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Venom of the Peruvian snake *Bothriopsis oligolepis*: Detection of antibacterial activity and involvement of proteolytic enzymes and C-type lectins in growth inhibition of *Staphylococcus aureus*

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ABSTRACT

There is a rising interest in snake venoms proteins (SVPs) because these macromolecules are related to pharmacological properties that manifest themselves during poisoning and can lead to secondary microbial infections. Interestingly, researchers have somehow neglected the antimicrobial activity of SVPs. The aims of this study were: (i) to verify whether the venom of the Peruvian snake *Bothriopsis oligolepis* displays such activity; (ii) to isolate and identify some of its antimicrobial constituents. Liquid growth inhibition assays revealed that the crude venom inhibited the growth of Gram-positive and Gram-negative bacteria, but not of *Candida* species. Fractionation of the venom by anion-exchange chromatography provided fractions P2, P4 and P8 active against *S. aureus*. Fractionation of P2 or P8 by gel-filtration chromatography and of P4 by RP-HPLC furnished the sub-fractions P2-I, P8-II and P4-II, respectively, being those fractions active against *S. aureus*. Analyses of these sub-fractions by SDS-PAGE under denaturing/reducing conditions evidenced SVPs with 59–73, 27 and 14–28 kDa, respectively. Their in-gel tryptic digestion gave peptide fragments, whose sequencing by MALDI-TOF/MS followed by protein BLAST analysis allowed identifying PIII metalloprotease(s) [SVMP(s)] in P2-I, serine protease(s) [SVSP(s)] in P4-II and lectin(s) in P8-II. Detection of gelatinolytic activity in P2-I and P4-II reinforced the existence of PIII-SVMP(s) and SVSP(s), respectively. Activation of the coagulation cascade intrinsic pathway by P8-II (probably by interaction with factors IX and/or X as some snake C-type lectins do) supported the presence of C-type lectin(s). Altogether, these new findings reveal that the venom of the Peruvian snake *Bothriopsis oligolepis* displays antibacterial activity and that the isolated SVMP(s), SVSP(s) and C-type lectin(s) are associated to its ability to inhibit the growth of *S. aureus*.

1. Introduction

Bacteria and fungi have become dangerous pathogens for humans mainly because of their ability to acquire resistance to antibiotics. The acquisition of such resistance is explained by changes in human demographics, globalization and, mostly, attempts taken in hospitals to cure microbial infections (de Oliveira Junior et al., 2013). In this context, the interest for novel antimicrobial agents has greatly increased over the last decades.

Many of the commercially available antibiotics were either directly isolated from microorganisms or derive from these compounds (Bérdy, 2012). However, animal secretions have also been studied to reveal their constituents with antimicrobial and microbicide activities or, alternatively, to find inspiration for the design of

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new compounds with these properties (de Oliveira Junior et al., 2013; Samy et al., 2017). Snake venoms, for instance, are secretions composed by biomolecules that help these animals to immobilize, kill their preys, digest them and defend themselves against pathogens. In fact, despite the envenomation by snakebite has been associated with low incidence of secondary bacterial infection, it is well-known that the oral cavity of snakes contains potentially pathogenic bacteria such as coagulase-negative *Staphylococci*, *S. epidermidis, Pseudomonas aeruginosa, Escherichia coli, Enterococcus* sp., *Proteus* sp. and *Clostridium* sp., which indicates the existence of antibacterial agents in snake venoms (Talan et al., 1991; Abrahamian and Goldstein, 2011; Dehghani et al., 2016). Although with low frequency, these bacteria have also been detected in wound infections secondary to snakebites of human victims. In contrast, *S. aureus,* usually found in human skin but not in the mouths of snakes, has been detected with high incidence in infected wounds or abscesses resultant from ophidian accidents (Saravia-Otten et al., 2007*)*. A large number of more recent studies focused on the microbiota of these lesions support such information. A good example is the work of Garg et al. (2009), who described that the infection produced by snakebite is associated with several pathogenic bacteria for humans, being *S. aureus* (32%) the most abundant (Garg et al., 2009; Abrahamian and Goldstein, 2011).

In these natural sources, proteins (SVPs) and peptides usually account for 90–95% of their dry weights and the mixture of amino acids, nucleotides, free lipids, carbohydrates and metal ions corresponds to the remaining 5% (De Lima et al., 2005). Most SVPs are enzymes and the remaining are disintegrins, myotoxins, helveprins/cysteine-rich secretory proteins (CRiSPs) and lectins (Mackessy, 2010). Among the snake venom enzymes, the most abundant are the serine proteases (SVSP), metalloproteases (SVMP), phospholipases A_2 (PLA_2) and L-amino acid oxidases (LAAO). There are also hyaluronidases, phosphomonoesterases, phosphodiesterases, phosphatases, 5'-nucleotidases, nucleases and acetylcholinesterases (De Lima et al., 2005; Mackessy, 2010). These enzymes have been associated with many pharmacological activities related to poisoning (snakebite and venom injection in the prey), including edema or hemorrhage induction, neurotoxicity, myotoxicity, cytotoxicity, antimicrobial activity and hypotension (Laraba-Djebari and Fatah, 2014).

