

A zymographic study of metalloprotease activities in extracts and extracellular secretions of *Leishmania (Viannia) braziliensis* strains

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SUMMARY

Proteolytic activities of 5 strains of *Leishmania (Viannia) braziliensis* isolated from Brazilian and Colombian patients, presenting distinct clinical manifestations, were characterized and compared using whole-promastigote extracts and extracellular secretions. Zymographic assays concerning whole-cell extracts and supernatants resulted in the detection of high molecular weight bands, ranging from 50 to 125 kDa. Proteolytic activities from both whole-cell extracts and supernatants were optimal in a pH range 5.5 to 9.0 for all analysed strains. Such protease activities were inhibited when 10 mM 1,10-phenanthroline was assayed, strongly suggesting that the enzymes responsible for hydrolysis of the substrate belong to the metalloproteases class. Distinct profiles of metalloproteases were observed among the studied *L. (V.) braziliensis* strains. Differences among the microorganisms might be related to the geographical origin of the strains and/or to the clinical presentation.

Key words: parasitic protozoa, trypanosomatids, *Leishmania (Viannia) braziliensis*, leishmaniasis, proteases, metalloproteases.

INTRODUCTION

The genus *Leishmania* includes parasitic protozoa responsible for a species-dependent spectrum of diseases extending from localized, self-healing cutaneous lesions to more severe visceral infections. In endemic areas of the Americas, *Leishmania (Viannia) braziliensis* is the major causative agent of tegumentary leishmaniasis (Grimaldi, Tesh and McMahan-Pratt, 1989; Canto-Lara *et al.* 1998). This parasite is genetically heterogeneous (Cupolillo *et al.* 2003), exhibiting substantial enzyme polymorphism. This protozoa has been associated to a broad range of clinical manifestations – from simple cutaneous ulcers to disseminated lesions and mucosal involvement, a very destructive form of leishmaniasis (Saravia *et al.* 1985; Marsden, 1986; Weigle *et al.* 1986; Grimaldi and Tesh 1993; Cupolillo *et al.* 2003). The plasticity of *L. (V.) braziliensis* and its interaction with the human host, producing distinct

clinical forms, represents a challenge to researchers looking for a better understanding of the infection's natural history.

The life-cycle of *Leishmania* parasites involves 2 principal developmental stages: the amastigote form, usually confined in mammalian macrophages, and the promastigote form, found within the sand fly insect vector. The digenetic parasite's mechanism of differentiation presented in diverse vertebrate and invertebrate hosts requires the parasite to possess the enzymatic tools necessary for quick and efficient response to dramatic environmental change. Proteases have been implicated to play crucial roles in parasitic cytoadherence, tissue invasion activities, survival and proliferation in host cells and virulence (Coombs, 1982; McGrath *et al.* 1995; Klemba and Goldberg, 2002; Sajid and McKerrow, 2002). Both metalloproteases and cysteine proteases have been implicated as putative determinants for infection and virulence factors of several species of *Leishmania* (Coombs, 1982; Chang and Chang, 1986; Russell and Wilhelm, 1986; Liu and Chang, 1992; Brittingham *et al.* 1995; Mottram *et al.* 1996, Mottram, Brooks and Coombs, 1998; Mahmoudzadeh-Niknam and McKerrow, 2004). In addition, differential expression of protease

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Table 1. Details of *Leishmania (Viannia) braziliensis* strains isolated from patients with diverse clinical presentations of tegumentary leishmaniasis

Isolates*	Zymodeme	Clinical manifestation	Geographical origin
MHOM/CO/1988/1407 C	1·1†-Z-108	Cutaneous	South East, Colombia
MHOM/CO/1988/1407 M	1·1†-Z-108	Mucosal	South East, Colombia
IOC-L 2463, MHOM/BR/2001/JOLIVAL	Z-27§-1·0	Mucosal	Bahia State, Brazil
IOC-L 2483, MHOM/BR/2000/LTCP 13396	Z-27§-1·0	Disseminated	Bahia State, Brazil
IOC-L 2481, MHOM/BR/2000/LTCP 13490	Z-27§-1·0	Cutaneous	Bahia State, Brazil

* The strains 1407 C and 1407 M were isolated from the same patient, the strains IOC-L 2463, IOC-L 2483, IOC-L 2481 were isolated from 3 distinct patients.

† Zymodeme classification adopted at the CIDEIM.

§ Zymodeme classification adopted at the Instituto Oswaldo Cruz.

profiles in virulent and avirulent promastigotes of *Leishmania (L.) amazonensis* has been described (de Araujo-Soares *et al.* 2003). In this manuscript, the protease activities of various *Leishmania (V.) braziliensis* strains, from Brazilian and Colombian patients presenting diverse clinical manifestations, were characterized and compared using whole-promastigote extracts and extracellular secretions.

MATERIALS AND METHODS

Chemicals

All chemicals were purchased from Sigma Chemical Company (USA). Stock solutions of 2,2-dipyridyl (20 mM), 1,10-phenantroline (200 mM) and pepstatin A (1 mg/ml) were solubilized in ethanol, whereas E-64 (6 mM), EDTA (500 mM) and EGTA (500 mM) were dissolved in water. PMSF (250 mM) was diluted in isopropanol, and TLCK (10 mM) was stocked in methanol. All these protease inhibitors were maintained at -20°C .

L. (V.) braziliensis strains and culture

Five *Leishmania (V.) braziliensis* strains were used: (i) 2 were isolated from both cutaneous and mucosal lesions of 1 Colombian patient (International Center for Training and Medical Research, CIDEIM, Cali, Colombia, kindly provided by Dr Nancy Saravia) and (ii) 3 from cutaneous, disseminated, and mucosal lesions of different patients (University Hospital of Federal University of Bahia, Brazil, strains deposited in the *Leishmania* Type Culture Collection, Instituto Oswaldo Cruz by Dr Edgar Carvalho). Clinical presentation, geographical origin and strain identity are summarized in Table 1. The promastigote forms of the parasite were grown at 25°C in Schneider's medium supplemented with 20% (v/v) heat-inactivated fetal bovine serum with passes every 4 days until reaching a density of 1×10^9 cells/ml. The cells were then harvested in late log phase of growth by centrifugation at 3000 g for 5 min and washed

twice in 0·01 M phosphate-buffered 0·9% NaCl (PBS), pH 7·2. Parasite density was estimated by counts in a haemocytometer, and the viability was assessed using the Trypan blue dye exclusion test.

