

RESEARCH ARTICLE

BoayPLI: Structural and functional characterization of the gamma phospholipase A2 plasma inhibitor from the non-venomous Brazilian snake *Boa constrictor*

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Abstract

Plasma in several organisms has components that promote resistance to envenomation by inhibiting specific proteins from snake venoms, such as phospholipases A₂ (PLA₂s). The major hypothesis for inhibitor's presence would be the protection against self-envenomation in venomous snakes, but the occurrence of inhibitors in non-venomous snakes and other animals has opened new perspectives for this molecule. Thus, this study showed for the first time the structural and functional characterization of the PLA₂ inhibitor from the *Boa constrictor* serum (BoayPLI), a non-venomous snake that dwells extensively the Brazilian territory. Therefore, the inhibitor was isolated from *B. constrictor* serum, with 0.63% of recovery. SDS-PAGE showed a band at ~25 kDa under reducing conditions and ~20 kDa under non-reducing conditions. Chromatographic analyses showed the presence of oligomers formed by BoayPLI. Primary structure of BoayPLI suggested an estimated molecular mass of 22 kDa. When BoayPLI was incubated with Asp-49 and Lys-49 PLA₂ there was no severe change in its dichroism spectrum, suggesting a non-covalent interaction. The enzymatic assay showed a dose-dependent inhibition, up to 48.2%, when BoayPLI was incubated with Asp-49 PLA₂, since Lys-49 PLA₂ has a lack of enzymatic activity. The edematogenic and myotoxic effects of PLA₂s were also inhibited by BoayPLI. In summary, the present work provides new insights into inhibitors from non-venomous snakes, which possess PLIs in their plasma, although the contact with venom is unlikely.

1. Introduction

Snake envenomation, reclassified as a neglected tropical disease by the World Health Organization (WHO), can have serious pathophysiological consequences [1–3]. The pharmacological

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actions of envenomation are related to the toxins' actions present in the venom, which consist mainly of proteins, whose activities can promote homeostatic, neuromotor, inflammatory and blood clotting disorders. Among the enzymatic proteins commonly found in the venoms are metalloproteases (SVMP), serine proteases (SVSP), phospholipases A₂ (PLA₂) and L-amino acid oxidases (LAAO) [4–6].

The PLA₂s are a group of low molecular mass enzymes (~ 13 to 15 kDa), which are related to calcium-dependent cleavage at the sn-2 position of phospholipids, releasing lysophospholipids and arachidonic acid, the precursor of the inflammatory cascade [7]. PLA₂s can be divided into several groups, being that those present in the Viperidae family snakes belong to group II and can be separated into two subgroups: Asp49-PLA₂ and Lys49-PLA₂. The variant Asp49-PLA₂ has a high enzymatic activity. When there is a substitution of the amino acid residue at position 49, the most common being Lys-49 substitution, there is a loss in the ability of calcium binding, resulting in a severe reduction of its enzymatic activity [8,9].

Nevertheless, PLA₂s pharmacological actions are not only related to their enzymatic activity, being responsible for myotoxicity, neurotoxicity and inflammatory disorders in snake bite envenomation. This protein is also responsible for local tissue damage, lethality and irreversible effects, such as muscle damage and loss of limbs, leading to individual incapacitation [10–12]. Furthermore, they also have anticoagulant, cardiotoxic, and platelet aggregation-inducing / inhibitory activity [8,13]. Several molecules have an inhibitory capacity against PLA₂s activity, some of which were identified by transcriptome of liver or isolated from snake plasma [14–17]. *In silico* techniques were also used to search for potential inhibitors [18].

The major hypothesis for the presence of PLA₂ inhibitors (PLIs) in venomous snakes is the protection against self-envenomation. However, such theory does not support their presence in non-venomous snakes [19–22], whose occurrence suggests that its physiological role is not restricted to protection against self-envenomation, but has a role not yet completely understood [23].

PLIs can be homo or hetero-oligomeric and are usually glycoproteins, but the carbohydrate is not essential for its inhibitory activity [14]. Due to their structural differences, such inhibitors can be classified into three groups: αPLI, βPLI, and γPLI, whose domains are related to the interaction between the inhibitor and PLA₂ [16].

Regarding the γPLIs, they are characterized by two structural units of highly conserved cysteine repeats, known as three finger motifs [24]. Another important feature of γPLIs is the highly conserved proline-rich region, that plays an important structural role, ensuring the integrity and conformation of protein interaction sites [25].

