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Purification of Phospholipases A₂ from American Snake Venoms

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1. Introduction

Snake venoms are a complex mixture of compounds with a wide range of biological and pharmacological activities, which more than 90% of their dry weight is composed by proteins, comprising a variety of enzymes, such as proteases (metalo and serine), phospholipases A₂, L-aminoacid oxidases, esterases, and others [1-5]. A great number of proteins were purified and characterized from snake venoms [1, 2]. Some of these proteins exhibit enzymatic activity, while many others are non-enzymatic proteins and peptides. Based on their structures, they can be grouped into a small number of super-families based on remarkable similarities in their primary, secondary and tertiary structures, however showing distinct pharmacologic effects [3].

One of the most important protein super-families present in snake venoms are the phospholipases A₂ (PLA₂, E.C. 3.1.1.4), a class of heat-stable and highly homologous enzymes, which catalyse the hydrolysis of the 2-acyl bond of cell membrane phospholipids releasing arachidonic acid and lysophospholipids (Figure 1). These proteins are found in a wide range of cells, tissues and biological fluids, such as macrophages, platelets, spleen, smooth muscle, placenta, synovial fluid, inflammatory exudate and animal venoms. There is a high medical and scientific interest in these enzymes due to their involvement in a variety of inflammatory diseases and accidents caused by venomous animals. Since the first PLA₂ activity was observed in Naja snake venom, PLA₂s were characterized as the major component of snake venoms, being responsible for several pathophysiological effects caused by snake envenomation, such as neurotoxic, cardiotoxic, myotoxic, cytotoxic, hypotensive and anti-coagulant activities [1-10].

Phospholipases constitute a diverse subgroup of lipolytic enzymes that share the ability to hydrolyse one or more ester linkages in phospholipids, with phosphodiesterase as well as acyl hydrolase activity. The amphipathic nature of phospholipids creates obstacles for the enzymes, as the substrates are assembled into bilayers or micelles and are not present in significant amounts as a single soluble substrate [11]. According to Waite [12], all phospholipases target phospholipids as substrates, they vary in the site of action on the phospholipid molecule, their function and mode of action, and their regulation. Phospholipases function in various roles, ranging from the digestion of nutrients to the formation of bioactive molecules. This diversity of function suggests that phospholipases are relevant for life; the continuous remodelling of cell membranes requires the action of one or more phospholipases. The most common phospholipids in mammalian cells are phosphatidylcholine (PC), phosphatidylserine (PS), phosphatidylinositol (PI) and phosphatidylethanolamine (PE). The plasma membrane of most eukaryotic cells contains predominantly PC and sphingomyelin in the outer leaflet, and PI, PE and PS in the inner leaflet [11].

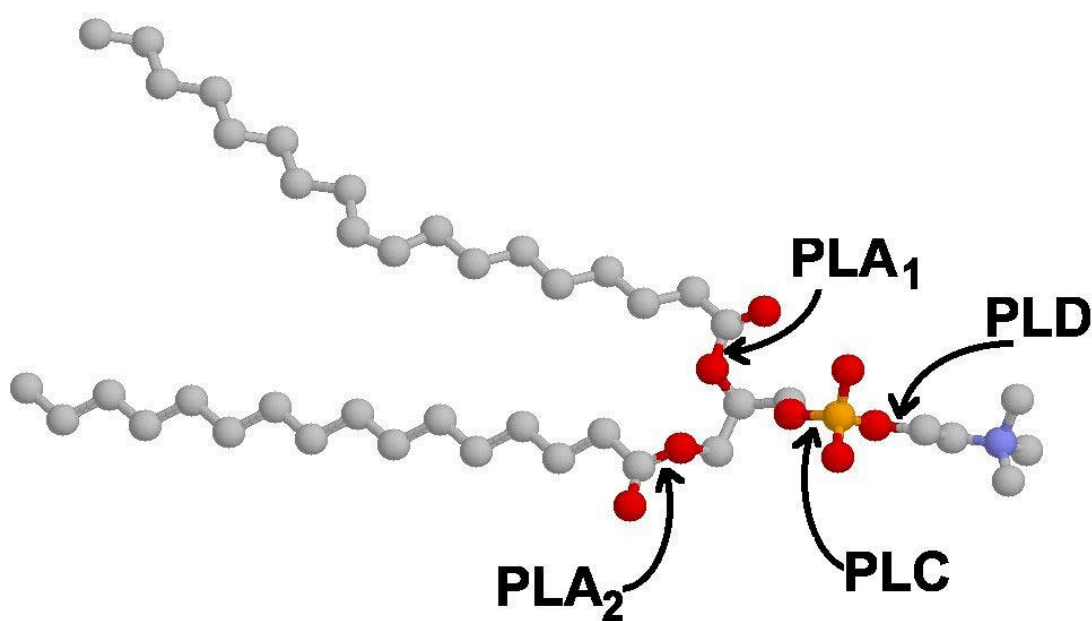


Figure 1. Phospholipase hydrolysis specificity sites in a 1,2-diacylglycerolphospholipid molecule (structure design from the ACD/I Lab. via Chem. Sketch – Freeware Version 1994 – 2009 software).

Phospholipases are classified according to their site of action in the phospholipid molecule. Thus, a phospholipase A₁ (PLA₁) hydrolyzes the 1-acyl group of a phospholipid, the bond between the fatty acid and the glycerine residue at the 1-position of the phospholipid. A phospholipase A₂ (PLA₂) hydrolyzes the 2-acyl, or central acyl, group and phospholipases C (PLC) and D (PLD), which are also known as phosphodiesterases, cleave on different sides of the phosphodiester linkage (Figure 1). The hydrolysis of a phospholipid by a PLA₁ or a PLA₂ results in the production of a lysophospholipid. The phospholipase metabolites are involved in diverse cellular processes including signal transduction, host defense (including antibacterial effects), formation of platelet activating cofactor, membrane remodeling and general lipid metabolism [12-14].

According to the latest classification [6], these proteins constitute a superfamily of different enzymes belonging to 15 groups and their subgroups including five distinct types of enzymes: the ones called secreted PLA₂ (sPLA₂), the cytosolic (cPLA₂), the Ca²⁺ independent (iPLA₂), the acetyl-hydrolases from platelet activating factors (PAF-AH) and the liposomal. The classification system groups these enzymes considering characteristics such as their origin, amino acid sequence and catalytic mechanisms, among others.

The sPLA₂s have a Mr. varying from 13,000 to 18,000, usually containing from 5 to 8 disulphide bond. They are enzymes that have a histidine in the active site and require the presence of the Ca²⁺ ion for the catalysis. Phospholipases A₂ from the IA, IB, IIA, IIB, IIC, IID, IIE, IIF, III, V, IX, X, XIA, XIB, XII, XIII, XIV groups are representative of the sPLA₂s. The cPLA₂s are proteins with Mr between 61,000 to 114,000 that also use a serine in the catalytic site (groups IVA, IVB, IVC, IVD, IVE, IVF). The iPLA₂s are enzymes which also use a serine for catalysis (groups VIA-1, VIA-2, VIB, VIC, VID, VIE, VIF). The PAF-AH are phospholipases A₂ with serine in the catalytic site that hydrolyze the acetyl group from the *sn*-2 position of the platelet activating factors (PAF), whose representative groups are VIIA, VIIB, VIIIA, VIIB. The liposomal PLA₂s are assembled in group XV and are enzymes with an optimum pH close to 4.5 that have preserved histidine and aspartate residues, suggesting the presence of the catalytic triad Ser/His/Asp and also a supposed sequence N-terminal sign and N-bond glycosylation sites [6].

With the discovery of a great variety of phospholipase A₂ in the last decade and the present expansion of the research in the area, more PLA₂s should be discovered yet. Phospholipase A₂ found in snake venoms (svPLA₂s) are classified into groups I and II. The phospholipase A₂ from group I have two to three amino acids inserted in the 52-65 regions, called "elapid loop", being isolated from the snake venoms of the Elapidae family (subfamily: Elapinae and Hydrophiinae). The ones from group II are characterized by the lack of the Cys11-Cys77 bond which is substituted by a disulphide bond between the Cys51-Cys133, and besides that had five to seven amino acids extending the C-terminal regions, being found in snake venoms of the Viperidae family (subfamily Viperinae and Crotalinae) [15,16].

The myotoxic PLA₂s of the IIA class have been subdivided in two main groups: The Asp49, catalytically active; and the Lys49, catalytically inactive. The essential co-factor for the phospholipase A₂ catalysis is Ca²⁺. The phospholipase A₂ Asp49 require calcium to stabilize the catalytic conformation, presenting a calcium binding site that is constituted by the β-carboxylic group of Asp49 and the C=O carbonylic groups of the Tyr28, Gly30 and Gly32. The presence of two water molecules structurally preserved complete the coordination sphere of Ca²⁺ forming a pentagonal pyramid [9,15].

The catalytic mechanism of the PLA₂-phospholipid involves the nucleophilic attack of a water molecule to the *sn*-2 bond of the phospholipid substrate (Figure 2). In the proposed model, the proton from position 3 of the imidazole ring of the His48 residue involved in a strong interaction with the carboxylate group of the Asp49 prevents the imidazole ring rotation to occur and keeps the nitrogen at position 1 of this ring, in an appropriate special position. A water molecule then promotes the nucleophilic attack to the carbon of the ester group of the substrate and, at this moment, the imidazole ring of the His48 receives a proton

from the water molecule, favoring the reaction. Subsequently to the acyl-ester bond hydrolysis at the sn-2 position of the phospholipid, this proton is donated by the imidazole ring to the oxygen, which then forms the alcohol group of the lysophospholipid to be released together with the fatty acid [15,17].

The Ca^{2+} ion, coordinated by the Asp49 residue, a water molecule and the oxygen atoms from the Gly30, Trp31 and Gly32 (not shown), are responsible for the stabilization of the reactive intermediary [15].

