A Serine Protease from a Detergent-soluble Extract of *Leishmania (Leishmania) amazonensis*

Raquel Elisa da Silva Lopez[a,*] and Salvatore Giovanni De Simone[a,b]

[a] Laboratório de Bioquímica de Proteínas e Peptídeos, Departamento de Bioquímica e Biologia Molecular, Instituto Oswaldo Cruz, FIOCRUZ, Av. Brasil 4365, 21045-900, Rio de Janeiro, RJ, Brasil. Fax: +55 21 590 3495. E-mail: rlopez@ioc.fiocruz.br

[b] Departamento de Biologia Celular e Molecular, Instituto de Biologia, Universidade Federal Fluminense, Niterói, RJ, Brasil

* Author for correspondence and reprint requests

Z. Naturforsch. **59 c**, 590–598 (2004); January 22/March 18, 2004

Proteases mediate important crucial functions in parasitic diseases, and their characterization contributes to the understanding of host-parasite interaction. A serine protease was purified about 43-fold with a total recovery of 60% from a detergent-soluble extract of promastigotes of *Leishmania amazonensis*. The purification procedures included aprotinin-agarose affinity chromatography and gel filtration high performance liquid chromatography. The molecular mass of active enzyme was 110 kDa by native gel filtration HPLC and by SDS-PAGE gelatin under non-reducing conditions. Under conditions of reduction using SDS-PAGE gelatin analyses the activity of enzyme was observed in two proteins of 60 and 45 kDa, suggesting that the enzyme may be considered as a dimer. The *Leishmania* protease was not glycosylated, and its isoelectric point (pI) was around 4.8. The maximal protease activity was at pH 7.0 and 28 °C, using α-*N*-ϱ-tosyl-$_L$-arginyl-methyl ester (L-TAME) as substrate. Assays of thermal stability indicated that this enzyme was totally denatured after pre-treatment at 42 °C for 12 min and preserved only 20% of its activity after pre-treatment at 37 °C for 24 h, in the absence of substrate. Hemoglobin, bovine serum albumin (BSA), ovalbumin and gelatin were hydrolyzed by *Leishmania* protease. Inhibition studies indicated that the enzyme belonged to a serine protease class because of a significant impediment by serine protease inhibitors such as benzamidine, aprotinin, and antipain. The activity of the present serine protease is negatively modulated by calcium and zinc and positively modulated by manganese ions. This is the first study that reports the purification of a protease from a detergent-soluble extract of *Leishmania* species.

Key words: Leishmania (Leishmania) amazonensis, Serine Protease, Characterization

Introduction

Protozoan parasites of the genus *Leishmania* are associated with a broad spectrum of diseases ranging from self-healing cutaneous lesions to lethal visceral consequences (Alexander and Russell, 1992). Leishmaniasis are endemic in the tropics and subtropics, and the increment of *Leishmania* transmission and dissemination has been mainly attributed to blood transfusion and human immunodeficiency virus (HIV) co-infection (Desjeux, 1999). *Leishmania* are dimorphic obligate intracellular parasites: Flagellated promastigotes replicate in the gut of the sandfly vector, and their transmission to humans or other vertebrate hosts occurs when the vector feeds on blood. These promastigotes are internalized by mononuclear phagocytic system cells and undergo transformation into the nonmotile amastigote form that maintain the infection, leading to the destruction of host tissues and invasion of new cells (Colmenares *et al.*, 2001). Consequently the expression of many molecules is responsible for mechanisms triggered by parasites in order to guarantee survival within the host. Among these molecules proteases have received paramount at-

Abbreviations: ACN, acetonitrile; BAME, *N*-α-benzoyl-$_L$-arginyl-methyl ester; BHI, brain heart infusion; Bz, benzoyl; CHAPS, 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; E-64, $_L$-*trans*-epoxysuccinyleucylamido-(4-guanidino) butane; HPLC, high performance liquid chromatography; PBS, phosphate buffer saline; pI, isoelectric point; PMSF, phenylmethylsulfonyl fluoride; ϱ-NA, paranitroanilide; SBTI, soybean trypsin inhibitor; SDS-PAGE, polyacrylamide gel electrophoresis containing sodium dodecyl sulfate; L-TAME, α-*N*-ϱ-tosyl-$_L$-arginyl-methyl ester; TLCK, *N*-tosyl-lysine chloromethyl ketone; TFA, trifluoroacetic acid; TPCK, *N*-tosyl-$_L$-phenylalanine chloromethyl ketone.

