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LmrTX, a basic PLA_2 (D49) purified from *Lachesis muta rhombeata* snake venom with enzymatic-related antithrombotic and anticoagulant activity

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ABSTRACT

A basic phospholipase A_2 (LmrTX) isoform was isolated from *Lachesis muta rhombeata* snake venom and partially characterized. The venom was fractionated by molecular exclusion chromatography in ammonium bicarbonate buffer followed by reverse-phase HPLC on a C-5 Discovery® Bio Wide column. From liquid chromatography–electrospray ionization/mass spectrometry, the molecular mass of LmrTX was measured as 14.277.50 Da. The amino acid sequence showed a high degree of homology between PLA_2 LmrTX from *L. muta rhombeata* and other PLA_2 from snake venoms, like CB1 and CB2 from *Crotalus durissus terrificus*; LmTX-I and LmTX-II from *Lachesis muta muta*. LmrTX had PLA_2 activity in the presence of a synthetic substrate and alkylation of histidine residues significantly inhibited ($P < 0.05$) the enzymatic activity of LmrTX and its anticoagulant and antithrombotic activity. In this study, we examined the ability of the LmrTX in altering thrombus formation in living mouse, using a photochemically induced arterial thrombosis model. The control animals that did not receive protein injection showed a normal occlusion time, which was around 57 ± 7.8 min. LmrTX, the PLA_2 from *L. muta rhombeata* venom, caused a change in the occlusion time to 99 ± 10 min with doses of 7.5 μg/mice. Additionally, LmrTX showed the anticoagulant activity *in vitro* and *ex vivo* and prolonging the time aggregation in wash platelet induced by ADP and Thrombin.

1. Introduction

The bushmaster is the largest venomous snake in the Americas and the second largest in the world, reaching 3.40 m. Individuals exceeding 2.80 m in length are rare in Brazil (Souza et al., 2007). The Lachesis accidents statistic misleads without a context: the 1.6–2.4% of total snakebites at the national level become 17% in the Amazon (Ramza, 1994) or more than that in highly fragmented and anthropized areas, such as the Atlantic Forest of southern Bahia (Souza et al., 2007). Much is said about the great capacity of adult inoculation of the *Lachesis*, but the severity of the accident is independent of the size of the animal (França and Cardoso, 1989), since, unlike what can be seen in *Bothrops*, where the size of the animal is the main prognostic factor of evolution accidents (França and Cardoso, 1989), even in surface scratches, inoculation with a single prey and accidents with young animals, characterized by low volume of the *Lachesis* venom inoculated, can still cause early serious and systemic effect (Souza et al., 2007). This is probably due to the cascade of

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effects triggered by the self pharmacological post inoculation, as well as the synergy between the various actions of the poison, namely: intense local pain, swelling, profuse bleeding at the site of the bite, diarrhea and abdominal pain, vomiting, bradycardia, hypotension/profuse sweating, inability to swallow or painful attempt to do so, dysphagia and shock (Souza et al., 2007).

Proteins found in snake venom that affect the clotting factors can be classified as those who work in coagulation factors, including activating factor V, factor X activator, prothrombin activators and enzymes Thrombin-like, anticoagulant factors, including proteins that bind to factors IX/X, protein C activators, inhibitors of thrombin and phospholipase A_2, and those that act in fibrinolysis, including fibrinolytic enzymes and plasminogen activators (Kini, 2005).

So far, five PLA_2 isoenzymes have been isolated from *Lachesis* spp. venoms: two acidic ($LmPLA_2I$ and $LmPLA_2II$) from *L. muta* (Fuly et al., 2003); two basic (LmTX-I and LmTX-II) from *L. muta muta* (Damico et al., 2005) and one (LsPA-1) from *Lachesis stenophrys* (de Assis et al., 2008). However, none have been purified from *L. muta rhombeata* and studied in relation to the anticoagulant activity.

In this study, we report for the first time, the purification, prediction of primary structure, anticoagulant and antithrombotic activity of the PLA_2 from *L. muta rhombeata* venom and its relation with its enzymatic activity.

2. Materials and methods

2.1. Venom and reagents

Venom was collected in Serra Grande Center (IBAMA authorization number 24945-1), Bahia State Brazil, the only facility in the country totally dedicated to study and preservation of the Atlantic Bushmaster, *L. muta rhombeata* (www.lachesisbrasil.com.br). All chemicals and reagents were of analytical or sequencing grade.

2.2. Animals

7–8 weeks C57BL6 mice were supplied by the Animal Services Unit of the State University of Campinas (UNICAMP). Mice were housed at room temperature on a 12 h light/dark cycle and had free access to food and water. All procedures were performed 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) number 1790-1.

2.3. Venom fractionation

One hundred mg of crude venom of *L. muta rhombeata* was dissolved in 1 ml of 0.2 M Ammonium bicarbonate buffer, pH 8.0. After centrifugation at 5.000× *g* for 5 min, the supernatant was loaded onto a Sephadex G75 column (1.5 cm × 90 cm), previously equilibrated with the same solution, under a flow rate of 12 ml/h. Three ml fractions were collected. Five mg from selected PLA_2 active fraction (FIII) was dissolved in 200 μl of 0.1% (v/v) trifluoroacetic acid (solvent A). The resulting solution was clarified by centrifugation and the supernatant was further submitted to a reversed phase chromatography on a C5 Discovery® Bio Wide Pore 10 μm (25 cm × 4.6 mm). Fractions were eluted using a linear gradient (0–100%, v/v) of acetonitrile (solvent B) at a constant flow rate of 1.0 ml/min over 50 min, and the resulting fractions were manually collected. The elution profile of both analyses was monitored at 280 nm, and the collected fractions were lyophilized and conserved at −20 °C. The homogeneity of the final material was assessed by mass spectrometry.

