Identification of Serine Proteases from *Leishmania braziliensis*

Herbert L. M. Guedes[a,b], João M. Rezende Neto[a], Mayra A. Fonseca[a], Cristiane M. C. Salles[a], Bartira Rossi-Bergmann[b], and Salvatore Giovanni De-Simone[a,c,*]

[a] Laboratório de Bioquímica de Proteínas e Peptídeos, Departamento de Bioquímica e Biologia Molecular, Fundação Oswaldo Cruz, Rio de Janeiro, RJ, Brazil. Fax: +55 21 25 90 34 95. E-mail: dsimone@ioc.fiocruz.br

[b] Laboratório de Imunofarmacologia, Instituto de Biofísica Carlos Chagas Filho, Universidade Federal do Rio de Janeiro, Rio de Janeiro, RJ, Brazil

[c] Departamento de Biologia Celular e Molecular, Instituto de Biologia, Universidade Federal do Rio de Janeiro, Niterói, RJ, Brazil

* Author for correspondence and reprint requests

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Leishmania (V.) braziliensis is one of the most important ethiologic agents of the two distinct forms of American tegumentary leishmaniasis (cutaneous and mucosal). The drugs of choice used in leishmaniasis therapy are significantly toxic, expensive and are associated with frequent refractory infections. Among the promising new targets for anti-protozoan chemotherapy are the proteases. In this study, serine proteases were partially purified from aqueous, detergent and extracellular extracts of *Leishmania braziliensis* promastigotes by aprotinin-agarose affinity chromatography. By zymography, the enzymes purified from the aqueous extract showed apparent activity bands of 60 kDa and 45 kDa; of 130 kDa, 83 kDa, 74 kDa and 30 kDa from the detergent extract; and of 62 kDa, 59 kDa, 57 kDa, 49 kDa and 35 kDa from the extracellular extract. All purified proteases exhibited esterase activity against N_α-benzoyl-L-arginine ethyl ester hydrochloride and N_α-*p*-tosyl-L-arginine methyl ester hydrochloride (serine protease substrates) and optimal activity at pH 8. 0. Proteases purified from the aqueous and extracellular extracts were effectively inhibited by benzamidine (trypsin inhibitor) and those from the detergent extract were inhibited by *N*-tosyl-L-phenylalanine chloromethyl ketone (chymotrypsin inhibitor) indicating that all these enzymes are serine proteases. These findings indicate that *L. braziliensis* serine proteases display some biochemical similarities with *L. amazonensis* serine proteases, demonstrating a conservation of this enzymatic class in the *Leishmania* genus. This is the first study to report the purification of a serine protease from *Leishmania braziliensis*.

Key words: Leishmania braziliensis, Serine Protease, Aprotinin-Agarose Affinity Chromatography

Introduction

The leishmaniases are parasitic diseases with a wide range of clinical symptoms caused by the protozoa of the genus *Leishmania*, which is transmitted by the bite of an infected phlebotomine female sandfly, and affects millions of people worldwide (Murray *et al.*, 2005). *Leishmania (V.) braziliensis* is one of the most important ethiologic agents of the two distinct American tegumentary leishmaniasis forms (cutaneous and mucocutaneous) (Hepburn, 2003). American cutaneous leishmaniasis is a granulomatous disease, clinically characterized by ulcerated skin lesions at the site of the insect bite that often heal spontaneously (Coutinho *et al.*, 1987). In other instances, in a small percentage of affected individuals, after the healing of the primary lesion, the amastigotes may spread developing to mucocutaneous leishmaniases, which leads to severe destruction of nasal, buccal and throat mucous membranes and surrounding tissues (Hepburn, 2003).

Despite numerous studies, the chemotherapy of the leishmaniases remains unsatisfactory. The drugs of choice are significantly toxic, expensive and frequently associated with refractory infections. Thus, the identification and research of new drug targets is necessary and the development of novel, more effective and less toxic drugs is an urgent priority (Croft and Coombs, 2003).