0939–5075/2004/0700–0590 $ 06.00

tention due to their crucial roles in the parasite life cycle and disease pathogenesis (Rosenthal, 1999; Sadij and McKerrow, 2002). They are involved in the host-parasite interaction, including 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) which are very important for parasite survival. *Leishmania* proteases have been studied for almost three decades, and some functions have been proposed for these enzymes (Coombs, 1982). The importance of *Leishmania* proteases has been confirmed by the findings that specific protease inhibitors killed *Leishmania* parasites and reduced the evolution of leishmaniatic lesions (Sadij and McKerrow, 2002).

Serine proteases (EC 3.4.21) are among the most extensively studied enzymes and participate in numerous physiological phenomena such as blood clotting and complement activation (Rawling and Barret, 1994). Serine proteases of pathogenic parasites play crucial roles in host-parasite interaction, and their characterization is crucial to elucidate their functions in parasite physiology and parasite-host interaction. The majority of parasite serine proteases has been isolated from water-soluble extracts and is involved in mechanisms of host invasion (Roggwiller *et al.*, 1996; Caler *et al.*, 1998). The most notorious protozoan serine proteases involved in host cell invasion are associated with *Plasmodium falciparum* and *Trypanossoma cruzi.* Malarial proteases digest cytoplasmatic membrane proteins of red blood cells (glycophorin and band 3 proteins) thereby affording invasion and infection by the parasite, whereas specific proteases inhibitors prevent such (Roggwiller *et al.*, 1996). Mammalian cell invasion by *T. cruzi* is mediated by a serine oligopeptidase B which is responsible for the generation of a peptide hormone-like factor that triggers Ca^{2+} signaling, facilitating parasite infection (Caler *et al.*, 1998). Deletion of the gene encoding oligopeptidase B results in a marked deficiency for host cell invasion, as well as reducing the establishment of infections in mice (Caler *et al.*, 1998).

Proteases of membrane or membrane-associated proteases require detergents for their extraction, isolation and purification, because of the low solubility in aqueous systems. *Schistosoma mansoni* has a membrane-associated serine protease of 28 kDa. This enzyme is anchored to the tegumental membrane of schistosomula by lipids and has great activity against human connective tissue proteins, enabling parasite penetration of the host tissues (Ghendler *et al.*, 1996). Furthermore, a chymotrypsin-like protease of *S. mansoni* cercariae is essential for host skin penetration and can be found both in cercariae secretions and membranes (Salter *et al.*, 2000). Herein we describe the purification procedure used and give some characteristics of serine protease from a detergent-soluble extract of *Leishmania amazonensis* promastigotes, an enzyme which definitely differs from the other known serine peptidases of this parasite (Ribeiro de Andrade *et al.*, 1998).

Materials and Methods

Parasites

Promastigote forms of *L. amazonensis* (IOC 575; IFLA/BR/67/PH8) were maintained at 28 °C in brain heart infusion (BHI) medium (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 Neubauer chamber.

Purification of Leishmania serine protease

The parasites (4.8×10^{10}) were harvested by centrifugation ($3{,}000 \times g$ for 15 min at 4 °C) at the log phase (4^{th} day of cultivation) and washed three times in cold phosphate buffer saline (PBS) (pH 7.2) by centrifugation ($3{,}000 \times g$ for 15 min at 4 °C). Parasite lysates were prepared in PBS by seven cycles of freezing and thawing (– 80 °C/37 °C). Finally, the cell lysate was centrifuged ($100{,}000 \times g$ for 60 min at 4 °C), the supernatant was discarded and the pellet was ressuspended using 10 ml of extraction buffer containing 0.5% CHAPS, 0.01 M Tris-HCl, pH 7.5, and centrifuged ($100{,}000 \times g$ for 60 min at 4 °C). The clear supernatant was dialyzed overnight at 4 °C against 0.01 M Tris-HCl, pH 7.5, containing 0.1% CHAPS. Insoluble material was removed by centrifugation ($100{,}000 \times g$, 60 min) and the clear supernatant loaded on to an aprotinin-agarose affinity column (2.5 ml gel) previously equilibrated with 0.01 M Tris-HCl, pH 7.5, 0.1% CHAPS containing 5 mM $CaCl_2$. After exhaustive washing (20 bed volumes) the active material was eluted with 0.01 M Tris-