2.4. Phospholipase A_2 activity

PLA_2 activity was measured using the assay described by Cho and Kezdy (1991) and Holzer and Mackessy (1996) modified for 96-well plates (Beghini et al., 2000). The standard assay mixture contained 200 μl of buffer (10 mM Tris–HCl, 10 mM $CaCl_2$ and 100 mM NaCl, pH 8.0), 20 μl of synthetic chromogenic substrate 4-nitro-3-(octanoyloxy) benzoic acid 3 mM, 20 μl of water, and 20 μl of PLA_2 in a final volume of 260 μl. After adding PLA_2 isoforms (20 μg), the mixture was incubated for up to 40 min at 37 °C, absorbance reading at intervals of 10 min. The enzyme activity, expressed as the initial velocity of the reaction (V_0), was calculated based on the increase of absorbance after 20 min.

2.5. Determination of the molecular mass of the purified protein by mass spectrometry

An aliquot (4.5 μl) of the protein was inject by C18 (100 μm × 100 mm) RP-UPLC (nanoAcquity UPLC, Waters) coupled with nano-electrospray tandem mass spectrometry on a Q-Tof Ultima API mass spectrometer (MicroMass/Waters) at a flow rate of 600 nl/min. The gradient was 0–50% acetonitrile in 0.1% formic acid over 45 min. The instrument was operated in MS continuum mode and the data acquisition was from *m*/*z* 100–3000 at a scan rate of 1 s and an interscan delay of 0.1 s. The spectra were accumulated over about 300 scans, and the multiple charged data produced by the mass spectrometer on the *m*/*z* scale were converted to the mass (molecular weight) scale using Maximum Entropy-based software (1) supplied with the Masslynx 4.1 software package. The processing parameters were: output mass range 6000–20,000 Da at a 'resolution' of 0.1 Da/channel; the simulated isotope pattern model was used with the spectrum blur width parameter set to 0.2 Da, the minimum intensity ratios between successive peaks were 20% (left and right). The deconvoluted spectrum was then smoothed (2 × 3 channels, Savitzky Golay smooth) and the centroid mass values were obtained using 80% of the peak top and a minimum peak width at half height of 4 channels.

2.6. Analysis of tryptic digests

The protein was reduced (DTT 5 mM for 25 min at 56 °C) and alkylated (Iodoacetamide 14 mM for 30 min) prior to the addition of trypsin (Promega-Sequence Grade Modified). After the trypsin addition (20 ng/μl in ambic 0.05 M),

the sample was incubated for 16 h at 37 °C. To stop the reaction, formic acid 0.4% was added and the sample centrifuged at 2500 rpm for 10 min. The pellet was discarded and the supernatant dried in a speed vac. The resulting peptides were separated by C18 (100 μm × 100 mm) RP-UPLC (nanoAcquity UPLC, Waters) coupled with nano-electrospray tandem mass spectrometry on a Q-Tof Ultima API mass spectrometer (MicroMass/Waters) at a flow rate of 600 nl/min. The gradient used was 0–90% acetonitrile in 0.1% formic acid over 20 min. Before performing a tandem mass spectrum, an ESI/MS mass spectrum (TOF MS mode) was acquired for each HPLC fraction over the mass range of 100–2000 *m*/*z*, in order to select the ion of interest,where these ions were subsequently fragmented in the collision cell (TOF MS/MS mode).

Raw data files from LC–MS/MS runs were processed using MassLynx 4.1 SCN662 software package (Waters) and analyzed using the Mascot Distiller v.2.3.2.0, 2009 (Matrix Science, Boston, MA) with SNAKES database (snakes_jun2011 was downloaded from NCBI Taxonomy) release from June 2011 (http://www.ncbi.nlm.nih.gov/protein/?term=txid8570[Organism:exp]), using the following parameters: peptide mass tolerance of ±0.1 Da, fragment mass tolerance of ±0.1 Da, oxidation as variable modifications in methionine and trypsin as enzyme.

2.7. Amino acid analysis

Amino acid analysis was performed on a Pico-Tag Analyzer (Waters Systems) as described by Henrikson and Meredith (1984). LmrTX PLA_2, sample (30 μg) was hydrolyzed at 105 °C for 24 h, in 6 M HCl (Pierce sequencing grade) containing 1% phenol (w/v). The hydrolysates were reacted with 20 μl of derivatization solution (ethanol:-triethylamine:water:phenylisothiocyanate, 7:1:1:1, v/v) for 1 h at room temperature, after which the PTC-amino acids were identified and quantified by HPLC, by comparing their retention times and peak areas with those from a standard amino acid mixture.

2.8. Modification with p-bromophenacyl bromide

Modification of histidine residues was carried out as previously described by Diaz-Oreiro and Gutiérrez (1997). Briefly, approximately 3 mg of Lmr-TX were dissolved in 1 ml of 0.1 M Tris-HCl buffer, pH 8.0, containing 0.7 mM EDTA before adding 125 μl of p-bromophenacyl bromide (pBPB) solution (1.5 mg/ml in ethanol). The mixture was incubated for 24 h and the excess reagent was removed by ultrafiltration using an AMICON Ultra-15 centrifugal filter unit (3000 NMWL), followed by lyophilization.