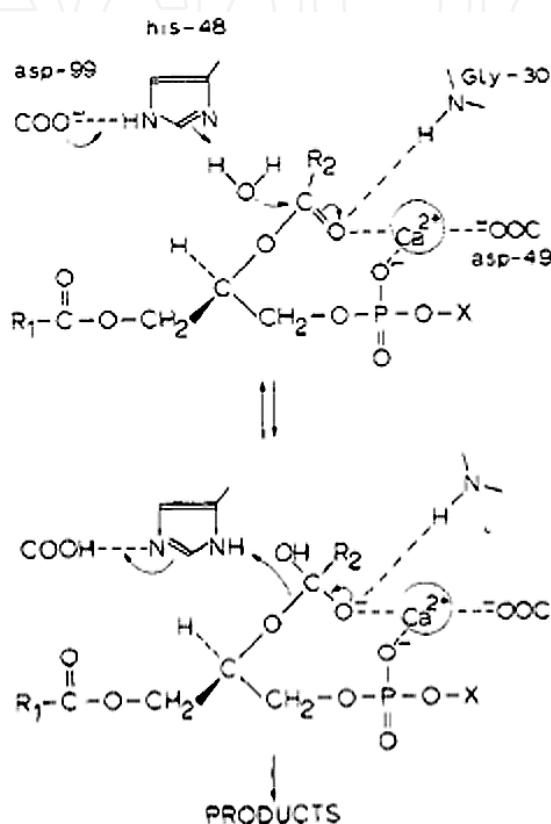


Figure 2. Schematic representation of the catalysis mechanism proposed for the PLA2s. Interaction of the residues from the catalytic site of sPLA2s and the calcium ion with the transition state of the catalytic reaction in which a water molecule polarized by the His48 and Asp99 residues binds to the carbonyl group of the substrate [18].

The substitution of the Asp49 residue by the Lys49 significantly alters the binding site of Ca^{2+} in the phospholipase A₂, preventing its binding and resulting in low or inexistent catalytic activity. Thus, the Asp49 residue is of fundamental importance for the catalytic mechanism of the phospholipase A₂. It is likely that this occurs due to its capability of binding and orienting the calcium ion, however, there is no relevant difference between Asp49 and Lys49 in relation to the structural conformation stability of these enzymes [9,15,19].

The absence of catalytic activity does not affect myotoxicity. Most snake PLA2s from the *Bothrops* genus already described are basic proteins, with isoelectric point between 7 to 10, showing the presence or absence of catalytic, myotoxic, edematogenic and anticoagulating activities [9,20].

On the other hand, acid PLA₂s present in Bothrops snake venoms were not studied as well as basic PLA₂s, resulting in little knowledge regarding the action mechanism of these enzymes [21-25].

PLA₂s catalytic activity represents a key role in envenomation pathophysiology, however, recent studies have shown that some effects are independent of PLA₂s catalytic activity, such as myotoxicity [19,26]. The absence of a tight correlation between PLA₂ catalytic and non-catalytic activities, together with the diversity of biological effects produced by these proteins increases the scientific interest in the understanding of the structural basis of PLA₂ mechanisms of action.

Evidences suggest that these activities can be mediated by interactions between PLA₂s and endogen acceptors on the target cell membrane [27-29].

2. PLA₂ purification

Snake venom components, obtained with high degree of purity, could be used for the understanding of the role of these components in the physiopathological processes resulted from poisoning, as well as biotechnological/nanotechnological applications. Hence, many purified PLA₂s from snake venoms, as well as epitopes of these molecules, are being mapped in order to identify determinants responsible for the deleterious actions seen, as well as possible applications in biotechnological models.

New advances in materials and equipments have contributed with protein purification processes, allowing the obtaining of samples with high degree of purity and quantity. These advances have allowed process optimization, providing reduction of steps, reagents use and thus avoiding the unnecessary exposure to agents that may, in some way, alter the sample's functionality or physical-chemical stability.

Thus, the selection of adequate techniques and chromatographic methods oriented by physical chemical properties and biological/functional characteristics, are of fundamental importance to obtain satisfactory results. The information pertinent to protein structure, such as the homology to others already purified, should be taken into consideration and could make the purification processes easier.

Ion exchange chromatography was introduced in 1930 [30] and still one of the main techniques used for protein purification. It has been extensively used in single step processes as well as associated to other chromatographic techniques. Ion exchange chromatography allows the separation of proteins based on their charge due to amino acid composition that are ionized as a function of pH.

Proteins with positive net charge, in a certain pH (bellow their isoelectric point), can be separated with the use of a cation exchange resin and on the other hand, proteins with negative net charge in a pH value above their isoelectric point, can be separated with an anion exchange resin.

Scientific publications have shown that the use of cation-exchange resins is a very efficient method to obtain PLA₂s from bothropic venoms, particularly those with alkaline pH (Table 1). The versatility of this technique can be observed in the work done by Andriao-Escarso et al. [21] who compared the fractioning of many bothropic venoms. In this work, the venoms were fractioned in a column containing CM-Sepharose® (2 x 20 cm), equilibrated with ammonium bicarbonate 50 mM pH 8.0 and eluted with a saline gradient of 50 to 500 mM of the same reagent. Under these conditions, MjTX-I and MjTX-II from *B. moojeni* snake venom were co-purified (isoforms of PLA₂ with pIs of 8.1 and 8.2 values, respectively). The same occurs with *B. jararacussu* venom, where the BthTX-I and BthTX-II were purified. However, the most expressive result was observed with *B. pirajai* venom, from which 3 isoforms of myotoxins, called as PrTX-I (pI 8.50), PrTX-II (pI 9.03) and PrTX-III (pI 9.16) were purified. In the above cases, it is important to note that the protein elution occurs always following pIs increasing value. In our lab we used this technique routinely in order to isolate myotoxins from bothropic venoms, which can be observed in the chromatograms shown in Figure 3.

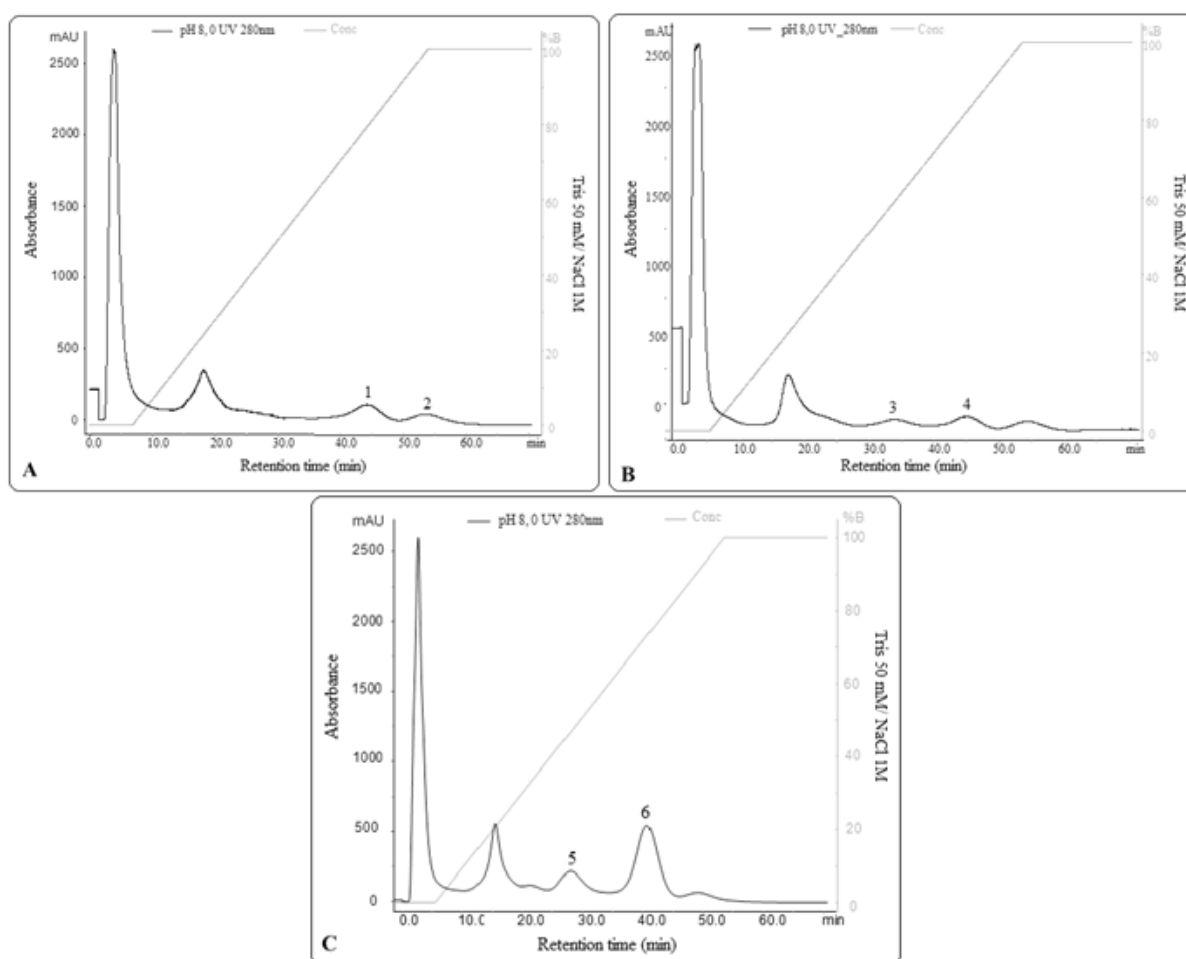


Figure 3. Chromatographic profile using CM-sepharose® Column 1ml (Hitrap) equilibrated with Tris 50 mM buffer (buffer A) and eluted with a linear gradient of Tris 50 mM/NaCl 1 M (buffer B) in pH 8.0. **A.** Chromatography of the crude venom from *Bothrops brazili* **B.** Chromatography of the crude venom from *Bothrops moojeni* **C.** Chromatography of the crude venom from *Bothrops jararacussu*. Absorbance read at 280 nm. ,2,3,4,5 and 6 marks indicate the fractions corresponding to the PLA₂s of each venom.

