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Venom characterization of the three species of *Ophryacus* and proteomic profiling of *O. sphenophrys* unveils Sphenotoxin, a novel Crotoxin-like heterodimeric β-neurotoxin

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A B S T R A C T

Venoms of the three species of *Ophryacus* (*O. sphenophrys, O. smaragdinus,* and *O. undulatus*), a viperid genus endemic to Mexico, were analyzed for the first time in the present work. The three venoms lacked procoagulant activity on human plasma, but induced hemorrhage and were highly lethal to mice. These venoms also displayed proteolytic and phospholipase A_2 activities in vitro. The venom of *O. sphenophrys* was the most lethal and caused hind-limb paralysis in mice. Proteomic profiling of *O. sphenophrys* venom showed a predominance of metalloproteinase (34.9%), phospholipase A_2 (24.8%) and serine protease (17.1%) in its composition. Strikingly, within its PLA_2 components, 12.9% corresponded to a Crotoxin-like heterodimer, here named Sphenotoxin, which was not found in the other two species of *Ophryacus*. Sphenotoxin, like Crotoxin, is composed of non-covalently bound A and B subunits. Partial amino acid sequence was obtained for Sphenotoxin B and was similar (78–89%) to other subunits described. The mouse i.v. LD_{50} of Sphenotoxin at 1:1 M radio was 0.16 μg/g. Also, like Crotoxin, Sphenotoxin induced a potent neuromuscular blockade in the phrenic nerve-diaphragm preparation. *Ophryacus* is the fifth genus and *O. sphenophrys* the third non-rattlesnake species shown to contain a novel Crotoxin-like heterodimeric β-neurotoxin.

Biological significance: *Ophryacus* is an endemic genus of semi-arboreal pitvipers from Mexico that includes three species with restricted distributions. Little is known about the natural history of these species and nothing is known about the properties of their venoms. Research on these species' venoms could generate relevant information regarding venom composition of Mexican pitvipers. Additionally, research into the presence of neurotoxic Crotoxin-like molecules outside of rattlesnakes (genera *Crotalus* and *Sistrurus*) has identified this molecule in several new genera. Knowing which genera and species possess neurotoxic components is important to fully understand the repercussions of snakebites, the interaction with prey and predators, and the origin, evolution, and phylogenetic distribution of Crotoxin-like molecules during the evolutionary history of pitvipers.

Our study expands current knowledge regarding venom's compositions and function from Mexican pitvipers, providing a comparative venom characterization of major activities in the three *Ophryacus* species. Additionally, the discovery and characterization of a novel Crotoxin-like molecule, here named Sphenotoxin, in *O. sphenophrys,* and the detailed protein composition of *O. sphenophrys* venom supports the hypotheses that Crotoxin-like -β-neurotoxins are more widespread than initially thought.

1. Introduction

Snake venoms consist of inorganic and organic molecules with proteins constituting 90 to 95% of the dry weight [1]. Most venom proteins cause toxic effects in prey or in envenomated individuals. Overall, 63 protein families have been reported in snake venoms [2] but the venom of each species usually contains representatives of 10–20 families. The presence of multiple variants within the same protein family results in a significant increase in snake venom complexity [3]. In Crotalinae (pitviper) venoms, the most abundant and frequently found proteins belong to a small number of protein families: phospholipase A_2 (PLA_2), snake venom metalloproteinases (SVMPs), snake venom Serine proteases (SVSPs), L-amino acid oxidase (LAAO), cysteine-rich secretory protein (CRiSP), C-type lectin/lectin-like (CTL),

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disintegrin (DIS) and natriuretic peptide (NP) [2,4,5]. Among these, SVMPs, SVSPs, and PLA_2 enzymes are prominent in abundance and primarily responsible for the local and systemic pathological effects of envenomation [2,6–8].

The application of proteomic tools to snake venom research has greatly broadened our view on the overall composition of venom, for an increasing number of species worldwide [2,3]. Understanding venom composition may help elucidate evolutionary relationships and ecological similarity among species [9,10] and, moreover, may predict toxin presence and venom activities in other species. Further, integrative approaches combining functional assays with additional features such as toxicity, immunogenicity, and antivenom neutralization are paving the way toward a better understanding of snake venoms and the improvement of antivenoms [3,5,8,11–13]. The breadth of venom diversity at all taxonomic scales and even in relatively well studied groups continues to expand as new taxa are analyzed. The increase in known diversity is more dramatic when taxa are analyzed for the first time.

For example, Crotoxin and Crotoxin-like molecules are being found in genera outside of rattlesnakes (*Crotalus* and *Sistrurus*), as more species are being analyzed venomically [14–17]. Crotoxin is a heterodimeric phospholipase A_2 (PLA_2) protein complex first described in the South American Rattlesnake, *Crotalus durissus terrificus* [18–21]. Crotoxin causes potent β-neurotoxicity and systemic myotoxicity and is comprised of two non-covalently bound subunits. The first, Crotapotin or subunit A, is a non-enzymatic, non-toxic, acidic PLA_2 (9490 Da). The second, subunit B, is a catalytically-active, toxic, basic PLA_2 (14,350 Da) [6,20,21]. The A subunit is a proteolytically-processed PLA_2 and functions as a chaperone which dissociates from the B subunit upon binding to its target to exert presynaptic neurotoxicity and myotoxicity [20,21]. Crotoxin has subsequently been reported in venoms of several closely related species of *C. durissus* including *C. simus* and *C. tzabcan* [22]. Additionally, Crotoxin-like molecules have been reported from other rattlesnake species including *C. scutulatus*, *C. tigris*, *C. horridus*, *Sistrurus tergeminus, C.* l. *klauberi* [11,15,16,20,23–28]. More recently, Crotoxin-like homologs of this potent neurotoxic complex were discovered in two non-rattlesnake venoms: Nigroviriditoxin in *Bothriechis nigroviridis* from Costa Rica [14,15], and gintexin in *Gloydius intermedius* from Northern China [16,29].

Because *Gloydius* is the hypothesized ancestor to New World pitvipers [16] and a Crotoxin-like molecule was identified in *G. intermedius*, Dowell et al. [30] hypothesized that Crotoxin-like molecules existed prior to the evolution of New World pitvipers. This was followed by subsequent loss of the heterodimer in taxa without it. Alternatively, the rarity of Crotoxin-like molecules among pitviper genera could be due to independent gains [15,30]. However, it is difficult to test these competing hypotheses because of the lack of knowledge of the presence and absence of Crotoxin-like molecules throughout the pitviper phylogeny. With the discovery of Nigroviriditoxin *in B. nigroviridis,* the likelihood of similar toxins occurring in other pitviper genera increased because *Bothriechis* is one of the genera that are part of the Central and South American lineage of pitvipers.

The genus *Ophryacus*, commonly known as Mexican Horned Pitvipers, includes three currently recognized species that, are all endemic to Mexico. All three species are considered semi-arboreal and have restricted distributions. *Ophryacus undulatus*, known as the Slender-horned Pitviper, is the species with the largest distribution, restricted to elevations above 1800 m, and occurs in both the southern Sierra Madre Oriental and the Sierra Madre del Sur, *O. smaragdinus,* the Emerald Horned Pitviper, is restricted to the humid windward slopes of the Sierra Madre Oriental in eastern Mexico and can be found at elevations of 1400 to 2400 m. Lastly, *O. sphenophrys,* the *Broad-horned Pitviper,* has the most restricted distribution and has only been found between the towns of La Soledad and Buenavista Loxicha, Oaxaca [31–33]. *Ophryacus* are considered among the least frequently encountered pitvipers in Mexico; thus, very little is known about their natural history and taxonomy [33]. Even less is known about the venoms of *Ophryacus* species, which until this study have remained completely unexplored.

The aim of the present work was to investigate, the biochemical and biological activities of the venoms of the three species of *Ophryacus* and test for the presence of a Crotoxin-like molecule in this genus. In this pursuit, we proteomically profiled the venom of *O. sphenophrys* and, as a result, characterized and named a novel Crotoxin-like β-neurotoxin, Sphenotoxin.

2. Materials and methods

2.1. Ethics statement

Animal experiments were approved by the Bioethics Committee of the UNAM (Universidad Nacional Autónoma de México) Biotechnology Institute. Snakes were collected under collection permit SGPA/DGVS/04788/17 issued by SEMARNAT (Secretariat of Environment and Natural Resources) in Mexico. Three specimens of *O. undulatus* used in this study belong to the Deval Herpetarium under registration number DGVS-CR-IN-0957-D.F./07. For all analyses using mice, we used the Institute of Cancer (ICR) strain which is also known as the CD-1 strain.

