Research Article

A Novel Phospholipase A\textsubscript{2} (D49) from the Venom of the *Crotalus oreganus abyssus* (North American Grand Canyon Rattlesnake)

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Currently, *Crotalus viridis* was divided into two species: *Crotalus viridis* and *Crotalus oreganus*. The current classification divides “the old” *Crotalus viridis* into two new and independent species: *Crotalus viridis* (subspecies: viridis and nuntius) and *Crotalus oreganus* (subspecies: abyssus, lutosus, concolor, oreganus, helleri, cerberus, and caliginis). The analysis of a product from cDNA (E6d), derived from the gland of a specie *Crotalus viridis viridis*, was found to produce an acid phospholipase A\textsubscript{2}. In this study we isolated and characterized a PL\textsubscript{A}_\textsubscript{2} (D49) from *Crotalus oreganus abyssus* venom. Our studies show that the PL\textsubscript{A}_\textsubscript{2} produced from the cDNA of *Crotalus viridis viridis* (named E6d) is exactly the same PL\textsubscript{A}_\textsubscript{2} primary sequence of amino acids isolated from the venom of *Crotalus oreganus abyssus*. Thus, the PL\textsubscript{A}_\textsubscript{2} from E6d cDNA is actually the same PL\textsubscript{A}_\textsubscript{2} presented in the venom of *Crotalus oreganus abyssus* and does not correspond to the venom from *Crotalus viridis viridis*. These facts highlight the importance of performing more studies on subspecies of *Crotalus oreganus* and *Crotalus viridis*, since the old classification may have led to mixed results or mistaken data.

1. Introduction

*Crotalus viridis* defines a large group of snakes, also named as Western Rattlesnakes, which inhabit the eastern region of the Rocky Mountains of the United States that stretch from southern Canada to northern Mexico (Figure 1) [1, 2]. Phylogenetic analyses on mitochondrial DNA sequences of snakes classified as *Crotalus viridis* show significant taxonomic variations between individuals from different areas of USA and indicate that this species has several subspecies [3]. To understand the variations in these subspecies, morphological analyses were carried out based on distance analysis of whole venom profiles and based on maximum parsimony (MP) analysis of cyt\textsubscript{b} and ND4 [1, 4, 5].
Initially, based only on morphology, Klau ber classified these snakes into nine subspecies of *Crotalus viridis*: *C. v. viridis*, *C. v. nuntius*, *C. v. abyssus*, *C. v. lutosus*, *C. v. concolor*, *C. v. oreganus*, *C. v. helleri*, *C. v. Cerberus*, and *C. v. caliginis* [1]. This classification was in place until the early 2000s, when reports by Pook et al. and Asthon and de Queiroz, based on the analysis of the molecular characteristics of DNA of the nine subspecies of *C. viridis*, showed that the subspecies could be grouped into two distinct and new groups: *Crotalus viridis* and *Crotalus oreganus* [3–5]. The new classification grouped the *C. viridis* into two new subspecies: *C. v. viridis* and *C. v. nuntius* and *Crotalus oreganus* into five new subspecies: *C. o. abyssus*, *C. o. lutosus*, *C. o. concolor*, *C. o. oreganus*, *C. o. helleri*, *C. o. Cerberus*, and *C. o. caliginis*. This is the current and official classification used for snakes previously classified as *C. viridis* (and its subspecies).

Secreted phospholipases A<sub>2</sub> (PLA<sub>2</sub>s) are a family of relatively stable enzymes, with low molecular mass (13–15 kDa) and 6 (or 7) conserved disulfide bonds. PLA<sub>2</sub>s employ calcium ions and the amino acid residues, Asp47, and His48, to catalyze the hydrolysis of the Sn-2 of glycerophospholipid esters bonds of membranes. This hydrolysis reaction releases glycerol and proinflammatory eicosanoids [6–10]. PLA<sub>2</sub> are present in snake venom and the biological fluids, cells, and tissues of these species and are widely studied due to their pharmacological diversity. These enzymes can act as regulators of the membrane phospholipid membrane homeostasis [8–10] and also present physiopathological processes that can be neurotoxic (pre- or postsynaptic), cardiotoxic [11–14], hypotensive [15–17], anticoagulant and platelet aggregating [18,19], genotoxic [20,21], myotoxic [22,23], antitumoral, and bacterial [24, 25]. Due to the toxic pharmacological effects produced by PLA<sub>2</sub>, several studies have researched or developed natural or synthetic compounds to aid in the treatment of the snake bites to inhibit the toxic effects of PLA<sub>2</sub> [26–33]. In addition, the amino acid sequences of hundreds of PLA<sub>2</sub>s from snake venom have been determined [34–36].

Tsai et al. studied PLA<sub>2</sub> from glands obtained from different samples of *C. viridis viridis*, arising from several regions of the United States (Figure 1(a)) [37]. They purified and sequenced five acidic PLA<sub>2</sub> sharing 78% or greater sequence identity. Interestingly, Tsai et al. observed that the product of the cDNA sequence named cvvE6d modified a PLA<sub>2</sub> with a molecular mass of 13782 ± 1 Da. This specific molecule of PLA<sub>2</sub> was found only in a unique snake from Southeastern Arizona. The authors correctly inferred and suggested that these individuals from Southeastern Arizona could actually represent a distinct population of *Crotalus viridis viridis* [37].

Recently, while studying the differences in total venoms from *C. viridis* and *C. oreganus* subspecies, Mackessy verified that all venoms display great variation, both in protein composition as well as in the activities of several enzymes, including the PLA<sub>2</sub> enzyme family [2]. The venom used by Macksey was obtained from *C. viridis* and *C. oreganus* subspecies from the locations shown in Figure 1(b) [2].

