JOURNAL OF PROTEOMICS 89 (2013) 15–23

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Venom proteomic characterization and relative antivenom neutralization of two medically important Pakistani elapid snakes (*Bungarus sindanus* and *Naja naja*)

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ARTICLE INFO

Article history:
Received 18 February 2013
Accepted 6 May 2013
Available online 25 May 2013

Keywords:
Elapid snake
2D PAGE
Polyvalent antivenom
Myotoxicity
LC MS

ABSTRACT

Intra- and interspecific variation in venom composition has been shown to have a major effect upon the efficacy of antivenoms. Due to the absence of domestically produced antivenoms, Pakistan is wholly reliant upon antivenoms produced in other countries, such as India. However, the efficacy of these antivenoms in neutralising the venoms of Pakistani snakes has not been ascertained. This is symptomatic of the general state of toxicological research in this country, which has a myriad of highly toxic and medically important venomous animals. Thus, there is a dire need for knowledge regarding the fundamental proteomics of these venoms and applied knowledge of the relative efficacy of foreign antivenoms. Here we present the results of our proteomic research on two medically important snakes of Pakistan: *Bungarus sindanus* and *Naja naja*. Indian Polyvalent Antivenom (Bharat Serums and Vaccines Ltd), which is currently marketed for use in Pakistan, was completely ineffective against either Pakistani species. In addition to the expected pre- and post-synaptic neurotoxic activity, the venom of the Pakistan population of *N. naja* was shown to be quite divergent from other populations of this species in being potently myotoxic. These results highlight the importance of studying divergent species and isolated populations, where the same data not only elucidates clinical problems in need of immediate attention, but also uncovers sources for novel toxins with potentially useful activities.

Biological significance

Pakistan *Bungarus sindanus* and *Naja naja* venoms are differentially complex. *Naja naja* is potently myotoxic. Neither venom is neutralized by Indian antivenom. These results have direct implications for the treatment of envenomed patients in Pakistan. The unusually myotoxic effects of *Naja naja* demonstrates the value of studying remote populations for biodiscovery.

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http://dx.doi.org/10.1016/j.jprot.2013.05.015

1. Introduction

The snakebite burden in Pakistan is difficult to determine accurately due to poor epidemiological record keeping and the fact that many victims prefer to rely on traditional remedies rather than going to hospital [1,2]. However, it is readily apparent that snakebite is of major medical concern in this developing country [3] and that four snakes dominate the clinical landscape: two from the family Elapidae (*Bungarus sindanus* and *Naja naja*) and two from the family Viperidae (*Daboia russelii* and *Echis carinatus sochureki*). While each of these (or a close relative) has been well studied in other regions, it is a well-established general principle that geographical variation in venom profile may have a dramatic effect upon relative neutralization by antivenom [4,5]. This is of particular concern for Pakistan, which relies upon foreign antivenoms such as those produced by India, despite their efficacy not having been ascertained for snakes in Pakistan.

Bungarus venoms have been shown to be rich in kunitz peptides, 3FTx (three finger toxins), PLA_2 (phospholipase A_2) and acetylcholinesterase. The 3FTx in *Bungarus* venoms are kappa-neurotoxins, which are disulphide-linked dimers that specifically target neuronal nicotinic acetylcholine receptors (cf. [2]). A different type of disulphide-linked dimer in the same venom is formed by the kunitz peptides and PLA_2 (cf. [6]). These toxins are presynaptically neurotoxic with both the kunitz peptide (blockage of L-type calcium channels) and the PLA_2 (destruction of membrane phospholipids) contributing to this toxicity (cf. [7]). The role of acetylcholinesterase in *Bungarus* venoms (cf. [8,9]) is likely to further reduce the amount of available neurotransmitter [10].

Like *Bungarus*, *Naja* venoms have been shown to be rich in 3FTx and PLA_2 but the specific pharmacology of the subclasses differs considerably. 3FTx in *Naja* venoms have been shown to be monomers that either block post-synaptic nicotinic acetylcholine receptors or are cytotoxic, while the PLA_2 are presynaptically neurotoxic (cf. [10,11]). *Naja* venoms are also rich in CVF (cobra venom factor), which is a mutated form of C3 (complement protein 3) that has been implicated in anaphylactic responses (cf. [12,13]).

The polyvalent snake antivenom from India (Bharat Polyvalent Antivenom (BPAV)) has been previously revealed to have extremely poor cross-reactivity against the following non-Indian snakes [14]: *Bungarus candidus* (geographical locality not given), *Bungarus fasciatus* (geographical locality not given), *Naja kaouthia* (Malaysia and Thailand), *N. naja* (Sri Lanka), *Naja siamensis* (geographical locality not given), *Naja philippinensis* (geographical locality not given), *Naja sputatrix* (Thailand), *Naja sumatrana* (Malaysia) and *Ophiophagus hannah* (geographical locality not given). BPAV was only moderately effective against *N. kaouthia* (Thailand) and *N. sumatrana* venom but not against any of the other venoms tested. This antivenom even performed extremely poorly against the Indian population of *N. naja*. However, despite these conspicuously ineffective results, even against Indian snakes, this antivenom continues to be marketed to countries outside of India as a treatment for snakebite. Pakistan is one such targeted country.

