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Identification and Partial Characterization of an Extracellular Acid Phosphatase Activity of *Leishmania donovani* Promastigotes

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An extracellular acid phosphatase was detected in the growth media of *Leishmania donovani* promastigotes. The enzyme was released at all stages of the growth cycle and in amounts which accounted for 90% of the total amount of this enzyme in the culture. The exoenzyme exhibited a pH optimum of 4.5 to 5.0 and was active with a variety of organic phosphates. The enzymatic activity was excluded from Sephacryl S-300 and was retained by ultrafilters with nominal molecular weight cutoffs of up to 300,000. The results of comparative studies indicated that the extracellular enzyme was distinct from a surface membrane-bound acid phosphatase of *L. donovani* promastigotes which has been previously described.

Live, intact promastigotes of the important human pathogen *Leishmania donovani* have the ability to hydrolyze impermeable organic phosphates, and this hydrolysis is catalyzed in part by a surface membrane-bound acid phosphomonoesterase (acid phosphatase; EC 3.1.3.2) which has been partially characterized (6). Further investigation of this activity has led to the observation that acid phosphatase is also readily detectable extracellularly in the growth media of promastigotes. We now report on the identification of this exoenzyme and its initial characterization. In particular, data on the comparison between the extracellular activity and the surface enzyme are presented.

An abstract of this work has been published previously (J. Protozool. 27:41A, 1981).

MATERIALS AND METHODS

Parasite maintenance and growth conditions. The strain of *L. donovani* used in these studies and its growth on fetal bovine serum-containing medium 199 have been described previously (6). In addition, the promastigotes were grown continuously on a chemically defined medium (24) with or without bovine serum albumin (BSA). For large-scale cultivation, the parasites were grown in 150 ml of medium in 150-cm^2 tissue culture flasks.

Isolation of surface membranes. The methods for the isolation and purification of surface membranes from promastigotes grown on serum-containing medium has been described previously (6).

Preparation and concentration of culture supernatants. Promastigotes were removed from the growth media by centrifugation at 6,000 × *g* for 20 min. Culture supernatants were filtered through a 0.22-μm membrane filter (Millipore Corp., Bedford, Mass.) before assay or concentration. Filtered media were concentrated by ultrafiltration with Amicon (Lexington, Mass.) filters and a stirring cell apparatus. The concentration was carried out at 4°C.

Enzyme assays. The acid phosphatase activity of membranes and culture supernatants was routinely assayed at 42°C as described previously (6) with *p*-nitrophenyl phosphate (PNPP) as a substrate. Enzyme activity with other phosphate ester substrates was determined by the estimation of inorganic phosphate by the method of Lanzetta et al. (9). Nucleotidase activities with 3'- and 5'-AMP were determined as previously described (5). Alanine aminotransferase (ALAT) was determined by the method of Wroblewski and LaDue (25).

For each set of experiments, separate controls lacking substrate and enzyme were run. The values obtained from these controls were subtracted.

Enzyme units for phosphatases are expressed as nanomoles of *p*-nitrophenol or P_i liberated per minute; units for ALAT are expressed as nanomoles of NADH oxidized per minute. Specific activities are expressed as enzyme units per milligram of protein or units per milliliter of medium. Protein concentration was determined by the method of Peterson (14) with a BSA standard.

Molecular sieve chromatography. Samples of concentrated culture supernatants were chromatographed on Sephacryl S-300 (Pharmacia Fine Chemicals, Piscataway, N.J.) columns. Columns (1.5 by 95 cm) were equilibrated with 20 mM Tris-hydrochloride (pH 8.0)–3 mM $MgCl_2$. In some cases, the equilibration buffer contained Triton X-100. A 2-ml amount of an Amicon PM-30 concentrated culture supernatant from a defined medium was applied. Elution was carried out

with equilibration buffer. Fractions of 2.5 ml were collected. The column chromatography was carried out at 4°C, and eluted fractions were analyzed for acid phosphatase activity with PNPP as a substrate. Void volumes for the molecular sieve columns were determined with blue dextran (Pharmacia Fine Chemicals) in a separate experiment performed under identical conditions.

Polyacrylamide gel electrophoresis. Concentrated culture supernatants were electrophoresed on a model 220 vertical slab-gel electrophoresis apparatus (Bio-Rad Laboratories, Richmond, Calif.). The samples were electrophoresed in the sodium dodecyl sulfate system described by Laemmli (7) with a 9% polyacrylamide separating gel. Some samples were run under identical conditions except that Triton X-100 was substituted for sodium dodecyl sulfate.

