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Purification and functional characterization of bothrojaractivase, a prothrombin-activating metalloproteinase isolated from *Bothrops jararaca* snake venom ☆

Markus Berger, Antônio F.M. Pinto, Jorge A. Guimarães*

Laboratório de Bioquímica Farmacológica, Centro de Biotecnologia Universidade Federal do Rio Grande do Sul (UFRGS), Av. Bento Gonçalves, 9500, P.O. Box 15005, 91501-970 Porto Alegre, RS, Brazil

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Abstract

Bleeding at the site of bite and/or systemic hemorrhage are symptoms frequently observed in envenomation by *Bothrops jararaca* snakes. In this study, we purified and characterized a prothrombin activator from *B. jararaca* that is probably involved in these clinical manifestations. The enzyme was isolated by a combination of gel filtration and ion exchange chromatographies and named bothrojaractivase. It has a single polypeptide chain with a molecular weight of 22,829 Da as measured by mass spectroscopy. Bothrojaractivase generates active thrombin from prothrombin, independently of cofactors. SDS-PAGE analysis of the prothrombin activation products shows that bothrojaractivase converts prothrombin into meizothrombin producing similar fragments to those generated by group A prothrombin's activators. In addition, bothrojaractivase degraded fibrinogen and fibrin. Chelating agents completely inhibited the enzymatic activity, whereas inhibitors of serine and cysteine proteinases had no effect. Amino acid sequence of four peptides demonstrated high similarity of bothrojaractivase with P-I class of snake venom metalloproteinases. Thus, our results indicate that bothrojaractivase is a new metalloproteinase that acts on different protein factors of the clotting cascade especially displaying a key and most relevant functional action in the generation of thrombin through prothrombin activation in a similar mode of action as that of group A activators.

Keywords: *Bothrops jararaca*; Snake venom; Prothrombin activator; Metalloproteinase; Bleeding disorders; Hemostasis

Abbreviations: Tris–HCl, tris(hydroxymethyl)aminomethane hydrochloride; PMSF, phenylmethanesulfonyl fluoride; E-64, *trans*-epoxysuccinyl-L-leucylamido(4-guanidino)-butane; EDTA, ethylene diamine tetraacetic acid; EGTA, ethylene glycol-bis(aminoethyl ether)-*N*, *N*, *N*′, *N*′-tetraacetic acid; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; S2222, Bz-Ile-Glu-Gly-Arg-pNA; S2238, H-D-Phe-Pip-Arg-pNa; FPLC, fast protein liquid chromatography

☆ *Ethical statement*: We inform that this work followed the appropriate and adequate ethical procedures. No animal or human experiments were used.

*Corresponding author. Tel.: +55 51 33086068; fax: +55 51 33087309.
E-mail address: guimar@cbiot.ufrgs.br (J.A. Guimarães).

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1. Introduction

Snake bite envenoming is an important public health problem in tropical and subtropical regions (Gutiérrez et al., 2006). In Central and South America, 300,000 snake bites are reported every year and the number of fatal accidents could exceed 5000 deaths per year (Chippaux, 1998). Epidemiological studies in Brazil indicate the occurrence of 20,000 snake bites annually (58% in south and southeast regions), with 85% of the accidents caused by *Bothrops* sp. snakes (Ribeiro and Jorge, 1997; Ministério da Saúde, 1998).

Envenomation induced by snakes of the *Bothrops* genus is often associated with local and systemic hemorrhage, together with blood-clotting disorders (Kamiguti et al., 1996; Gutiérrez and Rucavado, 2000; Braud et al., 2000). Phospholipases A2 (Gutiérrez and Lomonte, 1995) and hemorrhagic metalloproteinases (Bjarnason and Fox, 1994) are the major components responsible for edema formation, myonecrosis and local tissue damage. Serine proteinases (Nishida et al., 1994), metalloproteinases (Loria et al., 2003) and C-type lectins (Zingali et al., 1993) are some protein classes that interfere with plasmatic factors, causing their consumption or inhibition with consequent blood incoagulability. It is well known that during envenomation, these toxic proteins can act synergistically to produce the observed clinical profile. In fact, symptoms presented by envenomed patients include pain, ecchymosis, necrosis, renal failure, coagulopathy and bleeding (White, 2005).

Exogenous prothrombin activators are different proteinases that cleave specific sites in the prothrombin molecule generating active thrombin (Rosing and Tans, 1992; Kini, 2005). As a consequence of their enzymatic action, prothrombin activators contribute to the disruption of the blood coagulation balance. Kini et al. (2001) have proposed a classification for prothrombin activators considering their structural properties and cofactor requirements. Group A enzymes do not require the presence of any exogenous or plasmatic cofactors, e.g., ecarin from *Echis carinatus* and enzymes present in *Bothrops* snakes: activators from *Bothrops neuwiedi* (Govers-Riemslag et al., 1987), *B. atrox* (Hofmann and Bon, 1987), *B. erythromelas* (Silva et al., 2003) and *B. asper* (Loria et al., 2003) all belong to the P-III class of metalloproteinases, composed by the protease polypeptide chain and disintegrin-like and cysteine-rich domains. More recently, two prothrombin activators belonging to the P-I class of metalloproteinases (composed only by protease domain) were isolated from *B. insularis* (Modesto et al., 2005) and *B. cotiara* (Senis et al., 2006) venoms. Enzymes from group B comprise Ca^{+2}-dependent metalloproteinases such as carinactivase-1 from *Echis carinatus* (Yamada et al., 1996). Group C activators include serine proteinases that require Ca^{+2} and negatively charged phospholipids for activity. They have a structure similar to the factor Xa–factor Va complex and are present in the venom of some Australian elapids (Walker et al., 1980; Rao and Kini, 2002). Group D are serine proteinases similar to factor Xa, requiring Ca^{+2}, factor Va and negatively charged phospholipids. Enzymes from this group have been isolated from elapid venoms (Joseph et al., 1999; Joseph and Kini, 2001).

Bothrops jararaca venom is a rich source of several toxic proteins capable of deeply affecting blood coagulation (Cidade et al., 2006). Some of the venom components can act in a synergic way. Thus, it can be expected that both factor X and prothrombin activation should play a major role in the clinical profile resulting from envenomation following *B. jararaca*'s bite. Actually, these activators would be finally responsible for generation of intravascular thrombin, resulting in fibrinogen consumption, thrombus formation and thrombocytopenia observed in humans and rabbits upon envenomation (Sano-Martins et al., 1997; Santoro and Sano-Martins, 2004). Despite the profound hemostatic disturbance and clinical profile produced by *B. jararaca*'s envenomation the identification of the venom procagulant activity was reported long ago (Nahas et al., 1979) and there is no recent report on isolation and characterization of biochemical properties of a prothrombin activator present in this snake venom.

In this work, we describe for the first time the purification and characterization of a prothrombin-activating enzyme from *B. jararaca* snake venom, named bothrojaractivase. The enzyme is a low molecular weight metalloproteinase with procoagulant activity that belongs to group A activators. The present study can contribute to further improve our knowledge on the envenomation profile produced by these snakes.

