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ORIGINAL PAPER

Identification, purification, and characterization of a secretory serine protease in an Indian strain of *Leishmania donovani*

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Abstract An aprotinin sensitive serine protease was identified in the culture supernatant of the Indian strain of *Leishmania donovani* (MHOM/IN/1983/AG83). The protease was subsequently purified and characterized. The apparent molecular mass of the enzyme was 115 kDa in SDS-PAGE under non-reducing condition, while on reduction it showed a 56 kDa protein band indicating that the protease is a dimeric protein. The purified enzyme was optimally active at the pH and temperature of 7.5 and 28°C, respectively. Assays of thermal stability indicated that the enzyme preserved 59% of activity even after pretreatment at 42°C for 1 h. The purified protease was not glycosylated and its isoelectric pI was 5.0. *N*-α-*p*-tosyl-L-arginine methylester (TAME) appeared to be relatively better substrate among the commonly used synthetic substrates. The enzyme was inhibited by Ca^{2+} and Mn^{2+}, but activated by Zn^{2+}. The protease could play important role(s) in the pathogenesis of visceral leishmaniasis or kala-azar.

Keywords *Leishmania donovani* (MHOM/IN/1983/AG83) · Aprotinin · Extracellular serine protease · Extracellular matrix proteins

Abbreviations

ECM Extracellular matrix
BM Basement membrane
TAME α-*N*-*p*-Tosyl-L-arginine-methyle-ester
E-64 L-*trans*-Epoxysuccinyleucylamido-(4-guanidino) butane
PMSF Phenylmethyl sulfonyl fluoride
SBTI Soyabean trypsin inhibitor
TPCK *N*-Tosyl-L-phenylalanine chloromethyl ketone
TLCK *N*-Tosyl-lysine chloromethyl ketone

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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 [1]. Leishmaniasis is endemic in the tropics and subtropics. *Leishmania* are dimorphic obligate intracellular parasites—flagellated promastigotes replicate in the gut of the sandfly vector, and their transmission to human or other vertebrate hosts occurs when the vector feeds on blood. These promastigotes are internalized by mono-nuclear phagocytic cells and undergo transformation into the non-motile amastigote form that maintains the infection, leading to the destruction of host tissues and invasion of new cells [2]. Expression of many molecules is responsible for mechanisms triggered by parasites in order to guarantee their survival within the host. Among these molecules, proteases have received paramount attention due to their crucial roles in the parasite life cycle and disease pathogenesis [3, 4]. A variety of *Leishmania* promastigotes secrete proteins [5, 6] that have been shown to be clinically important for diagnosis and vaccine development [7]. Of significance was the observation that secretory parasite proteases play important roles in host-parasite interactions, infection maintenance and pathogenicity [5–7, 8].

Secreted serine proteases from different *helminthes* such as *Schistosoma mansoni* [9] and *Trichenella spirallis* [10] were identified and their functions in parasite-host tissue invasion and dissemination have been demonstrated. In some species of the genus *Crithidia*, metalloproteinase and cysteine proteinases were detected in the culture medium [11]. Protozoan exoproteases like secreted cysteine proteases of *Entamoeba hystolytica* [12–14] and secreted serine, cysteine, and metalloproteases of different *Acanthamoeba* species [15, 16] were shown to be active against extracellular matrix proteins. In *Leishmania*, the intracellular amastigotes after multiplication are released into the extracellular medium and differentiate into promastigotes. To gain entry into the different cell types, the promastigotes must cross the basal laminae and extracellular matrix and, therefore, interact with their components such as collagen [17, 18], fibronectin [19], and laminin [20]. The cysteine and metalloprotease activities of *Leishmania* species have been well explored and described [6, 21]. In contrast, description of serine protease profiles of *Leishmania donovani* is scarce and demands adequate attention. Serine proteases are among the most extensively studied enzymes, found in all organisms and participate in a vast number of physiological and pathological processes in different systems [22]. Importance of serine proteases in parasitic action of some species such as *Plasomodium* [3] and *Trypanosomes* [23] has now become evident.

Serine proteases are being extensively studied because of their possible roles in parasite survival and pathogenicity. Their biochemical characterization is of interest not only for understanding the biochemistry of these enzymes, but also to gain an insight into their possible roles in parasite infection and subsequently their exploitation as targets for chemotherapy of parasitic diseases [22, 24–27]. Lopez et al. [8] have identified and partially characterized a secretory protease in *L. amazonensis*, a Brazilian strain of *Leishmania*. However, to the best of our knowledge no report has so far been made about the identity and characteristics of serine proteases in Indian strains of *L. donovani*. This prompted us to undertake the present research. Herein, we report identification, purification, and characterization of a 115 kDa aprotinin sensitive serine protease secreted by the Indian strain of *L. donovani* ((MHOM/IN/1983/AG83). We suggest that the enzyme could play some roles in the pathogenesis of visceral leishmaniasis.

Materials and methods

Materials

Medium 199 was obtained from GIBCO, Grand Island, NY, USA. The deglycosylation kit was the product of Bio-Rad, CA, USA. Fetal calf serum, penicillin-streptomycin, HEPES, L-glutamine, bovine serum albumin, aprotinin-agarose, aprotinin, PMSF, benzamidine, TPCK, TLCK, antipain, chymostatin, SBTI, leupeptin, E-64, pepstatin-A, EDTA, 1,10-phenanthroline, α_2-macroglobulin, *N*-*p*-tosyl-L-arginine methyl ester, *N*-Bz-L-Arg-*p*-NA, *N*-Bz-Val-Gly-Arg-*p*-NA, *N*-Bz-L-Tyr-*p*-NA, *N*-Bz-Pro-Phe-Gly-Arg-*p*-NA, *N*-Succinyl-Ala-Ala-Pro-Phe-*p*-NA, *N*-Benzoyl-L-tyrosyl-*p*-nitroanilide, *N*-Carboxy-benzoyl-phenylalanyl-*p*-nitroanilide, collagen type IV, fibrinogen, hemoglobin, myoglobin, fibronectin, $CaCl_2$, $MgCl_2$, $ZnCl_2$, and $MnCl_2$ were purchased from Sigma chemicals Co, St. Louis, MO, USA. BCA protein assay kit was obtained from Pierce Biotechnology, Rockford, IL, USA. Immunoglobulin G was purified from goat serum in our laboratory. All other chemicals used were of highest purity grade available commercially.

Methods

Culture of L. donovani

Leishmania donovani strains (MHOM/IN/1983/AG83) were isolated from Indian patients with visceral leishmaniasis. Promastigotes were cultured at 22°C in medium 199 with Hank's salt containing HEPES, L-glutamine, 20% fetal calf serum, penicillin, and streptomycin at 50 U ml^{-1} and 50 μg ml^{-1}, respectively. On fifth day of cultivation, the cells were harvested and the culture supernatant was collected by centrifugation at 3,000*g* for 15 min at 4°C for enzyme purification and related studies. The cell viability was assessed by trypan blue exclusion method [28], and the cell growth was estimated by counting the parasites in the Neubaur Chamber.

