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Rapid purification of serine proteinases from *Bothrops alternatus* and *Bothrops moojeni* venoms

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ABSTRACT

Envenomation by Bothrops species results, among other symptoms, in hemostatic disturbances. These changes can be ascribed to the presence of enzymes, primarily serine proteinases some of which are structurally similar to thrombin and specifically cleave fibrinogen releasing fibrinopeptides. A rapid, three-step, chromatographic procedure was developed to routinely purify serine proteinases from the venoms of *Bothrops alternatus* and *Bothrops moojeni*. The serine proteinase from *B. alternatus* displays an apparent molecular mass of ~32 kDa whereas the two closely related serine proteinases from *B. moojeni* display apparent molecular masses of ~32 kDa and ~35 kDa in SDS–PAGE gels. The partial sequences indicated that these enzymes share high identity with serine proteinases from the venoms of other Bothrops species. These proteins coagulate plasma and possess fibrinogenolytic activity but lack fibrinolytic activity.

1. Introduction

Snake venoms are especially interesting since they contain high concentrations of proteins and peptides that are chemically and structurally similar to their mammalian counterparts and which, upon envenomation, trigger a wide spectrum of secondary effects that interfere with the maintenance and functioning of essential biological functions such as hemostasis, platelet aggregation and lipid digestion (Lewis and Gutmann, 2004) and thus, some of these proteins have been commercialized as diagnostic and clinical tools (Lewis and Garcia, 2003).

Crotalidae and Viperidae proteinases (Kang et al., 2011; Serrano, 2013; Takeda et al., 2012) are synthesized by the exocrine venom glands and are either metalloproteinases or serine proteinases and catalyze the cleavage of covalent peptide bonds in proteins. Snake venom serine proteinases (SVSPs) likely originated as digestive enzymes and subsequently evolved by gene duplication and sequence modifications to serve other functions. SVSPs encountered in Bothrops venoms are in many aspects functionally similar to endogenous blood clotting enzymes and they interfere with the maintenance and regulation of the blood coagulation cascade by proteolytically cleaving specific bonds and activating proteins involved in blood coagulation, fibrinolysis, and platelet aggregation and also in the proteolytic degradation of cells resulting in an imbalance of the hemostatic system (Kini, 2005; Serrano and Maroun, 2005).

Abbreviations: SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; SVSPs, snake venom serine proteinases; MCD, minimum coagulant dose.

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SVSPs are encountered in the venoms of a number of Bothrops species, for example two SVSPs, Bhalternin and Balterobin have been isolated from *Bothrops alternatus* venom (Costa Jde et al., 2010; Smolka et al., 1998), MSP 1, MSP 2, MMO3 and Batroxobin have been isolated from *Bothrops moojeni* venom (Oliveira et al., 1999; Serrano et al., 1993; Stocker and Barlow, 1976) and serine proteinases have been identified in the venoms of *Bothrops jararacussu* (Bortoleto et al., 2002; Hill-Eubanks et al., 1989), *Bothrops atrox* (Itoh et al., 1987; Kirby et al., 1979; Petretski et al., 2000), *Bothrops jararaca* (Mandelbaum and Henriques, 1964; Nishida et al., 1994; Serrano et al., 1995). The amino acid sequence homology shared between the SVSPs mentioned above is approximately 65%, however, the homology exhibited by these enzymes with mammalian serine proteinases such as thrombin and trypsin, ranges from 30% to 40%. SVSPs are structurally similar to the chymotrypsin family of proteinases, consist of approximately 232 amino acids and are made up of two homologous domains each containing a six-stranded β-barrel, the overall structures and the relative orientations of the three amino acids forming the catalytic triad, His57-Asp102-Ser195 are strictly conserved (Barrett and Rawlings, 1995; de Giuseppe et al., 2013). However, unique to SVSPs is an extended C-terminal domain which is stabilized by a disulphide bridge and is considered important for structural stability and allosteric regulation (Murakami and Arni, 2005). Amino acid substitutions and the positioning of carbohydrate moieties around the entrance to the catalytic site modulate the specificity of SVSPs, and hence SVSPs serve as diagnostic tools and are potentially interesting for the design of drugs aimed at reducing blood viscosity and for the prevention of thrombus formation. Leading examples are the SVSPs Ancrod (Arwin®) isolated from the venom of *Agkistrodon rodhostoma* and Batroxobin (Defibrase®) from the venoms of *B. moojeni* and *B. atrox*, respectively (Bell, 1997; Wang et al., 2009).