2. Materials and methods

2.1. Reagents and venom

Lyophilized *B. jararaca* crude venom and anti-bothropic serum were obtained from Instituto

Butantan, São Paulo, Brazil. Human citrated plasma was provided by Hospital de Clínicas, Porto Alegre, Brazil. Prothrombin and α-thrombin were purified from human plasma according to Stenflo (1976) and Ngai and Chang (1991), respectively. Bovine fibrinogen, human factor X, Tris buffer, EDTA, E-64, PMSF and collagen type IV were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Chromogenic substrates S2238 and S2222 were purchased from Chromogenix (Milano, Italy). Molecular weight markers were obtained from BioRad (Hercules, CA, USA) and Invitrogen (Carlsbad, CA, USA). FPLC system, Superdex 75 HR 10/30 and Mono S HR5/5 columns were from Amersham Biosciences (Uppsala, Sweden). All other chemicals were of the highest purity commercially available.

2.2. Purification of bothrojaractivase

Purification of the prothrombin activator was carried out in three steps, using a FPLC system (Amersham Biosciences, Uppsala, Sweden). Crude *B. jararaca* venom (352 mg) was dissolved in 2 mL of 20 mM Tris–HCl buffer containing 5 mM benzamidine and 0.2 M NaCl, pH 7.5. Insoluble material was removed by centrifugation at 5000*g* for 10 min. Aliquots (100 μL, 17 mg) were fractionated on a Superdex 75 HR 10/30 column, equilibrated in 20 mM Tris–HCl containing 0.2 M NaCl, at a flow rate of 0.25 mL/min. Eluted proteins were monitored at 280 nm and the fractions tested for their ability to activate prothrombin. Fractions displaying major activity (3.5 mg of protein) were dialyzed against 20 mM Tris–HCl, pH 7.5, at 4 °C then applied into a Mono S HR 5/5 column, equilibrated in the same buffer. The elution was performed with a linear NaCl gradient from 0 to 1 M in equilibrium buffer at flow rate of 0.5 mL/min. The fraction with prothrombin-activating activity corresponding to a single peak (0.66 mg of protein) was concentrated to 200 μL. This active sample was reapplied into a Superdex 75 column, equilibrated in 20 mM Tris–HCl containing 0.3 M NaCl, pH 7.5, and the elution was performed at a 0.25 mL/min. The fraction obtained from this last chromatographic step, named bothrojaractivase, was submitted to an SDS-PAGE analysis under non-reducing and reducing conditions, according to Laemmli (1970). The molecular weight of the purified protein was determined by matrix-assisted laser desorption/ionization-time-of-flight mass spectrometry (MALDI-TOF MS) on a PE Voyager DE Pro equipment. For determination of the isoelectric point, bothrojaractivase (5 μg) was also submitted to a 2D-gel electrophoresis. Briefly, isoelectric focusing was carried out in a PROTEAN IEF cell apparatus (BioRad) using 7 cm IPG strips (pH 3–10, linear gradient), as described by the manufacturer. The strips were then applied to 8–20% SDS-polyacrylamide gels, which were stained with Coomassie brilliant blue G-250. An aqueous solution (7.88 μM) of the purified bothrojaractivase was used for the molar absorption coefficient determination. The absorbance at 280 nm was measured with a SpectraMAX M2^{e} spectrophotometer.

2.3. Protein digestion and mass spectrometric analysis

Lyophilized bothrojaractivase was dissolved in 100 μL of 100 mM ammonium bicarbonate followed by reduction with 10 mM DTT and alkylation with 50 mM iodoacetamide prior to digestion with trypsin. Digested sample was injected into the mass spectrometer through a 75 μm × 8 cm C18 column flowing at 200 nL/min. Peptides were eluted from the column using an acetonitrile/0.1 M acetic acid gradient (2–80% acetonitrile over 20 min). The LTQ mass spectrometer was operated in the data-dependent mode in which an initial MS scan recorded the mass to charge (m/z) ratios on ions over the mass range 300–2000 Da, and then the 10 most abundant ions were automatically selected for subsequent collisionally activated dissociation and MS/MS spectrum recorded. All MS/MS data were searched against a *B. jararaca* protein database downloaded from NCBI database using the SEQUEST program (Thermo Electron Corp.).

2.4. Amidolytic assay

Purified bothrojaractivase and chromatographic fractions were assayed for their direct hydrolytic activity upon chromogenic substrate S2238. Reactions were initiated by addition of substrate S2238 (0.2 mM, final concentration) in a volume of 100 μL and the amount of *p*-nitroaniline produced was monitored at 405 nm in intervals of 14 s for 30 min using a SpectraMAX microplate reader (Molecular Devices, USA).

2.5. Fibrinogen clotting assay

Fibrinogen clotting activity expressed by purified bothrojaractivase and chromatographic fractions was measured in 20 mM Tris–HCl, pH 7.5, using a SpectraMAX microplate reader. Reactions were initiated by addition of fibrinogen (2 mg/mL, final concentration) in a volume of 100 μL. Fibrin formation was monitored at 650 nm in intervals of 14 s for 30 min.

2.6. Prothrombin activation assay

The activation of prothrombin by bothrojaractivase was indirectly determined by measuring thrombin formation from prothrombin. Samples containing bothrojaractivase activity or the purified enzyme (0.5–5 μg) were incubated with prothrombin (5 μg) in 20 mM Tris–HCl, pH 7.5, for 20 min at 25 °C. Generated thrombin was assayed by its amidolytic and/or fibrinogen-clotting activity, as described above. The amount of thrombin generated from prothrombin on incubation with the activator was estimated using a calibration curve made with known amounts of purified thrombin and thus expressed as μg of equivalent thrombin/μL reaction medium.

2.7. Analysis of the prothrombin cleavage

Fragments generated during prothrombin proteolysis were analyzed after SDS-PAGE on 8–20% (w/v) gradient gels according to Laemmli (1970) and identified after staining with Coomassie Brilliant Blue G-250. Prothrombin was incubated with bothrojaractivase at a 15:1 (w/w) ratio in 20 mM Tris–HCl, pH 7.5, containing 1 mM $CaCl_2$. Aliquots (5 μg) were removed from the reaction mixture at various incubation times (between 0 and 48 h) and the reaction was stopped by addition of 60% TCA in acetone and concentrated. Proteins were analyzed by SDS-PAGE under reducing and non-reducing conditions. Prothrombin was also incubated with bothrojaractivase in the presence of 50 mM benzamidine, for 8 h. Fragments formed during reaction were analyzed in the same conditions.

2.8. Effect of inhibitors

Purified bothrojaractivase was preincubated with 10 μM E-64, 15 μM Hg^{+2}, 10 mM β-mercaptoethanol, 2.5 mM PMSF, 5 mM benzamidine, 5 mM EDTA and 5 mM EGTA in 20 mM Tris–HCl, pH 7.5, at 25 °C for 20 min. One microgram of bothrojaractivase, previously incubated with specified inhibitors, was added to a prothrombin solution (5 μg). The residual activity was measured by prothrombin activation assay using fibrinogen as substrate.

2.9. Effect of pH

Prior to the addition of prothrombin, bothrojaractivase was preincubated at 25 °C for 20 min in the following buffer systems: 50 mM sodium acetate (pH 3.5–4.0), 20 mM MES (pH 6.0) and 50 mM Tris–HCl (pH 7.5–10.0). Samples containing 1 μg of enzyme were removed from the preincubation mixtures and added to prothrombin solution (5 μg). The residual activity was measured by prothrombin activation assay using S2238 as substrate.