2.9. Photochemically induction of thrombus formation

7–8 weeks old C57BL6 mice were anesthetized with xylazine (2%–16 mg/kg) and ketamine (10%–100 mg/kg) injected intramuscularly and placed in the supine position. Following a midline cervical incision, the right common carotid artery was isolated and a Doppler flow probe (model 0.5 VB; Transonic Systems, Ithaca, NY) was applied. A 1.5-mW, 540-nm laser beam (Melles Griot, Carlsbad, CA) was applied to the artery from a distance of 6 cm. PLA_2 was injected through lateral tail vein (3.75, 7.5 and 15 μg/animal) and after 10 min injury was initiated by injection into the lateral tail vein of rose bengal (50 mg/kg body weight; Fisher Scientific, Fair Lawn, NJ) dissolved in phosphate-buffered saline (PBS). Blood flow was monitored until complete and stable (5 min) occlusion occurred (Werneck et al., 2008).

2.10. Activated Partial Thromboplastin Time (APTT) and prothrombin time (PT)

Citrated Mouse plasma was obtained after blood was withdrawn from cava vein in citrate 3.2% and centrifuged at 3000 rpm for 15 min at 25 °C. For APTT a 50 μl aliquot plasma was warmed to 37 °C for 2 min, 50 μl APTT reagent was added and after a 2 min incubation at 37 °C, 0.25 M $CaCl_2$ was added and the clotting time was determined. For the PT test, 100 μl of CLOT PT reagent was incubated for 4 min at 37 °C and 50 μl of plasma was added, triggering the reaction. These analyses were performed in triplicate, using the APTT and PT Kit CLOT BIOS diagnosis (CLOT, Brazil) in a CLOTimer coagulometer. To assay APTT and PT *ex vivo*, C57BL6 mice were injected i.v. with LmrTX (15 μg/animal) and after 5, 15, 30, 60 and 90 min, blood was withdrawn and processed as described above.

2.11. Platelet aggregation assay

C57BL6 mice were injected i.v. with LmrTX (15 μg/animal). After 1 h mice were anesthetized (xylazine, 2%–16 mg/kg; ketamine 10%–100 mg/kg) and blood was collected from cava vein in citrate 3.2%. Whole blood was centrifuged at room temperature (95 g, for 12 min) to obtain the platelet-rich plasma (PRP). Five hundred microliters of platelet-rich plasma (PRP) were added to 700 μl of washing buffer (140 mM NaCl, 0.5 mM KCl, 12 mM trisodium citrate, 10 mM glucose, 12.5 mM saccharose, pH 6) and again centrifuged (800 g, 12 min). Platelets were gently suspended in Krebs solution containing (mM) 118 NaCl, 25 $NaHCO_3$, 1.2 KH_2PO_4, 1.7 $MgSO_4$, 5.6 glucose (pH 7.4). Platelet number was adjusted to 1.2×10^8 platelets/ml in the presence of 1 mM $CaCl_2$. Platelet aggregation was performed using an optical aggregometer (Chrono-log, Kordia Life Sciences, Leiden) at 37 °C with 400 μl of washed platelets placed in glass cuvettes containing a disposable stir bar for constant stirring. Platelet aggregation was carried out in ADP (20 μM) and thrombin (0.05 U/mL)-stimulated platelets.

2.12. Statistical analyses

Results were reported as mean ± SEM. The significance of differences among means was assessed by analysis of variance followed by ANOVA test, when several experimental groups were compared with the control group. Differences were considered statistically significant if $p < 0.05$.

3. Results

Four peaks were obtained after crude venom fractionation on the Sephadex G75 gel filtration column (Fig. 1A). All fractions were tested for the presence of PLA_2 activity. Peak 3 (FIII) displayed high PLA_2 activity. The proteins contained in this chromatographic peak were further purified using reverse phase-HPLC performed on a C5 column (Fig. 1B). All eluted peaks were manually collected, lyophilized and screened for PLA_2 activity. The main fraction, labeled as LmrTX, had PLA_2 activity. Analysis by ESI-MS of the intact protein indicated a molecular mass of the purified protein of 14277.50 Da (Fig. 2).

Mass spectrometric analysis was performed in order to obtain a molecular identification and homology study. Digestion of the protein (LmrTX) with trypsin, followed by LC/MS/MS, identified ten peptides. The deduced sequence and measured masses of alkylated peptides of LmrTX are summarized in Table 1. The sequence of each peptide was then submitted separately to the SNAKE database using the protein search program BLAST-p. Using the position matches of the '*de novo*' sequenced peptides with homologous proteins present in the database, it was possible to deduce their original position on the unknown protein LmrTX. Fig. 3 shows the result of BLAST alignment between LmrTX with the phospholipase A_2 from *Crotalus durissus terrificus*, *L. muta muta* and *L. stenophrys*.

Amino acid analysis revealed the following composition of LmrTX PLA_2: Asx/9, Glx/7, Ser/6, Gly/11, His/2, Arg/9, Thr/8, Ala/5, Pro/5, Tyr/11, Val/2, Met/2, Cys/14, Ile/5, Leu/6, Phe/6 and Lys/11. LmrTX showed a high content of Lys and Arg residues typical of a basic PLA_2 protein (data not show).

The enzymatic activity of whole venom, LmrTX PLA_2 and alkylated LmrTX (LmrTX (His)), was studied using the synthetic chromogenic substrate 4-nitro-3-(octanoyloxy) benzoic acid. The PLA_2 activity of *L. muta rhombeata* whole venom, Sephadex G75 fraction (FIII) and LmrTX was 122.70 ± 13.41; 191.65 ± 3.34 and 789.74 ± 6.59 nmol/mg/min, respectively, as shown in Fig. 4. The alkylation of histidine residues (with p-bromophenacyl bromide) from LmrTX, reduced significantly the toxin PLA_2 activity (only 11% of residual activity), 86.47 ± 13.41 nmol/mg/min ($p < 0.05$; Fig. 4).