The gels were stained for protein with Coomassie brilliant blue R-250 (Bio-Rad Laboratories). Portions of gels electrophoresed in the presence of Triton X-100 were stained for acid phosphatase activity with β-glycerophosphate and $Pb(NO_3)_2$ (10).

RESULTS

Presence of an extracellular acid phosphatase in growth medium. An extracellular acid phosphatase contributed a very substantial portion of the acid phosphatase produced by *L. donovani* promastigotes during growth. The results in Table 1 clearly show that >90% of this enzymatic activity from a log-phase culture was operationally extracellular; i.e., the activity was not removed by centrifugation and filtration of the cells. In distinct contrast, ALAT was completely removed from the growth medium by the centrifugation and filtration procedures. ALAT activity is known to be a cytosolic component of these (unpublished data) and related organisms (23). The absence of this soluble cytoplasmic marker from the culture supernatant indicates that the processing of the growth medium does not result in cell damage.

Evidence that the extracellular acid phosphatase was released or secreted by active, growing cells and was not a function of cell damage or lysis of aged cells is presented in Fig. 1. Culture supernatants of *L. donovani* promastigotes were obtained at all stages of the growth cycle, from a serum-containing medium (Fig. 1A) and from a chemically defined medium (Fig. 1B). The supernatants were assayed for acid phosphatase, and the results were plotted along with the culture density. The results demonstrate that the enzyme was present at all phases of the growth curve and was proportional to the density of the promastigotes.

Preliminary physicochemical characterization of extracellular acid phosphatase. We have begun to analyze some of the physicochemical properties of the extracellular acid phosphatase activity. The enzymatic activity of the culture supernatant was operationally soluble as it was completely recovered in the supernatant fraction after centrifugation at 100,000 × *g*.

TABLE 1. Presence of enzyme activities in growth medium of *L. donovani* promastigotes

Enzyme source[a]	Sp act[b] of acid phosphatase	Sp act[c] of ALAT
Culture medium (including cells)	81.3 ± 4.1	7.32 ± 0.04
Culture supernatant	74.7 ± 4.6 (92%)[d]	0 (0%)[d]

[a] Samples were obtained from the mid-log phase of the growth curve (2×10^7 promastigotes per ml) on defined medium and assayed for acid phosphatase activity with PNPP as the substrate and for ALAT activity as described in the text. The assays were carried out in the presence of Triton X-100 (0.1%) to ensure cell lysis. Culture supernatants were prepared by centrifugation and microfiltration.

[b] Nanomoles of *p*-nitrophenol released per minute per milliliter of culture.

[c] Nanomoles of NADH oxidized per minute per milliliter of culture.

[d] Percentage equals the specific activity in the culture supernatant divided by the specific activity in the total growth medium multiplied by 100.

The enzymatic activity in defined medium was concentrated by ultrafiltration with a stirred cell ultrafiltration apparatus. The enzymatic activity was completely retained by filters with nominal molecular weight cutoffs as high as 50,000; >80% of the enzymatic activity was retained by an Amicon XM-300 filter with a molecular weight cutoff of 300,000. Effective concentrations as high as 1,000× were achieved with ultrafiltration. A minimal loss of enzymatic activity, either by adsorption to the membrane or by denaturation, was encountered as the reduction in volume corresponded to the increase in activity. The concentration of the culture supernatants from defined medium resulted in an intense black solution. This appearance was the result of the concentration of hemin, either adsorbed to protein or in a polymerized form. The hemin was present in the medium as a necessary nutrient for the promastigotes. The color of the solution remained even after extensive dialysis under various conditions, including dialysis against 1 M NaCl and against 1% Triton X-100. The presence of the hemin made estimation of the protein concentration by the usual procedures impossible to interpret. Therefore, results are reported in terms of activity per milliliter of volume rather than activity per milligram of protein.

