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Immunocytochemical and biochemical detection of alpha-L-fucosidase in *Trypanosoma cruzi*

L.C. Miletti,
M. Almeida-de-Faria,
W. Colli and M.J.M. Alves

Departamento de Bioquímica, Instituto de Química,
Universidade de São Paulo, São Paulo, SP, Brasil

Abstract

The aim of the present study was to demonstrate the presence of α-L-fucosidase in *Trypanosoma cruzi*. Immunocytochemical and biochemical techniques were used to localize and characterize a membrane-associated, neutral-pH-optimum, α-L-fucosidase from the parasite. Light and electron microscopy localized the α-L-fucosidase specifically on the surface of the parasite and on membranes in the posterior region of the epimastigote stage. Although much less intense, labeling was also detected on the surface of trypomastigotes. At least 50% of the α-L-fucosidase activity was associated with epimastigote membrane solubilized with 1 M NaCl or 1% Triton X-100, suggesting that α-L-fucosidase is peripherally associated with membranes. The enzyme from epimastigotes had a neutral pH optimum (near 7) but displayed low specific activity when *p*-nitrophenyl-α-L-fucoside was employed as substrate (0.028 U/mg protein for epimastigotes and 0.015 U/mg protein for tissue culture-derived trypomastigotes). Polyacrylamide gel electrophoresis and Western blotting analysis both showed an expected 50-kDa polypeptide which was immunoreactive with anti-α-L-fucosidase antibodies.

Key words
- *Trypanosoma cruzi*
- Trypanosomatids
- Fucosidase
- Fucose
- Glycoproteins
- Glycolipids

Correspondence
M.J.M. Alves
Departamento de Bioquímica
Instituto de Química, USP
Av. Lineu Prestes, 748
Caixa Postal 26077
05513-970 São Paulo, SP
Brasil
Fax: +55-11-3815-5579
E-mail: mjmalves@iq.usp.br

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Introduction

Glycoconjugates from pathogens have been isolated and characterized due to their importance in a variety of biological functions. In the particular case of *Trypanosoma cruzi*, a protozoan parasite causing Chagas' disease, the most abundant surface glycoconjugates are a family of glycoinositolphospholipids (formerly lipopeptidophosphoglycan) with 2 x 10^7 molecules per parasite and a family of glycosylphosphatidylinositol-anchored mucin-like glycoproteins with 2 x 10^6 molecules per parasite, both forming a coat that almost covers the entire surface of the parasite (1). Although much less abundant, many other important surface glycoconjugates have been described in epimastigotes (a noninvasive and replicative form) and trypomastigotes (an invasive and nonreplicative form) of *T. cruzi*. In particular, two glycoproteins at least contain α-L-fucose in their structure: Gp-72, a glycoprotein isolated from epimastigote forms (2,3), suggested to be involved in *T. cruzi* differentiation (4), and the trypomastigote stage-specific glycoproteins (5-7) belonging to the Tc-85 family and implicated in parasite-host cell adhesion (8-10). Glycopeptides isolated from Gp-72 by affinity chromatography contain

rhamnose, fucose, xylose and galactose (1:1:2:3) linked to the peptide by a less usual phosphodiester linkage (11). Interestingly, no α-L-fucosidase was detected in one of the few studies concerning the degradation of these glycoconjugates in *T. cruzi*, although the existence of galactosidases, glucosidases and mannosidases has been described (12, 13).

The enzyme α-L-fucosidase is a ubiquitous glycosidase in eukaryotic cells, usually found as a soluble component within the lysosome, and acting as an acid hydrolase in the degradation of fucose-containing glycoconjugates. The enzyme is also present in body fluids and in the plasma membrane, as reported for sperm cells (14,15) or human erythrocytes (16).

The presence of α-L-fucosidase in epimastigote and trypomastigote forms of *T. cruzi* is demonstrated here using biochemical and immunological approaches. The enzyme has a neutral pH optimum and is partially localized on the plasma membrane of both forms of the parasite.

