Comparative Biochemistry and Physiology, Part A 195 (2016) 15–25

Contents lists available at ScienceDirect

Comparative Biochemistry and Physiology, Part A

journal homepage: www.elsevier.com/locate/cbpa

BbrzSP-32, the first serine protease isolated from *Bothrops brazili* venom: Purification and characterization

Kayena D. Zaqueo [a], Anderson M. Kayano [a], Thaisa F.S. Domingos [b], Laura A. Moura [b], André L. Fuly [b], Saulo L. da Silva [c], Gerardo Acosta [d], Eliandre Oliveira [e], Fernando Albericio [d,f,g], Fernando B. Zanchi [a], Juliana P. Zuliani [a], Leonardo A. Calderon [a], Rodrigo G. Stábeli [a,*], Andreimar M. Soares [a,*]

[a] Centro de Estudos de Biomoléculas Aplicadas à Saúde, CEBio, Fundação Oswaldo Cruz, FIOCRUZ Rondônia e Departamento de Medicina, Universidade Federal de Rondônia, Brazil
[b] Universidade Federal Fluminense, UFF, Niterói, RJ, Brazil
[c] Universidade Regional Amazónica IKIAM, Via Muyuna Km 7, Tena, Napo, Ecuador
[d] Institute for Research in Biomedicine (IRB Barcelona), 08028-Barcelona, Spain and CIBER-BBN, Barcelona Science Park, Barcelona 08028, Spain
[e] Proteomic Platform, Barcelona Science Park, Barcelona 08028, Spain
[f] Department of Organic Chemistry University of Barcelona, Barcelona 08028, Spain
[g] School of Chemistry, University of KwaZulu Natal, Durban 4001, South Africa

ARTICLE INFO

Article history:
Received 29 April 2015
Received in revised form 14 October 2015
Accepted 26 January 2016
Available online 28 January 2016

Keywords:
Snake venom
Enzyme
Biotechnology
Viperidae
Thrombin-like

ABSTRACT

Snake venom toxins are related not only in detention, death and the promotion of initial digestion of prey but also due to their different biochemical, structural and pharmacological effects they can result in new drugs. Among these toxins snake venom serine proteases (SVSPs) should be highlighted because they are responsible for inducing changes in physiological functions such as blood coagulation, fibrinolysis, and platelet aggregation. This article presents the first serine protease (SP) isolated from *Bothrops brazili*: BbrzSP-32. The new SP showed 36 kDa of relative molecular mass and its absolute mass was confirmed by mass spectrometry as 32,520 Da. It presents 79.48% identity when compared to other SVSPs and was able to degrade the α-chain of fibrinogen, in *in vitro* models, because of this it is considered a SVTLE-A. It showed dose-dependent activity in the process of degradation of fibrin networks demonstrating greater specificity for this activity when compared to its thrombolytic action. BbrzSP-32 demonstrated proteolytic activity on gelatin and chromogenic substrates for serine proteases and thrombin-like enzymes (S-2288 and S-2238 respectively), besides having coagulant activity on human plasma. After pre-incubation with PMSF and benzamidine the coagulant and proteolytic activities on the S-2288 and S-2238 substrates were reduced. BbrzSP-32 shows stability against pH and temperature variations, demonstrating optimum activity between 30 and 40 °C and in the pH range 7.5 to 8.5. A new SP with potential biotechnological application was isolated.

1. Introduction

The composition of snake venoms results from the interaction/action of various factors, leading to an efficient biological product and complex nature, which has as main objective the detention, death and the promotion of initial digestion of prey. And they deserve attention because of their numerous proteins and peptides with different biochemical, structural and pharmacological effects, acting through different mechanisms. Some toxins from snake venoms have been widely used as tools for functional analysis of molecular receptors on platelets and tumor cells, to understand the mechanisms of blood coagulation, ion channels and to understand the immune system (Calderon et al., 2014; Du et al., 2002; Gutiérrez et al., 2005; Six and Dennis, 2000; Theakston and Kamiguti, 2002).

Bothropic envenomation causes various pathophysiological disorders, including systemic disorders of hemostasis related to the activing of factor X and II, consumption of fibrinogen and inhibition of platelet aggregation, leading to characteristic systemic symptoms (Zelanis et al., 2007). Thus, cataloging the venom profile is an essential requirement to provide useful molecular tools for elucidating the mechanisms of action of toxins, as well as antagonists searching. The structural understanding of these agents enables changes in the structure to build a more effective and specific therapeutic activity. Some proteins from snake venoms are able to interact with factors of the coagulation cascade, while others may act on platelets and other components of the hemostatic system. And, such effects are usually attributed to serine

* Corresponding authors at: Fundação Oswaldo Cruz Rondônia, Centro de Estudos de Biomoléculas Aplicadas à Saúde, Rua da Beira, 7176, Bairro Lagoa, 76812-245 Porto Velho, Rondônia, Brazil.
E-mail addresses: stabeli@fiocruz.br (R.G. Stábeli), andreimar@fiocruz.br (A.M. Soares).

http://dx.doi.org/10.1016/j.cbpa.2016.01.021
1095-6433/

proteases, metalloproteases and disintegrins of snake venoms (Cominetti et al., 2004; Gay et al., 2005; Gutiérrez et al., 2005; Kamiguti et al., 1998).

One of the greatest relevance in the accident framework by venomous snakes is related to blood coagulant action, which proteases strongly participate by two independent mechanisms: activating some factors of coagulation cascade or converting fibrinogen to fibrin, leading to blood incoagulability, since fibrinogen was consumed (Ghorbanpur et al., 2009). These proteases are, in general called snake venom thrombin-like enzymes (SVTLE), because they present some functions similar to thrombin. SVTLE belong to a class of enzymes that has in the active site a highly conserved catalytic amino acids triad (His57, Asp102 and Ser195) that has analogous positions to mammalian serine proteases (Castro et al., 2004; Costa et al., 2008).

Bothrops brazili Hoge, 1954 (Brazil's Lancehead) is a rare venomous pitviper, and information about its venom composition has been restricted only to four myotoxins MTX-I, MTX-II (Costa et al., 2008), BbTX K49-II PLA_2 and BbTX III-PLA_2 (Huancahuire-Vega et al., 2009), one L-amino acid oxidase with cytotoxic action on cell cultures of *Staphylococcus aureus*, *Vibrio cholerae* e *Streptococcus faecalis* and one metalloproteinase (BbrzMP-I), with antiplasmodial activity (Kayano et al., 2015). It is worth noting that so far no serine protease has been described in the venom of *B. brazili*. So, the objective of this work was to isolate and perform a partial biochemical and enzymatic characterization of the first serine protease from *B. brazili* snake venom.

2. Material and methods

2.1. Isolation of BbrzSP-32

The new serine protease was isolated after chromatographic fractionation of *B. brazili* venom by bioaffinity and reverse phase chromatography. About 400 mg of crude venom was solubilized in 1 mL of 50 mM Tris–HCl pH 7.6 and centrifuged at 9,000 × *g* for 10 min at room temperature. The clear supernatant was applied to Column Tricorn 10/100 packed with Benzamidine-Sepharose resin (GE Healthcare), previously equilibrated with 50 mM Tris–HCl pH 7.6. The elution of proteins was performed using two steps: (i) 50 mM Tris–HCl pH 7.6 (ii) 50 mM Tris–HCl pH 7.6 plus 1 M NaCl and (iii) 20 mM Glycine at a flow of 1 mL/min. The collected samples were desalted in a Sephadex G-25 Superfine 5 mL columm (GE Healthcare) and lyophilized. The enriched-fraction in coagulant activity was lyophilized, dissolved in 0.1% trifluoroacetic acid (TFA) and a reversed-phase high performance chromatography was performed using a C2/C18 column (10 mm × 4.6 mm, 3 μm, 120 Å) (GE Healthcare), pre-equilibrated with 0.1% TFA. The elution was carried out in a linear gradient of 0–50% (99% acetonitrile + 0.1% TFA) at a flow rate of 0.75 mL/min. In both chromatographies, the elution of proteins was monitored at 280 nm. All chromatographic steps were performed in an Akta Purifier 10 system (GE Healthcare), capable of controlling pressure and temperature. The protein was quantified by Bradford's method (Hammond and Kruger, 1988).

