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

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

[a] Centro de Biotecnologia, IPEN, Av. Lineu Prestes 2242, CEP 05508-000, São Paulo, SP, Brazil
[b] Institut für Klinische Chemie, Universitätsklinikum Hamburg-Eppendorf, Haus Ost 26, Martinistr. 52, 20246, Hamburg, Germany
[c] Laboratório de Bioquímica e Biofísica, Instituto Butantan, Av. Vital Brasil, 1500, 05503-900, São Paulo, SP, Brazil
[d] Institut für Organische Chemie, Massenspektrometrie, Universität Hamburg, Martin-Luther-King-Platz 6, 20146, Hamburg, Germany
[e] Laboratório Especial de Ecologia e Evolução, Instituto Butantan, Av. Dr. Vital Brasil 1500, CEP 05503-900, São Paulo, SP, Brazil
[f] Institut für Biochemie und Molekularbiologie, Universität Hamburg, Martin-Luther-King-Platz 6, 20146, Hamburg, Germany
[g] Departamento de Física, IBILCE, UNESP, Rua Cristóvão Colombo, 2265, CEP 15054-000, São José do Rio Preto, SP, Brazil

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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 it venom: 2D SDS-PAGE and gel-filtration chromatography for protein mixture decomplexation; LC-MS^2 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.

1. Introduction

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

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_2, snake venom phospholipase A_2; 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 (P.J. Spencer).

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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_2 (PLA_2), 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 “Cascabel 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_{50} of 0.2 μg/g (i.v.) (Scannone et al., 1978) and 0.5 μg/g (i.m.) (Rodriguez-Acosta et al., 1998) in mice and that the hemorrhagic and neurotoxic activities (Gubenšek et al., 1978; Rodriguez-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 crotamine-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 formiate (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_2O 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 logP 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. Crotamine-like proteins detection using western blot immunoassays

A sample of *C. vegrandis* venom (33.3 μg/lane) was submitted to 15% SDS-PAGE under non-reducing conditions (Laemmli, 1970). The *C. d. terrificus* venom (33.3 μg/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^2. The membrane was blocked with 3% skim milk in pH 7.4 phosphate buffered saline and incubated with rabbit polyclonal antibodies against whole crotamine (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_2O_2 (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® (QIAGEN N.V., Netherlands) at −80 °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® 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′- TGCACCTCACCCTAAAACAGG -3′). A 20 μL 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 $MgCl_2$, and 0.2 mM dNTPs mix. The amplification process used an initial denaturation step of 4 min at 94 °C, followed by 30 cycles of 45 s at 94 °C, 45 s at 60 °C, 45 s at 72 °C, and, finally 1 min at 72 °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* DH5α (Ausubel et al., 2000), clones were purified using Zyppy 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′-GTAAAACGACGGCCAGT-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_2, 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. Crotamine-like immunodetection

The Western blot cross-species assay performed with an anti-crotamine 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 Crotalinae 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 Uracoan rattlesnake envenomation) and neurotoxic (causing death by respiratory paralysis) (Girón et al., 2005; Gubenšek et al., 1978; Rodriguez-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 crotamine-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

