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Isolation and characterization of a novel metalloprotease inhibitor from *Bothrops alternatus* snake serum

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ABSTRACT

Resistance of snakes and some other animals to snake envenomation has been attributed to soluble factors present in their tissues. Here we report the isolation of a novel metalloprotease inhibitor from *Bothrops alternatus* snake serum (named BaltMPI) with high purity, using a four-step chromatographic method. BaltMPI has molecular weights of 60.5 and 42.4 kDa, as determined by SDS-PAGE and mass spectrometry, respectively, and pI = 5.27. The first 60 amino acids from the N-terminal region of BaltMPI, determined by Edman's degradation, showed high homology (97%) with the snake venom metalloprotease inhibitor (SVMPI) BJ46a and other SVMPIs (78–82%). The chromatographic fractions and purified BaltMPI exhibited anti-hemorrhagic activity against Batroxase and BjussuMP-I. BaltMPI was stable over wide ranges of pH (1, 5, 8, and 9) and temperature (−80, −20, 4, 60, and 100 °C), and suppressed the fibrinogenolytic, fibrinolytic, and azocaseinolytic activities of Batroxase. BaltMPI specifically inhibited the activity of metalloproteases, without affecting the activity of serine proteases. Together, our results suggest that BaltMPI and other SVMPIs are promising molecules for the treatment of snake envenomation, in particular that caused by *Bothrops* sp.

1. Introduction

According to the World Health Organization (WHO), envenoming caused by snakebites is a worldwide tropical and subtropical neglected disease [1,2]. In Central America, it affects 5.500 patients per year [3]. In South America, especially in Brazil, the reported incidence is about 30,000 cases per year, with at least 100 deaths [4,5]. Snakes belonging to the genus *Bothrops* cause more than 70% of envenomation cases in Brazil with the highest morbidity rates [4,6,7]. Snake venom metalloproteases (SVMPs) provoke the severity of symptoms, such as intense proteolysis with rapid and drastic local and systemic injury [8–11].

SVMPs are zinc-dependent enzymes bearing three histidines in the catalytic site of their HEXXHXXGXXH *motif* and a *Met-turn*, a structurally and spatially conserved characteristic of these enzymes [12]. SVMPs are classified into three classes – P-I, P-II, and P-III –, according to the presence or absence of domains that are distinct from the protease domain [13]. The relevance of SVMPs to the physiopathology of bothropic envenomation is not limited to their hemorrhagic effect *per rhexis*. SVMPs also exert a dual action as coagulant proteins that directly activate prothrombin and factor X of the clotting cascade, and as anticoagulant proteins with fibrino(geno)lytic activity that degrade the α, β, and/or γ fibrinogen chains and the *Von Willebrand* factor, and thereby inhibit platelet aggregation. Proteolysis of the clotting factors accounts for the local and systemic effects of SVMPs [14,15].

Abbreviations: BaltMPI, metalloprotease inhibitor from *Bothrops alternatus* snake serum; BjSP, serine protease from *Bothrops jararaca* snake venom; BjussuMP-I, metalloprotease from *Bothrops jararacussu* snake venom; cMSF, Chinese mamushi serum factor; HSF, Habu (*Trimeresurus flavoviridis*) serum factor; jMSF, Japanese mamushi serum factor; MHD, minimal hemorrhage dose; MIHD, minimal inhibitory hemorrhage dose; MW, molecular weight; PBS, phosphate-buffered saline; pI, isoelectric point; SVMP, snake venom metalloprotease; SVMPI, snake venom metalloprotease inhibitor; TFA, trifluoroacetic acid.

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Interestingly, some animals exhibit natural resistance to snake envenomation, which is attributed to two mechanisms: (i) presence of neutralizing factors or inhibitors in their plasma, serum, or muscle that bind to the venom toxins [16–18]; and (ii) structural changes in receptors, which prevent binding of the venom toxins [19]. Both mechanisms are natural immune responses that these animals have developed after millions of years of phylogenetic evolution [20].

Literature reports the identification and/or isolation of several SVMP inhibitors (SVMPIs), which act as anti-hemorrhagic factors, from the plasma or serum of different venomous and non-venomous snakes, as well as from the plasma, serum, or muscle of mammals such as opossums, hedgehogs, mongooses, and squirrels [18,20]. These inhibitors protect venomous snakes against the effects of venom from themselves or from other snakes belonging to the same family [21–24]. Some non-venomous snakes (ophiophagus or not) are also resistant to the venom of other snakes [25].

Some well-characterized inhibitors are: the Habu serum factor (HSF), isolated from *Trimeresurus flavoviridis* snake serum – it is an endemic species of the Japanese island Ryukyu that belongs to the *Viperidae* family [26,27] –; BJ46a, isolated from *Bothrops jararaca* serum [28]; and Chinese mamushi serum factor (cMSF) and Japanese mamushi serum factor (jMSF) inhibitors, isolated from the serum of *Gloydius blomhoffii brevicaudus* and *Gloydius blomhoffii* snakes, respectively [29]. HSF is an acidic glycoprotein that inhibits the hemorrhagic activity of the two autologous metalloproteases HR1 and HR2, which belongs to the P-III and P-I metalloprotease groups, respectively. HSF has a molecular weight (MW) of 70 kDa in its native form, and of 47.8 kDa when determined by matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry; its pI is 4.0. The Habu (*T. flavoviridis*) serum inhibits the hemorrhagic activity of several venom toxins produced by snakes belonging to the same family [26,27,30]. The BJ46a factor is an acidic glycoprotein with MW = 46 kDa (79 kDa homodimer in its native state) and pI of 4.6. This factor inhibits the hemorrhagic activity of *B. jararaca* venom, Atrolysin C (a class P-I metalloprotease present in *Crotalus atrox* venom), and Jararhagin (a class P-III metalloprotease present in *B. jararaca* venom) [28]. cMSF and jMSF are acidic glycoproteins with MW of 40.5 kDa that exert anti-hemorrhagic action against several class P-I and P-III metalloproteases; cMSF also inhibits the hemorrhagic action of autologous venom [29].

SVMPIs are important and interesting subjects of study due to their ability to inhibit the hemorrhagic and/or proteolytic effects of venoms and/or isolated metalloproteases. SVMPIs are potential complements to serum therapy to either reduce or inhibit the drastic local effects of bothropic envenomation, which can not be treated or reversed with anti-ophidian serum administration. In addition, SVMPIs are important tools to elucidate the structural and functional characteristics of the inhibited SVMPs. In this sense, the present study aims to isolate and characterize biochemically a new metalloprotease inhibitor (BaltMPI) from *B. alternatus* snake serum.

2. Materials and methods

2.1. Animals

Female Swiss mice (18–20 g) provided by the Central Vivarium at the Ribeirão Preto Campus of the University of São Paulo (Ribeirão Preto, SP, Brazil) were used to assess the inhibition of the hemorrhagic activity of Batroxase. The Ethics Committee on Animal Use (CEUA) at the Ribeirão Preto Campus of the University of São Paulo approved the study protocol (n. 12.1.231.53.7), which complied with the guidelines of the Brazilian College of Animal Experimentation (COBEA).

2.2. B. alternatus *snake serum*

B. alternatus snake serum was kindly donated by the Center for the Study of Venoms and Venomous Animals (CEVAP), UNESP – Universidade Estadual Paulista, Botucatu, SP, Brazil, and by the Serpentarium of the Ribeirão Preto Medical School at the University of São Paulo (FMRP-USP), Ribeirão Preto, SP, Brazil.

2.3. *Venom and proteases*

The crude crystallized *B. atrox* snake venom was acquired from the Centre of Animal Toxins Extraction (CETA) (Morungaba, SP, Brazil) and was used to obtain Batroxase (a class P-I metalloprotease). BjussuMP-I (a class P-III metalloprotease present in *B. jararacussu* venom) and BjSP (a serine protease present in *B. jararaca* venom) were isolated following the protocols described by Mazzi et al. [31] and Carone et al. [32], respectively.