2.2. Molecular mass determination

The molecular mass was estimated by 12.5% SDS-PAGE (Laemmli, 1970) and determined by MALDI-TOF mass spectrometry using an AXIMA TOF^2 system (Shimadzu/Kratus Scientific). The mass spectrum was acquired in linear mode, using a saturated solution of sinapinic acid as ionization matrix.

2.3. Sequencing determination

2.3.1. Solution digestion

The protein was reduced by treatment with a solution of 20 mM DTT in 50 mM NH_4HCO_3 for 1 h at 30 °C and alkylated with a solution of 150 mM iodine acetamide in 50 mM NH_4HCO_3 for 1 h at 30 °C. The sample was then digested overnight at 37 °C with trypsin (Sequencing grade modified, Promega). Afterwards, tryptic peptides were cleaned up with a Proxeon Stage tip and eluted with 70% acetonitrile/0.1% trifluoroacetic acid. The eluted peptides were dried in a vacuum centrifuge and resuspended in 1% formic acid for LC–MS/MS analysis. Mass spectrometry was performed in a NanoAcquity (Waters) HPLC coupled to an OrbitrapVelos mass spectrometer (Thermo Scientific). An aliquot of the tryptic digest was injected and separated in a C18 reverse phase column (75 μm Oi, 10 cm, nanoAcquity, 1.7 μm BEH column, Waters). Bound peptides were eluted with the following gradients: 1 to 40% B in 20 min, followed by 40 to 60% B in 5 min.; flow was 250 nL/min (A: 0.1% formic acid in water; B: 0.1% formic acid in acetonitrile). Eluted peptides were ionized in an emitter needle (PicoTipTM, New Objective). Spray voltage applied was 1900 V. Peptide masses (m/z: 300–1700) were measured in full scan in the Orbitrap at a resolution of 60,000 at 400 m/z. Up to the 5 most abundant peptides (minimum intensity of 1500 counts) were selected from each MS scan and fragmented in the HCD collision cell using a normalized collision energy (NCE) of 40% with nitrogen as the collision gas. Fragments were detected in the Orbitrap with a resolution of 7500 FWHM at 400 m/z. Raw data were collected using ThermoXcalibur (v.2.1.0.1140).

2.3.2. Database search

Raw data were analyzed using Proteome Discoverer (v.1.3.0.339) software. A search was run with the search engine MASCOT against the NCBInrSerpentes database. Also, an. mgf file was generated in Proteome Discoverer and this file was used to search with PEAKS Studio (v.5.3.) against the same database. After that, the homology search bioinformatics tool SPIDER* was used to run a tag homology search. The search parameters were: Database/Taxonomy: NCBInrSerpentes; missed cleavage: 2; fixed modifications: carbamidomethyl of cysteine; variable modifications: oxidation of methionine and pyro-Glu (N-term Glutamine); peptide tolerance: 10 ppm for MS spectra and 0.05 Da for MS/MS spectra; and enzyme: trypsin.The Percolator node was used in the Proteome Discoverer Mascot search in order to discriminate correct from incorrect peptide spectrum matches using the q-value (FDR) to improve the number of confidently identified peptides at a given false discovery rate. The results have been filtered so that only high confidence peptides (FDR ≤ 0.01) are considered for identification results.

2.4. Molecular modeling and determination of glycosylation sites

To investigate the structural characteristics of its amino acids, we performed homology modeling of BbrzSP-32's sequence using as a template the crystal structure of AhV_TL-I, a glycosylated Snake-venom thrombin-like enzyme from *Agkistrodon halys* (PDB code: 4E7N) with a resolution of 1.75 Å (Zeng et al., 2013). For search and retrieval of the template structure, Protein Blast (http://blast.ncbi.nlm.nih.gov) (Altschul et al., 1990) and Protein Data Bank (PDB) (http://www.pdb.org) [Accessed 06 Feb. 2015] were used. The sequence alignments were carried out using MODELLER v9.10 (Sali and Blundell, 1993) and ClustalW (Thompson et al., 1994) softwares. Model building was carried out in MODELLER v9.10. A total of 1000 models were generated and the final model was selected based on the lowest DOPE scores calculated by MODELLER software. The overall stereo chemical quality of the final model for BbrzSP-32 was assessed by the program PROCHECK (Laskowski et al., 1993). Interactive visualization and comparative analysis of molecular structures were carried out in Swiss-PDB viewer (Guex and Peitsch, 1997) and structure images were taken with Persistence of Vision Raytracer (POV-ray) 3.62 (http://www.povray.org) [Accessed 06 Feb. 2015]. Potential sites of N-glycosylation were predicted in BbrzSP-32 by the program NetNGlyc v.1.0, available at the Center for Biological Sequence Analysis server (http://www.cbs.dtu.dk/).

2.5. Fibrinogenolytic activity

The fibrinogenolytic activity of BbrzSP-32 was determined according to Cominetti and coworkers (Cominetti et al., 2004) with modifications. Samples of bovine fibrinogen (10 mg/mL) were incubated with 2 μg BbrzSP-32 at 37 °C during different times (5, 10, 30 and 60 min). The control was coupled with a fibrinogen solution incubated with a dissolution sample buffer at 37 °C for 60 min. The reactions were stopped by adding 0.5 mM Tris–HCl pH 8.0, 20% glycerol (v/v), 4% SDS (v/v), 0.05% bromophenol blue (w/v) and 0.3% DL-dithiothreitol (w/v), in a 1:1 proportion. After overnight incubation, the digested fibrinogen was analyzed using 10% SDS-PAGE.

2.6. Fibrinolytic activity

Fibrinolytic activity was evaluated according to the method described by Cominetti and coworkers (Cominetti et al., 2004). First, enough agarose to prepare a 0.9% gel was solubilized in 50 mM Tris–HCl pH 7.4 plus 100 mM $CaCl_2$ and heated until melted. At a temperature of 37 °C, 0.3% bovine fibrinogen, dissolved in 50 mM Tris–HCl pH 7.4 plus 100 mM $CaCl_2$, and 1.2 U/mL bovine thrombin were added to the agarose solution. Afterwards, the mixture was polymerized in a Petri dish (0.9 cm × 15 cm) and 1, 2, 4, 8, 16 and 32 μg of BbrzSP-32 or crude venom (3 μg, positive control), and PBS (negative control) were incubated at 37 °C overnight in orifices as previously done. The halos that formed indicating fibrinolytic activity were analyzed by comparison to negative and positive controls. One activity unit was defined as the quantity of protein capable of producing a 1 mm halo on fibrin gel. The graph was expressed in millimeters.

2.7. Thrombolytic activity

In order to evaluate thrombolytic activity, human blood was collected from healthy donors according to the CAAE authorization: 14,204,413.5.0000.0011, in the absence of anticoagulants. For testing, blood clots were performed in 24-well plates by incubating 500 μL blood at room temperature for one hour. After this period, the clots were incubated for 24–48 h at 37 °C with different concentrations of BbrzSP-32 (83; 167 and 333 μg/mL), crude venom of *B. brazili* (167 μg/mL) and MilliQ water (300 μL) (samples, positive and negative control, respectively). After the incubation period, the sturdy clot was removed and the resulting volume was measured and the graph expressed in volume.

