PeerJ

Snake venomics of *Bothrops punctatus*, a semi-arboreal pitviper species from Antioquia, Colombia

Maritza Fernández Culma[1], Jaime Andrés Pereañez[1,2], Vitelbina Núñez Rangel[1,3] and Bruno Lomonte[4]

[1] Programa de Ofidismo/Escorpionismo, Universidad de Antioquia UdeA, Medellín, Colombia
[2] Facultad de Química Farmacéutica, Universidad de Antioquia UdeA, Medellín, Colombia
[3] Escuela de Microbiología, Universidad de Antioquia UdeA, Medellín, Colombia
[4] Instituto Clodomiro Picado, Facultad de Microbiología, Universidad de Costa Rica, San José, Costa Rica

ABSTRACT

Bothrops punctatus is an endangered, semi-arboreal pitviper species distributed in Panamá, Colombia, and Ecuador, whose venom is poorly characterized. In the present work, the protein composition of this venom was profiled using the 'snake venomics' analytical strategy. Decomplexation of the crude venom by RP-HPLC and SDS-PAGE, followed by tandem mass spectrometry of tryptic digests, showed that it consists of proteins assigned to at least nine snake toxin families. Metalloproteinases are predominant in this secretion (41.5% of the total proteins), followed by C-type lectin/lectin-like proteins (16.7%), bradykinin-potentiating peptides (10.7%), phospholipases A_2 (9.3%), serine proteinases (5.4%), disintegrins (3.8%), L-amino acid oxidases (3.1%), vascular endothelial growth factors (1.7%), and cysteine-rich secretory proteins (1.2%). Altogether, 6.6% of the proteins were not identified. *In vitro*, the venom exhibited proteolytic, phospholipase A_2, and L-amino acid oxidase activities, as well as angiotensin-converting enzyme (ACE)-inhibitory activity, in agreement with the obtained proteomic profile. Cytotoxic activity on murine C2C12 myoblasts was negative, suggesting that the majority of venom phospholipases A_2 likely belong to the acidic type, which often lack major toxic effects. The protein composition of *B. punctatus* venom shows a good correlation with toxic activities here and previously reported, and adds further data in support of the wide diversity of strategies that have evolved in snake venoms to subdue prey, as increasingly being revealed by proteomic analyses.

Subjects Biochemistry, Biodiversity, Toxicology
Keywords Snake venom, Viperidae, Proteomics, *Bothrops punctatus*

Submitted 11 December 2013
Accepted 27 December 2013
Published 22 January 2014

Corresponding author
Bruno Lomonte,
bruno.lomonte@ucr.ac.cr

Academic editor
Vladimir Uversky

Additional Information and Declarations can be found on page 12

DOI **10.7717/peerj.246**

INTRODUCTION

The Chocoan forest lancehead, *Bothrops punctatus*, known in Colombia as 'rabo de chucha', is a large semi-arboreal pitviper, ranging from 1.0 to 1.5 m in length. *Campbell & Lamar (2004)* described its distribution from the Pacific foothills and coastal plain of eastern Panamá through western Colombia to northwestern Ecuador, with an altitudinal range between 1350 and 2300 m. In Colombia, *Daza, Quintana & Otero (2005)* reported

How to cite this article Fernández Culma et al. (2014), Snake venomics of *Bothrops punctatus*, a semi-arboreal pitviper species from Antioquia, Colombia. **PeerJ 2:e246; DOI 10.7717/peerj.246**

the occurence of *B. punctatus* in the Cauca and Magdalena river basins of Antioquia to eastern Chocó. Although *Bothrops* species are clearly predominant in the epidemiology of snakebite accidents occuring in Colombia (*Otero, 1994*; *Paredes, 2012*), published reports of proven envenomings caused by *B. punctatus* appear to be rare. The protein composition of the venom of this species has not been investigated, although at least two reports characterized its toxicological properties, in comparative studies of snake venoms from Colombia (*Otero et al., 1992*) and Ecuador (*Kuch et al., 1996*), respectively. The lethal potency of this venom to mice was highest among the different *Bothrops* venoms analyzed in these two studies, being only second to that of *Crotalus durissus terrificus* venom (*Otero et al., 1992*; *Kuch et al., 1996*). Due to the lack of knowledge on the venom composition of *B. punctata*, this work aimed at characterizing its proteomic profile using the 'snake venomics' analytical strategy (*Calvete, Juárez & Sanz, 2007*; *Calvete, 2011*), in combination with the assessment of its enzymatic or toxic activities *in vitro*.

METHODS

Venom

Venom was obtained from two adult *Bothrops punctatus* specimens collected in the eastern region of the Department of Antioquia, and kept in captivity at the Serpentarium of Universidad de Antioquia, Medellín, Colombia, under institutional permission for Programa de Ofidismo/Escorpionismo. Venom samples were centrifuged to remove debris, pooled, lyophilized and stored at −20°C. In some functional assays, pooled venom obtained from more than 30 specimens of *Bothrops asper*, collected in the Departments of Antioquia and Chocó, was included for comparative purposes.

Proteomic profiling

For reverse-phase (RP) HPLC separations, 2.5 mg of venom was dissolved in 200 µL of water containing 0.1% trifluoroacetic acid (TFA; solution A), centrifuged for 5 min at 15,000 × g, and loaded on a C_{18} column (250 × 4.6 mm, 5 µm particle; Teknokroma) using an Agilent 1200 chromatograph with monitoring at 215 nm. Elution was performed at 1 mL/min by applying a gradient towards solution B (acetonitrile, containing 0.1% TFA), as follows: 0% B for 5 min, 0–15% B over 10 min, 15–45% B over 60 min, 45–70% B over 10 min, and 70% B over 9 min (*Lomonte et al., 2014*). Fractions were collected manually, dried in a vacuum centrifuge, and further separated by SDS-PAGE under reducing or non-reducing conditions, using 12% gels. Protein bands were excised from Coomassie blue R-250-stained gels and subjected to reduction with dithiothreitol (10 mM) and alkylation with iodoacetamide (50 mM), followed by in-gel digestion with sequencing grade bovine trypsin (in 25 mM ammonium bicarbonate, 10% acetonitrile) overnight on an automated processor (ProGest Digilab), according to the manufacturer. The resulting peptide mixtures were analyzed by MALDI-TOF-TOF mass spectrometry on an Applied Biosystems 4800-Plus instrument. Peptides were mixed with an equal volume of saturated α-CHCA matrix (in 50% acetonitrile, 0.1% TFA), spotted (1 µL) onto Opti-TOF 384-well plates, dried, and analyzed in positive reflector mode. Spectra were acquired using a laser intensity of 3000 and 1500 shots/spectrum, using as external standards CalMix-5 (ABSciex) spotted on the same plate. Up to 10 precursor peaks from each MS spectrum were selected for automated collision-induced dissociation MS/MS spectra acquisition at 2 kV, in positive mode (500 shots/spectrum, laser intensity of 3000). The resulting spectra were analyzed using ProteinPilot v.4 (ABSciex) against the UniProt/SwissProt database using the Paragon® algorithm at a confidence level of ≥95%, for the assignment of proteins to known families. Few peptide sequences with lower confidence scores were manually searched using BLAST (http://blast.ncbi.nlm.nih.gov). Finally, the relative abundance of each protein (% of total venom proteins) was estimated by integration of the peak signals at 215 nm, using Chem Station B.04.01 (Agilent). When a peak from HPLC contained two or more SDS-PAGE bands, their relative distribution was estimated by densitometry using the Image Lab v.2.0 software (Bio-Rad) (*Calvete, 2011*).

