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BbMP-1, a new metalloproteinase isolated from *Bothrops brazili* snake venom with *in vitro* antiplasmodial properties

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ABSTRACT

This study describes the biochemical and functional characterization of a new metalloproteinase named BbMP-1, isolated from *Bothrops brazili* venom. BbMP-1 was homogeneous on SDS-PAGE, presented molecular mass of 22,933Da and pI 6.4. The primary structure was partially elucidated with high identity with others metalloproteinases from Viperidae venoms. The enzymatic activity on azocasein was evaluated in different experimental conditions (pH, temperature). A significant reduction in enzyme activity after exposure to chelators of divalent cations (EDTA), reducing agents (DTT), pH less than 5.0 or temperatures higher than 45 °C was observed. BbMP-1 showed activity on fibrinogen degrading Aα chain quickly and to a lesser extent the Bβ chain. Also demostrated to be weakly hemorrhagic, presenting however, significant myotoxic and edematogenic activity. The *in vitro* activity of BbMP-1 against *Plasmodium falciparum* showed an IC_{50} of 3.2 ± 2.0 μg/mL. This study may help to understand the pathophysiological effects induced by this group of toxin and their participation in the symptoms observed in cases of snake envenomation. Moreover, this result is representative for this group of proteins and shows the biotechnological potential of BbMP-1 by the demonstration of its antiplasmodial activity.

1. Introduction

Snake venom metalloproteinases (SVMPs) (EC 3.4.24) comprise a complex family of zinc-dependent endoproteases, also called zinc metalloproteinases. They are considered some of the most abundant and numerous constituents, composing between 11 and 65% of total venom protein (Calvete et al., 2007; Fox and Serrano, 2008; Terra et al., 2009). Their presence is not restricted to Viperidae, being also observed, although to a lesser extent, in the Colubridae (OmPraba et al., 2010; Weldon and Mackessy, 2012) and Elapidae families (Fernández et al., 2011; Kumar et al., 2011).

SVMPs are phylogenetically most closely related to the mammalian ADAM (a disintegrin and metalloproteinase) and

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ADAMTS (ADAM with thrombospondin type-1 motif) families of proteins and, together with them, constitute the M12B clan of metalloendopeptidases (Takeda et al., 2012). SVMPs display a wide variety of biological activities such as hemorrhage, inhibition of platelet aggregation, coagulopathy, myonecrosis and inflammatory responses (Fox and Serrano, 2005; Gutiérrez et al., 2005; Teixeira et al., 2005). These activities are associated with the presence or absence of additional domains besides proteinase domains such as disintegrin and disintegrin-like domains, cysteine domains, and a lectin-binding domains that make up the three different classes of SVMPs called P-I, P-II and P-III (Fox and Serrano, 2008).

P-I metalloproteinases make up the smallest (molecular mass) SVMP class. This class of enzymes contains a pro-domain and a proteinase domain, presents molecular masses between 20 and 30 kDa and displays fibrinogen and fibrinolytic activities (Akao et al., 2010; Girón et al., 2013; Markland and Swenson, 2013). Although P-I SVMPs are known for their proteolytic, apoptotic, myonecrotic and inflammatory activities, the effect of P-I SVMPs against *Plasmodium falciparum* had not been reported until now. In this study, the biochemical and functional characterization of a new metalloproteinase (EC 3.4.24) BbMP-1, isolated from *Bothrops brazili* venom was described.

2. Material and methods

2.1. Venom

A venom pool obtained from adult specimens of the *B. brazili* snake was acquired from Serpentário Bioagents de Batatais –SP. Dehydrated crude venom was maintained at 8 °C. Prior to the experiments, the venom solutions were filtered through a cellulose ester membrane of 0.22 mm average pore size (Millipore Ind. Com. Ltda, Brazil).

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2.2. Animals

Male Swiss mice (18–20 g) were housed in temperature-controlled rooms and received water and food *ad libitum* until used. Animal care was in accordance with the guidelines of the Brazilian College for Animal Experimentation (COBEA) and was approved by Committee for Ethics in Animals Utilization of IPEPATRO/FIOCRUZ-RO (Process nº 001/2012).

