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ScienceDirect
Comparative Biochemistry and Physiology, Part A 151 (2008) 443–454
CBP
www.elsevier.com/locate/cbpa

BjussuSP-I: A new thrombin-like enzyme isolated from *Bothrops jararacussu* snake venom☆

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Received 6 August 2006; received in revised form 5 February 2007; accepted 28 February 2007
Available online 7 March 2007

Abstract

A thrombin-like enzyme named BjussuSP-I, isolated from *B. jararacussu* snake venom, is an acidic single chain glycoprotein with approximately 6% sugar, M*r*=61,000 under reducing conditions and pI∼3.8, representing 1.09% of the chromatographic A_{280} recovery. BjussuSP-I is a glycosylated serine protease containing both N-linked carbohydrates and sialic acid in its structure. BjussuSP-I showed a high clotting activity upon human plasma, which was inhibited by PMSF, leupeptin, heparin and 1,10-phenantroline. This enzyme showed high stability regarding coagulant activity when analyzed at different temperatures (−70 to 37 °C), pHs (4.5 to 8.0), and presence of two divalent metal ions (Ca^{2+} and Mg^{2+}). It also displayed TAME esterase and proteolytic activities toward natural (fibrinogen and fibrin) and synthetic (BAPNA) substrates, respectively, being also inhibited by PMSF and leupeptin. BjussuSP-I can induce production of polyclonal antibodies able to inhibit its clotting activity, but unable to inhibit its proteolytic activity on fibrinogen. The enzyme also showed crossed immunoreactivity against 11 venom samples of *Bothrops*, 1 of *Crotalus,* and 1 of *Calloselasma* snakes, in addition of LAAO isolated from *B. moojeni* venom. It displayed neither hemorrhagic, myotoxic, edema-inducing profiles nor proteolytic activity on casein. BjussuSP-I showed an N-terminal sequence (VLGGDECDINEHPFLA FLYS) similar to other thrombin-like enzymes from snake venoms. Based on its biochemical, enzymatic and pharmacological characteristics, BjussuSP-I was identified as a new thrombin-like enzyme isoform from *Bothrops jararacussu* snake venom.

Keywords: *Bothrops jararacussu*; Snake venoms; Enzymatic characterization; Coagulation; Proteolysis; Thrombin-like enzyme; Polyclonal antibody

Abbreviations: BAPNA, N2-benzoyl-dl-arginine-p-nitroanilide; BjussuSP-I, thrombin-like enzyme isolated from *B. jararacussu* snake venom; LAAO, l-amino acid oxidase; MCD, minimum coagulant dose; PMSF, phenylmethylsulfonyl fluoride; SVTLE, snake venom thrombin-like enzyme; TAME, Nα-p-tosyl-l-arginine methyl ester; TCA, trichloroacetic acid; TFA, trifluoroacetic acid.

☆ This paper is part of the 5th special issue of CBP dedicated to The Face of Latin American Comparative Biochemistry and Physiology organized by Marcelo Hermes-Lima (Brazil) and co-edited by Carlos Navas (Brazil), Rene Beleboni (Brazil), Carolina A. Freire (Brazil), Tania Zenteno-Savín (Mexico) and the editors of CBP. This issue is dedicated to the memory of Knut Schmidt-Nielsen (1915–2007), a great mind in comparative physiology, with books translated into 16 languages, including Spanish and Portuguese, and Cicero Lima (1934–2006), journalist, science lover and Hermes-Lima's dad.

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doi:10.1016/j.cbpa.2007.02.036

1. Introduction

The family Viperidae, with snakes spread all over the world, is undoubtedly the most important group of snakes regarding public health issues since they are responsible for most of the serious ophidian accidents reported not only in Brazil but also in other Western countries. *Bothrops* is considered, from a medical point of view, one of the most remarkable genus due to its 90% contribution to 20,000 ophidian annual accidents occurred in Brazil (Rosenfeld, 1971; Franca and Malaque, 2003).

Research on snake venoms has provided elucidation of several pharmacological mechanisms, and substantially helped the pharmaceutical industry in the search for new drugs (Ferreira, 2000; Marsh and Williams, 2005). Snake venoms are rich in proteolytic enzymes which belong to two groups: serine proteases and metalloproteases. Both groups affect the hemostatic system through several mechanisms (Yong-Hong et al., 2003; White, 2005).

Snake venom thrombin-like enzymes (SVTLEs) could be grouped into three categories: (i) SVTLE-AB, composed of enzymes that, like thrombin, trigger the clotting of fibrinogen by hydrolytic release of both fibrinopeptide A and B; (ii) SVTLE-A, characterized by cleaving only the Aα chain and (iii) SVTLEs-B that act only upon the Bβ chain (Castro et al., 2004). Several SVTLEs are already known, and were extensively studied and characterized. Examples are the enzymes isolated from *Agkistrodon caliginosus* venom named calobins: calobin I, M*r*=34,000, isoelectric point of 6.2, acts on fibrinogen to form fibrin, and also exhibits arginine esterase activity. The enzyme predominantly cleaves the Aα-chain of fibrinogen with little degradation of the Bβ-chain. The proteolytic activity of the enzyme acting on TAME as a substrate is higher than that of thrombin. However, it shows neither lysine esterase nor caseinolytic activity. The cDNA sequence indicates that calobin is synthesized as a pre-zymogen of 262 amino acid residues, including a putative secretory signal peptide of 18 residues, and a proposed zymogen peptide of 6 residues. Being calobin a glycoprotein, its possible glycosylation site NxT is located at residues 81–83 (Hahn et al., 1996); calobin II, M*r*=41,000, isoelectric point 7.4, shows little azocaseinolytic and fibrinolytic activity, and also exhibits arginine esterase activity. Amino acid sequencing of the N-terminal region established a primary structure composed of VIGGDECNINEHRFLVAxY (Cho et al., 2001).

Two thrombin-like enzymes were isolated from *Trimeresurus elegans* venom: Elegaxobin I, M*r*=30,000 and Elegaxobin II, M*r*=35,000. These enzymes clotted only rabbit fibrinogen, whereas human and bovine fibrinogens were unaffected. In fibrinogen–fibrin conversion, these enzymes released only fibrinopeptide A from rabbit fibrinogen, whereas it did not release fibrinopeptide B. Furthermore, Elegaxobin II released Lys-bradykinin when the enzyme was incubated with bovine plasma. The esterase activity was inhibited by *p*-amidinophenylmethanesulfonyl fluoride hydrochloride (*p*-APMSF), suggesting that this enzyme is a serine protease. The N-terminal sequence (VIGG) of this enzyme was identical to the typical sequence of serine proteinases, and its primary structure consisted of 233 amino acids showing conservation of the catalytic amino acid residues (His57, Asp102, and Ser195), with glucosamine at an N-linked glycosylation site (Oyama and Takahashi, 2000, 2003).

A thrombin-like enzyme was also isolated from *Bothrops jararacussu* venom by Zaganelli et al. (1996), M*r*=50,600 to 60,000, and pI 3.3–4.4. The inhibitory effect of PMSF and benzamidine on the amidolytic activity of this enzyme suggests that it is a serine proteinase. However, the results of inhibition with β-mercaptoethanol and dithiotreitol indicate that disulfide bonds seem to be essential for the full activity of the enzyme. This proteinase cleaved the Aα and Bβ chains of bovine fibrinogen, produced a fibrin clot and hydrolysed the chromogenic substrate S-2238.