HCl, pH 7.5, containing 1.5 M NaCl. Fractions of 1 ml were collected on ice, the absorption at 280 nm of effluents was monitored to detect the protein peak and the enzymatic activity of the fractions was assayed using α-*N*-ϱ-tosyl-$_L$-arginyl-methyl ester (L-TAME) as substrate. The enzyme active fractions were pooled and concentrated in Microcon (Amicon) concentrators (3-kDa cut-off membrane) at 4 °C. The material from affinity chromatography was applied to a high performance liquid chromatography (HPLC) gel filtration column [Shinpack Diol-150, 50 cm × 7.9 mm (internal diameter), Shimadzu, Kyoto, Japan] which was equilibrated with 10 mM phosphate buffer, pH 7.2. The proteins were fractionated on an automatic HPLC system (Shimadzu, Kyoto, Japan; 6A model) at a flow rate of 1 ml/min, during 28 min at 25 °C. The peaks were collected and analyzed by SDS-PAGE and the enzymatic activity was measured using L-TAME as substrate (Giovanni De Simone *et al.*, 1997). For molecular mass characterization the column was calibrated in the same buffer containing 0.2 M $(NH_4)_2SO_4$ and with the following markers: Myosin (200 kDa), β-galactosidase (105 kDa), bovine serum albumin (66 kDa), ovalbumin (45 kDa) and carbonic anhydrase (29 kDa), all from Sigma Chemical Co. (St. Louis, MO, USA).

Polyacrylamide gel electrophoresis and substrate gel electrophoresis

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to the method of Laemmli (1970). The gels were Coomassie Blue R-250 or silver stained (Sigma kit). Myosin (200 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 standards.

Gelatin substrate gel electrophoresis was carried out under reducing and non-reducing conditions as reported by Alves *et al.* (1993). After electrophoresis the gel was washed with 2.5% Triton X-100 for 1 h to remove SDS and incubated overnight at room temperature in 0.1 M Tris-HCl, 0.1% CHAPS, pH 7.0 (Tris-buffer) to allow proteolysis. Next day, the gel was stained with 0.1% amide black and destained using methanol/acetic acid/distilled water (3:1:6).

Determination of optimal pH, temperature and heat stability

The assays for pH dependency were carried out incubating the enzyme (2 μg) for 30 min at room temperature with 0.25 mM L-TAME in solutions with different pH values. The buffers used were as follows: 100 mM sodium citrate (pH 5.0/6.0) and Tris-HCl (pH 7.0/9.0), all buffers containing 0.1% CHAPS. To determine the effect of the temperature in enzyme activity, the reaction mixture was incubated in 100 mM Tris-HCl, 0.1% CHAPS, pH 7.0 (Tris-buffer) for 30 min at different temperatures ranging from 25 °C to 50 °C. The absorbance was monitored at 247 nm and each assay was carried out in triplicate. The specific activity was expressed in μmol of product per min per mg of protein. For thermal stability assays, the enzyme was previously incubated in Tris-buffer at 42 °C for up to 20 min and at 37 °C for up to 1 h. The reaction was triggered adding L-TAME (0.25 mM) at 28 °C. The residual activity was calculated taking the protease activity (at 28 °C) without previous incubation as 100%.

Deglycosylation assay

The presence of carbohydrate was investigated using an enzymatic deglycosylation kit (Bio-Rad, Richmond, CA, USA) according to the manufacturer's instructions. In this method, glycosidases are employed to cleave all *N*- and most *O*-linked oligosaccharides from glycoproteins or glycopeptides. Fetuin was used as an internal standard and the reaction products (before and after deglycosylation) were analyzed by SDS-PAGE as previously described.

Determination of isoelectric point

Isoelectric focusing (IEF) was conducted on a Phast System (Bio-Rad) using PhastGel IEF 3-9 according to the manufacturer's recommendation. The isoelectric point (pI) was determined comparing the migration of the isolated protein with the markers of the calibration kit (pI 3.5–9.3, Bio-Rad).

Enzyme assays and determination of kinetic parameters

Chromogenic substrates, such as *N*-benzoyl-L-arginyl-ϱ-nitroanilide, *N*-benzoyl-L-tyrosyl-ϱ-nitroanilide, *N*-benzoyl-valinyl-glycinyl-arginyl-ϱ-nitro-

anilide, *N*-succinyl-alanyl-alanyl-prolyl-phenylalanyl-ϱ-nitroanilide, *N*-benzoyl-prolyl-phenylalanyl-arginyl-ϱ-nitroanilide, α-*N*-benzoyl-arginyl-methyl-ester (BAME) and L-TAME, all purchased from Sigma Chemical Co. (0.25 mM), were digested in Tris-buffer at 28 °C. After addition of the enzyme (0.03 nM), digestion of substrate was followed by measuring the absorbance increase at 247 nm for L-TAME, 253 nm for BAME and 410 nm for all other substrates containing ϱ-nitroanilide (Sousa *et al.*, 2002).

The steady-state parameters K_M and *Kcat* were determined from initial velocity measurements at substrate concentrations between 1.25 μM and 2.5 mM. Plots of velocity *vs* substrate concentration were used to determine K_M values through non-linear regression (Prism, version 2.01, GraphPad Software, San Diego, CA, USA). The molecular mass of enzyme was assumed to be 110 kDa, with one catalytic site per enzyme molecule. *Kcat* was expressed as the number of formed product per min per enzyme molecule (Sousa *et al.*, 2002).

