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Isolation and characterization of two new Lys49 PLA_2s with heparin neutralizing properties from *Bothrops moojeni* snake venom

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ABSTRACT

Among the proteins and peptides already characterized in *Bothrops moojeni* venom, two novel phospholipases A_2 (PLA_2) have been purified and fully sequenced by ESI-MS/MS techniques. Both of them belong to the enzymatically non-active Lys49 variants of PLA_2. They consist of 122 amino acids and share a characteristic sequence in their C-terminal region composed of clusters of basic amino acids known to interact with heparin. Thus, as already established, heparin can be used as an antidote to antagonize some myotoxic PLA_2s from venoms of *Bothrops* genus. The two PLA_2 variants were shown to interact *in vitro* with unfractionated heparin (UFH) and low molecular weight heparin (LMWH), neutralizing their anticoagulant properties. Although the influences of PLA_2s from snake venoms on the blood coagulation system are known, their use to antagonize the anticoagulant effect of heparin *in vitro* or *in vivo* has never been proposed. These finding recommend diagnostic and therapeutic applications, which are currently investigated.

1. Introduction

Phospholipases A_2 (PLA_2) are widely distributed in Nature. They occur in venoms of snakes, scorpions, bees and in mammalian tissues, both as intracellular and extracellular forms (Soares et al., 1998; Kini, 2003). Snake venom PLA_2 enzymes, in addition to their role in digestion of the prey, belong to the most toxic and pharmacologically most potent venom components (Kini, 2003). They are able to induce several biological effects such as pre- or post-synaptic neurotoxicity, cardiotoxicity, myotoxicity, inhibition of platelet aggregation, edema, hemolysis,

Abbreviations: ACN, acetonitrile; aPTT, activated partial thromboplastin time; aq, aqueous; ATIII, antithrombin III; CT, clotting time; ESI-MS, electrospray ionisation-mass spectrometry; ex- TEM®, solution for activation extrinsic pathway of blood coagulation; FXa, factor Xa (activated factor X); FVa, factor Va (activated factor V); GF, gel filtration; 4-HCCA, α-cyano-4-hydroxycinnamic acid; in- TEM®, solution for activation intrinsic pathway of blood coagulation; ISTH/SSC, International Society on Thrombosis and Haemostasis/Scienticic and Standardization Committee; LMWH, low molecular weight heparin; MALDI-TOF, matrix assisted laser desorption ionisation – time of flight; MS, mass spectrometry; NHF, normal human fibroblasts; rPF4, recombinant platelet factor 4; PiCT, prothrombinase induced clotting time; PLA_2, phospholipase A_2; SA, sinapinic acid; star-TEM®, buffered $CaCl_2$ solution; UFH, unfractionated heparin; TFA, trifluoroacetic acid.

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anticoagulation, hemorrhage, convulsion and hypotension, as well as bactericidal activities (Kini and Iwanaga, 1986a,b; Paramo et al., 1998; Soares et al., 1998; Kini, 2003; Stábeli et al., 2006). Despite their diverse pharmacological properties, they share 40-99% identity in their amino acid sequences and a significant similarity in their three dimensional structures. Therefore, the functional differences among PLA_2 enzymes cannot be easily correlated with their structural differences (Kini, 2003).

Bothrops myotoxins are classified in group II PLA_2s, together with all crotalid/viperid venom lipases and the secreted non-pancreatic mammalian PLA_2 (Gutierrez and Lomonte, 1995). Moreover, they are divided into two major classes according to the amino acid residue at position 49 in the sequence, being a key residue for binding of the Ca^{2+}-cofactor. The enzymatically active Asp49 and the enzymatically non active Lys49 myotoxins can be distinguished (Soares et al., 1998; Lomonte et al., 2003a). Asp49Lys mutation, together with additional mutations in the calcium-binding loop, precludes an effective coordination of Ca^{2+} ions and, consequently, is responsible for the lack of enzymatic activity (Gutierrez and Lomonte, 1995; Ownby et al., 1999; Lomonte et al., 2003a). However, the enzymatic activity is apparently not necessary for the toxic actions. Lys49 myotoxins display also bactericidal properties, being lethal for a broad spectrum of Gram-negative and Gram-positive bacteria (Paramo et al., 1998; Santamaria et al., 2005). Recently, it has been shown (Stábeli et al., 2006) that *Bothrops moojeni* myotoxin II displays antiparasitic and antitumoral activities, supporting the idea of multiple bioactive sites in the protein.

