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

Vincent Louis Viala [a], Diana Hildebrand [b], Maria Trusch [c], Tamara Mieco Fucase [a], Juliana Mozer Sciani [d], Daniel Carvalho Pimenta [d], Raghuvir K. Arni [e], Hartmut Schlüter [b], Christian Betzel [c], Peter Mirtschin [f], Nathan Dunstan [f], Patrick Jack Spencer [a, *]

[a] Centro de Biotecnologia, IPEN, Av. Lineu Prestes 2242, 05508-000, São Paulo, SP, Brazil
[b] Institut für Klinische Chemie, Universitätsklinikum Hamburg-Eppendorf, Haus Ost 26, Martinistr. 52, 20246 Hamburg, Germany
[c] Institut für Biochemie und Molekularbiologie, Universität Hamburg, Martin-Luther-King-Platz 6, 20146, Hamburg, Germany
[d] Laboratório de Bioquímica e Biofísica, Instituto Butantan, Av. Vital Brasil, 1500, 05503-900, São Paulo, SP, Brazil
[e] Departamento de Física, IBILCE, UNESP, Rua Cristóvão Colombo, 2265 15054-000, São José do Rio Preto, SP, Brazil
[f] Venom Supplies Pty. Ltd., PO Box 547, 5352, Tanunda, South Australia, Australia

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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;

* Corresponding author.
E-mail address: pspencer@ipen.br (P.J. Spencer).

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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

reaction was performed using Taq DNA polymerase (Biotools B&M Labs S.A., Spain) in the following conditions: 1X 94 °C 5 min + 40X 94 °C 30 s, 51 °C 30 s and 72 °C 2min + 1X 72 °C 5 min (Thermal cycler CG1-96, Corbett Research, QIAGEN N.V., Netherlands). The resulting amplification bands were excised from the agarose gel, extracted with DNA gel extraction kit (Norgenbiotek, Canada) and cloned into pGEM-Teasy® (Promega, USA). *E. coli* DH5α electrocompetent cells (New England Biolabs Inc., USA) were transformed with the ligated plasmids. After ampicillin and white/blue selection of the recombinant colonies, plasmids were extracted by miniprep (QIAGEN N.V., Netherlands) and clones sequencing was performed on an ABI 3730 DNA Analyser with BigDye (Applied Biosystems) and universal forward and reverse M13 primers. As the forward and reverse sequences did not overlap, another forward primer Ptint1F (5′-ACTTCGGAGTCAGATGAGCC-3′) was designed to obtain the missing overlap sequence. Resulting sequences were aligned and the final consensus sequence was generated.

3. Results

By combining transcriptomics and proteomics high-throughput techniques in the analysis and identification of toxins, venom proteins and putative toxins, a big volume of data was generated. The data-mining results were evaluated and discussed in the light of toxinology, biochemistry, genetics, biology, and evolutionary knowledge. A previous proteomic analysis of this venom was performed by Birrell et al. (2006) in which the following toxins were identified: Pseutarin-C (catalytic and non-catalytic subunits), neurotoxic phospholipase A_2 (PLA_2) textilotoxin subunits and other PLA_2s, 3FTxs, textilinin, the pseudechetoxin-like cysteine rich secretory proteins (CRiSP) and an additional unknown protein in venoms, the Glucose Regulated Protein 78. Our combined analysis identified the following toxins in the *P. textilis* venome: prothrombinase complex coagulation factors, textilotoxin subunits, procoagulant PLA_2, short and long 3FTxs, the Kunitz-type protease inhibitor textilinin (bovine protease trypsin inhibitors family − BPTI), CRiSP and for the first time, a new splicing variant of the snake venom coagulation factor V_a ($VF5_a$), a yet undescribed long 3FTx (LNTX-2), C-type lectins (CLect), as well as evidences of lizard toxins from *Heloderma* genus and other toxin candidates such as calreticulin and dipeptidase 2. Some of those toxins were identified in a pool of venoms at the isoform level, supported by the observation of unique peptides. A venom metalloproteinase (SVMP), a class of toxins poorly investigated in elapidae, was detected by western blot, cloned and sequenced. Also, we identified at the transcript level a PLA_2 signal peptide switching mechanism driven by alternative splicing.

3.1. Venom gland transcriptome

Next Generation Sequencing of the transcriptome generated 104,878 reads (c.a. 37 Mb) with 34–944 base pairs (bp) (N50 = 466 bp) and 42.25% GC. The assembly generated 1518 isotigs (N50 = 916 bp, longer isotig = 6179 bp), clustered into 1266 isogroups, and 41,514 reads were considered singletons, totalizing 43,032 sequences. The BLASTx resulted in 192 transcripts of interest annotated as toxins, related to venom or considered putative new toxins. Coding and truncated toxins transcript/sequences were classified into 3FTx, BPTIs, $VF5_a$ and snake venom coagulation factor X_a ($VF10_a$), PLA_2, CLect, hyaluronidase, natriuretic peptides (NP), wey acidic proteins (WAP), veficolin, CRiSP, SVMP, calreticulin, transferrin, cobra venom factor (CVF), dipeptidylpeptidase IV and VIP/glucagon-like (vasoactive intestinal peptides). These sequences were individually checked for quality and UTRs, ORFs and their translated amino acid sequences, signal peptides, mutations and alternative splicing were identified. An in house transcriptomic annotated database was compiled and used for the next steps of venomics identification. This Transcriptome Shotgun Assembly project has been deposited at DDBJ/EMBL/GenBank under the accession GDAD00000000. The version described in this paper is the first version, GDAD01000000.

3.2. Venom proteome

The 2D gel decomplexed the crude venom into spots with a wide range of molecular mass (from 10 to >100 kDa) and a wide range of pI (3–10) (Fig. 1). 120 spots were collected manually from different regions of the 2D gel and protein clusters. Venom protein identification was successful in 86 spots. After data mining they were classified into eight toxin families ($VF5_a$; $VF10_a$; PLA_2: A and B chains from the multimeric PLA_2 textilotoxin and "acidic PLA_2"; 3FTx; BPTI textilinin; CLect; CRiSP and *Heloderma* peptides). The InChorus multi-algorithmic identification results are listed in the Supplementary Material. Nineteen sequenced transcripts encoding for $VF5_a$, $VF10_a$, Acidic PLA_2, PLA_2 textilotoxin A, CRiSP, LNTx-1, LNTx-2, SNTx, NXS7, textilinin, CLect and calreticulin were identified in 67 out of the 85 positive spots (Supplementary Material). The long 3FTx (LNTX-2) and the VF5a-3 are new variants that were identified at the protein level.

