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BjSP, a novel serine protease from *Bothrops jararaca* snake venom that degrades fibrinogen without forming fibrin clots

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ABSTRACT

Snake venom serine proteases (SVSPs) are commonly described as capable of affecting hemostasis by interacting with several coagulation system components. In this study, we describe the isolation and characterization of BjSP from *Bothrops jararaca* snake venom, a serine protease with distinctive properties. This enzyme was isolated by three consecutive chromatographic steps and showed acidic character (pI 4.4), molecular mass of 28 kDa and N-carbohydrate content around 10%. Its partial amino acid sequence presented 100% identity to a serine protease cDNA clone previously identified from *B. jararaca* venom gland, but not yet isolated or characterized. BjSP was significantly inhibited by specific serine protease inhibitors and showed high stability at different pH values and temperatures. The enzyme displayed no effects on washed platelets, but was able to degrade fibrin clots in vitro and also the Aα and Bβ chains of fibrinogen differently from thrombin, forming additional fibrinopeptides derived from the Bβ chain, which should be related to its inability to coagulate fibrinogen solutions or platelet-poor plasma. In the mapping of catalytic subsites, the protease showed high hydrolytic specificity for tyrosine, especially in subsite S1. Additionally, its amidolytic activity on different chromogenic substrates suggests possible effects on other factors of the coagulation cascade. In conclusion, BjSP is a serine protease that acts non-specifically on fibrinogen, generating different Bβ fibrinopeptides and thus not forming fibrin clots. Its distinguished properties in comparison to most SVSPs stimulate further studies in an attempt to validate its potential as a defibrinogenating agent.

1. Introduction

Accidents caused by snakes are a serious public health problem in tropical and subtropical countries, due to their incidence, severity and sequelae (Saad et al., 2012). In Brazil, the majority of the venomous snake accidents are caused by *Bothrops* species (Albuquerque et al., 2013) and the pathophysiology of these envenomations is characterized by three major actions of the venoms: (i) a proteolytic action that causes local lesions and tissue destruction; (ii) a coagulant action that induces blood incoagulability by the consumption of fibrinogen; and (iii) a hemorrhagic action promoted by lesions in the basal membrane of capillaries and the release of hypotensive substances (Cruz et al., 2009). As a result, some of the major clinical manifestations are related to the isolated or combined actions of several components of those venoms.

Several snake venom proteins act on hemostasis. Some are able to interact with specific factors of the coagulation cascade, while others can act on platelets and other components of the hemostatic system (Markland, 1998; Kini, 2011; Sajevic et al., 2011). Among the main classes of proteins related to these effects are the proteolytic enzymes, such as metalloproteases and serine proteases (Slagboom et al., 2017).

Snake venom serine proteases (SVSPs) are usually glycosylated

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monomeric enzymes of acidic character, which can act on various components of the coagulation cascade and of the fibrinolytic and kallikrein-kinin systems (Serrano and Maroun, 2005). Besides affecting hemostasis by activating coagulation factors and inducing platelet aggregation (Braud and Bon, 2000; Serrano and Maroun, 2005), some SVSPs may also present other biological activities, as those that act on the complement system (Yamamoto et al., 2002; Menaldo et al., 2013).

Most SVSPs are classified as thrombin-like enzymes (SVTLEs) because of their ability to promote blood coagulation similar to thrombin, i.e. cleaving the Aα and/or Bβ chains of fibrinogen (Pirkle, 1998; Castro et al., 2004). Nevertheless, none of the SVTLEs described to date presented all the functions of thrombin, and, as they preferentially cleave only one of the chains of fibrinogen and are not able to activate factor XIII, SVTLEs produce abnormal fibrin clots composed of small polymers that disperse quickly and are not linked by factor XIIIa, resulting in the inhibition of normal blood coagulation on victims (Braud and Bon, 2000; Matsui et al., 2000).

The majority of SVSPs are classified as fibrinogen-clotting enzymes, but there are also those that do not promote the coagulation of fibrinogen (Serrano et al., 1995; Paes-Leme et al., 2008). Such enzymes possess great therapeutic potential since they can cause depletion of plasma fibrinogen, debilitating the development of thrombi in vivo without forming fibrin clots. Furthermore, these SVSPs are usually not susceptible to endogenous inhibitors and exhibit high enzymatic stability due to their high degree of glycosylation (Serrano and Maroun, 2005; Soares and Oliveira, 2009), which increases their potential for clinical use.

Considering the functional characteristics of SVSPs and targeting possible uses for these molecules, recent studies have been exploring different potential applications. Brazilian researchers, for example, discovered and developed a new heterologous fibrin biopolymer based on SVSPs (Abbade et al., 2015; Barbizan et al., 2013, 2014; Barros et al., 2009, 2011; Benitez et al., 2014; Biscola et al., 2017; Ferreira Junior, 2014; Ferreira Junior et al., 2017; Gasparotto et al., 2014; Gatti et al., 2011; Orsi et al., 2017). These enzymes have also been evaluated regarding their therapeutic potential as defibrinogenating and/or thrombolytic agents (Gardiner and Andrews, 2008). One of the most promising SVTLEs for clinical use was a serine protease isolated from *Calloselasma rhodostoma* snake venom, named Ancrod (Viprinex™). Initial recommendations for its use in the treatment of acute ischemic stroke were very promising. However, Ancrod failed phase 3 clinical trials in 2008, and its development as a new drug was discontinued (Mackessy, 2010). Another promising SVTLE is Batroxobin (Defibrase®) from *Bothrops moojeni* venom, which has been investigated extensively for use in a variety of disorders, including cerebral and myocardial infarction and ischemic stroke (Xu et al., 2007; Mackessy, 2010).

In search of a new molecule with possible therapeutic applications, the present study describes the isolation and characterization of a novel serine protease from *Bothrops jararaca* snake venom, named BjSP, which presents distinctive actions on fibrinogen that could validate its use as a defibrinogenating agent.

2. Materials and methods

2.1. Venom and other components

B. jararaca snake venom was provided by the Center for the Study of Venoms and Venomous Animals from São Paulo State University (Botucatu, SP, Brazil), which extracted and pooled the venoms from various adult snakes of the same region (22°53′09″ S, 48°26′42″ W), as described by Saad et al. (2012). The chromatographic resins and reagents for the enzymatic and biochemical assays were obtained from GE Healthcare, Merck or Sigma-Aldrich. P-nitroanilide substrates S-2238, S-2222, S-2366, S-2302, S-2765 and S-2251 were from Chromogenix. Equipment and other materials were described in the methodology and reagents not otherwise specified were of analytical grade.

2.2. Human plasma

The human plasma used in the experiments of coagulation and effects on platelets was obtained from the blood of healthy male or female volunteers with ages between 20 and 40 years who had not used medication ten days prior to collection. Blood was collected by venipuncture using 3.8% sodium citrate (9:1, *v*/v) as anticoagulant. The plasma collected was centrifuged at approximately 170 x*g* for 10 min at room temperature to obtain platelet rich plasma (PRP) or at approximately 1050 x*g* for 15 min at room temperature to obtain platelet-poor plasma (PPP). All procedures involving human participants were in accordance with ethical standards and were approved by the Research Ethics Committee of FCFRP-USP (CEP/FCFRP n. 694.165).

2.3. BjSP purification procedure

The chromatographic fractionation of *B. jararaca* venom for obtaining BjSP was initiated by a molecular exclusion chromatography on Sephacryl S-200, followed by a chromatographic step on Asahipak ES502N 7C anion exchange column using an AKTA purifier system (GE Healthcare), and a final step on CLC-ODS C18 reverse phase column using a high-performance liquid chromatography system (HPLC) (Shimadzu). All chromatographic fractions were monitored by their absorbance at 280 nm and data were plotted on graphs using the Origin 8 software.

2.3.1. Molecular exclusion chromatography on Sephacryl S-200

The crystallized *B. jararaca* crude venom (100 mg) was suspended in 2 mL of 50 mM ammonium bicarbonate buffer (Ambic), pH 8.0, and then centrifuged at 10,000 x*g* for 10 min at room temperature. The clear supernatant was applied to a chromatography column (3 × 126 cm) containing Sephacryl S-200 resin (GE Healthcare), previously equilibrated and eluted with the same buffer. Fractions of 3 mL/tube were collected at a flow rate of 15 mL/h at room temperature. Fraction E was chosen according to its profile on sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and was pooled, lyophilized and stored at −20 °C until the following chromatography.

2.3.2. Anion exchange chromatography on ES502N 7C column

Fraction E from Sephacryl S-200 (~15 mg) was solubilized in 550 μL of 20 mM Tris-HCl pH 8.0, and centrifuged at 2000 x*g* for 10 min at room temperature. Subsequently, the supernatant was subjected to an ES502N 7C anion exchange column (0.76 × 10 cm, Shodex), previously equilibrated with 20 mM Tris-HCl pH 8.0. Elution was carried out with the same buffer and a segmented concentration gradient of 60 mM NaCl. In the first segment, the NaCl concentration ranged from 0% to 45% over 35 column volumes; in the second, from 45% to 100% in 5 column volumes, and in the third, concentration was maintained at 100% for 3 column volumes. Elution was carried out at a flow rate of 0.5 mL/min, collecting fractions of 3 mL/tube. Fraction g was chosen based on its SDS-PAGE profile and was rechromatographed after lyophilization.