Since high-resolution X-ray diffraction studies provide detailed information at the atomic level concerning factors that determine the stereo-specificity of SVSPs, a rapid, purification procedure was developed to obtain milligram quantities of SVSPs from the venoms of *B. alternatus* and *B. moojeni* for structural studies. This purification procedure can be used to obtain serine proteinases from other snake venoms.

2. Materials and methods

2.1. Materials

Desiccated crude venoms of *B. moojeni* (1 g) and *B. alternatus* (500 mg) were purchased from a local serpentarium (SANMARO, Taquaral Ltda. São Paulo, Brazil). Sephacryl S-100 Hiprep 16/60, ÄKTA purifier and Benzamidine Sepharose 4 Fast Flow (high sub) were obtained from GE Healthcare, Amicon ultra concentrator 10 kDa and Bovine fibrinogen were obtained from Millipore and Sigma Chemical Co. respectively.

Molecular weight standards (97 kDa Phosphorylase I, 66 kDa Albumin, 45 kDa Ovalbumin, 30 kDa Carbonic Anhydrase, 20.1 kDa trypsin inhibitors, 14.4 kDa α-lactalbumin) were purchased from Amersham Biosciences.

2.2. Sample preparation

Typically, samples of 250 mg of desiccated crude venoms of either *B. alternatus* or *B. moojeni* were solubilized in 1.5 ml of Tris–HCl buffer (0.02 M Tris; 0.15 M NaCl, pH 8.0) and centrifuged at 10,000 × g for 10 min. The clear supernatant (approximately 1 ml) of each sample was applied to a 16 × 60 Sephacryl S-100 column previously equilibrated with 0.02 M Tris–HCl pH 8.0 buffer containing 0.15 M NaCl. The proteins were eluted at a flow rate of 0.2 ml/min, and fractions of 1 ml/tube were collected.

The fractions obtained from peak 3a of the size-exclusion chromatography step were pooled and applied onto a Benzamidine Sepharose 4 Fast Flow (high sub) (5 ml bed volume) column, pre-equilibrated with 0.02 M Tris–HCl pH 8.0 containing 0.15 M NaCl, using a superloop (50 ml) at a flow rate of 0.5 ml/min. The unbound protein fractions were eluted with the same buffer. The non-specifically bound proteins were eluted with the aforementioned buffer which additionally contained 0.5 M NaCl. Once the baseline had stabilized, the tightly bound proteins were eluted by rapidly changing the pH to 3.0 using a 0.05 M glycine-HCl buffer. The pH of the eluted samples was immediately adjusted to pH 7.0 by adding a buffer containing 1 M Tris pH 9.0.

Fractions representing peak 3b from the affinity chromatography step were pooled and applied at a flow rate of 0.5 ml/min onto a cation-exchange column (Mono S 5/50 GL) previously equilibrated with 0.02 M pH 5.6 Na-acetate buffer. The unbound proteins were washed out with the same buffer and the bound protein fractions were eluted with a buffer which additionally contained 1 M NaCl using a non linear gradient from 0 to 100% NaCl. Fractions of 1 ml/tube were collected and the absorbance was monitored at 280 nm.

2.3. Eletrophoretic analysis: evaluation of purity and determination of relative molecular mass (Mr)

Electrophoresis (Laemmli, 1970) was carried out at 25 mA and 100 V/gel in Tris–glycine buffer, pH 8.3, containing 0.01% SDS. Gels were stained with Coomassie Brilliant Blue R-250 or with silver nitrate. Protein concentrations were determined according to the microbiuret method (Itzhaki and Gill, 1964), using bovine serum albumin as the standard.

2.4. Coagulant activities

The coagulant activity was performed qualitatively by evaluating the coagulation of human plasma *in vitro*. The minimum coagulant dose (MCD) was defined as the amount of enzyme able to clot plasma in 60 s (Theakston and Reid, 1983). The assay was conducted in triplicate with 200 μL of human plasma at 37 °C and 0.1 μg–6 μg of enzyme. As a control, plasma (200 μL) devoid of the enzyme was used.

2.5. Fibrinogenolytic activities

Fibrinogenolytic activity was determined using the method described by Edgar and Prentice (1973) with

modifications as indicated by Rodrigues et al. (2000). Samples of bovine fibrinogen (20 μg) dissolved in a buffer (0.1 M Tris–HCl pH 7.4, 0.01 M NaCl) were incubated with different concentrations of each enzyme (0.05–1.0 μg) at 37 °C for 30 min. The reaction was stopped by the addition of a reducing buffer (10% (v/v) glycerol, 10% SDS, 5% 2-mercaptoethanol, and 0.05% (w/v) bromophenol blue). Fibrinogen hydrolysis was evidenced by 12% SDS–PAGE gels.