3.3. Venom metalloproteinase detection, cDNA cloning and sequencing

When the crude venom was analysed by western blot using a polyclonal antibody raised against jararhagin, a single reactive band was detected around 45 kDa (data not shown), indicating the presence of a metalloproteinase. A full transcript was cloned and the sequence revealed to encode a P-III SVMP. The sequence was translated and the protein domains were identified by homology with similar sequences and a DCD motif was observed in the disintegrin domain (Fig. 2). The translated protein has 612 residues and calculated molecular mass (MM) of 68.5 kDa. After trimming out the signal peptide and the propeptide, the putative secreted protein has a predicted mass around 45 kDa and pI around 5.

4. Discussion

4.1. P. textilis toxins

4.1.1. Prothrombinase complex: coagulations factors Va and Xa

The venom coagulation factors $VF5_a$ and $VF10_a$ are present in large amounts in some Australian Elapidae venom such as *Oxyuranus microlepidotus*, *Oxyuranus scutellatus* and *Pseudonaja textilis* (Bos and Camire, 2010; Masci et al., 1988). In *P. textilis* venom, $VF5_a$ and $VF10_a$ form the prothrombinase complex named Pseutarin-C (Kini et al., 2001), with similar characteristics to the blood plasma prothrombinase complex, essential for blood clot formation by converting prothrombin into thrombin (Mann et al., 1990; Rao and Kini, 2002). The venom prothrombinase complex was recruited to the venom gland after successive gene duplication and rapid mutation accumulation (Minh Le et al., 2005), which enabled the toxins to be secreted already activated, modified to escape the protein C inactivation system and with no need for membrane interaction (Bos et al., 2009; Rao et al., 2003). Pseutarin-C is an effective *in vitro* procoagulant, but has the opposite effect *in vivo* by quickly exhausting the prey's blood plasma prothrombin, resulting in coagulopathy and spontaneous bleeding due to exhaustion of clotting factors (Tibballs et al., 1992, 1991).

4.1.1.1. $VF5_a$. Two full transcripts encoding for $VF5_a$ were identified

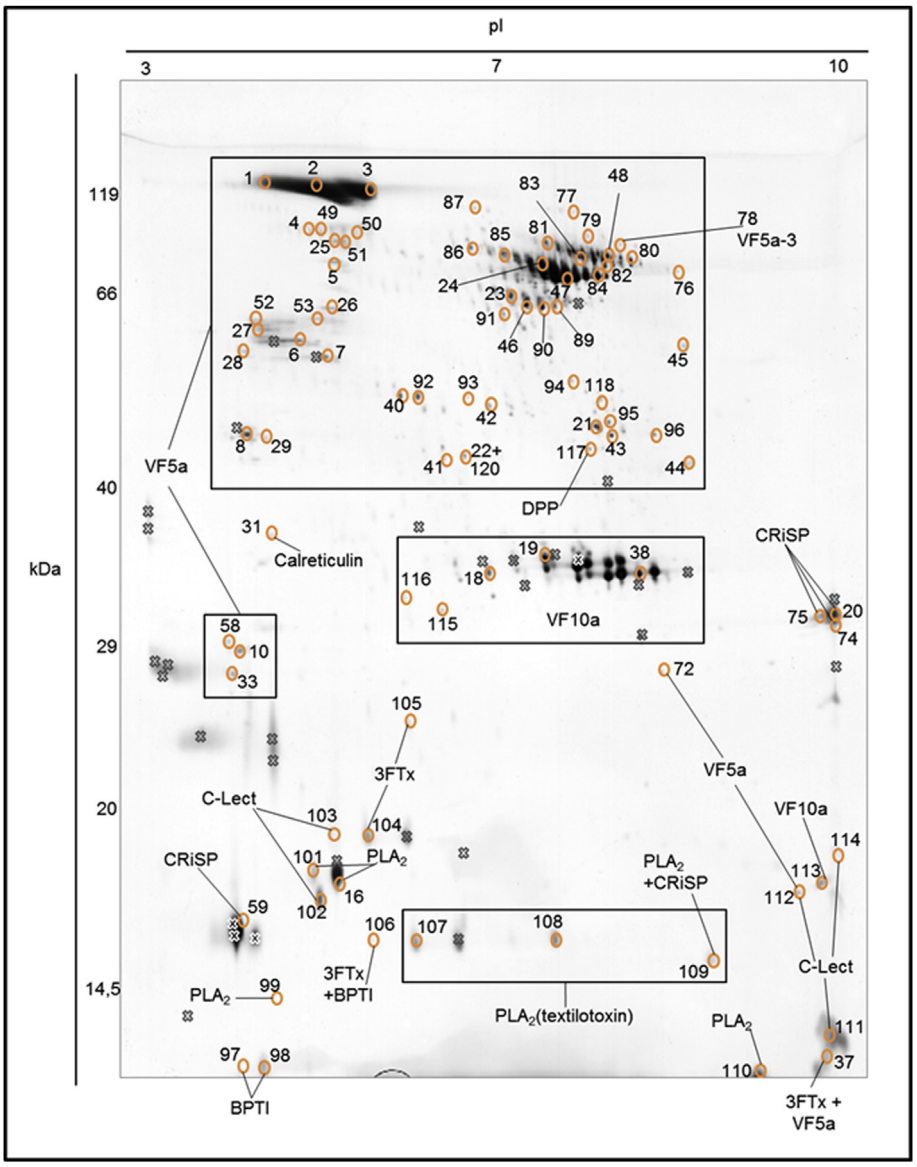

Fig. 1. 2D-PAGE of *Pseudonaja textilis* and the assigned spots (circles). Collected spots without positive match are crossed. Clusters of toxins are highlighted by labelled rectangles. VF5a: Coagulation venom factor 5a; VF10a: Coagulation venom factor 10a; CRiSP: Cysteine rich secretory protein; C-Lect: C type lectins; PLA_2: Venom phospholipase A_2; BPTI: Bovine protease trypsin inhibitor (textilinin); 3FTx: Three-finger toxin; DPP: Dipeptidylpeptidase-2.

(isotigs 199 and 200). The isotig 200 encodes a new isoform, named here VF5a2, similar to the venom prothrombin activator pseutarin-C non-catalytic subunit FA5V_PSETE (UniProt accession number) (Rao et al., 2003), the only one described so far in *P. textilis*. It contains minor mutations in the nucleotide and amino acid sequences, when compared to the previously described toxin FA5V_PSETE, but none at cleavage and known interaction sites (Camire and Bos, 2009). The isotig 199, named here VF5a3, encodes for the isoform VF5a2 increased by a 102 bp insertion at the ORF position 2528. Both $VF5_a2$ and $VF5_a3$ sequence were aligned (BLAST) against the *Ophiophagus hannah* genome (Vonk et al., 2013), as no *P. textilis* genomic sequence coding for $VF5_a$ is available in public databases, and both matched entirely and uniquely into the assembly scaffold 988.1 (Accession gb|AZIM01000987.1). The alignment shows that the $VF5_a3$'s 102 bp insertion is an extra exon, named here exon 15 (Fig. 3). Consequently, $VF5_a3$ might be a splicing variant. More interestingly, this unique additional amino acid sequence, translated from exon 15, was identified in the proteome analysis as described below.