2.2. Venoms and crotoxin control

Venom samples were collected from six specimens (4 adults and 2 juveniles) of *Ophryacus sphenophrys* from Cerro Perico, Oaxaca; seven adults from *O. smaragdinus*, of which four were from Huayacutla and three from Tlacolutan, Veracruz; and four adults from *O. undulatus*, from Sierra de Tecpa, Guerrero. The animals were collected in the wild and kept in captivity for a 2 month period for venom extraction and then released in the same place. Venom was manually extracted either two or three times from each snake using 50 mL plastic tubes covered with parafilm (supplemental Table 1 A). The individual venoms were centrifuged to remove cellular debris, lyophilized, and stored at −20 °C until use. The individual dry venoms from each species were added in similar amounts and reconstituted to generate three representative pools of venom. Additionally, we used the individual venom of all six *O. sphenophrys* to assess the presence of a Crotoxin-like molecule at the individual level. When needed, a venom pool of *Crotalus simus* or *C. durissus terrificus* was used as a control for the presence of Crotoxin. Additionally, Crotoxin, purified from the venom of *C. d. terrificus* was used as a standard when comparing neurotoxic activity. Whole venom of *C. d. terificus* was kindly provided by Dr. Adolfo de Roodt (Institutos de Salud “Dr Carlos G. Malbrán”, Argentina). Venom from *C. atrox* was used as a negative control for Crotoxin

2.3. Protein concentration

The protein concentration of the pooled samples of the three *Ophryacus* venoms was determined with the Pierce® Bicinchoninic Acid (BCA) Protein Assay (Thermo Scientific), using bovine serum albumin (BSA) as a standard, according to the manufacturer's protocols.

2.4. Hemorrhagic activity

Hemorrhagic activity for the three species of *Ophryacus* was determined using mice (25–28 g) in groups of five as previously described [34,35]. Different amounts of venoms were dissolved in 50 µL of PBS and injected into the shaved backs of the mice intradermally. After 3 h, mice were euthanized by CO_2 inhalation, their skin removed, and the perpendicular diameter of each lesion was measured with a Vernier caliper. Hemorrhagic activity was determined using the Minimum Hemorrhagic Dose (MHD) corresponding to the amount of venom that induces a hemorrhagic spot of 10 mm diameter

2.5. Coagulant activity

To determine the coagulant activity for each species, various amounts of venom was dissolved in 100 µL of 150 mM NaCl and added each to 200 µL of human citrated plasma obtained from healthy donors at 37 °C. The clotting time was recorded and coagulant activity was reported as the Minimum Procoagulant Dose (MPD) defined as the amount of venom needed to form a clot in 60 s [35].

2.6. Proteolytic activity

The hide powder azure (HPA) substrate was used to determine proteolytic activity for each species. Venom (100 µg) was added to HPA (5 mg/mL in 0.1 M Tris-HCl, pH 8.0) in a final volume of 1 mL and incubated for 2 h under constant mixing at room temperature in triplicate. After centrifugation at 15000 xg for 5 min, supernatants were separated and absorbances were measured at 595 nm. A standard curve was performed by adding 100, 200, or 300 µg of trypsin in 50 µL of HPA substrate. One unit of enzymatic activity (U) was defined as the amount of venom needed to degrade 1 mg of substrate over the 2 h incubation period at room temperature and reported as specific activity [26,27].

2.7. Phospholipase A_2 (PLA_2) activity

The synthetic substrate 4-nitro-3-(octanoyloxy)-benzoic acid (4-NOBA) was used to determine PLA_2 activity for the three species of *Ophryacus* [36,37]. Triplicate microplate wells were filled with 200 µL of reaction buffer (10 mM Tris, 10 mM $CaCl_2$, 0.1 M NaCl, pH 8.0) and 25 µL of substrate (4-NOBA; 1 mg/mL acetonitrile) to achieve a final substrate concentration of 0.32 mM and preincubated for 10 min at 37 °C. Then, 1 µg of venom in 25 µL of PBS was added per well and the reaction mixtures were incubated at 37 °C. Absorbances were read at 450 nm every 3 min for 30 min using an ELISA plate reader (Magellan R). Wells containing all reagents except venom were used as blanks. Units of specific enzymatic activity (U/mg) were defined as nanomoles of product (4-nitrobenzoic acid (4-NBA); $[M] = A_{450}/(l*\mathcal{E})$; $l = 0.82$ cm, $\mathcal{E} = 5039$) generated per minute per mg of venom. Data were analyzed by linear regression of the linear part of the curve to calculate slope using the software GraphPad Prism v 6.01.

2.8. Mouse phrenic nerve–hemidiaphragm neuromuscular preparation

Because Crotoxin acts on the presynaptic button of the neuromuscular junction, we used the mouse phrenic nerve-hemidiaphragm preparation to test for neurotoxic activity in the three venoms. The pure Crotoxin sample isolated from *C. d. terrificus* was used as a positive control. The preparation was mounted using a variation of the method originally described by Büllbring [38]. Briefly, an ICR mouse of 30–40 g body weight was euthanized using CO_2 inhalation and the left phrenic nerve with its attached half of the diaphragm muscle was immediately excised. The tissue was mounted in a 25 mL organ bath in Tyrode solution (0.4 mM NaH_2PO_4, 2.7 mM KCl, 1.8 mM $CaCl_2$, 1.0 mM $MgCl_2$, 137 mM NaCl and 11.9 mM $NaHCO_3$, pH 7.2) with 11 mM glucose, at 37 °C, as follows: the costal side of the diaphragm was held using a platinum hook to the bottom of the preparation, while the tendinous center of the diaphragm was tied to an isotonic force transducer (50 g, BioPac Systems UIM100 with TSD125C signal amplifier) which held the tissue to 5 g of resting tension and recorded muscle twitches. The whole preparation was constantly bubbled with carbogen solution (95% O_2, 5% CO_2) to oxygenate the tissue.

Nerve stimulation was performed using two platinum electrodes isolated from the Tyrode solution and directly in contact with the phrenic nerve. Direct muscle stimulation was performed using two platinum electrodes, one directly in contact with the diaphragm tissue and the other submerged in the Tyrode solution. Both stimulations consisted of supramaximal voltage pulses with duration of 0.1 ms (nerve) or 1 ms (muscle) and a frequency of 0.2 pulses/s. The mounted preparation was allowed to stabilize under nerve stimulation for 20 min. Then, 10 µM of δ-tubocurarine, a reversible blocker of neuromuscular transmission, was added to the solution, incubated for 5 min and then washed off to ensure that stimulation of the nerve and muscle were completely independent from each other. Finally, 5 µg/mL final concentration of venom was added to the Tyrode solution and left in contact with the tissue for 2 h or until 100% inhibition of muscle twitches was observed.

2.9. Lethality of venoms

Different amounts of venoms from each of the three *Ophryacus* species were dissolved in 0.5 mL of PBS. Samples were then injected through the tail vein of ICR mice (18–20 g) in groups of three [39]. After 24 h, the number of deaths was recorded and the corresponding median lethal dose (LD_{50}) was calculated [40]. LD_{50} values were estimated by non-linear regression using the software Prism v. 6 (GraphPad Software).

2.10. ELISA assay

A mouse monoclonal antibody (clone 4F6) and rabbit polyclonal antibodies to *C. d. terrificus* Crotoxin were tested for possible cross-recognition of components in the three *Ophryacus* venoms by ELISA. The assay was run as previously described [26,27]. Plates were coated with 100 µL of each venom (5 µg/mL) dissolved in 100 mM $NaHCO_3$ buffer, pH 9.5, at 37 °C for 1 h. Then, excess antigens were washed with 50 mM Tris, 150 mM NaCl, 0.05% Tween 20, pH 8.0 (wash buffer) and free binding sites were blocked with 200 µL of 0.5% gelatin in 50 mM Tris, 0.2% Tween 20, pH 8.0 (blocking buffer) over 2 h. Subsequently, serial 1:3 dilutions of monoclonal antibody 4F6 (starting at 30 µg/ml) or polyclonal antibodies to Crotoxin (starting at 15 µg/mL) were added to the wells, incubated for 1 h at 37 °C and followed by washing and addition of the corresponding conjugates (HRP-conjugated goat anti-mouse IgG or HRP-conjugated goat anti-rabbit IgG; diluted 1:4000) for 1 h at 37 °C. After washing, color was developed by adding 100 µL/well of 2,2-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid/H_2O_2) solution. Absorbance was read at 405 nm on a microplate spectrophotometer (Magellan R).

2.11. RP-HPLC (reversed-phase high-performance liquid chromatography) fractionation

Each venom pool (2–3 mg) was dissolved in 200 µL of solvent A (0.1% trifluoroacetic acid; TFA), centrifuged at 15000 rpm for 5 min, and separated by RP-HPLC on a C_{18} column (4.6 × 250 mm; Vydac®), and monitored at 214 nm. Elution was performed at 1 mL/min, by applying a gradient toward solvent B (100% acetonitrile, containing 0.1% TFA), as follows: 0% B for 5 min, 0–15% B over 10 min, 15–45% B over 60 min, 45–70% B over 10 min, and 70% B over 9 min [11,15,26]. Because *O. sphenophrys* venom caused flaccid paralysis, fractions were collected manually and dried by vacuum centrifugation (SpeedVac, Thermo) for further analyses.