2.4. *Purification of the metalloprotease inhibitor from B. alternatus serum*

Purification of the metalloprotease inhibitor from snake serum required four chromatographic steps: (1) ion-exchange chromatography using a DEAE Sepharose™ Fast Flow column (GE Healthcare Life Sciences; Pittsburgh, PA, USA); (2) molecular exclusion chromatography using a Superdex™ 200 10/300 GL column (GE Healthcare); (3) ion-exchange chromatography using a MonoQ™ 5/50 GL column (GE Healthcare); (4) reversed-phase chromatography using a CLC-ODS C18 column (Shimadzu; Kyoto, Japan).

In the four chromatographic steps, protein elution was monitored by recording absorbance at 280 nm. After plotting the data and analyzing the chromatographic profile – with the aid of the GraphPad Prism software (version 5 for Windows, GraphPad Software Inc., San Diego, CA, USA) –, the eluted peaks were pooled, lyophilized, and assessed for their anti-hemorrhagic activity (to determine the presence of a metalloprotease inhibitor) and purity (SDS-PAGE analysis). The fraction that exhibited anti-hemorrhagic effect against Batroxase was submitted to the next chromatographic step.

The particularities of each chromatographic step are described below.

2.4.1. *Ion-exchange chromatography using a DEAE sepharose™ fast flow column*

Fifty mg of *B. alternatus* lyophilized serum were dissolved in 2.0 mL of 0.05 M NH_4HCO_3 buffer pH 8.0 (buffer A) and centrifuged at 10,000*g* for 10 min. The resulting supernatant was applied onto a XK 26/20 (200 mm × 26 mm) column filled with DEAE Sepharose™ Fast Flow resin, previously equilibrated with buffer A, using the fast protein liquid chromatography (FPLC) system (ÄKTAFPLC, GE Healthcare). Elution was performed at a flow rate of 0.5 mL/min, using a segmented gradient of 1.0 M NH_4HCO_3 buffer pH 8.0 (buffer B): 0% B (0–220 min), 0–70% B (220–920 min), 100% B up to 970 min.

2.4.2. *Molecular exclusion chromatography using a superdex™ 200 column*

Ten mg of the bioactive fraction eluted from the ion-exchange column were dissolved in 4.0 mL of 0.05 M NH_4HCO_3 buffer pH 8.0 and centrifuged at 10,000*g* for 10 min. A 500 μL aliquot of the supernatant was applied onto a Superdex™ 200 10/300 GL column using the ÄKTA-FPLC system (GE Healthcare). Elution was

performed with 0.05 M NH_4HCO_3 pH 8.0, at a flow rate of 0.25 mL/min; fractions of 500 μL were collected.

2.4.3. Ion-exchange chromatography using a MonoQ™ 5/50 GL column

A 4.5-mg aliquot of the bioactive fraction eluted from the molecular exclusion column was dissolved in 1.5 mL of 0.05 M NH_4HCO_3 buffer pH 8.0 (buffer A) and centrifuged at 10,000*g* for 10 min. A 500-μL aliquot of the supernatant was applied onto a MonoQ™ 5/50 GL column using the ÄKTA-FPLC system (GE Healthcare). Elution was carried out at a flow rate of 0.5 mL/min, using a linear gradient (0–100%) of 1.0 M NH_4HCO_3 buffer pH 8.0 in 120 column volumes of eluent. Fractions of 2.0 mL were collected.

2.4.4. Reversed-phase chromatography using a CLC − ODS C18 column

One mg of the bioactive fraction eluted from the previous chromatographic step was dissolved in 1.5 mL of 0.1% trifluoroacetic acid (TFA; solution A) and centrifuged at 10,000*g* for 10 min. A 500-μL aliquot of the supernatant was applied onto a CLC-ODS C18 column (4.6 mm x 25 cm), previously equilibrated with solution A, using the high-performance liquid chromatography (HPLC) system equipped with the SPD 10AV-vp detector (Shimadzu). Elution was performed using a linear gradient of solutions A and B (70% acetonitrile + 0.1% TFA): 0–100% B in 65 min, at a flow rate of 1.0 mL/min.

2.5. Purity analysis

Purity of BaltMPI was analyzed by SDS-PAGE and reversed-phase HPLC as described below.

2.5.1. SDS-PAGE

SDS-PAGE analysis was performed using a 12% (w/v) polyacrylamide gel, according to the method of Laemmli [33]. The gel was stained with 0.2% Coomassie blue G for 30 min and destained with 10% acetic acid in 50:50 (v/v) water/ethanol solution. The following MW markers (Thermo Fischer Scientific, Waltham, MA, USA) were run in parallel with the samples: β-galactosidase (116 kDa), bovine serum albumin (66.2 kDa), ovalbumin (45 kDa), lacto dehydrogenase (35 kDa), rease Bsp981 (25 kDa), β-lactoglobulin (18.4 kDa), and lysozyme (14.4 kDa).

2.5.2. Reversed-phase HPLC

About 30 μg of BaltMPI was applied onto a C18 reversed-phase HPLC column (0.46 × 25 cm; Shimadzu), previously equilibrated with 0.1% (v/v) TFA. Elution was performed at a flow rate of 1 mL/min, using a linear concentration gradient of 5–95% of solvent B (70% acetonitrile + 0.1% TFA) for 30 min. Protein elution was monitored by recording absorbance at 280 nm.

2.6. Determination of protein concentration

Total protein concentration in the samples was determined by the 280/205 nm absorption method [34].

2.7. Assessment of hemorrhagic activity

The hemorrhagic activity of metalloproteases was assessed according to Kondo et al. [35], with modifications. Batroxase (10 μg) dissolved in 50 μL of phosphate-buffered saline (PBS) or an equivalent volume of PBS (negative control) was injected intradermally in the dorsum of mice previously anesthetized with a xylamine (10 mg/kg i.p)/ketamine (80 mg/kg i.p) solution (1:1). After 2 h, the animals were euthanized in a CO_2 chamber, their dorsal skin was removed, and the hemorrhagic halos were recorded photographically.

2.8. Assessment of anti-hemorrhagic activity

2.8.1. With pre-incubation

Different amounts of the isolated metalloprotease inhibitor BaltMPI or the chromatographic fractions were pre-incubated with Batroxase for 30 min, at 37 °C. Both the negative (PBS) and the positive control (Batroxase) were incubated under the same conditions. Thereafter, the samples were injected intradermally in the dorsum of mice previously anesthetized with a xylamine (10 mg/kg i.p)/ketamine (80 mg/kg i.p) solution (1:1). After 2 h, the animals were euthanized in a CO_2 chamber and their dorsal skin was removed to assess the degree of anti-hemorrhagic activity.

To determine the minimum inhibitory hemorrhagic dose (MIHD), the following BaltMPI concentrations were used to built a dose-response curve: 0.1, 0.5, 2.5, 5.0, 7.5, 10.0, and 12.5 μg. The MIHD was defined as the minimum amount of inhibitor required to completely inhibit the activity of a metalloprotease in its minimal hemorrhagic dose (MHD). MHD is defined as the minimum amount of protein required to produce a hemorrhagic halo of 10 mm of diameter in 2 h [36].

We also examined the anti-hemorrhagic activity of BaltMPI (10 μg) against BjussuMP-I, a class P-III metalloprotease isolated from *B. jararacussu* venom, assessed in its MHD (4 μg).

2.8.2. Without pre-incubation

We performed the same assay described above, without pre-incubating BaltMPI and Batroxase before injection in the mice dorsum. BaltMPI and Batroxase were assayed in duplicate at their MIHD (5 μg) and MHD (10 μg), respectively.

2.9. Fibrinogenolytic activity inhibition

The BaltMPI ability to suppress the fibrinogenolytic activity of snake venom toxins was evaluated according to the method described by Edgar and Prentice [37], including with the modifications reported by Rodrigues et al. [38]. Briefly, 50 μg of bovine fibrinogen (1 mg/mL in Milli-Q water) were incubated for 1 h, at 37 °C, with different concentrations of the toxins *Bothrops atrox* venom, Batroxase or BjSP (previously treated with 2 μg of BaltMPI for 30 min, at 37 °C). The reaction was interrupted by adding 10 μL of a denaturing buffer (0.05 M Tris-HCl pH 8.0, 10% (v/v) glycerol, 10% (v/v) β-mercaptoethanol, 2% (v/v) SDS, and 0.05% (w/v) bromophenol blue), followed by boiling at 100 °C for 5 min. The samples were analyzed by SDS-PAGE, as described in Section 2.5.1.