Protozoan proteases play crucial roles in the host-parasite interaction, and their characterization contributes to understand the protozoan disease mechanisms. Proteases are important for par-

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asite survival; they are involved in digestion of exogenous proteins for nutritive purposes (Rosenthal, 1999), invasion of host cells and tissues (Roggwiller *et al.*, 1996) and modification of host proteins (Caler *et al.*, 1998). The proteases have been considered as a pre-eminent target group in several pathologies and the importance of *Leishmania* proteases has been confirmed by the finding that specific protease inhibitors kill parasites and reduce the evolution of leishmaniatic lesions (Sadij and McKerrow, 2002).

Serine proteases are present in several organisms and participate in many biological functions (Rawlings and Barret, 1994). Protozoan serine proteases play crucial roles in host cell invasion. Subtilisin-like serine proteases from *Plasmodium falciparum* digest red blood cell cytoplasmatic membrane proteins, thereby affording the parasite invasion and infection, and *Trypanosoma cruzi* serine oligopeptidase B mediates mammalian cell invasion.

Previous studies on the genus *Leishmania* demonstrated the presence of serine proteases. Biochemical characterization of the *L. pifanoi* amastigotes antigen, P-8, demonstrated a serine protease with 56 kDa as an immunodominant component (Colmenares *et al.*, 2001). In *L. amazonensis* promastigotes, a 101 kDa serine oligopeptidase (Ribeiro de Andrade *et al.*, 1998), a 68 kDa serine protease purified from an aqueous extract (Silva-Lopez and De-Simone, 2004a), a 110 kDa serine protease from a detergent-soluble extract (Silva-Lopez and De-Simone, 2004b), and a 56 kDa serine protease from cell-free supernatant (Silva-Lopez *et al.*, 2004, 2005) have been characterized. A type-I signal peptidase from the *L. major* gene has also been cloned and expressed, however, the protein activity with a predicted molecular mass of 20.5 kDa was not measured (Rafati *et al.*, 2004, 2006).

In *L. braziliensis*, the identification of serine proteases, using the whole extract in gelatin substrate SDS-PAGE, was not possible (Alves *et al.*, 1993; Cuervo *et al.*, 2006). However, the treatment of alkaline antigens from *L. braziliensis* with a serine protease inhibitor (PMSF, phenylmethylsulfonyl fluoride) increased the antigen stability for diagnostic application (Celeste *et al.*, 1998). Furthermore, treatment with PMSF and TLCK (*N*-tosyl-lysine chloromethyl ketone) killed *L. braziliensis* and TLCK-killed parasites induced protection against *Leishmania* infection (O'Daly and Cabrera, 1986). These results indicated the presence of serine proteases in *L. braziliensis*. In this study, we identified serine proteases of the aqueous, detergent and extracellular extracts from *L. braziliensis* by aprotinin-agarose affinity chromatography.

Materials and Methods

Parasites

Cultures of *Leishmania (Viannia) braziliensis* (MHOM/BR/1975/M029; IOC-L566) promastigotes forms were maintained at 26 °C in brain heart infusion medium (BHI; Difco, Detroit, USA) supplemented with 10% (v/v) heat-inactivated fetal calf serum. For large-scale cultivation (2 L), the cultures were maintained at room temperature (25 °C) in Roller bottles using a Cel-Gro Rotator (Lab-Line Model, Thomas Scientific, New Jersey, USA). Cell growth was estimated by counting the parasites in a Newbauer chamber. Cellular viability was assessed by motility and trypan blue (de Melo *et al.*, 2002).