Zymographic assays

Protease activities present both in whole-parasites and extracellular secretions were investigated as previously described (Melo-Braga, da Rocha-Azevedo and Silva-Filho, 2003). Briefly, for proteolytic activities in whole-parasite extracts, 1×10^9 promastigotes were disrupted in 1% Triton X-100 containing 10 mM Tris-HCl at pH 6·8. For the investigation of the secreted protease analyses, 1×10^9 promastigotes were washed 3 times in cold PBS pH 7·2, resuspended in 1 ml of RPMI 1640 medium plus 25 mM HEPES and incubated for 30 min to 3 h at 25°C . Afterwards, cell suspensions were centrifuged, and the resulting supernatants were filtered in a $0\cdot22 \mu\text{m}$ pore membrane filter. Both whole-parasite extracts as well as filtered supernatants were fractionated on SDS-PAGE (10%) copolymerized with 0·1% fish gelatin (Lockwood *et al.* 1987). The gels were loaded with 30 μg of protein per slot. Following electrophoresis, the resulting gels were washed twice for 30 min at 4°C in 0·1 M sodium acetate buffer pH 3·5, 5·5, 7·0 or 9·0 containing 2·5% Triton X-100. Protease activity was detected by incubating the gels in the reaction buffer (0·1 M sodium acetate, 1 mM DTT) at pH 3·5, 5·5, 7·0 or 9·0 at 37°C during 4 or 24 h in the case of extracts or for 48 h in the case of supernatants. The resulting bands were visualized by staining the gels with 0·2% (w/v) Coomassie blue R-250, 50% (v/v) trichloroacetic acid and 10% (v/v) acetic acid. The approximate molecular weight for zymographic studies was calculated using a protein commercial molecular weight standard. All experiments were performed in triplicate, with similar results obtained in at least 3 separate cell suspensions.

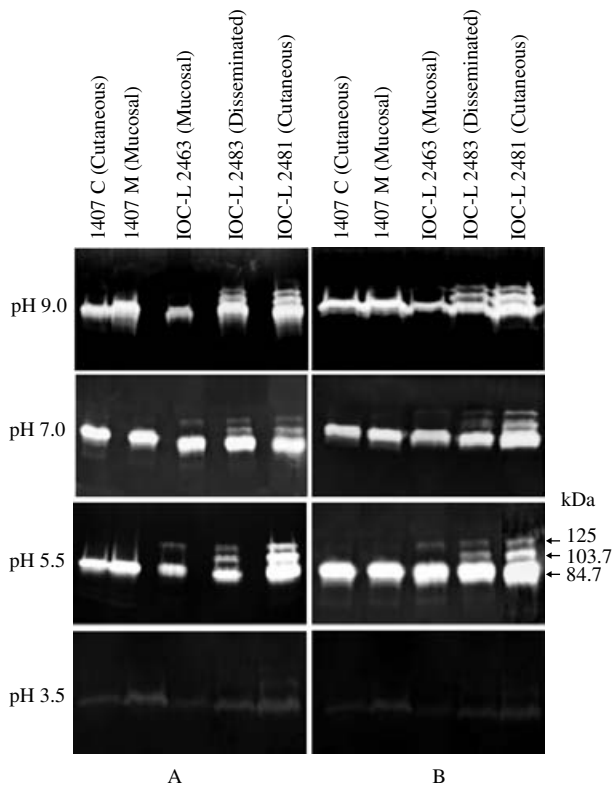


Fig. 1. Zymographic profiles and influence of pH on the protease activities of *Leishmania (V.) braziliensis* strains. Enzymatic activities detectable in each (A) whole-promastigote extracts after 4 h of reaction and (B) extracellular medium after 48 h of reaction.

Inhibition assays

The lysates of both promastigotes and supernatants were prepared as aforementioned. Each of the following protease inhibitors was added to the reaction buffer: 10 μ M E-64; 10 mM EDTA; 10 mM EGTA; 500 μ M PMSF; 10 μ M TLCK; 5 μ M pepstatin A and 10 mM 1,10-phenanthroline for 4 or 48 h for protease activities of extract and supernatant, respectively. Hydrolysis was detected as previously described.

RESULTS

Protease activities

Both extracts and supernatants from promastigotes of *L. (V.) braziliensis* were assessed for protease activities using gelatin SDS-PAGE. The whole-cell extracts of Colombian strains, 1407 cutaneous and 1407 mucosal isolated from the same patient, presented the same protease activity profile, with a prominent band of 84.7 kDa (Fig. 1A). The Brazilian strain, IOC-L 2463 isolated from a patient with mucosal leishmaniasis, displayed a protease profile with 2 bands of 84.7 and 125 kDa. Two clones of this strain were assayed and the same proteolytic activity profile was observed (data not shown). The strains isolated from disseminated (IOC-L 2483) and