The medical–scientific interest for these enzymes is growing markedly due to their specific differences when compared with thrombin itself, which is a multifunctional enzyme (Marsh and Williams, 2005). These enzymes are promising agents in medical clinics, acting as defibrinogenating agents, as the enzymes ancrod and batroxobin, which have been extensively used in patients victims of thrombosis, myocardium infarct, vascular peripheric diseases, acute ischemia, and renal transplant rejection (Bell, 1988; Stocker and Meier, 1988).

The objective of this work was the isolation and structural/functional characterization of a new thrombin-like enzyme from *Bothrops jararacussu* snake venom. Polyclonal antibodies were produced against this serine protease and their neutralizing potential of the coagulant activity and crossed immunoreactivity against other venoms and isolated enzymes were also evaluated.

2. Materials and methods

2.1. Material

Swiss male mice and rabbits from New Zealand were provided by the Central Bioterium of São Paulo University (Ribeirão Preto Campus). The human plasma came from Hemocentro of Ribeirão Preto. Resins for the isolation of the thrombin-like enzyme and IgG were from Merck and Pharmacia Biotech. Reagents for enzymatic and biochemical assays were from Sigma Chem. Co and Merck. Material for isoelectric focusing was from Invitrogen (ZOOM IPGRunner System Invitrogen), and CK-UV kit from Sigma-Aldrich Chem. Co.

2.2. Isolation of the thrombin-like enzyme

Samples of 1000 mg of desiccated crude venom from *B. jararacussu* were suspended in 8.0 mL of 0.05 M ammonium bicarbonate, pH 8.1 and centrifuged at 12,000 ×*g* for 10 min at 0 °C. The clear supernatant was applied on a molecular exclusion chromatographic column of Sephadex G-75 (110×4 cm), previously equilibrated and then eluted with the same buffer. Fractions of 10 mL/tube were collected at a flow rate of 30 mL/h at room temperature. The fraction showing clotting activity was applied on an affinity chromatography column of Benzamidine–Sepharose 6B (8.5×2.5 cm), previously equilibrated with 0.05 M Tris–HCl buffer, pH 7.4 and

then eluted with this same buffer containing 0.5 M NaCl and 0.02 M glycine, pH 3.2. Fractions of 3.0 mL/tube were collected at a flow rate of 30 mL/h at room temperature. These fractions were neutralized with 400 μL of 1 M Tris, pH 9.0. The clotting fraction was ultrafiltered in an AMICON System, using an YM 30 membrane. The purified enzyme was named BjussuSP-I.

2.3. Purity analysis and biochemical characterization

2.3.1. HPLC profile

Evidence of high purity of the isolated serine protease was obtained by reverse phase HPLC using a C18 column of 4.6×100 mm (Shimadzu) which was equilibrated with solvent A (5% acetonitrile, 0.1% trifluoroacetic acid), and eluted with a concentration gradient of solvent B (60% acetonitrile, 0.1% trifluoroacetic acid) from 0 to 100%, at a flow rate of 10 mL/min during 110 min. The peaks were monitored through the A_{280}, and registered by Dataq software (Dataq, Inc.).

2.3.2. Determination of Mr

(i) 12% SDS-PAGE was performed according to Laemmli (1970): Samples were heated at 100°C for 5 min, and then ran under both reducing (SDS+β-mercaptoethanol) and no reducing conditions. The gel was stained with 0.1% Coomassie Brilliant Blue G-250 in methanol: acetic acid: water (40:50:10, v/v) for 15 min, and distained in 10% acetic acid. M*r* was estimated by interpolation from a linear logarithmic plot of relative molecular mass versus distance of migration. Standard molecular weight markers (Sigma) were: phosphorylase b (97,000), bovine serum albumin (66,000), ovalbumin (45,000), glyceraldehyde-3-phosphate dehydrogenase (36,000), carbonic anhydrase (29,000), trypsinogen (24,000), soybean trypsin inhibitor (20,100), myoglobin (17,000), and α-lactalbumin (14,200). (ii) Molecular weight estimation by gel filtration: The molecular weight of BjussuSP-I was measured by gel filtration on HPLC Akta System carried out on a TSK-G2000sw column (0.8×30 cm) (Tosoh, Japan), previously equilibrated with PBS. The protein was eluted at flow rate of 1 mL/min with the same buffer. The molecular mass was calibrated with the following standard proteins (Pharmacia): bovine serum albumin (66,000), ovalbumin (44,000), chymotrypsinogen A (25,000), and bovine pancreatic ribonuclease A (13,700).

2.3.3. Enzyme treated with

2.3.3.1. PNGase F. The procedure was carried out according to manufacture's instructions (New England Biolabs Inc, Ipswich, MA) Briefly, 50 μg BjussuSP-I was denatured with glycoprotein denaturing buffer at 100 °C for 10 min. After the addition of NP-40 and G7 reaction buffer, 3 μL PNGase F was added, and the mixture was incubated at 37 °C for 2 h. The reaction product was visualized by SDS-PAGE.

2.3.3.2. Acid hydrolysis of sialic acid. Sialic acids are easily destroyed under acidic conditions. Briefly, 50 μg of BjussuSP-I was added to 0.2 M sulphuric acid 1:1 (V/V), heated for 1 h at 80 °C, dried in a SpeedVac (Savant), and redissolved in 50 μL of distilled water for analysis. The reaction product was visualized by SDS-PAGE.

2.3.4. Two-dimensional electrophoresis

M*r* and pI of the isolated protein were determined according to the method described by the manufacturer through the ZOOM IPGRunner Invitrogen system. *Step one*: The sample to be analyzed was dehydrated with 200 μL buffer solution (8.0 M urea, 2% CHAPS, 0.5% ampholytes from ZOOM Carrier Ampholytes Invitrogen pH: 3–10, 0.02% bromophenol blue, and 20 mM DTT), applied to immobilized pH gradient strips and maintained at room temperature during 16-hour rest on the cassette wells. After that, 600 mL of distilled water were added in the electrophoresis two-dimensional cube containing the support cassette and the sample cassette. Electrodes were placed, and the voltage adjusted constant 500V for 4 h. *Step two*: The strip was removed from the cassette and left in a solution of NuPAGE 1×+NuPAGE 10× (Invitrogen) under gentle agitation by 15 min. After that, the solution was discarded, replaced for another one containing NuPAGE 10×, and the strip was left for 15 min more under gentle agitation. The strip was placed in 12% SDS–polyacrylamide gel electrophoresis, and covered with 5% agarose solution containing Tris–Glycine running buffer at pH 8.4. After a 2-hour run at a constant voltage of 200 V, the gel was stained during 30 min with Coomassie Brilliant Blue and distained in 10% acetic acid.

2.3.5. Page for acidic proteins

A 10% (m/v) polyacrylamide gel was used, following the method of Davis (1964). The gel was stained with 0.1% Coomassie Brilliant Blue G-250 in methanol: acetic acid: water (40:50:10, v/v) for 15 min, and distained with 10% acetic acid.

2.3.6. Determination of total neutral sugars

Quantification of neutral sugars of the glycoprotein was performed according to the phenol–sulfuric colorimetric method described by Dubois et al. (1956).

2.3.7. N-terminal sequence

The N-terminal sequence of BjussuSP-I was determined using Shimadzu PPSQ-23 automatic protein sequencer based on Edman degradation, followed by derivatization of the released amino acids to PTH derivatives which were analyzed by a reverse phase C18 column, and identified through their characteristic retention time. The sequence and homology of the amino acids were analyzed by alignment using the BLAST program (Basic Local Alignment Search Tool) (Altschul et al., 1990).

