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Original Article

Characterization of Acid Phosphatase in the Promastigotes of Three Isolates of *Leishmania major*

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Abstract

Background: Acid phosphatase (ACP) is suggested to be one of the virulence factors in leishmania and a correlation has been reported between Leishmanial acid phosphatase (ACP) activity and the severity of the disease in all types of leishmaniasis including zoonotic cutaneous leishmaniasis (ZCL), which is a polymorphic disease.

Objective: Characterization of leishmania and its correlation with the severity of the skin lesions in zoonotic cutaneous leishmaniasis.

Methods: Promastigotes were isolated from 30 patients with scaly flat ulcers (LP1), volcano-shaped lesions (LP2) and papular forms (LP3) of ZCL. The K_m and V_{max} values of ACP in the supernatant of lysed promastigotes were calculated in presence and absence of the enzyme inhibitors, fluoride, tartarate, phosphate and salicylate.

Results: The Michaelis-Menten substrate saturation kinetics exhibited K_m values of 12.5, 16.0, 26.6 μM and V_{max} values of 8.78, 5.26 and 1.51 μM/min/mg protein for LP1, LP2 and LP3, respectively. In all isolates, the ACP was inhibited by all four inhibitors but the percentage inhibition was different and each inhibitor showed a particular inhibition pattern for each promastigote preparation. ACP content of LP1 was more sensitive to all four inhibitors and fluoride was more potent inhibitor of the enzyme in three isolates.

Conclusion: The differences in ACP kinetics may be interpreted as being consistent with the severity of the skin lesions in *Leishmania major* infection.
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Keywords • Leishmania • leishmaniasis • enzymes • acid phosphatase • leishmania major

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Introduction

Zoonotic cutaneous leishmaniasis (ZCL) due to *Leishmania major* is a major public health problem in many parts of the Middle East including Iran. The disease is particularly im-

portant in rural areas where it may cause considerable morbidity.[1,2] Leishmania amastigotes, the causative agents of human leishmaniasis, are intracellular parasites which infect cells of reticuloendothelial system of humans and other mammals. To establish an infection, the promastigotes must enter a macrophage, where they survive and replicate. One of the molecules believed to be incorporated in the entrance of the organism into the cell and establishment of its survival is acid phosphatase (ACP).[3-7] This enzyme inhibits production of superoxide anions (O_2^-) by neutrophils and macrophages.[8,-10] This, in turn, disarms host cells and in part, controls the clinical manifestations of the disease.[11] ACP activity has been detected not only in the cytoplasmic vacuoles and vesicles[12] but also on the outer surface membrane of both promastigotes and amastigotes of all leishmania species studied so far.[13,11,14,7,15] Moreover, this enzyme is reported to be the major protein secreted by some species,[13,16,17] however, qualitative and quantitative differences have been shown between virulent and avirulent forms of *L. donovani*[18,19] and *L. braziliensis* promastigotes.[14] The specific activity of ACP in the promastigotes of different species of leishmania[18,14] and *L. major* isolated from clinically different forms of ZCL is reported to be significantly different and it has been suggested that they might contain different forms of ACP.[20] In the present study, the characteristics of ACP were evaluated in the promastigotes isolated from clinically different cases of ZCL and the findings were correlated with the shape and severity of the skin lesions.

Materials and Methods

Parasites:

Leishmania major (HOM/IR/70/Nadim) is the causative agent of ZCL in Isfahan, Central Iran.[1,2] Leishmania promastigotes (LP) were isolated from cutaneous lesions of 30 selected patients living in rural districts of northern Isfahan, with clinically different forms of ZCL. The promastigotes isolated from 10 patients with scaly flat ulcers and satellite lesions measuring about 6 cm^2 were referred to as LP1. The second isolates (LP2) were taken from 10 patients with volcano-shaped lesions measuring about 2 cm^2 and the third isolates (LP3) were taken from another 10 patients with papular lesions measuring > 1 cm^2.

All of the patients were boys around 12 years of age with a disease history of about one month. They had neither previous record of infection nor medication for leishmaniasis and had not visited other endemic areas. They had a similar nutritional status with no apparent differences in their general state of health.