Effect of protease inhibitors

Inhibitors were incubated with the enzyme (0.03 nM) for 15 min at room temperature. The reaction commenced upon addition of the substrate (0.25 mM L-TAME) in Tris-buffer at 28 °C for 30 min, and the activity was measured as previously described. Appropriate controls were carried out in parallel using the same enzyme solutions free of inhibitors. Inhibition was expressed as the percentage of the appropriate control activity. All inhibitors were purchased from Sigma Chemical Co., except the 6-amino caproic acid (Dojinco, Japan). Canafistuline is a Kunitz type inhibitor purified from *Peltophorum dubium* seeds and was kindly provided by Dr. Ligia Macedo from USP, São Paulo.

Effect of ions on protease activity

The protease (0.03 nM) was incubated for 15 min at room temperature with 10 mM of ions of calcium, zinc, manganese and magnesium before the substrate (L-TAME, 0.25mM) was added. The reactions were performed as previously described. The percentage of inhibition was calculated considering the protease activity in the absence of added ions as an initial activity (100%). Each assay was carried out in triplicates.

Enzyme assays toward protein substrates

Protein substrates (0.1% w/v) of high grade (hemoglobin, BSA and ovalbumin, all from Sigma Chemical Co.) were dissolved in Tris-buffer (400 μl) and incubated with gentle agitation for 30 min at room temperature with 100 μl (2 μg) of purified enzyme. The reactions were stopped by addition of 500 μl of 10% (v/v) trichloroacetic acid. The tubes were centrifuged at 12,000 × *g* for 10 min and the absorbance of the supernatants was measured at 280 nm. The specific activity was defined as $A_{280} \cdot \text{min}^{-1} \cdot \text{mg of protein}^{-1}$. Protein content was determined by the Lowry's method (Lawry *et al.*, 1951) using BSA as a standard for calibration.

Results

Protease purification

The serine protease purification from *L. amazonensis* promastigotes using the procedure herein described is summarized in Table I. The overall purification of 42.85-fold yielded 60% detergent-soluble protease obtained from 4.8×10^{10} parasites. Fig. 1 shows the purification profile of the enzyme in the aprotinin-affinity column and the SDS-PAGE analysis (Fig. 1A and Fig. 1B). Under reducing conditions, gelatin-SDS-PAGE analyses demonstrated proteolytic activity in 60 and 45 kDa proteins (Fig. 1A), whereas under non-reducing conditions only one 110 kDa band of protein was observed (Fig. 1B). The purification of the pooled material from affinity chromatography, through gel filtration HPLC, showed that the highest en-

Table I. Purification of serine protease from *L. amazonensis*.

	Total protein[a] [mg]	Enzyme activity [μmol·min^{-1}]	Specific activity [μmol·min^{-1}·mg protein^{-1}]	Purification (-fold)	Yield (%)
Supernatant	10.33	2.00×10^{-3}	2.00×10^{-4}	100	–
Affinity column	0.57	1.40×10^{-3}	2.46×10^{-3}	12.30	70
HPLC	0.14	1.20×10^{-3}	8.57×10^{-3}	42.85	60

[a] From 4.8×10^{10} cells.

