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Extracellular release of acid phosphatase from blood stream forms of *Trypanosoma brucei brucei*.

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ABSTRACT: Acid phosphatase (ACP) activity was demonstrated in blood stream form of *Trypanosoma brucei brucei* harvested from infected Wister rats by Ion Exchange DEAE Cellulose 52 chromatography. Whole parasite extract (WPE) and Excretory Secretory Extract (ESE) were prepared and analyzed for acid phosphatase activity. A higher ACP activity (85.5 $\mu\text{mol}/\text{min}$) was recorded in WPE compared to ESE (36.8 $\mu\text{mol}/\text{min}$). ACP activity in ESE is suggestive of the presence of a cell rich enzyme. Phase separation of the extracts using the detergent Triton X-114 (TX-114), resulted in protein partitioning into aqueous and detergent phases. ACP activity was higher in the detergent phases (56.2 $\mu\text{mol}/\text{min}$ and 28.8 $\mu\text{mol}/\text{min}$) of WPE and ESE respectively. ACP activity recorded in the aqueous phases of WPE and EPE was 27.8 and 7.6 $\mu\text{mol}/\text{min}$ respectively. On a Size Exclusion chromatography column using Sephacryl-300, ESE emerged as five distinct protein peaks. ACP activity of the eluted fractions showed two peaks of relative molecular weights 195 and 325 KD. This study shows that *T. brucei* releases acid phosphatase extracellularly via a yet to be determined mechanism. Acid phosphatase activity in ESE is indicative of a soluble enzyme within the cell matrix which may also play an important role in the pathology of African Trypanosomiasis.

KEYWORDS: *Trypanosoma brucei*; acid phosphatase; enzyme secretion.

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INTRODUCTION

African trypanosomes are protozoan parasites of man and animals. Several species of trypanosomes infect mammals with the three most important belonging to the *Trypanosoma brucei* complex. *T. brucei brucei* causes "nagana" in cattle, *T. brucei gambiense* causes chronic human disease in Western and Central Africa and *T. brucei rhodesiense* results in acute human infections in Eastern Africa (Barrett, 1999). The disease in humans is termed the "Human African trypanosomiasis" (sleeping sickness). The transmission of the parasite and resulting disease in cattle renders a large area of sub Saharan Africa refractory to the production of cattle; causing nutrient deficiencies and significant economic losses in livestock. Trypanosomes cause devastating diseases resulting in high human mortality; sleeping sickness is invariably fatal if not treated (Kennedy, 2004).

Several enzymes, including acid phosphatase, acid pyrophosphate and adenylate cyclase have been identified in the flagellar pocket membrane of *Trypanosoma brucei* (Walter & Opperdoes, 1982). Acid phosphatases are a group of enzymes found in many plant and animal species (Bull *et al.*, 2004). They have the ability to catalyse the hydrolysis of orthophosphate monoesters under acidic conditions and are known to facilitate important physiological changes within cells (Bull *et al.*, 2004). Whether the enzyme is of plant, animal or microbial origin, acid phosphatase from these widely diverse origins can be divided into two main classes; high and low molecular weight enzymes (Tanizaki *et al.*, 1977), which hydrolyses orthophosphoric monoesters at acid pH. These two classes differ with respect to their intracellular distribution, substrate specificity, behaviour towards inhibitors, sequence length, amino acid homology, tissues and chromosomal origin (Bull *et al.*, 2004; Igarashi & Hollander, 1968; Chaimovich, & Nome, 1970). Acid