Snake venom phospholipases A_2 (PLA_2) are an extremely important and diverse group of proteins that affects hemostasis. In this study, we examined the ability of the LmrTX in altering the thrombus formation in living mouse. For this, we used a photochemically induced arterial thrombosis model in mice. Fig. 5 shows the effect of LmrTX in thrombus formation in the carotid artery of mice. Control animals that did not receive the protein injection showed a normal occlusion time, which was 57 ± 7.8 min. As shown in Fig. 5, LmrTX, the PLA_2 from *L. muta rhombeata* venom, caused a change in the normal occlusion time. With doses of 7.5 and 15 μg/mice, the occlusion time was 99 ± 10 min and 94 ± 11.5 min respectively. The dose of 3.25 μg/mice did not significantly differ from control values (62.6 ± 10 min). The animals that were treated with the modified protein showed the occlusion time similar to control animals (64.4 ± 14.0 min).

Anticoagulant PLA_2s (mainly Asp49 PLA_2) have been described in all major snake groups. In *in vitro* condition, LmrTX showed anticoagulant activity (APTT), prolonging the normal clotting time of platelet poor plasma (Fig. 6A). When mice were pretreated with LmrTX at different times before determining APTT, the protein showed significant anticoagulant activity with a rapid onset (maximal response obtained at 15 min pos-injection) which was sustained during 1 h (Fig. 6B). In the other hand, no significant effect on PT *in vitro* (Fig. 6C) and *ex vivo* (Fig. 6D) was observed.

We also verified if LmrTX could interfere with platelet aggregation. The animals that received the protein (15 μg) showed a partial inhibition in ADP and thrombin-induced washed platelet aggregation, 43 and 44%, respectively (Fig. 7).

In snake venom PLA_2 enzymes, His48 is conserved and plays a crucial role in the hydrolysis of phospholipids. Modification of PLA_2 enzyme from *L. muta rhombeata* venom on His48 residue by alkylation leads to the 89% of reduction in enzymatic activity, with concomitant loss of anticoagulant effects *in vitro*.

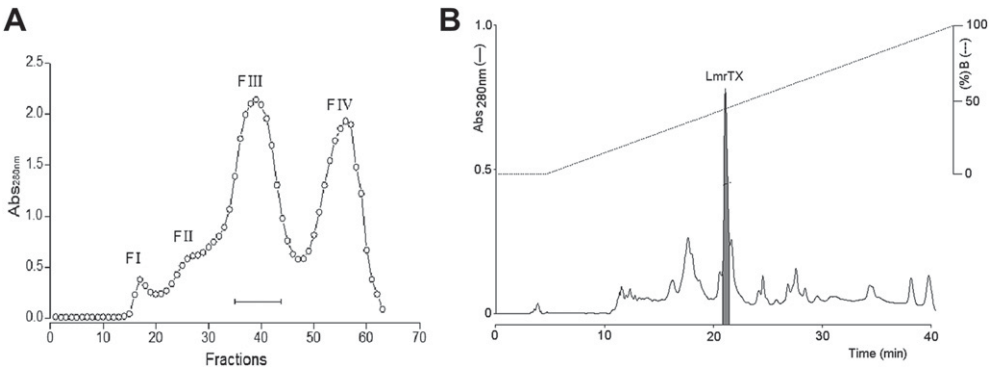

Fig. 1. (A) Elution profile of *L. muta rhombeata* venom by molecular exclusion chromatography on a Sephadex G75 column. Fraction III (FIII) contained PLA_2 activity. (B) Elution profile of FIII following RP-HPLC on C5 Discovery® Bio Wide column. The peak corresponding to the phospholipase A_2 (LmrTX) from *L. muta rhombeata* venom is indicated.

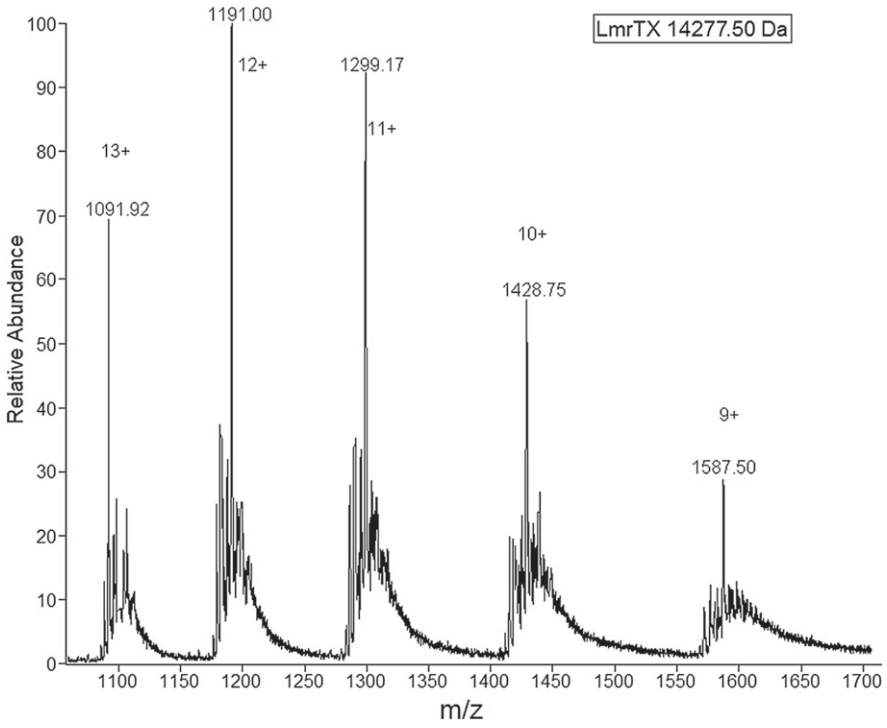

Fig. 2. Molecular mass determination of the native LmrTX PLA_2 by nano-electrospray tandem mass spectrometry, using a Q-Tof Ultima API mass spectrometer (MicroMass/Waters) with output mass range of 6000–20,000 Da at a "resolution" of 0.1 Da/channel.