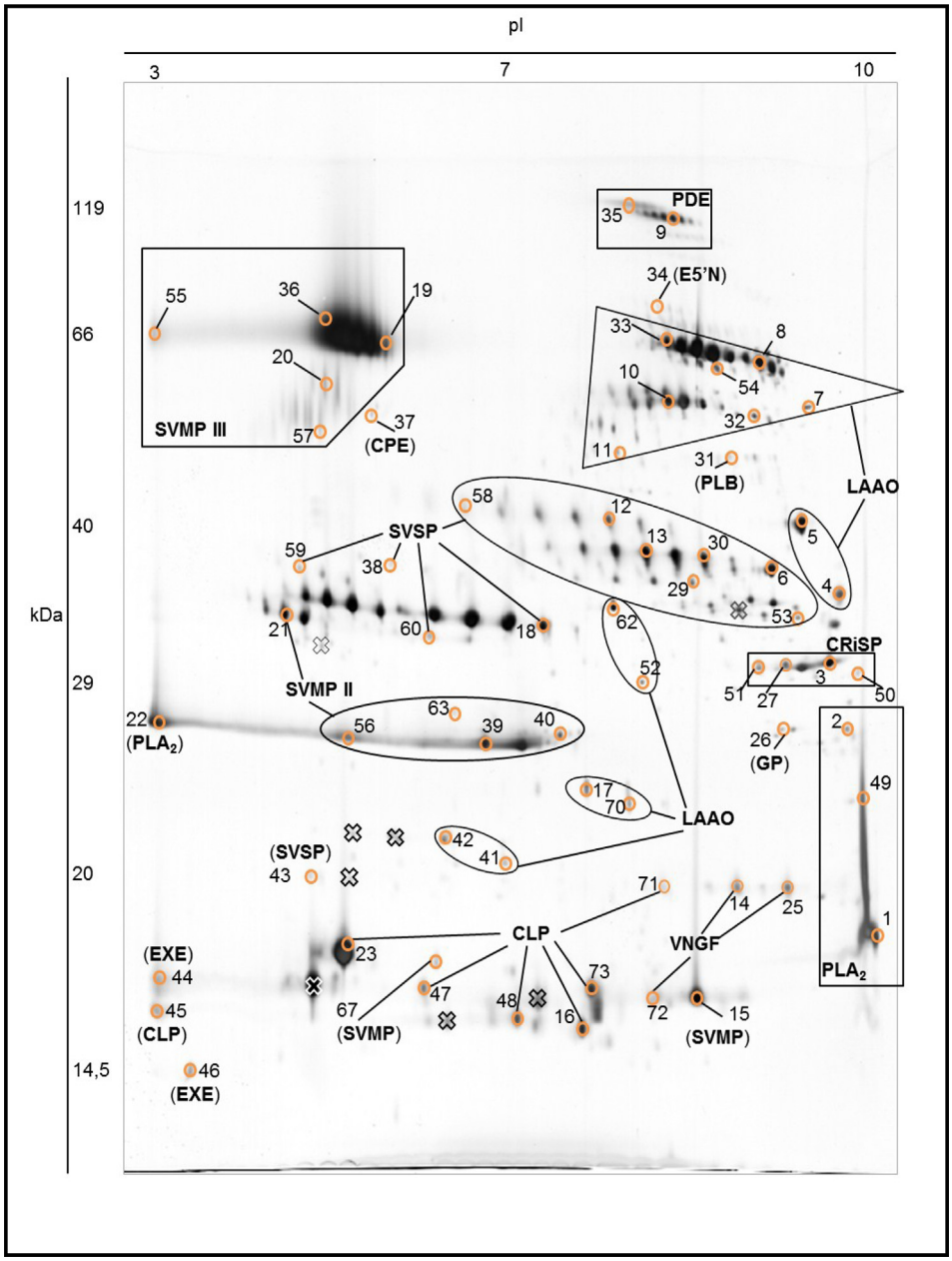

Fig. 1. 2D-PAGE of *Crotalus vegrandis* venom. Identified clusters of spots and individual spots are labeled. Circles represent identified spots and crosses represent collected spots with no significant result. pI: isoelectric point; kDa: molecular weight; SVMP III: PIII snake venom metalloproteinase; 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_2: snake venom phospholipase A_2; 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 ssp.* 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_2