In general, the proteome analysis detected $VF5_a$ tryptic peptides in 57 spots (1–8, 10, 21–29, 33, 37, 40–53, 58, 72, 76–87, 90–96, 112 and 120). A wide range of mass and pI distribution was observed in the 2D gel, with evident clusters, indicating the diversity of post-translational modifications (PTMs), and possibly proteolysis (Fig. 1).

The VF5a isoforms identified were: the *Oxyuranus scutellatus* (FA5V_OXYSU) (Welton and Burnell, 2005), *Pseudonaja textilis* venom and blood coagulation factor (FA5V_PSETE and FA5_PSETE) (Rao et al., 2003) and the two new isoforms described in this transcriptome (VF5a2 and VF5a3). The splicing variant VF5a3 contains an extra sequence of 34 amino acid residues (exon 15), after the Gln846 (KSQKLFWKIEESELESRKRIEKDKYIYSEENIKE). A set of two unique peptides IEESELESR and DKYIYSEENIK identified in spot 78 confirm that this product of alternative splicing is indeed translated and secreted in the venom (Supplementary Material). To our knowledge, this is the first time that an alternative splicing

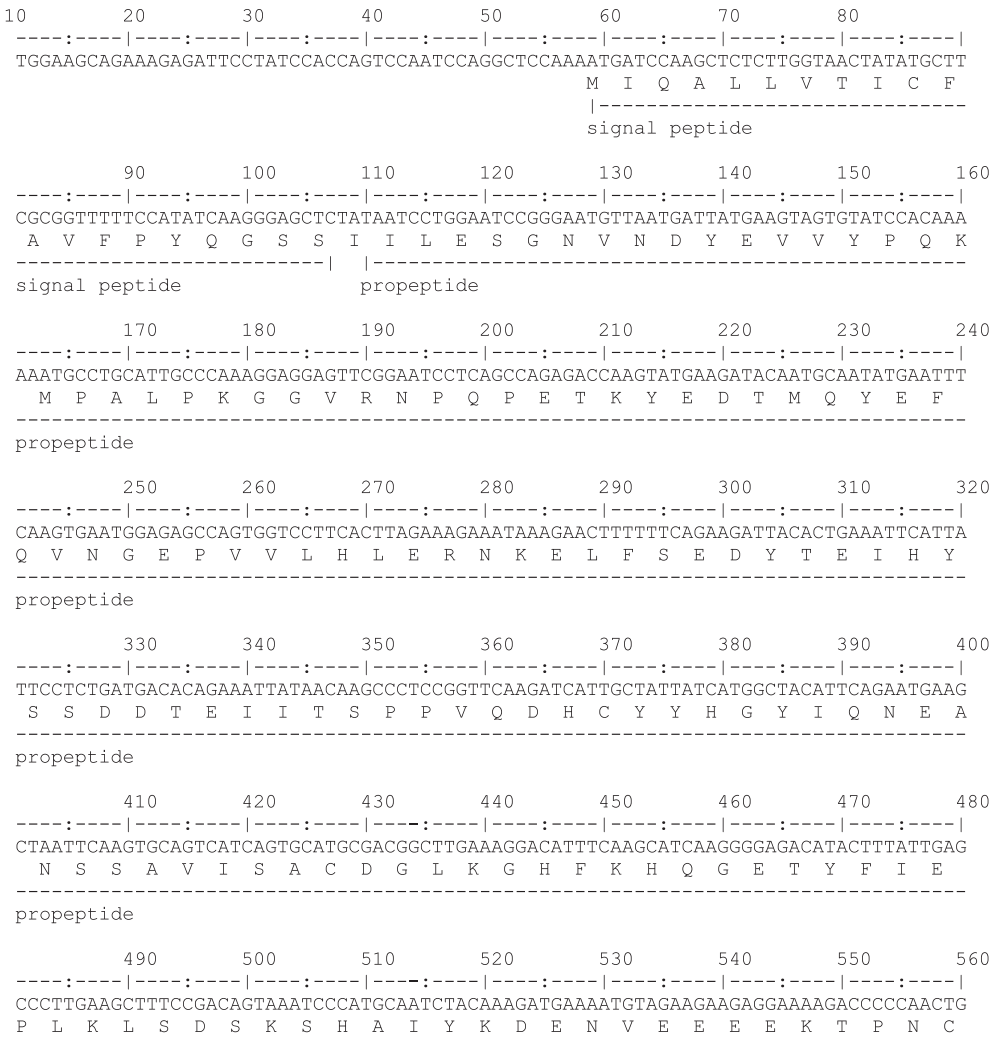

Fig. 2. cDNA sequence and translation of the cloned *P. textilis* P-III SVMP. Domains were identified by homology. The highlighted grey box indicates the DCD disintegrin tripeptide motif.

variant of snake venom toxin is detected at the protein level.

4.1.1.2. VF10$_a$. Two isotigs (188 and 189) encoding for VF10$_a$ were identified, with a slight difference only between their 3′UTR regions (17 pb gap). The ORF have 99% of identity with the only one described sequence (Accession: FAXC_PSETE) (Filippovich et al., 2005), but no mutations were found in known active sites. VF10$_a$ was identified in five spots (18, 38, 113, 115 e 116) from a single cluster with c.a. 35 kDa and pI between 6 and 8.5 (Fig. 1).

4.1.2. Phospholipases A$_2$

PLA$_2$s are ubiquitous enzymes in nature (Kini, 2003). Snake venom PLA$_2$s were recruited to the venom gland and lost their original function (Ohno et al., 1998). The great diversity of venom PLA$_2$s is best illustrated by the many different neurotoxic, myotoxic, cardiotoxic, haemolytic, haemorrhagic, hypotensive, anticoagulant and other functions (Kini, 2003). The most studied *P. textilis* PLA$_2$s are the textilotoxin subunits (A to D), which form a potent heterohexameric presynaptic neurotoxin (Aquilina, 2009; Pearson et al., 1993). Another PLA$_2$ with apparent procoagulant activity has also been described (Armugam et al., 2004).

Many transcripts were identified as snake venom PLA$_2$ (Table 1). The “acidic PLA$_2$” was found in isotigs 824, 263 and 264. Isotig 824 ORF is identical to the “acidic PLA$_2$ 1” (Accession: PA2A1_PSETE) (Armugam et al., 2004). The isotigs 263 and 264 encode for the same mature protein, with 99% identity to the “acidic PLA$_2$ isoform 2” (Accession: PA2A2_PSETE) (Armugam et al., 2004), and were named here as “PLA$_2$ isoform 3” and “PLA$_2$ isoform 4”, respectively. Isotig 264 5′UTR and signal peptide nucleotide sequences are different from the 263's (and consequently the signal peptide first 9 aa residues).

