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Expanding the neutralization scope of the Central American antivenom (PoliVal-ICP) to include the venom of *Crotalus durissus pifanorum*

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ABSTRACT

The proteomic, enzymatic, toxicological, and immunogenic profiles of the venom of *C. d. pifanorum* were studied. It was found that venom of *C. d. pifanorum* is composed of 63% phospholipases A_2 (PLA_2s), 13% serine proteinases (SVSPs), 8% bradykinin-potentiating peptides (BPPs), 4% L-amino acid oxidases (LAAOs), 3% metalloproteinases (SVMPs), and other minor components. This composition allows the venom to exert lethal, PLA_2, myotoxic, coagulant and defibrinogenating activities, but no azocaseinolytic or hemorrhagic activities. The addition of *C. d. pifanorum* venom to the group of venoms used as immunogens to produce the Central American antivenom PoliVal-ICP (i.e., venoms of *Bothrops asper*, *Crotalus simus* and *Lachesis stenophrys*) resulted in 1) the expansion of the neutralization scope of the antivenom to cover the venom of *C. d. pifanorum* and other antigenically related venom (i.e., *C. s. scutulatus* venom), 2) improvement of the neutralizing potency towards the venom of *C. simus*, and 3) no significant reduction of the neutralization of venoms of *B. asper* and L. *stenophrys*. It was concluded that supplementation of the immunogens used to produce PoliVal-ICP with the venom of *C. d. pifanorum* is a viable alternative to expand the neutralization scope of the antivenom.

Biological significance: The venom of *Crotalus durissus pifanorum* from Venezuela has a proteomic profile like those of other subspecies of the South American rattlesnake *C. durissus*, with predominance of phospholipases A_2 (especially crotoxin) and serine proteinases. This explains a toxicological profile characterized by neurotoxicity, myotoxicity, and coagulopathies, but being devoid of hemorrhagic activity. The antivenom used in Central America (PoliVal-ICP) includes the venom of *C. simus*, which has a different composition, in the immunizing strategy. Accordingly, this antivenom does not neutralize *C. d. pifanorum* venom. The addition of *C. d. pifanorum* venom to the immunizing mixture of PoliVal-ICP expands the neutralizing scope of this antivenom, to cover additional rattlesnake venoms, while not affecting the response to *C. simus*, *Bothrops asper* and *Lachesis stenophrys* venoms. This represents an improvement of the current PoliVal-ICP.

1. Introduction

Snakebite envenomation is an important public health problem in tropical and sub-tropical countries around the world [1]. In Central America, the envenomations with the highest incidence and severity are those caused by *Bothrops asper* and *Crotalus simus*. Therefore, these species are included in the group of snakes with the highest medical importance in the classification of the World Health Organization, i.e., Category 1 [1].

Other Central American species of the genera *Agkistrodon*, *Atropoides*, *Bothriechis*, *Cerrophidion*, *Lachesis*, *Metlapilcoatlus*, *Porthidium* and *Micrurus*, are also capable of causing morbidity, disability, and death. However, the incidence of envenomations by these species is low [2,3]. Therefore, they are considered as snakes with secondary medical importance, i.e., Category 2 [1].

To reduce the impact of snakebite envenomation in Central America, Costa Rica developed a polyspecific antivenom in 1967. This antivenom consisted of a formulation based on whole immunoglobulins purified by *salting out* from the plasma of horses immunized with a mixture of venoms of *B. asper*, *C. simus* and *Lachesis stenophrys* [4]. The method to produce this antivenom remained without major modifications until the 1990s, when the purification method was changed to caprylic acid

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precipitation [5] and the name PoliVal-ICP was coined to refer to this product. Since then, the manufacturing procedure has been gradually improved, but the venoms used as immunogens have remained the same.

The immunologic properties of venoms used as immunogens to produce PoliVal-ICP allow this antivenom to neutralize venoms of almost all medically important snakes of Central America [6,7]. However, the neutralization scope of PoliVal-ICP does not cover the venoms of neurotoxic rattlesnakes distributed in South America [8], some of which are frequently found in private snake collections in Costa Rica.

Recent cases of envenomation of snake-handlers by captive *Crotalus durissus pifanorum* specimens have evidenced the need to expand the neutralization scope of PoliVal-ICP to include this neurotoxic venom. However, as *Crotalus durissus* venoms normally contain high amounts of crotoxin [9], a heterodimeric phospholipase A_2 (PLA_2) with potent immunosuppressive activity [10–12], the inclusion of *C. d. pifanorum* venom into the group of venoms used as immunogens to produce PoliVal-ICP could reduce the antibody response of horses towards the other co-immunogen venoms. Therefore, modifications of the immunization procedure to achieve this goal must be carefully evaluated.

To expand the neutralization scope of PoliVal-ICP to include the venom of *C. d. pifanorum*, we studied the proteomic, enzymatic, and toxicological profiles of this venom, and determined its effect on the antibody response of horses towards the other co-immunogen venoms, and on the pre-clinical paraspecificity of PoliVal-ICP towards other rattlesnake venoms.

2. Materials and methods

This study meets the International Guiding Principles for Biomedical Research Involving Animals [13] and the ARRIVE Guidelines for the reporting of in vivo experiments [14]. All procedures involving animals were approved by the Institutional Committee for the Care and Use of Laboratory Animals of Universidad de Costa Rica (approval code CICUA 82-08).

2.1. Snake venoms

Venoms of *C. d. pifanorum, B. asper*, *C. simus*, and L. *stenophrys* were obtained from adult snakes maintained in captivity at the Serpentarium of Instituto Clodomiro Picado. Samples of venom from many specimens were mixed by species, stabilized by lyophilization, and stored at −20 °C. Venoms of *C. horridus* and *C. s. scutulatus* were provided by Charlotte L. Ownby (Oklahoma State University, USA). The venom of *C. d. terrificus* was kindly donated by the Serpentarium of Instituto Butantan. With the exception of Costa Rican *B. asper*, *C. simus* and L. *stenophrys*, we do not have information on the geographical origin of the specimens from which the venoms used in this study were obtained.

Solutions of venoms were prepared in 0.12 M NaCl, 0.04 M phosphate, pH 7.2 buffer (PBS) immediately before use.

2.2. Venomic analysis

The venomic analysis was performed following the method described by Calvete and collaborators [9], as follows:

2.2.1. Reverse-phase HPLC profiling

Five milligrams of venom of *C. d. pifanorum* were dissolved in 200 μL of 0.1% trifluoroacetic acid (TFA) and 5% acetonitrile buffer (buffer A). Insoluble material was removed by centrifugation and the proteins in the supernatant were separated by reverse-phase HPLC (HPLC system: Agilent 1100 series; Agilent Technologies), equipped with a C18 column (250 × 4.6 mm, 5 μm particle size; Agilent Technologies). The flow rate was set to 1 mL/min and the protein separation was performed with the following buffer gradient: 5% buffer B (buffer B: 95% acetonitrile, 0.1% TFA) for 10 min, followed by 5–15% B over 20 min, 15–45% B over 120 min, and 45–70% B over 20 min. Protein peaks detected at 215 nm were collected manually, dried in a centrifugal vacuum evaporator (Speed Vac, Savant) and stored at −20 °C until analysis.

2.2.2. SDS-PAGE separation

Proteins in HPLC-fractions were separated by SDS-PAGE, under reducing conditions, using an acrylamide concentration of 15% [15]. Gels were stained with Coomassie Brilliant Blue R-250 and the optical density of the electropherogram was determined with the Image-J® program (http://rsb.info.nih.gov/ij/).

