



## Proteomic analysis of the rare Uracoan rattlesnake *Crotalus vegrandis* venom: Evidence of a broad arsenal of toxins



Vincent Louis Viala<sup>a</sup>, Diana Hildebrand<sup>b</sup>, Tamara Mielo Fucase<sup>a</sup>, Juliana Mozer Sciani<sup>c</sup>, José Pedro Prezotto-Neto<sup>a</sup>, Maria Riedner<sup>d</sup>, Leonardo Sanches<sup>e</sup>, Paula Juliana Nishimura<sup>a</sup>, Nancy Oguiura<sup>e</sup>, Daniel Carvalho Pimenta<sup>c</sup>, Hartmut Schlüter<sup>b</sup>, Christian Betzel<sup>f</sup>, Raghuvir Krishnaswami Arni<sup>g</sup>, Patrick Jack Spencer<sup>a,\*</sup>

<sup>a</sup> Centro de Biotecnologia, IPEN, Av. Lineu Prestes 2242, CEP 05508-000, São Paulo, SP, Brazil

<sup>b</sup> Institut für Klinische Chemie, Universitätsklinikum Hamburg-Eppendorf, Haus Ost 26, Martinistr. 52, 20246, Hamburg, Germany

<sup>c</sup> Laboratório de Bioquímica e Biofísica, Instituto Butantan, Av. Vital Brasil, 1500, 05503-900, São Paulo, SP, Brazil

<sup>d</sup> Institut für Organische Chemie, Massenspektrometrie, Universität Hamburg, Martin-Luther-King-Platz 6, 20146, Hamburg, Germany

<sup>e</sup> Laboratório Especial de Ecologia e Evolução, Instituto Butantan, Av. Dr. Vital Brasil 1500, CEP 05503-900, São Paulo, SP, Brazil

<sup>f</sup> Institut für Biochemie und Molekularbiologie, Universität Hamburg, Martin-Luther-King-Platz 6, 20146, Hamburg, Germany

<sup>g</sup> Departamento de Física, IBILCE, UNESP, Rua Cristóvão Colombo, 2265, CEP 15054-000, São José do Rio Preto, SP, Brazil

### ARTICLE INFO

#### Article history:

Received 20 July 2015

Received in revised form

11 September 2015

Accepted 16 September 2015

Available online 25 September 2015

#### Keywords:

*Crotalus vegrandis*

Peptide

Uracoan rattlesnake

Toxin

Venom

Proteomics

### ABSTRACT

The investigation of venoms has many clinical, pharmacological, ecological and evolutionary outcomes. The *Crotalus* spp. venom can cause hemorrhage, neurotoxicity, myotoxicity, coagulopathy and hypotension. Although neurotoxicity and hemorrhage usually does not occur for the same species, the rare Venezuelan species *Crotalus vegrandis* presents both characteristic. Different from the other species it has a restricted ecological niche and geographical distribution. Nevertheless, it has a raising medical importance as this rattlesnake population is increasing. Few works describe its neurotoxic and hemorrhagic features, but other toxins might play an important role in envenomation. We combined proteomic methods to identify for the first time the main components of its venom: 2D SDS-PAGE and gel-filtration chromatography for protein mixture decomplexation; LC-MS<sup>2</sup> of low molecular mass fractions and tryptic peptides; bioinformatic identification of toxin families and specific protein species based on unique peptide analysis and sequence database enriched with species-specific venom gland transcripts; and finally polyclonal anti-crotamine Western-blotting. Our results point to a broad arsenal of toxins in *C. vegrandis* venom: PIII and PII metalloproteases, crotoxin subunits, other phospholipases, isoforms of serine proteases and lectins, L-amino-acid oxidase, nerve growth factor, as well as other less abundant toxins.

© 2015 Elsevier Ltd. All rights reserved.

## 1. Introduction

Rattlesnakes (Viperidae: Serpentes) are native predators from the Americas whose most popular feature is the noisy rattle used as

**Abbreviations:** UP, unique peptide; SVMP, snake venom metalloproteinase; SVSP, snake venom serine protease; LAAO, L-amino acid oxidase; CLP, C-type lectin-like proteins; PLA<sub>2</sub>, snake venom phospholipase A<sub>2</sub>; PDE, venom phosphodiesterase; PLB, phospholipase B; CRiSP, cysteine rich secretory protein; VNGF, venom nerve growth factor; CPE, carboxypeptidase-E; GP, glutathione peroxidase; EXE, exendin-like peptides.

\* Corresponding author.

E-mail address: [pspencer@ipen.br](mailto:pspencer@ipen.br) (P.J. Spencer).

a first defense against predators (Campbell and Lamar, 2004; Klauber, 1997). The study of their venoms led to the discovery of interesting new molecules, studied under a multidisciplinary fashion that integrates pharmacological, biotechnological, physiological and evolutionary points of view (Calvete, 2013; Campbell and Lamar, 2004; Harvey and Stöcklin, 2012). In general, toxins from *Crotalus* snakes can cause hemorrhage, neurotoxicity, myotoxicity, coagulopathy and hypotension (Azevedo-Marques et al., 2003; Gutiérrez, 2009; Mackessy, 2008; Markland, 1983). Based on the main systemic characteristics of envenomation, Mackessy (2008) observed distinct venom phenotypes and classified the *Crotalus* spp. in two groups, the type I, hemorrhagic, and the type II,

neurotoxic, apparently, with an inverse relationship, one excluding the other. Although type II appears to be more abundant in South America, while the type I in northern and central part of the continent, exceptions were described. South American exceptions to this classification are *Crotalus durissus ruruima* (Dos Santos et al., 1993), *C. d. cumanensis* (Cavalcante et al., 2015; Yoshida-Kanashiro et al., 2003) and *Crotalus vegrandis* (Girón et al., 2005), whose venoms display both hemorrhagic and neurotoxic activities. North and Central American exception are the species *Crotalus scutulatus scutulatus* (Massey et al., 2012), *Crotalus viridis/oreganus* complex (Mackessy, 2010), *Crotalus horridus* (Glenn et al., 1994), *Crotalus simus* (Castro et al., 2013) and *Crotalus tigris* (Calvete et al., 2012). Within a single species, populations can exhibit many different venom phenotypes, such as *C. scutulatus scutulatus* that displays six different phenotypes based on variations of three toxin families: neurotoxic phospholipase A<sub>2</sub> (PLA<sub>2</sub>), snake venom metalloproteinase (SVMP) and myotoxin (Massey et al., 2012), or such as *C. horridus* where some individuals even lack both hemorrhagic and neurotoxic features (Glenn et al., 1994). Not least than the toxicity vs. hemorrhagic phenotype discussion, exploring the hidden diversity of toxins that might play an important role in envenomation is of great relevance for the design and preclinical assessment of antivenoms (Calvete et al., 2014).

The “Casabel de Uracoa” has a restricted distribution at the central-west of the region of plateaus in Monagas and Anzoátegui States of Venezuela, in a small area of semiarid savannas (Pifano and Rodríguez Acosta, 1996). Its habitat is different from the other *Crotalus* species as it can only be found in a specific ecological niche: small relictual forested areas with a specific microclimate, not being found in desert areas, wetlands or in degraded areas, even in the dry ones. It is a nocturnal predator and its diet is mainly based on small lizards (*Anolis* sp. and *Tropidurus* sp.) but recent agricultural activities introduced vermin in its diet (Pifano and Rodríguez Acosta, 1996). Its ecology, biology and habits are poorly studied and its conservation status is “marginal at best” (quote from Pifano and Rodríguez Acosta, 1996).

Twenty deaths caused by snakebite accident were registered between 1980 and 2000 in the Monagas State (De Sousa et al., 2005). An epidemiological survey upon snake accidents registered at the Manuel Nuñez Tovar Hospital from Monagas State (Navarro et al., 2004), analyzed 53 crotalic accidents out of 158 snakebite accidents, over 10 years. In 36 out of the 53 crotalic accidents (68%), the snake was captured and identified as *C. vegrandis*. Based on previous studies, they concluded that the prevalence of crotalic envenomation was higher in Monagas state than in other Venezuelan regions. They discussed that in some regions the environment has changed increasing food availability, increasing the population size and the incidence of observed accidents.

Little is known about the *C. vegrandis* clinical features of envenomation. According to Rodríguez-Acosta et al. (1995), envenomed animals showed an intense general hemorrhage, respiratory difficulties and death by possible paralysis of the respiratory muscles. Few data indicate that this venom has an LD<sub>50</sub> of 0.2 µg/g (i.v.) (Scannone et al., 1978) and 0.5 µg/g (i.m.) (Rodríguez-Acosta et al., 1998) in mice and that the hemorrhagic and neurotoxic activities (Gubensek et al., 1978; Rodríguez-Acosta et al., 1998; Scannone et al., 1978) can be ascribed to metalloproteases (Aguilar et al., 2001) and to a crotoxin-like heterodimer (Girón et al., 2005) respectively. An accident case was reported, in which a great edema of the affected limb was described with no apparent neurological signals (Pifano and Rodríguez Acosta, 1995).

In this work, we analyzed for the first time the venom general composition of the Uracoan rattlesnake *C. vegrandis*. We performed a high throughput proteomic analysis based on mass spectrometry (MS), complemented with further experiments such as the

detection of crotoxin-like proteins by western-blotting and cDNA cloning and sequencing for snake venom serine proteases (SVSPs).

## 2. Materials and methods

### 2.1. Venom

Venoms of five captive individuals of *C. vegrandis* were pooled and provided by Venom Supplies Pty. Ltd. (Tanunda, Australia). After milking, the venoms were immediately freeze-dried and stored at –20 °C.

### 2.2. Venom decomplexation and MS analysis

#### 2.2.1. 2D gel, in-gel digestion and MS

Prior to use, the sample was dissolved up to 170 µg/mL (9 M urea, 70 mM DTT and 2% ampholytes), incubated (30 min at room temperature), centrifuged (45 min, 15000 g) and the supernatant removed and frozen at –80 °C. The 2D-PAGE was performed and stained using slightly adapted method from previous works (Viala et al., 2014). Selected spots were collected manually. In-gel digestion was performed with Trypsin (Promega) according to the manufacturer's instructions. The peptides were subsequently analyzed by LC/ESI/ion trap/MS–MS analysis (Agilent 1100 LC/MSD-trap XCT series system) (Viala et al., 2014).

#### 2.2.2. Gel filtration (GF) chromatography, in-solution digestion and MS

Thirty mg of crude venom were dissolved in 500 µL of ammonium formate (100 mM, pH 3.0). After centrifugation, the clear supernatant was injected in a Superdex 75 10/300 (GE Healthcare) column previously equilibrated with the same buffer, connected to an Äkta purifier system (GE Healthcare). Elution was performed at 0.6 mL/min and the absorbance was monitored at 220 and 280 nm. One milliliter fractions were collected and pooled according to the chromatogram. Lyophilized fractions were redissolved in 1 mL of ammonium bicarbonate (50 mM pH 7.4) and incubated 1 h at 45 °C in additional 50 µL of denaturation buffer (20 mM TrisHCl; 7 M Urea; 5 mM DTT; 2 mM EDTA). Disulfide bonds were reduced for 1 h by adding 10 µL of DTT (5 mM in 25 mM ammonium bicarbonate) and cysteine were alkylated in the dark for 1 h by adding 10 µL of iodoacetamide (55 mM in 25 mM ammonium bicarbonate). Proteins were digested overnight with 15 µL of 20 ng/µL Trypsin (Promega) (50 mM ammonium bicarbonate). The samples were dried and stored in –20 °C until further steps. Samples were redissolved in 0.1% formic acid (solvent A), injected in a C18 reverse phase (Supelco, 3 µm, 100 Å, 50 mm × 2.1 mm) and eluted with a 5–40% gradient of solvent B (90% acetonitrile/H<sub>2</sub>O with 0.1% formic acid) in 40 min, at a constant flow rate of 0.2 mL/min. The HPLC eluates were monitored by a Shimadzu SPD-M20A PDA detector scanning from 200 to 500 nm (1 nm steps). MS spectra were acquired on a IT-ToF (Shimadzu Co, Japan) under positive mode, interface voltage at 4.5 kV, detector voltage at 1.76 kV, interface temperature at 200 °C, and collected in the 50–2000 *m/z* range. MS/MS spectra was obtained by argon gas collision and obtained in a range of 50–2000 *m/z*. The fractions containing low molecular-weight proteins were not trypsinized.

#### 2.3. Protein identification and detection of protein species by unique peptides (UP) screening

The raw mass spectra files were converted to “.mgf” files for bioinformatic analysis. Protein identification was performed with PEAKS studio 7.0 (Ma et al., 2003) using the InChorus multi-

algorithmic tool (PEAKS + MASCOT) for better accuracy. The identification was based on public protein and genetic databases. The MS/MS identification was revised manually for score, quality of the mass spectra and correctness of assignments. In order to get the most of the data, an additional step was performed with PEAKS using all sample spectra together in a unique run. This is particularly useful for screening the data to locate UPs. UPs contain stretches of unique amino acid sequences present in a database of sequences. The PEAKS programmers define a UP as “[ ... ] a peptide with its  $-10 \log P$  score above the peptide filtering threshold that can be mapped to only one protein group” (Ma et al., 2003). Therefore, UPs were located in the resulting PEAKS tables within “Protein Groups” containing only one “Protein ID” entry. The sequences were then individually aligned to public protein databases to exclude false positives and to correctly assign specific protein species to the sample analyzed.

#### 2.4. Crotonamine-like proteins detection using western blot immunoassays

A sample of *C. vegrandis* venom (33.3  $\mu\text{g}/\text{lane}$ ) was submitted to 15% SDS-PAGE under non-reducing conditions (Laemmli, 1970). The *C. d. terrificus* venom (33.3  $\mu\text{g}/\text{lane}$ ) was used as a positive control. The gel was placed in the electroblot apparatus adjacent to nitrocellulose paper in buffer, as described by Towbin et al. (1979), and transferred for 90 min at 0.85 mA/cm<sup>2</sup>. The membrane was blocked with 3% skim milk in pH 7.4 phosphate buffered saline and incubated with rabbit polyclonal antibodies against whole crotonamine (diluted 1:8000) (Oguiura et al., 2000). The immunoreactive proteins were detected using peroxidase-labeled anti-rabbit IgG and the blot was developed with 0.05% diaminobenzidine in the presence of 0.03% H<sub>2</sub>O<sub>2</sub> (v/v).

#### 2.5. Venom gland cDNA library construction and SVSP transcript cloning and sequencing

The venom gland was extracted from an adult *C. vegrandis* male individual, born in captivity at Venom Supplies Pty. Ltd. (Tanunda, Australia). The venom gland was extracted three days after milking to obtain a tissue with a high level of toxin transcript expression. It was stored in RNA-Later<sup>®</sup> (QIAGEN N.V., Netherlands) at  $-80^\circ\text{C}$  until RNA extraction. 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. The total RNA was extracted with Trizol<sup>®</sup> reagent (Life Technologies, USA) in an RNase free environment. A cDNA library of the venom gland mRNA was built using In-Fusion SMARTer cDNA library construction kit (Clontech Laboratories Inc., USA). The SVSP cDNA was amplified by PCR using a set of primers designed based on conserved regions found in Viperidae SVSP: FUTRgyr (5'-CAGAGTTGAAGCTATGGTGCTGAT-3') and R6gyr (5'-TGCACCTCACCTAAAACAGG-3'). A 20  $\mu\text{L}$  reaction mix contained 14 ng DNA sample, 0.1 mM each primer, 0.5 U Taq DNA polymerase Platinum (Invitrogen), buffer with the addition of 1.5 mM MgCl<sub>2</sub>, and 0.2 mM dNTPs mix. The amplification process used an initial denaturation step of 4 min at 94  $^\circ\text{C}$ , followed by 30 cycles of 45 s at 94  $^\circ\text{C}$ , 45 s at 60  $^\circ\text{C}$ , 45 s at 72  $^\circ\text{C}$ , and, finally 1 min at 72  $^\circ\text{C}$ . The amplified DNA was purified, after electrophoresis on a 1% agarose gel, using the ZymoClean Gel DNA Recovery kit (ZymoResearch). The purified DNA was cloned into the pTZ57 R/T vector according to the manufacturer's instructions (Fermentas). After transformation of *E. coli* DH5 $\alpha$  (Ausubel et al., 2000), clones were purified using Zippy Plasmid Miniprep (ZymoResearch).

