Biologicals xxx (2017) 1–8

Contents lists available at ScienceDirect

Biologicals

journal homepage: www.elsevier.com/locate/biologicals

Cross-reactivity and inhibition myotoxic effects induced by *Bothrops* snake venoms using specific polyclonal *anti*-BnSP7 antibodies

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ARTICLE INFO

Article history:
Received 7 November 2016
Received in revised form
31 July 2017
Accepted 5 August 2017
Available online xxx

Keywords:
Bothrops pauloensis
Phospholipase A_2
BnSP-7-PLA_2
Polyclonal antibodies
Cross-reactivity
Inhibition

ABSTRACT

Polyclonal antibodies raised in Balb-c mice against BnSP-7, a Lys-49 phospholipase A2, were used to measure cross reactivity against other snake venoms. Using ELISA, these antibodies were able to recognize PLA_2s isoforms present in venoms of botropic snakes at 1:6400, 1:3200 and 1:100 ratios (w/w). These antibodies highly recognized proteins of low molecular weight (~14,000) from crude snake venom *Bp* and *Bm* by Western Blotting. PLA_2 these venoms, by alignment of primary structures demonstrated high identity with BnSP-7 PLA_2, especially in the C-terminal region. However, the crude snake venom *Bd* and *Bj*, showed low recognition. The PLA_2 activity of *Bothrops pauloensis*, *Bothrops moojeni* venoms or $BpPLA_2$-TXI was inhibited significantly when anti-BnSP-7 antibodies were incubated at 1:10 and 1:20 ratios (venoms or toxin:anti-BnSP-7, w/w), respectively. The myotoxic effect induced by the same venoms was also reduced significantly at 1:1, 1:10 and 1:20 ratios, by decreased creatine kinase levels. The *anti*-PLA2 polyclonal antibodies effectively recognized PLA2s from Bothrops pauloensis and Bothrops moojeni venoms, and neutralized specific catalytic and myotoxic activity.

1. Introduction

The World Health Organization recognizes envenomation by venomous snakes as a neglected tropical disease, particularly in the rural areas of tropical regions such as Africa, Asia and Latin America [1], with a mortality rate the same as other endemic diseases in distant regions and with difficult access to medical treatment [2]. In America, the snakes of the family Viperidae are responsible for most deaths from bites [3].

Snake venoms are glandular secretions enriched with wide variety of highly toxic enzymes and, when inoculated into the body of the victim, cause severe effects on the bite site and loss of internal balance. Among the molecules responsible for the symptoms of snake bite envenomation, phospholipases A_2 (PLA_2) exert the central role in this pathology. Phospholipases A_2 (EC 3.1.1.4) are widely distributed in living organisms and are responsible for the hydrolysis of phospholipids, releasing lyso-phospholipids and fatty acids [4]. These enzymes when analyzed in SDS PAGE under reducing and non-reducing conditions, or determined by mass spectrometry, reveal a molecular weight of approximately 14 kDa [5]. This versatile class of enzyme is classified into 6 types denominated: secreted, calcium-dependent, PAF-acetyl hydrolase, cytosolic, lipoprotein-associated and lysossomal PLA_2s [6]. Snake venom PLA_2s, described as secreted type, were the first protein discovered in this extensive superfamily and since the original classification were subdivided into two main groups, according to their structural features [7]. Group I, found in snake venom Elapidae/Hydrophidae and II Viperidae/Crotalidae [8]. The ones belonging group II are divided in two main subgroups, according to the residue at position 49 in the primary structure: Asp49 (D49) is enzymatically active while Lys49 (K49) presents low or no enzymatic activity [7,9–11].

Several snake venom PLA_2s have been isolated, among them

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http://dx.doi.org/10.1016/j.biologicals.2017.08.002
1045-1056/

highlights BnSP-7. It is a Lys49 homologue PLA_2 of approximately 14kDa from *B. pauloensis* venom. In regarding to functional properties, it is capable of inducing local tissue damage characterized by edema, necrosis and inflammatory response, as well as neuromuscular blockade [12].

These effects are considered medically challenging because they develop rapidly and the effectiveness of antivenom is limited, consequently, several studies aimed at identifying the main venom components responsible for this damage have been driven [13].

Therefore, several attempts have been made in order to improve antivenom therapy as well as the treatment of snakebites [2,14,15]. Several scientific studies have been developed and described in literature with the focus on neutralizing snake venom or isolated toxins, providing us more details on understanding the fundamental aspects of envenomation and treatment, in order to pursue other tools and/or alternative methods for the development of the most effective, safe, and affordable antiophidic therapeutic strategy [16].

Thus, with advanced technologies, recent studies began adapting as one of the therapeutic strategies in envenomation, the use of immunoglobulins (antibody IgG) or immunoglobulin fragments [$F(ab')_2$, Fab, scFv] obtained by enzymatic digestion or recombinant DNA techniques, and also using different combinations of these fragments (scFv dimer, nanobodies, human Fc nanobody) [17].

In the present study, polyclonal *anti*-BnSP-7 antibodies were produced in Balb/c mice and used to assess cross-reactivity against different crude snake venoms. Additionally, we also assayed their capacity to inhibit PLA_2 and myotoxic activities.

2. Materials and methods

2.1. Venoms and animals

Bothrops pauloensis, Bothrops diporus, *Bothrops jararaca, Bothrops alternatus* and *Bothrops moojeni* were obtained from Serpentarium Bioagents (Batatais, São Paulo, Brazil). After collection, the snake venoms were lyophilized and stored at −20 °C.

