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Biochemical characterization of venom from *Pseudoboa neuwiedii* (Neuwied's false boa; Xenodontinae; Pseudoboini)

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ABSTRACT

In this work, we examined the proteolytic and phospholipase A_2 (PLA_2) activities of venom from the opisthoglyphous colubrid *Pseudoboa neuwiedii*. Proteolytic activity (3 and 10 μg of venom) was comparable to that of *Bothrops neuwiedii* venom but less than *Bothrops atrox*. This activity was inhibited by EDTA and 1,10-phenanthroline but only slightly affected (≤30% inhibition) by PMSF and AEBSF, indicating it was mediated by snake venom metalloproteinases (SVMPs). The pH and temperature optima for proteolytic activity were 8.0 and 37 °C, respectively. The venom had no esterase activity, whereas PLA_2 activity was similar to *B. atrox*, greater than *B. neuwiedii* but less than *B. jararacussu*. SDS-PAGE revealed venom proteins > 100 kDa, 45–70 kDa, 21–24 kDa and ~15 kDa, and mass spectrometry of protein bands revealed SVMPs, cysteine-rich secretory proteins (CRISPs) and PLA_2, but no serine proteinases. In gelatin zymography, the most active bands occurred at 65–68 kDa (seen with 0.05–0.25 μg of venom). Caseinolytic activity occurred at 50–66 kDa and was generally weaker than gelatinolytic activity. RP-HPLC of venom yielded 15 peaks, five of which showed gelatinolytic activity; peak 7 was the most active and apparently contained a P-III class SVMP. The venom showed α-fibrinogenase activity, without affecting the β and γ chains; this activity was inhibited by EDTA and 1,10-phenanthroline. The venom did not clot rat citrated plasma but reduced the rate and extent of coagulation after plasma recalcification. In conclusion, *P. neuwiedii* venom is highly proteolytic and could potentially affect coagulation in vivo by degrading fibrinogen via SVMPs.

1. Introduction

Colubrid snakes belonging to the tribe Pseudoboini (family Dipsadidae, subfamily Xenodontinae) are considered to be a monophyletic group (Zaher et al., 2009; Vidal et al., 2010; Grazziotin et al., 2012) consisting of 11 genera (*Boiruna*, *Clelia*, *Drepanoides*, *Mussurana*, *Oxyrhopus*, *Paraphimophis*, *Phimophis*, *Pseudoboa*, *Rhachidelus*, *Rodriguesophis* and *Siphlophis*) and 47 species of moderate-size snakes with terrestrial, semi-arboreal and semi-fossorial habits (Pizzatto and Marques, 2002; Pizzatto, 2005; Bernarde and Abe, 2006; Scott et al., 2006). Most pseudoboine species inhabit forests and savannas and feed mainly on lizards, small mammals and occasionally other snakes (Alencar et al., 2013; Gaiarsa et al., 2013).

Human envenomation by pseudoboines has been reported for several species (Prado-Franceschi and Hyslop, 2002; Weinstein et al., 2011, 2013), including *Boiruna maculata* (Santos-Costa et al., 2000), *Clelia plumbea* and *Clelia clelia* (Pinto et al., 1991; Salomão et al., 2003) and *Oxyrhopus* spp. (often mistaken for truly venomous coralsnakes, *Micrurus* spp., because of their similar coloration patterns) (Salomão et al., 2003). This envenomation is characterized by a combination of edema, erythema, ecchymosis/local hemorrhage, 'stinging' pain, cyanosis around the wound and lymphadenopathy of varying degrees of intensity; systemic envenomation is generally absent.

Pseudoboa neuwiedii (Neuwied's false boa; ratonel) is a back-fanged nocturnal terrestrial species with a wide distribution throughout most of northern South America (Colombia, Guyana, French Guiana, Surinam, Venezuela and Peru) and much of the Brazilian Amazon. This species can reach up to 105 cm in length and dorsally is a uniform reddish brown or faded red (often with scattered black spots), except for the head and neck, which are black/brown with a variable single yellowish collar band in the region of the temples; the underside is a yellowish straw color (Fig. 1). *Pseudoboa neuwiedii* feeds on vertebrates, e.g., anurans, reptiles (including other snakes), and mice, killing them by constriction (Martins and Oliveira, 1998; Boos, 2001).

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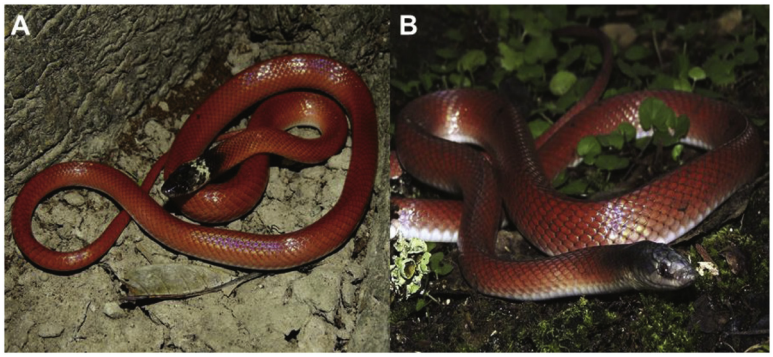

Fig. 1. *Pseudoboa neuwiedii* from Tolima, Colombia. A. Juvenile specimen. B. Adult specimen. There is no color variation between juvenile and adult specimens in this species. Photographs provided by James Herrán (Tolima, Colombia) and used with permission. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

As a back-fanged species, *P. neuwiedii* produces venom, but little is known of it composition or actions, and there are no reports of human envenomation by this species. This lack of reports on envenomation probably reflects a combination of (1) the docile, non-aggressive behavior of this species, even when handled by humans, (2) the fact that this species is most abundant in regions such as the Amazon basin where the human population density is generally very low, and (3) possible under-reporting of bites since envenomation by pseudoboines usually results in only mild local manifestations. We have recently shown that *P. neuwiedii* venom contains considerable proteolytic (caseinolytic) activity, low phospholipase A_2 (PLA_2) and negligible esterase activity; the venom also adversely affects neurotransmission in chick biventer cervicis neuromuscular preparations in vitro by producing moderate blockade and attenuating the muscle contractures to exogenously added acetylcholine and potassium chloride, in addition to producing mild muscle damage (Torres-Bonilla et al., 2017). In this report, we provide additional information on the biochemical characterization of *P. neuwiedii* venom, particularly in relation to its proteolytic activity and principal toxin families.