2.8. Clotting assays

The clotting activity of *B. brazili* venom and purified serine protease was determined using an Amelung Model KC4A coagulometer (Labcon, Germany). Different concentrations of *B. brazili* venom and serine protease were mixed with normal citrated human plasma (donated by the blood bank of Hospital Universitário Antônio Pedro, UFF) CAAE authorization: 14,204,413.5.0000.0011. The amount of venom and serine protease (μg/mL) that clotted plasma in 60 s was denoted as the Minimum Coagulant Dose (MCD). Inhibition of clotting activity was done by incubating one MCD of enzymes for 30 min at room temperature with the following substances: 5 mM/10 mM Benzamidine and 1 mM/3 mM PMSF (inhibitors of serine proteases) or with 5 mM/10 mM EDTA (inhibitor of metalloprotease). Then, the mixture was added to plasma and the clotting time recorded. Control experiments were performed by incubating venom or serine protease with DMSO or saline, instead of inhibitors. Results were expressed as clotting time (s)/protein concentration.

2.9. Hydrolytic activity upon chromogenic substrate

The ability of *B. brazili* venom and the isolated serine protease to hydrolyze chromogenic substrates, S-2238 (for thrombin-like enzymes) and S-2288 (for serine protease) (0.5 mM, final concentration) was tested in a Thermomax Microplate reader (Molecular Devices, USA) monitored at 405 nm for 20 min at 37 °C. Inhibitors (5 mM and 10 mM PMSF; 1 mM and 3 mM benzamidine or 5 mM and 10 mM EDTA) were incubated for 30 min at room temperature with crude venom of *B. brazili* and the new serine protease, then the enzymatic reaction was assayed with chromogenic substrates S-2238 and S-2288. Control experiments were performed by incubating *B. brazili* venom and serine protease with DMSO (1% v/v, final concentration) or saline. Results were expressed as % of inhibition.

2.10. Platelet aggregation assay

The platelet aggregation assay was carried out according to Fuly and coworkers (Fuly et al., 2002), with some modifications. Platelet aggregation was monitored turbidimetrically in an Aggregometer (Chrono Log model 490 2D, Havertown, USA) using Washed-Platelets (WP). WP were obtained from human whole blood of health volunteer donors (CAAE: 14,204,413.5.0000.0011). Different concentrations of the serine protease isolated from *B. brazili* were incubated with WP for 5 min at 37 °C under stirring. Then, platelet aggregation was triggered by adding collagen (24 μg/mL). Assays were performed at 37 °C in siliconized glass cuvettes in a final volume of 300 μL.

2.11. pH stability

The stability study of BbrzSP-32 was performed by preincubating the enzyme for 30 min at room temperature with in Tris–HCl buffer on different pH values (5.0–11.0) and then a reaction was triggered by adding the chromogenic substrate S-2238. Hydrolytic activity was assayed as mentioned in Section 2.10.

2.12. Thermal stability on bapna

The thermal stability was measured after preincubating 2 μg BbrzSP-32 diluted in Tris–HCl buffer (50 mM, pH 8.0) for 30 min at ranging temperature incubation values (20, 30, 37, 40, 50, 60 and 70 °C). The amidolytic activity was assayed in a 500 μL solution containing 1% N^{α}-Benzoyl-DL-Arginyl*p*-nitroanilide (BA*p*NA) in 50 mM Tris–HCl pH 8.0. The product of the reaction was analyzed at 405 nm using a value of 8800 M^{-1} cm^{-1} as the molar extinction coefficient of p-nitroanilide. A unit of enzymatic activity was defined as the quantity of enzyme capable of releasing 1 μmol p-nitroanilide/min, corresponding to the increase of 0.009 absorbance units measured at A405 nm.

2.13. Statistical analysis

Results are expressed as means ± SEM obtained with the indicated number of experiments performed. The statistical significance of differences among experimental groups was evaluated using Analysis of variance (ANOVA) and multiple comparisons by the Tukey's method and p values of ≤0.05 were considered statistically significant.

3. Results

The enzyme was isolated by two chromatographic steps: (i) bioaffinity chromatography in a Benzamidine-Sepharose column and (ii) Reverse Phase in a C2/C18 column. Three peaks having absorbance at 280 nm resulted from the fractionation of *B. brazili* venom on a Benzamidine-Sepharose (Fig. 1A), the last one showed the highest coagulant activity (data not shown) and then, it was subjected to reverse phase chromatography in a C2/C18 column, then resulting in

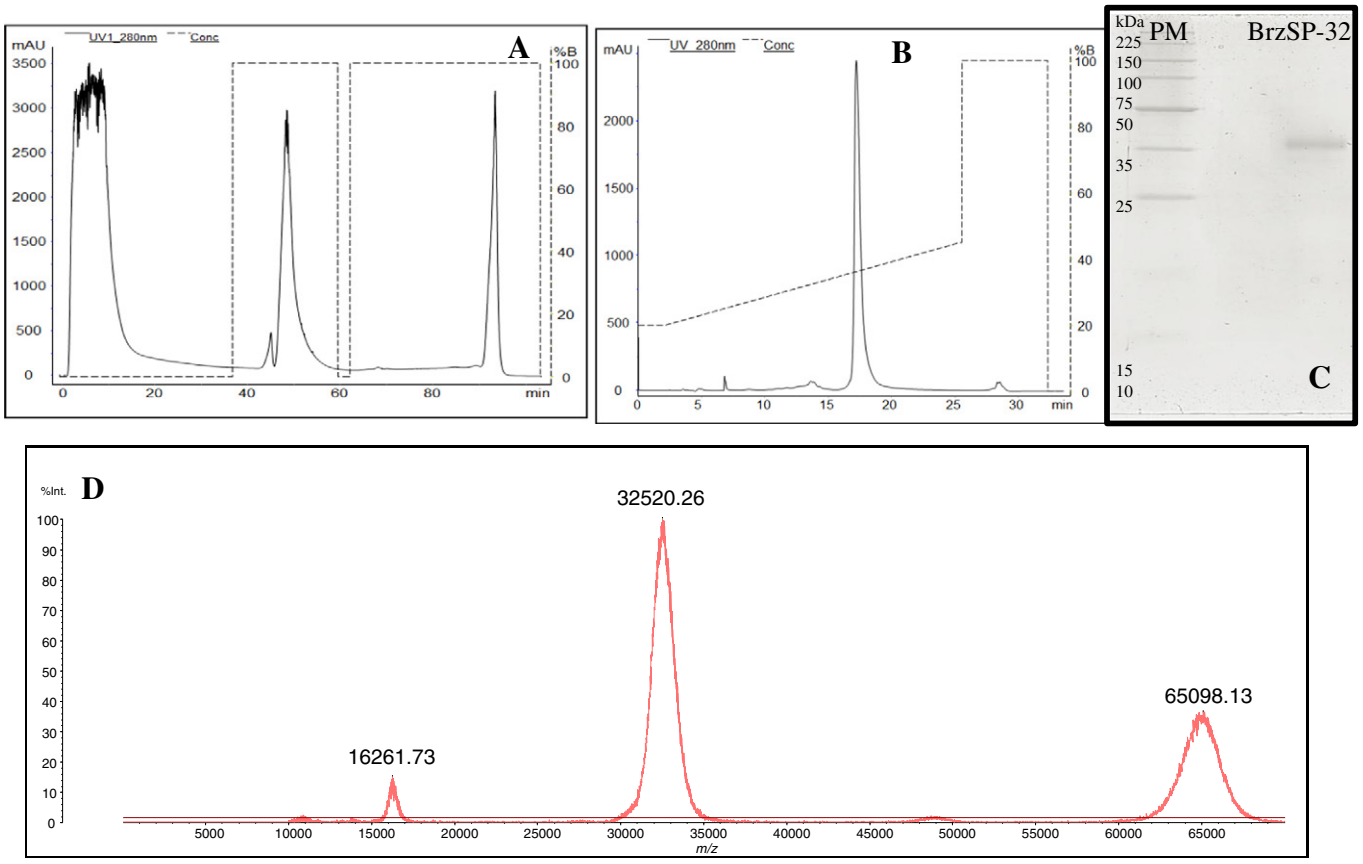

Fig. 1. Isolation and characterization of BbrzSP-32. A) Liquid chromatography in a Benzamidine Colum, the third peak was submitted to reversed phase. B) High Performance Liquid Chromatography using a C2/C18 column (10 mm × 4,6 mm, 3 μm, 120 Å). C) SDS-PAGE denaturing conditions. Line 1: molecular mass standard, V849A (Promega) (10–225 kDa), Lines: 2 — BbrzSP-32. D) MS spectrum of BprzSP-32 obtained by MALDI-TOF showing 32,520.26 m/z and its doubly-charged (16,261.73 m/z) and mono-charged dimer ions (65,098.13 m/z).

one peak (Fig. 1B). As shown in Fig. 1C, the isolated enzyme gave a single protein band of, approximately, 32 kDa on SDS-PAGE under reduced conditions, its molecular weight was confirmed by mass spectrometry in an AXIMA TOF^2 system as well as its homogeneity (Fig. 1D). The isolated serine protease was denoted BbrzSP-32.