The lesions were named according to Griffiths.[21] All of the isolates were identified as *L. major* by recombinant DNA probes using a pDK10 probe (an 1800-base-pair pstI fragment) and by comparing the hybridization patterns with those of the reference DNAs[22] of the London School of Hygiene and Tropical Medicine, UK.

Isolation and mass production of parasite

Promastigotes were isolated by culturing the aspirates of the cutaneous lesions in a modified NNN medium[23] containing 1.6% agar (w/v), 0.7% NaCl (w/v), 1% dextrose (w/v), 15% fresh defibrinated rabbit blood (v/v) and 500 U/mL penicillin. They were grown in sterile screw top tubes containing 10 mL culture media at 18–22 °C and subcultured weekly.

The promastigotes from the sixth *in vitro* passage were harvested during the stationary phase[20] by centrifugation of the liquid phase of the media at 2000 g for 30 min at 4 °C. They were resuspended in a salt mixture containing 150 mM NH_4Cl, 10 mM $KHCO_3$ and 0.1 mM EDTA for 10 min to disrupt erythrocyts.[24] After this procedure, the promastigotes were washed three times in isotonic ice-cold PBS (pH 7.4). The purity of promastigotes was examined microscopically to ensure that erythrocytes were not present. The pellets of purified promastigotes were kept at -25 °C until used.

Sample Preparation

One ml of 0.1 M sodium acetate buffer (pH 5.2) containing 1 mM $MgSO_4$ and 0.1% Triton-x-100 was added to the frozen promastigotes ($4.5–5\times10^6$ cells/mL). The resultant was homogenized at 4 °C by a glass Teflon homogenizer and centrifuged at 18000 g for 20 min at 4 °C. The supernatant was taken for enzyme assays. The protein content of the supernatant of the lysed promastigotes was measured by Lowery's method[25] using bovine serum albumin as standard.

Enzyme assays

The assays of ACP were carried out at 25 °C in 1 mL of the supernatant of the lysed promastigotes adjusted to pH 5.2. The enzymatic hydrolysis of P-nitrophenyl phosphate was measured spectrophotometrically at 410 nm by the release of P-nitro phenol. Determination of the Michaelis constant for the ACP experiments was made under a condition identical with those used for the standard assay, except that the substrate concentration was varied. The values of K_m and V_{max} of the enzyme preparations (supernatant of the lysed promastigotes) were calculated from the plots of reciprocal of initial velocity *vs.* the reciprocal of the μM concentration of

Table 1: Kinetic characteristics of acid phosphatase in promastigotes isolated from three different types of ZCL skin lesion

Promastigot,s Isolates	V_{max} (µM/min/mg protein)	K_m (µM substrate)
LP1	8.94±0.16	12.3±0.21
LP2	5.61±0.11	15.8±0.30
LP3	1.56±0.06	26.9±0.44

Supernatants of promastigotes isolated from scaly ulcers (LP1), volcano shapes (LP2) and papular shapes (LP3) were incubated for 30 min at 25°C at PH 5.2 in a range of P-nitrophenylphosphate concentrations between 0 to 200 µ M. Velocity was measured by the release of p-nitrophenol and V max and Km were obtained from linweaver-Burk plot of the data.