2.4. Clotting activity upon plasma

This activity was evaluated using 200 μL of human plasma at 37 °C, and different amounts of the enzyme (0.16–12.0 μg) in order to determine the minimum coagulant dose (MCD), which was then used for the study of the stability of this protein when preincubated for 30 min at different pHs (2.5 to 10.0), temperatures (−70 to 100 °C), and presence of different

divalent ions at 1.0 mM (Co^{2+}, Mn^{2+}, Zn^{2+}, Ca^{2+}, Fe^{2+} and Mg^{2+}), and inhibitors at 20 mM (2.0 mM leupeptin, PMSF, EGTA, EDTA, aprotinin, β-mercaptoethanol, 1,10- phenanthroline and heparin). The clotting time was characterized by the first sudden appearance of the fibrin net. Results were expressed in inverse proportion %, according to the following equation:

$$CT = \frac{1}{\Delta} \times 100$$

where CT is the clotting time, and Δ is the medium time in seconds.

2.5. Esterase activity upon tame by potentiometric titration

The method of Ehrempreis and Scheraga (1957) was followed using 7 mL of 0.01 TAME in 0.15 M KCl, pH 8.0. A given volume of standard KOH solution (0.0048 N was used) was initially added to bring the pH around 8.5. The enzyme was then added and the time elapsed to bring the pH back to 8.0 was measured. The standard KOH was delivered from a Gilmont microburet, and this operation was repeated 10 times in order to give a set of values which were then converted into medium time. One esterase activity unit represents the amount of enzyme able to release 0.1 μmol of acid in 10 min at 37 °C.

2.6. Proteolytic activity on natural and synthetic substrates

2.6.1. Fibrinogen

The method of Edgar and Prentice (1973) was followed with some modifications according to Rodrigues et al. (2000). Samples containing 30 μg of bovine fibrinogen were incubated with different amounts of enzyme (0.5–10 μg) at 37 °C for 2 h (1:1 v/v). The reaction was stopped by addition of 10 μL of 0.05 M Tris–HCl buffer, pH 8.0, containing 10% (v/v) glycerol, 10% (v/v) mercaptoethanol, 2% (m/v) SDS, 0.05% (m/v) bromophenol, and boiling at 100 °C for 5 min. The samples were analyzed by 12% SDS-PAGE. After the determination of the minimum amount of enzyme able to hydrolyze 30 μg of fibrinogen, a time-dependent curve was drawn at different experimental conditions including the stability of the proteolytic activity of BjussuSP-I in the presence of inhibitors (EDTA, EGTA, 1,10-phenantroline, β-mercaptoethanol, aprotinin and heparin PMSF and leupeptin), and different divalent ions (Ca^{++}, Mg^{++}, Mn^{++}, Co^{++}, Fe^{++} and Zn^{++}).

2.6.2. Fibrin

The method reported by Leitão et al. (2000), was used with some modifications. A 0.95% (m/v) agarose solution in barbital buffer (50 mM barbital, 1.66 mM $CaCl_2$, 0.68 mM $MgCl_2$, 93.96 mM NaCl, 0.02% (m/v) azide, pH 7.8) was prepared under heating up to the formation of a transparent colloid. After cooling, 0.3% fibrinogen was added in the above barbital buffer (1:1 v/v). To this solution, 100 μL of bovine thrombin (1 μg/μL) were added, and the final solution was poured into the Petri dish for clotting and formation of the fibrin net. The samples were applied in holes on the gel (2–64 μg), final volume=30 μL, followed by incubation at 37 °C for 48 h for later halos measuring.

2.6.3. BAPNA

A sample of 20 μg/5 μL was incubated in 600 μL of the reaction medium containing 1% BAPNA dissolved in 0.1 M Tris–HCl, pH 8.1 at 37 °C. The reaction was stopped by addition of 40 μL of 0.2 M HCl and the resulting product was analyzed measuring the A_{415}. Absorbance of the samples were measured and units/min were calculated considering 0.009 of A_{415}=1 U/min. Controls were made using the reagents only and replacing the solution of venom or protein by water in order to zero the spectrophotometer. One unit of enzymatic activity was defined as the amount of enzyme able to release 1 μmol of *p*-nitroanilide/min under the described conditions.

2.6.4. Casein

A modification of the method reported by Kunitz (1946) was used. Different amounts (10–60 μg) of the enzyme in 0.1 M Tris–HCl pH 9.0 were added, and the final volume was completed to 250 μL, followed by 750 μL of 1% (m/v) casein. The final 1000 μL were incubated for 15 min at 37 °C. The reaction was stopped by addition of 1.5 mL of 30% TCA. The resulting proteolysis products in the supernatant solution were evaluated spectrophotometrically at λ=280 nm after centrifugation at 1600 ×*g* for 10 min. One unit of caseinolytic activity corresponds to an increase of A_{280}=0.001/min.

2.7. Other biological activities

2.7.1. Edema inducing activity

The dose–response curve for this assay was drawn using both isolated enzyme and crude venom. Groups of six mice were injected subcutaneously in the subplantar region of the right hind paw with 50 μL solution containing 20 μg of the protein or crude venom dissolved in PBS. Control was made by injection of 50 μL PBS. The increase of the paw volume was measured with the aid of a low-pressure pachymeter (Mitutoyo, Japan), and expressed in % of induced edema (Soares et al., 2000).

2.7.2. Hemorrhagic activity

It was determined according to the method of Nikai et al. (1984). The mice were distributed in groups of six animals each. Hemorrhage was induced by intradermic injection in the dorsal region. Animals were then sacrificed, and the skins removed 2 h after injection. The hemorrhagic halo was then measured in mm.

2.7.3. Myotoxic activity

It was determined through quantitation of CK, using kit CK-UV from Sigma Chem. Co. The groups of six mice (18–22 g) were injected intramuscularly in the right gastrocnemius muscle. Thirty micrograms of the crude venom and 100 μg of BjussuSP-I were applied, dissolved in 50 μL PBS. Control mice received only PBS, and 3 h later the blood was collected from their tail in heparinized capillaries, and immediately centrifuged at 12,000 ×*g* for 6 min. The amount of creatine kinase was