2.2.3. Mass spectrometry analysis

The protein bands in F8a, F8b, F8c, F8d, F8e, F9a, F9b, F9c, F9e, F9f, F10a, F10b, F11a, F11b, F11c, F11d, F11e, F12a, F12b and F13b fractions, were excised from the SDS-PAGE gel and subjected to reduction with 10 mM dithiothreitol, alkylation with 50 mM iodoacetamide and in-gel digestion with bovine trypsin (sequencing grade) at 37 °C overnight. Resulting peptides were extracted from the gel with 50% acetonitrile and 0.1% TFA, concentrated by centrifugal vacuum evaporation (Vacufuge, Eppendorf) and mixed with an equal volume of matrix solution (saturated solution of α-cyano-4-hydroxycinnamic acid dissolved in 50% acetonitrile and 0.1% TFA). Then, one μL of the peptide preparation was spotted onto a MALDI plate (384 Opti-TOF, Applied Biosystems). After air-drying, spots were analyzed by MALDI-TOF/TOF using a 4800-Plus Proteomics Analyzer mass spectrometer (Applied Biosystems). Mass spectra were searched against the UniProt/SwissProt database (October 2019) for Serpentes using the Paragon algorithm of ProteinPilot v.4, or in few cases were analyzed manually to derive de novo sequences that were submitted to BLAST. In the case of fractions F4a, F7a, F9d, F11f, F12c, F13a and F13c, the resulting peptides were submitted to nESI-MS/MS on a Q-Exactive Plus® mass spectrometer (Thermo). Twelve μL of each tryptic digest were loaded on a 2 cm × 75 μm PepMap trap column, washed, and separated at 200 nL/min with a 3 μm particle, 15 cm × 75 μm C18 Easy-spray® analytical column using a nano-Easy® 1200 chromatograph. A gradient from 0.1% formic acid (solution A) to 80% acetonitrile with 0.1% formic acid (solution B) was developed: 1–5% B in 1 min, 5–26% B in 25 min, 26–79%B in 4 min, 79–99%B in 1 min, and 99% B in 4 min, for a total time of 35 min. MS spectra were acquired in positive mode at 1.9 kV, with a capillary temperature of 200 °C, using 1 scan at 400–1600 *m/z*, maximum injection time of 100 msec, AGC target of 3 × 106, and orbitrap resolution of 70,000. The top 10 ions with 2–5 positive charges were fragmented with AGC target of 1 × 105, maximum injection time of 110 msec, resolution 17,500, loop count 10, isolation window of 1.4 *m/z*, and a dynamic exclusion time of 5 s. MS/MS spectra were processed for assignment of peptide matches to known protein families by similarity with sequences contained in the UniProt/SwissProt database (Serpentes, October 2019), using Peaks X®. Cysteine carbamidomethylation was set as a fixed modification, while deamidation of asparagine or glutamine and methionine oxidation were set as variable modifications, allowing up to 3 missed cleavages by trypsin. Parameters for match acceptance included FDR < 0.1%, −10 lgP protein score ≥ 200, and unique peptides ≥0. Furthermore, unique peptides were used as selection criteria in this analysis. On the other hand in F1, F2, F3, F5 and F6 fractions, the material eluted from the reversed phase chromatography was dried in a centrifugal evaporator and used to the mass spectrometry analysis. Low molecular weight peptides were redissolved in 50% acetonitrile containing 0.1% formic acid and loaded into metal-coated capillaries (Proxeon) for direct infusion into a nano-electrospray II ion source coupled to a QTrap 3200 mass spectrometer (Applied Biosystems). Samples were scanned in enhanced multicharge mode and selected ion precursors were fragmented for MS/MS spectra acquisition using the enhanced product ion mode and collision energies of 20–45 eV as previously described [16]. Resulting spectra were manually interpreted to obtain de novo amino acid sequences.

2.2.4. Relative abundance of protein families

The relative abundance of the different protein families in the venom was calculated using the integration of the HPLC chromatogram (performed with the ChemStation v.B.04.01 Agilent software) and the densitometric analysis of the SDS-PAGE electropherogram of each HPLC fraction (performed with the software program Image Lab; v 2.0.1 build 18; Bio-Rad Laboratories).

2.3. Enzymatic and toxic activities

2.3.1. Lethality

Groups of five mice (*Mus musculus*; 18–20 g; CD-1 strain) were injected by the i.p. route with different doses of venom dissolved in 0.5 mL PBS. The ranges of doses were 0.59–3.00 μg/mouse for venom of *C. d. pifanorum*, 2.00–10.12 μg/mouse for venom of *C. horridus*, 0.26–1.33 μg/mouse for venom of *C. d. terrificus*, 0.59–3.00 μg/mouse for venom of *C. s. scutulatus*, 22.70–115.26 μg/mouse for venom of *B. asper*, 3.00–15.18 μg/mouse for venom of *C. simus*, and 51.23–259.33 for venom of L. *stenophrys*. Deaths that occurred during the following 48 h were recorded. The Median Lethal Dose (LD_{50}) was calculated by using the Probits [17]. Results were reported as LD_{50} and the 95% confidence interval.

2.3.2. PLA_2 activity

Egg yolk phospholipids were incubated with various amounts of venom for 30 min at 37 °C. Then, the released fatty acids were extracted and titrated according to Dole [18]. Activity was expressed as μEq of fatty acid released per mg protein per min. Results were reported as the mean ± SD of triplicate determinations.

2.3.3. Myotoxic activity

The myotoxic activity of venoms was determined by injecting groups of four mice (18–20 g; CD-1 strain) in the right gastrocnemius muscle, with 50 μL of PBS containing different amounts of venom, depending on the experiment and the specific venom. Three hours later, heparinized capillary tubes were used to collect samples of blood from the orbital plexus of mice under CO_2 anesthesia. Plasma was separated by centrifugation, and the CK activity in plasma was determined by using the kit CK-NAC Unitest (Wienner, St Ingbert, Germany). CK activity was expressed in U/L. Results were reported as the mean ± SD.

2.3.4. Coagulant activity in vitro

Coagulant activity was assessed by adding several amounts of venom dissolved in 100 μL of PBS, to 200 μL of human citrated plasma previously incubated at 37 °C and recording the clotting time. The minimum coagulant dose (MCD) was calculated as the amount of venom that causes the coagulation of plasma in 60 s [19]. Results were reported as the mean ± SD of triplicate determinations.

2.3.5. Defibrinogenating activity

Defibrinogenating activity was assessed following the method described by Theakston and Reid [19], as modified by Gené and co-workers [20]. Groups of four mice (18–20 g; CD-1 strain) were injected by the intravenous route with different amounts of venom dissolved in 200 μL PBS. One hour later, mice were bled from the orbital plexus under CO_2 anesthesia. Blood was collected in dry glass tubes and kept at room temperature (22–25 °C) for 1 h. Then, the tubes were tilted, and the formation of a clot was observed. The minimum defibrinogenating dose (MDD) was calculated as the lowest amount of venom that induced blood incoagulability in all injected animals.

2.3.6. Azocaseinolytic activity

Proteinase activity on azocasein was measured by a modification of the method used by Gutiérrez and co-workers [21]. First, various amounts of venom, dissolved in PBS, were added to 100 μL of substrate (10 mg/mL azocasein dissolved in 25 mM Tris, 150 mM NaCl, 5 mM $CaCl_2$, pH 7.4). Then, the mixtures were incubated at 37 °C for 90 min. After that, the reactions were stopped by the addition of 200 μL of 5% trichloroacetic acid, followed by centrifugation. Next, 150 μL of each supernatant was mixed with 150 μL of 0.5 M NaOH, and the absorbance at 450 nm was recorded in a spectrophotometer (Multiskan FC, Thermo Scientific). Azocaseinolytic activity was expressed as units/mg venom, considering that one unit corresponds to a change in absorbance of 0.2 per min. Results were reported as the mean ± SD of triplicate determinations.

2.3.7. Hemorrhagic activity

Hemorrhagic activity was determined by a modification of the method described by Kondo and co-workers [22–24]. Groups of four mice (18–20 g; CD-1 strain) were injected by the intradermal route with several amounts of venom dissolved in 100 μL PBS. Two hours later, mice were euthanized by CO_2 inhalation, the area of the hemorrhagic lesion on the inner surface of the skin was photographed and the area and intensity of the venom-produced hemorrhagic lesion, expressed in hemorrhage units (HaU), was determined using the Inkscape 0.91 program (https://inkscape.org/download/). A minimum hemorrhagic dose (MHD) was the amount of venom that generates 50 HaU [24].

2.4. Inhibition of PLA_2s with p-bromophenacyl bromide (p-BPB)

One milliliter of venom solution (3 mg/mL in PBS) was incubated with 150 μL of *p*-bromophenacyl bromide (*p*-BPB; Sigma-Aldrich, USA; 3 mg/mL in 99% ethanol) for 24 h at room temperature [25]. Samples of venom were inactivated immediately before assessment of their enzymatic and toxic activities by the methods previously described.

2.5. Inhibition of serine proteinases (SVSPs) with PMSF

One milliliter of venom solution (3 mg/mL in PBS) was mixed with 0.15 mL of phenylmethane sulfonyl fluoride (PMSF) in 99% ethanol (Sigma-Aldrich, USA; 3 mg/mL) and incubated for 24 h at room temperature. Samples of venom were inactivated immediately before assessment of their enzymatic and toxic activities by the methods previously described.

2.6. Inhibition of metalloproteinases (SVMPs) with Batimastat

Venom was inhibited with the peptidomimetic hydroxamate Batimastat following the method described by Rucavado and co-workers [26]. Briefly, 1 mg of venom of *C. d. pifanorum* was dissolved in 250 μL (PBS with 0.01% Tween 80), containing Batimastat (Sigma-Aldrich, USA) at a concentration of 40 μM for in vitro assays and 100 μM for in vivo assays. Then, mixtures were incubated for 30 min at 37 °C. Samples of venom were inhibited immediately before assessment of their enzymatic and toxic activities by the methods previously described.