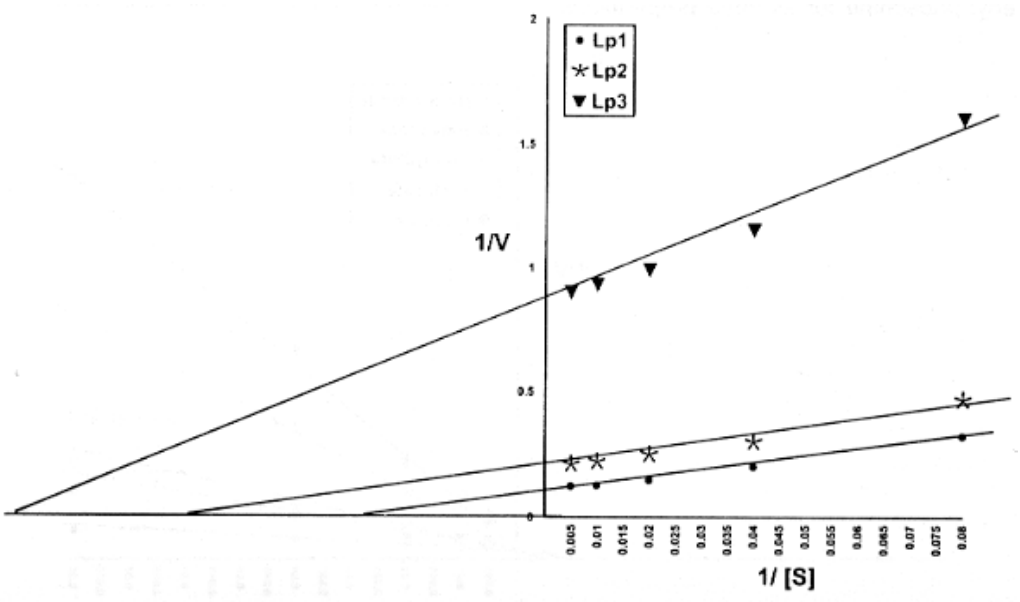
Results are expressed as the mean ± SD of triplicate determination of promastigotes isolated from 10 patients in each case

P-nitrophenyl phosphate by Linweaver-Burk plots[26] in the absence and presence of 0.3 mM of phosphate, tartarate, salycilate or fluoride.

Results

Initial rate kinetics of leishmania promastigote ACP was studied in the supernatant of the lysed promastigotes isolated from 30 patients with three clinically distinct forms of ZCL. The velocity of ACP in each preparation was a simple saturable process with respect to P-nitrophenyl phosphate substrate concentration, displaying Michaelis-Menten kinetics as demonstrated by the initial velocities of ACP against increasing concentration of substrate in the medium (Fig 1). The linear double reciprocal plots of the initial velocities against the substrate concentration indicated similar characteristics for ACP in all promastigotes isolated from clinically similar forms of skin lesions; significantly different characteristics with SD of each point being less than 5% for ACP in the promastigotes isolated from three clinically forms of ZCL studied namely, LP1, LP2 and LP3 (Figs 2–4). The apparent V_{max} and K_m values of the enzyme in the sample, as calculated from Lineweaver-Burke plots,[26] are given in Table 1. Preparations from promastigotes isolated from large scaly flat ulcers with satellite lesions (LP1) contained ACP with relatively highest V_{max} but the lowest K_m values as compared with V_{max} and K_m values of the ACP in the promastigotes isolated from the two other types of lesions (LP2 , LP3).

Relatively intermediate values were observed in the samples prepared from LP2, isolated from volcano-shaped lesions with intermediate size. The lowest V_{max} but the highest K_m values were found in the supernatant prepared from LP3, isolated from small papular lesions without ulceration.



Figre 1: Linweaver-Burk plot showing the effect of substrate concentrations on velocity of acid phosphatase in the promastigotes isolated from three different skin lesion types of leishmania major. Supernatant from LP1, LP2 and LP3 were incubated for 30 min at 25°C at pH 5.2 in a range of P-nitrophenylphosphate concentrations between 0 and 200 µM. Velocity was measured by the release of P-nitrophenol as described in Materials and Methods section. Each point represents the mean of three separate determinations.

Table 2: Percentage inhibition of the leishmania ACP preparations by four chemical inhibitors

Inhibitars	LP1 (% of inhibition)	LP2 (% of inhibition)	LP3 (% of inhibition)
Fluoride	76.5±1.8	40.3±1.8	31.2±3.0
Tartarate	70.6±2.5	34±1.8	4.8±1.2
Phasphate	36.9±1.5	28.7±0.8	2.9±0.6
Salycilate	27.1±0.6	10.9±1.8	1.0±0.2

ACP preparations from LP1, LP2, or LP3 promastigotes were incubated with P-nitrophenylphosphate (100 µM) in the absence or presence of one of the indicated inhibitors (0.3 mM) and specific activity of ACP was measured by the release of P-nitrophenol. Results are (mean±SD) percentage of inhibition of 3 determinations as compared to the specific activity of the enzyme in the absence of the inhibitors.