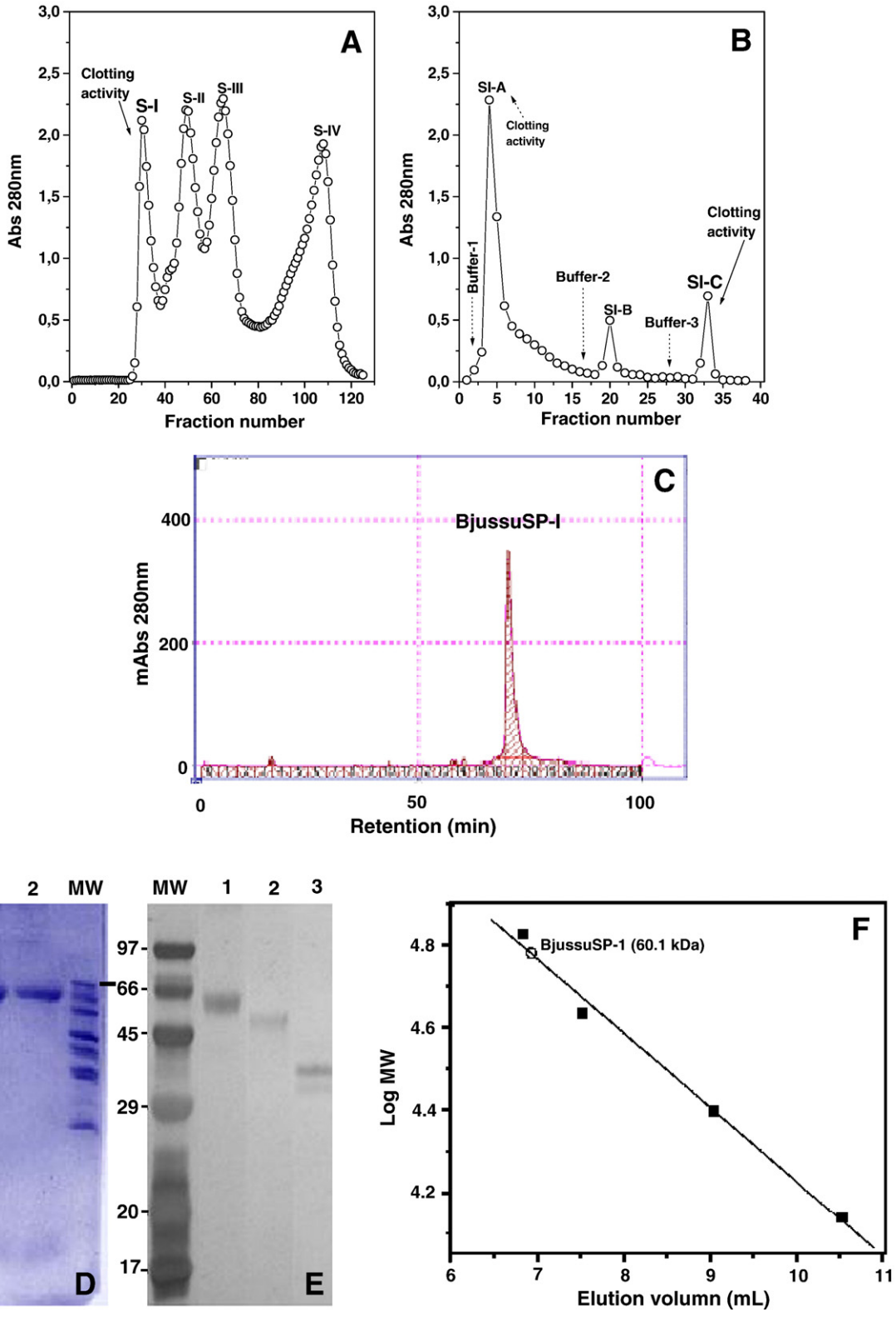

Fig. 1. Chromatographic profile of the purification procedure and purity assay of BjussuSP-I. (A): Molecular exclusion chromatography on Sephadex G-75. (B): Affinity chromatography on Benzamidine–Sepharose 6B. (C): Purity assay of BjussuSP-I by HPLC. (D): SDS-PAGE of BjussuSP-I (Lanes 1 and 2, reducing and non-reducing conditions respectively), and MW marker (Lane 3). (E): Electrophoresis of BjussuSP-I, stained with silver nitrate. MW: molecular mass markers (kDa). Lane 1: purified BjussuSP-I. Lane 2: BjussuSP-I with sialic acid. Lane 3: BjussuSP-I treated with PNGase F. (F): Molecular mass estimation of the BjussuSP-I by gel filtration on TSK-G2000sw column. The standards used were: bovine serum albumin (66.0 kDa), ovalbumin (44.0 kDa), chymotrypsinogen A (25.0 kDa) and bovine pancreatic ribonuclease A (13.7 kDa). The open circle shows the position of BjussuSP-I. Experimental details are in Materials and methods section.

expressed in units/L, one unit resulting from the phosphorylation of 1 nmol of creatine/min (Kaplan and Pesce, 1986).

2.8. Immunochemical characterization

Production of polyclonal antibodies: five doses of BjussuSP-I were applied in each male New Zealand rabbit weighing 2–3 kg ($n=4$). The first dose of 47 μg was dissolved in PBS and mixed (1:1) with complete Freund adjuvant (Sigma Chem. Co.). The subcutaneous injections were made in different regions of the animal's back in addition to one intramuscular. The second dose came 15–21 days later. The last three doses (63 μg protein in saline) were applied with intervals of 21 days each, and the incomplete Freund's adjuvant (Sigma Chem. Co.) was used. Five days after the last application, the animals were killed. The blood was collected, and the serum was separated and frozen at −20 °C.

2.8.1. Purification of IgG

(a) Preparation of the sample: serum was obtained, and the proteins precipitated with saturated $(NH_4)_2SO_4$ solution pH 6.5, followed by 3 washes with the same solution. The precipitate was dissolved in 0.15 M NaCl, and dialysed at 4 °C. (b) Ion-exchange on DEAE-cellulose (Sigma Chem. Co.): the sample was applied on a DEAE-cellulose column (1.5×16 cm) which was previously equilibrated in 0.02 M pH 7.5, sodium phosphate buffer at a flow rate of 40 mL/h at room temperature. Fractions of 3.0 mL/tube were collected.

2.8.2. Immunodiffusion

Immunodiffusion assays were carried out as described by Campbell et al. (1970). Slides containing 1.25% agarose+0.1% thimerosal in PBS were prepared, and samples were applied to reach a total volume of 10 μL holes, followed by incubation for 24–48 h in a wet chamber at room temperature. Immunoreaction lines were then observed.

2.8.3. Neutralization of the enzymatic activities

The neutralization of these activities was evaluated regarding the clotting and proteolytic action upon fibrinogen. The protein was incubated for 1 h at 37 °C with IgG, and its residual activity was evaluated at different concentrations as described by Escalante et al. (2000).

2.8.4. ELISA

Cross-immunoreactivity assays were performed in quadruplicate, and read in an ELISA reader (Molecular Devices) through the SoftMax program in WRP, OPD & Acid stop at $\lambda=490$ nm, using 2.0 and 5.0 μg of each venom and isolated enzyme, respectively. Samples were left standing overnight at 10 °C in 100 μL of 50 mM sodium bicarbonate pH 9.6 for sensitization of ELISA plates containing 96 wells (Costar®, Corning Inc.). The washes were made 3× with PBS, followed by incubations for 1 h with 22 μg of anti-BjussuSP-I IgG/well, and then 1:1000 anti-rabbit from she-goat conjugated with peroxidase in 1% MPBS on plates previously blocked with 2% MPBS for 2 h at 37 °C. After that 100 μL of OPD–H_2O_2 substrate (2 mg of OPD from Acros Organics) in 10 mL PBS and 30 μL of 10% H_2O_2 were added. The plates were left standing for 15 min protected from light up to the appearance of a characteristic color. The reaction was stopped by addition of 50 μL of 1 M H_2SO_4. The negative control was prepared with the anti-rabbit serum alone.

2.9. Statistic analysis

The results regarding biological activity were presented as medium±standard deviation (SD). Statistical comparison of differences was performed by ANOVA, Tukey's and Student's *t*-test. The value of $p<0.05$ was considered significant.