2.6. Fibrinolytic activity

The fibrinolytic activity was performed as described by Leitao et al. (2000) with some modifications. A 0.3% fibrinogen solution was prepared in barbital buffer (50 mM sodium barbital, 1.66 mM $CaCl_2$, 0.68 mM $MgCl_2$, 94 mM NaCl, 0.02% sodium azide, pH 7.8) and added to 0.95% agarose in barbital buffer under heating, until the formation of a transparent colloid. Upon cooling, the agarose solution (40 °C) was added to the solution of fibrinogen (fibrinogen: agarose, 1:1, v/v). 100 μL of bovine thrombin (1 μg/μL) was added to the solution, which was then poured into a Petri plate for clotting and fibrin formation. The samples were applied to pores in the gel at the desired concentrations (4–64 μg) in a final volume of 30 μL, followed by incubation at 37 °C for 24 h and subsequent measurement of the haloes.

2.7. Proteolytic activity on casein

The experiment for proteolytic activity was carried out by using the method described by Sant' Ana et al. (2008) with some modifications. Here, the pH was varied instead of the concentration of the protein. Casein solution (1% w/v) was prepared in different pHs (4.6, 5.4, 6.2, 7.0, 8.0, 8.6 and 10.2). The serine proteinases (1 mg/ml−10 μl) were added to this casein solution (100 μl) and incubated at 37 °C for 30 min. The reaction was stopped by adding 5% TCA (300 μl) to this solution. The samples were maintained at rest for 30 min and then centrifuged at 10,000 × g for 10 min. The absorbance of the supernatant was measured spectrophotometrically at 280 nm. The control experiment was carried out using the casein solution without the addition of serine proteinases. The caseinolytic activity was expressed as U/mg (caseinolytic unit per milligram of enzyme utilized). This experiment was repeated in triplicate.

2.8. Protein identification by mass spectrometry

After running SDS–PAGE gels, the protein bands were excised and in-gel trypsin digestion was performed according to Hanna et al. (2000). An aliquot (7.5 μL) of the resulting peptide mixture was separated onto an analytical C18 column (75 μm i.d. × 100 mm) (Waters, Milford, MA) for RP-HPLC coupled with nano-electrospray MS/MS on a Thermo Electron LTQ XL ion-trap mass spectrometer at a flow rate of 500 nL/min. The gradient was 2–80% acetonitrile in 0.1% formic acid over 45 min. The instrument was operated in the 'top ten' mode, in which one MS spectrum is acquired followed by MS/MS of the top ten most-intense peaks detected. Full dynamic exclusion was used to enhance dynamic range – one spectrum before exclusion for 120 s. The resulting fragment spectra were processed using the MS convert tool ProteoWizard (Kessner et al., 2008) for database searching with Mascot (Matrix Science, UK) search engine against the NCBI NR database restricted to the taxa Serpents with a parent tolerance of 1.50 Da and fragment tolerance of 1.0 Da. Iodoacetamide derivative of cysteine and oxidation of methionine were specified in MASCOT as fixed and variable modifications, respectively. The sequence similarity and amino acids were analyzed by alignment using BLAST (Altschul et al, 1997), Jalview 2.8 (Waterhouse et al., 2009) and Clustal W (Thompson et al., 1994).

3. Results

3.1. Purification of serine proteinase

An efficient protocol was developed for the rapid purification of serine proteinases from *B. alternatus* and *B. moojeni* venoms. Using three chromatographic steps with different strategies, highly pure serine proteinase samples were obtained (Fig. 1).

Since the serine proteinase from *B. alternatus* contained minor contaminants (molecular masses of about 40 and 60 kDa) (Fig. 2C), an additional cation-exchange chromatographic step was required (Fig. 2E) and the serine proteinase, which possessed coagulant activity was detected in the first peak (1c) and was labeled SPBA.

In the case of *B. moojeni,* two serine proteinases with apparent molecular masses of ~32 kDa and ~35 kDa were detected (Fig. 2D) and were subsequently purified by cation-exchange chromatography (Fig. 2F). The serine proteinase with a molecular mass of ~32 kDa eluted in the first peak (peak 1c, weakly bound) and was labeled BM-IIB32 kDa, whereas the serine proteinase with a molecular mass of ~35 kDa eluted in the second peak (2c) and was labeled BM-IIB35 kDa. The purity of each enzyme was confirmed by SDS–PAGE gels (Fig. 2F) and both displayed coagulant activities.

This procedure permitted us to obtain 12 mg of SPBA, 6 mg of BM-IIB32 kDa and 10 mg of BM-IIB35 kda from 250 mg of crude venom.