4. Discussion

Accidents caused by *Lachesis* venom present many symptoms like local pain, oedema, haemorrhage and necrosis at the site of the bite. Moreover, systemic complications such as nausea, vomiting, diarrhea, hypotension and bradycardia, coagulation disturbances and renal failure are observed during Lachesis envenomation (Jorge et al., 1997; Rucavado et al., 1999).

Only a few proteins were purified from the venom of *L. muta rhombeata,* among them a thrombin-like gyroxin enzyme (Aguiar et al., 1996); a kallikrein-like enzyme (Giovanni-De-Simone et al., 1997); a β-galactoside binding lectin (Giovanni-De-Simone et al., 2006) and also the expression of vascular apoptosis protein (VAP)-like metalloprotease from venom gland (Tavares et al., 2008), but there have been no reports on the purification of PLA_2 from this source.

In this paper, we described the purification of the first PLA_2 from the *L. muta rhombeata* venom. The isolated protein, now named *L. muta rhombeata* toxin (LmrTX), was able to prolong thrombosis time in a photochemically induced arterial thrombosis in mice, induced anticoagulant activity *in vitro* and *ex vivo* and reduced platelet aggregation in the presence of ADP and thrombin. LmrTX was purified through an experimental protocol that combined gel filtration and Reverse-phase HPLC chromatographies. The protein consists of a single polypeptide chain and a molecular mass of 14277.50 Da.

PLA_2 from *L muta rhombeata* (LmrTX) shows three regions that retain a significant degree of similarity between group II PLA_2, including the N-terminal region (forming the hydrophobic channel), the regions of the active site (formed by H48, D49, Y52 and D89) and binding of calcium (formed by Y27, G29, G31 and G32). The regions displaying a lower degree of amino acid homology correspond to structurally less conserved elements, and are likely determinants of the diverse pharmacology effects exhibited by venom PLA_2s (Arni and Ward, 1996).

The LmrTX sequence returns high homology with the sequence of a phospholipase A_2 present in the venom of *C. durissus terrificus* (crotoxin basic chain) (PA2B_CRODU

Table 1
Measured molecular masses and deduced amino acid sequences obtained by ESI-QTOF-MS/MS based on the alkylated tryptic peptides of LmrTX PLA_2. The peptides were separated by RP-HPLC and sequenced by mass spectrometry. *C* = alkylated cysteine, lysine and arginine residues shown in bold were deduced on the cleavage and missed cleavage by trypsin. All molecular masses are reported as monoisotopic.

Position	Amoniacid sequence	Measured mass (Da)
1–7	HL/IL/IK/QFN**K**	898,4634
15–33	**K**NAL/IPFYAFYGCYCGWGG**R**	2285,9459
43–53	*C*CFVHDCCYG**K**	1504,4855
61–69	**K**WDL/IYPYSL**K**	1183,5264
70–77	SGYL/ITCG**K**	884,3910
78–90	GTWCEEK/QL/ICECD**R**	1741,5744
91–97	VAAECL/I**R**	817,3577
98–104	RSL/ISTY**K**	853,4496
105–114	YGYMFYPDS**R**	1297,4471
115–122	*C***R**GPSET*C*	965,3567

