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Venom proteomes of South and North American opisthoglyphous (Colubridae and Dipsadidae) snake species: A preliminary approach to understanding their biological roles

María E. Peichoto [a,b,c,*], Flávio L. Tavares [c,d], Marcelo L. Santoro [b], Stephen P. Mackessy [c]

[a] Instituto Nacional de Medicina Tropical (INMeT), Neuquén y Jujuy s/n, 3370 Puerto Iguazú, Argentina
[b] Laboratório de Fisiopatologia, Instituto Butantan, Av. Vital Brazil, 1500, 05503-900 São Paulo, SP, Brazil
[c] School of Biological Sciences, University of Northern Colorado, 501 20th St., CB 92, Greeley, CO 80639-0017, USA
[d] União Dinâmica das Cataratas, Rua Castelo Branco, 349, 85852-010 Foz do Iguaçu, PR, Brazil

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ABSTRACT

Opisthoglyphous snake venoms remain under-explored despite being promising sources for ecological, evolutionary and biomedical/biotechnological research. Herein, we compared the protein composition and enzymatic properties of the venoms of *Philodryas baroni* (PbV), *Philodryas olfersii olfersii* (PooV) and *Philodryas patagoniensis* (PpV) from South America, and *Hypsiglena torquata texana* (HttV) and *Trimorphodon biscutatus lambda* (TblV) from North America. All venoms degraded azocasein, and this metalloproteinase activity was significantly inhibited by EDTA. PooV exhibited the highest level of catalytic activity towards synthetic substrates for serine proteinases. All venoms hydrolyzed acetylthiocholine at low levels, and only TblV showed phospholipase A_2 activity. 1D and 2D SDS-PAGE profile comparisons demonstrated species-specific components as well as several shared components. Size exclusion chromatograms from the three *Philodryas* venoms and HttV were similar, but TblV showed a notably different pattern. MALDI-TOF MS of crude venoms revealed as many as 49 distinct protein masses, assigned to six protein families. MALDI-TOF/TOF MS analysis of tryptic peptides confirmed the presence of cysteine-rich secretory proteins in all venoms, as well as a phospholipase A_2 and a three-finger toxin in TblV. Broad patterns of protein composition appear to follow phylogenetic lines, with finer scale variation likely influenced by ecological factors such as diet and habitat.

1. Introduction

Animal venoms represent rich sources of potent biological compounds, many with high potential for therapeutic drug development (Minea et al., 2012). While extensive research has explored the complexity of venoms from front-fanged snakes (e.g., families Viperidae and Elapidae), relatively little is known about the composition and biological activities of venoms from rear-fanged snakes (formerly lumped in the paraphyletic family Colubridae), which collectively comprise the most speciose group of advanced snakes. In part this is not only because snake bites are often medically insignificant (but see Weinstein et al., 2011), but also because it is much more difficult to extract venom for research purposes (Hill and Mackessy, 1997, 2000; Kamiguti et al., 2000; Mackessy, 2002). Dangerously venomous "colubrids" are much less commonly encountered than front-fanged species, and serious bites typically require longer contact time (Kuch and Mebs, 2002), so most species pose a minor problem to humans compared with the two major families of medically important front-fanged snakes. However, specific "colubrids" are far from harmless, and at least five genera (*Dispholidus*, *Thelotornis*, *Rhabdophis*, *Philodryas* and *Tachymenis*) contain species that have produced fatal human envenomations (Vellard, 1955; FitzSimons and Smith, 1958; Pope, 1958; Mittleman and Goris, 1976; Ogawa and Sawai, 1986; Salomão and Di-Bernardo, 1995). Other species of rear-fanged snakes have not caused human deaths but produce venoms with many characteristics similar to front-fanged snakes (Hill and Mackessy, 2000; Mackessy, 2002; Weinstein et al., 2011; Ching et al., 2012; Fry et al., 2012).

Philodryas is a genus of opisthoglyphous "colubrid" snakes which belongs to the family Dipsadidae (Vidal et al., 2007; Zaher et al., 2009), with a well-developed Duvernoy's gland connected to a grooved tooth. Most species have diurnal habits and are found in South America from Amazonia to Patagonia (Assakura et al., 1992; Assakura et al., 1994). *Philodryas baroni* is an arboreal rear-fanged dipsadid snake largely restricted to the north-central Gran Chaco of Argentina (Kuch and Jesberger, 1993), while *Philodryas olfersii olfersii* (also arboreal) and *Philodryas patagoniensis* (predominantly terrestrial) are widely distributed in Argentina, Bolivia, Brazil, Paraguay and Uruguay (Ribeiro et al., 1999; de Medeiros et al., 2010). Human envenomation by these rear-fanged snakes is characterized by pain, bleeding, erythema, edema

* Corresponding author at: Instituto Nacional de Medicina Tropical (INMeT), Neuquén y Jujuy s/n, 3370 Puerto Iguazú, Argentina. Tel./fax: +54 3757 425002.
E-mail addresses: mepeichoto@yahoo.com.ar, mepeichoto@conicet.gov.ar (M.E. Peichoto).

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and ecchymotic lesions on the bitten limb, often bearing a striking resemblance to the local signs and symptoms of *Bothrops* sp. envenomations (Kuch and Jesberger, 1993; Nishioka and Silveira, 1994; de Araujo and dos Santos, 1997; Ribeiro et al., 1999; de Medeiros et al., 2010).

Hypsiglena (family Dipsadidae) and *Trimorphodon* (family Colubridae) (Quijada-Mascareñas and Wüster, 2010), along with other genera of North American rear-fanged snakes, also have caused envenomations (Chiszar and Smith, 2002). *Hypsiglena torquata* is a small nocturnal rear-fanged snake that is found from southwestern Canada through much of the western United States to Baja California and the Mexican state of Guerrero (Rodriguez-Robles et al., 1999). *Trimorphodon biscutatus* is also a nocturnal rear-fanged snake, widespread in the deserts of the southwestern United States and northwestern Mexico (Goldberg, 1995). Both species are capable of causing envenomation in humans, inducing localized effects characterized by pain, edema, lymphadenopathy and ecchymosis; therefore, these bites have similar characteristics to those by small Viperidae (Chiszar and Smith, 2002). In addition, bites by *Hypsiglena* to other snakes can produce hemorrhagic lesions and fatalities (Hill and Mackessy, 2000).

