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Purification and Characterization of Jararassin-I, A Thrombin-like Enzyme from *Bothrops jararaca* Snake Venom

Débora F. VIEIRA[1], Leandra WATANABE[1], Carolina D. SANT´ANA[2,3], Silvana MARCUSSI[2,4], Suely V. SAMPAIO[3], Andreimar M. SOARES[2]*, and Raghuvir K. ARNI[1]*

[1]*Departamento de Física, Instituto de Biociências, Letras e Ciências Exatas, Universidade Estadual Paulista, UNESP, 15054-000, São José do Rio Preto, SP, Brazil;*
[2]*Unidade de Biotecnologia, Universidade de Ribeirão Preto, UNAERP, Ribeirão Preto, SP, Brazil;*
[3]*Departamento de Análises Clínicas, Toxicológicas e Bromatológicas, FCFRP, Universidade de São Paulo, USP, Ribeirão Preto, SP, Brazil;*
[4]*Departamento de Bioquímica e Imunologia, FMRP, Universidade de São Paulo, USP, Ribeirão Preto, SP, Brazil*

Abstract A thrombin-like serine protease, jararassin-I, was isolated from the venom of *Bothrops jararaca*. The protein was obtained in high yield and purity by a single chromatographic step using the affinity resin Benzamidine-Sepharose CL-6B. SDS-PAGE and dynamic light scattering analyses indicated that the molecular mass of the enzyme was about 30 kD. The enzyme possessed fibrinogenolytic and coagulant activities. The jararassin-I degraded the Bβ chain of fibrinogen while the Aα chain and γ chain were unchanged. Proteases inhibitors, PMSF and benzamidine inhibited the coagulant activity. These results showed jararassin-I is a serine protease similar to coagulating thrombin-like snake venom proteases, but it specifically cleaves Bβ chain of bovine fibrinogen. Single crystals of enzyme were obtained (0.2 mm×0.2 mm×0.2 mm) and used for X-ray diffraction experiments.

Key words snake venom; *Bothrops jararaca*; serine protease thrombin-like; fibrinogenolytic activity; crystallization

Snake venoms of the Viperidae family contain a number of proteins that cause hemostatic disturbances. Envenomation of this family is characterized by hemorrhage, edema, local tissue damage, myonecrosis, fibrinolytic and kinin releasing activities [1]. In southeastern Brazil, the viper *Bothrops jararaca* (Viperidae) is responsible for 90% of snakebite accidents [2].

The enzymes that have proteolytic, coagulate and hemorraghic activities can activate or interfere with the process of coagulation, and therefore referred to as thrombin-like enzymes [3]. These enzymes are mainly serine proteases and metalloproteases that resemble thrombin in their function to trigger the clotting of fibrinogen through fibrinopeptide release. Some other serine proteases lack the fibrinogen-clotting activity, but can directly aggregate platelets in platelet-rich plasma and washed platelet suspensions [4,5]. Due to their important roles in the coagulation process, these serine proteases have been studied as drugs in the clinical therapeutic area, because they can prevent thrombi formation and improve blood circulation by reducing blood viscosity. Such proteases have been purified from venoms of *B. jararaca* [4,6,7], *B. moojeni* [8] and *B. jararacussu* [9] and some have been utilized as drugs (Ancrod, Reptilase and Batroxobin).

The complete amino acid sequence of about 40 out of those proteases have been determined by protein sequencing or deduced from the nucleotide sequence of cDNA. Recently, the three-dimensional structures of five venom proteases, four of which are metalloproteases [5] and one serine protease [10], have been determined at high resolution by X-ray crystallography. It provided us a structural basis for understanding the steric requirements for the activities of these enzymes.