Three *B. moojeni* myotoxins have been isolated and described so far. MOO-1 myotoxin possesses high phospholipase activity as reported by Moura-da Silva et al. (Moura-da-Silva et al., 1991a,b). The two other myotoxins, MjTX-I and MjTX-II, are described as catalytically inactive Lys49 variants. Their amino acid sequences (Lomonte et al., 1990; de Azevedo et al., 1997; Soares et al., 1998, 2000a), as well as the native structures and the complex of MjTX-II with stearic acid (de Azevedo et al., 1997; Soares et al., 2000a; Marchi-Salvador et al., 2005; Watanabe et al., 2005) have been determined, revealing high homology with various Lys49 PLA_2-like proteins from other *Bothrops* venoms.

Lomonte et al. (Lomonte et al., 1994a,b) first described the binding of heparin to a lysine-rich site comprising residues 115–129 [numbering of (Renetseder et al., 1985)] of the myotoxin Lys49 MT-II from *B. asper*, resulting in the neutralization of its *in vitro* cytolytic and *in vivo* myotoxic activities. The polyanionic heparin can interact in a non-covalent, charge-dependent way with the basic myotoxin, forming an inactive acid-base complex, leading to inhibition of its myotoxic activity.

So far, it has been reported that heparin was used *in vivo* and *in vitro* to neutralize different toxic effects caused by some snake venoms and their PLA_2s (Melo and Suarez-Kurtz, 1988a,b; Melo et al., 1993; Lomonte et al., 1994a,b; Lin et al., 1999; Melo and Ownby, 1999; Ownby et al., 1999; Beghini et al., 2000; Soares et al., 2000b; Oshima-Franco et al., 2001; Calil-Elias et al., 2002a,b; Ketelhut et al., 2003; Stábeli et al., 2006). Heparin derivatives with little or no effect on the coagulation system, *i.e.* heparin with low affinity for antithrombin (Lomonte et al., 1994a,b) were considered as a complementary treatment of hemorrhages induced by snakes envenomations. Nevertheless, the therapeutic use of the heparin binding domain of a PLA_2 to prevent excessive bleeding mediated by heparin treatment had never been described. In the present work, two novel Lys49 PLA_2 variants from the venom of *B. moojeni* were isolated and fully sequenced by tandem mass spectrometry. They were found to interact with both unfractionated and low molecular weight heparins and to display promising properties for neutralization of anticoagulant effects.

2. Materials and methods

2.1. Crude venom

B. moojeni crude venom was collected, pooled and dried at Pentapharm do Brasil. It was transported and stored in the desiccated form and reconstituted in deionized water at Pentapharm Aesch. A 30% solution (250 mg/ml) in water was obtained and stored at −80 °C.

2.2. Protein separation

2.2.1. Size exclusion chromatography

Crude, reconstituted venom was separated into 18 gel filtration fractions (fractions Botmo GF 1 to 18) on two in-line Superdex-75 XK 26 columns (GE Healthcare). Up to 250 mg of venom was loaded per run and the elution was performed using a buffer of 50 mM ammonium acetate, 150 mM sodium chloride, pH 7.5 at a flow rate of 2.2 ml/min (25 cm/h). The absorbance was monitored at 280 nm. To ensure the good reproducibility of the process, the columns were regenerated every 5 runs using 0.5 M NaOH. Collected fractions were stored at −80 °C until further separation steps and bioassays were performed.

2.2.2. RP-HPLC

Fractions Botmo GF 10 and Botmo GF 12 (aliquot of 10 mg of protein) were submitted to solid phase extraction after acidification with aqueous 0.1% trifluoroacetic acid (TFA), pH 2.2 and loading on Sep-Pak C_{18} classic cartridges (Waters™). Solvatation, equilibration, sample application and elution were performed according to the manufacturer instructions. Elution was performed with a mobile phase of 40:60 aq. TFA (0.1 %): acetonitrile (ACN). The collected fraction were immediately dried in a SC210A SpeedVac™ Plus concentrator (ThermoSavant) and stored at −20 °C. Freeze-dried fractions were reconstituted in aq. 0.1% TFA and centrifuged at 11,000 rpm before separation by RP-HPLC on a Waters Alliance 2690 System using a semi-preparative HPLC column (Vydac #218TP510 protein & peptide C_{18}, 10 mm × 250 mm). Proteins and peptides were eluted using a 2–50% gradient of 90% ACN in aq. TFA (0.1%) in 75 min at a flow rate of 3 ml/min. The absorbance was monitored at 225 nm and the fractions were collected manually, following peak absorbance. Collected sub-fractions of interest were called Botmo GF10/71 (MjTX-III) and Botmo GF12/84 (MjTX-IV), stored at −20 °C and used for further measurements.