*International Journal of Current Research and Academic Review*

**ISSN: 2347-3215 Volume 4 Number 12 (December-2016) pp. 54-63**

**Journal home page:** http://www.ijcrar.com

doi: http://dx.doi.org/10.20546/ijcrar.2016.412.005

# Partial Purification and Properties of Adenosine Triphosphatase (ATPase) From *Leishmania tropica*

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**KEYWORDS**

ATPase, *Leishmania tropica*, gel filtration on sephadex G-200, DEAE-Cellulose chromatography.

**A B S T R A C T**

The plasma membrane of cells contains enzymes whose active sites face the external medium rather than the cytoplasm. The adenosine tri phosphatase (ATP phosphohydrolase, EC 3.6.1.3.; ATPase) is membrane – bound enzyme which transport protons across the plasma membrane using ATP as an enegy source. In this work, we extracted the adenosine tri phosphatase from promastigotes of *Leishmania tropica* by chloroform treatment and purified by means of ammonium sulphate fractionation, gel filtration on sephadex G-200 and DEAE-Cellulose chromatography. Kinetic experiments demonstrated a biphasic linear lineweaver - burk relationship (km= 0.25 and 1.1 mM) thus revealing the existence of two substrate binding enzyme site and has an apparent molecular weight of 365000 dalton by gel filtration. The result of this study firmly provided the first direct evidence for the existence of $Mg^{+2}$ - dependent ATPase in *L. tropica*, a fact which is of great interest from the phylogenetic point of view.

## Introduction

Adenosine triphosphatase (ATP phosphohydrolase, EC 3.6.1.3.; ATPase) is widely distributed in nature. In eukaryotic cells, ATPase considered as a marker for cytoplasmic membranes (Jesus *et al*., 2002; Meyer-Fernandes *et al.*, 2004). The main contribution of ATPase in membranes to cellular metabolism is to couples the energy released in intracellular hydrolysis of ATP to the export and the import of extracellular ion gradients across the cell membrane. The properties and roles of this enzyme in animal cells have been extensively investigated (Sarmila *et al*., 1992; Cortes *et al*., 2006). ATPase is also present in many parasitic protozoa and helminthes (Elandalloussi *et al*., 2005; de Sa Pinheiro *et al.*, 2008; Moore *et al.*, 2012) and it is grouped into several subfamilies depending on the extent of their sequence homology. In

parasitic protozoa, it has been postulated that the uptake of glucose, amino acids and such ions as protons, calcium, chloride and phosphate, may depend upon a H+– ion gradient generated by a parasite plasma membrane ATPase (Dan *et al.,* 1987; Tanushri *et al.,* 2005). Also, many intracellular parasites can maintain a membrane potential susceptible to protonophores probably through the same mechanism (Borges *et al*., 2007; Vesna *et al.,* 2008). This H+– ion pump may regulate parasite pH, possibly in conjunction with a K+/ H+ exchanger. Cation ATPase from Leishmania species have been studied both physiologically and by sequence analysis (Sarmila *et al*., 1992).

In this paper we will describe the purification of ATPase and its properties and physical characterisatics, as well as additional evidence concerning its surface location.

## Materials and Methods

*Organisms: Leishmania tropica* (MHOM / SU/ 74 / K27) promasigotes were grown at 26 °C and harvested as described previously (Hassan and Coombs, 1985). Washed parasites were stored as pellets at -70 °C until use.

## Preparation of crude homogenate and cellular fractionation

All procedures were performed at 4°C. Promastigotes were lysed in TSD buffer containing 50 mM Tris- Hcl (pH 7.6) containing 0.25M sucrose and 0.1m M dithiothritol by three cycles of 10 min. freezing in liquid nitrogen and thawing at 25 °C. The resulting homogenate was centrifuged for 1 hr. at 105 000 x g at 4 °C. The material sediment (pellet) was removed, resuspended in TSD buffer in a same volume as supernatant recovered. To investigate the subcellular localization of the enzyme, the promasigote were lysed as described above in TSD buffer containing 0.2% Triton x-100 and subjected to differential centrifugation as previously described (Hussain and Ali, 2014), yielding partial fraction P1 (2100 xg for 10 min.), P2 (15800xg for 10 min.) and P3(24000xg for 1 hr.).The four fractions produced were frozen and thawed thrice to disrupt organelles and were assayed for enzyme activity.