The amino acid sequence of BbrzSP-32 was almost completely identified using normalized collision energy (NCE) for higher energy collisionally activated dissociation (HCD) MS/MS spectra of BbrzSP-32 tryptic peptides, which were extracted and searched using MASCOT engine against the NCBInrSerpentes database. Sixteen tryptic peptides had theirs sequences identified and them alignment with amino acid sequences of others homologous thrombin-like enzymes from *Agkistrodon halys* (gi_383280398) *B. jararaca* (gi:14,285,806), *B. atrox* (gi:114,837), *B. leucurus* (gi:357,580,550 and *B. jararacussu* (gi:123,895,619) in order to elucidate BbrzSP-32 primary structure (Fig. 2 and Supplementary material). However, four little gaps in high conserved regions remained unelucidated, probably due to negatively net charge of the fragments, which promoted suppression effects in MS/MS analysis.

In order to create the molecular modeling of BbrzSP-32, LC–MS/MS sequencing was used and the gaps were filled in by homology with a thrombin-like enzyme from *Agkistrodon halys* venom (PDB ID: 4E7N) (Zeng et al., 2013). Glycosylation sites were predicted on residues Asn96 and Asn138 (Fig. 3).

According to the fibrinogenolytic activity by SDS-PAGE, BbzrSP-32 can be classified as SVTLE-A, because it acts on the α-chain of fibrinogen. The cleavage of the α-chain started from the first analysis time, i. e. five minutes (Fig. 4A). Besides degrading fibrinogen, thus, generating fibrinopeptides, BbrzSP-32 also has fibrinolytic activity (Fig. 4B), i.e., the enzyme has the ability to degrade fibrin networks formed by copolymerization agarose, in a dose-dependent way. The enzymatic activity on fibrinogen of BbrzSP-32 (8 μg) was higher than crude venom of *B. brazili* (40 μg).

The thrombolytic activity (Fig. 4C) is also related to the time of incubation; after 48 h of incubation the crude venom completely degraded the clot. You can also see that other proteins present in the crude venom of *B. brazili* also have thrombolytic activity or that the synergistic action of proteins consumes the clot more efficiently when compared to the action of BbrzSP-32 alone.

The crude venom and purified serine protease presented procoagulant activity in a concentration-dependent manner, as depicted in the reduced clotting time for human plasma. The MCD upon plasma of *B. brazili* venom and serine protease was 2.4 μg/mL (Fig. 5 A and B). Benzamidine inhibited plasma clotting induced by crude venom. On the other hand, the isolated serine protease was inhibited by benzamidine and PMSF (Fig. 5C and D). Neither DMSO (1.0% or 3.0% v/v, final concentration) nor saline alone interfered with clotting times of both assays.

Activity assays were performed on enzyme substrates S-2238 (for thrombin-like enzymes) and S-2288 (for serine proteases) aiming to evaluate the hydrolysis capability of said enzyme as well as crude venom, conducted both in the presence and absence of protease inhibitors: Benzamidine, EDTA and PMSF. *B. brazili* crude venom (6 μg/mL) and serine protease enzyme (25 μg/mL) were able to hydrolyze S-2238 (0.5 mM) and S-2288 (0.5 mM) (data not shown). Both *B. brazili* and serine protease activity upon S-2238 and S-2288 were significantly reduced by PMSF or benzamidine and in a lesser degree by EDTA (Fig. 6).