phosphatases have been identified in parasitic protozoa of the family *Trypanostomastidae*, including *Crithidia* species (McLaughlin *et al.*, 1976) salivarian Trypanosomes (Brooker & Vickerman, 1968) and *Trypanosoma cruzi* (Avila *et al.*, 1979; Steiger *et al.*, 1979). This enzyme has been cytochemically localized within these flagellates to the flagella pocket and vesicles adjacent to the base of the flagellum, endosome, lysosomal compartments and within reservoir surrounding emerging flagellum of Trypanosomes (Langreth & Balber, 1975; Schell *et al.*, 1990). There are reports of at least two different acid phosphatases (Langreth & Balber, 1975; Schell *et al.*, 1990) in *Trypanosoma brucei* and three acid phosphatases (Amlabu *et al.*, 2009) from the lysosome of blood stream forms of *Trypanosoma brucei brucei*. The presence of a membrane extracellular glycosylated acid phosphatase in *Leishmania donovani* suggested that these parasites secrete acid phosphatase into the surrounding medium (Menz *et al.*, 1991; Cazzulo *et al.*, 1990). Acid phosphatases are proposed to be involved in the regulation of pyridoxal phosphate requiring enzymes, in steroid transport, vitamin B6 metabolism and in lipid metabolism (Andrews & Turner, 1996; Kaplow & Burstone, 1964; Blank & Snyder, 1970). There are several possibilities to the physiological role of the major bound acid phosphatase. Some ACP located on the cell surface catalyses the hydrolysis of key glycolytic intermediate Fructose-1,6-phosphate and thus raises the possibility that once the parasite has been internalized by the host, the parasite derived phosphatase activity could disturb glycolysis specifically and energy metabolism in general. The parasite ACP while acting together with its proteases could cause tissue degradation thus easing tissue invasion, including the blood-brain barrier, by the trypanosome (Lonsdale-Eccles & Grab, 2002). ACP has also been proposed as one of the virulence factors in Leishmaniasis (Shakarian & Dwyer, 2000; Love *et al.*, 1998).

The flagellar pocket is the sole site of endocytosis and secretion in trypanosomes and several studies have suggested the presence of membrane bound ACP in *T. brucei*. This necessitates the investigation of the presence of secreted ACP from blood stream forms of trypanosomes and the likely role of such exocytosed molecules in the pathology of African Trypanosomiasis. This paper reports the acid phosphatase activity in the excretory secretory extract of *T. brucei brucei*.

MATERIALS AND METHODS

Parasite maintenance and harvest

Trypanosoma brucei brucei were collected from the Nigerian Institute for Trypanosomiasis Research (NITR), Vom, Nigeria and maintained in the laboratory in Albino rats in house breed at the Department of Zoology, University of Ibadan. Age and sex matched rats were employed in this study. The rats (10) were each inoculated intraperitoneally with 10^7 *T. b. brucei* in 0.5 ml of PSG. At peak parasitaemia (1×10^9 /ml), Trypanosomes were harvested from the rats by cardiac puncture using tri-Sodium citrate as anticoagulant. The parasites were separated from blood by DEAE-Cellulose ion exchange chromatography, using Phosphate Saline Glucose (PSG) pH 8.0 as column buffer (Lanhan & Godfrey, 1970).

Whole Parasite Extract (WPE) and Excretory-Secretory Extract (ESE)

Pelleted trypanosomes obtained by centrifuging eluent from DEAE Cellulose Chromatography were suspended in phosphate saline glucose buffer (PSG) pH 8.0. Trypanosomes were counted on a haemocytometer and 1×10^8 /ml was incubated in PSG at 4 °C for 30 minutes and then centrifuged at 1000 g for 10 minutes in a Beckman centrifuge (J-21). The resulting supernatant (ESE) was decanted and concentrated at 7500 g for 45 minutes using Centricon-10 (Millipore). The protein content of ESE collected was determined as described by Bradford using BSA as standard (Bradford, 1976).

To prepare the whole parasite extract, pelleted trypanosomes separated from blood by DEAE-Cellulose Chromatography were lysed using silicon carbide. Lysate was re-suspended in PSG and centrifuged at 1000 g for 10 minutes to remove silicon carbide. Supernatant lysate was incubated for 1 hour at 4 °C concentrated using Centricon 10. Acid phosphatase activity was determined for both WPE and ESE extracts.