2.12. Proteomic profiling of O. sphenophrys venom

RP-HPLC fractions of *O. sphenophrys* venom were further separated by SDS-PAGE on 15% gels under reducing conditions. Coomassie-stained protein bands were excised from the gel, reduced with dithiothreitol (10 mM), alkylated with iodacetamide (50 mM), and digested overnight with trypsin in an automated workstation (Intavis). The resulting peptides were mixed (1:1) with a saturated solution of α-cyanohydroxycinnamic acid in 50% acetonitrile with 0.1% TFA, and spotted (1 µL) onto an Opti-TOF 384 plate, dried, and analyzed by MALDI-TOF-TOF. Spectra were obtained after 500 laser shots at an

Table 1
Toxic and enzymatic activities of *Ophryacus* venoms.

Species	LD_{50} (μg/g)	MHD (μg)	HPA hydrolysis (U/mg)	HPA + EDTA hydrolysis (U/mg)	PLA_2 (U/mg)	MCD μg
O. sphenophrys	0.88 (0.85–0.9)	17.0 ± 1.0	14.9 ± 0.5	0.27 ± 0.10	55.2 ± 4.2	> 100
O. smaragdinus	1.45 (1.4–1.5)	2.5 ± 0.1	21.9 ± 0.1	0.12 ± 0.02	20.6 ± 2.0	> 100
O. undulatus	3.05 (3.0–3.1)	10.0 ± 1.2	22.7 ± 2.0	0.27 ± 0.10	10.6 ± 1.2	> 100

LD_{50}: Median Lethal Dose, dose of venom that induces death in 50% of injected mice (18–20 g) via intravenous (i.v.) injection. Values in parentheses represent the 95% confidence limits.
MHD: Minimum Hemorrhagic Dose - dose of venom that induces a hemorrhagic halo of 10 mm diameter in mice (25–28 g) after 3 h, via intradermal injection.
HPA: Hide Powder Azure - proteolytic activity on the HPA.
HPA + EDTA: proteolytic activity on hide powder azure substrate in the presence of ethylenediamine tetra-acetic acid (EDTA), pre-incubated for 30 min.
PLA_2: Phospholipase A_2 activity on 4-nitro-3-octanoyloxy-benzoic acid substrate.
MCD: Minimum Coagulant Dose - dose of venom that induces clotting of citrated human plasma in 60 s.
Values represent mean ± SD of three replicates.

intensity of 3200, in positive reflector mode, over the range of 900–3800 *m/z*. Up to 10 precursors from the TOF spectrum of each tryptic digest were selected for automated collision-induced dissociation TOF-TOF spectra acquisition at 2 kV, in positive reflector mode (500 shots, 3800 laser intensity). Resulting fragmentation spectra were searched against the UniProt/SwissProt database (Serpentes) using the ProteinPilot® v.4 software and the Paragon® algorithm (ABsciex) at ≥95% confidence, for the assignment of proteins to known families. The relative abundance of each protein (% of total venom proteins) was estimated by integration of the peak signals at 215 nm, using Chem Station B.04.01 (Agilent). When a chromatographic peak contained more than one SDS-PAGE band, the relative distribution was estimated by densitometry using the ImageLab® v.2.0 software (Bio-Rad).

2.13. Crotoxin-like purification

Three mg of venom from *O. sphenophrys* were dissolved in 250 μL of 20 mM ammonium acetate, pH 4.7, and fractionated by size-exclusion chromatography on a Cosmosil column (Diol-300, 7.5 × 600 mm) that was previously equilibrated with the same buffer. Elution was monitored at 280 nm, and manually collected fractions were freeze-dried. Fraction 3, contained the Crotoxin-like molecule and was redissolved in 1 mL of water +0.1% TFA and further subjected to C_{18} RP-HPLC as described in Section 2.11. Fractions were collected and dried in a SpeedVac concentrator (Thermo).

2.14. Reconstitution of the Crotoxin-like A + B complex

The purified A and B subunits were mixed at a 1:1 M ratio and incubated at 37 °C for 30 min in phosphate-buffered saline (PBS; 0.12 M NaCl, 0.04 M sodium phosphate, pH 7.2). We then used the complex to test PLA_2 activity as in Section 2.7, mouse phrenetic nerve as in 2.8, lethality as in 2.9, and ELISA as in 2.10.

2.15. Mass spectrometry and N-terminal amino acid sequencing

To determine the intact masses of Sphenotoxin and its isolated subunits, we used ESI-MS on an LCQ Fleet Ion Trap Mass Spectrometer [26]. Amino-terminal sequencing of Sphenotoxin-B was determined by automated Edman degradation on a PPSQ-31A Protein Sequencer (Shimadzu). Additional internal sequences of this protein were obtained by MS/MS analysis of tryptic peptides using MALDI-TOF-TOF on a 4800 Proteomics Plus mass spectrometer, as described previously [11]. Additional partial sequences were obtained by enzymatic digestion with GluC endopeptidase (Roche Applied Sciences), according to the manufacturer's protocol and subsequent Edman degradation.

2.16. Crotoxin neutralization assay

Different volumes of rabbit serum raised against *C. simus* venom from Veracruz, Mexico (containing about 16% of Crotoxin) were pre-incubated with 3xLD_{50} (50.4 μg) of *O. sphenophrys* venom, in a final volume of 500 μL of PBS. The mixture was preincubated for 30 min, injected intravenously in mice, and survivance was recorded after 24 h. We use the venom of *C. simus* as a standard for comparison.

3. Results

3.1. Toxic and enzymatic activities of Ophryacus venoms

The venoms from the three species of *Ophryacus* showed differences in their bioactivities. Regarding lethality in mice, the highest potency was obtained for *O. sphenophrys* venom (i.v. LD_{50} 0.88 μg/g). Upon injection of this venom, a relevant flaccid paralysis in the hind limbs was observed along with evident respiratory difficulties. The venoms of *O. smaragdinus* and *O. undulatus* exhibited lower lethal activity, with LD_{50} values estimated at 1.5 μg/g and 3.0 μg/g, respectively (Table 1), and no evidence of flaccid paralysis or respiratory difficulties was observed.

All three *Ophryacus* venoms induced hemorrhage in the mouse skin assay, but significant differences in potency were present. *Ophryacus smaragdinus* venom had the highest hemorrhagic activity with a MHD of 2.5 μg, *O. undulatus* had intermediate activity with MHD corresponding to 10 μg and *O. sphenophrys* had the least potent hemorrhagic activity with a MHD of 17 μg (Table 1).

Proteolytic activity on HPA substrate was similar for *O. smaragdinus* and *O. undulatus* (~22 U/mg), but lower for *O. sphenophrys* (~15 U/mg). All three venoms had their proteolytic activity nearly abolished (~95% decrease) when preincubated with the divalent cation chelator EDTA (Table 1). PLA_2 activity was highest in *O. sphenophrys* venom and was almost double that of *O. smaragdinus* and about five-fold higher than that of *O. undulatus* (Table 1). Finally, none of these venoms showed procoagulant activity on human plasma, even when the highest amount (100 μg) was tested (Table 1).

3.2. RP-HPLC profiles of Ophryacus venoms

Higher similarity between the elution profiles of *O. sphenophrys* and *O. smaragdinus* venoms was observed than between these and *O. undulatus* venom (Fig. 1). *Ophryacus sphenophrys* and *O. smaragdinus* had a higher number of prominent peaks eluting between 50 and 70 min, which generally correspond to SVSP and PLA_2 enzymes, than *O. undulatus,* which presented a slightly less complex elution profile. Also, *O. sphenophrys* and *O. smaragdinus* venoms presented larger areas for peaks eluting after 80 min which, in the gradient used here, generally correspond to SVMP enzymes [8]. In the case of *O. sphenophrys*, it was possible to analyze the chromatographic profiles of venoms of the six individuals sampled (Supplementary Fig.1A), which presented essentially the same pattern, there are slight differences in the proportion of the fractions, especially a smaller proportion of the metalloproteases