The γPLI from *Boa constrictor* was already identified by transcriptomic analysis [26], but its functional characterization has not been reported yet. Given the background, the biotechnological potential of these inhibitors may provide therapeutic molecular models with antiophidic activity to complement conventional serum therapy against these multifunctional enzymes, as well as its anti-inflammatory potential, since there is a structural and catalytic similarity between venom and human PLA₂s, besides contributing to the elucidation of the PLA₂-PLI interaction mechanism. In this context, we isolated a γPLI from *Boa constrictor* plasma, named as BoaγPLI, and characterized it structurally (primary and secondary structure, and its oligomerization) and functionally by enzymatic and pharmacological effects such as edema and myotoxicity inhibition of Asp-49 and Lys-49 PLA₂ activity.

2. Material and methods

2.1. Ethical committee

All animal experiments were approved by the Ethical Committee of Instituto Butantan (protocol number 6916110917) and experiments were in accordance with the Brazilian laws for the

use of experimental animals and with the ethical principles adopted by the Brazilian College of Animal Experimentation (COBEA).

2.2. Snake venom

Snake venoms from *Crotalus durissus terrificus* (three captive animals) and *Bothrops jararacussu* (three captive animals) were provided by the Laboratory of Herpetology at Instituto Butantan. Venoms were obtained by manual extraction, centrifuged at 1700 g for 15 minutes, lyophilized and stored at -20°C.

2.3. Serum

The blood of five *Boa constrictor* from captivity was collected by puncture of the paravertebral vein using plastic syringes and pooled. The volume of blood collected corresponded to 1% of snake total weight. Blood was maintained for 18 hours at 4°C for coagulation, prior to centrifugation (1200 g, 15 minutes) and stored at -20°C.

2.4. Purification of Asp-49 PLA₂ from *Crotalus durissus terrificus* and Lys-49 PLA₂ from *Bothrops jararacussu*

Purification of an Asp-49 PLA₂ from *C. d. terrificus* venom was performed according to Oliveira et al., (2002) [27]. The venom was fractionated on a gel-filtration chromatography, using a Superdex 75 column (GE Healthcare 10/300) and 50 mM Tris 100 mM NaCl pH 7.4 buffer, for 250 minutes at 1 mL/min. Subsequently, for some tests, crotoxin A (crotopotin) was dissociated from crotoxin B (PLA₂) by a reverse phase C5 column chromatography (Supelco C5 column, 0.10 cm × 25 cm) [28]. The chromatographic column was pre-equilibrated with solution A (0.1% TFA). Elution of PLA₂ was performed with a continuous linear gradient of solution B (66% acetonitrile in 0.1% TFA) and monitoring the chromatographic profile at 280 nm (detector UV-2077, Jasco, Japan). The samples were then lyophilized.

The purification of a Lys-49 PLA₂ from *Bothrops jararacussu* (Lys-49 PLA₂) was performed following Soares et al., (1998) [29]. The venom (100 mg) was fractionated on an ion exchange CM column (GE Healthcare, 5 mL), with a linear gradient (0–100%) of 0.05 M ammonium bicarbonate buffer to 1 M ammonium bicarbonate buffer pH 7.9, monitored by 215 nm. The fraction containing the purified PLA₂ was lyophilized.

2.5. Purification of PLI

The purification of PLI from *Boa constrictor* serum was performed in two chromatographic steps according to Serino-Silva et al., (2018) [30], with some modifications. In the first step, the *Boa constrictor* serum (5 mL) was diluted in 5 mL of 25 mM Tris buffer pH 7.5 (buffer A) and applied to an anion exchange column (HiTrap DEAE FF 5 mL, GE Healthcare), previously equilibrated with 95% buffer A (25 mM Tris, pH 7.5) and 5% buffer B (25 mM Tris, 1 M NaCl pH 7.5). Elution was performed maintaining 10% buffer B (100 mM NaCl) and then with a gradient of buffer B up to 50% (500 mM NaCl). The run was maintained at a flow rate of 1 mL/min, monitored at 280 nm, and the samples were fractionated every 5 mL (Akta purifier, GE Healthcare). After the chromatography, the protein fractions were pooled. Pool desalination was done by dialysis in PBS (140 mM NaCl, 2.6 mM KCl, 10 mM Na₂HPO₄, 1.7 mM KH₂PO₄, pH 7.4) for 24 hours at 4°C in a 10000 MWCO membrane. Then, the D2 fraction from DEAE chromatography was applied to an affinity column, previously prepared by the coupling of crotoxin into a CNBr-activated Sepharose matrix (GE Healthcare) and equilibrated with PBS. The non-adsorbed material was removed by extensive washing with PBS.

Finally, the proteins adsorbed to the resin were eluted with 0.1 M glycine pH 2.7, fractionated in microtubes, and the pH of the fractions was neutralized by the addition of 1 M Tris buffer pH 8.8 (9:1 v/v). The elution was manually measured in a spectrophotometer (Spectramax, Molecular Device) at 280 nm.