Molecular sieve chromatography was also used in an effort to estimate the molecular weight of the extracellular enzyme. The enzy-

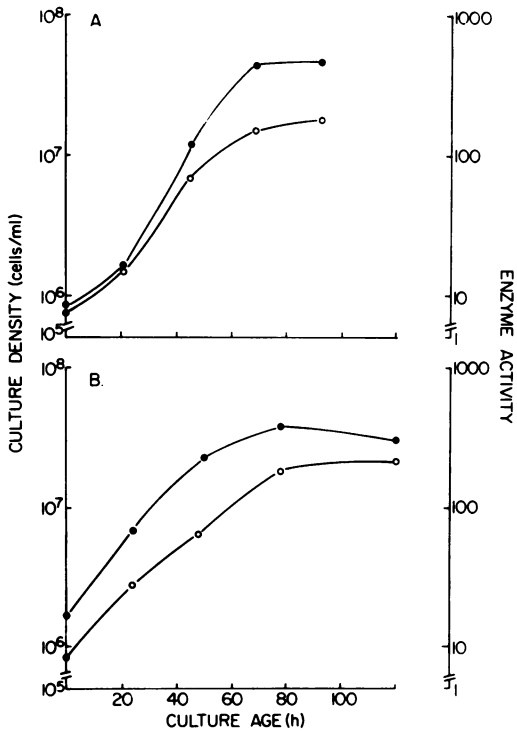

FIG. 1. Kinetics of growth of *L. donovani* promastigotes and the appearance of extracellular acid phosphatase. Culture flasks containing medium 199 and 20% fetal bovine serum (A) and defined medium with BSA (B) were inoculated with portions of late-log-phase cultures of *L. donovani* promastigotes grown on the respective media. Samples of the culture flasks were removed at the indicated times, and cell density (●) was estimated by hemacytometry. Portions of the removed samples were centrifuged in an Eppendorf microfuge, and the supernatant was filtered through a 0.22-μm filter. The culture supernatant was assayed for acid phosphatase activity (○) with PNPP as the substrate as described in the text. Enzyme activity is expressed as nanomoles per minute per milliliter of culture supernatant.

matic activity eluted with the void volume on a column of Sephacryl S-300. Sephacryl S-300 excludes proteins with molecular weights larger than 10^6. Identical results were obtained on columns equilibrated and eluted in the absence of Triton X-100. The latter was included in an attempt to dissociate possible aggregates.

The concentrated culture supernatants were subjected to polyacrylamide gel electrophoresis in a vertical slab-gel system under nondenaturing conditions. The resulting gel was cut into two sections and stained for protein and acid phosphatase activity. A typical result is shown in Fig. 2. The protein staining pattern revealed no discernible bands from concentrated culture supernatants from defined medium lacking any macromolecular constituents. Electrophoresis of concentrated culture supernatants from defined medium containing BSA revealed Coomassie blue-stained bands at positions corresponding to a standard BSA included on the same gel. The major bands presumably correspond to the position of BSA monomers and dimers. The enzymatic activity, visualized by a lead phosphate-staining procedure, was confined to the very upper portion of the separating gel. Concentrated culture supernatants from both BSA-containing and BSA-deficient media revealed a broad activity band. The BSA-containing super-

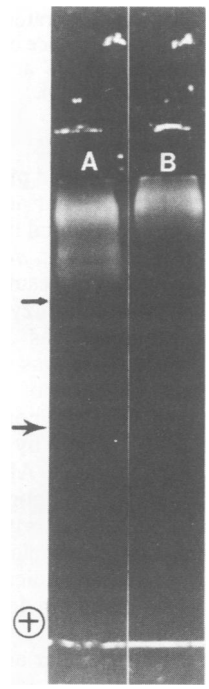

FIG. 2. Polyacrylamide gel electrophoresis of *L. donovani* extracellular acid phosphatase. Supernatants were obtained from *L. donovani* cultures grown on defined medium containing and lacking BSA and were concentrated by Amicon PM-30 ultrafiltration. The samples were applied to the slab-gel system made with Triton X-100 described in the text. Samples were electrophoresed until the readily visible hemin migrated to within 1 cm from the bottom of the gel. The gel was cut in half and stained for acid phosphatase activity, with β-glycerophosphate as the substrate (9). Lane A contained a sample from BSA-containing medium, and lane B contained a sample from BSA-deficient medium. This portion of the gels was stained for acid phosphatase. The remaining half of the gel was stained for protein, with Coomassie brilliant blue R-250. The arrows indicate the position of the BSA monomers (large arrow) and dimers (small arrows) on the remaining portion of the gel which was stained for protein. ⊕ indicates the position of the anode.

natant also revealed several minor bands which migrated more rapidly than the major band. Electrophoresis carried out in the absence of detergent yielded identical results. Electrophoresis carried out under reducing and denaturing conditions, i.e., in the presence of 2-mercaptoethanol and sodium dodecyl sulfate, resulted in the appearance of a single Coomassie blue-stained band at the position of BSA in samples obtained from BSA-containing medium. Enzymatic activity was not detectable in gels run under these conditions.