Preparation of aqueous, detergent and extracellular extracts

The parasites (2×10^{10} cells) at the log phase were harvested by centrifugation ($3{,}000 \times g$ for 15 min at 4 °C) and washed three times ($3{,}000 \times g$ for 15 min at 4 °C) in cold PBS, pH 7. 4. The cells were lysed by seven cycles of freezing and thawing (−80 °C/4 °C) in 10 mM 2-amino-2-(hydroxymethyl)-1,3-propanediol hydrochloride (Tris-HCl) buffer, pH 7.5, containing 5 mM $CaCl_2$. The cell lysate was centrifuged ($100{,}000 \times g$ for 30 min at 4 °C) and the supernatant was stored and called the aqueous extract. The pellet was resuspended in 10 mM Tris-HCl buffer, pH 7.5, containing 1% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS) and 5 mM $CaCl_2$, and the tube was shaken gently and maintained on ice for 30 min ($20{,}000 \times g$ for 30 min at 4 °C). The supernatant was then stored and denominated the detergent extract. 2 L of cell-free culture supernatant were precipitated overnight with 40% $(NH_4)_2SO_4$ and centrifuged ($10{,}000 \times g$ for 30 min at 25 °C). The pellet was resuspended in 100 mL of 10 mM Tris-HCl buffer, pH 7.5, containing 5 mM $CaCl_2$, dialyzed against the same buffer several times and centrifuged ($20{,}000 \times g$ for 30 min at 4 °C). The supernatant was stored and called the extracellular extract.

Purification of serine protease from aqueous, detergent and extracellular extracts

The clear supernatants of aqueous, detergent and extracellular extracts were loaded onto a pre-equilibrated (10 mM Tris-HCl buffer, pH 7.5, containing 5 mM $CaCl_2$) aprotinin-agarose affinity column (2.5 mL; SIGMA). After washing with 20 bed volumes, the material was eluted with 10 mM Tris-HCl buffer, pH 7.5, containing 1.5 M NaCl. For the detergent extract purification, 1% CHAPS was added to all buffers. Fractions of 1 mL were collected on ice and the effluents absorption at 280 nm was monitored to detect the protein peak. All protein peaks of each fraction were then pooled. The aprotinin-agarose fraction of the aqueous extract was called the aqueous fraction (AF), the detergent extract was denominated the detergent fraction (DF) and the extracellular extract was called the extracellular fraction (EF).

SDS-PAGE and gelatin substrate gel SDS-PAGE (zymography) of aprotinin-agarose fractions

The aprotinin-agarose fractions were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using the Laemmli method (1970) in non-reducing conditions. Gels were stained with Comassie Blue R-250. Myosin (205 kDa), β-galactosidase (116 kDa), phosphorylase (97 kDa), bovine serum albumin (66 kDa), ovalbumin (45 kDa), and carbonic anhydrase (29 kDa) were used as molecular weight/mass standards. Zymography was performed at 100 V for 2 h on ice in reducing and non-reducing conditions. After electrophoresis, gels were incubated in 2.5% Triton X-100 solution for 1 h and then incubated for 20 h in buffer (100 mM Tris-HCl buffer, pH 7.5; 100 mM citrate buffer, pH 5.5; or 100 mM acetate buffer, pH 3.5) at room temperature. Gels were stained with 0.1% Amido Black in 30% v/v methanol, 10% v/v acetic acid and destained with 30% v/v methanol, 10% v/v acetic acid.

Enzyme assays using chromogenic substrates

Chromogenic substrates, such as N_α-benzoyl-L-arginine 4-nitroanilide hydrochloride (L-BAPNA) (0.25 mM), N_α-benzoyl-L-arginine ethyl ester hydrochloride (L-BAEE) (0.25 mM) and N_α-*p*-tosyl-L-arginine methyl ester hydrochloride (L-TAME) (0.25 mM) were all purchased from Sigma. The substrates were digested in 10 mM Tris-HCl, pH 8.0, at 28 °C, for 60 min. After addition of the enzyme, digestion of the substrate was measured by the absorbance increase at 247 nm for L-TAME, 253 nm for L-BAEE and 410 nm for L-BAPNA (Sousa *et al.*, 2002). Results were expressed in specific activity. Specific activity is the amount of product formed by an enzyme in time under given conditions per milligram of enzyme (μmol of formed product per min per mg of protein). The reaction blank was set by the addition of the temperature-denatured protease fraction (100 °C, 10 min) under the same assay conditions.

