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CoaTx-II, a new dimeric Lys49 phospholipase A_2 from *Crotalus oreganus abyssus* snake venom with bactericidal potential: Insights into its structure and biological roles

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ABSTRACT

Snake venoms are rich and intriguing sources of biologically-active molecules that act on target cells, modulating a diversity of physiological functions and presenting promising pharmacological applications. Lys49 phospholipase A_2 is one of the multifunctional proteins present in these complex secretions and, although catalytically inactive, has a variety of biological activities, including cytotoxic, antibacterial, inflammatory, antifungal activities. Herein, a Lys49 phospholipase A_2, denominated CoaTx-II from *Crotalus oreganus abyssus*, was purified and structurally and pharmacologically characterized. CoaTx-II was isolated with a high degree of purity by a combination of two chromatographic steps; molecular exclusion and reversed-phase high performance liquid chromatography. This toxin is dimeric with a mass of 13868.2 Da (monomeric form), as determined by mass spectrometry. CoaTx-II is rich in Arg and Lys residues and displays high identity with other Lys49 PLA_2 homologues, which have high isoelectric points. The structural model of dimeric CoaTx-II shows that the toxin is non-covalently stabilized. Despite its enzymatic inactivity, *in vivo* CoaTx-II caused local muscular damage, characterized by increased plasma creatine kinase and confirmed by histological alterations, in addition to an inflammatory activity, as demonstrated by mice paw edema induction and pro-inflammatory cytokine IL-6 elevation. CoaTx-II also presents antibacterial activity against gram negative (*Pseudomonas aeruginosa* 31NM, *Escherichia coli* ATCC 25922) and positive (*Staphyloccocus aureus* BEC9393 and Rib1) bacteria. Therefore, data show that this newly purified toxin plays a central role in mediating the degenerative events associated with envenomation, in addition to demonstrating antibacterial properties, with potential for use in the development of strategies for antivenom therapy and combating antibiotic-resistant bacteria.

1. Introduction

Antimicrobial resistance is one of most serious and alarming public health problems, spreading faster than the discovery and introduction of new therapeutic molecules into clinical practice. The prevalence of this bacterial resistance and the side effects of conventional antibiotics have prompted the search and development of more efficient strategies, as well as powerful and safe compounds to aid in the fight against infectious diseases (Ling et al., 2015; Sudharshan and Dhananjaya, 2015). As such, proteins and peptides from snake venoms represent valuable and attractive sources of bioactive molecules against Gram-positive and Gram-negative bacteria (Oliveira-Junior et al., 2013; Corrêa et al., 2016).

Snake venoms constitute a complex and natural library of proteins and peptides that present valuable structural and functional diversity (Calvete et al., 2007; McCleary and Kini, 2013). A deeper

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understanding of the structural features, biological effects and biotechnological/medical applications of these compounds is instrumental in interpreting symptoms of snakebite envenomation and the identification and development of novel therapeutic agents (Angulo and Lomonte, 2009; Brahma et al., 2015). Among these numerous proteins and peptides, phospholipases A_2 (PLA_2s) are one of the most studied and well described group of proteins; these proteins generally present promising antibacterial activity (Lomonte et al., 2009; Gutierrez and Lomonte, 2013).

PLA_2s are stable, conserved, multifunctional, of low molecular weight and contain disulfide-rich enzymes that are involved in lipid metabolism and membrane remodeling (Van and De Haas, 1963; Schaloske and Dennis, 2006). This group of toxins shares significant sequential and structural similarity, however it exhibits an intriguing array of biological functions, which should be explored and studied from a toxicological, biotechnological and biomedical point of view (dos Santos et al., 2011; Carvalho et al., 2013; Gutierrez and Lomonte, 2013).

There are several isoforms of PLA_2, and these can be isolated from different sources. The most important known PLA_2s isolated from snakes venoms are: 1) The catalytically-active variant (Asp49 PLA_2), presenting a conserved aspartic acid residue at position 49, located in a catalytic center (key residue for binding essential Ca^{2+}), and 2) the catalytically-inactive homologues (Lys49 PLA_2), which lose the ability to cleave phospholipids (Ward et al., 2002; Lomonte et al., 2003a). Despite their catalytic inactivity, interestingly, Lys PLA_2s are biologically active and are involved in pathophysiology envenomation by snakes. These PLA_2s have been studied as structural models for the drug design of molecules with antitumor and antibacterial activities (Lomonte and Rangel, 2012; Azevedo et al., 2016). Therefore, the identification of new Lys PLA_2s from snake venom may be important for certain areas of health, such as serum therapy, treatment of cancer and infectious diseases (Lomonte et al., 2009; McCleary and Kini, 2013).

The *Crotalus oreganus abyssus* (Grand Canyon rattlesnake) is a venomous pitviper belonging to the Viperidae family and, like several other pitviper subspecies, biological and structural characterization of its venom composition is incomplete (Da Silva et al., 2011; Martins et al., 2014; Almeida et al., 2016). This pitviper subspecies also belongs to a group of snakes, commonly referred to as the Western Rattlesnake complex, and is considered a powerful tool for understanding venom profiles, variation and evolution, as described by Mackessy (2010). Given the molecular proprieties of Lys49 PLA_2s and the incomplete characterization of compounds from *C. o. abyssus* snake venom, we herein isolated and characterized a catalytically-inactive Lys49 PLA_2 (CoaTx-II) with antibacterial activity, in order to obtain information regarding its structure, biological roles and pharmacological potential.

2. Materials and methods

2.1. Venom and reagents

Crotalus oreganus abyssus snake venom was obtained from The National Natural Toxins Research Center (NNTRC) of Texas A&M University - Kingsville (Kingsville, TX, USA) and stored at −20 °C. All reagents were of analytical or sequencing grade.

2.2. Animals

Male Swiss mice (18–20 g; 7–8 weeks) used in assays were kept under specific pathogen-free conditions, a 12:12 h light–dark cycle and received water and food *ad libitum*. Mice were housed in laminar-flow cages maintained at a temperature of 22 ± 2 °C and a relative humidity of 50–60%. The animal experiments were carried out according to the general guidelines proposed by the Brazilian Council for Animal Experimentation (COBEA) and approved by the University's Committee for Ethics in Animal Experimentation (CEEA/UNICAMP).

2.3. Isolation of CoaTx-II from Crotalus oreganus abyssus snake venom

Lys49 PLA_2 was purified from the venom of *C. o. abyssus* using two chromatographic steps. The snake venom (100 mg) was fractioned by chromatography on a G75-Sephadex column, previously equilibrated with 0.20 M ammonium bicarbonate buffer (AMBIC - pH 7.8). Initially, the venom was dissolved in AMBIC 1M, homogenized and centrifuged at 9000*g* for 3 min. The supernatant obtained was loaded on a Sephadex G75 column (1.5 cm × 90 cm, Amersham Pharmacia Biotech), previously equilibrated with 80 mM ammonium bicarbonate buffer, pH 7.8, under a flow rate of 21 ml/h. Three peaks (Coa-I, Coa-II and Coa-III – Fig. 1A) were obtained and these were lyophilized and stored frozen at −20 °C. Fraction II (Coa-II peak – Fig 1A) was selected for the next purification step due to the presence of proteins with a molecular mass compatible with that of the protein of interest (Lys49 PLA_2).

For the HPLC separation, the Coa-II peak (pool of protein with molecular mass similar to Lys49 PLA_2s) was dissolved in 120 μl 0.1% (v/v) trifluoroacetic acid (solvent A) and 80 μl 1 M ammonium bicarbonate. The resulting solution was centrifuged at 9000*g* for 3 min and the supernatant was further submitted to a reverse-phase HPLC (model 2010, Shimadzu, Japan) using an analytical C18 column (μ-Bondapak, 0.78 × 30 cm). The C18 column was equilibrated in solvent A and the proteins eluted with a linear gradient from 0 to 100% of solvent B (66% acetonitrile, 0.1% TFA), at a flow rate of 1 ml/min, for 60 min. The elution profile of both chromatographic steps was monitored at 280 nm, and the collected fractions of 2 ml were lyophilized and conserved at −20 °C. All groups of peaks obtained were assayed for PLA_2 activity, and those without enzymatic activity were selected and tested for myotoxicity activity. The peak with the highest myotoxic activity, shown in Fig. 1B, was named CoaTx-II and chosen for this study. This peak was re-chromatographed to evaluate its purity, under the same conditions as described above (reverse-phase chromatography) and further functionally and structurally characterized.

