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P9a(*Cdt*-PLA$_2$) from *Crotalus durissus terrificus* as good immunogen to be employed in the production of crotalic anti-PLA$_2$ IgG

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HIGHLIGHTS

- We propose the use of a PLA$_2$ isoform from *C.d.t.* venom to be used as immunogen.
- P9a(*Cdt*-PLA$_2$) has the lowest toxicological activity among the isoforms from the venom.
- P9a(*Cdt*-PLA$_2$) IgG antibodies can be easily produced in laboratory animals.
- P9a(*Cdt*-PLA$_2$) IgG antibodies can cross react with the other isoforms from the venom.

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ABSTRACT

Four proteins with phospholipase A$_2$ (PLA$_2$) activity, designated P9a(*Cdt*-PLA$_2$), P9b(*Cdt*-PLA$_2$), P10a(*Cdt*-PLA$_2$) and P10b(*Cdt*-PLA$_2$) were purified from the venom of *Crotalus durissus terrificus* by two chromatographic steps: a gel filtration and reversed phase HPLC. The profile obtained clearly shows that three of them have a similar abundance. The molecular mass, 14193.8340 Da for P9a(*Cdt*-PLA$_2$), 14134.9102 Da for P9b(*Cdt*-PLA$_2$), 14242.6289 Da for P10a(*Cdt*-PLA$_2$) and 14183.8730 Da for P10b(*Cdt*-PLA$_2$), were initially evaluated by SDS-PAGE and confirmed by ESI-Q-TOF spectrometry, and all of them displayed a monomeric conformation. Also, partial amino acid sequence of each protein was obtained and their alignments with other crotalic PLA$_2$ revealed a high degree of identity among them. Additionally, we studied some pharmacological activities like neurotoxicity, myotoxicity and lethality, which prompted us to pick two of them, P9a(*Cdt*-PLA$_2$) and P10a(*Cdt*-PLA$_2$) that resulted to be less toxic that the others, and further characterize them to be used as immunogen. We next injected these last proteins in mice to produce antitoxins against them and ELISA and dot blots reveled that both toxins do not show immunogenic differences, unlike those other pharmacologic activities tested. Furthermore, the antibodies produced cross-reacted with all the isoforms purified demonstrating the feasibility of using only one of them and ensuring the cross-reaction of all.

The results obtained show that P9a(*Cdt*-PLA$_2$) isoform has the lowest toxicity and also a good purification performance; thus this protein may be a promising candidate to be employed in the production of crotalic antitoxins.

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1. Introduction

Crotoxin, the major lethal toxin of South American rattlesnake (*Crotalus durissus terrificus, C.d.t.*) venom, was the first snake venom protein to be purified and crystallized (Gutierrez, 2002). Crotoxin is a heterodimeric β-neurotoxin that consists of a toxic

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basic phospholipase A_2 (PLA_2) and a nonenzymatic, non-toxic acidic component (crotapotin) (Hendon and Fraenkel-Conrat, 1971). Crotoxin blocks the neuromuscular transmission; it acts primarily at the presynaptic level of neuromuscular junctions by impairing neurotransmitter release. Additionally, other biological activities normally attributed to crotoxin include myotoxicity, nephrotoxicity and cardiotoxicity. On the other hand, in recent years, there has been increasing evidence that crotoxin exerts a variety of other important actions unrelated to these activities. These actions include immunomodulatory, anti-inflammatory, antitumor, anti-microbial and analgesic activities (Sampaio et al., 2010).

Crotoxin PLA_2 subunit belong to an expanding superfamily of enzymes, which catalyzes hydrolysis of the ester bond at the *sn-2* position of 1,2-diacyl-*sn*-3-phosphoglycerides, generating lysophospholipids and free fatty acids (Kini, 2003). Crotalic PLA_2 displays similar pharmacological effects to those exhibited by crotoxin, however, in the case of lethality, higher doses of PLA_2 are required. Because of that, it is believed that crotapotin behaves as a carrier for the PLA_2, reducing its non-specific interaction, therefore, increasing the binding with its target and enhancing the toxicity of crotoxin (Hendon and Fraenkel-Conrat, 1971, 1976; Hendon and Tu, 1979). Additionally, since PLA_2 shows less toxicity than the whole venom, its use as immunogen has been proposed in order to obtain antitoxins to be use in crotalic envenomations (Fusco et al., 2014; Rodríguez et al., 2006).

Crotoxin has been shown to be a mixture of isoforms present in the whole venom. They result from combinations of several variants of each subunit. Purified PLA_2 isoforms consist of a single polypeptide chain of 121/2 amino acids, differing from each other by only few amino acids as determined by amino-acid composition (Faure et al., 1994). These differences create diversity in their biological functions, allowing the existence of an isoform among them that can be obtained with acceptable purity, would have low toxicity and good immunogenic capacity. Thus, this protein could be useful in the production of antitoxins.

