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Venomics of the Australian eastern brown snake (*Pseudonaja textilis*): Detection of new venom proteins and splicing variants

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ABSTRACT

The eastern brown snake is the predominant cause of snakebites in mainland Australia. Its venom induces defibrination coagulopathy, renal failure and microangiopathic hemolytic anemia. Cardiovascular collapse has been described as an early cause of death in patients, but, so far, the mechanisms involved have not been fully identified. In the present work, we analysed the venome of *Pseudonaja textilis* by combining high throughput proteomics and transcriptomics, aiming to further characterize the components of this venom. The combination of these techniques in the analysis and identification of toxins, venom proteins and putative toxins allowed the sequence description and the identification of the following: prothrombinase coagulation factors, neurotoxic textilotoxin phospholipase A_2 (PLA_2) subunits and "acidic PLA_2", three-finger toxins (3FTx) and the Kunitz-type protease inhibitor textilinin, venom metalloproteinase, C-type lectins, cysteine rich secretory proteins, calreticulin, dipeptidase 2, as well as evidences of *Heloderma* lizard peptides. Deep data-mining analysis revealed the secretion of a new transcript variant of venom coagulation factor 5a and the existence of a splicing variant of PLA_2 modifying the UTR and signal peptide from a same mature protein. The transcriptome revealed the diversity of transcripts and mutations, and also indicates that splicing variants can be an important source of toxin variation.

1. Introduction

Snake bites are an important public health problem in many parts of the rural, developing world, mostly in poor, tropical and subtropical areas (Harrison et al., 2009). There may be as many as 4.5 to 5 million snake bites/year globally, resulting in 2.5 million envenomings, 125,000 deaths and perhaps three times that number with permanent disabilities (Chippaux, 1998). However, an accurate estimation of snake bite envenoming is difficult to establish (WHO, 2007) and these numbers may be much higher, as many estimates rely on hospital returns, which are not available in many areas, and do not capture data for cases that do not present to public health services. The mainstay of treatment for snake bite envenoming is antivenom obtained and purified from hyperimmune plasma, mostly from horses. The value of antivenom is however determined by its ability to effectively neutralize all of the medically relevant toxins in a venom from which is raised, and some toxins may be poorly neutralized (Gutiérrez et al., 1981; Judge et al., 2006). A better knowledge of venom components and their role in the onset of the pathophysiological effects might circumvent the lack of efficacy of available antivenoms against some components of snake venoms. The currently available cutting-edge proteomic and transcriptomic tools have made such studies possible, enabling the detection of new and/or rare toxins (Calvete, 2013;

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Paiva et al., 2014) as well as intraspecific ontogenic and geographical variations of the venom (Castro et al., 2013; Madrigal et al., 2012). Such studies have improved our knowledge of venoms and how they vary within species and genera, providing tools to better understand the molecular evolution of toxins, as well as to identify potential targets for the design of more effective antisera. The combined proteomic/transcriptomics approach is also relevant to affirm new transcripts as real toxins, as common tissues can express toxin transcripts, even in non-venomous snakes such as pythons (Reyes-Velasco et al., 2014).

In the present work, our aim was to characterize the venom gland products from the eastern brown snake *Pseudonaja textilis* by combining transcriptomic and proteomic studies of the gland and its venom. The eastern brown snake is the predominant cause of accidents in mainland Australia (White, 2009), due to its abundance and adaptation to peridomicile, even in urban environments. Its venom has been described as containing toxins that induce defibrination coagulopathy, renal failure and microangiopathic hemolytic anemia (White, 2009). Although neurotoxicity is considered to be rare (Barber et al., 2012; White, 2009), a potent presynaptic neurotoxin named textilotoxin has been described (Aquilina, 2009; Pearson et al., 1993) as well as short and long three-finger postsynaptic toxins (3FTx) (Gong et al., 2001, 2000; St Pierre et al., 2007b). The low *P. textilis* venom yield (Mirtschin et al., 2002) associated to low neurotoxins concentrations (Barber et al., 2012) and the usual low venom/victim weight ratio (Mirtschin et al., 1998) can be considered as an explanation for this apparent paradox, although specificity of the toxin towards nerve terminals in a specific prey type should not be discounted as a possible explanation. According to Judge et al. (2006), there is an accumulating body of evidence to suggest that the efficacy of the brown snake antivenom is limited. These authors report that the antivenom does not recognize the low molecular mass protein components of the venoms of *P. textilis, P. affinis affinis* and *P. nuchalis* when assayed by western blot, nor was the antiserum able to neutralize the contractile response of tracheal nerve/muscle preparations. These observations suggest that either these toxins are poorly immunogenic or that they might not be present in the venom pool used to produce the antiserum. Indeed, according to the World Health Organization (2007) "Ineffective antivenoms may also be prepared because of an inappropriate selection of the venoms used as immunizing mixtures. This illustrates a lack of information on the snake fauna of the area or region as well as on the composition and immunochemistry of venoms". This highlights the need of further investigation of venom components and how they correlate with clinical observations.