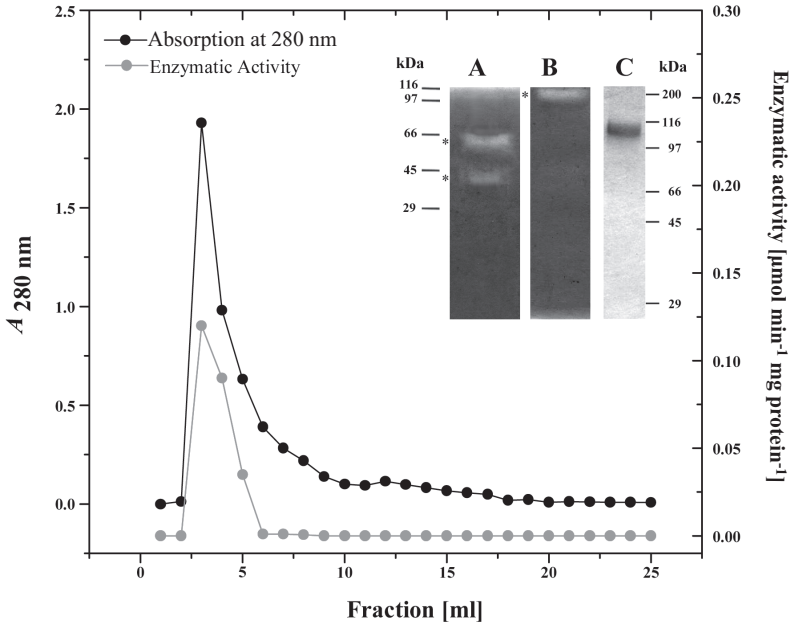

Fig. 1. Affinity chromatography purification and polyacrylamide gel electrophoresis of serine protease from *L. amazonensis*. The supernatant (10 mg) obtained by freeze-thawing was applied to an aprotinin-agarose column (2.5 ml) equilibrated with 0.01 M Tris-HCl buffer, pH 7.5, containing 5 mM $CaCl_2$. The column was eluted with the same buffer without $CaCl_2$, but containing 1.5 M NaCl. The peak fractions were pooled and the sample analyzed by gelatin-SDS-PAGE (A) under reducing conditions and gelatin-SDS-PAGE under non-reducing conditions (B). This material was fractionated by native HPLC gel filtration and the only one active fraction was purified and analyzed by SDS-PAGE under non-reducing conditions (C). The electrophoresis was conduced in 12% gels. The asterisks indicate the active serine protease. On both sides of the gel insert graph, the values of protein standard molecular mass (kDa) are shown.

zymatic activity (26.50 μmol $\cdot$ min^{-1} $\cdot$ mg protein^{-1}) was located in the ascendant portion of the peak centered at 10.54 min (data not showed), that corresponded to a molecular mass of about 110 kDa. This fraction (corresponded about 20% of the total absorbed material) was collected and concentrated for kinetic and electrophoretic analyses. No enzymatic activity was found on the other peaks and this material was not analyzed in this study. The SDS-PAGE under non-reducing conditions showed a single protein band of about 110 kDa, suggesting that the present enzyme is a dimeric protein (Fig. 1C).

Temperature and pH effects on the enzymatic activity

The pH dependence using L-TAME as substrate was analyzed and the maximum activity was observed at around pH 7.0. Furthermore, the enzyme preserved only 60% of activity at pH 9.0 and 50% of activity at pH 6.0. The optimum pH profile of aprotinin-agarose eluted material was similar to that of the purified protease (data not shown). The effect of temperature on the enzyme activity was analyzed at pH 7.0 and temperatures ranging from 25 °C to 50 °C. The enzyme demonstrated maximal activity at 28 °C, but no activity was detected at 50 °C. When protease was assayed in order to determine its thermal stability, a residual activity of 18% at 37 °C was observed under pre-incubation for 24 h. On the other hand the enzymatic activity was completely abolished after a 12-min pre-incubation at 42 °C, because the protease was completely denatured.

Substrates[a]	Specific activity [μmol·min^{-1}·mg protein^{-1}][b]
N-ϱ-tosyl-L-Arg-ME (L-TAME)	17.94 ± 0.84
N-Bz-L-Arg-ME (BAME)	64.35 ± 1.06
N-Bz-L-Arg-ϱ-NA	0.30 ± 0.00
N-Bz-Val-Gly-Arg-ϱ-NA	0.45 ± 0.01
N-Bz-L-Tyr-ϱ-NA	0.05 ± 0.02
N-Bz-Pro-Phe-Gly-Arg-ϱ-NA	0.34 ± 0.02
N-Succinyl-Ala-Ala-Pro-Phe-ϱ-NA	1.17 ± 0.03

Table II. Substrate specificity of *L. amazonensis* serine protease using synthetic substrates.

[a] Bz, benzoyl; ϱ-NA, paranitroanilide; ME, methyl ester.
[b] ±: Standard deviation of two independent experiments carried out in triplicate.

Carbohydrates detection and pI

According to the deglycosylation assays, the protease did not have carbohydrates in its molecule, since glycosidase activity was not observed (data not shown). In isoelectric focusing the protease migrated as a sharp band with pI about 4.8.

Substrate specificity

L. amazonensis enzyme showed proteolytic activity as demonstrated by substrate-SDS-PAGE (Fig. 1A and B), as well as when it was assayed using hemoglobin, BSA and ovalbumin as substrates and the activities obtained were 35.00 ± 5.29, 18.33 ± 2.60, 21.33 ± 1.23 respectively (A_{280}.min^{-1}·mg protein^{-1}). Among peptide substrates, L-TAME and BAME were better for this enzyme, because they contain an arginyl residue at the P1 site as an ester bond (Table II). The values of K_M, *Kcat* and *Kcat*/K_M calculated for L-TAME and BAME are summarized in Table III. The enzyme exhibited Michaelis-Menten kinetics in the substrate concentration range analyzed.