2.6. SDS-containing polyacrylamide gel electrophoresis (SDS-PAGE)

PLI samples were subjected to 12% SDS-PAGE, according to Laemmli, (1970) [31] under reducing by β - mercaptoethanol or non-reducing conditions. By lane, 20 μ g of protein was applied. The molecular marker used was Dual Color Precision Plus, BioRad. The gels were stained using Coomassie Blue R350 (GE Healthcare).

2.7. Mass spectrometry

Protein bands were excised from SDS-PAGE (under reducing conditions) which were dehydrated with acetonitrile addition and subjected to reduction with 5 mM dithiothreitol for 30 min at 60°C, alkylation with 15 mM iodoacetamide for 30 min under light protection at room temperature and overnight in-gel digestion with sequencing grade trypsin (Sigma), in 50 mM ammonium bicarbonate at 37°C. Digested peptides were analyzed by a Synapt G2 mass spectrometer coupled to a nanoAcquity UPLC system (Waters). Samples were injected into a trap column (C18 nanoAcquity trap Symmetry column 180 μ m x 20 mm, Waters) with 0.1% (v/v) formic acid. Peptides were eluted on a capillary analytical column (C18 nanoAcquity BEH 75 μ m x 150 mm, 1.7 μ m column) using a gradient of 93% A (0.1% formic acid) and 7% B (99.9% ACN 0.1% formic acid) to 35% B over 30 min in a flow of 275 nl/min. Data were acquired in MS^E mode [32,33] in duplicate. Protein identification, PTM and homology searches were performed in PEAKS Studio 7.5 software (Bioinformatics Solutions Inc.) by MS/MS search against the Serpentes databases obtained from Uniprot (2844 sequences, downloaded in October 25th, 2018). Analyses were carried out with precursor mass tolerance of 10 ppm, fragment mass tolerance of 0.025 Da and peptide cleavage by trypsin. Carbamidomethylation of the cysteines was considered as a fixed modification and the oxidation of methionines, N-terminal acetylation, and deamidation of asparagines and glutamines as variable modifications. Assignments for peptides and proteins were accepted at a false discovery rate < 1%. As the inhibitor was identified with γ PLIs, it was named as Boa γ PLI.

2.8. Size-exclusion chromatography protein analysis (SEC)

Boa γ PLI (20 μ g) were subjected to size-exclusion chromatography (BioSep SEC-s2000, Phenomenex) using 0.05 M Tris HCl, 0.05 M NaCl, pH 8, at a flow rate of 1 mL/min, and with monitoring at 280 nm (MD-2018, Jasco). For comparison of molecular mass, Gel Filtration Standard (BioRad) was used.

2.9. Circular dichroism

The Asp-49 and Lys-49 PLA₂ (30 μ g), Boa γ PLI (30 μ g) or the incubated mixture (PLA₂-PLI, 1:1) (w/w) was subjected to circular dichroism assay (J815 spectropolarimeter, Jasco). Proteins diluted in 0.002 M Tris 0.015 M NaCl 0.1 mM CaCl₂, pH 8 were run at wavelengths of 190 to 260 nm (1 nm/s) under 8 convolutions. All analyses were subtracted from the buffer used. The data were exported by Spectra manager and the relative percentages of secondary protein structures were determined by Circular Dichroism analysis using Neural Networks (CDNN) software.

2.10. Inhibition of the PLA₂ enzymatic activity

The PLA₂ activity was performed according to Holzer and Mackessy, (1996) [34]. The enzyme (*C.d. terrificus* Asp-49 PLA₂, 1 mg/mL) and the inhibitor (with a range of concentrations of 2 mg/mL, 1.5 mg/mL, 1 mg/mL and 0.5 mg/mL) were incubated for 10 minutes. Then, 20 µL of each solution were applied to the plate. The chromogenic substrate 4-nitro-3-octanoyloxy benzoic acid (NOB) (Enzo Life Sciences) (solubilized in 3 mM acetonitrile PA) was used (20 µL). Afterwards, the buffer 0.01 M Tris-HCl, 0.10 M NaCl and 0.01 M CaCl₂, pH 8 was added to the samples (200 µL). The controls were composed by 20 µL of saline 0.85%, 20 µL of inhibitor (2 mg/mL) or 20 µL of PLA₂ (1 mg/mL). The activity was analyzed by spectrophotometer Spectramax (Molecular Devices) at 425 nm, and the readings occurred over 90 minutes, with 5-minute intervals between readings. The absorbances of the last reading were transformed into the PLA₂ specific activity as nmol/mg/min per unit. Then, the percentage of inhibition was determined. The experiment was done in triplicate.

2.11. Paw edema inhibition

The paw edema was induced by a subplantar injection in male Swiss mice (18 to 21 g; n = 5) of 10 µg of Asp-49 or Lys-49 PLA₂, previously incubated for 30 min with 20 µg of BoaγPLI with a final volume injection of 20 µL. The contralateral paw was injected with the same volume of sterile 0.85% NaCl solution. Control groups were inoculated with 20 µg of BoaγPLI, 0.85% NaCl, or 10 µg of Asp-49 or Lys-49. Paw thickness was measured using a caliper reading to 0.01 mm at 0, 0.5, 1, 2, 4, 6 and 24 h after injection. Results were expressed as the difference in thickness of both paws and represented as the percentage increase in paw thickness.