2.4. N-terminal sequencing

The N-terminal sequence of 50 μg CoaTx-I provided by Martins et al. (2014) and CoaTx-II (purified in this work) were obtained directly by automated Edman sequencing on a PPSQ-33A (Shimadzu) automatic sequencer (Edman, 1950).

2.5. Phospholipase A_2 activity

Two methods using different substrates, a non-micellar (4-nitro-3-octanoyloxy benzoic acid - NOBA) and a micellar substrate (1-hexadecanoyl-2-(1-pyrenedecanoyl)-sn-glycero-3-phosphoglycerol - HPGP), were used to determine enzymatic activity.

2.5.1. Determination of PLA_2 activity using a non-micellar substrate (NOBA)

PLA_2 activity was determined using the assay method described by Holzer and Mackessy (1996), adapted for 96-well microplates according to Calgarotto et al. (2008). Enzymatic activity was expressed as the initial velocity of the reaction (Vo) and calculated based on absorbance after 20 min at 425 nm using a VersaMax 190 multiwell plate reader (Molecular Devices, Sunnyvale, CA). PLA_2

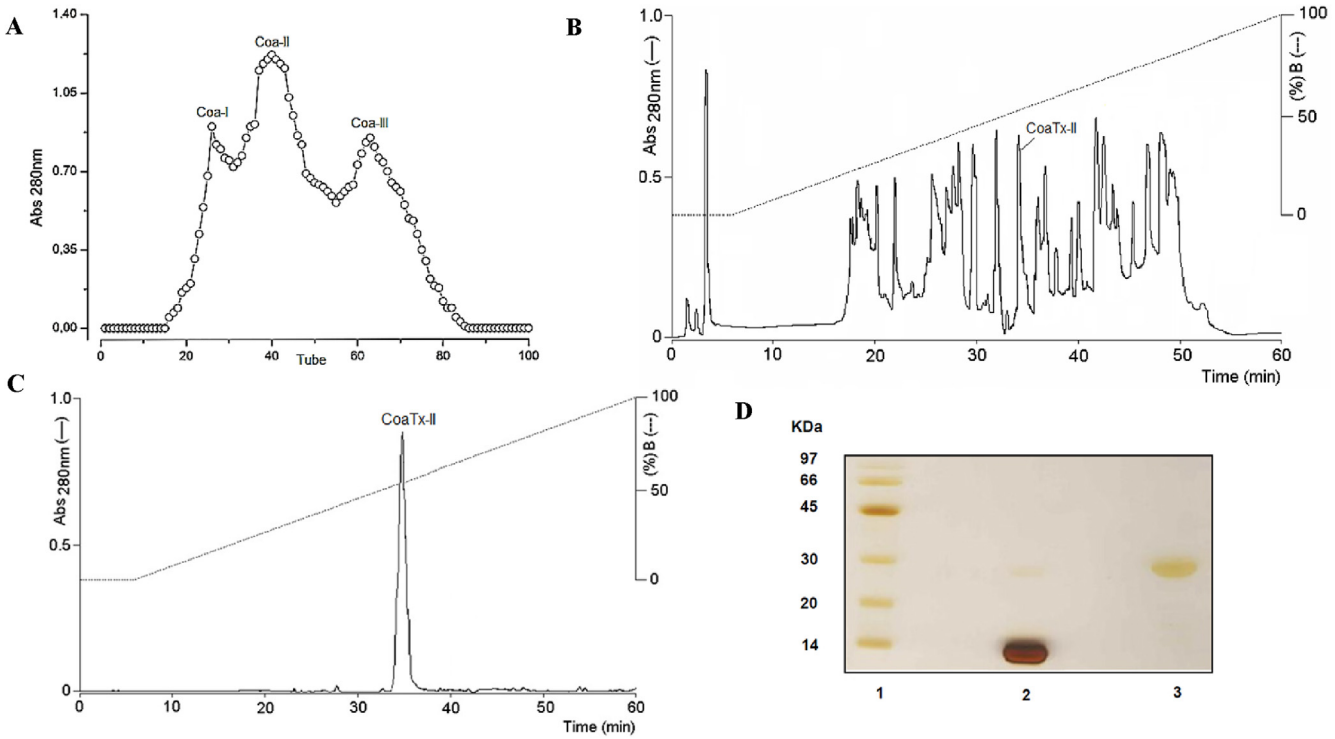

Fig. 1. Purification of CoaTx-II from *C. o. abyssus* venom. (A) Elution profile of venom by molecular exclusion chromatography on a Sephadex G-75 column. **(B)** Profile elution of Coa-II by RP-HPLC on an analytical C18 column. The peak corresponding to CoaTx-II (characterized in this study) is indicated. **(C)** Rechromatography of CoaTx-II on an RP-HPLC C18 column. **(D)** Protein analysis by 12% SDS–PAGE: Lines: (1) molecular mass markers (2) CoaTx-II reduced and (3) not reduced.

activity was described as the mean ± SD of three independent experiments performed in triplicate.

2.5.2. PLA_2 activity measured using a micellar substrate (HPGP)

The determination of PLA_2 activity using the 1-hexadecanoyl-2-(1-pyrenedecanoyl)-sn-glycero-3-phosphoglycerol (HPGP) substrate was performed using the microtiter plate assay, as previously described by Smart et al. (2004) and Da Silva et al. (2009). The fluorescence (excitation = 342 nm, emission = 395 nm) was read using a microtiter plate spectrophotometer (Fluorocount, Packard Instruments) and the PLA_2 activity was expressed as the initial velocity of the reaction (Vo), determined based on the absorbance at 20 min.

2.6. Electrophoresis

Polyacrylamide gel electrophoresis of CoaTx-II was performed according to Laemmli (1970) to evaluate homogeneity and estimate the molecular mass of the toxin. Samples were heated at 100 °C for 5 min and then ran. Silver staining was used to detect proteins after electrophoretic separation on polyacrylamide gels.

2.7. MALDI-TOF mass spectrometric analysis of CoaTx-II

In order to obtain the molecular mass, CoaTx-II was analyzed in a MALDI-TOF/TOF - Proteomics Analyzer 4700 (AB SCIEX), according to the methodology also used by Almeida et al. (2016). Briefly, 1 μl of sample (dissolved in a solution of 50% Acetonitrile/miliQ water (ACN/H_2O; v/v)) was mixed with1 μl of matrix (α-Cyano-4-hydroxycinnamic acid in 50% ACN/H_2O (v/v), 0.1% trifluoroacetic acid) on the MALDI plate. Before analysis, the samples were dried and the instrument calibrated using 4 pmol of BSA standard solution, under identical conditions.

2.8. Determination of primary structure

Mass spectrometry was performed using a NanoAcquity (Waters) UPLC, coupled to an Orbitrap Velos mass spectrometer (Thermo Scientific). CoaTx-II was reduced by treatment with a solution of 5 mM DTT (Dithiothreitol) for 25 min at 56 °C and alkylated with a solution of 14 mM Iodoacetamide for 30 min at room temperature, protected from light. After this procedure, the sample was digested for 16 h at 37 °C with sequencing grade trypsin, LysC or GluC in the proportion of 1:50 enzyme/substrate. To stop the reaction, 0.4% formic acid was added and the sample centrifuged at 2500 rpm for 10 min. The peptides from the enzymatic digestion were fractionated in a C18 reverse phase column (75 μm Øi, 10 cm, nano Acquity, 1.7 μm BEH column, Waters) using a concentration gradient of 1–40% B in 20 min, followed by a gradient of 40–60% B in 5 min; flow was 250 nl/min (A: 0.1% trifluoroacetic acid in water; B: 0.1% trifluoroacetic acid in acetonitrile). Fragments were detected in the Orbitrap with a resolution of 7500 FWHM at 400 m/z. The raw data were collected with a Thermo Xcalibur (v.2.1.0.1140) and analyzed using a Proteome Discoverer (v.1.3.0.339) software.

