

**Zeitschrift:** Acta Tropica  
**Herausgeber:** Schweizerisches Tropeninstitut (Basel)  
**Band:** 36 (1979)  
**Heft:** 4

**Artikel:** Acid hydrolases of trypanosomatid flagellates  
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**DOI:** <https://doi.org/10.5169/seals-312536>

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## Acid hydrolases of trypanosomatid flagellates

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### Summary

Homogenates from 5 species of Trypanosomatids were screened for the presence of a series of acid hydrolases. The insect flagellate, *Crithidia* sp., contains 5 enzymes reminiscent of plant parasitism, which were absent from or of very low specific activity in parasites of the genera, *Trypanosoma* and *Leishmania*. The latter mammalian parasites, on the other hand, exhibited higher acid proteinase and  $\alpha$ -D-mannosidase activity levels.

*Key words:* *Crithidia* sp.; *Trypanosoma brucei*; *T. cruzi*; *Leishmania donovani*; acid hydrolases.

### Introduction

A number of hydrolytic enzymes have been demonstrated in haemoflagellates as reviewed by Eeckhout (1973). Their respective rôles in the process of lysosomal digestion, however, as well as the digestive capacity of trypanosomatid flagellates in general, remain largely unknown. Most of the acid phosphatase (Jadin, 1971; Steiger, 1973; Langreth and Balber, 1975) and sucrase (Eeckhout, 1972) activities are believed to be associated with the flagellar pocket, while acid proteinase of *Trypanosoma rhodesiense* (Venkatesan et al., 1977), as well as the latter enzyme and acid DNase of *Crithidia* are likely to be lysosomal as evidenced by structural latency (Eeckhout, 1973).

The purpose of the present investigation was to screen 5 flagellate species of the genera, *Crithidia*, *Leishmania* and *Trypanosoma*, for acid hydrolases known to be of lysosomal origin in higher eukaryotes (Barrett and Heath, 1977). We hoped that such a study would unveil differences in the digestive capacity reflecting the particular nutritional host environment of these closely related organisms and that such enzymes could serve as markers for cell fractionation studies.

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Table 1. Activities of acid hydrolases<sup>1, 2</sup>

| Enzyme                                 | pH <sup>3</sup> | Buffer  | <i>Crithidia</i> | <i>L. donovani</i> | <i>T. cruzi</i> |                   | <i>T. brucei</i> |         |
|--|-----------------|---------|------------------|--------------------|-----------------|-------------------|------------------|---------|
|  |                 |         |                  |                    | blood           | culture           | blood            | culture |
| $\alpha$ -D-glucosidase . . . . .      | 4.6             | acetate | 4.2              | <0.1               | <0.1            | N.D. <sup>4</sup> | <0.1             | N.D.    |
| $\beta$ -D-glucosidase . . . . .       | 6.0             | citrate | 30               | 11                 | 0.1             | <0.1              | N.D.             | N.D.    |
| $\beta$ -D-xylosidase . . . . .        | 5.7             | citrate | 1.4              | <0.1               | <0.1            | N.D.              | <0.1             | N.D.    |
| $\alpha$ -N-ac'gal'aminidase . . . . . | 5.9             | citrate | <0.1             | <0.1               | <0.1            | N.D.              | N.D.             | —       |
| $\alpha$ -N-ac'glu'aminidase . . . . . | 5.9             | citrate | <0.1             | <0.1               | N.D.            | <0.1              | N.D.             | N.D.    |
| $\beta$ -N-ac'gal'aminidase . . . . .  | 4.5             | citrate | 0.2              | <0.1               | —               | N.D.              | 0.1              | 0.2     |
| $\beta$ -N-ac'glu'aminidase . . . . .  | 4.5             | citrate | <0.1             | 0.3                | 0.3             | <0.1              | 1.6              | 0.2     |
| $\alpha$ -D-mannosidase . . . . .      | 4.2             | citrate | 0.1              | 0.1                | 12              | <0.1              | 0.8              | 0.2     |
| $\alpha$ -D-galactosidase . . . . .    | 4.8             | acetate | <0.1             | <0.1               | <0.1            | N.D.              | <0.1             | N.D.    |
| $\beta$ -D-galactosidase . . . . .     | 3.6             | citrate | <0.1             | 0.2                | <0.1            | N.D.              | <0.1             | N.D.    |
| $\alpha$ -L-fucosidase . . . . .       | 4.5             | citrate | <0.1             | <0.1               | <0.1            | N.D.              | <0.1             | N.D.    |
| Cellobiase . . . . .                   | 5.7             | citrate | 0.5              | —                  | <0.1            | —                 | <0.1             | —       |
| Invertase . . . . .                    | 5.0             | acetate | 140              | 0.1                | 0.1             | —                 | <0.1             | —       |
| Acid phosphatase . . . . .             | 5.0             | acetate | 110              | 7                  | 6               | 8                 | 70               | 58      |
| Acid proteinase . . . . .              | 3.6             | acetate | 1.4              | 17                 | 50              | 7                 | 74               | 78      |
| Acid DNase . . . . .                   | 5.0             | acetate | —                | 4                  | 25              | 0.1               | 0.3              | —       |
| Lipase . . . . .                       | 3.8             | citrate | <0.1             | 0.2                | <0.1            | —                 | 0.1              | —       |
| Acid phosphodiesterase . . . . .       | 5.4             | acetate | 0.26             | <0.1               | 0.2             | 0.2               | 0.4              | N.D.    |