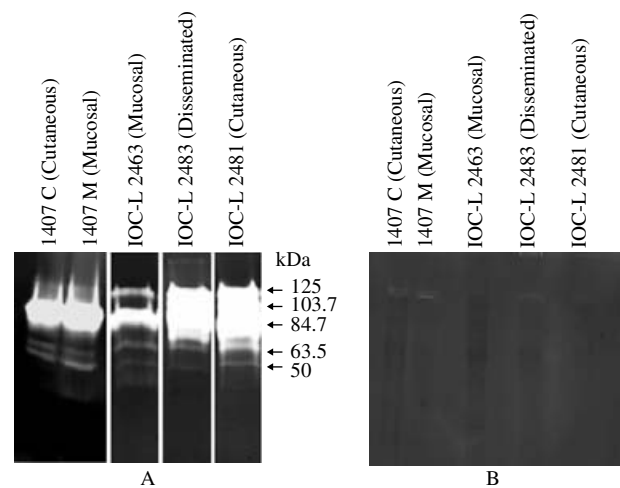


Fig. 2. Zymographic analysis of proteases obtained from extracts of whole-promastigotes after 24 h of reaction at pH 5.5 and 37 °C in the absence (A) of protease inhibitor and in the presence (B) of 10 mM 1,10-phenanthroline. Two additional bands between 50 and 65 kDa can be observed in all lanes in the absence of protease inhibitor.

cutaneous (IOC-L 2481) cases from the same locality in Brazil exhibited an extra band of 103.7 kDa (Fig. 1A). In addition, the reference strain (MHOM/BR/1973/M2903) was also analysed and an identical band profile to the disseminated (IOC-L 2483) and cutaneous (IOC-L 2481) strains was observed (data not shown). In order to verify the effect of pH on proteolytic profiles, a range of pH was evaluated (Fig. 1). Proteolytic activities of promastigote extracts were optimal at pH 5.5 for all the analysed strains. At pH 7.0 and pH 9.0 no significant increases or decreases in those enzymatic activities could be detected, and at pH 3.5, all activities were substantially reduced (Fig. 1A). All activities were verified after 4 h of proteolysis in reaction buffer. To check the possibility of protease secretion to extracellular medium, 10⁹ living promastigotes were incubated in RPMI medium without fetal bovine serum for a period of 3 h. Subsequently, the suspension was centrifuged for cell removal, and the supernatant was submitted to zymographic assays. The profile of secreted proteases was identical to that observed for whole-cell extracts in both Brazilian and Colombian strains. Nevertheless, when the secretion products from the parasites were loaded onto gelatin gels, enzyme activities were only detected after 48 h of incubation in reaction buffer (Fig. 1B).

To evaluate the possible presence of other proteases expressed in low concentration or with little activity, whole promastigote extracts were incubated for 24 h in reaction buffer pH 5.5. Post-incubation, at least 2 additional weak bands between 50 and 65 kDa were observed (Fig. 2A).

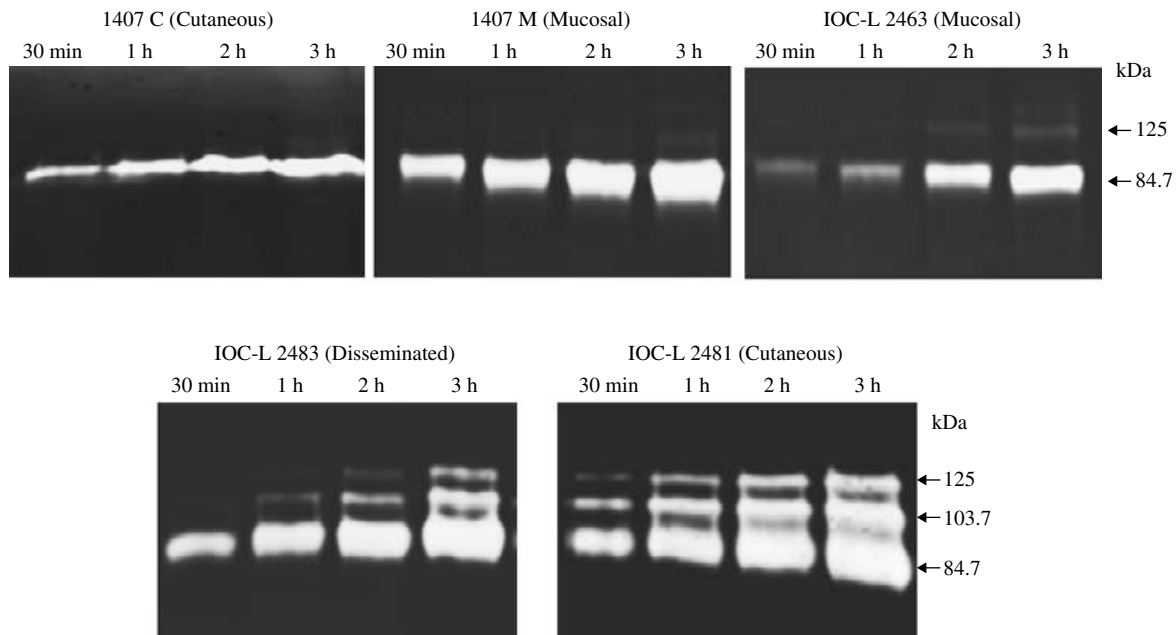


Fig. 3. Extracellular proteases secreted by *Leishmania (V.) braziliensis*. Supernatants of living parasites were obtained and assayed as detailed in the Materials and Methods section. Secreted proteases were detected after 30 min and 1, 2 and 3 h of reaction with fish gelatin substrate. A progressive increase of protease activities was observed for all analysed strains. Parasite viability $\geq 95\%$ was assessed before, during and after the time-course.

Time-course of secreted proteases

In order to check whether or not the release of proteases into the extracellular medium might possibly vary throughout the incubation times of the parasite in RPMI medium, 4 culture supernatants, each one corresponding to 10^9 parasites/ml, were collected after 30 min, 1 h, 2 h and 3 h of parasite incubation in serum-depleted RPMI medium. After 48 h of incubation in reaction buffer, proteolytic activities increased progressively from 30 min to 3 h of secretion (Fig. 3). After 30 min, the first band to appear in all strains was the 84.7 kDa. In the Brazilian IOC-L 2481 cutaneous strain, the 2 additional bands of 103.7 kDa and 125 kDa also appeared at this stage. These 2 bands were visualized clearly in the IOC-L 2483 disseminated strain just after 2 h of secretion (Fig. 3). In the mucosal IOC-L 2463 strain, the second band of 125 kDa was visualized only after 3 h of secretion (Fig. 3). No change in the molecular weight of the protease activities was observed during the time-course of secretion. In these assays, the viability and motility were tested before, during and after 3 h of parasite incubation in RPMI medium as previously described.