Venom activities

Phospholipase A_2 activity

Venom phospholipase A_2 (PLA_2) activity was determined on the monodisperse synthetic substrate 4-nitro-3-octanoyl-benzoic acid (NOBA) (*Holzer & Mackessy, 1996*), in triplicate wells of microplates. Twenty µL of venom solutions, containing 20 µg protein, were mixed with 20 µL of water, 200 µL of 10 mM Tris, 10 mM $CaCl_2$, 100 mM NaCl, pH 8.0 buffer, and 20 µL of NOBA (0.32 mM final concentration). Plates were incubated at 37°C, and the change in absorbance at 425 nm was recorded after 20 min in a microplate reader (Awareness Technology).

Proteolytic activity

Proteolysis was determined upon azocasein (Sigma-Aldrich) as described by *Wang, Shih & Huang (2004)*. Twenty µg of venoms were diluted in 20 µL of 25 mM Tris, 0.15 M NaCl, 5 mM $CaCl_2$, pH 7.4 buffer, added to 100 µL of azocasein (10 mg/mL) and incubated for 90 min at 37°C. The reaction was stopped by adding 200 µL of 5% trichloroacetic acid. After centrifugation, 100 µL of supernatants were mixed with an equal volume of 0.5 M NaOH, and absorbances were recorded at 450 nm. Experiments were carried out in triplicate.

L-amino acid oxidase activity

L-amino acid oxidase (LAAO) activity was determined by adding various concentrations of venom (2.5–20 µg) in 10 µL of water to 90 µL of a reaction mixture containing 250 mM L-Leucine, 2 mM *o*-phenylenediamine, and 0.8 U/mL horseradish peroxidase, in 50 mM Tris, pH 8.0 buffer, in triplicate wells of a microplate (*Kishimoto & Takahashi, 2001*). After incubation at 37°C for 60 min, the reaction was stopped with 50 µL of 2 M H_2SO_4, and absorbances were recorded at 492 nm.

Cytotoxic activity

Cytotoxic activity was assayed on murine skeletal muscle C2C12 myoblasts (ATCC CRL-1772) as described by *Lomonte et al. (1999)*. Venom (40 µg) was diluted in assay medium (Dulbecco's Modified Eagle's Medium [DMEM] supplemented with 1% fetal calf serum [FCS]), and added to subconfluent cell monolayers in 96-well plates, in 150 µL, after removal of growth medium (DMEM with 10% FCS). Controls for 0 and 100% toxicity consisted of assay medium, and 0.1% Triton X-100 diluted in assay medium, respectively. After 3 h at 37°C, a supernatant aliquot was collected to determine the lactic dehydrogenase (LDH; EC 1.1.1.27) activity released from damaged cells, using a kinetic assay (Wiener LDH-P UV). Experiments were carried out in triplicate.

ACE inhibitory activity

The angiotensin-converting enzyme (ACE) inhibitory activity of fraction 4 from the HPLC separation (see Table 1), which was identified as a bradykinin-potentiating peptide-like component, was assayed by the method of *Cushman & Cheung (1971)* with some modifications (*Kim et al., 1999*). Various concentrations of the fraction, diluted in 20 µL, were added to 100 µL of 10 mM N-hippuryl-His-Leu substrate diluted in 2 mM potassium phosphate, 0.6 M NaCl, pH 8.3 buffer, and 5 mU of ACE (EC 3.4.15.1; 5.1 UI/mg) diluted in 50% glycerol. The reaction was incubated at 37°C for 30 min, and stopped by adding 200 µL of 1 NHCl. The produced hippuric acid was extracted by vigorous stirring for 10 s, followed by the addition of 600 µL of ethyl acetate, and centrifugation for 10 min at 4000 × g. An aliquot of 500 µL of organic phase was dried at 95°C for 10 min. The residue was dissolved in 1 mL of water and, after stirring, the absorbance was measured at 228 nm. The percentage of ACE inhibition (% ACEi) was determined using the following formula; % ACEi = (Abs Control − Abs sample)/(Abs control − Abs blank). Control absorbance corresponded to hippuric acid formed after the action of ACE, while blank absorbance was enzyme without substrate.

Statistical analyses

The significance of differences between means was assessed by ANOVA, followed by Dunnett's test, when several experimental groups were compared with the control group, or by Student's t-test, when two groups were compared. Differences were considered significant if $p < 0.05$.

RESULTS AND DISCUSSION

B. punctatus has been included in the 'red list', a report categorizing conservation status, as a threatened species (*Carrillo et al., 2005*). Very scarce information on its venom is available in the literature. In comparative studies of snake venoms from Colombia (*Otero et al., 1992*) and Ecuador (*Kuch et al., 1996*), respectively, this venom was found to induce local effects such as hemorrhage, edema, and myonecrosis, as well as systemic alterations such as defibrination, in similarity to venoms from other *Bothrops* species. Developments in proteomic techniques have brought new possibilities to examine the detailed toxin composition of snake venoms, increasing knowledge on their evolution, toxicological properties, and correlation with clinical features of envenomings (*Calvete, Juárez & Sanz, 2007*; *Calvete, 2013*; *Fox & Serrano, 2008*; *Valente et al., 2009*; *Ohler et al., 2010*). Therefore, the venom of *B. punctatus* was analyzed for the first time using proteomic tools, to gain a deeper understanding on its protein composition and relationships to toxic and enzymatic actions.

Table 1 Assignment of the RP-HPLC isolated fractions of *Bothrops punctatus* venom to protein families by MALDI-TOF-TOF of selected peptide ions from in-gel trypsin-digested protein bands.