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 (crotapotin) acting as a chaperone, delivering the basic PLA_2 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_2	98.8	Basic phospholipase A2 A	PA2BA_CRODR	*Crotalus durissus ruruima*	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
						X.WDLYPY.X	856.35	1
						R.KNAIPFYAF	535.84	2
2	PLA_2	83.22	Phospholipase A2 crotoxin basic subunit CBc	PA2BC_CRODU	*Crotalus durissus terrificus*	K.YGYMFYPDSR.C	649.82	2
						K.WDIYPYSLK.S	592.85	2
3	SVSP	98.86	Kallikrein-CohLL-4	T1DEH3_CROOH	*Crotalus oreganus helleri*	R.FLVALYTFR.S	565.31	2
						R.IMGWGTISATK.E	582.82	2
						R.AAHGGLPATSR.T	519.3	2
						K.WDKDIMLIR.L	595.39	2
3	CRiSP	84.14	Cysteine-rich secereotory protein Ch-CRPKa (Fragment)	F2Q6E5_CROHD	*Crotalus horridus*	R.SVNPTASNMLK.M	581.3	2
						SVDFDSESPR.K	569.75	2
4	LAAO	99.18	L-amino acid oxidase (Fragment)	K9N7B7_CRODM	*Crotalus durissus cumanensis*	K.YILDKYDTYSTK.E	755.42	2
						K.SAAQLYVESLR.K	618.84	2
						K.NNPGILEYPVKPSEEGK.S	935.97	2
						R.VIEIQQNDR.E	557.83	2
						K.HDDIFGYEK.R	562.26	2
						K.DWYANLGPMR.L	611.84	2
						K.VQVHFNAR.V	485.86	2
						R.VIEIQQNDRETK.V	491.63	3
						K.SAAQLYVESLRK.V	682.93	2
						K.RFDEIVGGM(+15.99)DQLPTSM(+15.99)YEAIK.E	811.37	3
4	SVSP	61.97	Kallikrein-CohLL-4	T1DEH3_CROOH	*Crotalus oreganus helleri*	R.FLVALYTFR.S	565.33	2
5	LAAO	99.19	L-amino acid oxidase (Fragment)	K9N7B7_CRODM	*Crotalus durissus cumanensis*	K.SAAQLYVESLR.K	618.82	2
						K.NNPGILEYPVKPSEEGK.S	935.96	2
						R.ETDYEEFLEIAR.N	757.84	2
						K.YILDKYDTYSTK.E	755.42	2
						R.NGLTVTSNPK.H	515.77	2
						K.HDDIFGYEK.R	562.25	2
						R.VIEIQQNDR.E	557.78	2
						R.VIEIQQNDRETK.V	736.88	2
						K.DWYANLGPMR.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.HDDIFGYEKR.F	427.25	3
						K.YDTYSTK.E	439.24	2
						K.FEPPLPPKK.A	351.59	3
						K.FEPPLPPK.K	462.75	2
						R.ETDYEEFLEIAK.N	743.88	2
						R.IQFEPPLPPK.K	583.35	2
6	LAAO	99.18	L-amino acid oxidase (Fragment)	K9N7B7_CRODM	*Crotalus durissus cumanensis*	K.HVVIVGAGMAGLSAAYVLAGAGHQVTVLEASER.V	1078.56	3
						R.FDEIVGGMDQLPTSMYEAIK.E	1122.55	2
						K.NNPGILEYPVKPSEEGK.S	935.96	2
						K.EGNLSPGAVDMIGDLLNEDSGYYVSFIESLK.H	1111.51	3
						R.NGLTVTSNPK.H	515.77	2
						K.YILDKYDTYSTK.E	755.44	2
						K.SAAQLYVESLR.K	618.87	2
						R.ETDYEEFLEIAR.N	757.85	2
						K.FGLQLNEFFQENENAWYFIK.N	1269.08	2
						K.HDDIFGYEK.R	562.26	2
						R.VIEIQQNDR.E	557.78	2
						K.KDWYANLGPMR.L	675.83	2
						K.DWYANLGPMR.L	611.81	2
						R.VIEIQQNDRETK.V	736.89	2
						R.KKDWYANLGPMR.L	739.94	2
						K.VQVHFNAR.V	485.79	2
						K.DWYANLGPM(+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.FEPPLPPKK.A	526.9	2
						R.RIKFEPPLPPK.K	441.27	3
						R.IQFEPPLPPK.K	583.43	2
						R.IQFEPPLPPKK.Q	431.94	3
						R.RIQFEPPLPPK.K	441.27	3
						K.RFDEIVGGMDQLPTCM(+15.99)CRAIEEK.V	886.38	3
7	LAAO	99.16	L-amino acid oxidase (Fragment)	K9N7B7_CRODM	*Crotalus durissus cumanensis*	K.NNPGILEYPVKPSEEGK.S	936	2
						K.SAAQLYVESLR.K	618.87	2
						R.NGLTVTSNPK.H	515.81	2
						R.ETDYEEFLEIAR.N	757.87	2
						K.YILDKYDTYSTK.E	755.42	2
						R.VIEIQQNDR.E	557.83	2
						K.HVVIVGAGMAGLSAAYVLAGAGHQVTVLEASER.V	809.16	4
						K.SAAQLYVESLRK.V	682.96	2
						R.VIEIQQNDRETK.V	491.63	3
						R.KKDWYANLGPMR.L	493.63	3
7	PLA_2	84.12	Phospholipase A2 crotoxin basic subunit CBc	PA2BC_CRODU	*Crotalus durissus terrificus*	K.YGYMFYPDSR.C	649.81	2
						K.WDIYPYSLK.S	592.85	2
7	SVSP	61.3	Snake venom serine protease homolog (Fragment)	VSPH_CROAT	*Crotalus atrox*	R.FLVALYTFR.S	565.36	2
8	LAAO	99.18	L-amino acid oxidase (Fragment)	K9N7B7_CRODM	*Crotalus durissus cumanensis*	K.SAAQLYVESLR.K	618.87	2
						K.NNPGILEYPVKPSEEGK.S	935.98	2
						K.YILDKYDTYSTK.E	755.42	2
						R.VIEIQQNDR.E	557.81	2
						R.ETDYEEFLEIAR.N	757.83	2
						K.HVVIVGAGMAGLSAAYVLAGAGHQVTVLEASER.V	1078.56	3
						K.HDDIFGYEK.R	562.26	2
						K.DWYANLGPM(+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
						R.VIEIQQNDRETK.V	736.9	2
						K.VQVHFNAR.V	485.8	2
						K.RFDEIVGGMDQLPTSMYEAIK.E	800.72	3
						K.YDTYSTK.E	439.21	2
						R.FDEIVGGM(+15.99)DQLPTSM(+15.99)YEAIKEK.V	845.08	3