The properties of venom from *P. baroni* are unknown, but several biological activities of venoms from the other four "colubrid" snakes, particularly *P. patagoniensis* and *T. biscutatus*, have been reported (Vest, 1988; Hill and Mackessy, 2000; Acosta et al., 2003; Fry et al., 2003b; Huang and Mackessy, 2004; Lumsden et al., 2004; Peichoto et al., 2004, 2005, 2006, 2007, 2009, 2010; Zelanis et al., 2010). In addition, the venom of *P. olfersii* has been extensively studied at the transcriptome level (Ching et al., 2006), and several proteinases have been isolated and characterized (Assakura et al., 1994). However, with the exception of some data for *P. olfersii* (Ching et al., 2006), proteomic studies of these venoms have not been conducted, so the overall venom composition is poorly known. For this reason, we initiated a comparative study of the venom proteomes of the South American rear-fanged snakes *P. baroni* (PbV), *P. olfersii olfersii* (PooV) and *P. patagoniensis* (PpV), and the North American rear-fanged snakes *Hypsiglena torquata texana* (HttV) and *Trimorphodon biscutatus lambda* (TblV). In addition to shedding light on the pathophysiological mechanisms following envenomation, we determined the extent to which individual toxin families occur in common among the venoms of these opisthoglyphous snakes. Thus, this study provides important information concerning the evolution and diversification of venoms in New World "colubrid" snakes.

2. Materials and methods

2.1. Snake venoms

Pools of *P. patagoniensis* and *P. o. olfersii* venoms were obtained from wild specimens captured in northeastern Argentina and then maintained at the serpentarium of the local Zoo, Corrientes, Argentina. Specimens were extracted by introducing a 100-μL micropipette under each fang, according to a procedure described previously (Ferlan et al., 1983).

Hypsiglena torquata and *T. biscutatus* venoms were obtained from wild specimens captured in Colorado and Arizona, USA (scientific collecting permits 0456 and MCKSY000221), and maintained in the UNC Animal Facility. Four captive-born specimens of *P. baroni* were obtained from the Dallas Zoological Park and also maintained in the UNC Animal Facility. All housing and handling procedures were approved by the UNC-IACUC (protocols #9204.1 and 9401). Extraction of snakes utilized a previously published method (Rosenberg, 1992; Hill and Mackessy, 1997). Lyophilized pooled venom from adult *Bothrops jararaca* snakes was supplied by Laboratory of Herpetology, Instituto Butantan.

After extraction, all venoms were centrifuged, lyophilized and kept frozen at −20 °C. When required, venoms were dissolved in 0.01 M phosphate buffer saline (PBS), pH 7.4, and filtered through a 0.4 μm Millipore filter to remove insoluble material.

2.2. Protein concentration determination

Protein concentration was assayed in triplicate according to Bradford (1976) as modified by BioRad Inc. (San Diego, CA, USA), using bovine gamma globulin as a standard.

2.3. Enzyme assays

Endoprotease activity was determined with azocasein (Wang and Huang, 2002), and activity was expressed as ΔA_{450}/min per mg venom protein. Activity towards 4-nitroaniline-derived (pNA) synthetic substrates for thrombin (TosylGlyProArg-pNA and BzPheValArg-pNA) was assayed according to Mackessy (1993). Acetylcholinesterase activity was assayed according to Ellman et al. (1961) and activity was expressed as μmol product formed per minute per milligram venom protein. Phospholipase A_2 (PLA_2) activity was determined by the method of Holzer and Mackessy (1996), using 4-nitro-3-(octanoyloxy)benzoic acid as substrate. All enzyme assays were performed in triplicate. Negative controls were also performed in triplicate. Endoprotease, acetylcholinesterase and PLA_2 assays were also used to localize these enzymes following size exclusion HPLC (SE-HPLC; Section 2.6).

2.4. One-dimensional gel electrophoresis

Crude venoms were subjected to SDS-PAGE using NuPage Bis-Tris gels (Invitrogen, Inc., San Diego, CA, USA), MES/SDS running buffer and 24 μg venom/lane as described previously (Weldon and Mackessy, 2010). Following staining with Coomassie brilliant blue R-250 and destaining, the gel was imaged using an AlphaImager (Cell Biosciences, Inc., Santa Clara, CA, USA).

2.5. Two-dimensional gel electrophoresis (2DE)

The protocol used for two-dimensional electrophoresis was based on a previous report (Antunes et al., 2010). In brief, samples of five rear-fanged snake venom pools were dissolved in rehydration solution, and aliquots (125 μL, 80 μg protein) were used to hydrate precast strips for isoelectric focusing (IEF) (7 cm, linear pH 3–10, GE Healthcare, USA) for 18 h at room temperature. The first dimension IEF was performed in an Ettan IPGphor 3 instrument (GE Healthcare, USA), using the following protocol: 100 V–300Vh, 300 V–150Vh (gradient), 1000 V–300Vh (gradient), 5000 V–4000Vh (gradient) and 5000 V–3000Vh. After IEF, strips were reduced and alkylated, and directly applied to 12% SDS-PAGE gels in a SE260 cell (GE Healthcare), which was connected to a Multitemp III cooling bath (GE Healthcare) set at 10 °C. Gels were run at 15 mA/gel until the dye front reached the gel bottom. Thereafter gels were silver stained (Blum et al., 1987) and scanned on an Epson Imagescanner III, with a resolution of 600 dpi. In order to analyze statistically the variation in protein expression among gels, each sample of venom was run three times, and then images were analyzed with the software ImageMaster 2D 7.0 (GE Healthcare) for determination of relative molecular mass (MM) and isoelectric point (pI) of spots.

2.6. Size exclusion HPLC

Samples of venoms from the five rear-fanged snakes were dissolved in 25 mM HEPES buffer containing 100 mM NaCl (pH 6.8). Two hundred microliters (2 mg) of each venom was injected onto a TSKgel G2000 SWXL size exclusion column (7.8 mm i.d., 30 cm, 5 μm) (TOSOH Bioscience LLC, Tokyo, Japan) equilibrated with the same buffer and run at a flow rate of 0.2 mL/min using a Waters HPLC system, and chromatograms were recorded using Empower software. Fractions from each peak were run on a 1D gel and compared with results for the other crude venoms. Fractions were also assayed for endoprotease, acetylcholinesterase and PLA_2 activity as above.