3. Results

BjussuSP-I was isolated from *B. jararacussu* venom through the two chromatographic steps described in Material and Methods section. Four main fractions were initially obtained, namely S-I to S-IV, from which only S-I showed clotting activity upon human plasma. It was rechromatographed on Benzamidine–Sepharose 6B where from 3 new fractions were obtained and named SI-A, SI-B and SI-C, respectively (Fig. 1A and B). SI-C showed the highest clotting activity, and was submitted to ultrafiltration through an AMICON membrane as

Table 1
Comparative analysis of the N-terminal sequence of BjussuSP-I with those other serino protease enzymes from snake venoms

Enzymes	Snakes	N-terminal sequences 1 20	References
BjussuSP-I	*B. jararacussu*	**V**L**GG**DE**C**DINE**H**PF**L**AFL**Y**S	This work
Thrombin-like	*B. jararacussu*	**V**V**G**ADN**C**NFN----------	Andriao-Escarso et al. (1997)
Batroxobin	*B. moojeni*	**V**I**GG**DE**C**DINE**H**PF**L**AFM**Y**Y	Itoh et al. (1987)
Calobin	*A. caliginosus*	**V**I**GG**DE**C**NINE**H**RF**L**VAL**Y**N	Hahn et al. (1996)
Gabonase	*Bitis gabonica*	**V**V**GG**AE**C**KIDG**H**RC**L**ALL**Y**–	Pirkle et al. (1986)
Ancrod	*C. rhodostoma*	**V**I**GG**DE**C**NINE**H**RF**L**ALV**Y**A	Au et al. (1993)
Bilineobin	*A. bilineatus*	II**GG**DE**C**NINE**H**RF**L**VAL**Y**D	Nikai et al. (1995)
Flavoxobin	*T. flavoviridis*	**V**I**GG**DE**C**NINE**H**PF**L**VAL**Y**D	Shieh et al. (1988)
RVV-V gamma	*Vipera russelli*	**V**V**GG**DE**C**NINE**H**PF**L**VAL**Y**T	Tokunaga et al. (1988)

Note: conserved amino acid residues are shown in **bold.**

described. The assays for purity by HPLC and SDS-PAGE are shown in Fig. 1C, D, and E. The protein appeared as a single band on SDS-PAGE under both reducing and non-reducing conditions (Fig. 1D). The molecular weight of BjussuSP-I was about 61,000, and pI ~ 3.8, containing ~ 6% of neutral sugars. The first 20 amino acid residues from the N-terminal region of BjussuSP-I showed high similarity with other thrombin-like enzyme serine proteases (Table 1).

In order to investigate if BjussuSP-I is glycosylated, we submitted it removal of sialic acid residues and N-linked sugars, and performed an SDS-PAGE in reducing conditions. After that, BjussuSP-I appeared as a single band of 54 kDa and 37 kDa, respectively (Fig. 1E). These results demonstrate that this protein is glycosylated. The apparent molecular mass of BjussuSP-I determined by HPLC-gel filtration on TSK-G2000sw was approximately 60.1 kDa (Fig. 1F).

BjussuSP-I showed clotting activity upon human plasma. Its stability after pre-incubation at different pHs and temperatures was high at pHs between 4.5 and 8.0 (Fig. 2A), and temperatures ranging from −8 °C to 20 °C (Fig. 2B). Coagulant activity was inhibited by PMSF, leupeptin, heparin and 1,10-phenantroline (Fig. 2D) as well as by 1 mM Co^{++}, Mn^{++}, Zn^{++} and Fe^{++} (Fig. 2C). Its proteolytic activity was assayed upon different substrates as fibrinogen, whose Aα chain, but not the Bβ chain, was highly degraded, even at high concentrations, and prolonged reaction times (Fig. 3A and B). This activity was inhibited by PMSF, leupeptin and 1,10-phenantroline (Fig. 3D) but not by the divalent cations showed in Fig. 3C. It also displayed time and dose-dependent catalytic activity on fibrin and BAPNA inhibited by leupeptin (70%) and PMSF (50%) (Fig. 4A and B). The esterase activity upon TAME was ~ 10 U/mg, and both inhibitors (PMSF and leupeptin) were also able to inhibit this activity around 20% (Fig. 4C). BjussuSP-I did not display proteolytic activity upon casein.

BjussuSP-I was able to induce production of polyclonal antibodies in rabbits (Fig. 5A). Through immunodiffusion and

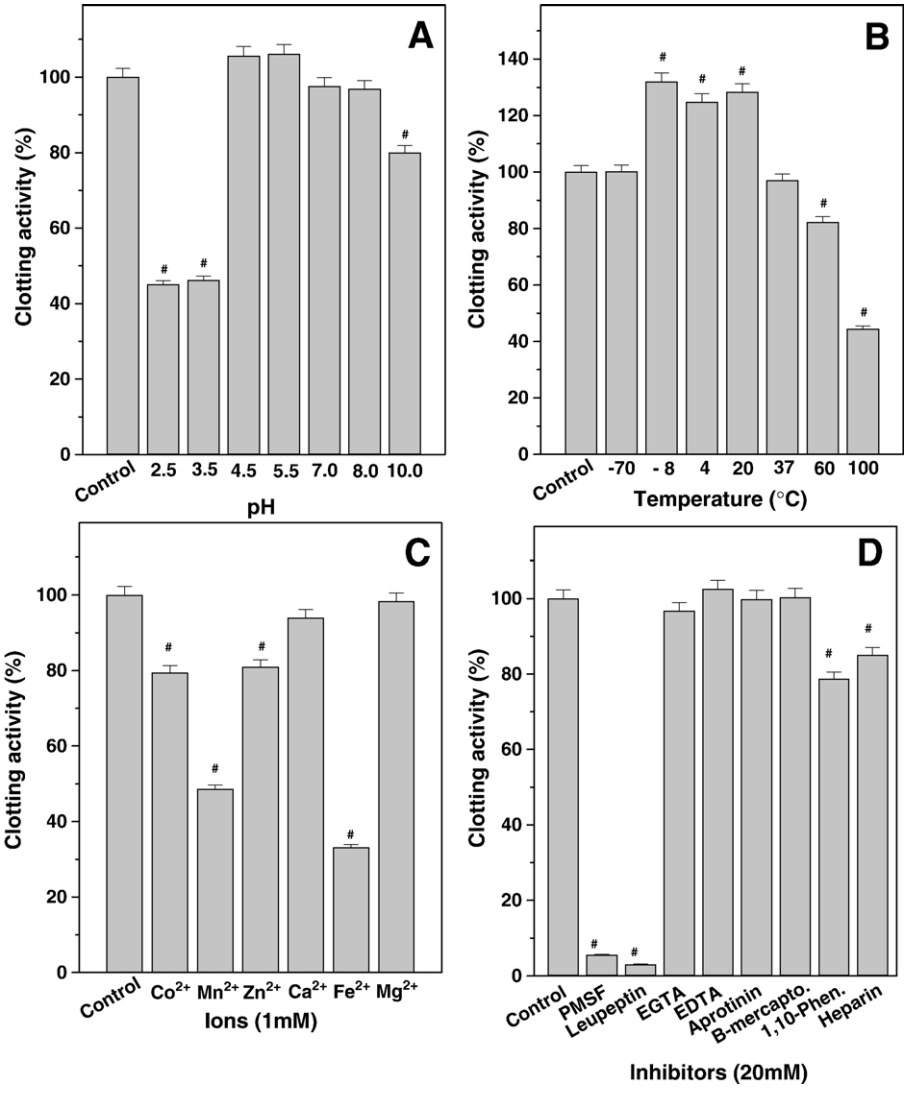

Fig. 2. Evaluation of the stability of BjussuSP-I regarding its clotting activity upon human plasma. As control, the % of clotting activity of 1.5 μg of the protein was determined at pH 6.5 and 37 °C. (A) Effect of pH; (B) Effect of temperature; (C) Effect of different ions; (D) Effect of inhibitors. Bars represent the mean of % clotting values activity ±SD ($n=6$), # ($p<0.001$) significance level related to BjussuSP-I (control).