2.7. Antivenom

We used 34 horses (5–6 years old; 400–450 kg), which had been previously immunized with venoms of *B. asper*, *C. simus* and L. *stenophrys*, according to the procedure described by Freitas et al. [27], as modified by Arroyo et al. [28]. During more than two years, these horses were periodically re-immunized for PoliVal-ICP production. To include the venom of *C. d. pifanorum* as an immunogen, the horses were taken out of their production routine and allowed to rest for two months before starting the additional immunization protocol. The first injection was composed of 6 mg of *C. d. pifanorum* venom emulsified in Freund's complete adjuvant. The second injection was composed of 6 mg of *C. d. pifanorum* venom emulsified in Freund's incomplete adjuvant. The following three injections were composed of 2 mg of venom of *C. d. pifanorum* dissolved in sterile saline solution. In the last booster, horses were immunized with 2 mg of *B. asper* venom, 1 mg of *C. simus* venom, 1

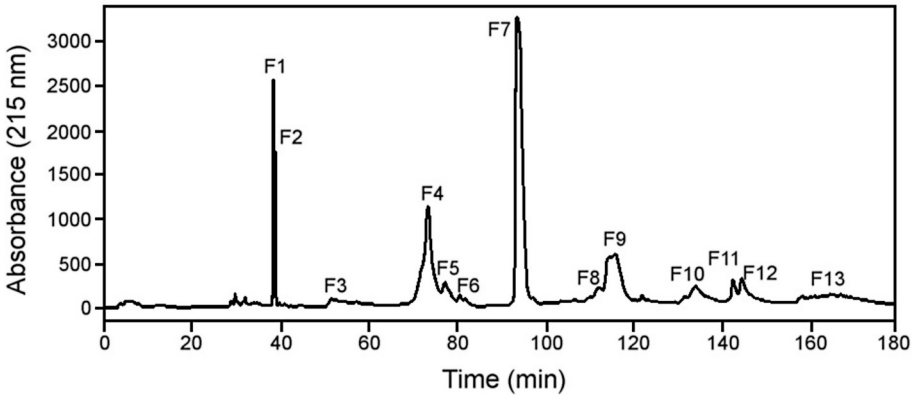

Fig. 1. RP-HPLC chromatogram of *C. d. pifanorum* venom. Venom fractions were identified by an alphanumeric code.

mg of *C. d. pifanorum* venom, and 2 mg of L. *stenophrys* venom. All injections were administered by the subcutaneous route, in one single injection, at two-week intervals between one injection and the next one. The horses were bled 13 days after the last booster, and plasma was separated from red blood cells and stored at 2–8 °C until use. For the preparation of the antivenom, immunoglobulins were purified by the caprylic acid precipitation method [5]. The experimental Quadrivalent antivenom was formulated at 55 g/L total protein, 9 g/L NaCl, 2.5 g/L phenol and pH 7.0; it was then sterilized by filtration and dispensed in 10 mL glass vials. Commercial Trivalent PoliVal-ICP (batch 5780716; 54 g/L total protein) was used as control.

2.8. Neutralization of enzymatic and toxic activities of venoms

The neutralizing ability of antivenoms was determined by mixing a constant challenge dose of venom with different dilutions of either antivenom (i.e., experimental Quadrivalent or commercial Trivalent antivenom). Mixtures were incubated at 37 °C for 30 min before determining the residual activity of venom by using the experimental systems described above. Venom solutions incubated with PBS instead of antivenom were used as controls. The challenge doses utilized for each activity were: for lethal activity, 4 LD_{50}s; for PLA_2 activity, 12.5 μg; for myotoxic activity, 5 μg or 25 μg; for coagulant activity, 2 MCDs; for defibrinogenating activity, 2 MDD; for azocaseinolytic activity, 3 μg; and for hemorrhagic activity, 10 MHDs. For lethal, PLA_2, azocaseinolytic, and hemorrhagic activities, neutralizing ability of antivenoms was expressed as the Median Effective Dose (ED_{50}), calculated by Probits [17], and defined as the ratio microliters antivenom per mg venom at which the activity of venom was reduced to 50%. Anti-lethal potency of antivenoms was expressed as the amount of venom in milligrams that is neutralized by 1.0 mL of antivenom, calculated according to the following formula: Potency = (Number of LD_{50}s in the challenge dose – 1 LD_{50})* LD_{50}*ED_{50}^{-1} [29]. In this formula, the value of ED_{50} corresponds to the volume of antivenom per challenge dose of venom, i.e., 4 LD_{50}s. In the case of myotoxic activity, only the ratio 333 μL antivenom per mg venom was used. For coagulant activity, neutralization was expressed as Effective Dose (ED), defined as the ratio microliters antivenom per mg venom at which the clotting time of plasma was prolonged three times as compared to the clotting time of plasma incubated with the venom alone [20]. In the case of defibrinogenating activity, neutralization was expressed as Effective Dose (ED), defined as the lowest ratio microliters antivenom per mg venom at which the blood samples of all mice clotted [20].

2.9. Statistical analysis

Statistical analysis was performed using IBM SPSS Statistics for Windows, version 24.0 (Armonk, NY: IBM Corp). The calculation of lethal activity and its respective neutralization was obtained using the Probit program [17]. Student's *t*-test was used to compare the means between the native venom and the inhibited venom for each activity, and for comparing neutralization by the two antivenoms. When several inhibitors were tested for the same activity, ANOVA tests were used, followed by a post-hoc analysis of the Tukey type. Values of $P < 0.05$, were considered significant.

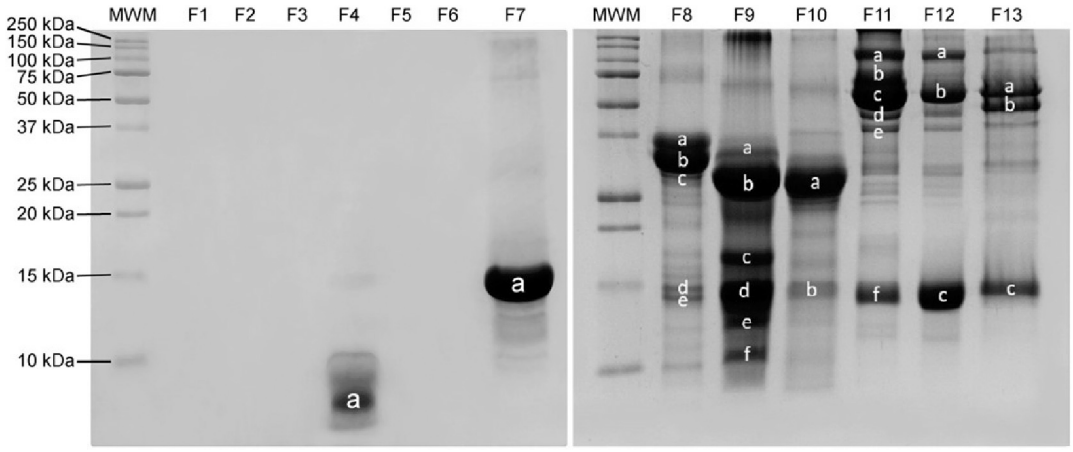

Fig. 2. SDS-PAGE of RP-HPLC fractions of the venom of *C. d. pifanorum*. Separation was performed under reducing conditions. Bands excised from the gel were identified by lowercase letters. MWM correspond to the Wide-range Molecular Weight marker.

Table 1
Assignment of the components of venom of *C. d. pifanorum* to protein families by mass spectrometry analysis of HPLC fractions or in-gel digested protein bands.