When carefully analysed, aligning the nucleotide sequences of isotigs 263 and 264 with the available gene sequences of this PLA$_2$ (GenBank Accession: AY027495.1) (Armugam et al., 2004), the isotig 264 was identified as a new splicing variant, with a different 5′UTR and a new start codon inside the usual first intron (Fig. 4). Interestingly, this new signal peptide matches with *Micrurus fulvius* (e.g. Accession: U3F5A1_MICFL) and *Bungarus multicinctus* (e.g. Accession: Q8AXW0_BUNMU) PLA$_2$ signal peptide. We identified unique peptides from this acidic PLA$_2$ in spots 16, 101 and 110.

Textilotoxin was also identified in the venome. Isotigs 814, 883, 819 and 800 encode for subunits A, B, C and D respectively, which translated amino acid sequences are all 100% identical to those already described in the literature, except for the isotig 883 (named here as subunit B isoform 2) with two substitutions out of the 121 residues. These data indicate a strong positive selection upon those

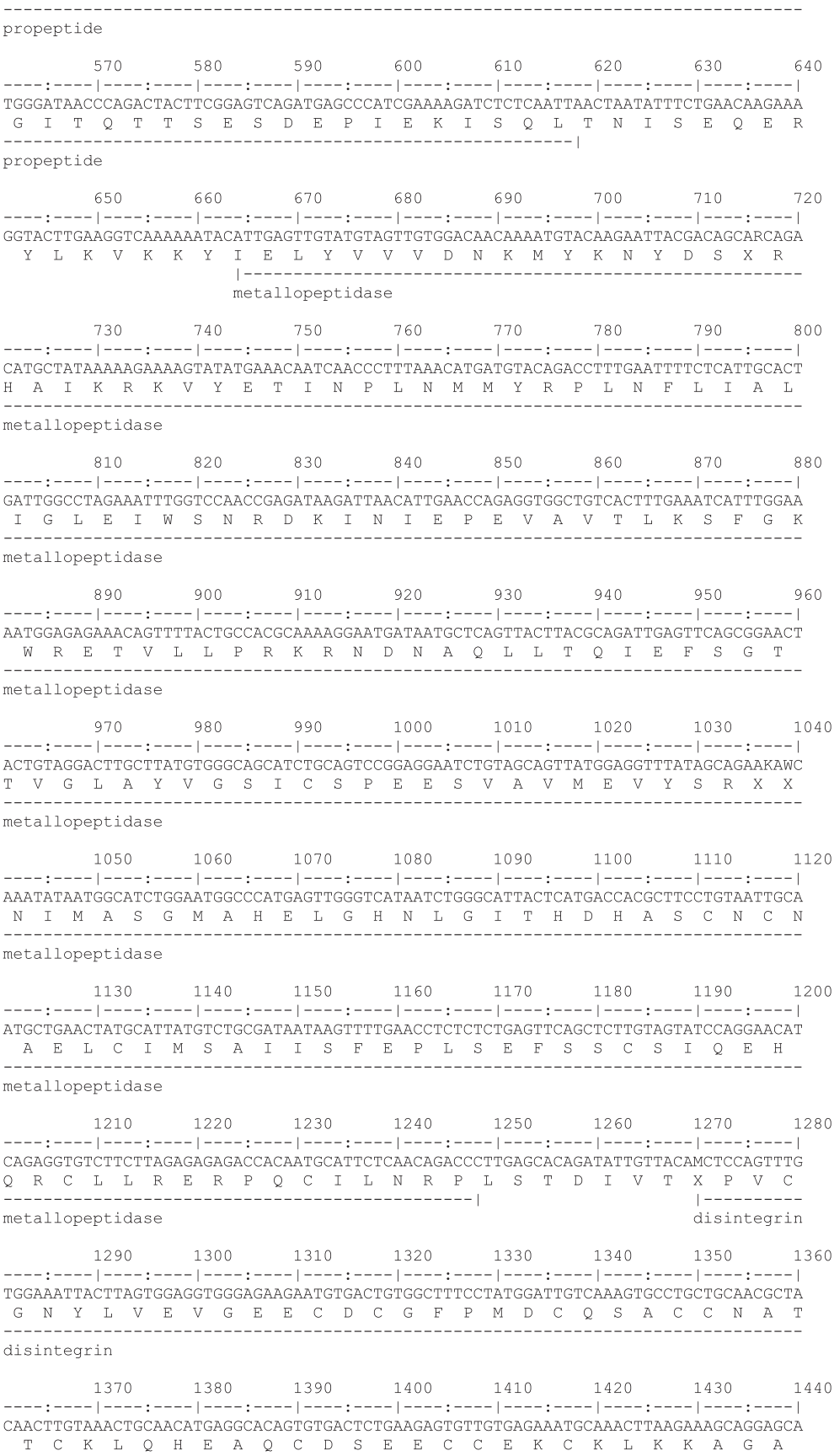

Fig. 2. (*continued*).

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------------------------------------------------------------------------------
disintegrin

         1450      1460      1470      1480      1490      1500      1510      1520
----:----|----:----|----:----|----:----|----:----|----:----|----:----|----:----|
GAATGCCGGGCAGCAAAGGATGACTGTGACTTGCCTGAAATCTGCACTGGCCAATCTGCTGAGTGTCCCATGGACAGCTT
E  C  R  A  A  K  D  D  C  D  L  P  E  I  C  T  G  Q  S  A  E  C  P  M  D  S  F
------------------------------------------------------------------------------
disintegrin

         1530      1540      1550      1560      1570      1580      1590      1600
----:----|----:----|----:----|----:----|----:----|----:----|----:----|----:----|
CCAGAGGAATGGACATCCATGCCAAAACAACCAAGGTTACTGCTACAATGGGAAATGCCCCATCATGACAAACCAATGTA
 Q  R  N  G  H  P  C  Q  N  N  Q  G  Y  C  Y  N  G  K  C  P  I  M  T  N  Q  C  I
-------|  |---------------------------------------------------------------------
         Cysteine rich

         1610      1620      1630      1640      1650      1660      1670      1680
----:----|----:----|----:----|----:----|----:----|----:----|----:----|----:----|
TCGATCTCTGGGGGCCAGGTGTAAATGTGTCTCCAGATATATGTTTTACGTTGAACCAGTATAGCCAAGGTTGTGGCTTC
  D  L  W  G  P  G  V  N  V  S  P  D  I  C  F  T  L  N  Q  Y  S  Q  G  C  G  F
------------------------------------------------------------------------------
Cysteine rich

         1690      1700      1710      1720      1730      1740      1750      1760
----:----|----:----|----:----|----:----|----:----|----:----|----:----|----:----|
TGCAGAATGGAAAATGATACAAAGATTCCGTGTGCAGCAAAGGATAAAATGTGTGGCAAGTTAATATGCGAAAAGGGAAA
C  R  M  E  N  D  T  K  I  P  C  A  A  K  D  K  M  C  G  K  L  I  C  E  K  G  N
------------------------------------------------------------------------------
Cysteine rich