Triton X-114 Phase Separation.

WPE and ESE were subjected to Triton-X 114 treatment by placing a mixture of 100 μ l each of test samples, 367 μ l of 10 mM Tris-HCl, 150 mM NaCl buffer and 1% Triton X-114, which had been maintained at 4 °C onto a 600 μ l of 0.06% Sucrose cushion. The mixture was incubated at 30°C until cloudy and centrifuged at 800 g for 5 minutes. Clearly defined detergent and aqueous phases were formed, which were aspirated into separate tubes and the sucrose cushion was discarded. Acid phosphatase activity was determined for each phase.

Determination of Acid Phosphatase Activity

Acid phosphatase activity was determined as described by Steiger *et al.* (1979). Briefly, to 1 ml of sample was added 1 ml of freshly prepared 0.1 M 4-Methylumbelliferly phosphate in 0.05 M Sodium acetate buffer (pH 5.0). The mixture was incubated at 37 °C for 10 minutes, enzyme reaction was terminated by addition 3 ml of 0.05 M Glycine-Sodium hydroxide buffer (pH 10.4). A reaction mixture in which substrate was incubated in the absence of test sample (enzyme) and the appropriate volume of enzyme added after the addition of glycine-sodium hydroxide buffer, compensating for non-enzymatic hydrolysis, served as control. A blank mixture was prepared containing the substrate and buffer in the absence of the enzyme. Enzyme activity (RF values) was determined on a spectroflurometer using 4-Methylumbelliferon as standard.

The standard was prepared by dissolving one μ mol/ml of 4-Methylumbelliferon in 0.05M Sodium Acetate-Acetic Acid buffer, pH 5.0. Serial dilutions (0.0-0.2 μ mol/ml) were prepared. To each dilution was added 3ml Glycine-Sodium hydroxide buffer pH 10.4 and mixed thoroughly. The Relative Fluorescence (RF) was determined for each dilution at Excitation (EX) = 364 nm, Emission (EM) = 448 nm at 50% calibration. The RF values obtained were plotted on a linear graph against sample concentration.

Enzyme activity ($\mu\text{m MUP}$) for each fraction collected was calculated from RF values;

$$\mu\text{m MUP} = \frac{\text{standard MUP}}{50\% \text{ calibration}} \times \text{RF}.$$

The specific activity of acid phosphatase was calculated using the formula:

$$\mu\text{m MUP/mg Protein} =$$

$$\frac{\frac{1000}{\text{vol of sample}} \times \frac{\mu\text{mMUP}}{1}}{\text{mgP}} \times \frac{1000}{\text{vol of sample}} \times \frac{\mu\text{mMUP}}{1}$$

Size Exclusion Chromatography

Partial purification of ESE was carried out by gel filtration on a pre-swollen Sephacryl-300 (BIO-RAD) column and equilibrated according to manufacturer instructions with 0.05 M Sodium acetate acetic acid buffer (pH 5). A 2 ml sample was layered on packed column and eluted with the same buffer at 4 °C at a flow rate of 18 ml/cm²-hr. The eluents were collected in 3ml fractions and absorbance of each fraction was measured at 280 nm to locate protein containing fractions. The molecular weight of each protein peak obtained was estimated after the calibration of the column and acid phosphatase activity was determined for each fraction collected and fractions with activity were pooled for electrophoresis.

Column Void volume

This was determined using Blue dextran 2000 and the elution volume (V_o) of Blue dextran 2000 is equal to column void volume. A fresh solution of Blue Dextran 2000 (1 mg/ml) in eluent buffer was prepared and layered on gel in column after elution of ESE. Blue Dextran was eluted at 18 ml/cm²-hr in 3 ml fractions. Absorbance of the eluents was monitored at A_{280} . The fraction with highest A_o value corresponds to V_o of the column.