Species	PLA ₂	PLA ₂ Activity	MW (kDa)	pI	Access Number (Uniprot)	Purification strategy	Ref.
<i>Agkistrodon bilineatus</i>	PLA ₂	Absence	14.0	10.2	Q9PSF9	Gel filtration chromatography on Sephadex G-75® and then submitted to ion-exchange on CM-Cellulose® column.	[78]
<i>Agkistrodon contortrix contortrix</i>	PLA ₂	Presence	14.0			Ion-exchange chromatography on DEAE-Cellulose® column, followed by affinity chromatography with immobilized BSA and then submitted to gel filtration on Cellulofine GCL-2000® column.	[79]
<i>Agkistrodon contortrix laticinctus</i>	MTI	Absence	14.0	9.0	49121	Anion-exchange chromatography on Waters DEAE-5PW® column and then submitted to cation-exchange on Protein Pak SP-SPW® column.	[80]
<i>Agkistrodon contortrix laticinctus</i>	ACL-I	Presence	14.0			Gel filtration chromatography on Superdex-200® column and then submitted to ion-exchange on CM-Sepharose FF® column.	[81]
<i>Atropoides nummifer</i>	Myotoxin IH	Absence	16.0			Cation-exchange chromatography on CM-Sephadex C-25® column.	[82]
<i>Atropoides nummifer</i>	Myotoxin I	Absence	16.0			Cation-exchange chromatography on CM-Sephadex C-25® column.	[83]
<i>Atropoides nummifer</i>	Myotoxin II	Absence	13.7	8.7	P82950	Cation-exchange chromatography on CM-Sephadex C-25® column.	[84]
<i>Bothriechis (Bothrops) schlegelii</i>	Miotoxina II	Presence	15.0	>9.5	P80963	Ion-exchange chromatography on CM-Sephadex® column.	[85]
<i>Bothrocophias hyoprora</i>	PhTX-I	Presence	14.2			Reverse Phase chromatography on Bondapack® C-18 column.	[86]
<i>Bothropoides insularis</i>	SIII-SPVI	Presence	15.0			Gel filtration chromatography on Sephadex G-150® column and then submitted to SP-Sephadex C25® column.	[87]
<i>Bothropoides insularis</i>	BinTX-I BinTX-II	Presence Presence	13.9 13.7	5.0 4.4	Q8QG87 P84397	Reverse Phase chromatography on Vydac® C18 column.	[88]
<i>Bothropoides insularis</i>	Bi PLA ₂	Presence	13.9	8.6		Gel filtration chromatography on Superdex 75® column and then submitted to cation-exchange on Protein pack SP-5PW® column and Reverse Phase chromatography on μ -Bondapack® C18 column.	[89]
<i>Bothropoides jararaca</i>	BjPLA ₂	Presence	14.0		P81243	Ion-exchange chromatography on DEAE Sephacel® column and then submitted to Reverse Phase chromatography on Ultrapore RPRC-C3® column.	[90]
<i>Bothropoides jararaca</i>	PLA ₂	Presence	14.2	4.5	Q9PRZ0	Gel filtration on Sephacryl S-200® column and then submitted to reverse phase on Pep-RPC HR 5/5® column.	[91]
<i>Bothropoides pauloensis</i>	BpPLA ₂	Presence	15.8	4.3	D0UGJ0	Cation-exchange chromatography on CM-Sepharose® column followed by Phenyl-Sepharose CL-4B® column and then submitted to reverse phase chromatography on C8 column.	[23]
<i>Bothropoides pauloensis</i>	BnSP-7	Absence	13.7	8.9	Q9IAT9	Cation-exchange chromatography on CM-Sepharose® column or heparin agarose® column.	[26]
<i>Bothrops alternatus</i>	BA SpII RP4	Presence	14.1	4.8	P86456	Gel filtration chromatography Sephadex G-75® column followed by reverse phase chromatography on C18 column.	[92]
<i>Bothrops alternatus</i>	PLA ₂	Presence	15.0	5.0		Gel filtration chromatography on Sephadex G-50® column followed by ion-exchange on SP Sephadex C-50® column and then submitted to gel filtration chromatography on Sephadex G-75® column.	[93]
<i>Bothrops alternatus</i>	BaTX	Absence	13.8	8.6	P86453	Gel filtration chromatography on Superdex 75® column followed by reverse phase chromatography on μ -Bondapack® C18 column.	[94]
<i>Bothrops asper</i>	MTX-I MTX-II MTX-III MTX-IV Basp-I-PLA ₂	Presence Absence Presence Absence Presence	14.1 14.2 14.2 Nd 14.2	8.1- 8.3 8.1- 8.3 8.1- 8.3 8.1- 8.3 4.6		Ion-exchange chromatography on CM-Sepharose® column followed by hydrophobic interaction chromatography on Phenyl-Sepharose® column.	[95]
<i>Bothrops asper</i>	Myotoxin I	Presence	10.7	nd		Ion-exchange chromatography on CM-Sephadex C-25® column followed by gel filtration chromatography on Sephadex G-75® column.	[7]
<i>Bothrops asper</i>	Myotoxin II	Absence	13.3	nd	P24605	Ion-exchange chromatography on CM-Sephadex C-25® column.	[96]
<i>Bothrops asper</i>	Myotoxin III	Presence	13.9	>9.5	P20472	Ion-exchange chromatography on CM-Sephadex C-25® column.	[97]
<i>Bothrops asper</i>	Myotoxic PLA ₂	Presence	14.1	nd		Gel filtration chromatography on Sephadex G-75® followed by ion-exchange chromatography on CM-cellulose® column.	[98]
<i>Bothrops asper</i>	BaspPLA ₂ -II	Presence	14.2	4.9	P86389	Ion-exchange on CM-Sephadex C-25® followed by chromatography on DEAE Sepharose® column, active fractions subjected to reverse phase chromatography on C8 column and finally chromatography with CM-	[22]

8 Chromatography – The Most Versatile Method of Chemical Analysis

<i>Bothrops atrox</i>	BaPLA ₂ I BaPLA ₂ III	Presence Presence	15.0 15.0	9.1 6.9		Gel filtration chromatography on Sephacryl S-100 HR® column followed by reverse phase on C4 column.	[99]
<i>Bothrops atrox</i>	Basic Myotoxin	Presence	13.5			Ion-exchange chromatography on CM-Sephadex C-25® column and then re-chromatographed on the same column and same conditions.	[32]
<i>Bothrops atrox</i>	Myotoxin I	Absence	13.8	8.9	Q6JK69	Ion-exchange chromatography on Carboxymetil-Sephadex C-25® followed by reverse phase chromatography on C8 column.	[100]
<i>Bothrops brazili</i>	MTX-I MTX-II	Presence Absence	14.0 14.0			Ion-exchange chromatography on CM-Sephacryl® column.	[101]
<i>Bothrops brazili</i>	BbTX-II BbTX-III	Absence Presence	13.9 13.6	8.7 8.4		Reverse phase chromatography on C18 column.	[102]
<i>Bothrops erythromelas</i>	BE-I-PLA ₂	Presence	13.6			Gel filtration chromatography on Superdex 75® followed by chromatography on monoQ® column, fractions being subjected to reverse phase chromatography on C4 column afterwards.	[103]
<i>Bothrops jararacussu</i>	BthTX-I BthTX-II	Absence Presence	13.0 13.0	8.2	Q90249 P45881	Gel filtration chromatography on Sephadex G-75®, followed by cation-exchange chromatography on SP-Sephadex C-25® column.	[34]
<i>Bothrops jararacussu</i>	BJ IV	Presence	15.0		P0CAR8	Ion-exchange chromatography on Protein Pack SP 5PW® column followed by reverse phase chromatography on µ-Bondapack® C18 column.	[104]
<i>Bothrops jararacussu</i>	BthA-I-PLA ₂	Presence	13.7	4.5	Q8AXY1	Ion-exchange chromatography on CM-Sephacryl® column, followed by reverse phase chromatography on C18 column.	[58]
<i>Bothrops jararacussu</i>	SIISPIIA SIISPIIB SIISPIIIA SIISPIIIB	Presence Presence Presence Presence	15.0 15.0 15.0 15.0	5.3 5.3 5.3 5.3		Gel filtration chromatography on Sephadex G-75® column, followed by ion-exchange chromatography on SP-Sephadex C-25® column, and finally HPLC on C18 column.	[105]
<i>Bothrops lanceolatus</i>	PLA ₂ -1 PLA ₂ -2 PLA ₂ -3	Presence Presence Presence	15.0 13.0 18.0	5.3 5.3 5.3		Reverse phase chromatography on Lichrosfera RP100® C18 column.	[106]
<i>Bothrops leucurus</i>	BLK-PLA ₂ BLD-PLA ₂	Absence Presence	14.0 14.0		P86974 P86975	Gel filtration chromatography on Sephacryl S-200®, followed by ion-exchange on Q-Sepharose and then submitted to reverse phase chromatography on HPLC Vydac® C4.	[38]
<i>Bothrops leucurus</i>	BI-PLA ₂	Presence	15.0	5.4	P0DJ62	Ion-exchange chromatography on CM- Sepharose® column, followed by hydrophobic interaction chromatography on Phenyl-Sepharose® column.	[43]
<i>Bothrops marajoensis</i>	BmjeTX-I BmjeTX-II	Presence Presence	13.8 13.8		P86803 P86804	Ion-exchange chromatography on Protein Pack SP 5PW®, followed by reverse phase chromatography on µ-Bondapack® C18 column.	[107]
<i>Bothrops marajoensis</i>	BmarPLA ₂	Absence	14.0	nd	P0DI92	Ion-exchange chromatography on Protein Pack SP 5PW®, followed by reverse phase chromatography.	[108]
<i>Bothrops marajoensis</i>	Bmaj-9	Presence	13.7	8.5	B3A0N3	Reverse phase chromatography on µ-Bondapack® C18 column.	[109]
<i>Bothrops moojeni</i>	BthA-I	Presence	13.6	5.2	G3DT18	Ion-exchange on CM-Sephacryl® column, followed by hydrophobic interaction chromatography on Phenyl-Sepharose® column.	[24]
<i>Bothrops moojeni</i>	MjTX-III MjTX-IV	Absence Absence	14.6 14.6			Gel filtration chromatography on Superdex -75XK® column, followed by reverse phase chromatography on C18 column.	[110]
<i>Bothrops moojeni</i>	MjTX-I ou Miotoxina-I	Absence	13.4	8.2	P82114	Ion-exchange chromatography on CM-Sephacryl® column.	[26]
<i>Bothrops moojeni</i>	MjTX-II	Absence	14.0	8.2	Q9I834	Ion-exchange chromatography on CM-Sephacryl® column.	[111]
<i>Bothrops moojeni</i>	BmooTX-I	Presence	15.0	4.2		Ion-exchange on DEAE-Sepharose®, gel filtration on Sephadex G-75® column and hydrophobic interaction chromatography on Phenyl-Sepharose®.	[42]
<i>Bothrops moojeni</i>	BmTX-I	Presence	14.2	7.8	P0C8M1	Reverse phase chromatography on µ-Bondapack® C18 column.	[112]
<i>Bothrops moojeni</i>	BmooMtx	Absence	16.5			Ion-exchange chromatography on DEAE-Sepharose® column and then submitted to gel filtration on Sephadex G-75® column.	[113]
<i>Bothrops pirajai</i>	Piratoxin-I	Absence	13.8	8.3	P58399	Gel filtration chromatography on Sephadex G-75® column, followed by ion-exchange chromatography on Sephadex C25® column.	[114]
<i>Bothrops pirajai</i>	Piratoxin-III ou MPIII 4R	Presence	13.8		P58464	Ion-exchange chromatography on semi-preparative µ-Bondapack® column, followed by ion-exchange chromatography on Protein Pack SP 5PW® column.	[115]
<i>Bothrops pirajai</i>	Bpir-I PLA ₂	Presence	14.5		C9DPL5	Ion-exchange chromatography on CM- Sepharose FF® column, followed by reverse phase chromatography on C18 column.	[25]
<i>Bothrops pirajai</i>	Piratoxin -II	Absence	13.7	9.0	P82287	Gel filtration chromatography on Sephadex G-75® column and ion-exchange chromatography on Sephadex C25® column.	[116]