Material and Methods

Parasites

Epimastigote forms of *T. cruzi*, CL-14 strain (17) kindly supplied by Dr. E. Chiari, were grown in liver infusion tryptose (LIT) medium supplemented with 10% fetal calf serum (FCS), at 28ºC (18,19). Metacyclic trypomastigote forms of *T. cruzi*, Y strain, were obtained in a mixture of 55% Grace medium, 20% LIT medium and 25% preconditioned LIT medium supplemented with 10% FCS at 28ºC. Trypomastigotes of *T. cruzi* were obtained by infecting LLC-MK_2 tissue-cultured cells, as described (20).

Preparation of cell extract

Cells were harvested by centrifugation and washed three times with phosphate-buffered saline (PBS), pH 7.2 (5,000 *g*, 10 min at 4ºC). The pellet was resuspended in PBS (1 x 10^9 cells/ml) containing 1 mM phenylmethylsulfonyl fluoride and 1 mM N-*p*-tosyl-L-lysine chloromethyl ketone, and the parasites were immediately lysed by sonication (3 x 30 s in ice). The insoluble material was removed by centrifugation (10,000 *g*, 15 min at 4ºC) and the supernatant employed in the assays. The protein content was determined as described (21).

Tunicamycin treatment of *Trypanosoma cruzi*

Parasites (5 x 10^8) were incubated with or without 10 µg of tunicamycin in 10 ml of methionine-free Dulbecco modified Eagles' medium supplemented with 2% FCS. After 3 h, 300 µCi of [^{35}S]-methionine was added and the incubation continued for another 2 h and 30 min. The medium was removed by centrifugation and the cells were washed with PBS, as described above. All incubations were carried out at 28ºC for epimastigotes and at 37ºC for trypomastigotes.

Enzymatic assays

Extracts were prepared as described above. The extract corresponding to 5 x 10^9 epimastigotes was centrifuged at 80,000 *g* for 2 h at 4ºC. The supernatant was separated and the pellet resuspended in 66 mM NaH_2PO_4/Na_2HPO_4 buffer, pH 7.0, or treated for 60 min with 1 M NaCl. The material solubilized with NaCl was separated by centrifugation at 80,000 *g* for 2 h at 4ºC and the pellet was resuspended and dialyzed against 66 mM NaH_2PO_4/Na_2HPO_4 buffer, pH 7.0, for 18 h at 4ºC. α-L-fucosidase activity was determined by measuring the amount of *p*-nitrophenol released by the hydrolysis of *p*-nitrophenyl-α-L-fucopyranoside (Sigma, St. Louis, MO, USA), as described (22,23). The reaction mixture containing 0.5 mM *p*-nitrophenyl-α-L-fucopyranoside in 66 mM NaH_2PO_4/Na_2HPO_4 buffer, pH 7.0, and the

desired amount of protein was incubated for 60 min at 37ºC. The reaction was stopped by the addition of 1 ml of 0.25 M Na_2CO_3/$NaHCO_3$ and absorbance was read at 410 nm. The amount of *p*-nitrophenol was calculated using the molar extinction coefficient of the *p*-nitrophenolate ion, 1.75 x 10^4 M^{-1} cm^{-1}. One enzyme unit (U) is defined as µmol of *p*-nitrophenolate ion formed per minute and specific activity is defined as U/mg protein. Alternatively, the amount of L-fucose as the reaction product was determined by high-performance liquid chromatography for carbohydrates using a CarboPac PA-100 anion exchange column (Dionex Co., Sunnyvale, CA, USA).

The stability of the α-L-fucosidase activity was determined after storage of the enzyme at 4ºC or -20ºC in the presence of 50% glycerol, 50 µg/ml bovine serum albumin (BSA) or 2 mM ß-mercaptoethanol. The inactivation plot was obtained by preincubation of the epimastigote extract at 28º or 37ºC for different periods of time followed by the standard enzymatic assay. The optimal pH for α-L-fucosidase was determined in 66 mM sodium acetate buffer, pH 5.0-6.5, or 66 mM NaH_2PO_4/Na_2HPO_4 buffer, pH 7.0-8.0. Standard assay conditions, pH 7.0, were used to determine the optimal temperature of the reaction.