2.9. Tridimensional structure modeling

Modeling of the CoaTx-II 3D-structure was performed using the crystal structure of a Lys49 PLA_2, from the venom of the Cottonmouth snake (PDB ID: 1PPA), as a template (Holland et al., 1990). The sequence identity between the CoaTx-II and the template was 85%. The CoaTx-II – PLA_2 homology model was built and optimized using Maestro and Prime software (Jacobson et al., 2004) and the model was built as a monomer and assembled as a dimer using the crystal structure of Myotoxin II Lys49 PLA_2 from *Bothrops moojeni* (PDB ID: 4KF3) (Salvador et al., 2013). The homology model was validated using PROCHECK (Laskowski et al., 1993). The

Schrödinger Master software (version 9.2) interface was used to add hydrogen atoms by assigning the bonds and charges to the homology model. The model was embedded in an orthorhombic box of pre-equilibrated Simple Point Charge (SPC) water molecules, with periodic boundary conditions. The system was subjected to a conjugate gradient energy minimization and 10 ns Molecular Dynamic simulation (MDs) in Desmond software using an OPLS force field (Kaminski et al., 2001) to equilibrate the protein. A restriction was applied to the backbone atoms of the protein using a spring constant force of 0.5 kcal $\times$ mol^{-1} $\times$ Å^{-2}.

2.10. Toxicological characterization

2.10.1. Local and systemic myotoxicity

Eight distinct groups of six male Swiss mice (18–20 g) were used to study the myotoxicity activity of the protein (four groups were used in local myotoxicity analyses and four groups in a systemic myotoxicity study). The animals of the local myotoxicity groups received an intramuscular injection in their right gastrocnemius, while the animals of the groups used to evaluate systemic myotoxicity received an intravenous injection. Both groups (local and systemic myotoxicity) received injections with different doses of toxin (5, 10 and 20 μg/mice) dissolved in PBS. The control groups received an identical intramuscular injection (local myotoxicity) or intravenous injection (systemic myotoxicity) of PBS alone. At different intervals of time after injections, a blood sample was collected from the tail into heparinized capillary tubes, centrifuged and a plasma aliquot was utilized to quantify the creatine kinase (CK) activity using a kinetic assay (CK-Nac Bioliquid). Enzyme activity was expressed in U/L, where one unit equals the production of 1 mmol of NADH per minute at 25 °C (Almeida et al., 2016).

2.10.2. Histological study

Myotoxicity was also assayed on the basis of the morphologic changes triggered by the injection of 20 μg/mice of CoaTx-II in the right gastrocnemius muscle of groups of six Swiss mice (18–20 g). PBS (50 μl) was injected in the muscle of control animals. At 1 h following injection, the mice were sacrificed by an overdose of ketamine/xylazine and gastrocnemius samples were removed for histological investigation, which was performed according to the methodology used by Mamede et al. (2013). Briefly, the excised muscles were fixed in 10% formaldehyde, dehydrated, processed for hematoxylin-eosin staining of paraffin-embedded sections and examined under a light microscope.

2.10.3. Edema-inducing activity

The edema formation caused by CoaTx-II was assessed in groups of six Swiss mice (18–20 g) and determined according to the methodology used by Almeida et al. (2016). Fifty μl of PBS with different amounts of CoaTx-II (5, 10, 20 μg/paw) were injected into the subplantar region of the right footpad. The left footpads of the same mice received an injection of 50 μl PBS. The footpad thickness was measured with a low-pressure pachymeter (Mitutoyo, Japan) before and at various intervals after injection (0.5, 1, 3, 6, 9 and 24 h). Edema-forming activity was defined as the percentage increase in the volume of the footpad relative to values measured in the footpad before injection.

2.10.4. Interleukin-6 (IL-6) measurement

To understand inflammatory responses, three groups of six Swiss mice (18–20 g) received injections of 5, 10 or 20 μg/50 μl CoaTx-II in the tibialis anterior muscle, respectively, while a fourth control group received PBS alone (vehicle used to dissolve toxins). Blood samples from tail were collected into heparinized capillary tubes at different time intervals (0.5, 1, 3, 6, 9, 12 and 24 h after toxin injection) and plasma aliquots were used for quantification of interleukin-6 (IL-6) levels by enzyme-linked immunosorbent assay (Mouse IL-6 ELISA Set BD Biosciences) (Calgarotto et al., 2008).

2.11. Evaluation of the antibacterial activity of CoaTx-II

2.11.1. Bacterial strains

Four bacterial strains were used: *Pseudomonas aeruginosa* 31NM (Bergamini et al., 2012), *E. coli* ATCC 25922, *Staphyloccocus aureus* BEC9393 (Soares et al., 2001) and *Staphyloccocus aureus* Rib1 (Cury et al., 2009), all provided by Dr. Marcelo Lancellotti – Biotechnology Laboratory (UNICAMP). These Gram-negative and Gram-positive bacteria were maintained in Mueller Hinton Broth (MHB) plus 20% glycerol and frozen at −20 °C until use (experiments), when they were thawed and cultured on Mueller Hinton Agar (MHA) at 37 °C for 24 h.

2.11.2. Preparation of bacterial suspensions and determination of antibacterial activity

Following the growth of bacterial strains in MHA, bacteria were transferred to sterile MHB and adjusted to 4×10^5 CFU/ml (colony-forming units/mL). This bacterial concentration was confirmed by spectrophotometric reading at 620 nm and used as a target for bactericidal activity determination in our experiments. Ninety microliters of bacterial strain suspension were incubated for 24 h at 37 °C with variable amounts of CoaTx-II dissolved in MHB (10 μl). After incubation, viable bacteria remaining were counted by the plate dilution technique, as previously described by Paramo et al. (1998) and adapted according to the recommendations of the Clinical and Laboratory Standards Institute (2012).

2.12. Statistical analysis

Results are expressed as means ± standard deviations (SD). Statistical significance of differences between groups was evaluated by ANOVA tests and confirmed by Tukey's Post Hoc using the Software Origin Pro 8 (OriginLab Corporation). Differences were considered statistically significant if $p < 0.05$.

3. Results

3.1. Purification of CoaTx-II

The Lys49 PLA_2, CoaTx-II, was purified from *Crotalus oreganus abyssus* snake venom by two chromatographic methods; molecular exclusion and reverse-phase chromatography. The profile of *C. o. abyssus* venom on Sephadex G-75 presented three main fractions, denominated Coa-I, Coa-II and Coa-III (Fig. 1A). The CoaTx-II fraction was selected for separation by HPLC. This fraction contained several proteins, as shown in Fig. 1B. The peaks or groups of peaks obtained in this chromatography step were screened for PLA_2 activity. Those peaks, or groups of peaks, unable to hydrolyze the synthetic substrate were tested for their ability to induce muscle damage *in vivo*. The peak with the highest myotoxic activity was denominated CoaTx-II and was selected for structural and functional characterization.

Homogeneity of CoaTx-II was confirmed by rechromatography on an analytical RP-HPLC, which showed the presence of a single peak (Fig. 1C), and by electrophoresis (Fig. 1D), which revealed one electrophoretic band under reducing and non-reducing conditions with relative molecular masses of approximately 14 and 28 kDa, respectively. These data are consistent with the molecular mass of 13868.2 Da, determined by MALDI-TOF mass spectrometry (Fig. 2).

The ability of both toxins to hydrolyze the substrates NOBA (4-nitro-3-octanoyloxy benzoic acid) and HPGP (1-hexadecanoyl-2-

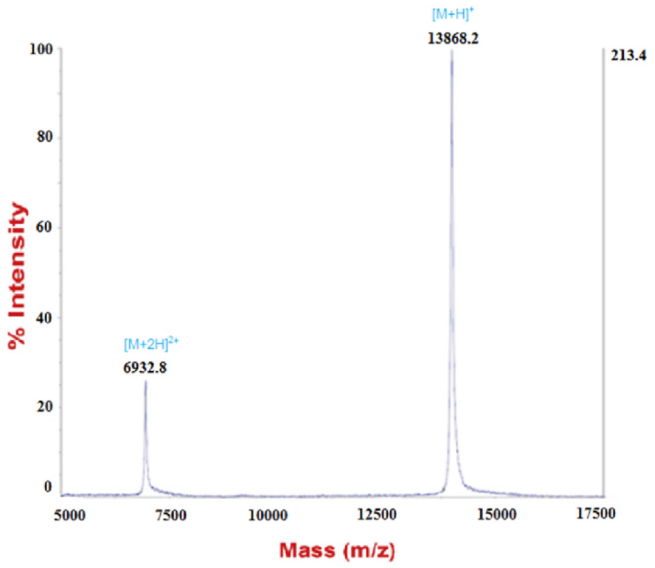

Fig. 2. MALDI-TOF MS analysis of CoaTx-II. The mass spectrum shows the mass/charge ratio (*m*/*z*) of CoaTx-II, determined by matrix-assisted laser desorption/ionization-time-of-flight using a MALDI-TOF/TOF – Proteomics Analyzer 4700 (AB SCIEX) demonstrating a MW of 13868.2 Da for a monoprotic ion and 6932.8 Da for a diprotic ion.