In this study we compare the proteomic profiles of *B. sindanus* and *N. naja* venoms from Pakistan and determine the relative neutralization of these venoms by the Indian antivenin BPAV, as it is the most commonly available antivenom in Pakistan. The results not only contribute to the theoretical body of knowledge regarding venom diversification, but also have immediate implications for care of the envenomed patient.

2. Materials and methods

2.1. Snake venoms and antivenom

B. sindanus and *N. naja* were collected from the Pakistan province of Sindh (districts Tharpakar and Sajawal, respectively). Venom was milked into sterile containers and stored at −20 °C until use. The Indian polyvalent antivenom "BPAV" (i.e. serum globulins), raised against the venoms of the four most medically-significant snakes (listed as "cobra", "common krait", "Russell's viper" and "saw-scaled viper") in the region (Reg. No: 053882, Mfg. Lic. No: KD-4, Batch No: A5311023, Mfg Date: 06/11, Exp. Date: 05/15) was purchased from a hospital medical supply store sanctioned by the Pakistan government.

2.2. Proteomics

2.2.1. 1D gels

The 1D gel method followed the established Laemmli protocol (cf. [15]), with the following specific conditions: 1 mm 12% SDS-PAGE gels with resolving gel layer (3.3 mL Milli-Q H_2O, 4 mL 30% acrylamide mix, 2.5 mL 1.5 M Tris–HCl buffer, pH 8.8, 100 μL 10% SDS, 4 μL TEMED, 100 μL 10% APS); 20 μg venom sample per lane after dissolving in 3 μL of 4× sample loading buffer (12 μL total volume) with DTT; reducing conditions were 3 min incubation at 100 °C; gels were run at room temperature at 120 V for 20 min and then 140 V for 60 min; runs were stopped when dye front was less than 10 mm from the base of the gel (Mini Protean3, Bio-Rad Lab). Gels were stained with colloidal Coomassie brilliant blue G250 (34% methanol, 3% phosphoric acid, 170 g/L ammonium sulphate, 1 g/L Coomassie blue G250) overnight and then destained in 1% acetic acid.

2.2.2. 2D gels

For two-dimensional gel electrophoresis, venom samples (~2 mg) were directly solubilized in 300 μL of rehydration buffer (8 M urea, 100 mM DTT, 4% CHAPS, 110 mM DTT, and 0.5% ampholytes (Biolytes pH 3–10, Bio-Rad Lab)) and 0.01% bromophenol blue. The sample was mixed and centrifuged (5 min, 4 °C, 14 000 rpm) to remove any insoluble material and the supernatant was loaded onto IEF strips (Bio-Rad ReadyStrip, non-linear pH 3–10, 17 cm IPG) for 24 h of passive rehydration. Proteins were focused in a PROTEAN i12 IEF CELL (Bio-Rad Lab). The IEF running conditions were as follows: 100 V for 1 h, 500 V for 1 h, 1000 V for 1 h and 8000 V for 98,400 V/h. A constant current of 50 μA per strip at 20 °C was applied. After running IEF, IPG strips were equilibrated for 10 min in a reducing equilibration buffer (50 mM Tris–HCl, pH 8.8, 6 M urea, 2% SDS, 30% glycerol, 2% DTT) followed by a second incubation for 20 min in an equilibration buffer that had a DTT replaced with 2.5% iodoacetamide (alkylating buffer). IPG strips were briefly

rinsed in SDS-PAGE running buffer and embedded on top of 12% polyacrylamide gels (Protean-II Plus, 18 × 20 cm, Bio-Rad Lab) and covered with 0.5% agarose. Second dimension gels were run at 4 °C with a current of 10 mA/per gel for 20 min followed by 30 mA/per gel for 4 h or until the bromophenol dye front was within 1 cm of the base of the gel. Finally, gels were briefly washed in MilliQ water and stained with 0.2% colloidal Coomassie brilliant blue G250 overnight and destained in 1% acetic acid/H_2O. Visible spots were subsequently picked from gels and digested overnight (at 37 °C) using sequencing grade trypsin (Sigma) and according to the following protocol: gel spots were washed with ultrapure water, destained (40 mM NH4CO3/50% acetonitrile (ACN)) and dehydrated (100% ACN); gel spots were rehydrated in 10 μL of 20 μg/ml TPCK trypsin and incubated at 37 °C overnight. Digested peptides were eluted by washing the gel spots for 20 min with each of the following solutions: 20 μL of 1% formic acid (FA), followed by 20 μL of 5% ACN/0.5% FA. The cleaved peptides were eluted and thereafter subjected to LC–MS/MS analysis.

2.2.3. *Shotgun sequencing*

Specimens were milked by BGF into sterile specimen jars. Polyethylene equipment was used to collect and process samples in all cases. Samples were subsequently filtered using 20 Å syringe filters to remove large mucoidal strands and then lyophilised.

For shotgun sequencing, reduction and alkylation were performed by dissolving 3 μg of sample in 50 μL of 100 mM ammonium carbonate. 50 μL of 2% iodoethanol/0.5% triethylphosphine in acetonitrile was then added to the dissolved samples. The reduced and alkylated sample was re-suspended in 20 μL of 40 mM ammonium bicarbonate, before being incubated overnight (at 37 °C) with 750 ng sequencing grade trypsin (Sigma). Digestion was stopped by addition of 1 μL of concentrated formic acid. 0.75 μg was processed by LC–MS/MS.