<i>Cerrophidion goodmani</i>	Myotoxin I Myotoxin II	Presence Absence	14.3 13.4	8.2 8.9		Ion-exchange chromatography on CM-Sephadex® column.	[117]
<i>Cerrophidion goodmani</i>	GodMT-II	Absence	13.7			Ion-exchange chromatography on CM-Sephadex® column.	[118]
<i>Cerrophidion goodmani</i>	Pgo K49	Absence	13.8			Gel filtration chromatography on Sephadex G-75 HR® column, followed by reverse phase chromatography on Vydac® C8 column.	[119]
<i>Crotalus atrox</i>	PLA ₂ -1 PLA ₂ -2	Absence Presence	15.3 15.5	4.6 8.6		Gel filtration chromatography on DEAE-cellulose® column.	[119]
<i>Crotalus atrox</i>	Cax-K49	Absence	13.8		Q81VZ7	Gel filtration chromatography on DEAE-cellulose® column.	[119]
<i>Crotalus durissus cascavella</i>	PLA ₂	Presence	15.0			Gel filtration chromatography (pharmacia), followed by reverse phase on μ-Bondapak® C-18 column.	[120]
<i>Crotalus durissus collilineatus</i>	F6a	Presence	14.9	5.8	P0CAS2	Reverse phase chromatography on μ-Bondapak® C18 column.	[121]
<i>Crotalus durissus cumanensis</i>	Cdc-9 Cdc-10	Presence Presence	14.1 14.2	8.25 8.4	P86805 P86806	Reverse phase chromatography on μ-Bondapak® C18 column.	[122]
<i>Crotalus durissus cumanensis</i>	Cdcum6	Presence	14.3	Nd	P0CAS1	Gel filtration chromatography followed by reverse phase chromatography.	[123]
<i>Crotalus durissus ruruima</i>	PLA2A	Presence	14.2		P86169	Gel filtration chromatography followed by reverse phase chromatography.	[124]
<i>Crotalus durissus ruruima</i>	Cdr-12 Cdr-13	Presence Presence	14.3 14.2	8.1 8.1	P0CAS3 P0CAS4	Reverse phase chromatography on μ-Bondapak® C18 column.	[121]
<i>Crotalus durissus terrificus</i>	CdtF16	Presence	14.8		P0CAS6	Gel filtration chromatography on Superdex 75® column, followed by reverse phase chromatography on μ-Bondapak® C18.	[125]
<i>Crotalus durissus terrificus</i>	Crotoxin B	Presence	14.5	5.1		Gel filtration chromatography on Sephadex G75® column, followed by chromatography on Mono-Q® and finally ion-exchange chromatography followed by DEAE-cellulose® column.	[126]
<i>Crotalus durissus terrificus</i>	CdtF17	Presence	14.6	8.15	P0CAS7	Reverse phase chromatography on μ-Bondapak® C-18 column.	[127]
<i>Crotalus durissus terrificus</i>	CdtF15	Presence	14.5	8.8	P0CAS5	Gel filtration chromatography on Superdex 75® column followed by reverse phase chromatography on μ-Bondapak® C-18 column.	[128]
<i>Crotalus scutulatus scutulatus</i>	MTX-a MTX-b		14.5 14.4	9.2 7.4	P18998 P62023	Reverse phase on Vydac® C8 column.	[129]
<i>Lachesis muta</i>	LmTX-I LmTX-II	Presence Presence	14.2 14.1	8.7 8.6	P0C942 P0C943	Gel filtration chromatography on Superdex 75® column, followed by reverse phase chromatography on μ-Bondapak® C-18 column and finally reverse phase chromatography on C8 column.	[130]
<i>Lachesis muta</i>	LM-PLA ₂ -I LM-PLA ₂ -II	Presence Presence	17.0 18.0	4.7 5.4	P0C932 P0C933	Gel filtration chromatography on Sephacryl S-200® column, followed by reverse phase chromatography on C2 column and finally reverse phase chromatography on C18 column.	[131]
<i>Lachesis stenophrys</i>	LSPA-1	Presence	13.8	nd	P84651	Gel filtration chromatography on Sephacryl S-200® column followed by ion-exchange chromatography using MonoQ HR 5/5® column and finally reverse chromatography on Sephasil® C-18 column.	[132]
<i>Porthidium nasutum</i>	PnPLA ₂	Presence	15.8	4.6		Reverse phase chromatography on C18 column.	[133]
<i>Micrurus tener tener</i>	MitTx-beta	Presence	16.7		G9I930	Reverse phase chromatography on C18 Vydac® column followed by reverse phase on Vydac® C18 column.	[134]
<i>Micrurus tener microgalbineus</i>	PLA ₂ -1	Presence			P25072	Gel filtration chromatography on Sephadex G-50® followed by ion-exchange chromatography on CM-cellulose column.	[135]
<i>Micrurus pyrrhocryptus</i>	PLA ₂ A1 PLA ₂ B1 PLA ₂ D5 PLA ₂ D6	Presence Presence Presence Presence			P0CAS8 P0CAS9 P0CAT0 P0CAT1	Gel filtration chromatography on Superdex G 75 HR® followed by reverse phase chromatography on Vydac® C18 column.	[136]
<i>Micrurus nigrocinctus</i>	Nigroxin A Nigroxin B	Presence Presence			P81166 P81167	Ion-exchange chromatography on Mono Q FF® column followed by reverse phase chromatography on Vydac® C4 column.	[137]
<i>Micrurus nigrocinctus</i>	PLA ₂ -1 PLA ₂ -2 PLA ₂ -3	Presence Presence Presence			P21790 P21791 P21792	Gel filtration chromatography on Sephadex G-75® column followed by ion-exchange chromatography on CM-cellulose® column.	[138]
<i>Micrurus dumerilli carinicauda</i>	MiDCA1	Presence	15.5	8.0		Reverse phase chromatography on Sephasil Peptide® C18 column followed by reverse phase chromatography on μ-Bondapak® C18 column.	[139]

Table 1. PLA₂s isolated from American snake venoms and respective chromatographic methods used.

Some authors have proposed changes to the methodology described above. Spencer et al. [31] described the purification of BthTX-I with the use of Resource S® (methyl-sulphonate

functional group), equilibrated in pH 7.8 (phosphate buffer 25 mM). Sample elution was done in increasing ionic strength conditions (NaCl 0 to 2 M), under 2.5 ml/min flow. In this model, the BthTX-I was eluted in NaCl 0.42M with a high degree of purity. However, the chromatographic profile in the conditions tested differs significantly from the observed in other works that describe the fractioning of this venom. This difference is due to the resin composition. This is corroborated with data obtained in experiments performed in our lab, where the effect of pH in the separation of myotoxin isoforms from *B. jararacussu* venom was used, as shown in Figures 4. SDS-PAGE showed that fractions corresponding to myotoxins showed protein bands with apparent molecular mass compatible with PLA₂s class II (Figure 5).

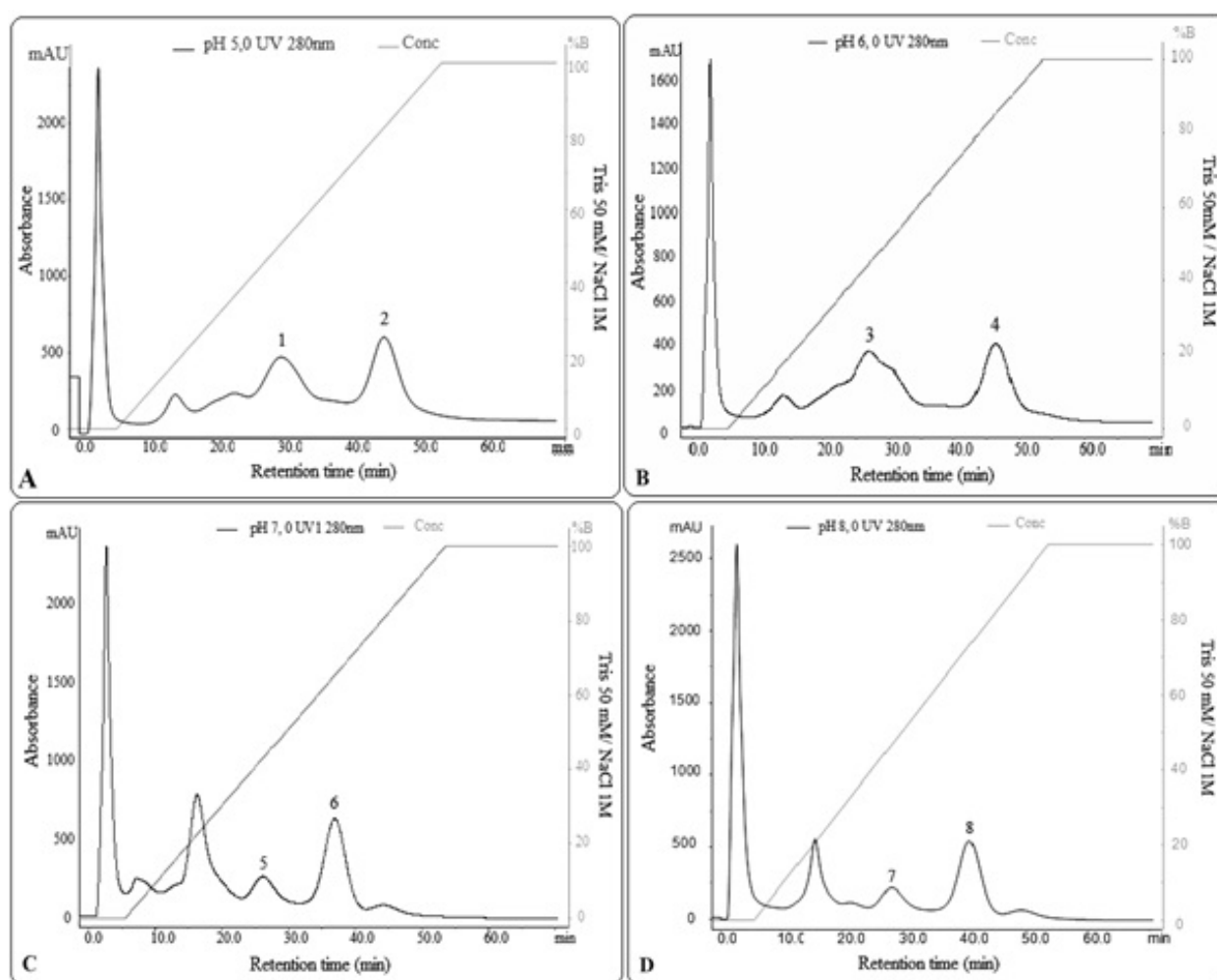


Figure 4. Chromatographic profile of the *B. jararacussu* venom in CM-sepharose® column 1 ml (Hitrap) equilibrated with Tris 50 mM buffer (buffer A) and eluted with a linear gradient of Tris 50 mM/NaCl 1M (buffer B) in different pH conditions. **A.** pH 5.0 **B.** pH 6.0 **C.** pH 7.0 **D.** pH 8.0. Absorbance was read at 280 nm. Fractions numbered (1 to 8) indicate the fractions selected for SDS-PAGE analysis in order to confirm the presence of PLA₂s (BthTx I e BthTx II).