2.7. Mass spectrometry

Approximately 1 μg crude venoms in 50% ACN containing 0.1% TFA was spotted onto a MALDI sample holder, mixed with an equal volume of 10 mg/mL sinapinic acid in 50% ACN containing 0.1% TFA, and allowed to dry. Mass spectra were obtained using a Bruker Ultraflex II MALDI-TOF/TOF mass spectrometer (Proteomics and Metabolomics Facility, CSU, Fort Collins, CO, USA) in linear mode using a 25 kV accelerating voltage and calibrated with an external protein standard (5 proteins, 6–140 kDa). Putative protein families of common venom proteins known to occur in rear-fanged snake venoms (e.g., Mackessy, 2002; Fry et al., 2003b, 2008; Weldon and Mackessy, 2010, 2012) are based on characteristic masses. For comparison of proteins between species, we used a mass difference of >2% to categorize proteins as distinctly different in mass. Proteins were considered to be the same isoform if masses differed by less than 0.5% (within experimental error).

For unequivocal identification of some venom proteins, gel bands of interest were excised from a 1D SDS-NuPAGE gel, destained and subjected to reduction with DTT, alkylation with iodoacetamide, and then in-gel digestion with mass spectrometry grade Trypsin Gold (Promega, Madison, WI, USA), following the manufacturer's instructions. The tryptic peptide mixtures were purified and concentrated using ZipTip® C18 pipette tips (Millipore Corporation, Billerica, MA, USA). The peptides eluted from the ZipTip® tips were dried in a Speed-Vac and redissolved in 5 μL of 50% ACN containing 0.1% TFA. Digests (1 μL) were spotted onto a MALDI sample holder, mixed with an equal volume of 10 mg/mL α-cyano-4-hydroxycinnamic acid in 50% ACN containing 0.1% TFA, dried, and analyzed with a Bruker Ultraflex II MALDI-TOF/TOF mass spectrometer (Proteomics and Metabolomics Facility, CSU). Singly-charged ions of selected peptides from the MALDI-TOF mass fingerprint spectra were sequenced by collision-induced dissociation tandem mass spectrometry. Spectra were interpreted using the on-line form of the MASCOT program at http://www.matrixscience.com against the NCBInr protein database, with carbamidomethylation as fixed modification and oxidation of methionine as variable modification, with a parent tolerance of 1.2 Da and a fragment tolerance of 0.6 Da. Peptide sequences with expected values lower than 0.05 ($p<0.05$) indicated identity or extensive homology. The expected cutoff value of 0.05 was applied in the MASCOT ion score to avoid peptide identifications out of 95% confidence interval from being selected.

3. Results

3.1. Enzyme assays

The most common enzyme activities present in "colubrid" venoms (Mackessy, 2002) were tested in this study in order to determine the relative enzymatic composition of the five crude venoms investigated (Table 1). All five venoms degraded azocasein, and in all cases activity was significantly inhibited (>75%) by 5 mM EDTA, demonstrating that this activity is prominently due to metalloproteinases; HttV showed the highest protease levels. PooV exhibited the highest level of catalytic activity towards both synthetic substrates for serine proteases, whereas HttV presented the lowest levels. The five venoms hydrolyzed acetylthiocholine at low levels, but PpV and TblV exhibited the highest level of acetylcholinesterase activity; only TblV showed PLA_2 activity.

3.2. Electrophoretic profiles

One-dimensional electrophoretic profiles showed many band similarities among all venoms studied (Fig. 1); minimally, four to five protein families are present in most venoms. However, individual protein bands in the range 31.0–37.0 kDa, (under both reducing and non-reducing conditions, demonstrating that they are single-chain proteins) were only expressed in PooV, PpV and TblV. More intensely-stained bands were observed in the ranges of 6.0–21.0 kDa, under reducing and non-reducing conditions, primarily in TblV (Fig. 1).

Two-dimensional electrophoresis (Fig. 2) enlarged the scope of the one-dimensional analysis. Proteins in the range of 50–80 kDa, which were more densely stained in one-dimensional gels, were also observed in the form of several acidic spots (pI 3.5–7.0) in two-dimensional gels from all venoms (red box, Fig. 2). On the other hand, acidic protein spots with molecular masses of 30–40 kDa were preferentially expressed in PooV, PpV and TblV. It is known that serine proteases affecting hemostasis are the snake venom components that dominate these mid-mass ranges (Mackessy, 2010).

There are three marked distinctions between the 2DE gel images (Fig. 2) of TlbV (family Colubridae senso stricto) and the four other venoms of rear-fanged snakes that belong to the family Dipsadidae (formerly Colubridae). The former showed several basic protein spots in the range of 25.0–30.0 kDa (orange box, Fig. 2). In addition, it revealed one spot with molecular mass of ~15.0 kDa (pI of ~4.5) and another of less than 15.0 kDa (pI of ~9.0), both corresponding to proteins differentially expressed in TblV (blue and pink boxes, respectively, Fig. 2). The 2DE profile of the four venoms from snakes belonging to the family Dipsadidae showed a high level of similarity. However, it is important to note that basic proteins in the range of 50–75 kDa were only observed in the venoms of the two arboreal species studied in this work, *P. baroni* and *P. o. olfersii* (violet box, Fig. 2). The *B. jararaca* gel (Fig. 2) showed many more distinct proteins than the other gels, demonstrating the comparatively lower complexity of the proteome of the venoms studied in this work, typical of many "colubrid" snakes (Mackessy et al., 2006; Weldon and Mackessy, 2010).

3.3. Size exclusion HPLC

Size exclusion chromatography resolved 2–5 major protein peaks (Fig. 3), and acetylcholinesterase and endoprotease activity was limited to peak I of all five venoms. Based on 1D SDS-PAGE, a ~25 kDa protein, which was identified as a CRISP using mass spectrometry (see below), was present in peak II of all chromatograms.

Table 1
Enzymatic activities of five rear-fanged snake venoms.