Gel band	% Relative abundance in whole venom	Ions		Peptides derived from Mass Spectrometry	Peptide ID Score	% Algorithm confidence	Protein family	ID code
		m/Z	Z					
F1	3.4	413.1	1	ZNW	Manual	NA	SVMPs inhibitory tripeptide	NA
		1238.6		ZRWPHPQIPP	Manual	NA	PPB	P68515
F2	2.2	444.1	1	ZBW	Manual	NA	SVMPs inhibitory tripeptide	NA
		931.4		ZRWPHPK	Manual	NA	PPB	P68515
F3	1	1238.6	1	ZRWPHPQIPP	Manual	NA	PPB	P68515
F4a	20.3	558.270	2	PTTDVYTYR	80.30	NA	PLA_2 (Acidic Subunit Crotoxin)	P08878
		915.634	4	RQEDGEIVCGEDDPCGTQICECDKAAAICFR	58.83	NA		
		621.269	2	LTGCDPTTDVY	57.46	NA		
		686.799	2	AAAICFRNSMDT	54.96	NA		
		953.103	4	SSYGCYCGAGGQGWPQDASDRCCFEHDCCYAK	43.83	NA		
F5	4.1	1238.6	1	ZRWPHPQIPP	Manual	NA	PPB	P68515
F6	NA	905.6	1	UNIDENTIFIED	NA	NA	Unidentified	Unidentified
F7a	40.3	891.890	4	SGYITCGKGTWCEEQICECDRVAAECLRR	107.45	NA	PLA_2 (Basic Subunit Crotoxin)	P62022
		639.306	4	CNTKWDIYPYSLKSGYITCGK	96.93	NA		
		456.0	4	HLLQFNKMIKFETR	87.85	NA		
		682.077	4	KNAIPFYAFYGCYCGWGGRGRPK	86.26	NA		
		594.755	4	DATDRCCFVHDCCYGKLAK	79.65	NA		
		831.321	2	RCCFVHDCCYGK	71.80	NA		
		738.102	4	VAAECLRRSLSTYKYGYMFYPDSR	68.94	NA		
		500.761	4	LAKCNTKWDIYPYSLK	68.33	NA		
		736.078	4	SLSTYKYGYMFYPDSRCRGPSETC	61.36	NA		
		691.770	2	FYGCYCGWGGR	57.06	NA		
F8a	0.7	1395.6	1	AAYPEYGLPATSR	13	99	SVSP (Kallikrein type)	T1E7K6
		890.5	1	IHLGVHSK	10	99		
F8b	3	1395.6	1	AAYPEYGLPATSR	17	99	SVSP (Kallikrein type)	T1E7K6
		2889.4	1	LDSPVSNSEHIAPLSLPSSPPSVGSVCR	7	99	SVSP	T2HPV4
F8c	0.8	2142.0	1	LDDAVCQPPYPELPATSR	8	99	SVSP	T1DH10
		2889.4	1	LDSPVSNSEHIAPLSLPSSPPSVGSVCR	11	99		
		1395.6	1	AAYPEYGLPATSR	14	99	SVSP (Kallikrein type)	T1E7K6
F8d	0.2	1762.8	1	QFPAENCREEPEPC	6	97.6	PLA_2 (Acidic)	C9E7C4
F8e	NA	903.2	1	UNIDENTIFIED	NA	NA	Unidentified	Unidentified
F9a	0.2	2294.0	1	AAYPWNPVTSTTLCAGSQQGGK	6	99	SVSP	A0A0U2UH64
		1488.6	1	VVGGHPCNINEHR	9	99		
		2889.4	1	LDSPVSNSEHIAPLSLPSSPPSVGSVCR	8	99		
		1395.6	1	AAYPEYGLPATSR	16	99	SVSP (Kallikrein type)	T1E7K6
F9b	1.2	3359.4	1	DTCGADSGGPLICNGQFQGIVSWGGHPCGQAR	8	99	SVSP	A0A0U2UH64
		2294.0	1	AAYPWNPVTSTTLCAGSQQGGK	10	99		
		1488.7	1	VVGGHPCNINEHR	15	99		
		3681.8	1	IMGWGTISPTKETYPDVPHCANINILDHAVCR	9	99	SVSP	T1E3B5
		2494.1	1	ETYPDVPHCANINILDHAVCR	12	99		
		2889.4	1	LDSPVSNSEHIAPLSLPSSPPSVGSVCR	22	99		
		2142.0	1	LLDDAVCQPPYPELPATSR	21	99	SVSP	T1DH10
		1395.6	1	AAYPEYGLPATSR	11	99	SVSP (Kallikrein type)	T1E7K6
F9c	0.1	2294.0	1	AAYPWNPVTSTTLCAGSQQGGK	10	99	SVSP	A0A0U2UH64
		2889.4	1	LDSPVSNSEHIAPLSLPSSPPSVGSVCR	22	99		
		1745.6	1	QFPAENCREEPEPC	7	99	PLA_2 (Acidic)	C9E7C4
		1762.7	1	QFPAENCREEPEPC	8	99		
		2142.0	1	LLDDAVCQPPYPELPATSR	7	96.8	SVSP	T1DH10
F9d	0.18	411.717	2	VAAVCFR	58.96	NA	PLA_2 (Acidic)	Q800C3
		447.198	2	CFPAENCR	48.93	NA		
		473.746	2	VSYTYSVK	47.17	NA		
		758.984	3	AYGCYCGWGGQGRPQDATDR	43.13	NA		
		694.631	3	YKRFPAENCREEPEPC	38.90	NA		
		480.971	4	VKNGEIICEDDDPCKK	38.90	NA		
		401.686	2	PQDATDR	35.65	NA		
		688.597	3	DATDRCCFVHDCCYGK	35.09	NA		
		749.835	2	VVGGDECNINEHR	91.74	NA	SVSP	F8S116
		540.230	2	AHCDSTNFK	58.19	NA		
		559.795	2	TLCAGILEGGK	53.97	NA		
		535.258	2	EKFFCPNK	53.68	NA		
		422.740	2	GSITPIEK	42.85	NA		
		495.588	3	HIMGWGSITPIEK	41.57	NA		
		421.475	4	KKVPNEDEQTRNPK	41.18	NA		
		634.807	2	LDSPVSNSEHIA	39.40	NA		
		416.722	2	KDDVLDK	39.27	NA		
F9e	0.3	1762.6	1	QFPAENCREEPEPC	12	99	PLA_2 (Acidic)	C9E7C4
		2081.8	1	YRQFPAENCREEPEPC	15	99		
		2142.0	1	LLDDAVCQPPYPELPATSR	8	99	SVSP	T1DH10
		1395.6	1	AAYPEYGLPATSR	7	93.7	SVSP (Kallikrein type)	T1E7K6

(*continued on next page*)

Table 1 (*continued*)

Gel band	% Relative abundance in whole venom	Ions		Peptides derived from Mass Spectrometry	Peptide ID Score	% Algorithm confidence	Protein family	ID code
		m/Z	Z					
F9f	0.1	1505.5	1	CCFVHDCCYGK	11	99	PLA_2 (Acidic)	C9E7C4
F10a	6.7	1747.8	1	ILCAGVLEGGIDTCNR	14	99	SVSP	A0A193CHI5
		2318.1	1	NNEHIAPLSLPSSPPSVGSVCR	9	99		
		1136.5	1	SVQFDKEQR	11	98.6		
		1512.6	1	VIGGDECNINEHR	9	99	SVSP (Kallikrein type)	T1E7I9
F10b	0.4	2081.7	1	YRQFPAENCREEPEPC	6	84.6	PLA_2 (Acidic)	C9E7C4
F11a	0.3	1563.7	1	CSSITELEKVNQR	8	99	Phosphodiesterase	T1DJT5
		2332.1	1	EQSSPLSCPFGPVPSPDVSGCK	8	99		
F11b	0.1	1580.7	1	DLHPELSEDEIKR	9	99	Hyaluronidase	T1D6Q3
		1472.7	1	VIEIQQNDRETK	6	96.8	LAAO	K9N7B7
F11c	2.4	1870.8	1	NNPGILEYPVKPSEEGK	8	99	LAAO	K9N7B7
		1472.7	1	VIEIQQNDRETK	10	99		
		970.4	1	VQVHFNAR	13	99		
		1580.7	1	DLHPELSEDEIKR	8	99	Hyaluronidase	T1D6Q3
F11d	0.1	970.4	1	VQVHFNAR	8	99	LAAO	K9N7B7
		1580.7	1	DLHPELSEDEIKR	9	99	Hyaluronidase	T1D6Q3
F11e	0.1	970.4	1	VQVHFNAR	6	99	LAAO	K9N7B7
F11f	0.4	472.883	3	TCLGLEQDTNHK	81.19	NA	C-type lectin	B0VXV2
		647.305	2	VQGEEKQCSTK	80.85	NA		
		439.232	2	INPFVCK	47.25	NA		
		453.681	2	NWDDAER	46.15	NA		
		449.734	2	GGHLVSIES	44.43	NA		
		443.209	2	SGYITCGK	76.81	NA	PLA_2 (Basic)	P62022
		665.302	3	SLSTYKYGYMFYPDSR	65.57	NA		
		563.275	3	CNTKWDIYPYSLK	59.11	NA		
		456.0	4	HLLQFNKMIKFETR	55.67	NA		
		487.761	2	VAAECLRR	54.79	NA		
		483.689	2	CRGPSETC	46.48	NA		
		871.830	2	GTWCEEQICECDR	44.80	NA		
		499.761	2	DIYPYSLK	40.81	NA		
		688.596	3	DATDRCCFVHDCCYGK	40.14	NA		
F12a	0.6	1563.7	1	CSSITELEKVNQR	8	99	Phosphodiesterase	T1DJT5
		2332.1	1	EQSSPLSCPFGPVPSPDVSGCK	8	99		
F12b	2.3	1870.9	1	NNPGILEYPVKPSEEGK	7	99	LAAO	K9N7B7
		1472.1	1	VIEIQQNDRETK	8	99		
F12c	4.5	415.885	3	DRVAAECLRR	46.48	NA	C-type lectin	J3S3U6
		427.739	2	RSLSTYK	46.25	NA		
		579.183	2	CCFVHDCC	46.11	NA		
		458.203	2	MFYPDSR	44.22	NA		
		538.894	3	YGYMFYPDSRCR	44.10	NA		
		723.818	2	CNTKWDIYPYS	43.12	NA		
		615.660	3	MIKFETRKNAIPFYA	43.03	NA		
		624.253	3	KGTWCEEQICECDR	42.62	NA		
		442.249	2	IYPYSLK	42.37	NA		
		607.606	3	FYGCYCGWGGRGRPK	41.28	NA	Phospholipase B	F8S101
		1056.921	2	GTWCEEQICECDRVAAE	41.06	NA		
		534.795	2	MIKFETRK	40.21	NA		
		522.695	2	DATDRCCF	39.82	NA		
		680.303	2	CNTKWDIYPY	39.66	NA		
		508.196	2	CYCGWGGR	39.19	NA		
		563.798	2	LKSGYITCGK	39.11	NA		
		479.260	2	GHLLQFNK	38.99	NA		
		688.315	2	SLSTYKYGYMF	38.28	NA		
		486.255	3	FETRKNAIPFYA	38.24	NA		
		759.817	2	SGYITCGKGTWCE	37.59	NA		
		843.317	2	TWCEEQICECDR	36.05	NA	PLA_2 (Basic)	P62022
		466.257	4	GHLLQFNKMIKFETR	35.71	NA		
		577.909	3	FYPDSRCRGPSETC	35.01	NA		
		553.790	4	MIKFETRKNAIPFYAFYG	34.10	NA		
		605.550	4	LRRSLSTYKYGYMFYPDSR	33.97	NA		
		853.116	4	SGYITCGKGTWCEEQICECDRVAAECLR	33.68	NA		
		598.771	2	CNTKWDIYP	33.19	NA		
		449.160	2	DATDRCC	32.76	NA		
		420.224	2	CYGKLAK	30.90	NA		
F13a	1.4	621.335	4	VREVKNNPGILEYPVKPSEEGK	100.82	NA	LAAO	C0HJE7
		503.995	4	TNCKYILDKYDTYSTK	93.99	NA		
		772.354	2	AHDRNPLEECFR	82.03	NA		
		539.783	4	YILDKYDTYSTKEYLLK	76.71	NA		
		736.885	2	VIEIQQNDRETK	75.48	NA		
		682.877	2	SAAQLYVESLRK	75.14	NA		
		484.262	4	KKDWYANLGPMRLPTK	72.97	NA		
		579.647	3	VLAGAGHQVTVLEASER	71.55	NA		