Sequencing was performed at the Biotechnology Center in the Butantan Institute, on an ABI Prism 3500 Genetic Analyzer (Applied Biosystems), using the M13 (5'-GTAAACGACGGCCAGT-3') and T7 (5'-TAATACGACTCACTATAGGG-3') primers.

### 3. Results

#### 3.1. Venom protein identification by MS

The 2D electrophoresis detected components ranging from *pI* 3 to 10 and molecular mass ranging from 14 to 120 kDa (Fig. 1).

Multiple horizontal trains of spots are observed. A total of 73 spots were collected for analysis from different regions of the gel. Protein families were assigned to 65 spots such as basic and acidic PLA<sub>2</sub>, including crotoxin subunits; SVMP (PIII and PII); SVSP, such as gyroxin-like and others; C-type lectin-like proteins (CLP), including a convulxin-like toxin; cysteine-rich secretory protein (CRISP); ecto-5'-nucleotidase (E5'N); venom nerve growth factor (VNGF); phospholipase B (PLB); venom phosphodiesterase (PDE) and exendin-4 like peptides (EXE). Glutathione peroxidase (GP) and carboxypeptidase E-like (CPE) were identified as well (Table 1).

The complementary MS analysis of GF fractionated venom (Fig. 2) resulted in the additional identification of bradykinin-potentiating peptides (BPPs), bradykinin inhibitory peptide (BIP), C-type natriuretic peptide (CNP) and the crotoxin acidic subunit (Table 2).

#### 3.2. Crotonamine-like immunodetection

The Western blot cross-species assay performed with an anti-crotonamine polyclonal antibody (Oguiura et al., 2000) was positive indicating the existence of a homologous toxin in *C. vegrandis* venom (Fig. 3).

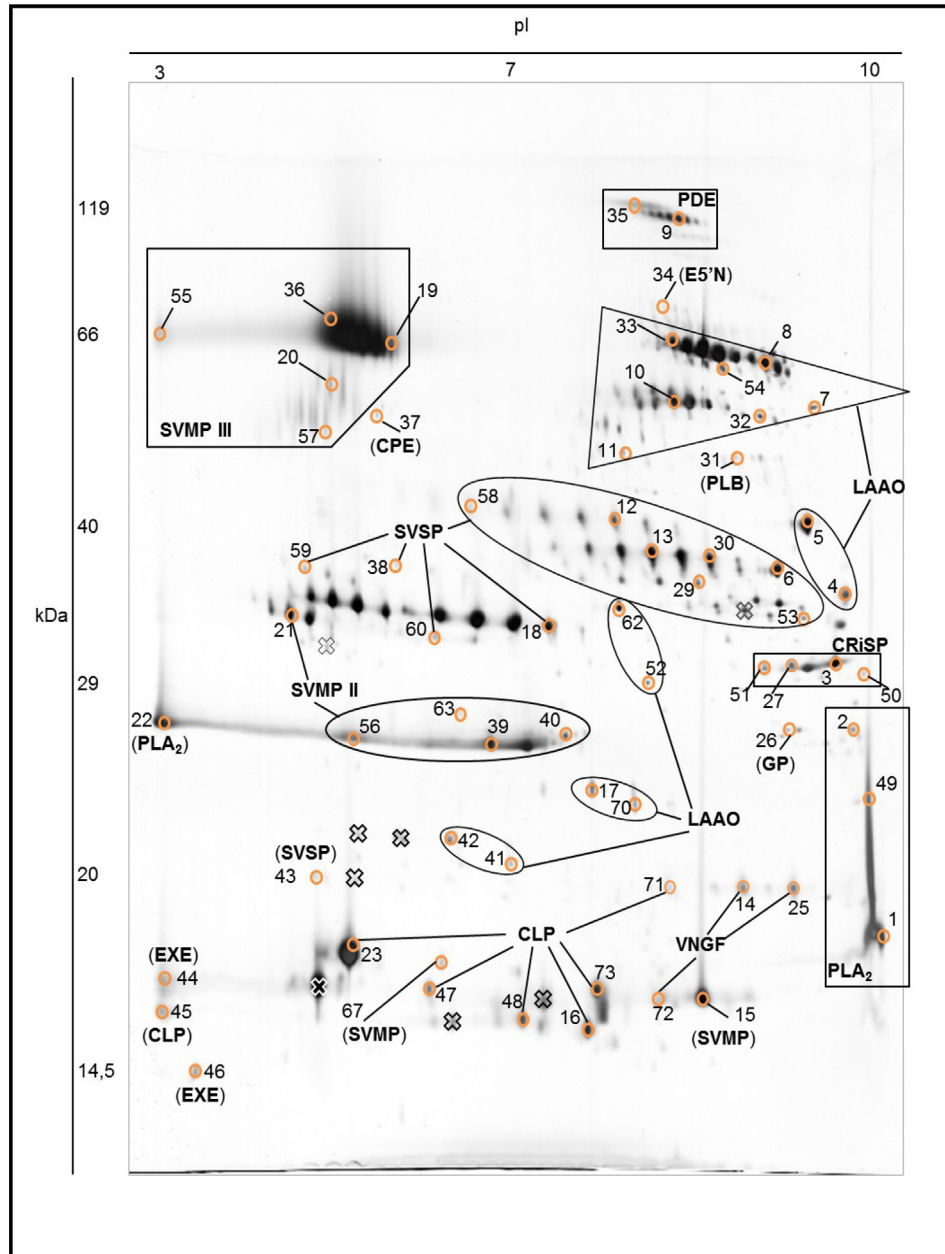
#### 3.3. SVSP cloning and sequencing

A 1027 bp PCR fragment, named SVSPcv01, was sequenced (GenBank: KT266708) (Supplementary material). It includes the open reading frame and partial 3' untranslated region. A search at BLASTn, using the cDNA sequence, matches to other SVSP of Crotoninae snakes with c.a. 90% of identity. The translated primary sequence is 258 residues long (Fig. 4). Domain prediction analysis (Jones et al., 2014; Petersen et al., 2011) identified it as a peptidase S1 trypsin-like cysteine/serine protein, and detected an 18 residue long signal peptide and three peptidase S1A chymotrypsin-type subfamily domain signature (Fig. 4).

### 4. Discussion

The *C. vegrandis* venom is known to be highly hemorrhagic (local and systemic bleeding are major signs of Urocoan rattlesnake envenomation) and neurotoxic (causing death by respiratory paralysis) (Girón et al., 2005; Gubenšek et al., 1978; Rodríguez-Acosta et al., 1998; Scannone et al., 1978). A neurotoxic fraction described by Kaiser and Aird (1987) contains crotoxin-like proteins. A hemorrhagic fraction containing SVMP was also described (Aguilar et al., 2001). Myotoxic activity was also reported (Pulido-Mendez et al., 1999) and Bober et al. (1988) suggested the existence of a crotonamine-like toxin.

A more accurate description of this venom composition is important for appropriate treatment of accidents (Castro et al., 2013; Gutiérrez et al., 2014; Saviola et al., 2015; Sunagar et al., 2014). Additionally to the epidemiological and medical importance, many snake venom molecules are targets for innovative development in pharmacology and biotechnology (e.g. Barnwal and



**Fig. 1.** 2D-PAGE of *Crotalus vegrandis* venom. Identified clusters of spots and individual spots are labeled. Circles represent identified spots and crosses represent collected spots with no significant result. pI: isoelectric point; kDa: molecular weight; SVMP III: PIII snake venom metalloproteinase; SVMP II: PII snake venom metalloproteinase; SVMP: unclassified snake venom metalloproteinase; SVSP: snake venom serine protease; LAAO: L-amino acid oxidase; CLP: C-type lectin-like proteins; PLA<sub>2</sub>: snake venom phospholipase A<sub>2</sub>; PDE: venom phosphodiesterase; PLB: phospholipase B; CRiSP: cysteine rich secretory protein; VNGF: venom nerve growth factor; CPE: carboxypeptidase-E; GP: glutathione peroxidase; EXE: exendin-like peptides.

Kini, 2013; McCleary and Kini, 2012). Besides describing the main components of *C. vegrandis* venom from a qualitative point of view, our data might contribute to a better understanding of *Crotalus* spp. ecology and evolution. Other *Crotalus* spp. from adjacent geographical location, such as *C. d. ruruima* and *C. d. cumanensis*, have similar venom compositions, showing both hemorrhagic and neurotoxic activities (Aguilar et al., 2007; Calvete et al., 2010; Cavalcante et al., 2015; Dos Santos et al., 1993). Thus, there must be some positive evolutionary pressure to favor this phenotype.

#### 4.1. Crotoxin-like toxins and other PLA<sub>2</sub>

The neurotoxic crotoxin was first described by Slotta and

Fraenkel-Conrat (1938) as the main component of *C. d. terrificus* venom. Crotoxin is a beta-neurotoxin, acting pre-synaptically on muscular junctions blocking the signal transduction (Degn et al., 1991). Crotoxin is heterodimeric, composed by a nontoxic acidic subunit (crotopotin) acting as a chaperone, delivering the basic PLA<sub>2</sub> subunit to the synaptic cleft, blocking the release of acetylcholine. Although non-toxic, the acidic subunit is essential to neurotoxic activity as the isolated basic subunit has low toxicity (Bon, 1982). Peptides matching to crotoxin basic subunit were detected in spots 1, 2 and 49, in a vertical cluster, ranging from ~17 to ~28 kDa at pI 10 (Fig. 1) and in other isolated spots (7 and 43). LC/MS-MS analyses of the GF low molecular mass fraction (not trypsinized) showed the presence of peptides derived from N and

**Table 1**  
Summary table of *Crotalus vegrandis* venom LC-MS/MS identification on 2D-PAGE spots. Data generated by PEAKS + MASCOT (InChorus) analysis. Bold peptide sequences are Unique Peptides. M(+15.99): Oxidation of methionine. m: mass; z: charge; SVMP: snake venom metalloproteinase; SVSP: snake venom serine protease; LAAO: L-amino acid oxidase; CLP: C-type lectin-like proteins; PLA2: snake venom phospholipase A2; PDE: venom phosphodiesterase; PLB: phospholipase B; CRiSP: cysteine rich secretory protein; VNGF: venom nerve growth factor; CPE: carboxypeptidase-E; GP: glutathione peroxidase; EXE: exendin-like peptides.

Spot	Prot. family	Score (%)	Top hit description	Accession	Hit species	Peptide	m/z	z
1	PLA <sub>2</sub>	98.8	Basic phospholipase A2 A	PA2BA_CRODR	<i>Crotalus durissus ruruima</i>	K.YGYMFYPDSR.C	649.76	2
						HLLQFNK.M	450.25	2
						K.WDIYPYSLK.S	592.79	2
						R.RSLSTYK.Y	427.78	2
						R.SLSTYK.Y	349.71	2
						<b>X.WDLYPY.X</b>	856.35	1
2	PLA <sub>2</sub>	83.22	Phospholipase A2 crotoxin basic subunit CBc	PA2BC_CRODU	<i>Crotalus durissus terrificus</i>	R.KNAIPFYAF	535.84	2
						K.YGYMFYPDSR.C	649.82	2
3	SVSP	98.86	Kallikrein-CohLL-4	T1DEH3_CROOH	<i>Crotalus oreganus helleri</i>	K.WDIYPYSLK.S	592.85	2
						R.FLVALYTFR.S	565.31	2
3	CRiSP	84.14	Cysteine-rich secretory protein Ch-CRPKa (Fragment)	F2Q6E5_CROHD	<i>Crotalus horridus</i>	R.IMGWGTISATK.E	582.82	2
						R.AAHGGLPATSR.T	519.3	2
						K.WDKDIMLIR.L	595.39	2
						R.SVNPTASNMLK.M	581.3	2
4	LAAO	99.18	L-amino acid oxidase (Fragment)	K9N7B7_CRODM	<i>Crotalus durissus cumanensis</i>	SVDFDSESPR.K	569.75	2
						K.YILDKYDTYSTK.E	755.42	2
						K.SAAQLYVESLR.K	618.84	2
						K.NNPGILEYVPKPSEEGK.S	935.97	2
						R.VIEIQQNDRE	557.83	2
						K.HDDIFGYEK.R	562.26	2
						K.DWYANLGPML.L	611.84	2
						K.VQVHFNAR.V	485.86	2
						<b>R.VIEIQQNDRETK.V</b>	491.63	3
						K.SAAQLYVESLRK.V	682.93	2
						K.RFDEIVGGM(+15.99)DQLPTSM(+15.99)YEAIK.E	811.37	3
						R.FLVALYTFR.S	565.33	2
						4	SVSP	61.97
R.FLVALYTFR.S	565.33	2						
5	LAAO	99.19	L-amino acid oxidase (Fragment)	K9N7B7_CRODM	<i>Crotalus durissus cumanensis</i>	K.SAAQLYVESLR.K	618.82	2
						K.NNPGILEYVPKPSEEGK.S	935.96	2
						R.ETDYEEFLEIAR.N	757.84	2
						K.YILDKYDTYSTK.E	755.42	2
						<b>R.NGLTVTSNPK.H</b>	515.77	2
						K.HDDIFGYEK.R	562.25	2
						R.VIEIQQNDRE	557.78	2
						<b>R.VIEIQQNDRETK.V</b>	736.88	2
						K.DWYANLGPML.L	611.82	2
						K.RFDEIVGGM(+15.99)DQLPTSM(+15.99)YEAIK.E	811.39	3
						R.FDEIVGGM(+15.99)DQLPTSM(+15.99)YEAIK.E	759.37	3
						K.VQVHFNAR.V	485.79	2
						K.SAAQLYVESLRK.V	682.88	2
						K.HDDIFGYEK.R	427.25	3
						K.YDTYSTK.E	439.24	2
						K.FEPPLPPK.A	351.59	2
						K.FEPPLPPK.K	462.75	2
						R.ETDYEEFLEIAK.N	743.88	2
						<b>R.IQFEPPLPPK.K</b>	583.35	2
						6	LAAO	99.18
R.FDEIVGGMDQLPTSMYEAIK.E	1122.55	2						
K.NNPGILEYVPKPSEEGK.S	935.96	2						
K.EGNLSPGAVDMIGDLLNEDSGYVYVFIK.H	1111.51	3						
<b>R.NGLTVTSNPK.H</b>	515.77	2						
K.YILDKYDTYSTK.E	755.44	2						
K.SAAQLYVESLR.K	618.87	2						
R.ETDYEEFLEIAR.N	757.85	2						
<b>K.FGLQLNEFFQENENAWYFIK.N</b>	1269.08	2						
K.HDDIFGYEK.R	562.26	2						
R.VIEIQQNDRE	557.78	2						
K.KDWYANLGPML.L	675.83	2						
K.DWYANLGPML.L	611.81	2						
<b>R.VIEIQQNDRETK.V</b>	736.89	2						
R.KKDWYANLGPML.L	739.94	2						
K.VQVHFNAR.V	485.79	2						
K.DWYANLGPML(+15.99)R.L	619.83	2						

Table 1 (continued)