According to Macksey, as the Western Rattlesnake occurs across a broad geographical area, it represents an ideal species group to investigate variations in venom composition, and to understand how these differences evolve and how composition affects the biological role(s) of venom [2]. In this study, to further the understanding of the biological diversity of the subspecies of *C. oreganus*, we biochemically isolated and characterized a PLA<sub>2</sub> (D49) from *C. oreganus abyssus* venom. Moreover, we sequenced the primary structure of
PLA₂, performed pharmacological and biochemical characterization assays, and used molecular modeling to analyze the structure obtained.

2. Material and Methods

2.1. Material. All reagents were purchased from Aldrich or Sigma Co (USA). Crotalus oreganus abyssus (Coa), Crotalus viridis viridis (Cev), and Crotalus viridis nuntius (Cvn) venoms were obtained from The National Natural Toxins Research Center (NNTRC) of Texas A&M University-Kingsville (Kingsville, Texas, USA). The substrate, 1-hexadecanoyl-2-(1-pyrenedecanoyl)-sn-glycero-3-phosphoglycerol (HPGP), was supplied by Molecular Probes (USA). The substrate 4-nitro-3-octanoyloxy benzoic acid (NOBA) was synthesized following the methodology described by Cho et al. [38].

2.2. Isolation of the Phospholipase A₂ from Crotalus oreganus abyssus (CoaPLA₂). Venom from C. o. abyssus (200 mg) was fractionated by chromatography on a G75-Sephadex column, previously balanced with 0.05 M ammonium bicarbonate buffer (AMBIC—pH 8.0). Elution was performed using 1.0 M ammonium bicarbonate (AMBIC—pH 8.0) at a flow rate of 0.5 mL/min. Fraction II, presenting phospholipase activity, was collected and ultrafiltered using the MidJet apparatus (Ge Healthcare, USA) equipped with the UFP-10-C-MM01A cartridge (superficial area of 26 cm², cut off: 10,000 Da—Ge Healthcare, USA). The filtrate was lyophilized and stored frozen at −20 °C.

Lyophilized fraction II (25 mg), containing PLA₂ activity, was dissolved in 250 μL of 5% (v/v) acetonitrile in 0.1% (v/v) trifluoroacetic acid (TFA), homogenized and centrifuged at 480 x g for 5 min, and then subjected to a reverse phase HPLC (model 2010, Shimadzu, Japan) using an analytical C18 column (Supelco, 250 mm × 4.6 mm). The analytical C18 column was equilibrated in solvent A (5% acetonitrile, 0.1% TFA) and elution proceeded with a concentration gradient from 0 to 100% of solvent B (60% acetonitrile, 0.1% TFA) at a flow rate of 1 mL/min, for 60 min.

To help remove any other impurities that might be present, the fraction with PLA₂ activity was again subjected to ultrafiltration using the MidJet apparatus (Ge Healthcare, USA), equipped with the UFP-10-C-MM01A cartridge (superficial area of 26 cm², cut off: 10,000 Da—Ge Healthcare, USA). A PLA₂ named CoaPLA₂ was isolated, and the filtrate was lyophilized and rechromatographed to evaluate its purity, under the same conditions as described above (Figure 2). The fractions were monitored by spectrophotometry at 280 nm. The purity level of the CoaPLA₂ was also evaluated using native polyacrylamide gel (PAGE) and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) [18, 21–23].

2.3. Biochemical Characterization of CoaPLA₂

2.3.1. SDS-PAGE and PAGE Electrophoresis. Electrophoresis analysis was performed to evaluate the purity and estimate molecular mass of CoaPLA₂, under reducing and nonreducing conditions. The standard molecular weight proteins were purchased from BioRad Co. (Phosphorylase b—97,400; Serum albumin—66,200; Ovalbumin—45,000; Carbonic anhydrase—30,000; Trypsin inhibitor—20,100; Lysozyme—14,400 MW). CoaPLA₂ pl was determined by isoelectric focusing, according to a previously described method [24–26].

2.3.2. Phospholipase A₂ Activity. Enzymatic activity was measured by two methods using two different substrates; a nonmicellar (4-nitro-3-octanoyloxy benzoic acid—NOBA) and a micellar substrate (1-hexadecanoyl-2-(1-pyrenedecanoyl)-sn-glycero-3-phosphoglycerol—HPGP).

(1) Phospholipase A₂ Activity Measured Using a Nonmicellar Substrate (4-nitro-3-octanoyloxy benzoic acid—NOBA). The phospholipase A₂ activity of CoaPLA₂ (both in the isolated protein and in total venoms) was measured using the assay described by Holzer and Mackessy [43], but modified for 96-well plates [17, 31–34]. The standard assay mixture contained 200 μL of buffer (10 mM Tris-HCl, 10 mM CaCl₂ and 100 mM NaCl, pH 8.0), 20 μL of substrate (3 mM 4-nitro-3-octanoyloxy benzoic acid), 20 μL of water, and 20 μL of PLA₂ (10 mg/mL) in a final volume of 260 μL. After adding PLA₂ (or total venom) (20 μg), the mixture was incubated for up to 40 min at 37 °C, with the reading absorbance at intervals of 10 min until 60 min. Enzyme activity, expressed as the initial velocity of the reaction (V₀), was calculated based on the absorbance at 20 min. After this time, the velocity did not change (maximum velocity was achieved). Enzyme activity was expressed as mean ± SD of three independent experiments and each experiment was carried out in triplicate.