BbrzSP-32 did not induce platelet aggregation on washed-platelets as well as did not inhibit collagen-induced platelet aggregation at the

```
                                  10        20        30        40        50        60
                                   |         |         |         |         |         |
BbrzSP_32                 ------------------------VIGGDECNINEHRFLVALYKS--HGYFCGLTLINQE
gi_383280398_Agk_pdb_4E7N ------------------------IIGGDECNINEHRFLVALYTSRSRTLFCGGTLINQE
gi_14285806_jararaca      ------------------------VIGGDECDINEHPFLAFMYYS--PQYFCGMTLINQE
gi_114837_atrox           MVLIRVIANLLILQVSYAQKSSELVIGGDECDINEHPFLAFMYYS--PRYFCGMTLINQE
gi_357580550_leucurus     ------------------------VIGGDECDINEHPFLAFMYYS--PRYFCGMTLINQE
gi_123895619_jararacussu  ------------------------VLGGDECDINEHPFLAFLYS---HGYFCGLTLINQE
                                                  ::*****:**** **. :*       *** ******
                                  70        80        90        100       110       120
                                   |         |         |         |         |         |
BbrzSP_32                 WVLTAAHCDRRFMRIYLGIHTRKVLNEDEQTRYPKEKFICPN--KNVITDKDIMLIRLDS
gi_383280398_Agk_pdb_4E7N WVLTAAHCDRKNFRIKLGMHSKKVPNEDEQTRVPKEKFFCLSSKNYTLWDKDIMLIRLDS
gi_14285806_jararaca      WVLTAAHCDKTYMRIYLGIHTRSVANDDEVIRYPKEKFICPNKKKNVITDKDIMLIRLNR
gi_114837_atrox           WVLTAAHCNRRFMRIHLGKHAGSVANYDEVVRYPKEKFICPNKKKNVITDKDIMLIRLDR
gi_357580550_leucurus     WVLTAAHCNRRFMRIXXXXHAGSVANYDEVVRXXXXXFICPNKKKNVITDKDIMLIRLDR
gi_123895619_jararacussu  WVVTAAHCDSTNFQMQLGVHSKKVLNEDEQTRNPKEKFICPNKNMSEVLDKDIMLIKLDK
                          **:*****:   :::    *: .* * **  *     *:* .     : *******:*:

                                 130       140       150       160       170       180
                                   |         |         |         |         |         |
BbrzSP_32                 PVSNSEHIAPLSLPSSPPSVGSVCRIMGWGTITP-SKTNPDVPHCANINLLDDAVCREAY
gi_383280398_Agk_pdb_4E7N PVKNSKHIAPFSLPSSPPSVGSVCRIMGWGRISPTEGTYPDVPHCVNINLLEYEMCRAPY
gi_14285806_jararaca      PVKNSTHIAPISLPSNPPSVGSVCRIMGWGAITTSEDTYPDVPHCANINLFNNTVCREAY
gi_114837_atrox           PVKNSEHIAPLSLPSNPPSVGSVCRIMGWGAITTSEDTYPDVPHCANINLFNNTVCREAY
gi_357580550_leucurus     PVKNSEHIAPLSLPSNPPSVGSVCRIMGWGAITTSEDTYPDVPHCANINLFNNTVCREAY
gi_123895619_jararacussu  PISNSKHIAPLSLPSNPPSVGSVCRIMGWGSITIPNETYPDVPYCANINLVDYEVCQGAY
                          *:.** ****:****.************** *:  . * ****:*.****.:  :*: .*

                                 190       200       210       220       230       240
                                   |         |         |         |         |         |
BbrzSP_32                 AVDPAKT----LCAGILQGGIDSCKGGDSGGPLICNGQFQGIVGS--DPCAEPRLKPAIY
gi_383280398_Agk_pdb_4E7N PEFELPATSRTLCAGILEGGKDTCKG-DSGGPLICNGQFQGIASWGDDPCAQP-HKPAAY
gi_14285806_jararaca      NGLPAKT----LCAGVLQGGIDTCGG-DSGGPLICNGQFQGILSWGSDPCAEP-RKPAFY
gi_114837_atrox           NGLPAKT----LCAGVLQGGIDTCGG-DSGGPLICNGQFQGILSWGSDPCAEP-RKPAFY
gi_357580550_leucurus     NGLPAKT----LCAGVLQGGIDTCGG-DSGGPLICNGQFQGILSWGSDPCAEP-RKPAFY
gi_123895619_jararacussu  NGLPAKTT---LCAGVLEGGKDTCVG-DSGGPLICNGQFQGIVSYGAHSCGQG-PKPGIY
                                :    ****:*:** *:* * *************** .   ..*.:   **. *

                                 250       260
                                   |         |
BbrzSP_32                 TKVFDYLDRERSIIAGNTAAACPP
gi_383280398_Agk_pdb_4E7N TKVFDHLDWIENIIAGNTDASCPP
gi_14285806_jararaca      TKVFDYLPWIQSIIAGNKTATCPP
gi_114837_atrox           TKVFDYLPWIQSIIAGNKTATCP-
gi_357580550_leucurus     TKVFDYLPWIQSIIAGNKTATCP-
gi_123895619_jararacussu  TNVFDYTDWIQRNIAGNTDATCPP
                          *:***:    .  ****. *:**
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Fig. 2. Multiple sequence alignment between BbrzSP-32 tryptic peptides and thrombin-like enzymes (EC:3.4.21) from *Agkistrodon halys* (gi_383280398) *B. jararaca* (gi:14,285,806), *B. atrox* (gi:114,837), *B. leucurus* (gi:357,580,550 and *B. jararacussu* (gi:123,895,619). Asterisk indicates identical amino acid residues. *Colon* indicates conservation between groups of strongly similar properties. Period indicates conservation between groups of weakly similar properties. "-" means an unknown amino acid residue.

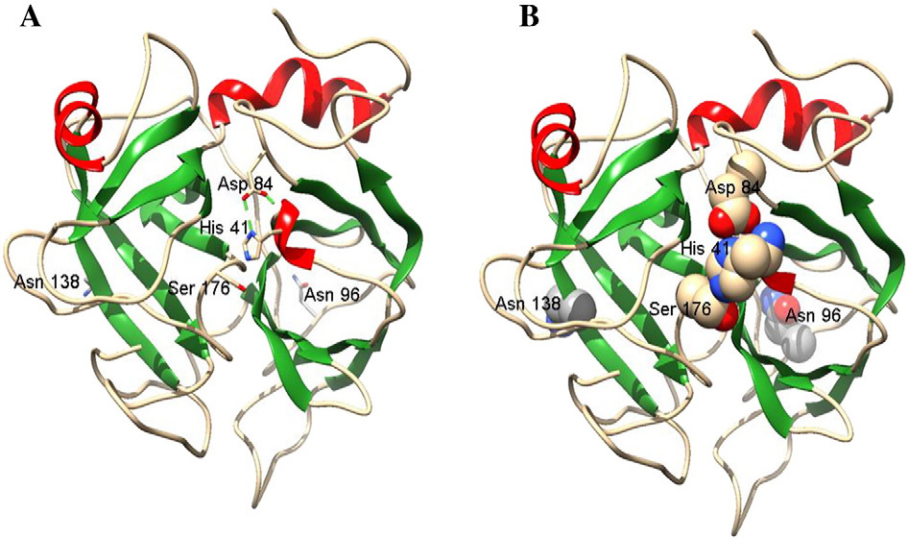


Fig. 3. Representation in "ribbon" of BbrzSP-32 performed on the Swiss-PDB Viewer and rendered with POV-ray. The catalytic triad (His41, Asp84, and Ser176) and the predicted N-linked oligosaccharides sites (Asn96 and Asn138) were detached in Sticks (A) and Spheres (B).

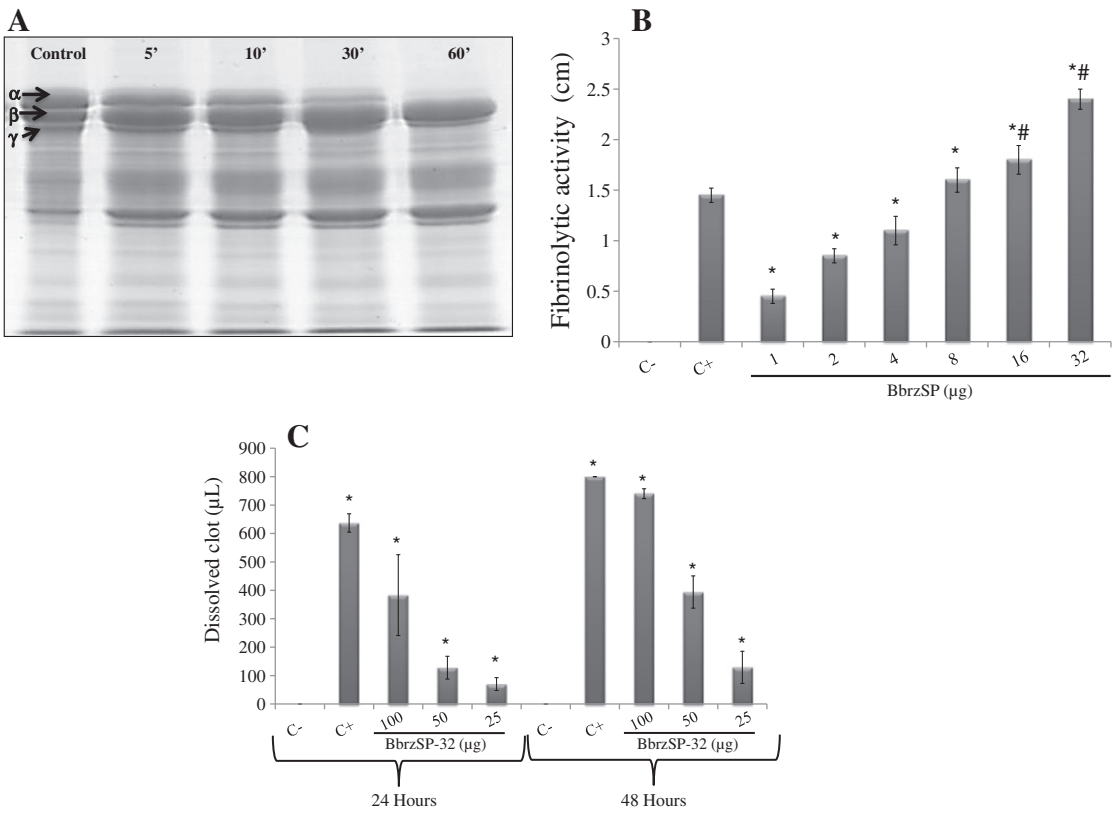