Spot	Prot. family	Score (%)	Top hit description	Accession	Hit species	Peptide	m/z	z
						K.YDTYSTK.E	439.23	2
						K.FEPPLPPK.K	462.78	2
						K.RFDEIVGGMDQLPTSM(+15.99)YEAIKEK.V	891.75	3
						K.FEPPLPPK.K.A	526.9	2
						R.RIKFEPPLPPK.K	441.27	3
						<b>R.IQFEPPLPPK.K</b>	583.43	2
						<b>R.IQFEPPLPPK.Q</b>	431.94	3
						<b>R.RIQFEPPLPPK.K</b>	441.27	3
7	LAO	99.16	L-amino acid oxidase (Fragment)	K9N7B7_CRODM	<i>Crotalus durissus cumanensis</i>	K.RFDEIVGGMDQLPTCM(+15.99)CRAIEEK.V	886.38	3
						K.NNPGILEYVPKPSSEEGK.S	936	2
						K.SAAQLYVESLR.K	618.87	2
						<b>R.NGLTVTSNPK.H</b>	515.81	2
						R.ETDYEEFLEIAR.N	757.87	2
						K.YILDKYDTYSTK.E	755.42	2
						R.VIEIQQNDR.E	557.81	2
						K.HVVIVGAGMAGLSAAYVLAGAGHQVTVLEASER.V	809.16	4
						K.SAAQLYVESLRK.V	682.96	2
						<b>R.VIEIQQNDRETK.V</b>	491.63	3
						R.KKDWWYANLGPML.R	493.63	3
7	PLA <sub>2</sub>	84.12	Phospholipase A2 crotoxin basic subunit Cbc	PA2BC_CRODU	<i>Crotalus durissus terrificus</i>	K.YGYMFYPSDR.C	649.81	2
7	SVSP	61.3	Snake venom serine protease homolog (Fragment)	VSPH_CROAT	<i>Crotalus atrox</i>	K.WDIYPYSLK.S	592.85	2
						R.FLVALYTFR.S	565.36	2
8	LAO	99.18	L-amino acid oxidase (Fragment)	K9N7B7_CRODM	<i>Crotalus durissus cumanensis</i>	K.SAAQLYVESLR.K	618.87	2
						K.NNPGILEYVPKPSSEEGK.S	935.98	2
						K.YILDKYDTYSTK.E	755.42	2
						R.VIEIQQNDR.E	557.81	2
						R.ETDYEEFLEIAR.N	757.87	2
						K.HVVIVGAGMAGLSAAYVLAGAGHQVTVLEASER.V	1078.56	3
						K.HDDIFGYEK.R	562.26	2
						K.DWYANLGPML(+15.99)R.L	619.83	2
						K.FWEDDGIR.G	519.27	2
						K.SAAQLYVESLRK.V	682.93	2
						K.SGLTAAR.D	338.22	2
						R.KFWEDDGIR.G	583.33	2
						<b>R.VIEIQQNDRETK.V</b>	736.9	2
						K.VQVHFNAR.V	485.8	2
						K.RFDEIVGGMDQLPTSMYEAIKE	800.72	3
						K.YDTYSTK.E	439.21	2
						R.FDEIVGGM(+15.99)DQLPTSM(+15.99)YEAIKEK.V	845.08	3
						K.STDLPSR.F	438.72	2
						K.IFLTCSK.K	406.22	2
9	PDE	99.18	Venom phosphodiesterase 2	PDE2_CROAD	<i>Crotalus adamanteus</i>	K.DFYTFDSEGIK.N	710.84	2
						K.TFLPIFVNPVN	630.92	2
						K.YGPVSGEIIK.A	531.82	2
						K.NPFYTPSPAK.E	561.25	2
						R.VRDVELLTGLNFYSLK.Q	642.05	3
						R.AGYLENWDSLM(+15.99)PNINK.L	940.93	2
						R.LWNYFHHTLIPK.Y	511.61	3
						K.QPLPETLQK.T	583.87	2
						K.SMQAIFLAHGPGFNEK.N	582.99	3
						K.AATYFWPGSEVK.I	678.39	2
						R.TLGMMLMEGLK.Q	546.85	2
						K.GGTHGYDNEFK.S	612.81	2
						K.RLHYANNIR.I	386.26	3
						K.ALQMADR.T	402.72	2
						R.VTEVLK.W	344.79	2
						K.WLDLPK.A	386.27	2
10	LAO	99.18	L-amino acid oxidase (Fragment)	K9N7B7_CRODM	<i>Crotalus durissus cumanensis</i>	K.SAAQLYVESLR.K	618.88	2
						R.ETDYEEFLEIAR.N	757.85	2
						K.NNPGILEYVPKPSSEEGK.S	935.98	2
						<b>R.NGLTVTSNPK.H</b>	515.79	2
						K.YILDKYDTYSTK.E	755.43	2
						R.VIEIQQNDR.E	557.84	2
						K.HDDIFGYEK.R	562.28	2
						K.SAAQLYVESLRK.V	682.95	2
						K.FWEDDGIR.G	519.27	2
						K.RFDEIVGGM(+15.99)DQLPTSM(+15.99)YEAIKE	811.4	3
						K.STDLPSR.F	438.74	2

(continued on next page)

Table 1 (continued)

Spot	Prot. family	Score (%)	Top hit description	Accession	Hit species	Peptide	m/z	z
10	SVMP	98.68	Zinc metalloproteinase-disintegrin-like crostastatin (Fragment)	VM31_CRODC	<i>Crotalus durissus cascavella</i>	R.MYDIVNVITPIYHR.M K.LFLVADYIM(+15.99)YLK.Y R.ATDLSR.K <b>K.DHQEFLIK.N</b>	578.7 752.91 388.24 515.25	3 2 2 2
11	LAAO	98.87	L-amino acid oxidase (Fragment)	K9N7B7_CRODM	<i>Crotalus durissus cumanensis</i>	K.YILDKYDTYSTK.E K.FWEDDGIR.G R.KFWEDDGIR.G R.VIEIQQNDRE.E K.HDDIFGYEK.R <b>R.VIEIQQNDRETK.V</b> K.STTDLPSRF	755.37 519.28 583.3 557.83 562.3 736.93 438.77	2 2 2 2 2 2 2
12	SVSP	98.38	Snake venom serine proteinase 2	VSP2_CROAD	<i>Crotalus adamanteus</i>	R.AAYPEYGLPATS.R.T K.IHLGVHSHK.K K.WNKDIMLIR.L K.DIMLIR.L	698.35 445.83 594.93 380.76	2 2 2 2
13	SVSP	84.94	Snake venom serine protease catroxase-1	VSP1_CROAT	<i>Crotalus atrox</i>	R.AAYPEYGLPATS.R.T K.WNKDIMLIR.L K.IHLGVHSHK.K K.DIMLIR.L	698.35 594.95 445.79 380.75	2 2 2 2
14	VNGF	82.01	Venom nerve growth factor	NGFV_CRODU	<i>Crotalus durissus terrificus</i>	K.ALTM(+15.99)EGNQASWR.F K.TTATDIR.G	690.37 389.24	2 2
15	SVMP	61.63	SVMP-CohPH-3	T1E6U1_CROOH	<i>Crotalus oreganus helleri</i>	R.M(+15.99)YELANTVNDIYR.Y	809.43	2
16	CLP	61.41	Snaclec crotoctetin	SL_CRODU	<i>Crotalus durissus terrificus</i>	<b>K.QDMTWEDAEEK.F</b>	626.84	2
17	LAAO	98.45	L-amino acid oxidase (Fragment)	K9N7B7_CRODM	<i>Crotalus durissus cumanensis</i>	K.FWEDDGIR.G R.KFWEDDGIR.G K.SGLTAAR.D K.FWEDDGIRGGK.S R.KFWEDDGIRGGK.S K.STTDLPSRF	519.25 583.33 338.21 427.23 469.94 438.74	2 2 2 3 3 2
18	SVSP	98.87	Snake Venom Serine Protease clone 1	SVSP_clone 1	<i>Crotalus vegrandis</i>	K.NFQMQQLGVHSHK.K K.KVLNEDEQTR.D K.VLNEDEQTR.D K.VLNEDEQTRDPK.E K.DDEKDKDIMLIR.L K.DKDIMLIR.L R.IMGWGTISPTK.E	644.86 616.34 552.27 481.92 745.9 502.33 595.81	2 2 2 3 2 2 2
19	SVMP	98.88	SVMP-CohPH-3	T1E6U1_CROOH	<i>Crotalus oreganus helleri</i>	R.MYELANTVNDIYR.Y K.HDNAQLLTAIDLDR.V K.MFYSDNEDEHKGMVLPGTK.C R.FVELVLVVDK.A R.KKHDNAQLLTAIDLDR.V K.MFYSDNEDEHK.G K.KHDNAQLLTAIDLDR.V R.KTDLTR.K K.GMVLPGTK.C K.TDLTR.K	801.37 797.96 695.04 580.87 617.71 650.27 575.03 423.77 802.43 359.74	2 2 3 2 3 2 2 1 2
20	SVMP	98.81	SVMP-CohPH-3	T1E6U1_CROOH	<i>Crotalus oreganus helleri</i>	R.M(+15.99)YELANTVNDIYR.Y R.FVELVLVVDK.A R.KKHDNAQLLTAIDLDR.V R.KTDLTR.K	809.39 580.87 617.72 423.75	2 2 3 2
21	SVMP	98.66	Metalloproteinase P-II	Q2QA03_CRODD	<i>Crotalus durissus durissus</i>	<b>R.VAVTMTHELGHNLGIR.H</b> <b>R.YVELFIVVDHGMFTK.Y</b> R.VHQMVNIMK.E K.YNGSDSKIR.Q R.FVELVLVVDK.A <b>K.MVSYTYSVK.N</b>	874.45 599.98 550.35 534.28 580.85 539.27	2 3 2 2 2 2
22	PLA <sub>2</sub>	77.26	Acidic PLA2	C9E7C4_CRODC	<i>Crotalus durissus cascavella</i>	<b>R.DNIPSYDK.K</b> <b>R.DNIPSYDKK.Y</b> K.TWDDAER.F	951.48 540.26 446.72	1 2 2
23	CLP	52.54	Snaclec coagulation factor IX-binding protein subunit A	SLA_PROFL	<i>Protobothrops flavoviridis</i>	K.TWDDAER.F	446.72	2

Table 1 (continued)

Spot	Prot. family	Score (%)	Top hit description	Accession	Hit species	Peptide	m/z	z
25	VNGF	61.8	Venom nerve growth factor	NGFV_CRODU	<i>Crotalus durissus terrificus</i>	R.ALTMEGNQASWR.F	682.36	2
26	GP	98.35	Glutathione peroxidase (Fragment)	V8P395_OPHHA	<i>Ophiophagus hannah</i>	QYFM(+15.99)ETK.M	481.89	2
						K.QEPGQNSEILQGIK.H	770.97	2
						K.FLVNPQGGKPVMR.W	693.46	2
						K.HVRPGGGFVPPNFQLFQK.G	643.42	3
27	CRiSP	98.38	Cysteine-rich secretory protein Ch-CRiPKa (Fragment)	F2Q6E5_CROHD	<i>Crotalus horridus</i>	K.QEPGQNSEILQGIK.H	770.97	2
						K.HVRPGGGFVPPNFQLFQK.I	643.42	3
						K.MEWYPEAANAER.W	769.41	2
						SVDFDSESPR.K	569.74	2
27	LAAO	95.82	L-amino acid oxidase (Fragment)	K9N7B7_CRODM	<i>Crotalus durissus cumanensis</i>	R.SVNPTASNMLK.M	581.48	2
						K.IVDLHNSLR.R	533.94	2
						K.FWEDDGIR.G	519.27	2
						K.STTDLPSR.F	438.81	2
27	SVSP	95.27	Serine proteinase 5	T1DGZ7_CROHD	<i>Crotalus horridus</i>	K.SGLTAAR.D	338.26	2
						R.IMGWGTISATK.E	583.02	2
						R.AAHGGLPATSRT	519.27	2
						K.WDKDIMLRL	595.41	2
29	SVSP	99.08	Serine proteinase 6	T1D6M5_CROHD	<i>Crotalus horridus</i>	K.TNNEWKDIMLRL	831.44	2
						R.RLNPGFYTK.V	548.37	2
						R.AAYPWVWPVTR.I	674.38	2
						K.NFQQLGVHVK.K	635.96	2
						K.EIYPNVPR.C	494.33	2
						R.IMGWGTISSTK.E	590.93	2
						<b>R.IMGWGTISSTK.V</b>	590.87	2
						R.AAYPEYGLPATSRT	698.43	2
30	SVSP	98.36	Serine proteinase 8 (Fragment)	F8S120_CROAD	<i>Crotalus adamanteus</i>	R.AAYPEYGLPATSRT	698.4	2
						K.IHLGVHVK.K	445.86	2
						K.WNKDIMLRL	594.94	2
						K.DIM(+15.99)LIR.L	388.76	2
31	PLB	99.11	Phospholipase B	PLB_CROAD	<i>Crotalus adamanteus</i>	R.HGLEFSYEMAPRA	718.9	2
						R.SLEDGTLIIEQVPK.L	852.94	2
						K.FTAYAIANGPPVEK.G	703.92	2
						R.IANMMADSGK.T	519.29	2
						R.YNNYKEDPYAK.H	702.94	2
						K.VTDMESMK.S	470.82	2
						K.TWAETFEK.Q	506.25	2
						K.QNSGTYNQYMILDTK.K	945.44	2
						K.VADISM(+15.99)AAK.F	461.29	2
						K.SAAQLYVESLR.K	618.96	2
32	LAAO	99.08	L-amino acid oxidase (Fragment)	K9N7B7_CRODM	<i>Crotalus durissus cumanensis</i>	R.VIEIQQNDR.E	557.86	2
						K.YILDKYDTYSTK.E	755.36	2
						K.SAAQLYVESLRK.V	682.93	2
						<b>R.VIEIQQNDRETK.V</b>	736.91	2
						K.FWEDDGIR.G	519.23	2
						K.NNPGILEYVPKPEEGK.S	624.39	3
						K.STTDLPSR.F	438.76	2
						K.NNPGILEYVPKPEEGK.S	935.99	2
						K.SAAQLYVESLR.K	618.89	2
						R.ETDYEEFLEIAR.N	757.87	2
33	LAAO	99.17	L-amino acid oxidase (Fragment)	K9N7B7_CRODM	<i>Crotalus durissus cumanensis</i>	K.YILDKYDTYSTK.E	755.43	2
						<b>R.NGLTVTSNPK.H</b>	515.88	2
						K.HDDIFGYEK.R	562.31	2
						K.DWYANLGPML.L	611.95	2
						K.SAAQLYVESLRK.V	455.64	3
						<b>R.VIEIQQNDRETK.V</b>	736.91	2
						K.VQVHFNAR.V	485.81	2
						R.VIEIQQNDR.E	557.84	2
						K.FWEDDGIR.G	519.29	2
						R.KFWEDDGIR.G	583.32	2
						K.FWEDDGIRGGK.S	640.4	2
						K.RFDEIVGGM(+15.99)DQLPMSYEAIK.E	806.04	3
						K.STTDLPSR.F	438.76	2
						K.SGLTAAR.D	338.4	2
						K.EKVQVHFNAR.V	410.03	3
						<b>R.IQFEPPLPPK.K</b>	583.44	2

(continued on next page)