2.10. Fibrinolytic activity inhibition

The BaltMPI ability to inhibit the fibrinolytic activity of Batroxase was evaluated in petri plates by following the method described by Jespersen and Astrup [39] and the modifications proposed by Leitão et al. [40]. Briefly, 0.3% (w/v) bovine fibrinogen and 0.76% (w/v) agarose solutions were prepared in 40 mM sodium barbital buffer (pH 7.8) containing 2.1 mM $CaCl_2$, 1.0 mM $MgCl_2$, and 95 mM NaCl. The agarose solution was heated on a hotplate until the formation of a transparent colloid. After cooling to 40 °C, the fibrinogen solution was added to it at a ratio of 1:1 (v/v). Further, 100 μL of thrombin solution (1 μg/μL) was added to this mixture, which was poured into plastic petri plates (85 × 10 mm) for the formation of the fibrin clot. After a 60 min incubation at room temperature (25 °C), 5 mm diameter holes were bored and 30 μL of the samples were applied into them: 10 μg Batroxase and 5 μg BaltMPI, either alone or in combination, and previously incubated for 30 min, at 37 °C; 1 μg/μL plasmin (positive control); PBS (negative control).

The plates were incubated for 18 h, at 37 °C. Finally, the lysis halos were analyzed visually and their diameter was measured in mm.

2.11. Azocaseinolytic activity inhibition

The BaltMPI ability to suppress the proteolytic activity of Batroxase, using azocasein as substrate, was assessed using the method described by Siigur et al. [41], with modifications. First, Batroxase (10 μg) was pre-incubated with BaltMPI (2.5, 5.0, 10, 15, and 20 μg) or EDTA (20 mM; positive control) for 30 min, at 37 °C. Next, Batroxase alone or in combination with EDTA or BaltMPI was added to 1 mL of azocasein solution (1 mg/mL) in Tris-HCl buffer pH 7.6 (0.2 M Tris, 0.5 M HCl, and 4 mM $CaCl_2$). After a 60 min. incubation at 37 °C, non-degraded azocasein was precipitated by adding 500 μL of 80% (w/v) trichloroacetic acid and incubating for 10 min. The mixture was centrifuged for 10 min at 10,000*g*, and the absorbance of the filtrate was recorded at 366 nm using the Beckman DU® 640 spectrophotometer (Beckman Coulter, Brazil).

One unit of activity was defined as the amount of enzyme that increased absorbance by 0.01 units at 366 nm. The specific activity in U/mg was calculated and used to determine the residual activity of Batroxase after treatment with the inhibitors.

2.12. Determination of MW

The MW of BaltMPI was determined by MALDI/TOF/TOF mass spectrometry, (AXIMA-Performance, Kratos-Shimadzu Biotech, Manchester, UK) using linear TOF in the positive ionization mode. The mass spectrometer was calibrated using protein standards including bovine serum albumin, insulin, cytochrome C, ribonuclease, apomyoglobin, and aldolase. The samples were diluted in 50 μL of Milli-Q water, and 5 μL aliquots were mixed at a 1:1 ratio with a matrix of sinapinic acid (10 mg/mL) in 50% acetonitrile and 0.1% TFA. The resulting mixtures were applied to the MALDI target plate. An average of 100 scans were collected using 10 shots/scan. The range of MW analyzed was 5000–100,000 *m*/*z*.

2.13. Amino acid sequencing and multiple alignment

The partial amino acid sequence of BaltMPI was determined using the Edman's degradation and mass spectrometry techniques. N-terminal sequencing was performed in an automatic protein sequencer (PPSQ-33A system, Shimadzu), using the purified protein at a concentration of ~10 pmol. The sample was identified and quantified by comparison with standard amino acids (25 pmol) analyzed in the beginning of the sequencing.

For the mass spectrometry analysis of tryptic peptides, the SDS-PAGE gel band corresponding to BaltMPI was excised and treated as follows. First, SDS and Coomassie blue dye were removed from the gel band by washing it three times with 400 μL of a solution composed of 0.1 M NH_4HCO_3 and 50% acetonitrile. Second, the sample was dehydrated with pure acetonitrile and dried in a rotary centrifuge under vacuum (Savant™ SpeedVac™; Thermo Fischer Scientific, Waltham, MA, USA). Third, the dried gel band was rehydrated with trypsin solution (0.5 μg/20 μL) (Promega, Madison, WI, USA) and, after its complete swelling, the gel was covered with 0.1 M NH_4HCO_3 solution and incubated for 24 h, at 37 °C. Next, the reaction was stopped by adding 5 μL of formic acid, and the mixture was incubated at room temperature for peptide extraction. The extract was kept frozen at −20 °C until mass spectrometry analysis.

The tryptic peptides of the sample were purified using a ZipTip column filled with POROS R2 reverse-phase resin (PerSeptive Biosystems, USA), previously activated with methanol and equilibrated with 0.2% formic acid. The sample was loaded onto the column and purified from salts and other hydrophilic components by carrying out three washes with 100 μL of 0.2% formic acid. The peptides were eluted from the resin using 30 μL of a 60% methanol solution in 5% formic acid, and further dried in the SpeedVac™ system and prepared for LC-ESI-Q-TOF-MS analysis.

Asp-N endoproteinase (Promega, sequencing grade) was added to 5 μg of reduced and alkylated BaltMPI, at a final protease/protein ratio of 1:20 (w/w), in 0.1 M NH_4HCO_3 pH 8.0–this protease cuts at N-terminal of ASP and GLU. The mixture was vortexed, centrifuged, and incubated for 2–18 h, at 37 °C. ASP-N peptides were desalted as described in the previous paragraph and analyzed by LC-ESI-Q-TOF-MS.

The tryptic and ASP-N peptides were analyzed on a Q-TOF-Ultima™ mass spectrometer (Waters, Manchester, UK) coupled to a nanoAcquity™ capillary HPLC system (Waters) equipped with a reverse-phase column (10 cm × 75 μm i.d.; 3-μm diameter particle; Waters). The analysis conditions were: linear gradient of 5% acetonitrile/0.1% formic acid to 80% acetonitrile/0.1% formic acid, at a flow rate of 300 nL/min through the electrospray source, which was equipped with a fused silica capillary emitter of 20 μm i.d., 3.5 kV and 40 V cone voltage, and cone temperature of 100 °C. The sample was injected into the trap column (C18; 5-μm diameter particle) via an autosampler, at a flow rate of 2 μL/min. The mass spectra were collected as data dependent acquisition (DDA) of top 3 ions in the MS and MS/MS modes, and processed using the MassLynx software v.4.1 (Waters, Manchester, UK) to generate a peak list that was used to search against the SwissProt database. The mass data were collected using MS1 scanning (400–1500 amu), which aided determination of the peptide mass fingerprint. Each peptide ion detected was subjected to CID-MS/MS via DDA, yielding *b*- and *y*-type ion fragments that were used to match the respective amino acid sequences of the peptides. The list of peptide ions are reported in Table S1 and S2 (Suppl. Material).

The homologous sequences of BaltMPI were obtained from the National Center for Biotechnology Information database (NCBI, www.ncbi.nlm.nih.gov). The alignment between the sequences was performed using the BLAST and the Constraint-based Multiple Alignment Tool (COBALT).

2.14. Isoelectric focusing

The isoelectric focusing of BaltMPI was performed according to the method reported by Vesterberg [42], with modifications. In this study, we used a 7.5% polyacrylamide gel containing carrier ampholytes covering the pH range from 3 to 10 (Pharmalyte® 3–10; Sigma-Aldrich, St. Louis, MO, USA).