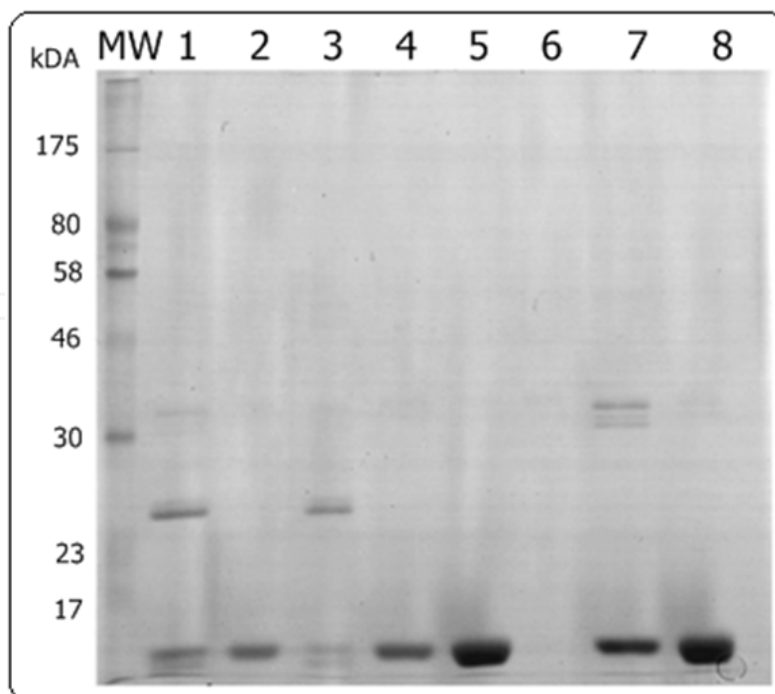


Figure 5. SDS Page analysis. Lines 1 and 2 (pH 5.0); 3 and 4 (pH 6.0); 5 and 6 (pH 7.0); 7 and 8 (pH 8.0). BthTx I was obtained in high degree of purity with pHs 5.0, 6.0, and 8.0. BthTx II was obtained with pH 7.0.

Resolution differences were also observed by other authors. As performed by Lomonte et al. [26], the isolation of two basic myotoxins, MjTX-I e MjTX-II, from the *B. moojeni* venom was obtained using CM-Sephadex C-25 equilibrated with Tris-HCl 50 mM pH 7.0 and eluted in saline gradient up to 0.75 M of Tris-HCl. Also, Soares et al. [33] described the isolation of MjTX-II with high purity using the combination of CM-Sepharose resin and ammonium bicarbonate buffer. According to the authors, the increase of pH to 8.0 has favored the elution of several fractions, allowing MjTX-II to be eluted separately with ionic strength equal to 0.35 M of ammonium bicarbonate. Moreover, the use of CM-Sepharose® seems to have also contributed a lot in the increasing of resolution for this chromatographic separation.

The combination of chromatographic techniques has also been used to purify these toxins. The association of the Ion-exchange chromatography and molecular exclusion has been one of the most recurrent in isolation and purification of phospholipases from bothropic venoms. Gel filtration chromatography is a technique based in particle size to obtain the separation. In this type of separation there is no physical or chemical interaction between the molecules of the analyte and the stationary phase, being currently used for separation of molecules with high molecular mass. The sample is introduced in a column, filled with a matrix constituted by small sized silica particles (5 to 10 μm) or a polymer containing a uniform net pores of which solvent and solute molecules diffuse. The retention time in the column depends on the effective size of the analyte molecules, the higher sized being the first ones to be eluted. Different from the higher molecules, the smaller penetrate the pores being retained and eluted later. Between the higher and lower molecules, there are the

intermediary sized molecules, whose penetration capacity in the pores depends on their diameter. In addition to that, this technique has also some very important characteristics, such as operational simplicity, physical chemical stability, inertia (absence of reactivity and adsorptive properties) and versatility, since it allows the separation of small molecules with mass under 100 Da as well as extremely big molecules with various kDa.

The work performed by Homsí-Brandeburgo et al. [34] is an example of combination of different chromatographic techniques for the isolation of myotoxins with PLA₂ structure. It describes for the first time the BthTX-I purification using the combination of molecular exclusion chromatography in Sephadex G-75® resin followed by Ionic exchange chromatography in SP-Sephadex C-25®. In the first step, four fractions were obtained, called S_I, S_{II}, S_{III} and S_{IV}. The Functional analysis of these fractions showed that the proteolytic activity over casein and fibrinogen was detected on fraction S_I, while the phospholipase activity was concentrated in fraction S_{III}. The apparent molecular mass profile of this fraction showed that it was composed by proteins between 12,900 and 28,800 Da, compatible with the mass profile of the class II PLA₂s.

On the second step, S_{III} fraction was submitted to ionic exchange chromatography and five fractions were obtained, identified as S_{III}SP_I to S_{III}SP_{IV}. The pIs and apparent molecular mass evaluation showed the following profile: S_{III}SP_I (pI 4.2 and 22.400 Da), S_{III}SP_{II} (pI 4.8 and 15.500 Da), S_{III}SP_{III} (pI 6.9 and dimeric structure, each monomer with a molecular mass of 13.900 Da), S_{III}SP_{IV} (pI 7.7 and 13.200 Da) e S_{III}SP_V called BthTX-I that presented pI 8,2 and 12.880 Da. Pereira et al. [35] obtained the complete sequence of BthTX-II, a myotoxin homologous to the BthTX-I, which corresponds to the S_{III}SP_{IV} fraction described by Homsí-Brandeburgo et al. [34].

Another chromatographic technique regularly used in PLA₂s purification procedures is the Reverse-phase associated with High performance liquid chromatography (RP- HPLC). This technique is characterized by its high resolution capacity and is normally used in a more refined step of the purification process, being very useful in separating isoforms. The retention principle of reverse-phase chromatography is based in hydrophobicity and is mainly due to the interactions between hydrophobic domains of the proteins and the stationary phase. This technique has many advantages, such as: use of less toxic mobile phases together with lower costs, such as methanol and water; stable stationary phases; fast column equilibrium after mobile phase change; easy to use gradient elution; faster analysis and good reproducibility.

Rodrigues et al. [36] described the isolation of two PLA₂s isoforms from the *B. neuwiedi pauloensis* venom using the combination of ion (cation) exchange chromatography and molecular exclusion setting up a preparative phase. Subsequently, a reverse-phase chromatography was used for the analytical phase of the procedure. Initially, the venom was fractioned in a column containing CM-Sepharose® equilibrated with ammonium acetate solution 0.05 M, pH 5.5 and eluted in linear gradient up to 1 M of the same buffer, resulting in six fractions. The pH, more acid than the ones used in the work previously mentioned, has increased the surface residual charge, intensifying the interaction force

between the protein and the resin, thus altering the elution profile when compared to the performed by Rodrigues et al. [37]. Proceeding with purification, the sample with phospholipase activity (S-5) was submitted to a new fractioning in a Sephadex G-50® column yielding 3 fractions, of which the denominated S-5-SG-2 showed catalytic activity. It was then submitted to RP- HPLC in C18 column to obtain toxins with high purity degree.

Also, with the use of a multiple step procedure [38] successfully isolated two isoforms of PLA₂s from *B. leucurus* venom. After a first molecular exclusion chromatography using Sephacryl S-200®, 7 fractions were obtained, from which the named "P6" showed to be composed by proteins with apparent molecular mass bellow 30 kDa, and a major fraction of approximately 14 kDa concentrated the phospholipase activity. This fraction was re-chromatographed in a Q-Sepharose® resin (ion exchange) and equilibrated with Tris-HCl 20 mM pH 8.0, yielding 6 fractions. The fraction corresponding to the negatively charged fraction was eluted without significant interaction with the resin, hence with a positive residual charge (basic pI) was selected, showing to be a homogeneous fraction of 14 kDa and presenting phospholipase activity. This fraction was submitted to a RP- HPLC in C4 column, yielding as result two major fractions with close hydrophobicity (eluted with 33% and 36% acetonitrile) and apparent molecular mass of 14 kDa.

Myotoxins with PLA₂s structure from bothropic venoms that have acid pI have being more difficult to isolate. Different from cation exchange resins (CM Sepharose®, Resource S® and CM Sephadex®), anion exchange resins have not been so efficient in the separation of components from bothropic venoms, which requires, complementary steps to obtain these toxins with a satisfactory purity degree, as shown in Table 1.

Daniele et al. [32] described the fractioning of the *B. neuwiedii* venom using a combination of double molecular exclusion chromatography followed by anion exchange chromatography. The first step of the molecular exclusion chromatography was done using Sephadex G-50® where a single fraction with PLA₂s activity was eluted. This fraction was re-chromatographed in Sephacryl S-200® resin, yielding 2 active fractions. The first fraction was re-chromatographed in Mono Q® column (functional group quaternary ammonium) yielding a PLA₂s named P-3. From the second fraction, submitted to the same chromatographic procedure, two other PLA₂s isoforms were isolated, named P-1 and P-2. Although showing different behavior over the molecular exclusion resin, the three isoforms showed very close apparent molecular mass (15 kDa) when assayed by SDS-PAGE. This difference could be resulted from differential interactions of aromatic residues located on the protein surface with the stationary phase [40, 41] and can be also verified in other acid PLA₂s, like the one obtained from *B. jararacussu* venom by Homsí-Brandeburgo et al. [34].