(2) Phospholipase A₂ Activity Measured Using a Micellar Substrate (1-hexadecanoyl-2-(1-pyrenedecanoyl)-sn-glycero-3-phosphoglycerol—HPGP). The measurements of enzymatic activity using the substrate 1-hexadecanoyl-2-(1-pyrenedecanoyl)-sn-glycero-3-phosphoglycerol (HPGP) were carried out using a microtiter plate assay [10, 20, 44]. One hundred μL of solution A in assay buffer (27 μL bovine serum albumin, 50 mM KCl, 1 mM CaCl₂, 50 mM Tris-HCl pH 8.0) were added to a 96-well microtiter plate. Solution B presented the same composition as Solution A but with PLA₂ (0.5 μg/mL) or total venom (1.0 μg/mL) and was delivered in 100 μL portions to four wells, except for the first one. As a control, instead of Solution B, an additional 100 μL of Solution A was added to the first of the four wells in the assay. Solution B was prepared immediately prior to each set of assays to avoid loss of enzymatic activity. After the addition of Solution B, the assay was rapidly initiated by the addition of 100 μL of Solution C (420 mM 1-hexadecanoyl-2-(1-pyrenedecanoyl)-sn-glycero-3-phosphoglycerol vesicles in assay buffer) with a repeating pipette to all four wells. The fluorescence (excitation = 342 nm, emission = 395 nm) was read with a microtiter plate spectrophotometer (Fluorocount, Packard Instruments). Enzyme activity, expressed as the initial velocity of the reaction (V₀), was calculated based on the absorbance at 20 min. After this time, the velocity did not...
change (the maximum velocity was achieved). Enzyme activity was expressed as mean ± SD of three independent experiments and each experiment was carried out in triplicate.

2.3.3. Optimal pH and Temperature Determination of the Enzymatic Activity. Optimal pH and optimal temperature of the PLA$_2$ activity (using methodology described in Section 2.3.2(1)) of the CoaPLA$_2$ were determined by incubating the enzyme in four buffers of different pH values (4–10) and at different temperatures (25, 30, 35, 40, and 45°C), respectively, as described above (Section 2.3.2). The effect of substrate concentration (10, 5, 2.5, 1.25, 0.625, and 0.312 μM) on enzyme activity was determined by measuring the increase of absorbance after 20 min and absorbance values at 425 nm were measured with a VersaMax 190 multiwell plate reader (Molecular Devices, S., CA). Enzyme activity was expressed as mean ± SD of three independent experiments and each experiment was carried out in triplicate.

2.3.4. Determination of Influence of Ca$^{2+}$ (and Other Ions) on PLA$_2$ Activity. Three experiments were carried out to determine the influence of calcium ions on CoaPLA$_2$ activity (using methodology described in Section 2.3.2(1)). The activity was described above (Section 2.3.2). Initially, Ca$^{2+}$ concentrations of 0, 1, and 10 mM were used. After this procedure, the other three experiments were carried out: (1) without Ca$^{2+}$, but in the presence of 10 mM of Mg$^{2+}$, Cd$^{2+}$, and Mn$^{2+}$; (2) 1 mM of Ca$^{2+}$ in the presence of 10 mM of Mg$^{2+}$, Cd$^{2+}$, and Mn$^{2+}$, and (3) 10 mM of Ca$^{2+}$ in the presence of 10 mM of Mg$^{2+}$, Cd$^{2+}$, and Mn$^{2+}$. The influences of the ions on the enzyme activity were measured by determining absorbances at 425 nm with a VersaMax 190 multiwell plate reader (Molecular Devices, S., CA). Enzyme activity was expressed as
Figure 3: Phospholipase \( A_2 \) activities of CoaPLA\(_2\) from Crotalus Oreganus abyssus. (a) Phospholipase \( A_2 \) activities of CoaPLA\(_2\) and total venom of Crotalus Oreganus abyssus, Crotalus viridis viridis, and Crotalus viridis nuntius using a nonmicellar substrate (4-nitro-3-octanoyloxy benzoic acid); (b) Phospholipase \( A_2 \) activities of CoaPLA\(_2\) and total venom of Crotalus Oreganus abyssus, Crotalus viridis viridis, and Crotalus viridis nuntius using a micellar substrate (1-hexadecanoyl-2-(1-pyrenedecanoyl)-sn-glycero-3-phosphoglycerol); (c) influence of pH variations on the enzymatic activity of CoaPLA\(_2\); (d) influence of temperature variations on the enzymatic activity of CoaPLA\(_2\); (e) analysis of the influence of calcium ions and other divalent cations on the phospholipase \( A_2 \) activity of CoaPLA\(_2\). Results are expressed as mean ± SD of three independent experiments performed in triplicate (\( n = 3 \)).
mean ± SD of three independent experiments and each experiment was carried out in triplicate.

2.4. Biological Activity

2.4.1. Animals. Groups of 6 Swiss male mice (6–8 weeks old) were matched for body weight (18–22 g). The animals were housed for at least one week before the experiment in laminar-flow cages maintained at a temperature of 22 ± 2°C and a relative humidity of 50–60%, under a 12:12 h light-dark cycle. The animal experiments were carried out with the approval of the Institutional Ethics Committee, in accordance with protocols following the recommendations of the Canadian Council on Animal Care. The mice used in this study were kept under specific pathogen-free conditions.

2.4.2. 50% Lethal Dose. To evaluate the 50% lethal dose (dose that causes death in 50% of animals) of CoaPLA$_2$, and total venoms from C. o. abyssus, C. v. viridis, and C. v. nuntius, groups of six Swiss male mice (18–22 g) received an intravenous injection of 100 μg of enzyme or total venom, dissolved in 100 μL of PBS. As a control, six mice were similarly injected with 100 μL of PBS alone. Animals were observed for up to 24 h after injection to record deaths. Lethal dose (LD) was expressed as mean ± SD of three independent experiments, performed in triplicate (n = 6) (Figure 3) [17, 18, 28, 29, 31, 33, 34].