						K.STTDLPSR.F	438.72	2
						K.IFLTCSK.K	406.22	2
9	PDE	99.18	Venom phosphodiesterase 2	PDE2_CROAD	*Crotalus adamanteus*	K.DFYTFDSEGIVK.N	710.84	2
						K.TFLPIFVNPVN	630.92	2
						K.YGPVSGEIIK.A	531.82	2
						K.NPFYTPSPAK.E	561.25	2
						R.VRDVELLTGLNFYSGLK.Q	642.05	3
						R.AGYLENWDSLM(+15.99)PNINK.L	940.93	2
						R.LWNYFHTTLIPK.Y	511.61	3
						K.QPLPETLQLK.T	583.87	2
						K.SMQAIFLAHGPGFNEK.N	582.99	3
						K.AATYFWPGSEVK.I	678.39	2
						R.TLGMLMEGLK.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	LAAO	99.18	L-amino acid oxidase (Fragment)	K9N7B7_CRODM	*Crotalus durissus cumanensis*	K.SAAQLYVESLR.K	618.88	2
						R.ETDYEEFLEIAR.N	757.85	2
						K.NNPGILEYPVKPSEEGK.S	935.98	2
						R.NGLTVTSNPK.H	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)YEAIK.E	811.4	3
						K.STTDLPSR.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 crotastatin (Fragment)	VM31_CRODC	*Crotalus durissus cascavella*	R.MYDIVNVITPIYHR.M	578.7	3
						K.LFLVADYIM(+15.99)YLK.Y	752.91	2
						R.ATDLLSR.K	388.24	2
						K.DHQEFLIK.N	515.25	2
11	LAAO	98.87	L-amino acid oxidase (Fragment)	K9N7B7_CRODM	*Crotalus durissus cumanensis*	K.YILDKYDTYSTK.E	755.37	2
						K.FWEDDGIR.G	519.28	2
						R.KFWEDDGIR.G	583.3	2
						R.VIEIQQNDR.E	557.83	2
						K.HDDIFGYEK.R	562.3	2
						R.VIEIQQNDRETK.V	736.93	2
						K.STTDLPSR.F	438.77	2
12	SVSP	98.38	Snake venom serine proteinase 2	VSP2_CROAD	*Crotalus adamanteus*	R.AAYPEYGLPATSR.T	698.35	2
						K.IHLGVHSK.K	445.83	2
						K.WNKDIMLIR.L	594.93	2
						K.DIMLIR.L	380.76	2
13	SVSP	84.94	Snake venom serine protease catroxase-1	VSP1_CROAT	*Crotalus atrox*	R.AAYPEYGLPATSR.T	698.35	2
						K.WNKDIMLIR.L	594.95	2
						K.IHLGVHSK.K	445.79	2
						K.DIMLIR.L	380.75	2
14	VNGF	82.01	Venom nerve growth factor	NGFV_CRODU	*Crotalus durissus terrificus*	K.ALTM(+15.99)EGNQASWR.F	690.37	2
						K.TTATDIR.G	389.24	2
15	SVMP	61.63	SVMP-CohPH-3	T1E6U1_CROOH	*Crotalus oreganus helleri*	R.M(+15.99)YELANTVNDIYR.Y	809.43	2
16	CLP	61.41	Snaclec crotocetin	SL_CRODU	*Crotalus durissus terrificus*	**K.QDMTWEDAEK.F**	626.84	2
17	LAAO	98.45	L-amino acid oxidase (Fragment)	K9N7B7_CRODM	*Crotalus durissus cumanensis*	K.FWEDDGIR.G	519.25	2
						R.KFWEDDGIR.G	583.33	2
						K.SGLTAAR.D	338.21	2
						K.FWEDDGIRGGK.S	427.23	3
						R.KFWEDDGIRGGK.S	469.94	3
						K.STTDLPSR.F	438.74	2
18	SVSP	98.87	Snake Venom Serine Protease clone 1	*SVSP_clone 1*	*Crotalus vegrandis*	K.NFQMQLGVHSK.K	644.86	2
						K.KVLNEDEQTR.D	616.34	2
						K.VLNEDEQTR.D	552.27	2
						K.VLNEDEQTRDPK.E	481.92	3
						K.DDEKDKDIMLIR.L	745.9	2
						K.DKDIMLIR.L	502.33	2
						R.IMGWGTISPTK.E	595.81	2
19	SVMP	98.88	SVMP-CohPH-3	T1E6U1_CROOH	*Crotalus oreganus helleri*	R.MYELANTVNDIYR.Y	801.37	2
						K.HDNAQLLTAIDLDR.V	797.96	2
						K.MFYSNEDEHKGMVLPGTK.C	695.04	3
						R.FVELVLVVDK.A	580.87	2
						R.KKHDNAQLLTAIDLDR.V	617.71	3
						K.MFYSNEDEHK.G	650.27	2
						K.KHDNAQLLTAIDLDR.V	575.03	3
						R.KTDLLTR.K	423.77	2
						K.GMVLPGTK.C	802.43	1
						K.TDLLTR.K	359.74	2
20	SVMP	98.81	SVMP-CohPH-3	T1E6U1_CROOH	*Crotalus oreganus helleri*	R.M(+15.99)YELANTVNDIYR.Y	809.39	2
						R.FVELVLVVDK.A	580.87	2
						R.KKHDNAQLLTAIDLDR.V	617.72	3
						R.KTDLLTR.K	423.75	2
21	SVMP	98.66	Metalloproteinase P-II	Q2QA03_CRODD	*Crotalus durissus durissus*	**R.VAVTMTHELGHNLGIR.H**	874.45	2
						R.YVELFIVVDHGMFTK.Y	599.98	3
						R.VHQMVNIMK.E	550.35	2
						K.YNGDSDKIR.Q	534.28	2
						R.FVELVLVVDK.A	580.85	2
22	PLA_2	77.26	Acidic PLA2	C9E7C4_CRODC	*Crotalus durissus cascavella*	**K.MVSYTYSVK.N**	539.27	2
						R.DNIPSYDK.K	951.48	1
						R.DNIPSYDKK.Y	540.26	2
23	CLP	52.54	Snaclec coagulation factor IX-binding protein subunit A	SLA_PROFL	*Protobothrops flavoviridis*	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	*Crotalus durissus terrificus*	R.ALTMEGNQASWR.F	682.36	2
						QYFM(+15.99)ETK.M	481.89	2
26	GP	98.35	Glutathione peroxidase (Fragment)	V8P395_OPHHA	*Ophiophagus hannah*	K.QEPGQNSEILQGIK.H	770.97	2
						K.FLVNPQGKPVMR.W	693.46	2
						K.HVRPGGGFVPNFQLFQK.G	643.42	3
						K.QEPGQNSEILQGIK.H	770.97	2
						K.HVRPGGGFVPNFQLFQK.I	643.42	3