Other procedures used hydrophobic interaction chromatography to isolate these PLA₂s. This is a method that separates the proteins by means of their hydrophobicity: the hydrophobic domains of the proteins bind to the hydrophobic functional groups (phenyl and aryl) of the stationary phase. Proteins should be submitted to the presence of a high saline concentration, which stabilize then and increases water entropy, thus amplifying hydrophobic interactions. In the presence of high salt concentrations, the matrix functional groups interact and retain the

proteins that have surface hydrophobic domains. Hence, elution and protein separations can be controlled altering the salt or reducing its concentration.

Santos-Filho et al. [42], working with *B. moojeni* venom, applied three sequential steps to obtain BmooTX-I, a PLA₂ with apparent molecular mass of 15 kDa and pI 4.2. In this work, the crude venom was chromatographed in DEAE-Sepharose® (Diethylaminoethyl) resin, equilibrated with ammonium bicarbonate 50mM, pH 7.8 and brought to a saline gradient of 0.3M of the same salt. A fraction named E3 showed phospholipase activity, being then submitted to molecular exclusion chromatography in Sephadex G-75® resin. Three fractions were obtained, from which one named S2G3 was submitted to hydrophobic interaction chromatography in Phenyl-Sepharose® resin, the BmooTX-I being eluted in the end of the process.

In a work published in 2011, Nunes et al. [43] described the isolation of an acid phospholipase named BL-PLA₂, obtained from *Bothrops leucurus* through two sequential chromatographic steps. On the first step, the acid proteins were separated from the others with the use of a cation exchange column (CM-Sepharose®) equilibrated with ammonium bicarbonate, pH 7.8. The acid fraction (eluted without interaction with the resin) was lyophilized and applied to a Phenyl-Sepharose CL-4B® column (1 x 10 cm), previously equilibrated with a Tris-HCl 10mM buffer, NaCl 4M, pH 8.5. The elution occurred under decreasing NaCl gradient in a buffered environment (Tris-HCl 10 mM, pH 8.5), concluding the process in an electrolyte free environment. An enzymatically active fraction (BL-PLA₂), (with pI 5.4 and apparent molecular mass of approximately 15 kDa) was obtained at the end of the process.

The bioaffinity chromatography differs from others chromatographic methods because it is based in biological or functional interactions between the protein and the ligand. The nature of these interactions varies, being the most used those which are based on the interactions between: enzymes and substrate analogous and inhibitors; antigens and antibodies; lectins and glycoconjugates; metals and proteins fused with histamine tails. The high selectivity, the easiness of performance together with the diversity of ligands that can be immobilized in a chromatographic matrix make this method a useful tool for the purification of phospholipases. Based on the neutralization of myotoxic effects of the venom from *B. jararacussu* by heparin [44-46], the use of a column containing Agarose-heparin® could be used for the purification of myotoxins. They also ratify the interactions between heparin and myotoxin through the reduction of many biological effects, such as: edema induction, myotoxicity (*in vivo*) and cytotoxicity over mice myoblasts culture (L.6 – ATCC CRL 14581) and endothelial cells.

Following this strategy, Soares et al. [26] described the purification of BnSP-7, a myotoxin Lys-49 from *B. neuwiedi pauloensis*, with the use of chromatographic process based in this heparin functionality, which corroborates previous results obtained by Lomonte et al. [46], that showed the efficient inhibitory activity of heparin against myotoxicity and edema induced by myotoxin II, a lysine 49 phospholipase A₂ from *Bothrops asper*. Also in this study, it was possible to infer the participation of the C-terminal region of the protein in the damaging effects on the cytoplasmic membrane.

Snake venom components share many similar antigenic epitopes that can induce to a crossed recognition by antibodies produces against a determined toxin. In this context, Stabeli et al. [47] showed that antibodies that recognize a peptide (Ile1-Hse11) from Bm-LAAO present crossed immunoreactivity with components not related to the LAAOs group present in venoms from *Bothrops*, *Crotalus*, *Micrurus* e *Lachesis* snake venoms. Also, Beghini et al. [48] showed that the serum produced against crotoxin and phospholipase A₂ from *Crotalus durissus cascavella* was able to neutralize the neurotoxic activity produced by *B. jararacussu* venom and BthTX-I.

Based on this information, pertinent to the crossed immunoreactivity existent between venom components, Gomes et al. [49] described the co-purification of a lectin (BJcuL) and a phospholipase A₂ (BthTX-1) using a immunoaffinity resin containing antibodies produced against the crotoxin. 20 mg of crotoxin was solubilized in coupling buffer (sodium bicarbonate 100 mM, NaCl mM, pH 8.3) and incubated overnight at 4 °C with 1 g of Sepharose® activated by cyanogen bromide (CNBr). After washing with the same buffer, the resin was blocked with Tris-HCl 100 mM buffer. This resin was packed and thoroughly washed with saline phosphate buffer (PBS) pH 7.4. Crotalic counter-venom hiperimmune horse plasma (20 mg) was applied over the resin at a flow of 10 mL/hr and re-circulated overnight through the column. Then, it was washed until the absorbance went back to basal levels, showing that the material was retained (IgG anti-Ctx), then eluted with glycine-HCl 100 mM pH 2.8. The IgG anti-Ctx was then immobilized in CNBr activated Sepharose® resin through a procedure analogous to the above cited, generating a new resin called Sepharose-Bound Anti-CtxIgG. 20 mg of the crude venom from *B. jararacussu* was applied over this resin, yielding two fractions: the first, composed by proteins that were not recognized by the immobilized antibodies and a second fraction composed by components of venom from *B. jararacussu* that reacted crosswise with the Anti-Ctx antibodies, called Bj-F. A posterior analysis of this fraction, done by mass spectrometry, amino-terminal sequencing by Edman degradation and search by homology in the NCBI *protein data bank*, showed that it was composed by lectin and BthTX-I.

Different authors used substrate analogous or reversible inhibitors coupled to the chromatographic resin. Rock and Snyder [50] were the first ones to use phospholipid analogous to build a bioaffinity matrix [Rac-1-(9-carboxy)-nonil-2-exadecilglycero-3-phosphocoline]. In addition to them, Dijkman [51] described the synthesis of an analogous of acylamino phospholipid[(R)-1-deoxy-1-thio-(ω -carboxy-undecyl)-2-deoxy-(n-decanoylamino)-3-glycerophosphocholine] which was coupled to a Sepharose 6B® resin containing a spacer arm. With the use of this resin it was possible to purify phospholipases from horse pancreas, and venoms from *Naja melanonleuca* and *Crotallus adamanteus*.

3. Characterization

Venomic can be defined as an analysis in large scale of the components present in the venom of a certain species. In this context, the proteomic approach has allowed a better understanding of the venom components, through the application of many instruments that

enables the analysis of their expression, structure, post-translational modifications and classification by homology or function. An approach developed by Calvete [52] for the analysis of snake venom consists in an initial fractioning step of the crude venom using RP - HPLC, followed by characterization of each fraction by a combination of amino-terminal sequencing, SDS-PAGE, IEF or 2DE and mass spectrometry to determine molecular mass and cysteine content. Additionally, the modern venom analysis uses techniques such as Peptide Mass Fingerprint and the search for sequence similarity in data banks.

SDS-PAGE is a method related to the migration of charged particles in a medium under the influence of a continuous electric field [53]. From the electrophoretic point of view, the most important properties of the proteins are molar mass, charge and conformation. Mono dimensional polyacrylamide gel electrophoresis permits the analysis of the protein in its native or denatured form. In the first case, there are no alterations in conformation, biological activity and between protein subunits. This system is called non-dissociating or native, which proteins are separated based on their charge, using the isoelectric focusing method (IEF), or else, in vertical gel without SDS. During the IEF, a pH gradient is formed and the charged species move through the gel until they reach a specific pH. In this pH, the proteins have no effective charge (known as protein pI). The IEF shows high resolution, being able to separate macromolecules with pI differences of just 0.001 pH units [54, 55]. In dissociating or denaturing systems, the proteins are solubilized in buffer containing the reagent used to promote protein denaturation. SDS-PAGE, originally described by Laemmli [56], is an electrophoresis technique in polyacrylamide gel (PAGE) that uses SDS as a denaturing agent, which interacts with the proteins giving them negative charges, allowing them to migrate, through a polyacrylamide gel towards a positive electrode and be separated by the differences related to their mass.

Teixeira et al [25] described the purification of an acid phospholipase from *B. pirajai* (BpirPLA₂I). As a biochemical characterization step, polyacrylamide gel electrophoresis in denaturing conditions (SDS-PAGE) was done. Using this approach, carried out in reducing and non-reducing conditions, the author could infer that the purified protein had the form of a monomer with apparent molecular mass of 14 kDa, both in reducing conditions as well as in non-reducing conditions the proteins presented the same mass, being confirmed afterwards by mass spectrometry.

Moreover, Torres [57] fractionated *B. marajoensis* venom using a cationic ion exchange column followed by an analytical phase in RP- HPLC, obtaining a phospholipase BmarPLA₂ that was submitted to SDS-PAGE in reducing conditions showing apparent molecular mass of 14 kDa. However, in non-reducing conditions, the author observed the appearance of a single band at 28 kDa, concluding that BmarPLA₂ was a dimeric structured protein joined by disulphide bridges. Thus, the above-cited examples demonstrate the importance of this procedure (SDS-PAGE) as a protein characterization step.

The determination of the isoelectric point is another important biochemical characterization of phospholipases A₂. Previous studies involving phospholipases from snake venoms have shown that the acid phospholipases are catalytically more active than their basic isoforms

[22, 42, 58]. Therefore, many authors have included, as a biochemical characterization parameter, the determination of the isoelectric point of the by isoelectric focusing. Due to pI determination importance, Teixeira [25] used the methodology proposed by Vesterberg and Eriksson [59] to evaluate pI of BpirPLA₂-1. In order to obtain the pI value, a 7% polyacrylamide gel was prepared and polymerized over a glass plate of 12 x 14 cm using a U shaped rubber as support. A millimeter plate was previously greased with glycerin for better refrigeration of the gel. Two strips of Pharmacia Biotech were used to connect the gel and the platinum electrodes. The cathode was in contact with NaOH 1 M solution and the anode was in phosphoric acid 1 M. The platinum electrodes were centered over the paper strips and the system was then closed. The high voltage source was adjusted to the maximum values of 500 V, 10 mA, 3 watts and 30 minutes for a pre-run. Following, the samples were applied always in the intersection of two blue lines, exactly over the more central line of the gel. Then the source was programed for 1500 V, 15 mA, 10 watts and 5 h. The end of the run was determined when the source showed a high voltage and low amperage (around 1 mA). After isoelectric focusing, about 1 cm width (lengthwise) were sliced from each extremity of the gel and placed in test tubes containing 200 µL of distilled water for the pH reading after 2 hours of rest. Next, the pH gradient determination graph was plotted. The remaining gel containing the samples was fixed in solution of trichloroacetic acid for 30 minutes, followed by silver staining.