Gel electrophoresis and immunoblotting

Samples were submitted to SDS-PAGE (0.1% SDS, 9% polyacrylamide) as described (24). After electrophoresis, the gels were fixed and stained with 0.2% Coomassie blue (25) or with the $AgNO_3$ stain (26). For immunoblotting, the gels were incubated in 25 mM Tris/192 mM glycine and 40% methanol for 15 min and the proteins were transferred onto a nitrocellulose membrane at 50 volts for 18 h at 4ºC (27). After blockage with 50 mM TBS-0.03% Tween 20, pH 8.0, and 1% BSA, the membrane was incubated with mouse anti-bovine epididymis α-L-fucosidase polyclonal antibodies in the same buffer. After washing, the blot was incubated with horseradish peroxidase-conjugated anti-mouse IgG and developed with an ECL Western blot chemiluminescense kit (AmershamBioscience, Piscataway, NJ, USA).

Antibodies. Anti-bovine epididymis α-L-fucosidase antibodies were obtained by injecting BALB/c mice with the gel band containing the enzyme after submitting a commercial preparation (Oxford Glycosystems, Abingdon, Oxon, England) to SDS-PAGE. The band was sliced in PBS, sonicated (30 s, 10 times) and mixed with Freund's adjuvant before injection. The specificity of the antibodies was confirmed by Western blot analysis against commercial α-L-fucosidase.

Immunoprecipitation

Extracts from epimastigotes and trypomastigotes previously labeled with [^{35}S]-methionine were preincubated with 50 µl Sepharose-Protein A (AmershamBioscience) in PBS. The samples were centrifuged and the supernatants incubated with 50 µl Sepharose-Protein A and 10 µl of anti-bovine epididymis α-L-fucosidase at 4ºC. After overnight incubation, the samples were exhaustively washed with 100 mM Tris, pH 8.6, 300 mM NaCl, 10 mM EDTA, 0.05% Nonidet P-40, 1% BSA, and 0.02% NaN_3 before the addition of SDS sample buffer (25 mM Tris-HCl, pH 6.8, 38% glycerol, 0.2 M EDTA, 2% SDS, 4% ß-mercaptoethanol, and bromophenol blue).

Indirect immunofluorescence

Epimastigotes and trypomastigotes were washed three times in PBS, pH 7.2, and fixed in 2% paraformaldehyde in PBS, pH 7.2, for 1 h at 4ºC. The cells were incubated with anti-bovine epididymis α-L-fucosidase polyclonal antibodies at the desired dilution

(1:500) for 30 min at 37ºC, washed in PBS and incubated with fluorescein-conjugated anti-mouse IgG (Sigma) for 30 min at 37ºC.

Electron microscopy. Parasites were washed in PBS and fixed in a solution of 0.5% glutaraldehyde, 4% *p*-formaldehyde, and 0.2% picric acid in 0.1 M sodium cacodylate buffer, pH 7.4. After 90 min, the cells were washed in 50 mM glycine in 0.1 M sodium cacodylate buffer, pH 7.4, embedded in 10% gelatin, cut into 1-mm^3 pieces, and maintained overnight at 4ºC in 2.3 M sucrose. After freezing in liquid nitrogen, the samples were dehydrated with methanol by cryosubstitution at -90ºC for 48 h and embedded in Lowicryl HM20 resin at -45ºC. Ultrathin sections were collected on 300-mesh nickel grids and incubated for 30 min with 50 mM glycine in PBS, followed by 30 min in PBS containing 0.1% BSA. The material was incubated for 16 h at 4ºC in the same medium containing anti-bovine epididymis α-L-fucosidase serum, washed with PBS-1% BSA and incubated for 60 min at room temperature with protein A coupled to 10-nm colloidal gold particles. The grids were washed, stained with 2% uranyl acetate, and observed with a Jeol 1200 EXII transmission electron microscope.

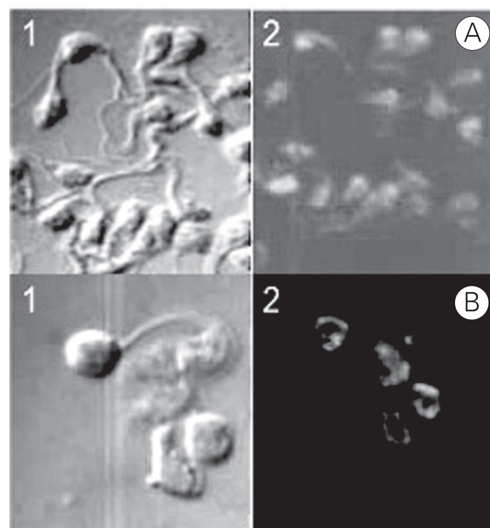

Figure 1. Reactivity of anti-α-L-fucosidase antibodies with epimastigote and trypomastigote forms of *Trypanosoma cruzi*. The reactivity was confirmed by indirect immunofluorescence with *p*-formaldehyde-fixed parasites and visualized under a confocal microscope. *A*, Epimastigotes; *B*, trypomastigotes. *1*, Phase-contrast microscopy; *2*, fluorescence microscopy.