3.2. Coagulant activities

The serine proteinases isolated from *B. alternatus* (SPBA) and from *B. moojeni* (BM-IIB34 kDa + BM-IIB32 kDa) were capable of clotting human plasma with MCD of 6 and 1 μg respectively.

3.3. Fibrinogenolytic and fibrinolytic activities

After incubation of fibrinogen with the *B. alternatus* serine proteinase (SPBA), degradation of the Aα and Bβ chains was observed (Fig. 3A). In the case of serine proteinases from *B. moojeni*, BM-IIB32 kDa completely cleaved both the Aα and Bβ chains (Fig. 3B) whereas BM-IIB35 kDa completely cleaved only the Aα chain and only partially

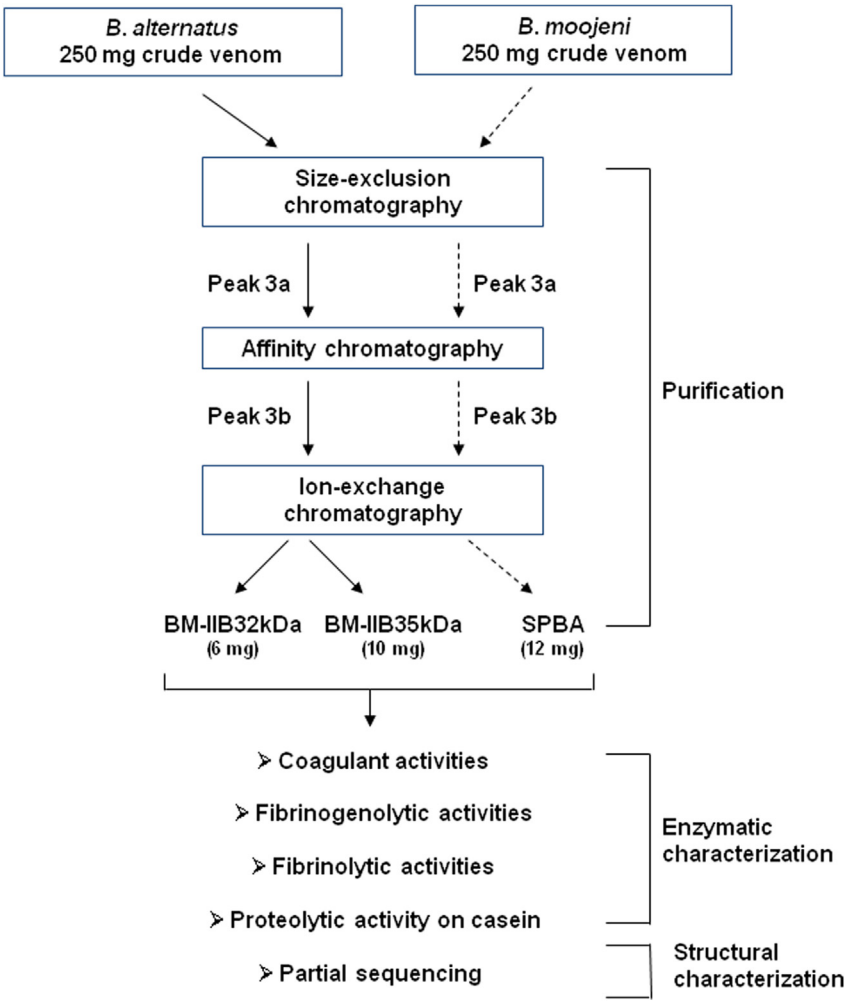

Fig. 1. Schematic of the purification protocol developed to obtain pure samples of serine proteinases BM-IIB32 kDa, BM-II35 kDa and SPBA from *B. moojeni* and *B. alternatus* crude venoms.

cleaved the Bβ chain (Fig. 3C). The purified serine proteinases did not display fibrinolytic activity on fibrin clots formed in agarose gels by the reaction of fibrinogen with thrombin after incubation for 48 h at 37 °C (results not shown).

3.4. Proteolytic activity

The proteolytic activity results indicate that the serine proteinases isolated display maximum proteolytic activity on casein at pH 8.6. However, they display only moderate activity at either pH 8.0 or at pH 10.2.

3.5. Partial sequencing

The partial amino acid sequence analysis of isolated enzymes SPBA and BM-IIB32 kDa (Table 1) and their alignments with HS114 serine proteinases from *B. jararaca* venom (Saguchi et al., 2005) revealed 100% identity (Fig. 4) whereas, with Batroxobin (Itoh et al., 1987), HS112 (Saguchi et al., 2005), KN-BJ (Serrano et al., 1998) and PA-BJ (Serrano et al., 1995) the identity was between 61 and 70%. The partial sequence of BM-II35 kDa is approximately 80–85% identical to Batroxobin, HS112 and Bothrombin (Table 2). All partial sequences of the enzymes here isolated share 20–31% identity with Trypsin (Emi et al., 1986) and Thrombin (MacGillivray and Davie, 1984) (Fig. 4).