This paper reports the purification by a single step chromatographic procedure, the characterization of some

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Abbreviations: SVTLE, snake venom thrombin-like enzyme; SDS, sodium dodecyl sulphate; EDTA, ethylene diaminetetraacetic acid
*Corresponding authors:
Andreimar M. SOARES: Tel, 55-16-603-6892; Fax, 55-16-603-7030; E-mail, andreimar@unaerp.br
Raghuvir K. ARNI: E-mail, arni@df.ibilce.unesp.br

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aspects of the biochemistry, and the crystallization of a thrombin-like enzyme, jararassin-I, from the venom of *B. jararaca*, a snake found in southeastern Brazil. Elucidation of its three-dimensional structure by the X-ray diffraction technique and preliminary crystallographic characterization are in progress.

Materials and Methods

Materials

Crude venom from *B. jararaca* was obtained as a lyophilized powder from a local serpentarium. SDS, EDTA and Benzamidine-Sepharose CL-6B were obtained from Sigma (St. Louis, MO, USA) or from Amersham Pharmacia Biotech (USA).

Purification of jararassin-I

300 mg crude venom powder were dissolved in 1.0 ml of 0.02 M phosphate buffer (PB), pH 7.8, and centrifuged at 3500 *g* for 10 min. The clear supernatant was filtered through a 0.22 μM filter and injected onto a Benzamidine-Sepharose CL-6B column, which was previously equilibrated with the aforementioned PB buffer. The column was washed with the same buffer till the baseline returned to zero and kept stable. Nonspecifically bound proteins were eluted with the washing buffer (PB containing 0.5 M NaCl). The serine protease was eluted by rapidly changing the pH to 3.2 using the 0.02 M glycine buffer. Fractions of 1 ml/tube were collected at a flow rate of 1.2 ml/min using FPLC system (Pharmacia) and the absorbance was measured at 280 nm. The pH of the eluted fraction was then immediately adjusted back to 7.0, dialyzed against water (24 h) and concentrated by using Micro-concentrator (Amicon). For the purity assay, 1 mg sample was applied on an HPLC C18 reverse phase column (0.46 cm×15 cm) equilibrated with 0.1% trifluoracetic acid (TFA, *V*/*V*) and eluted by an acetonitrile concentration gradient from 20% to 60% (*V*/*V*) in 0.1% TFA for 50 min.

Electrophoretic analyses

The fractions were analyzed by 12% SDS-PAGE [11] under reducing conditions. Molecular weight markers (phosphorylase B, bovine serum albumin, ovalbumin, carbonic anhydrase, trypsin inhibitor and lactalbumin) were run in parallel. After electrophoresis, the gel was developed with silver staining. Isoelectric focusing was carried out according to the method described previously [12] with some modifications. A 5.5% polyacrylamide gel with ampholine at pH 3.5–10.0 was used in a preliminary run for 1 h at 150 V. After this, a sample of 2 mg/ml of enzyme (20 μl) in aqueous solution with 10% glycerol was applied in the gel. Isoelectric focusing markers (trypsinogen, basic lentil lectin, middle lentil lectin, acidic lentil lectin, basic horse myoglobin, acidic horse myoglobin, human carbonic anhydrase B, bovine carbonic anhydrase B, lactaglobulin A, soybean trypsin inhibitor and amyloglucosidase) were run in parallel to the sample for 3 h at 200 V. The electrophoretic buffer was NaOH and H_3PO_4 and the experiments were carried out at 4 °C. The pI bands were detected by Coomassie R staining. Molecular weight markers, pI markers and ampholine were from Pharmacia.

Fibrinogenolytic activity

The method described previously [13] was used with minor modification. 50 μl bovine fibrinogen (1 mg/ml, dissolved in PBS, pH 7.2) were incubated with different amount of jararassin-I (10 to 50 μg) at 37 °C for 20 min. Fibrinogen without jararassin-I treatment was used as control. The reaction was stopped with the addition of 25 μl 0.05 M Tris-HCl buffer [pH 8.8, containing 10% glycerol (*V*/*V*), 10% β-mercaptoethanol (*V*/*V*), 2% SDS (*V*/*V*), and 0.05% bromophenol blue (*W*/*V*)]. The reaction mixtures were then analyzed by 12% SDS-PAGE.