In respect to SVPs with antimicrobial activity, LAAO from *B. mattogrossensis* venom was reported to act either on the Gram-negative bacteria *Klebsiella pneumoniae*, *E. coli* and *P. aeruginosa* or on the Gram-positive bacteria *S. aureus* and *Streptococcus pyogenes* (Okubo et al., 2012); LAAO from *Crotalus durissus cumanensis* venom (CdcLAAO) was more active against *S. aureus* than against Gram-negative bacteria; CdcLAAO was effective against *Acinetobacter baumannii* and inactive against *E. coli* (Vargas et al., 2013). A PLA_2 isolated from *B. jararacussu* venom was also able to kill *E. coli* and *S. aureus* (Roberto et al., 2004); $BmarPLA_2$ from *B. marajoensis* venom inhibited the growth of *S. aureus*, *C. albicans*, *P. aeruginosa* and *Leishmania* (Torres et al., 2010). The acidic $PnPLA_2$ from *Porthidium nasutum* venom inhibited *S. aureus* growth (Vargas et al., 2012), but the basic PLA_2 fraction VIIIa (VRV-PL-VIIIa) from *Daboia russelii pulchella* venom was more active against the Gram-positive bacteria *S. aureus* and *Bacillus subtilis* than against Gram-negative bacteria (Sudharshan and Dhananjaya, 2015). The lectin BlL from *B. leucurus* venom was also effective on *S. aureus, B. subtilis,* and *Enterococcus faecalis* (Nunes Edos et al., 2011); the C-type lectin from *B. jararacussu* venom inhibited the growth of several Gram-positive (*S. aureus*, *S. chromogenes*, *S. hyicus*, *Streptococcus agalactiae*) and Gram-negative (*E. coli*) biofilms without affecting cell viability (Klein et al., 2015); cathelicidin-BF from *Bungarus fasciatus* venom showed activity against Gram-negative and multidrug-resistant bacteria, pathogenic and saprophytic fungi (Wang et al., 2008; Samy et al., 2017). In contrast, to date, only Samy et al. (2008) described a zinc-dependent metalloprotease from *Agkistrodon halys* venom as active against Gram-positive bacteria. More interestingly, there is no report of SVSPs involvement in the antimicrobial activity of snake venoms. Overall, this information turns snake venoms proteins into potential targets or sources of inspiration for the development of new drugs and antimicrobial agents.

The snakes from the subfamily Crotalinae are commonly known as pitvipers because they have a pit organ or fossa in the loreal area on either side of the head. They are distributed in eastern Europe and Asia (*Calloselasma*, *Deinagkistrodon*, *Gloydius*, *Hypnale*, *Ovophis*, *Trimeresurus* and *Tropidolaemus*), North America (*Agkistrodon*, *Sistrurus* and *Crotalus*) as well as in Central and South Americas (*Bothrops*, *Bothriechis*, *Bothriopsis*, *Bothrocophias*, *Porthidium, Lachesis*, *Rhinocerophis*, *Ophryacus*, *Atropoides*, *Cerrophidion*) (Campbell and Lamar, 2004; Carrasco et al., 2012). The validity of the genus *Bothriopsis* is controversial because while some authors use it (Castoe and Parkinson, 2006; Fenwick et al., 2009), others synonymize *Bothrops* with *Bothriopsis* (Salomão et al., 1997; Carrasco et al., 2012).

Bothriopsis oligolepis is a venomous forest pitviper found in southeastern Peru (Cuzco, Junín and Puno) and northwestern Bolivia (Wallach et al., 2014). In 2015, Guerra-Duarte et al. described that the venom of this snake presents hyaluronidase, LAAO, SMVP and SPSV activities, but no PLA_2 activity (Guerra-Duarte et al., 2015). Since then, neither this venom nor any of its constituents was studied. Targeting to find snake venoms constituents with potential to inspire the development of new antimicrobial peptide agents, we performed the present study intended to: (i) first detect antimicrobial activity in the crude venom of the Peruvian snake *B. oligolepis*, (ii) first fractionate it, (iii) first isolate proteins/enzymes directly related to this important biological function.

2. Material and methods

2.1. B. oligolepis venom

The venom employed was from two adult specimens caught in Pichanaki, Department of Junin, Peru, and kept at the Laboratory of Biochemistry from the Faculty of Health Sciences, University of Callao, Peru. The pool of the venom collected corresponds to two milking per months during six months. The venom was pooled, lyophilized and stored at −20 °C.

2.2. Chemicals

SDS-PAGE Standard Low Range and Precision Plus Protein™ dual color standards were purchased from Bio-Rad (Hercules, CA, USA). All solutions and buffers were prepared with water purified by the Milli-Q® system (Millipore-Merck, Billerica, MA, USA). All the solvents and other chemicals used were of analytical grade, being either from Sigma-Aldrich (St Louis, MO, USA) or from Bio-Rad Laboratories (Hercules, CA, USA). Luria-Bertani broth was from Sigma-Aldrich (St Louis, MO, USA), PDB broth was from BD Difco (Franklin Lakes, NJ). Trypsin Gold of mass spectrometry grade was from Promega (Madison, WI).

2.3. Determination of protein content

Protein concentrations in the crude venom and in active fractions were determined using Bio-Rad Protein Assay Dye Reagent Concentrate (Bio-Rad, CA, USA) and bovine serum albumin (BSA) as standard protein (Bradford, 1976). The samples were prepared in