2.3.3. Reverse phase chromatography on C18 column

Fraction g from the anion exchange step was subjected to a CLC-ODS C18 reverse phase column (0.46 × 25 cm, Shimadzu) using a HPLC system (Shimadzu). The column was previously equilibrated with a solution of 0.1% trifluoroacetic acid (TFA) (solution A), and fraction g (~2 mg) was diluted in the same solution and applied to the system using a 500 μL loop. Elution was performed collecting 1 mL/tube at a flow rate of 1 mL/min, using a segmented concentration gradient of solution B (70% acetonitrile and 0.1% TFA), as follows: 0% B for 10 min; 0–60% B for 10 min; 60–65% B for 20 min; 70–100% B for 5 min and 100% B for 10 min.

The fraction containing the serine protease of interest (BjSP) was identified according to its profile on SDS-PAGE, lyophilized and

quantified (as described below) for use in the following experiments.

2.4. Protein quantification

Protein concentrations were determined by the bicinchoninic acid assay using the Pierce™ BCA Protein Assay Kit (Thermo Fischer Scientific), according to the manufacturer's instructions and using bovine serum albumin (Bio-Rad) as standard.

2.5. Esterase activity

The esterase activity of chromatographic fractions and the isolated protein was determined using the substrate Nα-p-tosyl-L-arginine methyl ester (TAME), as described by Menaldo et al. (2012). Briefly, the esterase activity was assessed by the absorbance at 247 nm after 30 min of reaction of samples (5 μg) with 1 mM TAME (final concentration) at 37 °C. One TAME unit represents an increase of 0.001 absorbance units per minute of reaction. The results were expressed as specific activity, related to TAME units per milligram of protein (U/mg).

2.6. SDS-PAGE

SDS-PAGE was performed under reducing or non-reducing conditions using a 12% polyacrylamide gel, according to method described by Laemmli (1970). Samples were heated at 100 °C for 5 min with reducing or non-reducing buffers (presence or absence of β-mercaptoethanol, respectively), and then applied to the gels along with a molecular mass standard (Thermo Scientific Unstained Protein Molecular Weight Marker #26610). The electrophoresis system used was a Mini VE 10 × 10 cm Vertical Gel System (GE Healthcare) with a power supply EPS 301 (GE Healthcare).

2.7. Deglycosylation assays

The enzyme N-glycosidase F (PNGase F, New England BioLabs) was used to evaluate the degree of N-glycosylation of BjSP, according to the manufacturer's instructions. BjSP (5 μg) was denatured by heating at 100 °C in the presence of 1 μL of denaturing buffer (5% SDS and 0.4 M dithiothreitol - DTT) to a final volume of 10 μL. Then, 2 μL of reaction buffer (0.5 M sodium phosphate, pH 7.5), 2 μL of 10% NP40 solution (SDS neutralizing agent), 1 μL of PNGase F (50 U) and 5 μL of ultrapure water were added to a final reaction volume of 20 μL. Reaction samples were incubated at 37 °C for 24 h and then heated at 100 °C for 10 min in the presence of reducing buffer (10 μL), followed by analysis on 12% SDS-PAGE (as described in section 2.6).

2.8. Determination of the molecular mass

The molecular mass of native and deglycosylated BjSP was initially estimated by SDS-PAGE under reducing conditions. The estimation was made by the interpolation of a linear logarithmic curve of the relative molecular mass of standard proteins versus the distance of migration of sample proteins in the gel.

Molecular mass of BjSP was also determined by mass spectrometry using an AXIMA Performance MALDI-TOF/TOF mass spectrometer (Shimadzu Biotech) and mass spectra were acquired in linear mode. The molecular mass range evaluated was from 15,000 to 70,000 *m/z*. BjSP was diluted in 50 μL of ultrapure water, mixed in a 1:1 ratio with a matrix consisting of sinapinic acid (10 mg/mL) in 50% acetonitrile and 0.1% TFA, and then applied on the MALDI plate using the dried-droplet method.

2.9. Isoelectric focusing

The pI of BjSP was determined by isoelectric focusing as described by Arantes et al. (1989), using a 7% polyacrylamide gel containing carrier ampholytes covering the range from pH 3 to 10 (Pharmalyte, Sigma-Aldrich). An isoelectric focusing standard of pI range from 4.45 to 9.6 (Bio-Rad) was run in parallel to the samples under the same conditions. After focusing, sections of the gel (1 × 2 cm) were cut along the gel sides, immersed individually in 500 μL of ultrapure water for 2 h, and measured for their pH. The pI of BjSP was calculated from the curve of pH versus the distance of migration in the gel.

2.10. Amino acid sequence analysis

2.10.1. N-terminal sequencing

Sequencing of the N-terminal region of BjSP was carried out by Edman degradation (Edman and Begg, 1967). The purified enzyme was placed directly on a glass membrane (Wako, Japan) at a concentration of 10 pmol. Then, the automatic protein sequencer (PPSQ-33A system, Shimadzu) examined the primary structure of the serine protease. The identification and quantification of the sample was performed by comparison with standard amino acids (25 pmol) analyzed in the beginning of the sequencing.

2.10.2. Mass spectrometry sequencing

SDS-PAGE and lyophilized samples of BjSP were used for determining its amino acid sequence by mass spectrometry (MS). Initially, gel bands of BjSP were excised and transferred to 1.5 mL conical tubes. Then, SDS and Coomassie Blue dye were removed by three successive washes with 100 mM Ambic containing 50% acetonitrile (ACN). The samples were then dehydrated with pure ACN and dried in a rotary vacuum centrifuge (SpeedVac, Savant).

BjSP dried gel bands or lyophilized samples were incubated at 37 °C for 24 h with one of the following proteases in 100 mM Ambic: modified trypsin, Glu-C (*Staphylococcus aureus* V8) or Asp-N (Promega). After the incubation period, the reaction was stopped by adding 1% formic acid and kept at room temperature for the extraction of peptides. Samples were applied on ZipTip columns containing a reverse phase resin (POROS R2, Perseptive Biosystems) previously activated with methanol and equilibrated with 0.2% formic acid. The removal of salts and other hydrophilic components from samples was achieved by 3 washes with 0.2% formic acid, and the peptides attached to the resin were eluted with a solution of 60% methanol in 5% formic acid. The eluted peptides were dried in a SpeedVac so that they could be diluted in a suitable matrix for analysis by MALDI-TOF/TOF MS.

Samples of peptides were suspended in α-cyano-4-hydroxycinnamic acid matrix (5 mg/mL in 0.1% trifluoroacetic acid/50% ACN) and applied to the MALDI plate for analysis in a MALDI-TOF/TOF mass spectrometer (Axima Performance, Shimadzu), with automatic acquisition of MS and MS/MS spectra for the most abundant ions. MS spectra were acquired automatically in Reflectron Positive mode and were processed and submitted to the NCBInr or SwissProt databases for identification of proteins using the Mascot software (http://www.matrixscience.com/search_form_select.html).

2.10.3. In silico analysis

The amino acid sequences obtained from digested peptides and Edman degradation were subjected to multiple sequence alignment with other sequences of homologous molecules deposited in the main databases using the programs FASTA (www.ebi.ac.uk/Tools/fasta33/index.html) and BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Multiple sequence alignments were performed using the Clustal X version 2.02 software.

2.11. Fibrinolytic activity

The fibrinolytic activity of BjSP was assessed on fibrin clots formed in Petri dishes, according to methodology of Leitão et al. (2000). Different doses of BjSP (5, 10 and 20 μg in PBS to a final volume of 30 μL) were applied in cavities (5 mm diameter) made on the fibrin gel along

with a negative control of phosphate-buffered saline (PBS, 30 μL) and positive controls of plasmin (5 μg) and *B. jararaca* venom (20 μg). The plate was incubated for 24 h at 37 °C and the fibrinolytic activity was evaluated visually by the presence or absence of fibrinolysis halos.

2.12. Fibrinogenolytic activity

The fibrinogenolytic activity of BjSP was determined according to the methodology described by Edgar and Prentice (1973), with modifications. Human fibrinogen (15 μg in 50 mM Tris-HCl buffer, pH 7.4, containing 70 mM NaCl) was incubated at 37 °C for 10, 30, 60 and 120 min with BjSP (5 μg). The enzyme was also preincubated with the inhibitors benzamidine (5 mM) and ethylenediaminetetraacetic acid (EDTA, 5 mM) for 30 min at 37 °C prior to the addition of fibrinogen and incubation at 37 °C for 120 min. After the incubation periods, reactions were stopped by addition of denaturing buffer containing β-mercaptoethanol and heating at 100 °C for 5 min. All samples were subjected to 12% SDS-PAGE (as described in section 2.6) for the analysis of fibrinogen degradation.