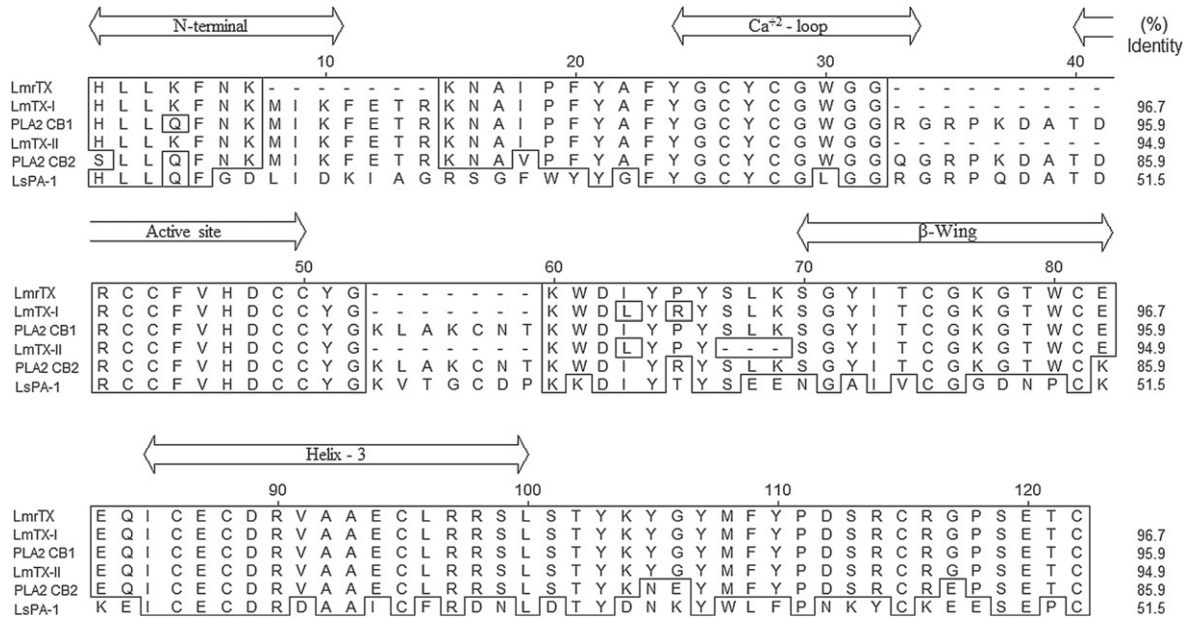

Fig. 3. Amino acid alignment of the deduced amino acid sequence of the new phospholipase A_2 LmrTX with: CB1 (P62022) and CB2 (P24027) from *Crotalus durissus terrificus* venom; PLA_2A (P86169) from *Crotalus durissus ruruima*; LmTX-I (P0C942) and LmTX-II (P0C943) from *Lachesis muta muta* venom and LsPA-1 (P84651) from *Lachesis stenophrys*. The PLA_2 functional domains are show by arrows.

Accession Number P62022) and *L. muta muta* (LmTX-I and LmTX-II) (PA2T1_LACMU Accession Number P0C942; PA2T2_ LACMU Accession number P0C943). It is not surprising that LmrTX has a high degree of structural identity with LmTX-I and LmTX-II, since *L. muta rhombeata* and *L. muta muta* are closely related subspecies. Zamudio and Greene (1997), used mitochondrial genes determinate, that these are, in fact, two subspecies closely related; especially between *L. muta rhombeata* and populations of *L. muta muta* from southern regions of its distribution (e.g. Mato Grosso, Brazil). These authors also point it out that the speciation process between this two subspecies it is a recently event (300–800 thousand years ago). Interestingly, it was found that the positively selection evolution process for the PLA_2 family from venoms of Crotalinae subfamily take, at least 300 thousand years (Gibbs and Rossiter, 2008). Therefore, the higher degree of structural identity between these proteins it is an expected phenomena.

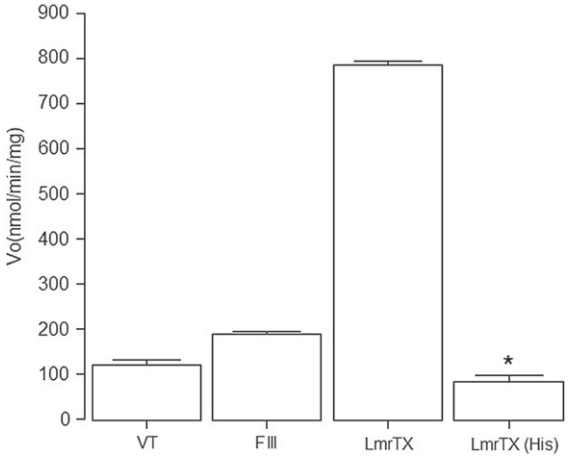

Fig. 4. PLA_2 activity of *L. m. rhombeata* venom, Fraction III, LmrTX and LmrTX(His), modified on the histidine residues by bromophenacyl bromide, on the chromogenic substrate 4-nitro-3-octanoyloxybenzoic acid. Error bars indicate mean plus or minus standard deviation ($n = 3$ for each group). $^*P < 0.05$.

Nevertheless, LmrTX show biochemical and structural differences with LmTX-I and LmTX-II from *L. muta muta* (Damico et al., 2005). As presented in our results LmrTX has a shorter retention time at similar conditions on HPLC-RP (21 min) compare with the two PLA_2 isoforms ($\geq$24 min) purified from *L. muta muta*. Also the catalytic activity of LmrTX (15.79 nmol/min) it is more than twice that showed by the isoforms LmTX-I and LmTX-II (6.1 nmol/min and 5.7 nmol/min, respectively). Both data clearly indicate that exits some degree of structural differences between these proteins.

Along with the biochemical data, the molecular mass of LmrTX (14,277.50 Da) is different from LmTX-I and LmTX-II (14,245.4 and 14,186.2, respectively). The molecular mass difference found is in accordance with the aminoacid composition, which shown variation in the number of Pro, Thr and Ala residues.

Despite the biochemical and structural differences between LmrTX and the isoforms LmTX-I and LmTX-II, the high degree of identity suggest that this toxin could exert similar pharmacologic activities, i.e neurotoxic activity *ex vivo*.

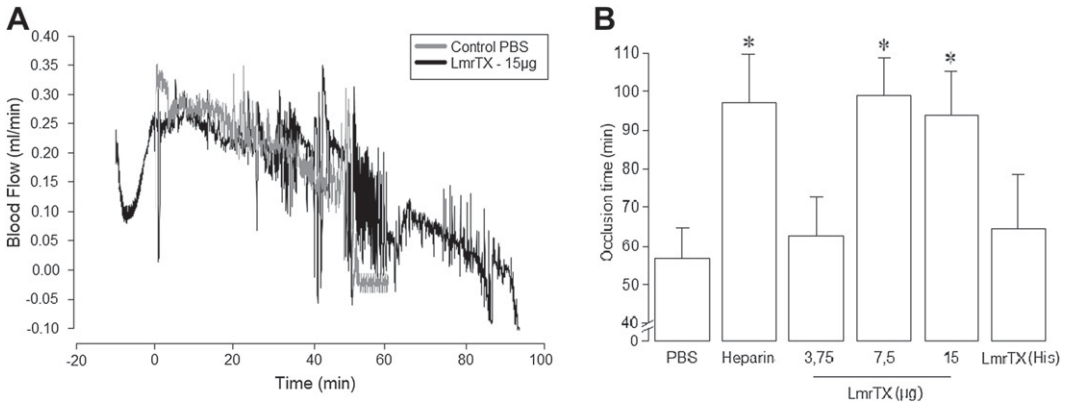

Fig. 5. Effect of LmrTX on thrombotic occlusion of the carotid artery. (A) Representative blood flow recordings showing the delayed occlusion time and stochastic flow pattern in treated animals with purified PLA_2. Rose bengal dye was injected at time = 0 min. (B) The time to occlusion of blood flow following injury is shown. Error bars indicate mean plus or minus standard deviation ($n = 5$ for each group). $^*P < 0.05$.

