

Is surface membrane acid phosphatase an essential requirement for the survival of *Leishmania* in macrophages ?

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Abstract

The occurrence of acid phosphatase activity amongst the leishmanias has been investigated with the aim of determining whether the enzymes are adaptations for survival and growth in insect or mammals. The results of the study suggest that there are no marked differences between the acid phosphatase activity of *L.major* mid log and stationary phase promastigotes. The activities of both, however, differed considerably from those of *L.tropica* and *L.donovani* . In contrast to *L.tropica* and *L.donovani*, is the apparent lack of surface acid phosphatase on *L.major*, and so it is suggested that the surface enzyme is not essential for survival of all leishmanias in macrophage.

Introduction

Acid phosphatase (phosphoric monoester hydrolase,(EC 3.1.3.2) occurs in most eukaryotic cells and is a normal constituent of lysosomes (Mullin et al, 2001,Chatterjea & Shinde 2005). The distribution of acid phosphatase activity in leishmanias, however, is unusual, especially the presence of surface –located enzymes(Mc Gwire et al, 2003 ; Paugam et al, 2003). Apparently there are three acid phosphatase complex and it has been reported that one inhibits the oxidative metabolism of neutrophils (Remaley et al ;1984 ,Hassan & Coombs1987 ,Spath et al, 2003). This finding led to the suggestion that surface acid phosphatase of leishmanias plays a crucial role in the parasite`s survival in macrophages (Mc Gwire et al 2002 ; Araujo Soares *et al* 2003;Yao et al, 2003). A second unusual feature of leishmania acid phosphatases is the large quantity of enzyme that is secreted (Mc Conville et al ,2002).The functional significance of this secreted activity is unclear, but again a role in invasion of macrophages is possible. It is now well established that *L. major* differs from all other leishmanias investigated in not secreting acid phosphatase; this suggests that either the way in which *L. major* survives in macrophages is different from the mechanisms used by other leishmanias or that secreted acid phosphatase is not related to survival in macrophages.The present study was undertaken to provide a fascinating insight in to the functional

significance of acid phosphatase by which leishmanias may be adapted for survival in macrophages and how this apparently varies with species .In an attempt to provide more information on acid phosphatase and the part it play ,I have investigated their occurrence in various forms of Iraqi leishmanias .

Materials and methods

Parasites:

The Iraqi strains of *Leishmania donovani* (MHOM / IQ / 89 / PRL) , *L.major* (MHOM / IQ / 81 / BRC) and *L.tropica* (MHOM /IQ /93 /MRCI) were cultured in HOMEM medium (Berens et al, 1976) supplemented with 10% (v/v) heat inactivated foetal calf serum and 25 µg gentamycin sulfate /ml.

Acid phosphatase assay :

For enzyme assay , the cells were harvested by centrifugation at 5000xg and washed thrice with 0.9% NaCl in 50mM Tris buffer , pH 7.2 (TBS) , by similar centrifugation .The washed cells were suspended in TBS containing 1mM B.mercaptoethanol and homogenized by sonication using soniprep 150 . The homogenate was centrifuged at 105000xg for 60 min and the resultant pellets were resuspended in TBS to the volume of supernatant fraction . The 105000xg supernatant and pellet were used as the enzyme source . All these steps were carried out at 4C . Acid phosphatase was assayed according to the method described by (Glew et al ,1982) using P-nitrophenylpyrophosphate as substrate . Protein was estimated by the method of (Lowery et al ,1951) .Specific activity of the acid phosphatase is expressed as nmol /min / mg protein.

Ultrastructural localisation of acid phosphatase : °µ

Parasites ($5 \times 10^7 - 1 \times 10^8$ /ml) were initially fixed in 1% (v/v) glutaraldehyde in 0.1M sodium cacodylate , pH 7.2 , containing 5% (w/v) sucrose (SCS) for 30 min at 4 C°. Thereafter , they were washed twice in SCS , twice in 50 mM sodium acetate , pH 5.0 , containing 5% (w/v) sucrose (SAS) and incubated for 30 min at 37C° in SAS containing 3.3 mM Pb(NO₃)₂ and 12.5 mM disodium glycerol-2-phosphate as substrate . After incubation , the cells were washed twice in SAS , postfixed in 1% (v/v) OsO₄ for 1 hr at 4C° , washed with distilled water , stained with 0.5% (w/v) uranyl acetate and then dehydrated in ethanols , embedded in epon , sectioned and examined unstained or after staining with uranylacetate and lead citrate.