Enzyme assays for protein substrates

Aprotinin-agarose fractions were incubated with 100 μL of protein substrates, 0.1% (w/v) [hemoglobin, bovine serum albumin (BSA) and casein; all from Sigma Chemical], in 100 mM Tris-HCl, pH 7.5, for 30 min at 28 °C. The reactions were discontinued by adding 150 μL of 20% (v/v) trichloroacetic acid (TCA) and the mixtures centrifuged at $12{,}000 \times g$ for 10 min. Supernatant absorbance was monitored at 280 nm (Genesis 10uv spectrophotometer). Activity was expressed in OD per min per mg of protein. The reaction blank was set by the addition of protease fractions that had their reactions stopped with TCA.

Optimal pH value and temperature of aprotinin-agarose fractions

To evaluate the optimum pH value, the aprotinin-agarose fractions were incubated with 0.25 mM L-BAEE for 60 min at room temperature in 100 mM phosphate buffer (pH 6.0 and 6.5) and in 100 mM Tris-HCl (pH 7.0 and 9.0). Absorbance was monitored at 253 nm and each assay performed in triplicate. Fractions were incubated in the best buffer for each, at 25 °C, 28 °C, 33 °C and 37 °C to evaluate the optimum temperature.

Effect of protease inhibitors on aprotinin-agarose fractions

Inhibitors of the known protease classes were used to determine the protease types in the zymography assay and enzyme assay, using proteins and chromogenic susbtrates. In both procedures, 10 μM aprotinin, 1 mM 6-amino-*n*-caproic acid (ACA), 100 μM *N*-tosyl-lysine chloromethyl ketone (TLCK), 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM benzamidine (Bz), 100 μM *N*-tosyl-

L-phenylalanine chloromethyl ketone (TPCK) were used for serine protease identification; 10 μM pepstatin were used for aspartic protease identification; 1 mM 1,10-phenanthroline (O-fen) and 1 mM ethylenediaminetetraacetic acid (EDTA) were used for metallo-protease identification; and 10 μM E-64 was used for cysteine protease identification. All inhibitors were purchased from Sigma Chemical, except for 6-amino-*n*-caproic acid (Wako Pure Chemical, Tokyo, Japan). Zymography was performed as previously described with or without the addition of the protease inhibitor in the incubation step, in order to analyze proteolysis inhibition. The activity was measured, as previously described using inhibitors in the assay, and controls were carried out using the same enzyme solutions that were free of inhibitors.

Determination of protein concentration

Protein content was determined by the method of Lowry *et al.* (1951) BSA as a standard for calibration.

Results

Aprotinin-agarose affinity chromatography

The purification profile of serine proteases from aqueous, detergent and extracellular extracts using aprotinin-agarose affinity column chromatography is shown in Fig. 1. We observed a single eluted fraction. However, SDS-PAGE analysis demonstrated that the affinity chromatography purification step did not produce homogeneous material (Fig. 2A). Table I demonstrates that serine proteases are present in the three extracts, but that they are minor proteins.

Gelatin substrate gel electrophoresis

Purified fractions were studied using gelatin gel electrophoresis to determine their proteolytic activity (Fig. 2B). At pH 7.5 in non-reducing conditions, aqueous fraction serine proteases demonstrate activity bands of 60 kDa and 45 kDa, detergent fraction serine proteases bands of 130 kDa, 83 kDa, 74 kDa and 30 kDa, and extracellular fraction serine proteases bands of 62 kDa, 59 kDa, 57 kDa, 49 kDa and 35 kDa. At pH 5.5, at pH 3.5 and in reducing conditions, proteolytic activity was not observed (results not shown).