2. Material and methods

2.1. Reagents

The reagents for electrophoresis were obtained from Sigma Chemical Co. (St. Louis, MO, USA) or GE LifeSciences (Piscataway, NJ, USA). Molecular mass markers were from BioRad Laboratories (Hercules, CA, USA). 4-(2-Aminoethyl)benzenesulfonyl fluoride (AEBSF), azocasein, *N*-benzoyl-L-arginine *p*-nitroanilide (BA*p*NA), casein, ethylenediaminetetraacetic acid (EDTA), gelatin, glycine, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 4-nitro-3-(octanoyloxy) benzoic acid, 1,10-phenanthroline, phenylmethylsulfonyl fluoride (PMSF), plasminogen-free bovine fibrinogen, Tris hydrochloride, Triton X-100 and trypsin were from Sigma. Isoflurane was from Cristália (Itapira, SP, Brazil). The other reagents were of analytical grade obtained from GE LifeSciences, Mallinckrodt/J.T. Baker (Mexico City, DF, Mexico) and Merck S.A. (Rio de Janeiro, RJ, Brazil).

2.2. Venoms

Pseudoboa neuwiedii venom was a pool collected from two adult snakes (1M, 1F) maintained at the Laboratorio de Herpetología, Eco-Fisiología y Etología of the Universidad del Tolima (Ibagué, capital city of Tolima, Colombia) under Environmental License No. 2046 (June 13, 2012) provided by Corporación Autónoma Regional del Tolima (CORTOLIMA). The venom was collected using micropipettes placed over the enlarged rear fangs and then lyophilized and stored at −20 °C (Ferlan et al., 1983; Assakura et al., 1992). The mean volume and yield of venom per extraction was 200 μl and 2 mg of dried venom, respectively, based on two extractions from each of the specimens used here. When required, the venom was freshly dissolved in saline (0.9% NaCl) solution prior to use. Venoms from adult *Bothrops atrox*, *Bothrops jararacussu* and *Bothrops neuwiedii* snakes of both sexes were obtained from the Centro de Extração de Toxinas Animais (CETA, Morungaba, SP, Brazil) and stored lyophilized at −20 °C.

2.3. Animals

Male Wistar rats (300–400 g) obtained from the Multidisciplinary Center for Biological Investigation (CEMIB) at UNICAMP were housed in ventilated racks (Alesco®) in standard plastic cages with a wood shaving substrate (5 rats/cage) at 23 °C on a 12 h light/dark cycle (lights on at 6 a.m.) and had free access to standard rodent chow (Nuvital®) and water. The animal protocols were approved by an institutional Committee for Ethics in Animal Use (CEUA/UNICAMP, protocol no. 4479-1/2017) and the experiments were done according to the general ethical guidelines for animal use established by the Brazilian Society of Laboratory Animal Science (SBCAL; http://www.sbcal.org.br/conteudo/view?ID_CONTEUDO=65) and Brazilian legislation (Federal Law no. 11,794, of October 8, 2008), in conjunction with the guidelines for animal experiments established by the Brazilian National Council for Animal Experimentation (CONCEA, http://www.mct.gov.br/index.php/content/view/310553.html; http://www.mct.gov.br/upd_blob/0234/234054.pdf) and EU Directive 2010/63/EU for the Protection of Animals Used for Scientific Purposes.

2.4. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and zymography

SDS-PAGE was done in a discontinuous system with a 4% polyacrylamide stacking gel prepared in 0.5 M Tris-HCl, pH 6.8, containing 0.4% SDS, and a 10% running gel prepared in 1.5 M Tris-HCl, pH 8.8, containing 0.4% SDS (Laemmli, 1970). The samples were diluted in sample buffer containing 4% bromophenol blue, 0.06 M Tris-HCl, 2% SDS and 10% glycerol and boiled for 5 min prior to loading onto the stacking gel. The gels (10 cm × 12.5 cm) were run in a mini-VE 206E system (GE Life Sciences) at constant voltage (100 mV), with 0.625 M Tris-HCl, 1.92 M glycine and 1% SDS, pH 6.8, as the running buffer. Molecular mass markers were included in all runs. After electrophoresis, the gels were stained with Coomassie brilliant blue G250 and documented.

Zymography was done as previously described (Miyazaki et al., 1990). Briefly, samples were mixed with SDS sample buffer in the absence of a reducing agent, incubated for 20 min at 37 °C and separated on a 10% polyacrylamide gel containing gelatin (final concentration: 0.1%) or casein (final concentration: 0.8%). After electrophoresis, gels were soaked in 2.5% Triton X-100 for 40 min then digested by incubating the gel in 50 mM Tris-HCl, pH 7.4, containing 0.1 mM NaCl, 1 M dehydrated $CaCl_2$ and 0.02% NaN_3 at 37 °C for 16 h. The gels were stained with 0.1% Coomassie brilliant blue G250 and proteolytic activity was detected as clear bands against a blue background.