2.2.4. *LC–MS/MS*

LC–MS/MS of both digested gel spots and digested whole venoms (shotgun) was performed using an Agilent Zorbax stable bond C18 column (2.1 mm × 100 mm, 1.8 μm particle size, 300 Å pore size) at a flow of 400 μL/min and a gradient of 1–40% solvent B (90% acetonitrile, 0.1% formic acid) in 0.1% formic acid over 15 min or 4 min for shotgun samples and 2D-gel spots, respectively, on a Shimadzu Nexera UHPLC coupled with an AB SCIEX 5600 Triple TOF mass spectrometer. MS^2 spectra were acquired at a rate of 20 scans/s, with a cycle time of 2.3 s, and optimised for high resolution. Precursor ions were selected between 80 and 1800 m/z, with a charge state of 2–5, and of an intensity of at least 120 counts per second, with a precursor selection window of 1.5 Da, and excluding isotopes within 2 Da for MS^2. MS^2 spectra were searched against known translated cDNA libraries or UniProt databanks with Proteinpilot v4.0 (ABSciex) using a thorough identification search, specifying alkylation method (iodoacetamide or iodoethanol), tryptic digestion, and allowing for biological and chemical modifications and amino acid substitutions, including artefacts induced by the preparation or analysis processes. This was done to maximize the identification of protein sequences. Spectra were inspected manually to eliminate false positives and only fragments with common artefacts e.g. ethanolyl(C) or deamidated(N) was retained. Full search conditions are shown in Supplementary File 1.

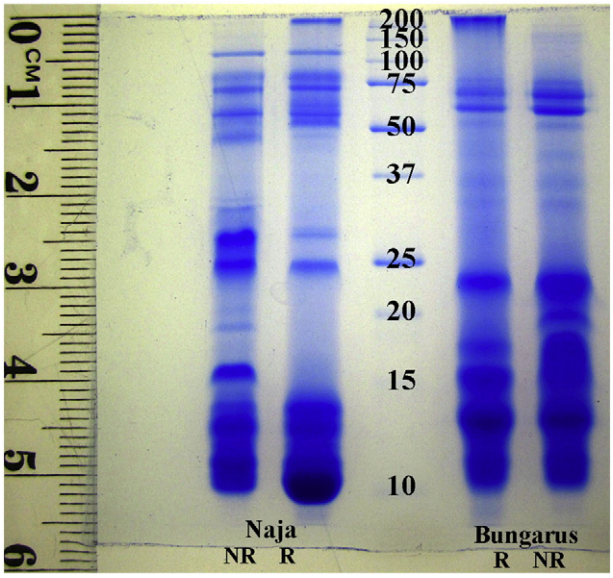

Fig. 1 – 12% SDS-PAGE analysis carried out under dissociating reducing (R) and non-reducing (NR) conditions. Gel was stained in 0.2% colloidal Coomassie brilliant blue G250.

2.2.5. *LC/MS*

LC-MS was done using an Agilent Zorbax C18 column (2.1 x 250 mm, 5 μm, 300 Å) at a flow rate of 250 μl/min and a gradient of 1-60% B (90% acetonitrile, 0.1% formic acid) in 0.1% formic acid over 60 min on an Agilent 1100-series LC coupled to an ABSciex Pulsar Q-TOF mass spectrometer scanning between 400-2000 m/z with a cycle time of 1 sec.

2.3. *Pharmacology*

2.3.1. *Neurotoxicity studies*

Male chicks (4–10 days) were killed by CO_2 and exsanguination. Chick biventer cervicis nerve muscle preparations were isolated and mounted on wire tissue holders under 1 g resting tension in 5 mL organ baths containing Krebs solution (NaCl, 118.4 mM; KCl, 4.7 mM; $MgSO_4$, 1.2 mM; KH_2PO_4, 1.2 mM; $CaCl_2$, 2.5 mM; $NaHCO_3$, 25 mM and glucose, 11.1 mM), maintained at 34 °C and bubbled with 95% O_2/5% CO_2. Indirect twitches were evoked by electrical stimulation of the motor nerve (supramaximal voltage, 0.2 ms, 0.1 Hz) using a Grass S_{88} stimulator (Grass Instruments, Quincy, MA). d-Tubocurarine (10 μM) was added, and subsequent abolition of twitches confirmed selective stimulation of the motor nerve, after which thorough washing with Krebs solution was applied to re-establish twitches. In the absence of electrical stimulation, contractile responses to acetylcholine (ACh; 1 mM for 30 s), carbachol (CCh; 20 μM for 60 s) and potassium (KCl; 40 mM for 30 s) were obtained prior to the addition of venom and at the conclusion of the experiment. The preparation was equilibrated for 30 min or until a stable twitch tension was observed