2.15. BaltMPI stability

Analysis of BaltMPI stability relied on the maintenance of the anti-hemorrhagic activity against Batroxase (determined as described in Section 2.8.1) of BaltMPI samples pre-incubated for 30 min at different conditions of temperature and pH, when compared with the control condition (temperature = 37 °C and pH = 7.4). The assessed temperatures were −80, −20, 4, 60, and 100 °C, and the assessed pH were 1.0 (0.1% TFA), 5.0 (0.5 M sodium acetate), 8.0 (0.1 M sodium phosphate), and 9.0 (0.5 M Tris–HCl).

2.16. Statistical analysis

The experiments were performed in duplicate and the results were expressed as mean ± standard deviation. Statistical analyses were performed by Analysis of Variance (ANOVA) followed by the Tukey's test, using the GraphPad Prism software (version 5 for Windows). $p < 0.05$ was considered significant.

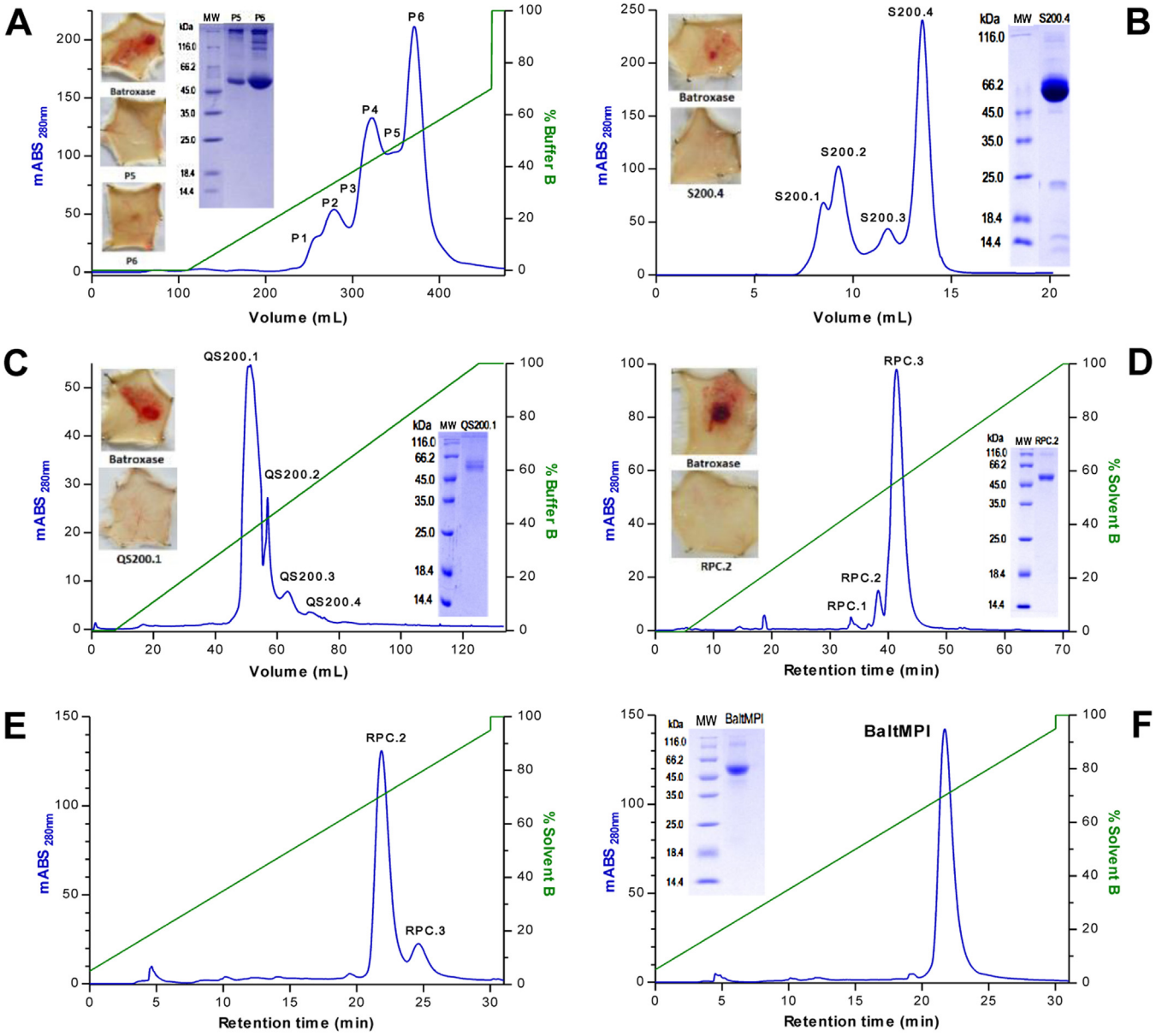

Fig. 1. Purification of the metalloprotease inhibitor from *Bothrops alternatus* snake serum (BaltMPI). A) Ion exchange chromatography using a DEAE-Sepharose™ column. Lyophilized *B. alternatus* snake serum was chromatographed using a gradient of buffer B (1.0 M NH_4HCO_3, pH 8.0) at a flow rate of 0.5 mL/min. Fractions of 2 mL were collected. Inset, SDS-PAGE analysis of fractions P5 and P6 and assessment of their anti-hemorrhagic activity against Batroxase. B) Fractionation of fraction P6 using a Superdex™ 200 molecular exclusion column. Elution was performed with 0.05 M NH_4HCO_3 pH 8.0, at a flow rate of 0.25 mL/min; fractions of 500 μL were collected. Inset, SDS-PAGE analysis of fraction S.200.4 and assessment of its anti-hemorrhagic activity against Batroxase. C) Fractionation of fraction S.200.4 using a MonoQ™ 5/50 ion exchange column. Elution was carried out using a linear gradient of 0.05 M NH_4HCO_3 (Buffer A) and 1.0 M NH_4HCO_3 (Buffer B), pH 8.0. Inset, SDS-PAGE analysis of fraction QS200.1 and assessment of its anti-hemorrhagic activity against Batroxase. D) HPLC analysis of fraction QS200.1 using a CLC-ODS C18 reverse-phase column. Elution was performed with 0.1% TFA (solvent A) and 70% acetonitrile + 0.1% TFA (solvent B), at a flow rate of 1.0 mL/min. Inset, SDS-PAGE analysis of fraction RPC.2 and assessment of its anti-hemorrhagic activity against Batroxase. E) Reverse-phase HPLC analysis of fraction RPC.2. Elution was carried out using a linear gradient of solvent B (70% acetonitrile + 0.1% TFA), from 5 to 95% in 30 min, at a flow rate of 1 mL/min; 0.1% TFA was the solvent A of the system. F) Purity analysis of fraction RPC.2. The chromatographic conditions were the same as described in E. The single peak corresponds to BaltMPI, the metalloprotease inhibitor from *B. alternatus* snake serum. Inset, SDS-PAGE analysis of BaltMPI.

3. Results

3.1. Purification of BaltMPI

To isolate the metalloprotease inhibitor from *B. alternatus* serum, we employed a four-step chromatographic process using the DEAE Sepharose™ ion exchange, Superdex™ 200 molecular exclusion, MonoQ™ 5/50GL ion exchange, and CLC-ODS C18 reverse-phase columns. Such procedure yielded the inhibitor, which we named BaltMPI, with high degree of purity, as assessed by HPLC and SDS-PAGE (Fig. 1). The selected chromatographic fractions and isolated BaltMPI exhibited strong anti-hemorrhagic activity against Batroxase (Fig. 1 – insets).

3.2. Biochemical and structural characterization of BaltMPI

BaltMPI displayed acidic pI (pI = 5.27; Fig. S1 – Supplementary material) and low MW – its MW was 60.5 kDa and 43.4 kDa, as determined by SDS-PAGE (Fig. 1F) and MALDI/TOF/TOF mass spectrometry (Fig. 2), respectively. BaltMPI preserved its anti-hemorrhagic activity against Batroxase in a wide range of pH (1–9) and temperature (−80–100 °C) (Fig. 3).