Since the three different preparations demonstrated various kinetic mechanisms, it was of interest to determine kinetic characteristics of the enzyme preparation in the presence of the defined inhibitors of ACP.[27,28] Kinetic data from the initial rate experiments in Figures 2–4 illustrate plots of 1/velocity against 1/(substrate concentrations) at fixed level of tartarate (0.3 M), salicylate (0.3 M), phosphate (0.3 M) and fluoride (0.3 M).

It is clear from these illustrations that double-reciprocal plots are convergent and that the chemicals are acting as the linear competitive inhibitor of P-nitrophenyl phosphate for all three preparations. However, each inhibitor shows a particular inhibition pattern for each of ACP preparation (Figs 2–4). Table 2 summarizes the percentage inhibition of the ACP sample preparations as determined by addition of 0.3 M of the inhibitors to the standard assay mixtures. It is evident that ACP content of LP1 is more sensitive to all four inhibitors as compared to that of other preparations. LP2 and LP3 are intermediate and less sensitive, respectively. As can be seen in Table 2, fluoride is a more potent inhibitor of the enzyme in the three different preparations as compared to other inhibitors.

Discussion

Zoonotic cutaneous leishmaniasis is a polymorphic disease which may show various clinical manifestations, ranging from asymptomatic infection without apparent lesion to extensive lesions which may cause severe disfiguring.[29] This clinical variation which has been reported even within small endemic foci, may reflect either variability in the parasite virulence or variability in the host immune response. There are evidences to support either possibilities,[30,20,29,31] however, the relative importance of each one is still debatable. Since, ACP is proposed to be one of the prominent virulence factors in leishmania,[16,5,32,18,19] the present study was undertaken to compare the kinetic characteristics of ACP in *L. major* promastigotes isolated directly from clinically different cases of naturally infected

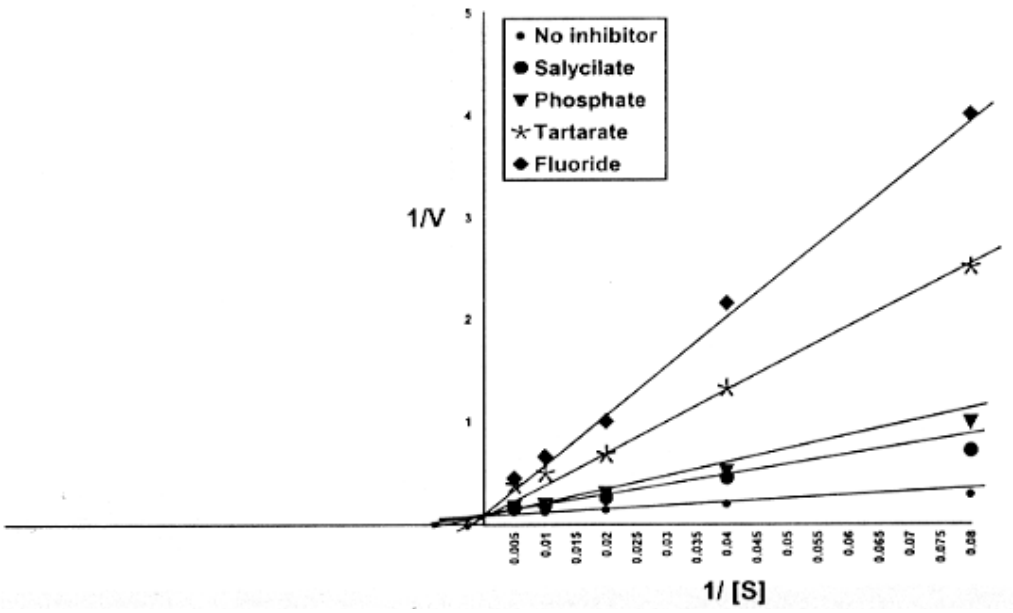


Figure 2: Inhibition of acid phosphatase in leishmania promastigotes isolated from scaly flat ulcers (LP1). Plot of reciprocal of initial velocity vs.the reciprocal of µM concentrations (0-200) of P-nitrophenylphosphate in the absence and presence of 0.3 mM of fluoride, tartarate, phosphate and salycilate. Velocity was determined by the release of P-nitrophenol as described in materials and methods. Triplicate determination of promastigotes isolated from 10 patients with SD<5%.