         1770      1780      1790      1800      1810      1820      1830      1840
----:----|----:----|----:----|----:----|----:----|----:----|----:----|----:----|
CTCGACATGCACCTGCTTTCCTACAACAGATGACCCAGATGATGGAATGGTTGAACCTGGAACAAAATGTGGAGATGGAA
 S  T  C  T  C  F  P  T  T  D  D  P  D  D  G  M  V  E  P  G  T  K  C  G  D  G  M
------------------------------------------------------------------------------
Cysteine rich

         1850      1860      1870      1880      1890      1900      1910      1920
----:----|----:----|----:----|----:----|----:----|----:----|----:----|----:----|
TGGTGTGCAGCAACAGGCAGTGTGTTGATGTGCAGACAGCCTACTGATCAAGCACTGGCTTCTCTCAATTTGATTTTGAA
  V  C  S  N  R  Q  C  V  D  V  Q  T  A  Y  *
-------------------------------------------|
Cysteine rich

         1930      1940      1950      1960      1970      1980      1990
----:----|----:----|----:----|----:----|----:----|----:----|----:----|----
GATCCTCATCCAGAAAGCTTCCCTCAAGTCCAAAGAGACCCATCTGTCTTTATCCTACAATCACTAGTGAATTC
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Fig. 2. (*continued*).

toxins. The textilotoxin B subunit nucleotide sequence is being described for the first time. The proteomics identified textilotoxin A and B subunits in spots 107 to 109.

A tryptic peptide with a sequence only found in the "basic PLA_2 CM-II" (UniProt Acession number: PA2B2_NAJMO) described in *Naja mossambica* (Joubert, 1977) was identified in the spots 90, 91 and 99. CM-II causes myonecrosis when injected intramuscularly, causes neuromuscular blockade with a gradual contracture and a decreased sensitivity to ACh and KCl (Lin et al., 1987).

4.1.3. Three-finger toxins

The 3FTx are non-enzymatic polypeptides with 60–74 aa residues. They are commonly known as 3FTx due to the three finger-like loops emerging from a hydrophobic core and linked together by four conserved disulfide bonds. Despite this similar structure, 3FTx can have different biological activities including the blockade of nicotinic acetylcholine receptors, distinct muscarinic acetylcholine receptor subtypes, L type Ca^{2+} channels, inhibition of acetylcholinesterase, platelet aggregation, or as analgesic and membrane

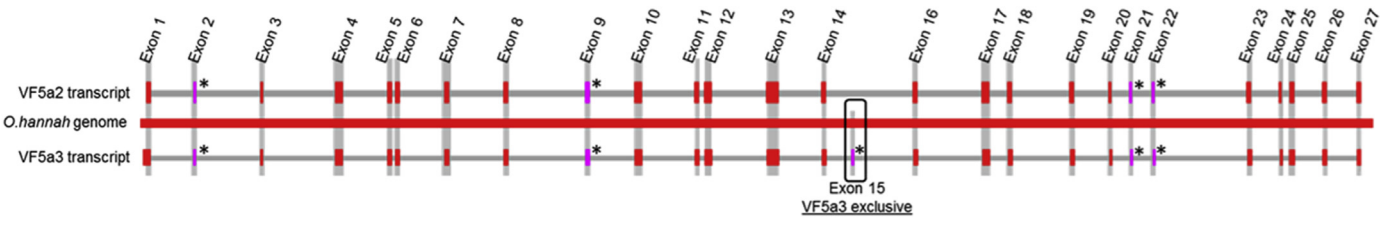

Fig. 3. Graphical overview of *Pseudonaja textilis* VF5a2 and VF5a3 transcripts alignment (BLAST) with the king cobra (*Ophiophagus hannah*) genome scaffold 988.1 (Accession gb|AZIM01000987.1), described by Vonk et al. (2013). An alternative splicing of this gene is demonstrated by the existence of exclusive exon 15 on the VF5a3 transcript (box). Alignments are coded by BLAST score. Exons labelled with * have a similarity score ranging from 80 to 200, unlabelled exons have similarity scores above 200. Horizontal lines connect exon sequences.

Table 1
Venomic PLA_2 identification details. The numbering of the spots (proteome) and the isotigs (transcriptome) are cited in columns 2 and 3 respectively. ND: not detected. Hit description: name obtained from SwissProt database. IA and IB are subclasses of class I PLA_2, depending on the presence or absence of the pancreatic loop.

Hit description	Spot	Istotig	Hit's activity	Pancreatic loop
Basic PLA_2 CM-II	90, 91, 99	ND	Myonecrotic and neurotoxic	No (IA)
Textilotoxin A chain	107, 109	814	Hexameric neurotoxin	
Textilotoxin B chain		883		
Textilotoxin C chain	ND	819		
Textilotoxin D chain		800		Yes (IB)
Acidic A2 1	ND	824	pro-coagulant	
Acidic A2 2	16, 101, 110	263		
Acidic A2 2		264		

pore formation agents (Hegde et al., 2009; Kini and Doley, 2010; Utkin, 2013). 3FTx are common component of elapid snake venoms and are often responsible for their lethal effects. The α-neurotoxin antagonists of postsynaptic nicotinic acetylcholine receptors are the most studied 3FTx. They promote paralysis, respiratory failure and death (Servent et al., 1997). The molecular accelerated evolution, classification and nomenclature of these toxins are subject to debate (Doley et al., 2009; Sunagar et al., 2013). Although a huge diversity of genes and transcripts have been described (Gong et al., 1999; Jackson et al., 2013; St Pierre et al., 2007b; Tyler et al., 1987), the usual simplified short-chain neurotoxin (SNTx) or long-chain neurotoxin (LNTx) classification is used here (Hegde et al., 2009).

This toxin family presented the greatest number of isotigs and isogroups in the transcriptome, after assembly. A closer analysis into the sequences ORFs and UTRs enabled the subclassification of isogroup 2 into three more subgroups (Table 2).

+The isogroups 2 and 27 are clusters of transcripts encoding for SNTx. Isotig 74 is the exact NXS7 variant (Gong et al., 2000) and isotigs 67, 68, 72 and 76 to 79 encode for new SNTx isoforms. Isotigs 185 and 187 encode for new SNTxs similar to 3FTxs from *Pseudonaja modesta* "Pse-290" (Jackson et al., 2013) (~90%) and *Oxyuranus microlepidotus* "3FTx-Oxy6" (Fry et al., 2008) (84%). Other transcripts had nonsense mutations (e.g.: insertions, deletions, intron insertions) suggesting that the 3FTx gene family undergoes wide-ranging alternative splicing and deserves further investigation.