**Table 1** (continued)

Spot	Prot. family	Score (%)	Top hit description	Accession	Hit species	Peptide	m/z	z
34	E5'N	99.08	Snake venom 5'-nucleotidase	V5NTD_CROAD	<i>Crotalus adamanteus</i>	K.IIALGHSGFSEDQR.I	510.63	3
						K.IINVGSEK.V	430.4	2
						R.QVPVVQAYAFGK.Y	654	2
						K.VVYDLRSR.K	426.27	2
						K.VFPAVEGR.M	437.97	2
						R.VPTYVPLEK.E	523.33	2
35	PDE	98.53	Venom phosphodiesterase 2	PDE2_CROAD	<i>Crotalus adamanteus</i>	K.YLGYLNVIFDDKGNVIK.S	657.69	3
						SFELTILHTNDVHAR.V	585.1	3
						K.YGVPVSGEIIK.A	531.84	2
						K.TFLPIFVNPVN	630.88	2
						R.TLGMLMEGLK.Q	546.84	2
						K.AATYFWPGSEVK.I	678.42	2
36	SVMP	92.28	SVMP-CohPH-3	T1E6U1_CROOH	<i>Crotalus oreganus helleri</i>	K.KGTHGYDNEFK.S	612.82	2
						R.IQJHTAR.V	419.91	2
						R.MYELANTVNDIYR.Y	801.37	2
						K.MFYSNEDEHKGMVLPGTK.C	695.07	3
						K.MFYSNEDEHK.G	650.34	2
						R.KTDLLTR.K	423.93	2
37	CPE	98.98	Carboxypeptidase E-like	J3RPY4_CROAD	<i>Crotalus adamanteus</i>	K.K.GM(+15.99)VLPGTK.C	409.87	2
						<b>K.NNDDLKIK.T</b>	537.83	2
						K.VTASASGYLAITK.K	641.4	2
						R.SNAQGDILNR.N	544.35	2
						K.YVGNM(+15.99)HGNEAVGR.E	473.91	3
						K.VAVPFSPAIR.V	529.06	2
38	SVSP	76.64	Serine proteinase 7	T1E3B5_CROHD	<i>Crotalus horridus</i>	R.IVYVNER.E	446.94	2
						R.EGGPNNHLLK.N	539.85	2
						K.VTASAPGYLAITK.K	646.44	2
						K.KVLNEDEQTR.D	616.37	2
						K.VLNEDEQTR.D	552.3	2
						K.VLNEDEQTRDPK.E	481.96	3
39	SVMP	98.82	Metalloproteinase P-II	Q2QA03_CRODD	<i>Crotalus durissus durissus</i>	K.NFQM(+15.99)QLGVHSK.K	652.88	2
						<b>R.VAVTMHELGHNLGIR.H</b>	583.42	3
						<b>R.YVELFIVVDHGM(+15.99)FTK.Y</b>	605.36	3
						R.VHQMVNIM(+15.99)K.E	558.39	2
						K.YNGSDSKIR.Q	534.31	2
						<b>R.ETDLLQR.R</b>	437.83	2
40	SVMP	98.16	Metalloproteinase P-II	Q2QA03_CRODD	<i>Crotalus durissus durissus</i>	<b>R.VAVTM(+15.99)THELGHNLGIR.H</b>	588.68	3
						R.VHQMVNIM(+15.99)K.E	558.41	2
						K.YNGSDSKIR.Q	534.3	2
						K.FWEDDGIR.G	519.41	2
						R.KFWEDDGIR.G	583.34	2
						K.SGLTAAR.D	338.35	2
41	LAAO	97.94	L-amino acid oxidase (Fragment)	K9N7B7_CRODM	<i>Crotalus durissus cumanensis</i>	K.KFWEDDGIR.G	583.34	2
						K.KFWEDDGIR.G	519.29	2
						K.KFWEDDGIR.G	519.29	2
						K.STTDLPSRF	438.87	2
						K.KVPNEDEQTR.V	608.35	2
						<b>R.HGEGFTSDLSK.Q</b>	639.86	2
42	LAAO	74.61	L-amino acid oxidase (Fragment)	K9N7B7_CRODM	<i>Crotalus durissus cumanensis</i>	K.QMEEEEAVR.L	496.31	2
						<b>K.QEMTWADA EK.F</b>	604.89	2
						<b>R.HGEGFTSDLSK.Q</b>	639.83	2
						K.QMEEEEAVR.L	496.32	2
						K.YVYVWIGLR.I	535.3	2
						<b>K.STFFWIGANNIWNK.C</b>	849.44	2
43	SVSP	61.3	Snake venom serine protease catrocase-2	VSP2_CROAT	<i>Crotalus atrox</i>	<b>K.QEMTWADA EK.F</b>	604.89	2
						<b>R.HGEGFTSDLSK.Q</b>	639.83	2
						K.QMEEEEAVR.L	496.32	2
						K.YVYVWIGLR.I	535.3	2
						<b>K.STFFWIGANNIWNK.C</b>	849.44	2
						<b>K.QEMTWADA EK.F</b>	604.89	2
44	EXE	62.31	Exendin-4	EXE4_HEL SU	<i>Heloderma suspectum</i>	<b>K.MTHQSLK.S</b>	422.75	2
						<b>K.MTHQSLK.S</b>	422.75	2
						K.YGYMFYPSR.C	649.93	2
						K.WDIYPYSLK.S	592.95	2
						HLLQFNK.M	450.29	2
						K.MIKFETR.K	462.81	2
45	CLP	59.03	Snaclec convulxin subunit beta	SLB_CRODU	<i>Crotalus durissus terrificus</i>	R.SLSTYK.Y	349.88	2
						<b>R.HGEGFTSDLSK.Q</b>	639.86	2
						K.QMEEEEAVR.L	496.31	2
						<b>K.QEMTWADA EK.F</b>	604.89	2
						<b>K.MTHQSLK.S</b>	422.75	2
						K.YGYMFYPSR.C	649.93	2
46	EXE	60.95	Exendin-4	EXE4_HEL SU	<i>Heloderma suspectum</i>	K.WDIYPYSLK.S	592.95	2
						HLLQFNK.M	450.29	2
						K.MIKFETR.K	462.81	2
						R.SLSTYK.Y	349.88	2
						<b>R.HGEGFTSDLSK.Q</b>	639.83	2
						K.QMEEEEAVR.L	496.32	2
47	CLP	61.38	Snaclec crotoctin-1	SL1_CRODU	<i>Crotalus durissus terrificus</i>	<b>K.QEMTWADA EK.F</b>	604.89	2
						<b>K.MTHQSLK.S</b>	422.75	2
						K.YGYMFYPSR.C	649.93	2
						K.WDIYPYSLK.S	592.95	2
						HLLQFNK.M	450.29	2
						K.MIKFETR.K	462.81	2
48	CLP	97.74	Snaclec convulxin subunit beta	SLB_CRODU	<i>Crotalus durissus terrificus</i>	R.SLSTYK.Y	349.88	2
						<b>R.HGEGFTSDLSK.Q</b>	639.86	2
						K.QMEEEEAVR.L	496.31	2
						<b>K.QEMTWADA EK.F</b>	604.89	2
						<b>K.MTHQSLK.S</b>	422.75	2
						K.YGYMFYPSR.C	649.93	2
49	PLA <sub>2</sub>	93.51	Phospholipase A2 crotoxin basic subunit Cbc	PA2BC_CRODU	<i>Crotalus durissus terrificus</i>	K.WDIYPYSLK.S	592.95	2
						HLLQFNK.M	450.29	2
						K.MIKFETR.K	462.81	2
						R.SLSTYK.Y	349.88	2
						<b>R.HGEGFTSDLSK.Q</b>	639.86	2
						K.QMEEEEAVR.L	496.31	2

Table 1 (continued)

Spot	Prot. family	Score (%)	Top hit description	Accession	Hit species	Peptide	m/z	z
50	CRiSP	85.93	Cysteine-rich venom protein 2	CRVP_SISCA	<i>Sistrurus catenatus edwardsii</i>	K.IVDLHNSLR.R	533.85	2
51	CRiSP	98.38	Cysteine-rich secretory protein Ch-CRiPKa (Fragment)	F2Q6E5_CROHD	<i>Crotalus horridus</i>	K.MEWYSEAAAANAER.W R.SVNPTASNMLK.M K.MEWYPEAAAANAER.W	764.41 581.36 769.42	2 2 2
51	LAO	90.45	L-amino acid oxidase (Fragment)	K9N7B7_CRODM	<i>Crotalus durissus cumanensis</i>	SVDFDSESPR.K R.SVNPTASNMLK.M R.KFWEDDGIR.G	569.79 581.27 583.36	2 2 2
52	LAO	99.07	L-amino acid oxidase (Fragment)	K9N7B7_CRODM	<i>Crotalus durissus cumanensis</i>	K.STIDLPSR.F R.IKFEPPLPPK.K K.KFWEDDGIR.G K.NNPGILEYVPVKPSEEGK.S	438.74 583.41 583.36 936.03	2 2 2 2
53	SVSP	97.65	Kallikrein-CohLL-4	T1EH3_CROOH	<i>Crotalus oreganus helleri</i>	R.ETDYEEFLEIAR.N K.YILDKYDYSTK.E K.SAAQLYVESLR.K <b>R.NGLTVTSNPK.H</b> K.HDDIFGYEK.R K.SAAQLYVESLRK.V R.KKDWWYANLGP(+15.99)R.L K.HVVIVGAGMSGLSAAYVLGAGHEVTLEASER.A R.FLVALYTFR.S	757.88 755.46 619.06 515.83 562.31 682.97 498.98 1084.23 565.44	2 2 2 2 2 2 3 2
54	LAO	99.14	L-amino acid oxidase (Fragment)	K9N7B7_CRODM	<i>Crotalus durissus cumanensis</i>	R.AAHGGLPATSRT K.WDKDIM(+15.99)LIR.L R.ETDYEEFLEIAR.N	519.34 603.46 757.87	2 2 2
55	SVMP	98.17	SVMP-CohPH-3	T1E6U1_CROOH	<i>Crotalus oreganus helleri</i>	K.YILDKYDYSTK.E K.NNPGILEYVPVKPSEEGK.S <b>R.NGLTVTSNPK.H</b> <b>R.VIEIQNDRETK.V</b> K.FWEDDGIR.G K.STIDLPSR.F K.VQVHFNAR.V R.KFWEDDGIR.G R.MYELANTVNDIYR.Y	755.46 936.06 515.89 736.97 519.43 438.78 485.79 583.37 801.34	2 2 2 2 2 2 2 2
56	SVMP	84.27	Metalloproteinase P-II	Q2QA03_CRODD	<i>Crotalus durissus durissus</i>	R.FVELLVVDK.A K.GMVLPGTK.C R.KTDLLTR.K <b>K.NNDDLKIK.T</b> <b>R.VAVTM(+15.99)THELGHNLGIR.H</b>	580.91 401.79 423.82 537.81 588.71	2 2 2 2 3
57	SVMP	85.43	SVMP-CohPH-3	T1E6U1_CROOH	<i>Crotalus oreganus helleri</i>	R.VHQMVNIMK.E <b>R.ETDLLQR.R</b> R.M(+15.99)YELANTVNDIYR.Y	550.35 437.81 809.44	2 2 2
58	SVSP	61.29	Serine proteinase 8 (Fragment)	F8S120_CROAD	<i>Crotalus adamanteus</i>	R.FVELLVVDK.A R.AAYPEYGLPATSRT	580.93 698.4	2 2
59	SVSP	98.26	Snake venom serine protease	VSP_CRODD	<i>Crotalus durissus durissus</i>	<b>R.AAKPELPTSRT.T</b> <b>R.LKDVQTGVSK.D</b> K.DDEKDKDIM(+15.99)LIR.M K.DKDIM(+15.99)LIR.M K.KDDEKDKDIM(+15.99)LIR.M R.SVQFDKEQR.R	584.91 537.86 753.97 510.35 545.67 568.86	2 2 2 2 3 2
60	SVSP	98.32	Thrombin-like enzyme gyroxin B1.4	VSP14_CRODU	<i>Crotalus durissus terrificus</i>	<b>R.THFLIYGVHDR.S</b> R.TALPQLR.L <b>R.HGEGTFTSDLSK.Q</b>	729.09 399.78 639.85	2 2 2
60	EXE	61.48	Exendin-4	EXE4_HELSU	<i>Heloderma suspectum</i>	K.NNPGILEYVPVKPSEEGK.S	935.99	2
62	LAO	99.14	L-amino acid oxidase (Fragment)	K9N7B7_CRODM	<i>Crotalus durissus cumanensis</i>	K.SAAQLYVESLR.K K.YILDKYDYSTK.E R.ETDYEEFLEIAR.N <b>R.NGLTVTSNPK.H</b> R.VIEIQNDR.E K.HDDIFGYEK.R <b>R.VIEIQNDRETK.V</b> K.VQVHFNAR.V K.HDDIFGYEK.R.F	619.04 755.43 757.87 515.83 557.86 562.29 736.89 485.83 427.26	2 2 2 2 2 2 2 2 3

(continued on next page)

Table 1 (continued)

Spot	Prot. family	Score (%)	Top hit description	Accession	Hit species	Peptide	m/z	z
63	SVSP	78.43	Serine proteinase 7	T1E3B5_CROHD	<i>Crotalus horridus</i>	K.SAAQLYVESLRK.V	682.95	2
						R.FDEIVGGM(+15.99)DQLPTSM(+15.99)YEAIKEK.V	845.15	3
						K.EKVQVHFNAR.V	409.94	3
						K.DDEKDKDIM(+15.99)IIR.L	502.96	3
						K.VLNEDEQTR.D	552.25	2
						K.KDDEKDKDIM(+15.99)IIR.L	545.7	3
						K.NFQM(+15.99)QLGVHVK.K	652.86	2
						K.VLNEDEQTRDPK.E	481.92	3
67	SVMP	61.66	Metalloproteinase P-II	Q2QA03_CRODD	<i>Crotalus durissus durissus</i>	<b>R.VAVTM(+15.99)THELGHNLGIR.H</b>	588.68	3
						<b>R.VAVTMHELGHNLGIR.H</b>	583.41	3
70	LAAO	70.4	L-amino acid oxidase (Fragment)	K9N7B7_CRODM	<i>Crotalus durissus cumanensis</i>	K.STTDLPSRF	438.78	2
71	CLP	98.37	Snaclec crotoctin-1	SL1_CRODU	<i>Crotalus durissus terrificus</i>	K.WSDGSSVNYENLLK.S	806.41	2
						K.YYVWIGLR.I	535.34	2
72	VNGF	61.53	NGF-CohID-1 (Fragment)	T1DEA2_CROOH	<i>Crotalus oreganus helleri</i>	<b>K.GAHLVSVESAGEADFVAQLVAENIK.Q</b>	852.19	3
						R.ALTM(+15.99)EGNQASWR.F	690.4	2
73	CLP	60.6	Snaclec crotoctin-1	SL1_CRODU	<i>Crotalus durissus terrificus</i>	<b>K.GAHLVSVESAGEADFVAQLVAENIK.Q</b>	852.18	3

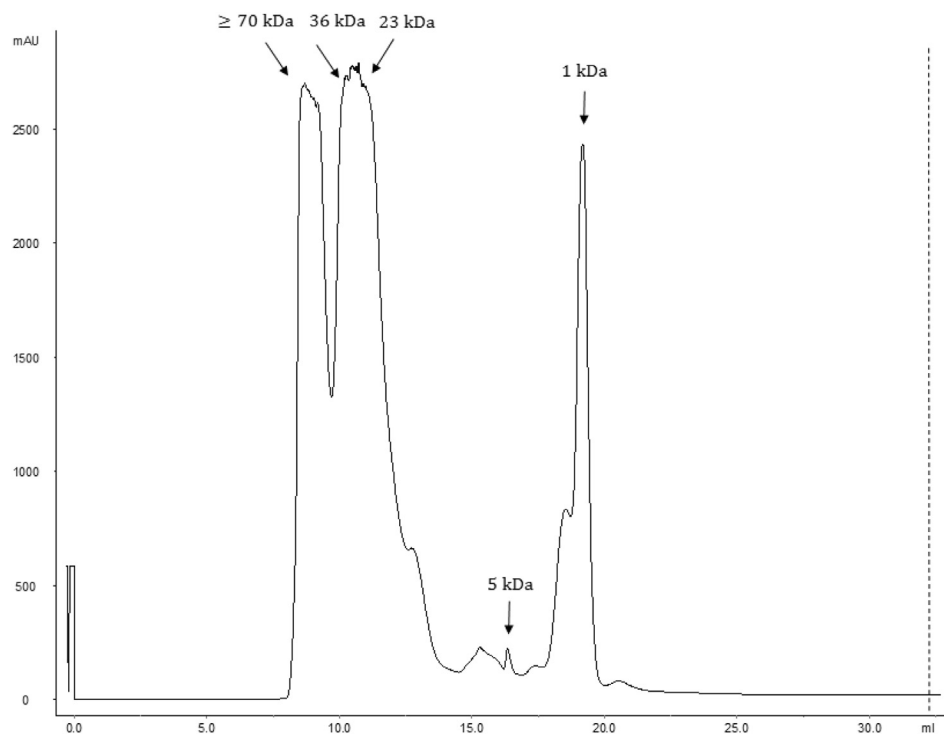


Fig. 2. Gel filtration chromatogram of 30 mg of *Crotalus vegrandis* crude venom, fractionated in a Superdex 75 10/300 (GE) column. Arrows indicate the molecular weight range.

C-terminal ends of crotoxin basic subunit. However, due to sample preparation and manipulation, one cannot currently assure whether the presence of these peptides is an artifact or that these fragments are cryptides (Pimenta and Lebrun, 2007) with a yet unknown function.

The crotoxin acidic subunit was identified in the GF venom fractions. The tryptic peptide (QICECDKAAICFR) identical to crotoxin acidic subunit (crotopotin) was identified in the GF fraction IV (Table 2) and, in the low molecular fractions a UP corresponding to its N-terminal end (G.SLVEFETLMMKIAGR.S) was detected.