2.12. Myotoxicity inhibition

The myotoxicity inhibition by BoaγPLI was determined according to Belchor et al. (2017) [35], by the injection of 40 µL of Asp-49 or Lys-49 PLA₂ (10 µg) incubated for 30 min with BoaγPLI (20 µg) on the right *gastrocnemius* muscle (18 to 21 g male Swiss mice, n = 5). Control groups received 20 µg of Asp-49 or Lys-49 PLA₂ or 20 µg of BoaγPLI or 40 µL of 0.85% saline. Mice blood samples were collected from the tip of the tail [36] into tubes containing citrate as anticoagulant, centrifuged at 1200 g for 15 minutes and the plasma was separated. The amount of Creatine Kinase (CK) present in the samples was estimated with a commercial CK kit (Sigma), according to the manufacture's instructions.

2.13. Statistical analyses

The data were expressed as mean ± standard deviation (SD). The enzymatic and myotoxic assays were analyzed with one-way ANOVA, with Tukey as a *posteriori* test, while the edematogenic test was analyzed with a two-way ANOVA, with Tukey as a *posteriori* test. The inhibition of the edema activity was specified by the area under the curve analysis. Values of p < 0.05 was considered significant.

3. Results and discussion

This work shows for the first time the structural and functional characterization of the BoaγPLI, a PLI from *Boa constrictor*, a non-venomous Brazilian snake. Contrasting with the number of PLIs isolated from venomous snakes [14,16], only a small number of this molecule were isolated from non-venomous snakes [19,22], suggesting that the PLI is not restricted to protection against self-venomation, but it may be involved in other unknown physiological mechanisms that has yet to be determined [23].

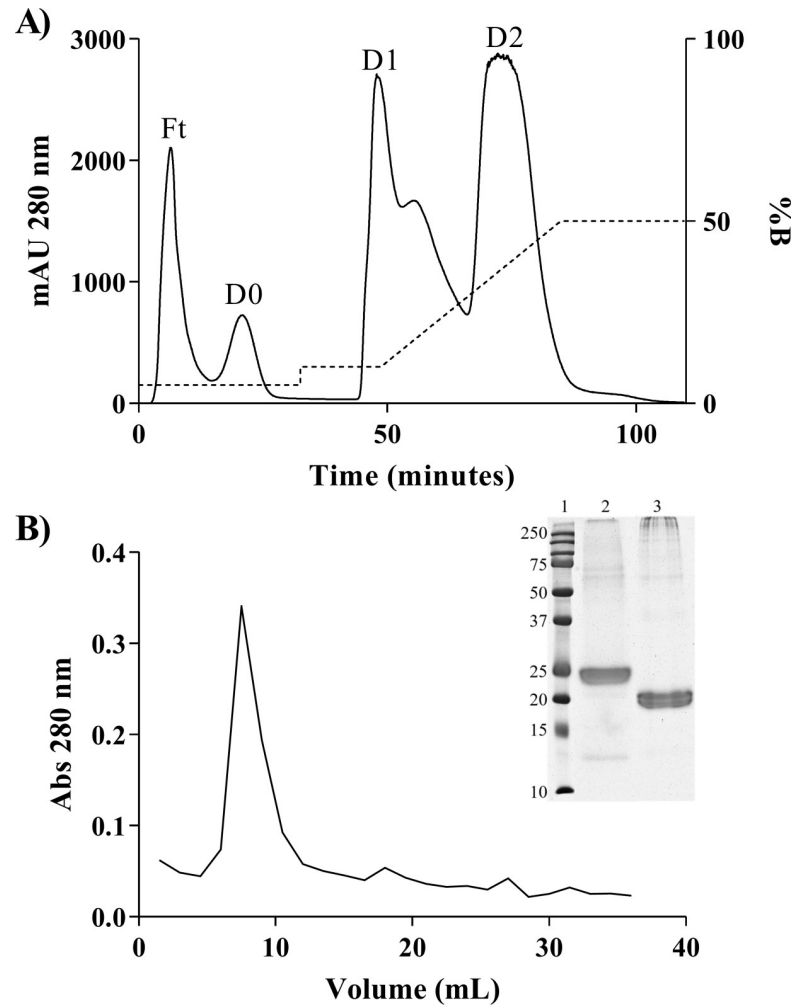


Fig 1. The purification process of the BoayPLI. A) Fractionation of *B. constrictor* serum. HiTrap DEAE column ion exchange chromatography (5 mL), flow 1 mL / min. Five ml of serum diluted in buffer A were applied to the chromatography. Three resulting fractions were evidenced: D0, D1, and D2. B) Elution of D2 fraction retained on CNBr-activated Sepharose resin-coupled crotoxin affinity chromatography (GE Healthcare). Elution was performed with 0.1 M glycine, pH 2.7, pH neutralized with the addition of 1 M Tris, pH 8.8. Elution was manually dosed at 280 nm. Insert: SDS-Page 12% of the BoayPLI in reducing (2) and non-reducing conditions (3). 1) Molecular mass marker Dual Color Precision Plus, Biorad.