(*continued on next page*)

Table 1 (*continued*)

Gel band	% Relative abundance in whole venom	Ions		Peptides derived from Mass Spectrometry	Peptide ID Score	% Algorithm confidence	Protein family	ID code
		m/Z	Z					
		614.331	2	EKVQVHFNAR	69.43	NA		
		515.778	2	NGLTVTSNPK	68.17	NA		
		594.286	3	EDIQTFCRPSMIQR	67.25	NA		
		577.814	2	SGTKIFLTCK	66.10	NA		
		559.790	2	GGKSTTDLPSR	65.46	NA		
		602.310	2	FHGWIDSTIK	64.09	NA		
		673.076	4	RFDEIVGGMDQLPTSMYEAIKEK	62.34	NA		
		469.904	3	KFWEDDGIRGGK	61.79	NA		
		441.269	3	RIKFEPPLPPK	60.63	NA		
		500.008	4	FIESLKHDDIFGYEKR	60.34	NA		
		479.494	4	TYRKKDWYANLGPMR	60.27	NA		
		582.340	2	DLSLIHQLPK	58.36	NA		
		642.815	2	ADYVIVCTTSR	56.42	NA		
		695.809	2	EGNLSPGAVDMIGD	53.37	NA		
		602.979	3	QFQHFSEALTAPFKR	48.59	NA		
		503.762	2	EEFLEIAR	47.90	NA		
		573.261	2	DVNRASENPSG	47.15	NA		
		474.783	2	IVREYIR	46.48	NA		
		486.254	2	VQVHFNAR	45.16	NA		
		613.830	2	HVVIVGAGMAGLS	43.76	NA		
		407.712	2	VQVHFNA	42.13	NA		
		436.288	2	VVKELKR	38.81	NA		
		434.730	2	FQALDFK	34.27	NA		
		500.516	4	KNNPGILEYPVKPSEEGK	33.53	NA		
		570.315	2	HVVIVGAGMAGL	33.43	NA		
		452.243	2	RPSMIQR	33.31	NA		
		410.239	2	KFGLQLN	33.12	NA		
		510.593	3	IIALGHSGFSEDQR	78.90	NA	Nucleotidase	F8S0Z7
		713.331	2	DEVEELQNHANK	73.48	NA		
		653.860	2	QVPVVQAYAFGK	67.67	NA		
		524.267	3	HGEGMGELLQVSGIK	66.40	NA		
		426.233	2	VVYDLSR	63.95	NA		
		526.633	3	HANFPILSANIRPK	63.36	NA		
		523.299	2	VPTYVPLEK	61.94	NA		
		521.796	2	SSGNPILLNK	61.70	NA		
		672.274	2	CTGQDCYGGVAR	57.25	NA		
		696.026	3	SSGNPILLNKDISEDQDIK	56.24	NA		
		535.263	3	DISEDQDIKAEVNK	54.31	NA		
		476.277	2	VGIIGYTTK	54.07	NA		
		506.261	3	IQLHNYSSQEIGK	51.36	NA		
		423.256	2	LTTLGVNK	49.39	NA		
		555.773	2	QAFEHSVHR	48.39	NA		
		430.246	2	IINVGSEK	47.40	NA		
		413.555	3	EVVKFMNSLR	33.88	NA		
		843.386	3	NPCNIYYSPNDEDKGMVLPGTK	77.98	NA	SVMP	C5H5D1
		694.628	3	LRQGAQCAEGLCCDQCR	77.87	NA		
		566.898	3	AAKDECDMADVCTGR	73.88	NA		
		538.873	3	TCRDPCCDAATCK	69.92	NA		
		684.815	2	LYCFPNSPENK	68.80	NA		
		515.270	2	DHQEFLIK	67.48	NA		
		502.515	4	TRMYDIVNVITPIYHR	67.39	NA		
		629.931	3	NGQPCKNNNGYCYNGK	56.72	NA		
		433.861	3	STECTDRFQR	56.44	NA		
		513.243	2	FKGAGTECR	52.22	NA		
		642.280	2	EGNHYGYCRK	50.51	NA		
		471.714	2	ACFQFNR	49.20	NA		
		528.950	3	KKPLKTDVVSPAVCGN	47.02	NA		
		510.253	2	NMPQCILK	45.16	NA		
		614.229	2	DTCTCGTRPC	44.49	NA		
		709.814	2	EASFLFSDCSQK	44.11	NA		
		529.228	2	DACFQFNR	40.58	NA		
		559.307	2	WRATDLLSR	39.82	NA		
		478.226	3	IACEPQDVKCGR	35.66	NA		
		481.238	2	DYIMYLK	35.15	NA		
		902.364	2	YFVEVGEECDCGSPR	34.22	NA		
F13b	1.4	1155.4	1	EGNHYGYCR	8	99	SVMP (Disintegrin-like	Q076D1
		1299.5	1	STECTDRFQR	7	95.4	crotastatina)	
F13c	1.2	502.515	4	TRMYDIVNVITPIYH	84.43	NA	SVMP	C5H5D1
		572.231	3	AAKDECDMADVCTGR	76.23	NA		
		684.815	2	LYCFPNSPENK	70.56	NA		
		716.835	2	IACEPQDVKCGR	64.83	NA		
		433.861	3	STECTDRFQR	62.64	NA		

(*continued on next page*)

Table 1 (*continued*)

Gel band	% Relative abundance in whole venom	Ions		Peptides derived from Mass Spectrometry	Peptide ID Score	% Algorithm confidence	Protein family	ID code
		m/Z	Z					
		602.244	2	NNNGYCYNGK	58.43	NA		
		471.714	2	ACFQFNR	58.41	NA		
		843.382	3	NPCNIYYSPNDEDKGMVLPGTK	58.15	NA		
		642.280	2	EGNHYGYCRK	56.26	NA		
		513.243	2	FKGAGTECR	52.84	NA		
		515.270	2	DHQEFLIK	50.91	NA		
		807.806	2	TCRDPCCDAATCK	47.76	NA		
		559.307	2	WRATDLLSR	43.30	NA		
		510.252	2	NMPQCILK	41.50	NA		
		906.844	2	QGAQCAEGLCCDQCR	38.63	NA		
		586.256	3	TWDDAERFCTEQAK	77.79	NA	C-type lectin	Q7LZ71
		691.257	2	DCPSGWSSYEGH	49.95	NA		
		671.315	2	GGHLVSIESAGEAD	49.22	NA		

PLA_2s, phospholipases A_2; SVSPs, serine proteinases; BPPs, bradykinin-potentiating peptides; LAAOs, L-amino acid oxidases; SVMP-ITs, SVMP-inhibitor tripeptides; SVMPs, Zn^{2+}-dependent metalloproteinases; PLB, phospholipases B.
NA: Not assigned (see details of the analysis in methodology).