Isoelectric focusing:

Cells used for isoelectric focusing were washed 3 times (3000xg for 15 min at 4C°) in 250mM sucrose, pelleted either used immediately or stored at -70C°. Parasite homogenates were prepared by resuspending the pellets in 250mM sucrose and sonicating as described above.

Flat-bed isoelectric focusing (IEF) was performed using carrier ampholytes as outlined in the Pharmacia guide to IEF. The gel contained 0.28g agarose in 25ml deionised, distilled water. The anode and cathode electrode strips were soaked in 0.05M H₂SO₄ and 1M NaOH, respectively. The samples applied contained equal amounts of protein (300-800 µg) unless indicated otherwise. A constant power supply set to deliver a maximum of 1500V, 50mA and 20W was applied and electrophoresis was continued for a total of 2.5h, by which time the human haemoglobin markers had focused.

Protein bands were stained using 0.2% (w/v) PAGE blue 53 dye. Acid phosphatase were stained using the methods described by (Harris & Hopkinson, 1976). Approximate pIs of isoenzymes were determined by comparison to pI standards.

Results and Discussion

Each of the Leishmanias investigated, listed in Table 1, were found to contain relatively high levels of the acid phosphatase activities. The specific activities in the homogenates of *L.major* mid log phase and stationary promastigotes were quite similar to those investigated for other leishmanias. The activity of mid log phase promastigotes was slightly higher than that of stationary promastigotes and in preliminary experiments a similar situation was seen with *L.tropica* and *L.donovani* mid log phase and stationary phase promastigotes (Table 1). This is consistent with the report of (Hassan & Coombs, 1987) that acid phosphatase activity in *L.m.mexicana* promastigotes was slightly higher than in amastigotes. The distribution of the acid phosphatase activity of Leishmanias investigated between the soluble and particulate fractions is shown also in Table 1. All activities except for *L.major* acid phosphatase were sedimentable more than 50% by centrifugation of cell homogenates at 105,000 Xg for 1 hour. In contrast, only about 21% and 25% of the acid phosphatase activity of mid log phase and stationary promastigotes of *L.major* was sedimentable by centrifugation. Respectively.

In this respect , *L.major* differs from other species of *Leishmania* that have been investigated for which most of the acid phosphatase activity was found to be particulate(Gottlieb & Dwyer,1981, 1982;Glewr et al, 1982, Hassan &Coombs,1987).

Isoelectric focusing of homogenates of *L.major* consistently revealed just one isoenzyme with an apparent pI of 5.6 in mid log phase and stationary promastigotes (Fig.1) .This contrasts with *L.donovani* and *L.tropica* promastigotes ,which possess two isoenzymes as revealed by isoelectric focusing (Fig.2) As indicated isoenzymes of acid phosphatase of *L. donovani*(pIs 4.7 and 5.2) and *L. tropica* (pIs 5.3 and 5.6) were at higher activity in mid log phase promastigotes than in stationary . Interestingly *L.m.mexicana* amastigotes contain different and fewer isoenzyme (Coombs et al ,1987) . Overall , these data show that mid log phase and stationry promastigotes of *L.major* possess very similar acid phosphatase activities and suggest that parasites do not acquire more or additional acid phosphatase activities concomitant with transforming to the metacyclic form .

Greater variation was found when the occurrence of acid phosphatase activities on the surface of the organisms was investigated.As shown in Fig.3 .acid phosphatase activity has occur on the surface membrane of mid log phase as wells as stationary promastigotes of *L.donovani* ; this was apparently the same as with *L.m.mexicana* (Hassan & Coombs,1988) and *L.m. amazonensis* (Pimenta & de Souza, 1986) .In contrast ,however ,*L.major* promastigotes (mid log phase and stationary) lacked surface acid phosphatase (Fig.3) under the conditions employed in this study.