Fig. 4. A) Fibrinogenolytic activity of *B. brazili* serine protease. 5 μg of BbrzSP-32 was incubated with 50 μL of fibrinogen solution (2 mg/mL) at 37 °C for different times (5, 10, 30 and 60 min). B) Fibrinolytic activity. Results after incubation were assessed by measurement of degradation halos (cm) for varying amounts of BbrzSP-32 (1, 2, 4, 8, 16, and 32 μg) and 40 μg of crude venom of *B. brazili* (C+) or milliQ water (C-) with the fibrin network copolymerized with gelatin *p ≤ 0.05 compared to the negative control, and #p ≤ 0,05 compared to the positive control (ANOVA). C) Thrombolytic activity assay was performed with human blood incubated for 24 to 48 h at 37 °C with different concentrations of BbrzSP-32 (25, 50 and 100 μg in 300 μL), crude venom of *B. brazili* (50 mg/300 μL) (C+) and MilliQ water (300 μL) (C-) * p ≤ 0,05 compared to the negative control.

conditions assayed. The inedited serine protease isolated from *B. brazili* snake venom showed pH stability upon chromogenic substrate S-2238. At pHs ranging from 6.8 to 11 (Fig. 7A) the enzyme showed the highest activity. Furthermore, when tested in a temperature range (−20, −8, 20, 30, 37, 40 and 50) the enzyme was able to produce *p*-nitroanilide, thus showing a thermal stability (Fig. 7B). But, at temperature above 70 °C, its enzymatic activity was lost.

4. Discussion

Viperidae and Crotalidae snakes have venoms that generally induce persistent bleeding, since their venoms contain a large variety of proteins and peptides that have activities upon the hemostatic system. These compounds are known as coagulant, anticoagulant or fibrinolytic factors and some of these proteins may induce a considerable degradation of fibrinogen and other coagulation factors preventing clot formation (Braud et al., 2000; Costa et al., 2010, 2007).

Snake Venom Serine Proteases (SVSPs) are known enzymes that directly affect the hemostatic system. This article reports an efficient procedure for the isolation of an new serine protease from the crude venom of the Amazonian *B. brazili* snake using two chromatographic procedures (Fig. 1A and B) followed by the characterization of its structural and biological properties. Its molecular mass was showed to be 32,519.25 Da by MALDI-TOF/MS, which is in accordance with molecular mass of others SVSPs, that are single-chained proteins, with molecular weight ranging from 26 to 67 kDa, depending on the amount of carbohydrate (Kini, 2005; Serrano and Maroun, 2005).

BbrzSP-32 showed around 79% homology with other SVSPs (Fig. 2 and Supplementary material). Homology modelling is usually the method of choice when a clear relationship of homology between the sequence of the target protein and at least one known structure is found. This approach gives reasonable results based on the assumption that the tertiary structures of two proteins will be similar if their sequences are related (Kroemer et al., 1996).

To evaluate the validity of 4E7N as a structural template for BbrzSP-32 modelling, we examined the sequence homologies. The alignment of BbrzSP-32 yielded 71% identity with 4E7N. The percentage of residues lying in the favored regions of a Ramachandran plot (Ramachandran et al., 1963) is one of the best guides to check the stereo chemical quality of a protein model based on the assumption that a good template should have more than 90% of the residues in the allowed regions (Laskowski et al., 1993). The analysis of the Ramachandran plot for the structure of BbrzSP-32 showed that 90.8% of the amino acids are in favorable regions (Fig. 8). The quality of the model was also assessed by comparing the predicted structure with the template structure via superimposition and RMS deviation (RMSD) assessment of atomic positions. The fact that the RMSD of Cα trace between the homology structure and the template is 1.93 Å supports that the generated model is reasonably good and quite similar to the template. The structure has two α helices on the outside and ten uniformly distributed β sheets. Most SVSPs are glycoproteins showing a variable number of N-or O-glycosylation sites in sequence positions that differ between SVSPs (Serrano and Maroun, 2005).

With respect to biological activities, BbrzSP-32 was able to initiate the degradation of the α-chain of bovine fibrinogen since the first time tested. Many serine proteases from Viperidae snake are capable of converting fibrinogen into fibrin by cleaving fibrinopeptides A and/or B (FPB) (Pirkle and Theodor, 1990; Stocker et al., 1982). By mimicking this function of thrombin, these enzymes are referred to as thrombin-like and are classified according to their preference on the catalytic

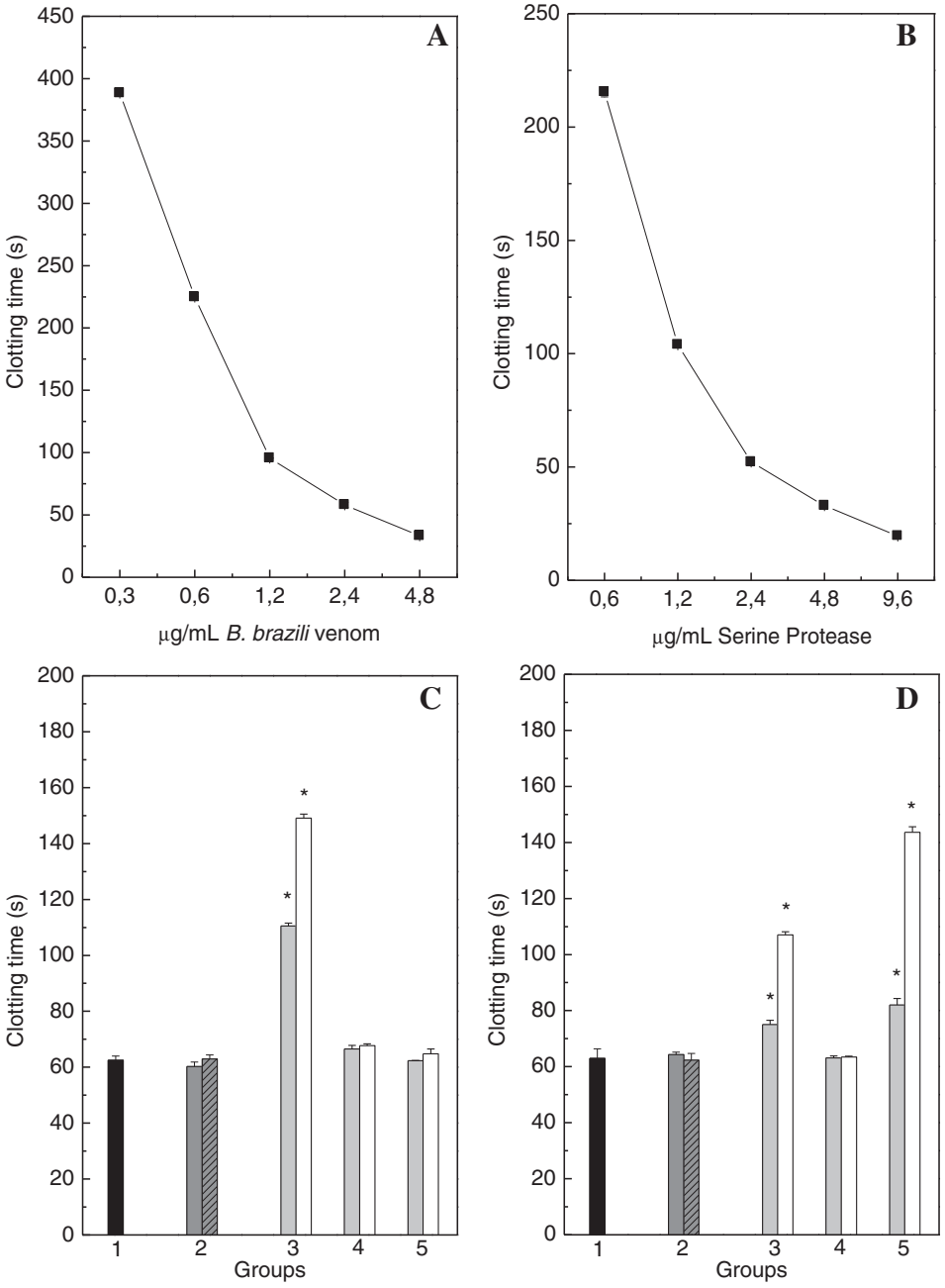