2.4.3. Edema-Inducing Activity. Groups of six Swiss male mice (18–22 g) were injected in the subplantar region with various amounts of total venom or CoaPLA$_2$ (in a volume of 50 μL) prepared in PBS, pH 7.2. Subsequently, paw volume was measured at different time intervals (30, 60, 120, and 180 min), subtracting the initial paw volume (time 0 h). Paw edema was measured with a low-pressure pachymeter (Mutoyco, Japan). Edema-inducing activity was expressed as mean ± SD of three independent experiments and each experiment was carried out in triplicate (n = 6) (Figure 4) [17, 18, 28, 29, 31, 33, 34].

2.4.4. Myotoxic Activity. Groups of six Swiss male mice (18–22 g) were injected in the right gastrocnemius muscle with total venom or PLA$_2$ (50 mg/50 mL of PBS) or PBS alone (50 mL). After 3 h, blood was collected from the tail in heparinized capillary tubes and centrifuged for plasma separation. Activity of creatine kinase (CK) was then determined using 4 mL of plasma, which was incubated for 3 min at 37°C with 1.0 mL of the reagent according to the kinetic CK-UV protocol from Bioclin, Brazil. The activity was expressed in U/L, where one unit corresponds to the production of 1 mmol of NADH per minute (Figure 6). Myotoxic activity was expressed as mean ± SD of three independent experiments and each experiment was carried out in triplicate (n = 6) (Figure 5) [17, 18, 28, 29, 31, 33, 34].

2.5. Structural Analysis

2.5.1. MALDI-TOF Analysis of CoaPLA$_2$. The molecular mass of CoaPLA$_2$ was analyzed by matrix-assisted laser desorption/ionization-time-of-flight (MALDI-TOF) mass spectrometry using a MALDI-TOF/TOF—Proteomics Analyzer 4800 (Applied Biosystems). Sample treatment: CoaPLA$_2$ was analyzed at a concentration of 0.8 μL of matrix and mixed with 0.5 μL of sample on the MALDI plate. Samples were allowed to dry before analysis. The matrix consisted
of 10 mg/mL sinapinic acid in 50% acetonitrile/miliQ water (v/v) and 0.1% trifluoroacetic acid (TFA). To calibrate the apparatus, a BSA standard solution was prepared following the same procedure, and 4 pmols were analyzed under the same conditions.

### 2.5.2. Sequencing Procedure

1. **In Solution Digestion.** Proteins were reduced by treatment with a solution of 20 mM DTT (Dithiothreitol) in 50 mM NH$_4$HCO$_3$ for 1 h at 30°C and alkylated with a solution of 150 mM iodoacetamide in 50 mM NH$_4$HCO$_3$ for 1 h at 30°C. The sample was then digested overnight at 37°C with trypsin (sequencing grade modified, Promega). Tryptic peptides were then cleaned-up with a Proxeon Stage tip. Peptides were eluted from the tip with 70% acetonitrile/0.1% trifluoroacetic acid. The eluted peptides were dried in a vacuum centrifuge and resuspended in 1% formic acid for LC-MS/MS analysis.

2. **LC-MS/MS Analysis.** Mass spectrometry was performed in a NanoAcquity (Waters) HPLC coupled to an Orbitrap Velos mass spectrometer (Thermo Scientific). An aliquot of the tryptic digest was injected and separated in a CI8 reverse phase column (75 μm OI, 10 cm, nano-Acquity, 1.7 μm BEH column, Waters). Bound peptides were eluted from the column with the following gradient: 1 to 40% B in 20 minutes, followed by a gradient of 40 to 60% B in 5 minutes; flow was 250 nL/min (A: 0.1% formic acid in water; B: 0.1% formic acid in acetonitrile). Eluted peptides were ionized in an emitter needle (PicoTipTM, New Objective). The spray voltage applied was 1900 V. Peptide masses (m/z: 300–1700) were measured in full scan in the Orbitrap at a resolution of 60,000 at m/z 400. The 5 most abundant peptides (minimum intensity of 1500 counts) were selected from each MS scan and fragmented in the HCD collision cell using a normalized collision energy (NCE) of 40% with nitrogen as the collision gas. Fragments were detected in the Orbitrap with a resolution of 7500 FWHM at 400 m/z. Raw data were collected with Thermo Xcalibur (v.2.1.0.1140). We obtained eight fragments, as shown in Table 1 [45, 46].

3. **Database Search.** Raw data were analyzed using Proteome Discoverer (v.1.3.0.339) software. A search was run with the search engine MASCOT against NCBI nr Serpentes database. The Percolator node was used in the Proteome Discoverer Mascot search in order to discriminate correct from incorrect peptide spectrum matches using the q-value (FDR) to improve the number of confidently identified peptides at a given false discovery rate. The results have been filtered so only high confidence peptides (FDR ≤ 0.01) have been considered for identification results [46, 47].

### 2.6. Tridimensional Structure Modeling

1. **Threading Modeling.** The initial 3D model of PLA$_2$ from *C. oreganus abyssus* venom was generated employing the threading modeling technique [39, 40, 48–57] implemented in the HHpred webserver (http://toolkit.tuebingen.mpg.de/hhpred). Initially, we generated 31 primary structure alignments using X-Ray structures available at the Protein Data Bank (PDB, http://www.rcsb.org/pdb/home/home.do) employing the global alignment option and scoring the predicted secondary structure alignment. From the 5 best ranked alignments, we evaluated the crystallographic quality (resolution and R-free value, Ramachandran distribution, and b-factor) of the target proteins. The phospholipase A$_2$ from *Crotalus durissus terrificus* (PDB ID: 3ROL chain D) [58] was chosen as a template to thread the modeling, presenting 51.1% of identity and 100% of hit probability, with an E-value of 2.1$^{-41}$ and score of 255.95.