2.10. Effects of divalent cations

Different concentrations (0.1–15 mM) of the cations Ca^{+2} and Zn^{+2} were preincubated with bothrojaractivase at 25 °C for 20 min. Samples containing 1 μg of enzyme were removed from the preincubation mixtures and added to prothrombin solution (5 μg). The residual activity was measured by prothrombin activation assay using S2238 as substrate.

2.11. Analysis of the fibrin(ogen)olytic activity

The ability of bothrojaractivase to degrade fibrinogen and fibrin was also accessed. Briefly, each protein and the enzyme were incubated at a 15:1 (w/w) protein ratio for 20, 40 min, 1, 2 or 24 h. Aliquots from the reaction mixtures were submitted to an 8–20% SDS-PAGE under reducing conditions, as described by Pinto et al. (2004).

2.12. Assay for clotting activity

The procoagulant action of bothrojaractivase was verified using the recalcification time assay adapted to a SpectraMAX microplate reader (Ribeiro et al., 1995). For the assay, aliquots (50 μL) of citrated human plasma were transferred to a 96-wells microplate and incubated with protein samples (0.1–5 μg) to be tested in 20 mM Tris–HCl, pH 7.5, at 37 °C in the presence or absence of antibothropic serum in a final volume of 150 μL. After

5 min, 10 μL aliquots of 150 mM $CaCl_2$ were added to each well and the clot formation was monitored during 30 min at 650 nm.

3. Results

3.1. Purification and molecular characterization of the prothrombin activator

Several peaks were obtained after crude venom fractionation on the superdex 75 gel filtration column (Fig. 1A). All fractions were tested for the presence of amidolytic, fibrinogen-clotting and prothrombin-activating activities. Peaks 1 and 2 displayed the highest fibrinogen-clotting activity, while in peak 3, prothrombin-activating activity was observed together with a contaminating amidolytic activity. The fraction corresponding to peak 3 was further submitted to a cation exchange chromatography (Mono S) and fractionated into three peaks (Fig. 1B). Prothrombin-activating activity was eluted in the major peak (at 0.3 M NaCl), being well separated from the amidolytic activity (eluted at 0.7 M NaCl). The active fraction was concentrated to 200 μL and applied into a second gel filtration chromatography in the same column, resulting in a homogeneous peak (Fig. 1C). When analyzed by gel electrophoresis a single band was observed with both non-reducing and reducing conditions (Fig. 1C, insert). Molecular mass was estimated by SDS-PAGE in 18,800 Da under non-reducing and 22,700 Da under reducing conditions. The protein has a molar coefficient of absorption at 280 nm of 19.3×10^3. Analysis by MALDI-TOF MS indicated a molecular mass of the purified protein of $22{,}829 \pm 45$ Da, similar to the value found by SDS-PAGE under reducing conditions. Two-dimensional gel electrophoresis showed a single spot of protein with an apparent isoelectric point of 8.1 (results not shown). The protein thus obtained and displaying prothrombin-activating activity was designated bothrojaractivase, in order to follow the recommendation for the nomenclature of exogenous hemostatic factors by the International Society on Thrombosis and Haemostasis (Kini et al., 2001).

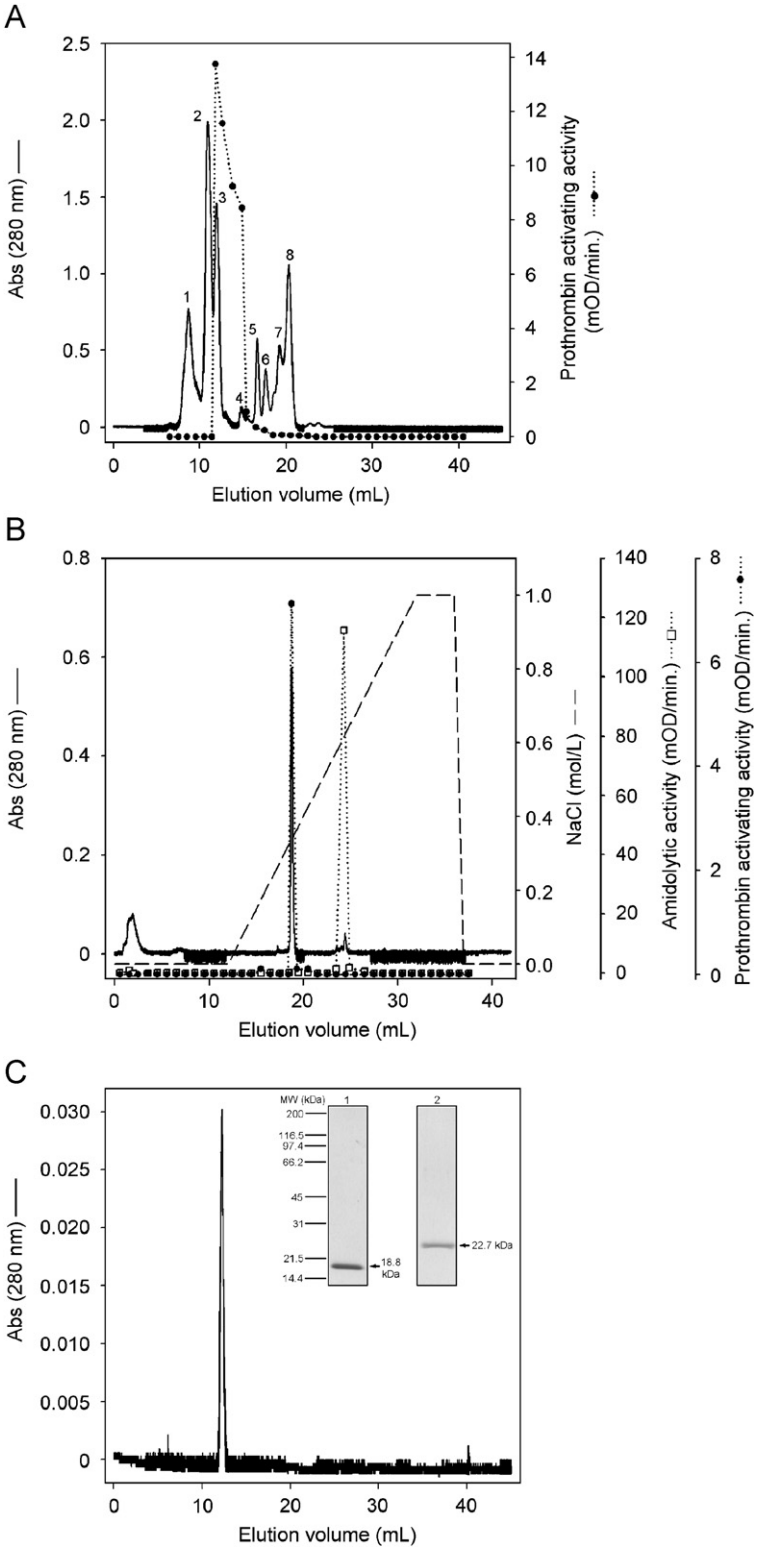
Mass spectrometric analysis was performed in order to obtain a molecular identification and partial sequence of bothrojaractivase. Digestion of the protein with trypsin, followed by LC/MS/MS identified four peptides (Fig. 2), all of them showing similarity with metalloproteinases from viperidae snake venoms. Bothrojaractivase tryptic peptides show high identity pattern with the P-I class of metalloproteinases, among them BaP1 (Watanabe et al., 2003), a hemorrhagic and fibrin(ogen)olytic enzyme; insularinase (Modesto et al., 2005), a group A prothrombin-activating enzyme; and the fibrin (ogen)olytic enzymes neuwiedase (Rodrigues et al., 2000) and leucurolysin-A (Bello et al., 2006). On the other hand, a minor identity was observed with P-III members of metalloproteinases, ecarin (Nishida et al., 1995) and berythractivase (Silva et al., 2003), both group A prothrombin activators (Fig. 2).