Comparison of extracellular acid phosphatase with plasma membrane-bound acid phosphatase. Previously (6) we have shown that *L. donovani* promastigotes contain significant acid phosphatase activity, approximately half of which is recovered with the isolated plasma membrane fraction. In an effort to compare this activity with that of the extracellular acid phosphatase, we examined a number of enzymatic parameters. The activities of both enzymes as a function of pH were very similar; the optimum activity was observed at pH 4.5 (Fig. 3). There was no evidence for alkaline phosphatase activity either with the cellular enzyme or with the culture supernatants. Both enzymes were sensitive to fluoride ions, with complete inhibition observed at 0.5 mM. A striking difference between the two enzymatic activities was observed when assays were conducted in the presence of sodium tartrate. The membrane-bound activity was 400 times more resistant to the effects of this inhibitor than was the extracellular enzyme. Indeed, the exoenzyme was very sensitive to tartrate, with a 50% diminution in activity occurring at 20 μM.

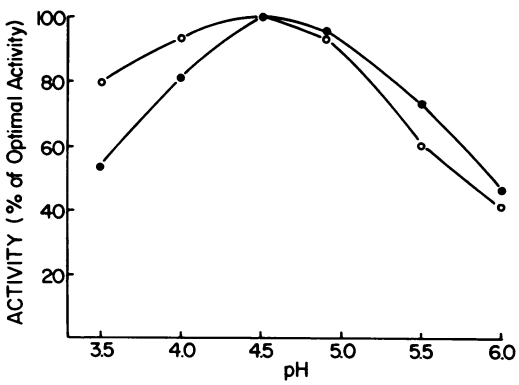

FIG. 3. pH optimum of *L. donovani* plasma membrane and extracellular acid phosphatases. Reaction mixtures were prepared containing 50 mM sodium acetate at the indicated pH, 5 mM PNPP and the appropriate enzyme, either isolated plasma membranes (○) or culture supernatant (●) from defined medium. Mixtures were incubated for 30 min at 42°C and assayed for liberated *p*-nitrophenol as described in the text. The results are expressed as the percentage of the activity obtained at the indicated pH divided by the optimum activity. Optimum activities were 192 nmol of *p*-nitrophenol liberated per min per ml of culture medium and 490 nmol of *p*-nitrophenol liberated per min per mg of plasma membrane protein.

TABLE 2. Comparison of substrate specificities of plasma membrane and extracellular acid phosphatases of *L. donovani* promastigotes

Substrate	Relative substrate specificity[a]	
	Membrane enzyme	Extracellular enzyme
PNPP	1.00	1.00
Glycerophosphate	0.02	0.88
Glucose 6-phosphate	0.03	0.31
Glucose 1-phosphate	0.11	0.91
Fructose 6-phosphate	0.29	0.92
Fructose 1,6-diphosphate	0.98	1.50
Naphthyl phosphate	0.24	0.80
5'-AMP	0.17	0.28
3'-AMP	1.38	0.00

[a] Reaction mixtures were prepared which contained 50 mM sodium acetate buffer (pH 5.0), and the appropriate source of enzyme, either isolated plasma membrane from promastigotes or a suitable dilution of a 1,000-fold–concentrated Amicon XM-50 ultrafiltrate of promastigote defined medium culture supernatant. The indicated substrates were added to a final concentration of 5 mM, and the mixtures were incubated for 30 min at 42°C. Samples of each mixture were removed and assayed for P_i by the method of Lanzetta et al. (9). The results for controls lacking substrate and lacking enzyme were subtracted. The results are presented as the relative substrate specificity, which is expressed as the ratio of the specific activity of the enzyme with the indicated substrate divided by specific activity with PNPP. The actual specific activities obtained with PNPP as the substrate were 365 nmol of P_i released per min per mg of protein for the membrane enzyme and 7.09×10^4 nmol of P_i released per min per ml of the concentrated extracellular enzyme.