Results

Anti-α-L-fucosidase antibodies react with epimastigote and trypomastigote forms of *Trypanosoma cruzi*

Antibodies raised against a commercial preparation of α-L-fucosidase were employed to check for the presence of the enzyme in *T. cruzi*, since data from the literature showed an almost undetectable amount of enzymatic activity in extracts of the parasite. Epimastigote and trypomastigote forms of *T. cruzi* presented intense reactivity with the anti-fucosidase antibodies by indirect immunofluorescence (Figure 1). No fluorescence was observed when serum from a nonimmunized mouse or PBS was used as negative controls. When fucosidase was located by immunocytochemistry at the ultrastructural level, a strong labeling was observed in epimastigotes over the posterior region of the cell, apparently associated with membranes and also widespread on the plasma membrane (Figure 2A). In comparison, a less intense labeling was detected in the perinuclear region with poor plasma membrane labeling when trypomastigotes were analyzed (Figure 2B).

Activity of α-L-fucosidase in different developmental stages of *Trypanosoma cruzi*

Extracts of *T. cruzi* epimastigotes and trypomastigotes hydrolyze the synthetic substrate *p*-nitrophenyl-α-L-fucopyranoside. In spite of a low enzymatic activity detected in both stages, a higher specific activity was measured in epimastigote extracts (0.03 U/mg protein) when compared to trypomastigotes (0.002 U/mg protein). For this reason epimastigote extracts were employed in most of the experiments. The activity of α-L-fucosidase in epimastigotes was proportional to the amount of protein (data not shown). A significant variability was observed among different preparations (Table 1) which was

independent of the cell density of the culture or the amount of metacyclic trypomastigotes that appear by spontaneous differentiation from epimastigotes. Metacyclic trypomastigotes also showed a low enzymatic activity (0.015 U/mg protein).

Stability and kinetic parameters of α-L-fucosidase from epimastigotes

Preliminary experiments indicated that α-L-fucosidase from epimastigote extracts and from NaCl-solubilized membrane fractions (see below) were unstable when stored at 4º or -20ºC in the presence or absence of 50% glycerol, 50 µg/ml BSA or 2 mM ß-mercaptoethanol (data not shown). The enzyme is inhibited by Tris buffer, as described for other fucosidases (23). For this reason, all the enzymatic assays were performed with fresh extracts.

Incubation for 60 min was used for most of the experiments (Figure 3) at either 28º or 37ºC due to the low amount of activity of the enzyme. Optimum temperature was established as 37ºC (data not shown). The enzyme showed maximal activity at neutral pH (pH 6.0-7.5) and lost activity at acidic or basic pH (Figure 4). When the reaction product was analyzed by anion-exchange chromatography, a peak coincident with L-fucose was also observed when the reaction was carried out at pH 5.0, although much less intense in comparison to the peak observed at pH 7.0 (data not shown). Even though fresh extracts were employed in all experiments, a rapid inactivation of α-L-fucosidase was observed. Figure 5 shows the decrease of the enzymatic activity measured after preincubation of epimastigote extracts for different periods of time at 28º or 37ºC prior to the standard enzyme assay. The enzyme preparation lost 50% of its activity after 2 h of preincubation and no trace activity was detected after 4 h. Addition of glycerol or ß-mercaptoethanol during preincubation did not protect the enzyme activity from inactivation.