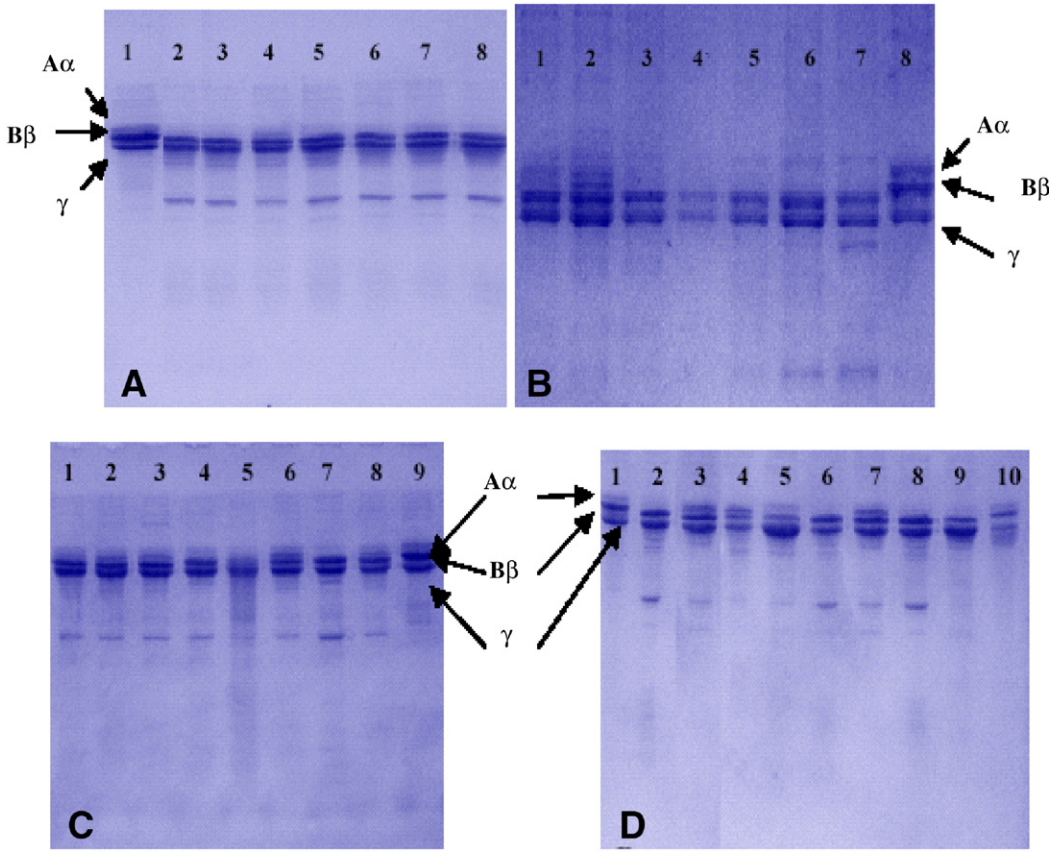

Fig. 3. Determination of the fibrinogenolytic activity of BjussuSP-I and stability under different conditions. (A) BjussuSP-I at different concentrations incubated at 37 °C for 2 h with 30 μg bovine fibrinogen. Lane 1: control (fibrinogen), Lane 2: 0,5 μg, Lane 3: 1,0 μg, Lane 4: 2,0 μg, Lane 5: 4,0 μg, Lane 6: 6,0 μg, Lane 7: 8,0 μg and Lane 8: 10,0 μg; (B) 2 μg of BjussuSP-I at different interval times incubated at 37 °C with 30 μg fibrinogen; Lane 1: 15 min, Lane 2: 30 min, Lane 3: 60 min, Lane 4: 120 min, Lane 5: 240 min, Lane 6: 360 min, Lane 7: 1440 min, and Lane 8: control (fibrinogen), 1440 min; (C) 2 μg of BjussuSP-I+different ions (1 mM) (pre-incubation during 30 min), after+30 μg of fibrinogen incubated during 60 min; Lane 1: Mn^{2+}, Lane 2: Mg^{2+}, Lane 3: Ca^{2+}, Lane 4: Co^{2+}, Lane 5: Fe^{2+}, Lane 6: Zn^{2+}, Lane 7: Zn^{2+}(2mM) Lane 8: control (BjussuSP-I diluted in water+fibrinogen) and Lane 9: control (fibrinogen); (D) 2 μg of BjussuSP-I+different inhibitors (20 mM) (pre-incubation during 30 min), after +30 μg of fibrinogen incubated during 60 min; Lane 1: control (fibrinogen), Lane 2: control (BjussuSP-I diluted in water+fibrinogen), Lane 3: PMSF, Lane 4: Leupeptin (2 mM), Lane 5: Heparin, Lane 6: β-mercaptoethanol, Lane 7: 1,10-phenantroline, Lane 8: Aprotinin, Lane 9: EDTA and Lane 10: EGTA.

ELISA (Fig. 5B, C and D) the cross-reactivity was evaluated with snake, scorpion, toad, and bee venoms. We noted no occurrence of cross immunoreactivity between anti-BjussuSP-I and scorpion, toad and bee venoms. The occurrence of cross immunoreactivity is noted between anti-BjussuSP-I and *Bothrops* snake venoms with more intensity when compared to other venoms, mainly those from *B. jararacussu*, *B. marajoensis*, *B. bilineatus*, *B. newviedi pauloensis*, *B. atrox* and *B. moojeni* (Fig. 5B and C). These antibodies were also assayed against different toxin classes isolated from venoms (LAAO, metalloproteases, myotoxic PLA_{2s} and crotoxin). Only *B. moojeni* LAAO showed positive immunoreactivity, although low when compared with the BjussuSP-I signal (Fig. 5D).

Neutralization of BjussuSP-I clotting activity through previous incubation with anti-BjussuSP-I for 1 h at 37°C showed a completely inhibited activity at 1:60 (mol/mol) (Table 2). The proteolytic activity upon fibrinogen was evaluated at 1:1 (mol/mol) and no inhibition was observed (data not show).

BjussuSP-I is a new thrombin-like serine protease, isolated from *B. jararacussu* venom (Table 3), without myotoxic or hemorrhagic activities even at high concentrations. It shows only a low to moderate edema-inducing effect when compared with that induced by *B. jararacussu* venom.

4. Discussion

This article reports an efficient and relatively simple procedure for the isolation of a highly purified thrombin-like serine protease from *B. jararacussu* venom, which was named BjussuSP-I.

BjussuSP-I is a single chain glycosylated serine protease, M*r*~61,000, with ~6% of neutral sugars, pI~3.8 that contain both N-linked carbohydrates and sialic acid in its structure. These features are the object of further investigations to explore the role of glycosylation on protein activity. Thrombin-like enzymes so far described usually show similar chemical characteristics as low pI, and presence of linked carbohydrate (Herzig et al., 1970; Ouyang et al., 1974; Pirkle et al., 1986; Andriao-Escarso et al., 1997; Bortoleto et al., 2002). As already observed in other SVSPs, the primary structure of BthaTL revealed two putative glycosylation sites, both of them situated far from the active site (Vitorino-Cardoso et al., 2006). The