Coagulant activity

Bovine plasma was prepared by centrifuging citrated blood twice at 1000 *g* at 5 °C. For the assay, 100 μl of plasma was incubated with different amount of enzyme (2.5, 5.0, 10.0, 20.0 or 50.0 μg) in PB and the coagulation time was recorded. As a control, plasma aliquot was incubated with 0.5 μg of *B. jararaca* venom and the coagulation time was determined similarly. Inhibition of coagulant activity was assayed after preincubation of 20 μg of enzyme in 0.1 M Tris-HCl buffer, pH 8.0 for 30 min at 37 °C, which containing either 2 mM PMSF or 2 mM benzamidine.

Crystallization

The serine protease was concentrated in the micro-concentrator to a concentration of 5.0 mg/ml and was screened for crystallization conditions at 18 °C using the hanging-drop vapour-diffusion method. Initial trials were carried out using the screens produced by Hampton Research [14]. In a typical crystallization experiment, 0.5 μl of protein solution was mixed with the same volume of screening solution and allowed to equilibrate against 500 μl of the latter.

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Results

Purification and biochemical characteristics

After dissolving the crude venom powder in PB buffer and removal of the undissolved material by centrifugation and filtration, the purification was carried out by a single affinity chromatography step, the results were presented in Fig. 1. The first large peak in Fig. 1(A) indicated the nonspecifically bound proteins eluted by the washing with the initial buffer. The second peak indicated the proteins (proteases, PLA_2s and other) eluted by PB containing 0.5 M NaCl. Once the baseline was stable, 0.02 M glycine buffer (pH 3.2) was applied and the serine protease was eluted as a single sharp peak. Jararassin-I isolated showed high purity as analyzed by using C18 reverse phase HPLC [Fig. 1(B)].

The molecular mass of purified enzyme jararassin-I was estimated to be 30,000 Da by SDS-PAGE [Fig. 2 (A)]. Jararassin-I product appeared to be a band of approximately between 7.4 and 7.8 in isoelectric focusing [Fig. 2(B)].

Fibrinogenolytic and coagulant activities

The fibrinogenolytic activity of 10–50 μg jararassin-I on the Bβ chain was detected. The results showed that jararassin-I degraded the Bβ chain of fibrinogen while the Aα chain and γ chain were unchanged as shown in Fig. 3.

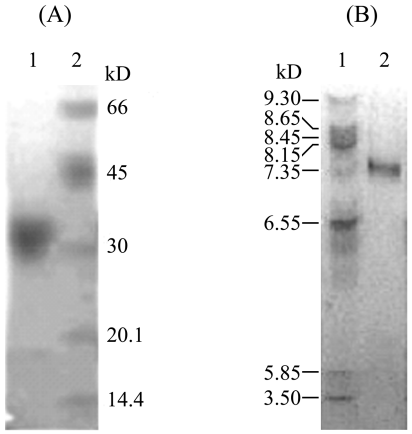

Fig. 2 Electrophoretic analyses

(A) 12% SDS-PAGE analysis. The gel was silver stained. 1, serine protease; 2, molecular weight marker. (B) Isoelectric focusing on a 5.5% polyacrylamide gel with a pH gradient from 3.5 to 10.0, at 200 V for 3 h. 1, pI markers; 2, fraction of serine protease.

Fibrinogen without jararassin-I treatment was used as control (Lane 1).

Coagulant activity assay showed that all doses of jararassin-I sample had serine protease activity, but showed different coagulation time (Fig. 4). The protease inhibitors PMSF and benzamidine (2.0 mM) inhibited the coagulant activity of the enzyme (20 μg) by 59.2% and 86.7%, respectively (data not shown).