2.13. Identification of fibrinopeptides

Fibrinopeptides originated by the proteolytic activity of BjSP were identified as described by Magalhães et al. (2007), with modifications. The fibrinopeptides were generated by incubation of human fibrinogen (3 mg/mL in 50 mM Tris-HCl buffer, pH 7.4, containing 70 mM NaCl) with BjSP (20 and 400 μg/mL) at 37 °C for 120 min. Insoluble proteins were removed with addition of 2% TCA followed by centrifugation at 2000 ×*g* for 10 min and the supernatants containing fibrinopeptides were analyzed by reverse phase HPLC at 214 nm, using a C18 column (0.46 × 25 cm, CLC-ODS, Shimadzu, Japan) and a gradient of solutions A (0.1% TFA) and B (70% acetonitrile in 0.1% TFA), as follows: A for 15 min; then 0–100% B for 30 min, and 100% B for 10 min, at a flow rate of 1 mL/min. Thrombin (5 μg/mL) was incubated with fibrinogen for 120 min as a positive control, and solutions of fibrinogen, thrombin, BjSP and TCA were also chromatographed individually as assay controls. For comparative purposes, purified fibrinopeptides A and B (Sigma F3254 and F3379) were passed through the column to determine their elution times. Then, the fibrinopeptides generated by thrombin and BjSP were sequenced by Edman degradation (Section 2.10.1).

2.14. Coagulant activity

2.14.1. Clotting of fibrinogen solutions

Clotting of bovine fibrinogen was assayed as previously described (Da Silva et al., 2012), with minor modifications. Briefly, 50 μL of BjSP solutions (800 μg/mL) was incubated with 150 μL of human fibrinogen solution (2 mg/mL) at 37 °C for 30 min. If no coagulation could be observed in that period, 50 μL of *B. jararaca* crude venom (400 μg/mL) was added and solutions were monitored for additional 60 min. BjSP samples were compared to positive controls consisting of 50 μL of thrombin (5 U/mL) or *B. jararaca* venom (400 μg/mL).

2.14.2. Clotting of human plasma

Human citrated platelet-poor plasma (200 μL) was incubated for 30 min at 37 °C in the presence of 50 μL of solutions of BjSP (800 μg/mL). If no coagulation could be observed in that period, 50 μL of 250 mM $CaCl_2$ was added to the solutions and the clotting time was then monitored for additional 60 min. $CaCl_2$ was evaluated under the same conditions as a positive control of coagulation. The minimum coagulant concentration of *B. jararaca* crude venom (50 μg/mL), defined as the concentration required to clot plasma in 60 s, was also used as a positive control of coagulation.

2.15. Effects on platelets

The assays were performed using a platelet aggregometer (Chronolog Corporation, model 490 2D) and the software AggroLink, evaluating the platelet aggregation on washed platelets by turbidimetry.

Washed platelets were obtained by centrifugation of PRP at 1050 x*g* for 15 min to obtain PPP and a platelet pellet. The pellet was resuspended in 15 mL of washing buffer (103 mM NaCl, 5 mM KCl, 1 mM $MgCl_2.6H_2O$, 36 mM citric acid, 5 mM glucose, 0.35% BSA, 2 mM EGTA), pH 6.5, homogenized and centrifuged. This process was repeated twice and then the pellet of washed platelets was resuspended in resuspension buffer (103 mM NaCl, 5 mM KCl, 1 mM $MgCl_2.6H_2O$, 36 mM citric acid, 5 mM glucose, 0.35% BSA, 3.8 mM Hepes), pH 7.35. An aliquot of this suspension (10 μL) was separated for the platelet count held in a Neubauer chamber, being accordingly adjusted to a concentration of approximately 350 cells/μL.

Assays were performed using 450 μL of the solution of washed platelets with 5 μL of 250 mM $CaCl_2$ and 25 μL of a solution of BjSP (50 μg). This mixture was incubated for up to 10 min at 37 °C and its turbidity was monitored in aggregometer. As there were no significant changes in turbidity during this period of time, 25 μL of thrombin (13.4 U/mL) was added. The aggregation was compared with the incubation of 450 μL of washed platelets with 25 μL of thrombin (13.4 U/mL) alone.

2.16. Amidolytic activity on chromogenic substrates

The assay consisted of incubating BjSP (10 μg/mL) in the presence of the chromogenic substrates S-2238 (for thrombin), S-2366 (for factor XIa and activated protein C), S-2251 (for plasmin and streptokinase-activated plasminogen), S-2302 (for plasma kallikrein, factor XIa and factor XIIa), S-2222 and S-2765 (both for Factor Xa) at a final concentration of 0.4 mM. The reaction was carried out in 96-well microplates for 60 min at room temperature, using 50 mM Tris-HCl buffer, pH 7.4, containing 100 mM NaCl. The kinetics of the reaction was monitored by measuring the absorbance at 405 nm using an ELX808 microplate reader (BioTek). The absorbance of the amidolytic activity of substrates alone was subtracted from the values obtained after reaction with BjSP, resulting in a curve of absorbance vs. time. One unit of amidolytic activity (U) was defined as the increase of one absorbance unit at 405 nm per minute of the hydrolysis reaction, and the results were presented as specific activity (U/mg of BjSP).

2.17. Prothrombin, factor X and protein C activation

Activation of prothrombin (factor II), factor X and protein C was indirectly determined using the isolated coagulation factors and the specific chromogenic substrates for thrombin, factor Xa and activated protein C (S-2238, S-2222 or S-2366, respectively), according to Modesto et al. (2005). The reactions, consisting of coagulation factors (12.5 μg/mL, Calbiochem), BjSP (10 μg/mL) and the respective chromogenic substrate (0.4 mM) in 50 mM Tris-HCl pH 7.4 containing 100 mM NaCl, were carried out for 60 min at room temperature in microplates, being monitored at 405 nm, as described in section 2.16. The activities of BjSP alone on the chromogenic substrates were used as controls to determine the amidolytic activity of activation products formed by BjSP. Values correspond to final concentrations.

2.18. Effects of inhibitors

To evaluate the effect of inhibitors on the activity of BjSP, the protease (4 μg/mL) was incubated with different inhibitors: benzamidine (20 mM), phenylmethylsulfonyl fluoride - PMSF (20 mM), leupeptin (5 mM), EDTA (20 mM), SDS (20 mM), DTT (20 mM) and polyvalent bothropic/crotalic antiserum (1:20 and 1:200 m/m, Lema Injex Biologic). Incubations were performed at 37 °C for 30 min. After that,