The previously described "LNTx-1" (St Pierre et al., 2007b) and an isoform were identified in isogroup 23. The isogroup 83 encodes for "Pseudonajatoxin b homologue" (Gong et al., 2001) and isotig 1328 is a new LNTx transcript, named here "LNTx-2", with 93% translation similarity to a *Pseudonaja modesta* LNTx (Accession: R4G319_9SAUR) (Jackson et al., 2013), 85% to α-neurotoxin "LNTx 20" from *Drysdalia coronoides* (Accession: 3FL20_DRYCN) (Chatrath et al., 2011) and only 78% similar to "Pseudonajatoxin b homologue". Some singletons showed truncated sequences of diverse LNTxs.

The proteome analysis identified SNTx variants in spots 37, 105 and 106, namely "NXS2", "NXS3" and "NXS7". The "LNTx-1" was identified in spot 37 and the new "LNTx-2" (isotig 1328) was identified in spot 104 (Supplementary Material).

4.1.4. Serine protease inhibitors peptides: Kunitz-like and WAP

Kunitz-like serine protease inhibitors (I2 family), also known as Bovine Protease Trypsin Inhibitors (BPTI), are present in a great variety of tissues in almost all organisms (except for Archeae and Fungi). A variety of BPTIs with different activities are found in venoms of different creatures, ranging from anemones (e.g. Schweitz et al., 1995), wasps (e.g. Hisada et al., 2005), spiders (e.g. Chung et al., 2002), shrews (e.g. Kita et al., 2004), scorpions (e.g. Chen et al., 2012), viperid (e.g. Cheng et al., 2012) and elapid (e.g. Masci et al., 2000) snakes. Textilinin, a selective and reversible inhibitor of plasmin was previously described in *Pseudonaja textilis* venom, and has applications in the control of blood clotting (Earl et al., 2012; Masci et al., 2000).

Among the transcripts, isotig 173 was the only one with a functional venom BPTI sequence, identical to "textilinin-4" (Accession: VKT4_PSETT) (Filippovich et al., 2002). Others have nonsense

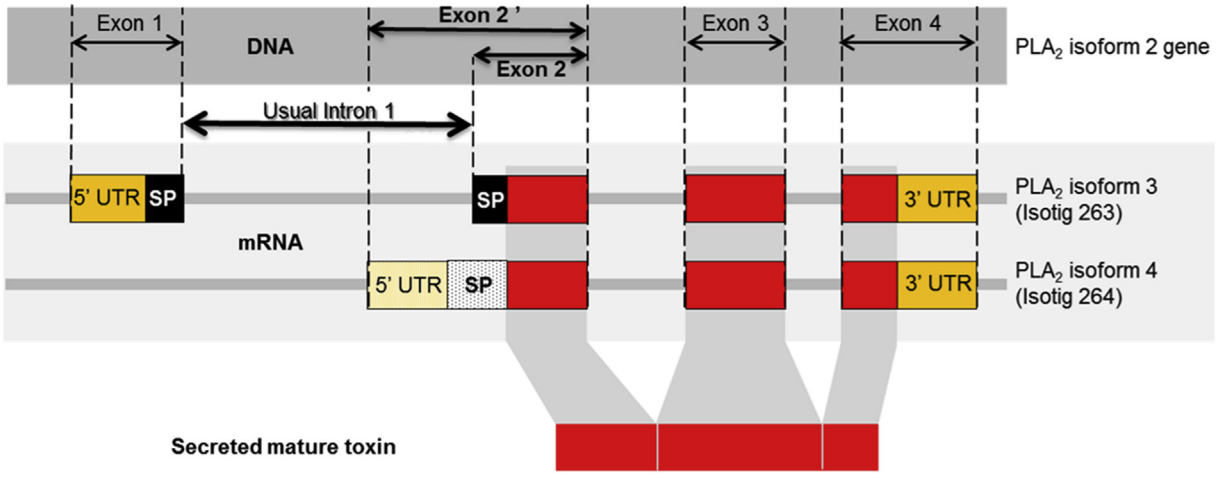

Fig. 4. Graphical overview of the alternative splicing occurring between two PLA_2 transcripts. The alignment with the gene, described in *Pseudonaja textilis* (Armugam et al., 2004), highlights an alternative splicing occurring at exon 2. Although the alternative splicing generates two different signal peptides (SP), the secreted mature toxin is the same. UTR: untranslated region.

Table 2
Diversity of 3FTx transcripts of the *P. textilis* venom gland transcriptome. Isogroups are derived from assembly. Subgroups were assigned based on sequence similarity. Blast2Go annotations are based on public NCBI protein database annotations. NA: Not Available.

Isogroup	Subgroup	Istotig	Blast2Go annotation	Note
2	1	67, 68, 72, 74, 76 to 79	Short neurotoxin precursor	SNTx isoforms
		75	NA	Deletion nonsense
	2	60 to 62	Short neurotoxin precursor	Premature stop codon
	3	57 to 59	Uncharacterized transposon-derived protein	Loss of signal peptide by insertion
		63 to 66, 69 to 71, 73	Short neurotoxin precursor	
27	4	185 and 187	Short neurotoxin precursor	New SNTx
		186	NA	Deletion nonsense
23	5	174 and 176	Long chain neurotoxin precursor	LNTx-1 (100% similar)
		175		LNTx-1 new isoform
83	6	295 and 296	LNTx-1 precursor	LNTx Pseudonajatoxin b homologue (100% similar)
1076	7	1328	Long neurotoxin	LNTx-2 (new)
6	8	112 to 118	NA	Non-coding, with gene introns.

mutations in the beginning or the end of the transcript (e.g. isotig 171 and 172). The proteomic analysis identified textilinin 1, 2 and 3 at the isoform level because of unique peptides at spots 97, 98 and 106, but no other kind of serine protease inhibitors were identified in this venom.

Since there is an interest in understanding the structure, function and pharmacology of textilinin and other venom serine-protease inhibitors as well as the molecular evolution mechanism of toxin recruitment in the venom gland, it is important to survey all sequences related to this family. A great variety of BPTI transcripts were already described in elapid venom gland transcriptomes and some of them present an additional BPTI or whey-acidic-protein (WAP) domain (Jackson et al., 2013; St Pierre et al., 2008). WAP are peptides from the I17 serine proteases inhibitors family, found in large amounts in many mammals milk, and contain a domain found in many other proteins (Bingle et al., 2002; Hennighausen and Sippel, 1982). Snake waprins were isolated from *Oxyuranus microlepidotus* and *Naja nigricollis* venom, and displayed antimicrobial activity but no haemolytic, no toxicity in mice and no elastase and cathepsin G inhibition (Nair et al., 2007; Torres et al., 2003). WAP transcripts were also sequenced from snake venom glands, such as textwaprin from *P. textilis* (Fry et al., 2008; St Pierre et al., 2008) but the function in the venom is still unknown. Transcripts of fused Kunitz inhibitors and WAP domains (KuWAP) were described in *Sistrurus catenatus edwardsii* (Pahari et al., 2007) and *Suta fasciata* (Jackson et al., 2013) venom gland, but with no further characterization. In humans the Epididymal Protease Inhibitors (EPPIN) containing a BPTI and a WAP domain are found in spermatozoa surface and have an antimicrobial activity and contraceptive function (Wang et al., 2007; Yenugu et al., 2004). In snake venom glands, both inhibitors seem to have a common evolutionary pathway as they share signal peptide and the first exon (Jackson et al., 2013; St Pierre et al., 2008). In this transcriptome, a great variety of BPTI transcripts (isotigs and fragments encoded on singletons) were identified (mono, double and triple domain as well as WAPs and fused BPTI/WAP) and knowledge of their sequences can help to better understand this family of protease inhibitors in snakes and their toxin version in their venom. Surprisingly, a partial sequence of a double BPTI domain serine protease inhibitor similar to those found in leech (Simakov et al., 2013) and tick (Macedo-Ribeiro et al., 2008) saliva was identified in singleton BC91X. In the *Rhipicephalus microplus* saliva, the Boophilin-H2 (Acession: BOOH2_RHIMP) is a double BPTI domain serine protease inhibitor that inhibits the host blood clotting as the animal feeds (Macedo-Ribeiro et al., 2008). Further genetic cloning and venom characterization should elucidate the function of this inhibitor in venom.