<sup>1</sup> Expressed as nanomoles of substrate hydrolyzed  $\times \text{min}^{-1} \times \text{mg}^{-1}$  protein; for acid proteinase: nanograms of haemoglobin  $\times \text{min}^{-1} \times \text{mg}^{-1}$ ; for acid DNase: nanomoles of nucleotides liberated  $\times \text{min}^{-1} \times \text{mg}^{-1}$ .

<sup>2</sup> Values represent means of at least 2 experiments.

<sup>3</sup> Optimal acid pH's; specific activities listed are those measured at the optimal acidic pH, for exceptions see Results.

<sup>4</sup> N.D. = not detected, at the lower limit of detection of 0.2 picomoles  $\times \text{min}^{-1} \times \text{mg}^{-1}$  protein for the fluorometric assays using derivatives of 4-methylumbelliferone.

## Materials and methods

*Crithidia fasciculata* (Strain ATCC 11.745), *C. luciliae* («Steinert»), *Leishmania donovani* (ITMAP 263) promastigotes, *Trypanosoma cruzi* (Tehuantepec) epimastigotes and *T. brucei* (EA-TRO 1125) procyclic forms were grown at 27° C in GLSH (Jadin and Pierreux, 1960) and RE medium (Steiger and Steiger, 1976) supplemented with 10% heat-inactivated foetal bovine serum; blood forms of monomorphic *T. brucei* (LUMP 127) and *T. cruzi* (FK) were raised in NMRI mice and chinchillas, respectively. Cultured parasites were harvested in late log phase, and bloodforms during the terminal parasitaemia. The blood parasites were purified by DEAE-cellulose column chromatography (Lanham and Godfrey, 1970). The organisms were washed twice by centrifugation ( $1000 \times g$  for 10 min) and resuspension in cold PSG buffer (Lanham and Godfrey, 1970) and then in 2 changes of cold 1 mM HEPES buffered 0.15 M NaCl (pH 7.2). Aliquots of pelleted parasites were stored frozen at -20° C or in liquid nitrogen. Thawed material used for the incubations was homogenized ( $3 \times 10$  sec, 75 W) with a Branson B-12 sonifier (Branson Sonic Power Co., Danbury, Conn.) and then supplemented with 0.1% (final concentration) Triton X-100.