27	CRiSP	98.38	Cysteine-rich secereto ry protein Ch-CRPKa (Fragment)	F2Q6E5_CROHD	*Crotalus horridus*	K.MEWYPEAAANAER.W	769.41	2
						SVDFDSESPR.K	569.74	2
						R.SVNPTASNMLK.M	581.48	2
						K.IVDLHNSLR.R	533.94	2
27	LAAO	95.82	L-amino acid oxidase (Fragment)	K9N7B7_CRODM	*Crotalus durissus cumanensis*	K.FWEDDGIR.G	519.27	2
						K.STTDLPSR.F	438.81	2
						K.SGLTAAR.D	338.26	2
27	SVSP	95.27	Serine proteinase 5	T1DGZ7_CROHD	*Crotalus horridus*	R.IMGWGTISATK.E	583.02	2
						R.AAHGGLPATSR.T	519.27	2
						K.WDKDIMLIR.L	595.41	2
29	SVSP	99.08	Serine proteinase 6	T1D6M5_CROHD	*Crotalus horridus*	K.TNNEWEKDIMLIR.L	831.44	2
						R.RLNPGFYTK.V	548.37	2
						R.AAYPWWPVTTR.I	674.38	2
						K.NFQIQLGVHSK.K	635.96	2
						K.EIYPNVPR.C	494.33	2
						R.IMGWGTISSTK.E	590.93	2
						R.IMGWGTISTSK.V	590.87	2
						R.AAYPEYGLPATSR.T	698.43	2
30	SVSP	98.36	Serine proteinase 8 (Fragment)	F8S120_CROAD	*Crotalus adamanteus*	R.AAYPEYGLPATSR.T	698.4	2
						K.IHLGVHSK.K	445.86	2
						K.WNKDIMLIR.L	594.94	2
						K.DIM(+15.99)LIR.L	388.76	2
31	PLB	99.11	Phospholipase B	PLB_CROAD	*Crotalus adamanteus*	R.HGLEFSYEMAPR.A	718.9	2
						R.SLEDGTLYIIEQVPK.L	852.94	2
						K.FTAYAINGPPVEK.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.QNSGTYNNQYMILDTK.K	945.44	2
						K.VADISM(+15.99)AAK.F	461.29	2
32	LAAO	99.08	L-amino acid oxidase (Fragment)	K9N7B7_CRODM	*Crotalus durissus cumanensis*	K.SAAQLYVESLR.K	618.96	2
						R.VIEIQQNDR.E	557.86	2
						K.YILDKYDTYSTK.E	755.36	2
						K.SAAQLYVESLRK.V	682.93	2
						R.VIEIQQNDRETK.V	736.91	2
						K.FWEDDGIR.G	519.23	2
						K.NNPGILEYPVKPSEEGK.S	624.39	3
						K.STTDLPSR.F	438.76	2
33	LAAO	99.17	L-amino acid oxidase (Fragment)	K9N7B7_CRODM	*Crotalus durissus cumanensis*	K.NNPGILEYPVKPSEEGK.S	935.99	2
						K.SAAQLYVESLR.K	618.89	2
						R.ETDYEEFLEIAR.N	757.87	2
						K.YILDKYDTYSTK.E	755.43	2
						R.NGLTVTSNPK.H	515.88	2
						K.HDDIFGYEK.R	562.31	2
						K.DWYANLGPMR.L	611.95	2
						K.SAAQLYVESLRK.V	455.64	3
						R.VIEIQQNDRETK.V	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)DQLPTSMYEAIK.E	806.04	3
						K.STTDLPSR.F	438.76	2
						K.SGLTAAR.D	338.4	2
						K.EKVQVHFNAR.V	410.03	3
						R.IQFEPPLPPK.K	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	*Crotalus adamanteus*	K.IIALGHSGFSEDQR.I	510.63	3
						K.IINVGSEK.V	430.4	2
						R.QVPVVQAYAFGK.Y	654	2
						K.VVYDLSR.K	426.27	2
						K.VFPAVEGR.M	437.97	2
						R.VPTYVPLEK.E	523.33	2
						K.YLGYLNVIFDDKGNVIK.S	657.69	3
						SFELTILHTNDVHAR.V	585.1	3
35	PDE	98.53	Venom phosphodiesterase 2	PDE2_CROAD	*Crotalus adamanteus*	K.YGPVSGEIIK.A	531.84	2
						K.TFLPIFVNPVN	630.88	2
						R.TLGMLMEGLK.Q	546.84	2
						K.AATYFWPGSEVK.I	678.42	2
						K.GGTHGYDNEFK.S	612.82	2
						R.IQIHTAR.V	419.91	2
36	SVMP	92.28	SVMP-CohPH-3	T1E6U1_CROOH	*Crotalus oreganus helleri*	R.MYELANTVNDIYR.Y	801.37	2
						K.MFYSNEDEHKGMVLPGTK.C	695.07	3
						K.MFYSNEDEHK.G	650.34	2
						R.KTDLLTR.K	423.93	2
						K.GM(+15.99)VLPGTK.C	409.87	2
						K.NNDDLDKIK.T	537.83	2
37	CPE	98.98	Carboxypeptidase E-like	J3RYP4_CROAD	*Crotalus adamanteus*	K.VTASASGYLAITK.K	641.4	2
						R.SNAQGIDLNR.N	544.35	2
						K.YVGNM(+15.99)HGNEAVGR.E	473.91	3
						K.VAVPFSPAIR.V	529.06	2
						R.IVYVNER.E	446.94	2
						R.EGGPNNHLLK.N	539.85	2
						K.VTASAPGYLAITK.K	646.44	2
38	SVSP	76.64	Serine proteinase 7	T1E3B5_CROHD	*Crotalus horridus*	K.KVLNEDEQTR.D	616.37	2
						K.VLNEDEQTR.D	552.3	2
						K.VLNEDEQTRDPK.E	481.96	3
						K.NFQM(+15.99)QLGVHSK.K	652.88	2
39	SVMP	98.82	Metalloproteinase P-II	Q2QA03_CRODD	*Crotalus durissus durissus*	**R.VAVTMTHELGHNLGIR.H**	583.42	3
						R.YVELFIVVDHGM(+15.99)FTK.Y	605.36	3
						R.VHQMVNIM(+15.99)K.E	558.39	2
						K.YNGDSDKIR.Q	534.31	2
						R.ETDLLQR.R	437.83	2
40	SVMP	98.16	Metalloproteinase P-II	Q2QA03_CRODD	*Crotalus durissus durissus*	**R.VAVTM(+15.99)THELGHNLGIR.H**	588.68	3
						R.VHQMVNIM(+15.99)K.E	558.41	2
						K.YNGDSDKIR.Q	534.3	2
41	LAAO	97.94	L-amino acid oxidase (Fragment)	K9N7B7_CRODM	*Crotalus durissus cumanensis*	K.FWEDDGIR.G	519.41	2
						R.KFWEDDGIR.G	583.34	2
						K.SGLTAAR.D	338.35	2
						K.STTDLPSR.F	438.84	2