## Purification of the ATPase

The promastigotes were suspended in TSMA buffer containing 50 mM Tris-HCl, pH7.6, 0.25 M sucrose, 2.5 mM magnesium chloride and 2% bovine serum albumin and disrupted by 3 cycles of freezing and thawing. The resulting homogenate was centrifuged at 10000xg for 10 min. at 4 °C. The supernatant was removed and the pellet was resuspended in TSMA buffer and used as source for membrane bound ATPase.

The chloroform (2 vol.) was added to the particles suspended in TSMA buffer, thoroughly mixed and the precipitate protein eliminated by centrifugation for 60 min. at 10500x g at 4°C. The supernatant containing the solubilized ATPase was fractionated with solid ammonium sulphate. The 0 - 70% saturation fraction, obtained by centrifugation at 30000xg for 15 min. at 4°C was dissolved in 10 mM Tris-HCl (pH7.6) containing 1.0 mM EDTA (TE buffer), and was loaded on to Sephadex G-200(1.6×34cm) equilibrated with TE buffer and eluted with the same buffer at a flow rate of 8 ml / hr. Fractions of 4 ml were collected.

The active fractions from the Sephadex G-200 column were pooled and loaded on to a column (1.3×45cm)of DEAE- cellulose

equilibrated with the same TE buffer and eluted with continuous gradient of KCl(0-0.5M). The active fractions were pooled, concentrated by precipitation with ammonium sulphate at 70% saturation and used for the experiments described.

### Molecular weight determination

The approximate molecular weight of the native enzyme was determined by gel filtration (Andrew, 1965) on a column (1.6×34cm) of Sephadex G-200 equilibrated with TE buffer, using ovalbumin (43000), bovine serum albumin (67000), lactate dehydrogenase (140000), catalase (248000), Ferritin (440000) as protein markers, under experimental conditions similar to those of the sephadex step of the purification procedure. The void volume of the column was determined with Blue Dextran 2000.

### Enzyme assay

ATPase activity was conducted at 37°C in a 1 ml reaction volume of 50 mM Tris-HCl buffer (pH7.2), 1.0 mM MgCl2, 1.0 mM ATP, and sufficient enzyme to yield an appropriate reaction rate. Assays were terminated after 30 min. by the addition of 1 mL of trichloro acetic acid 10% (w/v) and the resulting mixture was centrifuged at 2000 g for 10 min. at 4°C. Liberated inorganic phosphate (ip) was determined by the method of Fisk and Subbarow. One unit of ATPase activity is defined as the amount of enzyme which hydrolyses 1.0 µmol of ATPase per min. per mg protein.

### Statistical analysis

All experiments were performed in triplicate, with similar results obtained in at least three separate cell suspensions. Apparent *K*m and *V*max values were calculated using a computerized nonlinear regression analysis of the data to the Michaelis– Menten equation. Statistical significance was determined by Student's *t*-test.

Significance was considered as $P < 0.05$.

## Results and Discussion

The activity of ATPase recovered in the various cell fractions of L. *tropica* is shown in the Tables 1 and 2. ATPase was recovered in all fractions but the highest amount were in fractions P1, P2 and P3.. By the same procedure, succinate dehydrogenase which is known to be a particulate enzyme was recovered exclusively in the pellet fraction. It was concluded that the ATPase is particulate enzyme. Table (3) shows the results of a representation partial purification of ATPase. The yield of the chloroform extraction was 61%; this might be due to some latency of the ATPase activity in the particles. The solubilized enzyme by precipitation with ammonium sulphate was found to be efficient to eliminate the fat and contaminating proteins. Although precipitation with ammonium sulphate made it possible to concentrate the solubilized enzyme into a small volume with no effect on the enzyme specific activity. The enzyme was further purified with Sephadex G-200 and DEAE Cellulose to eliminate the trace amount of contaminated protein. It has been shown that about 95% of the solubilized enzyme was recovered after gel filtration on Sephadex G-200 and about 77% of this fraction were eluted from DEAE- Cellulose column chromatography by KCl. The enzyme, was purified by 97 fold with a yield of about 30% and 44 µ mol/min./mg protein. The molecular weight of the native enzyme determined by gel filtration on Sephadex G-200 was about 365000 dalton (Figure 1).

