



Biochemical and functional properties of a thrombin-like enzyme isolated from *Bothrops pauloensis* snake venom

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ABSTRACT

In the present study, a thrombin-like enzyme named BpSP-I was isolated from *Bothrops pauloensis* snake venom and its biochemical, enzymatic and pharmacological characteristics were determined. BpSP-I is a glycoprotein that contains both N-linked carbohydrates and sialic acid in its structure, with $M_r = 34,000$ under reducing conditions and $pI \sim 6.4$. The N-terminal sequence of the enzyme (VIGGDECDINEHPFL) showed high similarity with other thrombin-like enzymes from snake venoms. BpSP-I showed high clotting activity upon bovine and human plasma and was inhibited by PMSF, benzamidine and leupeptin. Moreover, this enzyme showed stability when examined at different temperatures (-70 to 37°C), pH values (3–9) or in the presence of divalent metal ions (Ca^{2+} , Mg^{2+} , Zn^{2+} and Mn^{2+}). BpSP-I showed high catalytic activity upon substrates, such as fibrinogen, TAME, S-2238 and S-2288. It also showed kallikrein-like activity, but was unable to act upon factor Xa and plasmin substrates. Indeed, the enzyme did not induce hemorrhage, myotoxicity or edema. Taken together, our data showed that BpSP-I is in fact a thrombin-like enzyme isoform isolated from *Bothrops pauloensis* snake venom.

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Abbreviations: BpSP-I, *B. pauloensis* serine proteinase; PMSF, phenyl-methylsulfonyl fluoride; MCD, minimum coagulant dose; TAME, *N- α -p-tosyl-L-arginine methyl ester; TFA, trifluoroacetic acid; PNGase F, peptide *N*-glycosidase.*

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1. Introduction

Snake components can stimulate or inhibit various stages of hemostasis, including the blood coagulation cascade, fibrinolysis, hypotension, vascular integrity and platelet function (Braud et al., 2000; Matsui et al., 2000; White, 2005).

The main active components that interfere with hemostasis can be grouped as: (1) procoagulants (factors V, X, IX and prothrombin activators, and proteases with action similar to thrombin, known as thrombin-like enzymes); (2)

anticoagulants (protein C activator, proteins that activate the formation of the complex factor IX/X, thrombin inhibitor and phospholipases A₂); (3) fibrin(ogen)olytic agents (fibrin and fibrinogen degradation, plasminogen activator); (4) vessel wall interaction agents (hemorrhagins); (5) agents that interfere on platelet functions (including toxins which are inducers or inhibitors of platelet aggregation); and (6) plasma protein activators (SERPIN inhibitors) (White, 2005).

Many of these snake toxins that interfere with hemostasis are enzymes, as phospholipases A₂, nucleotidases, phosphodiesterases, L-amino acid oxidases, serine proteinases and metalloproteinases, while others, such as disintegrins and C-type lectins, have no enzymatic activity (Markland, 1998).

The two main groups of proteases synthesized by exocrine venom glands of snakes and that are responsible for changes in hemostasis are known as metalloproteinases and serine proteinases. These groups comprise toxins that own highly homologous sequences, conserved during evolution.

Serine proteinases (SVSPs) responsible for coagulant activity *in vitro* are found in various snake venoms from different genera. These toxins are often referred to as thrombin-like enzymes (SVTLEs) by resembling the best known functions of thrombin, such as the ability to clot fibrinogen (Stocker et al., 1982; Kornalik, 1985; Pirkle, 1998). SVTLEs can be interesting molecular models for the development of drugs or therapeutic agents, mainly because they are resistant to physiological protease inhibitors, as SERPINS (Matsui et al., 2000; Serrano and Maroun, 2005). These enzymes have many therapeutic applications, including treatment of arterial and venous thrombosis, myocardial infarct, acute ischemia and disorders of the blood coagulation system (Kornalik, 1985; Markland, 1998; Henriques et al., 2004; Marsh and Williams, 2005).