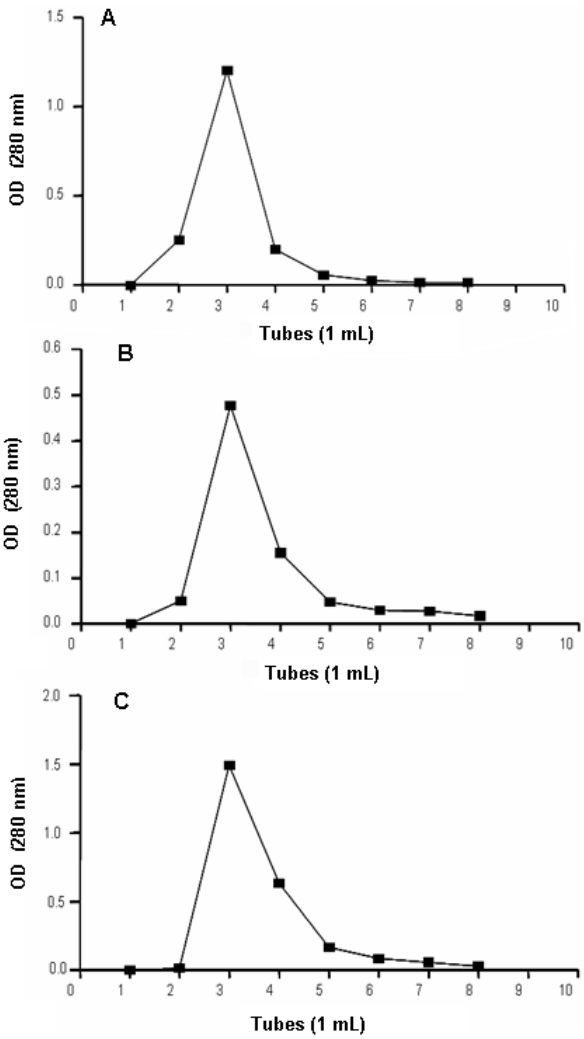

Fig. 1. Aprotinin-agarose fraction affinity chromatography profile. The supernatants [(A) aqueous extract, (B) detergent extract, (C) extracellular extract] were loaded onto an aprotinin-agarose affinity column (2.5 mL), pre-equilibrated with 10 mM Tris-HCl buffer, pH 7.5, containing 5 mM $CaCl_2$, and with 1% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS) for the detergent extract. The washing step was performed with the same buffer. The fractions (1 mL) were eluted with 10 mM Tris-HCl buffer, pH 7.5, containing 1.5 M NaCl, collected and stored on ice. The absorbance at 280 nm of effluents (aqueous, detergent and extracellular extracts) was monitored to detect the protein peak.

Substrate specificity

Aprotinin-agarose fractions showed proteolytic activity at pH 7.5 using hemoglobin, BSA and ovalbumin as natural protein substrates; results

Table I. Proteinases from *Leishmania (V.) braziliensis* purification.

Fraction	Total protein [mg]			Recovery			Specific activity[a] [μmol min^{-1} mg protein^{-1}]			Purification			Yield (%)		
	AF	DF	EF	AF	DF	EF	AF	DF	EF	AF	DF	EF	AF	DF	EF
Supernatant	25	14.5	2.260	–	–	–	0.012	0.024	0.018	–	–	–			
Affinity column	0.450	0.3	1.44	1.8	2	0.064	0.261	0.1	0.227	21.75	7.08	12.6	42	15	0.85

[a] Specific activity using BAEE as substrate.