Peak	%	Mass (kDa)	Peptide ion *m/z*	*z*	MS/MS-derived amino acid sequence*	Protein family; ∼ related protein
1	0.2		-	-	-	unknown
2	0.3		-	-	-	unknown
3	1.6		-	-	-	unknown
4	10.7	-	967.5	1	ZBWAPVBK	BPP-like; ∼ Q7T1M3
5	0.8	▼10	2259.1	1	XARGDDM^{ox} DDYCNGXSAGCPR	Disintegrin; ∼ Q7SZD9
			2051.0	1	XRPGABCAEGXCCDBCR	
			2459.0	1	EAGEECDCGTPGNPCCDAATCK	
6	3.0	▼10	1902.9	1	GDDMDDYCNGXSAGCPR	Disintegrin; ∼ Q0NZX5
			2243.1	1	XARGDDMDDYCNGXSAGCPR	
			2051.0	1	XRPGABCAEGXCCDBCR	
			2459.1	1	EAGEECDCGTPGNPCCDAATCK	
7	0.3		-	-	-	unknown
8	1.7	▼11	2062.0	1	CGGCCTDESXECTATGBR	VEGF; ∼ Q90X23
			3134.9	1	ETXVSXXEEHPDEVSHXFRPSCVTAXR	
9	1.2	▼22 ■18	2526.1	1	SGPPCGDCPSACDNGXCTNPCTK	CRISP; ∼ Q7ZT99
			1537.8	1	MEWYPEAAANAER	
			1828.9	1	YFYVCBYCPAGNMR	
10a	0.4	▼38	1561.9	1	SVPNDDEEXRYPK	Serine proteinase; ∼ Q5W960
10b	0.2	▼29 ■28	1206.8	1	XMGWGTXSPTK	Serine proteinase; ∼ Q072L6
			1683.2	1	TYTBWDBDXMXXR	
			2534.5	1	VSYPDVPHCANXNXXDYEVCR	
			1069.8	1	FXVAXYTSR	
			1512.8	1	VXGGDECNXNEHR	
			3387.8	1	DSCBGDSGGPXXCNGBFBGXXSWGVHPCGBR	
10c	0.3	▼12 ■22	-	-	-	unknown
11	1.5	▼28 ■20	1288.7	1	NFBMBXGVHSK	Serine proteinase; ∼ Q072L6
			1190.7	1	XMGWGTXSPTK	
			2305.4	1	AAYPWBPVSSTTXCAGXXBGGK	
			1140.6	1	VSDYTEWXK	
			2477.5	1	VSNSEHXAPXSXPSSPPSVGSVCR	
			2477.4	1	VXGGDECNXNEHR	
12a	1.8	▼35	1083.7	1	FXAFXYPGR	Serine proteinase; ∼ Q6IWF1
12b	0.4	▼29 ■22	1517.9	1	NDDAXDBDXMXVR	Serine proteinase; ∼ Q5W959
			1499.8	1	VVGGDECNXNEHR	
			2294.3	1	TNPDVPHCANXNXXDDAVCR	
			1279.7	1	AAYPEXPAEYR	
			2889.7	1	XDSPVSNSEHXAPXSXPSSPPSVGSVCR	
			1083.7	1	FXAFXYPGR	
13–15	0.8		-	-	-	unknown

(*continued on next page*)

Table 1 (*continued*)

Peak	%	Mass (kDa)	Peptide ion *m/z*	*z*	MS/MS-derived amino acid sequence*	Protein family; ∼ related protein
16	3.1	▼16 ■16	1505.7	1	CCFVHDCCYGK	Phospholipase A_2, D49; ∼ P86389
			934.6	1	YWFYGAK	
			1966.1	1	YXSYGCYCGWGGXGBPK	
			2064.1	1	DATDRCCFVHDCCYGK	
			2027.2	1	DNBDTYDXBYWFYGAK	
			2626.4	1	XDXYTYSBETGDXVCGGDDPCBK	
			1786.0	1	BXCECDRVAATCFR	
17a	0.4	▼14 ■21	1928.9	1	DCPPDWSSYEGHCYR	C-type lectin/lectin-like; ∼ P22030
17b	1.7	▼15 ■16	2027.1	1	DNBDTYDXBYWFYGAK	Phospholipase A_2, D49; ∼ C9DPL5
17c	0.4	■13	1720.8	1	E^{pa} NGDVVCGGDDPCBK	Phospholipase A_2, D49; ∼ P86389
			1505.7	1	CCFVHDCCYGK	
			2064.0	1	DATDRCCFVHDCCYGK	
18	2.8	▼13	2064.0	1	DATDRCCFVHDCCYGK	Phospholipase A_2, D49; ∼ Q9I968
19	0.3		-	-	-	unknown
20	6.2	▼13 ■19	1928.9	1	DCPSDWSPYEGHCYR	C-type lectin/lectin-like; ∼ Q9PS06
21	0.8		-	-	-	unknown
22a	0.9	■120	1537.8	1	ACSNGBCVDVNRAS	Metalloproteinase; ∼ Q8AWI5
			1269.7	1	SAECTDRFBR	
22b	3.1	▼53 ■48	3185.9	1	VVXVGAGMSGXSAAYVXANAGHBVTVXEASER	L-amino acid oxidase; ∼ Q6TGQ9
			2605.5	1	BFGXBXNEFSBENENAWYFXK	
			2271.3	1	XYFAGEYTABAHGWXDSTXK	
			1388.8	1	BFWEDDGXHGGK	
			1352.8	1	SAGBXYEESXBK	
22c	0.9	▼13	1636.0	1	NXBSSDXYAWXGXR	C-type lectin/lectin-like; ∼ P22029
			1928.9	1	DCPPDWSSYEGHCYR	
23–25a	1.3	▼13	1533.7	1	SYGAYGCNCGVXGR	Phospholipase A_2, K49; ∼ Q9PVE3
23–25b	1.1	▼28, ■20	1279.7	1	AAYPEXPAEYR	Serine proteinase; ∼ Q5W959
			14.997	1	VVGGDECNXNEHR	
			2294.1	1	TNPDVPHCANXNXXDDAVCR	
23–25c	0.9	▼13, ■19	1635.8	1	NXBSSDXYAWXGXR	C-type lectin/lectin-like; ∼ P22029
26	14.4	▼23 ■42	2040.2	1	YXYXDXXXTGVEXWSNK	Metalloproteinase; ∼ P86976
			1114.6	1	XHBMVNXMK	
			2257.3	1	DXXNVBPAAPBTXDSFGEWR	
			1828.0	1	YVEXFXVVDHGMFMK	
27	2.0		-	-	-	unknown
28a	18.3	▼46 ■42	1552.7	1	VCSNGHCVDVATAY	Metalloproteinase; ∼ Q8QG88
			2953.3	1	ASM^{ox} SECDPAEHCTGBSSECPADVFHK	
			2154.2	1	XTVBPDVDYTXNSFAEWR	

(*continued on next page*)

Table 1 (*continued*)

Peak	%	Mass (kDa)	Peptide ion *m/z*	*z*	MS/MS-derived amino acid sequence[*]	Protein family; ∼ related protein
28b	2.1	■21	3261.7	1	TDXVSPPVCGNYFVEVGEDCDCGSPATCR	Metalloproteinase;
			1457.0	1	XVXVADYXM^{ox} FXK	∼ O93517
28c	6.2	▼14	1635.9	1	NXBSSDXYAWXGXR	C-type lectin-like;
			1193.6	1	TTDNBWWSR	∼ P22029
29a	3.2	▼46	2154.2	1	XTVBPDVDYTXNSFAEWR	Metalloproteinase;
			1609.9	1	XYEXVNTXNVXYR	∼ Q8QG88
			1775.0	1	YVEFFXVVDBGMVTK	
29b	2.1	▼14	992.5	1	MNWADAER	C-type lectin/lectin-like;
			1928.8	1	DCPPDWSSYEGHCYR	∼ M1V359
			1842.9	1	MNWADAERFCSEQAK	
30	2.6	▼38	1327.8	1	YXEXVXVADHR	Metalloproteinase; ∼ Q8AWX7

Notes.