4. Discussion

Snake venom serine proteinases demonstrate high substrate specificities and are capable of converting fibrinogen into fibrin (thrombin-like enzymes) (Huang et al., 1999; Matsui et al., 1998), release bradykinin from kininogen (Nikai et al., 1998; Serrano et al., 1998), increase capillary permeability (Sugihara et al., 1980), activate Factor X (Hofmann et al., 1983), induce platelet aggregation (Basheer et al., 1995; Serrano et al., 1995) and activate prothrombin (Kitano et al., 2013) among various other activities.

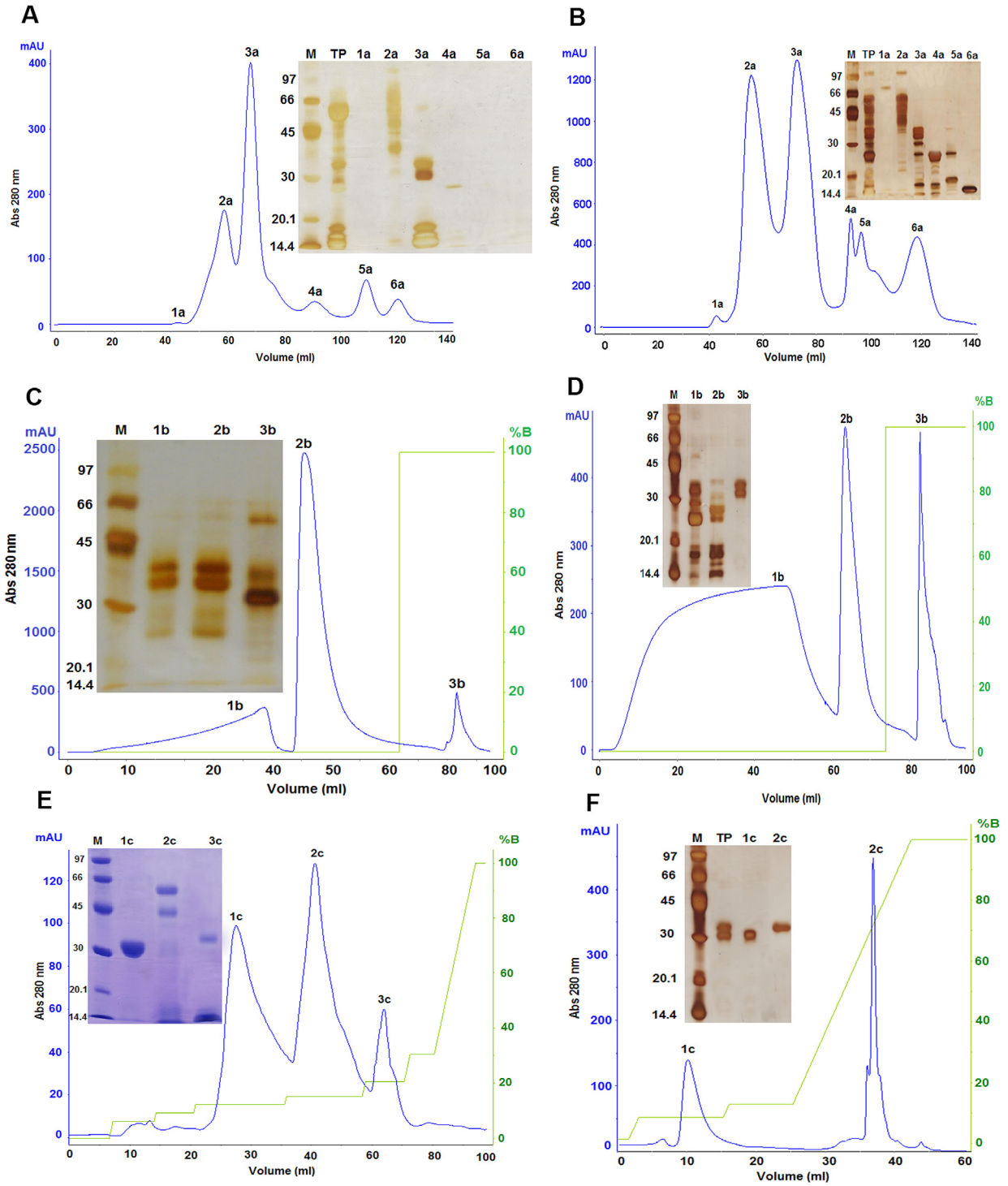

Fig. 2. Size-exclusion chromatography profile of crude (A) *B. alternatus* and (B) *B. moojeni* venoms on Sephacryl S-100. Inset, silver stained gel of peak fractions. Affinity chromatography profile on Benzamidine Sepharose 4 Fast Flow. (C) *B. alternatus* and (D) *B. moojeni*; green line and scale on the left indicate the relative salt concentration. Inset, silver stained PAGE gel of corresponding peak fractions. Cation-exchange chromatographic profile on Mono S 5/50 GL column (E) *B. alternatus* and (F) *B. moojeni*; green line and scale on the left indicate the relative salt concentration. Inset, Coomassie Brilliant Blue stained gel of peak fractions. M: Molecular weight standards; TP: Total Protein (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.).

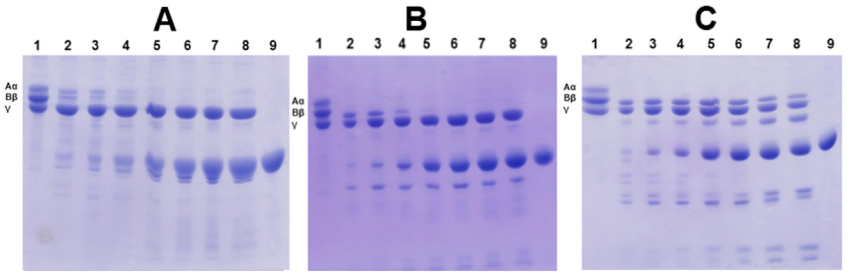

Fig. 3. Fibrinogenolytic activity of (A) *B. alternatus,* (B) *B. moojeni* 32 kDa and (C) *B. moojeni* 34 kDa serine proteinases respectively. 12% SDS–PAGE gel analysis of bovine fibrinogen degradation. 1: fibrinogen (20 μg) control without serine proteinase; 2: fibrinogen (20 μg) + serine proteinase (0.05 μg); 3: fibrinogen (20 μg) + serine proteinase (0.1 μg); 4: fibrinogen (20 μg) + serine proteinase (0.2 μg); 5: fibrinogen (20 μg) + serine proteinase (0.4 μg); 6: fibrinogen (20 μg) + serine proteinase (0.6 μg); 7: fibrinogen (20 μg) + serine proteinase (0.8 μg); 8: fibrinogen (20 μg) + serine proteinase (1 μg); 9: serine proteinase (0.6 μg) control without fibrinogen.