Assay	*P. patagoniensis* venom (PpV)	*P. baroni* venom (PbV)	*P. o. olfersii* venom (PooV)	*H. torquata texana* venom (HttV)	*T. biscutatus lambda* venom (TblV)
Proteolytic activity on azocasein ($\Delta A_{450\ nm}$/min/mg protein)	0.174 ± 0.015	0.299 ± 0.023	0.250 ± 0.019	0.405 ± 0.052	0.087 ± 0.007
Inhibition by 5 mM EDTA (%)	83	81	76	83	95
Activity toward:					
TosylGlyProArg-pNA	19.5 ± 1.8	3.4 ± 0.3	157.9 ± 9.7	0.3 ± 0.1	10.9 ± 0.7
BzPheValArg-pNA (nmol/min/mg protein)	3.4 ± 0.2	1.3 ± 0.1	66.1 ± 7.2	0.4 ± 0.1	23.1 ± 1.9
Acetylcholinesterase activity (μmol/min/mg protein)	1.21 ± 0.12	0.08 ± 0.01	0.09 ± 0.01	0.03 ± 0.01	2.33 ± 0.20
Phospholipase A_2 activity (nmol/min/mg protein)	0	0	0	0	25.7 ± 1.1

Values are mean ± SEM of three determinations. 0, no activity detected.

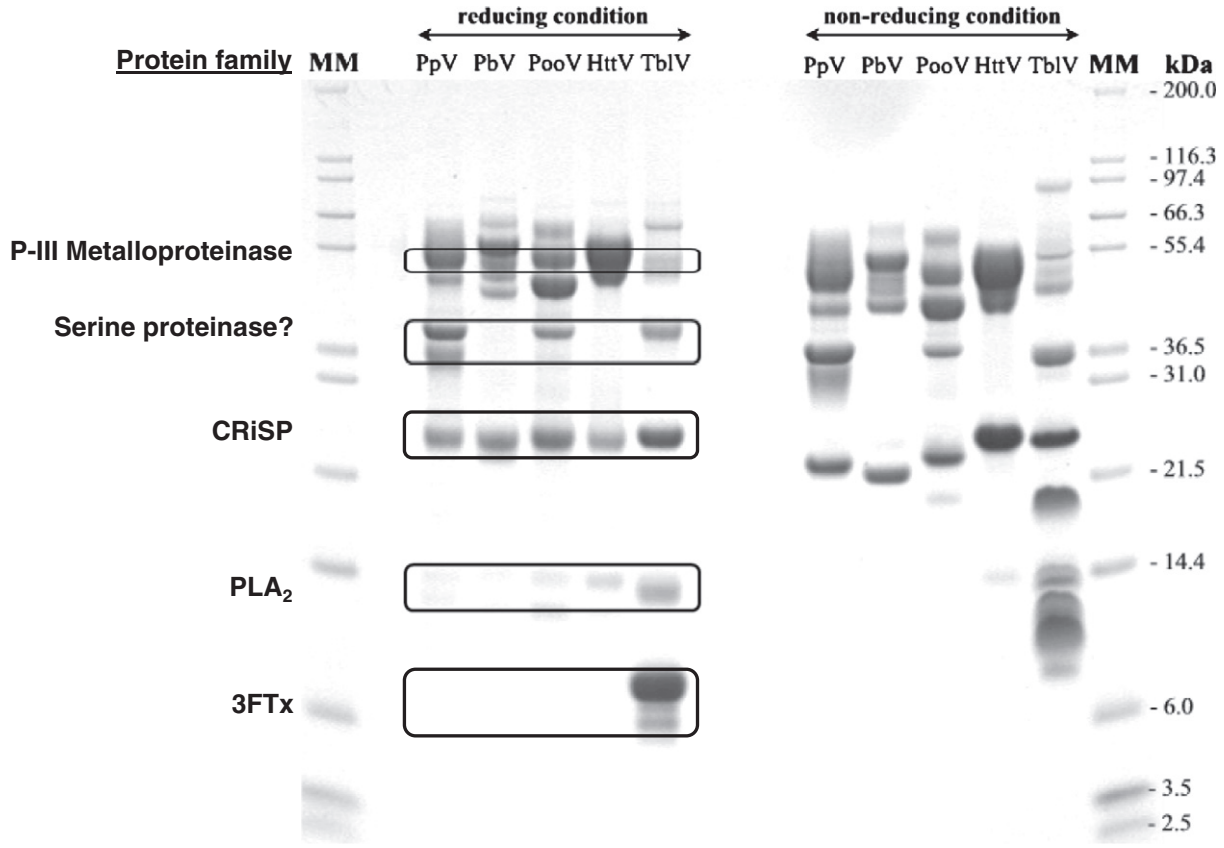

Fig. 1. One-dimensional electrophoretic profiles of venoms from five rear-fanged snake species (24 μg) under reducing and non-reducing conditions on NuPAGE Novex 12% Bis-Tris gel; stained with Coomassie brilliant blue. Novex Mark 12 molecular mass standards (MM) are on the right lane of the gel. Putative venom protein families are indicated for the reduced venom samples (left side).

Chromatograms (Fig. 3) from the three *Philodryas* venoms and HttV were similar but greater variation was seen in TblV, in which three additional protein peaks were revealed. One of these corresponded to the PLA_2-active fraction of the venom, and the other two corresponded to proteins of ~9 and 18 kDa, both of which showed homology with 3FTx-Tri2 (Fry et al., 2008), a three-finger toxin from TblV (see below). Minor peaks corresponding to 6–20 kDa protein bands (data not shown) were also revealed in chromatograms from the three *Philodryas* venoms and HttV (Fig. 3), indicating that these peptides are expressed at much lower levels in these venoms.