2. **Molecular Dynamics.** A molecular dynamics (MD) simulation was carried out aiming to refine the constructed PLA$_2$ model. The MD simulation was performed employing GROMACS 4.5.4 package [48, 49], running with a 8G RAM Intel Xeon processor. Explicit water molecules were used employing the Simple Point Charge (SPC) model [50]. Protonation states of charged groups were set according to pH 7.0 and counter ions were added to neutralize the system. Gromos force field [51] was chosen to perform the MD simulation. The MD simulation was performed at constant temperature and pressure in a periodic truncated cubic box with a volume that was equal to 259.14 nm$^3$ and at a minimum distance of 5 Å between any atom of the protein and the box wall. Sodium ions were added as counter ions to neutralize the system.

Initially, an energy minimization using the steepest descent algorithm was performed. After, 20 ps of MD simulation with position restraints applied to the protein were performed.
Table 1: Peptide fragments obtained by sequencing procedure. The CoaPLA$_2$ was reduced and digested overnight with trypsin. Eluted tryptic peptides were dried in a vacuum centrifuge and resuspended in 1% formic acid for LC-MS/MS analysis.

| Number | Peptide fragment                          | Molecular mass (MH$^+$/Da) |
|--------|------------------------------------------|-----------------------------|
| 1      | SLVQFEML1MKVAKR                         | 1793.01230                  |
| 2      | SGLFSYAYSAGCGWGWHGR                     | 2241.91338                  |
| 3      | PQDATDHCFVHDCGKY                        | 2269.8394                   |
| 4      | TASYTYSEENGEIVCGGDPCKK                  | 2580.07798                  |
| 5      | QVCECDR                                 | 966.37218                   |
| 6      | VAAICFR                                 | 836.44066                   |
| 7      | DNIPTYDNK                               | 1079.49590                  |
| 8      | FPPENCQEEPEPC                           | 1632.62198                  |

at 300 K to relax the system. Finally, an unrestrained MD simulation was performed at 300 K during 10 ns to assess the stability of the structures. During the simulation, temperature and pressure (1.0 bar) were maintained by coupling to an external heat and an isotropic pressure bath.

2.6.3. Structural Analysis and Validation. After the MD simulation, several tools of structural analysis contained in the GROMACS package were employed to evaluate the final 3D model. All figures were generated employing PyMOL 0.99c software [52]. Other validation methods were also used, such as a pseudoenergy profile, which was analyzed with Verify 3D [53, 54] and ProSA-web [55, 56], as well as the Ramachandran plot [57], ERRAT program [39], and ANOLEA web server [40].

2.7. Statistical Analysis. Results are presented as mean ± SD obtained with the indicated number of animal samples or in vitro assays. The statistical significance of differences between groups was evaluated using the Student’s unpaired t-test and ANOVA analysis of variance. Significance levels were considered at a confidence interval of 0.1 > $P$ > 0.05.

3. Results and Discussion

3.1. Isolation and Purification of the Phospholipase A$_2$ from Crotalus oreganus abyssus (CoaPLA$_2$). The process used to obtain the pure protein (CoaPLA$_2$) is shown in Figure 2. Gel filtration (Figure 2(a)) demonstrated the presence of fraction II containing PLA$_2$ activity, which was further purified. Figure 2(b) shows the HPLC profile obtained using a reverse phase C18 column and the detachment of the peak containing CoaPLA$_2$. This peak was also further purified by rechromatography and subjected to electrophoresis (SDS-PAGE and PAGE). As shown in Figure 2(c), the purification process was efficiently purified. Nondenaturation electrophoresis showed that CoaPLA$_2$ was a dimeric protein with a molecular mass of approximately 28 kDa (lines 3 and 6), but under denaturing conditions, it was a monomer with a molecular mass of approximately 14 kDa (lines 2 and 5). This information was subsequently confirmed by MALDI-TOF mass spectrometry.

3.2. Biochemical Characterization of CoaPLA$_2$. We biochemically analyzed and characterized CoaPLA$_2$. Figure 3 shows phospholipase A$_2$ activity under several conditions. We measured the phospholipase A$_2$ activity of the isolated enzyme and total venom (using two different methods related to substrate type), as well as the optimal temperature and pH, and the influence of ions on the activity of the enzyme. Figures 3(a) and 3(b) show the PLA$_2$ activity of the CoaPLA$_2$ and of the total venom from Crotalus oreganus abyssus, Crotalus viridis viridis, and Crotalus viridis nuntius. Figure 3(a) shows that the phospholipase A$_2$ activity (using the nonmicellar substrate, 4-nitro-3-octanoyloxy benzoic acid) of the CoaPLA$_2$ is approximately 48 nmol/min/mg, while the total venom from Crotalus oreganus abyssus has a PLA$_2$ activity (approximately 22.5 nmol/min/mg) that is very different to the PLA$_2$ activity of venom from Crotalus viridis viridis and Crotalus viridis nuntius (approximately 53 and 9 nmols/min/mg, resp.). Conversely, using the micellar substrate, 1-hexadecanoyl-2-(1-pyrene-decanoyl)-sn-glycero-3-phosphoglycerol, Figure 3(b) shows that the activity of the CoaPLA$_2$ was approximately 590 µmol/min/mg, while the total venom from Crotalus oreganus abyssus has a PLA$_2$ activity (approximately 276 µmol/min/mg) that was significantly different to the PLA$_2$ activity of venom from Crotalus viridis viridis and Crotalus viridis nuntius (approximately 606 and 51 µmol/min/mg, resp.). Both methods clearly demonstrate that the venom from Crotalus oreganus abyssus that we used to isolate CoaPLA$_2$ was not derived from Crotalus viridis viridis or Crotalus viridis nuntius.