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In this study, was performed using two chromatographic steps (Fig 1) and the percentage of recovery was 0.63% (Table 1). a reasonable value considering that the inhibitor is a minor protein of the serum, especially in a non-venomous species, whose contact with venom is unlikely.

Table 1. Purification table of the *Boa constrictor* PLI.

	Total mg*	% of recovery
Initial	125.15	-
D2	57.81	46.19%
Eluted (BoayPLI)	0.80	0.63%

*measured by A₂₈₀ nm.

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However, the value was superior to the percentage of recovery from *Python reticulatus*' PLI, which was 0.25% [37], although the distinct methodology applied could have affected the recovery.

In comparison, the purification of the γ PLI from *Bothrops jararaca* (γ BjPLI) presented 1% of recovery, using the same methodology applied in this case [30], while under different approaches the γ PLI from *Crotalus durissus collilineatus* (γ CdcPLI) presented 2.69% [38]. The higher recovery of PLIs from venomous snakes may be related to the constant contact with the venom, which may stimulate the presence of inhibitors in the plasma [39].

In fact, a difference in the PLIs recovery percentage between two non-venomous snakes of the same genus (*Elaphe quadrivirgata* and *Elaphe climacophora*) was observed. This behavior can be related to its dietary habits, since *E. quadrivirgata* may be ophiophagus, which would result in an indirect contact with venom. Consequently, this animal can have its inhibitors positively regulated [19]. Nevertheless, contact with venom may not be the only mechanism related to the presence of PLIs in snake's plasma.

The SDS-PAGE of Boa γ PLI after elution of affinity chromatography, showed a band at 25 kDa, under reducing conditions, and 20 kDa, under non-reducing conditions. Actually, data in the literature suggest a monomerization when inhibitors had contact with PLA₂ [40]. Thus, since affinity chromatography has crotoxin coupled in the resin, the contact of the D2 fraction with it probably monomerizes the inhibitor, explaining the result found in the electrophoresis (Fig 1, insert). Boa γ PLI had an anomalous migration under reducing conditions, in which it has a higher molecular mass than under non-reducing conditions. Molecular interactions of PLI, such as non-covalent bonds and disulfide bonds, might facilitate migration under non-reducing conditions. When the molecule is linearized, in turn, a slower migration occurs [40]. In addition, the same phenomenon was observed with γ BjPLI [30].

The partial amino acid sequence of Boa γ PLI has been identified (best MS/MS spectrum match shown in Fig 2), with 58% and 30% of coverage with the γ PLI subunit from *Lachesis muta* (P60591), 30% with PLI from *C. d. terrificus* (Q90358), 32% coverage with *Glodyus brevicaudus siniticus* (P82143) and 17% with *Protobothrops flavoridis* (O57690) and *Elaphe quadrivirgata* (Q9PW14), the last being a non-venomous snake (Table 2), reinforcing the classification of the Boa γ PLI as a γ PLI.

When the inhibitor was identified by mass spectrometry, the peptides identified presented similarities with 6 previously described PLIs and it is possible to observe that the identified sequence of Boa γ PLI is well conserved compared to other PLIs (Table 2). In addition, the γ PLI sequence of *Lachesis muta* was identified through its transcript [41], having its signal peptide of 19 amino acids, which is absent in a purified plasma protein. Disregarding the signal peptide, the coverage increases to 64%.

The oligopeptide ¹⁰⁴QPFPGPLSRPNGYY¹¹⁸ was suggested as a site of interaction between γ PLIs and PLA₂s in *Bothrops sp* [42] and this amino acid sequence was partially identified in Boa γ PLI (part of this sequence shown in Fig 2), which reinforces the conserved primary structure of these inhibitors [30,40]. Some authors also suggest that there may be three phosphorylation sites, at position ²¹S, ²²S and ¹¹¹T, and that these sites may be involved in other physiological roles, beyond the inactivation with PLA₂, which would reinforce the hypothesis about their presence in plasma of non-venomous snakes. So far, no other role has been assigned to PLIs, and these phosphorylation sites were not found in Boa γ PLI partial sequence [30,40,42].