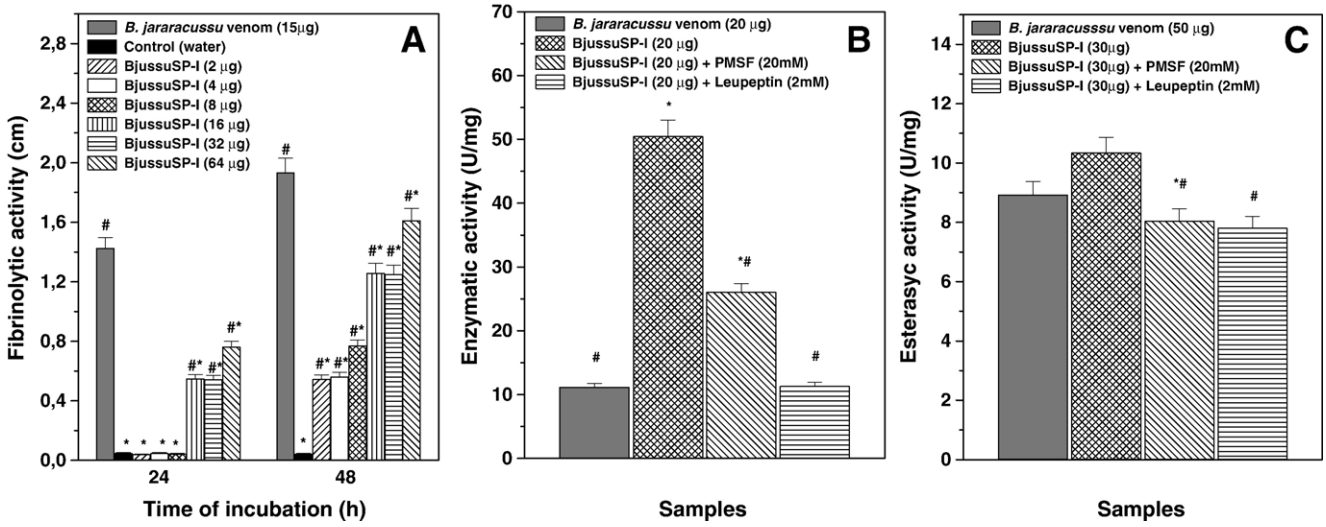

Fig. 4. Determination of BjussuSP-I activity upon different substrates. (A) Dose- and time-dependent fibrinolytic activity of BjussuSP-I through measurement of the resulting halos, *($p<0.001$) significance level related to snake venom, #($p<0.01$) significance level related to water (control); (B) Amidolytic activity of crude *B. jararacussu* venom and BjussuSP-I upon BAPNA, and inhibitors influence, *($p<0.001$) significance level related to snake venom, #($p<0.01$) significance level related to BjussuSP-I; (C) Esterase activity of crude *B. jararacussu* venom and BjussuSP-I upon TAME, and inhibitors influence, *($p<0.05$) significance level related to snake venom, #($p<0.01$) significance level related to BjussuSP-I, &($p<0.001$) significance level related to BjussuSP-I. Bars represent the medium of values ±SD ($n=6$). Experimental details are in Materials and methods section.

importance of the carbohydrate moiety on the structure-function relationship from SVTLEs is not yet established. Oyama and Takahashi (2003) showed that N-deglycosylation affects interaction of elegaxobin II with fibrinogen and kininogen, but not with smaller molecules.

This thrombin-like BjussuSP-I showed biochemical characteristics (M*r*, pI and % carbohydrates) distinct from other enzymes of the same class already studied (Zaganelli et al., 1996; Andrião-Escarso et al., 1997; Bortoleto et al., 2002; among others). In addition, enzymatic activities as coagulant, proteolytic upon different substrates (*in natura* and artificially), and esterase characterize it as a new thrombin-like enzyme isolated from *B. jararacussu venom* (Table 3).

The N-terminal region of all these enzymes show high homology, from 70% to 85%, when compared with BjussuSP-I, as is the case with ancrod with 75% homology (Au et al., 1993); batroxobin, 85% (Itoh et al., 1987); flavoxobin, 75% (Shieh et al., 1988); and calobin, 70% (Hahn et al., 1996). There are highly conserved amino acids along their peptide chains, namely: Val1, Gly3, Gly4, Glu6, Cys7, Ile9, His12, Leu15, and Tyr19 (Table 1).

BjussuSP-I showed clotting activity upon human plasma and high stability at different temperatures, pHs, and presence of several divalent cations and inhibitors. It was sensible to Mn^{++}, Zn^{++}, Co^{++}, Fe^{++}, PMSF, leupeptin, heparin and 1,10-phenantroline. It is different from the thrombin-like enzyme isolated from the same venom by Andrião-Escarso et al. (1997), which did not clot plasma.

Similar to most serine proteases extensively studied, which do not show any activity upon factor XIII, and consequently produce a soft clot, BjussuSP-I probably does not activate this factor since it also produces a soft clot different from that produced by thrombin. An exception to this rule is *Bitis gabonica*, isolated by Pirkle et al. (1986), which activates factor XIII (Stocker et al., 1982; Marsh and Willians, 2005).

Most of these serine proteases act upon several natural and synthetic substrates. Their enzymatic properties are generally affected by specific inhibitors such as PMSF and leupeptin. BjussuSP-I shows proteolytic activity on BAPNA, being inhibited by PMSF and leupeptin; on fibrinogen, inhibited by PMSF, leupeptin, and 1,10-phenantroline, and on fibrin, showing specificity toward argininyl bonds of the assayed substrates (BAPNA and fibrinogen) in addition to esterase activity on TAME.

BjussuSP-I was able to induce production of polyclonal antibodies in rabbits using successive doses of the protein. Its high M*r* made easier the production of antibodies due to large contact surface, thus showing more immunogenic regions. The clotting activity of BjussuSP-I was completely inhibited by the purified IgG at high concentrations. IgG lacks specificity for the enzyme, and thus the epitope able to induce production of these antibodies was not identified. The proteolytic activity was however not inhibited (data not shown).

The cross immunoreactivity showed between anti-BjussuSP-I and *Bothrops*, *Crotalus* and *Calloselasma* venoms suggests common epitopes or epitopes able to assume a strategic conformation thus making a possible recognition of some venom proteins by the antibodies (Arevalo et al., 1994; Trinh et al., 1997; Oldstone, 1998). Anti-BjussuSP-I was also assayed against some isolated enzymes (LAAO, PLA_2s and metalloproteases). Only LAAO from *B. moojeni* venom showed cross-reactivity, apparently one of the venom proteins displaying epitopes able to assume such conformation, and then the antibody recognition (Stabeli et al., 2005). The concept that immunological cross-reactivity of snake venoms is mediated by antibodies that recognize venom components bearing either amino acid sequence