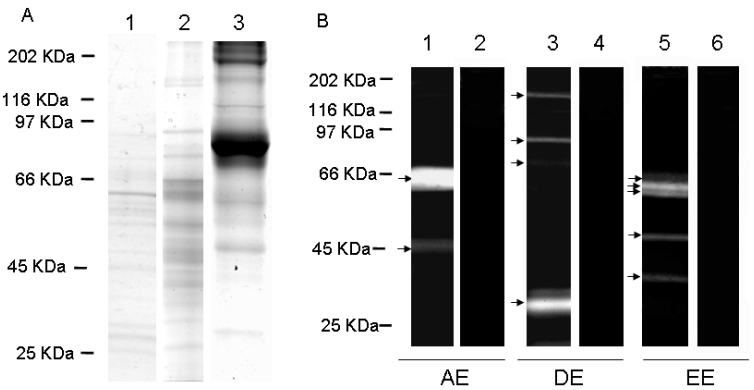

Fig 2. SDS-PAGE and gelatin substrate SDS-PAGE analysis of aprotinin-agarose fractions. (A) The samples collected by affinity chromatography were analyzed by SDS-PAGE 10% acrylamide gels under non-reducing conditions. Lane 1, aqueous fraction; lane 2, detergent fraction; lane 3, extracellular fraction. (B) Analysis of gelatin substrate SDS-PAGE 10% acrylamide gel aprotinin-agarose fractions was performed as described in Materials and Methods. Lanes 1 and 2, aqueous fraction; lanes 3 and 4, detergent fraction; lanes 5 and 6, extracellular fraction. Lanes 2 and 6 were incubated with benzamidine and lane 4 with TPCK in order to verify their effect on enzyme activity. The arrows indicate a higher proteolytic activity. The molecular mass protein standard values (kDa) are on the left side of the gels.

Table II. Protease activity on natural substrates. The AF (5 μg), DF (5 μg) and EF (5 μg) were incubated with 100 mM Tris-HCl buffer, pH 7.5, at 28 °C, for 30 min with hemoglobin, casein and albumin. The reaction was assayed as described in Materials and Methods. Results represent the average of 3 separate experiments carried out in duplicate.

Substrate	Specific activity [OD min^{-1} mg protein^{-1}]		
	AF	DF	EF
Hemoglobin	0.933 ± 0.077	0.020 ± 0.005	0.021 ± 0.002
Casein	1.26 ± 0.025	0	0.024 ± 0.005
Albumin	1.046 ± 0.064	0.53 ± 0.008	0.131 ± 0.010

Table III. Substrate specificity of serine proteases on synthetic substrates. The AF (5 μg), DF (5 μg) and EF (5 μg) were incubated with 100 mM Tris-HCl buffer, pH 8.0, at 28 °C, for 60 min with L-TAME, L-BAPNA and L-BAEE. The reaction was assayed as described in Materials and Methods. Results represent the average of 3 separate experiments carried out in duplicate.

Substrate	Specific activity [μmol min^{-1} mg protein^{-1}]		
	AF	DF	EF
L-TAME	0.101 ± 0.006	0.113 ± 0.008	0.105 ± 0.004
L-BAPNA	0.0005 ± 0.0001	0.0022 ± 0.0003	0.004 ± 0.0007
L-BAEE	0.261 ± 0.003	0.17 ± 0.002	0.227 ± 0.001

are summarized in Table II. Our results indicated that the aqueous extract had more activity against these substrates than the other fractions; the detergent fraction preferred albumin and the extracellular fraction had low activity against these substrates. Considering all the substrates used (substrates containing the arginyl residue in the P1 site of the amide bond for L-BAPNA and in the

Table IV. Effects of different types of protease inhibitors on aprotinin fractions at pH 8.0.

Fraction	Aprotinin	BZ	TLCK	TPCK	PMSF	ACA	E-64	Pepstatin	EDTA	O-fen
AE	50	9	51	57	80	100	> 100	100	> 100	100
DE	10	100	90	32	92	100	100	100	100	100
EE	43	0	95	78	49	100	100	100	> 100	100

The AF (5 μg), DF (5 μg) and EF (5 μg) were incubated with 100 mM Tris-HCl buffer, pH 8.0, at 28 °C, for 30 min with casein, albumin and L-BAEE, respectively, as described in Materials and Methods in the presence and absence of protease inhibitors. The remaining activity of serine protease was estimated as the percentage of the activity on substrate without inhibitors. Results represent the average of 3 separate experiments carried out in duplicate. Standard error was less than of 5%.

ester bond for L-TAME and L-BAEE), the three fractions preferentially cleaved the ester bond of L-BAEE (Table III).