The lack of surface bound activity in *L.major* correlate well with the finding that less of the acid phosphatase activity of this species is particulate compared to other mammalian leishmanias .It is possible that *L.major* possesses a surface acid phosphatase so different from that of other trypanosomatid that it was not detected by the method used. Nevertheless, the results show that with respect to surface acid phosphatase, *L.major* differs significantly from the other mammalian parasites and suggest that surface localized acid phosphatase is not essential for survival of all leishmanias in macrophages. The finding of the apparent lower activity of acid phosphatase present on the surface of *L.m.mexicana* amastigotes compared with promastigotes also point to a role for the enzyme in the insect host (Hassan & Coombs, 1987) .Similarly ,the finding of high activity present on the surface of an insect trypanosomatid, *H.m.muscarum*,

(Coombs et al ,1987) is especially intriguing and also provides strong evidence for a role of this enzyme in the invertebrate .At present ,there is no sufficient information to understand why only some insect trypanosomatids possess the enzyme or its role when present . These findings ,although providing no support for the proposal ,do not rule out the possibility that the surface acid phosphatase of *L.donovani* and *L.mexicana* plays some crucial part in survival of the parasites in macrophages(Remaley et al ,1984,1985; Hassan & Coombs ,1987; Mottram et al ,2004) .Literatures ,however, suggested that other factors must also be involved and that the presence of a similar enzyme on the ancestral insect forms could have been a useful preadaptation for parasitism of mammals. The finding that *C.fasciculata* and *L.donovani* release acid phosphatase into the medium can be interpreted in a similar fashion(Mottram et al, 2004) . A need for more information on the environmental condition of the various trypanosomatids in their insect hosts to enable us to understand the parts the enzymes play . The lack of detectable secretion of acid phosphatase by *L.major* also distinguishes this species from the other mammalian Leishmanias(Dwyer & Gottlieb,1985) .Indeed the results of this study suggest that acid phosphatase is not central to the survival of *L.major* in mammals .It remain to be seen whether this is also true for other species , but currently available evidence indicates that different species of *Leishmania* may differ, at least quantitatively, in the ways in which they are adapted for survival in macrophages.

References

- Araujo Soares , R., Santos , A., Bonaldo , M. and Goldenberg , S.,(2003): *Leishmania amazonensis* ; deferential expression of protienases and cell surface polypeptides in a virulent and virulent promastigotes. Exp.Parasitol.,Vol.104 ,pp. 104-112.
- Berens,R.,Brun,R.and Krassner,S.,(1976): A simple momophasic medium for axenic culture of hemoflagellates.J.parasitol,Vol. 62, pp. 360-365
- Chatterjea , M. and Shinde , R., (2005): Medical Biochemistry , Jaypee Brothers Med. Pub . Ltd , New Delhi. *
- Coombs ,G.,Hassan , H., Lockwood ,B., and Tetley ,L.,(1987): Phosphomonoesterases and proteinases of leishmanias . In Host parasite cellular and Molecular interactions (Chang,K and Snary ,D., eds.) Springer Verlay ,Berlin.

- Dwyer,D.and Gottlieb,M.,(1985): The biochemistry of *Leishmania* surface membranes . In Leishmaniasis (Chang.K. and Brey ,R.eds). pp 32-47.Elsevier, Amsterdam.
- Glew, R., Diven, W.,Berens ,R. and Katsoulis,D.,(1982): Partial purification of particulate acid phosphatase of *Leishmania donovani* .Comp.Biochem.Physiol.,Vol. 72,pp. 581-590.
- Gottlieb ,M. and Dwyer, D.,(1982): Localisation and partial characterization of extracellular acid phosphatase of *Leishmania donovani* . Mol.Cell.Biol.,Vol. 2,pp. 76-81.
- Gottlieb, M.and Dwyer, D.,(1981):*Leishmania donovani* surface membrane acid phosphatase , Exp.Parasitol, Vol. 52,pp. 117-128.
- Harris, H.and Hopkinson ,D.,(1976): Handbook of enzyme electrophoresis in human genetics Amsterdam,North Holland Pub.Co.
- Hassan ,H.and Coombs,G.,(1987): Phosphomonoesterase of *Leishmania mexicana* and other flagellates. Mol.Biochem.Parasitol,Vol. 23,pp.285-296.
- Hassan ,H.and Coombs,G.,(1988): Purine and pyrimidine metabolism in parasitic protozoa. FEMS Microbiol ,Rev., Vol .54,pp. 47-84.
- Lowery,D.,Rosebrough,N.,Farr,A. and Randall,R., (1951): Protein measurement with folin phenol reagent.J.Biol.Chem., Vol. 193, pp. 265-275.
- Mc Gwire, B., Oconnell,W., and Engman, D.,(2002): Extra cellular release of glycosylphosphatidylinositol linked *Leishmania* surface metalloprotease .J.Biol.Chem., Vol.277, pp.8802-8809.
- Mc Gwire,B.Chang,K. and Engman ,D.(2003): Migration through the extracellular matrix by parasitic protozoa *Leishmania* is enhanced by surface metalloprotease .Infect. Immun ., Vol .71,pp. 1008-1010.
- McConville ,M., Mullin ,K., Ilgoutz , S. and Teasdale ,R., (2002): Secretory pathway of trypanosomatid parasites . Microbiol .Mol. Biol. Rev., Vol .66 , pp.122- 154.
- Mottram , J., Coombs , G. and Alexander ,J., (2004) : Cysteine peptidases as virulence factors of *Leishmania* . Curr.Op. Microbiol ., Vol .7 ,pp. 375-381.