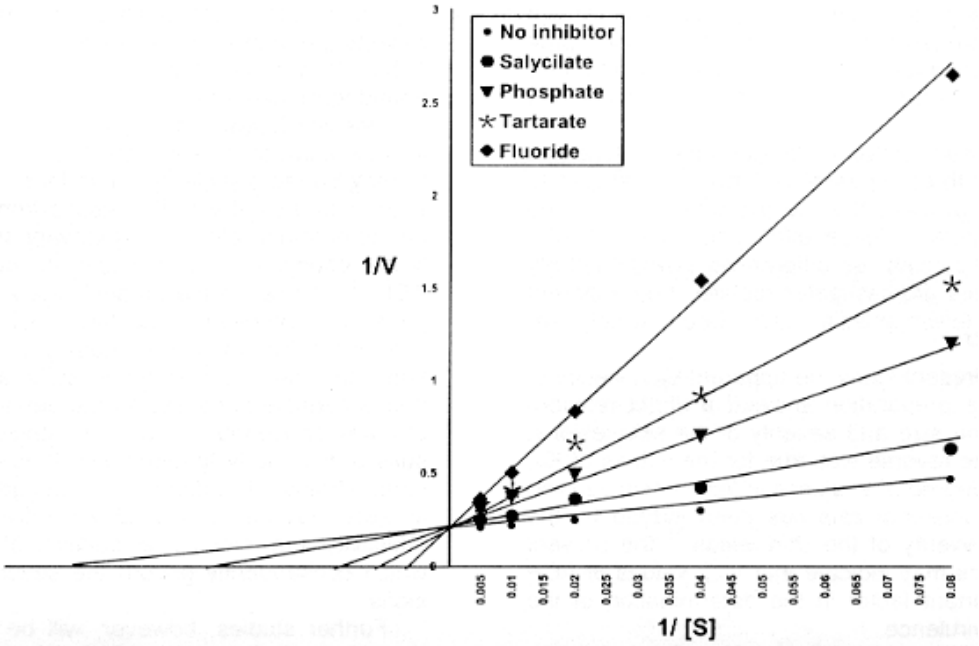


Figure 3: Inhibition studies of acid phasphatase in leishmania promastigotes isolated fom volcano-shaped lesions (LP2). Plot of reciprocal of initial velocity *vs* the reciprocal of µM concentrations (0-200) of P-nitrophenylphosphate in the absence and presence of 0.3 mM of flouride tartarate, phosphate and salycilate. Velocity was determined by the release of P-nitrophenol as described in Materials and Methods section. Triplicate determination of promastigotes isolated from 10 patients with SD<5%.