Table 1
Mass-to-charge ratio (m/z) of the fragments obtained from CoaTx-II sequencing. The CoaTx-II was reduced and digested overnight with trypsin, LysC and GluC. The fragments from enzymatic the digestion were separated by RP-HPLC and sequenced by mass spectrometry.

Number	Sequenced fragment	Monoisotopic		Accuracy (ppm)
		Measured	Theoretical	
1	SLVELGK	745.4446	745.4454	1.07
2	MILQETGK	935.4823	935.4866	4.60
3	SVLELGKMILQE[a]	1375.7504	1375.7501	−0.22
4	NAIPSYGFYGCNCGWGGR	2035.8538	2035.8537	−0.05
5	DATDRCCFVHK	1408.6013	1408.6096	5.89
6	KLTDCDPK	976.4725	976.4768	4.40
7	RCCFVHKCCYKKLTD[a]	2074.9440	2074.9442	1.00
8	DATDRCCFVHKCCYK	2019.8256	2019.8292	1.78
9	TDIYSYSWK	1162.5364	1162.5415	4.39
10	TIICDVNNPCLK	1446.6966	1446.7079	7.81
11	IYSYSWKNKTIICD[a]	1790.8785	1790.8782	−0.17
12	EMCECDK	971.3235	971.3267	3.29
13	AVAICLR	802.4581	802.4603	2.74
14	CDKAVAICLRE[a]	1334.6545	1334.6555	0.75
15	ENLDTYNKK	1124.5577	1124.5582	0.44
16	YRIYPK	839.4774	839.4775	0.12
17	FLCKKPDTC	1168.5395	1168.5489	8.04
18	YRIYPKFLCK	1387.7693	1387.7555	−9.94

[a] Peptides from digestion with GluC. The other peptides resulted from digestion with trypsin or LysC.

(1-pyrenedecanoyl)-sn-glycero-3-phosphoglycerol) is shown in Fig. 3. CoaTx-I, provided by our collaborators, presented a high catalytic activity using micelar (HPGP) and non-micelar (NOBA) substrates, while CoaTx-II was unable to hydrolyze these substrates using the same conditions (Fig. 3A and B).

3.2. Structural characterization

In order to characterize the molecular identification, homology and structure of purified non-catalytic PLA_2, Edman degradation, mass spectrometry, bioinformatic analysis and molecular modeling were carried out. Digestion of CoaTx-II with trypsin, LysC and GluC, followed by LC/MS/MS allowed the identification of eighteen peptides (Table 1) and enabled the purified toxin to be classified as a catalytically-inactive homologue protein, Lys49 PLA_2 (fragment 4 – DATDRCCFVH**K** - Table 1). The determination of the N-terminal sequence of CoaTx-II also revealed the presence of the amino acid, Lys, at position 49, which is a feature of enzymatically-inactive phospholipases A_2 and corroborates with its inability to hydrolyze synthetic substrates (Fig. 2).

The multiple sequence alignment of the new Lys49 PLA_2 described herein (CoaTx-II) demonstrated a primary structure that is closely related to those of other Lys49 PLA_2s of viperine species (Fig. 4). The amino acid sequence comprises the general features of inactive Lys49 PLA_2 homologues (group II secreted PLA_2s), including the conserved residues Leu5, Gly6, Lys7, Gln11, Glu12, Thr13, Asn28 (conserved residue in the Ca^{2+}-binding loop region) and Lys49. CoaTx-II presented a high number of amino acid residues that are typical of basic proteins and, therefore, an elevated pI

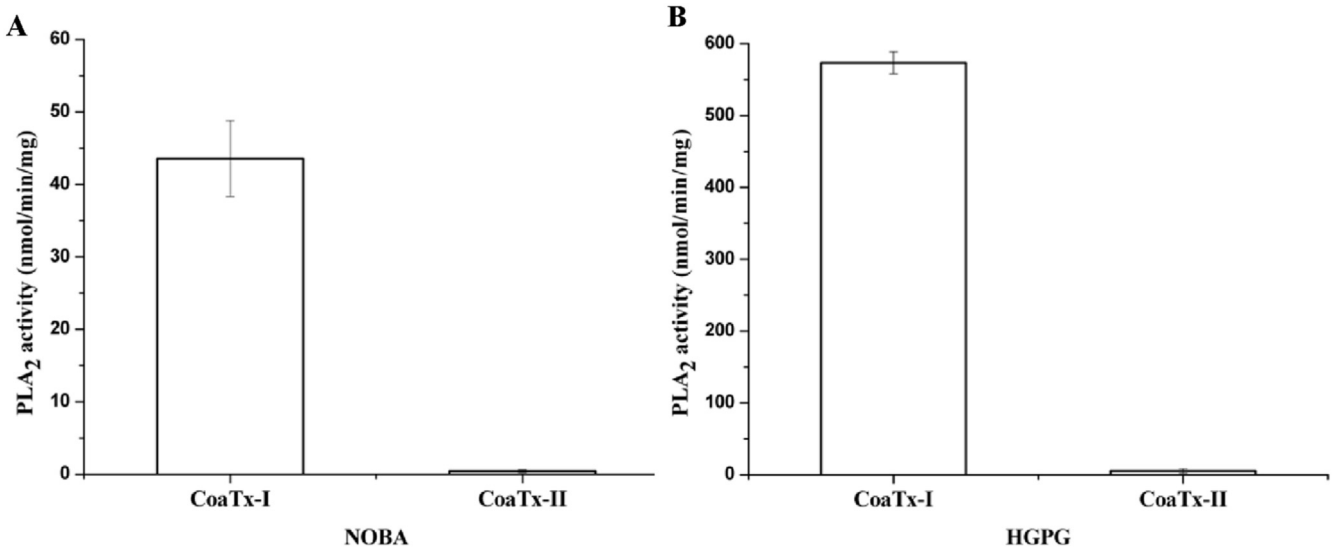

Fig. 3. Phospholipase A_2 activities of CoaTx-I and CoaTx-II. (A) The phospholipase A_2 activity of toxins purified from *C. o. abyssus* snake venom was determined using a non-micelar substrate (4-nitro-3-octanoyloxy benzoic acid) and **(B)** a micelar substrate (1-hexadecanoyl-2-(1-pyrenedecanoyl)-sn-glycero-3-phosphoglycerol).