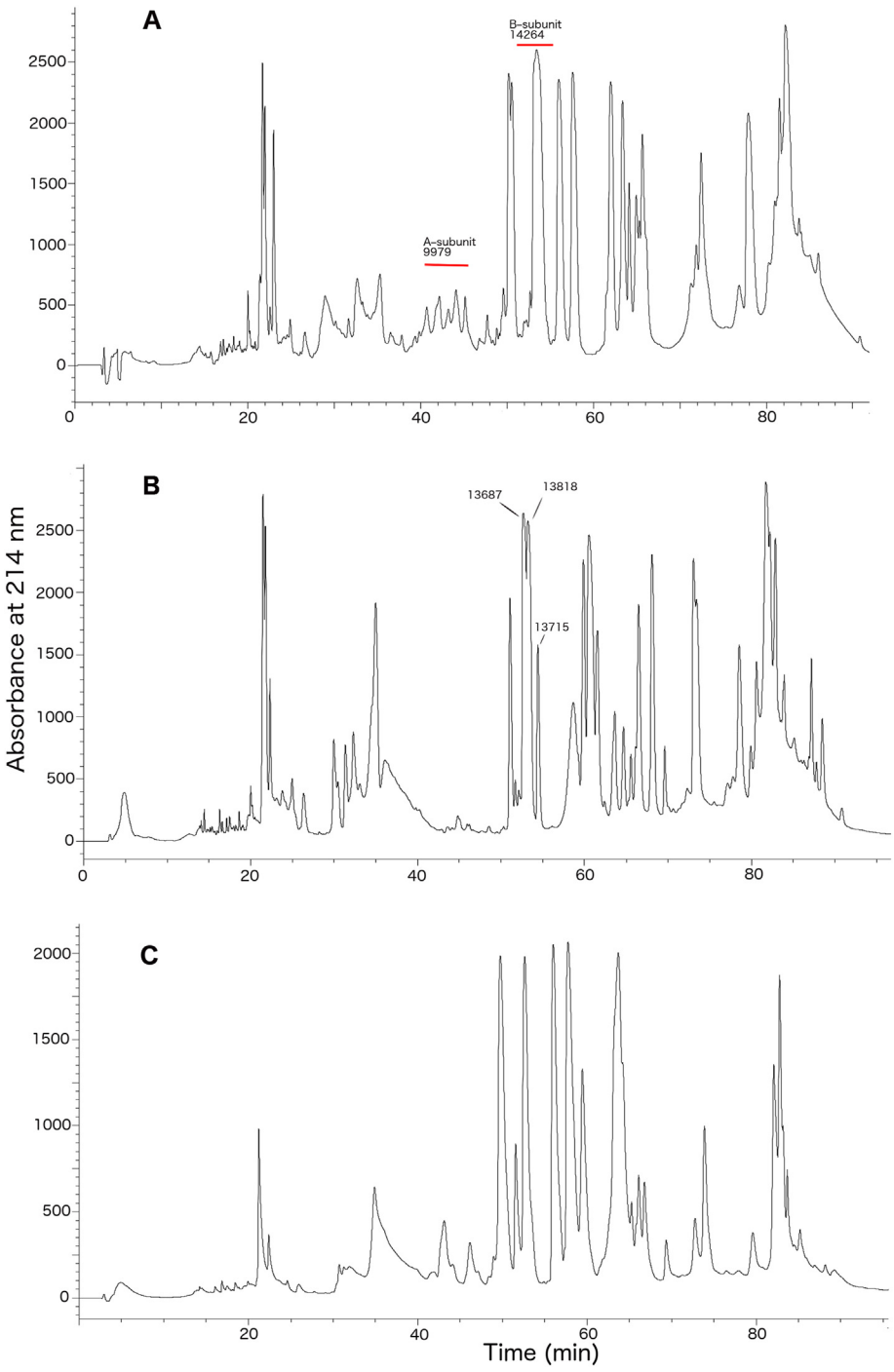

Fig. 1. Comparative Reverse-phase HPLC venom profiles of the three species of *Ophryacus:* (A) *O. sphenophrys*; (B) *O. smaragdinus*; (C) *O. undulatus*. Venoms (3 mg) were separated on a C_{18} column as described in Section 2.11 and the gradient line is omitted for better visualization. In (A), the red bars indicate the region where the A (acidic) and B (basic) subunits of Sphenotoxin elute and the molecular masses of 9979 and 14,261 Da are indicated. In the case of *O. smaragdinus* (B), three fractions elute at retention times similar to that of Sphenotoxin B, but their molecular masses differed substantially. In the case of *O. undulatus* (C), none of the fractions eluted with the same retention times as the Sphenotoxin subunits. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

(after 80 min) of the two juvenile specimens (Fig. B and C of Supplementary Fig. 1A), however, it is necessary to carry out studies with a greater number of samples of juveniles and adults to confirm or discard the presence of ontogenetic variation. RP-HPLC fractions in the elution profile of *O. sphenophrys* venom that were subsequently identified as having sequence similarity to the acidic and basic subunits of Crotoxin are labeled in Fig. 1.

3.3. Proteomic profiling of O. sphenophrys venom

Because *O. sphenophrys* was the only species that caused flaccid paralysis while carrying out LD_{50} experiments, we further analyzed the proteome for this species. A striking result of the proteomic profile of *O. sphenophrys* venom was the finding of two protein fractions with amino acid sequence similarity to the A and B subunits, respectively, of the heterodimeric Crotoxin complex. Therefore, the isolation of such Crotoxin-like protein (here named Sphenotoxin) was pursued. RP-HPLC venom fractions were separated by SDS-PAGE and the resulting protein bands were analyzed by MS/MS. Peaks 1 and 3–10 did not present proteins after the SDS-PAGE and were not analyzed further (Fig. 2). Protein assignments for the remaining chromatographic peaks (2 and 11–35) were done to protein family by MALDI-TOF-TOF analysis of tryptic peptides (Table 2). Overall, venom proteins were assigned to one of seven protein families with relative abundances shown in Fig. 3. The most abundant components of *O. sphenophrys* venom were SVMPs (34.9%), PLA_2 (24.8%; including 12.9% represented by Sphenotoxin), and SVSPs (17.1%) enzymes. Other proteins detected include members of the vascular endothelial growth factor (VEGF), Vespryn, L-amino

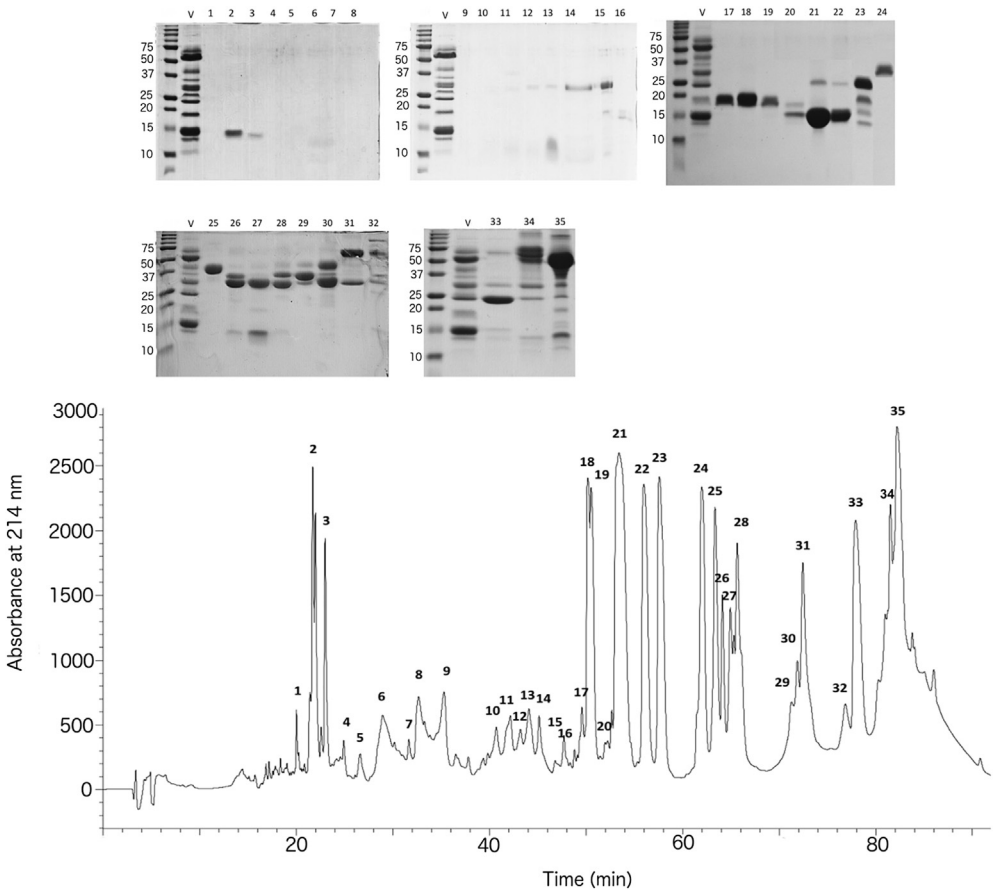

Fig. 2. Reverse-phase HPLC profile of *O. sphenophrys* venom on C_{18}. Each fraction was further separated by SDS-PAGE (15%) under reducing conditions, for trypsin digestion and MALDI-TOF/TOF analysis.

acid oxidase (LAAO), and cysteine-rich secretory protein (CRISP) families. The remaining 10.7% of proteins were unidentified/unassigned.