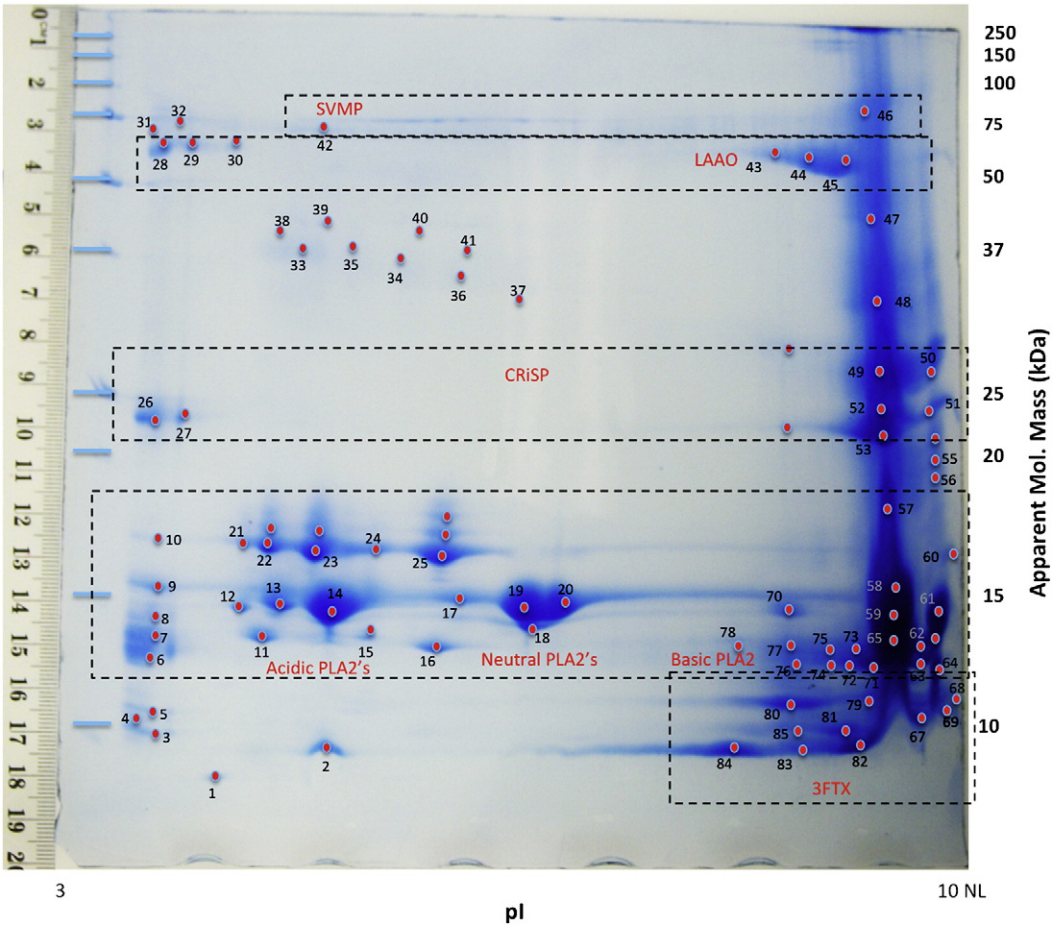

Fig. 2 – 2-dimension gel of the *B. sindanus* crude venom stained with colloidal Coomassie brilliant blue G250. First dimension: isoelectric focusing (pH 3–10 non-linear gradient); second dimension: 12% SDS PAGE. The pH gradient and the molecular weight marker positions are shown. The marked spots were analysed by MS2 and the positive results are summarized in Supplementary Table S2.

prior to the addition of venom. Venoms were left in contact with the preparation for a maximum of 3 h to test for slow developing effects. Efficacy of Indian Polyvalent Antivenom (BPAV), with a stated potency of 16.7 μL neutralizing 10 μg of four major snake venoms (i.e. *Naja*, *Bungarus*, *Echis* and *Daboia* species), was assessed via a 10 min pre-incubation in the organ bath using a 10× concentration of antivenom relative to stated neutralizing dose.

2.3.2. *Data analysis and statistics*

Twitch tension was measured from the baseline in two-minute intervals. Responses were expressed as a percentage of twitch tension prior to the addition of the venom. Contractile responses to agonists obtained at the conclusion of the experiment were measured and expressed as a percentage of the response obtained prior to the addition of venom. The time taken to inhibit 90% of twitch contractions (t_{90}) was measured as a quantitative means of measuring neurotoxicity. Values for t_{90} were measured by the time elapsed to reach 10% twitch tension amplitude following addition of venom. Where indicated, a two-way analysis of variance (ANOVA) followed by a Bonferroni-corrected post-hoc test was used to determine statistical significance of responses. Statistical analysis was performed using the Prism 5 (GraphPad Software, San Diego, CA, USA) software package. Unless otherwise indicated, data are expressed as mean ±S.E.M.

3. Results and discussion

B. sindanus and *N. naja* venoms were quite different proteomically (Figs. 1–4, Table 1, Supplemental Tables 1–4), both in types of toxins present and relative diversity within each class. Both venoms contained 3FTx, kunitz, PLA_2, CRiSP and L-amino oxidase. Consistent with previous investigations of venoms from each genus, both venoms additionally contained large molecular weight proteins: acetylcholinesterase (*Bungarus*) and snake venom metalloprotease (*Naja*). The region consistent with Cobra Venom Factor did not sequence but based results for a 2D gel from a related *Naja* species [16], we provisionally identify this region as cobra venom factor. While both venoms contained a diverse array of proteins, the combined results reveal domination by different toxin types with each technique revealing different information (Figs. 1–4).