Another important technique as a step to characterize components from snake venoms is the bidimensional electrophoresis (2D). This one was initially developed by O'Farrell [60]. The original methodology consisted of the preparation of polyacrylamide cylindrical gels, in which a pH gradient was established through a pre-run with specific amphoterics (also called ampholytes), that present high buffering capability in pHs close to their isoelectric points (pIs). The proteins were then submitted to an isoelectric focusing (IEF) and subsequently to an electrophoresis in the presence of SDS by a conventional system described by Laemmli [56]. Then, proteins were separated in the first dimension according to their pIs (IEF) and in the second dimension based on their molecular mass (SDS-PAGE).

Bidimensional electrophoresis is laborious, time consuming and difficult to be reproduced in different laboratories and depended on the ability of the researcher to obtain consistent results. Nowadays, many of these problems were solved with the development of new technologies. An important advance which has contributed to the increase of the 2D electrophoresis reproducibility was developed by Gorg [61] of the strip form gels with immobilized pH gradient (IPG - *immobilized pH gel*). The strips are made by the copolymerization of acrylamide with the *Immobiline*® (Amersham Biosciences/GE Healthcare) reagent, which contains acid and alkaline buffering groups. Another important technological progress was the improvement of the protein samples preparation methods, together with the discovery of new non-ionic detergents, such as CHAPS surfactants and SB 3-10, used with reducing agents adequate for IEF, like Dithiothreitol (DTT) and Tributyl Phosphine (TBP). Studies performed by Herbert [62] demonstrated that these advances had strongly contributed to the solubilization of a greater number of proteins to be analyzed in bidimensional electrophoresis.

The proteomic analysis of snake components has made use of the 2D electrophoresis as a tool, due to its high-resolution capability that allows, in a single process, the determination of apparent molecular mass and isoelectric point of the venom constituents. Fernandez et al. [22] described the determination of the isoelectric point and apparent molecular mass of Basp-PLA₂-II using this technique. In order to do it, the protein was focused in IPG Immobiline® Dry Strip of 7 cm and pH 3-10, under a 200 V tension for 1 min, followed by a second stage of 3500 V for 120 minutes. The second dimension was done in SDS-PAGE 12% and then subsequently dyed with Coomassie blue. It was demonstrated that Basp-PLA₂-II had a pI of 4.9, which is close to the theoretical isoelectric point value (pI 5.05) defined by the primary sequence, evaluated using the Compute pI/MW tool (www.expasy.ch/tools) software and apparent molecular weight between 15 and 16 kDa, consistent with the molecular weight (MW 14,212±6 Da) obtained by ESI/MS (Electrospray Ionization/Mass Spectrometry).

The advantage of this technique is the high resolution. Alape-Giron [63] working with *B. asper* venom, performed an ontogenic analysis and an analysis based on the snake's capture location in different regions of Costa Rica. Using tryptic digestion, MALDI-TOF mass fingerprinting analysis and aminoacid sequencing by MALDI-TOF submitted to similarity search by BLAST, the author showed the intra-specific variability in venom composition. It was hence evidenced that among the venoms obtained from adult species collected in the Caribe area and the Pacific area, there are around 30 proteins that are found in a snake group from a place which find no correspondents in the other.

In our lab, this technique has been used as follows: The proteins are separated by the isoelectric point in 13 cm strips with pH values varying between 3 and 10 in a nonlinear form. These strips contain polyacrylamide gel, where the gradient pH is formed by the presence of ampholytes. To re-hydrate the strips, 250 µL of sample [400 µg of proteins plus re-hydration solution (7 M of urea, 2 M of Thiourea, 2% of Triton X-100 (v/v), 1% of IPG Buffer® (v/v) and DTT)] is applied in a channel of the apparatus over which the strips are set. The strip's gel is re-hydrated at room temperature for about 12 hours. After this period, the strips are taken to the focusing system in the following conditions: (1) 500 V step until accumulates 500 Vh; (2) 500 to 1000 V gradient until it accumulates 800 Vh; (3) 1000 to 8000 V gradient until it accumulates 11300 Vh and (4) 8000 V step until it accumulates 3000 Vh. In average, the program run during 5.5 hours, but the time of the final step can be lengthened, if the sample does not reach to the end of the strip during the running according the initial program, it could be confirmed by a bromophenol blue line. At the end of focusing, the strips are equilibrated in two steps. On the first, 10 mL of the solution containing 6 M of urea, 2% of SDS (m/v), 30% of glycerol (v/v), 75 mM of Tris-HCl (pH 8,8), 0,002% of bromophenol blue and 1% DTT (m/v) for each strip is used. In the second, the same solution is used, but DTT is replaced by 2.5% of iodoacetamide (m/v). Each strip equilibrium step run during 15 minutes, under light stirring. Following that, the strips are applied on 10 % polyacrylamide gels previously prepared on 180 X 160 X 1.0 mm plates. After each strip and the standard stay appropriately accommodated in the polyacrylamide gel, a 0,5% agarose (m/v) heated (40 °C) solution is added. The agarose polymerization, provides an effective contact between the strip and the gel, thus avoiding the appearance of air bubbles. Protein

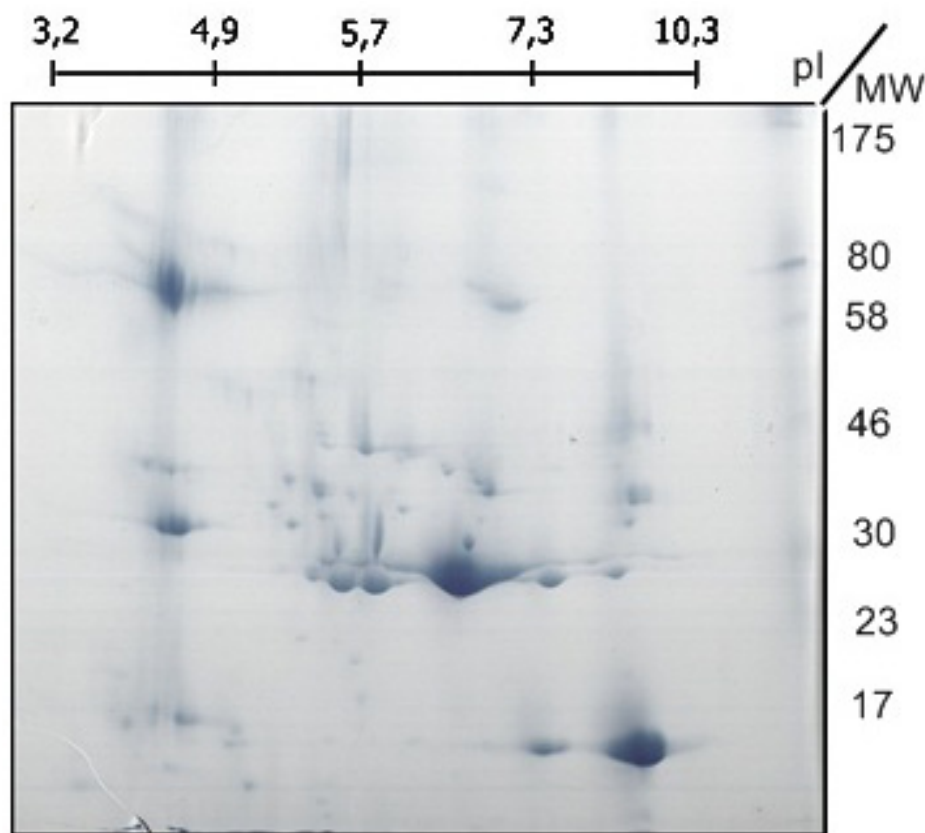


Figure 6. Electrophoretic profile in 2D-PAGE 10%, 13 cm strip pH 3-10 non-linear of proteins from crude venom from *Bothrops moojeni*. Molecular weight (MW) –Color Plus Prestained Protein Marker – Broad Range (7-175 kDa) (P7709S). Coomassie G-250.

separation, according to molecular mass, is done by applying 25 mA per gel and 100 W during approximately 5.5 hours. After this period, the gel is washed with deionized water. Then, the proteins are fixed using a solution containing acetic acid 10% (v/v) and ethanol 40% (v/v) during one hour. Then, the fixing solution is removed and the gel is washed again with deionized water 3 times during 10 minutes. The proteins present in the gel are exposed using traditional methods for protein coloring, such as Coomassie blue or Silver nitrate. An example of the practical application of this methodology can be seen in Figure 6.

4. Functional characterization

Many biological activities are related to myotoxins with PLA₂ structure obtained from snake venoms. In bothropic snake bite accidents and in experimental models with the use of these venoms, the noxious activity induced by these toxins on the striated muscles is striking [64]. The detection of the myotoxic activity associated to the phospholipase activity detection (in the case of Snake venom PLA₂ Asp49) is used as an important auxiliary biological marker in the purification procedures, monitoring its presence.

The myotoxic activity assay can be done in two ways: *in vivo* and *in vitro*. The analysis can be done through the quantification of the released intracellular enzymes activity to the

periphery blood or to the supernatant of the culture medium of cellular lineages. There are two main enzymes used to this end:

Creatine Kinase (EC 2.7.3.2): is a dimeric protein formed by the combination of subunits (B and M) and in its cytosolic form is found in many tissues, especially in skeletal muscle tissue (CK-MM), cardiac (CK-MB) and in the brain (CK-BB).

Lactate dehydrogenase (EC 1.1.1.27): is an enzyme widely distributed in many tissues and organisms. It is presented in the form of homo or hetero tetramers of subunits M and H, being present in muscular tissue in the homotetrameric form of subunit M.

In vivo, the CK activity quantification in murine models has been the most used to assay the presence of myotoxic PLA₂, especially due to their low cost, ease of performance and high specificity as skeletal muscular tissue lesion markers when exposed to myotoxins.

As for the *In vitro* assays, myoblast lineages C2C12 (ATCC CRL-1772), differentiated until the formation of myotubules, have been used as models to assay the cellular toxicity, through the quantification of LDH levels in the supernatant of cell cultures exposed to toxins.