3.2. Prothrombin activation

Upon incubation with purified prothrombin, bothrojaractivase was able to rapidly generate free thrombin, being however unable to show direct fibrinogen-clotting activity (Fig. 3A). In order to ensure specific thrombin formation during prothrombin activation, fibrinogen was used as substrate. Bothrojaractivase showed a dose-dependent prothrombin-activating activity (Fig. 3B). In addition, this activity was independent of any plasmatic or exogenous cofactors added to the assay (data not shown).

As shown in Table 1, bothrojaractivase's activity was only partially inhibited (62.2% and 69.1%) by the serine-proteinase inhibitors PMSF (2.5 mM) and benzamidine (5 mM), respectively. A similar effect was seen with the reducing agent β-mercapethanol where just a partial (75.2%) inhibitory effect was found. On the other hand, Hg^{+2} (15 μM) and E-64 (10 μM), both known effective cysteine-proteinase inhibitors, exert poor inhibitory effects on bothro-

Fig. 1. Purification of bothrojaractivase. (A) Crude *Bothrops jararaca* venom (17 mg) was fractionated on a superdex 75 HR 10/30 column equilibrated with 20 mM Tris–HCl, pH 7.5, containing 0.2 M NaCl. Elution was performed at 0.25 mL/min and the fractions were subject to prothrombin-activating assay using fibrinogen as substrate. (B) Cation-exchange chromatography of peak 3 on a Mono S HR 5/5 column equilibrated with 20 mM Tris–HCl, pH 7.5, and eluted with a linear gradient of 0–1 M NaCl. Fractions obtained were tested for the presence of prothrombin-activating activity and amidolytic activity. (C) Gel filtration of the prothrombin activator on a Superdex 75 HR 10/30 column equilibrated with 20 mM Tris–HCl, pH 7.5, containing 0.3 M NaCl. The inset is the SDS-PAGE analysis of bothrojaractivase (10 μg) under non-reducing (lane 1) and reducing conditions (lane 2). Estimated molecular mass of enzyme and molecular weight markers using as standards are indicated.


A
Abs (280 nm) ——
Prothrombin activating activity (mOD/min.) ·····●·····
Elution volume (mL)
B
Abs (280 nm) ——
NaCl (mol/L) — —
Amidolytic activity (mOD/min.) ···□···
Prothrombin activating activity (mOD/min.) ·····●·····
Elution volume (mL)
C
Abs (280 nm) ——
MW (kDa)
200
116.5
97.4
66.2
45
31
21.5
14.4
18.8 kDa
22.7 kDa
Elution volume (mL)

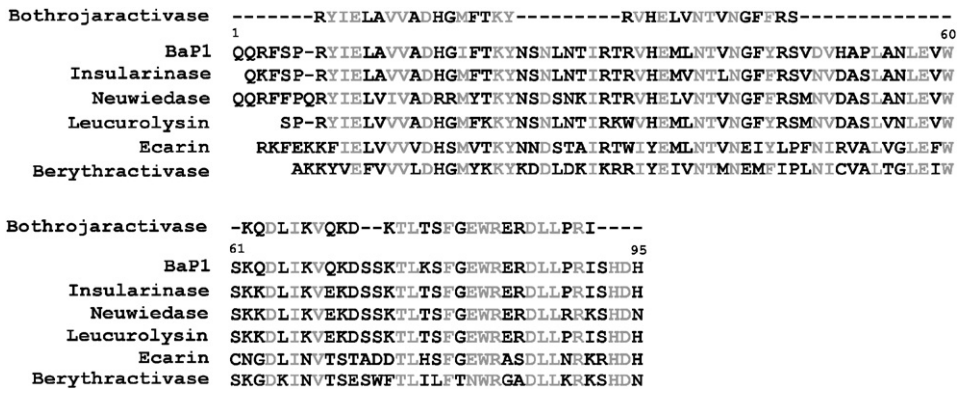


Fig. 2. Sequence alignments of bothrojaractivase tryptic fragments analyzed by mass spectrometry. Four peptides were identified by LC–MS/MS analysis. Amino acid sequences show similarity with other snake venom metalloproteinases: BaP1 (Watanabe et al., 2003), neuwiedase (Rodrigues et al., 2000), insularinase (Modesto et al., 2005) and leucurolysin (Bello et al., 2006) are P-I snake venom metalloproteinases from *B. asper*, *B. neuwiedi*, *B. insularis* and *B. leucurus*, respectively. Berythractivase (Silva et al., 2003) and ecarin (Nishida et al., 1995) are P-III snake venom metalloproteinases from *B. erythromelas* and *Echis carinatus*, respectively. Residues in gray indicate high consensus.

jaractivase. Contrary to this, the prothrombin-activating capacity of the enzyme was completely blocked by chelators agents EGTA (5 mM) and EDTA (5 mM) (Table 1).

3.3. Analysis of the prothrombin cleavage fragments

The identity of the possible intermediates formed during prothrombin activation is well established in the literature (Rhee et al., 1982; Rosing and Tans, 1992). In this work, we identified the products generated during prothrombin cleavage produced by bothrojaractivase based on their molecular masses and gel migration patterns. Prothrombin was incubated in the presence and absence of bothrojaractivase for 48 h. The products of the reaction were analyzed by SDS-PAGE under non-reducing and reducing conditions. As shown in Fig. 4, a complete consumption of prothrombin was observed between 2 and 8 h of incubation, with the appearance of expected intermediates and final products. Under non-reducing conditions (Fig. 4A), an increase in intensity of the band in 50–60 kDa indicated the formation of prethrombin-1 and/or meizothrombin lacking fragment 1, called meizothrombin(des)F1. In addition, a gradual increase in the intensity of the band corresponding to thrombin formation (~36 kDa) can be observed. At the same time, fragments 1 and 2 start to appear in the gel. Under reducing conditions (Fig. 4B), the increasing consumption of the band with a molecular mass of 50–60 kDa during incubation indicates the conversion of prethrombin-1 into thrombin B-chain (~32 kDa) and thrombin A-chain + fragment-2 (~20 kDa). These fragments are part of meizothrombin(des)F1, which is disassembled due to the presence of a reducing agent. Other bands corresponding to fragments-1 and -2 can also be visualized.

These experiments were also performed in the presence of the serine-proteinase inhibitor benzamidine. In a medium containing 50 mM benzamidine, prothrombin was incubated for 8 h in the presence and absence of bothrojaractivase. The reaction products were analyzed by SDS-PAGE under reducing and non-reducing conditions.