The size-exclusion chromatography showed two peaks corresponding to oligomer and monomer, respectively (Fig 3A). This result is corroborated by the γ PLI homologue from *Python sabaes*, that also showed oligomers and monomers in its spectrum [43]. On the other hand, γ BjussuMIP [40] showed only the oligomer, as well as the purified γ PLI of

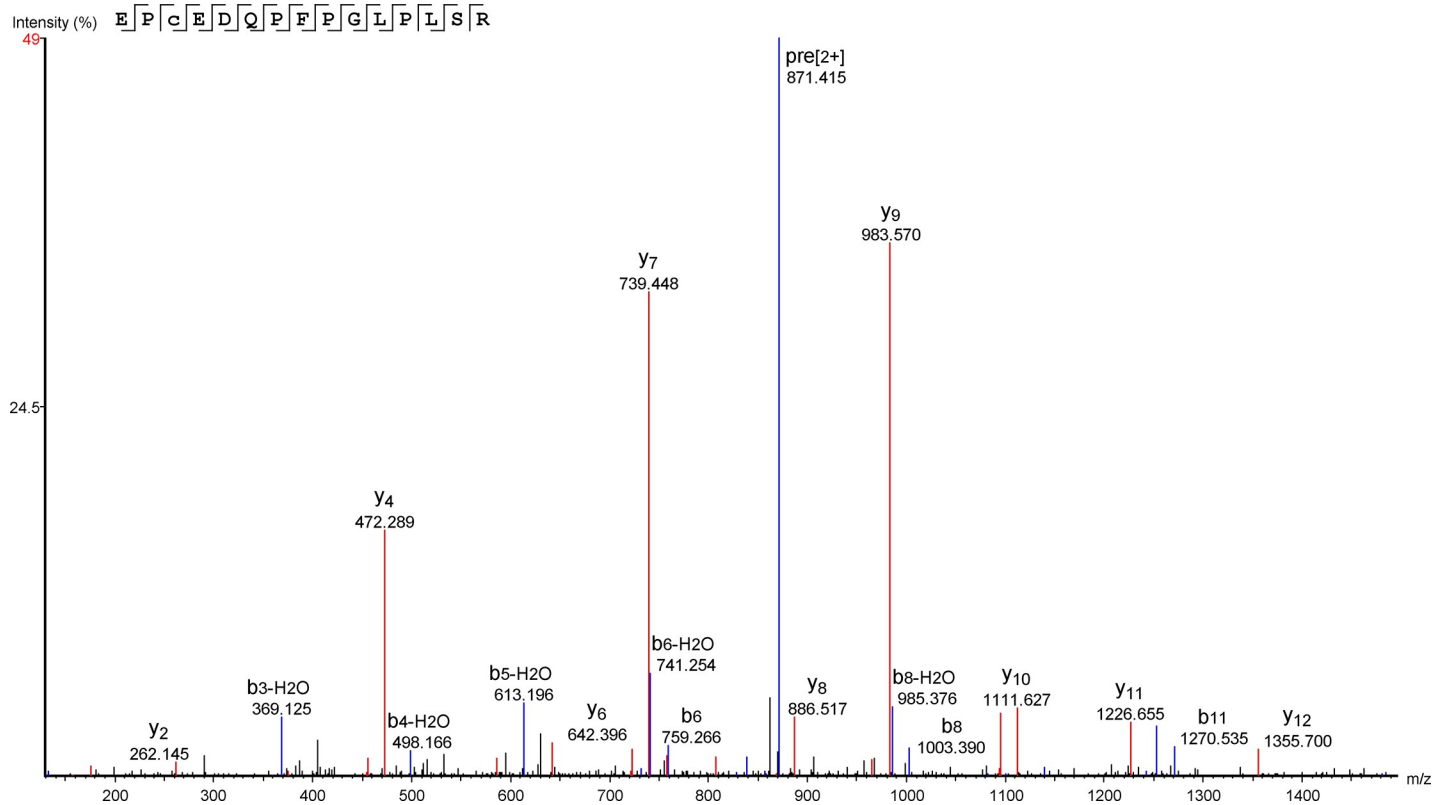


Fig 2. MS/MS spectrum of the BoayPLI peptide. Annotated MS/MS spectrum of the tryptic peptide EPCEDQPFGLPLSR from the BoayPLI. The peptide was detected at m/z 871.42⁺² and identified by database search in PEAKS Studio 7.5.

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Macropisthodon rudis [20,40]. Our group has found only the monomeric form of γ BjPLI. Moreover, the interaction of γ BjPLI with Asp-49 PLA₂ was also evidenced in this work [30].

BoayPLI secondary structure was investigated by circular dichroism (Fig 3B), presenting 21.4% of alpha-helices, 39.4% of beta-sheets and 38.7% of random coils. Besides that, incubation of BoayPLI with Asp-49 and Lys-49 PLA₂s does not significantly alter the spectrum shown in circular dichroism, which was also evidenced in γ CdcPLI and γ BjussuMIP [38,40]. This observation suggests a weak interaction of BoayPLI with PLA₂, most likely non-covalently. The PLI of *Macropisthodon rudis* also showed non-covalent binding characteristics evidenced by SDS-PAGE when incubated with PLA₂ under non-reducing conditions [20].