The 1D and 2D gels (Figs. 1–3) indicated that the relative abundance of 3FTx was much greater in *N. naja*, while *B. sindanus*

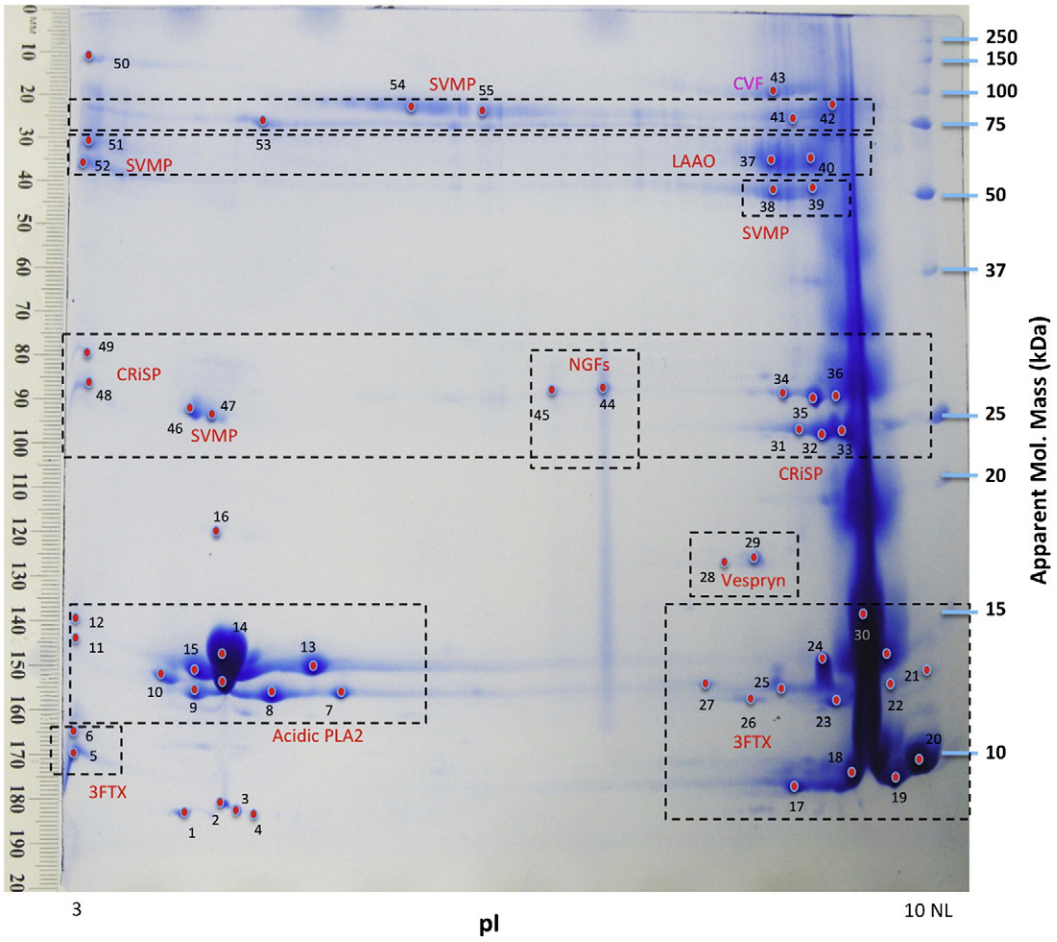

Fig. 3 – 2-dimension gel of the *N. naja* crude venom stained with colloidal Coomassie brilliant blue G250. First dimension: isoelectric focusing (pH 3–10 non-linear gradient); second dimension: 12% SDS PAGE. The pH gradient and the molecular weight marker positions are shown. The marked spots were analysed by MS2 and the positive results are summarized in Supplementary Table S4. CVF (cobra venom factor) spot sequencing failed so the provisional identification is based upon location similarity to CVF identified in a 2D gel from a related *Naja* species [16].

had much larger amounts and greater diversity of PLA_2. Although *N. naja* contained PLA_2, only acidic types were present, whereas *B. sindanus* had acidic, neutral and basic PLA_2. The latter result agrees broadly with a previous transcriptomic analysis of *Bungarus multicinctus*, *Bungarus flaviceps* and *Naja atra* venom glands [17,18] in which numerous PLA_2 transcripts were recovered from the *B. multicinctus* but none were recovered from *N. atra*. In the aforementioned transcriptomic study, however, a greater number of 3FTx transcripts were recovered from *B. multicinctus* than *N. atra*, but may not reflect the proportional quantities in the venoms. On contrast, our proteomic analyses indicated a greater quantity of 3FTx in the *N. naja* venom. LC–MS showed that while 3FTx may be present in greater relative amounts in the *Naja* than the *Bungarus* venom, both possessed extreme isotypic diversity of this peptide type (Fig. 4A–B). There was a general trend in that *Bungarus* venom contained higher molecular weight 3FTx than that of *Naja*. This is consistent with the mass spectrometry sequencing results, with identified *Bungarus* 3FTx being mostly kappa-neurotoxins and *Naja* 3FTx being primarily cytotoxins.