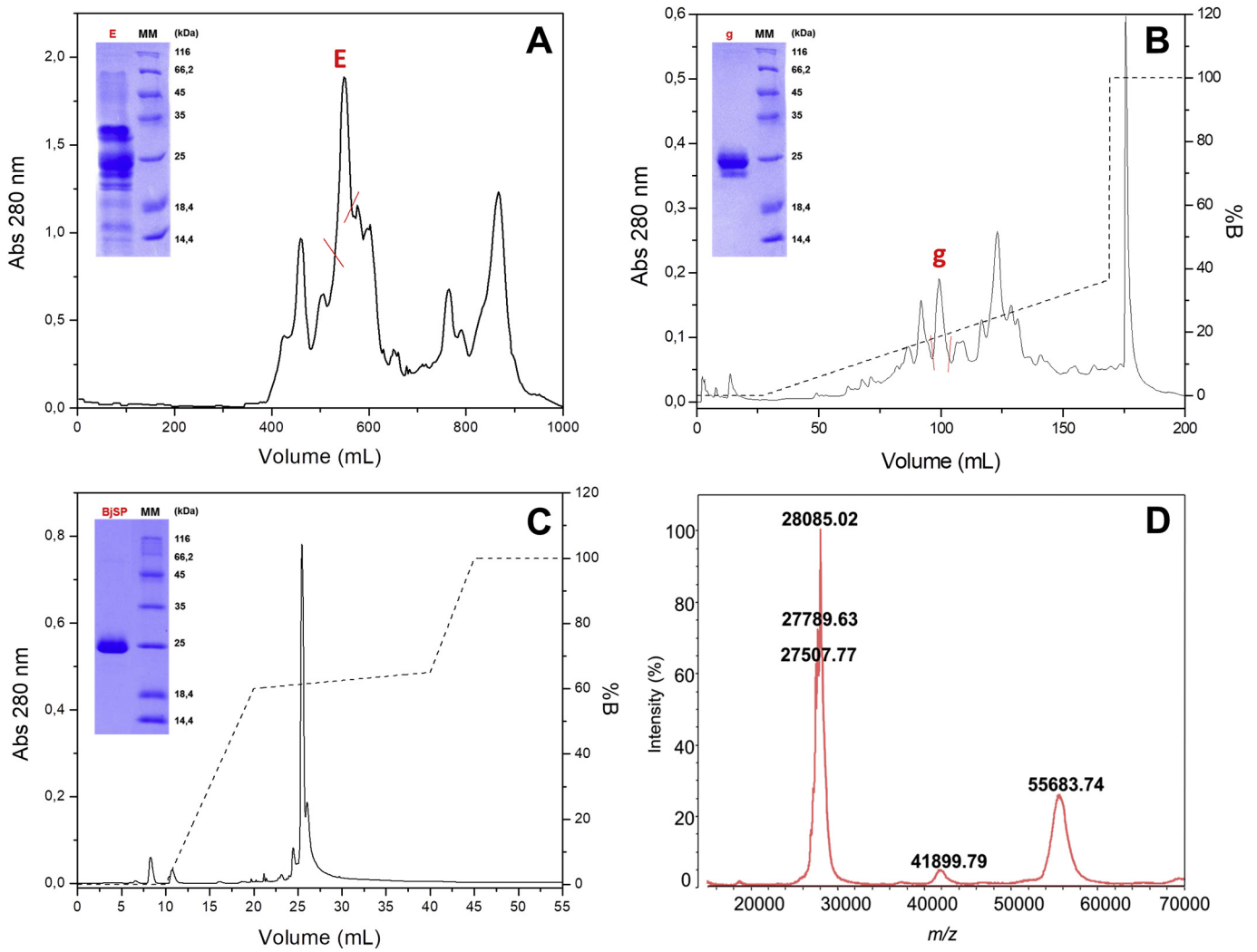

Fig. 1. Isolation of BjSP from *B. jararaca* snake venom. (A) Crude venom (100 mg) was applied to a Sephacryl S-200 molecular exclusion column (3 × 126 cm), previously equilibrated and eluted with 0.05 M ammonium bicarbonate buffer (Ambic), pH 8.0. Fractions of 3 mL/tube were collected at a flow rate of 15 mL/h at room temperature. Inserted, 12% SDS-PAGE of the fraction of interest (*E*) and a molecular mass standard (MM). (B) Fraction E (~15 mg) was subjected to an ES502N 7C anion exchange column (0.76 × 10 cm) using an AKTA purifier system. Elution was carried out with 20 mM Tris-HCl buffer pH 8.0 and a segmented concentration gradient of 0.6 M NaCl (solution B), at a flow rate of 0.5 mL/min and collecting fractions of 3 mL/tube. Inserted, 12% SDS-PAGE of the fraction of interest (*g*) and a molecular mass standard (MM). (C) Fraction g (~2 mg) was rechromatographed on a CLC-ODS C18 reverse phase column (0.46 × 25 cm) using a HPLC system (Shimadzu). The column was previously equilibrated with 0.1% trifluoroacetic acid (TFA) (solution A), and elution was performed using a segmented concentration gradient of solution B (70% acetonitrile and 0.1% TFA), collecting 1 mL/tube at a flow rate of 1 mL/min. Inserted, 12% SDS-PAGE of the isolated enzyme (BjSP) and a molecular mass standard (MM). (D) MALDI-TOF MS spectrum for the determination of the molecular mass of BjSP (~28 kDa).

the amidolytic activity of samples on substrate S-2366 (0.4 mM, final concentration) was evaluated as described in Section 2.16, and the results were expressed as the percentage of relative activity, comparing the values obtained for the reaction of BjSP and the chromogenic substrate (100% relative activity) with the values obtained for the reactions in the presence of inhibitors.

2.19. Optimum pH and temperature and determination of catalytic specificity

Optimum conditions of pH, temperature and catalytic specificity for BjSP were determined using fluorescence resonance energy transfer (FRET), within the conditions of pseudo first-order ([S] $<<$ k_M) at excitation and emission wavelengths of 320 nm and 420 nm, respectively.

Reaction conditions of pH and temperature were evaluated with substrate Abz-KLRYSKQ-EDDnp (P_1Y). The optimum pH was determined by testing a range of pH values from 6 to 9 and the buffers used were MES (pH 6 and 6.5), HEPES (pH 7, 7.5 and 8) and BICINE (pH 8.5 and 9) all at 100 mM. The optimum temperature was determined at optimum pH using a range from 30 to 70 °C with increment of 5 °C. Results were expressed as relative activity (%).

Specificity study was performed using four series of FRET substrates, with a combinatorial replacement of amino acids in the determined positions P_1; P_2; P_3 and P'_1 (Abz-KLXSSKQ-EDDnp, Abz-KXRSSKQ-EDDnp, Abz-XLRSSKQ-EDDnp, Abz-KLRXSKQ-EDDnp). The catalytic efficiency (k_{cat}/k_M) was evaluated as the determinant parameter of specificity for the interaction of the enzyme with peptide substrates. We also evaluated the hydrolysis of two fibrinogen clotting sequences – Abz-GVRGPRQ-EDDnp (Aα chain) and Abz-SARGHRQ-EDDnp (Bβ chain) – by incubating the protease with the peptides for 2 h at 50 °C.

In conditions of pseudo first-order ([S] $<<$ k_M), the catalytic efficiency was obtained by the relation between nonlinear regression of hydrolysis data and enzyme concentration. The substrate and enzyme final concentrations used in all assays were 0.6 μmol and 0.116 μmol,

respectively. The data were fitted with GraphPad Prism v. 5.01 software and enzyme concentration was obtained by active-site titration using antipain inhibitor (Klemencic et al., 2000).

The enzymatic reaction was performed in a Lumina fluorescence spectrometer (Thermo Scientific) with agitation and temperature control using a Peltier system 4-Position Cell Holder Fluorescence with quartz cuvettes (10 mm optical path length).

2.20. Stability evaluation

The stability of BjSP was assayed using its esterase activity on TAME (section 2.5). For that, BjSP was preincubated for 30 min at different temperatures (4, 25, 37, 45, 60 and 100 °C) and pH buffers (3.0, 4.5, 6.0, 7.5, 9.0 and 10.5) at 37 °C, followed by a 30 min reaction of the serine protease (5 μg) with 1 mM TAME (final concentration) at 37 °C. Then, the absorbances were determined at 247 nm and the results were expressed as relative activity (%).

2.21. Statistical analysis

Functional and enzymatic activities were assessed by two individual experiments in triplicate ($n = 3$), and the results were presented as mean values ± SEM. Statistical analysis of data was performed by one-way ANOVA followed by Tukey post-test, calculated on GraphPad Prism v. 5.01 software. P-values < 0.05 were considered as statistically significant.

3. Results

3.1. Isolation of BjSP

The isolation of the serine protease from *B. jararaca* crude venom was performed using consecutive chromatographic steps, beginning with a molecular exclusion on Sephacryl S-200 (Fig. 1A), followed by an anion exchange step on ES502N 7C column (Fig. 1B) and a reverse phase step on a C18 column (Fig. 1C). As we can see in the gel inserted in Fig. 1C, the enzyme of interest, named BjSP, appeared as a homogeneous protein band around 25 kDa under non-reducing conditions. Table 1 shows the total protein and enzymatic recovery of *B. jararaca* crude venom, the chromatographic fractions and the purified BjSP.

3.2. Molecular mass determination and deglycosylation assays

The molecular mass of BjSP estimated by SDS-PAGE under reducing conditions was of 34 kDa, while that determined by MALDI-TOF MS was of approximately 28 kDa (Fig. 1D, see supplementary files for further details on the MS spectra). When the enzyme was deglycosylated with PNGase F, a value of 30.8 kDa was estimated by SDS-PAGE, indicating that this difference of approximately 10% in the molecular masses of native and deglycosylated BjSP should be related to the presence of N-linked carbohydrates in its structure (Fig. 2A).

3.3. Isoelectric focusing

The pI calculated for BjSP was of approximately 4.4 (Fig. 2B), showing that this serine protease is an acidic enzyme.

Table 1
Recovery rates of *Bothrops jararaca* venom fractions and enzymatic activity.

	Protein concentrations		Esterase activity on TAME
	Total (mg)	Recovery (%)	Specific activity (U/mg)
Crude venom	81.96	100.00	1620
Fraction E	15.00	18.30	3028
Fraction g	1.33	1.62	3874
BjSP	0.27	0.33	4782

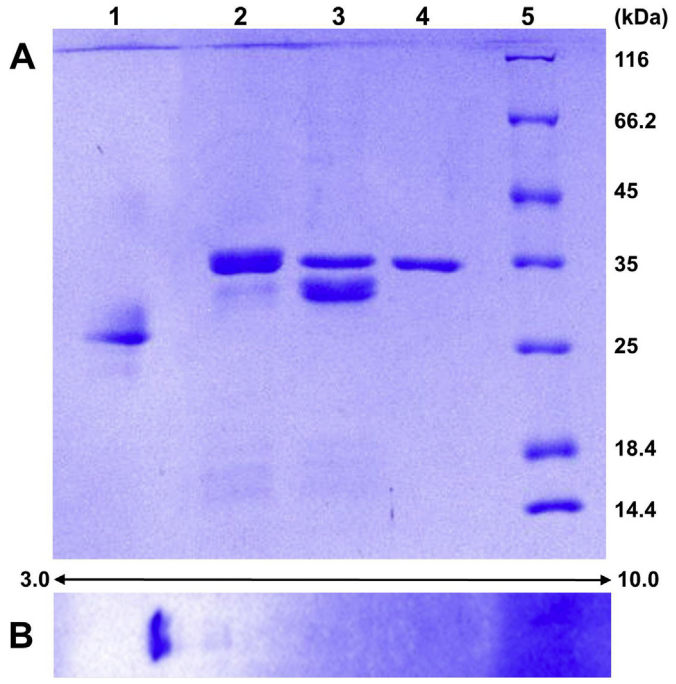

Fig. 2. (A) Deglycosylation of BjSP. BjSP (5 μg) was incubated with PNGase F (50 U) for 24 h at 37 °C and deglycosylation was evaluated on 12% SDS-PAGE under reducing conditions. Lanes: 1. Non-reduced BjSP (5 μg); 2. Reduced BjSP (5 μg); 3. BjSP (5 μg) + PNGase F (50 U); 4. PNGase F (50 U); 5. Molecular mass standard. (B) Isoelectric focusing of BjSP. BjSP (20 μg) was applied to a 7% polyacrylamide gel and isoelectric focusing was performed. A curve of migration distance vs. pH was constructed for determining the isoelectric point of BjSP (pI~4.4).