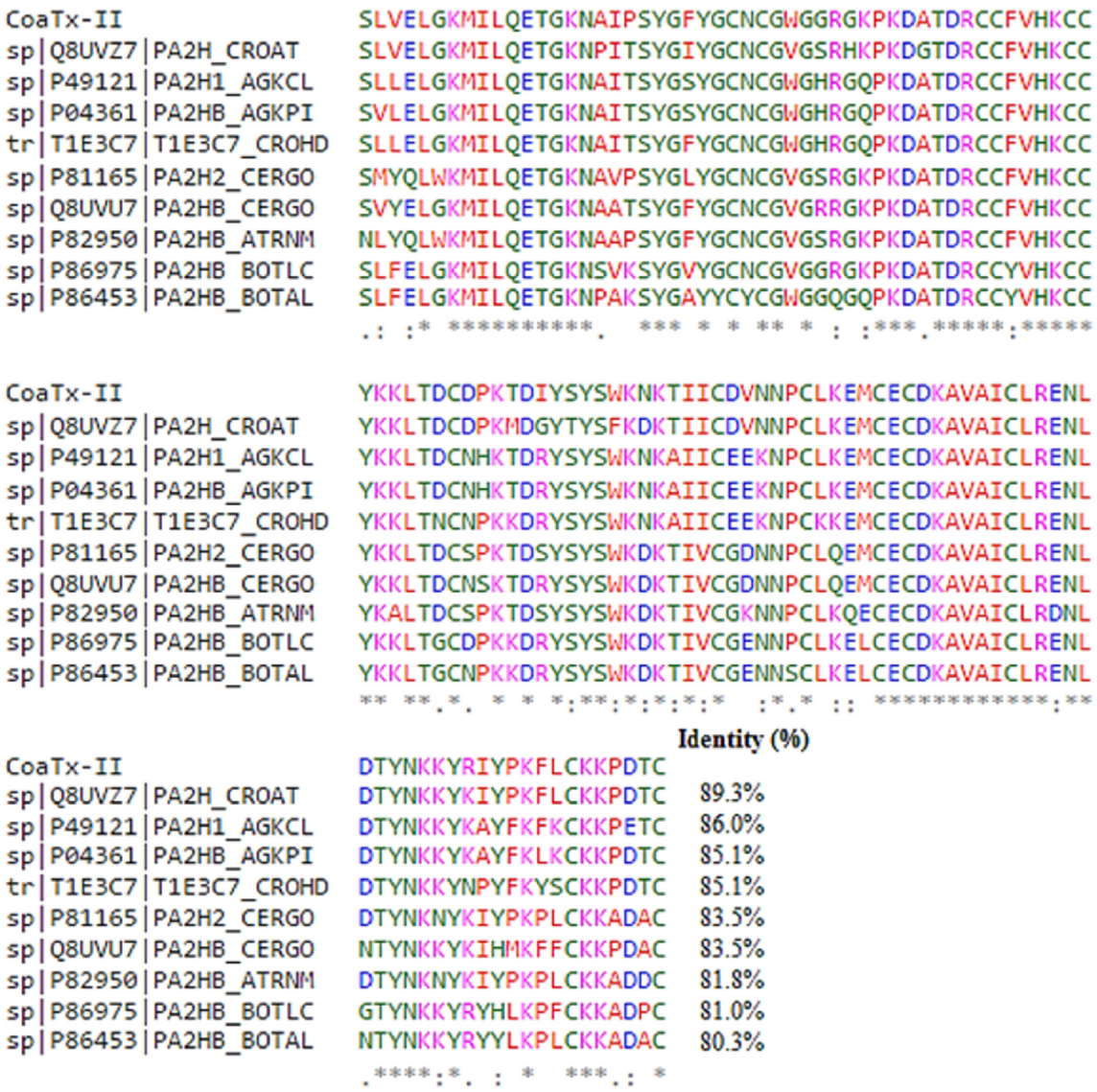

Fig. 4. Multiple alignment of the primary structure of CoaTx-II with similar proteins after a BLAST search and processing with Software T-Coffee. Asterisks indicate identical amino acids. Two dots represent conservation between groups with highly similar properties, while dots indicate conservation between groups with weakly similar properties. Accession codes in the UniProt database are indicated at the left of each sequence and the theoretical pI shown in the legend was calculated using the Compute pI/Mw tool available in ExPASy, according to the primary structure described in the alignment. Q8UVZ7: *Crotalus atrox* (pI: 8.60) (Tsai et al., 2001); P49121: *Agkistrodon contortrix laticinctus* (pI: 8.89) (Selistre de Araujo et al., 1996); P04361: *Agkistrodon piscivorus piscivorus* (pI: 8.89) (Scott et al., 1992); T1E3C7: *Crotalus horridus* (pI: 8.96) (Rokyta et al., 2013); Q8UVU7: *Bothrops godmani* (pI: 8.90) (Tsai et al., 2001); P81165: *Bothrops godmani* (pI: 8.61) (De Sousa et al., 1998); P82950: *Atropoides nummifer* (pI: 8.71) (Angulo et al., 2002); P86975: *Bothrops leucurus* (pI: 9.03) (Higuchi et al., 2007) and P86453: *Bothrops alternatus* (pI: 8.95) (Ponce-Soto et al., 2007).

(theoretical: 8.72). CoaTx-II shows a high identity with Lys49 PLA_2s from North American snake venoms (Pit Vipers), such as, *Crotalus atrox*, *Agkistrodon contortrix laticinctus*, *Agkistrodon piscivorus piscivorus* and *Crotalus horridus* (Fig. 4).

The structural model of CoaTx-II was obtained using homology modeling. Residues in the 3D structure were numbered according to Renetseder et al. (1985). The model was built as a monomer and assembled as a dimer. CoaTx-II presents all the secondary structures of a typical PLA_2 (three parallel α-helixes and a β-wing - one double-stranded anti-parallel β-sheet). Ramachandran plot analysis to characterize the structure of CoaTx-II showed that most of the amino acids are in favorable regions with an acceptable stereochemistry, generating a model of high quality (data not shown). Molecular dynamic validation and modeling demonstrated that the dimers are held together by hydrophobic interactions (Fig. 5).

3.3. Toxicological characterization

The toxicological characterization of Lys49-PLA_2, isolated from *Crotalus oreganus abyssus*, was performed using measurements of myotoxicity (CK levels) and inflammatory responses (mice paw edema and IL-6 levels). Insights into the functional role of CoaTx-II showed that this toxin is capable of causing muscle injury, as demonstrated by increased CK levels (Fig 6). However, CoaTx-II triggered CK release from cells only when administered locally by intramuscular injection (Fig. 6A), while intravenous administration did not elevate plasma CK (Fig. 6B).

The high plasma levels of CK observed after CoaTx-II toxin injection were associated with histological alterations, indicative of myotoxic activity (Fig. 7). At 1 h following injection, CoaTx-II induced obvious pathological changes in the gastrocnemius muscles of mice (Fig. 7B–F), when compared to the control group

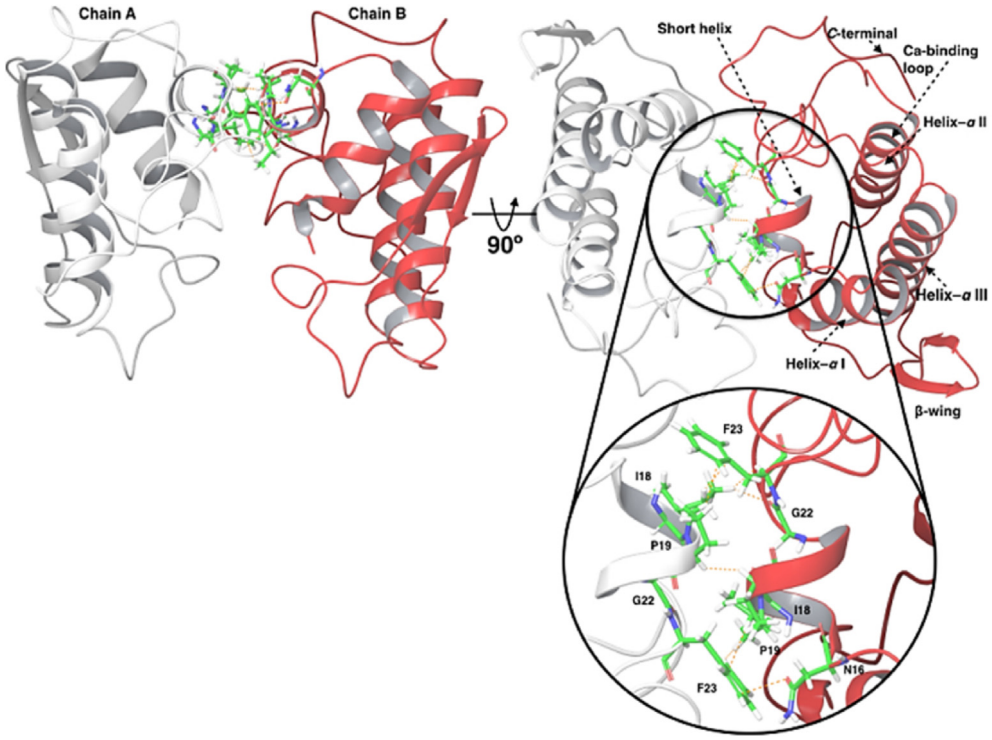

Fig. 5. Dimeric configuration of CoaTx-II, as determined by homology modeling. The model was built as a monomer and assembled as a dimer using the crystal structure of Myotoxin II Lys49 PLA_2 from *Bothrops moojeni*. The non-covalent interactions between the two subunits are highlighted.