42	LAAO	74.61	L-amino acid oxidase (Fragment)	K9N7B7_CRODM	*Crotalus durissus cumanensis*	K.FWEDDGIR.G	519.29	2
						K.STTDLPSR.F	438.87	2
43	SVSP	61.3	Snake venom serine protease catroxase-2	VSP2_CROAT	*Crotalus atrox*	K.KVPNEDEQTR.V	608.35	2
44	EXE	62.31	Exendin-4	EXE4_HELSU	*Heloderma suspectum*	**R.HGEGTFTSDLSK.Q**	639.86	2
						K.QMEEEAVR.L	496.31	2
45	CLP	59.03	Snaclec convulxin subunit beta	SLB_CRODU	*Crotalus durissus terrificus*	**K.QEMTWADAEK.F**	604.89	2
46	EXE	60.95	Exendin-4	EXE4_HELSU	*Heloderma suspectum*	**R.HGEGTFTSDLSK.Q**	639.83	2
						K.QMEEEAVR.L	496.32	2
47	CLP	61.38	Snaclec crotocetin-1	SL1_CRODU	*Crotalus durissus terrificus*	K.YYVWIGLR.I	535.3	2
48	CLP	97.74	Snaclec convulxin subunit beta	SLB_CRODU	*Crotalus durissus terrificus*	**K.STFFWIGANNIWNK.C**	849.44	2
						K.QEMTWADAEK.F	604.89	2
						K.MTHQSLK.S	422.75	2
49	PLA_2	93.51	Phospholipase A2 crotoxin basic subunit CBc	PA2BC_CRODU	*Crotalus durissus terrificus*	K.YGYMFYPDSR.C	649.93	2
						K.WDIYPYSLK.S	592.95	2
						HLLQFNK.M	450.29	2
						K.MIKFETR.K	462.81	2
						R.SLSTYK.Y	349.88	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	*Sistrurus catenatus edwardsii*	K.IVDLHNSLR.R	533.85	2
						K.MEWYSEAAANAER.W	764.41	2
						R.SVNPTASNMLK.M	581.36	2
51	CRiSP	98.38	Cysteine-rich secretory protein Ch-CRPKa (Fragment)	F2Q6E5_CROHD	*Crotalus horridus*	K.MEWYPEAAANAER.W	769.42	2
						SVDFDSESPR.K	569.79	2
						R.SVNPTASNMLK.M	581.27	2
51	LAAO	90.45	L-amino acid oxidase (Fragment)	K9N7B7_CRODM	*Crotalus durissus cumanensis*	R.KFWEDDGIR.G	583.36	2
						K.STTDLPSR.F	438.74	2
						R.IKFEPPLPPK.K	583.41	2
						K.KFWEDDGIR.G	583.36	2
52	LAAO	99.07	L-amino acid oxidase (Fragment)	K9N7B7_CRODM	*Crotalus durissus cumanensis*	K.NNPGILEYPVKPSEEGK.S	936.03	2
						R.ETDYEEFLEIAR.N	757.88	2
						K.YILDKYDTYSTK.E	755.46	2
						K.SAAQLYVESLR.K	619.06	2
						R.NGLTVTSNPK.H	515.83	2
						K.HDDIFGYEK.R	562.31	2
						K.SAAQLYVESLRK.V	682.97	2
						R.KKDWYANLGPM(+15.99)R.L	498.98	3
						K.HVVIVGAGMSGLSAAYVLAGAGHEVTVLEASER.A	1084.23	3
53	SVSP	97.65	Kallikrein-CohLL-4	T1DEH3_CROOH	*Crotalus oreganus helleri*	R.FLVALYTFR.S	565.44	2
						R.AAHGGLPATSR.T	519.34	2
						K.WDKDIM(+15.99)LIR.L	603.46	2
54	LAAO	99.14	L-amino acid oxidase (Fragment)	K9N7B7_CRODM	*Crotalus durissus cumanensis*	R.ETDYEEFLEIAR.N	757.87	2
						K.YILDKYDTYSTK.E	755.46	2
						K.NNPGILEYPVKPSEEGK.S	936.06	2
						R.NGLTVTSNPK.H	515.89	2
						R.VIEIQQNDRETK.V	736.97	2
						K.FWEDDGIR.G	519.43	2
						K.STTDLPSR.F	438.78	2
						K.VQVHFNAR.V	485.79	2
						R.KFWEDDGIR.G	583.37	2
55	SVMP	98.17	SVMP-CohPH-3	T1E6U1_CROOH	*Crotalus oreganus helleri*	R.MYELANTVNDIYR.Y	801.34	2
						R.FVELVLVVDK.A	580.91	2
						K.GMVLPGTK.C	401.79	2
						R.KTDLLTR.K	423.82	2
						K.NNDDLDKIK.T	537.81	2
56	SVMP	84.27	Metalloproteinase P-II	Q2QA03_CRODD	*Crotalus durissus durissus*	**R.VAVTM(+15.99)THELGHNLGIR.H**	588.71	3
						R.VHQMVNIMK.E	550.35	2
						R.ETDLLQR.R	437.81	2
57	SVMP	85.43	SVMP-CohPH-3	T1E6U1_CROOH	*Crotalus oreganus helleri*	R.M(+15.99)YELANTVNDIYR.Y	809.44	2
						R.FVELVLVVDK.A	580.93	2
58	SVSP	61.29	Serine proteinase 8 (Fragment)	F8S120_CROAD	*Crotalus adamanteus*	R.AAYPEYGLPATSR.T	698.4	2
59	SVSP	98.26	Snake venom serine protease	VSP_CRODD	*Crotalus durissus durissus*	**R.AAKPELPVTSR.T**	584.91	2
						R.LKDVQTGVSK.D	537.86	2
						K.DDEKDKDIM(+15.99)LIR.M	753.97	2
						K.DKDIM(+15.99)LIR.M	510.35	2
						K.KDDEKDKDIM(+15.99)LIR.M	545.67	3
60	SVSP	98.32	Thrombin-like enzyme gyroxin B1.4	VSP14_CRODU	*Crotalus durissus terrificus*	R.SVQFDKEQR.R	568.86	2
						R.THFLIYVGVHDR.S	729.09	2
						R.TALPQLR.L	399.78	2
60	EXE	61.48	Exendin-4	EXE4_HELSU	*Heloderma suspectum*	**R.HGEGTFTSDLSK.Q**	639.85	2
62	LAAO	99.14	L-amino acid oxidase (Fragment)	K9N7B7_CRODM	*Crotalus durissus cumanensis*	K.NNPGILEYPVKPSEEGK.S	935.99	2
						K.SAAQLYVESLR.K	619.04	2
						K.YILDKYDTYSTK.E	755.43	2
						R.ETDYEEFLEIAR.N	757.87	2
						R.NGLTVTSNPK.H	515.83	2
						R.VIEIQQNDR.E	557.86	2
						K.HDDIFGYEK.R	562.29	2
						R.VIEIQQNDRETK.V	736.89	2
						K.VQVHFNAR.V	485.83	2
						K.HDDIFGYEKR.F	427.26	3