3.4. Purification of Sphenotoxin

In order to preserve the integrity of the Sphenotoxin complex, *O. sphenophrys* venom was fractionated by size-exclusion chromatography, resulting in the separation of nine peaks that were analyzed via SDS-PAGE under reducing conditions (Fig. 4A). Peaks 1 and 2 predominantly contained proteins above 30 kDa and the remaining seven peaks exhibited proteins of ≤25 kDa. Proteins of ~14 kDa were observed in peaks 3–6 which appeared to increase their molecular weight when analyzed in an unreduced state. A screening for toxicity was performed by injecting 20 µg of each fraction intravenously (i.v.) in mice in groups of three. Only peaks 3 and 4 were lethal. Peak 3 also generated paralysis in the hind limbs suggesting neurotoxin activity. This fraction was further separated by RP-HPLC, which is known to dissociate the non-covalently bound subunits of Crotoxin or Crotoxin-like complexes. Peaks eluting at 41–44 min (Fig. 4B) showed molecular masses of 9.97, 9.65, 9.92, 9.31 and 9.26 kDa (Fig. 4C), suggesting that they likely correspond to isoforms of the A-subunit of Sphenotoxin, having similar retention times to A-subunits from Crotoxin or Crotoxin-like proteins. In addition, a prominent peak eluted at 54 min (Fig. 4B), with a molecular mass of 14.26 kDa (Fig. 4D) which is likely the B-subunit of Sphenotoxin. Most of the amino acid sequence of this protein was obtained by a combination of N-terminal and MS/MS sequencing (107/122 residues), which presented clear homology to the B subunit of Crotoxin in a multiple alignment of Crotoxin and Crotoxin-like molecules (Fig. 5). The partial sequence of Sphenotoxin B is 85% similar to Crotoxin B from *C. d. terrificus* (P08878) and 78% similar to Nigroviriditoxin B from *B. nigroviridis* (C0HJL8).

3.5. Functional characterization of Sphenotoxin

The main feature of Crotoxin/Crotoxin-like proteins is their high lethal potency in mice. The purified B subunit of Sphenotoxin induced i.v. lethality with an LD_{50} of 0.49 µg/g, and as mentioned above, caused flaccid paralysis of the hind limbs and breathing difficulties, ultimately resulting in death. When the B subunit was combined with the A subunit to reconstitute the Sphenotoxin complex, at a 1:1 ratio, the lethal potency increased (LD_{50} 0.11 µg/g) with the same signs of paralysis occurring. As expected, injection of the A subunit alone, up to a dose of 5.2 µg/g, did not cause lethality nor evidence of neurotoxic effects in mice (Table 3).

The B subunit exhibited relatively high PLA_2 activity and, as expected, the A subunit of Sphenotoxin did not display PLA_2 activity. When combined, the reconstituted A + B Sphenotoxin complex showed a 5-fold lower PLA_2 activity compared to the B-subunit alone (Table 3).

The β-neurotoxic activity of Sphenotoxin was clear in the mouse phrenic nerve-hemidiaphragm preparation. In similarity to Crotoxin, used as a positive control, Sphenotoxin was slightly less potent and blocked the indirect twitch response, with a T_{90} of 74.6 min whereas Crotoxin had a T_{90} of 58.9 min. (Fig. 6). In addition, whole venom of *O. sphenophrys* also displayed a β-neurotoxic blockade in this assay with a T_{90} of 10.3 min. In contrast, *O. smaragdinus* and *O. undulatus* venoms did not induce a blocking effect (Supplementary Fig.2A).

Table 2
Assignment of the RP-HPLC isolated fractions of *Ophryacus sphenophrys* venom to protein families by MALDI-TOF-TOF of selected peptide ions from in-gel trypsin-digested protein bands, or by N-terminal Edman sequencing.

Peak	%	Mass (kDa)	Peptide ion		MS/MS-derived or N-terminal sequence (*)	Conf	Score	Protein family	Related protein
			m/z	z	–	–	–	–	–
1	–	–	–	–	no proteins observed in gel	–	–	–	–
2	3.8	14.1▼	Edman	–	SVEHVMCSMYNN	–	–	Metalloproteinase	SVMP fragment, ~ ADI47592 *Echis carinatus*
3	1.3	–	–	–	unidentified	–	–	–	–
4-9	–	–	–	–	no proteins observed in gel	–	–	–	–
11-13	4.2	10▼ (9,319; 9,651; 9,979)	2192.8 1092.4 1634.6 1506.2	1 1 1 1	SYGCYCGAGGQGWPQDASDR YGCYCGAGGQ CCFEHDCCYAK CCFEHDCCYR	99.0 99.0 99.0 manual	9 14 11 –	Phospholipase A_2	Crotoxin acidic subunit, ~ P08878 *Crotalus d. terrificus*
14	1	33.1▼	–	–	n.a.	–	–	–	–
15	0.8	34.1▼	–	–	n.a.	–	–	–	–
16	0.7	17▼	–	–	n.a.	–	–	–	–
17	0.5	20▼	–	–	n.a.	–	–	–	–
18	2.7	20▼ (13,783)	974.5 2108.0	1 1	YWFYPAK YWFYPAKNCQEESEPC	99.0 82.2	9 6	Phospholipase A_2	PLA_2, ~ P86907 *Bothrops ammodytoides*
19	3.1	19▼	1505.5 2107.8 974.4	1 1 1	CCFVHDCCYGK YWFYPAKNCQEESEPC YWFYPAK	99.0 99.0 99.0	11 14 9	Phospholipase A_2	PLA_2, ~ P86907 *Bothrops ammodytoides*
20	0.4	▼	–	–	n.a.	–	–	–	–
21a	1	27.5▼ (13,697)	2107.8 1505.5 974.4	1 1 1	YWFYPAKNCQEESEPC CCFVHDCCYGK YWFYPAK	99.0 99.0 99.0	17 15 10	Phospholipase A_2	PLA_2, ~ P86907 *Bothrops ammodytoides*
21b	8.7	14.4▼ (14,264)	2159.0	1	NAIPFYAFYGCYCGWGGR	98.3	6	Phospholipase A_2	Crotoxin basic subunit ~ P62022 *Crotalus d. terrificus*
22	5.1	14.8▼ (13,789)	1969	1	TYPDIFCDAT	–	–	Phospholipase A_2	PLA2 *T.flavoviidis* ~Q02517
23a	3.5	27▼ (24,829)	2680.4	1	VNPTASNMLKMEWYPEAAANAER	85.9	5	CRISP	ablomin, ~ Q8JI40 *Gloydius blomhoffii*
23b	1.4	20▼ (24,829)	3180.5 2680.1 1537.6	1 1 1	SVDFDSESPRKPEIQNEIVDLHNSLRR SVNPTASNMLKMEWYPEAAANAER MEWYPEAAANAER	99.0 99.0 88.9	10 8 5	CRISP	CRISP, ~ T2HPR8 *Ovophis okinavensis*
24a	4.9	31▼ (33,595)	Edman 1869.8	– 1	VVGGDECNINE EWVLTAAHCDRENIR	– 99.0	– 10	Serine protease	albolabrase, ~ P0DJF4 *Trimeresurus albolabris*
24b	0.2	12	2201.0 1519.7	1 1	ADVTFDSNTAFSSLVVSANKK NVGVPQVVPDNPER	99.0 88.3	11 5	Ohanin/Vespryn	Vespryn, ~ T1E3B3 *Crotalus horridus*
25a	2.8	46.2▼	1746.9 2904.3 1512.6 2889.3	1 1 1 1	KKDDEKDKDIMLIR LNSPVSNSEHIAPLSLPSSPP^{ox}SVGSVCR VIGGDECNINEHR LDSPVKN^{da}SAHIAPLSLPSSPPSVGSDCR	99.0 99.0 99.0 98.8	6 12 7 9	Serine protease	SP-2a, ~ A0A194ALW4 *Agkistrodon c.contortrix* pallabin, ~ Q9YGJ2 *Gloydius halys*
25b	0.8	32.7▼ (27,509)	2221.8	1	IEVMQFTEHTECECRPR	99.0	11	VEGF	VEGF-1a, ~ A0A194ARM3 *Sistrurus c. tergeminus*
26	1.7	40▼	1746.9 2506.2 2904.3	1 1 1	KKDDEKDKDIMLIR FICPNKKKDDEKDKDIMLIR LNSPVSNSEHIAPLSLPSSPP^{ox}SVGSVCR	99.0 98.7 99.0	11 6 19	Serine protease	kallikrein-CohCI-5, ~ T1DP86 *Crotalus o. helleri* SVSP-2a, ~ A0A194ALW4 *Agkistrodon c. contortrix*
27	1.8	33.7▼	1129.5 1164.5	1 1	FLVALYTFR IMGWGTISATK	99.0 99.0	7 8	Serine protease	kallikrein-CohLL-4, ~T1DEH3 *Crotalus o. helleri*
28a	1.7	38▼ (30,829)	1129.7	1	FLVALYTFR	72.7	4	Serine protease	kallikrein-CohLL-4, ~T1DEH3 *Crotalus o. helleri*
28b	2.4	34▼ (27,616)	1782.9	1	NVPNEDEQTRVPKEK	96.6	6	Serine protease	serine protease, ~ T2HS45 *Ovophis okinavensis*
29	2.4	30	–	–	n.a.	–	–	–	–
30	1.4	48	–	–	n.a.	–	–	–	–
31a	3.7	67▼ (60,205)	2106.9	1	SAGQLYEESLGKVAEELKR	99.0	6	L-amino acid oxidase	Lm29, ~ J7H670 *Lachesis muta*
31b	1.6	34.4▼ (30.1)	1883.9 2106.9 1678.6 3263.4	1 1 1 1	KNPGLLKYPVKPSEEGK SAGQLYEESLGKVAEELKR NEKDGWYVNLGPMR HIVIVGAGMSGLSAAYVLAGAGHKVTVLEASER	99.0 99.0 99.0 99.0	8 8 14 10	L-amino acid oxidase	LAAO, ~ A0A098LWS4 *Python regius* Lm29, ~ J7H670 *Lachesis muta* LAAO, ~ Q4JHE1 *Pseudechis australis* LAAO, ~ G8XQX1 *Daboia russelli*
31c	0.4	34.4▼ (30,130)	1498.6 2889.7	1 1	VVGGDECNINEHR LDSPVSNSEHIAPLSLPSSPPSVGSVCR	89.4 70.2	5 6	Serine protease	SVSP-10, ~ T1DH10 *Crotalus horridus*
32	2.2	67▼	–	–	n.a.	–	–	–	–
33a	1.2	66.3▼	1678.7	1	NEKDGWYVNLGPMR	99.0	12	L-amino acid oxidase	LAAO, ~ Q4JHE1 *Pseudechis australis* *Bothrops jararaca*
33b	0.9	34.9▼	1717.7	1	VAVTMAHELGHNLGIR	99.0	24	Metalloproteinase	SVMP, ~ Q98SP2
33c	4.3	26▼ (46,250) (69,450)	1099.5 1007.5	1 1	VHQMVNIMK NLNPEHQR	99.0 99.0	13 12	Metalloproteinase	SVMP P-II, ~ Q2QA03 *Crotalus d. durissus*
34,35a	25.9	60▼ (48,647)	2154.1 1552.6 2942.1 1052.4 1633.7 1783.6	1 1 1 1 1 1	ITVKPDVDYTLNSFAEWR VCSNGHCVDVATAY ASMSECDPAEHCTGQSSECPADVFHK GNYYGYCR MYELANTVNDIYR SGSQCGHGECCEQCK	99.0 99.0 99.0 62.9 99.0 99.0	27 10 10 8 17 14	Metalloproteinase	SVMP, atroxlysin-III, ~ A0A1S6K7T1 *Bothrops atrox* CohPH-3, ~ T1E6U1 *Crotalus o. helleri* SVMP, ~ V5Z141 *Deinagkistrodon acutus*
35b	1.4	28▼	1103.4 1544.7 1672.7 1746.7 1498.5	1 1 1 1 1	TLCAGIVQGGK NNEVLDKDIMLIK KNNEVLDKDIMLIK KKDDEKDKDIMLIR VVGGDECNINEHR	99.0 99.0 83.8 99.0 77.6	9 10 7 7 5	Serine protease	SVSP-PA ~ Q71QH7 *Trimeresurus stejnegeri* kallikrein CohCI-5, ~ T1DP86 *Crotalus o. helleri*