The two enzymes could also be distinguished on the basis of substrate specificity. The data for this distinction are shown in Table 2. The results are expressed as activity with a given substrate relative to activity with the artificial substrate PNPP. On this basis, the extracellular enzyme was more active with a wider range of substrates than the membrane-bound activity. Both enzymes were more active with fructose phosphates than with the glucose derivatives tested. The membranes hydrolyzed 3'-AMP and other 3'-nucleotides, whereas the extracellular enzyme was completely inactive toward these substrates. Actually, the membrane activity is the result of a distinct 3' nucleotidase (5) which is readily distinguished by various criteria from the membrane-nonspecific acid phosphatase. When assayed under conditions (5) which optimized

this nucleotidase activity, the membranes were much more active with 3'-AMP. In contrast, under these conditions, the exoenzyme was still inactive with this substrate.

DISCUSSION

Among microbial organisms, enzyme secretion is recognized in bacteria and fungi (8, 15), free-living protozoa (11, 16), and algae (12, 13). To our knowledge, exoenzymes have not previously been characterized from parasitic protozoa. Eeckhout (3) noted that phosphatase and invertase are detectable in the culture medium of *Crithidia luciliae*, a trypanosomatid of insects.

The data presented in this paper suggest that an extracellular acid phosphatase of *L. donovani* promastigotes was released as the result of a specific process and was not the product of cell "leakiness" caused by the experimental manipulations. This conclusion was based upon the relative levels of the extracellular and intracellular enzymes and the absence of detectable amounts of ALAT, a known cytosolic enzyme, in the culture supernatants. Furthermore, the exoenzyme was released at all stages of growth, including the early log phase. However, we can not rule out entirely at this point the possibility that the extracellular activity is the result of the dissociation of a very loosely associated acid phosphatase at the cell surface.

The mechanism of the release, as well as the origin, of the exoenzyme is as yet unknown. The exoenzyme may arise from the shedding of the membrane-bound acid phosphatase. However, despite similarities in some enzymatic characteristics, the differences in the relative substrate specificity and sensitivity to sodium tartrate suggest that the two enzymes are distinct. The distinctions between the extracellular and membrane-bound activities were maintained when a Triton X-100-solubilized preparation of membranes was used.

Alternatively, the exoenzyme may originate from the intracellular pool of acid phosphatase. Half of the intracellular acid phosphatase content of the promastigote is not associated with the surface membranes (6), and a portion of this activity is soluble, i.e., not sedimented at 100,000 × *g* (unpublished data). Studies are in progress to elucidate the relationship between the intracellular soluble enzyme and the exoenzyme with regard to various enzymatic characteristics, especially substrate specificity and tartrate sensitivity.

In addition to the cytochemical localization of acid phosphatase activity at the cell surface, fine-structure cytochemistry has revealed the localization of acid phosphatase activity within vesicles, perhaps lysosomes, in the anterior end of the promastigote (6). The enzyme contained within these vacuoles may be destined for exocytosis and secretion into the flagellar reservoir and subsequently into the extracellular medium. This possibility, however, is speculative, and definitive conclusions must await further experimentation.

Promastigotes can be grown in a P_i-deficient defined medium, provided that some form of organic phosphate is included (unpublished data). Thus, at least part of the physiological role of the leishmanial surface and extracellular acid phosphatases is nutritional. This is supported by the finding that the exoenzyme has a rather broad substrate specificity. The enzymatic activity may also provide the necessary organic moieties from phosphorylated derivatives which are not readily transported into the cell. Other nonnutritive functions of the acid phosphatases are not known. In particular, the role of these enzymes in the host-parasite relationship requires further investigation.

Although this is the first demonstration of an exoenzyme associated with promastigotes, various groups (1, 2, 4, 17–22) have studied the constituents released by *Leishmania* sp. in culture media. The studies have focused almost exclusively on a carbohydrate-rich factor(s) which is immunoprecipitated with sera from rabbits previously immunized with promastigotes. This component(s) is alternatively labeled excretion factor, exometabolite, or antigenically active glycoproteins and is used in serotyping leishmanial strains (17). Our preliminary studies have indicated that the exoenzyme is not part of the excretory antigen, as it is not immunoprecipitated by antisera which recognize extracellular antigen.

In summary, our results demonstrated the presence of an acid phosphatase released by *L. donovani* promastigotes. The presence of similar activity in amastigotes, the form found intracellularly within macrophages in the mammalian host, must await additional investigation.

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