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Biochemical properties of a new PI SVMP from *Bothrops pauloensis*: Inhibition of cell adhesion and angiogenesis

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ABSTRACT

In the present work, we demonstrate some biochemical and functional properties of a new PI snake venom metalloproteinase (SVMP) isolated from *Bothrops pauloensis* snake venom (BpMP-II), in addition we evaluated its capacity to inhibit endothelial cell adhesion and *in vitro* angiogenesis. BpMP-II was purified after a combination of three chromatography steps and showed molecular mass of 23,000 Da determined by MALDI-TOF, an isoelectric point of 6.1 and the sequence of some fragments obtained by MS/MS (MALDI TOF\TOF) presented high structural similarity with other PI-SVMPs. BpMP-II showed proteolytic activity against azocasein, was able to degrade bovine fibrinogen and was inhibited by EDTA, 1.10 phenantroline and β-mercaptoethanol. BpMP-II did not induce local hemorrhage in the dorsal region of mice even at high doses and did not affect plasma creatine kinase (CK) levels when administered intramuscularly into the gastrocnemius muscle of mice. Moreover, this metalloproteinase decreased tEnd cells viability at concentrations higher than 20 μg/mL. With sub-toxic doses this metalloproteinase affected tEnd cell adhesion and was also able to inhibit *in vitro* angiogenesis. BpMP-II showed very important functional properties suggesting considerable therapeutic potential for this class of protein.

1. Introduction

Metalloproteinases or metalloendopeptidases (MP) are hydrolytic enzymes classified as endopeptidases (E.C.3.4.24) dependent on metal binding, usually zinc, for the catalytic site to be activated. They are important in physiological and pathological processes such as cellular adhesion, fertilization, signal transduction, intoxication by venomous animals, *etc.* These enzymes occur widely in all five kingdoms of life [1,2], the most studied of which are present in snake venom (SVMPs – **S**nake **V**enom **M**etallo**p**roteinases).

The group of SVMPs is characterized by a large molecular mass spectrum (20 a 110 kDa) according to the number of structural domains present in the structure. These domains have been characterized due to their specific functions and are named as catalytic domain, disintegrin or disintegrin-like domain, cysteine-rich domain and lectin-like domain. Fox and Serrano (2008) [3] classified SVMPs into 11 subclasses (PIa, PIIa, PIIb, PIIc, PIId, PIIe, D-I, PIIIa, PIIIb, PIIIc and PIIId) based on studies of SVMP precursors and products of post-translational modifications.

The structural complexity of SVMPs gives them the capacity to interfere in various physiological processes that resulting in several physiopathological changes such as hemorrhage, myonecrosis, inflammation and inhibition of platelet aggregation [4,5]. The main effect is related to their capacity to degrade extracellular matrix (ECM) proteins, cellular membrane proteins and plasma proteins [4,6–9]. Beyond those effects, metalloproteinases are also capable of interacting with specific receptors such as integrins on the surface of platelets [10,11], fibroblasts [12] and endothelial cells [13,14] thereby activating or inhibiting cellular responses.

SVMPs are reported to induce apoptosis and disrupting the angiogenic process of endothelial cells [15–20]. Angiogenesis is important in the pathogenesis of a broad range of disorders such as

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arthritis and cancer. The microvascular endothelial cell recruited by a tumor is an important target in cancer therapy and treating both the cancer cell and the endothelial cell in a tumor may be more effective than treating the cancer cell alone [21,22]. Currently, anticarcinogenic activities of snake crude venoms and their components have been recognized [23]. Thus the biodiversity of venoms and toxins makes them a unique source from which novel therapeutics may be developed.

Bothrops pauloensis venom toxins have been isolated in recent years and their functional and structural characteristics have been demonstrated [24–26]. Additionally, the transcriptomics and proteomics studies of the *Bothrops pauloensis* venom gland revealed the presence of many peptides and proteins that have not yet been isolated and characterized in this venom, such as metalloproteinases [27]. Due the great diversity of metalloproteinases present in *B. pauloensis* venom [27] and their great therapeutic potential [28], the present work aimed to isolate and to evaluate the biochemical and enzymatic properties of a new PI-metalloproteinase from *B. pauloensis*, as well as to evaluate its capacity to inhibit endothelial cell adhesion and *in vitro* angiogenesis.

2. Materials and methods

2.1. Venom and animals

Bothrops pauloensis venom was obtained from specimens kept at Serpentarium Bioagents, LTDA, Batatais-SP, Brazil. This serpentarium is registered in the Brazilian Institute of the Environment and Renewable Natural Resources (no. 471301).

Male BALB/c mice were obtained from CBEA (Centro de Bioterismo e Experimentação Animal) of Federal University of Uberlandia and maintained under standard conditions (12 h light/dark cycle, temperature 22 ± 1 °C, diet and water *ad libitum*). The procedures protocol was approved by the Committee of Ethics for Use of Animals of the Federal University of Uberlandia, Minas Gerais, Brazil (protocol number 046/09), and was in agreement with the ethical principles of animal experimentation according to the Brazilian Society of Science for laboratory animals (COBEA/SBCAL).

