.1% TFA. Eluates were dried in vacuum and stored at −80 °C prior to LC–MS/MS analysis.

2.3. LC–MS/MS analysis

Analysis was performed on a TripleTOF 5600+ mass-spectrometer with a NanoSpray III ion source (ABSciex) coupled with a NanoLC Ultra 2D+ nano-HPLC system (Eksigent). The HPLC system was configured in a trap-elute mode. For sample loading solvent and solvent A, the mix of 98.9% water, 1% methanol, 0.1% formic acid (v/v) was used. Solvent B was 99.9% acetonitrile, 0.1% formic acid (v/v). Samples were loaded on a Chrom XP C18 trap column (3 μm 120 Å 350 μm × 0.5 mm; Eksigent) at a flow rate of 3 μl/min for 10 min and eluted through a 3C18-CL-120 separation column (3 μm, 120 Å, 75 μm × 150 mm; Eksigent) at a flow rate of 300 nl/min. The gradient was from 5 to 40% of solvent B in 120 min. Between samples, a blank 45 min run consisting of 5 × 5 min waves (5%–95%–95%–5%B) was acquired to wash the column and the precolumn between two consecutive samples.

Information-dependent mass-spectrometer experiment consisted of one survey MS1 scan followed by 50 dependent MS2 scans for the most intense ions. MS1 acquisition parameters were as follows: mass range 300–1250 m/z, signal accumulation time 250 ms, intensity threshold for parent ion selection was 400 cps and the charge state was from 2 to 5. MS2 acquisition parameters were as follows: resolution of quadrupole was set to UNIT (0.7 Da), mass range was 200–1800 m/z. Collision-activated dissociation was performed with nitrogen gas with collision energy ramping from 25 to 55 V within signal accumulation time of 50 ms for each MS2 spectrum. Analyzed parent ions were sent to dynamic exclusion list for 15 s.

2.4. LC–MS/MS data analysis

Raw data was recalibrated with ProteinPilot (version 4.5) software by the search against keratin protein contaminants, followed by mgf peaklist export. For thorough peptide identification, the generated peaklists were analyzed with MASCOT (version 2.2.07) and X! Tandem (CYCLONE, 2013.2.01) search engines against NCBI protein data base, taxon Serpentes (downloaded from http://www.ncbi.nlm.nih.gov/protein 24.04.2015) with concatenated randomized decoy dataset (with 408906 entries altogether). Precursor and fragment mass tolerance were set to 20 ppm and 0.04 Da, respectively. Database-searching parameters included the following: tryptic digestion with one possible misscleavage, fixed modification for carbamidomethyl (C). For X! Tandem we also selected parameters that allowed quickly check for protein N-terminal residue acetylation, peptide N-terminal glutamine ammonia loss or peptide N-terminal glutamic acid water loss. Result files were submitted to the Scaffold 4 software (version 4.2.1) for validation and meta-analysis. For the evaluation of peptide and protein hits, we used local false discovery rate (LFDR) scoring algorithm with 5% LFDR selected for both. The estimation of the relative protein contents in venom samples was performed on the bases of exponentially modified protein abundance index (emPAI, Ishihama et al., 2005) using Scaffold 4 software. For MS1 based quantification, peptide extracted ion chromatograms (XIC) for all the identified peptides were obtained and integrated in a SkyLine software with manual checking for misassignment.

2.5. Gel-filtration of venoms

Gel filtration of crude venoms was carried out using Superdex 75 column (10 × 300 mm, GE Healthcare) in 0.1 M ammonium acetate buffer, pH 6.2 at a flow rate of 0.5 ml/min.

2.6. Anticomplementary activity

Anticomplementary activity was determined essentially as described by Shoibonov et al., 2005. In brief, freeze-dried fractions obtained after separation of 10 mg venom on Superdex 75 column were dissolved in 500 μl of isotonic veronal buffer containing Ca^{2+} and Mg^{2+} (VBS^{2+}) and 20 μl of solution obtained were incubated with 1 μl of guinea pig serum, 200 μl sheep erythrocytes sensitized with rabbit IgG (1.5 × 10^8 cell/ml) in total volume 0.5 ml of VBS^{2+} for 30 min at 37° C. After incubation, the mixture was diluted with