3.4. Mass spectrometry

MALDI-TOF mass spectra of the five crude venoms revealed a diversity of proteins (Fig. 4), complementing 2D SDS-PAGE, and approximately 40 proteins with unique masses (difference of >2%) were resolved; protein family identity was assigned based on characteristic masses (Supplementary Table 1). Three finger toxins (masses ~7.8–8.5 kDa) were present in the venoms of Poo, Htt and Tbl, but they were major components only in Tbl venom. Consistent with enzyme assays, proteins assigned to PLA_2 (masses ~13.8–14.2 kDa) were present only in Tbl venom, but proteins with similar masses (<13.5, >14.5 kDa) were present in the other venoms. Proteins with masses of C-type lectins (masses ~15.3–16.2 kDa) were found in venoms of Pp, Poo and Tbl. Cysteine-rich secretory proteins (CRISPs, masses ~25–26 kDa) were present in all venoms. Snake venom metalloproteinases (SVMPs) were also found in all venoms, with putative PII SVMPs only in venoms of Pp, Poo and Tbl (masses ~40 kDa) and PIII SVMPs (masses ~49–53 kDa) in all venoms.

In order to identify the major component present in each of the three differentially expressed protein peaks of the chromatogram from TblV, 1D SDS-PAGE-separated protein bands were excised, in-gel digested with trypsin and the resulting peptides were analyzed by MALDI-TOF peptide mass fingerprinting followed by MALDI-TOF/TOF. For the major protein present in the peak with PLA_2 activity, the MS/MS spectrum of the fragmented singly-charged peptide ion ($m/z = 2419.692$) was matched by MASCOT to an internal sequence, NVNCEGDNDECGAFVCECDR, from a PLA_2 sequenced from the venom gland transcriptome of *T. biscutatus* (Fry et al., 2008). This enzyme, named trimorphin, was previously isolated in native form from the venom of the Sonoran Lyre Snake (*T. b. lambda*) by Huang and Mackessy (2004). The MALDI-TOF mass fingerprint spectrum obtained from the 9 kDa protein significantly matched with theoretical peptide masses of the venom toxin 3FTx-Tri2 from *T. biscutatus* (Fry et al., 2008). Similarly, the MS/MS spectrum of the fragmented singly-charged peptide ion ($m/z = 1791.842$) from the 18 kDa protein was matched by MASCOT to an internal sequence, QAIGPPFTRCSQCNR, from the toxin 3FTx-Tri2 sequenced from the venom gland transcriptome of *T. biscutatus* (Fry et al., 2008).

The 25 kDa protein peak present in chromatograms of all the five venoms was identified by mass spectrometry as a venom CRISP. The MS/MS spectrum of two fragmented singly-charged peptide ions from the 25 kDa protein of the three *Philodryas* venoms was matched by MASCOT to two internal sequences, MEWYAEAAANAER and VLDGIQCGENIYMSSNPR, from CRISP-PHI1 sequenced from the venom gland transcriptome of *P. olfersii* (Fry et al., 2006). Similarly, the MS/MS spectrum of the fragmented singly-charged peptide ions with m/z of 1759.230 and 2569.541 from the *T. b. lambda* protein, was matched to two internal sequences, SLVQQNSCQHDWTR and SGPTCGDCPSACDNGLCTNPCLR respectively, from CRISP-TRI1 sequenced from the venom gland transcriptome of *T. biscutatus* (Fry et al., 2006), and that of the fragmented