The enzyme assays were optimized with regard to pH, effectors (cations, dithiothreitol, EGTA), substrate concentration ( $K_m$ ) and linearity, and carried out at 27° C (culture parasites) or 37° C (bloodforms):  $\alpha$ -D-glucosidase,  $\beta$ -D-glucosidase,  $\beta$ -D-xylosidase,  $\alpha$ -hexosaminidases ( $\alpha$ -N-acetylgalactosaminidase and  $\alpha$ -N-acetylglucosaminidase),  $\beta$ -hexosaminidase ( $\beta$ -N-acetylgalactosaminidase and  $\beta$ -N-acetylglucosaminidase),  $\alpha$ -D-mannosidase,  $\alpha$ -D-galactosidase,  $\beta$ -D-galactosidase,  $\alpha$ -L-fucosidase and cellobiase were measured fluorimetrically in a Perkin-Elmer 1000 fluorescence spectrophotometer (Beaconsfield, Buckinghamshire, England) using the respective 4-methylumbelliferyl derivatives (Koch-Light Laboratories Ltd., Colnbrook, Buckinghamshire, England) essentially according to published procedures (Barrett and Heath, 1977; Van Hoof, 1972; Van Hoof and Hers, 1968), and so were lipase (elaidate esterase, acyl esterase) (Jacks and Kircher, 1967), except for fixing the assay with 0.1 M acetate buffer (pH 5.0), and acid phosphodiesterase (Welman and Peters, 1976) using both 4-methylumbelliferyl pyrophosphate diester and *bis*(4-methylumbelliferyl)phosphate as substrates. Sucrase (invertase,  $\beta$ -fructofuranosidase) activity was determined according to Dahlquist (1964), acid phosphatase with the use of p-nitrophenyl-phosphate (Eeckhout, 1973), acid proteinase according to the technique described by Eeckhout (1972), except for using [ $^3H$ ]acetyl haemoglobin (1.3 mCi/mmol) as substrate (Barrett, 1972), and acid DNase by measuring acid-soluble radioactivity (Hulhoven et al., 1978) liberated from  $^{125}I$ -labelled (0.17 mCi/mmol) moderately denatured herring sperm DNA (Orosz and Wetmur, 1974). Protein concentrations were determined with fluorescamine (Böhlen et al., 1973) after dissolving the samples in 0.2 M carbonate buffered (pH 11.3) 1% deoxycholate. Bovine serum albumin served as standard. The specific enzyme activities were expressed as indicated in Table 1.

## Results

*Crithidia fasciculata* and *C. luciliae* exhibited identical activity levels of the enzymes tested. The type of culture medium used had no significant influence on the enzyme activity levels. The data obtained with both species were therefore pooled. As summarized in Table 1, the enzyme activity pattern of the mammalian parasites differs distinctly from that of *Crithidia*: acid  $\alpha$ -D-glucosidase,  $\beta$ -D-glucosidase,  $\beta$ -D-xylosidase, cellobiase and invertase are barely detectable in the mammalian group of parasites, but show elevated specific activities in *Crithidia*. The opposite is true for  $\alpha$ -D-mannosidase and acid proteinase. The acid pH profiles of the enzymes are comparable for all 5 parasite species, with the exception that  $\alpha$ -D-glucosidase and acetylcetesterase of *T. brucei* bloodforms

had only neutral pH optima. Culture forms of *T. brucei* and *T. cruzi* resemble the corresponding blood stages, except for  $\beta$ -hexosaminidases and  $\alpha$ -D-mannosidase, which were more active in the latter forms. Moreover, acid proteinase activity of *T. cruzi* is higher in blood than in culture forms. Preliminary investigations on the action of inhibitors and activators were carried out with sonicates of *T. brucei* bloodforms. In analogy to the non-lysosomal mammalian acid phosphohydrolase (reviewed by Barrett and Heath, 1977), acid phosphatase is only slightly (10%) stimulated by 5 mM  $Mg^{2+}$ , and the respective additions of 20 mM L(+)tartrate and NaF led to a 56% and 92% inhibition of the enzyme. More than 50 mM EGTA was required to obtain a 50% inhibition. Acid proteinase previously incorrectly referred to as "cathepsin D" (Venkatesan et al., 1977) did not behave like the classical mammalian liver cathepsin (Barrett, 1977): it was not affected by pepstatin, but stimulated 25% by 1 mM EGTA and about twofold by 10 mM cysteine. Activity was completely blocked by 1 mM p-chloromercuribenzoate; the parasite enzyme therefore behaves like a thiol endopeptidase (EC 3.4.22.-).

A number of hydrolases could not be detected in any of the flagellate species, namely lysozyme, trehalase,  $\beta$ -D-fucosidase,  $\beta$ -D-glucuronidase, sulfatases A, B and C, collagenase, cathepsin B<sub>1</sub>, true glucose-6-phosphatase and alkaline phosphodiesterase.