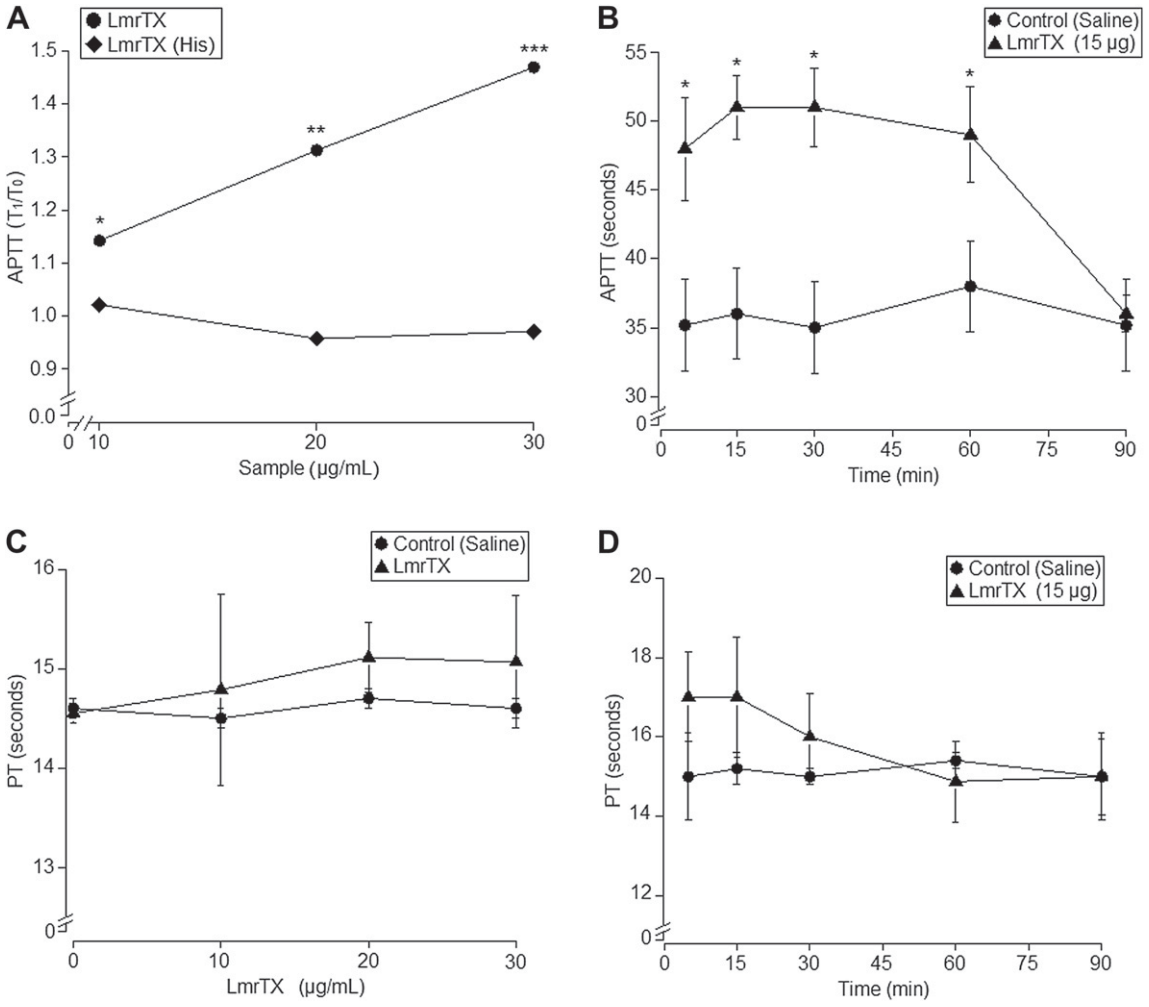

Fig. 6. Activated Partial Thromboplastin Time (aPPT) blood test of LmrTX (◇) and histidine-alkylated LmrTX (○) on platelet poor plasma. Results are expressed as a ratio of normal condition test. Error bars indicate mean plus or minus standard deviation ($n = 3$ for each group).

