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BthMP: a new weakly hemorrhagic metalloproteinase from *Bothrops moojeni* snake venom

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ABSTRACT

In this work, a new weakly hemorrhagic metalloproteinase (BthMP) was purified from *Bothrops moojeni snake venom*. This enzyme was homogeneous by native and SDS-PAGE. It showed a polypeptide chain of 23.5 kDa, pI = 7.1, and N-terminal blocked. BthMP is comprised of high proteolytic activity on casein, fibrin and bovine fibrinogen, with no coagulating, esterase or phospholipase A_2 activities; it was inhibited by EDTA, EGTA and 1,10-phenanthroline and maintained its activity on pH from 7.0 to 9.0 and temperature from 5–40 °C. Assays with metal ions showed that Ca^{2+} is an activator, whereas Zn^{2+} and Hg^{2+} inhibited about 50 and 80% of its activity, respectively. The edema evidenced the important role of the toxin in the inflammatory activity of the venom. BthMP also caused unclotting, and provoked histological alterations in the gastrocnemius muscle of mice inducing hemorrhage, necrosis and leukocytic infiltrate. The molecular mass and the inhibition assays suggest that the metalloproteinase BthMP belongs to class P-I of SVMPs.

1. Introduction

The snake venoms are constituted of a true biochemical arsenal, containing a large variety of biologically active peptides and proteins. They are responsible for the development of local and systemic damage, and alterations at the site of the bite, which develop rapidly after the accident.

Among other components from snake venoms, the metalloproteinases (SVMPs) and the fibrin(ogen)olytic enzymes play a relevant role, being directly involved with several pathologies, such as: hemorrhage, edema, pain, necrosis as well as disturbance in blood coagulation system (Swenson and Markland, 2005; Gutiérrez et al., 2005). SVMPs are synthesized in the venom gland as large multidomain proteins, including a proenzyme domain and a highly

Abbreviations: AMBIC, ammonium bicarbonate buffer; BthMP, metalloproteinase from *Bothrops moojeni*; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene-glycol-bis(β-aminoethylether)tetraacetic acid; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate buffered saline; PVDF, polyvinylidene difluoride; SDS, sodium dodecyl sulfate; SVMPs, snake venom metalloproteinases; TAME, Nα-p-tosyl-L-argenine ester; TCA, trichoroacetic acid; TEMED, N,N,N′,N′-tetramethylethylenediamine; TCA, trichloroacetic acid.

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conserved zinc-proteinase domain (*HEBXHXBGBXH*) (Fox and Serrano, 2005; Ramos and Selistre-de-Araujo, 2006; Wang et al., 2005). They are classification according to their multidomain in classes P-I, P-II, P-III and P-IV. Class P-I includes the small SVMPs with or without hemorrhagic effect, whereas the classes P-II, P-III and P-IV have disintegrin-like, disintegrin-like and cysteine-rich, and disintegrin-like, cysteine-rich and lectin-like domains, respectively (Bello et al., 2006). Recently Fox and Serrano (2005) updated this classification of the SVMPs including the following precursor classes: P-IIa, P-IIb, P-IIIa and P-IIIb.

Snakes from the genus *Bothrops* (family Viperidae) are responsible for most of the envenomations that occur in Brazil, being responsible for 90% of all recorded snakebites in which the snake is identified (Ministério da Saúde, Ministério Nacional de Saúde, 2001; Camey et al., 2002; Silva et al., 2003; Bello et al., 2006). Snake venom metalloproteinases are able to hydrolyze proteins of the basal membrane, including fibronectin, laminin and type IV collagen. They also degrade fibrinogen and fibrin (Gutiérrez and Rucavado, 2000; Gay et al., 2005; Moraes and Selistre-de-Araujo, 2006; Mourada-Silva et al., 2008). Several of these enzymes have been largely studied and characterized from venoms of different *Bothrops* species, such as: *Bothrops asper* (Gutiérrez et al., 1995; Franceschi et al., 2000), *B. neuwiedi* (Rodrigues et al., 2000; Baldo et al., 2008), *B. moojeni* (Assakura et al., 1985; Serrano et al., 1993a,b; Bernardes et al., 2008), *B. cotiara* (Senis et al., 2006), *B. jararacussu* (Marcussi et al., 2007; Mazzi et al., 2004, 2007), *B. jararaca* (Assakura et al., 2003; Maruyama et al., 2002), *B. leucurus* (Bello et al., 2006; Magalhães et al., 2007; Sanchez et al., 2007). In this work, we describe the purification and biochemical characterization of BthMP, an α-fibrin(ogen)ase metalloproteinase from *B. moojeni* snake venom.