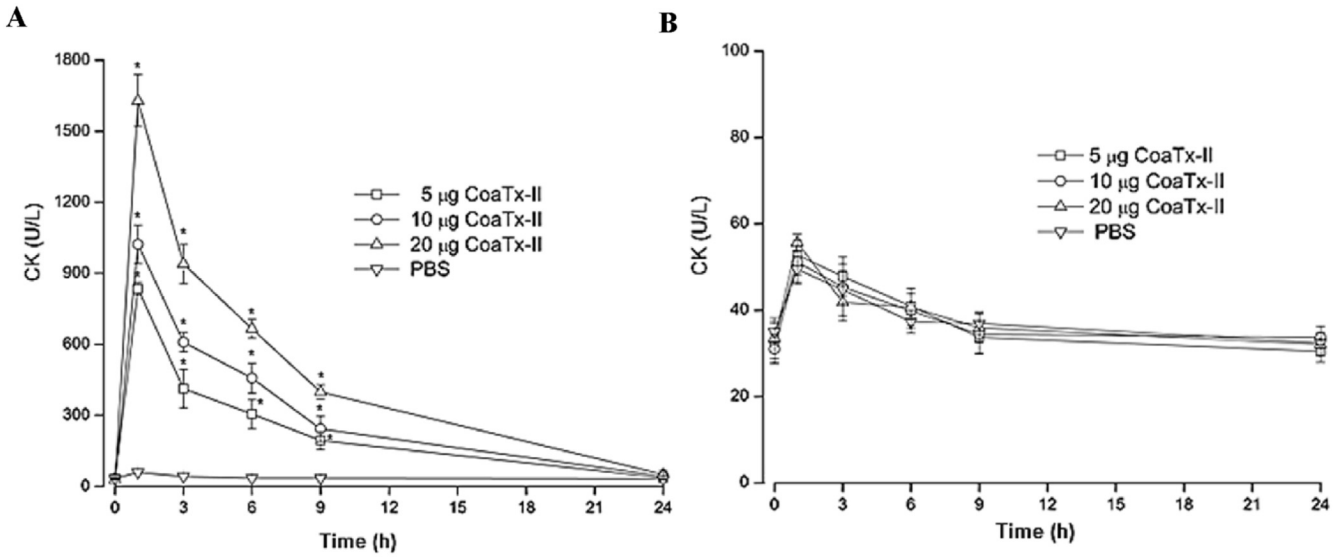

Fig. 6. Local myotoxicity induced by CoaTx-II. (A) Mice received an intramuscular administration of CoaTx-II (local myotoxicity groups) in the right gastrocnemius or **(B)** intravenous CoaTx-II (systemic myotoxicity groups). Animals in each group (local and systemic) received one of three concentrations of purified toxin (5, 10, 20 μg/mice), respectively, while the control groups received an identical intramuscular injection or intravenous injection of PBS alone. Plasma creatine kinase levels were determined at the indicated time intervals by an enzymatic assay. Asterisks indicate a statistically significant ($p < 0.05$) difference in values, compared to the PBS control group.

(Fig. 7A). CoaTx-II induced a number of degenerative events, including edema, widespread necrosis of muscle fibers and abundant leukocyte infiltration (Fig. 7B–F). Extravasation of erythrocytes in the interstitial space was not observed.

The inflammatory events induced by CoaTx-II were further studied using a mouse paw edema assay and the measurement of pro-inflammatory cytokine (interleukin-6). The subplantar injection of CoaTx-II triggered a dose-dependent local inflammation, characterized by an increase in mice paw volume, when compared to PBS-injected animals (Fig. 8A). The paw edema evoked by the toxin was characterized by a profile of rapid onset, maximal response at 30 min after injection with return to basal levels by 24 h. The analysis of the inflammatory response, as assessed by enzyme-linked immunosorbent assay, showed that CoaTx-II promoted a rapid increase in plasma IL-6 levels, reaching maximum concentrations at 1 h after intramuscular injection (Fig. 8B).

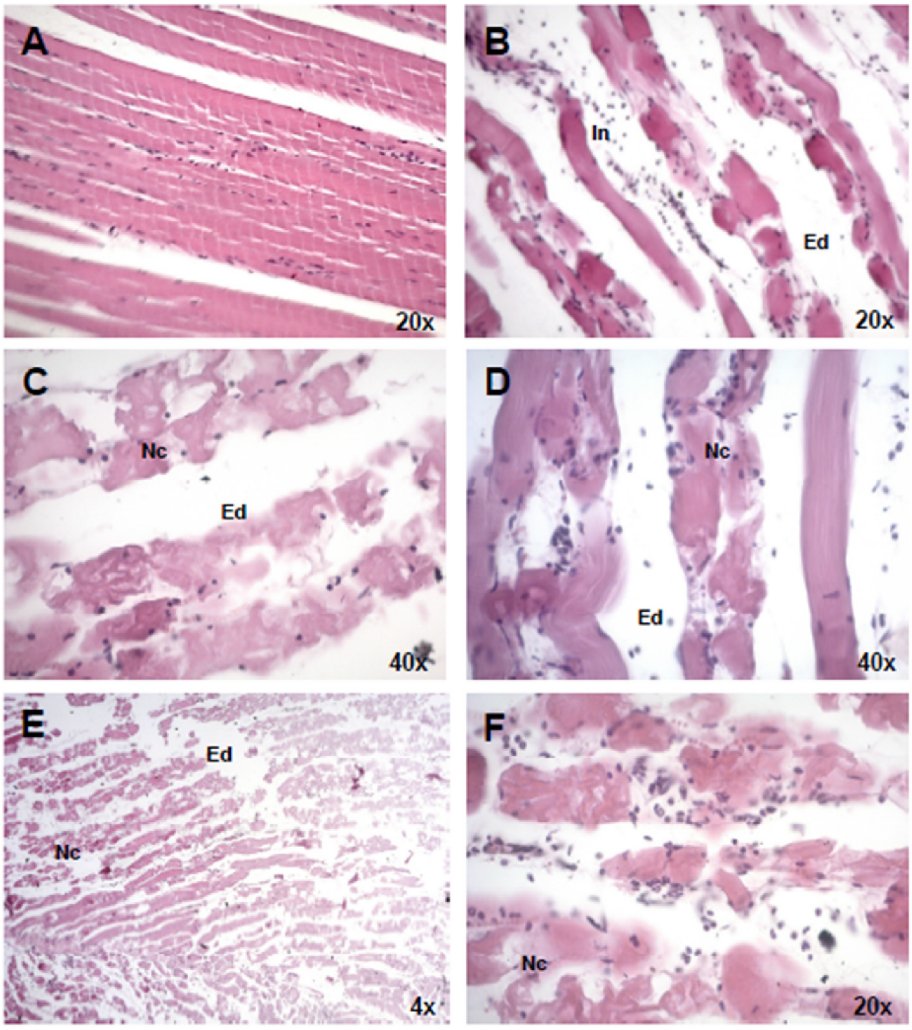

Fig. 7. Histological evaluation of the local myotoxic effect caused by CoaTx-II. Light micrograph of muscle tissue at 1 h after intramuscular injection of PBS (control group) or 20 μg of CoaTx-II. Hematoxylin and eosin staining was performed and the magnification is indicated in each figure. **(A)** Control mice injected with PBS alone exhibited normal integral fibers. **(B–F)** Muscle sections of mice injected with CoaTx-II showing muscular necrosis (Nc), edema (Ed) and intense infiltration (In) of inflammatory cells.

3.4. Antibacterial activity

CoaTx-II demonstrated antibacterial activity against Gram-negative bacteria (Fig. 9A); *Pseudomonas aeruginosa* 31NM and *Escherichia coli* ATCC 25922 and Gram-positive methicillin-resistant *Staphylococcus aureus* (Rib1 and BEC9393) (Fig. 9B).

4. Discussion

The snake venom PLA_2s present well known toxic activities that can lead to death in snake-bite victims (Gutierrez and Ownby, 2003; Carvalho et al., 2013). However, these toxins have aroused great medical interest, particularly with regard to their potential as antibacterial agents (Samy et al., 2012; Ben Bacha et al., 2013). In this study, the combination of two chromatographic steps was sufficient for the purification of a new catalytically-inactive phospholipase (CoaTx-II) with antibacterial potential, which we have structurally and functionally characterized.