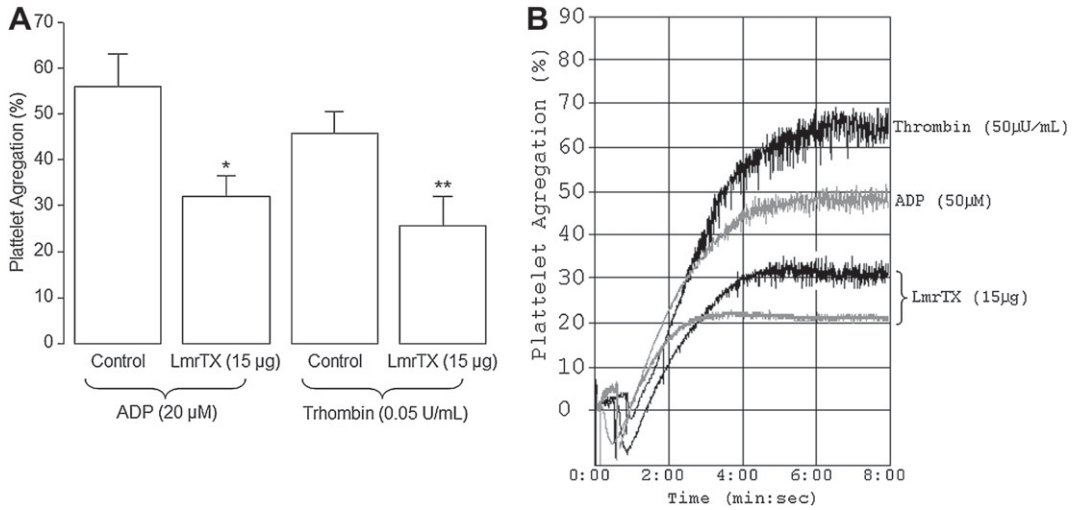

Fig. 7. Effect of LmrTX on platelet aggregation. (A) Inhibitory effect of LmrTX on platelet aggregation induced by ADP (20 µM) and Thrombin (0.05 U/mL) in treated animals with purified PLA_2 (15 µg) (▲) or control (●). (B) Representative recordings showing the aggregation curves with agonist alone or LmrTX. Each point represent the average from five experiments ± SEM. * and ** shown significant difference ($p < 0.05$) compare with the respective control.

Phospholipase enzymes can exert their anticoagulant effects by the hydrolysis and physical destruction of the membrane surface required for the formation of coagulation complexes or by their interaction with blood coagulation proteins and not phospholipid hydrolysis (Kini, 2005). APTT is used to measure the integrity of components of the intrinsic pathaway and PT measures the integrity of the extrinsic pathaway. LmrTx interfered only with APTT, prolonging this time. The protein could be acting in the enzymatic cleavage of the available phospholipids required to intrinsic pathaway, since it was seen that chemical modification of histidine residues neutralized its anticoagulant activity.

Based on the comparison of the three dimensional structure of class II PLA_2 enzymes, three independent groups supported the predicted anticoagulant site (Carredano et al., 1998; Zhao et al., 2000; Singh et al., 2001). This region shows conformational similarity and the presence of positively charged residue free for intermolecular interactions at the corner of molecule corresponding to the stretch of residues 55–67 seems to be a common feature of most of the anticoagulant PLA_2 enzymes (Carredano et al., 1998; Zhao et al., 2000; Singh et al., 2001). For the RVV-VD, a PLA_2 from the venom of Russell's viper (*Vipera russeli russeli*), a strong anticoagulant PLA_2 of this class, this region has several lysine residues (Carredano et al., 1998). In LmrTx this region has not been fully determined, only two residues positively charged in this segment was showed, which are favorably oriented to induce the anticoagulant effect.

When performing chemical modification of histidine residues (alkylation with p-bromophenacyl bromide), LmrTX showed a reduction in its catalytic activity in 89% and there was an inactivation of the anticoagulant activity. The present study supports that anticoagulant activity *in vitro* of LmrTX is dependent on its catalytic activity.

The same chemical modification, when made in PrTX-III (from *Bothrops pirajai*) and BthTX-II (from *Bothrops jararacussu*), two basic Asp49-PLA_2s, almost completely inhibited the catalytic, anticoagulant, myotoxic and cytotoxic activity (Diaz-Oreiro and Gutiérrez, 1997; Andrião-Escarso et al., 2000; Soares and Giglio, 2003). These results show that His48 is essential for the hydrolysis of phospholipids and that these pharmacological effects are dependent, even partially, of the catalytic activity (Soares and Giglio, 2003).

A photochemically induced model of arterial thrombosis in mice was used to determine the antithrombotic activity of PLA_2 *in vivo* (LmrTX). The process of thrombosis after the injection of rose bengal (dye) is related to the formation of reactive oxygen species from the stimulation of the dye by laser light, leading to endothelial injury. The laser remains on until the thrombus formation, and the blood flow is monitored by an ultrasound probe. When we compared control animals with animals that received the LmrTX, the time required to the thrombus formation (occlusion time = zero blood flow) was extended by approximately 40 min.

PLA_2 enzymes interfere with several physiological processes, including platelet aggregation. LmrTX produced a partial inhibition of thrombin and ADP-induced aggregation in washed platelets. PLA_2 enzymes can inhibit platelet aggregation by physical destruction of the integrity of the platelet membrane via hydrolysis of the membrane phospholipids, which could affect the functions of receptors that play important roles in platelet aggregation (Kini and Evans, 1997). As platelet aggregation was performed with mice washed platelets, probably the mechanism of action of LmrTx is through its interaction with the platelet membrane.

The data of the primary structure, together with the results of the anticoagulant activity (APTT) *in vitro* and *ex vivo*, lead us to infer that this enzyme can be classified as

a PLA_2 with anticoagulant activity related on its catalytic activity. In conclusion, the results strongly suggest that LmrTX exerts its anticoagulant effect thought intrinsic pathway (interaction with coagulation factors of this way) or by enzymatically hydrolyzing the plasma phospholipids.

However, further experiment of interaction of LmrTX with coagulation factors are necessary for better understanding of action mechanism of this enzyme in cascade coagulation.

Conflict of interest statement

The author declares that there are no conflicts of interest.

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