Serine proteinases from the venoms of *B. alternatus* and *B. moojeni* were isolated through a combination of three steps – size-exclusion, affinity and ion-exchange chromatographies (Fig. 1).

Ohler et al. (2010) performed two-dimensional electrophoresis of the venom of *B. alternatus* and isolated proteins of apparent molecular masses of 30 kDa and 28 kDa and these isoforms share high sequence identity with BthaTL, a serine proteinase present in the venom of *B. alternatus* that affects the hemostatic system. We have successfully purified and characterized the 32 kDa enzyme referred to as SPBA, from *B. alternatus* venom.

The partial amino acid sequences of SPBA and its alignment with serine proteinase HS114 of *B. jararaca* (Saguchi et al., 2005) revealed 100% identity.

Several serine proteinases of molecular masses of about 30 kDa share significant homology with HS114 (a cDNA that encodes an SVSP of *B. jararaca*) (Saguchi et al., 2005). According to their specificity for cleaving fibrinogen chains, the serine proteinases have been classified as α, β and γ-fibrinogenases. SVSPs preferentially cleave the Bβ-chain (Herzig et al., 1970; Markland, 1998; Menaldo et al., 2012; Vieira et al., 2004), however, some of them such as Batroxobin from *B. atrox*, Bothrombin from *B. jararaca*, Bhalternin from *B. alternatus* (Costa Jde et al., 2010; Sant' Ana et al., 2008; Stocker and Barlow, 1976) also cleave the Aα-chain. The SPBA enzyme isolated from *B. alternatus* cleaved the Bβ-chain and was also able to degrade the Aα-chain, similar to thrombin and some SVSPs, such as Jararacussin-I from *B. jararacussu* (Zaganelli et al., 1996), MOO3 from *B. moojeni* (Oliveira et al., 1999) and BpirSP41 from *Bothrops pirajai* (Menaldo et al., 2012).

Based on its enzymatic, structural and biochemical characteristics, it can be inferred that SPBA is a serine proteinase with significant proteolytic activity on casein and fibrinogen. However, unlike most venom serine proteinases studied (Bortoleto et al., 2002; Costa Jde et al., 2010; Perez et al., 2008; Sant' Ana et al., 2008), SPBA showed low coagulant activity, with an MCD of 6 ug.