[*] Cysteine residues determined in MS/MS analyses are carbamidomethylated.

X, Leu/Ile; B, Lys/Gln; ^{ox}, oxidized; ^{pa}, propionamide.

▼, reduced, or ■, non-reduced SDS-PAGE mass estimations, in kDa. Abbreviations for protein families as in Fig. 2.

RP-HPLC of the crude venom resulted in the separation of 30 fractions (Fig. 1C), which were further subjected to SDS-PAGE (Fig. 1B), in-gel digestion of the excised bands, and MALDI-TOF-TOF analysis of the resulting peptides. The amino acid sequences obtained allowed the unambiguous assignment of 29 out of the 37 components analyzed, to known protein families of snake venoms (Table 1). Protein family relative abundances were estimated by integration of the chromatographic areas, combined with gel densitometric scanning. Results showed that the predominant proteins in this secretion are metalloproteinases (41.5%; SVMP), followed by C-type lectin/lectin-like proteins (16.7%; CTL), bradykinin-potentiating peptide-like peptides (10.7%; PEP), phospholipases A_2 of both the D49 (8.0%) and K49 (1.3%) subtypes (for a combined 9.3%; PLA_2), serine proteinases (5.4%; SP), disintegrins (3.8%; DIS), L-amino acid oxidases (3.1%; LAO), vascular endothelial growth factor (1.7%; VEGF), and cysteine-rich secretory proteins (1.2%; CRISP), as summarized in Fig. 2 and Table 1. An estimated 6.6% of the proteins remained unidentified, and owing to the scarcity of the venom, their assignment could not be further pursued.

A recent phylogenetic analysis of the genus *Bothrops* (*sensu lato*) by *Fenwick et al. (2009)* grouped *B. punctatus* within the same clade as *Bothrops atrox* and *Bothrops asper*. Since the proteomic profile of the venoms of the latter two species has been reported (*Núñez et al., 2009*; *Alape-Girón et al., 2008*), a comparison of their venom compositions, together with those of two other pitviper species distributed in Colombia, *Bothrops ayerbei* (*Mora-Obando et al., 2014*) and *Bothriechis schlegelii* (*Lomonte et al., 2008*), was compiled (Table 2). Venoms from these five species have been analyzed by the same methodological strategy, therefore allowing reliable comparisons. The composition of *B. punctatus* venom resembles that of the other *Bothrops* species listed in Table 2 only

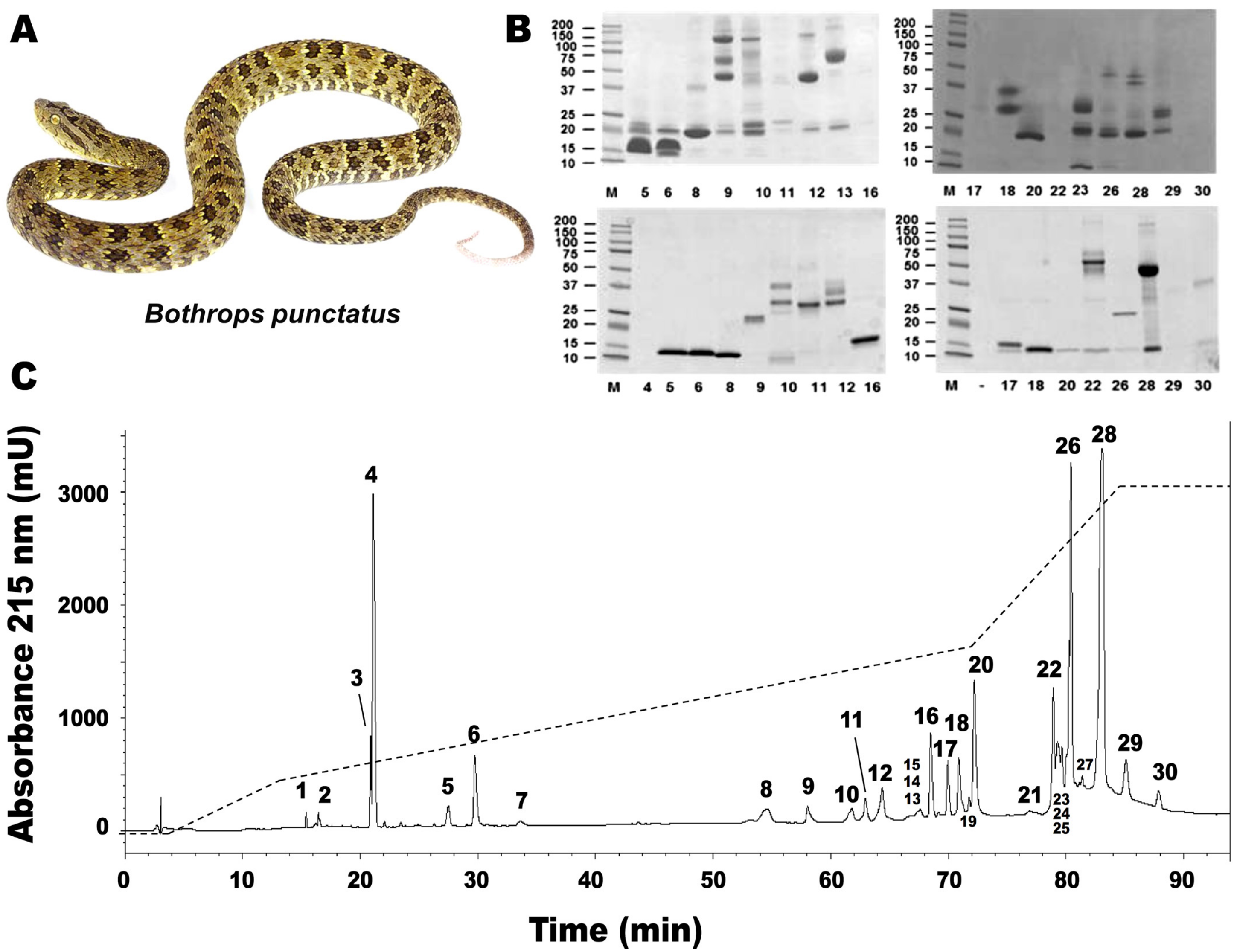

Figure 1 **Separation of *Bothrops punctatus* (A) venom proteins by RP-HPLC (C) and SDS-PAGE (B).** Venom was fractionated on a C_{18} column (C) by applying an acetonitrile gradient from 0 to 70% (dashed line), as described in Methods. Each fraction was analyzed by SDS-PAGE (B) under non-reducing (top gels) or reducing (bottom gels) conditions. Molecular weight markers (M) are indicated in kDa, at the left. Tryptic digests of the excised protein bands were characterized by MALDI-TOF/TOF, as summarized in Table 1. The photograph of *B. punctatus* was obtained with permission from www.tropicalherping.com.