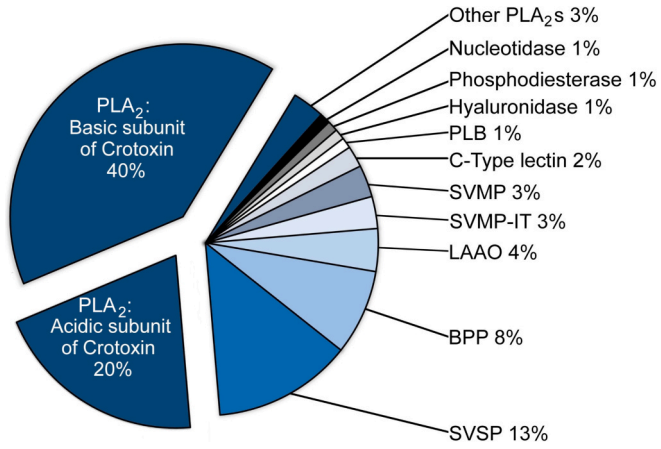

Fig. 3. Relative abundance of the main protein families of *C. d. pifanorum* venom identified by mass spectrometry from gel-digested protein bands. In the case of peptides present in the first HPLC peaks, identification was performed by direct mass spectrometry analysis. PLA_2s, phospholipases A_2; SVSPs, serine proteinases; BPPs, bradykinin-potentiating peptides; LAAOs, L-amino acid oxidases; SVMP-ITs, SVMP-inhibitor tripeptides; SVMPs, Zn^{2+}-dependent metalloproteinases; PLB, phospholipases B.

3. Results and discussion

3.1. Characterization of the proteome of C. d. pifanorum venom

The separation of the venom by RP-HPLC resulted in 13 fractions, which were numbered according to their order of elution (Fig. 1). The analysis by SDS-PAGE evidenced the absence of protein components in fractions F1, F2, F3, F5 and F6, whereas the rest of the fractions showed protein bands (Fig. 2). Table 1 shows the peptides identified by mass spectrometry in each band and fraction.

PLA_2s constitute 63% of the venom (Fig. 3). Most of these PLA_2s correspond to the highly toxic basic subunit of crotoxin (subunit B; 40%) and the acidic subunit of crotoxin (subunit A; 20%). Crotoxin is a component with potent neurotoxic and systemic myotoxic activities [30,31], which has also been shown to have immunomodulatory properties that reduce the immune response towards co-immunogens [10–12].

SVSPs comprise 13% of venom proteins (Fig. 3), including thrombin-like and other kallikrein-like enzymes. Thrombin-like enzymes contribute to defibrinogenation by releasing fibrinopeptides A or B from fibrinogen, and therefore reducing plasma fibrinogen concentration [32]. This causes the formation of micro-clots that are easily degraded by the fibrinolytic system. A thrombin-like enzyme has been characterized from the venom of *C. d. terrificus* [33]. Kallikrein-type SVSPs contribute to hypotension through the generation of bradykinin [34]. Whether the SVSPs present in *C. d. pifanorum* venom exert these activities remains to be determined.

Other protein families which are also present in the venom of *C. d. pifanorum* are 8% Bradykinin-potentiating peptides (BPPs), 4% L-amino acid oxidases (LAAOs), 3% SVMP inhibitor tripeptides, 3% SVMPs, 2% C-type lectin-like proteins, 1% phospholipases B, 1% hyaluronidases, 1% phosphodiesterases, and 1% nucleotidases (Fig. 3).

Based on the SVMPs content and overall toxicity, Mackessy classified venoms of rattlesnakes as type I (i.e., venoms with high content of SVMPs and relatively low toxicity), or type II (i.e., venoms with low

Table 2
Enzymatic and toxic activities of the native and inhibited venom of *C. d. pifanorum*.

Activity	Venom of *C. d. pifanorum*			
	Native	Inhibited with *p*-BPB	Inhibited with PMSF	Inhibited with Batimastat
Lethality (LD_{50})[a]	1.6 (1.0–2.9)	3.3 (1.4–4.4)	2.7 (2.0–3.5)	1.1 (0.7–1.6)
PLA_2[b]	4.2 ± 1.7	1.7 ± 1.1	ND	ND
Myotoxic[c]	1297 ± 137	426 ± 65[h]	ND	ND
Coagulant (MCD)[d]	6.4 ± 0.9	6.4 ± 0.2	195.4 ± 0.4[h]	6.6 ± 0.9
Defibrinogenating (MDD)[e]	1	4	>8[h]	ND
Azocaseinolytic[f]	0.02 ± 0.01	ND	ND	ND
Hemorrhagic (MHD)[g]	>8	ND	ND	ND

ND: Not determined.
[a] LD_{50}: The dose of venom (μg) at which the venom kills 50% of challenged mice.
[b] PLA_2 activity is reported as μEq of fatty acid/mg venom/min.
[c] Myotoxic activity is reported as CK units/L. Venom injected = 10 μg.
[d] MCD: The dose of venom (μg) that causes the coagulation of plasma in 60 s.
[e] MDD: The lowest amount of venom (μg) that induced blood incoagulability in all injected animals.
[f] Azocaseinolytic activity is reported as units/mg venom.
[g] MHD: The dose of venom (μg) that generates 50 HaU.
[h] Significant differences when compared to the native venom.

content of SVMPs and high toxicity) [35]. Examples of type I venoms are those of *C. basiliscus* (14% PLA_2s, 11% SVSPs and 68% SVMPs) [36], and adult *C. simus* (8% PLA_2s, 5% SVSPs and 72% SVMPs) [9]. Examples of type II venoms are *C. horridus* (32% PLA_2s, 58% SVSPs and 0% SVMPs) [37], *C. d. terrificus* (59% PLA_2s, 8% SVSPs and 5% SVMPs) [9], and phenotype A *C. s. scutulatus* (63% PLA_2s, 31% SVSPs, and 0% SVMPs) [38]. The relative abundance of protein families in the venom of *C. d. pifanorum* indicates that this venom corresponds to a type II rattlesnake venom.

3.2. Enzymatic and toxic activities of venom of C. d. pifanorum

The venom of *C. d. pifanorum* showed lethal, PLA_2, myotoxic, coagulant and defibrinogenating activities, but no azocaseinolytic and hemorrhagic activities (Table 2). In the broad spectrum of rattlesnake venoms, ranging from neurotoxic no hemorrhagic venoms to hemorrhagic no neurotoxic venoms [38], enzymatic and toxicological profiles of *C. d. pifanorum* places it within the group of type II rattlesnake venoms, which is in accordance with the proteomic profile of the venom.

The high toxicity of *C. d. pifanorum* venom, together with the abundance of the two subunits of crotoxin in this venom, strongly suggests that lethality is mostly due to the neurotoxic paralytic activity of this heterodimeric presynaptic neurotoxin. In agreement, mice injected with the venom showed signs of muscle paralysis and respiratory difficulties. Therefore, it was of interest to assess whether the inhibition of PLA_2 activity by *p*-BPB would reduce toxicity.

Results indicate that there was only a slight, but not significant, reduction in both PLA_2 activity and lethality upon incubation of venom with this inhibitor (Table 2). It has been previously observed that *p*-BPB is able to inhibit the enzymatic activity of the subunit crotoxin B, but not of the whole crotoxin heterodimer [39,40]. The structural explanation of this finding has to do with the fact that subunit A of crotoxin partially masks the catalytic site of subunit B and sterically occludes the access of phospholipids to catalytic residues [41]. Therefore, based on these structural data, it is likely that subunit A also affects the accessibility of *p*-BPB to the His residue in the catalytic site of subunit B [39,40].

The reduction in enzymatic activity after incubation with *p*-BPB may be due to the inhibition of PLA_2s other than crotoxin, including monomeric subunit B whose capacity to form heterodimeric complexes is limited by the amount of subunit A [42], as evidenced by proteomic analysis. Inhibition of *C. d. pifanorum* venom with *p*-BPB resulted in a partial but significant reduction of the myotoxic activity ($t = 2.342$; df = 4; $P = 0.001$), indicating that PLA_2s different from crotoxin may play a role in the myotoxic activity of this venom.

No significant reduction in lethality was observed when *C. d. pifanorum* venom was incubated with the serine proteinase inhibitor PMSF (Table 2), implying that SVSPs do not play a key role in venom lethality. On the other hand, the significant reduction of coagulant and defibrinogenating activities after inhibition of venom with PMSF ($F = 114{,}379.905$; df = 3; $P = 0.001$; Table 2), indicates that SVSPs determine these activities, most likely due to their thrombin-like effect. Thrombin-like SVSPs have been isolated from the venoms of other subspecies of *C. durissus* [33,43].

Inhibition of SVMPs with Batimastat did not affect the lethality or coagulant activity of the venom in mice (Table 2), which is in accordance with the low content of SVMPs in this venom. Proteomic and enzyme inhibition results suggest that the overall toxicity of this venom is due to the action of crotoxin, and that venom-induced coagulopathies, are due to thrombin-like SVSPs.