After incubation under non-reducing conditions no alteration in the migration pattern was observed for prothrombin alone (control) or prothrombin + bothrojaractivase (Fig. 5, lane 2). However, the addition of β-mercaptoethanol to the same reaction mixture shows the presence of thrombin B-chain and an increased intensity of the ~55 kDa band (Fig. 5, lane 3). These results indicate that bothrojaractivase cleave prothrombin (~72 kDa) generating meizothrombin, a prothombin derivative with the same molecular mass (~72 kDa). Consequently, neither thrombin (~36 kDa molecular mass) nor fragment-1 (~27 kDa) were visualized under non-reducing conditions. On the other hand, in the presence of the reducing agent, meizothrombin, the first product of prothrombin + bothrojaractivase reaction, originated two fragments, thrombin B-chain (~32 kDa) and thrombin A-chain + fragment 1.2 (~55 kDa) (Fig. 5). Thus, the other bands previously observed in the absence of benzamidine

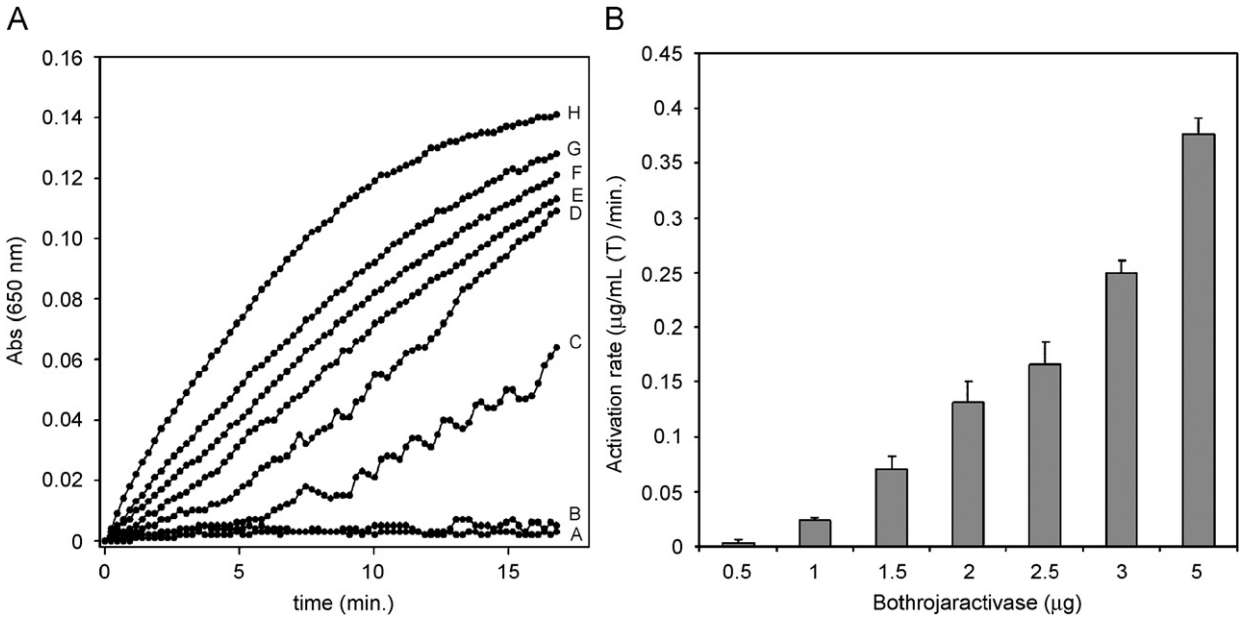


Fig. 3. Prothrombin activation. (A) Purified bothrojaractivase (0.5–5 µg) was incubated for 20 min in the presence and absence of prothrombin (5 µg) or fibrinogen (0.2 mg). Thrombin generated during incubation time was measured by addition of fibrinogen, in a final volume of 100 µL, as described in Section 2. (Lane A) Bothrojaractivase (5 µg) + fibrinogen, (lane B) 1 µg, (lane C) 1.5 µg, (lane D) 2.0 µg, (lane E) 2.5 µg, (lane G) 3.0 µg and (lane H) 5.0 µg of bothrojaractivase + prothrombin. (B) Thrombin concentration produced during prothrombin activation with different amounts of bothrojaractivase was also estimated. The data represent the average of results found in three independent measurements.

Table 1
Effects of inhibitors on prothrombin activating activity

Inhibitor	Concentration	Rate of activation (%)[a]
None	–	100
Hg^{+2}	15 µM	95.4
E-64	10 µM	90.4
β-Mercaptoethanol	10 mM	75.2
PMSF	2.5 mM	62.2
Benzamidine	5 mM	69.1
EGTA	5 mM	0
EDTA	5 mM	0

[a]Bothrojaractivase (1 µg) was pre-incubated in the presence and absence of different proteinase inhibitors for 20 min. The rate of activation was measured after addition of prothrombin (5 µg) and expressed as percent of the control value (in the absence of inhibitors). Results represent mean of three experiments.

(Fig. 4) were probably formed by autolysis of the meizothrombin and/or catalytic action of thrombin and meizothrombin(des)F1, as described for other prothrombin activators (Hofmann and Bon, 1987; Rhee et al., 1982; Modesto et al., 2005).

3.4. Effect of pH and divalent cations on prothrombin-activating activity

As shown in Fig. 6A, bothrojaractivase presents maximum activity in pH 8.0. Incubation of bothrojaractivase at pH values below 6.0 and above 8.8 results in an abrupt decrease in its prothrombin-activating activity.

Bothrojaractivase did not require any cofactors, but the rates of prothrombin activation varied at different calcium and zinc concentrations (Fig. 6B). In the presence of these cations, the rate of activation increased by 1.5- and 3.0-fold at optimal zinc (0.1 mM) and calcium (1 mM) concentrations, respectively. Higher concentrations of the cations produced inhibitory effects upon the enzymatic activity (Fig. 6B).

3.5. Degradation of fibrinogen and fibrin

Fibrinogen degradation by bothrojaractivase was determined by incubation for 20, 40 min, 1, 2 and 24 h. The products obtained from the reaction mixtures were analyzed by SDS-PAGE under reducing conditions. Fig. 7A shows that the enzyme degraded fibrinogen Aα-chain after 20 min of incubation, while