(*continued on next page*)

Table 1 (*continued*)

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

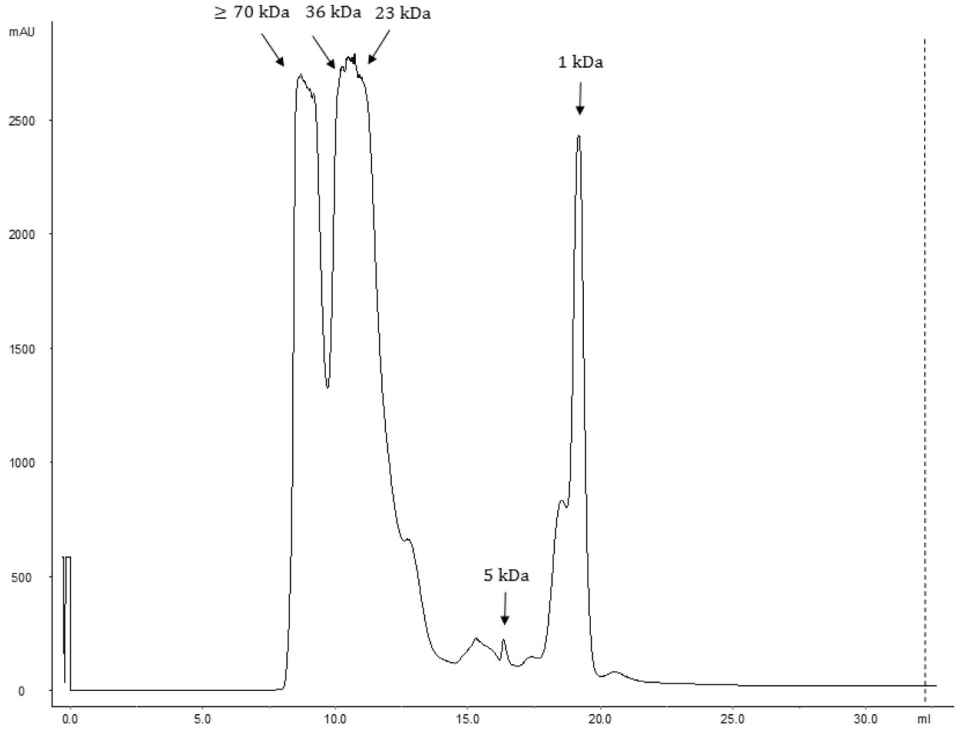

Fig. 2. Gel filtration chromatogram of 30 mg of *Crotalus vegrandis* crude venom, fractionated in a Superdex 75 10/300 (GE) column. Arrows indicate the molecular weight range.

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 (QICECDKAAAICFR) identical to crotoxin acidic subunit (crotapotin) 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_2S, different from the classical crotoxin in sequence and activity, can also be found in rattlesnake venoms. The basic PLA_2 Cdc-9 and Cdc-10 from *C. d. cumanensis* present myotoxic and edema-inducing activity (Romero-Vargas et al., 2010). An acidic PLA_2 was identified in spot 22 (Fig. 1). Its sequence matches to a PLA_2 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_2 named LmTX-II from *Lachesis muta muta* (Damico et al., 2005).

