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Biochemical and functional characterization of BmooSP, a new serine protease from *Bothrops moojeni* snake venom

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ABSTRACT

In this work, we describe the purification and characterization of a new serine protease enzyme from *Bothrops moojeni* snake venom (BmooSP). On SDS-PAGE, BmooSP was found to be a single-chain protein with an apparent molecular mass of 36,000 and 32,000 under reduced and non-reduced conditions, respectively. Mass spectrometry analysis showed that the BmooSP is composed by two isoforms with molecular mass of 30,363 and 30,070, respectively. The purified enzyme consists of 277 amino acid residues, disregarding the cysteine and tryptophan residues that have been degraded by acid hydrolysis, and its N-terminal sequence showed similarity with other serine protease enzymes. BmooSP induced blood-clotting *in vitro*, defibrination *in vivo*, caseinolytic and fibrin(ogen)olytic activities. The enzyme is stable at high temperatures (up to 100 °C) and shows maximum activity at pH around 7.0. Preliminary results show that BmooSP can induce the formation of a stable fibrin clot for more than 10 days. BmooSP presents medical interest because it can be used as biodegradable fibrin glue and for the treatment and prevention of cardiovascular disorders because of its ability to promote the defibrination *in vivo*, decreasing blood viscosity and improving blood circulation.

1. Introduction

Snake venom proteins present powerful action on the components of the hemostatic system (Braud et al., 2000; Cecchini et al., 2005; Garcia et al., 2004; Lu et al., 2005; Sanchez et al., 2005; Torres et al., 2012). Serino proteases, metalloproteases, phospholipases A_2, L-amino acid oxidases, 5'-nucleotidases, disintegrins and C-type lectin-like present in snake venoms are protein which can affect hemostasis at many different points (Sajevic et al., 2011). They may act on coagulation cascade factors, vascular integrity or platelet aggregation (Marsh and Williams, 2005).

Snake venom serine proteases (SVSPs) are usually single chain glycoprotein with highly conserved catalytic region (Ser^{195}, His^{57} and Asp^{102}) and a large diversity of substrates (Serrano and Maroun, 2005). SVSPs may act directly on fibrinogen and/or fibrin chains, triggering to proteolysis by depletion of some coagulation factors and making the blood incoagulable (Swenson and Markland Jr., 2005). Thrombin-like enzymes are SVSPs that are, like the thrombin, able to cleave the fibrinopeptide A or B from fibrinogen, but do not usually activate other coagulation factors. This enzyme triggers the formation of a "loose" fibrin clot that can be quickly degraded by fibrinolytic system (Castro et al., 2004). Paradoxically, these enzymes cause clotting *in vitro*, but they cause defibrination in *vivo* (Pérez et al., 2008; Sajevic et al., 2011; Stocker and Barlow, 1976). These SVSPs have been used in medicine for prevention and treatment of thrombosis due to its ability to reduce circulating fibrinogen levels (Vu et al., 2013).

These enzymes are of medical interest because they are able to degrade or to prevent fibrin clot formation and are not susceptive to proteases endogenous inhibitors in humans. Studies have shown that the thrombin-like enzymes induce a rapid and efficient thrombolysis (Amel and Fatima, 2015; Castro and Rodrigues, 2006; You et al., 2004; Yitao et al., 2014). The absence of undesirable

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physiological changes attributable to these enzymes show that they can be promising for clinical use. They function independently of the endogenous fibrinolytic system and provide a safe, effective, fast and specific mechanism to the dissolution of the fibrin clot, which can be an alternative for therapeutic use as an antithrombotic agent or in combination with other thrombolytic agents currently used (Serrano and Maroun, 2005).

Bothrops moojeni snake venom is rich in fibrin(ogen)olytic enzymes (Bernardes et al., 2008; Gomes et al., 2009; Morais et al., 2012; Oliveira et al., 2013; Serrano et al., 1993a; Torres et al., 2012). In this work, we describe the purification, biochemical and functional characterization of the BmooSP, a new serine protease enzyme from this venom.

2. Material and methods

2.1. Venom and animals

Desiccated *B. moojeni* venom was purchased from Bioagents Serpentarium (Batatais-SP, Brazil). Male Swiss mice (*Mus musculus*) weigh 20 at 30 g, were kindly provided by the Institute Vallé (Minas Gerais, Brazil). These animals were housed in a temperature-controlled room (23 °C) on an automatic 12 h light/dark cycle (light phase 6 a.m.–6 p.m.) with diet and water *ad libitum*. The bovine plasma samples were kindly donated by the veterinary hospital of the Federal University of Uberlândia. The experimental protocol was approved by the Ethics Committee on Animal Experimentation of the Federal University of Uberlândia (CEUA/UFU, Protocol number 028/09).