Susceptibility to protease inhibitors and divalent cations

Various substances were examined for the ability to inhibit the activity of the protease under L-TAME hydrolysis, within their analyzed concentration range. The enzyme activity was strongly inhibited by serine protease inhibitors such as aprotinin and benzamidine (Table IV). Inhibitors such as antipain inhibited 70% of the protease activity. TPCK (a known competitive inhibitor of chymotrypsin) inhibited 40% of the enzyme activity. Chymostatin, TLCK (a known irreversible inhibitor of trypsin) and 6-amino-caproic acid demonstrated no inhibition of enzyme activity. Canafistuline inhibited 50% of the enzymatic activity. Contrarily, SBTI and leupeptin, which are known to be competitive inhibitors of trypsin-like protease and α_2-macroglobulin (a general endoprotease inhibitor from human serum), did not decrease the enzymatic activity. Likewise, pepstatin and the chelator agents, such as EDTA and 1,10-phenanthroline, did not affect the protease. The thiol protease inhibitors such as DTT did not affect the enzymatic activity, and E-64 (10 μM) exhibited a non-significant inhibitory effect (Table IV). Divalent cations, such as Ca^{2+} and Zn^{2+} added to the reaction mix-

Table III. Kinetic parameters of *L. amazonensis* serine protease on L-TAME and L-BAME.

Kinetic parameter	L-TAME	L-BAME
K_M [μM]	6.40 ± 0.36[a]	1.21 ± 0.13
Kcat [s^{-1}]	8.26 ± 0.10	29.46 ± 0.16
Kcat/K_M [μM·s^{-1}]	1.30 ± 0.21	24.37 ± 1.23

[a] The values refer to the mean ± S. D. of two experiments carried out in triplicate.

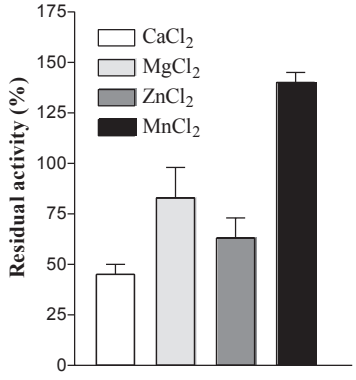

Fig. 2. Effects of cations on the enzymatic activity of *L. amazonensis* serine protease. In all the experiments the purified enzyme (0.03 nM) was pre-incubated with each ion (10 mM) in Tris-buffer for 15 min at room temperature. Values are the residual activity of serine protease as a percentage of the activity on L-TAME without ions and represent the average of three separate experiments carried out in duplicate.

Table IV. Effects of different types of protease inhibitors on the *L. amazonensis* serine protease activity.

Type of target protease	Inhibitor	Concentration	Residual activity (%)[a]
Serine	Antipain	100 μM	30
	Aprotinin	100 μM	0
	TPCK	100 μM	60
	TLCK	100 μM	100
	Canafistuline	0.38 μM	50
	Chymostatin	100 μM	100
	PMSF	10 μM	80
	Benzamidine	1 mM	0
	Leupeptin	1 mM	100
	6-Amino-caproic acid	1 mM	100
	SBTI	0.38 μM	100
Cysteine	E-64	10 μM	90
	DTT	10 μM	100
Aspartyl	Pepstatin	1 μM	100
Metalloprotease	EDTA	10 mM	100
	1,10-Phenanthroline	10 mM	100
General	α_2-Macroglobulin	0.38 μM	100

[a] Values are the remaining activity of serine protease as a percentage of the activity on L-TAME without compounds, measured as described in "Materials and Methods" and represent the average of three separate experiments carried out in duplicate.

ture, induced a negative effect on the enzymatic activity of about 66% and 40%, respectively, while Mn^{2+} positively modulated the enzyme (about 50% higher than the control), and Mg^{2+} did not show any effect on enzymatic activity (Fig. 2).

Discussion

Previous serine oligopeptidase purified from intracellular soluble extract of *L. amazonensis* promastigotes exhibited a molecular mass of about 101 kDa (Ribeiro de Andrade *et al.*, 1998), however, in the presence of reducing agents this protein did not alter its molecular mass, consequently proving to be a single polypeptide chain. The enzyme showed maximal proteolytic activity at pH 7.0 and 28 °C. Although the maximum activity was detected at pH 7.0, the enzyme preserved considerable activity between pH 6.0 and 8.5. This observation could suggest that our protease may be distributed in many cellular compartments of *Leishmania.* Moreover, the maximal activity at 28 °C could be an adaptation, as *L. amazonensis* promastigotes inhabit in the gut of the sandfly at a temperature of about 28 °C. The poor thermal stability exhibited by this enzyme could indicate that its action is limited to the host's organism environments, where the temperatures alteration is negligible. Another observation for consideration is that enzymatic activity decayed significantly when the enzyme was incubated at 37 °C up to 24 h. This protease could be short-lived or does not participate in the amastigotes metabolism, since the temperature would inactivate it.