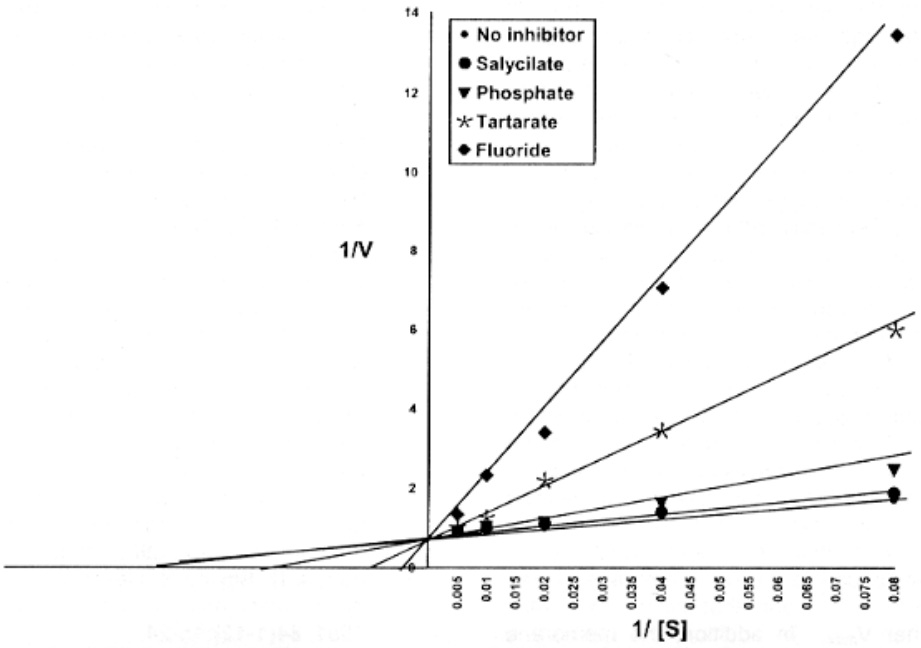


Figure 4: Inhibition of acid phosphatase in leishmania promastigotes isolated from papular lesions (LP3) . Plot of reciprocal of initial velocity vs. the reciprocal of µM concentrations (0-200) of P-nitrophenylphosphate in the absence and presence of 0.3 M M of fluoride, tartarate phosphate and salycilate. Velocity was determined by the release of P-nitrophenol as described in Materials and Methods section. Triplicale determination of promastigotes isolated from 10 patients with SD<5%.

human host in a single geographical area without possible interference of additional *in vitro* passages, mice infection, cryopreservation and heterogeneity reported in the stocks of leishmania strains. [33,-36]

The results reveal considerable differences among the three types of isolates with respect to the values of V_{max}, K_m and the effects of defined ACP inhibitors. These differences are not altogether unexpected, as differences in ACP activity of leishmania promastigotes isolated from different forms of leishmaniasis have been widely reported.[20,18,14]

In the present study the apparent V_{max} values of the enzyme preparation showed a direct relationship with the size and severity of the skin lesions, whereas the reverse was true for the values of K_m. Since the degree of virulence in leishmania causing cutaneous leishmaniasis has been judged by the size and severity of the skin lesion,[21] the present observations may indicate that V_{max} values of ACP is an important factor in the determination of the degree of virulence.

Although the character of the lesions in CL partly depends upon the reaction of the human host, the observed clinical differences appear to be rather independent of host factors, because similar promastigotes isolated from different cases of ZCL have been reported to produce clinically different forms of skin lesions and different courses of infection in genetically defined inbred BALB/C mice.[20] Moreover, the age, sex, race, apparent state of health, socio-economic condition, general mode of life in all the patients studied were identical and no difference was observed in the ACP kinetics of promastigotes isolated from different patients with clinically similar forms of skin lesions. The present results support the suggestion that *L. major* isolated from the field may differ in virulence, which influences the course of the disease and type of immune response elicited by the host.[29] These findings are in accord to the report that promastigotes of the virulent form of *L. donovani* possess a higher level of membrane bound ACP relative to that of avirulent forms.[18,19] In the present study , different kinetic properties of ACP in the promastigotes isolated from different cases of ZCL may be interpreted as being consistent with compart–mentalization of ACP in the leishmania causing ZCL. There have been a number of reports that ACP in the promastigotes of leishmania species may be either bound to the cell membrane or free in the cytosol[13,21,11,14] and that bound form may show a higher V_{max}. In addition, the membrane bound enzyme in the promastigotes of *L. donovani*[35] and epimastigote of *Trypanosoma cruzi*[37] have been found to be strongly inhibited by tartarate and fluoride. The highest V_{max} and lowest K_m values of ACP in LP1 isolates, which was very sensitive to the addition of tartarate and fluoride (Fig 2, Table 1) suggest that this enzyme might be a bound form, whereas the enzyme measured in LP3 isolates which has a low V_{max} and a high K_m, that is less sensitive to tartarate and fluoride (Fig 4, Table 1) may be the soluble cytosolic form. This is however, inconsistent with the results from the studies on the promastigotes of *L. donovani*, that the membrane bound form were shown to be a resistant ACP.[38] Similar to the present study, previous reports in *L. donovani* found three distinct ACP with different molecular weights from which the cell surface form has a rather broad substrate specificity and is capable of catalyzing the dephosphorylation of many phosphoproteins.[38] In conclusion, the results of this study together with those reported for other strains of leishmania[30,7] suggest that ACP kinetics may be associated with strain-dependent infectivity and intracellular survival of the parasite which consequently govern the severity of the lesions.

Further studies, however, will be needed and studies are in progress to identify the molecular basis of virulence variability among *L. major* isolated from clinically different cases.

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