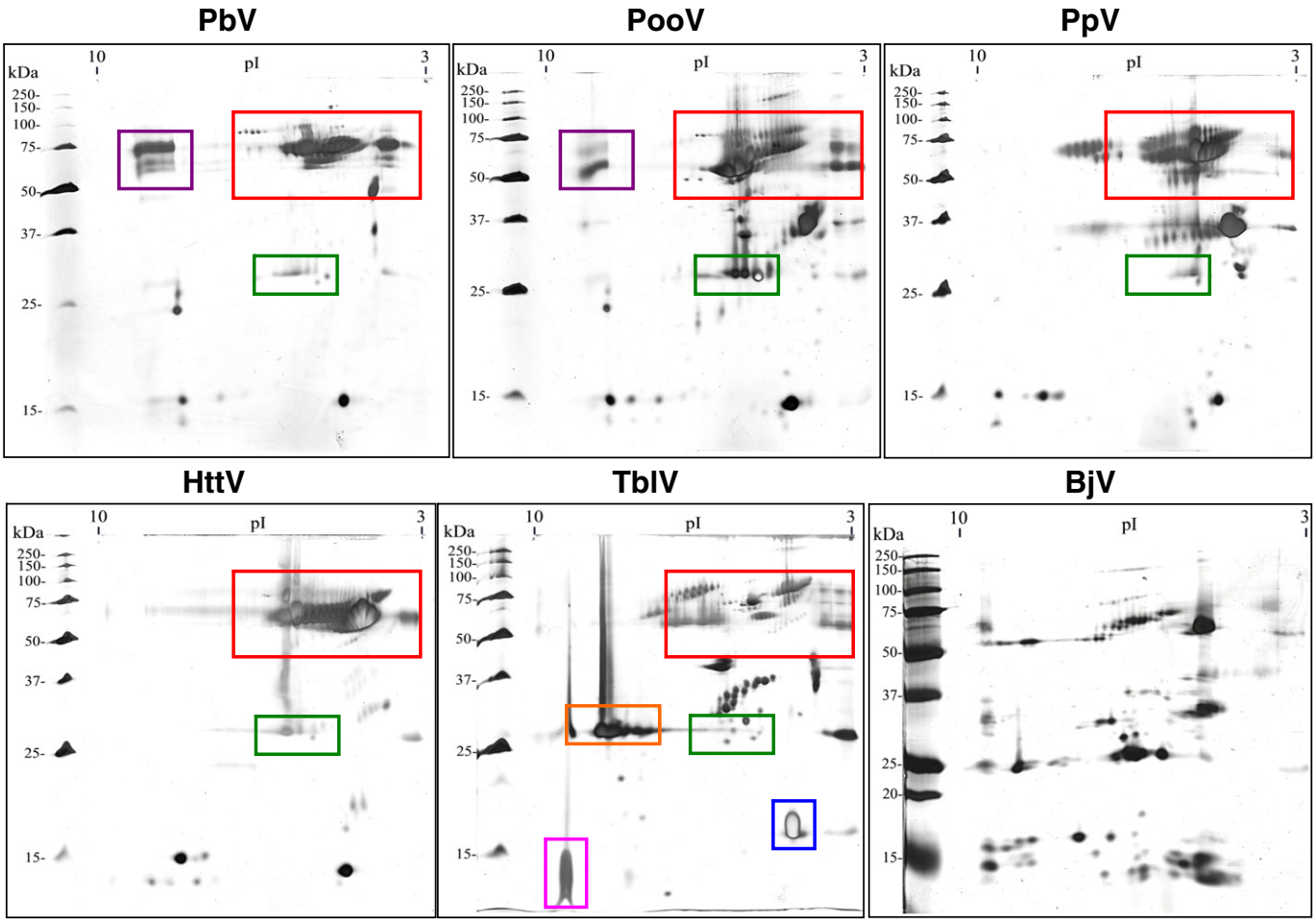

Fig. 2. Comparison of two-dimensional gel electrophoresis profiles of venoms from five rear-fanged snake species (80 μg protein). 2DE gel pattern of *Bothrops jararaca* venom (BjV) is shown for comparison. Gels were run under identical conditions and silver stained. The red and green boxes show protein spots expressed in all "colubrid" venoms, while the blue, orange and pink boxes show spots only expressed in TblV. The violet box shows protein spots only expressed in the two arboreal species.

singly-charged peptide ion (m/z = 1527.751) from the *H. t. texana* protein was matched to MEWYSEAAANAER from CRISP-2 sequenced from the venom gland transcriptome of the Desert Massasauga rattlesnake (*Sistrurus catenatus edwardsii*) (Pahari et al., 2007). All of these results confirmed that a CRISP was the major protein present in peak II from the five venoms studied.

4. Discussion

The paraphyletic family "Colubridae" is the largest group of modern snakes, containing at least 700 venomous species world-wide (Cadle, 1994; Mackessy, 2002; Vidal, 2002), but only a few venom proteomes are currently known (Ching et al., 2006; Mackessy et al., 2006; Weldon and Mackessy, 2010; Ching et al., 2012). Herein, we compare the venom proteomes of five rear-fanged snakes potentially capable of causing envenomation in humans, three from South America and two from North America. Although the rear-fanged snakes in general are not regarded as clinically important due to their less efficient (to humans) venom delivery apparatus, a growing number of accidents caused by these snakes have been reported in epidemiological studies (Ribeiro et al., 1999; Salomão et al., 2003; de Medeiros et al., 2010), and reviewed in Weinstein et al. (2011). However, the vast majority of "colubrid" venoms are still unstudied, mainly because of the exceedingly small quantities of raw material obtained upon extraction (Mackessy, 2002). As more attention is devoted toward these venoms, particularly utilizing proteomic and genomic techniques, it is becoming clear that rear-fanged snake venom components are of high pharmacological, medical and scientific importance (Mackessy, 2002; Peichoto et al., 2007, 2009, 2010, 2011a, 2011b; Ching et al., 2012; Fry et al., 2012; Weldon and Mackessy, 2012). In addition, variation in rear-fanged snake venom composition is reflected in the clinical symptoms of envenomation, most commonly characterized by minor to significant bleeding (Kuch and Mebs, 2002; Weinstein et al., 2011). Thus, comparative analyses of these venoms will provide a better understanding of their toxicity and may help to reveal novel compounds with biomedical applications. For example, a novel dimeric and taxon specific 3FTx was found in the venom of *Boiga irregularis* (Pawlak et al., 2009). Ching et al. (2012) have recently revealed three new types of components for the venom of *Thamnodynastes strigatus*, and Fry et al. (2012) have reported novel compounds in the venoms of *Liophis miliaris*, *Rhabdophis tigrinus*, *T. biscutatus*, *Dispholidus typus*, *Leioheterodon madagascarensis*, and *Enhydris polylepis*. As was noted previously (Mackessy, 2002), it is apparent that we have only begun to unravel the diversity of venom components present in these understudied taxa of modern snakes.

Protease activity is widely distributed among rear-fanged snake venoms (Hill and Mackessy, 2000) – see Mackessy (2002) for a review – and endoproteolytic activity, prominent in venoms analyzed here, was markedly inhibited by EDTA, revealing that metalloproteinases are responsible for this activity. SVMPs play a very important role in the pathophysiology of "colubrid" envenomation, as they are responsible for the hemorrhagic activity exhibited by many rear-fanged snake venoms (e.g.,