Other PLA<sub>2</sub>S, different from the classical crotoxin in sequence and activity, can also be found in rattlesnake venoms. The basic PLA<sub>2</sub> Cdc-9 and Cdc-10 from *C. d. cumanensis* present myotoxic and edema-inducing activity (Romero-Vargas et al., 2010). An acidic

PLA<sub>2</sub> was identified in spot 22 (Fig. 1). Its sequence matches to a PLA<sub>2</sub> cloned from the *C. d. cascavella* venom gland (UniProtKB: C9E7C4) (Guarnieri et al., 2009). The identification of this protein species is supported by two UPs. Examining the data for UPs also enabled us to distinguish another protein species in spot 1 (Fig. 1; Table 1), a basic PLA<sub>2</sub> named LmTX-II from *Lachesis muta muta* (Damico et al., 2005).

#### 4.2. SVMP

Snake venom metalloproteinases are a multigene enzymatic toxin family in which the diversity of gene products can induce hemorrhage, fibrin(ogen)olysis, apoptosis, as well as activate blood coagulation factor X, inactivate blood serine protease inhibitors,

**Table 2**

Summary table of *Crotalus vegrandis* venom LC-MS/MS identification of gel-filtration fractions. Data generated by PEAKS + MASCOT (InChorus) analysis. Bold peptide sequences are Unique Peptides. M(+15.99): Oxidation of methionine; C(+57.02): Carbamidomethylation of cysteine; m: mass; z: charge; SVMP III: PIII snake venom metalloproteinase; SVMP II: PII snake venom metalloproteinase; SVSP: snake venom serine protease; LAAO: L-amino acid oxidase; PLA2: snake venom phospholipase A2; VNGF: venom nerve growth factor; BPP: bradykinin-potentiating peptide; BIP: bradykinin inhibitory peptide; CNP: C-type natriuretic peptide.

Prot. Family	Score (%)	Top hit description	Accession	Hit species	Peptide	m/z	z
Fraction I							
LAAO	99	L-amino acid oxidase (Fragment)	K9N7B7_CRODM	<i>Crotalus durissus cumanensis</i>	R.SGTKIFLTC(+57.02)K.R	577.81	2
					R.KKDWYANLGPMLPTK.H	640.02	3
					R.RIKFEPPLPPK.A	483.97	3
					R.IKFEPPLPPKKAHALR.S	614.70	3
					R.DVNRASENPSG.I	573.26	2
					R.SVHYR.S	661.35	1
					R.KVVKELKR.T	500.32	2
SVMP III	83.09	SVMP-CohPH-3	T1E6U1_CROOH	<i>Crotalus oreganus helleri</i>	R.KTDLLTR.K	423.76	2
					R.KKHDNAQLLTAIDLDR.V	617.66	3
					R.KTDLLTRK.K	487.80	2
					R.KKHDNAQLLTAIDLDRVIGLAYVGSMD (+57.02)HPK.R	1121.88	3
SVMP II	81.59	Metalloproteinase P-II	Q2QA03_CRODD	<i>Crotalus durissus durissus</i>	<b>R.VAVMTMHELGHNLGIR.H</b>	583.32	3
					L.NLNPEHQR.Y	504.25	2
SVSP	67.93	Snake venom serine proteinase 2	VSP2_CROAD	<i>Crotalus adamanteus</i>	R.AAYPEYGLPATSR.T	698.34	2
Fraction II							
LAAO	98.58	L-amino acid oxidase (Fragment)	K9N7B7_CRODM	<i>Crotalus durissus cumanensis</i>	R.KKDWYANLGPMLPTK.H	640.02	3
					R.IKFEPPLPPKKAHALR.S	614.71	3
					R.KVVKELKR.T	500.33	2
					K.EYLLK.E	665.38	1
					K.SGLTAAR.D	675.37	1
SVMP III	94.77	Zinc metalloproteinase-disintegrin-like	VM3_CRODD	<i>Crotalus durissus durissus</i>	R.KTDLLTR.K	423.75	2
					R.KKHDNAQLLTAIDLDR.V	617.68	3
					R.KTDLLTRK.K	487.80	2
					R.ETDLLQR.R	437.75	2
					R.ETDLLTR.K	424.10	2
SVMP II	81.46	Metalloproteinase P-II	Q2QA03_CRODD	<i>Crotalus durissus durissus</i>	<b>R.VAVMTMHELGHNLGIR.H</b>	583.31	3
					R.ETDLLK.R	718.40	1
					L.NLNPEHQR.Y	504.25	2
Fraction III							
BIP + BPP propeptide	97.89	Bradykinin-potentiating and C-type natriuretic peptides	BNP_CRODO	<i>Crotalus durissus collilineatus</i>	K.AAAAAPQRLSK.S	542.31	2
					D.TPPAGPDGGPR.G	511.25	2
					K.SKGASATSAASRD.L	604.79	2
LAAO	96.15	LAAO-CohPH-1	T1E6V1_CROOH	<i>Crotalus oreganus helleri</i>	K.VQVHFNAR.V	485.77	2
					K.EYLLK.E	665.39	1
					R.SVHYR.S	661.34	1
Crotoxin PLA <sub>2</sub> basic	84.02	Basic phospholipase A2 CB1 (Fragment)	PA2B1_CRODV	<i>Crotalus durissus vegrandis</i>	K.MIKFETR.K	462.76	2
					HLLQFNK.M	450.26	2
					HLLQFNKMIKFETR.K	602.33	3
SVMP II	59.58	Metalloproteinase P-II	Q2QA03_CRODD	<i>Crotalus durissus durissus</i>	<b>R.VAVMTMHELGHNLGIR.H</b>	583.32	3
SVMP III	56.51	Snake venom metalloprotease	C6JUN4_PHIOL	<i>Philodryas olfersii</i>	R.ETDLLQR.R	437.75	2
					K.EYLLK.Y	665.39	1
Fraction IV							
Crotoxin PLA <sub>2</sub> basic	97.52	Basic phospholipase A2 Mtx-b	PA2B1_CROSS	<i>Crotalus scutulatus scutulatus</i>	K.MIKFETR.K	462.74	2
					G.HLLQFNK.M	450.26	2
					A.VLLVG.V	500.37	1
BIP and CNP	82.49	Bradykinin-potentiating and C-type natriuretic peptides	BNP_CRODO	<i>Crotalus durissus collilineatus</i>	D.TPPAGPDGGPR.G	511.26	2
					T.PPAGPDGGPR.G	460.73	2
					L.KLDRIGSMSGLGC(+57.02)	697.36	2
crotoxin PLA <sub>2</sub> acidic	60.91	Phospholipase A2 1b	T1E3Y3_CROHD	<i>Crotalus horridus</i>	K.QIC(+57.02)EC(+57.02)DKAAAIC (+57.02)FRE	871.41	2
LAAO	56.31	L-amino acid oxidase (Fragment)	K9N7B7_CRODM	<i>Crotalus durissus cumanensis</i>	F.EPPLPPK.K	777.44	1

(continued on next page)

Table 2 (continued)

Prot. Family	Score (%)	Top hit description	Accession	Hit species	Peptide	m/z	z
Fraction V BIP	59.8	Bradykinin-potentiating and C-type natriuretic peptides	BNP_CRODO	<i>Crotalus durissus collilineatus</i>	D.TPPAGPDGGPR.G	511.25	2
VNGF	59.22	NGF-CohPH-1	T1E7J7_CROOH	<i>Crotalus oreganus helleri</i>	T.DQHYPAPK.K	478.23	2
Fraction VI + VII + VIII crotoxin PLA <sub>2</sub> basic	98.10	Phospholipase A2 crotoxin basic subunit CBB	PA2BB_CRODU	<i>Crotalus durissus terrificus</i>	K.MIKFETR.K HLLQFNK.M R.C(+57.02)RGPSETC(+57.02)	462.74 450.24 483.68	2 2 2
crotoxin PLA <sub>2</sub> acidic	59.73	Phospholipase A2 homolog crotoxin acid subunit CA	PA1A_CRODU	<i>Crotalus durissus terrificus</i>	<b>G.SLVEFETLMMKIAGRS</b>	862.94	2
BIP	61.35	Bradykinin-potentiating and C-type natriuretic peptides	BNP_CRODO	<i>Crotalus durissus collilineatus</i>	D.TPPAGPDGGPR.G	511.25	2

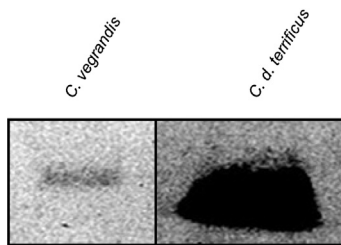


Fig. 3. Western blotting of *Crotalus vegrandis* (right lane) and *C. durissus terrificus* (control) crude venoms (both 33.3 µg/lane) against polyclonal anti-crotamine (1:8000 dilution).

inhibit platelet aggregation and have pro-inflammatory activity (Markland and Swenson, 2013). SVMPs are classified in PI, PII and PIII classes according to their domain composition. So far, the only SVMP described in *C. vegrandis* venom is a hemorrhagic fraction purified by Aguilar et al. (2001), named Uracoin-1, with an estimated mass of 58 kDa under denaturing conditions. In this work, PIII and PII SVMPs were observed. PIII peptides from *Crotalus* species were identified in venom fractions and in a cluster of spots ranging from ~50 kDa to ~70 kDa and *pI* around 5 (Fig. 1, Table 1, Table 2). A UP was detected in spot 10, indicating the existence of a crostastatin-like isoform. Crostastatin, also known as VAP1, is a vascular apoptosis-inducing protein first described in *Crotalus atrox* (Masuda et al., 1997). The expression of homologous transcripts was also detected in *C. d. cascavella*, *Bothrops atrox* and *L. m.*

*rhombata* (Tavares et al., 2008). Another UP matching to a PIII was detected in spots 36 and 55. It matches to a transcript described in *C. d. durissus* venom gland (Azofeifa-Cordero et al., 2008).

PII peptides were identified in venom fractions and in the 2D gel, in spot 21 and in a cluster formed by the spots 39, 40, 56 and 63 (Fig. 1, Table 1, Table 2). In all those spots, the identification of a *C. d. durissus* PII gene product (UniProtKB: Q2QA03) was supported by UPs. Interestingly, the spots 39 and 56, belonging to this PII spot cluster, contain a UP derived from a PIII cloned from the Colubridae *Philodryas olfersii* (Ching et al., 2006).

Two spots (15 and 67), with much lower mass, were also identified as SVMPs. Their position in the gel suggests the existence of PI SVMPs in the venom, but their tryptic peptide sequences align better with PII and PIII sequences than with PI. Those spots could be products of PII and PIII proteolysis.

#### 4.3. SVSP

Snake venom serine proteases are a multigene family that is present in almost all families of venomous snakes. Despite their apparent similarity, the isoforms have diversified activities on the coagulation cascade, i.e. factor V, factor X, protein C and plasminogen activation, thrombin-like activity on fibrinogen, induction of platelet aggregation, and also prothrombin activation and action on the kallikrein-kinin system (Serrano, 2013). SVSPs were identified in spots 3, 4, 12, 13, 18, 27, 29, 30, 38, 53, 58, 59, 60 and 61. The spot 60 contains a UP from the isoform B1.4 of gyroxin (Yonamine et al., 2009). Gyroxin is a toxin from *C. d. terrificus*, which has similar to

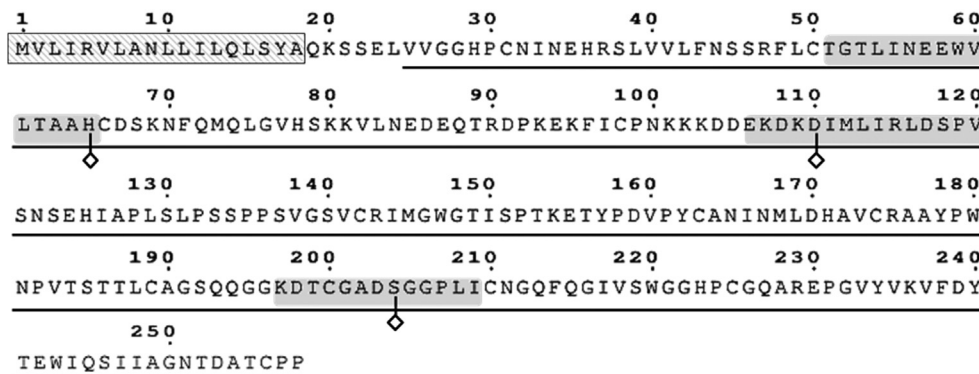


Fig. 4. Translated amino acid sequence (258 residues) of the SVSPc01 transcript cloned from a *Crotalus vegrandis* venom gland (GenBank: KT266708). Domain prediction analysis identified: peptidase S1 trypsin-like cysteine/serine (underlined sequence); signal peptide (crosshatched box); and peptidase S1A chymotrypsin-type subfamily domain signatures (gray boxes). The catalytic triad residues are indicated with  $\diamond$  symbol.

trypsin specificity, releasing only fibrinopeptide A in the conversion of fibrinogen to fibrin (Barrabin et al., 1978; Barrio, 1961). It also induces the barrel rotation syndrome in mice, probably by increasing reversibly the permeability of the blood brain barrier (Alves da Silva et al., 2011). Based on two UPs, we also identified in spot 59 a specific gene product cloned from the Central American *C. d. durissus* (UniProtKB: Q2QA04).

The gene product SVSPcv01 we have cloned from *C. vegrandis* venom gland transcripts was identified in spot 18. The identification is supported by the UP sequence R.EPGVYVK.V (residue 230 to 236;  $m/z = 791.46$ ;  $z = 1$ ) indicating it is secreted in the venom. The first five hits from the protein BLAST analysis of the SVSPcv01 full sequence resulted in matches with *Crotalus* SVSP transcripts. These hit sequences were obtained from *C. horridus* and *Crotalus oreganus* venom glands without further characterization (Rokyta et al., 2013; Fry, unpublished). Identity values range from 94.6% to 87.6%. We looked for sequences with described activity and found it has 86.5% identity with the thrombin-like enzyme asperase found in *Bothrops asper* venom (Pérez et al., 2008). The most similar *Crotalus* sequence hit (82% of identity), is catroxase-2 from *C. atrox* venom, described as a kallikrein-like SVSP (Bjarnason et al., 1983).

In general, the matching SVSPs we identified by proteomics are expected to have fibrinolytic activity, as they show high homology with other SVSP with such features such as shedaoenase from *Agkistrodon shedaoenesis* (Jiao et al., 2005), calobin from *Gloydius ussuriensis* (Hahn et al., 1996), and crotalase from *Crotalus adamanteus* that additionally shows kinin-releasing activities (Markland et al., 1982).

#### 4.4. CLP

Snake C-type lectins are non-enzymatic toxins classified into snake lectins, which are classic sugar binding proteins, and snake CLPs mostly found in Viperidae, lacking the sugar-binding loop. CLPs are composed by heterodimers with homologous  $\alpha$  and  $\beta$  subunits (Du and Clemetson, 2009). CLPs are important components in the highly hemorrhagic venoms of the Viperidae that activate or inhibit a wide range of plasma components and blood cells such as coagulation Factor X, Factor IX, von Willebrand factor, GPIb-complex, GPVI, integrin  $\alpha 2\beta 1$  and the fibrinogen receptor integrin  $\alpha IIb\beta 3$  (Arlinghaus and Eble, 2012; Clemetson, 2010). Seven spots (16, 23, 45, 47, 48, 71 and 73) ranging from 15 to 20 kDa and from very acidic to very basic  $pI$  (Fig. 1) matched with CLPs. Convulxin subunits were identified in spots 15, 45 and 48. Convulxin is a ~84 kDa heterodimeric, disulfide linked tetramer ( $\alpha 4\beta 4$ ) that induces platelet aggregation in mammals (Murakami et al., 2003; Polgár et al., 1997). The alpha subunit was identified in this venom by the detection of a UP (seq.: GAHLVSIK;  $m/z = 412.76$ ;  $z = 2$ ; score = 94.7%; #Spec = 2). The beta subunit was identified in spots 45 and 48 supported by three UP (Table 1). Crotoctin was also identified, supported by a UP, in spots 16, 71 and 73. Crotoctin was originally cloned from *C. d. terrificus* venom gland (UniProtKB: Q719L8). The *Trimeresurus mucrosquamatus* crotoctin homologue, mucroctin, has *in vitro* platelet aggregation activity (Huang et al., 2004).