Regarding the phospholipase activity detections, it can be done by direct and indirect methods. Directly, it is possible to detect the presence of PLA₂s with the use of chromogenic substrates, such as 4N3OBA(4-nitro-3-octanoyloxybenzoic acid) that induce the formation of detectable product at 425 nm [65] and fluorescent substrates (NBD) coupled to phospholipids that are used to quantitatively and qualitatively survey the PLA₂s activity isolated from snake venom [23].

Indirectly, the approach used consists in the potentiometric assay of the fatty acids released after the enzymatic hydrolysis of the phospholipids, through the quantification with standard alkaline solution [66]. Moreover, fatty acids released by the enzymatic degradation can be quantified through the alteration of the optical density of the pH indicator solution, such as phenol red [67], brilliant yellow [68] and bromothymol blue [69]. Another indirect method to assay PLA₂ activity present in samples consists in the detection of hemolysis induced by lysophospholipids derived from phospholipids submitted to enzymatic digestion. This can be done through the quantification of hemoglobin present in solution or through the visualization of hemolytic halo in agarose matrix with immobilized red blood cells.

4.1. *In vivo* assay of the myotoxic activity

Mice is used for the *in vivo* assay of the myotoxic activity. Swiss males weighing between 18 g and 22 g, kept in controlled environment (12 h in the light and 12 h in the dark), with food and water *ad libitum* up to the moment of use. PBS solubilized sample and control (PBS) are filtered through 44 µm pores immediately prior to use. Reagents for CK activity dosage are prepared and used according to manufacturer's instructions.

A Sample (50 µL) or control (50 µL) will be injected in mice gastrocnemius muscle using adequate device in order to guarantee a precise volume control. After a time lap (3 and/or 6 h), blood sample is collected in heparinized tubes and centrifuged to separate plasma. CK

concentration is determined according to manufacturer's instructions and expressed in U/L, where one unit corresponds to the production of 1 mmol of NADH per minute [26,70-72].

4.2. *In vitro* myotoxic activity assay

In order to assay myotoxic *in vitro* activity, myoblast lineage cells are used, such as murine skeletal muscle C2C12 myoblasts (ATCC CRL-1772) as described by Lomonte et al. [73], cultivated in modified Dubelco Eagle medium, supplemented with 1% bovine fetal serum. PBS solubilized sample, negative control (PBS) and positive control (Triton X-100) should be filtered through 22 µm pore filters immediately prior to use. Reagents for LDH activity dosage are prepared and used according to manufacturer's instructions.

In 96 well plate, 2X10⁵ cells/150 µL are set, sample and/or control (50 µL) are incubated in humid atmosphere at 37 °C and 5% CO₂ for a 3 hour period. Afterwards, collect supernatant aliquot and quantify LDH activity released by cells with cytoplasmic membrane integrity compromised, according to manufacturer's instructions and expressed in U/I, where one unit corresponds to the production of 1 mmol of lactate per minute.

5. Phospholipasic activity

5.1. 4N3OBA Substrate enzymatic hydrolysis

Phospholipase A₂ activity can be measured according to the technique described by Holzer and Mackessy [65], modified for 96 wells plate [74].

Prepare aliquots of 100 µL of 4N3OBA 0.1% solution in acetonitrile and lyophilize. Keep the aliquots at -20° C until ready to use. The color reagent is prepared solubilizing the contents of one aliquot of 4N3OBA in 1ml of reagent containing Tris 10 mM, CaCl₂ 10 mM, NaCl 100 mM, and pH 8.0. For the test in micro plates, add 180 µL of color reagent and 20 µL of sample or water (blank), incubate the mixture at 37 °C for 5 minutes, measuring the optical density at 425 nm and 600 nm (to correct sample turbidity) at 30 second intervals. The activity will be expressed according to the equation (1) where 1 unit of phospholipase activity corresponds to the production of 1 µmol of 4-nitro-3-hydroxy-benzoic acid per minute.

$$\text{PLA}_2 \text{ activity } [\mu\text{mol} \cdot \text{min}^{-1} \cdot \mu\text{g}^{-1}] = \frac{[\text{OD}_{425\text{nm}} - \text{OD}_{600\text{nm}} / \text{min}] \times 0.07862 [\mu\text{mol} / \text{OD}_{425\text{nm}}]}{1 / \text{protein } (1 / \mu\text{g})} \quad (1)$$

5.2. Enzymatic hydrolysis of fluorescent substrates (NBD)

The phospholipase activity can also be assayed with the used of chromogenic substrates, using acyl-NBD reagents: NBD-PC (Phosphatidylcholine), NBDPG (phosphatidylglycerol), NBD-PE (phosphatidylethanolamine) or NBD-PA (phosphatidic acid). A solution of fluorescent lipids should be previously prepared in a 1 mg/ml concentration in chloroform. 100 µL aliquots are distributed and then dried under nitrogen flow. The dried lipid will be solubilized in 1 ml of NaCl 0.15 M and sonicated until the obtention of a limpid solution. For

the test, the lipids should be diluted in a solution containing Tris-HCl 50 mM, CaCl₂ 1 mM pH 7.5. Initially, incubate the solution at 37 °C and, after 2 minutes, make an initial reading, configuring the equipment for excitation at 460 nm and emission at 534 nm. Following, apply the sample and make a second reading after 12 minutes. The change in fluorescence intensity is converted to nanomoles of product per minute (nmoles/min) using a calibration curve, prepared by hydrolyzing completely a substrate solution through sodium hydroxide treatment. The fluorescence intensity unit was converted to nmoles/min [33].

5.3. Potentiometric titration of fatty acids

The phospholipase activity can be assayed by potentiometric titration as described by de Haas [75], using as substrate an egg yolk emulsion in the presence of sodium deoxycholate 0.03 M and CaCl₂ 0.6 M. Fatty acids released enzymatically are titrated with a standard solution of NaOH 0.1 N at pH 8.0 at room temperature. The phospholipase activity is generally done with different concentrations of toxin, and calculated per amount of microequivalents of alkali consumed per minute, by mg of protein. One unit of phospholipase activity can be defined as the quantity of enzyme that releases 1 μmol of fatty acid per minute, in the reaction conditions.

5.4. Phenol red

The spectrophotometric detections of phenol red solution, induced by the increase of free fatty acids concentration can also be used to assay the phospholipase activity in samples, as described by Radvanyi [67].

In order to use this technique, prepare the reagent solution containing Phosphatidylcholine 0.25% (w/v) TritonX-100 0.4% (v/v), phenol chloride 32 mM. In a thermostatic cuvette at 37 °C, add 1mL of reagent solution and 10 μL of sample. After stabilization for 20 seconds, determine the optical density measuring at 558 nm for 3 minutes, in kinetic intervals of 15 seconds. One unit of phospholipase can be defined as the quantity of enzyme necessary to convert 0.001 UA 558 nm per minute.

5.5. Indirect hemolysis

In this test, phospholipids (from egg yolk, soy lecithin or other sources) are used as substrates, with the production of fatty acid and corresponding lysophospholipids. These lysophospholipids have membrane activity over red blood cells, producing hemolysis that can be detected through the quantification of hemoglobin present in solution or through a hemolysis halo present in agarose gel containing intact red blood cells [76].

For the test, collect blood in a heparinized tube, wash the red blood cells with PBS, centrifuging at 800 xg for 5 minutes and prepare the suspension at 3%. Prepare solution containing phosphate buffer 20 mM, sodium chloride 100 mM and CaCl₂ 10 mM, erythrocyte suspension 3% (1:30 v/v) and egg yolk solution 0.1% (1:30 v/v). Add 10 μL of sample or PBS (control 0%) or Triton X-100 0.1% (control 100%) and incubate at 37 °C for 30

minutes. Then, centrifuge, collect the supernatant and determine the optical density at 405 nm, using PBS as blank. The results will be expressed in % of hemolysis compared with the positive control.

Hemoglobin dosage present in solution with the use of the Drabkin reagent (potassium ferrocyanide in buffered environment) [77] can be done by comparing the optical density of the samples with the standard curve made with the hemoglobin cyanide solution, according to manufacturer instructions.

To assay the hemolytic activity in agarose gel, carefully heat the suspension containing agarose 2% in PBS until complete fusion. After partial cooling (45 °C), add an equal volume of PBS containing CaCl₂ 0.02 M; egg yolk suspension (1:30 m/v), erythrocytes washed in PBS (1:30 m/v), pouring over Petri plate until the formation of a layer approximately 2 mm thick. After solidification of the gel, orifices of uniform diameter (0.2 cm diameter) to apply the sample are made. The gel is incubated for 12 hours, at 37 °C and humid environment. The formation of a translucent halo around the gel application point is indicative of phospholipase activity, contrasting with the rest of the gel which remains with a reddish tone due to the presence of integral red blood cells retained in the gel net.

Abbreviations

PLA₂s: Phospholipases A₂

sPLA₂: secreted PLA₂

cPLA₂: cytosolic PLA₂

iPLA₂: Ca²⁺ independent PLA₂

PAF-AH: acetyl-hydrolases from platelet activating factors and liposomal

Mr: relative mass

PAF: platelet activating factors

svPLA₂s: phospholipase A₂ found in snake venoms

SDS-PAGE: Sodium dodecyl sulfate – PolyAcrylamide Gel Electrophoresis

MW: Molecular weight

RP-HPLC: reverse-phase chromatography

DEAE-Sepharose: Diethylaminoethyl Sepharose

tEnd cells: endothelial cells

CNBr: cyanogen bromide

PBS: phosphate buffer saline

IEF: isoelectric focusing

2DE: bi-dimensional electrophoresis

IPG: immobilized pH gel

pIs: isoelectric points

DTT: Dithiothreitol

TBP: Tributyl phosphine

ESI: Electrospray Ionization

MS: Mass Spectrometry

CK-MM: Creatine Kinase - skeletal muscle tissue

CK-MB: Creatine Kinase – cardiac

CK-BB: Creatine Kinase - brain

CK: Creatine Kinase

LDH: lactate dehydrogenase

NBD: N-4-Nitrobenzo-2-Oxa-1,3-Diazole

NBD-PC: N-4-Nitrobenzo-2-Oxa-1,3-Diazole Phosphatidylcholine

NBD-PG: N-4-Nitrobenzo-2-Oxa-1,3-Diazole

NBD-PE: N-4-Nitrobenzo-2-Oxa-1,3-Diazole phosphatidylglycerol

NBD-PA: N-4-Nitrobenzo-2-Oxa-1,3-Diazole Phosphatidic acid

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