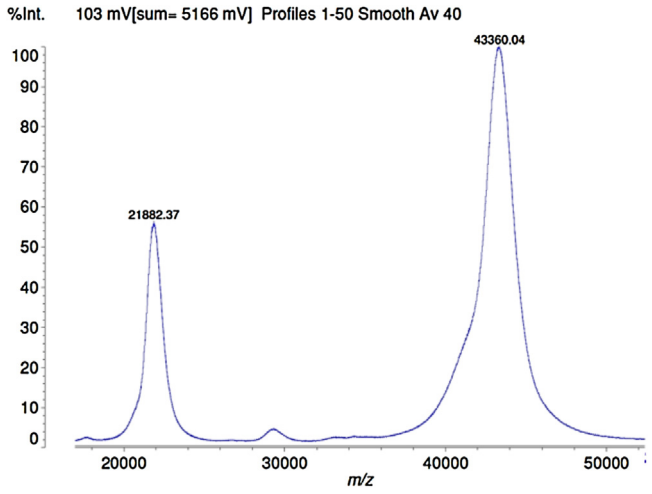

Fig. 2. Determination of the molecular weight of BaltMPI. Linear spectrum of BaltMPI acquired in a MALDI/TOF/TOF mass spectrometer, in the positive linear mode.

Data from the Edman's degradation revealed that the first 60 amino acid residues of the N-terminal region of BaltMPI are: SQVRGDLECDEKDAKEWTDIGVRYINEHKLHGYKYAL NVIKNIVDVP-WDGDWVAVFLKLN. Multiple alignment of the identified sequence of BaltMPI with BJ46a, cMSF, jMSF, and HSF, which are SVMPIs isolated from *Viperidae* snakes sera, resulted in extremely high identity scores (78–97%). BaltMPI shared the highest identity with BJ46a (97%) – they differed only by two amino acids: the Ile-25 and Asp-45 in BaltMPI corresponded to Thr-25 and Val-45 in BJ46a, respectively (Fig. 4).

BaltMPI was also submitted to digestion with trypsin and Asp-N endoproteinase, and the resulting peptides were analyzed by mass spectrometry. The sequences of these tryptic and Asp-N peptides (Table S1 and Table S2 – Supplementary material), which constitute the partial sequence of BaltMPI, were aligned with the BJ46a sequence (Fig. 5). The partial BaltMPI sequence covered 53% of the BJ46a sequence, including the N- and C-terminal regions.

3.3. Anti-hemorrhagic activity

First, we determined the MIHD of BaltMPI, which represents its minimum amount necessary to completely inhibit the hemorrhagic activity of Batroxase, a class P-I metalloprotease, in its MHD (10 μg). The MIHD of BaltMPI was 5 μg (Fig. 6A), indicating that inhibition resulted from a 1:2 stoichiometric relationship between the inhibitor and the metalloprotease. BaltMPI also exerted anti-hemorrhagic effect against BjussuMP-I, a class P-III metalloprotease (Fig. 6B).

1.2 Pre-incubation of BaltMPI with Batroxase for 30 min, at 37 °C, before injection in the dorsum of the animals (Fig. 6A) improved the anti-hemorrhagic effect, when compared with the sequential administration of Batroxase and BaltMPI, without previous incubation (Fig. 6C). In the last condition, BaltMPI significantly but not completely suppressed the hemorrhagic process.

3.4. Inhibition of proteolysis of fibrinogen, fibrin, and azocasein

We assessed whether BaltMPI interfered in the Batroxase-catalyzed proteolysis of fibrinogen, fibrin, and azocasein. We found that BaltMPI inhibited: (ii) the fibrinogenolytic activity of Batroxase at a 1:1 molar ratio of inhibitor (2 μg): metalloprotease (1 μg) (Fig. 7A); (ii) the fibrinolytic activity of Batroxase (Fig. 7B); and (iii) the azocaseinolytic activity of Batroxase in a concentration-dependent manner (Fig. 7C). The amount of Batroxase used (1 μg) completely cleaved the α chain and partially cleaved the β chain of fibrinogen (Fig. 7A).

To examine the specificity of BaltMPI activity, we compared its ability to inhibit the fibrinogenolytic activity of Batroxase and BjSP (a serine protease). Interestingly, BaltMPI specifically suppressed

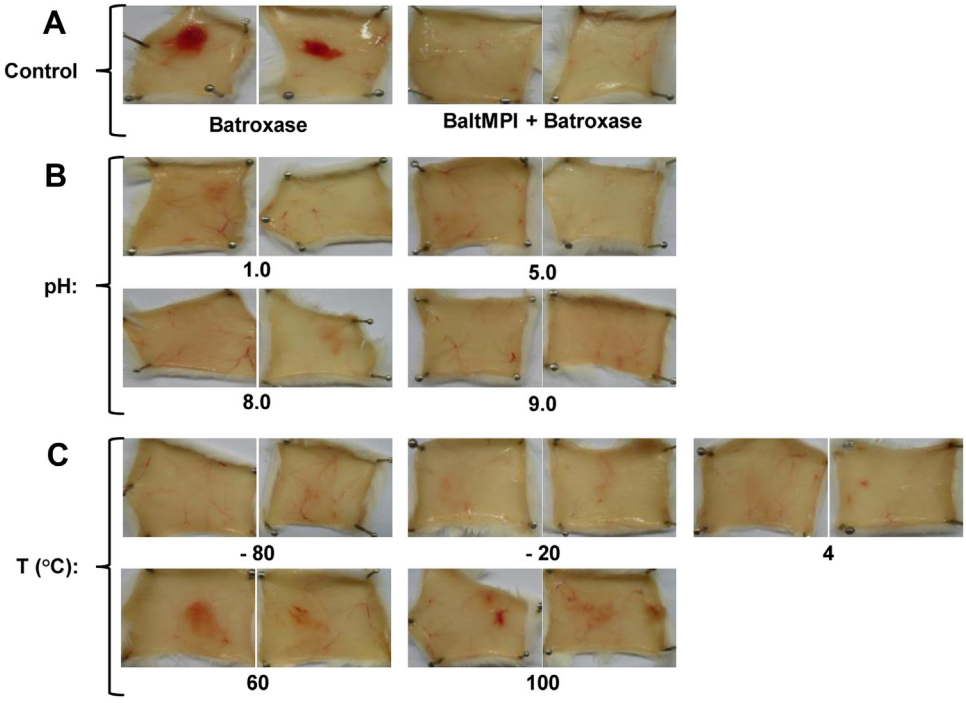

Fig. 3. Stability of BaltMPI at different pH and temperature. BaltMPI samples (5 μg) were incubated for 30 min at different conditions of temperature and pH. The anti-hemorrhagic activity of the samples against Batroxase (10 μg) was assessed. A) Control condition (pH = 7.0 and temperature = 37 °C). B) Different temperatures: −80, −20, 4, 60, and 100 °C. C) Different pH: 1.0 (0.1% TFA), 5.0 (0.5 M sodium acetate), 8.0 (0.1 M sodium phosphate), and 9.0 (0.5 M Tris–HCl).

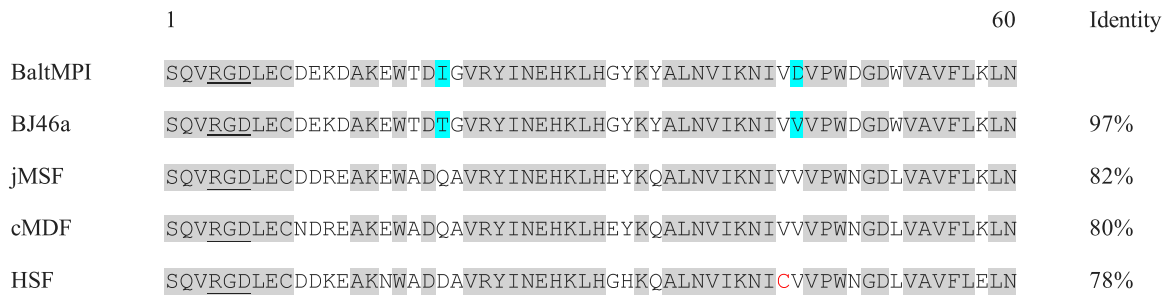

Fig. 4. Comparison of the N-terminal sequence of BaltMPI (60 residues) with other related inhibitor sequences. Homology of the N-terminal region of BaltMPI against the same region of other anti-hemorrhagic factors: BJ46a (*Bothrops jararaca* ID: Q9DGI0), jMSF (*Gloydius blomhoffii*, ID: Q5KQS1), cMSF (*Gloydius brevicaudus*, ID: Q5KQS4), and HSF (*Protobothrops flavoviridis*, ID: P29695). Gray: Homologous amino acid residues in all the sequences; Turquoise: amino acid residues that differ in relation to BJ46a; Underlined: the RGD domain. The ID corresponds to the sequence identifier for each protein in the NCBI database.