3.3. Neutralization of C. d. pifanorum venom

Lethality of *C. d. pifanorum* venom was neutralized by the Quadrivalent antivenom (i.e., raised towards *B. asper*, *C. simus*, *L. stenophrys*, and *C. d. pifanorum* venoms), whereas it was not neutralized by the Trivalent antivenom (i.e., raised towards *B. asper*, *C. simus*, and L. *stenophrys* venoms) (Table 3). This result indicates that lethal toxins of *C. d. pifanorum* venom, most likely crotoxin, have antigenic determinants that are no shared with their homologs in venoms of *B. asper*, *C. simus* and L. *stenophrys*.

Table 3
Neutralization of venom of *C. d. pifanorum* by the two antivenoms.

Activity	Antivenom	
	Trivalent	Quadrivalent
Lethality (ED_{50})[a]	>3846	1665[h] (1149–2439)
Potency[b]	<0.20	0.45[h] (0.31–0.65)
PLA_2 (ED_{50})[c]	2249 ± 131	2061 ± 250
Myotoxic[d]	290 ± 82	189 ± 104
Coagulant (ED)[e]	515 ± 2	440 ± 13[h]
Defibrinogenating (ED)[f]	500	500
Azocaseinolytic (ED_{50})	ND	ND
Hemorrhagic (ED_{50})	ND	ND

ND: Not determined.
[a] ED_{50}: The ratio microliters antivenom per mg venom at which the lethality of venom was reduced to 50%.
[b] Potency was expressed as mg venom neutralized per mL antivenom.
[c] ED_{50}: The ratio microliters antivenom per mg venom at which the PLA_2 activity of venom was reduced to 50%.
[d] Neutralization of myotoxic activity is expressed as the residual plasma CK activity (IU/L) at a ratio of 333 μl antivenom per mg venom. CK activity in mice injected with a challenge dose of venom (i.e., 25 μg) without antivenom was 1308 ± 246 IU/L.
[e] ED: The ratio microliters antivenom per mg venom at which the clotting time of plasma was prolonged three times as compared to the clotting time of plasma incubated with the venom alone.
[f] ED: The lowest ratio microliters antivenom per mg venom at which the blood samples of all mice clotted.
[h] Significant differences when compared with the Trivalent antivenom.

Table 4
Enzymatic and toxic activities of some heterologous rattlesnake venoms.

Activity	Venoms		
	C. horridus	*C. d. terrificus*	*C. s. scutulatus*
Lethality (LD_{50})[a]	6.1 (4.7–8.7)	0.6 (0.4–0.8)	1.0 (0.6–1.5)
PLA_2[b]	6.5 ± 1.0	6.5 ± 1.0	5.0 ± 1.0
Myotoxic[c]	729 ± 92	1216 ± 292	1069 ± 106
Coagulant (MCD)[d]	5356 ± 5	22 ± 1	>152
Defibrinogenating (MDD)[e]	>8	2	>8
Azocaseinolytic[f]	0.24 ± 0.04	0.10 ± 0.04	0.34 ± 0.01
Hemorrhagic (MHD)[g]	6.93 ± 0.17	>8	>8

[a] LD_{50}: The dose of venom (μg) at which the venom kills 50% of challenged mice.
[b] PLA_2 activity is reported as μEq of fatty acid/mg venom/min.
[c] Myotoxic activity is reported as CK units/L. Venom injected = 10 μg.
[d] MCD: The dose of venom (μg) that causes the coagulation of plasma in 60 s.
[e] MDD: The lowest amount of venom (μg) that induced blood incoagulability in all injected animals.
[f] Azocaseinolytic activity is reported as units/mg venom.
[g] MHD: The dose of venom (μg) that generates 50 HaU.

PLA_2s and myotoxic activities were similarly neutralized by both antivenoms (Table 3). This result suggests that *C. d. pifanorum* PLA_2s responsible for the enzymatic and myotoxic activities share antigenic determinants with some PLA_2s present in the venoms of *B. asper*, *C. simus* and/or L. *stenophrys*. While coagulant activity was better neutralized by the Quadrivalent antivenom ($t = 9.523$; df = 4; $P = 0.001$), no differences were observed between antivenoms in the neutralization of defibrinogenating activity (Table 3), which could mean that there are antigenic differences between coagulant SVSPs of *C. d. pifanorum* with those of *B. asper*, *C. simus* and/or *L. stenophrys*, that are not evidenced by the lower sensitive technique used to evaluate in vivo defibrinogenation.

Table 5
Neutralization of some heterologous rattlesnake venoms.

Venom	Activity	Antivenom	
		Trivalent	Quadrivalent
C. horridus	Lethality (ED_{50})[a]	341 (239–485)	400 (303–555)
	Potency[b]	2.20 1.55–3.14)	1.88 (1.35–2.48)
	PLA_2 (ED_{50})[c]	1172 ± 97	652 ± 19[i]
	Myotoxic[d]	268 ± 86	286 ± 70
	Coagulant (ED)[e]	ND	ND
	Defibrinogenating (ED)[f]	ND	ND
	Azocaseinolytic (ED_{50})[g]	432 ± 69	466 ± 83
	Hemorrhagic (ED_{50})[h]	33 ± 5	72 ± 20[i]
C. d. terrificus	Lethality (ED_{50})[a]	1923 (1205–3030)	1786 (1428–2500)
	Potency[b]	0.39 (0.25–0.62)	0.42 (0.30–0.52)
	PLA_2 (ED_{50})[c]	1012 ± 6	978 ± 60
	Myotoxic[d]	794 ± 202	439 ± 307
	Coagulant (ED)[e]	248 ± 18	221 ± 1
	Defibrinogenating (ED)[f]	125	250
	Azocaseinolytic (ED_{50})[g]	ND	ND
	Hemorrhagic (ED_{50})[h]	ND	ND
C. s. scutulatus	Lethality (ED_{50})[a]	3030 (1923–10,000)	1250[i] (909-1665)
	Potency[b]	0.25 (0.08–0.39)	0.60[i] (0.45–0.82)
	PLA_2 (ED_{50})[c]	1085 ± 107	943 ± 36
	Myotoxic[d]	932 ± 79	649 ± 48
	Coagulant (ED)[e]	ND	ND
	Defibrinogenating (ED)[f]	ND	ND
	Azocaseinolytic (ED_{50})[g]	ND	ND
	Hemorrhagic (ED_{50})[h]	ND	ND

ND: Not determined.
[a] ED_{50}: The ratio microliters antivenom per mg venom at which the lethality of venom was reduced to 50%.
[b] Potency was expressed as mg venom neutralized per mL antivenom.
[c] ED_{50}: The ratio microliters antivenom per mg venom at which the PLA_2 activity of venom was reduced to 50%.
[d] Neutralization of myotoxic activity is expressed as the residual plasma CK activity (IU/L) at a ratio of 333 μl antivenom per mg venom. CK activity in mice injected with a challenge dose of venom (i.e., 5 μg of *C. horridus* venom, or 25 μg of *C.s. scutulatus* or *C. d. terrificus* venoms) without antivenom was 594 ± 179 IU/L for *C. horridus*, 1250 ± 303 IU/L for *C. d. terrificus* and 1140 ± 149 IU/L for *C. s. scutulatus*.
[e] ED: The ratio microliters antivenom per mg venom at which the clotting time of plasma was prolonged three times as compared to the clotting time of plasma incubated with the venom alone.
[f] ED: The lowest ratio microliters antivenom per mg venom at which the blood samples of all mice clotted.
[g] ED_{50}: The ratio microliters antivenom per mg venom at which the azocaseinolytic activity of venom was reduced to 50%.
[h] ED_{50}: The ratio microliters antivenom per mg venom at which the hemorrhagic activity of venom was reduced to 50%.
[i] Significant differences when compared with the Trivalent antivenom.

3.4. Cross-neutralization of some heterologous rattlesnake venoms

Similar antigenicity is expected between venoms of phylogenetically related snakes. Thus, it was hypothesized that the Quadrivalent antivenom would show an improved cross-neutralization of some heterologous type II rattlesnake venoms, as compared with the Trivalent antivenom. To test this hypothesis, we determined the enzymatic and toxic activities of venoms of *C. horridus*, *C. d. terrificus*, and *C. s. scutulatus* (Table 4) and compared the ability of both Trivalent and Quadrivalent to neutralize these activities.

No significant differences were observed between the antivenoms in the neutralization of several activities of the venoms of *C. horridus* and *C. d. terrificus*, except for the PLA_2 activity of *C. horridus* ($t = 5.128$; df = 4; $P = 0.007$; Table 5). Moreover, the hemorrhagic activity of *C. horridus* venom was better neutralized by the Trivalent antivenom ($t = -3.181$; df = 4; $P = 0.034$).