The molecular homogeneity accessed by electrophoresis and rechromatography confirmed the purity of CoaTx-II by the presence of a single eletrophoretic band and peak, respectively. CoaTx-II, similarly to other Lys49 PLA_2s purified from snake venoms (Francis et al., 1991; Lomonte and Rangel, 2012), migrated as a single electrophoretic band of 14 and 28 KDa in the presence and absence of a reducing agent, respectively. Homodimeric assembly has been reported as an essential feature for the toxicological activity of many Lys49 PLA_2s, since it presents a large hydrophobic surface, which mediates the interaction of the toxin and its effects (Angulo et al., 2005). Many studies have shown that the different quaternary structures of PLA_2s can result in variable toxic potencies (dos Santos et al., 2011). The molecular mass, 13868.2 Da, determined by MALDI-TOF mass spectrometry, is closely related to the mass of other PLA_2s purified from snake venoms (Higuchi et al., 2007; Martins et al., 2014).

The enzymatic assays revealed that CoaTx-II is not able to cleave non-micelar and micelar substrates. These results were confirmed by spectrometric studies that indicated the presence of a Lys residue at position 49 of CoaTx-II and other residues commonly observed in the primary structure of this toxin group. The inability of catalytically-inactive PLA_2 homologues to hydrolyze phospholipids results from their failure to bind the divalent ion, Ca^{2+}, which is essential for enzymatic catalysis (Lomonte et al., 2003a; Fernandes et al., 2010). The calcium-binding domain in the catalytically active Asp49 PLA_2s is composed of amino acids 26–34 and 49, among which four highly conserved residues, Tyr28, Gly30, Gly32 and Asp49, directly participate in calcium-binding (Scott

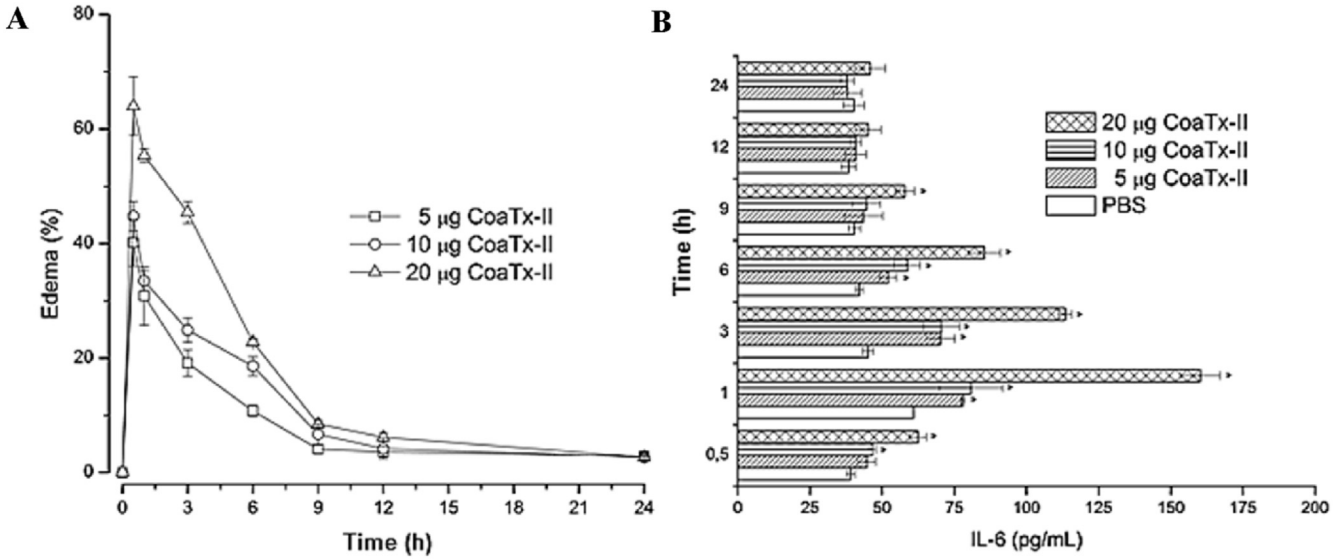

Fig. 8. Inflammatory effect of CoaTx-II in mice. (A) Edema-forming activity of CoaTx-II. Mice received subcutaneous injections of different doses of CoaTx-II or PBS vehicle alone and their footpad thickness was measured at different time intervals, as described in Materials and Methods. **(B)** Interleukin-6 response induced by CoaTx-II after its injection in the tibialis anterior muscle of mice. Interleukin-6 levels were determined at the indicated time points by enzyme-linked immunosorbent assay. Asterisks indicate a statistically significant ($p < 0.05$) difference in values, in comparison to the PBS control group.

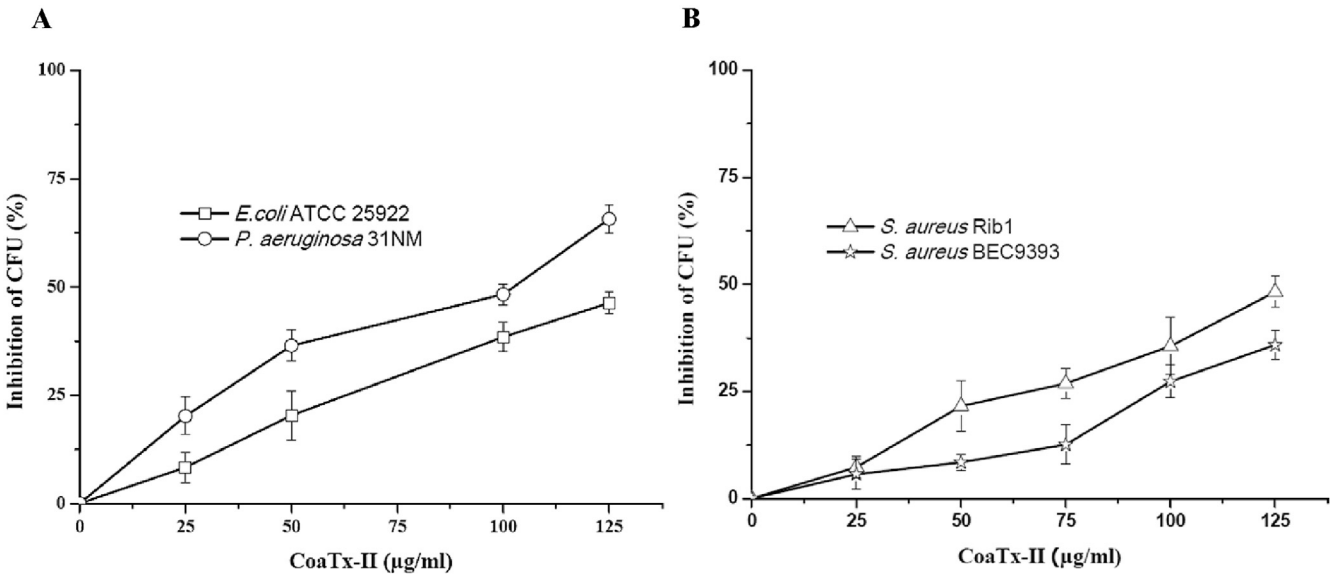

Fig. 9. Bactericidal effect of different concentrations of CoaTx-II. The antibacterial abilities of CoaTx-II against **(A)** Gram-negative (*E. coli* ATCC25922 and *P. aeruginosa* 31NM) and **(B)** Gram-positive (*S. aureus* BEC9393 and Rib1) bacteria were evaluated using the plate dilution technique. CFU: colony-forming units.

et al., 1990; Wei et al., 2006). In a study performed by Fernandes et al. (2010), the structural comparison of the Ca^{2+} binding domains of PLA_2s from snake venom highlighted the conservation of the polar amino acid Tyr28 in Asp49 PLA_2s, which was considered to be essential for the structural integrity of the calcium-binding loop in these toxins. Its substitution by the Asn residue, together with others such as Lys49 instead of Asp49, contributes to the destabilization of the Ca^{2+}−binding loop and, consequently, the lack of divalent cation binding and enzymatic activity in Lys49 PLA_2s. In CoaTx-II, the amino acids in this molecular region are Asn28, Gly30, Gly32 and Lys49. Thus, the alteration observed in the calcium loop (Asn28 and Lys49) of CoaTx-II is in line with data reports in the literature (Tsai et al., 2001; Rokyta et al., 2013) and justifies the loss of hydrolytic ability for non-micelar and micelar substrates observed in our experiments.