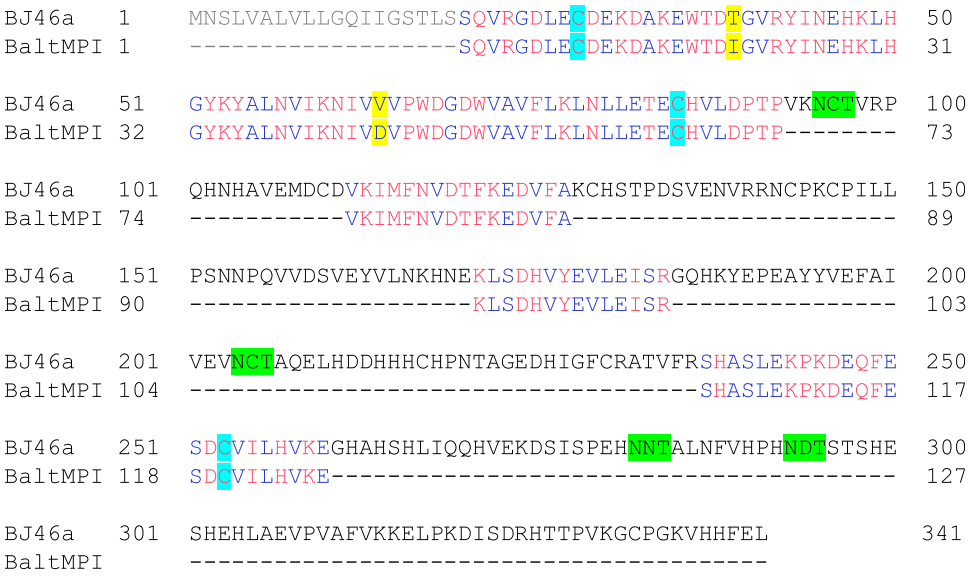

Fig. 5. Comparative analysis between the partial sequence of BaltMPI and the sequence of the anti-hemorrhagic factor BJ46a. Red: highly conserved amino acid residues; Blue: less conserved amino acid residues; Yellow: amino acid residues that differ between the two proteins but occupy the same position; Turquoise: cysteine residues that are identical in both proteins; Green: glycosylation sites in BJ46a; Gray: amino acid residues corresponding to the signal sequence. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

the fibrinogenolytic activity of the former but not of the latter protease (Fig. 7D).

4. Discussion

SVMPIs present in snake sera can bind metalloproteases and prevent their proteolytic action, which is strong in bothropic envenomation. In this study, we isolated for the first time a metalloprotease inhibitor from *B. alternatus* snake serum that we named BaltMPI, using a four-step chromatographic procedure. SVMPIs have been successfully isolated from the serum of small mammals and snakes using different multi-step chromatographic procedures [16,17,43–46]. For instance, the anti-hemorrhagic factor HSF was isolated from *T. flavoviridis* snake serum through fractionation with ammonium and ethanol sulfate, followed by chromatography using Sephadex G-200 and DEAE Cellulose columns [26]; BaSAH was isolated from *B. asper* snake serum through ammonium sulfate fractionation followed by chromatography using Sephacryl S200 and DEAE Sepharose at pH 7.2 and 8.3, respectively, and Phenyl Sepharose CL4B [47]; BJ46a was isolated from *B. jararaca* snake serum through fractionation with ammonium sulfate followed by chromatography using Phenyl Sepharose and C4 reverse-phase columns [28]; and cMSF and jMSF were isolated from *G. blomhoffii brevicaudus* and *G. blomhoffii* snake sera, respectively, through fractionation with ethanol and reversed-phase chromatography [29].

The aforementioned SVMPIs share several characteristics with BaltMPI: they are acidic glycoproteins with pI between 3.5 and 5.4, they are thermostable and functional in a wide pH range, and their MW lay in the range 40–95 or 700–1090 kDa; the low-MW SVMPIs are more abundant [18,20]. The identified amino acid sequence of BaltMPI shared high identity with SVMPIs isolated from *Viperidae* snakes sera, in particular with BJ46a. The lower identity scores between the N-terminal region of BaltMPI and the other SVMPIs can be attributed to the phylogenetic distance among the species from which the SVMPIs were isolated. The fact that cMSF and jMSF share higher homology with HSF than with BJ46a corroborates this hypothesis [29]. The first 19 amino acids of the BJ46a sequence correspond to the signal sequence of the expressed protein, but it is absent in the mature protein [20,28]. BJ46a bears the NXS/T *motif*, which can be glycosylated at four positions (76, 185, 263, and 274); although the BaltMPI partial sequence did not display this *motif*, it is highly possible that it is a glycosylated protein.

SVMPIs belong to the cystatin superfamily, which is characterized by the presence of 12 cysteine residues that form six disulfide bridges. HSF, the first SVMPI to be identified, is the only known exception: it bears 13 cysteine residues – the additional residue is located at position 44 [48,49] –, while BJ46a [28] and BaltMPI