Effects of temperature and pH value on enzymatic activity

The pH dependence was determined using L-BAEE as substrate (result not shown). The maximum activities of the three aprotinin-agarose fractions were observed around pH 8. 0. Furthermore, the activity showed significant reduction at pH values less than of pH 7.0 and higher than pH 9. 0. The effect of temperature (25 °C, 28 °C, 33 °C and 37 °C) on the enzymatic activity was analyzed using pH 8.0 and no significant difference in activity was determined (result not shown).

Proteinase class determination

Through zymography analysis (Fig. 2B), proteases of the aqueous and extracellular fractions were inhibited by benzamidine (trypsin inhibitor) and detergent fraction was inhibited by TPCK (chymotrypsin inhibitor), although the activity was reduced by benzamidine (data not shown). The enzymatic activity in solution demonstrates that the most important inhibitor was benzamidine for aqueous and extracellular aprotinin-agarose fractions and TPCK for the detergent aprotinin-agarose fraction (Table IV). E-64, EDTA, pepstatin and 1,10-phenanthroline did not affect the protease activity.

Discussion

The knowledge of proteases in protozoa is important for a better host-parasite interaction characterization and for the development of improved drugs (Vermelho *et al.*, 2007). These enzymes have been found in several *Leishmania (Viannia)* species (Coombs and Mottram, 1997); however, protease characterization in these species is scarce, demanding further investigation (Leon *et al.*, 1994). Cysteine and metallo-proteases are important virulence factors for *Leishmania* and play major roles in infection, survival, pathogenicity and inhibition of the host-immune response (Mottram *et al.*, 2004; Yao *et al.*, 2003). Zymography of the total lysate and extracellular products protease profiles of *L. braziliensis* demonstrated the presence of cysteine and metallo-proteases (Alves *et al.*, 1993; Cuervo *et al.*, 2006), but serine and aspartic proteases activity was not detected.

In this study, aqueous, detergent and extracellular *L. braziliensis* culture extracts were used for partial serine protease purification, as described for *L. amazonensis.* Aprotinin, a reversible serine protease inhibitor that forms a complex with the serine residue from the active site, has been used as ligand in affinity chromatography for serine protease purification. It was possible to identify, by aprotinin-agarose chromatography, serine proteases in aqueous, detergent and extracellular extracts from *L. braziliensis* cultures, allowing to determine the concentration of these proteins. This method improved the *Leishmania* serine protease purification yield in comparison to other parasite serine peptidases purification protocols (Ribeiro de Andrade *et al.*, 1998). It is well known that protease activity is influenced specially by the cultivation temperature, pH value and culture medium (de Melo *et al.*, 2002). *L. braziliensis* cultivation was established as the same as in the *L. amazonensis* protocol to compare serine proteases.

Protease zymography detection at pH 7.5, under non-reducing conditions, demonstrated two proteases in the aqueous fraction, four proteases in the detergent fraction and five proteases in the extracellular fraction. In the aprotinin-agarose fraction of *L. amazonensis*, a single protease band was found in the aqueous and detergent fractions.

These results suggested a greater complexity and variability of serine proteases in *L. braziliensis*; however these enzymes had great similarity. A protease with approximately 110 kDa was observed in the detergent fraction of *L. amazonensis* in non-reducing condition that could be the same protease observed in the *L. braziliensis* fraction with a molecular weight of 130 kDa. A 56 kDa band, observed in *L. amazonensis,* may be the same 57 kDa protease found in *L. braziliensis.* Similarly to the serine proteases from *L. amazonensis*, the *L. braziliensis* aqueous fraction hydrolyzed all assayed natural substrates; however, detergent and extracellular fractions seemed to be more selective.