Gelatin-zymographic study for identification of extracellular protease

Gelatin substrate gel electrophoresis was carried out by following the procedure described by Das et al. [29] under non-reducing conditions of the culture supernatant of *L. donovani* (MHOM/IN/1983/AG83). Briefly, after electrophoresis, the gel was washed with 2.5% Triton X-100 for 1 h with two changes to remove SDS and incubated overnight at 28°C in 50 mM Tris–HCl (pH 7.5) in the absence and presence of different classes of proteolytic enzyme inhibitors at appropriate concentrations: EDTA (10 mM), 1,10-phenanthroline (10 mM), pepstatin (1 mM), E-64 (10 μM), DTT (1 mM), aprotinin (1 μM). The next day, the gels were stained with coomassie blue and destained using methanol/acetic acid/distilled water (3:1:6).

Purification of the extracellular protease

Cells were removed by centrifugation at 3,000*g* for 15 min at 4°C and the supernatant was filtered through a 0.22 μm pore size membrane filter. Ammonium sulfate (solid) was added to the culture supernatant to 45% saturation with gentle stirring at 4°C overnight. The suspension was then centrifuged at 12,000*g* for 60 min at 4°C. The pellet obtained was resuspended in 10 mM Tris–HCl buffer (pH 7.5) at one-tenth of the original volume and dialyzed overnight against the same buffer at 4°C. The insoluble material was removed by centrifugation at 33,000*g* for 60 min at 4°C. The supernatant was then filtered through a 0.22 μm membrane filter and loaded on to 2.0 ml aprotinin-agarose affinity column which was previously equilibrated with 10 mM Tris–HCl (pH 7.5) containing 5 mM $CaCl_2$ [8, 30]. After repeated washings (20 bed volumes), the protease was eluted (at a flow rate of 15 ml h^{-1}) with the same buffer containing 1.5 M NaCl without calcium. In each tube, 1 ml fraction was collected, kept in ice, and the protein peak was detected after measuring the absorbance of each fraction at 280 nm. The enzymatic activity of the fractions was determined with α-*N*-*p*-tosyl-L-arginine methylester (TAME) as the substrate. The fractions containing the protease activity were pooled and concentrated (in Amicon concentrator: 3 kDa cut-off membrane) at 4°C and then performed continuous elution electrophoresis (Prep Cell; Model 491-Bio-Rad) [29]. Briefly, a 10 cm separating gel of 7.5% polyacrylamide and 2.5 cm stacking gel of 4% polyacrylamide were cast in the 28 mm diameter gel tube of the instrument and the electrophoresis was performed, following the method of Laemmli [31]. The elution chamber contained 800 ml elution buffer (50 mM Tris–HCl buffer, pH 7.5). The system was then equilibrated to 4°C. To the sample, SDS-sample buffer (62.5 mM Tris–HCl, pH-6.8) containing 10% glycerol, 0.05% bromophenol blue, 5% 2-mercaptoethanol, and 2% SDS) was added and the reaction mixture was not heated. The sample was then loaded onto the top of the stacking gel. Electrophoresis was performed at 40 mA (max 300 V) under cooling conditions, with an anode buffer (25 mM Tris, 192 mM Glycine, pH 8.3). Electrophoresis was continued until the dye front reached the bottom of the separating tube ($\sim$3 h). Then the elution was started after an additional electrophoretic run for 1 h. The proteins were eluted at a flow rate of 1 ml min^{-1} and the fractions of 1 ml were collected for 5 h. The enzymatic activity of the fractions was analyzed in gelatin-SDS-PAGE [29]. Fractions containing the proteolytic activity were pooled, concentrated by ultra filtration (Amicon, YM-10 membrane) and dialyzed overnight against 100 mM Tris–HCl (pH 7.5). The enzymatic activity was determined using TAME as the substrate. The enzyme activity in the substrate gel and the protein profile in SDS-PAGE at different stages of the purification process were determined. Additionally, SDS-PAGE of the purified protein was performed under reducing condition [31].

Determination of optimum pH, temperature, and thermal stability

The optimum pH was determined using buffers over a wide pH range between 5.0 and 9.0. Sodium citrate buffer (100 mM) was used for the pH 5.0 and 6.0; while Tris–HCl buffer (100 mM) was used for the pH range between 7.0 and 9.0. About 250 μM of the substrate (TAME) was added to 2 μg of the enzyme and incubated for 15 min in different buffer solutions, and the protease activity was measured by monitoring absorbance at 247 nm. To determine the optimum temperature, the reaction mixture was incubated for 15 min at temperatures between 25 and 50°C. After that readings were taken at 247 nm, and specific activity was expressed as μ mole product formed/min/mg protein [8]. The pH and temperature with the highest activity were considered as 100%. To determine heat stability, the enzyme (2 μg) was incubated in 100 mM Tris–HCl buffer (pH 7.5) at 42°C for different time intervals up to 1 h, and then the enzymatic reaction was started at 28°C by adding TAME (250 μM). The activity of the fraction that was not heat-treated was considered as 100%, and from this the residual protease activity at different preincubation time was determined [8, 32].

Deglycosylation study of the protease

The enzymatic deglycosylation kit (Bio-Rad) that cleave all N- and most of the O-linked oligosaccharides from glycoproteins was used to ascertain whether or not the protease contains any carbohydrate moiety. We used fetuin as the standard. After the reaction, the experimental as well as the standard were subjected to SDS-PAGE, and the gel profiles were then analyzed [8, 33].

Determination of pI of the protease

Isoelectric focusing was carried out on IEF strip (pH 3–9) on a Phast System (Bio-Rad, CA) with the standard markers: human carbonic anhydrase B (pI 6.5), bovine carbonic anhydrase B (pI 6), β-lactoglobulin B (pI 5.1), soyabean trypsin inhibitor (pI 4.5). The pI of the protease was determined by comparing its migration with the standard IEF markers [34].

SDS-PAGE analysis of digestion of natural substrates

The activities of the purified protease toward natural substrates were analyzed. The substrates used were: collagen

type IV, fibrinogen, immunoglobulin G, and hemoglobin. Five hundred microgram of each substrate, dissolved in 0.1 M sodium acetate buffer (for type IV collagen) or 100 mM Tris–HCl (pH 7.5) (for other substrates) at a concentration 100 μg ml^{-1} were mixed with 1 μg of the purified protease and incubated at 28°C. Forty micro-liter aliquots were withdrawn at 6 and 24 h, respectively, from the reaction mixture and electrophoresed in SDS-PAGE. After electrophoresis, the gel was stained with 0.1% coomassie blue.

Enzyme assays using natural substrates

Protein substrates 0.1% (w/v) of collagen type IV, fibrinogen, hemoglobin, immunoglobulin G (IgG), fibronectin, and myoglobin were dissolved in 400 μl of 100 mM Tris–HCl (pH 7.5) and incubated with gentle agitation for 30 min at 28°C with 1 μg of purified enzyme. The reaction was stopped by addition of 500 μl of ice-cold TCA (10%). The tubes were centrifuged at 12,000*g* for 5 min at 4°C and the absorbance of the supernatants was measured at 280 nm in a spectrophotometer. One unit of enzymatic activity was defined as the amount of enzyme required to cause an increase of 0.1 in the absorbance under standard conditions [33].