2. Material and methods

2.1. Material and animals

B. moojeni venom was purchased from Pentapharm of Brazil (Uberlândia-MG, Brazil). DEAE-Sephacel and Sephadex G-75 were purchased from Amersham Biosciences. Acrylamide, bromophenol blue, ethylenediaminetetraacetic acid (EDTA), benzamidine, bovine fibrinogen, human thrombin, β-mercaptoethanol, N,N′-methylene-bis-acrylamide, molecular weight markers and N,N,N′,N′-tetramethylethylenediamine (TEMED), and sodium dodecyl sulfate (SDS) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). All other chemicals were of analytical reagent grade. Male Swiss mice were kindly provided by the Institute Vallé and by Pentapharm of Brazil (Uberlândia-MG, Brazil).

2.2. Purification of the metalloproteinase BthMP

BthMP was purified from *B. moojeni* venom by a combination of ion-exchange chromatography on DEAE-Sephacel and gel-filtration on Sephadex G-75. Crude venom of *B. moojeni* (200 mg) was dissolved in 50 mM ammonium bicarbonate buffer (pH 7.8) and clarified by centrifugation at 10,000 × *g* for 10 min. The supernatant solution was chromatographed on a DEAE-Sephacel column (1.4 × 10 cm), previously equilibrated with 50 mM, pH 7.8, ammonium bicarbonate buffer (AMBIC) and eluted with a concentration gradient (5–50 mM) of the same buffer at a flow rate of 1.5 ml/min (the gradient was established in tube 60). Absorbance of the effluent solution was recorded at a wavelength of 280 nm. The fraction D2 showing strong proteolytic activity were collected, lyophilized, dissolved in 50 mM, pH 7.8 ammonium bicarbonate and applied on a 1 × 100 cm Sephadex G-75 column, previously equilibrated with the same buffer. The fraction (now named BthMP) was collected, concentrated and stored at 4 °C for subsequent biochemical analysis.

2.3. Biochemical characterization

Protein concentration was determined by the method of Itzhaki and Gill (1964), using bovine serum albumin as standard. SDS-PAGE without or after reduction with 4% β-mercaptoethanol was performed by the method of Laemmli (1970) using 14% gels and stained with coomassie blue R-250. The relative molecular mass of the purified enzyme was estimated from reducing gels by comparison with the molecular weight marker Dalton Mark VII-L (Sigma), consisting of bovine albumin (66 kDa), egg albumin (45 kDa), glyceraldehydes-3-phosphate dehydrogenase (36 kDa), carbomic anhydrase (29 kDa), trypsinogen (24 kDa), trypsin inhibitor (20 kDa) and α-lactalbumin (14.20 kDa). Moreover, electrophoresis for native protein was performed by the method of Reisfeld et al. (1962). The isoelectric focusing of the metalloproteinase was done according to Vesteberg (1972).

2.4. Enzymatic activity

2.4.1. Proteolytic activity upon fibrinogen

Fibrinogenolytic activity was assayed as described by Rodrigues et al. (2000), with slight modifications. Samples of 50 μL of bovine fibrinogen (1 mg/mL AMBIC pH 7.8) were incubated with 5 μg of the enzyme at 37 °C and different time intervals (5, 10, 15, 30, 45 and 60 min). The reaction was stopped with the addition of an equal volume of a denaturing buffer containing 2% SDS; 5% β-mercaptoethanol, 10% glycerol and 0.005% bromophenol blue. Reaction products were analyzed by 14% SDS-PAGE.

The effect of inhibitors on the fibrinogenolytic activity was assayed after preincubation of the enzyme (5 μg) with 10 μL of 10 mM EDTA, 1,10-phenanthroline, aprotinin, β-mercaptoethanol and leupeptin. Similarly, 5 μg of the enzyme were incubated with 50 μg bovine fibrinogen (1 mg/mL AMBIC pH 7.8) at different temperatures (5 to a 90 °C), and pH (3 to a 9) values.

2.4.2. Proteolytic activity upon fibrin and TAME esterase

These activities were assayed as described by Rodrigues et al. (2000).

2.4.3. Proteolytic activity upon casein

These activities were assayed as described by Rodrigues et al. (2000), using casein as substrate. Proteolytic activity was also assayed in the presence of divalent ions (Mg^{++},