Interestingly, the enzymatic activity, obtained by Tsai et al., 2003, when cloning E6d was around 680 µmol/min/mg and when using the micellar substrate L-dipalmitoyl-glycero-phosphatidyl-choline, being relatively close to the value found in the present study. We used a different, but similar, micellar substrate (1-hexadecanoyl-2-(1-pyrene-decanoyl)-sn-glycero-3-phosphoglycerol) and obtained a value of enzymatic activity of approximately 590 µmol/min/mg.

The optimal temperature of CoaPLA$_2$ was determined to be 37.3°C (Figure 3(d)) and optimal pH was 7.9 (Figure 3(c)). These values are in accordance with other PLA$_2$ measurements described in the literature [12–34]. The influence ions on the enzyme activity was determined in the presence and absence of Ca$^{2+}$ and other divalent cations (also in the presence and absence of Ca$^{2+}$). Figure 3(e) shows that the PLA$_2$ activity of CoaPLA$_2$ is calcium-dependent. In the presence of 10 mM calcium, the PLA$_2$ activity was 45.8
Table 2: Multiple alignment of fragment of CoaPLA2.

| Fragments obtained | - - - Frag 1 - - - | - - - Frag 2 - - - - - - | - - Frag 3 |
|--------------------|-------------------|---------------------------|-----------|
| CoaPLA2 (1)        | SLVQFELIMKVAKR    | SLVQFELIMKVAKR             | PQQDATDHCC|
| Cvv_E6D (1)        | MRTLWIVALLLGVEG   | SLVQFELIMKVAKR             | PQQDATDHCC|
| Cvv_E6E (1)        | MRTLWILAVLLLGVEG  | SLVQFELLIMKVAKR             | PQQDATDHCC|
| Cvv_E6H (1)        | MRTLWILAVLLLGVEG  | SLVQFETIMKIAKR             | PQQDATDRCC|
| Cvv_E6G (1)        | MRTLWIVAVLLLGVEG  | SLVQFEMMIKVAKR             | PQQDATDHCC|
| Cvv_E6A (1)        | MRTLWIVAVLLLGVEG  | SLVQFETIMKIAKR             | PQQDATDRCC|
| Cadam (1)          | MRTLWIVAVLLLGVEG  | SLVQFEMMIKVAKR             | PQQDATDRCC|
| Chorrid (1)        | MRTLWILAVLLLGVEG  | SLVQFEMMIKVAKR             | PQQDATDRCC|
| Chhorr (1)         | MRTLWIVAVLLLGVEG  | SLVQFEMMIKVAKR             | PQQDATDRCC|
| Scterg (1)         | MRTLWILAVLLLGVEG  | SLVQFEMMIKVAKR             | PQQDATDRCC|
| Catrox (1)         | SLVQFETILIMKIAGR  | SGLLWSAYGCGWGHHGLQ         | PQQDATDRCC|
| FVHDCCYGK (45)     | TASYTSEENEGIEVGGDPCKK | QVCECDR VAACFR DNIPTYD|
| Cvv_E6D (61)       | FVHDCCYGK VDCNPK  | TASYTSEENEGIEVGGDPCKK     | QVCECDR VAACFR DNIPTYD|
| Cvv_E6E (61)       | FVHDCCYGK VDCNPK  | TASYTSEENEGIEVGGDPCKK     | QVCECDR VAACFR DNIPTYD|
| Cvv_E6H (61)       | FVHDCCYGK ATDCNPK | TASYTSEENEGIEVGGDPCKK     | QVCECDR VAACFR DNIPTYD|
| Cvv_E6G (61)       | FVHDCCYGK ATDCNPK | TASYTSEENEGIEVGGDPCKK     | QVCECDR VAACFR DNIPTYD|
| Cvv_E6A (61)       | FVHDCCYGK ATDCNPK | TASYTSEENEGIEVGGDPCKK     | QVCECDR VAACFR DNIPTYD|
| Cadam (61)         | FVHDCCYGK ATNCNPK | TASYTSEENEGIEVGGDPCKK     | QVCECDR VAACFR DNIPTYD|
| Chorrid (61)       | FVHDCCYGK ATDCNPK | TASYTSEENEGIEVGGDPCKK     | QVCECDR VAACFR DNIPTYD|
| Chhorr (61)        | FVHDCCYGK ATDCNPK | TASYTSEENEGIEVGGDPCKK     | QVCECDR VAACFR DNIPTYD|
| Scterg (61)        | FVHDCCYGK ATDCNPK | TASYTSEENEGIEVGGDPCKK     | QVCECDR VAACFR DNIPTYD|
| Catrox (45)        | FVHDCCYGK ATDCNPK | TASYTSEENEGIEVGGDPCKK     | QVCECDR VAACFR DNIPTYD|
| NKYWR FPPENCQEEPEPC|                  |                           |           |
| CoaPLA2 (98)       | NKYWR FPPENCQEEPEPC|                      |           |
| Cvv_E6D (121)      | NKYWR FPPENCQEEPEPC|                      |           |
| Cvv_E6E (121)      | NKYIQ FPACKQEEPEPC|                      |           |
| Cvv_E6H (121)      | NNYKR FPACNCREEEPEPC|                    |           |
| Cvv_E6G (121)      | NNYKR FPACNCREEEPEPC|                    |           |
| Cvv_E6A (121)      | NNYWL FLPKNCRREEPEPC|                    |           |
| Cadam (121)        | NNYWL FPPKNCRREEPEPC|                    |           |
| Chorrid (121)      | NNYWL FPPKNCRREEPEPC|                    |           |
| Chhorr (121)       | NNYWL FPPKNCRREEPEPC|                    |           |
| Scterg (121)       | NNYWR LPTENQEEPEPC|                      |           |
| Catrox (105)       | NNYWL FPPKDCRREEPEPC|                    |           |