Fig. 5. Effect of *B. brazili* (panel A) and Bbrz-32 (panel B) on clotting. Different concentrations (0.3–4.8 μg/mL) of *B. brazili* venom and of purified serine protease (0.6–9.6 μg/mL) were tested upon plasma as described in the experimental section. Data expressed as mean ± SD of two individual experiments (n = 3). Effect of inhibitors on clotting activity induced by *B. brazili* venom and by the purified serine protease. Inhibitors were incubated for 30 min. at room temperature with 2.4 μg/mL of *B. brazili* venom and 2.4 μg/mL of the serine protease, respectively (panel C and D). Groups 1: *B. brazili* venom or BbrzSP-32 incubated with NaCl (black columns); Groups 2: *B. brazili* venom or BbrzSP-32 incubated with 1% DMSO (gray column) or 3% DMSO (hatched gray column); Groups 3: *B. brazili* venom or BbrzSP-32 incubated with benzamidine 5 mM (gray column) or 10 mM (white column); Groups 4: *B. brazili* venom or BbrzSP-32 incubated with 5 mM (gray column) or 10 mM (white column) EDTA; Groups 5: *B. brazili* venom or BbrzSP-32 incubated with 1 mM (gray column) or 3 mM (white column) PMSF. Data expressed as mean ± SD of two individuals experiments (n = 3). * Significance level ($p < 0.05$), when compared to columns 1 and 2.

activities of fibrinogen chains α, β or α and β in SVTLE-A,-B or SVTLE SVTLE-AB, respectively (Castro et al., 2004; F.S.J. Markland, 1998). BbrzSP-32 is considered an α-thrombin-like (SVTLE-A); most SVSPs are classified as clotting enzymes and the majority releases only fibrinopeptides A (FPA) from fibrinogen (Serrano and Maroun, 2005).

The proteases which cleave preferentially only α or β chains of fibrinogen lead to an increase of fibrinopeptides A or B, respectively, thereby causing abnormal clot formation. Furthermore, in most cases, these proteins did not have activity on coagulation factor XIII, so the produced clot will be an abnormal fibrin clot (F.S. Markland, 1998) of short polymers that are rapidly dispersed and perhaps lack the susceptibility to serpins. This has generated interest in their potential therapeutic application for the treatment of patients with occlusive arterial or venous thrombotic diseases without stimulating the endogenous fibrinolysis system (Matsui et al., 2000; White, 2005).There are serine proteases isolated from snakes being applied in clinical medicine as defibrinogenating agents, as is the case of batroxobin, isolated from *B. atrox moojeni* (now *B. moojeni*) (Stocker and Barlow, 1976) registered under the trade name Defibrase, already been used in patients suffering from thrombosis, myocardial infarction, peripheral vascular disease,

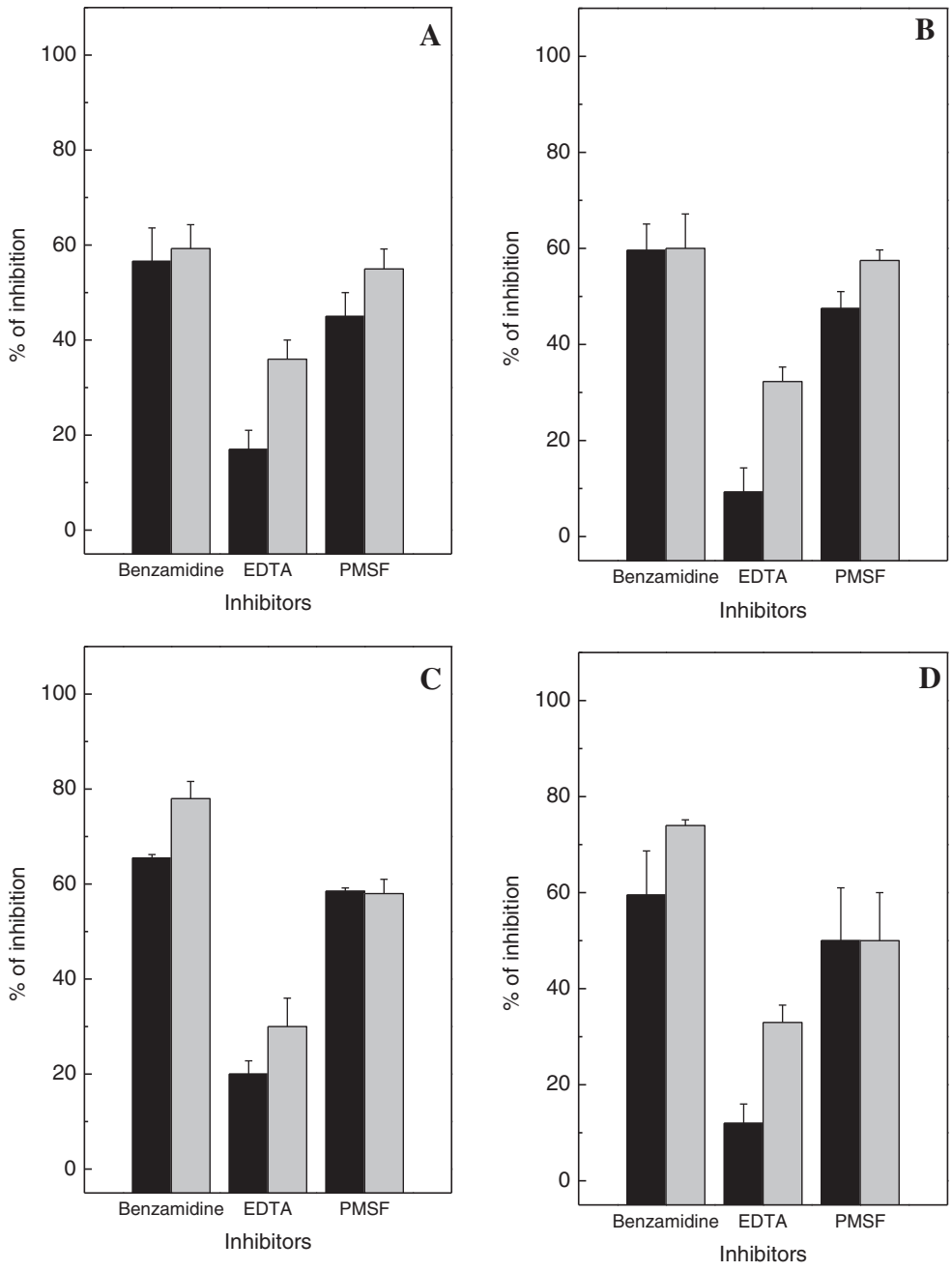


Fig. 6. Effect of inhibitors upon *B. brazili* and BbrzSP-32 activity. Inhibitors were incubated for 30 min. at room temperature with *B. brazili* venom (6 μg/mL), and then the enzymatic activity upon S-2238 (Panel A) or S-2288 (Panel B) was performed; and with BbrzSP-32 (25 μg/mL), and then the enzymatic activity upon S-2238 (Panel C) or S-2288 (Panel D) was performed. Inhibitors: Benzamidine: 5 mM (black column) or 10 mM (gray column); EDTA: 5 mM (black column) or 10 mM (gray column); PMSF: 1 mM (black column) or 3 mM (gray column). Data expressed as mean ± SEM of two individual experiments (n = 3).

acute ischemia and renal transplant rejection (Niewiarowski et al., 1979; Stocker et al., 1982). The same enzyme is also used in the preparation of fibrin adhesives, which are used as an alternative or complement to conventional suturing processes (Dascombe et al., 1997).

The majority of serine proteases may act on several natural and synthetic substrates. The clotting activity of Viperidae family venoms is associated with specific proteases groups: metalloproteases and serine proteases. And their enzymatic activities are generally affected by specific inhibitors such as benzamidine, PMSF and in a lesser degree by EDTA (metalloprotease inhibitor/Ca^{2+} chelator). The clotting activity of the crude venom was only reduced after pre-incubation with benzamidine; this result is explained by the presence of other proteases that have clotting activity and did not have their activities reduced/inhibited by the inhibitors tested. On the other hand, BbrzSP-32's dose-dependent clotting activity was reduced by PMSF and benzamidine; this reduction is acceptable since serine proteases can undergo reduction or avoidance of its activities after treatment with specific inhibitors, these inhibitors in turn can act reversibly or irreversibly. PMSF is an example of an inhibitor which has the capacity to bind in a specific serine residue at the catalytic site of serine, forming an irreversible bond that inactivates the activity of these enzymes. The inhibitors leupeptin and benzamidine bind reversibly and compete for the active site of serine proteases. Serine proteases has the amino acids arginine and lysine, which are residues for which the benzamidine have greater specificity; benzamidine and leupeptin can be used as insulating material to aid the enzyme in question. Generally, snake

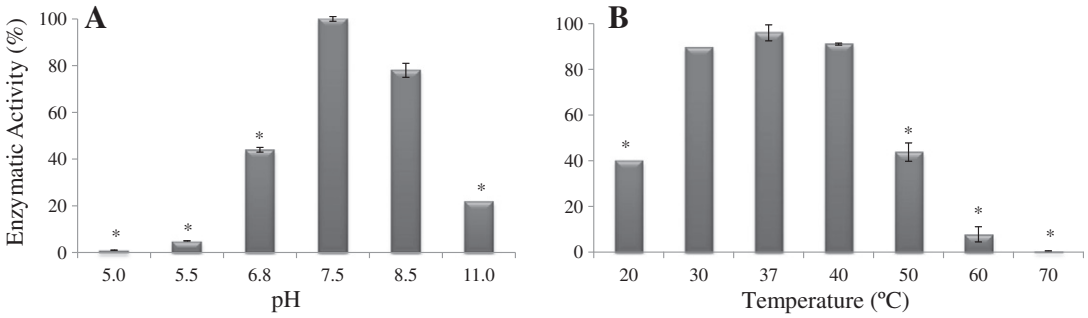