Preliminary assay of ATPase was conducted to ensure that the reaction rate (liberation of inorganic phosphate) was a linear function of assay time and protein concentration in the assay mixture; under such conditions these reaction rates should provide a reasonable estimate of initial reaction velocities.

The pH optimum of ATPase activity was 7.0 and further ATPase assays were conducted at this pH. Under these assay conditions, the ATPase activity displayed a biphasic double reciprocal plot which allowed the calculation of two different Km values, namely 0.25 and 1.1 mM (Figure 2).

**Table.1** Cellular fractionation of ATPase from *L.tropica*

| Fractions | Total volum (ml) | Specific activity µmol/min/mg protein | Total activity | %activity |
|---|---|---|---|---|
| Crude homogenate | 6.0 | 0.687 | 164.8 | |
| Pellet (p) | 5.3 | 0.510 | 108.1 | 67 |
| Supernatant | 4.8 | 0.285 | 54.7 | 33 |

**Table.2** Distribution of ATPase in subcellular fractions of *L.tropica*

| Fractions | Total volum(ml) | Activity* | Total activity | % Racovered activity |
|---|---|---|---|---|
| Crude homogenate | 4.0 | 0.024 | 3.84 | |
| Pellet (p1) | 3.8 | 0.019 | 2.93 | 29 |
| Pellet (p2) | 3.6 | 0.018 | 2.68 | 26 |
| Pellet (p3) | 3.5 | 0.019 | 2.70 | 26 |
| Supernatant | 3.4 | 0.014 | 1.90 | 19 |

*** The activities given are in µmol of inorganic phosphate liberated from hydrolysis of ATP.**

**Table.3** Purification of the ATPase from *L.tropica*

| Step | Total protein(mg) | Total avtivity µmol /min | Specific activity µmol/min./mg protein | Purification fold | Yield% |
|---|---|---|---|---|---|
| 800-4500x | 11.520 | 5.40 | 0.46 | | |
| Chlrorfrom exraction | 6.720 | 3.31 | 0.49 | 1.056 | 61 |
| Ammonium sulphate precipitation | 1.440 | 2.24 | 1.55 | 3.360 | 41 |
| Sephadex G-200 | 0.123 | 2.11 | 17.01 | 36.970 | 39 |
| DEAE-cellulose chromatography | 0.036 | 1.60 | 44.44 | 96.900 | 30 |

**Table.4** The effects of various potential effectors on ATPase activity of *Leishmania tropica*

| | Assay mixture | ATPase Activity* |
|---|---|---|
| 1- | Exract +1Mmatp | 48 |
| 2- | + 2.5m M EDTA | 0.9 |
| 3- | + $Mg^{+2}$ | 163.2 |
| 4- | + $Ca^{+2}$ | 87.7 |
| 5- | + $Mn^{+2}$ | 120.4 |
| 6- | + $Ca^{+2}$ , $Mg^{+2}$ | 163.6 |
| 7- | + $Mg^{+2}$, $Mn^{+2}$ | 109.5 |
| 8- | + $Ca^{+2}$ , $Mn^{+2}$ | 131.3 |
| 9- | +1Mm ouabain | 48.1 |
| 10- | +50mM $Na^{+}$ +10mM $K^{+}$ | 48 |
| 11- | + 50mM $Na^{+}$ +10mM $K^{+}$ +1mM ouabain | 48.2 |
| 12- | + $Mg^{+2}$ +10m M fluorid | 159.4 |
| 13- | + $Mg^{+2}$ +10m M Molybdate | 38.2 |

***The activities given are in µmol of inorganic phosphate liberated from hydrolysis of ATP, and values represent the mean of triplicate determinations using a single purified enzyme.**

**Fig.1** Estimation of the molecular weight of ATPase by gel filtration on Sephadex G-200. Standard proteins F=Ferritin; BSA=Bovine Serum albumi; OV= Ovalumin; C=Catalase ; LDH=Lactate dehydrogenase. The elution volume determined by Blu dextran 2000