Possibly, serine proteases present in the aqueous and detergent fractions may be involved as digestive proteases and these hemoglobin-degrading enzymes may serve as heme and iron sources. Gelatin hydrolysis by extracellular serine proteases indicated a high participation of serine proteases in migration through the extracellular matrix, as observed for GP63 (McGwire *et al.*, 2003).

Serine protease from both *Leishmania* species did not hydrolyze the L-BAPNA substrate (they did not demonstrate any amidase activity) and preferentially cleaved the ester bond. Serine protease fractions from *L. amazonensis* demonstrated a greater activity against L-BAME and *L. braziliensis* fractions against L-BAEE, compared to L-TAME. This substrate specificity is present in trypsin, oligopeptidase B, the subtilisin family (furin, proprotein convertase 1–5, PrcA peptidase and kexin) and the 20 S proteasome peptidase complex (Rawlings *et al.*, 2006).

L. amazonensis serine proteases showed maximum activity at neutral to basic pH values; this same characteristic is observed in *L. braziliensis* proteases that prefer basic pH values. *L. amazonensis* serine proteases were found in many cell compartments, mostly localized in vesicular structures near the flagellar pocket region (morphologically similar to endocytic/exocytic pathways) and megasomes. This fact demonstrates the activity depending on the pH values (Silva-Lopez *et al.*, 2004; Morgado-Diaz *et al.*, 2005)

Aprotinin is the major inhibitor of *L. amazonensis* serine proteases, and was used as a ligand in affinity chromatography for *L. braziliensis* serine proteases purification. Aprotinin inhibited several serine protease families, above all, trypsin, thrombin, chymotrypsin, plasmin and subtilisin. To characterize the present proteases in three fractions, an inhibitor panel against different protease classes was used. PMSF, a non-selective serine protease inhibitor, inhibited all fractions. Benzamidine, a trypsin-like inhibitor, inhibited aqueous and extracellular fraction proteases. TPCK, which has a greater specificity for chymotrypsin-like proteases, inhibited detergent fraction proteases. Inhibition with ACA, a plasmin inhibitor, was not detected, indicating that proteases in our fractions were not from this protease family. The protease inhibitor analysis indicated the presence of trypsin and chymotrypsin-like protease activities.

In the *L. major* genome, peptidases represent ~2% of protein-coding genes in the Tritryps; however no trypsin/chymotrypsin family serine peptidases were found in Tritryps (Ivens *et al.*, 2005). Possibly, in *L. amazonensis* and *L. braziliensis,* the trypsin/chymotrypsin-like activities observed involved other serine proteases. In the genome, a subtilisin-like prolyl oligopeptidase (including oligopeptidase B), a type-I signal peptidase, a 26S regulatory proteasome subunit, a nucleoporin homologue, and several orthologs of the rhomboid-like intramembrane serine peptidase family were present.

Curiously, we observed that detergent fractions were inhibited by TPCK (chymotrypsin inhibitor) and cleaved a substrate with arginine in P1 (trypsin substrate), a subtilisin family particularity. Subtilisin is a membrane-associated enzyme, found in eukaryotes with an approximate molecular weight of 110 to 140 kDa (Withers-Martinez *et al.*, 2004; Kim, 2004). Possibly, a 130 kDa protease in the detergent fraction could be a subtilisin-like serine protease.

In the extracellular fraction, we observed a major band with 88 kDa, with no activity in zymography. The same band was observed in the purified culture supernatant of *L. amazonensis* (Silva-Lopez *et al.*, 2004). Absence of activity in zymography indicated that these serine proteases did not show activity against gelatin or that they are serine oligopeptidases. Interestingly, these bands displayed similarity with the molecular mass of the *L. major* oligopeptidase B, a serine oligopeptidase, described in the genome project (Ivens *et al.*, 2005).

These serine protease activities were the first serine proteases described in *Leishmania (V.) braziliensis.* Further investigations on determination of serine protease class, biological function and the

evaluation of its potential as a drug target is our main group object.

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