(*) Cysteine residues determined in MS/MS analyses are carbamidomethylated. Few peptides were obtained by N-terminal Edman degradation sequencing (Edman). ▼: molecular weight estimated by reduced SDS-PAGE, in kDa; isotope-averaged molecular masses of selected peaks, determined by MALDI-TOF, are indicated in parentheses. Confidence (Conf) and Score values are calculated by the Paragon® algorithm of ProteinPilot® v.4 software. n.a.: not analyzed. Unverified amino acid modifications suggested by the matching algorithm are indicated as: (ox): oxidation; (da) deamidation.

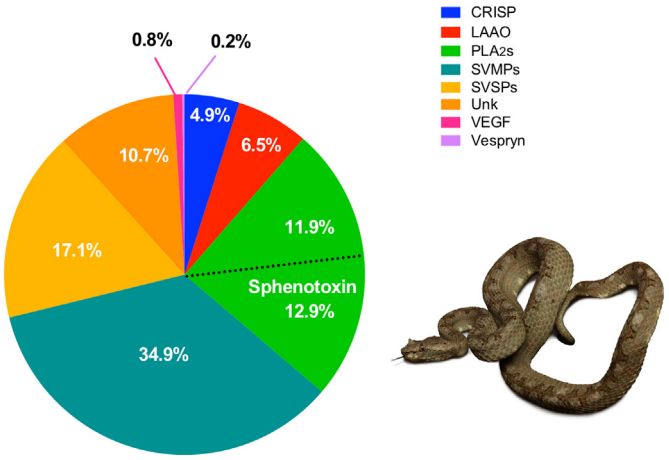

Fig. 3. Proteomic composition of *O. sphenophrys* venom according to relative proportion of each protein families. Relative abundances were estimated from the integration of chromatographic peak areas of the RP-HPLC separation, combined with densitometric distribution of protein bands in SDS-PAGE as described in Section 2.12. SVMPs: snake venom metalloproteinases; SVSPs: snake venom serine proteinases; LAAO: L-amino acid oxidase; CRISP: cysteine-rich secretory proteins; Sphenotoxin: Crotoxin-like; PLA_2: phospholipase A_2; VEGF: vascular endothelial growth factor; Vesp: vespryn/ohanin; Unk: unknown proteins. Photograph of *Ophryacus sphenophrys* courtesy of Ivan Ahumada.

3.6. Immunological cross-reactivity and neutralization by anti-Crotoxin antibodies

Monoclonal antibody 4F6, capable of recognizing Crotoxin molecules from rattlesnake, such as *C. d. terrificus*, *C. simus*, *C. s. scutulatus* [26,27] did not bind to any of the *Ophryacus* venoms including that of *O. sphenophrys* which contains Sphenotoxin (Fig. 7B). However, polyclonal antibodies raised against Crotoxin from *C. simus* produced a significant binding signal against *O. sphenophrys* venom but not against the venoms of *O. smaragdinus* or *O. undulatus* (Fig. 7A). In addition, immune serum raised in rabbits against *C. simus* venom was capable to cross-neutralize the lethal effect of *O. sphenophrys* venom at 2.82 mg/mL (venom/serum), while neutralization of the homologous *C. simus* venom was achieved at 0.88 mg/mL (Table 3).

4. Discussion

By characterizing the biological and proteolytic activity of all three species in the pitviper genus *Ophryacus*, we identified and describe Sphenotoxin, a novel β-neurotoxin similar to Crotoxin in *O. sphenophrys*. *Ophryacus* is the fifth genus of pitviper to contain a heterodimeric PLA_2 and *O. sphenophrys* is only the third non-rattlesnake species to contain a Crotoxin-like molecule. The venoms of the three small-bodied snake species [41] displayed variable i.v. lethal activity in mice. Consistent with the presence of a β-neurotoxin, *O. sphenophrys* was the most potent, followed by *O. smaragdinus* and *O. undulatus*. Our study is the first to examine the venom of the semi-arboreal Mexican Horned Pitvipers, *Ophryacus*, and we also identified high variability in the venom activity of this endemic Mexican genus.

The three *Ophryacus* venoms lacked procoagulant activity upon human plasma, an effect most frequently mediated by specific SVSP enzymes, for example thrombin-like SVSPs [42–44]. Hemorrhage was induced by the venoms of all three species with *O. smaragdinus* being the most potent and *O. sphenophrys* being the weakest. Hemorrhage is an effect mediated by zinc-dependent SVMPs. Enzymes responsible for proteolysis of HPA were shown to correspond largely to SVMPs, and not SVSPs, since addition of EDTA to the assay reaction reduced this activity by > 95%. Lastly, *O. sphenophrys* venom had the highest PLA_2 activity of the three, suggesting either the presence of a large proportion of PLA_2s, and/or of highly active PLA_2s. Based on our results this is driven by the presence of the neurotoxic PLA_2, Sphenotoxin. Interestingly, it has been shown that in rattlesnake species there is an inverse correlation between the expression of neurotoxicity and hemorrhagic activity, leading to a classification of their venoms as type I (highly hemorrhagic) or type II (highly neurotoxic) [4]. *Ophryacus* follows this trend as the most lethal venom (*O. sphenophrys*) also displayed the lowest hemorrhagic activity.