in terms of their high content of metalloproteinases (41.5–53.7%), but overall, its composition departs from the relative protein abundances observed in any of the other four pitvipers. The high proportion of CTL proteins in *B. punctatus* is of note, doubling the abundance observed in *B. atrox*, and close to that of *B. ayerbei*, while in contrast such proteins are expressed only in trace amounts in *B. asper*, and have not been detected in *B. schlegelii* (Table 2). Further, *B. punctatus* venom presents a modest amount of VEGF (1.7%), which has not been found in any of the venoms listed in Table 2. Similar to the venom of the arboreal snake *B. schlegelii*, but also with the terrestrial species *B.*

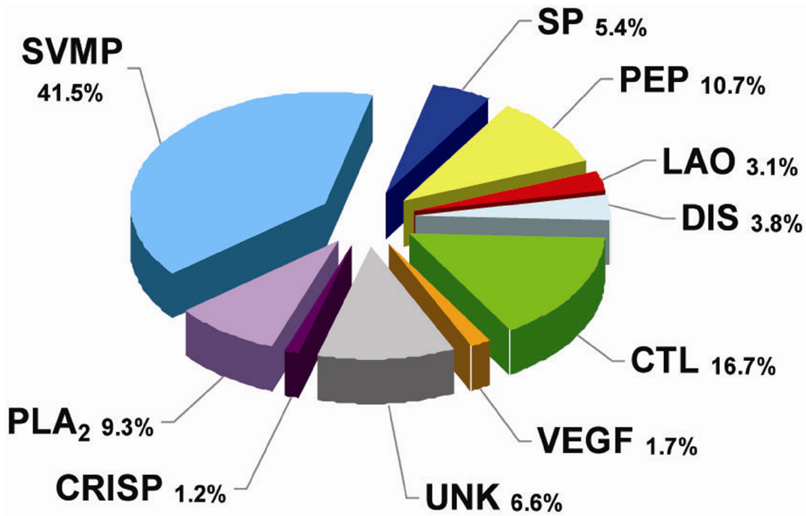

Figure 2 **Composition of *Bothrops punctatus* venom according to protein families, expressed as percentages of the total protein content.** SP, serine proteinase; PLA_2, phospholipase A_2; CRISP, cysteine-rich secretory protein; DIS, disintegrin; PEP, bradykinin-potentiating peptide-like (BPP-like); LAO, L-amino acid oxidases; SVMP, metalloproteinase; VEGF, vascular endothelium growth factor; CTL, C-type lectin/lectin-like; UNK, unknown/unidentified.

ayerbei, the venom of *B. punctatus* presents a high content of BPP-like peptides, strikingly differing from *B. asper* and *B. atrox* venoms in this regard. The possible trophic relevance of these vasoactive peptides among viperids remains elusive, and no clear correlations with prey types or habitats have been disclosed thus far. BPPs are oligopeptides of 5–14 amino acid residues, rich in proline residues and often presenting a pyroglutamate residue, which display bradykinin-potentiating activity. Their pharmacological effect is related to the inhibition of angiotensin I-converting enzyme (ACE) (*Ianzer et al., 2007*). Peak 4 of the HPLC separation of *B. punctatus* venom components (Fig. 1C) was identified as a BPP (Table 1), and its inhibitory activity on ACE was confirmed, showing a half-maximal inhibition of this enzyme at 0.9 mg/mL (Fig. 3A). Interest in snake venom BPPs stems from their potential in the development of hypotensive drugs, as exemplified by Captopril®. Overall, the comparison of *B. punctatus* venom with those of other pitvipers distributed in Colombia (Table 2) highlights the remarkable divergence of compositional profiles that have arisen through the evolution and diversification of snakes (*Casewell et al., 2013*).

The protein composition of *B. punctatus* venom correlates with the enzymatic activities assayed, as well as with those described in earlier studies (*Otero et al., 1992*; *Kuch et al., 1996*). L-amino acid oxidase (Fig. 3C), proteolytic (Fig. 4A), and PLA_2 (Fig. 4B) activities of this venom were corroborated. Interestingly, its proteolytic activity was higher than that of *B. asper* venom (Fig. 4A), and this might be related to the stronger hemorrhagic potency that was reported for *B. punctatus* venom in comparison to *B. asper* venom (*Otero et al., 1992*). Hemorrhage induced by viperid venoms is mainly dependent on the proteolytic action of SVMPs upon the microvasculature and its extracellular matrix support (*Bjarnason & Fox, 1994*; *Gutiérrez et al., 2005*), and this effect can be enhanced by venom components affecting haemostasis, such as procoagulant SPs with thrombin-like

Table 2 **Comparison of the venom composition of *Bothrops punctatus* with venoms from pitviper species distributed in Colombia.**[*]

Protein family	Snake species				
	Bothrops punctatus[a]	***Bothrops atrox***[b]	***Bothrops asper***[c]	***Bothriechis schlegelii***[d]	***Bothrops ayerbei***[e]
Metalloproteinase	41.5	48.5	44.0	17.7	53.7
Phospholipase A_2	9.3	24.0	45.1	43.8	0.7
Serine proteinase	5.4	10.9	10.9	5.8	9.3
BPP-like	10.7	0.3	-	13.4	8.3
CRISP	1.2	2.6	0.1	2.1	1.1
C-type lectin/lectin-like	16.7	7.1	0.5	-	10.1
VEGF	1.7	-	-	-	-
L-amino acid oxidase	3.1	4.7	4.6	8.9	3.3
Disintegrin	3.8	1.7	1.4	-	2.3
Kazal type inhibitor	-	-	-	8.3	-
Phosphodiesterase	-	-	-	-	0.7
Nerve growth factor	-	-	-	-	0.1
unknown	6.6	-	-	-	1.7
Number of families	**9**	**8**	**7**	**7**	

Notes.

[*] Although *B. asper* and *B. schlegelii* are found in Colombia, data correspond to venoms from specimens found in Costa Rica.

[a] Present work.

[b] *Núñez et al. (2009)*.

[c] *Alape-Girón et al. (2008)*, specimens of Pacific versant.

[d] *Lomonte et al. (2008)*.

[e] *Mora-Obando et al. (2014)*.