Enzyme assays using synthetic substrates and determination of kinetic parameters

We studied the protease activity by using different synthetic substrates. The substrates that we used were as follows: *N*-Benzoyl-L-arginyl-*p*-nitroanilide, *N*-Benzoyl-valinyl-glycinyl-arginyl-*p*-nitroanilide, *N*-Succinyl-alanyl-alanyl-prolyl-phenylalanyl-*p*-nitroanilide, *N*-Benzoyl-prolyl-phenylalanyl-glycinyl-arginyl-*p*-nitroanilide, *N*-Benzoyl-L-tyrosyl-*p*-nitroanilide (BTPNA), *N*-Carboxy-benzoyl-phenylalanyl-*p*-nitroanilide (CBPPNA) and α-*N*-*p*-tosyl-L-arginine-methyl ester (TAME) (250 μM) in 100 mM Tris–HCl buffer (pH 7.5) at 28°C [8, 35]. The reaction was started by adding 100 μl (0.2 μM) of the protease with 400 μl of the substrate solutions for 1−10 min. For TAME, the activity was monitored at 247 nm, while for the other chromogenic substrates, the absorbance was measured at 410 nm. The specific activity of the enzyme was expressed as μmol^{-1} min^{-1} mg protein.

The kinetic parameters K_m and K_{cat} values for the protease were determined with TAME, BTPNA and CBPPNA as the substrates. The substrates were prepared with concentrations between 1–400 μM. K_m values were calculated from velocity versus substrate concentration plots. For the determination of K_{cat}, we considered the mol mass of the protease as 115 kDa and also assumed that per enzyme only one catalytic site exists [8, 36].

Effect of different protease inhibitors on the enzyme activity

Effects of different classes of protease inhibitors were tested to ascertain the type of protease using TAME as the substrate. Stock solutions of aprotinin, antipain, chymostatin, α_2-macroglobulin, SBTI, and EDTA were prepared in distilled water; leupeptin and TLCK solutions were prepared in dimethylsulfoxide; while the stock solutions of PMSF, pepstatin-A, TPCK, and 1,10-phenanthroline were prepared in ethanol. The inhibitors were incubated with 2 μg of the *L. donovani* purified protease for 15 min at room temperature. The reaction was initiated with the addition of the substrate TAME (250 μM) in 100 mM Tris–HCl buffer (pH 7.5) at 28°C for 30 min, and the absorbance was monitored at 247 nm. Appropriate blanks were prepared for every enzyme-inhibitor and adjusted for relevant experiments done simultaneously. The activity was considered as 100% where no inhibitor was present. Inhibition was expressed as the percent change in the appropriate control activity.

Effect of some divalent metal ions on the enzyme activity

To determine the effect of divalent metal ions: Ca^{2+}, Mn^{2+}, Mg^{2+}, and Zn^{2+}, the purified enzyme (2 μg) was added to 10 mM of the divalent metal solutions for 15 min at room temperature. The reaction was initiated with the addition of TAME (250 μM) in 100 mM Tris–HCl buffer (pH 7.5) at 28°C for 15 min at room temperature, and the protease activity was measured. A control was included without adding the divalent metal ions. The activity under the control condition was considered as 100%, and based on this the percent change due to the addition of divalent metal ions were calculated [37]. The protease activity was also observed in the substrate gel electrophoresis, by incubating the gels with the above metal ions (10 mM).

Cell growth and activity of the protease

At different stages of growth of the *L. donovani* promastigote, the secretory serine protease activity was determined using TAME as the substrate. Growth of the parasite was ascertained by counting in a Neubaur chamber for 10 consecutive days. In each day, the culture supernatant was collected and centrifuged twice. Initially, it was centrifuged at 3,000*g* for 10 min at 4°C, then finally at 12,000*g* for 10 min at 4°C. The enzymatic activity was determined using 100 μl of the culture supernatant against TAME (250 μM) as the substrate [8].

Protein measurement

Protein content was estimated by Pierce Micro BCA protein assay kit using bovine serum albumin as the standard.

Statistical analyses

Data were analyzed by the analysis of variance, followed by the test of least significant differences for comparison between the groups [38]. The value of $P < 0.05$ was considered as significant.

Results

Identification of a serine protease in culture supernatant

Gelatin zymogram of the culture supernatant of *L. donovani* (AG83) revealed a proteolytic band having an apparent molecular mass of 115 kDa, which was completely inhibited by aprotinin (Fig. 1).

Purification of the protease

Purification of an extracellular protease from the culture supernatant of *L. donovani* was performed with a combination of ammonium sulfate precipitation, followed by affinity chromatography and continuous elution electrophoresis by Prep-Cell (Bio-Rad, CA). The proteins obtained by precipitation after 45% saturation with solid ammonium sulfate were applied to aprotinin-agarose affinity chromatography column. The peak fractions of enzymatic activity eluted from aprotinin-agarose affinity column (Fig. 2A) were pooled, concentrated, dialyzed, and applied to Prep-Cell for continuous elution electrophoresis (Fig. 2B). The proteolytically active fractions from Prep-Cell were then pooled, concentrated, and used for further studies. The purification steps for this proteolytic enzyme from the culture supernatant of *L. donovani* (AG83 strain) are summarized in Table 1. The specific activity of the purified enzyme was 753-fold higher than that of the culture supernatant, with 46% yield (Table 1).

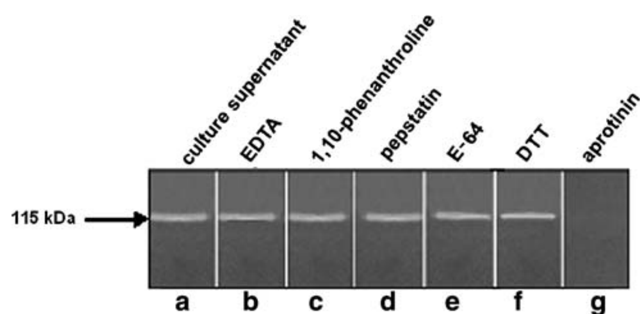

Fig. 1 Identification of an aprotinin sensitive protease of the culture supernatant of Indian strain of *L. donovani* in gelatin-zymogram. Different inhibitors were added in the incubation buffer containing the substrate gel. Lane a: control (culture supernatant without any inhibitor); lane b: EDTA (10 mM); lane c: 1,10-phenanthroline (10 mM); lane d: pepstatin (1 mM); lane e: E-64 (10 μM); lane f: DTT (1 mM); lane g: aprotinin (1 μM)

SDS-PAGE and substrate gel electrophoresis

After each purification step, samples were run for SDS-PAGE to assess their purity. Figure 2C shows purified enzyme on SDS-PAGE, which demonstrated the molecular mass of the enzyme to be 115 kDa under non-reducing condition**.** The affinity chromatography step was not able to purify the enzyme to homogeneity (Fig. 2C). However, the enzyme was purified to homogeneity after continuous elution electrophoresis as evidenced by SDS-PAGE (Fig. 2C). The SDS-PAGE analysis under reducing condition revealed a different electrophoretic pattern (Fig. 3), where the purified protease showed a 56 kDa homogeneous protein band (Fig. 3), indicating that a reducing agent appears to have important influence on the structure of this protein. Our results suggested that the protein might exist as dimer. Gelatin substrate gel electrophoresis (Fig. 2D) revealed significant proteolytic activity at 115 kDa both in affinity chromatography eluate as well as in the eluate of continuous elution electrophoresis.