By comparing the chromatograms of the three *Ophryacus* venoms studied, fractions eluting with retention times similar to that of Sphenotoxin B in *O. smaragdinus* seemed unlikely to correspond to this protein, since their masses were lower (13.6, 13.7, 13.8 kDa). The elution profile of *O. undulatus* venom differed from that of *O. sphenophrys* showing no peaks with retention time similar to Sphenotoxin. Moreover, *O. smaragdinus* and *O. undulatus* venoms did not induce neuromuscular blockade in the ex vivo assay, and did not induce signs of paralysis when injected into mice. In addition, rabbit antibodies raised against Crotoxin, which bound to *O. sphenophrys* venom by ELISA, did not display significant binding to *O. undulatus* or *O. smaragdinus* venoms. Altogether, these observations suggest that Sphenotoxin, or related Crotoxin-like components, are not present in the venoms of *O. undulatus* or *O. smaragdinus*, at least in significant amounts. However, further studies at the genomic, transcriptomic, and/or proteomic level for additional individuals in more locations would be necessary to rule out the possibility of a Crotoxin-like molecule being present in these two species.

Due to the presence of Sphenotoxin, the venom of *O. sphenophrys* was characterized using a bottom-up proteomics approach that combines RP-HPLC and 1-D SDS-PAGE as decomplexation steps [3]. Venom proteins were predominately assigned to three enzyme families (SVMP, PLA_2, SVSP) which accounted for ~77% of the total protein content (Table 2 and Fig. 3). Our results demonstrate that the venom of *O. sphenophrys* contains a novel Crotoxin-like heterodimeric complex, Sphenotoxin, which displays β-neurotoxicity and comprises ~12.9% of the venom proteins. The observed cross-recognition of Sphenotoxin by the polyclonal rabbit antibodies to Crotoxin in the ELISA assay confirms structural similarity. However, the lack of cross-recognition of Sphenotoxin by a monoclonal antibody to classical Crotoxin indicates that its corresponding epitope is not shared between the two toxins. However antigenic similarity of Sphenotoxin with classical Crotoxin is further supported by the cross-neutralization of lethality obtained using an anti-*C. simus* serum with antibodies against Crotoxin, albeit at a lower potency than against the homologous antigen. Importantly, results of the proteomic analysis determined that Sphenotoxin shared partial amino acid sequences to the A and B chains of Crotoxin. Sphenotoxin dissociated into ~9 kDa and ~14 kDa subunits under the conditions of RP-HPLC, in agreement with the expected presence of A and B subunits. Partial (107/122) amino acid sequencing of Sphenotoxin B confirmed its high similarity to Crotoxin or Crotoxin-like proteins described in *Crotalus*, *Sistrurus*, *Gloydius*, and *Bothriechis* species, ranging from 78 to 89% (Fig. 5).

Crotoxin and its homologs have been widely studied in rattlesnake (*Crotalus* and *Sistrurus*) venoms [20,21,23–28,45–52] but, only in recent years, have Crotoxin-like molecules been reported in venoms of species from other genera including *Gloydius* [29] and *Bothriechis* [15]. Mouse lethal potencies (expressed as LD_{50}) have been reported in the range of 0.08 to 0.65 μg/g (i.v.) for Crotoxin in *C. d. terrificus* Crotoxin, 0.45 μg/g (i.p.) for Gintexin in *G. intermedius*, 2.2 μg/g (i.v.) for Nigroviriditoxin in *B. nigroviridis*, and 0.16 μg/g (i.v.) for Sphenotoxin in *O. sphenophrys*

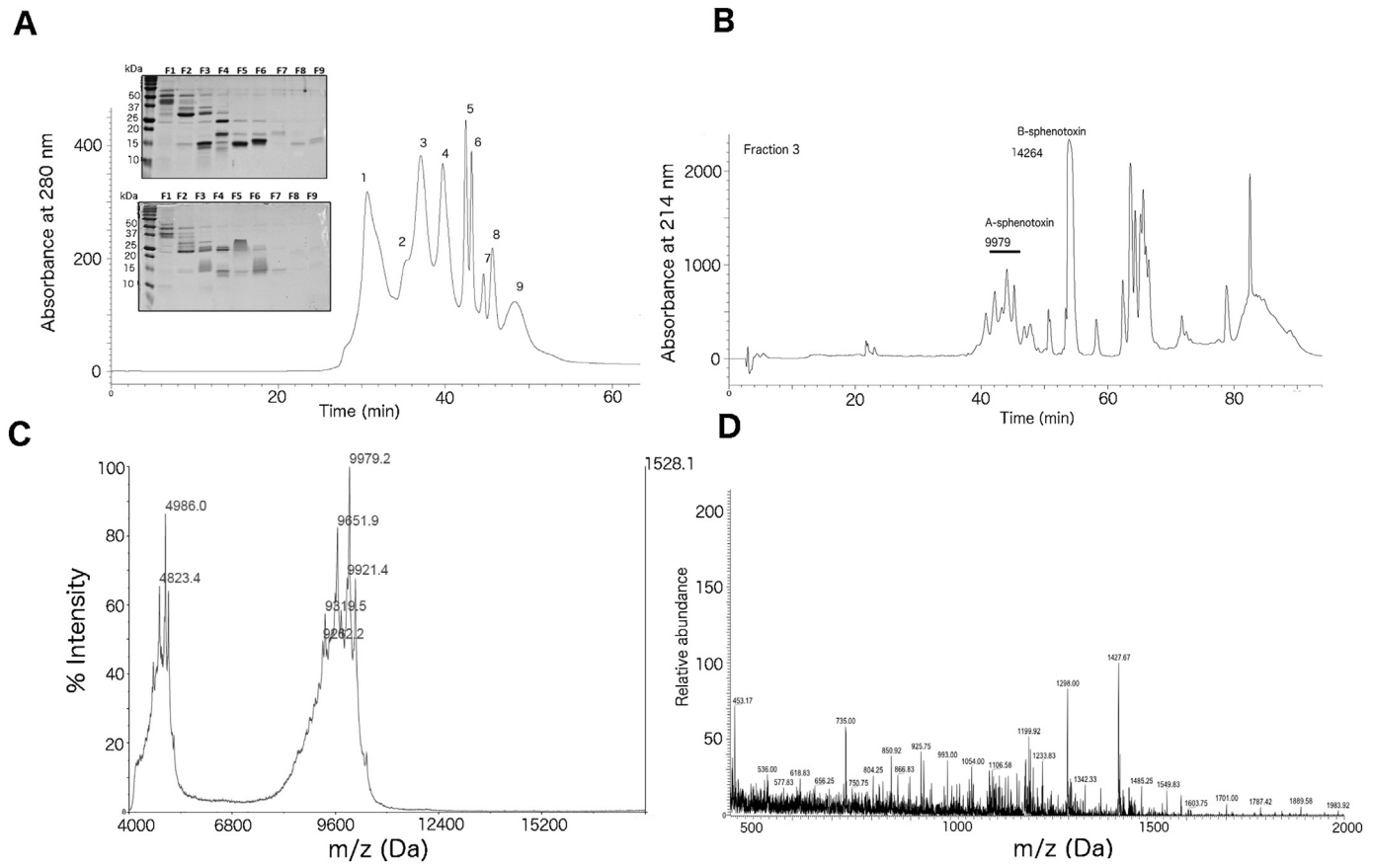

Fig. 4. Sphenotoxin purification and mass determination. (A) Size-exclusion chromatography of *O. sphenophrys* venom. Each fraction was analyzed by SDS-PAGE (15%) under reducing conditions (upper) and non-reducing conditions (lower). (B) Reverse-phase HPLC of fraction three from size-exclusion was analyzed; the two subunits of Sphenotoxin were marked. The A subunit (C) and B subunit (D) were analyzed by mass spectrometry. Five possible isoforms were identified for the A subunit and a single mass for the B subunit.

described in this study. Crotoxin B isoforms from *C. d. terrificus* venom have been reported with lethal potency values of 0.48, 0.5, and 0.7 μg/g (i.v.) [15,16,20]. All of these are similar to that obtained for Sphenotoxin B 0.49 μg/g (i.v.) (Table 3). The potent paralyzing and lethal actions of Crotoxin-like molecules suggest that its presence provides an important trophic adaptation but the precise cause remains unknown. For *O. sphenophrys* specifically, it is the most arboreal of the three species of *Ophryacus* and also has the most limited distribution. It is

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Sphenotoxin-B   O. sphenophrys     HLLQFNKMIKEETGKNAIPFYAFYGCYCGWGGSGKPKDATDRCCFEHDCCYGKLTNCNTK
AID56659        G. intermedius     HLLQFNKMIKVETGKNAIPFYAFYGCYCGWGGRGRPKDGTDRCCFVHDCCYGKLPNCNTK
P62022          C. d. terrificus   HLLQFNKMIKFETRKNAIPFYAFYGCYCGWGGRGRPKDATDRCCFVHDCCYGKLAKCNTK
ABY77917        S. tergeminus      NLLQFNKMIKFETNKNAIPFYAFYGCYCGWGGRGRPKDATDRCCFVHDCCYGKLPNCDTK
AUS82469        C. s. scutulatus   HLLQFNKMIKFETRRNAIPFYAFYGCYCGWGGRGRPKDATDRCCFVHDCCYGKLAKCNTK
C0HJL8          B. nigroviridis    NLLQFNRMIKLETKKNAVPFYAFYGCYCGWGGQGQPKDATDRCCFEHDCCYGKLTKCNTK