In keeping with the differences in toxin composition, the venoms differed in their bioactivity. *N. naja* (10 μg/mL) and *B. sindanus* (10 μg/mL) caused a rapid blockade of nerve-stimulated twitches in the chick biventer cervicis nerve-muscle preparation (Fig. 5A, $n = 3$). Time required to inhibit 90% of twitch contractions (t_{90}) for *N. naja* and *B. sindanus* were 33 ± 1 min ($n = 3$) and 25 ± 3 min ($n = 3$), respectively. Both venoms (10 μg/mL) produced significant inhibition of contractile responses to exogenous agonists (Fig. 5B–C; $P < 0.0001$, $n = 3$) but, unlike that of *N. naja*, the venom of *B. sindanus* did not significantly affect the response to potassium chloride (KCl, 40 mM) (Fig. 5B, $P > 0.05$). Inhibition of the response to KCl is indicative of post-synaptic neurotoxicity and myotoxicity being caused by venom of *N. naja*.

Prior incubation of venom with a 10× concentration of polyvalent antivenom BPAV (16.7 μL, 10 min) did not neutralise the neurotoxic effects of either venom (Fig. 5A) or restore contractile responses to exogenous agonists (Fig. 5B). If this antivenom is not able to neutralize these activities under idealized laboratory conditions, then the clinical usefulness is

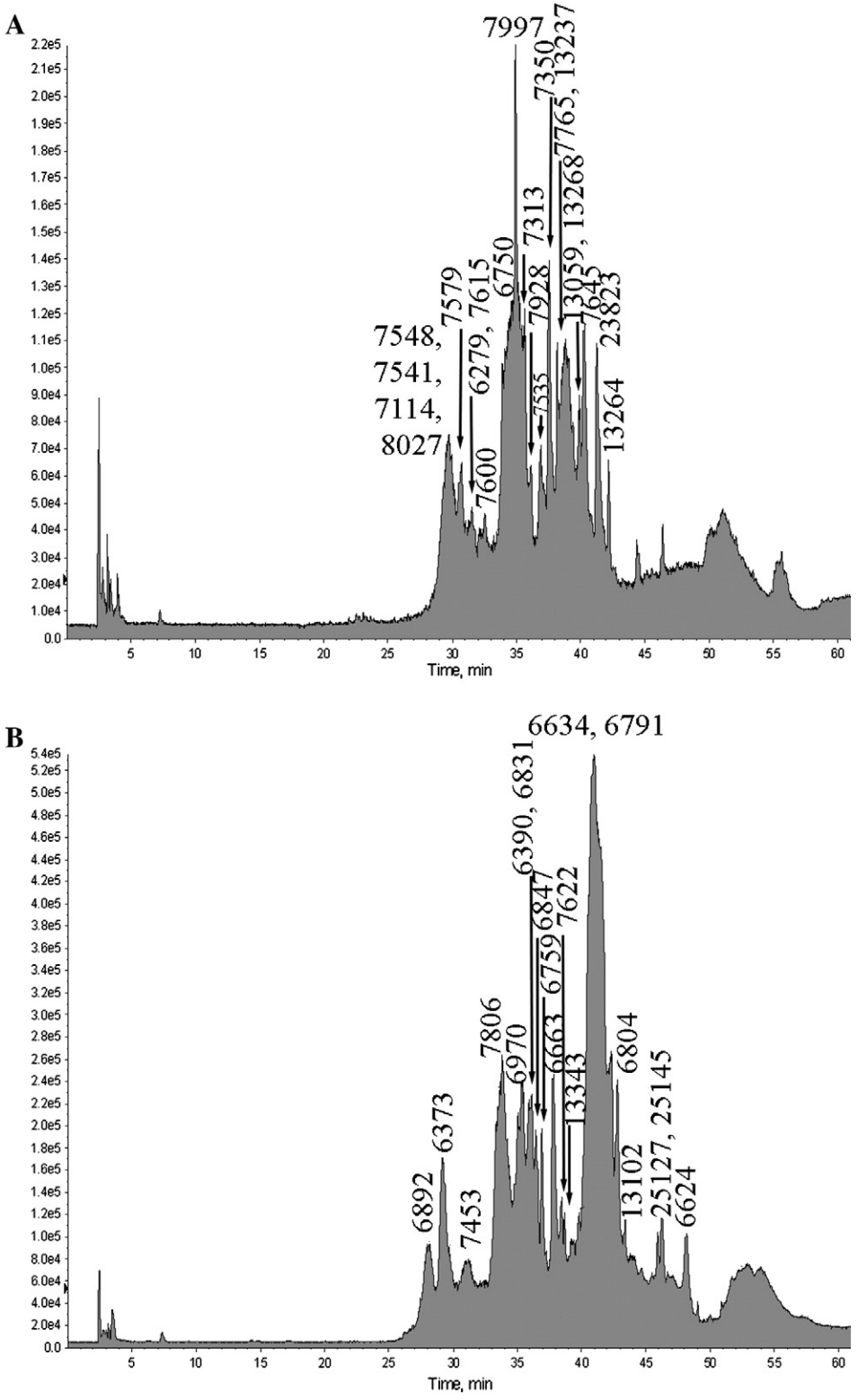

Fig. 4 – LC/MS analysis of (A) *B. sindanus* and (B) *N. naja* venoms. In the presented total ion current (TIC) profiles x-axis is the time (min), while y-axis is the relative intensity (cps). Reconstructed masses in Daltons (Da) are shown above each peak.