Optimum pH and temperature

Figure 4A demonstrated the purified protease activity profile at different pH. The optimum pH of the enzyme appeared to be 7.5 (Fig. 4A). At pH 5.0 and 9.0, the enzyme retained, respectively, 49% and 32% of its activity (Fig. 4A).

The protease showed optimum activity at 28°C and retained about 79% of its activity at 50°C (Fig. 4B). For thermal stability determination, preincubation of the enzyme at 42°C for 1 h showed a residual activity of 59.7% (Fig. 4C).

Glycosylation analysis and pI determination

The *L. donovani* extracellular protease appeared to be devoid of carbohydrate moieties in its molecule as evidenced from the deglycosylation assays, since neuraminidase II, O-glycosidase DS and N-glycosidase F were not observed (data not shown). The protease migrated as a band at pH 5.0 in the isoelectric focusing experiment (data not shown).

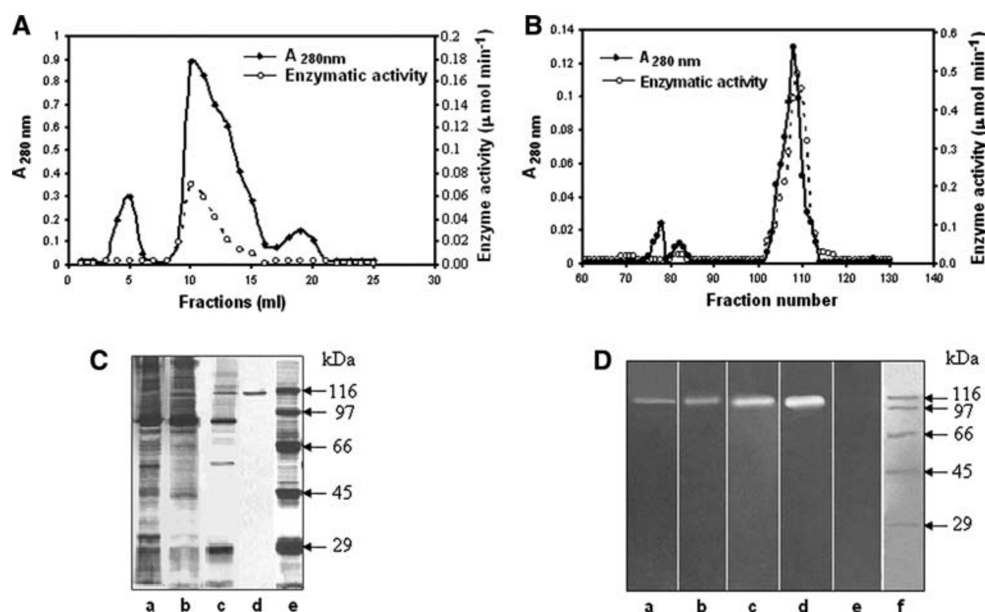

Fig. 2 Purification profile of the secretory protease from the culture supernatant of Indian strain of *L. donovani*. **A** Chromatogram of aprotinin-agarose affinity chromatography of the ammonium sulfate-precipitated culture supernatant. **B** Chromatogram of continuous elution electrophoresis of the enzymatically active fractions from **A**. **C** 10% SDS-PAGE of purification profile of the extracellular protease. Lane a: culture supernatant; lane b: ammonium sulfate (0–45%) precipitate; lane c: pooled and concentrated active fractions from the aprotinin-agarose affinity column; lane d: pooled and concentrated active fractions eluted from Prep-Cell (non-reduced); lane e: molecular mass standards. **D** Gelatin-zymogram of the extracellular protease. Lane a: culture supernatant; lane b: ammonium sulfate (0–45%) precipitate; lane c: pooled and concentrated fractions eluted from the aprotinin-agarose affinity column; lane d: pooled and concentrated active fractions eluted from Prep-Cell (non reduced); lane e: treatment of purified protease with aprotinin; lane f: molecular mass standards

Table 1 Purification of *Leishmania donovani* extracellular serine protease

Purification steps	Total protein[a] (mg)	Enzyme activity (μmol/min)	Specific activity (μmol/min/mg protein)	Purification (-fold)	Yield (%)
Supernatant of culture	490	1.27×10^{-3}	2.60×10^{-6}	–	100
$(NH_4)_2SO_4$	91	1.02×10^{-3}	1.12×10^{-5}	4	66
Affinity chromatography	0.6	0.8×10^{-3}	1.33×10^{-3}	470	48
Prep cell	0.12	0.77×10^{-3}	1.96×10^{-3}	753	46

[a] From 2×10^{10} cells

Degradation of natural substrates in SDS-PAGE

The purified extracellular serine protease of *L. donovani* degraded collagen type IV, an extracellular matrix protein, into many small-sized fragments (Fig. 5A). The α- and β-chains of type IV collagen were almost completely degraded within 24 h upon treatment with the enzyme (Fig. 5A). The major fibrinogen protein bands were gradually disappeared from 6 to 24 h indicating that this protein was degraded significantly into smaller molecular weight fragments by the protease (Fig. 5B). Small amounts of the hemoglobin dimer almost disappeared within 6 h (Fig. 5C); the monomer was degraded slowly, but completely disappeared by 24 h of incubation with the enzyme (Fig. 5C). The protease degraded most of the heavy chains of IgG within 6 h of its treatment (Fig. 5D). However, the light chain of the immunoglobulin was less sensitive to the enzyme up to 6 h, while it was degraded almost completely by 24 h of treatment with the enzyme (Fig. 5D).

Degradation of natural and synthetic substrates and determination of kinetic parameters

The purified protease exhibited proteolytic activity when it was assayed using hemoglobin, collagen type IV, IgG, and fibrinogen as the natural substrates (Table 2). The enzyme was unable to cleave fibronectin and myoglobin.