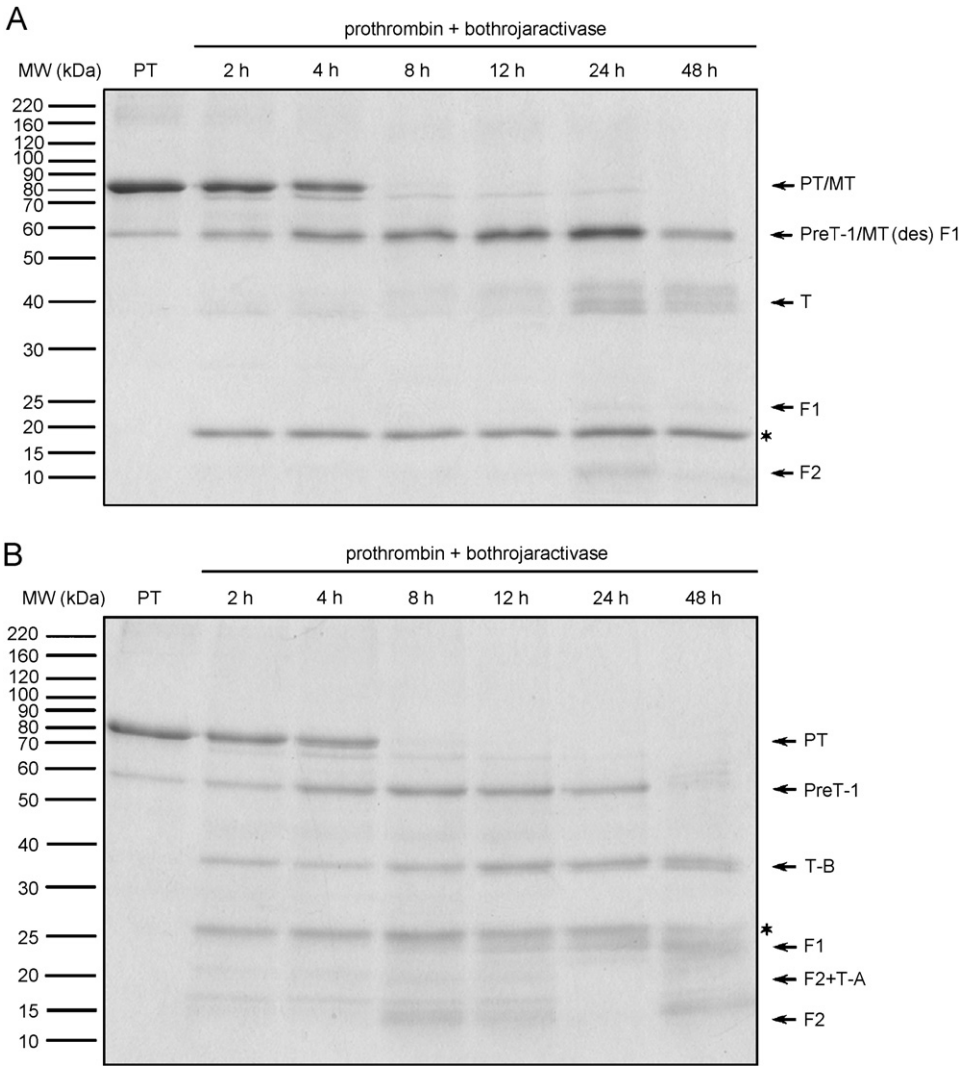


Fig. 4. Analysis of the prothrombin cleavage in the absence of serine-proteinase inhibitor. Prothrombin was incubated in the presence and absence of bothrojaractivase at a substrate to enzyme ratio of 15:1 (w/w) for a total time of 48 h. Aliquots from the incubation mixture were removed at indicated times. Migration pattern of the fragments formed during reaction were analyzed by 8–20% SDS-PAGE under non-reducing (A) and reducing (B) conditions. MW represent the molecular weight markers used as standards and (*) the bothrojaractivase band. Prothrombin-derived fragments are indicated as follows: P, prothrombin; MT, meizothrombin; T, thrombin; PreT-1, prethrombin-1; MT(des)F1, meizothrombin (des) fragment-1; F1, fragment-1; F2, fragment-2; T-B, B-chain of thrombin; F2 + T-A, fragment-2 + A-chain of thrombin.

the Bβ-chain was completely degraded upon 24 h. The γ-chain of the molecule was just partially degraded at the same incubation time. Fibrin degradation was analyzed by the same procedure. As seen in Fig. 7B, bothrojaractivase was able to degrade fibrin, generating fragments with 31–45 kDa.

3.6. Procoagulant activity

Purified prothrombin activator displayed a dose-dependent procoagulant activity on human plasma (Fig. 8). Nonetheless, in the presence of anti-bothropic serum the pro-coagulating action of bothrojaractivase was fully neutralized (Fig. 8).

4. Discussion

A profound blood coagulation disorder is produced as a consequence of *Bothrops jaracaca* bite. Actually it can be considered the most important cause of the severe pathological process developed during *B. jaracaca* envenomation (Maruyama et al.,

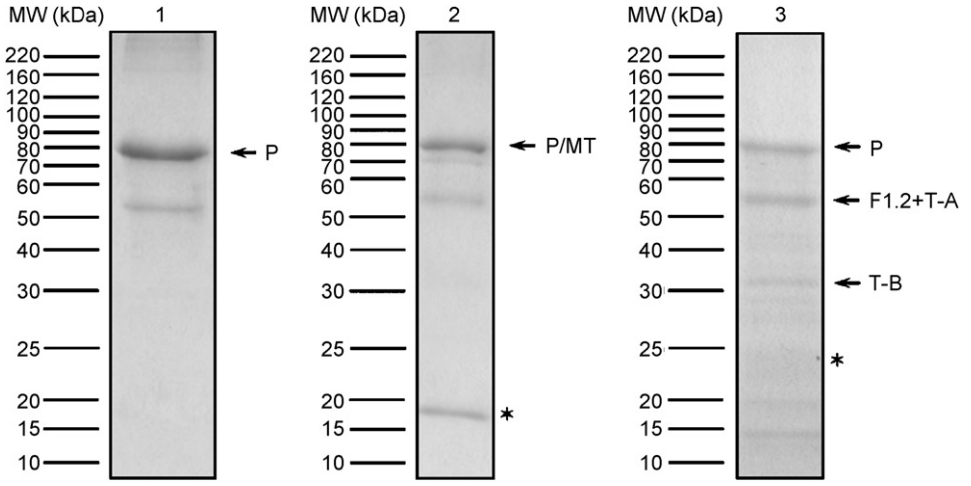


Fig. 5. Analysis of the prothrombin cleavage in the presence of serine-proteinase inhibitor. Prothrombin was incubated with the serine-proteinase inhibitor benzamidine (50 mM) in the presence and absence of bothrojaractivase at a substrate to enzyme ratio of 15:1 (w/w) for a total time of 8 h. Aliquots from the incubation mixture were removed and submitted to 8–20% SDS-PAGE. (Lane 1) Prothrombin under reducing conditions, (lane 2) prothrombin + bothrojaractivase under non-reducing conditions and (lane 3) prothrombin + bothrojaractivase under reducing conditions. MW represent the molecular weight markers used as standards and (*) the bothrojaractivase band. Prothrombin-derived fragments are indicated as follows: P, prothrombin; MT, meizothrombin; T-B, B-chain of thrombin; F1.2 + T-A, fragment 1.2 + A-chain of thrombin.