#### 4.5. Crotoamine

Crotoamine is a myotoxic peptide that induces spastic paralysis of the hind limbs in mice (Gonçalves and Vieira, 1950) and it was first described in *C. d. terrificus* venom by Gonçalves and Polson (1947). Crotoamine features were reviewed in Oguiura et al. (2005), and its potential biotechnological and therapeutic applications by Kerkis et al. (2014). Crotoamine shows antimicrobial activity that can be explained by its  $\beta$ -defensin structure (Oguiura et al., 2011; Yamane

et al., 2013) and interacts with vesicles which mimic bacterial membranes (Costa et al., 2014) as well as affects voltage gated potassium (KV) channels (Peigneur et al., 2012). Its low molecular mass (4.9 kDa) and high isoelectric point (9.5) may impair its detection using the 2D-PAGE MS<sup>2</sup> approach, due to the limitations of the 2D gel to small peptides (less than 14 kDa). Thus, the immuno-detection of this toxin was an alternative to circumvent this issue. A previous study (Scannone et al., 1978) suggests that *C. vegrandis* has crotoamine in lower levels than *C. d. cumanensis*. We detected a weak reactive band in the Western blot assay with anti-crotoamine polyclonal antibodies (Fig. 3).

#### 4.6. Bradykinin-potentiating peptides, Bradykinin inhibitor peptides and C-type natriuretic peptides

BPPs are angiotensin converting enzymes (ACE) inhibitors found in Viperidae venom, that can lead victims of envenoming to hypotensive shock (Camargo et al., 2012). They were first discovered in *Bothrops jararaca* by Ferreira and Rocha e Silva (1965), whose further studies led to the development of the first drug based on a snake venom molecule: Captopril. It is a synthetic inhibitor of ACE used for treatment of hypertension and heart failure (Cushman and Ondetti, 1991). Even with this accumulated history of success, new features are being discovered for BPPs in many different Viperidae venoms (e.g. Gomes et al., 2007; Rioli et al., 2008) and, even in Elapidae venom such as *Naja mossambica* (Munawar et al., 2014). Usually, BPP precursors are composed by BPPs, BIP and CNP, separated by propeptides that are cleaved into the active peptides. BIP antagonizes the vasodilator actions of bradykinin at the B2 bradykinin receptor (Graham et al., 2005). CNP exhibits hypotensive and vasodepressor activity (Schweitz et al., 1992). In the size exclusion fractions, we identified a BIP, a CNP and propeptide sequences, indicating the presence of BPP precursors similar to those of *C. d. collilineatus* and *C. d. terrificus* venoms. The identity of five BPPs detected in the low molecular mass fractions by their proline—proline fragment signature ( $MH^+ = 213.1$ ) still needs to be confirmed (Menin et al., 2008).

#### 4.7. CRiSP

Cysteine-rich secretory protein was identified in a cluster of basic spots (3, 27, 50 and 51), ~30 kDa (Fig. 1). Although CRiSP function is still unclear in the majority of snake venoms, some researchers have shown that venom CRiSP can have an ion channel blocking activity, such as the first CRiSP described from *Heloderma horridum* lizard (Mochca-Morales et al., 1990). Smooth muscle contraction inhibition and cyclic nucleotide-gated ion channels inhibition activity were described for the pseudodechotoxin (*Pseudechis australis*) and pseudodecin (*Pseudechis porphyriacus*) (Yamazaki and Morita, 2004). Natrin, from *Naja atra* venom, has proinflammatory modulator activity (Wang et al., 2010). In a related species *C. atrox*, a weak muscle contraction inhibition was observed as well (Yamazaki et al., 2003).

#### 4.8. LAAO

L-amino-acid oxidase was identified in spots 4–8, 10, 11, 17, 27, 32, 33, 41, 42, 51, 52, 54, 62 and 70 (Fig. 1, Table 1, Table 2). The 2D gel displays a visible cluster of LAAO spots at MW ~65 kDa and  $pI$  from 8 to 9. Some others spots dispersed in the gel are probably proteolytic fragments. LAAO was identified in the venom fraction analysis as well. This toxin may contribute to the toxicity of the venom mainly because of hydrogen peroxide production, but recent studies indicate differently. The purified LAAO from *C. d. cascavella* induces platelet aggregation or inhibits agonist-induced

aggregation, depending on the experimental conditions (Toyama et al., 2006). UP analysis evidenced at least two different gene product in this venom (Table 1): (1) the L-amino acid oxidase Cdc18 cloned and isolated from *C. d. cumanensis* (UniProtKB: K9N7B7), with antibacterial activity (Vargas et al., 2013), is supported by the detection of three UPs, and (2) an isoform described in the venom of the European *Vipera ammodytes ammodytes* (UniProtKB: P0D184) (Georgieva et al., 2008).

#### 4.9. VNGF

Venom nerve growth factors are neurotrophic factors that belong to the NGF-beta family found in snake venom (Kostiza and Meier, 1996). VNGF was identified in acidic spots 14, 25 and 72, below 20 kDa (Fig. 1, Table 1), as well as in fraction V (Table 2). VNGF was described in related species venom such as *C. d. terrificus* (Georgieva et al., 2010), *C. simus* (Castro et al., 2013) and venom gland transcripts of *C. adamanteus* (Margres et al., 2014) and *C. horridus* (Rokyta et al., 2013).

#### 4.10. E5'N

Seven peptides matching to *Crotalus* venom ecto-5'-nucleotidase were detected in spot 34 (~70 kDa and *pI* 8) (Fig. 1). Although E5'N biological activity and further role in snake venom are not well understood, the presence of free purines supports the potential of venom-induced hypotension and paralysis via purine receptors (Dhananjaya and D'Souza, 2010). According to Hart et al. (2008) E5'N can inhibit platelet aggregation.

#### 4.11. PLB

The analysis of spot 31 (~50 kDa and *pI* from 8.5 to 9) identified nine peptides matching to phospholipase B (Table 1). Common PLB are enzymes that catalyze the hydrolysis of glycerophospholipids and their toxin variants are present in bee as well as in snake venoms. Venom PLB was first isolated from *Pseudechis colletti* (Bernheimer et al., 1987) and, recently, identified in *Drysdalia coronoides* (Chatrath et al., 2011), *Protobothrops flavoviridis*, *Ovophis okinavensis* (Aird et al., 2013), *Pseudechis guttatus* (Viala et al., 2014) and *C. d. terrificus* (Melani et al., 2015). The PLBs found in snake and bee venoms exist as monomers and dimers with molecular masses of approximately 16 kDa and 35 kDa, respectively. They are known to cause hemolysis (Bernheimer et al., 1986; Doery and Pearson, 1964).

#### 4.12. PDE

Venom phosphodiesterase are a superfamily of metalloenzymes with diverse physiological and cellular signaling functions which have become new therapeutic targets for treatment of various diseases, such as Alzheimer's disease, inflammation, erectile dysfunction, and cardiac or vascular-related diseases (Peng et al., 2013). Snake venom PDEs are poorly investigated on what refers to potential pharmacological activities, but some of these enzymes purified from *Trimeresurus stejnegeri*, *Daboia russelli russelli* and *B. jararaca* inhibited ADP-induced platelet aggregation in human platelet-rich plasma by hydrolyzing ADP (Mitra and Bhattacharyya, 2014; Peng et al., 2011; Santoro et al., 2009). Spots 9 and 35 generated at least 16 peptides covering 22% of *C. adamanteus* and *C. horridus* venom PDEs. The spots are situated at a high mass position in the 2D gel (~120 kDa and *pI* ranging from 7.5 to 8.5) in a clear and isolated train of spots (Fig. 1).

#### 4.13. Exendin4-like peptides

Exendin-4 was identified in the spots 44, 46 and 60, based on two peptides, one of them a UP. This peptide, described in the *Heloderma suspectum* lizard venom (Eng et al., 1992), is a polypeptidic hormone that mimics glucagon and induces hypotension in prey. Exendins are a family of peptides derived from secretin-like hormones (Irwin, 2012). The antidiabetic drug Byetta® was developed based on its ability to stimulate the insulin production (Furman, 2012).

#### 4.14. Other venom proteins

This study also revealed other venom proteins such as glutathione peroxidase and carboxypeptidase E-like. Their function in the venom is unknown. Those proteins were recently identified among others in *C. d. terrificus* venom (Melani et al., 2015).

The GP was identified in spot 26, located between 25 and 30 kDa and *pI* ~9.0 (Fig. 1). GPs (EC:1.11.1.9) are enzymes that catalyze the reduction of hydroxyperoxides by glutathione, which main function is to protect against oxidative damage of hydroxyperoxides (Barnett and King, 1995).

CPE was identified in spot 37, CPE is classically known to be involved in carboxyterminal peptide processing by releasing C-terminal arginine or lysine residues from polypeptides, and could be involved in post-translational modification of toxins and other venom proteins. Recent studies support that it is a multifunctional protein that subserves many essential nonenzymatic roles acting on many physiological and neuronal pathways (Cawley et al., 2012) and could be engineered as a prognostic biomarker for metastasis in endocrine and nonendocrine tumors (Lee et al., 2011).

## 5. Conclusion

Using the mass spectrometry approach for protein identification we assessed for the first time the full venom composition of the medically important snake *C. vegrandis*. Besides reinforcing previous data that described both neurotoxic and hemorrhagic components in the *C. vegrandis* venom, we provide a more detailed profile of this venom, identifying other PIII and PII SVMPs isoforms, other PLA2s, SVSPs isoforms (including a newly sequenced transcript), CLPs isoforms, LAAO isoforms, VNGF, CRISP, E5'N, PDE, GP, CPE and peptides such as crotamine-like, BPP, BIP and CNP. The venom components identified in this work match the local and systemic symptoms described in the scarce clinical cases available in the literature: neurotoxic and myotoxic effects can be ascribed to the presence of crotoxin and crotamine-like molecules. Moreover, hemostatic disturb, such as hemorrhage and coagulopathies can be induced directly or indirectly by SVMPs, SVSPs, LAAOs and even E5'N. On what refers to edema, the generation and/or release of inflammatory mediators by the action of PLA2s, as well as other pro-inflammatory molecules either from the venom or activated by its components might be considered the causative agent of that inflammatory symptom. These data might be helpful from both the clinical and evolutionary point of view.

## 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.

## Acknowledgments

We would like to acknowledge Mr. Joaquín Sánchez and Ms. Adelina Jara from the Brenesia scientific journal of the Museo Nacional de Costa Rica for providing valuable literature used in this manuscript; the Biotechnology Center at the Butantan Institute for DNA sequencing; grant and scholarship from FAPESP (SP, Brazil) (2010/08585-8 and 2009/10305-8) and CAPES/DAAD no 364/11 (Brazil/Germany research program).

## Appendix A. Supplementary data

Supplementary data related to this article can be found at <http://dx.doi.org/10.1016/j.toxicon.2015.09.023>.

## Transparency document

Transparency document related to this article can be found online at <http://dx.doi.org/10.1016/j.toxicon.2015.09.023>.