Kinetic characterization was performed using L-TAME and BAME (both known to contain Arg residues in ester bound) as substrates, because these were the best substrates for the enzyme (Table II). The most efficient enzymatic parameter is the catalytic efficiency ($Kcat/K_M$). $Kcat/K_M$ quotients for L-TAME and BAME were calculated (Table III). Although the higher values of *Kcat* were obtained for L-TAME (substrate used in general analysis), the protease exhibited greater catalytic efficiency for BAME. Unfortunately, it was difficult to compare our kinetic results with those from other trypanosomatid serine peptidases, due to the utilization, by other authors, of *p*-nitroanilide or other substrates containing an amide bond (Ribeiro de Andrade *et al.*, 1998; Salter *et al.*, 2000). Serine proteases from snake venom showed similar esterase activity (Giovanni De Simone *et al.*, 1997; Aguiar *et al.*, 1996) to that of the *L. amazonensis* detergent-soluble serine protease.

One previous study reported the purification and characterization of a soluble 101-kDa serine oligopeptidase from *L. amazonensis* (Ribeiro de Andrade *et al.*, 1998). In this study a 110-kDa serine protease was obtained from the same parasite but from a detergent-soluble extract, being a protease associated to membranes. Both enzymes have particular characteristics. The 110-kDa enzyme here described hydrolyzed long chain peptides, such as hemoglobin, gelatin, albumin and ovalbumin, whereas the 101-kDa oligopeptidase showed strong amidolytic activity but did not

cleave proteins (Ribeiro de Andrade *et al.*, 1998). These results might indicate that *Leishmania* promastigotes have different forms of serine peptidases, oligopeptidase and protease. While the 101-kDa oligopeptidase activity was inhibited significantly by TLCK (irreversible trypsin-like serine protease inhibitor) and 1,10-phenanthroline (metalloprotease inhibitor), neither affected the activity of the 110-kDa protease (Table IV). However both enzymes were inhibited by TPCK (chymotrypsin-like serine protease inhibitor). The effects of inhibitors provide the most reliable information concerning catalytic type proteases. The assays performed in the presence of different inhibitors showed aprotinin, benzamidine, and antipain to be specific for serine proteases, thus guaranteeing its serine protease classification.

The activity of the classical mammalian serine proteases is modulated by ions. Pancreatic trypsin and chymotrypsin require calcium for peptide bond hydrolysis (Berezin and Martinek, 1970). In contrast the presence of calcium ions decreased the activity of both enzymes from *L. amazonensis*, serine oligopeptidase (Ribeiro de Andrade *et al.*, 1998) and serine protease as demonstrated in Fig. 2. The enzyme here obtained seems to be a serine protease negatively modulated by Ca^{2+} and positively modulated by Mn^{2+}. Thus, *Leishmania* serine protease differs from mammalian serine proteases, such as trypsin and chymotrypsin, due to the negative calcium modulation of enzymatic activity.

In general, membrane proteases or membrane-associated proteases are involved in the hydrolysis of specific peptide bonds, mediating regulatory effects in the physiology of the organisms (Lu *et al.*, 1999). A prolyl endopeptidase from a detergent-soluble extract of herring spermatozoa is involved in flagellar activation movement (Yoshida *et al.*, 1999). Proteases mediate important functions in their life cycles of medically important parasites. The biochemical characterization of *Leishmania* serine proteases improves knowledge of parasite physiology, and the interaction with the hosts and inhibitors of these enzymes have potent antiparasitic effects. In conclusion, this is the first report of purification and characterization of membrane serine protease from trypanosomatid species. The physiological function of the enzyme remains unknown. However, the wide activity between pH 6.0 and 8.5 may suggest that the enzyme could act in membranes of many intracellular vesicles, processing substrates or allowing complementary properties. In order to accurately determine the role of the enzyme it is necessary to examine the cellular localization, identify the natural substrate(s) and evaluate the effects of serine protease inhibitors in the parasite physiology.

Acknowledgments

This work was supported by grants from Fundação de Amparo a Pesquisa do Estado do Rio de Janeiro (FAPERJ), Fundação Oswaldo Cruz (FIOCRUZ, PAPES) and Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq). We are gratefully indebted to Dr. C. R. Alves for helping at the beginning of this work, Mr G. A. de Souza and C. A. Branco for technical assistance on *Leishmania* cultures and HPLC analysis and Dr. L. Macedo for the generous gift of an amount of canafistuline.