Inhibition assays

To characterize the classes of the observed proteases in *L. (V.) braziliensis*, several compounds known to inhibit different proteases were tested for their effects on the activity of both extract and supernatant proteases. It was found that 10 μM E-64, 10 mM EDTA, 10 mM EGTA; 500 μM PMSF; 10 μM TLCK and

5 μM pepstatin A had no inhibitory effect on the activity of promastigote extract and secreted proteases (Fig. 4). In contrast, 10 mM 1,10-phenanthroline, a strong and specific metalloprotease inhibitor, completely abrogated protease activities in all *L. (V.) braziliensis* strains (Fig. 4). This inhibitor also completely abrogated the 2 additional activities between 50 and 65 kDa (Fig. 2B). The fact that the parasite extract and secreted protease activities were blocked by 1,10-phenanthroline indicates that these enzymes are metalloproteases or metal-dependent proteases.

SDS-PAGE analysis

Analysis of both whole-promastigote extract and supernatant extractions was performed by SDS-PAGE and silver staining. Neither in the extract nor supernatant protein profile was any difference observed (Fig. 5).

DISCUSSION

Proteases of protozoan parasites play major roles in infection, survival and pathogenicity (Rosenthal, 1999; Sajid and McKerrow, 2002). These enzymes have been found in several *Leishmania* species (Fong and Chang, 1981; North and Coombs, 1981; Coombs, 1982; Pupkis and Coombs, 1984) and are involved in the establishment of intracellular parasitism as well as in the inhibition of host immune responses (Chang and Chang, 1986; Russell and Wilhelm, 1986; Liu and Chang, 1992; Mottram *et al.* 1996; Alexander, Combs and Mottram, 1998;

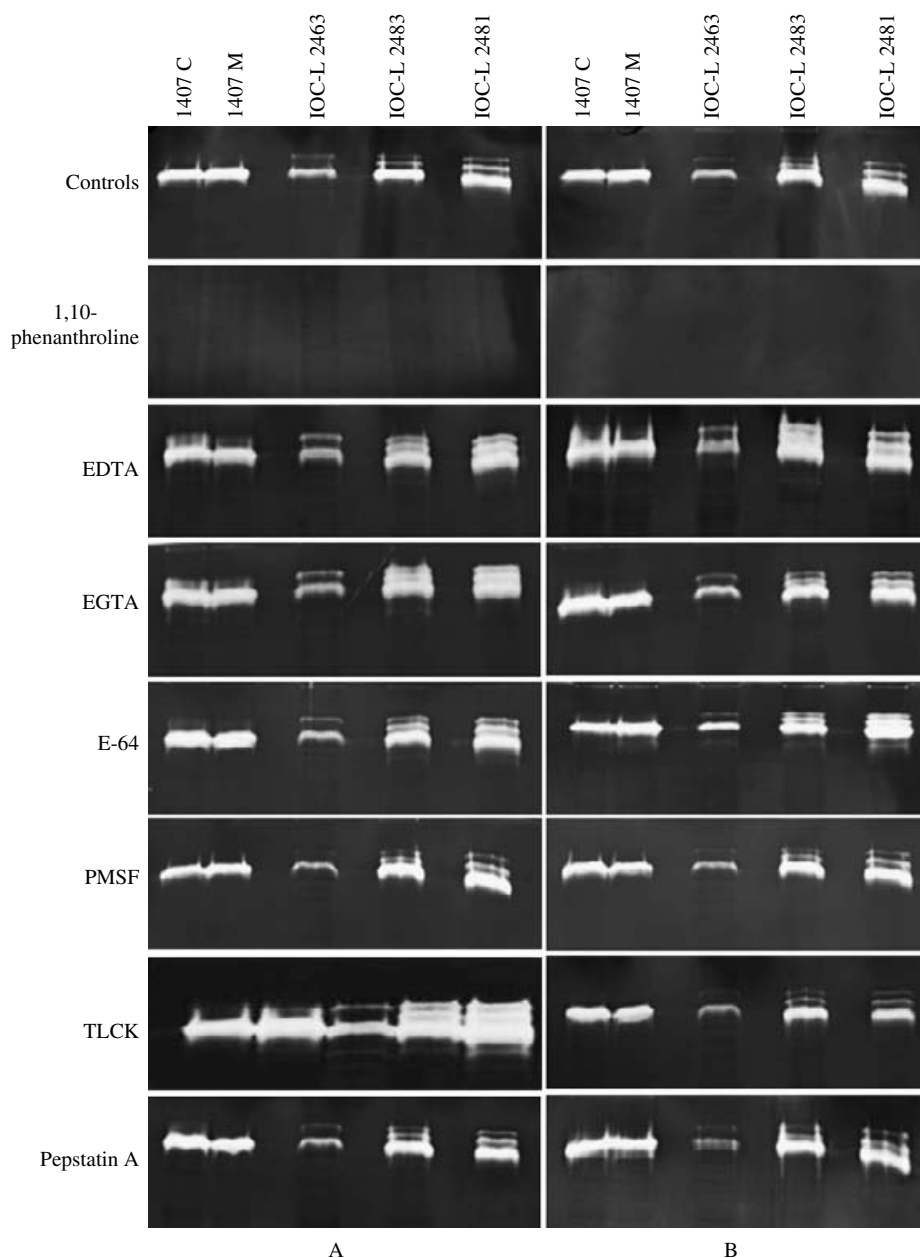


Fig. 4. Protease activities detected in (A) whole-promastigotes extracts and (B) the supernatants of *Leishmania (V.) braziliensis* strains in the absence (controls) or presence of 10 mM 1,10-phenanthroline, 10 mM EDTA, 10 mM EGTA, 10 μ M E-64, 500 μ M PMSF, 10 μ M TLCK, 5 μ M pepstatin A, for 4 or 48 h for protease activities of extract and supernatant, respectively.