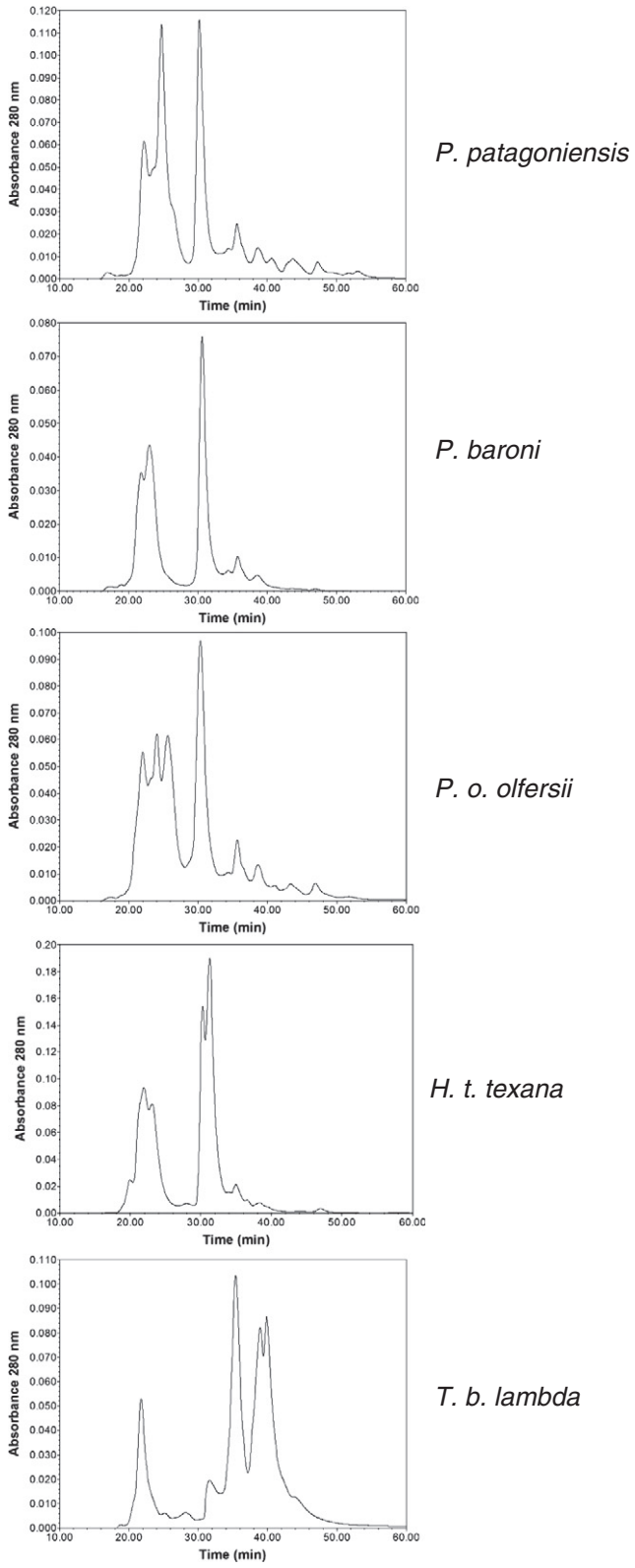

Fig. 3. Size exclusion HPLC separation of venoms from five rear-fanged snake species. Samples of 2 mg of each venom were fractionated, and components of several peaks were identified via enzyme assays and SDS-PAGE analysis of fractions.

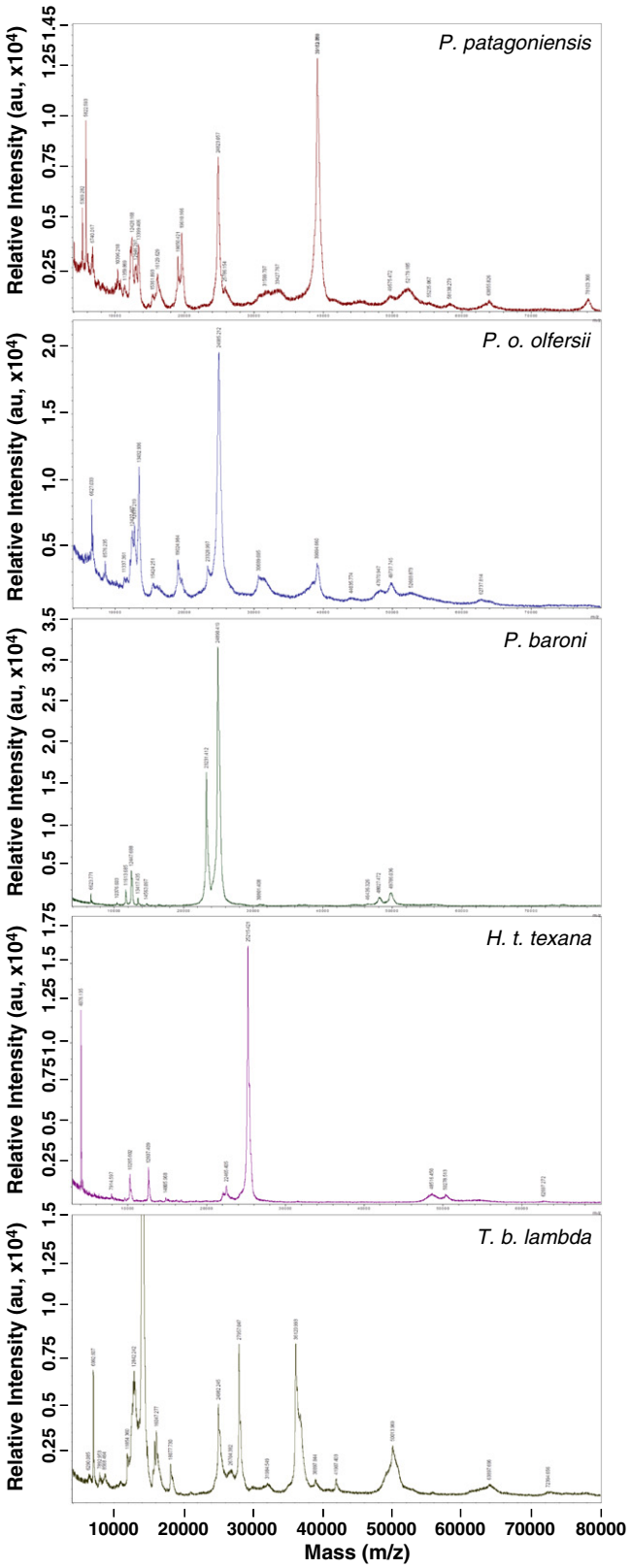

Fig. 4. MALDI-TOF mass spectra of venom samples of five rear-fanged snake species. Approximately 49 distinct protein/peptide masses were observed for all venoms collectively (see also Supplementary Table 1).

Peichoto et al., 2007; Weldon and Mackessy, 2010, 2012). These enzymes degrade basement membrane structure and weaken and disrupt the capillary wall, which leads to bleeding (Acosta et al., 2003) as well as inflammatory effects (Peichoto et al., 2011b). It is important to note that all venoms studied in this work showed to be constituted mainly by proteins with molecular masses in the range of class P-III SVMPs, which is in line with the fact that predominantly P-III class of SVMP precursors has been identified in the transcriptome of *P. olfersii* (Ching et al.,

2006). Another thing worth noting is that snake venom matrix metalloproteinases (svMMPs), major components recently discovered in the venom of the dipsadid *T. strigatus* (Ching et al., 2012), do not seem to be abundant components in the venoms analyzed here, including those from species belonging to the same family of *T. strigatus* (Pp, Poo, Pb and Htt).

Serine proteases have also been identified in several "colubrid" venoms (Assakura et al., 1994; Mackessy, 2002), and all are in much lower abundance than is commonly observed among viperid (rattlesnake) venoms (Mackessy, 2008). Consistent with an earlier report (Assakura et al., 1994), PooV showed the highest levels of activity, likely due to the serine protease PoFib S. Most of the venoms tested in this study showed higher activity toward TosylGlyProArg-pNA than toward BzPheValArg-pNA, similar to that observed for *Alsophis portoricensis* venom (Weldon and Mackessy, 2010), whereas most rattlesnake venoms show the opposite trend (Mackessy, 1993, 2008). From the results of 2D SDS-PAGE, PooV, PpV and TblV exhibited considerable diversity of protein spots in the masses typical of serine proteases (~27–35 kDa).