4.2. SVMP

Snake venom metalloproteases 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	*Crotalus durissus cumanensis*	R.SGTKIFLTC(+57.02)K.R	577.81	2
					R.KKDWYANLGPMRLPTK.H	640.02	3
					R.RIKFEPPLPPKK.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	*Crotalus oreganus helleri*	R.KTDLLTR.K	423.76	2
					R.KKHDNAQLLTAIDLDR.V	617.66	3
					R.KTDLLTRK.K	487.80	2
					R.KKHDNAQLLTAIDLDRVIGLAYVGSMC(+57.02)HPK.R	1121..88	3
SVMP II	81.59	Metalloproteinase P-II	Q2QA03_CRODD	*Crotalus durissus durissus*	**R.VAVTMTHELGHNLGIR.H**	583.32	3
					L.NLNPEHQR.Y	504.25	2
SVSP	67.93	Snake venom serine proteinase 2	VSP2_CROAD	*Crotalus adamanteus*	R.AAYPEYGLPATSR.T	698.34	2
Fraction II							
LAAO	98.58	L-amino acid oxidase (Fragment)	K9N7B7_CRODM	*Crotalus durissus cumanensis*	R.KKDWYANLGPMRLPTK.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	*Crotalus durissus durissus*	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	*Crotalus durissus durissus*	**R.VAVTMTHELGHNLGIR.H**	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	*Crotalus durissus collilineatus*	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	*Crotalus oreganus helleri*	K.VQVHFNAR.V	485.77	2
					K.EYLLK.E	665.39	1
					R.SVHYR.S	661.34	1
Crotoxin PLA_2 basic	84.02	Basic phospholipase A2 CB1 (Fragment)	PA2B1_CRODV	*Crotalus durissus vegrandis*	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	*Crotalus durissus durissus*	**R.VAVTMTHELGHNLGIR.H**	583.32	3
SVMP III	56.51	Snake venom metalloprotease	C6JUN4_PHIOL	*Philodryas olfersii*	R.ETDLLQR.R	437.75	2
					K.EYLLK.Y	665.39	1
Fraction IV							
Crotoxin PLA_2 basic	97.52	Basic phospholipase A2 Mtx-b	PA2B1_CROSS	*Crotalus scutulatus scutulatus*	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	*Crotalus durissus collilineatus*	D.TPPAGPDGGPR.G	511.26	2
					T.PPAGPDGGPR.G	460.73	2
					L.KLDRIGSMSGLGC(+57.02)	697.36	2
crotoxin PLA_2 acidic	60.91	Phospholipase A2 1b	T1E3Y3_CROHD	*Crotalus horridus*	K.QIC(+57.02)EC(+57.02)DKAAAIC(+57.02)FR.E	871.41	2
LAAO	56.31	L-amino acid oxidase (Fragment)	K9N7B7_CRODM	*Crotalus durissus cumanensis*	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	*Crotalus durissus collilineatus*	D.TPPAGPDGGPR.G	511.25	2
VNGF	59.22	NGF-CohPH-1	T1E7J7_CROOH	*Crotalus oreganus helleri*	T.DQHYPAPK.K	478.23	2
Fraction VI + VII + VIII							
crotoxin PLA_2 basic	98.10	Phospholipase A2 crotoxin basic subunit CBb	PA2BB_CRODU	*Crotalus durissus terrificus*	K.MIKFETR.K	462.74	2
					HLLQFNK.M	450.24	2
					R.C(+57.02)RGPSETC(+57.02)	483.68	2
crotoxin PLA_2 acidic	59.73	Phospholipase A2 homolog crotoxin acid subunit CA	PA1A_CRODU	*Crotalus durissus terrificus*	**G.SLVEFETLMMKIAGR.S**	862.94	2
BIP	61.35	Bradykinin-potentiating and C-type natriuretic peptides	BNP_CRODO	*Crotalus durissus collilineatus*	D.TPPAGPDGGPR.G	511.25	2

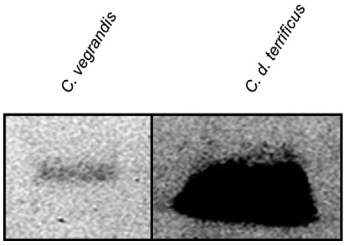

Fig. 3. Western blotting of *Crotalus vegrandis* (right lane) and *C. durissus terrificus* (control) crude venoms (both 33.3 μg/lane) against polyclonal anti-crotamine (1:8000 dilution).

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 Uracoine-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 crotastatin-like isoform. Crotastatin, 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. rhombeata* (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

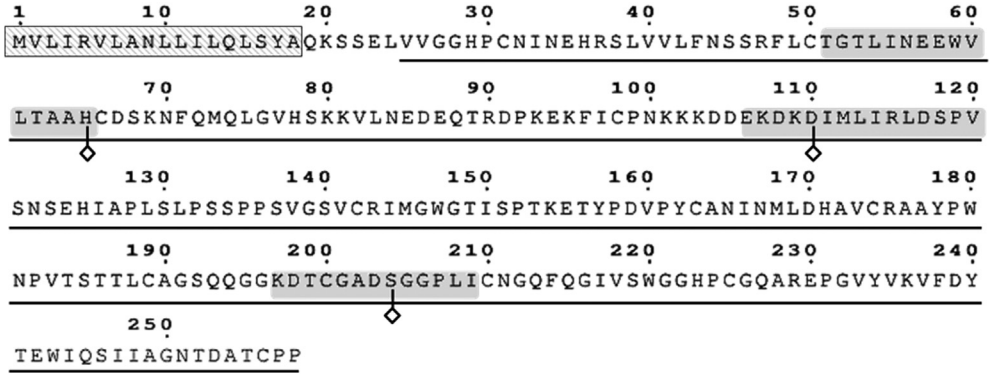

Fig. 4. Translated amino acid sequence (258 residues) of the SVSPcv01 transcript cloned from a *Crotalus vegrandis* venom gland (*GenBank: KT266708*). Domain prediction analysis identified: peptidase S1 trypsin-like cysteine/serine (underlined sequence); signal peptide (crosshatched box); and peptidase S1A chymotrypsin-type subfamily domain signatures (gray boxes). The catalytic triad residues are indicated with ◊ 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 fibrino(gen)olityc activity, as they show high homology with other SVSP with such features such as shedaoenase from *Agkistrodon shedaoenthesis* (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 α and β 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 α2β1 and the fibrinogen receptor integrin αIIbβ3 (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 (α4β4) 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). Crotocetin was also identified, supported by a UP, in spots 16, 71 and 73. Crotocetin was originally cloned from *C. d. terrificus* venom gland (*UniProtKB: Q719L8*). The *Trimeresurus mucrosquamatus* crotocetin homologue, mucrocetin, has *in vitro* platelet aggregation activity (Huang et al., 2004).

4.5. Crotamine

Crotamine 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). Crotamine features were reviewed in Oguiura et al. (2005), and its potential biotechnological and therapeutic applications by Kerkis et al. (2014). Crotamine shows antimicrobial activity that can be explained by its β-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^2 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 crotamine in lower levels than *C. d. cumanensis*. We detected a weak reactive band in the Western blot assay with anti-crotamine 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 horridum* lizard (Mochca-Morales et al., 1990). Smooth muscle contraction inhibition and cyclic nucleotide-gated ion channels inhibition activity were described for the pseudechetoxin (*Pseudechis australis*) and pseudecin (*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: P0DI84*) (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 PLA_2s, 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.

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Appendix A. Supplementary data

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

Transparency document

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