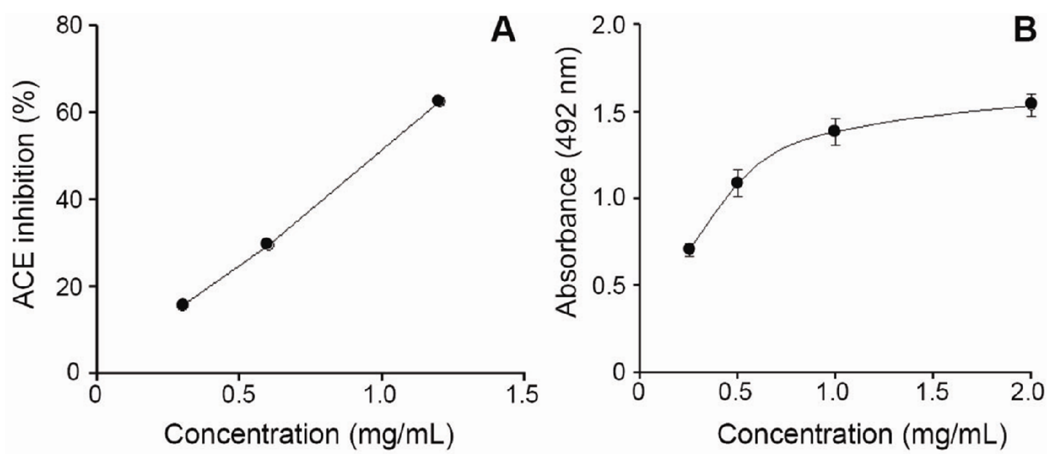

Figure 3 **Bothrops punctatus venom activities.** (A) Inhibition of angiotensin-converting enzyme (ACE) by peak 4 of *B. punctatus* venom, identified as a BPP-like peptide (Table 1). Each point represents the mean ± SD of three replicates. (B) L-amino acid oxidase activity of *B. punctatus* venom. Each point represents the mean ± SD of three replicates.

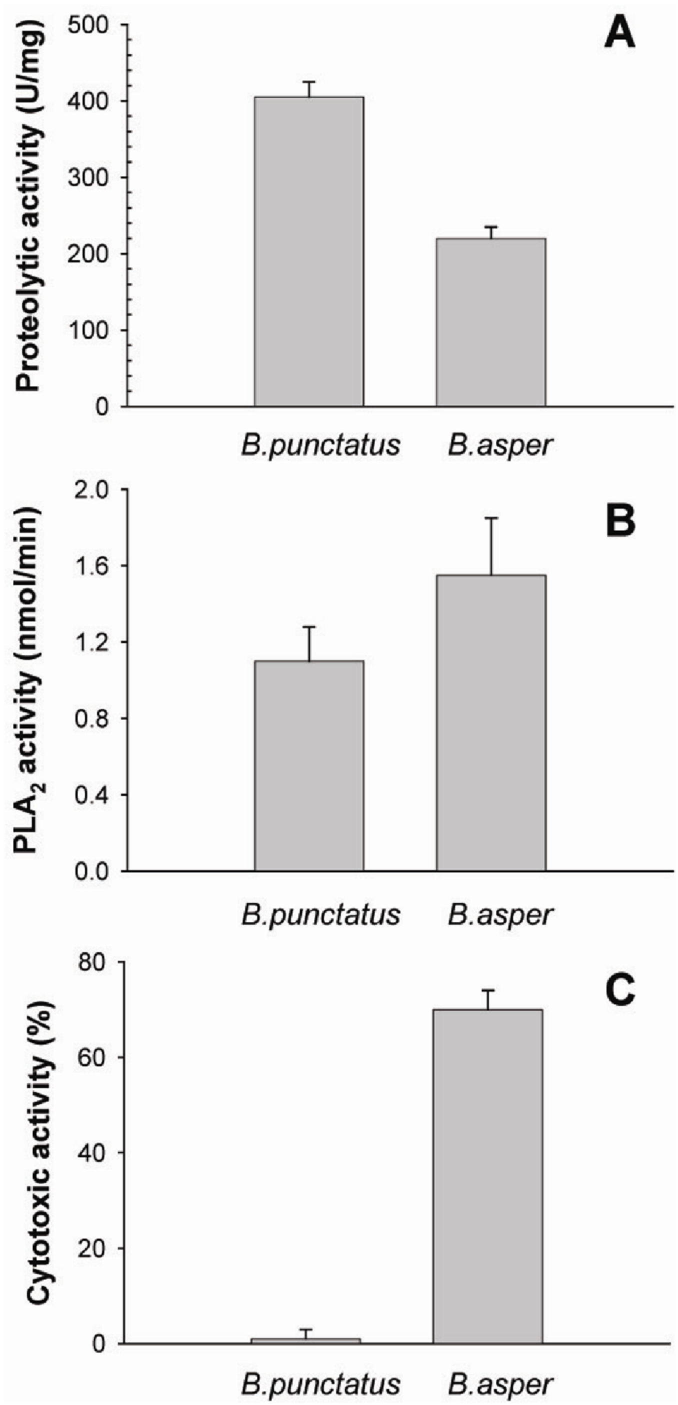

Figure 4 Proteolytic (A), phospholipase A$_2$ (B), and cytotoxic (C) activities of *Bothrops punctatus* venom, compared to the venom of *Bothrops asper*. Proteolytic activity was determined on azocasein, using 20 µg of each venom. Phospholipase A$_2$ activity was determined on 4-nitro-3-octanoyloxy-benzoic acid, using 20 µg of each venom. Cytotoxic activity was determined on C2C12 murine myoblasts, using 40 µg of each venom, as described in Methods. Bars represent mean ± SD of three replicates. For each activity, differences between the two venoms were significant ($p < 0.05$).

activity, or some CTL components and disintegrins that potently interfere with platelets, among others (*Gutiérrez, Escalante & Rucavado, 2009*; *Calvete et al., 2005*). Considering that the proportion of SVMPs is lower in *B. punctatus* than in *B. asper* venom (Table 2), the higher hemorrhagic action reported for the former (*Otero et al., 1992*) suggests that its abundant CTL components (16.7%) might include toxins that affect platelets, a hypothesis that deserves future investigation. On the other hand, the PLA_2 activity of *B. punctatus* venom was lower than that of *B. asper* (Fig. 4B), in agreement with their corresponding relative contents of these enzymes (Table 2). However, a major contrast was evidenced in the cytotoxic activity of these two venoms upon myogenic cells in culture, *B. punctatus* being essentially devoid of this effect, while *B. asper* causing overt cytolysis and LDH release under identical conditions (Fig. 4C). Since cytolysis of myogenic cells, an *in vitro* correlate for *in vivo* myotoxicity (*Lomonte et al., 1999*), has been shown to be mediated mainly by basic PLA_2s in the case of viperid venoms (*Gutiérrez & Lomonte, 1995*; *Lomonte & Rangel, 2012*), this finding anticipates that the catalytically active (D49) PLA_2s present in *B. punctatus* venom are likely to belong to the acidic type of these enzymes, which despite frequently having higher enzymatic activity than their basic counterparts, usually display very low, or even no toxicity (*Fernández et al., 2010*; *Van der Laat et al., 2013*). In contrast, the venom of *B. asper* is rich in basic D49 and K49 PLA_2s/PLA_2 homologues with strong cytolytic and myotoxic effects (*Angulo & Lomonte, 2005*; *Angulo & Lomonte, 2009*) that would explain the present findings. Although at least one PLA_2 component of *B. punctatus* venom was shown to belong to the K49 type of catalytically-inactive, basic PLA_2 homologues (fraction 23–25a; Table 1), its low abundance (1.3%) in the venom would be in agreement with the observed lack of cytotoxicity (Fig. 4C).

