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Expression and partial biochemical characterization of a recombinant serine protease from *Bothrops pauloensis* snake venom

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ABSTRACT

Snake venom serine proteases (SVSPs) are enzymes capable of interfering at several points of hemostasis. Some serine proteases present thrombin-like activity, which makes them targets for the development of therapeutics agents in the treatment of many hemostatic disorders. In this study, a recombinant thrombin-like serine protease, denominated rBpSP-II, was obtained from cDNA of the *Bothrops pauloensis* venom gland and was characterized enzymatically and biochemically. The enzyme rBpSP-II showed clotting activity on bovine plasma and proteolytic activity on fibrinogen, cleaving exclusively the Aα chain. The evaluation of rBpSP-II activity on chromogenic substrates demonstrated thrombin-like activity of the enzyme due to its capacity to hydrolyze the thrombin substrate. These characteristics make rBpSP-II an attractive molecule for additional studies. Further research is needed to verify whether rBpSP-II can serve as a template for the synthesis of therapeutic agents to treat hemostatic disorders.

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1. Introduction

Snake venom is composed of a large variety of biologically active molecules that function to kill or weaken the prey. Snake venoms from Viperidae family species have large amounts of proteolytic toxins, capable of performing several actions on many metabolic processes, including hemostatic mechanisms (Markland, 1998; Higuchi et al., 2007; Sanchez and Swenson, 2007).

Proteomic studies of *Bothrops pauloensis* snake venom revealed that 10% of the protein corresponds to serine proteases (Rodrigues et al., 2012). Snake venom serine proteases (SVSPs) are classified in the clan PA, family S1 (chymotrypsin), subfamily A of proteolytic enzymes, according to the MEROPS database (Rawlings et al., 2012). These enzymes are glycoproteins synthesized as zymogens and present a single peptide chain and molecular weight ranging between 26 and 67 kDa according to the number of glycosylation sites in its structure. These proteases present a highly conserved catalytic region (His^{57}, Asp^{102} and Ser^{195}), and exhibit the ability to cause hemostatic disorders by interfering in various parts of the blood coagulation cascade (Braud et al., 2000; Serrano and Maroun, 2005; Serrano, 2013).

Structural cDNA analysis established that all SVSPs present a catalytic mechanism that involves a highly reactive Ser residue essential for the formation of the acyl-enzyme complex, stabilized by the presence of His and Asp residues at the active site. The SVSPs have 12 Cys residues in the chain, essential for maintaining the three-dimensional structure for holding disulfide bonds, and have their proteolytic activity inhibited by organophosphorus compounds such as DIFP and PMSF (Barrett and Rawlings, 1995; Itoh et al., 1987; Serrano, 2013).

Some of these SVSPs perform functions similar to those of thrombin and therefore such toxins are usually called Snake Venom Thrombin-Like Enzymes (SVTLEs). The SVTLEs are important molecular models in the development of drugs and therapeutic agents, since they exhibit all the main functions of thrombin and resistance

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to physiological inhibitors (Kornalik, 1985; Pirkle, 1998; Matsui et al., 2000; Serrano and Maroun, 2005). Some SVTLEs, such as Ancrod (Knoll, Ludwigshafen, Germany) isolated from *Callosellasma rhodostoma* and Batroxobin (Reptilase, Pentapharm, Basel, Switzerland) isolated from *Bothrops atrox*, have been used in patients with deep vein thrombosis, myocardial infarction and peripheral arterial thrombosis (Stocker and Barlow, 1976; Yu et al., 2007).

For many years, these proteins have been purified directly from the crude venom by traditional methods, which do not always result in sufficient yields. However, with advances in molecular biology techniques, several studies have been published showing advantages in heterologous protein expression (Maeda et al., 1991; Yu et al., 2007). Thus, research in the field of molecular biology, biotechnology and bioinformatics seeks to elucidate the relationship between the structure and function of these molecules, and study the development of drugs that can improve the quality of life of the population.

For many years, *Escherichia coli* was employed as the main host organism in heterologous protein expression systems because of its ease of use. However, proteins derived from eukaryotic genomes were not properly expressed in this system, since such species do not possess the cellular structures necessary for post-translational modifications. A wide variety of proteins that are not correctly expressed in *E. coli* were expressed in the methylotrophic yeast *Pichia pastoris* with satisfactory results both in yield and functionality (Daly and Hearn, 2005).

Currently, snake venom toxins have been used as tools for the investigation of blood clotting mechanisms and as molecular models for the development of new therapeutics drugs for the treatment of some pathologies (Trento et al., 2001). The study of the structural and functional enzymes in native and recombinant form showing various pharmacological and biotechnological applications can provide relevant information for the development of new and more effective therapeutic strategies. Serine proteases from different glands of snakes such as *Crotalus durissus terrificus* (Yonamine et al., 2009), *Bothrops moojeni* (You et al., 2004), *Agkistrodon acutus* (Zha et al., 2003), *Bothrops insularis* (Junqueira-De-Azevedo and Ho, 2002) were cloned from cDNA libraries in the eukaryotic expression system to corroborate its importance in developing recombinant proteins that have large pharmacological impact.