Many proteinases have been purified and characterized from *B. moojeni*, for example, the clotting enzyme Batroxobin (Lochnit and Geyer, 1995; Stocker and Barlow, 1976), three serine proteinases, MSP1, MSP2 (Serrano et al., 1993) and MOO3 (Oliveira et al., 1999) and some metalloproteinases, such as BmMP-III (Ullah et al., 2012) and Moojenin (de Morais et al., 2012). Through a combination of three chromatographic steps, two serine proteinases from the venom of *B. moojeni* have been isolated in this study.

Serrano et al. (1993) isolated a serine proteinase from *B. moojeni* venom named MSP1, that displayed two bands around 32 kDa and 34 kDa. However, unlike the MSP1 which presents only traces of coagulant activity, the serine proteinases (BM-IIB32 kDa + BM-IIB34 kDa protein) from *B. moojeni* isolated in this study induces high blood clotting *in vitro* with an MCD of 1 ug. Other potent coagulant enzymes from Bothrops snake venom presented MCD higher than the enzyme from *B. moojeni* isolated in this study, 4.1 ug (Perez et al., 2008) and 3.5 ug (Menaldo et al., 2012). This capacity of both enzymes BM-IIB32 kDa and BM-

Table 1
Data of peptides identified by mass spectrometry.

	SPBA	BM-IIB32 kDa	BM-IIB35 kDa
Peptides	AAYPELPAEYR; DLMLVR; IFCPNK; IMGWGSITPIQK; KNDDALDKDLMLVR; LDSPVSDSEHIAPLSLPSSPPSVGSVCR; NDDALDKDLMLVR; TNPDVPHCANINLLDDAVCR	AAYPELPAEYR; IMGWGSITPIQK; LDSPVSDSEHIAPLSLPSSPPSVGSVCR; NDDALDKDLMLVR; TNPDVPHCANINLLDDAVCR	KNVITDKDIMLIR; NSEHIAPLSLPSNPPSVGSVCR; NVITDKDIMLIR
ID (NCBI)	gi/82233395	gi/82233395	gi/114837
Mascot score	1298	588	865
Protein description	Venom serine proteinase HS114; Flags: precursor	Venom serine proteinase HS114; Flags: precursor	Thrombin-like enzyme batroxobin