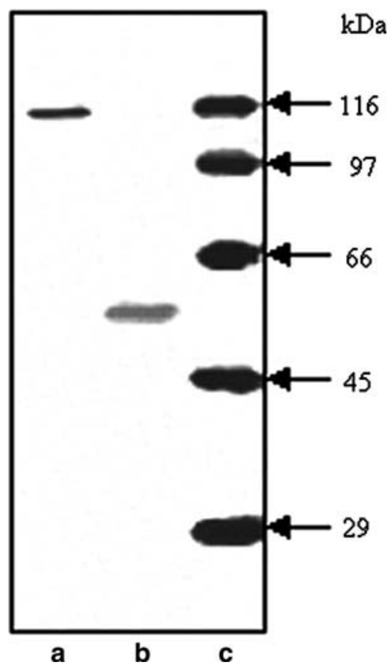

Fig. 3 10% SDS-PAGE profile of the pooled and concentrated proteolytically active fractions eluted from the Prep-Cell under (a) non-reducing conditions and (b) reducing conditions; (c) molecular mass standards

The enzyme did not show discernible activity towards *N*-benzoyl-L-arginyl-*p*-nitroanilide, *N*-benzoyl-valinyl-glycinyl-arginyl-*p*-nitroanilide, *N*-succinyl-alanyl-alanyl-prolyl-phenylalanyl-*p*-nitroanilide, and *N*-benzoyl-prolyl-phenylalanyl-glycyl-arginyl-*p*-nitroanilide (Table 3); although the enzyme elicited significant activity against *N*-benzoyl-L-tyrosyl-*p*-nitroanilide (BTPNA) and *N*-carboxy-benzoyl-phenylalanyl-*p*-nitroanilide (CBPPNA), both of which possess aromatic residue at the P1 site (Table 3). The values of K_m, K_{cat}, and K_{cat}/K_m calculated for TAME are represented in Table 4. The enzyme followed Michaelis–Menten kinetics within the substrate concentration range analyzed. Considering the synthetic substrates used, TAME was determined to be the relatively better substrate for the protease (Tables 3, 4).

Effect of different inhibitors and divalent cations on the enzyme

Different types of protease inhibitors were used to examine the nature of the *L. donovani* secretory protease. Among the inhibitors used, only aprotinin was able to completely inhibit the enzyme (Table 5). Benzamidine reduced 70% of the protease activity (Table 5); while PMSF and TPCK (chymotrypsin site-specific reagent) reduced 64% and 55% of the protease activity, respectively. In contrast, TLCK (trypsin site specific inhibitor), chymostatin (competitive inhibitor of chymotrypsin), and antipain inhibited the enzyme activity by only 22%, 5%, and 10%, respectively (Table 5). On the other hand, α_2-macroglobulin (general protease inhibitor in serum) was found to be ineffective to inhibit the enzyme activity (Table 5). Additionally, pepstatin A and the chelating agents, EDTA and 1,10-phenanthroline did not have any discernible effect on the enzyme activity. E-64 (a cysteine protease inhibitor

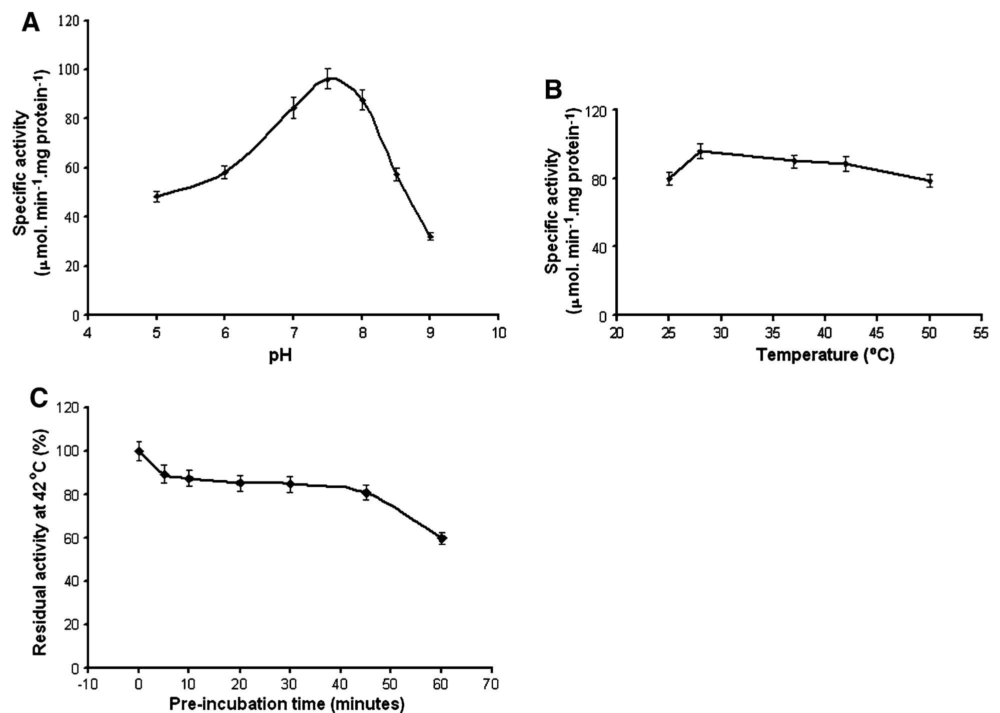

Fig. 4 Effects of pH and temperature on the purified extracellular serine protease activity of *L. donovani*. **A** Effects of pH using 100 mM buffers in reaction mixture. Buffers used were: 100 mM sodium citrate (pH 5.0–6.5) and 100 mM Tris–HCl (pH 7.0–9.0). **B** Effect of temperature on the purified enzyme activity. **C** Determination of heat stability of the enzyme. Heat stability was verified by preincubating the enzyme at 42°C for 1 h and then its activity was assayed at 28°C. Values are mean ± SD ($n = 4$)