- Mullin,k.,Foth ,B., Ilgontz , S. and McConville , M.,(2001): Regulated degradation of endoplasmic reticulum membrane protein in tubular lysosome in *Leishmania mexicana*.*Mol. Biol . Cell* ,Vol.12 , pp. 2364-2377 .
- Paugam ,A.,Bulteau ,A. and Friguet , B., (2003): Characterization and role of protozoan parasite proteosomes. *T rends parasitol.*,Vol. 19 , pp. 55-59.
- Pimenta ,P. and de souza, (1986): Cytochemical localization of acid phosphatase in *Leishmania m. amazonensis*.*J.Submicrosc.Cyt.*,Vol.18, pp. 127-132.
- Remaley,A.Das,S. and Glew,R.,(1985): Character isition of leishmania donovani acid phosphate .*J.Biol.Chem.*,Vol. 260,pp.880-886.
- Remaley,A.,Kuhus,D.,Glew R. and Kaplan, S.,(1984): Leishmanial phosphatase blocks neutrophil O₂ production .*J.Biol.Chem.*, Vol.259,pp. 11173-11175.
- Spath,G., Garrawy.L.and Beverley ,S.,(2003) : The role of lipophosphoglycan in the establishment of leishmania major infection in mammalian host . *Proc.Nat.Acad Sci.Usa.*, Vol. 100,pp. 9536-9541.
- Yao, C.,Doneison , J. and Wilson , W.,(2003): The major surface protease of *Leishmania* sp. *Mol.Biochem. parasitol.*, Vol.132,pp.1-16.

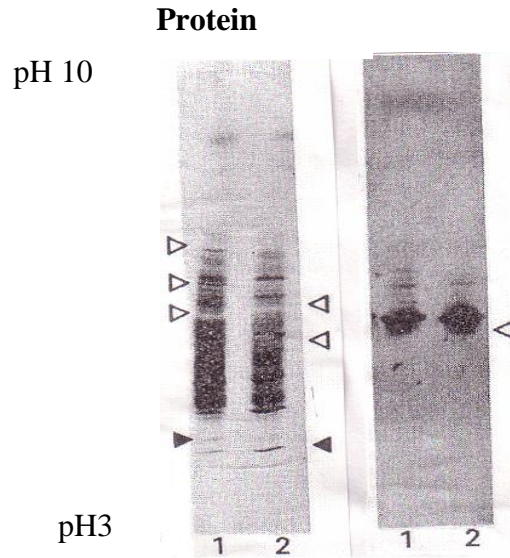


Fig . 1. Isoenzymel Of acid phosphatase and protein profiles of *L. major* mid log phase and stationary promastigotes . Lane 1 , mid log phase promastigotes ; Lane 2 , stationary promastigotes . The results shown are representative of the 3 or more samples of each form studied . Closed arrow indicate quantitative differences between the two promastigote forms , open arrows indicate apparent qualitative differences .

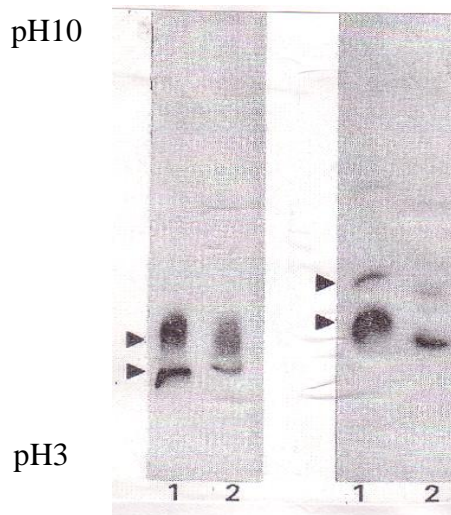


Fig . 2. Isoenzymel profiles of mid log phase promastigotes (Lane 1) and stationary promastigotes (Lane 2) of *L. donovani* (a) and *L. tropica* (b) . The results shown are representative of the two or more samples of each form studied . Closed arrow indicate quantitative differences between the two promastigote forms .