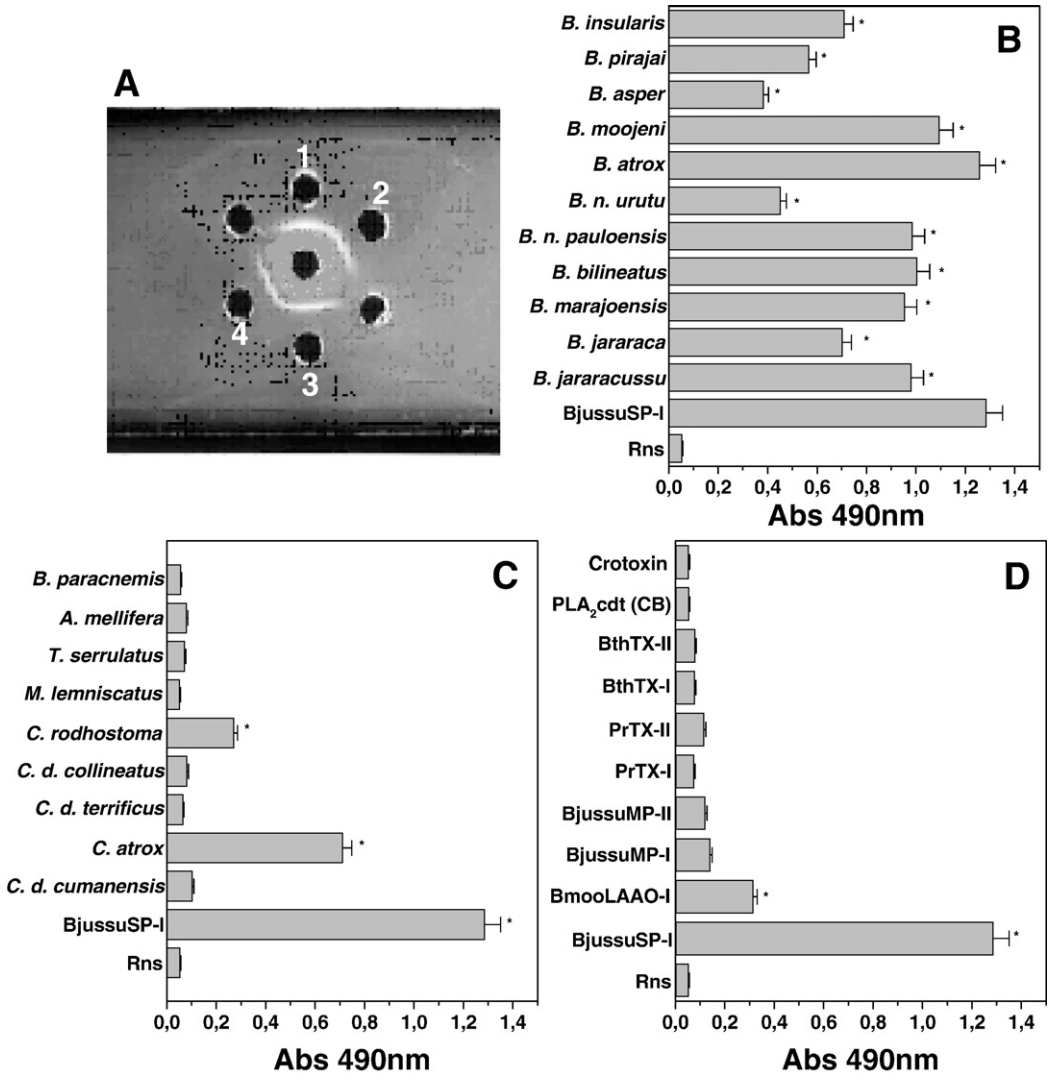

Fig. 5. Immunochemical analysis by enzyme diffusion and ELISA. On Panel (A), reactivity between anti-BjussuSP-I produced in rabbits (220 μg, central well) with different doses of enzyme BjussuSP-I (wells 1 and 2 = 10 μg; wells 3 and 4 = 15 μg). (B) Cross-reactivity against anti-BjussuSP-I with different snake venoms from *Bothrops* genus. (C) Cross-reactivity against anti-BjussuSP-I with different animal venoms (snakes, bees, scorpions, and toads) and (D) with isolated enzymes from snake sources (MP, metalloproteases, LAAO, l-amino acid oxidases, PLA_2, phospholipase A_2 and SP, serine proteases).*($p < 0.001$) significance level related to Rabbit normal serum (Rns) (control).

homology or similar biological functions is widely accepted. Polyspecific *Bothrops* antivenom is a source of cross-reactive antibodies that interact with venom proteins of distinctive primary structures, and biological functions. As we can see in the study of Stábeli et al. (2005), the homoserine lactone derivative of the undecapeptide IQRWSLDKYAM (Ile^1–Hse^{11}) excised from the l-amino acid oxidase (LAAO) of *Bothrops moojeni* venom was the ligand affinity resin used to isolate specific anti-Ile^1–Hse^{11} antibodies which were instrumental in revealing immunological cross-reactivity among unrelated venom proteins. The results indicate that all venoms tested had at least three reactive components toward anti-Ile^1–Hse^{11} antibodies, among which two serine proteases were identified: one phospholipase A_2 homologue and LAAO. We hypothesized that the cross-reactivity of the anti-Ile^1–Hse^{11} antibodies to unrelated venom proteins derives from their mechanism of antigen recognition, whereby complementarity is achieved through reciprocal conformational adaptation of the reacting molecules.

Table 2
Inhibition of the clotting activity of BjussuSP-I by anti-BjussuSP-I at different concentrations

BjussuSP-I/Anti-BjussuSP-I (mol/mol)	Residual clotting activity (minutes)
Control *	1.09
Control **	0
1:1	1.37
1:10	1.45
1:30	1.59
1:60	10.0

* Control consisted of 1.0 μg of BjussuSP-I directly upon plasma.
** Control consisted of 333 μg of anti-BjussuSP-I directly upon plasma.

Thrombin-like enzymes from snake venoms are being extensively explored as diagnostic reagents for detection of fibrin clots in patients with clotting troubles, as haemostatic drugs, and as defibrinogenating agents. The enzymes ancrod (Arwin®) from

Table 3
Comparison of the biochemical, enzymatic, and pharmacological properties of thrombin-like enzymes so far isolated from *Bothrops jararacussu* venom

SVTLE from *Bothrops jararacussu*	Protein recovery (%)	M*r*	pI	Carbohydrate content (%)	Optimal pH and temperature	Enzymatic activity				
						Clotting	Esterase	Proteolysis on BAPNA	Proteolysis on fibrinogen	Subclass
BjussuSP-I	1.09	61,000	3.8	6	4.5–8.0 and −8 at 37 °C	+	+	+++	+	Aα chain SVTLE-A
Zaganelli et al. (1996)	21	50,600–60,000	3.3–4.4	19	8 and 37 °C	+	ND	++	+	Aα and Bβ chains SVTLE-AB
Andrião-Escarso et al. (1997)	6.2	37,500	5.18–5.2	5.3	ND	−	+	+	+	Aα chain SVTLE-A
Bortoleto et al. (2002)	ND	28,000	5.0	5.4	7.4–8.0 and 37 °C	+	+	+	+	Aα and Bβ chains SVTLE-AB

ND not determined, (+) active, (−) inactive.

A. rodhostoma (Nolan et al., 1976), and batroxobin (Defibrase®) from *B. moojeni* (Stocker and Barlow, 1976), are used in medical care for patients showing thrombosis, myocardium infarct, vascular peripheric diseases, acute schemia and renal transplant rejection (Bell, 1988; Stocker and Meyer, 1988).

Therefore the enzyme BjussuSP-I, which does not induce neither hemorrhage nor pronounced edema at high doses, can be considered a new thrombin-like enzyme, subclass SVTLE-A (cleave fibrinogen only A*a* chain), with promising clinical potential to be used as a defibrinating agent. Recently, Pérez et al. (in press) showed that BjussuSP-I does not induce any of the local pathological effects characteristic of *Bothrops* sp venoms, but, it has a potent defibrino(ogen)ating activity *in vivo*. We can suggest that this protein does not contribute significantly to the toxic effect when analyzed separately, but it does when associated with other enzymes of the venom due to its high proteolytic activity. The toxicological role of clotting proteases from snake venoms is not significant in the formation of thrombi or fibrinogen consumption. Therefore they do not contribute directly to lethality by ophidian envenomation (Stocker et al., 1982; Marsh and Williams, 2005).

Acknowledgements

The authors express their gratitude to the Fundação de Amparo a Pesquisa do Estado de São Paulo (FAPESP), and the Conselho Nacional de Desenvolvimento Cientifico e Tecnológico (CNPq) for financial support. Authors express their gratitude to Ieda M. R. Prado from FCFRP/USP for her collaboration in the sugar analysis; to Prof. Dr. Paulo S. Pereira (Biotecnologia-UNAERP) for their collaboration in the HPLC; to Silvana C. Silva and Prof. Dr. Bernardo Mantovani, Andre O. M. Junior and Prof. Dr. Jose E. Barbosa from FMRP/USP for their collaboration in the immunochemical analysis; to Daniela T. Bertazzi and Prof. Dr. Eliane C. Arantes from FCFRP/USP for pI determination.

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