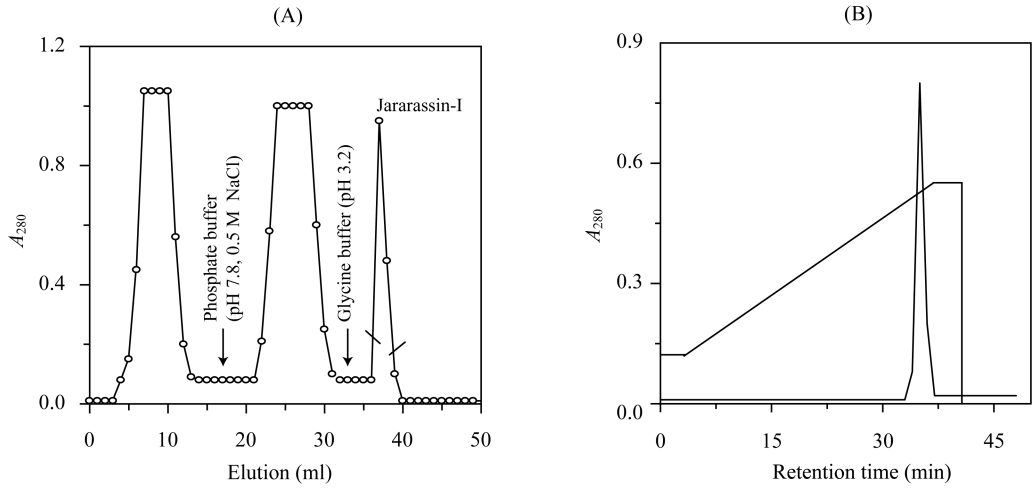

Fig. 1 Purification of *Bothrops jararaca* jararassin-I

(A) Affinity chromatography of *B. jararaca* venom (300 mg) on a column of Benzamidine-Sepharose CL-6B previously equilibrated with 0.02 M, pH 7.8, phosphate buffer. The first elution was obtained with PB buffer and the second with the washing buffer. For the elution of serine protease was utilized 0.02 M, pH 3.2, glycine buffer. Fractions of 1 ml/min. were collected at a flow rate of 1.2 ml/min. (B) Chromatography profile of purified jararassin-I (1 mg) on a C18 RP-HPLC column.

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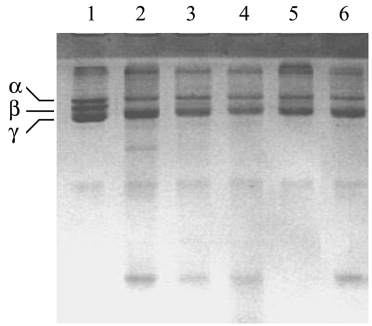

Fig. 3 Fibrinogenolytic activity of jararassin-I

12% SDS-PAGE analysis of bovine fibrinogen digest. 1, fibrinogen control without jararassin-I treatment; 2, fibrinogen+jararassin-I (50 μg); 3, fibrinogen+jararassin-I (40 μg); 4, fibrinogen+jararassin-I (30 μg); 5, fibrinogen+jararassin-I (20 μg); 6, fibrinogen+jararassin-I (10 μg).

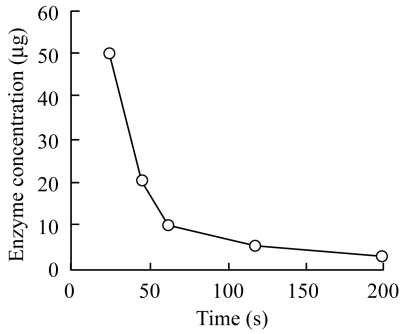

Fig. 4 Coagulant activity of jararassin-I

Coagulation time was recorded.