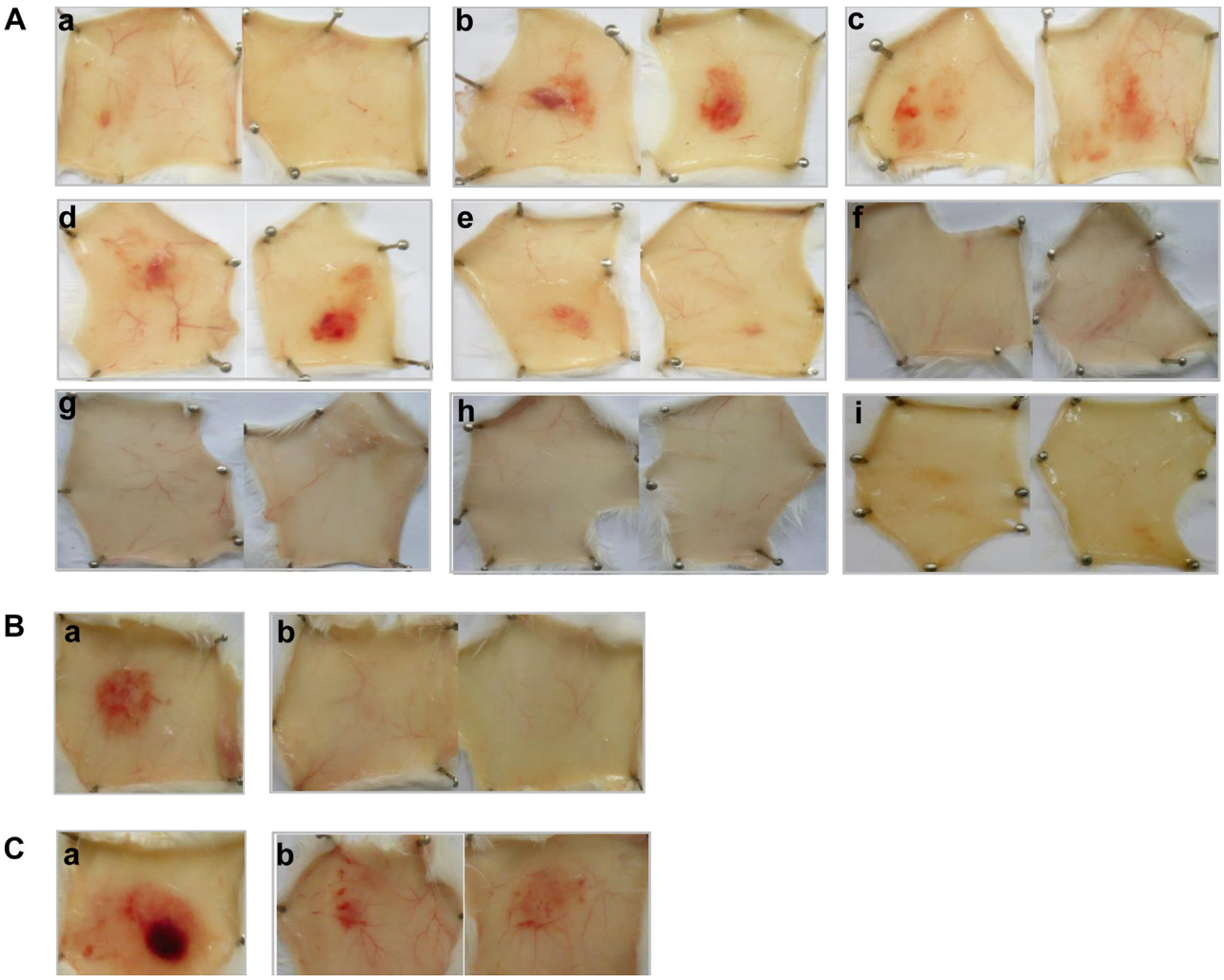

Fig. 6. Assessment of the anti-hemorrhagic activity of BaltMPI. All the assays were performed in duplicate. A) Determination of the minimal inhibitory hemorrhage dose (MIHD) of BaltMPI. The MIHD of BaltMPI was determined by assessing its ability, at different concentrations, to inhibit the hemorrhagic activity of Batroxase at its MHD (10 μg). Batroxase alone or in combination with BaltMPI was pre-incubated for 30 min, at 37 °C, before injection in the dorsum of female Swiss mice. a) PBS (negative control); b) Batroxase (10 μg, positive control); c–i) Batroxase (10 μg) + 0.1, 0.5, 2.5, 5.0, 7.5, 10, and 12.5 μg BaltMPI, respectively. B) Anti-hemorrhagic effect of BaltMPI against BjussuMP-I. BjussuMP-I was pre-incubated alone or in combination with BaltMPI for 30 min, at 37 °C, before injection in the dorsum of mice.); a) BjussuMP-I in its MHD (4 μg) (positive control); b) 4 μg BjussuMP-I + 10 μg BaltMPI. C) Anti-hemorrhagic effect of BaltMPI not previously incubated with Batroxase before injection in the dorsum of mice. a) 10 μg Batroxase; b) 10 μg Batroxase + 5 μg BaltMPI.

bear a Val residue at the same position. Hence, BJ46a and BaltMPI probably bear only 12 cysteine residues. It is worth to note that these SVMPIs, although belonging to the cystatin superfamily, do not catalyze proteolysis of either cystatins or metalloproteases [20]. A research group has proposed a new diversification of this cystatin superfamily, which would become the IH clan of cystatin-like proteins, divided into subfamilies I25A, I25B, and I25C; the SVMPIs and other cysteine-like proteins that do not exhibit proteolytic inhibitory activity on cysteine proteases would be classified in the I25C subfamily [50].

The mechanisms by which SVMPIs inhibit hemorrhage and other effects of SVMPs are not clear. However, some authors argue that the metalloprotease-inhibitor interaction is non-covalent, another characteristic of this group of inhibitors [20,27,28,44,51,52]. In this sense, an experiment with HSF, previously cleaved with papain and cyanogen bromide, demonstrated that: (i) the D1 domain, the N-terminus or the cystatin domains are essential for the inhibition of the hemorrhagic activity of SVMPs; (ii) during formation of the inhibitor-metalloprotease complex, three Lys at positions 15, 41, and 103 are protected against chemical modifications, indicating that these residues participate in the interaction with SVMPs [52]. The BaltMPI sequence also had Lys at the positions 15 and 41 (Fig. 5). Another hypothesis is that the inhibitor also interacts with the catalytic domain of the metalloprotease; it is supported by the findings that: (i) BJ46a inhibits the hemorrhagic activity of both Atrolisyn C and Jararhagin [28] – the former is a class P-I metalloprotease that bears only the metalloprotease catalytic domain, while the latter is a P-III class metalloprotease that bears disintegrin- and cysteine-rich domains, in addition to the catalytic domain [13] –; (ii) the lack of complexation between BJ46a and Jararhagin-C, which is identical to Jararhagin but lacks the catalytic domain [28].

SVMPIs specifically diminish the activity of SVMPs [21,28,47]. BaltMPI suppressed the hemorrhagic activity of Batroxase and BjussuMP-I at a 1:2 inhibitor/metalloprotease ratio, but did not affect the proteolytic activity of the serine protease BjSP; this ratio is in line with that reported for BJ46a and the metalloproteases Atrolysin C and Jararhagin [28]. In contrast, the anti-hemorrhagic factor HSF inhibits HR1 (class P-III) and HR2 (class P-I), which are metalloproteases from the autologous venom, at a 1:1 ratio [27], while the BaSAH factor inhibits the hemorrhagic action of BaH1 (a metalloprotease from *B. asper* snake venom) at a 2:1 ratio [47]. The