nmols/min/mg. When the calcium concentration was 1 mM calcium, the phospholipase A2 activity was slightly reduced to 38.1 nmols/min/mg. A complete absence of calcium ions drastically reduced the enzyme activity to values of approximately 3 nmols/min/mg. When 10 mM of other divalent cations (Mg^{2+}, Cd^{2+} and Mn^{2+}) was employed, the activity of the PLA2 was completely suppressed. However, phospholipase A2 activity was recovered when Ca^{2+} was mixed with these divalent cations (Mg^{2+}, Cd^{2+} and Mn^{2+}), both at concentrations of 1 mM and 10 mM (Figure 3(e)).

3.3. Biological Characterization of CoaPLA2. The biological characterization of CoaPLA2, isolated from Crotalus oreganus abyssus, was carried out using measurements of lethal activity (LA_{50}%,—dose that causes death in 50% of animal subjects), edema-inducing and myotoxic activities. We tested the lethal activity (LA_{50}%) of CoaPLA2 and of the total venom of Crotalus oreganus abyssus; Cvv_E6D, Cvv_E6E, Cvv_E6H, Cvv_E6G, and Cvv_E6A (cDNA from Crotalus viridis viridis [37]), Cadam: Crotalus adamanteus [39]; Chorrid and Chhorrid: Croatus horridus and Crotalus horridus horridus [40]; Scterg: Sistrurus catenatus tergeminus [41]; Catrox: Crotalus atrox [42].
Crotaulus oreganus abyssus, Crotaulus viridis viridis, and Crotaulus viridis nuntius. Figure 4 shows that LA\textsubscript{50}% of the venom from Crotaulus oreganus abyssus is approximately 2.2 ± 0.4 μg of venom/g of mouse, and this value is bigger in relation to C. v. viridis and C. v. nuntius. CoaPLA\textsubscript{2} has a LA\textsubscript{50}% at a dose of about 1.8 μg ± 0.2 of venom/g mouse weight and was higher than that of the total venom of C. v. viridis and C. v. nuntius. The total C. o. abyssus venom is more lethal than CoaPLA\textsubscript{2} alone, as venom contains other enzymes that also exhibit lethality, such as serine proteases and metalloproteases [12–34].

The edema-inducing activity of CoaPLA\textsubscript{2} was measured using different dosages of the enzyme (25, 50, and 100 μg). From Figure 5, we can see that the edema-inducing activity of CoaPLA\textsubscript{2} is dose-dependent. The increase in the amount of enzyme increases the percentage of edema formed, principally in the first 24 h. After this time, the edema-inducing activity is significantly reduced and the edema is suppressed.

Similarly to the edema-inducing activity results obtained, the myotoxic activity induced by CoaPLA\textsubscript{2} was also dose-dependent. When we increased the quantity of CoaPLA\textsubscript{2} (25, 50, and 100 μg), its myotoxic effects were augmented.

The phospholipases A\textsubscript{2} are a group of enzymes present in most venoms or oral secretions of snakes. In addition to the digestive function of the prey, these enzymes interfere with the physiological processes and cause many pharmacological and pathophysiological effects, such as neurotoxic, cardiotoxic, anticoagulant, antiplatelet, hemolytic, hemorrhagic, and inflammatory activities [59–61].

Both the crude venom of Crotaulus oreganus abyssus and the isolated CoaPLA\textsubscript{2}, were able to induce experimental toxicity, such as myonecrosis, edema, and mortality. Due to the neurotoxic potential of this kind of snake, it was observed that the LD\textsubscript{50}% of the crude venom of C. oreganus abyssus and its CoaPLA\textsubscript{2} showed low values of lethal doses, when compared to Bothrops genera venoms and its isolated PLAs\textsubscript{5} [36].

The CoaPLA\textsubscript{2} also induced myotoxic activity, similarly to other PLAs isolated from snake venoms. The myotoxicity was evaluated by the activity levels of creatine kinase (CK) in the plasma of animals. Creatine kinase is an enzyme used in muscular energy metabolism and, in cases of cell damage, is released and can be detected in plasma as a marker [36, 60]. The catalytic activity of the PLAs on the membrane suggests an important role these enzymes in the toxicity of snake venoms (svPLAs\textsubscript{5}). The breakdown of phospholipids causes severe changes in the structural and functional integrity of the plasmatic membrane with a consequent influx of calcium ions [62], release of calcium-dependent proteases [63], activation of endogenous PLAs\textsubscript{5} [64], and mitochondrial collapse [65]. The sum of all these molecular changes could lead to cell death.

The CoaPLA\textsubscript{2} was able to induce edema in mice paws. Local inflammation is a feature of poisoning by snakes of the subfamilies of Viperidae and Crotalidae [60, 61]. The catalytically active mechanism by which PLAs induces edema is probably due to the release of precursors of eicosanoids due to the hydrolysis of phospholipids. Release of biogenic amines from mast cells is also proposed as a possible mechanism of induction of edema by PLAs\textsubscript{2} [66, 67].

3.4. Structural Characterization of CoaPLA\textsubscript{2}. The molecular mass of CoaPLA\textsubscript{2} (Da) was analyzed by matrix-assisted laser desorption/ionization-time-of-flight (MALDI-TOF mass spectrometry—Proteomics Analyzer 4800). The peak with a molecular mass of 13793.8 Da corresponds to s monoisotopic ion (m/z, where z = +1) and the peak with a molecular mass of 6890.0 Da corresponds to a diisotopic ion (m/z, where z = +2).