Once the possible interaction between BoayPLI and PLA₂s was evidenced, an inhibition assay was performed (Fig 4A), resulting in a dose-dependent inhibition, reaching 48.7% when

Table 2. Coverage of the peptides from BoayPLI. Identification of the proteins with a higher coverage from the peptides obtained from SDS-PAGE band excised and analyzed by mass spectrometry.

Access number	Coverage (%)	Peptides	Mass (Da)	Description
P60592	58	30	22207	Phospholipase A ₂ inhibitor LNF2 OS = <i>Lachesis muta muta</i>
P60591	30	12	22235	Phospholipase A ₂ inhibitor LNF1 OS = <i>Lachesis muta muta</i>
Q90358	30	12	22267	Phospholipase A ₂ inhibitor CNF OS = <i>C. durissus terrificus</i>
P82143	32	11	22232	Phospholipase A ₂ inhibitor subunit γ B OS = <i>G. b.siniticus</i>
O57690	17	9	22395	Phospholipase A ₂ inhibitor 1 OS = <i>Protobothrops flavoviridis</i>
Q9PW14	17	7	22547	Phospholipase A ₂ inhibitor subunit γ A OS = <i>E. quadrivirgata</i>

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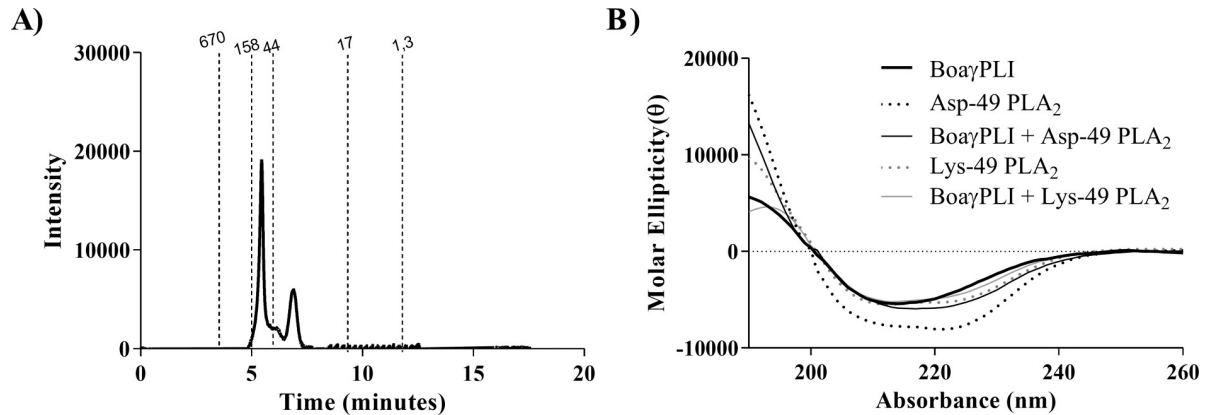


Fig 3. Structural analysis of the BoγPLI. A) Chromatographic profile on BioSep SEC S-2000 size-exclusion chromatography of BoγPLI (20 μg), at a flow of 1 mL / min, monitored by 280 nm. B) Spectra of Asp-49 and Lys-49 PLA₂s isolated, BoγPLI isolated and PLA₂s + BoγPLI incubated (30 μg) obtained by circular dichroism. The data were expressed in molar ellipticity.

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40 μg of inhibitor were used, values similar to found for γBjPLI [30]. In contrast, a γPLI inhibitor from *Python sabaes* showed a low enzymatic activity inhibition of PLA₂ from bee venom. In turn, PIP, the γPLI isolated from *Python reticulatus*, showed a high inhibition at 1:1 molar ratio, reaching 90%, measured by egg yolk acidimetric method, with PLA₂ of *D. r. russelli* [37].

The inhibitory potential of BoγPLI was also verified considering edematogenic and myotoxic effects of Asp-49 and Lys-49 PLA₂s. Wherein, when incubated with the inhibitor, edema was significantly lower compared to isolated PLA₂s. In order to highlight the differences between the curves of treatments, the area under the curve was estimated. As expected, edema profile induced by isolated PLA₂s resulted in a higher area under the curve when compared to the groups that received PLA₂s pre-incubated with BoγPLI (Control: 58.66; BoγPLI: 43.51; Asp-49 PLA₂: 206.0; BoγPLI + Asp-49 PLA₂: 134.8; Lys-49 PLA₂: 126.5; BoγPLI + Lys-49 PLA₂: 105.7). (Fig 4B). Creatine kinase levels, which reflects myotoxicity, were also reduced when PLA₂s were incubated with the inhibitor (Fig 4C). Other γPLIs from venomous and non-venomous snakes also demonstrated the inhibitory potential in the pharmacological activities caused by PLA₂ [30,38,40]. In addition, our data showed no statistical differences of