## References

- Aguilar, I., Girón, M.E., Rodríguez-Acosta, A., 2001. Purification and characterisation of a haemorrhagic fraction from the venom of the Uraoan rattlesnake *Crotalus vegrandis*. *Biochim. Biophys. Acta* 1548, 57–65.
- Aguilar, I., Guerrero, B., Maria Salazar, A., Girón, M.E., Pérez, J.C., Sánchez, E.E., Rodríguez-Acosta, A., 2007. Individual venom variability in the South American rattlesnake *Crotalus durissus cumanensis*. *Toxicon* 50, 214–224. <http://dx.doi.org/10.1016/j.toxicon.2007.03.012>.
- Aird, S.D., Watanabe, Y., Villar-Briones, A., Roy, M.C., Terada, K., Mikheyev, A.S., 2013. Quantitative high-throughput profiling of snake venom gland transcriptomes and proteomes (*Ovophis okinavensis* and *Protobothrops flavoviridis*). *BMC Genom.* 14, 790. <http://dx.doi.org/10.1186/1471-2164-14-790>.
- Alves da Silva, J.A., Oliveira, K.C., Camillo, M.A.P., 2011. Gyroxin increases blood-brain barrier permeability to Evans blue dye in mice. *Toxicon* 57, 162–167. <http://dx.doi.org/10.1016/j.toxicon.2010.06.027>.
- Arlinghaus, F.T., Eble, J.A., 2012. C-type lectin-like proteins from snake venoms. *Toxicon* 60, 512–519. <http://dx.doi.org/10.1016/j.toxicon.2012.03.001>.
- Ausubel, F.M., Brent, R., Kingston, R., Moore, D., Seidman, J., Smith, J., Struhl, K., 2000. *Current Protocols in Molecular Biology*. John Wiley & Sons Inc., New York.
- Azevedo-Marques, M.M., De Hering, S.E., Cupo, P., 2003. *Acidente crotálico*. In: Cardoso, J.L.C., França, F.O. de S., Wen, F.H., Málague, C.M.S., Haddad Jr., V. (Eds.), *Animais Peçonhentos No Brasil – Biologia, Clínica E Terapêutica Dos Acidentes*. Sarvier, São Paulo, pp. 91–98.
- Azofeifa-Cordero, G., Arce-Estrada, V., Flores-Díaz, M., Alape-Girón, A., 2008. Immunization with cDNA of a novel P-III type metalloproteinase from the rattlesnake *Crotalus durissus durissus* elicits antibodies which neutralize 69% of the hemorrhage induced by the whole venom. *Toxicon* 52, 302–308. <http://dx.doi.org/10.1016/j.toxicon.2008.05.023>.
- Barnett, Y.A., King, C.M., 1995. An investigation of antioxidant status, DNA repair capacity and mutation as a function of age in humans. *Mutat. Res.* 338, 115–128. [http://dx.doi.org/10.1016/0921-8734\(95\)00017-Z](http://dx.doi.org/10.1016/0921-8734(95)00017-Z).
- Barnwal, B., Kini, R.M., 2013. Characterization of inflammin, the first member of a new family of snake venom proteins that induces inflammation. *Biochem. J.* 455, 239–250. <http://dx.doi.org/10.1042/Bj20130599>.
- Barrabin, H., Martiarena, J.L., Vidal, J.C., Barrio, A., 1978. Isolation and characterization of gyroxin from *Crotalus durissus terrificus* venom. In: Rosenberg, P. (Ed.), *Toxins: Animal, Plant and Microbial*. Pergamon Press, New York, pp. 113–133.
- Barrio, A., 1961. Gyroxin, a new neurotoxin of *Crotalus durissus terrificus* venom. *Acta Physiol. Latinoam.* 11, 224.
- Bernheimer, A.W., Linder, R., Weinstein, S.A., Kim, K.S., 1987. Isolation and characterization of a phospholipase B from venom of Collett's snake, *Pseudechis colletti*. *Toxicon* 25, 547–554.
- Bernheimer, A.W., Weinstein, S.A., Linder, R., 1986. Isoelectric analysis of some Australian elapid snake venoms with special reference to phospholipase B and hemolysis. *Toxicon* 24, 841–849.
- Bjarnason, J.B., Barish, A., Drenzo, G.S., Campbell, R., Fox, J.W., 1983. Kallikrein-like enzymes from *Crotalus atrox* venom. *J. Biol. Chem.* 258, 12566–12573.
- Bober, M.A., Glenn, J.L., Straight, R.C., Ownby, C.L., 1988. Detection of myotoxin alpha-like proteins in various snake venoms. *Toxicon* 26, 665–673.
- Bon, C., 1982. Synergism of the two subunits of crotoxin. *Toxicon* 20, 105–109.
- Calvete, J.J., 2013. Snake venomomics: from the inventory of toxins to biology. *Toxicon* 75, 44–62. <http://dx.doi.org/10.1016/j.toxicon.2013.03.020>.
- Calvete, J.J., Pérez, A., Lomonte, B., Sánchez, E.E., Sanz, L., 2012. Snake venomomics of *Crotalus tigris*: the minimalist toxin arsenal of the deadliest Neartic rattlesnake venom. Evolutionary clues for generating a pan-specific antivenom against crotalid type II venoms. *J. Proteome Res.* 11, 1382–1390. <http://dx.doi.org/10.1021/pr201021d>.
- Calvete, J.J., Sanz, L., Cid, P., Torre, P., De Flores-di, M., Santos, M.C., Dos Borges, A., Brema, A., Angulo, Y., Lomonte, B., Alape-giro, A., 2010. Snake venomomics of the Central American rattlesnake *Crotalus simus* and the South American *Crotalus durissus* complex points to neurotoxicity as an adaptive paedomorphic trend along *Crotalus* dispersal in South America research articles, pp. 528–544.
- Calvete, J.J., Sanz, L., Pla, D., Lomonte, B., Gutiérrez, J.M., 2014. Omics meets biology: application to the design and preclinical assessment of antivenoms. *Toxins (Basel)* 6, 3388–3405. <http://dx.doi.org/10.3390/toxins6123388>.
- Camargo, A.C.M., Ianzer, D., Guerreiro, J.R., Serrano, S.M.T., 2012. Bradykinin-potentiating peptides: beyond captopril. *Toxicon* 59, 516–523. <http://dx.doi.org/10.1016/j.toxicon.2011.07.013>.
- Campbell, J.A., Lamar, W.W., 2004. *The Venomous Reptiles of the Western Hemisphere*. Comstock Pub. Associates, Ithaca.
- Castro, E.N., Lomonte, B., del Carmen Gutiérrez, M., Alagón, A., Gutiérrez, J.M., 2013. Intraspecific variation in the venom of the rattlesnake *Crotalus simus* from Mexico: different expression of crotoxin results in highly variable toxicity in the venoms of three subspecies. *J. Proteom.* 87, 103–121. <http://dx.doi.org/10.1016/j.jprot.2013.05.024>.
- Cavalcante, W.L.G., Ponce-Soto, L.A., Marangoni, S., Gallacci, M., 2015. Neuromuscular effects of venoms and crotoxin-like proteins from *Crotalus durissus ruruima* and *Crotalus durissus cumanensis*. *Toxicon* 96, 46–49. <http://dx.doi.org/10.1016/j.toxicon.2015.01.006>.
- Cawley, N.X., Wetsel, W.C., Murthy, S.R.K., Park, J.J., Pacak, K., Loh, Y.P., 2012. New roles of carboxypeptidase E in endocrine and neural function and cancer. *Endocr. Rev.* 33, 216–253. <http://dx.doi.org/10.1210/er.2011-1039>.
- Chatrath, S.T., Chapeaurouge, A., Lin, Q., Lim, T.K., Dunstan, N., Mirtschin, P., Kumar, P.P., Kini, R.M., 2011. Identification of novel proteins from the venom of a cryptic snake *Drysdalia coronoides* by a combined transcriptomics and proteomics approach research articles, pp. 739–750.
- Ching, A.T.C., Rocha, M.M.T., Paes Leme, A.F., Pimenta, D.C., de Fátima, D., Furtado, M., Serrano, S.M.T., Ho, P.L., Junqueira-de-Azevedo, I.L.M., 2006. Some aspects of the venom proteome of the colubridae snake *Philodryas olfersii* revealed from a Duvernoy's (venom) gland transcriptome. *FEBS Lett.* 580, 4417–4422. <http://dx.doi.org/10.1016/j.febslet.2006.07.010>.
- Clemetson, K.J., 2010. Snaclecs (snake C-type lectins) that inhibit or activate platelets by binding to receptors. *Toxicon* 56, 1236–1246. <http://dx.doi.org/10.1016/j.toxicon.2010.03.011>.
- Costa, B.A., Sanches, L., Gomide, A.B., Bizerra, F., Dal Mas, C., Oliveira, E.B., Perez, K.R., Itri, R., Oguiura, N., Hayashi, M.A.F., 2014. Interaction of the rattlesnake toxin crotoxin with model membranes. *J. Phys. Chem. B* 118, 5471–5479. <http://dx.doi.org/10.1021/jp411886u>.
- Cushman, D.W., Ondetti, M.A., 1991. History of the design of captopril and related inhibitors of angiotensin converting enzyme. *Hypertension* 17, 589–592.
- Damico, D.C.S., Lilla, S., de Nucci, G., Ponce-Soto, L.A., Winck, F.V., Novello, J.C., Marangoni, S., 2005. Biochemical and enzymatic characterization of two basic Asp49 phospholipase A2 isoforms from *Lachesis muta muta* (*Suruucu*) venom. *Biochim. Biophys. Acta* 1726, 75–86. <http://dx.doi.org/10.1016/j.bbagen.2005.05.022>.
- De Sousa, L., Vásquez, D., Salazar, D., Valecillos, R., Vásquez, D., Rojas, M., Parrilla-Álvarez, P., Quiroga, M., 2005. Mortalidad en humanos por envenenamientos causados por invertebrados y vertebrados en el Estado Monagas, Venezuela. *Invest. Clin.* 46, 229–240.
- Degn, L.L., Seebart, C.S., Kaiser, I.I., 1991. Specific binding of crotoxin to brain synaptosomes and synaptosomal membranes. *Toxicon* 29, 973–988.
- Dhananjaya, B.L., D'Souza, C.J.M., 2010. The pharmacological role of nucleotidases in snake venoms. *Cell Biochem. Funct.* 28, 171–177. <http://dx.doi.org/10.1002/cbf.1637>.
- Doery, H.M., Pearson, J.E., 1964. Phospholipase B in snake venoms and bee venom. *Biochem. J.* 92, 599–602.
- Dos Santos, M.C., Ferreira, L.C., Da Silva, W.D., Furtado, M.F., 1993. Characterization of the biological activities of the “yellow” and “white” venoms from *Crotalus durissus ruruima* compared with the *Crotalus durissus terrificus* venom. Neutralizing activity of *Crotalus durissus ruruima* antivenins. *Toxicon* 31, 1459–1469.
- Du, X.-Y., Clemetson, K.J., 2009. Reptile C-type lectins. In: Mackessy, S.P. (Ed.), *Handbook of Venoms and Toxins of Reptiles*. CRC Press, Boca Raton, p. 521.
- Eng, J., Kleinman, W.A., Singh, L., Singh, G., Rauffman, J.P., 1992. Isolation and characterization of exendin-4, an exendin-3 analogue, from *Heloderma suspectum* venom. Further evidence for an exendin receptor on dispersed acini from guinea pig pancreas. *J. Biol. Chem.* 267, 7402–7405.
- Ferreira, S.H., Rocha e Silva, M., 1965. Potentiation of bradykinin and eledoisin by BPF (bradykinin potentiating factor) from *Bothrops jararaca* venom. *Experientia* 21, 347–349. <http://dx.doi.org/10.1007/BF02144709>.
- Furman, B.L., 2012. The development of Byetta (exenatide) from the venom of the Gila monster as an anti-diabetic agent. *Toxicon* 59, 464–471. <http://dx.doi.org/10.1016/j.toxicon.2010.12.016>.
- Georgieva, D., Arni, R.K., Betzel, C., 2008. Proteome analysis of snake venom toxins: pharmacological insights. *Expert Rev. Proteom.* 5, 787–797. <http://dx.doi.org/10.1586/14789450.5.6.787>.
- Georgieva, D., Ohler, M., Seifert, J., von Bergen, M., Arni, R.K., Genov, N., Betzel, C., 2010. Snake venomomics of *Crotalus durissus terrificus*—correlation with pharmacological activities. *J. Proteome Res.* 9, 2302–2316. <http://dx.doi.org/10.1021/pr901042p>.
- Girón, M.E., Aguilar, I., Romero, L., Sánchez, E.E., Pérez, J.C., Rodríguez-Acosta, A.,



2005. A low-cost method to test cytotoxic effects of *Crotalus vegrandis* (Serpentes: Viperidae) venom on kidney cell cultures. *Rev. Inst. Med. Trop. Sao Paulo* 47, 147–152. <http://dx.doi.org/10.1590/S0036-46652005000300006>.
- Glenn, J.L., Straight, R.C., Wolt, T.B., 1994. Regional variation in the presence of canebrake toxin in *Crotalus horridus* venom, 107, 337–346.
- Gomes, C.L., Konno, K., Conceição, I.M., Ianzer, D., Amanouye, N., Prezoto, B.C., Assakura, M.T., Rádis-Baptista, G., Yamane, T., Santos, R.A., de Camargo, A.C.M., Hayashi, M.A.F., 2007. Identification of novel bradykinin-potentiating peptides (BPPs) in the venom gland of a rattlesnake allowed the evaluation of the structure-function relationship of BPPs. *Biochem. Pharmacol.* 74, 1350–1360. <http://dx.doi.org/10.1016/j.bcp.2007.07.014>.
- Gonçalves, J.M., Polson, A., 1947. The electrophoretic analysis of snake venoms. *Arch. Biochem.* 13, 253–259.
- Gonçalves, J.M., Vieira, L., 1950. Estudos sobre venenos de serpentes brasileiras. I. Análise eletroforética. *An. Acad. Bras. Cienc.* 22, 141–150.
- Graham, R.L.J., Graham, C., McClean, S., Chen, T., O'Rourke, M., Hirst, D., Theakston, D., Shaw, C., 2005. Identification and functional analysis of a novel bradykinin inhibitory peptide in the venoms of new world Crotalinae pit vipers. *Biochem. Biophys. Res. Commun.* 338, 1587–1592. <http://dx.doi.org/10.1016/j.bbrc.2005.10.130>.
- Guarnieri, M., Melo, E., Melo, K., Albuquerque-Modesto, J., Prieto-da-Silva, A., Rádis-Baptista, G., 2009. Cloning of a novel acidic phospholipase A2 from the venom gland of *Crotalus durissus* cascavella (Brazilian northeastern rattlesnake). *J. Venom. Anim. Toxins Incl. Trop. Dis.* 15, 745–761. <http://dx.doi.org/10.1590/S1678-91992009000400012>.
- Gubenssek, F., Turk, V., Drujon, B., 1978. Proteolytic and clotting enzymes in some Venezuelan snake venoms. In: *Proc. 2nd Symp. Eur. Sect. Int. Soc. Toxicol.*, 80, pp. 101–105.
- Gutiérrez, J.M., 2009. Snake bite envenomation in Central America. In: Mackessy, S.P. (Ed.), *Handbook of Venoms and Toxins of Reptiles*. CRC Press, Boca Raton, pp. 491–507.
- Gutiérrez, J.M., Lomonte, B., Sanz, L., Calvete, J.J., Pla, D., 2014. Immunological profile of antivenoms: preclinical analysis of the efficacy of a polyspecific antivenom through antivenomics and neutralization assays. *J. Proteom.* 105, 340–350. <http://dx.doi.org/10.1016/j.jprot.2014.02.021>.
- Hahn, B.S., Yang, K.Y., Park, E.M., Chang, I.M., Kim, Y.S., 1996. Purification and molecular cloning of calobin, a thrombin-like enzyme from *Agkistrodon caliginosus* (Korean viper). *J. Biochem.* 119, 835–843.
- Hart, M.L., Köhler, D., Eckle, T., Kloor, D., Stahl, G.L., Eltzschig, H.K., 2008. Direct treatment of mouse or human blood with soluble 5'-nucleotidase inhibits platelet aggregation. *Arterioscler. Thromb. Vasc. Biol.* 28, 1477–1483. <http://dx.doi.org/10.1161/ATVBAHA.108.169219>.
- Harvey, A.L., Stöcklin, R., 2012. From venoms to drugs: introduction. *Toxicon* 59, 433. <http://dx.doi.org/10.1016/j.toxicon.2011.09.016>.
- Huang, K.-F., Ko, T.-P., Hung, C.-C., Chu, J., Wang, A.H.-J., Chiou, S.-H., 2004. Crystal structure of a platelet-agglutinating factor isolated from the venom of Taiwan habu (*Trimeresurus mucrosquamatus*). *Biochem. J.* 378, 399–407. <http://dx.doi.org/10.1042/BJ20031507>.
- Irwin, D.M., 2012. Origin and convergent evolution of exendin genes. *Gen. Comp. Endocrinol.* 175, 27–33. <http://dx.doi.org/10.1016/j.ygcen.2011.11.025>.
- Jiao, H.-M., Yang, L.-X., Lu, B., Wu, Y.-Q., Zhou, Y.-C., 2005. Shedaonase, a novel fibrinogenase from the venom of *Agkistrodon shedaonensis* Zhao. *Acta Biochim. Biophys. Sin. Shanghai* 37, 835–842.
- Jones, P., Binns, D., Chang, H.-Y., Fraser, M., Li, W., McAnulla, C., McWilliam, H., Maslen, J., Mitchell, A., Nuka, G., Pesseat, S., Quinn, A.F., Sangrador-Vegas, A., Scheremetjew, M., Yong, S.-Y., Lopez, R., Hunter, S., 2014. InterProScan 5: genome-scale protein function classification. *Bioinformatics*. <http://dx.doi.org/10.1093/bioinformatics/btu031>.
- Kaiser, I.L., Aird, S.D., 1987. A crotaxin homolog from the venom of the Uracoan rattlesnake (*Crotalus vegrandis*). *Toxicon* 25, 1113–1120.
- Kerkis, I., Hayashi, M.A.F., Prieto da Silva, A.R.B., Pereira, A., De Sá Júnior, P.L., Zaharenko, A.J., Rádis-Baptista, G., Kerkis, A., Yamane, T., 2014. State of the art in the studies on crotamine, a cell penetrating peptide from South American rattlesnake. *Biomed. Res. Int.* 2014, 675985. <http://dx.doi.org/10.1155/2014/675985>.
- Klauber, L.M., 1997. *Rattlesnakes: Their Habits, Life Histories, and Influence on Mankind*, vol. 1. University of California Press.
- Kostiza, T., Meier, J., 1996. Nerve growth factors from snake venoms: chemical properties, mode of action and biological significance. *Toxicon* 34, 787–806.
- Laemmli, U.K., 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227, 680–685.
- Lee, T.K., Murthy, S.R.K., Cawley, N.X., Dhanvantari, S., Hewitt, S.M., Lou, H., Lau, T., Ma, S., Huynh, T., Wesley, R.A., Ng, I.O., Pacak, K., Poon, R.T., Loh, Y.P., 2011. An N-terminal truncated carboxypeptidase E splice isoform induces tumor growth and is a biomarker for predicting future metastasis in human cancers. *J. Clin. Invest.* 121, 880–892. <http://dx.doi.org/10.1172/JCI40433>.
- Ma, B., Zhang, K., Hendrie, C., Liang, C., Li, M., Doherty-Kirby, A., Lajoie, G., 2003. PEAKS: powerful software for peptide de novo sequencing by tandem mass spectrometry. *Rapid Commun. Mass Spectrom.* 17, 2337–2342. <http://dx.doi.org/10.1002/rcm.1196>.
- Mackessy, S.P., 2008. Venom composition in rattlesnakes: trends and biological significance. In: Hayes, W.K., Beaman, K.R., Cardwell, M.D., Bush, S.P. (Eds.), *The Biology of Rattlesnakes*.
- Mackessy, S.P., 2010. Evolutionary trends in venom composition in the western rattlesnakes (*Crotalus viridis* sensu lato): toxicity vs. tenderizers. *Toxicon* 55, 1463–1474. <http://dx.doi.org/10.1016/j.toxicon.2010.02.028>.
- Margres, M.J., McGivern, J.J., Wray, K.P., Seavy, M., Calvin, K., Rokyta, D.R., 2014. Linking the transcriptome and proteome to characterize the venom of the eastern diamondback rattlesnake (*Crotalus adamanteus*). *J. Proteom.* 96, 145–158. <http://dx.doi.org/10.1016/j.jprot.2013.11.001>.
- Markland, F.S., 1983. Rattlesnake venom enzymes that interact with components of the hemostatic system. *Toxin Rev.* 2, 119–160. <http://dx.doi.org/10.3109/1569548309012695>.
- Markland, F.S., Kettner, C., Schiffman, S., Shaw, E., Bajwa, S.S., Reddy, K.N., Kirakossian, H., Patkos, G.B., Theodor, I., Pirkle, H., 1982. Kallikrein-like activity of crotalase, a snake venom enzyme that clots fibrinogen. *Proc. Natl. Acad. Sci. U. S. A.* 79, 1688–1692.
- Markland, F.S., Swenson, S., 2013. Snake venom metalloproteinases. *Toxicon* 62, 3–18. <http://dx.doi.org/10.1016/j.toxicon.2012.09.004>.
- Massey, D.J., Calvete, J.J., Sánchez, E.E., Sanz, L., Richards, K., Curtis, R., Boesen, K., 2012. Venom variability and envenoming severity outcomes of the *Crotalus scutulatus* scutulatus (Mojave rattlesnake) from Southern Arizona. *J. Proteom.* 75, 2576–2587. <http://dx.doi.org/10.1016/j.jprot.2012.02.035>.
- Masuda, S., Araki, S., Yamamoto, T., Kaji, K., Hayashi, H., 1997. Purification of a vascular apoptosis-inducing factor from hemorrhagic snake venom. *Biochem. Biophys. Res. Commun.* 235, 59–63. <http://dx.doi.org/10.1006/bbrc.1997.6728>.
- McCleary, R.J.R., Kini, R.M., 2012. Non-enzymatic proteins from snake venoms: a gold mine of pharmacological tools and drug leads. *Toxicon* 1–19. <http://dx.doi.org/10.1016/j.toxicon.2012.09.008>.
- Melani, R.D., Araujo, G.D.T., Carvalho, P.C., Goto, L., Nogueira, F.C.S., Junqueira, M., Domont, G.B., 2015. Seeing beyond the tip of the iceberg: a deep analysis of the venom of the Brazilian Rattlesnake, *Crotalus durissus* terrificus. *EuPA Open Proteom.* <http://dx.doi.org/10.1016/j.euprot.2015.05.006>.
- Menin, L., Perchuc, A., Favreau, P., Perret, F., Michalet, S., Schöni, R., Wilmer, M., Stöcklin, R., 2008. High throughput screening of bradykinin-potentiating peptides in *Bothrops moojeni* snake venom using precursor ion mass spectrometry. *Toxicon* 51, 1288–1302. <http://dx.doi.org/10.1016/j.toxicon.2008.02.019>.
- Mitra, J., Bhattacharyya, D., 2014. Phosphodiesterase from *Daboia russelli* russelli venom: purification, partial characterization and inhibition of platelet aggregation. *Toxicon* 88C, 1–10. <http://dx.doi.org/10.1016/j.toxicon.2014.06.004>.
- Mochca-Morales, J., Martin, B.M., Possani, L.D., 1990. Isolation and characterization of helohermine, a novel toxin from *Heloderma horridum* horridum (Mexican bearded lizard) venom. *Toxicon* 28, 299–309.
- Munawar, A., Trusch, M., Georgieva, D., Hildebrand, D., Kwiatkowski, M., Behnken, H., Harder, S., Arni, R., Spencer, P., Schlüter, H., Betzel, C., 2014. Elapid snake venom analyses show the specificity of the peptide composition at the level of genera *Naja* and *Notechis*. *Toxins Basel* 6, 850–868. <http://dx.doi.org/10.3390/toxins6030850>.
- Murakami, M.T., Zela, S.P., Gava, L.M., Michelan-Duarte, S., Cintra, A.C.O., Arni, R.K., 2003. Crystal structure of the platelet activator convulxin, a disulfide-linked alpha4beta4 cyclic tetramer from the venom of *Crotalus durissus* terrificus. *Biochem. Biophys. Res. Commun.* 310, 478–482.
- Navarro, J., Caraballo, A., Sánchez, E., Rodríguez-Acosta, A., 2004. Epidemiological and clinical aspects of snakebite in Monagas state, Venezuela. *Rev. La Fac. Med.* 27, 106–110.
- Oguiura, N., Boni-Mitake, M., Affonso, R., Zhang, G., 2011. In vitro antibacterial and hemolytic activities of crotamine, a small basic myotoxin from rattlesnake *Crotalus durissus*. *J. Antibiot. Tokyo* 64, 327–331. <http://dx.doi.org/10.1038/ja.2011.10>.
- Oguiura, N., Boni-Mitake, M., Rádis-Baptista, G., 2005. New view on crotamine, a small basic polypeptide myotoxin from South American rattlesnake venom. *Toxicon* 46, 363–370. <http://dx.doi.org/10.1016/j.toxicon.2005.06.009>.
- Oguiura, N., Camargo, M.E., da Silva, A.R.P., Horton, D.S.P.Q., 2000. Quantification of crotamine, a small basic myotoxin, in South American rattlesnake (*Crotalus durissus* terrificus) venom by enzyme-linked immunosorbent assay with parallel-lines analysis. *Toxicon* 38, 443–448. [http://dx.doi.org/10.1016/S0041-0101\(99\)00157-9](http://dx.doi.org/10.1016/S0041-0101(99)00157-9).
- Peigneur, S., Orts, D.J.B., Prieto da Silva, A.R., Oguiura, N., Boni-Mitake, M., de Oliveira, E.B., Zaharenko, A.J., de Freitas, J.C., Tytgat, J., 2012. Crotamine pharmacology revisited: novel insights based on the inhibition of KV channels. *Mol. Pharmacol.* 82, 90–96. <http://dx.doi.org/10.1124/mol.112.078188>.
- Peng, L., Xu, X., Guo, M., Yan, X., Wang, S., Gao, S., Zhu, S., 2013. Effects of metal ions and disulfide bonds on the activity of phosphodiesterase from *Trimeresurus stejnegeri* venom. *Metallomics* 5, 920–927. <http://dx.doi.org/10.1039/c3mt00031a>.
- Peng, L., Xu, X., Shen, D., Zhang, Y., Song, J., Yan, X., Guo, M., 2011. Purification and partial characterization of a novel phosphodiesterase from the venom of *Trimeresurus stejnegeri*: inhibition of platelet aggregation. *Biochimie* 93, 1601–1609. <http://dx.doi.org/10.1016/j.biochi.2011.05.027>.
- Pérez, A.V., Rucavado, A., Sanz, L., Calvete, J.J., Gutiérrez, J.M., 2008. Isolation and characterization of a serine proteinase with thrombin-like activity from the venom of the snake *Bothrops asper*. *Braz. J. Med. Biol. Res.* 41, 12–17.
- Petersen, T.N., Brunak, S., von Heijne, G., Nielsen, H., 2011. SignalP 4.0: discriminating signal peptides from transmembrane regions. *Nat. Methods* 8, 785–786. <http://dx.doi.org/10.1038/nmeth.1701>.
- Pifano, F., Rodríguez Acosta, A., 1996. Ecological niche and redescription of *Crotalus vegrandis* (Serpentes: Crotalidae) in Venezuela. *Brenesia* 169–175.
- Pifano, F., Rodríguez Acosta, A., 1995. Sobre el emponzoñamiento producido por la cascabel de uracoa (*Crotalus vegrandis* Klauber 1941), serpiente neotropical. *Rev. Fac. Med.* 18, 194–196.