In summary, the general compositional profile of *B. punctatus* venom was obtained through the analytical strategy known as 'snake venomics'. The present data add to the growing body of knowledge on the remarkable diversity of compositional strategies in snake venom 'cocktails', in spite of the reduced number of gene families that encode their proteins/toxins (*Casewell et al., 2013*; *Calvete, 2013*). Due to the key adaptive role of venoms, this knowledge, in combination with toxicological, ecological, and natural history information, could lead to a deeper understanding of the evolutionary trends and selective advantages conferred by particular venom compositions in the divergence of snakes. In addition, compositional data may offer a more comprehensive basis to foresee the features of envenomings by this pitviper species, largely unreported in the literature.

ACKNOWLEDGEMENTS

We thank Leidy Gómez, Paola Rey, and Wan-Chih Tsai for their valuable collaboration in the laboratory.

ADDITIONAL INFORMATION AND DECLARATIONS

Funding

This work was funded by grants obtained from Universidad de Antioquia Sostenibilidad (2013–2014), and from Vicerrectoría de Investigación, Universidad de Costa Rica (project

"Network for proteomic characterization of snake venoms of medical and biological relevance in Latin America"; 741-B3-760). The Proteomics Laboratory of the Instituto Clodomiro Picado is partially supported by the Vicerrectoría de Investigación, UCR. Maritza Fernández received a Young Researcher Fellowship from COLCIENCIAS. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Grant Disclosures

The following grant information was disclosed by the authors:
Universidad de Antioquia Sostenibilidad (2013–2014).
Vicerrectoría de Investigación, Universidad de Costa Rica.

Competing Interests

Bruno Lomonte is an Academic Editor for PeerJ. There are no other competing interests to declare regarding this manuscript.

Author Contributions

- Maritza Fernández Culma and Jaime Andrés Pereañez performed the experiments, analyzed the data, wrote the paper.
- Vitelbina Núñez Rangel conceived and designed the experiments, analyzed the data, wrote the paper.
- Bruno Lomonte analyzed the data, wrote the paper.

Animal Ethics

The following information was supplied relating to ethical approvals (i.e., approving body and any reference numbers):

The "Programa de Ofidismo/Escorpionismo" has institutional permission from Universidad de Antioquia to have a serpentarium to keep live snakes for scientific research purposes.

REFERENCES

Alape-Girón A, Sanz L, Escolano J, Flores-Díaz M, Madrigal M, Sasa M, Calvete JJ. 2008. Snake venomics of the lancehead pit viper *Bothrops asper*: geographic, individual, and ontogenetic variations. *Journal of Proteome Research* **7**:3556–3571 DOI 10.1021/pr800332p.

Angulo Y, Lomonte B. 2005. Differential susceptibility of C2C12 myoblasts and myotubes to group II phospholipase A_2 myotoxins from crotalid snake venoms. *Cell Biochemistry and Function* **23**:307–313 DOI 10.1002/cbf.1208.

Angulo Y, Lomonte B. 2009. Biochemistry and toxicology of toxins purified from the venom of the snake *Bothrops asper*. *Toxicon* **54**:949–957 DOI 10.1016/j.toxicon.2008.12.014.

Bjarnason JB, Fox JW. 1994. Hemorrhagic metalloproteinases from snake venoms. *Pharmacology & Therapeutics* **62**:325–372 DOI 10.1016/0163-7258(94)90049-3.

Calvete JJ. 2011. Proteomic tools against the neglected pathology of snake bite envenoming. *Expert Reviews on Proteomics* **8**:739–758 DOI 10.1586/epr.11.61.

Calvete JJ. 2013. Snake venomics: from the inventory of toxins to biology. *Toxicon* **75**:44–62 DOI 10.1016/j.toxicon.2013.03.020.

Calvete JJ, Juárez P, Sanz L. 2007. Snake venomics, strategy and applications. *Journal of Mass Spectrometry* **42**:1405–1414 DOI 10.1002/jms.1242.

Calvete JJ, Marcinkiewicz C, Monleón D, Esteve V, Celda B, Juárez P, Sanz L. 2005. Snake venom disintegrins: evolution of structure and function. *Toxicon* **45**:1063–1074 DOI 10.1016/j.toxicon.2005.02.024.

Campbell JA, Lamar WW. 2004. *The venomous reptiles of the western hemisphere*. Ithaca, New York: Cornell University Press.

Carrillo E, Aldás S, Altamirano MA, Ayala-Varela F, Cisneros-Heredia DF, Endara A, Márquez C, Morales M, Nogales-Sornosa F, Salvador P, Torres ML, Valencia J, Villamarín-Jurado F, Yánez-Muñoz MH, Zárate P. 2005. *Lista roja de los reptiles del Ecuador*. Quito, Ecuador: Fundación Novum Milenium, Ministerio de Educación y Cultura.

Casewell NR, Wüster W, Vonk FJ, Harrison RA, Fry BG. 2013. Complex cocktails: the evolutionary novelty of venoms. *Trends in Ecology and Evolution* **28**:219–229 DOI 10.1016/j.tree.2012.10.020.

Cushman DW, Cheung HS. 1971. Spectrophotometric assay and properties of the angiotensin-converting enzyme of rabbit lung. *Biochemical Pharmacology* **20**:1637–1648 DOI 10.1016/0006-2952(71)90292-9.

Daza JM, Quintana JC, Otero R. 2005. *Bothrops punctatus* (Chocoan forest pitviper). *Herpetological Review* **36**:338.

Fenwick AM, Gutberlet Jr RL, Evans JA, Parkinson CL. 2009. Morphological and molecular evidence for phylogeny and classification of South American pitvipers, genera *Bothrops*, *Bothriopsis*, and *Bothrocophias* (Serpentes: *Viperidae*). *Zoological Journal of the Linnean Society* **156**:617–640 DOI 10.1111/j.1096-3642.2008.00495.x.

Fernández J, Gutiérrez JM, Angulo Y, Sanz L, Juárez P, Calvete JJ, Lomonte B. 2010. Isolation of an acidic phospholipase A_2 from the venom of the snake *Bothrops asper* of Costa Rica: biochemical and toxicological characterization. *Biochimie* **92**:273–283 DOI 10.1016/j.biochi.2009.12.006.

Fox JW, Serrano SMT. 2008. Exploring snake venom proteomes: multifaceted analyses for complex toxin mixtures. *Proteomics* **8**:909–920 DOI 10.1002/pmic.200700777.

Georgieva D, Ohler M, Seifert J, von Bergen M, Arni RK, Genov N, Betzel C. 2010. Snake venomic of *Crotalus durissus terrificus* - correlation with pharmacological activities. *Journal of Proteome Research* **9**:2302–2316 DOI 10.1021/pr901042p.