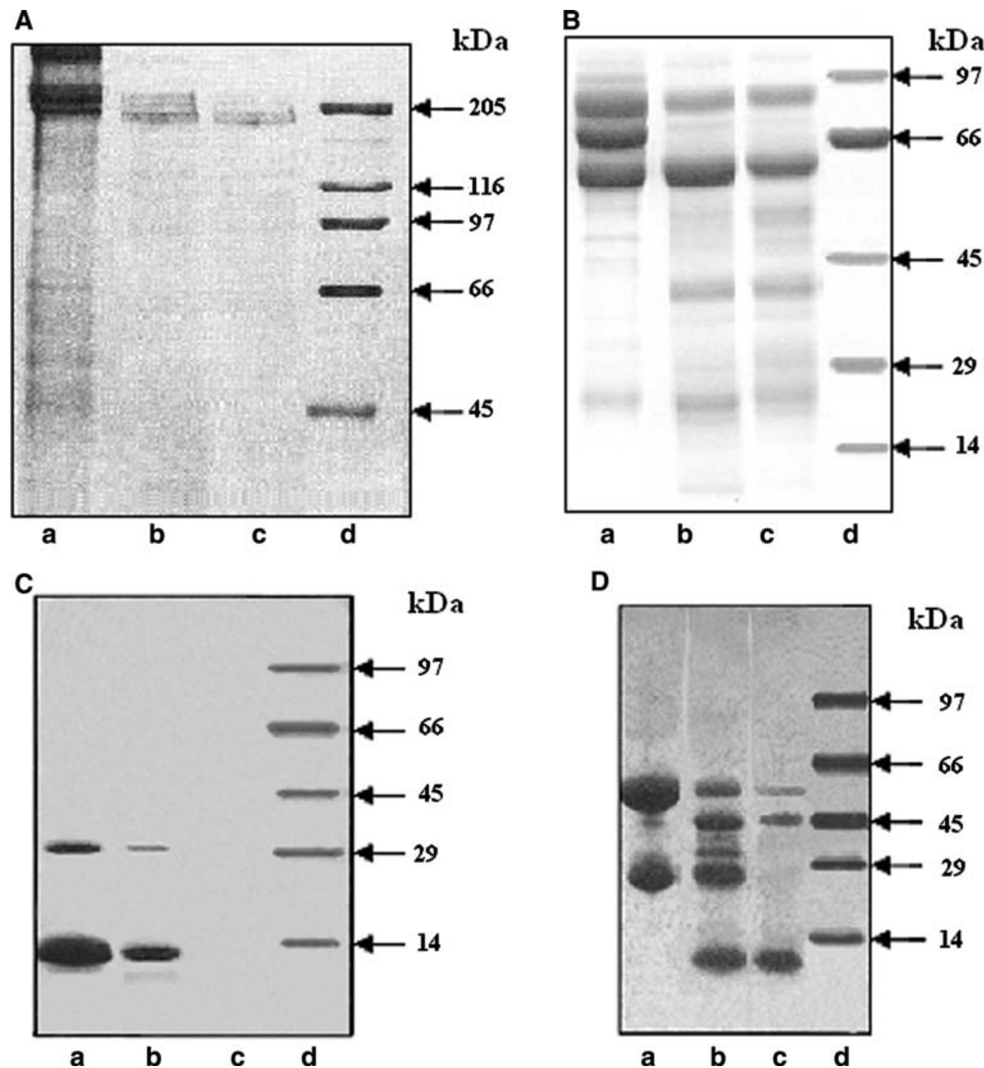

Fig. 5 Effect of the purified extracellular serine protease of *L. donovani* on degradation of some natural substrates. **A** Degradation of collagen by the protease. Lane a, collagen type IV solution incubated without enzyme for 24 h; lane b, collagen type IV solution incubated with the enzyme for 6 h; lane c, collagen type IV solution incubated with the enzyme for 24 h; lane d, molecular mass standards. **B** Degradation of fibrinogen by the purified protease. Lane a: fibrinogen solution incubated without the enzyme for 24 h; lane b: fibrinogen solution incubated with the enzyme for 6 h; lane c: fibrinogen solution incubated with the enzyme for 24 h; lane d: molecular mass standards. **C** Degradation of hemoglobin by the purified protease. Lane a: hemoglobin solution incubated without the enzyme for 24 h; lane b: hemoglobin solution incubated with the enzyme for 6 h; lane c: hemoglobin solution incubated with the enzyme for 24 h; lane d: molecular mass standards. **D** Degradation of IgG by the purified protease. Lane a: IgG solution incubated without the enzyme for 24 h; lane b: IgG solution incubated with the enzyme for 6 h; lane c: IgG solution incubated with the enzyme for 24 h; lane d: molecular mass standards

relatively specific for calpains) elicited only 7% inhibition. Likewise, the thiol protease inhibitor, DTT did not produce any discernible inhibition on the enzyme activity (Table 5).

Upon addition of Ca^{2+} and Mn^{2+}, the enzyme activity was reduced by 68% and 60%, respectively (Fig. 6). Addition of Zn^{2+} increased the protease activity by 33% indicating its role as a positive modulator of the enzyme (Fig. 6), while addition of Mg^{2+} did not produce any appreciable alteration of the enzyme activity (Fig. 6).

Measurement of extracellular serine protease activity with cell growth

During 10 consecutive days of the growth of *L. donovani*, the culture supernatant was collected along with counting of the parasite. The enzyme activity of the culture supernatants was measured using TAME as the substrate. It appears from Fig. 7 that on day 5 of the culture, maximum enzyme activity was observed. However, the decline of the enzyme activity did not closely resemble with the parasite growth.

Table 2 Degradation of some natural substrates by the purified extracellular protease of *Leishmania donovani*

Substrates	Specific activity (unit mg protein^{-1})
Collagen type IV	0.15 ± 0.03
Hemoglobin	2.10 ± 0.15
IgG	0.09 ± 0.002
Fibrinogen	0.05 ± 0.003
Fibronectin	ND
Myoglobin	ND

Results are mean ± SD ($n = 4$). ND, not detected

Table 3 Degradation of some synthetic peptide substrates by the purified *Leishmania donovani* extracellular protease

Substrate	Specific activity (μmol min^{-1} mg protein^{-1})
N-*p*-Tosyl-L-Arg-ME (L-TAME)	96.11 ± 4.18
N-Carboxy-Bz-Phe-*p*-NA (CBPPNA)	54.39 ± 2.47
N-Bz-L-Tyr-*p*-NA (BTPNA)	47.37 ± 2.02
N-Bz-Pro-Phe-Gly-Arg-*p*-NA	ND
N-Succinyl-Ala-Ala-Pro-Phe-*p*-NA	ND
N-Bz-L-arg-pNA	ND
N-Bz-Val-Gly-Arg-*p*-NA	ND

Bz, benzoyl; *p*-NA, *p*-nitroanilide; ME, methyl ester. Results are mean ± SD ($n = 4$). ND, not detected

Table 4 Substrate specificity of *Leishmania donovani* extracellular protease on synthetic peptide substrates

Kinetic parameters	TAME	BTPNA	CBPPNA
K_m (M)	2.43×10^{-6}	6.3×10^{-4}	3.53×10^{-4}
K_{cat} (S^{-1})	2.52	1.15	3.16
K_{cat}/K_m (M S)$^{-1}$	1.04×10^{6}	1.96×10^{3}	8.93×10^{3}

Results are mean of three sets of experiments

No activity was detected against *N*-Bz-Pro-Phe-Gly-Arg-*p*-NA, *N*-Succinyl-Ala-Ala-Pro-Phe-*p*-NA, *N*-Bz-L-arg-pNA, *N*-Bz-Val-Gly-Arg-*p*-NA

Table 5 Effects of different types of protease inhibitors on *Leishmania donovani* extracellular serine protease activity

Type of protease	Inhibitor	Concentration	Residual activity (%)
Serine protease	Aprotinin	1 μM	0
	Benzamidine	1 mM	22
	PMSF	1 mM	36
	TLCK	100 μM	78
	TPCK	100 μM	45
	Antipain	100 μM	90
	SBTI	1 μM	99
	Chymostatin	100 μM	95
Metallo protease	EDTA	10 μM	100
	1,10-Phenanthroline	10 μM	100
Cysteine protease	Leupeptin	10 μM	100
	E-64	10 μM	93
Thiol protease	DTT	1 mM	100
Aspartic protease	Pepstatin A	1 mM	100
General protease	α_2-Macroglobulin	1 μM	100

The percentage of the remaining activity of protease on the substrate (TAME) compared with a control reaction without preincubation

Values are the mean of four independent experiments

Discussion

This work describes the purification and some biochemical properties of an extracellular serine protease isolated from culture supernatants of an Indian strain of *L. donovani*. The enzyme was classified as serine protease based on its complete inhibition by aprotinin, a serine protease inhibitor. An extracellular serine protease has been identified and characterized in a Brazilian strain of *Leishmania*, the *L. amazonensis* [8]. However, until now, no report is available indicating that the Indian strain of *L. donovani* secretes serine protease in the culture medium.