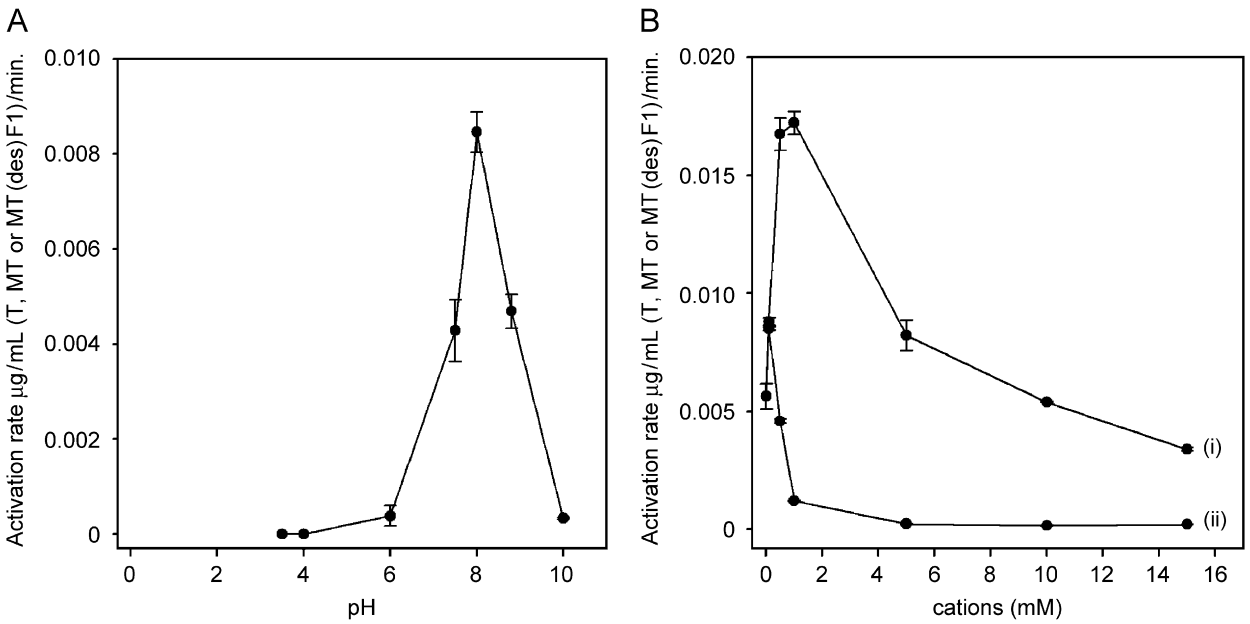


Fig. 6. Effects of pH and divalent cations on prothrombin activating activity. (A) Bothrojaractivase (1 µg) in different pH values was incubated with prothrombin (5 µg), as described in Section 2. The rate of the prothrombin activation was measured by the addition of chromogenic substrate S2238. (B) Prior to the addition of prothrombin (5 µg) bothrojaractivase (1 µg) was pre-incubated with calcium (i) and zinc (ii) at various cations concentrations (0.1–15 mM). Residual activity was measured by addition of S2238 as substrate. The results are expressed in concentration of catalytically active products (MT, MT(des)F1 or T) formed during prothrombin activation. Data represent mean ± SD of three independent determinations. MT, meizothrombin; MT(des)F1, meizothrombin (des) fragment-1; T, thrombin.

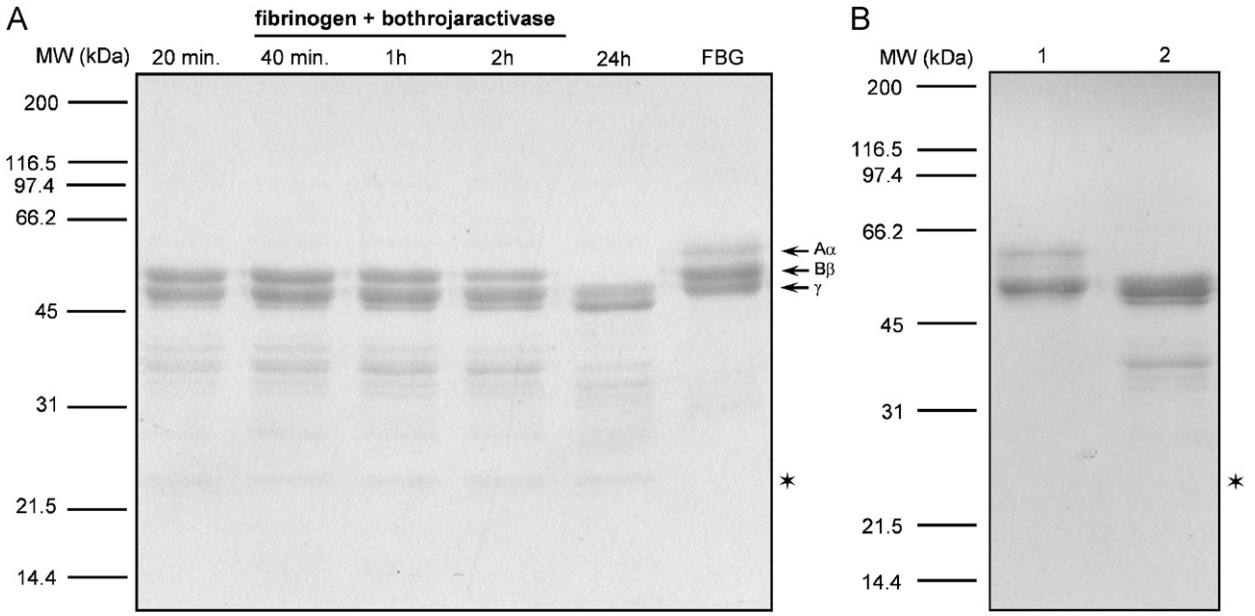


Fig. 7. Degradation pattern of fibrinogen and fibrin by bothrojaractivase. (A) Bovine fibrinogen was incubated with bothrojaractivase at a substrate to enzyme ratio of 15:1 (w/w) for the indicated time intervals. (B) Fibrin was incubated with the enzyme in the same proportions for 24 h. Samples were subjected to 8–20% SDS-PAGE under reducing conditions. (Lane 1) Fibrin, (Lane 2) fibrin + bothrojaractivase. MW represent the molecular weight markers used as standards and (*) the bothrojaractivase band. The polypeptide chains of fibrinogen (Aα, Bβ and γ) are also indicated.

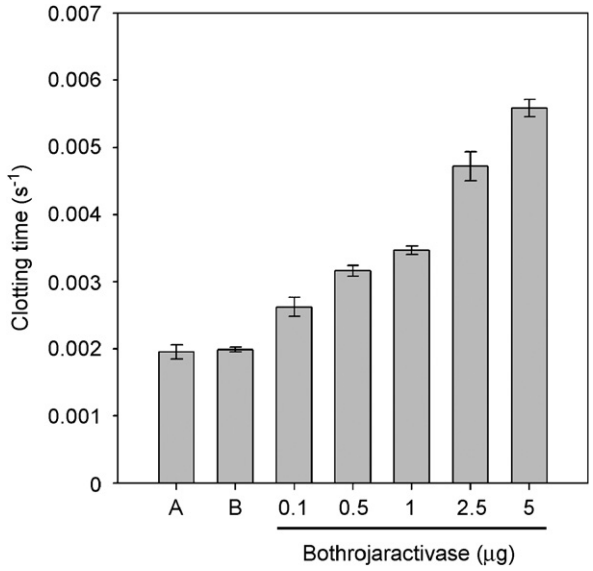


Fig. 8. Activity on human plasma. Human plasma was incubated with bothrojaractivase (0.1–5 µg) in the presence or absence of anti-bothropic serum for 5 min. After addition of 10 mM $CaCl_2$ (final concentration) the clot formation was monitored for 30 min at 650 nm. (A) Control plasma and (B) bothrojaractivase (5 µg) in the presence of anti-bothropic serum. The data represent the average of the results found in three independent measurements.

1990; Sano-Martins et al., 1997; Santoro and Sano-Martins, 2004). For instance, rabbits that received *B. jararaca* venom showed a drastic reduction in fibrinogen levels and platelet counts. Moreover, envenomed animals presented symptoms of disseminated intravascular coagulation with formation of thrombus deposits in lungs and kidneys (Kamiguti et al., 1991; Santoro and Sano-Martins, 2004). In addition, in envenomed patients, high levels of circulating thrombin–antithrombin III complex were also observed (Maruyama et al., 1990). All these results strongly suggest that *B. jararaca* venom was able to generate significant amounts of thrombin in blood circulation, causing the formation of fibrin and platelet aggregation, leading to fibrinogen level and platelet count reduction.