Balb/c male mice were obtained maintained under standard conditions (12 h light/dark cycle, temperature 22 ± 1 °C, relative humidity 60± 5%), according to CBEA (Centro de Bioterismo e Experimentação Animal) with diet and water *ad libitum*. The experimentation protocol was approved by the Committee of Ethics for Use of Animals of the Federal University of Uberlândia, Minas Gerais, Brazil (Protocol number: CEUA/UFU 008/12) and is in agreement with the ethical principles of animal experimentation adopted by the Brazilian Society of Science.

2.2. Purification of the phospholipases BnSP-7 and $BpPLA_2$-TXI

Phospholipases A_2 (BnSP-7 and $BpPLA_2$-TXI) were isolated from *Bothrops pauloensis* as previously described by Rodrigues et al. 1998 [18] and Ferreira et al. (2013) [19], respectively. The protein concentration was estimated by the method of Bradford, 1976 [20].

2.3. Electrophoresis in polyacrylamide gel (SDS-PAGE)

12.5% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to Laemmli, 1970 [21]. Snake venoms or toxins were heated at 100 °C for 5 min and then run under reducing (10% β-mercaptoethanol) conditions. The gel was stained with Coomassie brilliant blue R-250 and bovine serum albumin (66kDa), ovalbumin (45kDa), glyceraldehyde-3-phosphate dehydrogenase (36kDa), trypsinogen (24kDa), trypsin inhibitor (20.1kDa), α-lactalbumin (14.2kDa) were used as molecular marker.

2.4. Immunochemical assays

2.4.1. Production and purification of polyclonal antibodies

78 μg of BnSP7 (corresponding to half of LD_{50}) [12] was emulsified with complete Freund's Adjuvant in the first inoculation at 1:1 (v/v; immunogen/adjuvant) ratio and administered by *i.p.* route. After, 15 days a booster dose with the same amount of proteins but emulsified with incomplete Freund's Adjuvant was injected.

After seven days of the last immunization, the animals were sacrificed using ketamine® 10% (0.05 mL/kg) + xylazine® 2% (0.025 mL/kg) and blood samples, obtained by cardiac puncture and in the absence of anticoagulant, were centrifuged at 950 *g* for 10 min at 4 °C. The serums obtained from centrifugation were filtered using a sterile syringe coupled to a filter of 0.45μm in porosity. Sequentially, this material was applied to an affinity HiTrap Protein G Sepharose column (GE Healthcare) in order to isolate polyclonal antibodies. This chromatographic procedure was performed according to the manufacturer's instructions, equilibrating the resin with 0.02 M sodium phosphate buffer, pH 7.8 and finally eluting with 0.1 M Glycine-HCl buffer, pH 2.7. The IgGs were monitored at 280nm and collected in tubes containing the neutralization buffer 1.0 M Tris-HCl pH 9.0, to protect the native state of these immunoglobulins and the IgGs concentration was estimated by the method of Bradford, 1976 [20].

2.4.2. ELISA assay

In this assay 96-*well* microplates were coated with 100μL of 10 μg/mL (*Bothrops pauloensis*, *Bothrops jararaca*, *Bothrops diporus*, *Bothrops alternatus*, *Bothrops moojeni*) crude venom in 0.06mM $NaHCO_3$, pH 9.6. The plates were incubated overnight at 4 °C, then washed four times with TBS-T (Tris-buffered saline containing 0.05% Tween 20) and blocked with 5% (w/v) non-fat dry milk in TBS-T for 1 h at 37 °C. Polyclonal anti BnSP-7 antibodies at dilutions of 1:50, 1:100, 1:200, 1:400, 1:600, 1:800, 1:1600, 1:3200 and 1:6400 were added (100μL/*well*) in duplicate and incubated for 1 h at 37 °C. The plates were again washed four times with TBS-T, then incubated for 1 h at 37 °C with 100μL/*well* of anti-mouse IgG-horseradish peroxidase (Sigma-Aldrich, Fab specific diluted 1:5000), washed and revealed for 15–30 min with OPD substrate (*o*-Phenylenediamine, Sigma–Aldrich, UK). The reaction was stopped with 50% 0.1 M H_2SO_4 and the absorbance was recorded at 490nm using an automated ELISA reader (BioTek Instruments).

2.4.2.1. Western blotting. Bothrops pauloensis, *Bothrops jararaca*, *Bothrops diporus*, *Bothrops alternatus*, and *Bothrops moojeni* crude venoms (20 μg/μL) were submitted to 12.5% polyacrylamide gel electrophoresis in (SDS-PAGE), and the gel bands were transferred onto a nitrocellulose membrane (Sigma-Aldrich; 0.22μm pore size) for 2 h at 150mA. Subsequently, the membrane was blocked with 5% (w/v) non-fat dry milk in TBS (0.01 M Tris-HCl, 0.17 M NaCl, pH 7.6) for 1 h at 37 °C, washed three times with TBS buffer and incubated with primary antibodies (*anti*-BnSP-7, dilution 1:100 in TBS) for 4–5 h at room temperature. After incubation, three more washes were done and the membrane was incubated with 4μL anti-mouse IgG-horseradish peroxidase (Sigma–Aldrich, Fab specific diluted 1:5000) in 20mL TBS, for 1 h in agitation (30 *rpm*; at room temperature). Finally, after another three washes, the membrane was revealed with DAB + H_2O_2 (Sigma–Aldrich, 3,3′-Diaminobenzidine) and the reaction was stopped with deionized water.

2.5. Neutralization assays

Neutralization of PLA_2 and myotoxic activities were determined

Please cite this article in press as: Melo LL, et al., Cross-reactivity and inhibition myotoxic effects induced by *Bothrops* snake venoms using specific polyclonal *anti*-BnSP7 antibodies, Biologicals (2017), http://dx.doi.org/10.1016/j.biologicals.2017.08.002