3.4. Partial amino acid sequencing

The partial amino acid sequence of BjSP was determined by Edman degradation and MALDI-TOF mass spectrometry methods, resulting in 188 amino acid residues determined from an estimated total of 234 residues. The multiple alignment of this sequence with other serine proteases previously described from *B. jararaca* venom showed that BjSP presented 100% identity to the cDNA clone HS114 described by Saguchi et al. (2005), besides showing percentages of identity between 60 and 70% with other serine proteases, such as BPA, PA-BJ, KN-BJ2 and Bothrombin (Fig. 3). Among the sequenced amino acid residues, there are 9 Cys residues related to the formation of disulfide bonds (from a total of 12 cysteine residues that form 6 disulfide bonds), Asp86 and Ser180 residues, which are part of the highly conserved catalytic triad of serine proteases (formed by residues His41, Asp86 and Ser180), and also residue Asn20, which is part of a NXT/S glycosylation site sequon and thus should be related to the N-glycosylation we previously described for BjSP (Fig. 2A).

3.5. Fibrinolytic activity

After 24 h of incubation of fibrin plates at 37 °C, BjSP at 20 μg induced a fibrinolysis halo comparable to that induced by plasmin at 5 μg. This indicates that BjSP presents fibrinolytic activity in vitro, but its effects on fibrin are considerably lower in comparison to plasmin (see supplementary files for figures).

3.6. Degradation of fibrinogen and formation of fibrinopeptides

As evaluated by SDS-PAGE, BjSP was able to degrade the Aα chains

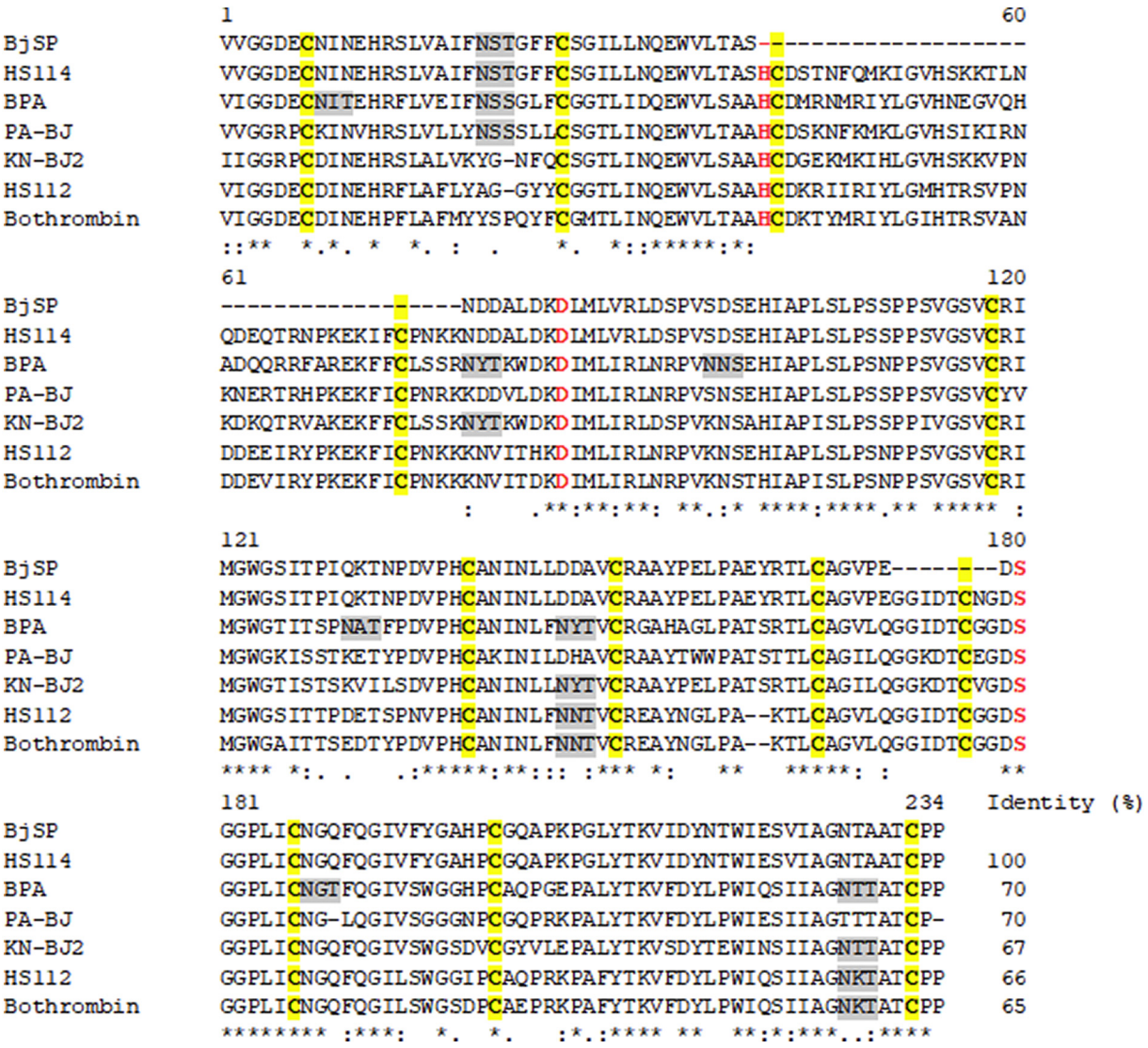

Fig. 3. Comparative analysis of the partial amino acid sequence of BjSP with other serine proteases from *B. jararaca* venom. The highlighted amino acid residues are part of disulfide bonds (yellow), the catalytic site (red) or possible N-glycosylation sites (NXT/S, where X is any amino acid except proline) (grey). NCBI GI numbers: HS114 gi 82233395, BPA gi 13959657, PA-BJ gi 999161, KN-BJ2 gi 13959622, HS112 gi 82233396 and Bothrombin gi 14285806. The multiple sequence alignment was made by the program ClustalX v. 2.0.11. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

of fibrinogen since the first minutes of reaction, with degradation of the Bβ chains predominantly occurring after longer incubation periods and no apparent effects on the γ chain (Fig. 4). Almost complete degradation of both Aα and Bβ chains were observed after 120 min of reaction (Fig. 4, lane 5). In addition, preincubation of BjSP with benzamidine apparently inhibited this degradation, but the same did not occur with EDTA (Fig. 4, lanes 6 and 7).

After that, we evaluated the ability of BjSP to form fibrinopeptides, using commercial fibrinopeptides A and B (Fig. 5A) and the fibrinogen degradation promoted by thrombin (Fig. 5B) as controls for comparison. Our results showed that, at lower concentrations (20 μg/mL), BjSP preferentially formed fibrinopeptide A (Fig. 5C), but when in excess (400 μg/mL), the serine protease also formed other fibrinopeptides with elution times that did not match fibrinopeptides A or B (Fig. 5D). To determine the amino acid sequences of such peptides, we performed the N-terminal sequencing of samples *a*, *b*, b_1 and b_2 (Fig. 5D) by Edman degradation. The sequence of sample *a* matched that of fibrinopeptide A (ADSGEGDFLAEGGGVR), while sample *b* could not be sequenced by Edman degradation, suggesting that this peptide has a blocked N-terminal (pyro-Q) that would match the sequence of fibrinopeptide B (QGVNDNEEGFFSAR). Regarding samples b_1 and b_2, both presented sequences derived from the Bβ chain of fibrinogen (GHRPLDKKREEAPSLRPAPPPISGGGYR), indicating that BjSP presented different proteolytic properties on fibrinogen in comparison to thrombin.

3.7. Fibrinogen-clotting activity

In accordance with the results of fibrinogenolysis and formation of fibrinopeptides, BjSP at the high concentration of 800 μg/mL was not capable of forming fibrin clots from fibrinogen solutions up to a 30 min evaluation period, differently from thrombin (5 U/mL) and *B. jararaca* crude venom (400 μg/mL), which were able to promote fibrinogen coagulation in about 37 and 15 s, respectively. Additionally, the fibrinogen solutions incubated with BjSP (800 μg/mL) did not coagulate up to 60 min after addition of *B. jararaca* venom (400 μg/mL) to the solutions (see supplementary files for detailed data).