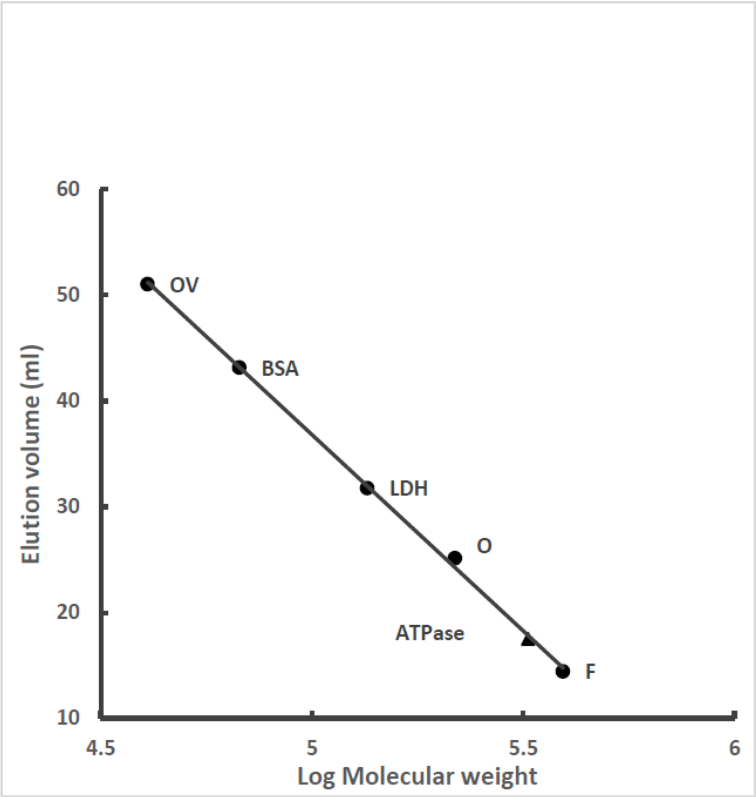



**Fig.2** Line weaver-Burke plot of the effect of substrate concentration (ATP) on purified ATPase. The reaction mixture contain Tris-HCl 50 mM, pH8.0, Mg Cl2 5Mm, ATP(0.05-20) mM

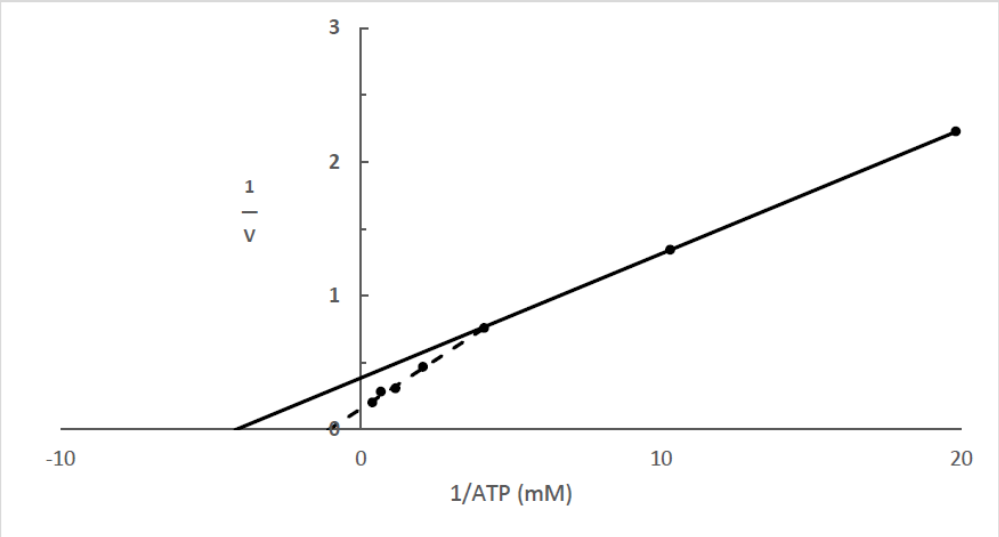


**Fig.3** Line weaver –Burke plot of the effect of the estimation of Km (Ca+2) value to ATPase in *Leishmania tropica*. The reaction mixture contains Tris- Hcl 50mM,PH7.6,ATP (1) Mm,Ca Cl2(0.1-1) mM.