2.3. Purification of the metalloproteinase

Approximately 50 mg of *B. brazili* venom was solubilized in 1 mL of 50 mM ammonium bicarbonate, pH 8.0, clarified by centrifugation at 5.900 × g for 10 min at room temperature and applied to a column containing CM-Sepharose FF resin (GE Healthcare Lifesc.) (1.0 × 40.0 cm) previously equilibrated with the same buffer. Samples were eluted in a linear gradient from 50 to 500 mM ammonium bicarbonate at a flow of 1.0 mL/min. The eluted fractions were monitored at 280 nm. The purity of BbMP-1 was assessed by high pressure liquid chromatography on a reverse phase column (RP-HPLC). Therefore, the fraction was solubilized in aqueous trifluoroacetic acid (TFA) 0.1% (v/v) and applied to a C18 column (Discovery 4.6 cm × 25.0 cm, 4 mM – Supelco) previously equilibrated with this buffer solution and eluted using a linear gradient from 0 to 70% solution of TFA 0.1% (v/v) acetonitrile for 10 column volumes (CV) at a flow rate of 1 mL/min. The eluted fractions were monitored at 215 nm. The purification procedure was performed in an ÄKTA purifier 10 (GE Healthcare Lifesc.).

2.4. Biochemical characterization

The molecular mass (Mr) of BbMP-1 was determined by SDS-PAGE (12.5%) as described by (Laemmli, 1970) after staining with Coomassie Blue R-250 2.5%. Moreover, the molecular mass was confirmed by mass spectrometry performed in MALDI (matrix assisted laser desorption ionization) with two TOF analyzers (AXIMA TOF^2 Shimadzu Biotech) operating in linear mode using a saturated solution of sinapinic acid as a matrix ionization.

The isoelectric point of BbMP-1 was determined by two-dimensional electrophoresis as previously described (Da Silva et al., 2012) with modifications. Briefly, the sample was solubilized in rehydration solution (7 M urea, 2 M thiourea, 2.0% Triton X-100, 1.0% immobilized pH gradient (IPG buffer®, GE Healthcare Lifesc.) buffer and 0.002% bromophenol blue) and applied on 13 cm polyacrylamide strips with a pH gradient from 3.0 to 10.0 immobilized in a non-linear way (Immobiline® DryStrip). Isoelectric focusing was performed in a III IPGphor (GE Healthcare LifeSc.) System. After isoelectric focusing, the sample was reduced and alkylated and the strip positioned in the upper portion of 12.5% SDS-PAGE gel to perform the second dimension (30 mA, 210 min). The gel was stained with 2.5% Coomassie Blue R-250 and the image obtained with the Image Scanner III® and analyzed using the software IQTL (GE Healthcare LifeSc.).

2.5. Enzymatic hydrolysis

BbMP-1 was reduced with 20 mM dithiothreitol (DTT) in 50 mM ammonium bicarbonate for a period of 1 h at 30 °C. For alkylation of the thiol groups of cysteine residues the reduced protein was incubated with 150 mM iodoacetamide in 50 mM ammonium bicarbonate for 1 h at 30 °C protected from light. After this step, the protein was subjected to digestion by modified trypsin (Sequencing grade modified®) for 18 h at 37 °C protected from light. The excess of the reagent and electrolytes were removed with the use of a C-18 resin (Proxeon Stage tip®) and eluted with 70% acetonitrile/0.1% TFA. The samples were lyophilized and dissolved in 0.1% formic acid.

2.6. Protein sequence identification

Spectrometric analysis was performed on a NanoAcquity HPLC (Waters) coupled to a mass spectrometer OrbitrapVelos (Thermo Scientific). Protein was digested with trypsin and an aliquot of the digest was injected and separated on a C18 column (75 micron Hi, 10 cm, NanoAcquity, 1.7 mM BEH column, Waters). The peptides were eluted with the following gradient: 1–40% B in 20 min, followed by 40%–60% B gradient in 5 min a flow of 250 nL/min. Solution A consisted of 0.1% formic acid (v/v) in water and, solution B 0.1% formic acid (v/v) in acetonitrile. The peptide masses (m/z 300–1700) were measured in the Orbitrap 60,000 at a resolution of m/z 400. The most abundant peptides were selected and fragmented in the collision cell HCD with normalized collision energy (NCE) of 40% and nitrogen as the collision gas. The fragments were detected in the Orbitrap with a resolution of 7500FWHM 400 m/z. Data were collected with Thermo Xcalibur (v.2.1.0.1140).

Database search was performed against NCBiSerpentes database in the Proteome Discoverer software (Thermo Scientific) using Mascot as search engine. The search parameters were: 2 missed cleavages, fixed modifications: carbamidomethyl of cysteine; variable modifications: oxidation for methionine and pyro-Glu for N-terminal glutamine; peptide tolerance: 10 ppm for MS spectra and 0.05 Da for MS/MS spectra; and semy trypsin as enzyme. The