3.8. Coagulant activity on plasma

BjSP at the concentration of 800 μg/mL was unable to coagulate the

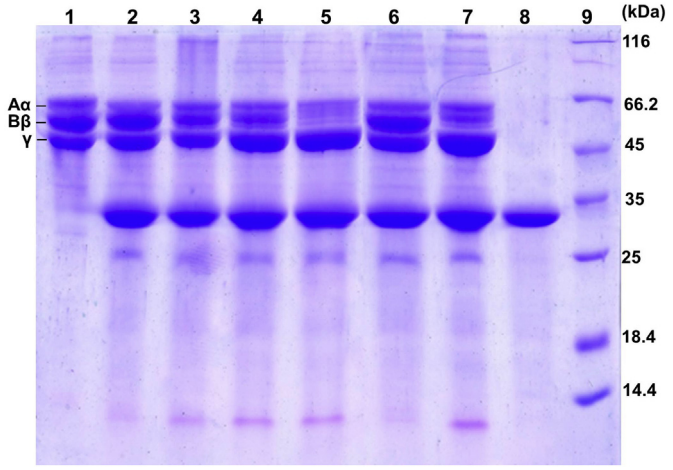

Fig. 4. Effects of BjSP on fibrinogen. Fibrinogenolytic activity was evaluated on 12% SDS-PAGE after incubation of BjSP (5 μg) with human fibrinogen (15 μg) at 37 °C for different time periods. When evaluated with inhibitors, BjSP was preincubated at 37 °C for 30 min in the presence of benzamidine or EDTA (5 mM) prior to the addition of fibrinogen. Lanes: 1. Fibrinogen control; 2. Fibrinogen + BjSP (10 min); 3. Fibrinogen + BjSP (30 min); 4. Fibrinogen + BjSP (60 min); 5. Fibrinogen + BjSP (120 min); 6. Fibrinogen + BjSP + Benzamidine (120 min); 7. Fibrinogen + BjSP + EDTA (120 min); 8. BjSP; 9. Molecular mass standard.

platelet-poor plasma in up to 30 min of reaction. Following that period, addition of $CaCl_2$ to the solution also resulted in no coagulation during the additional 60 min period of evaluation, differently from that observed for the $CaCl_2$ control, which coagulated the plasma in about 220 s (see supplementary files for detailed data).

3.9. Effects on platelets

BjSP (50 μg) showed no effects on washed platelets in the assayed conditions, not being able to induce platelet aggregation or to inhibit the aggregation promoted by thrombin (see supplementary files for figures).

3.10. Amidolytic activity on chromogenic substrates

The cleavage specificity of BjSP was assessed using different chromogenic substrates. At a concentration of 10 μg/mL, the enzyme showed higher efficiency of cleavage for substrates S-2366 and S-2238, used to determine the activity of activated protein C/factor XIa and thrombin, respectively. In comparison, low cleavage activities were observed for the other tested substrates (S-2222, S-2765, S-2302 and S-2251), as evidenced by the specific activity calculated for each substrate (Fig. 6).

3.11. Activation of coagulation factors

BjSP (10 μg/mL) was not able to induce activation of prothrombin, factor X and protein C, as observed by the unaltered amidolytic activity on substrates S-2238, S-2222 and S-2366 after incubation of BjSP and the isolated coagulation factors in comparison to the values obtained after incubation of BjSP and substrates only.

3.12. Effects of inhibitors

Substrate S-2366 was also used to evaluate the effects of different inhibitors on the activity of BjSP, showing that specific serine protease inhibitors such as PMSF, benzamidine and leupeptin, and denaturing agents such as SDS and DTT, were able to significantly inhibit its enzymatic activity, whereas EDTA and commercial antivenom did not

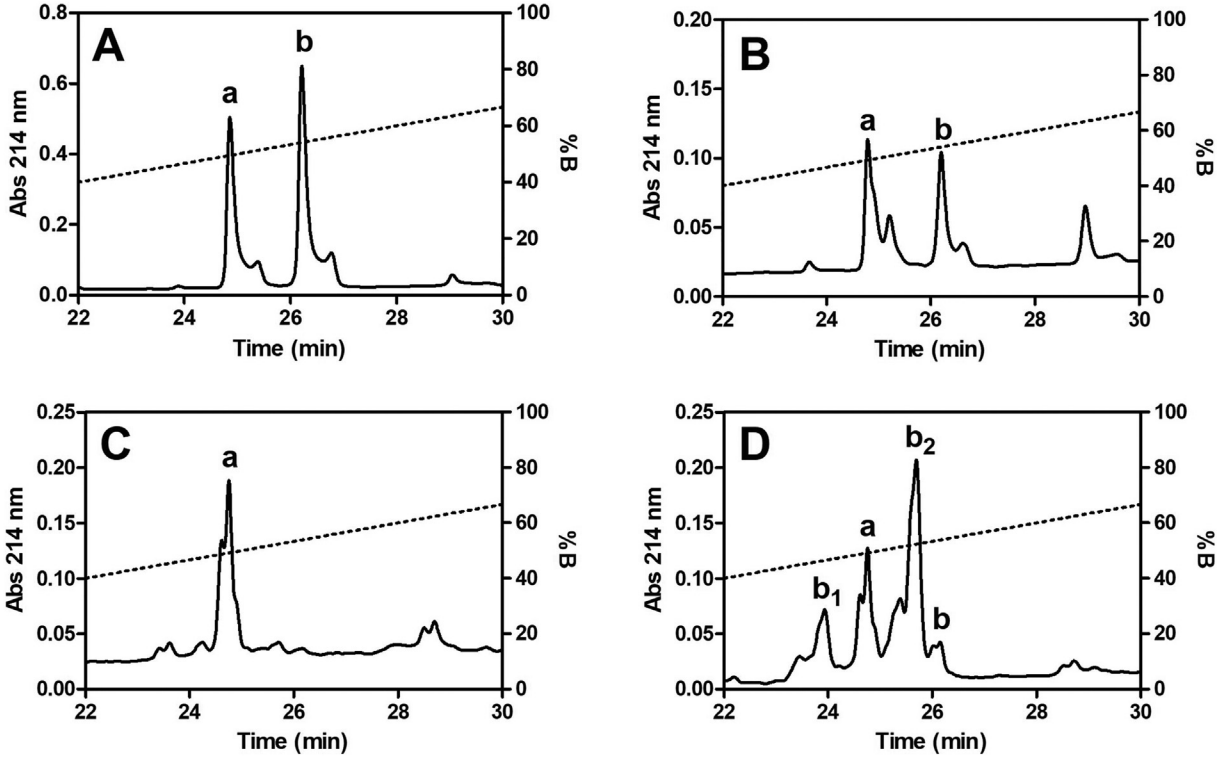

Fig. 5. RP-HPLC profiles of peptides released from fibrinogen. Standard fibrinopeptides A (*a*) and B (*b*) were used as controls (A). The fibrinopeptides were generated by incubation of human fibrinogen (3 mg/mL) with (B) thrombin (5 μg/mL), BjSP at 20 μg/mL (C) or BjSP at 400 μg/mL (D) at 37 °C for 120 min, followed by protein removal with 2% TCA and centrifugation at 2000 x*g* for 10 min, and analysis of supernatants by RP-HPLC at 214 nm. Amino acid sequences of peptides *a*, *b*, b_1 and b_2 were then determined by Edman degradation.

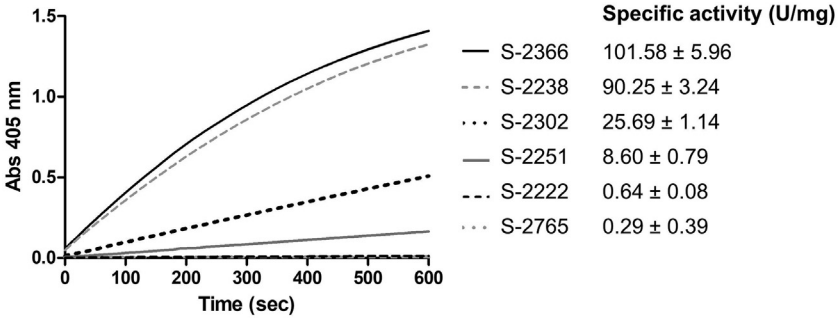

Fig. 6. BjSP activity on different chromogenic substrates. BjSP (10 μg/mL) was incubated with various chromogenic substrates (0.4 mM, final concentration) and the kinetics of the reactions was monitored by measuring the absorbance at 405 nm for 10 min at room temperature. The absorbance of the amidolytic activity of substrates alone was subtracted from the values obtained after reaction with BjSP, resulting in a curve of absorbance vs. time. Results were also presented as specific activity (U/mg), by converting absorbance values at 405 nm in amidolytic activity units (U) per milligrams of BjSP. Results expressed as mean values ± SEM ($n = 3$).

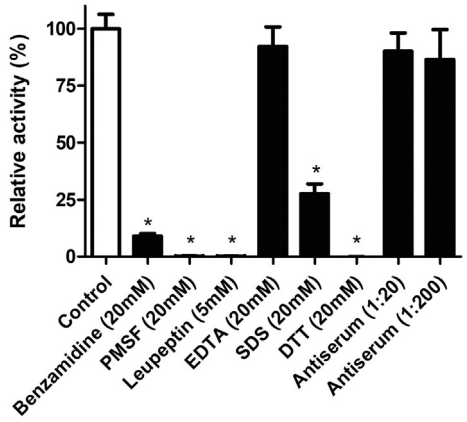

Fig. 7. Influence of inhibitors on the amidolytic activity of BjSP. BjSP was incubated at 37 °C for 30 min with different inhibitors. Then, the amidolytic activity of samples on substrate S-2366 (0.4 mM, final concentration) was monitored at 405 nm for 30 min at room temperature, and the results were expressed as the percentage of relative activity, comparing the control values obtained for the reaction of BjSP and substrate S-2366 (100% relative activity) with the values obtained for the reactions in the presence of inhibitors. Results expressed as mean values ± SEM (n = 3) of relative activities (%). (*) Values significantly different from the control ($p < 0.05$).

result in inhibition (Fig. 7).