Gutiérrez JM, Lomonte B. 1995. Phospholipase A_2 myotoxins from *Bothrops* snake venoms. *Toxicon* **33**:1405–1424 DOI 10.1016/0041-0101(95)00085-Z.

Gutiérrez JM, Rucavado A, Escalante T, Díaz C. 2005. Hemorrhage induced by snake venom metalloproteinases: biochemical and biophysical mechanisms involved in microvessel damage. *Toxicon* **45**:997–1011 DOI 10.1016/j.toxicon.2005.02.029.

Gutiérrez JM, Escalante T, Rucavado A. 2009. Experimental pathophysiology of systemic alterations induced by *Bothrops asper* snake venom. *Toxicon* **54**:976–987 DOI 10.1016/j.toxicon.2009.01.039.

Holzer M, Mackessy SP. 1996. An aqueous endpoint assay of snake venom phospholipase A_2. *Toxicon* **34**:1149–1155 DOI 10.1016/0041-0101(96)00057-8.

Ianzer D, Santos RA, Etelvino GM, Xavier CH, de Almeida Santos J, Mendes EP, Machado LT, Prezoto BC, Dive V, de Camargo AC. 2007. Do the cardiovascular effects of angiotensin-converting enzyme (ACE) I involve ACE-independent mechanisms? new insights from proline-rich peptides of *Bothrops jararaca*. *Journal of Pharmacology and Experimental Therapeutics* **322**:795–805 DOI 10.1124/jpet.107.120873.

Kim YK, Yoon S, Yu DY, Lönnerdal B, Chung BH. 1999. Novel angiotensin-I-converting enzyme inhibitory peptides derived from recombinant human alpha s1-casein expressed in *Escherichia coli*. *Journal of Dairy Research* **66**:431–439 DOI 10.1017/S0022029999003556.

Kishimoto M, Takahashi T. 2001. A spectrophotometric microplate assay for L-amino acid oxidase. *Analytical Biochemistry* **298**:136–139 DOI 10.1006/abio.2001.5381.

Kuch U, Mebs D, Gutiérrez JM, Freire A. 1996. Biochemical and biological characterization of Ecuadorian pitviper venoms (genera *Bothriechis, Bothriopsis, Bothrops*and *Lachesis*). *Toxicon* **34**:714–717 DOI 10.1016/0041-0101(96)00016-5.

Lomonte B, Rangel J. 2012. Snake venom Lys49 myotoxins: from phospholipases A_2 to non-enzymatic membrane disruptors. *Toxicon* **60**:520–530 DOI 10.1016/j.toxicon.2012.02.007.

Lomonte B, Angulo Y, Rufini S, Cho W, Giglio JR, Ohno M, Daniele JJ, Geoghegan P, Gutiérrez JM. 1999. Comparative study of the cytolytic activity of myotoxic phospholipases A_2 on mouse endothelial (tEnd) and skeletal muscle (C2C12) cells *in vitro*. *Toxicon* **37**:145–158 DOI 10.1016/S0041-0101(98)00171-8.

Lomonte B, Escolano J, Fernández J, Sanz L, Angulo Y, Gutiérrez JM, Calvete JJ. 2008. Snake venomics and antivenomics of the arboreal neotropical pitvipers *Bothriechis lateralis* and *Bothriechis schlegelii*. *Journal of Proteome Research* **7**:2445–2457 DOI 10.1021/pr8000139.

Lomonte B, Tsai WC, Ureña-Díaz JM, Sanz L, Mora-Obando D, Sánchez EE, Fry BG, Gutiérrez JM, Gibbs HL, Calvete JJ. 2014. Venomics of New World pit vipers: genus-wide comparisons of venom proteomes across *Agkistrodon*. *Journal of Proteomics* **96**:103–116 DOI 10.1016/j.jprot.2013.10.036.

Mora-Obando D, Guerrero-Vargas J, Prieto-Sánchez R, Beltrán J, Rucavado A, Sasa M, Gutiérrez JM, Ayerbe S, Lomonte B. 2014. Proteomic and functional profiling of the venom of *Bothrops ayerbei* from Cauca, Colombia, reveals striking interspecific variation with *Bothrops asper* venom. *Journal of Proteomics* **96**:159–172 DOI 10.1016/j.jprot.2013.11.005.

Núñez V, Cid P, Sanz L, De La Torre P, Angulo Y, Lomonte B, Gutiérrez JM, Calvete JJ. 2009. Snake venomics and antivenomics of *Bothrops atrox* venoms from Colombia and the Amazon regions of Brazil, Perú and Ecuador suggest the occurrence of geographic variation of venom phenotype by a trend towards paedomorphism. *Journal of Proteomics* **73**:57–78 DOI 10.1016/j.jprot.2009.07.013.

Ohler M, Georgieva D, Seifert J, von Bergen M, Arni RK, Genov N, Betzel C. 2010. The venomics of Bothrops alternatus is a pool of acidic proteins with predominant hemorrhagic and coagulopathic activities. *Journal of Proteome Research* 9: 2422-2437.

Otero R. 1994. *Manual de Diagnóstico y Tratamiento del Accidente Ofídico*. Medellín, Colombia: Editorial Universidad de Antioquia.

Otero R, Osorio RG, Valderrama R, Giraldo CA. 1992. Efectos farmacológicos y enzimáticos de los venenos de serpientes de Antioquia y Chocó (Colombia). *Toxicon* **30**:611–620 DOI 10.1016/0041-0101(92)90855-Y.

Paredes AE. 2012. *Informe del Evento Accidente Ofídico. Vigilancia y Control en Salud Pública*. Colombia: Instituto Nacional de Salud.

Valente RH, Guimarães PR, Junqueira M, Neves-Ferreira AG, Soares MR, Chapeaurouge A, Trugilho MRO, León IR, Rocha SL, Oliveira-Carvalho AL, Wermelinger LS, Dutra DLS, Leão LI, Junqueira-de-Azevedo ILM, Ho PL, Zingali RB, Perales J, Domont GB. 2009. *Bothrops insularis* venomics: a proteomic analysis supported by transcriptomic-generated sequence data. *Journal of Proteomics* **72**:241–255 DOI 10.1016/j.jprot.2009.01.001.

Van der Laat M, Fernández J, Durban J, Villalobos E, Camacho E, Calvete JJ, Lomonte B. 2013. Amino acid sequence and biological characterization of BlatPLA$_2$, a non-toxic acidic phospholipase A$_2$ from the venom of the arboreal snake *Bothriechis lateralis* from Costa Rica. *Toxicon* **73**:71–80 DOI 10.1016/j.toxicon.2013.07.008.

Wang WJ, Shih CH, Huang TF. 2004. A novel P-I class metalloproteinase with broad substrate-cleaving activity, agkislysin, from *Agkistrodon acutus* venom. *Biochemical and Biophysical Research Communications* **324**:224–230 DOI 10.1016/j.bbrc.2004.09.031.