2.2. Reagents

CM-Sepharose, Sephacryl S300, HiTrap Capto-Q and Reverse phase C18 (Whatman®) chromatographic columns were obtained from Amersham Bioscience of Brazil. Acrylamide, N,N′-methylenebisacrylamide, N,N,N′,N′, tetramethylethylenediamine (TEMED), ammonium persulfate (PSA), sodium dodecyl sulfate (SDS), bromophenol blue, bovine fibrinogen, ethylenediaminetetraacetic acid (EDTA), bovine serum albumin (BSA), aprotinin, benzamidine, β-mercaptoethanol, 1,10-phenanthroline and MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium) bromide were obtained from Sigma–Aldrich Chemical Co. (St. Louis, MO, USA). Molecular weight markers were purchased from GE Healthcare Bio-Sciences (Pittsburgh, USA).

2.3. BpMP-II Isolation

Metalloproteinase BpMP-II was purified from *B. pauloensis* snake venom according to methods previously described by our research group [26,29] with some modifications. Initially, desiccated crude venom (200 mg) was dissolved in 2 mL of 50 mM ammonium bicarbonate (AMBIC) buffer (pH 7.8) and centrifuged at 3000 × *g* for 10 min at 4 °C. The supernatant was recovered (137 mg) and submitted to a CM-Sepharose column previously equilibrated with the same buffer. Fractions of 1 mL/tube were collected in a linear gradient from 0.05 M to 0.5 M AMBIC buffer pH 7.8 at a flow rate of 6.5 mL/h and monitored by an Ultrospec 1000 Spectrophotometer (Amersham Pharmacia Biotech) with an absorbance of 280 nm. The fractions were lyophilized and stored at −20 °C.

Fraction CM1 (40 mg) obtained from chromatography using the CM-Sepharose column was then dissolved in 0.05 M AMBIC and applied onto a HiPrep 26/60 Sephacryl S300 column previously equilibrated with AMBIC buffer (0.05 M, pH 7.8). Elution was performed using the same buffer with a flow rate of 0.2 mL/min on the Akta Prime Plus (Amersham Biosciences) system. Fractions were collected, lyophilized and stored at −20 °C. The fraction CM1S3 obtained from the previous step was then submitted to anion exchange chromatography using a Capto-Q column previously equilibrated with 0.05 M AMBIC buffer. Fractions of 1 mL/tube were collected in a linear gradient with 0.5 M AMBIC buffer at a flow rate of 0.4 mL/min. The main peak from the previous step, named BpMP-II, was diluted in 1 mL of trifluoracetic acid (TFA, 0.1%, (v/v)) and submitted to an HPLC system using a reverse phase column (Partisphere C18 Whatman®) (4.6 mm × 125 mm). Sample elution was initially made with 0.1% trifluoroacetic acid followed by a linear gradient of 80% acetonitrile at a flow rate of 1 mL/min at room temperature. The peak was isolated and lyophilized for further structural and chemical characterization.

The protein concentration was determined according to Bradford (1976) [30]. In this method, each sample was dissolved in 100 μL of deionized water and 3 mL of Bradford (Sigma–Aldrich Chemical Co.) reagent was added. The concentration was determined spectrophotometrically at 595 nm in triplicate.

2.4. Biochemical characterization

2.4.1. Determination of molecular Mr

BpMP-II *Mr* was estimated by 12.5% (w/v) SDS-PAGE [31]. Samples of protein (10 μg) were heated at 100 °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 bovine serum albumin (66 kDa), ovalbumin (45 kDa), carbonic anhydrase (29 kDa), trypsinogen (24 kDa), β-lactoglobulin (18 kDa) and α-lactalbumin (14.4 kDa) (Amersham Biosciences). Protein molecular mass was estimated by interpolation from a linear logarithmic plot of the relative molecular mass *versus* the distance of migration.

2.4.2. Molecular mass determination by MALDI-TOF

BpMP-II molecular mass was also determined by matrix-assisted laser desorption ionization time of flight mass spectrometry (MALDI-TOF-MS). Analyses were made by MALDI double TOF mass spectrometry (MALDI-TOF/TOF, AutoFlex III Bruker Daltonics, Bremen, Germany). Protein (80 μg) was resuspended in 10 μL of 0.1% TFA. Afterwards, 0.5 μL of this solution were mixture with 0.5 μL of sinapinic acid (0.1% TFA, 50% ACN) and added to the MALDI target plate (Bruker Daltonics) where they were homogenized and dried at room temperature. According to their mass ranges, protein was recorded in linear positive mode.

2.4.3. Isoelectric focusing

Isoelectric focusing was performed according to the manufacturer's instructions (GE Healthcare). Twenty-five micrograms of protein were dissolved in rehydration buffer (7 M urea, 2 M thiourea, 2% CHAPS (w/v), 10 mM DTT, 0.002% bromophenol blue) also containing IPG buffer (GE Healthcare). Polyacrylamide strips with immobilized pH (pH 3 to 10) were rehydrated in the IPGbox for 16 h. Afterwards, isoelectric focusing was performed on strips in the Ettan IPGphor 3 system using the following program: 200 V/1 h, 3000 V/1 h, 4000 V/1 h, 1250 V/1 h and 50 V/1 h.

Between the first and second dimensions, focused strips were equilibrated in 10 mL of 1.5 M Tris–HCl pH 8.8, 6 M urea, 30%