3.13. Kinetic study and catalytic specificity

As displayed in Table 2, in general, BjSP showed moderate proteolytic activity when alanine (A), lysine (K), asparagine (N), valine (V), tryptophan (W), isoleucine (I), methionine (M) or serine (S) were evaluated on subsites S_1, S_2, S_3 and S'_1, with very low hydrolysis occurring when acidic amino acids (D and E) appeared at those subsites. Notorious values of catalytic efficiency (K_{cat}/K_M) were shown at P_1A (1883 ± 21), P_2M (2612 ± 21), P_3S (2089 ± 19) and to tyrosine, especially at S_1 subsite (3470.5 ± 35).

On the enzyme accommodation to the substrate, we observed that there was no displacement of enzyme in the anchoring to the substrates, with only one cleavage point at $P_1\downarrow P'_1$ to all substrates, except to P_2I.

Additionally, we also performed the specificity study of BjSP on fibrinogen peptides derived from the Aα chain (Abz-GVRGPRQ-EDDnp) and Bβ chain (Abz-SARGHRQ-EDDnp). The proteolytic hydrolysis confirmed that BjSP preferentially acts on the Aα chain, as shown by the catalytic efficiency values of 23,371.5 ± 90 (Aα chain) and 6166.5 ± 60 (Bβ chain). These results demonstrate a superiority of approximately 4 times in the hydrolysis of the Aα chain, reinforcing our previous findings on the assays of fibrinogenolysis and formation of fibrinopeptides.

Table 2
Kinetic parameters for the hydrolysis of FRET peptides by BjSP.

X	P_1 Position Abz-KLXSSKQ-EDDnp K_{cat}/K_M ($mM^{-1}.s^{-1}$)	P_2 Position Abz-KXRSSKQ-EDDnp K_{cat}/K_M ($mM^{-1}.s^{-1}$)	P_3 Position Abz-XLRSSKQ-EDDnp K_{cat}/K_M ($mM^{-1}.s^{-1}$)	P'_1 Position Abz-KLRXSKQ-EDDnp K_{cat}/K_M ($mM^{-1}.s^{-1}$)
A	1883 ± 21	1215 ± 2	1520 ± 30	1288 ± 36
D	76.5 ± 5	211 ± 5	179.5 ± 13	187.5 ± 15
E	130 ± 12	152.5 ± 21	171 ± 8	175 ± 16
H	372.5 ± 16	361.5 ± 30	664.5 ± 32	522 ± 30
I	853 ± 30	963 ± 50	993 ± 26	930 ± 30
K	1031 ± 16	819 ± 26	1393 ± 25	709.5 ± 4
M	588 ± 30	2612 ± 21	1012.5 ± 33	1258 ± 31
N	1105 ± 40	813 ± 35	930 ± 25	638 ± 35
S	506 ± 19	795.5 ± 51	2089 ± 19	1393 ± 25
V	935 ± 40	1020 ± 7	1086 ± 46	918 ± 26
W	1107 ± 27	1133 ± 21	561 ± 45	914.5 ± 40
Y	3470.5 ± 35	2819 ± 39	1468.5 ± 31	2849 ± 33
Fibrinogen clotting sequence		K_{cat}/K_M ($mM^{-1}.s^{-1}$)		
Abz-GVRGPRQ-EDDnp (Aα chain)		23,371.5 ± 90		
Abz-SARGHRQ-EDDnp (Bβ chain)		6166.5 ± 60		

Hydrolysis conditions: 100 mM Hepes buffer pH 7.0 at 50 °C. These values are the average of three independent experiments ± standard deviations. All peptide substrates presented only one hydrolysis point (Abz-KLX↓SSKQ-EDDnp; Abz-KXR↓SSKQ-EDDnp; Abz-XLR↓SSKQ-EDDnp and Abz-KLRX↓SKQ-EDDnp), except Abz-KIR↓SSK↓Q-EDDnp: 57% (R↓S) – 43% (K↓Q). Fibrinogen clotting sequences also presented only one cleavage point: Abz-GVR↓GPRQ-EDDnp (Aα chain); Abz-SAR↓GHRQ-EDDnp (Bβ chain).

3.14. Optimum pH and temperature conditions

Using the FRET substrate P_1Y, BjSP showed higher enzymatic activity at neutral pH values, with optimum catalysis at pH 7.5 (Fig. 8A). Regarding ideal temperature conditions, the enzyme showed optimum activity at 50 °C (Fig. 8B) and maintained relative activity up to 75% in the range of 45–60 °C.

3.15. Esterase activity and stability

BjSP showed activity on the artificial substrate TAME, which is commonly used for the analysis of the enzymatic activity of enzymes such as trypsin, thrombin and serine proteases in general. This activity was used to evaluate the stability of BjSP under different conditions of pH and temperature after 30 min of incubation. The enzyme showed high stability at pH values ranging from 6.0 to 10.5, with significant decreases in its TAME activity observed only at the acidic pH values of 3.0 and 4.5 (Fig. 9A). Regarding its thermostability, the enzymatic activity of BjSP was stable at temperatures up to 45 °C, with significant decreases observed only at higher temperatures such as 60 and 100 °C (Fig. 9B).

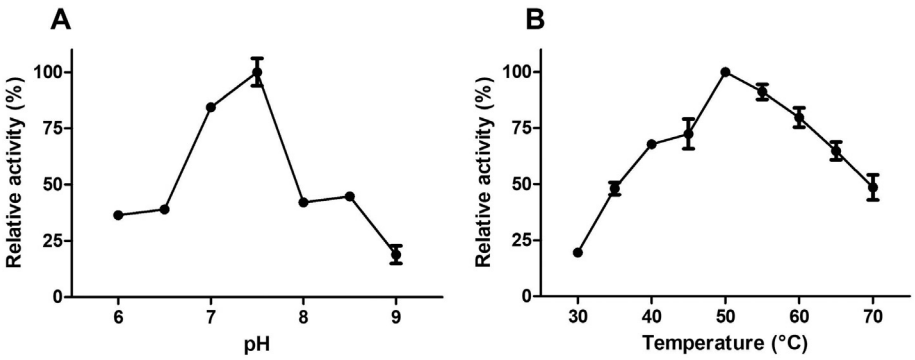

Fig. 8. Determination of the optimum pH (A) and temperature (B) for the enzymatic activity of BjSP on a FRET substrate. The enzymatic reaction between peptide Abz-KLRYSKQ-EDDnp (0.6 μmol) and BjSP (0.116 μmol) was evaluated within the conditions of pseudo first-order ([S] < < Km) using excitation and emission wavelengths of 320 nm and 420 nm, respectively. Results were expressed as mean values ± SEM (n = 2) of relative activities (%).

4. Discussion

This study described the isolation and characterization of a new SVSP from the venom of *B. jararaca*, called BjSP. Although several serine proteases have been isolated from the venom of this snake species, including BPA (Reichl et al., 1983), Bothrombin (Nishida et al., 1994), PA-BJ (Serrano et al., 1995), KN-BJ1 and 2 (Serrano et al., 1998), TL-BJ1, 2 and 3 (Serrano et al., 2000) and Jararassin-I (Vieira et al., 2004), the partial amino acid sequencing of BjSP revealed that this enzyme showed total identity to the cDNA clone HS114 obtained by Saguchi et al. (2005) from *B. jararaca* venom gland and then also identified in the transcriptomes and venom proteomes from different populations of this snake species (Gonçalves-Machado et al., 2016), but which had not yet been isolated and characterized. Considering that we have determined about 80% of BjSP sequence, our results strongly suggest that it corresponds to the cDNA clone HS114.

The presence of different serine proteases in the venom of *B. jararaca* may be related to the different activities that these enzymes exert on the hemostatic system. Thus, although some of these enzymes show around 70% structural identity, they display specific biological activities, e.g. Bothrombin, TL-BJ isoforms and Jararassin-I are fibrinogen-coagulating enzymes (Nishida et al., 1994; Serrano et al., 2000; Vieira et al., 2004); PA-BJ is a platelet-aggregating serine protease (Serrano et al., 1995), KN-BJ 1 and 2 display coagulant and bradykinin-releasing properties (Serrano et al., 2000), and BPA is a potent non-coagulant fibrinogenolytic enzyme (Paes-Leme et al., 2008). Other factors that may explain variations in the composition of snake venoms include age, sex, feeding and habitat (Mackessy, 2009).