Buxbaum *et al.* 2003). The cysteine, serine and metalloproteases activities of *Leishmania (Leishmania)* species have been well explored and described. In contrast, description of protease profiles of *Leishmania (Viannia)* is scarce, demanding further investigation (Leon *et al.* 1994).

Gp63 is the most abundant surface metalloprotease in *Leishmania* spp. (Etges, Bouvier and Bordier, 1986) and is expressed in both amastigote and promastigote stages. It is more abundant in the promastigotes, being released into the extracellular environment (Gardiner, Jaffe and Dwyer, 1984; Bouvier, Etges and Bordier, 1985; Colomer-Gould *et al.* 1985; Etges *et al.* 1985; Medina-Acosta *et al.*

1989; Medina-Acosta, Karess and Russell, 1993; McGwire *et al.* 2002; Jaffe and Dwyer, 2003). Gp63 is specifically involved in evasion of humoral lytic factors by rendering promastigotes resistant to complement-mediated cytotoxicity (Brittingham *et al.* 1995); attachment of promastigotes to macrophages via receptor-mediated endocytosis (Chang and Chang, 1986; Russell and Wilhelm, 1986); protection against degradation within macrophage phagolysosome (Chaudhuri *et al.* 1989) and migration through the extracellular matrix (McGwire, Chang and Engman, 2003).

Our results demonstrate that all analysed strains of *L. (V.) braziliensis* express abundant proteolytic

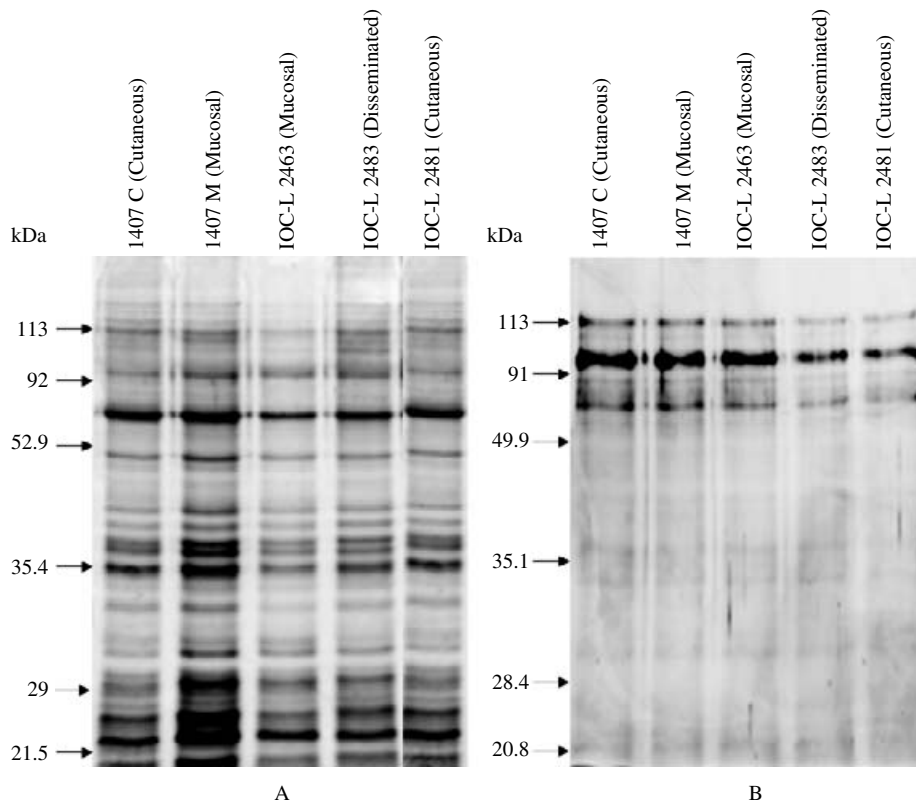


Fig. 5. SDS-PAGE of whole-promastigote extracts (A) and supernatants (B) of *Leishmania (V.) braziliensis*.

activities between 84.7 and 125 kDa, probably corresponding to metal-dependent proteases as hydrolysis bands were abrogated by 1,10-phenanthroline – a potent inhibitor of metalloproteases. However, none of the herein enzymatic activities were affected either by EGTA or EDTA, both of which have been considered to be metalloprotease inhibitors of eukaryotic cells (Beynon and Bond, 2001), including parasitic protozoa. We, as yet, do not have a clear explanation to this fact. Nevertheless, we cannot exclude the possibility that the enzymes might be in such a configuration that during our reported assays the active sites of the enzymes may have been inaccessible to the chelators. Such an issue is under investigation.

The occurrence of metalloprotease activities in promastigotes of *L. (V.) braziliensis* as well as other *Leishmania* species (de Araujo-Soares *et al.* 2003), may be clearly observed among parasites collected from the late log phase of growth. It must be pointed out that in zymographic studies, the proteins are not completely denatured and reduced, and therefore, the molecular weight of proteolytic activities is estimated, thus perhaps not representing the true protease weight.

Considering that the supernatant after centrifugation and filtration displayed the same zymographic profile as the extract and that there was a progressive increase of protease activity during the secretion time-course, the presence of 2 different forms of this metalloprotease was demonstrated, an

intracellular form and a soluble form which was secreted into the extracellular environment. The release of other enzymes, including metalloprotease gp63, has been reported in *Leishmania* promastigotes (Bates, Hermes and Dwyer, 1989; Jacobson and Schlein, 1993; Blum and Opperdoes, 1994; McGwire *et al.* 2002; Yao *et al.* 2002; Jaffe and Dwyer, 2003).