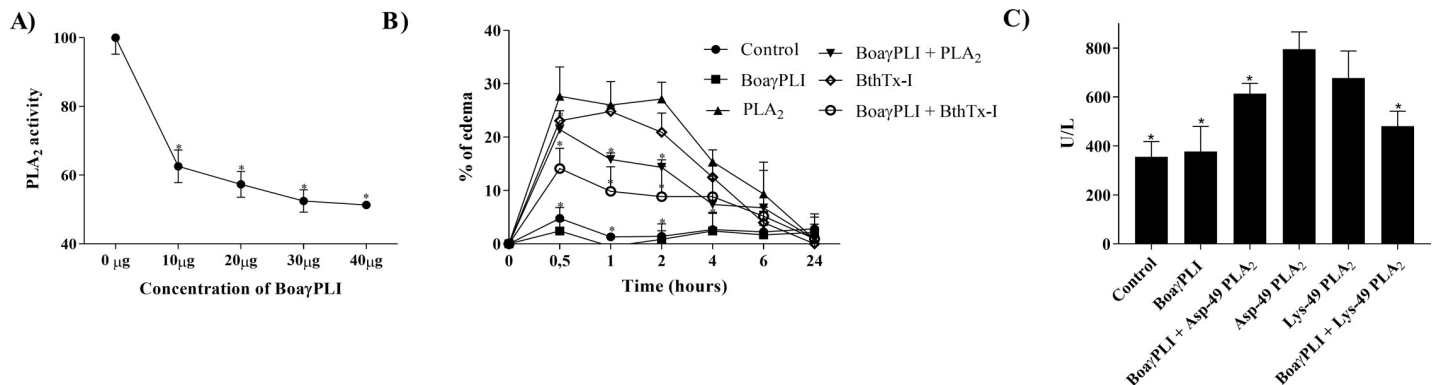


Fig 4. Inhibitory potential of the BoγPLI. A) Enzymatic activity of Asp-49 PLA₂ incubated with different concentrations (0 μg, 10 μg, 20 μg and 40 μg) of BoγPLI with NOB as a substrate. * represents statistical differences when compared to 0 μg of inhibitor. B) Edematogenic activity of Asp-49 and Lys-49 PLA₂ isolated and when previously incubated for 30 min with 20 μg of BoγPLI, injected into the right sub plantar region of mice paw (Swiss). C) Myotoxic activity of Asp-49 and Lys-49 PLA₂ isolated and when previously incubated for 30 min with 20 μg of BoγPLI, injected in the *gastrocnemius* muscle of mice (Swiss). * represents statistical differences when compared to PLA₂s.

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the inhibition potential of Boa γ PLI against Asp-49 and Lys-49 PLA₂s, unlike γ BjussuMIP, which possess higher affinity for Asp-49 PLA₂ [40]. An interesting point of the study is that, even being a non-venomous snake, the functional and structural characteristics of Boa γ PLI were well conserved when compared with γ PLIs from venomous snakes.

4. Concluding remarks

In summary, the study showed the purification of a γ PLI of the non-venomous snake *Boa constrictor* (Boa γ PLI), which has a molecular mass of approximately 22 kDa, oligomerization capacity, and a primary structure similar to the PLI of *Lachesis muta*. The direct interaction of Boa γ PLI with Asp-49 PLA₂ of *C. d. terrificus* and Lys-49 PLA₂ from *Bothrops jararacussu* was evidenced by circular dichroism, and the molecule displayed inhibition upon enzymatic, edematogenic and myotoxic activities of PLA₂s.

An interesting point of this study is that our data add another piece of evidence pointing the wide distribution of these inhibitors, that appears in venomous and non-venomous snakes, and, beyond that, their structure and inhibitory activity seems to be well conserved between them. That evidence amplifies the primary hypothesis for the PLI presence, that was the protection against the self-envenomation. Therefore, the inhibitor is not restricted to such function.

In addition, since γ PLIs inhibit PLA₂s from IIA group, which includes proinflammatory PLA₂s from mammalian and from viperids, the isolation and characterization of distinct γ PLIs may contribute to the enrichment of information for the bioprospection that can be useful, not only in antivenom therapy, but also in other inflammatory processes triggered by human PLA₂s, whereas their catalytic site is well conserved [8,42]. In this context, the present study may contribute to the elucidation of the presence of PLA₂ inhibitors in non-venomous snakes and to provide new perspectives for PLA₂ inhibitors from snake plasma by characterizing and comparing the similarities and differences between PLI from venomous and non-venomous snakes.

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