Aguiar A. S., Alves C. R., Melgarejo A., and Giovanni De Simone S. (1996), Purification and partial characterization of a thrombin-like/gyroxin enzyme from bushmaster (*Lachesis Muta rhombeata*) venom. Toxicon **34**, 555–565.

Alexander J. and Russell D. G. (1992), The interaction of *Leishmania* species and macrophages. Adv. Parasitol. **31**, 175–254.

Alves C. R., Marzochi M..C. A., and Giovanni De Simone S. (1993), Heterogeneity of cysteine protease in *Leishmania braziliensis* and *Leishmania major*. Braz. J. Med. Biol. Res. **26**, 167–171.

Berezin I. and Martinek K. (1970), Specificity of [α]-chymotrypsin. FEBS Lett. **8**, 261–267.

Caler E. V., Avalos S. V., Haynes P. A., Andrews N. W., and Burleigh B. A. (1998), Oligopeptidase B-dependent signaling mediates host cell by *Trypanosoma cruzi*. The EMBO J. **17**, 4975–4986.

Colmenares M., Tiermeyer M., Kima P., and McMahon-Pratt D. (2001), Biochemical and biological characterization of the protective *Leishmania pifanoi* amastigote antigen P-8. Infect Immun. **69**, 6776–6784.

Coombs G. H. (1982), Proteinases of *Leishmania mexicana* and other flagellate protozoa. Parasitology **84**, 149–155.

Desjeux P. (1999), Global control and *Leishmania* HIV co-infection. Clin. Dermatol. **17**, 317–325.

Ghendler Y., Parizade M., Arnon R., McKerrow J. H., and Fishelson Z. (1996), *Schistosoma mansoni*: Evidence for a 28-kDa membrane-anchored protease on *Schistosomula*. Exp. Parasitol. **83**, 73–82.

Giovanni De Simone S., Aguiar A. S., Gimenez A. R., Novelino K. A., and Moura R. S. (1997), Purification, properties and *N*-terminal amino acid sequence of a kalikrein-like enzyme from the venom of *Lachesis Muta rhombeata* (Bushmaster). J. Prot. Chem. **16**, 809–818.

Laemmli U. K. (1970), Cleavage of structural proteins during the assembly of the head bacteriophage T4. Nature **227**, 680–685.

Lowry O. H., Rosebrough N. J., Farr A. L., and Randall R. J. (1951), Protein measurement with the Folin reagent. J. Biol. Chem. **193**, 264–275.

Lu D., Futterer K., Korolev S., Zheng X., Kar T., Waksman G., and Sadler J. E. (1999), Crystal structure of enteropeptidase light chain complexed with an analog of the trypsinogen activation. J. Mol. Biol. **292**, 361–373.

Rawling N. D. and Barret A. J. (1994), Families of serine peptidases. Methods Enzymol. **244**, 19–45.

Ribeiro de Andrade A., Santoro M. M., Norma de Melo M., and Mares-Guia M. (1998), *Leishmania (Leishmania) amazonensis*: purification and enzyme characterization of a soluble serine oligopeptidase from promastigotes. Exp. Parasitol. **89**, 153–160.

Roggwiller E., Betoulle M. E., and Blisnick T. (1996), A role for erythrocyte band 3 by the parasite gp76 serine proteinase in the formation of the parasitophorous vacuole during the invasion of erythrocytes by *Plasmodium falciparum*. Mol. Biol. Parasitol. **82**, 13–24.

Rosenthal P. J. (1999), Proteases of protozoan parasites. Adv. Parasitol. **43**, 105–159.

Sadij M. and McKerrow J. H. (2002), Cysteine proteases of parasitic organisms. Mol. Biochem. Parasitol. **120**, 1–21.

Salter J. P., Lim K. C., Hansell E., Hsieh I., and McKerrow J. H. (2000), Schistosoma invasion of human skin and degradation of dermal elastin are mediated by a single serine protease. J. Biol. Chem. **275**, 38667–38673.

Sousa M. O., Miranda T. S. L., Maia C. N., Bittar E. R., Santoro M. M., and Figueiredo A. F. S. (2002), Kinetic peculiarities of human tissue kallikrein: 1-Substrate activation in the catalyzed hydrolysis of H-D-valyl-leucyl-L-arginine 4-nitroanilide and H-D-valyl-leucyl-L-lysine 4-nitroanilide; 2-Substrate inhibition in the catalyzed hydrolysis of *N*-ϱ-tosyl-$_L$-arginine methyl ester. Arch. Biochem. Biophys. **400**, 7–14.

Yoshida K., Inaba K., Ohtake H., and Morisawa M. (1999), Purification and characterization of prolyl endopeptidase from the Pacific herring, *Clupea pallasi*, and its role in the activation of sperm motility. Develop. Growth Different. **41**, 217–225.