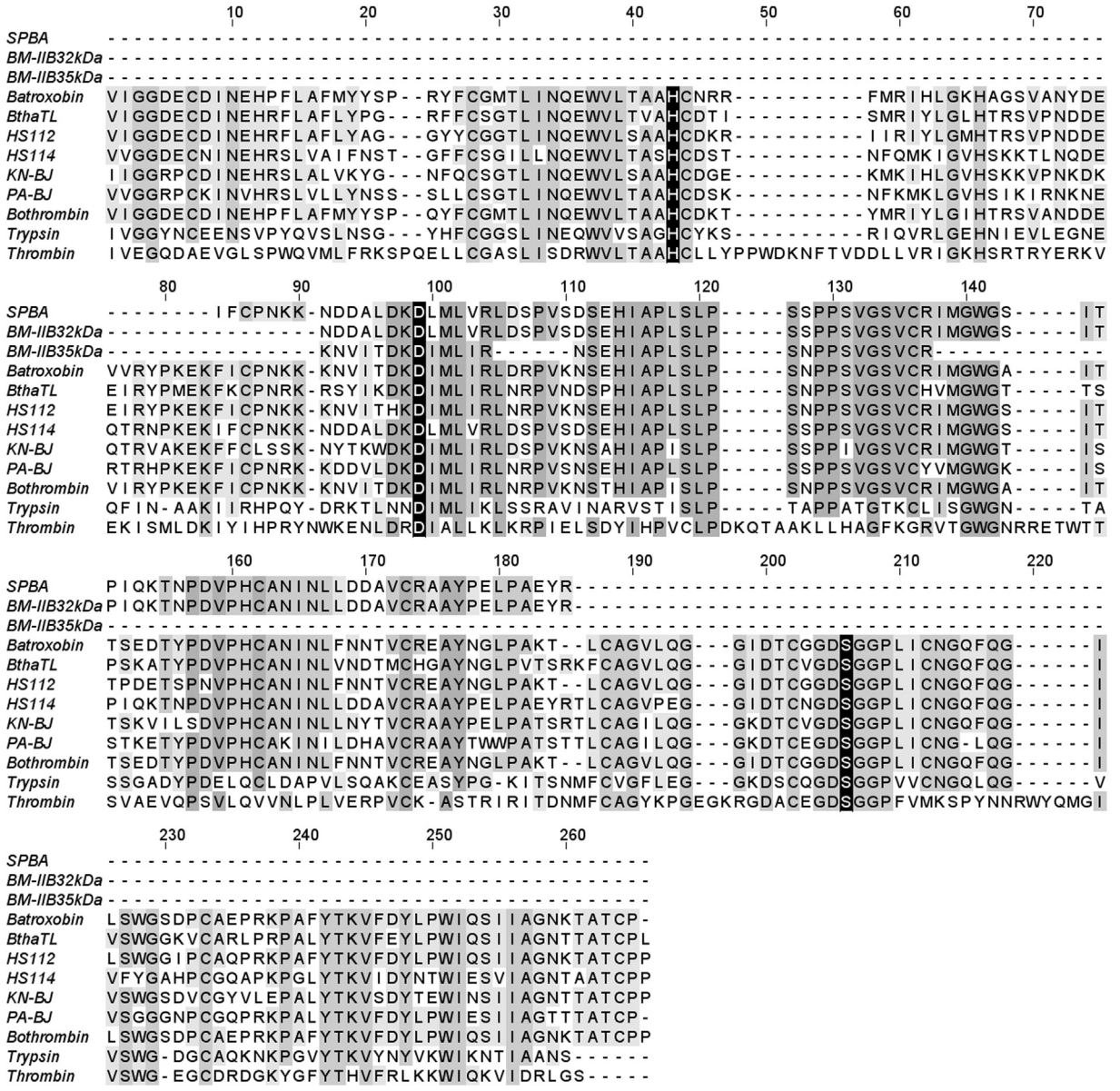

Fig. 4. The multiple sequence alignments of the partial amino acid sequences of SPBA, BM-IIB32 kDa and BM-IIB35 kDa with Batroxobin, BthaTL, HS112, HS114, KN-BJ, PA-BJ, Bothrombin, Trypsin and Thrombin. The multiple sequence alignments were performed using the programs Jalview 2.8 (Waterhouse et al., 2009) and Clustal W (Thompson et al., 1994). Residues forming the catalytic triad are highlighted using a dark background. References: Batroxobin (Itoh et al., 1987), BthaTL (Vitorino-Cardoso et al., 2006), HS112 (Saguchi et al., 2005), HS114 (Saguchi et al., 2005), KN-BJ (Serrano et al., 1998), PA-BJ (Serrano et al., 1995), Bothrombin (Nishida et al., 1994), human Trypsin (Emi et al., 1986) and Thrombin (MacGillivray and Davie, 1984).

IIB34 kDa to promote clotting *in vitro*, can be an indication of their ability to form soluble, non-cross-linked clots *in vivo*, thereby highlighting their clinical application as defibrinogenating agents.

High partial sequence identity (63–100%) was observed between the two enzymes isolated from *B. moojeni* and the Bothrops serine proteinase venoms compared. In addition, BM-IIB32 kDa and BM-IIB35 kDa were able to degrade casein and presented thrombin-like activities. Our results indicate that BM-IIB23 kDa enzyme from *B. moojeni* is an αβ-fibrinogenase, and BM-IIB34 kDa is an α-fibrinogenase.

We present here, a protocol to obtain milligram quantities of highly pure serine proteinases suitable for structural and other biophysical and biochemical studies. The crystal structure of Jararacussuin-I, a thrombin like enzyme from *Bothrops jararacussu*, has been reported and it has been proposed that the amino acid substitutions in the loops surrounding the active site make this protein highly

Table 2
Percentage of identity between partial sequences of SPBA, BM-IIB32 kDa, BM-IIB35 kDa, Trypsin, Thrombin and serine proteinase peptides from Bothrops snake venoms.

Enzyme	SPBA %	BM-IIB32 kDa %	BM-IIB35 kDa %
SPBA	–	100	63.41
BM-IIB32 kDa	100	–	63.41
BM-IIB35 kDa	63.41	63.41	–
Batroxobin	68.13	67.86	85.37
BthaTL	61.54	61.90	68.29
HS112	65.93	65.48	82.93
HS114	100	100	63.41
KN-BJ	68.13	70.24	66.67
PA-BJ	69.23	70.24	70.73
Bothrombin	64.84	64.29	80.49
Trypsin	29.67	30.95	31.71
Thrombin	24.75	25.53	20.00

negatively charged, a feature that may be relevant for its macromolecular selectivity (Ullah et al., 2013). The crystal structures of these enzymes from *B. alternatus* and *B. moojeni* venoms which we are currently pursuing may provide important insights into the structures, functions and specificities of SVSPs.

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Conflict of interest statement

The authors declare that there are no conflicts of interest.

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