Fig. 7. A) Effect of pH on enzymatic activity. BbrzSP-32 was incubated for 30 min at room temperature with buffers of different pH values, and then the reaction was triggered adding S-2238. B) Effect of temperature. BbrzSP-32 was incubated for 30 min at different temperatures values, and then a reaction was triggered by the addition of BA*p*NA.

serine proteases are not sensitive to the action of endogenous inhibitors of serpin, such as antithrombin III and aprotinin (Serrano and Maroun, 2005).

Similar data could be observed by testing with substrates S-2238 and S-2288 where both the crude venom and BbrzSP-32 had reduced their activities after pre-incubation with benzamidine and PMSF. This result corroborates with other serine proteases isolated from other species: such as: BjussuSP-I, a thrombin-like isolated from *B. jararacussu* snake venom that showed its clotting, esterasic and amidolytic activity to be reduced after incubation with PMSF (Sant' Ana et al., 2008), BpiSP27 and BpirSP41 isolated from *B. pirajai* snake venom that had reduced their hydrolytic activities upon S-2238 substrate by PMSF and benzamidine (Menaldo et al., 2012), Agacutase, isolated from *Deinagkistrodon acutus* snake venom, showed its clotting activity reduced by PMSF (Tang et al., 2013), Da-36, isolated from *Deinagkistrodon acutus* snake venom that had reduced its hydrolytic activity upon TAME by PMSF and weakly decreased by benzamidine (Zheng et al., 2013), among others.

Platelets form a vital part of the haemostatic process. They act as the 'front line' in plugging any vascular deficiency, as well as providing activating surfaces for the coagulation cascade. They are metabolically active and subject to many forms of attack. There are two likely principal effects of snake venoms on platelets; (I) inhibition of platelet activity, thus reducing their effectiveness in hemostasis; and (II) promotion of platelet activity, increasing their contribution to hemostasis (White, 2005). Both activities were tested by incubating BbrzSP-32 with washed-platelets and the enzyme was not able to influence in platelet aggregation neither inducing nor inhibiting it. Basic serine proteases usually display direct platelet-aggregating activity while acidic proteins are described to have a variety of proteolytic activities on substrates related to hemostasis (Serrano and Maroun, 2005).

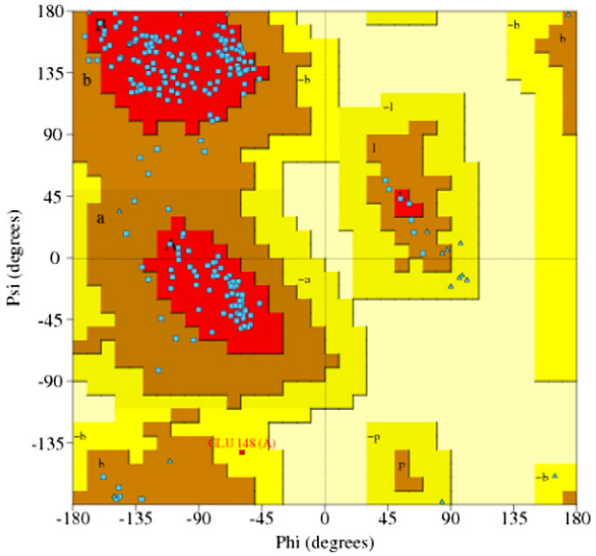
BbrzSP-32 demonstrated to be a resistant enzyme when tested in heat and pH ranges. In general, serine fibrinogenolytic enzymes are proteins that show a large resistance to inactivation by heat and pH extremes and this resistance to denaturation is probably provided by covalently bound carbohydrates (Swenson and Markland, 2005); based on its enzymatic activities, the protein proved to be a new serine protease from snake venom and the first isolated from *B. brazili*.

In conclusion, based on its biochemical and enzymatic characteristics, BbrzSP-32 was identified as the first thrombin-like enzyme reported from *B. brazili* snake venom.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.cbpa.2016.01.021.

Conflict of interest

There are no conflicts of interest.



1. Ramachandran Plot statistics

		No. of residues	%-tage
Most favoured regions	[A,B,L]	174	90.6%
Additional allowed regions	[a,b,l,p]	16	8.3%
Generously allowed regions	[~a,~b,~l,~p]	2	1.0%
Disallowed regions	[XX]	0	0.0%
Non-glycine and non-proline residues		192	100.0%
End-residues (excl. Gly and Pro)		1	
Glycine residues		19	
Proline residues		17	
Total number of residues		229	

Fig. 8. Analysis of the Ramachandran of BbrzSP-32. The result shows 90.8% of the amino acids in favorable regions.

Acknowledgment

The authors express their gratitude to Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq, No. 550854/2010-0 and No. 404358/2012-8), Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES, EDITAL 047/2012 PRÓ-AMAZÔNIA), Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP), Fundação de Amparo à Pesquisa do Estado de Minas Gerais (FAPEMIG), Instituto Nacional de Ciência e Tecnologia em Toxinas (INCT-Tox), Secretaria de Estado do Planejamento e Coordenação Geral (CNPq-SEPLAN-RO), CICYT (CTQ2009-07758) and the *Generalitat de Catalunya* (2009SGR 1024) for financial support, and to Conselho de Gestão do Patrimônio Genético (CGEN/MMA) for the authorization number 010627/2011-1. This study was authorized by CGEN/CNPq (010627/2011-1), IBAMA (27131-2) and human research committee CAAE: 14204413.5.0000.0011. The authors thank the Program for Technological Development in Tools for Health-PDTIS-FIOCRUZ for use of its facilities. The authors thank the Dra. Maria Antonia O. Caballol (CIBER-BBN, Barcelona Science Park, Barcelona, Spain) for the technical assistance in Proteomic Platform. Amy Grabner provided the English editing of the manuscript.

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