SVTLEs can be found in venoms from several snake species, including *Bothrops pauloensis*. This species was first described by Amaral (1925) as *Bothrops neuwiedi pauloensis*, as one out of 12 subspecies of *B. neuwiedi*. Following the systematic revision of the *B. neuwiedi* complex by Silva (2004), the 12 subspecies resulted in seven distinct species as accepted by the Brazilian Society of Herpetology (BSH, 2005; Jansen, 2006). *Bothrops pauloensis* snake inhabits throughout the Brazilian territory, except in the Amazonian regions of Brazil. They are more frequently found in areas of agricultural activity of coffee and soy, pasture areas, reforestations and close to springs (Valle and Brites, 2008). In the present work, we isolated and biochemically and functionally characterized the first SVTLE from *Bothrops pauloensis* snake venom.

2. Materials and methods

2.1. Materials

Bothrops pauloensis snake venom, vacuum dried and stored at 4 °C, was obtained from specimens kept in the serpentarium of Pentapfarm, Minas Gerais, Brazil. CM-Sepharose, Phenyl-Sepharose CL-4B and molecular weight markers were purchased from Amersham Life Science Inc.

p-Nitroanilide substrates, H-D-Phe-pipecolyl-Arg-pNA.2HCl (S-2238), H-D-Ile-Pro-Arg-pNA.2HCl (S-2288), Z-D-Arg-Gly-Arg-pNA.2HCl (S-2765), H-D-Val-Leu-Lys-pNA.2HCl (S-2251), H-D-Val-Leu-Arg-pNA.2HCl (S-2266) and H-D-Pro-Phe-Arg-pNA.2HCl (S-2302) all came from Chromogenix. All other reagents used for chemical and biological characterization were of analytical grade and purchased from Sigma Chem. Co.

2.2. Animals

Male Swiss mice were obtained from Valle Institute (Minas Gerais, Brazil) and maintained under standard conditions (temperature 22 ± 1 °C, relative humidity 60 ± 5%, 12 h light/dark cycle) with diet and water *ad libitum*. The experimentation protocol was approved by the Committee of Ethics for the Use of Animals of the Federal University of Uberlândia-Minas Gerais, Brazil (protocol number 08-2008) and is in agreement with the ethical principles of animal experimentation adopted by the Brazilian Society of Science in laboratory animals.

2.3. Isolation of the thrombin-like enzyme

Bothrops pauloensis crude venom (122 mg) was applied on a CM-Sepharose column (2.0 × 20 cm), previously equilibrated with 0.05 M ammonium bicarbonate (AMBIC), pH 7.8. Elution was carried out using the same buffer at a flow rate of 20 mL/h, collecting 3 mL/tube. The fraction showing clotting activity was applied on a Phenyl-Sepharose CL-4B column (1.0 × 10 cm) equilibrated with 10 mM Tris-HCl buffer plus 3 M NaCl, pH 8.5. Elution occurred at room temperature, with 10 mL of the same buffer, followed by decreasing concentrations of NaCl (3, 2, 1 and 0.5 M) in 10 mM Tris-HCl buffer, pH 8.5, ending the elution process with water.

The active fraction was concentrated by ultrafiltration in AMICON YM10 System and then applied on reverse phase HPLC using a CLC-ODS C18 column of 2.0 × 2.5 cm (Shimadzu), which was equilibrated with solvent A (0.1% trifluoroacetic acid and 5% acetonitrile) and eluted with a concentration gradient of solvent B (70% acetonitrile, 0.1% trifluoroacetic acid) from 0 to 100% at a flow rate of 1 mL/min at room temperature. The protein contents were monitored at 280 nm and the single peak was separated and lyophilized to determine the N-terminal sequence and biochemical properties. Proteins were estimated by the method of Bradford (1976) and the purified enzyme was named BpSP-I.

2.4. Biochemical characterization

2.4.1. Determination of M_r

14% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to Laemmli (1970). Samples were heated at 100 °C for 5 min and then ran under reducing (SDS + 10% β-mercaptoethanol) and non-reducing conditions. The gel was stained with Coomassie brilliant blue R-250 or with silver staining (Pharmacia Biotech). M_r was estimated by interpolation from a linear logarithmic plot of relative molecular mass versus distance of migration. The molecular mass markers used were: bovine serum albumin

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