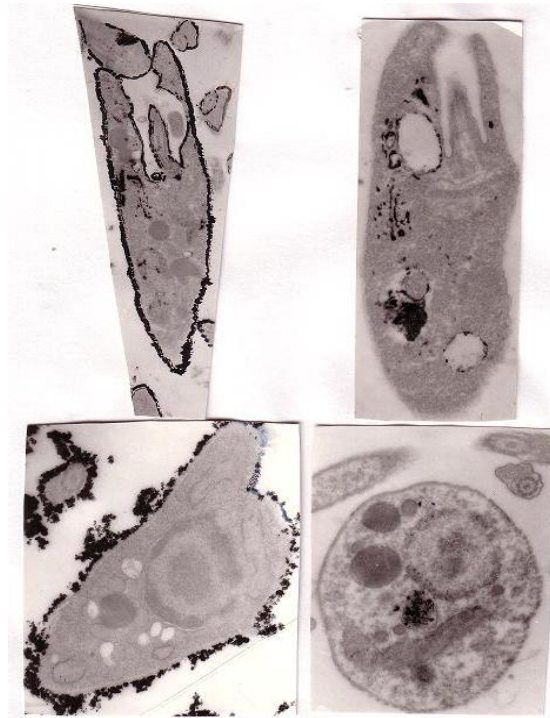


Fig .3. Ultrastructural localization of acid phosphatase in *L.donovani* mid log phase promastigote (a) and stationary phase promastigote (b) and in *L.major* mid log phase promastigote (c) and stationary phase promastigote (d) . Note the absence of reaction product from the surface yet the presence of such within the *L.major* (c and d) (arrowed). Abbreviation : f , flagellum ; fp , flagellar pocket ; k, kinetoplast ; m, mitochondria ; n, nucleus.

Table.1. Activity of acid phosphatase in a range of *Leishmania*

	Species	Form	Specific activity ^a	% Sedimentability ^b	
				Supernatant	Pellet
1.	<i>L.major</i>	Mid-log promastigotes	82 ± 4	21±2	79±6
		Stationary promastigotes	65±2	25±3	75±5
2	<i>L.tropica</i>	Mid-log promastigotes	59 ±6	62 ±4	38 ±4
		Stationary promastigotes	44 ±3	66 ±5	34 ±5
3.	<i>L.donovani</i>	Mid-log promastigotes	96 ±8	71±6	29±2
		Stationary promastigotes	71±5	67±5	33±4

- a. The activities given are those in parasite homogenates , are expressed in nmol/min/mg protein and are the means ± SD from three determinations .
- b. The activity sedimented in the 105,000xg pellet as a % of the total activity recovered .The figures are the means ±SD from replicate experiments

هل الفوسفاتيز الحامضي الغشائي السطحي ضرورة ملحة لنمو الليشمانيا داخل الخلايا البلعمية ؟

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الخلاصة

تم اجراء الكشف عن تواجد فعالية انزيم الفوسفاتيز الحامضي في انواع الليشمانيا بهدف
عما اذا كان الانزيم بمثابة تكييف لنمو وحيوية الطفيلي في الحشرة او في اللبائن . تدل نتائج هذه
الدراسة بعدم وجود اختلاف في فعالية انزيم الفوسفاتيز الحامضي بين الطور اللوغارثيمي والطور
الثباتي لبروماستيجوت الليشمانيا الكبيرة (ليشمانيا ميجر) ، وكا تشير النتائج الى وجود اختلاف في
الفعالية الانزيمية بين ليشمانيا ميجر والليشمانيا الاستوائية (ليشمانيا تروبيكا) والليشمانيا الاحشائية
(ليشمانيا دونوفاني) . وعلى العكس من ليشمانيا تروبيكا وليشمانيا دونوفاني ، فان الفوسفاتيز الحامضي
لا تقع في الغشاء السطحي لبروماستيجوت ليشمانيا ميجر وبذلك تقترح الدراسة بان الفوسفاتيز الحامضي
ليست ضرورية لنمو وتكاثر جميع انواع الليشمانيا في الخلايا البلعمية .