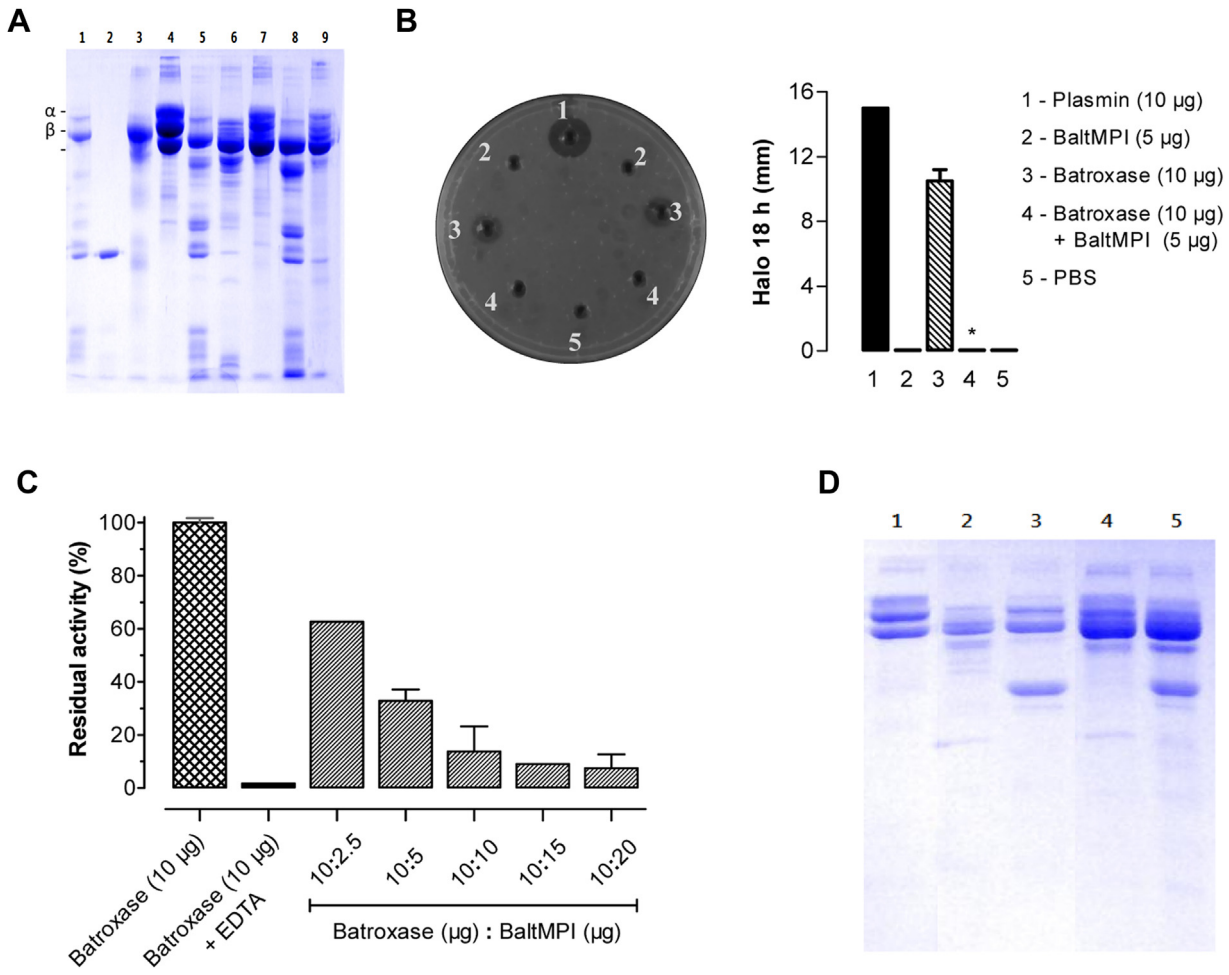

Fig. 7. Inhibitory effect of BaltMPI on different proteases. A) Inhibition of the fibrinogenolytic activity of Batroxase. The samples 8 and 9 were pre-incubated with BaltMPI for 30 min, at 37 °C, before addition of bovine fibrinogen to all the samples. After a 1 h incubation at 37 °C, the samples were analyzed by SDS-PAGE (12%). 1) Bot *hrops atrox* venom (1 μg); 2) Batroxase (1 μg); 3) BaltMPI (2 μg); 4) Fibrinogen (50 μg); 5) Fibrinogen + *B. atrox* venom; 6) Fibrinogen + Batroxase; 7) Fibrinogen + BaltMPI; 8) Fibrinogen + (*B. atrox* venom + BaltMPI); 9) Fibrinogen + (Batroxase + BaltMPI). Identification of the α-, β-, and γ-chain refers to sample 4. B) Inhibition of the fibrinolytic activity of Batroxase. The fibrinolytic activity was assessed by measuring the diameters (in millimeters) of lysis halos formed after incubation of fibrin plates with the samples for 18 h. Plasmin (10 μg) and PBS were used as the positive and negative controls, respectively. Results expressed as mean ± standard deviation (n = 2). *$p < 0.05$ vs. Batroxase (ANOVA followed by the Tukey's test). C) Residual proteolytic activity of Batroxase in the presence of inhibitors. Batroxase (10 μg) alone or in combination with BaltMPI at different concentrations was incubated for 30 min, at 37 °C, before addition to azocazein solution. The percentage of residual activity was calculated in relation to Batroxase alone (100% activity). EDTA (20 mM) was used as the positive control. Results are expressed as mean ± standard deviation (n = 2). D) Specificity of the inhibitory action of BaltMPI on proteases. BaltMPI was pre-incubated with the proteases Batroxase or BjSP for 30 min, at 37 °C, before addition of bovine fibrinogen. After a 1 h incubation at 37 °C, the samples were analyzed by SDS-PAGE (12%). 1) Fibrinogen; 2) Fibrinogen + Batroxase (1 μg); 3) Fibrinogen + BjSP (2 μg); 4) Fibrinogen + [Batroxase (1 μg) + BaltMPI (2 μg)]; 5) Fibrinogen + [BjSP (2 μg) + BaltMPI (2 μg)]. BjSP is a serine protease isolated from *Bothrops jararaca* snake venom.

SVMPIs HSF, BJ46a, and MSFS also specifically inhibit the activity of metalloproteases [28,29,48].

In addition to the hemorrhagic activity, SVMPs may exert fibrinogenolytic, fibrinolytic, myonecrotic, inflammatory, proteolytic, non-hemorrhagic, and pro-apoptotic activity, among others [13,53–55]. The proteolytic activity of SVMPs seems to correlate positively with their hemorrhagic effect due to the fact that they catalyze proteolysis of different components of the basement membrane, which mediates their hemorrhagic effect. In fact, the class P-III metalloproteases, which bear additional catalytic domains when compared with the class P-I metalloproteases, exert stronger hemorrhagic effect [54,56].

Many SVMPs exhibit fibrinogenolytic activity due to their ability to cleave both the α and β chains of fibrinogen; some SVMPs preferentially cleave the α chain [53]. Batroxase selectively cleaves the fibrinogen α-chain or both, the α- and β-chain, depending on the concentration of this metalloprotease added to the medium [57]. We found that BaltMPI inhibited the Batroxase-catalyzed proteolysis of fibrinogen at a 1:1 molar ratio of inhibitor/metalloprotease; it corroborates a literature report on the complete suppression of the fibrinogenolytic activity of Jararhagin by the anti-hemorrhagic factor PO41, using equimolar amounts of the inhibitor and the metalloprotease [17]. BaltMPI also suppressed the fibrinolytic and azocaseinolytic activities of Batroxase.

Other studies have reported the ability of some SVMPIs to suppress the proteolytic activity of SVMPs or crude snake venom. For instance, the metalloprotease inhibitors DM40 and DM43, isolated from *Didelphis marsupialis* serum, suppressed the fibrinogenolytic and fibrinolytic activities of *B. jararaca* venom [16]; the anti-hemorrhagic factors cMSF and jMSF inhibited the caseinolytic effect of class P-I and P-III metalloproteases [29]; the anti-hemorrhagic factor BJ46a suppressed the Atrolysin C- and Jararhagin-catalyzed proteolysis of the fluorogenic substrate Abz-Ala-Gly-Leu-Ala-Nba, which is structurally similar to the active site of several vertebrate collagenases [28]; the anti-hemorrhagic factor PO41 inhibited the Jararhagin-catalyzed proteolysis of Abz-Ala-Gly-Leu-Ala-Nba [17].

Although it remains unclear how SVMPIs suppress the proteolytic activity of metalloproteases, and how SVMPs exert their

hemorrhagic and proteolytic effects, some authors have proposed that the structural domains that mediate the different biological activities are not the same [56,58]. Hence, it is not a single type of interaction, but multiple non-covalent interactions among different structural sites of both proteins upon the metalloprotease-inhibitor complex formation that result in the inhibitory action of SVMPIs on several biological activities of SVMPs [20].

One of the most interesting aspects of SVMPIs is their potential to complement current antivenom therapies, especially in the cases where these therapies are insufficient to prevent or treat the severe local effects of bothropic envenomation. In this study, we found that subcutaneous administration of BaltMPI immediately after injection of Batroxase in the dorsum of the animals inhibited the hemorrhagic process less effectively, when compared with injection of BaltMPI pre-incubated with Batroxase for 30 min; probably, inactive metalloprotease-inhibitor complexes were formed during the incubation period.

Considering that the local effects are important complicating factors in snake envenomation, especially in that caused by snakes belonging to the genus *Bothrops*, it is essential to seek molecules that prevent the action of the enzymes responsible for the harmful effects, such as metalloproteases. Although the mechanisms by which BaltMPI and other SVMPIs act deserve further investigation, it is evident that these molecules are potential candidates for the development of complementary therapies to treat snake envenomation, as well as they are potential tools for basic and applied research.

5. Conclusion

The present work successfully isolated and characterized a novel specific metalloprotease inhibitor from the serum of *B. alternatus* snake, whose venom is only partially characterized to date. The main features of the inhibitor that we named as BaltMPI are: pI = 5.7, MW = 43.4 kDa, high homology (97%) with the metalloprotease inhibitor BJ46a, high stability over a wide range of pH and temperature, and high specificity as metalloprotease inhibitor. Therefore, BaltMPI is an interesting molecule with promising biotechnological and molecular applications for the treatment of ophidic accidents.

Conflict of interest

The authors declare that there is no conflict of interest regarding this work.

Author contributions

T.Z.P. designed and performed the experiments, and processed and analyzed the data. J.C.R. performed the mass spectrometry analyses, and processed and interpreted their results. R.S.F.-Jr and B.B. designed the chromatographic analyses and biological assays, and analyzed their data. N.A.S.-F. and S.V.S. conceived the project, designed the study, and searched for funding. All the authors drafted, read, and approved the final manuscript.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ijbiomac.2017.01.131.

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