2. Material and methods

2.1. Biological samples

The venom gland was extracted from an adult *Pseudonaja textilis* male individual captured at Barossa Valley, near Adelaide, South Australia. The venom gland was extracted three days after milking to obtain a tissue with a high level of toxin transcript expression, and stored in RNA-Later® (QIAGEN N.V., Netherlands) at −80 °C until RNA extraction. The venom of *P. textilis* used for proteomics and toxin purification was a pool from five captive individuals from the same region. The crude venom was lyophilized and stored at −20 °C until use. The animal was euthanized for tissue collection in accordance with Euthanasia of Animals Used for Scientific Purposes guidelines (2001), Australian and New Zealand Council for the Care of Animals in Research and Teaching, under the monitoring of the SA Pathology/CHN Animal Ethics Committee, Project Approval 93/12.

2.2. Venom gland de novo transcriptome

The venom gland *de novo* transcriptome was obtained by shotgun pyrosequencing (GS-FLX, Roche) of a normalized cDNA library (GATC Biotech, Konstanz, Germany). After trimming, the resulting reads were aligned and assembled with Newbler™ (Roche). Resulting isotigs and singletons were identified and annotated with BLAST2GO (Conesa et al., 2005; Götz et al., 2008). All steps were manually eye-checked and fixed when necessary.

2.3. Venom 2D-PAGE, in-gel digestion and MS^2 analysis

Prior to use, the venom sample was dissolved to 170 μg/ml in 9 M urea, 2% ampholytes and 70 mM DTT. After 30 min room temperature incubation and centrifugation (45 min, 15,000 *g*) the supernatant was removed and frozen at −80 °C. The protein mixture was decomplexed by 2D-PAGE using a slightly adapted method from previous works (Georgieva et al., 2011; Meganathan et al., 2012). The selected spots were collected and in-gel digested with Trypsin (Promega, USA). Peptides were analysed by liquid chromatography (LC) followed by electrospray ionization (ESI) and detected in an ion trap mass spectrometry system (Agilent 1100 LC/MSD-trap XCT series system) (Viala et al., 2014). The most intense ions were fragmented by collision-induced dissociation (CID) and MS^2 spectra were acquired. The protein identification was performed based on the public protein database enriched with our in house *P. textilis* transcriptome, using the InChorus multi-algorithmic tool from PEAKS (Bioinformatics Solutions Inc., Canada) that integrates PEAKS and MASCOT (Matrix Science Inc., USA) identification results. Identity was considered when significant score was achieved. All MS/MS assignments and automatic *de novo* sequencing results were manually revised for correctness as well as the quality of the mass spectra of peptides from near-threshold identification.

2.4. Anti-jararhagin western blot

Crude lyophilized *P. textilis* venom was redissolved (2 mg/mL in PBS pH 7), centrifuged and 30 μL of the supernatant were diluted in 10 μL of non-reducing buffer and submitted to 15% SDS-PAGE (Laemmli, 1970). The gel was placed in the electroblot apparatus and transferred to nitrocellulose paper in transfer buffer for 90 min at 0.85 mA/cm^2 (Towbin et al., 1979). The nitrocellulose paper was then incubated with polyclonal anti-jararhagin antibodies (diluted 1:5000). Jararhagin is a P-III metalloproteinase from *Bothrops jararaca* and the antibodies were gently provided by Dr. Maísa Splendore Della Casa (Instituto Butantan, São Paulo, Brazil). The immunoreactive proteins were detected using peroxidase-labelled anti-rabbit IgG and the blot was developed with orthophenyldiamine in the presence of 0.03% H_2O_2 (v/v).

2.5. Metalloproteinase cDNA cloning and sequencing

The metalloprotease transcript was cloned from a *P. textilis* venom gland cDNA library, built using In-Fusion SMARTer cDNA library construction kit (Clontech Laboratories Inc., USA). RNA was extracted with Trizol® reagent (Life Technologies, USA) in an RNAse free environment. The 20-mers primers (5′UTR: 5′-TTGGAAGCAGAAAGAGATTC-3′ and 3′UTR: 5′-GTAGGATAAAGACAGATGGG-3′) were designed based on conserved regions found by aligning metalloproteases untranslated region (UTR) sequences from Elapidae, Colubridae and Viperidae species, available in public databases (GenBank, NCBI). The 5′UTR and 3′UTR sequences were first separated from the open reading frame (ORF), then the UTRs were aligned independently (Hall, 1999; Lassmann et al., 2009). PCR