MALDI-TOF MS analysis showed that BjSP presents a molecular mass of approximately 28 kDa, and deglycosylation assays indicate the presence of N-linked carbohydrates in the molecule, since PNGase F promotes hydrolysis of bonds between asparagine residues and N-linked oligosaccharides (N-glycans) (Tarentino et al., 1985), which occurs in consensus sequence sites of Asn-X-Ser/Thr, where X is any amino acid except proline (Gavel and Von Heijne, 1990). Most SVSPs are glycoproteins with varying number of sites for N- or O-glycosylation in their amino acid sequences, and this degree of glycosylation is responsible for the wide range of molecular masses reported for these enzymes (Matsui et al., 2000; Serrano and Maroun, 2005), which can vary from 25 kDa (for flavoxobin, a serine protease lacking glycosylation sites) (Shieh et al., 1988) to 67 kDa (for BPA, a highly glycosylated serine protease with > 60% of its molecular mass corresponding to N- and O-linked carbohydrates) (Murayama et al., 2003). BjSP partial amino acid sequence (and also the full sequence of HS114) showed only one possible N-glycosylation site, which could explain the low percentage of its mass (~10%) that was related to N-glycosylation.

Studies on the structure-function relationship of glycoproteins have shown that glycosylation confers important and specific biological roles that may include immunogenicity, protection from proteolysis, stimulation and preservation of a biologically active protein conformation, among others (Soares and Oliveira, 2009). In the case of SVSPs, the importance of glycosylation in the structure-function relationship of these enzymes has not been fully understood, with some studies showing that glycosylation can influence their catalytic activity (Zhu et al., 2005), while others report that carbohydrates are more important for the stability of these molecules (Komori and Nikai, 1998).

With pI 4.4, BjSP presented an acidic character as most of the SVSPs described to date, including some from *B. jararaca* venom: BPA (Reichl et al., 1983), Bothrombin (Nishida et al., 1994), and the isoforms of TL-BJ (Serrano et al., 2000) and KN-BJ (Serrano et al., 1998). Although less frequent, some serine proteases can show neutral or basic character as well, as Jararassin-I (Vieira et al., 2004) and PA-BJ (Serrano et al., 1995), also from *B. jararaca* venom.

BjSP showed interesting effects on hemostasis: despite not inducing or inhibiting aggregation of washed platelets, this serine protease was able to degrade fibrin clots in vitro and also the Aα and Bβ chains of fibrinogen, generating fibrinopeptides A, B and other peptides derived from the Bβ chains. The conversion of fibrinogen to fibrin by thrombin is made by limited proteolysis, with cleavage of the peptide bond between residues Arg16-Gly17 of the two Aα chains and residues Arg14-Gly15 of the two Bβ chains (Blombäck et al., 1967). This cleavage results in the release of four fibrinopeptides (two fibrinopeptides A and two B) and originates fibrin monomers with new N-terminal sequences responsible for the polymerization reactions that occur subsequently,

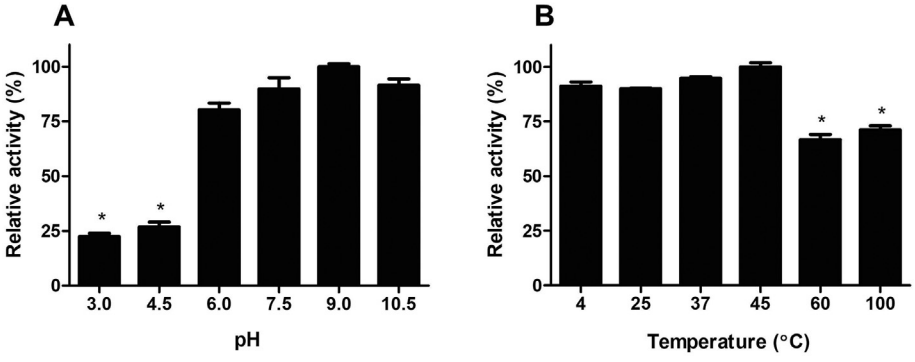

Fig. 9. Influence of pH (A) and temperatures (B) on the TAME esterase activity of BjSP. The enzyme (5 μg) was previously incubated at different pH values and temperatures for 30 min and then the reaction with TAME (1 mM, final concentration) occurred for 30 min at 37 °C. The results were obtained at 247 nm and expressed as mean values ± SEM (n = 3) of relative activities (%). (*) Values significantly different from the group with higher activity (100% relative activity) ($p < 0.05$).

forming insoluble fibrin clots (Davie et al., 1991).

Since BjSP degraded fibrinogen but was unable to coagulate plasma or fibrinogen solutions, we can safely propose a nonspecific action of BjSP on the fibrinogen chains, differently from that promoted by thrombin. In fact, N-terminal sequencing of the fibrinopeptides formed by BjSP (other than A or B) revealed identity to Bβ15–42, a fragment from the N-terminal of Bβ chain which is usually generated by the plasmin cleavage of fibrin and possesses potential anti-inflammatory effects (Jennewein et al., 2011). This particular proteolysis is not common for SVSPs, which are usually described as able to form fibrinopeptides A and/or B, or, at best, C* (corresponding to the N-terminal fragment 1–42 from the Bβ chain of fibrinogen) (Nishida et al., 1994; Magalhães et al., 2003, 2007; Boldrini-França et al., 2015).

Some other SVSPs have displayed no coagulant activity on fibrinogen, e.g. PA-BJ and BPA, both isolated from *B. jararaca* venom (Serrano et al., 1995; Paes-Leme et al., 2008). PA-BJ, however, was able to promote platelet aggregation in platelet-rich plasma or washed platelet suspensions (Serrano et al., 1995). In comparison, BjSP did not clot fibrinogen or platelet-poor plasma and was also unable to induce platelet aggregation. Thus, this enzyme seems to be a promising defibrinogenating agent, as it apparently depletes fibrinogen without causing coagulation, as shown when *B. jararaca* crude venom was added to plasma or fibrinogen solutions previously incubated with BjSP and was incapable of inducing coagulation. Furthermore, it also promoted direct fibrinolysis, which would be an interesting additional feature for a defibrinogenating agent.

Catalytic specificity assay of this protease showed a wide activity spectrum at different peptide sequences, pointing out a greater preference for tyrosine, especially at S_1 subsite. BjSP also exhibited a high hydrolysis specificity for the fibrinogen clotting sequences GVR↓GPR (Aα chain) and SAR↓GHR (Bβ chain), with notorious preference of hydrolysis for the Aα chain. Although this hydrolysis specificity is similar to that of thrombin, interestingly, BjSP showed no ability to form fibrin clots, as discussed earlier.

In an attempt to elucidate possible factors of the coagulation cascade on which BjSP could be acting, different chromogenic substrates were used. The results showed that BjSP has high enzymatic activity on the substrates for activated protein C and factor XIa (S-2366), and for thrombin (S-2238). On the other hand, the enzyme had minor effects on the substrate for plasma kallikrein (S-2302), plasmin (S-2251) and factor Xa (S-2222 and S-2765), and also on certain isolated factors, such as prothrombin, factor X and protein C, not inducing the formation of their activated forms.

The effects of BjSP on substrate S-2238 endorse that this serine protease shows affinity for thrombin substrates, such as fibrinogen, although, as mentioned earlier, other results suggest a nonspecific action on this substrate. Also, the significant effects observed on substrate S-2366 could indicate that BjSP acts on factors of the intrinsic pathway of the coagulation cascade, possibly degrading factor IX as factor XIa does. Furthermore, as S-2366 is also a substrate for activated protein C, these results suggest a possible regulatory effect of BjSP on coagulation, since activated protein C enzymatically cleaves factor Va and factor VIIIa into forms that will no longer support coagulation. This control promoted by protein C and its activated form is a very effective mechanism of inhibition of the coagulation cascade (Boon, 1993). Nevertheless, those are propositions that should be further evaluated in order to enable clearer conclusions on the mechanisms of action of BjSP.

5. Conclusions

Our results showed that BjSP possesses distinguishing effects on hemostasis in comparison to the majority of the SVSPs described in the literature. Considering its characteristics, BjSP presents great therapeutic potential as a defibrinogenating agent, since it could promote depletion of plasma fibrinogen without forming fibrin clots, most likely debilitating the formation of thrombi. Furthermore, this serine protease showed high enzymatic stability under a broad range of pH values and temperatures, which raises its potential as a candidate agent for future clinical trials.

Acknowledgments

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Conflict of Interest Statement

Sante E. I. Carone, Danilo L. Menaldo, Marco A. Sartim, Carolina P. Bernardes, Renato C. Caetano, Ronivaldo R. da Silva, Hamilton Cabral, Benedito Barraviera, Rui S. Ferreira Junior, Suely V. Sampaio, authors of the manuscript “BjSP, a novel serine protease from *Bothrops jararaca* snake venom that degrades fibrinogen without forming fibrin clots”, declare that (i) all researchers involved in this study are included in the author's list and agreed to have these results published; (ii) all authors revised and approved the final version of the manuscript; (iii) all organizations that funded this research were mentioned in the Acknowledgements section of the manuscript (including grant numbers where appropriate); and (iv) this manuscript is original and accurate, it does not contain clinical studies or patient data, and it was not submitted for publication elsewhere. Thus, there are no conflicts of interest regarding this work.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.taap.2018.08.018.

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