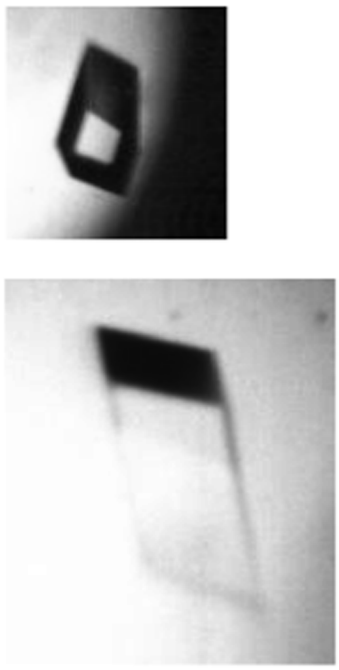

Fig. 5 Crystal of serine protease

The crystal used for X-ray diffraction experiment (0.2 mm×2 mm×0.2 mm).

Crystallization

Single crystals with a minimum dimension of 0.2 mm were obtained in a crystal growing solution containing 0.1 M MES, pH 6.2, 0.01 M zinc sulphate and 15% PEG MME 550. The crystals grew up after 6 days (Fig. 5).

Discussion

Snake venom thrombin-like enzymes (SVTLEs) are directly involved in the envenomation process with a range of life-threatening activities that cause several deaths per year. Particularly in the rural tropics, snakebite morbidity and mortality has a significant human and veterinary medical impact. On that account, there is an urgent need to understand the envenomation process and the molecules involved in it.

Many reports have described thrombin-like enzymes from snake venoms [5]. Some of them show thrombin-like coagulant activity whereas others have different substrates specificities [7]. Their primary structures are highly similar to each other, implying that they have evolved from the same ancestor and gradually adapted to each target protein of the snake's prey.

The single step procedure described here for the purification of serine protease jararassin-I from the venom of *B. jararaca* is relatively rapid and efficient (Fig. 1). The serine protease obtained is of high purity with a correspondingly high yield. Electrophoretic analyses by SDS-PAGE and isoelectric focusing indicated that the protease possessed a molecular mass of 30 kD and a pI between 7.4 and 7.8. This is similar to the molecular masses of other serine proteases isolated from snake venoms as the PA-BJ, MSP1, MSP2, TL-BJ [4,6,8] and bothrombin [7]. In addition, present data revealed that the enzyme possessed fibrinogenolytic and coagulant activities as observed for bothrombin [7], TL-BJ [6] and LM-TL [15]. All four enzymes belong to the group referred to SVTLE of high sequence and structural homology.

Jararassin-I purified in this work was found to have fibrinogenolytic and coagulant activities. This enzyme was

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able to specifically cleave Bβ chain of fibrinogen while the Aα chain and γ chain were unchanged *in vitro* with a 20 min incubation span. However, Guan *et al.* [16] described that thrombin-like enzyme were also able to degrade the Aα chain when time was long enough. Similar Bβ chain fibrinogenase has been isolated from *Crotalus atrox* [17], *B. jararaca* [7] and *Agkistrodon halys blomhoffii* [18]. *B. jararaca* bothrombin hydrolyzes the same site of the Bβ chain as *A. blomhoffii* halystase and also the Aα chain to release fibrinopeptide A [18].

In this study, we described a simple and efficient method for purifying this serine protease from *B. jararaca* venom. With the aim of understanding the structural basis for the differences observed in specificities of these enzymes, we have undertaken a crystallographic study of these enzymes. The ability of these enzymes to form fibrin clots made up of short polymers has potential therapeutic application for the treatment of patients with occlusive arterial or venous thrombolytic diseases without stimulating the endogenous fibrinolysis system [19,20].