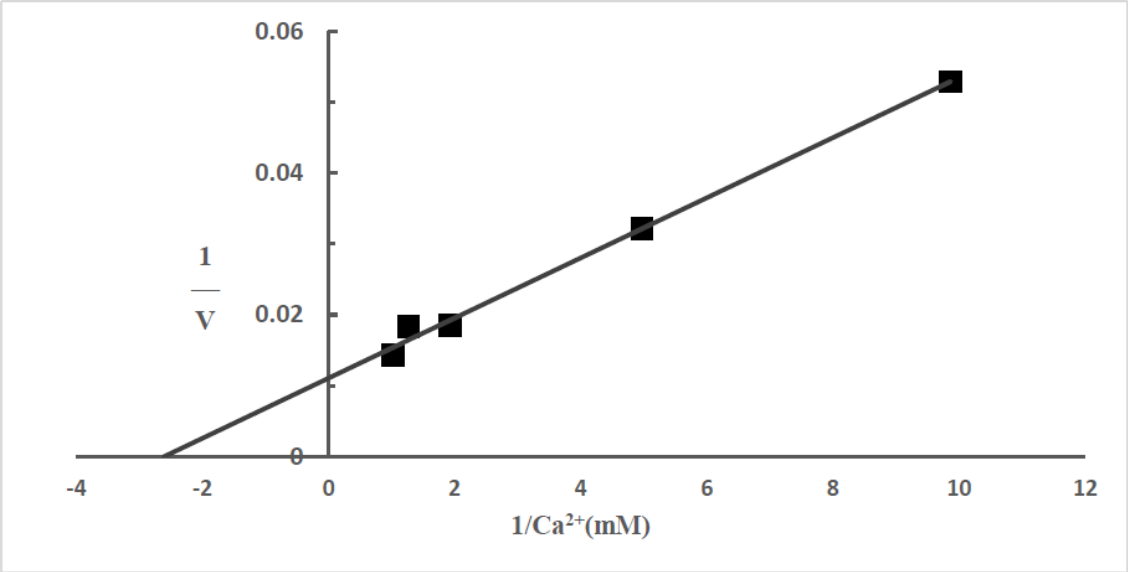
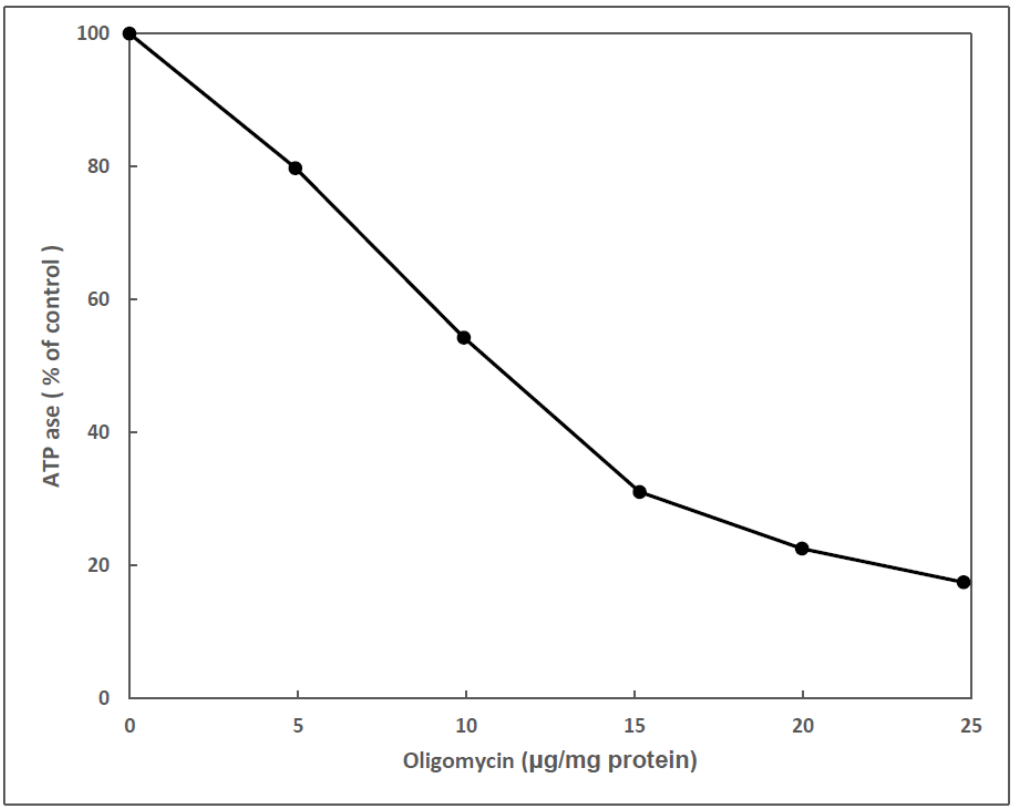