Although the tested range of pH was suitable for serine, cysteine and metalloprotease detection (de Araujo-Soares *et al.* 2003; Silva-Lopez *et al.* 2004; Alves *et al.* 2005), only the third was observed, suggesting either metalloprotease predominance or that the other two were at extremely low levels, being undetectable and/or not expressed in promastigotes. In addition, it is important to emphasize that this metalloprotease activity as well as the inexistence of other proteases is limited to gelatin digestion. Therefore, we cannot exclude the possibility of *L. (V.) braziliensis* possessing cysteine proteases of which the activities might be detectable using other substrates. In this context, some proteases, such as cysteine and metalloproteases, have been characterized as developmentally regulated both in *Leishmania* spp. (Medina-Acosta *et al.* 1989; Robertson and Coombs, 1992; Medina-Acosta, Karess and Russell, 1993; Leon *et al.* 1994) and in other parasites (Kembla and Goldberg, 2002; Coppi *et al.* 2005).

Two additional hydrolysis bands between 50 and 65 kDa were observed in whole-cell extract assays

when the reaction time was increased to 24 h, and these activities were also inhibited by 1,10-phenanthroline, similar to gp63. Finally, attention must be given to the fact that we cannot exclude the possibility of the bands located between 50–65 kDa, as well as any other of the bands observed, being the result of post-translational modification of the same protein.

The time of isolation and maintenance in culture did not influence the protease profiles observed in this study. The reference strain of *L. (V.) braziliensis*, which has been maintained in culture for several years, exhibits the same profile as the other Brazilian strains. These strains were passed in hamsters and were all cryopreserved while still infective (3rd passage). For the analysis conducted in this study, the stocks were maintained *in vitro* for less than 2 months while being processed. The Colombian strains exhibiting a different protease profile were maintained *in vitro* for at least 5 years, and they are no longer infective, considering that they produced no lesions whatsoever in hamsters under observation for up to 9 months (CIDEIM, unpublished data).

The nutritional requirement of the parasite in the insect vector has been suggested as the primary function for gp63 in *Leishmania* as well as other trypanosomatids (Etges, 1992; Schlein, 1993; Schneider and Glaser, 1993; Jaffe and Dwyer, 2003). It has recently been demonstrated that down-regulation of the metalloprotease gp63 affects the early development of *L. (L.) amazonensis* in experimental infections of *Lutzomyia longipalpis* (Hajmová *et al.* 2004). Considering these results, it is possible to hypothesize that differences observed among the metalloprotease profiles of the Colombian and Brazilian strains analysed herein might indicate geographical specialization of such enzymes to distinct vectors involved in the transmission of the parasite in these countries. The Brazilian and Colombian strains belonging to distinct zymodemes (IOC/Z27 and IOC/Z108 respectively) differ in 3 of the 18 loci employed in the MLEE characterization (unpublished results). These zymodemes probably correspond to clones adapted to different environmental conditions (Cupolillo *et al.* 2003). In Brazil, *L. (V.) braziliensis* is transmitted by *L. intermedia* and *L. whitmani*, whereas in Colombia *L. spinacrasa* is the incriminated vector for this *Leishmania* species (Young *et al.* 1987). Finally, we hypothesize that the invasive and immunomodulatory roles attributed to metalloproteases and other proteases in several protozoan infections (Bontempi and Cazzulo, 1990; Brittingham *et al.* 1999; Que and Reed, 2000; Buxbaum *et al.* 2003; McGwire *et al.* 2003) may be reflected in the profiles encountered among the Brazilian mucosal, cutaneous and disseminated strains isolated from different patients in the same geographical region and from the same zymodeme.

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REFERENCES

- Alexander, J., Coombs, G. H. and Mottram, J. C.** (1998). *Leishmania mexicana* cysteine proteinase-deficient mutants have attenuated virulence for mice and potentiate a Th1 response. *Journal of Immunology* **161**, 6794–6801.
- Alves, C. R., Corte-Real, S., Bourguignon, S. C., Chaves, C. S. and Saraiva, E. M.** (2005). *Leishmania amazonensis*: early proteinase activities during promastigote-amastigote differentiation *in vitro*. *Experimental Parasitology* **109**, 38–48.
- Bates, P. A., Hermes, I. and Dwyer, D. M.** (1989). *Leishmania donovani*: immunochemical localization and secretory mechanism of soluble acid phosphatase. *Experimental Parasitology* **68**, 335–346.
- Beynon, R. and Bond, J. S.** (2001). *Proteolytic Enzymes*, 2nd Edn. Oxford University Press, New York.
- Blum, J. J. and Opperdoes, F. R.** (1994). Secretion of sucrase by *Leishmania donovani*. *Journal of Eukaryotic Microbiology* **41**, 228–231.
- Bontempi, E. and Cazzulo, J. J.** (1990). Digestion of human immunoglobulin G by the major cysteine proteinase (cruzipain) from *Trypanosoma cruzi*. *FEMS Microbiology Letters* **58**, 337–341.
- Bouvier, J., Etges, R. J. and Bordier, C.** (1985). Identification and purification of membrane and soluble forms of the major surface protein of *Leishmania* promastigotes. *The Journal of Biological Chemistry* **260**, 15504–15509.
- Brittingham, A., Morrison, C. J., McMaster, W. R., McGwire, B. S., Chang, K. P. and Mosser, D. M.** (1995). Role of the *Leishmania* surface protease gp63 in complement fixation, cell adhesion, and resistance to complement-mediated lysis. *Journal of Immunology* **155**, 3102–3111.
- Brittingham, A., Chen, G., McGwire, B. S., Chang, K. P. and Mosser, D. M.** (1999). Interaction of *Leishmania* gp63 with cellular receptors for fibronectin. *Infection and Immunity* **67**, 4477–4484.
- Buxbaum, L. U., Denise, H., Coombs, G. H., Alexander, J., Mottram, J. C. and Scott, P.** (2003). Cysteine protease B of *Leishmania mexicana* inhibits host Th1 responses and protective immunity. *Journal of Immunology* **171**, 3711–3717.
- Canto-Lara, S. B., Cardenas-Maruffo, M. F., Vargas-Gonzalez, A. and Andrade-Narvaez, F.** (1998). Isoenzyme characterization of *Leishmania* isolated from human cases with localized cutaneous leishmaniasis from the State of Campeche, Yucatan Peninsula, Mexico. *American Journal of Tropical Medicine and Hygiene* **58**, 444–447.
- Chang, C. S. and Chang, K. P.** (1986). Monoclonal antibody affinity purification of a *Leishmania* membrane glycoprotein and its inhibition of leishmania-macrophage binding. *Proceedings of the National Academy of Sciences, USA* **83**, 100–104.