In general terms, the neutralizing ability of the Trivalent antivenom towards these two venoms was not improved by the inclusion of *C. d. pifanorum* in the group of immunogens. The ability of Trivalent antivenom to neutralize lethality of *C. d. terrificus* venom could be explained by the presence of crotoxin in the standard venom of *C. simus* used as immunogen (our unpublished observations).

In contrast, the lethal activity of the venom of *C. s. scutulatus* was better neutralized by the Quadrivalent antivenom (Table 5). This result suggests that there are antigenic similarities between Mojave toxin of *C. s. scutulatus* [38] and the crotoxin-like toxins in venom of *C. d. pifanorum*. Interestingly, no significant differences were found in the ability of both antivenoms to neutralize PLA_2 or myotoxic activities of *C. s. scutulatus* venom (Table 5). This might be due to the presence of PLA_2s in the venoms of *B. asper*, *C. simus* and L. *stenophrys* with immunological similarities with isoforms in *C. d. pifanorum* venom.

Table 6
Enzymatic and toxic activities of venoms used to produce the Trivalent antivenom.

Activity	Venoms		
	B. asper	*C. simus*	*L. stenophrys*
Lethality (LD_{50})[a]	62.2 (46.9–96.6)	10.4 (7.6–17.3)	119.5 (85.1–163.8)
PLA_2[b]	14.9 ± 0.8	17.8 ± 0.8	22.1 ± 0.8
Myotoxic[c]	735 ± 234	890 ± 104	448 ± 124
Coagulant (MCD)[d]	0.2 ± 0.2	41.1 ± 1.8	10.8 ± 0.2
Defibrinogenating (MDD)[e]	3	2	6
Azocaseinolytic[f]	1.43 ± 0.38	1.35 ± 0.35	2.02 ± 0.22
Hemorrhagic (MHD)[g]	3.19 ± 0.39	4.05 ± 0.74	3.34 ± 0.42

[a] LD_{50}: The dose of venom (μg) at which the venom kills 50% of challenged mice.
[b] PLA_2 activity is reported as μEq of fatty acid/mg venom/min.
[c] Myotoxic activity is reported as CK units/L. Venom injected = 10 μg.
[d] MCD: The dose of venom (μg) that causes the coagulation of plasma in 60 s.
[e] MDD: The lowest amount of venom (μg) that induced blood incoagulability in all injected animals.
[f] Azocaseinolytic activity is reported as units/mg venom.
[g] MHD: The dose of venom (μg) that generates 50 HaU.

3.5. Neutralization of *B. asper*, *C. simus* and *L. stenophrys* venoms

Since immunosuppressive properties of crotoxin have been demonstrated [10–12], we hypothesized that the use of *C. d. pifanorum* venom to immunize animals for antivenom production could result in a reduction of the ability of the antivenom to neutralize the toxic effects of the other co-immunogen venoms. To test this hypothesis, we determined the enzymatic and toxic activities of venoms of *B. asper*, *C. simus* and *L. stenophrys* (Table 6) and compared the ability of both Trivalent and Quadrivalent antivenoms to neutralize these activities.

Contrary to our predictions, Quadrivalent antivenom did not have a lower ability to neutralize lethality of the co-immunogen venoms (Table 7). In fact, the lethal activity of *C. simus* was better neutralized by the Quadrivalent antivenom (Table 7). Elucidation of the mechanism by which complementation of the group of immunogens used to produce PoliVal-ICP with the venom of *C. d. pifanorum* results in improvement of the neutralizing potency of the antivenom towards the venom of *C. simus* deserves additional research.

The Quadrivalent antivenom has higher ability than Trivalent antivenom to neutralize PLA_2 activity ($t = 9.931$; df = 4; $P = 0.001$), coagulant activity ($t = 24.584$; df = 4; $P = 0.001$), and proteinase activity ($t = 4.740$; df = 4; $P = 0.009$) of *B. asper* venom; coagulant activity of *C. simus* venom ($t = 5.661$; df = 4; $P = 0.005$); and myotoxic activity of L. *stenophrys* venom ($t = 5.645$; df = 4; $P = 0.005$). In contrast, the

Table 7
Neutralization of the venoms used to produce the Trivalent antivenom.

Venom	Activity	Antivenom	
		Trivalent	Quadrivalent
B. asper	Lethality (ED_{50})[a]	291 (224–388)	244 (170–355)
	Potency[b]	2.58 (1.93–3.35)	3.07 (2.11–4.41)
	PLA_2 (ED_{50})[c]	3922 ± 68	1638 ± 393[i]
	Myotoxic[d]	550 ± 84	371 ± 159
	Coagulant (ED)[e]	277 ± 13	87 ± 2[i]
	Defibrinogenating (ED)[f]	250	250
	Azocaseinolytic (ED_{50})[g]	492 ± 47	307 ± 26[i]
	Hemorrhagic (ED_{50})[h]	26 ± 6	55 ± 6[i]
C. simus	Lethality (ED_{50})[a]	356 (267–457)	179[i] (132–232)
	Potency[b]	2.11 (1.64–2.81)	4.19[i] (3.23–5.68)
	PLA_2 (ED_{50})[c]	453 ± 222	486 ± 14
	Myotoxic[d]	836 ± 75	677 ± 325
	Coagulant (ED)[e]	188 ± 10	149 ± 6[i]
	Defibrinogenating (ED)[f]	250	250
	Azocaseinolytic (ED_{50})[g]	543 ± 76	499 ± 73
	Hemorrhagic (ED_{50})[h]	175 ± 80	235 ± 160
L. stenophrys	Lethality (ED_{50})[a]	196 (139–268)	184 (130–260)
	Potency[b]	3.83 (2.80–5.40)	4.08 (2.88–5.77)
	PLA_2 (ED_{50})[c]	1172 ± 104	1199 ± 47
	Myotoxic[d]	437 ± 16	223 ± 64[i]
	Coagulant (ED)[e]	300 ± 3	511 ± 1[i]
	Defibrinogenating (ED)[f]	1000	1000
	Azocaseinolytic (ED_{50})[g]	413 ± 26	477 ± 18[i]
	Hemorrhagic (ED_{50})[h]	69 ± 15	84 ± 22

[a] ED_{50}: The ratio microliters antivenom per mg venom at which the lethality of venom was reduced to 50%.
[b] Potency was expressed as mg venom neutralized per mL antivenom.
[c] ED_{50}: The ratio microliters antivenom per mg venom at which the PLA_2 activity of venom was reduced to 50%.
[d] Neutralization of myotoxic activity is expressed as the residual plasma CK activity (IU/L) at a of 333 μl antivenom per mg venom. CK activity in mice injected with a challenge dose of venom (i.e., 5 μg) without antivenom was 564 ± 284 IU/L for *B. asper*, 850 ± 145 IU/L for *C. simus* and 444 ± 168 IU/L for L. *stenophrys*.
[e] ED: The ratio microliters antivenom per mg venom at which the clotting time of plasma was prolonged three times as compared to the clotting time of plasma incubated with the venom alone.
[f] ED: The lowest ratio microliters antivenom per mg venom at which the blood samples of all mice clotted.
[g] ED_{50}: The ratio microliters antivenom per mg venom at which the azocaseinolytic activity of venom was reduced to 50%.
[h] ED_{50}: The ratio microliters antivenom per mg venom at which the hemorrhagic activity of venom was reduced to 50%.
[i] Significant differences when compared with the Trivalent antivenom.

Trivalent antivenom has higher ability to neutralize the hemorrhagic activity of *B. asper* venom ($t = -5.836$; df = 4; $P = 0.004$), and the coagulant ($t = -107.4897$ df = 4; $P = 0.001$) and proteinase activities ($t = -3.497$; df = 4; $P = 0.025$) of *L. stenophrys* venom. Both antivenoms showed similar neutralizing activity towards the rest of activities tested (Table 7). Thus, the addition of *C. d. pifanorum* venom to the immunizing mixture did not result in a reduction in the ability to neutralize the other three venoms.

4. Concluding remarks

The venom of *C. d. pifanorum* is mainly composed of PLA_2s, with predominance of crotoxin subunits and SVSPs, with low amounts of SVMPs, hence corresponding to type II rattlesnake venom. The incorporation of this venom into the antigenic mixture to generate the PoliVal-ICP antivenom improved its neutralizing scope towards *C. d. pifanorum* and *C. scutulatus* venoms, and the neutralization of *C. simus* venom, without affecting the potency against *B. asper* and L. *stenophrys* venoms. It is therefore concluded that such enrichment of the immunizing mixture with a venom containing high amounts of crotoxin generates an antivenom that could be effective against predominantly neurotoxic rattlesnake venoms, hence potentially amplifying the geographical coverage of PoliVal-ICP and ensuring therapeutic efficacy against envenomings by neurotoxic *Crotalus sp* kept in private serpentariums in Costa Rica.

Ethical statement

This manuscript presents an experimental study performed following the standard procedures of scientific ethics, including those related to the use and care of animals.

Data availability

No data was used for the research described in the article.

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