Sphenotoxin-B   O. sphenophrys     WDIYSYSLKDGYITCGKGTWCEKEVCECDK----CLRRNLRTYKYGYMFYL---------
AID56659        G. intermedius     WDIYPYSLKDGYITCGKGTWCEKQICECDRVAAECLRRNLSTYKYRYMFYLDSRCTGPSE
P62022          C. d. terrificus   WDIYPYSLKSGYITCGKGTWCEEQICECDRVAAECLRRSLSTYKYGYMFYPDSRCRGPSE
ABY77917        S. tergeminus      WDIYSYSLKSGFITCGKGTWCEEQICECDRVAAECLRRSLSTYKYGYMFYLDSRCKGPSE
AUS82469        C. s. scutulatus   WDIYRYSLKSGYITCGKGTWCEEQICECDRVAAECLRRSLSTYKYGYMFYPDSRCRGPSE
C0HJL8          B. nigroviridis    SDLYSYSSKYGFLLCGKGTWCEEQICECDRIAATCLRRSLDTYKLKYMFYLDSYCKGPSE

                                                 n     id%
                                                 ---   ---
Sphenotoxin-B   O. sphenophrys     --            107   100
AID56659        G. intermedius     KC            122    89
P62022          C. d. terrificus   TC            122    85
ABY77917        S. tergeminus      TC            122    85
AUS82469        C. s. scutulatus   QC            122    84
C0HJL8          B. nigroviridis    KC            122    78
```

Fig. 5. Multiple alignment conducted in Clustal Omega of the partial amino acid sequence of Sphenotoxin B with related homologs from *Crotalus d. terrificus*, *Sistrurus tergeminus*, *Bothriechis nigroviridis*, and *Gloydius intermedius*. Undetermined amino acid residues in the Sphenotoxin B sequence are indicated by (−). The Uniprot access codes for the proteins are indicated to the left and the identity values calculated with the partial sequence is shown to the right. Grey bars indicate the cysteines conserved.

Table 3
Functional characteristics of Sphenotoxin and its isolated subunits.

Sample	LD_{50}	PLA_2	Neutralization
	(μg/g)	(U/mg)	mg V/mL S
Sphenotoxin A	> 5.2	0	n.a.
Sphenotoxin B	0.49 (0.47–0.5)	174 ± 17.5	n.a.
Sphenotoxin (A + B)	0.16 (0.1–0.19)	34 ± 8.3	n.a.
C. simus venom	0.16 (0.12–0.18)	n.a.	0.88
O. sphenophrys venom	0.88 (0.85–0.9)	55.2 ± 4.2	2.82

LD_{50}: Median Lethal Dose - dose of venom that induces death in 50% of injected mice (18–20 g) by intravenous (i.v.) route. Values in parentheses represent the 95% confidence limits.
PLA_2: Phospholipase A_2 activity on 4-nitro-3-(octanoyloxy)-benzoic acid substrate.
Neutralization is expressed as the preincubated venom/serum ratio (mg V/mL S) at which mice were protected from lethality.
Sphenotoxin (A + B) was reconstituted at a 1:1 M ratio.
Values represent mean ± SD of three replicates.
n.a: not analyzed.

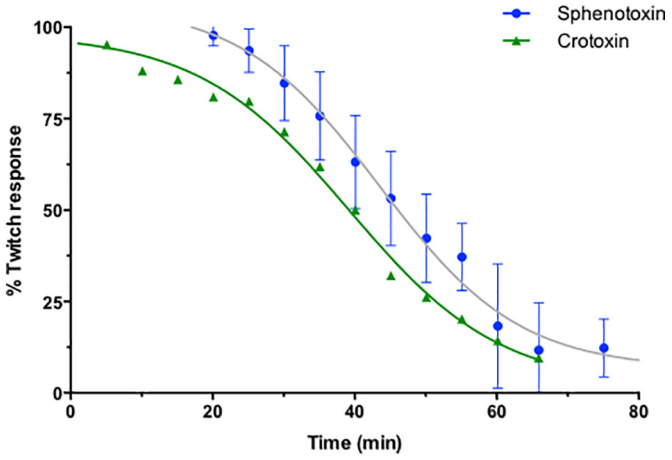

Fig. 6. Neurotoxic effect of Sphenotoxin and Crotoxin, at 3.5 μg/mL on the mouse phrenic-hemidiaphragm neuromuscular preparation. Points represent mean ± SD of three replicates.

possible that their diet is specialized and, through strong local adaptation, they have diverged in venom composition from their sister species, *O. undulatus*.

On the basis of the discovery of a β-neurotoxic PLA_2 heterodimeric complex in *Gloydius intermedius* [17,29], it has been recently proposed that Crotoxin originated in an Old World ancestor which migrated to North America from Asia through the Bering Strait bridge ~22 mya [3,53]. In silico studies on ancestor-state reconstructions have predicted that a single nucleotide mutation could suffice to explain the complete proteolytic processing of Mojave toxin A (Crotoxin A homolog) and its subsequent association with the B subunit [54]. In addition, the scattered pattern for the presence of Crotoxin among rattlesnake species has been shown to originate on recent lineage-independent loss of genes rather than on convergent evolution [53]. However, it is still unclear why some species possess mRNA transcripts encoding for both subunits of Crotoxin, yet in proteomic analyses do not show presence of this neurotoxin in their venoms [11,25]. Recent research indicates the presence of iRNA in the regulation mechanisms of protein expression in snake venoms [25], could explain why certain populations within the same species, for example *C. tzabcan*, produce venoms that contain Crotoxin, determined by ELISA, whereas others lack this toxin [22].

The presence of Crotoxin-like molecules in non-rattlesnake genera, as in the case of *O. sphenophrys* described here and *B. nigriviridis* [15], is of interest from an evolutionary perspective because it adds to the dataset for the presence and absence of heterodimeric β-neurotoxins. If Crotoxin-like molecules are found in more taxa, this will further support the hypothesis that Crotoxin genes were already present in venom glands of the New World pitviper ancestor and, eventually, they were lost in some lineages [53]. Therefore, it would be highly informative to investigate other species of pitvipers in the New World [33]. Testing for the presence of a homologous toxin to Sphenotoxin in the three species of *Mixcoatlus* could help inform the gene gain vs gene loss hypotheses of Crotoxin-like molecules in this lineage because *Mixcoatlus* is likely the sister genus for *Ophryacus*. Additionally, now that Crotoxin-like molecules have been found in two New World genera other than rattlesnakes, it is possible that β-neurotoxins will be found in other Middle American pitviper genera like *Bothriechis* and *Ophryacus* such as *Atropoides*, *Cerrophidion* or *Porthidium*, as more individuals and populations are analyzed venomically.

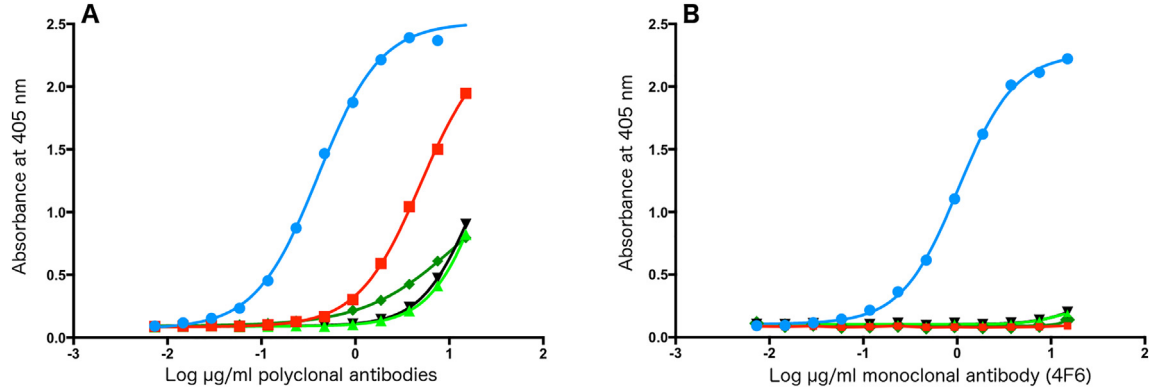

Fig. 7. Cross-recognition screening assay by ELISA. Wells coated with crude venoms of either *Ophryacus sphenophrys* (■), *O. undulatus* (▼), or *O. smaragdinus* (▲) were incubated with (A) mouse monoclonal 4F6 against *C. d. terrificus* Crotoxin, or (B) rabbit polyclonal antibodies raised against *C. d. terrificus* Crotoxin. The venom of *Crotalus atrox* (♦), which lacks Crotoxin, was used as a negative control, whereas *C. d. terrificus* venom (●), containing Crotoxin, was a positive control. Bound antibodies were detected with corresponding anti-IgG-HRP conjugates, as described in Section 2.10.

Conflict of interest statement

The authors declare no conflicts of interest in this study.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jprot.2018.09.002.

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