This study aimed to achieve the cloning, expression and partial biochemical characterization of a recombinant thrombin-like serine protease from *B. pauloensis* venom.

2. Materials and methods

2.1. Materials for cloning and expression

The snakes were kept in captivity at the Serpentarium Bioagents of Batatais – SP, Brazil. Gel loading buffer was purchased from Thermo Scientific. The Wizard® SV Gel and PCR Clean-Up System and Wizard Plus SV minipreps – DNA purification System kits were purchased from Promega. The PCR buffer, dNTP mix, Taq DNA polymerase enzyme and the Zeocin™ antibiotic were purchased from Invitrogen. pPICZαA vectors were purchased from Invitrogen. Thermo Scientific InsTAclone PCR Cloning Kit (pTZ57R/T cloning vector) was purchased from Fermentas. T4 ligase enzyme, *Xho*I and *Sal*I restriction enzymes and specific primers were purchased from Fermentas. *P. pastoris* KM71H strain, pPICZαA expression plasmid and Zeocin™ were purchased from Invitrogen™ (Carlsbad, US). The *E. coli* cells were stored in LB medium with 50% glycerol and yeast colonies were stored in YPD (1% yeast extract, 2% peptone, and 2% dextrose) with 15% glycerol at −80 °C.

2.2. Construction of recombinant expression vector

The gene encoding rBpSP-II was obtained from the cDNA library of the *B. pauloensis* venom gland (Rodrigues et al., 2012). The gene was amplified by PCR using the cDNA prepared from venom gland mRNA templates employing Taq DNA polymerase (Invitrogen), with forward (5′- AAACTCGAGAAAAGACAAAAGTCTTCTGAACTGG – 3′) and reverse (5′- AAAGTCGACCGGGGGGCAAGTCGCAGTTTT – 3′) primers. The fragment was purified from the 1% agarose gel, digested with *Xho*I and *Sal*I and ligated into pTZ57R/T cloning vector digested by the same enzymes. The ligated vector was transformed into *E. coli* DH5α cells, according to the manufacturer's protocol manual. The pTZ57R/T vector was extracted from *E. coli* cells and digested by the same restriction enzymes whereas the rBpSP-II gene was ligated with T4 ligase enzyme into a pPICZαA expression vector treated with the same restriction enzymes. The expression vector was transformed into *E. coli* DH5α cells, grown on LB low salt medium with Zeocin™ and confirmed by PCR.

The correct in-frame connection of the insert was confirmed by sequencing using ET Dye Terminator kit in the equipment MegaBACE 1000 - automated DNA sequencer (GE Healthcare, Chalfont St. Giles, UK).

2.3. Expression and purification of recombinant protein in *P. pastoris*

The pPICZαA/rBpSP-II vector was linearized with *PmeI* and transformed into *P. pastoris* KM71H by electroporation in a GenePulser (Bio-Rad, US) at 1500 V, 25 mF and 200 Ω. The transformants were selected and placed onto YPDS-agar medium (1% yeast extract, 2% peptone, 2% dextrose, 1 M sorbitol, 2% agar) containing 100 mg/mL of Zeocin™, and incubated at 30 °C for 3–5 days for selection of transformants. The recombinant colonies were subjected to colony PCR, and the product was visualized on a 1% agarose gel to confirm that the insert was incorporated in the yeast genomic DNA.

The transformant yeasts were screened for protein induction in 24-well plates, as described by Boettner et al. (2002). Recombinant yeast was selected for enzyme production and purification. Expression induction was carried out as described by Generoso et al. (2012), differentiated only by the use of BMGY (2.0% Peptone, 1.0% Yeast extract, 100 mM Potassium phosphate pH 6.0, 1.34% Yeast Nitrogen Base, 0.4 μg/mL Biotin, 1.0% Glycerol), with Zeocin™ under constant stirring of 250 rpm at 30 °C until a culture OD600 of 2 was reached. BMMY medium (2.0% Peptone, 1.0% Yeast extract, 100 mM Potassium phosphate pH 6.0, 1.34% Yeast Nitrogen Base, 0.4 μg/mL Biotin, 1.0% Methanol) was incubated at 26 °C at 250 rpm. Methanol was replaced every 24 h at a final concentration of 0.75%, for the maintenance of induction.

The recombinant rBpSP-II was purified by affinity chromatography using Ni-NTA agarose resin Superflow (Qiagen®). The fractions containing the recombinant rBpSP-II were pooled and desalted on Snake Skin pleated Dialysis Tubing 7000 MWCO, 22 mm dry diameter (Thermo Scientific) with PBS buffer (pH 8.0). The rBpSP-II was resuspended in ultrapure water and quantified by the Bradford method (1976), at 595 nm.

2.4. SDS-polyacrylamide gel electrophoresis

The rBpSP-II *Mr* was estimated by 12.5% (w/v) SDS-PAGE according to Laemmli (1970). Protein samples (10 μg) were heated at 100^{O}C for 5 min and the run was performed under reducing conditions. Afterwards, the gel was stained with Coomassie Brilliant Blue R-250. The molecular mass marker used contained β-galactosidase (116 kDa), bovine serum albumin (66.2 kDa), ovalbumin

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