To extend our structural study of CoaPLA\textsubscript{2}, we determined its primary structure using LC/MS-MS, after in-solution digestion (trypsic digestion). The fragments obtained were analyzed using the Proteome Discoverer
Figure 9: RMSF values per residue after protein stabilization.

Figure 10: (a) Stereoview of the final model; (b) superimposition of the initial (green), final (blue), and template (red) models; (c) residues with high energy predicted by ANOLEA are displayed in wireframe model.

(v.1.3.0.339) software, where a search was run with the MASCOT engine against the NCBIinr Serpentes database. The search found eight fragments (Table 1). The analysis using the ClustalW multiple sequence alignment showed that fragments recovered from the PLA$_{2}$ sequence produced by the cDNA E6d and described by Tsai et al. [37] displayed 94% sequential homology (Table 2), except for a unique fragment that was not found (VTDCNPK). From Table 2 we can see that this fragment is extremely conserved in all sequences analyzed and in the model proposed by us (Figure 10). We have inserted the sequence VTDCNPK in the gap of the fragment as not found, as shown in the E6D sequence.

The comparison between the E6D sequence and the CoaPLA$_{2}$ sequence obtained is interesting because both are exactly equal, except for the VTDCNPK fragment (not found in this study). Tsai et al., [37] suggested that the specimen, initially considered as *Crotalus viridis viridis*, may be a distinct population present in Southwestern Arizona, considered the natural habitat of *Crotalus oreganus abyssus* (Figure 1(b)). Thus, we infer that, probably, the specimen used by Tsai et al., [37] that produced the E6D cDNA was actually a *Crotalus oreganus abyssus* snake, and not a *Crotalus viridis viridis*. In addition to this information, and to enforce our conclusion, it should be remembered that the value of the enzymatic activity of CoaPLA$_{2}$ found in this work is very near to the value found by Tsai et al., 2003 (around 680 $\mu$mol/min/mg for the E6d clone and approximately 590 $\mu$mol/min/mg for CoaPLA$_{2}$).
| Table 3: Results for ANOLEA, ERRAT, Verify 3D, ProSA, and Ramachandran analyses for the initial and final models. |
|-------------------------------------------------|-----------------|-----------------|
| **Initial model**                               | **Final model** |
| ANOLEA: residues with high energy               | 35 (26.32%)     | 25 (18.80%)     |
| Overall quality factor**^\prime\prime^: 64.516  | Overall quality factor**^\prime\prime^: 99.029 |
| ERRAT: error per residue                         |                 |
| Error value * (%)                                | 99              | 95              |
| 120 100 80 60 40 20                               |                 |
| Residue number (window center)                   |                 |
| Verify 3D: residues with scores of lower than 0 have low structural quality | 17 residues < 0 | 0 residues < 0 |
| Ramachandran plot                                |                 |
| ψ(°)                                            |                 |
| −180 0 +180                                     |                 |
| φ(°)                                            |                 |
| −180 0 +180                                     |                 |
| Residues in the outlier region                   | 3 GLY (2.29%)   | 2 GLY (1.53%)   |

This fact supports the need for more studies on *Crotalus oreganus* (all subspecies) because for many years all subspecies of *Crotalus viridis* and *Crotalus oreganus* were treated as a single serpent specimen. However, as subsequent studies have shown, the old classification was incorrect and the "old" *Crotalus viridis* can in fact be divided into two subspecies of *Crotalus viridis* (*viridis* and *nuntius*) and seven subspecies of *Crotalus oreganus* (*abyssus, lutosus, concolor, oreganus, helleri, Cerberus, and caliginis*).

3.5. Molecular Modeling. To increase the understanding of the CoaPLA2 structure, we conducted molecular modeling studies using Molecular Dynamics (MD) simulation. We calculated the values of root mean squared distance (RMSD) considering the protein backbone atoms, which are displayed in Figure 8. When analyzing these results, we noted that the PLA2 model was stabilized after approximately 1300 ps of simulation.

From the root mean squared fluctuation (RMSF) values of the alpha carbons per residue, we can see that the fluctuation of PLA2 residues from 1300 ps to 5000 ps is very low (except for the residues in the terminal loop, 119–133), indicating that there are no significant changes in the conformation of the residues (Figure 9).

Comparing the initial 3D model and the final model obtained after the MD simulation, it can be easily seen that the MD simulation is fundamental to refine the PLA2 model. The ProSA energy profile indicates that both initial and final models have energy values per residue of lower than 0, indicating a good pseudoenergy profile. Table 3 displays the results obtained from ANOLEA, ERRAT, Verify 3D, ProSA, and Ramachandran analyses for the initial and final models.

The ANOLEA results indicate that the MD simulation decreased the number of high energy residues by 8%. With all high energy residues of the final model being located in the loop region (Figure 9(c)). The ERRAT results indicate that the MD simulation improved the quality of the structural model from 64% to 99%. From the Verify 3D results, the initial model had 17 residues with poor structural quality (score lower than 0) and all residues of the final model had score values of higher than 0. Finally, the Ramachandran plot analysis indicates that both initial and final models had 3 and 2 glycine residues located at an outlier region, respectively. Figure 9(a) shows the Stereoview of the final model. Figure 9(a) shows that our model displays the typical phospholipase conformation, containing three parallel α-helixes and a β-wing (one double-stranded antiparallel β-sheet) [20].

Data reinforce the necessity of rearranging and clarifying all information available regarding the two subspecies of
Crotalus oreganus and Crotalus viridis, considering the length of time during which these species were considered as one and the same.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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