The tridimensional structure generated demonstrates protein folding compatible with that of other PLA_2s isolated from snake venoms and previously characterized by X-ray crystallography (dos Santos et al., 2011). The disulfide bonds present in this structure have been suggested to contribute to the high stability of the PLA_2s. From the three-dimensional structural model of CoaTx-II, it was possible to identify structures similar to those of other PLA_2 Lys49 homologues (Delatorre et al., 2011), such as the N-terminal region (represented by an a-helix I; the short helix; the calcium binding loop; a-helix II; the antiparallel β sheet (b-wing); α-helix III), which binds to a flexible C-terminal region. The dimeric model of CoaTx-II proposed is in line with those of other studies and suggests that the subunits in this toxin groups are held together by non-covalent

interactions (Murakami et al., 2007; Lomonte and Rangel, 2012; Ullah et al., 2012).

Similarly to the Asp49 PLA_2, CoaTx-I, described by Martins et al. (2014), the CoaTx-II toxin purified herein triggered local inflammation and myotoxicity, as shown by increases in IL-6 and CK levels, respectively. Thus, these findings, together with those described for CoaTx-I (Martins et al. (2014), suggest that both the catalytic and non-catalytic PLA_2 toxins from this venom are involved in and mediate the events that occur during envenomation by *C. o. abyssus* and therefore contribute to the pathophysiological alterations induced by snakebites.

Microscopic observations of the effects promoted by CoaTx-II on skeletal muscle, confirmed local myotoxicity, as supported by data showing an efflux of a muscle damage marker (creatine kinase), cellular necrosis, edema and leukocyte infiltrate. These data are accordance with the morphological alterations induced by *B. atrox* myotoxin I (Nunez et al., 2004), promutoxin from *Protobothrops mucrosquamatus* (Wei et al., 2006) and BnSP-7 from *B. (neuwiedi) pauloensis* (Oliveira et al., 2009). The pattern of histological alterations evoked by Lys49 PLA_2 homologues, such as CoaTx-II, can be used as a model for studies of injury and repair processes of skeletal muscle, since these are disorders with similar features. These toxin effects may also serve as a pharmacological model for the development of more efficient antivenoms (Montecucco et al., 2008; Lomonte and Rangel, 2012).

CoaTx-II exerts its myotoxic effect only locally, at the anatomical region where it was injected, in contrast to crotalic D49 PLA_2 (systemic), which induces both local and systemic myotoxicity. The envenomation by *Crotalus* species is characterized by local and systemic events, which are identified by the presence of a skeletal muscle damage biomarker in mice plasma (Gutiérrez et al., 2008). However, CoaTx-II only contributed to local pathological damage, and does not induce systemic myotoxicity. Local inflammation is a typical feature of envenomation caused by crotalid species (Teixeira et al., 2003). CoaTx-II administration induced the accumulation of numerous inflammatory cells at the site of injection, as can be seen in histological sections, in association with paw edema and high levels of IL-6. The extensive inflammatory infiltrate identified in the histological analyses is a common consequence of acute local pathology and, together with the production of endogenous molecules, such as IL-6, may represent a mechanism of response to envenomation (Gutierrez et al., 2009).

The comparative structural and toxicological study of catalytic and non-catalytic PLA_2s consists of an important platform to address the intriguing structure-function relationships that rule this highly diverse group of toxins. These studies may provide further understanding of the evolutionary advantages and biological roles of these toxins (Lomonte et al., 2009), as the rationale for the emergence of Lys49 PLA_2s in venoms is still unclear. The comparison between the toxicological effects of CoaTx-II described in our study and the results of previously investigated CoaTx-I (Martins et al., 2014), revealed that the local myotoxic potency of CoaTx-II was higher than that of Asp49 PLA_2, CoaTx-I. This gain in myotoxic potency over Asp49 was also observed for PLA_2 toxins from *Protobothrops flavoviridis* (Kihara et al., 1992). These data suggest that the gain in myotoxic potency may represent a possible explanation for the emergence of Lys49 PLA_2s in these snake venoms. However, this primary structure alteration in Lys49 does not always provide such an elevation in potency, as in other venoms, Asp49 PLA_2s can be more potent or display the same activity (in potency) when compared to Lys49 PLA_2s (Lomonte et al., 2009). More studies regarding this interesting observation are necessary, as well as an evaluation of the synergism between the catalytically active and inactive toxins.

The toxicological activities mediated by basic Lys49 PLA_2, CoaTx-II, are independent of phospholipid hydrolysis. A number of studies have proposed models to understand the molecular basis of the biological effects of Lys49 PLA_2s and aimed to specify the structural determinants of their effects (Murakami et al., 2007; Lomonte and Rangel, 2012; Ullah et al., 2012). CoaTx-II presents a theoretical pI of 8.72. A common characteristic shared by the non-catalytic PLA_2s is their high pI, which reflects their high number of Lys and Arg amino acid residues (de Araujo et al., 1996). Studies have proposed that this cationic character may have a crucial role in cell target recognition and, consequently, in the induction of the protein's toxic effects (Falconi et al., 2000; Gutiérrez et al., 2008). In addition, the 115–129 segment of the C-terminal region of non-catalytic PLA_2s, which is constituted mainly by positively-charged and hydrophobic residues has been considered responsible for local myotoxicity (Nunez et al., 2001). The C-terminal region (115–129) of CoaTx-II comprises a similar cationic/hydrophobic composition, suggesting that this may be the effector region of myotoxic activity. However, further studies are required to confirm this hypothesis, as the C-terminal region alone does not always exert this effect (Lomonte et al., 2003b).

The emergence and spread of multidrug-resistant bacteria is a world health problem and has prompted the discovery, design and development of new antibacterial agents (Buke et al., 2007; Cardozo et al., 2013). Snake venoms are important sources of bioactive molecules for this purpose, among which Lys49 PLA_2s have shown to represent a promising tool against bacterial infections (Núñez et al., 2004; Stabeli et al., 2006; Samy et al., 2012; Corrêa et al., 2016). The new Lys49 PLA_2 from *C. o. abyssus* displays antibacterial effects against both Gram-positive and Gram-negative bacteria, including methicillin-resistant strains, which are responsible for several difficult-to-treat infections in humans (Bergamini et al., 2012; Cardozo et al., 2013). Some authors have proposed that the effect exerted by Lys49 PLA_2s constitutes an important adaptive role for snakes, since it may help prevent venom gland bacterial contamination and/or avoid the bacterial-induced decomposition of their prey (Kochva, 1987; Tsai et al., 2007; Lomonte et al., 2009).

The mechanism of the bactericidal effect exerted by Lys49 PLA_2s is not fully elucidated, but studies have reported that the bacterial effect of these non-catalytic toxins involves their recognition and interaction with anionic sites on target cells (Koduri et al., 2002; Samy et al., 2012). It is of importance to understand the mechanism by which Lys49 PLA_2s exerts its effects in order to identify the structural determinants or molecular regions of the toxin's amino acid sequence that are responsible for its therapeutic effect and that are not toxic to humans. In this context, some works have demonstrated that small peptides derived from the C-terminal region (115–129) of the PLA_2 primary structure reproduce the antibacterial effect of parent toxins (Santamaria et al., 2005a, 2005b; Lomonte et al., 2010). It is possible that CoaTx-II exerts its antibacterial effect via the C-terminal region, since its amino acid sequence (115–129) demonstrates great similarity to the antibacterial C-terminal of other PLA_2s. Modifications of these antibacterial peptides from Lys49 PLA_2s also can be performed during their chemical synthesis, enabling the acquisition of promising molecules with high activity and good stability, and that are resistant to proteolytic degradation and, in particular, are non-toxic to man.

Our study describes the purification and structural and toxicological characterization of a dimeric Lys49 phospholipase A_2 homologue from *C. o. abyssys* with antibacterial activity. This toxin plays a role in envenomation by this species, contributing to inflammatory and myotoxic events, but also exhibits a promising bactericidal activity. These insights may provide a useful model for the development of new and specific strategies for the treatment of local effects of envenomation by snake venom, as well as for the

design and discovery of effective molecules against multi-resistant bacteria.

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Transparency document

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Conflict of interest

The authors declare no relevant conflicts of interest.

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