4.1.5. C-type lectins

The most commonly described C-type lectins (CLects) in Elapidae are galactose or mannose binding with ~26 kDa and usually structured into homo or heterodimers (Abreu et al., 2006; Du et al., 2002; Earl et al., 2011; Zha et al., 2001). C-type lectins are common body proteins and the diversity of transcripts observed in the venom gland might be related to house-keeping functions, thus, abundance of transcript may not equate to toxin diversity *per se*. In fact, between the diversity of CLects sequenced, only the isogroup 13 set of transcripts was identified in the proteome analysis in spots 102, 103, 111, 112 and 114. This isogroup is formed by 4 variants of a galactose-binding CLect (isotigs 145 to 148, named "Venom C-type lectin galactose binding variant" numbered from 3 to 6, respectively) differing from each other by some point mutations, not involved in the QPD sugar binding site or metal binding regions. Some other galactose binding CLect described in elapid snake venoms were also identified in this proteome. CLects are being described for the first time at the protein level on *P. textilis* venom.

4.1.6. CRiSP

Venom Cysteine Rich Secretory Proteins (CRiSPs) have conserved sequence between Colubridae, Viperidae and Elapidae species, with ~23 kDa and 16 structural cysteines. Although the 3D structure is resolved, the active sites and function are poorly elucidated (Heyborne and Mackessy, 2009). The first venom CRiSP described was "helothermine" from the venomous lizards *Heloderma*, which modifies the ionic channels in mice causing lethargy, partial paralysis of the hind limbs and hypothermia (Mochca-Morales et al., 1990; Morrissette et al., 1995).

Pseudechetoxin, a CRiSP from *Pseudechis australis* venom inhibits smooth muscle contraction by blocking cyclic nucleotide-gated ion channels (Yamazaki and Morita, 2004). In other species such as *P. textilis*, a pseudechetoxin-like sequence was described but has not been characterized (St Pierre et al., 2005).

The transcriptome revealed a unique truncated sequence (isotig 418) of CRiSP. An adenosine homopolymer might have impaired the exact sequence signal during the pyrosequencing resulting on the insertion of an artificial stop codon at the ORF position 639. However, a more careful analysis of the sequence allowed us to observe the frame shift and, after correction, to identify the full sequence of a pseudechetoxin-like toxin. CRiSP peptides were identified in spots 20, 74, 75 and 109 (Fig. 1).

4.1.7. Snake venom metalloproteinases (SVMPs)

Snake venom metalloproteinases are a polygenic family of enzymes, whose sequence, structure and function are well characterized. Most viper venoms contain an abundance of SVMPs that

can be responsible for bite site haemorrhage, oedema, myonecrosis, blister formation, dermo-necrosis and inflammatory reactions. Systemic effects of SVMPs in snake venoms include coagulopathy, fibrinolysis, apoptosis induction, and activation of factor X and prothrombin (Markland and Swenson, 2013).

The transcriptome revealed a variety of truncated sequences of a P-III class SVMP. These variations of non-sense mutations can be interpreted as (1) individual genetic variation of the specimen, (2) negative selection of this toxin (loss of trophic adaptation) in the species or population, or even (3) a pre-adaptation or neo-functionalization process. Although the venomics analysis did not provide reliable evidences of SVMP in the venom, western blotting revealed an immunoreactive band against anti-jararhagin polyclonal antibodies indicating the presence of P-III SVMP. Furthermore, a full P-III SVMP transcript was cloned and sequenced from a venom gland cDNA library. All those truncated sequences identified in the transcriptome match to this cloned P-III SVMP cDNA. The sequence similarity analysis (BLAST) of the cloned SVMP shows a higher similarity to related species (98–62% of identity with Elapidae) than to other venomous snakes (62–52% of identity with Viperidae).

4.2. Transcripts of interest not identified in the venom

Some transcripts identified in the transcriptome as toxins or putative toxin transcripts were not identified at the protein level in the venom. This might be explained by the fact that (1) not all transcripts are translated into proteins, and (2) when translated it is not necessarily present in the venom, or (3) individual and regional genetic and epigenetic variations can result into different patterns of expression. Additionally, biased results derived from technical limitations (e.g. 2D-PAGE limitation, spot sampling and peptide ionization issues) can lead to underrepresentation of some venom components. Even so, we discuss these toxin transcripts families below.

4.2.1. Natriuretic peptides

Venom NPs are homologous to physiological ones and act by decreasing the blood volume in the prey vessels, inducing immediate hypotension, impairing locomotion and facilitating predation (Vink et al., 2012). A C-terminal fragment of a new isoform of the "PtNP-a" (St Pierre et al., 2006) was identified in the isotig 227, called "PtNP-b" as well as a fragment of a new NP in isotig 1037.

4.2.2. Cobra venom factor

The CVF is a structural and functional analogous to the C3b complement system (Fritzinger et al., 1994). It role in envenomation is not yet clear, nevertheless it has been used for the therapeutic depletion of the immune system in humans (Vogel and Fritzinger, 2010). Partial sequences were identified in singletons A5YBF, A9LX7 e AWSDT.

4.2.3. Hyaluronidase

The isogroup 46 encodes for a hyaluronidase, an enzyme responsible for degrading the hyaluronan, a glycosaminoglycan component of the extracellular matrix in connective tissues and blood vessels. Thus, it role in envenomation is primarily described as a venom spreading factor (Kemparaju et al., 2009). Isotig 221 encodes a snake venom hyaluronidase with high sequence identity (>80%) to hyaluronidases from *Micrurus fulvius* (Margres et al., 2013) and *Ophiophagus hannah* (Vonk et al., 2013). The isotig 222 encodes an unusual truncated version, already described in *Echis*, *Cerastes* and *Bitis* species (Harrison et al., 2007).