Column Calibration and Molecular Weight Estimation

Standard proteins (Aldolase 5 mg/ml, Ferritin 1 mg/ml, and Bovine Serum Albumin 5 mg/ml) of known molecular weights were used for calibration by dissolving each protein in the eluent buffer and allowed to stand at room temperature for 10 minutes as recommended by (Pharmacia) the manufacturer. A 1 ml of the dissolved proteins was layered on the column and eluted at 18 ml/cm²-hr and collected in 3 ml fractions. V_o for each protein was measured at A_{280} . The molecular weights of these standard proteins were: Aldolase 158 kD, Ferritin 440 kD, Bovine serum albumin (BSA) 58 kD. The relative molecular weight (K_{av}) of the eluted proteins were calculated.

$$K_{av} = \frac{V_e - V_o}{V_t - V_o}$$

$$\frac{V_e}{V_o} = \text{relative mobility}$$

where V_e = Elution volume ,

V_t = Total bed volume,

V_o = Column void volume

The K_{av} values for each standard protein was plotted on a linear scale against their corresponding molecular weights and the points on the calibration curve which corresponds to the calculated K_{av} values for the protein peaks identified in ESE was equivalent to the estimated molecular weight of the peak.

RESULTS AND DISCUSSION

In the acid phosphatase assay of ESE and WPE, enzyme activity was recorded in both the WPE (85.8 $\mu\text{mol/min}$) and ESE (36.8 $\mu\text{mol/min}$). The specific activity of acid phosphatase recorded was 99.88 $\mu\text{mol/mgprotein/min}$ and 46.82 $\mu\text{mol/mg protein/min}$ for ESE and WPE respectively (Table 1).

When the extracts were subjected to phase separation with TX-114, ACP activity was recorded in the aqueous and detergent phases of both extracts. The enzyme activity recorded was higher in the detergent phase of both ESE and WPE compared with aqueous phases. The enzyme activity in both phases of WPE was 97.9% of total activity in extract before Tx-114 treatment while acid phosphatases activity recorded in ESE after treatment with TX-11 was 99% of untreated extract (Table 2).

The absorbance profile (A_{280}) of protein fractions obtained from size exclusion chromatography (Figure 1) showed five protein peaks of relative molecular weights 31, 50, 72, 160 and 298 kD. Acid phosphatase activity of the protein fractions showing 2 peaks of relative molecular weight 195 and 325 kD is shown in Figure 1. The column void volume was 80 ml.

The possible physiological function for the extracellular acid phosphatase in *Trypanosoma brucei* is yet unknown. It may allow the intake of necessary orthophosphate from the organic phosphates in the blood circulation. In this study acid phosphatase activity in ESE is suggestive of extracellular release into the incubation medium indicating the presence of a cell rich acid phosphatase. This is in consonance with the reports of Brooker (1971) which proposed that the acid phosphatase activity observed in the flagellar pocket reservoir was as a result of exocytotic defecation processes. It was however reported that ACP in *T. congolense* did not appear to be secreted into the surrounding medium by living parasites (Tosomba et al., 1996).

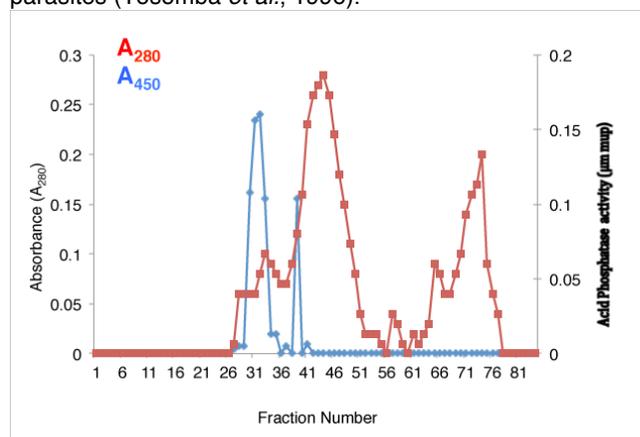


Figure 1: Protein profile and Acid phosphatase activity in eluted fractions from column chromatography of excretory secretory extract of *Trypanosoma brucei* blood stream forms.