We employed ammonium sulfate precipitation, aprotinin agarose affinity chromatography followed by continuous elution electrophoresis by Prep-Cell (Bio-Rad, CA) to purify the *L. donovani* extracellular serine protease. The enzyme exhibited a molecular mass of 115 kDa; however, in presence of reducing agent this protein alters its molecular mass to 56 kDa indicating its dimeric nature.

The *L. donovani* extracellular protease elicited maximum activity at pH 7.5, and the optimum temperature for the enzyme activity was determined to be 28°C. Although maximum activity detected at pH 7.5, the enzyme preserved considerable proteolytic activity between pH 7.0–8.0. This observation could suggest that the purified extracellular protease may be distributed in many cellular compartments of *L. donovani* promastigotes [39].

In *L. amazonensis*, subcellular localization studies of serine protease by immunoelectron microscopy suggested that the promastigote serine protease is predominantly located in the flagellar pocket and in vesicular structures that are morphologically similar to the compartments of mammalian endocytic as well as exocytic pathways [40]. In amastigotes, the enzyme was found to be located in electron dense structures corresponding to megasomes in addition to subcellular structures like flagellar pocket and cytoplasmic vesicles as in promastigotes [40]. It is well known that the flagellar pocket membrane is an obligatory intermediary station for membrane-bound molecule

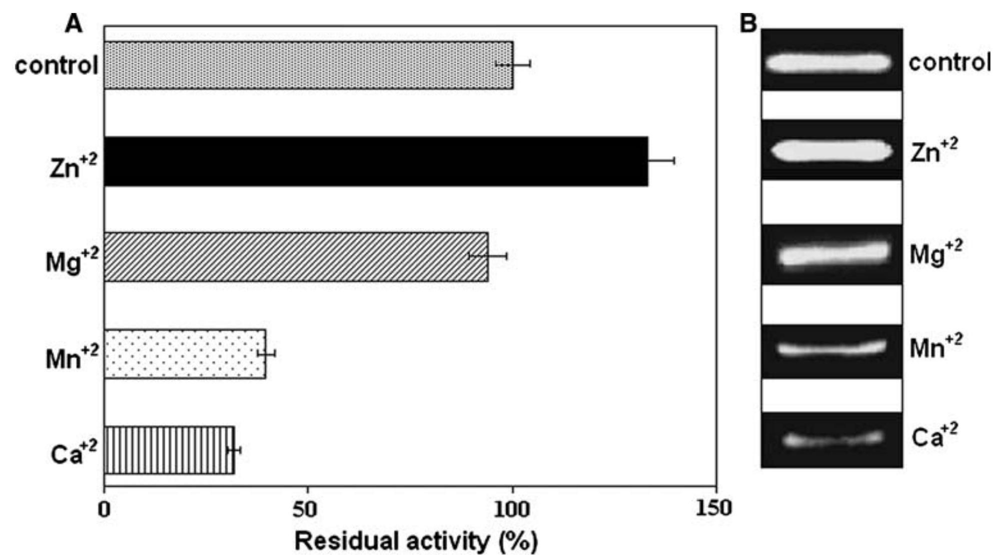

Fig. 6 Effect of some divalent cations on the extracellular serine protease activity of *L. donovani*: **A** using TAME as the synthetic substrate; and **B** in gelatin zymogram. Values are mean ± SD ($n = 4$). $P < 0.05$ compared the effect of the metal ions with the control. Residual activity was calculated by defining the protease activity at 28°C without any treatments

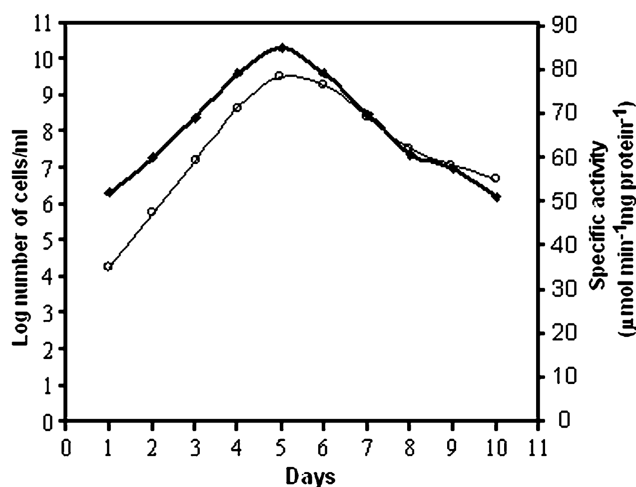

Fig. 7 *Leishmania donovani* cell growth and the purified extracellular protease activity. The culture supernatant was collected for 10 consecutive days for the enzyme assay using TAME as the substrate and the numbers of the parasites were counted. Values are mean ± SD ($n = 4$)

trafficking between intracellular membranes and cell surface and vice versa [41]. The processing and trafficking of lysosomal cysteine protease in *L. mexicana* was found to be channeled via flagellar pocket to megasomes [42] that are significant sites of proteolytic activity of *Leishmania* and have active roles in differentiation process and intracellular survival of the parasite [43]. All these earlier studies as well as our observations regarding the sensitivity of the purified protease in the broad range of pH lead us to study the localization of this novel extracellular protease, which is currently in progress. The temperature in the alimentary tract of the sandfly vector where *L. donovani* present has been found to be around 28°C. Thus, the optimum temperature of the protease at 28°C could be due to the adaptation of the promastigote in the alimentary tract of the sandfly vector [8]. With the exception of a few serine proteases of *A. healy* [44] and *Streptomyces* species [30], majority of the serine proteases are heat labile. The thermal stability of the *L. donovani* extracellular serine protease appears unusual and could be related to its adaptation in the extracellular environment [8].

The presence of carbohydrate in a protein provides protection against the attack of proteolytic enzymes and could generate information about its possible cellular location and intracellular functions. Representative examples of glycosylated proteases are cathepsins, which is present in the lysosomes [45]. In the amastigotes of Brazilian species of *L. mexicana*, presence of nonglycosylated serine protease has been detected in the megasome [40] that shows lysosome-like properties [46]. However, our purified *L. donovani* extracellular protease does not have carbohydrate moiety in its molecule, indicating that *Leishmania* needs different enzymes working in different compartments in order to execute distinct functions in both invertebrate and vertebrate hosts.