4.2.4. Transferrin

Transferrins have been identified in the venom proteomes of other Australian elapids including *Pseudechis australis* (Georgieva et al., 2011) and *Pseudechis guttatus* (Viala et al., 2014). Antimicrobial activity was detected and suggested as a probable activity in the venom (Georgieva et al., 2011).

4.2.5. Dipeptidylpeptidase-IV

Dipeptidylpeptidases-IV (DPP4) were previously described in snake venom and cloned from venom glands (Faiz et al., 1996; Gasparello-Clemente and Silveira, 2002; Ogawa et al., 2006; St Pierre et al., 2007a). They may act by interfering in prey homeostasis by inactivating peptides like glucagon or acting upon the immune or neuroendocrinous system (Ogawa et al., 2006), or simply processing zymogen toxins. Truncated and fragmented isotigs and singletons were identified.

4.2.6. Veficolin

Veficolins are venom ficolins, first described in *Cerberus rynchops* (Homoplasidae) venom as ryncolins, which contains an N-terminal collagen domain and C-terminal fibrinogen domain (Ompraba et al., 2010). Ompraba et al. (2010) identified this protein in the venom by MS considering "hidroxylation of proline" as a variable post-translation modification. No ficolin was identified in *Pseudonaja* venom using this approach. The singleton AUJZW was identified as a fragment of transcript encoding for veficolin, similar to ryncolins. Other singletons with truncated sequences were also identified.

4.3. Proteins of interest: venom proteins and toxin candidates

4.3.1. VIP/glucagon-like peptides and Heloderma lizard toxins

Peptides derived from four toxins found in Gila-monster lizards (*Heloderma*) were identified in *P. textilis* venom proteome: "exendin-2 long" (Accession: EXE2_HELSU), "exendin-4" (Accession: EXE4_HELSU), "helokinestatin-1" (Accession: HKS_HELHO) and "kallikrein-toxin" (Accession: C6EVG4_HELSC) (Supplementary Material). Exendins form a peptide family similar to secretin hormones (Irwin, 2012). "Exendin-2 long" is an intestinal vasoactive peptide that induces hypotension in prey mediated by the relaxation of the myocardium (Tsueshita et al., 2004) and "exendin-4", also hypotensive, mimetizes glucagon which led to the development of the antidiabetic Byetta®, an insulin production stimulator (Furman, 2012). "Helokinestatin-1", derived from a decapeptide, antagonizes the bradykinin vasodilatation activity on B2 receptors of bradykinin (Kwok et al., 2008). Finally, "kallikrein-toxin", described at the cDNA level (Fry et al., 2010), is a kallikrein serine protease of unknown activity, with a sequence similar to gilatoxin (Utaisincharoen et al., 1993). Gilatoxin, similar to batroxobin (Itoh et al., 1987) and crotalase (Henschen-Edman et al., 1999), has kallikrein activity and can act as a haemorrhagic potentiator of *Heloderma* toxins (Utaisincharoen et al., 1993). The origin of those peptides in the venom is unknown, as no transcripts were identified in the transcriptome. No evident spot clustering was observed in the 2D-PAGE for these toxins and the identification of the peptides was always associated with other toxins ($VF5_a$, PLA_2, $VF5_a + PLA_2$ and CRiSP).

4.3.2. Calreticulin

Five peptides from spot 31 corresponded to calreticulin, also identified in the transcriptome (isotig 391, with 82% of identity with humans). This calreticulin sequence contains a Concanavalin A-like lectin/glucanases domain (Con A-like) followed by a calreticulin/calnexin P domain. InterPro resource (Mitchell et al., 2014) predicted the whole sequence as non-cytoplasmic and without

transmembrane motifs, but its function in the venom is unknown. Toxic activity was previously reported in Con A-like domain containing proteins from bacteria, viruses, lion-fish and snakes: e.g. *Clostridium* neurotoxins responsible for the neuroparalytic effects of botulism and tetanus (Swaminathan and Eswaramoorthy, 2000); *Pseudomonas* exotoxin A virulence factor (arrest of protein synthesis in eukaryotic cells) (Wedekind et al., 2001); *Vibrio cholerae* neuraminidase (Crennell et al., 1994) and the rotaviral outer capsid protein VP4 (cell attachment and membrane penetration) (Dormitzer et al., 2002); the *Dendrochirus zebra* (former *Pterois*) lion-fish toxin (UniProt Accession: A0A068BD83_DENZE); and finally, ohanin and thaicobrin are neurotoxins found in snakes (*Ophiophagus hannah* and *Naja kaouthia* respectively) that induce hypolocomotion and hyperalgesia in mice (Pung et al., 2005). Additionally, Kuwabara et al. (1995) show that tick salivary secreted CRT binds to coagulation factors without affecting their coagulant properties and interacts with the endothelium to stimulate release of nitric oxide and inhibit clot formation.

4.3.3. Dipeptidase 2

The identification of dipeptidase-2 in the proteome was unexpected. Based on 3 peptides in spot 117 (Fig. 1, Supplementary material), the peptides matched with DPP-2 described in the *O. hannah* genome (Uniprot Acession: V8NS94_OPHHA) (Vonk et al., 2011). To our knowledge, there is no description of such a venom component in lizard or snake databases, except for a transcript from *Crotalus adamanteus* venom gland (Acession: J3SEA2_CROAD) (Rokyta et al., 2012). DPP2 are M19 membrane peptidases with a renal dipeptidase active site. Those peptidases are involved in renal metabolism of glutathione and their conjugates (Adachi et al., 1993). *O. hannah* and *C. adamanteus* dipeptidase-2 have a predicted N-terminal signal peptide but also a transmembrane region at the C-terminal region, indicating its original membrane function in the venom gland cells.

5. Conclusion

We reported here the first full venomic study of the medically important Australian snake *Pseudonaja textilis*. The combination of proteomics and transcriptomics allowed us to distinguish isoforms, a common limitation in proteomics due to limited tryptic peptide sequence coverage (Birrell et al., 2006). The venom gland *de novo* transcriptome analysis revealed 113 different toxin transcripts, including 17 new sequences, such as the textilotoxin B subunit so far described only at the protein level and most of the toxins UTRs, and splicing variants such as the VF5a-3 (with an additional exon) and the "acidic PLA_2 isoform 4" (with an alternative signal peptide). The new splicing variant VF5a-3 and a new transcript of long neurotoxin LNTx-2 were identified at the protein level. Additionally, a P-III SVMP was cloned and detected in the venom by western blot. Based on those new observations upon toxin transcripts splicing variants, we suggest that the alternative splicing is an important source of variation within toxin families, therefore, genomic sequences and phenotypic identification of those variants should have more attention in venomics works.

Ethical statement

The animal used in this work 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.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.toxicon.2015.06.005.

Transparency document

Transparency document related to this article can be found online at http://dx.doi.org/10.1016/j.toxicon.2015.06.005.

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