**Fig.4** Inhibition of ATPase by Oligomycin in L. tropica,




In the absence of divalent cations the purified ATPase displayed significant activity. It is evident (Table4) that the ATPase activity was neither inhibited nor

stimulated by the addition of Na+ and K+ (Na+ / K+ ratio 5/1) to the assay mixture.

Also, in the presence and absence of Na+ / K+ the ATPase activity was not inhibited by 1mM Ouabin (Table 4). On the other hand, the Km value was found to be 3.8 mM for Ca –ATPase on different Ca+2 at fixed ATP concentration(1Mm) (Figure3), As shown in (Table 4). (Figure4) shows the inhibition of ATPase activity by the antibiotic oligomycin. The maximum inhibition was about 81%.

The present investigation has shown that in several respects the ATPase of *L.tropica* appear to be similar to those of parasitic protozoa (Jesus *et al*., 2002; Meyer-Fernandes *et al.,* 2004; Cortes *et al*., 2006) and parasitic helminthes (Wani and Srivastava, 1995; Cunha *et al.*, 1996; Moore *et al.,* 2012).

The recovery of a significant proportion of ATPase activity in the particulate fractions suggest that this enzyme may be associated with tegument as has been reported in the tapeworm *Hymenolepis diminuta* (Wani and Srivastava, 1995). The ATPase of *L. tropica* appear to function in the hydrolysis of nucleotide and the resultant nucleotide could be further catabolised by the surface located 5¢- nucleatidase (unpublished data) to yield nucleotides, which could be taken into the cells more easily and so it is possible that the ATPase may play a part in the interaction of the parasite with its host. The specific activity of purified ATPase in this investigation was considerably higher than most ATPase attained values available from other parasite such as *Crithidia fasiculata ; Trypanosoma cruzi* (Higa and Cazzulo, 1981; Meyer-Fernandes *et al.*, 2004). The molecular weight of ATPase from *L. tropica* are shown to be fairly consistent with those reported for other ATPase, among them the enzymes from *T. cruzi* 2 and *C. fasiculata* (Higa and Cazzulo, 1981). The calculation of the different Km value for purified *L. tropica* are very similar to those reported for the purified *T. cruzi* enzyme (Frasch *et al*., 1978).

Comparison of the ATPase activity reported in this study with that of other animal cell membranes indicates that the ATPase in the *L. tropica* does not resemble the more intensively studied ATPases. The ouabain insensitivity of the enzyme (in the absence or present of added Na+ and K+) in the *L. tropica* suggest that it is not due to Na+ - K+ activated ATPase. In contrast, the existence of two (Na+, K+) ATPase isoforms has been reported in the related trematode *Scistosoma mansoni*. The absence of Ca+2 activation also indicate the absence of Ca+2 transport function usually associated with the Ca2+ activated, Mg+2 - dependent ATPase (Ghosh *et al*., 1990). Unlike mitochondria Mg+2 - ATPase (Berrêdo-Pinho *et al*., 2001) the ATPase of *L. tropica* was only partially sensitive to oligomycin at high inhibitor concentrations. The small inhibition by oligomycin may be due to contamination of membrane fraction with mitochondria and so it is possible to suggest that the origin of the major portion of ATPase activity in this was not mitochondrial. The result of this study firmly provided the first direct evidence for the existence of Mg+2 – dependent ATPase in *L. tropica*, a fact which is of great interest from the phylogenetic point of view.

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**How to cite this article:**

Husain F.Hassan, Abeer Abbas Ali. 2016. Partial Purification and Properties of Adenosine Triphosphatase (ATPase) From *Leishmania tropica*. *Int.J.Curr.Res.Aca.Rev.*4(12): 54-63.
doi: http://dx.doi.org/10.20546/ijcrar.2016.412.005