expected to be equally poor. As previously mentioned, this antivenom has been demonstrated to perform poorly against the venoms of a variety of medically significant species of venomous snakes occurring throughout Asia, including the Indian cobra (*N. naja*), one of the immunizing species used in the preparation of the antivenom [14]. Despite these previously published results, due to its low cost, BPAV remains one of the most widely available antivenoms in Asia, and is

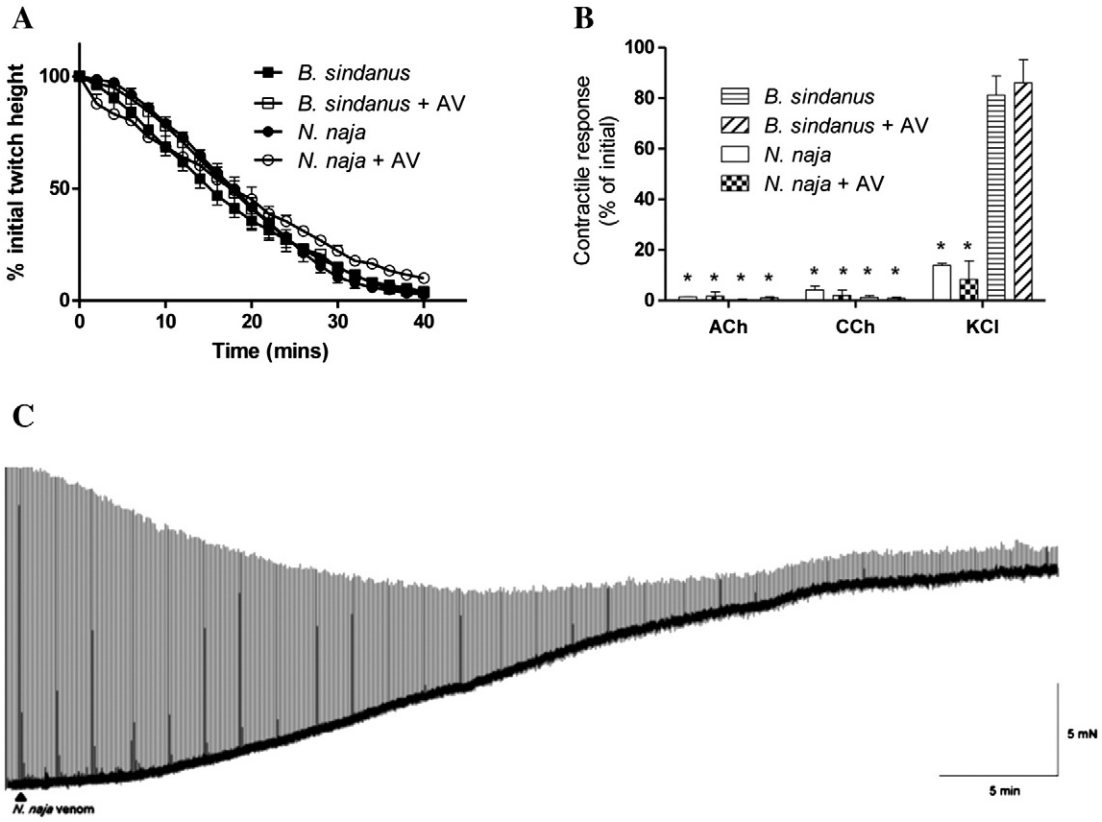

Fig. 5 – Effects of *B. sindanus* and *N. naja* crude venom (10 μg/mL) in the absence and presence of 10× dose BPAV on (A) nerve-mediated twitches of the chick biventer nerve-muscle preparation and (B) responses to exogenous ACh (1 mM), CCh (20 μM) and KCl (40 mM). * P < 0.0001, two-way ANOVA, compared to initial response, *n* = 3. (C) *N. naja* crude venom induced rise in base-line tension indicative of myotoxicity.

currently the only antivenom available in Pakistan. On the other hand, Vins polyvalent antivenom (VPAV manufactured by Vins Bioproducts Limited, Hyderabad, India) was demonstrated in the aforementioned study to be moderately effective against all Asian species of *Naja* and several species of *Bungarus* [14]. VPAV is currently unavailable in Pakistan, but a future study should assess the efficacy of this antivenom in neutralizing the toxic effects of the venoms of Pakistan's dangerous species of snake. Although antivenom made in Pakistan with antibodies raised against the venoms of Pakistani snakes is the ideal, it is worth investigating the possibility that VPAV may be preferable to BPAV until such a product becomes available. Further studies could also assess the specific antigenicity of antivenoms via enzyme-linked immunosorbent assay (ELISA) and/or immunoblotting. It has previously been speculated that components of the venom of the monocled cobra of Thailand (*N. kaouthia*), which are highly toxic but poorly immunogenic, such as 3FTx, and may not be effectively neutralized by currently available antivenoms [16]. This possibility should also be investigated with regard to Pakistani snake species like *N. naja* and *B. sindanus*.