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References

1 Mandelbaum FR, Reichel P, Assakura MT. Isolation and characterization of a proteolytic enzyme from the venom of the snake *Bothrops jararaca* (Jararaca). Toxicon, 1982, 20(6): 955–972

2 Rosenfeld G. In: Bucherl W, Buckley E, Deulofeu V eds. Venomous Animals and Their Venoms II. New York: Academic Press, 1971

3 Selistre HS, Giglio JR. Isolation and characterization of a thrombin-like enzyme from the venom of the snake *Bothrops insularis* (Jararaca ilhoa). Toxicon, 1987, 25(11): 1135–1144

4 Serrano SM, Mentele R, Sampaio CA, Fink E. Purification, characterization, and amino acid sequence of a serine proteinase, PA-BJ, with platelet-aggregating activity from the venom of *Bothrops jararaca*. Biochemistry, 1995, 34(21): 7186–7193

5 Matsui T, Fujimura Y, Titani K. Snake venom proteases affecting hemostasis and thrombosis. Biochim Biophys Acta, 2000, 1477(1-2): 146–156

6 Serrano SM, Sampaio CA, Mentele R, Camargo AC, Fink E. A novel fibrinogen-clotting enzyme, TL-BJ, from the venom of the snake *Bothrops jararaca*: Purification and characterization. Thromb Haemost, 2000, 83(3): 438–444

7 Nishida S, Fujimura Y, Miura S, Ozeki Y, Usami Y, Suzuki M, Titani K *et al.* Purification and characterization of bothrombin, a fibrinogen-clotting serine protease from the venom of *Bothrops jararaca*. Biochemistry, 1994, 33(7): 1843–1849

8 Serrano SMT. The isolation and structural and functional characterization of serine proteinases in the venom of *Bothrops jararaca*. J Venom Anim Toxins, 1999, 5(2): 226

9 Bortoleto RK, Murakami MT, Watanabe L, Soares AM, Arni RK. Purification, characterization and crystallization of jararacussin-I, a fibrinogen-clotting enzyme isolated from the venom of *Bothrops jararacussu*. Toxicon, 2002, 40(9): 1307–1312

10 Parry MA, Jacob U, Huber R, Wisner A, Bon C, Bode W. The crystal structure of the novel snake venom plasminogen activator TSV-PA: A prototype structure for snake venom serine proteinases. Structure, 1998, 6(9): 1195–1206

11 Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature, 1970, 227(5259): 680–685

12 López LM, Sequeiros C, Natalucci CL, Brullo A, Maras B, Barra D, Caffini NO. Purification and characterization of macrodontain I, a cysteine peptidase from unripe fruits of *Pseudananas macrodontes* (Morr.) harms (*Bromeliaceae*). Protein Expr Purif, 2000, 18(2): 133–140

13 Astrup T, Mullertz S. The fibrin plate method for estimating fibrinolytic activity. Arch Biochem Biophys, 1952, 40(2): 346–351

14 Jancarik J, Kim SH. Sparse matrix sampling: A screening method for crystallization of proteins. J Appl Crystallogr, 1991, 24: 409–411

15 Castro HC, Silva DM, Craik C, Zingali RB. Structural features of a snake venom thrombin-like enzyme: thrombin and trypsin on a single catalytic platform? Acta Biochim Biophys, 2001, 1547(2): 183–195

16 Guan LF, Chi CW, Yuan M. Study on the thrombin-like enzyme preferentially releasing fibrinopeptide B from the snake venom of *Agkistrodon halys Pallas*. Thromb Res, 1984, 35: 301–310

17 Pirkle H. Thrombin-like enzymes from snake venoms: An updated inventory. Thromb Haemost, 1998, 79(3): 675–683

18 Matsui T, Sakurai Y, Fujimura Y, Hayashi I, Oh-Ishi S, Suzuki M, Hamako J *et al.* Purification and amino acid sequence of halystase from snake venom of *Agkistrodon halys blomhoffii*, a serine protease that cleaves specifically fibrinogen and kininogen. Eur J Biochem, 1998, 252(3): 569–575

19 Markland FS. Snake venoms and the hemostatic system. Toxicon, 1998, 36(12): 1749–1800

20 Marsh NA. Use of snake venom fractions in the coagulation laboratory. Blood Coagul Fibrinolysis, 1998, 9(5): 395–404

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