- Chaudhuri, G., Chaudhuri, M., Pan, A. and Chang, K. P.** (1989). Surface acid proteinase (gp63) of *Leishmania mexicana*. A metalloenzyme capable of protecting liposome-encapsulated proteins from phagolysosomal degradation by macrophages. *The Journal of Biological Chemistry* **264**, 7483–7489.
- Colomer-Gould, V., Glvao Quintao, L., Keithly, J. and Nogueira, N.** (1985). A common major surface antigen on amastigotes and promastigotes of *Leishmania* species. *The Journal of Experimental Medicine* **162**, 902–916.
- Coombs, G. H.** (1982). Proteinases of *Leishmania mexicana* and other flagellate protozoa. *Parasitology* **84**, 149–155.
- Coppi, A., Pinzon-Ortiz, C., Hutter, C. and Sinnis, P.** (2005). The *Plasmodium* circumsporozoite protein is proteolytically processed during cell invasion. *The Journal of Experimental Medicine* **201**, 27–33.
- Cupolillo, E., Brahim, L. R., Toaldo, C. B., de Oliveira-Neto, M. P., de Brito, M. E., Falqueto, A., de Farias Naiff, M. and Grimaldi, G. Jr.** (2003). Genetic polymorphism and molecular epidemiology of *Leishmania (Viannia) braziliensis* from different hosts and geographic areas in Brazil. *Journal of Clinical Microbiology* **41**, 3126–3132.
- De Araujo Soares, R. M., dos Santos, A. L., Bonaldo, M. C., de Andrade, A. F., Alviano, C. S., Angluster, J. and Goldenberg, S.** (2003). *Leishmania (Leishmania) amazonensis*: differential expression of proteinases and cell-surface polypeptides in avirulent and virulent promastigotes. *Experimental Parasitology* **104**, 104–112.
- Etges, R.** (1992). Identification of a surface metalloproteinase on 13 species of *Leishmania* isolated from humans, *Crithidia fasciculata*, and *Herpetomonas samuelpessoai*. *Acta Tropica* **50**, 205–217.
- Etges, R., Bouvier, J. and Bordier, C.** (1986). The major surface protein of *Leishmania* promastigotes is a protease. *The Journal of Biological Chemistry* **261**, 9098–9101.
- Etges, R. J., Bouvier, J., Hoffman, R. and Bordier, C.** (1985). Evidence that the major surface proteins of three *Leishmania* species are structurally related. *Molecular and Biochemical Parasitology* **14**, 141–149.
- Fong, D. and Chang, K. P.** (1981). Tubulin biosynthesis in the developmental cycle of a parasitic protozoan, *Leishmania mexicana*: changes during differentiation of motile and nonmotile stages. *Proceedings of the National Academy of Sciences, USA* **78**, 7624–7628.
- Gardiner, P. R., Jaffe, C. L. and Dwyer, D. M.** (1984). Identification of cross-reactive promastigote cell surface antigens of some leishmanial stocks by 125I labeling and immunoprecipitation. *Infection and Immunity* **43**, 637–643.
- Grimaldi, G. Jr. and Tesh, R. B.** (1993). Leishmaniasis of the New World: current concepts and implications for future research. *Clinical Microbiology Reviews* **6**, 230–250.
- Grimaldi, G. Jr., Tesh, R. B. and McMahon-Pratt, D.** (1989). A review of the geographic distribution and epidemiology of leishmaniasis in the New World. *American Journal of Tropical Medicine and Hygiene* **41**, 687–725.
- Hajmova, M., Chang, K. P., Kolli, B. and Volf, P.** (2004). Down-regulation of gp63 in *Leishmania amazonensis* reduces its early development in *Lutzomyia longipalpis*. *Microbes and Infection/Institut Pasteur* **6**, 646–649.
- Jacobson, R. L. and Schlein, Y.** (1997). Cellulase activity of *Leishmania major* in the sandfly vector and in culture. *Journal of Eukaryotic Microbiology* **44**, 216–219.
- Jaffe, C. L. and Dwyer, D. M.** (2003). Extracellular release of the surface metalloprotease, gp63, from *Leishmania* and insect trypanosomatids. *Parasitology Research* **91**, 229–237.
- Klemba, M. and Goldberg, D. E.** (2002). Biological roles of proteases in parasitic protozoa. *Annual Review of Biochemistry* **71**, 275–305.
- Leon, L. L., Temporal, R. M., Soares, M. J. and Grimaldi, G. Jr.** (1994). Proteinase activities during temperature-induced stage differentiation of species complexes of *Leishmania*. *Acta Tropica* **56**, 289–298.
- Liu, X. and Chang, K. P.** (1992). Extrachromosomal genetic complementation of surface metalloproteinase (gp63)-deficient *Leishmania* increases their binding to macrophages. *Proceedings of the National Academy of Sciences, USA* **89**, 4991–4995.
- Lockwood, B. C., North, M. J., Scott, K. I., Bremner, A. F. and Coombs, G. H.** (1987). The use of a highly sensitive electrophoretic method to compare the proteinases of trichomonads. *Molecular and Biochemical Parasitology* **24**, 89–95.
- Mahmoudzadeh-Niknam, H. and McKerrow, J. H.** (2004). *Leishmania tropica*: cysteine proteases are essential for growth and pathogenicity. *Experimental Parasitology* **106**, 158–163.
- Marsden, P. D.** (1986). Mucosal leishmaniasis (“espundia” Escomel, 1911). *Transactions of the Royal Society of Tropical Medicine and Hygiene* **80**, 859–876.
- McGrath, M. E., Eakin, A. E., Engel, J. C., McKerrow, J. H., Craik, C. S. and Fletterick, R. J.** (1995). The crystal structure of cruzain: a therapeutic target for Chagas’ disease. *Journal of Molecular Biology* **247**, 251–259.
- McGwire, B. S., O’Connell, W. A., Chang, K. P. and Engman, D. M.** (2002). Extracellular release of the glycosylphosphatidylinositol (GPI)-linked *Leishmania* surface metalloprotease, gp63, is independent of GPI phospholipolysis: implications for parasite virulence. *The Journal of Biological Chemistry* **277**, 8802–8809.
- McGwire, B. S., Chang, K. P. and Engman, D. M.** (2003). Migration through the extracellular matrix by the parasitic protozoan *Leishmania* is enhanced by surface metalloprotease gp63. *Infection and Immunity* **71**, 1008–1010.
- Medina-Acosta, E., Karess, R. E. and Russell, D. G.** (1993). Structurally distinct genes for the surface protease of *Leishmania mexicana* are developmentally regulated. *Molecular and Biochemical Parasitology* **57**, 31–45.
- Medina-Acosta, E., Karess, R. E., Schwartz, H. and Russell, D. G.** (1989). The promastigote surface protease (gp63) of *Leishmania* is expressed but differentially processed and localized in the amastigote stage. *Molecular and Biochemical Parasitology* **37**, 263–273.
- Melo-Braga, M. B., da Rocha-Azevedo, B. and Silva-Filho, F. C.** (2003). *Tritrichomonas foetus*: the role played by iron during parasite interaction with epithelial cells. *Experimental Parasitology* **105**, 111–120.