- Pimenta, D.C., Lebrun, I., 2007. Cryptides: buried secrets in proteins. *Peptides* 28, 2403–2410. <http://dx.doi.org/10.1016/j.peptides.2007.10.005>.
- Polgár, J., Clemetson, J.M., Kehrel, B.E., Wiedemann, M., Magnenat, E.M., Wells, T.N., Clemetson, K.J., 1997. Platelet activation and signal transduction by convulxin, a C-type lectin from *Crotalus durissus terrificus* (tropical rattlesnake) venom via the p62/GPVI collagen receptor. *J. Biol. Chem.* 272, 13576–13583.
- Pulido-Mendez, M., Rodríguez-Acosta, A., Finol, H.J., Aguilar, I., Girón, M.E., 1999. Ultrastructural pathology in skeletal muscle of mice envenomed with *Crotalus vegrandis* venom. *J. Submicrosc. Cytol. Pathol.* 31, 555–561.
- Rioli, V., Prezoto, B.C., Konno, K., Melo, R.L., Klitzke, C.F., Ferro, E.S., Ferreira-Lopes, M., Camargo, A.C.M., Portaro, F.C.V., 2008. A novel bradykinin potentiating peptide isolated from *Bothrops jararacussu* venom using catalytically inactive oligopeptidase EP24.15. *FEBS J.* 275, 2442–2454. <http://dx.doi.org/10.1111/j.1742-4658.2008.06389.x>.
- Rodríguez-Acosta, A., Aguilar, I., Giron, M., Rodríguez-Pulido, V., 1998. Haemorrhagic activity of neotropical rattlesnake (*Crotalus vegrandis* Klauber, 1941) venom. *Nat. Toxins* 6, 15–18.
- Rodríguez-Acosta, A., Mondolfi, A., Orihuela, A., Aguilar, M., 1995. Qué hacer frente a un accidente ofídico. *Venediciones*, Caracas.
- Rokytka, D.R., Wray, K.P., Margres, M.J., 2013. The genesis of an exceptionally lethal venom in the timber rattlesnake (*Crotalus horridus*) revealed through comparative venom-gland transcriptomics. *BMC Genom.* 14, 394. <http://dx.doi.org/10.1186/1471-2164-14-394>.
- Romero-Vargas, F.F., Ponce-Soto, L.A., Martins-de-Souza, D., Marangoni, S., 2010. Biological and biochemical characterization of two new PLA2 isoforms Cdc-9 and Cdc-10 from *Crotalus durissus cumanensis* snake venom. *Comp. Biochem. Physiol. C. Toxicol. Pharmacol.* 151, 66–74. <http://dx.doi.org/10.1016/j.cbpc.2009.08.011>.
- Santoro, M.L., Vaquero, T.S., Paes Leme, A.F., Serrano, S.M.T., 2009. NPP-BJ, a nucleotide pyrophosphatase/phosphodiesterase from *Bothrops jararaca* snake venom, inhibits platelet aggregation. *Toxicon* 54, 499–512. <http://dx.doi.org/10.1016/j.toxicon.2009.05.016>.
- Saviola, A.J., Pla, D., Sanz, L., Castoe, T.A., Calvete, J.J., Mackessy, S.P., 2015. Comparative venomomics of the Prairie Rattlesnake (*Crotalus viridis viridis*) from Colorado: Identification of a novel pattern of ontogenetic changes in venom composition and assessment of the immunoreactivity of the commercial antivenom CroFab®. *J. Proteom.* 121, 28–43. <http://dx.doi.org/10.1016/j.jprot.2015.03.015>.
- Scannone, H., Rodríguez, O.G., Lancini, A.R., 1978. Enzymatic activities and other characteristics of *Crotalus vegrandis* snake venom. In: Rosenberg, P. (Ed.), *Toxins: Animal, Plant and Microbial*. International Symposium on Animal, Plant and Microbial Toxins. Pergamon Press, San José, Costa Rica.
- Schweitz, H., Vigne, P., Moinier, D., Frelin, C., Lazdunski, M., 1992. A new member of the natriuretic peptide family is present in the venom of the green mamba (*Dendroaspis angusticeps*). *J. Biol. Chem.* 267, 13928–13932.
- Serrano, S.M.T., 2013. The long road of research on snake venom serine proteinases. *Toxicon* 62, 19–26. <http://dx.doi.org/10.1016/j.toxicon.2012.09.003>.
- Slotta, K.H., Fraenkel-Conrat, H., 1938. Crotoxin. *Nature* 144, 290–291.
- Sunagar, K., Undheim, E.A.B., Scheib, H., Gren, E.C.K., Cochran, C., Person, C.E., Koludarov, I., Kelln, W., Hayes, W.K., King, G.F., Antunes, A., Fry, B.G., 2014. Intraspecific venom variation in the medically significant Southern Pacific Rattlesnake (*Crotalus oreganus helleri*): biodiscovery, clinical and evolutionary implications. *J. Proteom.* 99, 68–83. <http://dx.doi.org/10.1016/j.jprot.2014.01.013>.
- Tavares, N.A.C., Correia, J.M., Guarnieri, M.C., Lima-Filho, J.L., Prieto-da-Silva, A.R.B., Rádis-Baptista, G., 2008. Expression of mRNAs coding for VAP1/crotastatin-like metalloproteases in the venom glands of three South American pit vipers assessed by quantitative real-time PCR. *Toxicon* 52, 897–907. <http://dx.doi.org/10.1016/j.toxicon.2008.08.022>.
- Towbin, H., Staehelin, T., Gordon, J., 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc. Natl. Acad. Sci. U. S. A.* 76, 4350–4354.
- Toyama, M.H., Toyama, D., de, O., Passero, L.F.D., Laurenti, M.D., Corbett, C.E., Tomokane, T.Y., Fonseca, F.V., Antunes, E., Joazeiro, P.P., Beriam, L.O.S., Martins, M.A.C., Monteiro, H.S.A., Fonteles, M.C., 2006. Isolation of a new L-amino acid oxidase from *Crotalus durissus cascavella* venom. *Toxicon* 47, 47–57. <http://dx.doi.org/10.1016/j.toxicon.2005.09.008>.
- Vargas, L.J., Quintana, J.C., Pereañez, J.A., Núñez, V., Sanz, L., Calvete, J., 2013. Cloning and characterization of an antibacterial L-amino acid oxidase from *Crotalus durissus cumanensis* venom. *Toxicon* 64, 1–11. <http://dx.doi.org/10.1016/j.toxicon.2012.11.027>.
- Viala, V.L., Hildebrand, D., Trusch, M., Arni, R.K., Pimenta, D.C., Schlüter, H., Betzel, C., Spencer, P.J., 2014. Pseudechis guttatus venom proteome: insights into evolution and toxin clustering. *J. Proteom.* 110C, 32–44. <http://dx.doi.org/10.1016/j.jprot.2014.07.030>.
- Wang, Y.-L., Kuo, J.-H., Lee, S.-C., Liu, J.-S., Hsieh, Y.-C., Shih, Y.-T., Chen, C.-J., Chiu, J.-J., Wu, W.-G., 2010. Cobra CRISP functions as an inflammatory modulator via a novel Zn<sup>2+</sup>- and heparan sulfate-dependent transcriptional regulation of endothelial cell adhesion molecules. *J. Biol. Chem.* 285, 37872–37883. <http://dx.doi.org/10.1074/jbc.M110.146290>.
- Yamane, E.S., Bizerra, F.C., Oliveira, E.B., Moreira, J.T., Rajabi, M., Nunes, G.L.C., de Souza, A.O., da Silva, I.D.C.G., Yamane, T., Karpel, R.L., Silva, P.I., Hayashi, M.A.F., 2013. Unraveling the antifungal activity of a South American rattlesnake toxin crotamine. *Biochimie* 95, 231–240. <http://dx.doi.org/10.1016/j.biochi.2012.09.019>.
- Yamazaki, Y., Hyodo, F., Morita, T., 2003. Wide distribution of cysteine-rich secretory proteins in snake venoms: isolation and cloning of novel snake venom cysteine-rich secretory proteins. *Arch. Biochem. Biophys.* 412, 133–141. [http://dx.doi.org/10.1016/S0003-9861\(03\)00028-6](http://dx.doi.org/10.1016/S0003-9861(03)00028-6).
- Yamazaki, Y., Morita, T., 2004. Structure and function of snake venom cysteine-rich secretory proteins. *Toxicon* 44, 227–231. <http://dx.doi.org/10.1016/j.toxicon.2004.05.023>.
- Yonamine, C.M., Prieto-da-Silva, A.R.B., Magalhães, G.S., Rádis-Baptista, G., Morganti, L., Ambiel, F.C., Chura-Chambi, R.M., Yamane, T., Camillo, M.A.P., 2009. Cloning of serine protease cDNAs from *Crotalus durissus terrificus* venom gland and expression of a functional Gyroxin homologue in COS-7 cells. *Toxicon* 54, 110–120. <http://dx.doi.org/10.1016/j.toxicon.2009.03.022>.
- Yoshida-Kanashiro, E., Navarrete, L.F., Rodríguez-Acosta, A., 2003. On the unusual hemorrhagic and necrotic activities caused by the rattlesnake (*Crotalus durissus cumanensis*) in a Venezuelan patient. *Rev. Cuba. Med. Trop.* 55, 38–40.