The *L. donovani* extracellular protease showed hydrolytic activity against both natural and synthetic substrates. In the present study, we determined that the enzyme possesses high degree of proteolytic activity against a great variety of specific substrates like hemoglobin, collagen type IV, fibrinogen, and IgG which are normal host proteins. This activity suggests that the secreted protease may also play important roles in parasite biology [33]. Indeed, the proteolytic activity against the natural substrates like collagen type IV and fibrinogen, the components of extracellular matrix and basement membrane, suggests that some of the *L. donovani* proteases could play a significant role in tissue degradation to facilitate parasite migration to the host cell. Since the ultimate goal of invading

Leishmania is to get entry into the intracellular milieu, so we can speculate that the secreted protease may enhance migration by degrading ECM components at the site of inoculation and promote parasite binding to and phagocytosis by macrophages. In addition, migration through the ECM and BM may facilitate the access of the parasite to the blood or lymph circulation for dissemination to distant sites [47]. It has been reported that the secreted serine proteases of *Acanthamoeba* species [16, 48] and *Toxoplasma gonodii* [49] appear to be important virulence factors and cause the pathogenesis by degrading ECM components. The strong activity of the *L. donovani* secreted protease against collagen type IV thus could facilitate tissue invasion by the *L. donovani* parasites. The broad substrate specificity and strong activity of the serine protease purified in the present study indicated a possible role of the enzyme in the invasion of the parasite to the host and thus for the pathogenesis of visceral leishmaniasis. It may be mentioned that the proteolytic degradation of fibrinogen and collagen type IV by the *L. donovani* extracellular protease was not similar compared to that of plasmin [50] and collagenase [51].

It is also noteworthy that the specific activity of the enzyme using hemoglobin as the substrate appears to be significant in comparison to the other studied natural substrates. For growth and nutrition, the *Leishmania* parasite requires hemin, which they acquire from hemoglobin [52, 53]. The hemoprotein is internalized by promastigotes in vesicles in the flagellar pocket membrane and, subsequently, degraded by fusion with other vesicles [53]. However, it is unclear whether or not the cell membrane attachment of the hemoprotein requires modification by the secreted protease. Thus, the degradation of hemoglobin and IgG by the *L. donovani* extracellular proteases led us to postulate other roles for the protease e.g. in nutrition and evasion of host's immune system as has been described for the poteases of *S. mansoni* and *S. solidus* plerocercoids [44, 54].

TAME appeared to be the suitable substrate for the *L. donovani* secreted protease, suggesting that this enzyme is trypsin-like protease. Chymotrypsin-like serine proteases utilize substrates, which have phenylalanine and tyrosine at the P1 site. Since the parasite secreted protease can hydrolyze CBPPNA and BTPNA that contain phenyl alanine and tyrosine, respectively, at the P1 site, it appears conceivable that the enzyme also behaves as chymotrypsin-like serine protease suggesting its broad substrate specificity.

The *Leishmania* secreted serine protease was almost completely inhibited by aprotinin, while relatively low but discernible inhibition by benzamidine and TPCK confers this extracellular serine prtotease in a selective class of serine protease. Aprotinin and benzamidine are inhibitors of trypsin-like serine protease while TPCK is specific for chymotrypsin-like serine protease, since it has phenylalanine linked to the chloromethyleketone group. Moreover, the mild sensitivity of the enzyme toward PMSF indicates that the enzyme could be trypsin-like serine protease since PMSF reacts slowly with trypsin [8]. Thus, the *L. donovani* extracellular protease appears to have broad substrate specificity.

The ability of the classical mammalian serine protease is modified by divalent metal ions. Pancreatic trypsin and chymotrypsin require Ca^{2+} for peptide bond hydrolysis [55]. In contrast, the presence of Ca^{2+} ion decreased the activity of the secretory serine protease of *L. donovani*. So, the secretory enzyme seems to be negatively modulated by Ca^{2+}. The difference between the *Leishmania* serine protease and the mammalian enzymes appears significant. Ca^{2+} is known to play important roles in the transformation of *Leishmania* promastigotes to amastigotes during temperature changes [56] and in *Leishmania*-macrophage attachment process [57]. In this context, it may be worthy to mention that the secretory protease of *L. donovani* could be involved in some functions of the parasite physiology and pathophysiology rather than host parasite interactions, because the enzyme activity has been determined to be significantly inhibited by Ca^{2+} [8].

Enzymes released in host's blood-stream play major roles in disease pathology. An alkaline peptidase from *Trypanosoma cruzi* cleaves and activates cytoplasmic factors involved in calcium signaling mechanisms that mediate parasite invasion of mammalian cells [58]. Cysteine proteases are important in the pathogenesis of several diseases and have been identified as targets for a structure-based strategy for drug design [59]. Cruzipain, a major cysteine protease of *T. cruzi*, has been selected as a model for anti-parasite drug development [60]. In *T. congolense*, Fish et al. [61] described a life cycle regulated cysteine protease of pathological importance. Thus, it appears conceivable that different classes of proteases have significant role in the pathogenesis and, therefore, could be potential targets for drug therapy.

In summary, we have identified, purified, and characterized a novel aprotinin sensitive serine protease in the culture supernatant of the Indian strain of *L. donovani*. The novelty of the protease has been ascertained by the following characteristics of the enzyme: (i) the enzyme exhibits optimum activity at pH 7.5 and 28°C, respectively, having pI of 5.0; (ii) the enzyme does not possess any carbohydrate moiety suggesting that it is non glycosylated and typically a secretory protease, and not arises due to the rupture of the parasite; (iii) TAME and aprotinin are, respectively, the appropriate substrate and inhibitor of the enzyme, indicating that the enzyme is trypsin-like, although it has a tendency to behave chymotrypsin-like

since TPCK inhibits the enzyme to a marked extent; (iv) Ca^{2+} negatively modulated, while Zn^{2+} positively regulated the enzyme activity. Since Ca^{2+} is known to require for macrophage attachment of the parasite, it appears that the enzyme could be involved in different functions in the parasite physiology or other pathophysiological processes; (v) the enzyme cannot hydrolyze myoglobin and fibronectin. But, it hydolyzed hemoglobin more readily than collagen type IV, fibrinogen, and IgG. This indicates that the protease helps to make available hemin from hemoglobin for the growth of the parasite; and (vi) the secretory protease exhibits properties to degrade the ECM components and IgG, thus indicating the role of this protease in invasion of host as well as evasion of host's immune response by the *L. donovani* promastigotes.

The pathogenic *Leishmania* infection is probably a multistep process that includes adhesions, degradation, and invasion. Adhesion is the primary step, followed by invasion and degradation of extracellular matrix proteins and host cells. *Leishmania* is known to release some proteases-like histidine acid phosphatase, a filamentous proteoglycan modified phosphomonoesterase [5] and the surface metalloprotease gp63 [6] to the extracellular environment. These enzymes employ different mechanisms in order to reach the extracellular milieu. The class of proteases responsible for host protein degradation has been demonstrated by several independent studies but the pathophysiological role of the *L. donova*ni serine protease is currently not clear and needs further exploration.

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