Table 1: Acid Phosphatase activity in extracts from *Trypanosoma brucei brucei*

	Enzyme Activity ($\mu\text{mol}/\text{min}$)*	Specific Activity ($\mu\text{mol}/\text{mgProtein}/\text{min}$)	Protein content (mg/ml)
Whole Parasite Extract (WPE)	85.8	99.88	0.859
Excretory Secretory Extract (ESE)	36.8	46.82	0.786

* $\mu\text{mol}/\text{min}$ is amount of substrate hydrolyzed per minute

Table 2: Acid Phosphatase activity in aqueous and detergent phases of TX-114 treated extracts of *Trypanosoma brucei brucei*

	Relative Fluorescence (RF)	Enzyme Activity ($\mu\text{mol}/\text{min}$)	Specific Activity ($\mu\text{mol}/\text{mgProtein}/\text{min}$)	% yield
Whole Parasite Extract (WPE)	21.45	85.8	99.88	-
Aqueous phase	6.95	27.8	32.36	32.4
Detergent phase	14.05	56.2	65.42	65.5
Excretory Secretory Extract (ESE)	9.2	36.8	46.82	-
Aqueous phase	1.9	7.6	9.67	20.7
Detergent phase	7.2	28.8	36.64	78.3

The acid phosphatase activity recorded in WPE suggests the presence of membrane bound acid phosphatase which are released after fractionation of the parasite membrane. Williamson & McLauren (1981) suggested that ACP in *Trypanosoma rhodesiense* was membrane bound while Zingales *et al.* (1979) reported an enrichment of acid phosphatase activity in the plasma membrane fractions and there were suggestions that portions of these enzymes make up constituents of plasma membrane of *Leptoma collosoma* and *Leishmania donovani* (Gottlieb and Dwyer, 1981). Observations from this study suggest the presence of two acid phosphatase types, a membrane bound and a cytosolic form. There were reports of a membrane and an extracellularly glycosylated acid phosphatase in *L donovani* (Menz *et al.*, 1991). Dietma Schnell *et al.* (1990) also reported the presence of two different acid phosphatases in *T brucei*. They further suggested that acid phosphatase activity

appeared to be confined to the intracellular compartments, which directly communicate with the flagellar pocket.

The higher enzyme activity recorded in the detergent fractions of the extract after Tx-114 treatment is in consonance with Langreth & Balber (1975), who had found that more than half of the acid phosphatase activity of trypanosome blood stream forms was not detected unless activated in some way by a detergent. The presence, in this study, of enzyme activity in the hydrophobic aqueous phase is suggestive of a soluble enzyme. This could be attributed to the diffusion of the enzyme along a gradient resulting in extra-cellular release of acid phosphatase. Eeckhont (1972) had reported the accessibility of the enzyme to the exterior through a diffusion barrier across the endoplasmic reticulum in cells.

The eluted protein fractions had acid phosphatase peaks of molecular weight 195 and 325 kD, which could suggest that the enzyme may be made up of subunits similar to those reported by Menz *et al.* (1991) in which the presence of dimeric units of acid phosphatases in *L donovani* were suggested. Allen *et al.* (1984) reported a soluble 128 Kd acid phosphatase composed of 65,000 and 68,000 subunits.

Contrary to the report of Tosomba *et al.* (1996) that acid phosphatase did not appear to be secreted into the surrounding medium by living parasites, this study has shown a high enzyme activity in the medium (ESE) in which live parasites were incubated. Thus it was shown in this study that *T brucei* releases acid phosphatase extracellularly although the mechanisms, pathway and its role in the pathology of trypanosomiasis remains to be thoroughly investigated.

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