High levels of acetylcholinesterase activity have been reported from venoms of several *Boiga* species (Broaders and Ryan, 1997). In this study, PpV and TblV also exhibited relatively high acetylcholinesterase activity. However, similar to the venoms of *B. irregularis* (Hill and Mackessy, 2000) and *A. portoricensis* (Weldon and Mackessy, 2010), PbV, PooV and HttV displayed low levels of activity when assayed for acetylcholinesterase. As with serine protease activity, acetylcholinesterase activity is much lower in "colubrid" venoms than that observed in front-fanged (elapid) snake venoms (Mackessy et al., 2006).

The most striking enzymatic feature of TblV is the presence of PLA_2 activity, which is in stark contrast with the other rear-fanged snake venoms studied in this work. Previously, Hill and Mackessy (2000) had demonstrated that TblV exhibits high PLA_2 activity and that many other "colubrid" venoms appear to lack this common venom component. This PLA_2, trimorphin, was previously isolated from TblV (Huang and Mackessy, 2004) and N-terminal sequencing showed that it contains all of the hallmark characteristics of a type IA snake venom PLA_2 (Kini, 1997), including the highly conserved C11 residue, the Ca^{2+} binding sites and the catalytic H48 residue (Verheji et al., 1980; Scott et al., 1990a, 1990b). In the present study, the level of PLA_2 activity in the crude venom was approximately 2.5-fold lower than that observed for a different, larger snake (also Tbl) in an earlier study (Hill and Mackessy, 2000), demonstrating that intraspecific variation in expression levels of venom proteins, as observed in many other snake species, also occurs in *Trimorphodon*.

One dimension SDS-PAGE analysis demonstrated that the "colubrid" venoms studied in this work contained numerous protein bands, and profile comparisons demonstrated species-specific components as well as several shared components. Species belonging to the same family, such as *P. baroni*, *P. o. olfersii*, *P. patagoniensis* and *H. t. texana*, showed very similar protein banding patterns. As observed in venoms of other rear-fanged species (Weldon and Mackessy, 2010), the venoms studied in this work have greater complexity in the higher molecular mass region on 1D SDS-PAGE.

2DE has been the preferred technique used by several researchers to decipher the complexity of venoms due to its capability of separating a mixture of proteins based on two parameters, pI and mass (Vejayan et al., 2010), and 2DE resolved many more proteins in the present study than 1D SDS-PAGE. Comparison of the venom proteomes of two arboreal "colubrid" species of the same genus, *P. baroni* and *P. o. olfersii*, reveals a remarkable similarity between them, which may be related to their common habitats and similarities in diet. A striking feature in both proteomes that differentiates them from the others (terrestrial "colubrids") is the presence of basic proteins in the range of 50.0–75.0 kDa. Although a 2DE gel pattern of *P. olfersii* venom was previously shown (Ching et al., 2006), no proteins with such characteristics were observed in that work. As with most rear-fanged snake venoms (Mackessy, 2002), the venom proteome was less complex than that of front-fanged viperids (such as *B. jararaca*), but based on mass spectrometry and PAGE data, there are numerous proteins present which are characteristic of front-fanged snake venoms. The region of greatest complexity was primarily restricted to the 50–80 kDa acidic region of the gel. These proteins likely represent different isoforms of one or more metalloproteinases and/or acetylcholinesterases present in the venoms, as indicated by enzyme assays, MALDI-TOF, SE-HPLC and SDS-PAGE of fractions.

Cysteine-rich secretory proteins (CRISPs) were found in all venoms studied in this work, consistent with the observation that this family of proteins is a common component of most reptile venoms (Mackessy, 2002; Yamazaki and Morita, 2004). CRISPs are thought to interact with voltage-gated or/and cyclic nucleotide calcium channels (e.g., Fry et al., 2008), but the function of most CRISPs is not fully understood and their biological roles in venoms are unknown. However, the nearly ubiquitous presence in reptile venoms strongly suggests a common ancestry and a core importance to venom function.

Three-finger toxins (often neurotoxins), commonly found in some rear-fanged snake venoms (Levinson et al., 1976; Broaders et al., 1999; Mackessy, 2002; Fry et al., 2003a; Lumsden et al., 2005; Pawlak et al., 2006, 2009), were only observed in *T. b. lambda* venom following routine SDS-PAGE and in *T. b. lambda* and *P. olfersii* venoms following MALDI analysis. However, very low intensity bands in the 6–20 kDa mass range were seen in SDS-PAGE following SE-HPLC of the other venoms, and minor spots were observed in 2D gels, suggesting that if 3FTXs are present in them, they are only minor constituents of the venom proteome. But if we take into consideration that Ching et al. (2006) reported full-length sequences of CTLs from the venom gland transcriptome of *P. olfersii*, it is much more likely that those spots correspond to C-type lectins (CTLs). Also supporting this suggestion is the fact that Ching et al. (2012) have recently identified two major isoforms of CTL in the venom gland transcriptome and also in the venom proteome of the rear-fanged dipsadid snake *T. strigatus*. Thus, CTLs may be common components of venoms from "colubrids" belonging to the family Dipsadidae.

The severity of an envenomation depends on the nature of the venom components and, as demonstrated in this and other studies, "colubrid" snakes possess many venom proteins in common with the venoms of front-fanged snakes. In addition, with the vast majority of rear-fanged snake venoms still unstudied, there is an opportunity to discover novel biological molecules with possible medical implications. Thus, the preliminary comparative characterization of the overall venom protein composition of the five opisthoglyphous snakes presented in this paper certainly contributes to the current knowledge of venomous proteins and gives insight into future directions for research on them. Further, because of the complex phylogenetic history of "colubrids" (Vidal et al., 2007; Zaher et al., 2009), understanding the composition and evolution of venoms from rear-fanged snakes is central to understanding the evolution of squamate venoms generally.

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Conflict of interest

The authors declare that there are no conflicts of interest.

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