Of particular note in this study is the fact that *N. naja* venom (at 10 μg/mL) induced strong muscle contractures and reduced the response to an elevated level of potassium (K^+), features of which are indicative of the presence of myotoxic components in the venom [19]. Although myotoxicity is uncommon as a clinical symptom following envenoming by most elapid snakes, myotoxic PLA_2 enzymes have been found in certain Australian elapids, with *Pseudechis australis* venom being particularly rich in them (cf. [20,21]). Myotoxic PLA_2 in the venoms of Australian elapid snakes are known to induce severe muscle damage, resulting in clinical rhabdomyolysis and dark brown urine [22]. In contrast, the source of occasional myotoxic symptoms resulting from bites from non-Australian elapids has been difficult to ascertain. Within the *Naja* genus, low-to-moderate myotoxicity has been reported for *Naja haje*, *N. kaouthia*, *N. naja*, *Naja nigricollis* and *Naja nivea* [23–25] with the effects speculated to be caused by PLA_2. However, injection of purified PLA_2 isolated from *N. naja*, *N. nivea*, *N. haje* and *N. nigricollis* did not result in myotoxic activity in vivo, instead producing presynaptic neurotoxicity. Furthermore, inhibition of the PLA_2 activity of *N. kaouthia* venom in vitro did not affect the direct myotoxicity observed. It was thus concluded that cardiotoxic 3FTx were an integral part of the myotoxic effects seen in vitro [25]. Thus it is strongly suggestive that the unusually potent myotoxic effects of the *N. naja* venom in this study may not be due to PLA_2, which are in low amounts, but rather due to the rich pool of cytotoxic 3FTx that we have shown to be present in this venom (Supplementary Tables 3–5) and which have previously been shown to be present in the venom of *N. atra* [26]. Cytotoxic 3FTx are also the predominant component of the venoms of African spitting cobras which are known to cause severe local tissue damage and haemorrhaging (*Naja katiensis*, *Naja mossambica*, *N. nigricollis*, *Naja nubiae* and *Naja*

Table 1 – Orthologous proteins of combination of 2D Gel spots and shotgun sequencing.

Toxin type	*Bungarus sindanus* matches to other *Bungarus* toxins			*Naja naja* matches to other *Naja* toxins		
	Uniprot	Matched peptides	% coverage	Uniprot	Matched peptides	% coverage
3FTx	O12962	9	58.6	P01441	55	96.7
	Q8AY56	16	71.3	P01447	53	96.7
	A2CKF6	1	11.6	P01391	32	93
	Q8AY51	5	46.5	P29180	3	56.9
	A2CKF6	1	11.6	P01391	32	93
	Q8AY51	5	46.5	P29180	3	56.9
	Q9YGI8	17	59.3	P01427	11	80.3
	Q27J48	3	14.7	P82885	4	62
				Q9W716	21	74.7
				Q98959	39	74.1
				P01400	6	87.7
				P29181	7	75.4
				Q9PTT0	1	31.3
				P82463	5	75.4
				Q9W727	1	11.6
ACN	Q92035	4	13.9			
CRiSP	Q7ZZN8	6	28.6	Q7T1K6	5	23.4
	Q8UW11	4	26.5	Q2XXP4	2	10.2
	Q8AVA4	2	9.2			
Kunitz	Q8AY43	1	16.9	P19859	3	84.2
				P20229	11	70.2
LAO	A8QL51	3	14.1	A8QL58	15	25.2
NGF				Q5YF89	3	14.1
PLA_2	P14615	6	38.3	P60045	10	80.2
	P81166	6	25.4	Q91133	17	69.2
	Q9I843	2	27.6	Q91900	2	10.5
	P0C551	3	19	P15445	3	22.7
	Q8QFW4	15	68.7	P00598	3	78.8
	Q8QFW3	14	65.3	P15445	4	46.46
SVMP				P82942	10	18.5

ACN = acetylcholineseterase; LAO = L-amino acid oxidase; NGF = nerve growth factor; PLA_2 = phospholipase A_2; SVMP = snake venom metalloprotease.

pallida) [27] and have been demonstrated to be potently cytotoxic in vitro [28]. It is likely, therefore, that this abundance of cytotoxic 3FTx (aka: cardiotoxins) is common to the venoms of the majority of species of *Naja* worldwide and is responsible for many of the clinical sequelae resultant from bites by these snakes, which often leave survivors permanently crippled as a result of local tissue destruction.

The results of the present study indicate a path for further research attempting to positively identify the myotoxic components of *N. naja* venom, which may be of considerable biodiscovery potential. On a more practical level, these findings also reveal that myotoxicity, as well as cytotoxicity, may be a clinically significant feature of envenomations from Pakistani *N. naja*, and perhaps from the same species elsewhere. In addition and of most immediate concern, our results reveal the failure of polyvalent antivenom (BPAV) to effectively neutralize the venoms of *Bungarus* or *Naja* in Pakistan. This finding highlights the dire need for effective antivenom to be made available in Pakistan. To this end, the polyvalent antivenom manufactured by Vins Bioproducts should be tested against the venoms of Pakistani snakes, as it may prove to be a more effective substitute for BPAV until such time as a polyvalent antivenom raised against the venoms of Pakistani snakes is manufactured.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.jprot.2013.05.015.

Acknowledgements

SAA was the recipient of postdoctoral fellowship (PDRF Phase II Batch-V) from Higher Education Commission (HEC Islamabad) Pakistan. BGF was funded by an Australian Research Council Future Fellowship and by the University of Queensland. TNWJ was funded by an Australian Postgraduate Award. EABU acknowledges funding from the University of Queensland (International Postgraduate Research Scholarship, UQ Centennial Scholarship, and UQ Advantage Top-Up Scholarship) and the Norwegian State Education Loans Fund.

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