In this work we isolated and characterized the prothrombin activator from *B. jararaca* venom. The isolated protein, now named bothrojaractivase, was able to rapidly generate thrombin from prothrombin and is probably involved in the genesis of the envenomation process. Bothrojaractivase was purified through an experimental protocol

that combined gel filtration and ion exchange chromatographies. The protein consists of a single polypeptide chain with an isoelectric point of 8.1 and a molecular mass of 22,829 Da. The enzyme shows an optimal activity at pH 8.0 and its catalytic activity was independent of cofactors, although the presence of low concentrations of calcium and zinc ions enhanced its ability to convert prothrombin into active thrombin. On the other hand, complete inhibition of the prothrombin-activating activity by chelating agents indicates that the enzyme is a metalloproteinase. Mass spectrometric studies indicate that bothrojaractivase is a new prothrombin activator and its partial amino acid sequence presents high similarity with other P-I classes of snake venom metalloproteinases. Altogether these properties differentiate bothrojaractivase from the physiological serine-proteinase prothrombin activator such as factor Xa. Recently, a transcriptome study from *B. jararaca* venom gland showed that the metalloproteinases are the most expressed group of proteins in this venom (Cidade et al., 2006). Other *Bothrops* species also contain low molecular weight metalloproteinases that activate prothrombin and have similar functional characteristics presented by bothrojaractivase (Modesto et al., 2005; Senis et al., 2006).

In this work, we also characterized the molecular mechanism by which bothrojaractivase promotes prothrombin cleavage, generating active thrombin. Analysis of prothrombin degradation by gel electrophoresis indicates that bothrojaractivase cleaves

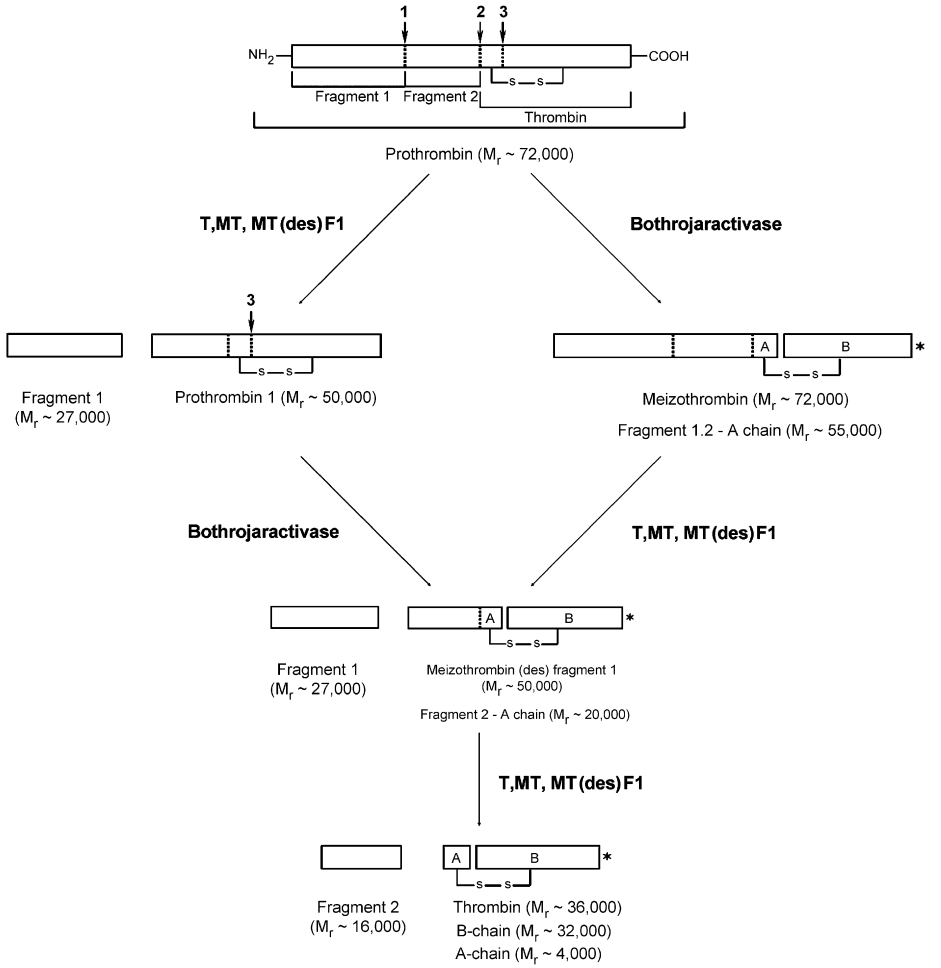


Fig. 9. Schematic representation of the prothrombin cleavage by bothrojaractivase. Prothrombin and the possible sites of cleavage are represented. Bothrojaractivase and prothrombin activators of group A seems to cleave at site 3. Other fragments observed during activation are probably formed by hydrolysis of the meizothrombin, meizothrombin (des) fragment-1 and /or thrombin. (*) indicate products with catalytic activity.

prothrombin through a similar sequence of events as that of ecarin (Rhee et al., 1982) and other group A prothrombin activators (Rosing and Tans, 1992). As shown in Fig. 9, the enzyme probably first cleaves prothrombin at site 3, directly generating meizothrombin. This intermediate can display partial enzymatic activity and thus sequentially generate by autolysis meizothrombin(des)F1 and then active thrombin. These products with partial catalytic activity can also cleave prothrombin at site 1, releasing fragment 1 and prethrombin-1, as observed in SDS-PAGE analysis (Fig. 4). Through this route, prethrombin-1 is further cleaved at site 3 by bothrojaractivase, generating meizothrombin (des)F1 and then active thrombin as seen above. Distinctly from these routes, in the case of prothrombin activation by factor Xa (Wu et al., 2002), an additional site is hydrolyzed in the prothrombin molecule (site 2, Fig. 9), thus forming prethrombin-2 (~36 kDa), fragment 1.2 (~50 kDa) and thrombin (~36 kDa). Evidence depicted in Fig. 5 indicates that bothrojaractivase is not able to cleave prothrombin at site 2, since no bands with molecular masses of ~36 kDa were visible under non-reducing conditions in the presence of serine-proteinase inhibitor.

Another feature of bothrojaractivase was shown by its ability to degrade fibrinogen and fibrin. These characteristics are common between other P-I class metalloproteinases that display high sequence identity with bothrojaractivase (Fig. 2). Despite its fibrin(ogen)olytic activity, the enzyme produces consistent and stable plasma clots, indicating that this metalloproteinase is a potent procoagulant enzyme, displaying a dose-dependent procoagulant activity in human plasma. However, bothrojaractivase did not present direct thrombin-like or amidolytic activities upon substrates S2238 and S2222 (not shown).

In this work, the isolation and functional characterization of the prothrombin activator from *B. jararaca* venom was described and named bothrojaractivase. In addition, a mechanism of molecular activation of prothrombin is proposed. Altogether the data indicate that bothrojaractivase is a new low molecular weight snake venom metalloproteinase belonging to group A prothrombin activators. Further determination of full-length amino acid sequence is necessary for precise structural details of this enzyme. Moreover, the role of bothrojaractivase in the envenomation process remains to be explored.

Acknowledgments

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