- Mottram, J. C., Brooks, D. R. and Coombs, G. H.** (1998). Roles of cysteine proteinases of trypanosomes and *Leishmania* in host-parasite interactions. *Current Opinion in Microbiology* **1**, 455–460.
- Mottram, J. C., Souza, A. E., Hutchison, J. E., Carter, R., Frame, M. J. and Coombs, G. H.** (1996). Evidence from disruption of the *lmcpb* gene array of *Leishmania mexicana* that cysteine proteinases are virulence factors. *Proceedings of the National Academy of Sciences, USA* **93**, 6008–6013.
- North, M. J. and Coombs, G. H.** (1981). Proteinases of *Leishmania mexicana* amastigotes and promastigotes: analysis by gel electrophoresis. *Molecular and Biochemical Parasitology* **3**, 293–300.
- Pupkis, M. F. and Coombs, G. H.** (1984). Purification and characterization of proteolytic enzymes of *Leishmania mexicana mexicana* amastigotes and promastigotes. *Journal of General Microbiology* **130**, 2375–2383.
- Que, X. and Reed, S. L.** (2000). Cysteine proteinases and the pathogenesis of amebiasis. *Clinical Microbiology Reviews* **13**, 196–206.
- Robertson, C. D. and Coombs, G. H.** (1992). Stage-specific proteinases of *Leishmania mexicana mexicana* promastigotes. *FEMS Microbiology Letters* **73**, 127–132.
- Rosenthal, P. J.** (1999). Proteases of protozoan parasites. *Advances in Parasitology* **43**, 105–159.
- Russell, D. G. and Wilhelm, H.** (1986). The involvement of the major surface glycoprotein (gp63) of *Leishmania* promastigotes in attachment to macrophages. *Journal of Immunology* **136**, 2613–2620.
- Sajid, M. and McKerrow, J. H.** (2002). Cysteine proteases of parasitic organisms. *Molecular and Biochemical Parasitology* **120**, 1–21.
- Saravia, N. G., Holguin, A. F., McMahon-Pratt, D. and D'Alessandro, A.** (1985). Mucocutaneous leishmaniasis in Colombia: *Leishmania braziliensis* subspecies diversity. *American Journal of Tropical Medicine and Hygiene* **34**, 714–720.
- Schlein, Y.** (1993). *Leishmania* and sandflies: interactions in the life-cycle and transmission. *Parasitology Today* **9**, 255–258.
- Schneider, P. and Glaser, T. A.** (1993). Characterization of two soluble metalloexopeptidases in the protozoan parasite *Leishmania major*. *Molecular and Biochemical Parasitology* **62**, 223–231.
- Silva-Lopez, R. E., Morgado-Diaz, J. A., Alves, C. R., Corte-Real, S. and Giovanni-De-Simone, S.** (2004). Subcellular localization of an extracellular serine protease in *Leishmania (Leishmania) amazonensis*. *Parasitology Research* **93**, 328–331.
- Weigle, K. A., Saravia, N. G., de Davalos, M., Moreno, L. H. and D'Alessandro, A.** (1986). *Leishmania braziliensis* from the Pacific coast region of Colombia: foci of transmission, clinical spectrum and isoenzyme phenotypes. *American Journal of Tropical Medicine and Hygiene* **35**, 722–731.
- Yao, C., Leidal, K. G., Brittingham, A., Tarr, D. E., Donelson, J. E. and Wilson, M. E.** (2002). Biosynthesis of the major surface protease GP63 of *Leishmania chagasi*. *Molecular and Biochemical Parasitology* **121**, 119–128.
- Young, D. G., Morales, A., Kreutzer, R. D., Alexander, J. B., Corredor, A., Tesh, R. B., Ferro de Carrasquilla, C. and de Rodriguez, C.** (1987). Isolation of *Leishmania braziliensis* (Kinetoplastida: Trypanosomatidae) from cryopreserved Colombian sand flies (Diptera: Psychodidae). *Journal of Medical Entomology* **24**, 587–589.