## Discussion

It appears from ultrastructural and biochemical studies that acid hydrolases of trypanosomatid flagellates are localized in at least 2 different subcellular compartments, namely in the flagellar pocket (acid phosphatase) and in lysosomes (acid proteinase). More recently, using cell fractionation techniques (Steiger et al., in preparation), we found that in *T. brucei* bloodforms acid phosphatase and acid phosphodiesterase behave entirely differently from  $\alpha$ -D-mannosidase and acid proteinase. This observation is consistent with the idea that acid phosphohydrolases are located separately (possibly in the flagellar pocket) from  $\alpha$ -D-mannosidase and acid proteinase, which are probably true lysosomal enzymes. In contrast to the situation in *Crithidia* (Eeckhout, 1972), acid DNase of *T. brucei* turned out to be soluble; the same applies to neutral acetyl esterase (Steiger et al., in preparation).

Apart from the fact that the monogenetic parasite, *Crithidia*, uniquely exhibits enzymatic activities with acid pH optima appropriate to the breakdown of predominantly plant-derived polysaccharides, the localization and characteristics of the other hydrolases studied may differ within the group of haemoflagellates. Examples for this are the presence of distinct acid phosphohydrolase isoenzymes in the cytosol and the particulate fraction of *Crithidia* (McLaughlin et al., 1975) and the exclusive presence of a neutral  $\alpha$ -D-glucosidase in *T. brucei*. The comparatively higher specific activities of acid proteinase,  $\beta$ -N-acetylgluco-

saminidase and  $\alpha$ -D-mannosidase in the mammalian trypanosomes might reflect an adaptation to the mammalian host related to the utilization of blood proteins (Fairlamb and Bowman, 1977) and the turnover of the surface coat glycoproteins (Cross and Johnson, 1976), respectively.

From the presence or absence and the specific activities of the acid hydrolases screened for in the present study one can conclude that lysosomes of haemoflagellates have a limited digestive capacity as compared to those in tissues of higher eukaryotes, such as the rat liver (Tappel, 1969; Barrett and Heath, 1977). This appears to be even more so when taking into account that some of the hydrolytic enzymes tested may be localized in compartments other than lysosomes (cytosol, flagellar pocket). The predominance of glycosidases in *Crithidia* and acid proteinase in *T. brucei*, however, points to a distinct degree of specialization in nutrient utilization. To what extent the digestive capacity of *Crithidia* can be correlated with nutrient availability in and the hydrolytic capacity of the natural Dipteran hosts (Wallace, 1966) remains uncertain. Though it is known that the hosts in question feed, at least occasionally, on plant-derived foodstuff and that glycosidases and proteinases are produced by such insect vectors (reviewed by Gooding, 1975), quantitative enzymatic data related to the adequacy and digestibility of vegetative substrates, such as glucans and xylans, are scarce.

The flagellar pocket of trypanosomes is believed to represent the site of exogenous substrate predigestion in trypanosomes (Jadin, 1971). It can therefore be assumed that, in addition to acid phosphatase and  $\beta$ -fructofuranosidase, other acid carbohydrases of *Crithidia* are associated with the above structure. This would corroborate earlier data on the carbohydrate utilization of insect flagellates (Cosgrove, 1963). The same author noted, for instance, the absence of lactose utilization. This correlates with the observation that the appropriate glycosidase,  $\beta$ -D-galactosidase, is of very low activity in our material.

It can tentatively be concluded that the localization of acid hydrolases in the flagellar pocket of trypanosomes is prerequisite to making substrates, such as oligosaccharides and proteins, available for the parasite, since it is known that these parasites possess highly efficient sugar (Min, 1965; Gruenberg et al., 1978) and amino acid transport systems (Manjra and Dusanic, 1972; Owen and Voorheis, 1976).

*Note added in proof:* Very recently, Camargo et al. (1978) similarly found acidic protease activity in extracts of *Trypanosoma* and *Leishmania*.

*Acknowledgments:* We wish to thank Profs. C. de Duve and A. Trouet for their encouraging interest, Dr. F. R. Opperdoes for invaluable criticism as well as Mrs. N. Lapieza and Mr. J. Van Roy for excellent technical assistance. This research was supported by the «WHO Special Programme for Research and Training in Tropical Diseases».



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