PLA_2s from *C.d.t.* venom were isolated and sequenced from Brazilian specimens by several authors (Damico et al., 2005; Marchi-Salvador et al., 2007; Ponce-Soto et al., 2006, 2007a; Romero-Vargas et al., 2010; Toyama et al., 2000), however most of these studies have been limited to an exhaustive biochemical characterization, and none of them focuses on their potential use as immunogens.

There is no available information about PLA_2 sequences isolated from *C.d.t.* venom from Argentina and considering regional variations not only among species, but also within a single species, this represents an exceptional reservoir to explore and find useful alternatives to be employed in technological applications (Fernandez et al., 2010; Saravia et al., 2002). Thus, to address this challenge a comprehensive understanding of the sequences-structure-biological function relationship result essential.

In this work, we describe the biochemical, pharmacological and immunological characterization of PLA_2 isoforms, isolated from *C. d.t.* snake venom, in order to provide evidence for selecting the best candidates to be employed in the production of crotalic anti-PLA_2 IgG.

2. Material and methods

2.1. Venom and animals

C.d.t. venom was pooled from 10 specimens of 8/10-year-old adult snakes held in the serpentarium of the local Zoo, Corrientes, Argentina. The venom was lyophilized and kept frozen at −20 °C. Male Swiss white mice weighing 20–22 g were supplied by the Animal Services Unit of the State University of Campinas (UNICAMP). The mice were housed at 25 °C on a 12 h light/dark cycle. Male young chickens (4–8 days old, HY-LINE W36 lineage) were supplied by Granja Ito S/A (Campinas, SP, Brazil). All animals had free access to food and water and were conducted in accordance with guidelines of the Ethics Committee of the Biology Institute-UNICAMP (Campinas, Brazil).

2.2. Toxins purification

2.2.1. Molecular exclusion chromatography

Venom (25 mg) was dissolved in 50 mM ammonium bicarbonate (pH 8.0), and fractionated on a Sephadex G75 column (1 × 75 cm), eluted with the same buffer at a constant flow of 0.5 ml/min. Elution was monitored at 280 nm, and the fractions were immediately lyophilized and stored at −20 °C.

2.2.2. Reverse phase (RP) HPLC

Four mg of the fraction obtained from Sephadex G75 (Peak II) were dissolved in 200 μl of solvent A (0.1% (v/v) trifluoroacetic acid; TFA). The resulting solution was clarified by centrifugation and the supernatant was applied to a μ-Bondapack C18 column (0.78 × 30 cm; Waters 991-PDA system). Proteins were eluted with a gradient (0–20%, 20–60%, 60–100%) of 66% (v/v) acetonitrile in solvent B, at a flow rate of 1 ml/min. The elution profile was monitored at 280 nm and the fractions were collected, lyophilized and stored at −20 °C.

2.3. Molecular mass determination and protein sequencing

2.3.1. Molecular mass determination

An aliquot (4.5 μl) of the purified protein was injected by C18 (100 μm × 100 mm) RP-UPLC (nanoAcquity UPLC, Waters) coupled with nano-electrospray tandem mass spectrometry on a Q-TOF Ultima API mass spectrometer (Micromass/Waters) at a flow rate of 600 nl/min. The gradient was 0–50% acetonitrile in 0.1 % formic acid over 45 min. The instrument was operated in MS continuum mode and the data acquisition was from *m/z* 100–3,000 at a scan rate of 1 s and an interscan delay of 0.1 s. The spectra were accumulated over about 300 scans and the multiple charged data by the mass spectrometer on the *m/z* scale were converted to the mass (molecular weight) scale using maximum entropy-based software supplied with Masslynx 4.1 software package. The processing parameters were: output mass range 6,000–20,000 Da at a "resolution" of 0.1 Da/channel; the simulated isotope pattern model was used with the spectrum blur width parameter set to 0.2 Da, the minimum intensity ratios between successive peaks were 20% (left and right). The deconvoluted spectrum was then smoothed (2 × 3 channels, Savitzky Golay smooth) and the mass centroid values obtained using 80% of the peak top and a minimum peak width at half height of 4 channels.

2.3.2. Enzymatic hydrolysis

The purified proteins were hydrolyzed with sequencing grade bovine pancreatic trypsin in 0.4 % ammonium bicarbonate (pH 8.5), for 4 h at 37 °C, at an enzyme/substrate ratio of 1:100 (w/w). The reaction was ceased by lyophilization.

2.3.3. Analysis of tryptic digests

The proteins were reduced with 5 mM DTT (Dithiothreitol) for 25 min at 56 °C and alkylated with 14 mM iodoacetamide for 30 min prior to the addition of trypsin (Promega-Sequence Grade Modified). After the trypsin addition (20 ng/μl in ambic 0.05 M), the sample was incubated for 16 h at 37 °C. To stop the reaction, formic acid 0.4% was added and the sample centrifuged at 2500 × *g* for 10 min. The pellet was discarded and the supernatant dried. The resulting peptides were separated by C18 (100 μm × 100 mm)

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