2.2. Reagents

Acrylamide, ammonium bicarbonate, ammonium persulfate, aprotinin, benzamidine, bovine fibrinogen, bovine thrombin, bromophenol blue, casein, ethylenediaminetetraacetic acid (EDTA), glucose, leupeptin, N,N,N0,N0-tetramethylethylenediamine (TEMED), N,N0-methylene-bis-acrylamide, phenanthroline, phenylmethylsulphonyl fluoride (PMSF), sodium dodecyl sulfate (SDS) and β-mercaptoethanol, were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Glycine, Tris, molecular mass markers for electrophoresis and all chromatographic media (DEAE Sephacel and Sephadex G-75) were purchased from GE Healthcare (Sweden). All other reagents used were of analytical grade.

2.3. Purification of the BmooSP

The crude venom of *B. moojeni* (200 mg) was dispersed in 2.0 mL of 0.05 M ammonium bicarbonate buffer (AMBIC, pH 7.8), clarified by centrifugation at 10,000 × g for 10 min and applied on a DEAE Sephacel column (1.5 × 15 cm). Chromatography was carried out at a flow rate of 20 mL/h, with a convex concentration gradient of the same buffer (0.05–0.45 M). Fractions of 2.0 mL/tube were collected, their absorbances at 280 nm were read and those corresponding to the peak D3 were pooled, lyophilized, dissolved in 50 mM AMBIC and applied on a Sephadex G-75 column (1 × 100 cm), previously equilibrated with the same buffer. The fraction named D3G2 was lyophilized and rechromatographed on a Sephadex G-75 column under the same conditions.

2.4. Estimation of protein concentration

Protein concentration was determined according to a microbiuret method described by Itzhaki and Gill (1964), using bovine serum albumin as standard. The isolated enzyme was named BmooSP.

2.5. Biochemical characterization

2.5.1. Electrophoretic analysis: molecular mass determination

BmooSP molecular mass was determined using polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS-PAGE) according to Laemmli (1970). The molecular mass standard proteins used were phosphorylase b (97,000 Da), bovine serum albumin (66,000 Da), ovoalbumin (45,000 Da), carbonic anhydrase (30,000 Da), soybean trypsin inhibitor (20,100 Da), and alpha-lactalbumin (14,400 Da). The relative molecular mass of the BmooSP was estimated by Kodak 1D image analysis software.

2.5.2. Mass spectrometry

Electrospray ionization mass spectrometry (ES/MS) was also used to determine the molecular mass and purity of BmooSP. A Perkin–Elmer SCIEX API300 triple quadruple mass spectrometer equipped with an electrospray ionization interface was operated in the positive-ion mode. The samples were dissolved in methanol/water/acetic acid (65/35/5) and applied into the mass spectrum by an automatic syringe injection at a flow rate of 20 μL/h. The parameters used were: ion spray voltage 5 KV, curtain gas (ultrapure nitrogen) flow 0.6 L/min, ring voltage 280 V and orifice voltage 50 V.

2.5.3. Isoelectric focusing

Isoelectric focusing of BmooSP was determined according to the method previously described by Vesterberg (1972). Buffalyte, pH range 3.0–10.0 was used to generate the pH gradient.

2.5.4. Determination of amino acid composition

Determination of amino acid composition was performed using a Hitachi U-8500 analyzer, which employs a system of post-column derivatization with ninhydrin. BmooSP was hydrolysis with 6N HCl at 109 °C for 24 h.

2.5.5. N-terminal sequence determination

N-terminal sequencing was performed on an automated sequenator (477/120A Applied Biosystems), according to Edman and Begg (1967). The identity of the primary sequence of the BmooSP was compared with other proteins using BLAST (Basic Local Alignment Search) (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

2.6. Functional characterization

2.6.1. Proteolytic activity

Proteolytic activity upon fibrinogen, fibrin and casein was assayed as previously described by Oliveira et al. (1999), with some modifications. Fibrin was prepared by the addition of 10 UNIH thrombin to 50 μL of bovine fibrinogen solution (1.5 mg/mL saline). Samples of 50 μL of bovine fibrinogen (1.5 mg/mL saline), fibrin or casein solutions (1.5 mg/mL saline) were incubated with 5 μg of the BmooSP for different periods of time at 37 °C. The reaction was stopped with 25 μL of Tris–HCl buffer (0.05 M, pH 8.8) containing 10% (v/v) glycerol, 10% (v/v) β-mercaptoethanol, 2% (w/v) SDS, and 0.05% (w/v) bromophenol blue. The samples were then analyzed by 14% (w/v) SDS- PAGE.

2.6.2. Coagulant activity

Coagulant activity was determined by mixing 5 μg of BmooSP with 200 mL of citrated bovine plasma or bovine fibrinogen solution at 37 °C in triplicate. Bovine plasma samples were mixed with 3.8% sodium citrate (9:1, v/v) and centrifuged at 2.500 × g for 15 min at 4 °C to obtain platelet-poor plasma. The activity was characterized by the immediate appearance of the fibrin clot. The fibrinogen-clotting times (NIH) were determined by the standard