JOURNAL OF PROTEOMICS 89 (2013) 15–23

Available online at www.sciencedirect.com

ScienceDirect

www.elsevier.com/locate/jprot

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

Syed A. Ali[a,b,c], Daryl C. Yang[d], Timothy N.W. Jackson[a,b], Eivind A.B. Undheim[a,b], Ivan Koludarov[a], Kelly Wood[a], Alun Jones[b], Wayne C. Hodgson[d], Sean McCarthy[e], Tim Ruder[a], Bryan G. Fry[a,b,*]

[a]Venom Evolution Lab, School of Biological Sciences, University of Queensland, St. Lucia, Qld 4072, Australia
[b]Institute for Molecular Bioscience, The University of Queensland, St. Lucia, Queensland 4072, Australia
[c]HEJ Research Institute of Chemistry, International Centre for Chemical and Biological Sciences (ICCBS), University of Karachi, Karachi 75270, Pakistan
[d]Department of Pharmacology, Monash University, Victoria, Australia
[e]Snakehandlers Pty Ltd, Hastings, Victoria, Australia

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.

* *Corresponding author at:* Venom Evolution Lab, School of Biological Sciences, University of Queensland, St. Lucia, Qld 4072. Tel.: +61 400193182.
E-mail address: bgfry@uq.edu.au (B.G. Fry).

1874-3919/$ – see front matter © 2013 Elsevier B.V. All rights reserved.
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