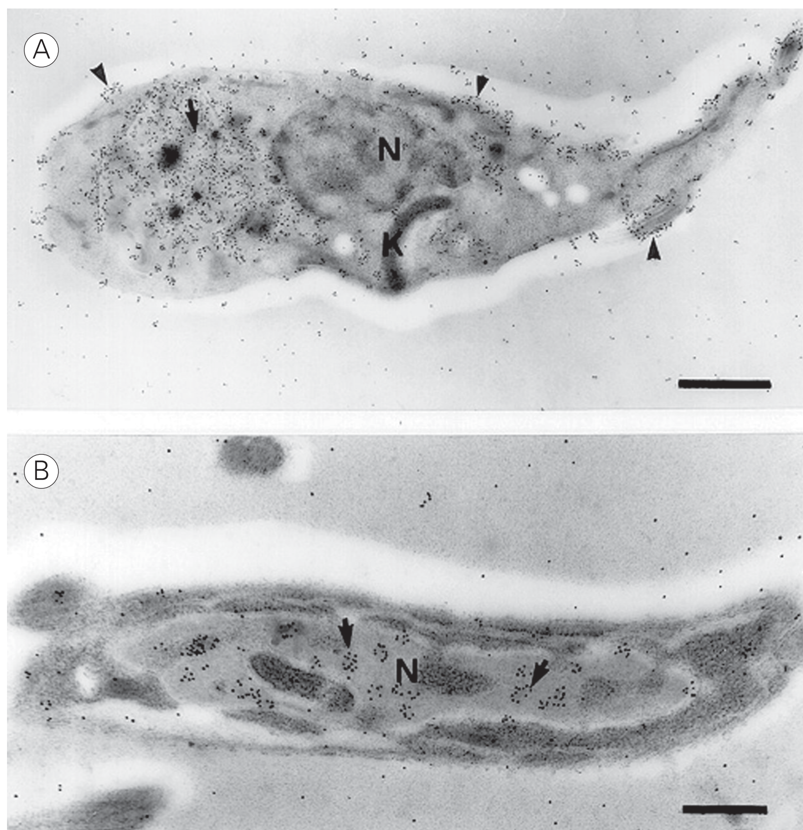

Figure 2. Immunocytochemical localization of α-L-fucosidase in *Trypanosoma cruzi*. Ultrathin sections of epimastigote (A) and trypomastigote (B) forms of *T. cruzi* were first incubated with anti-α-L-fucosidase and then with gold-labeled protein A. Gold particles are seen in the cytoplasm (arrows) and on the surface of the plasma membrane (arrowheads). N: nucleus; K: kinetoplast. Bar: 1 µm.

Table 1. Activity of α-L-fucosidase in different preparations of *Trypanosoma cruzi* epimastigote extracts.

Experiment	Cell density	Metacyclic forms (%)	Specific activity
1	120	8.0	0.04
2	110	7.0	0.10
3	112	10.0	0.16
4	130	8.0	0.03
5	90	7.5	0.23
6	100	8.0	0.04

All incubations were performed for 60 min. Cell density is the number of parasites (10^6/ml) in culture. All samples correspond to parasites in the stationary phase of growth. Specific activity is reported as U/mg protein.

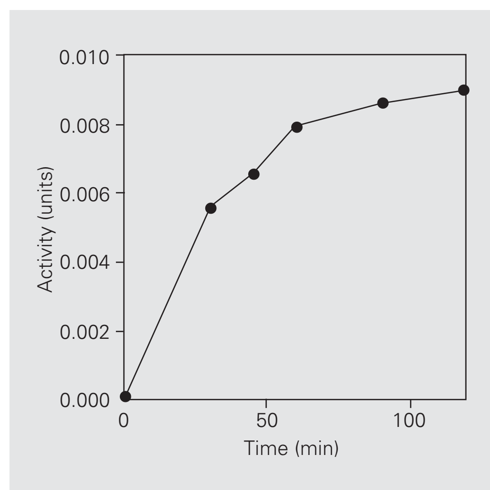

Figure 3. α-L-fucosidase activity as a function of incubation time. An epimastigote extract (0.4 mg) was incubated with *p*-nitrophenyl-α-L-fucopyranoside for the times indicated on the abscissa in 66 mM NaH_2PO_4/Na_2HPO_4 buffer, pH 7.0.

Table 2. Association of α-L-fucosidase from *Trypanosoma cruzi* with membrane fractions.

Fraction	Total units	Specific activity
Extract	0.24	0.05
80,000 *g* pellet	0.12	0.31
1 M NaCl insoluble fraction	0.01	0.02
1 M NaCl solubilized fraction	0.10	0.12
1% Triton X-100 insoluble fraction	0.03	0.04
1% Triton X-100 solubilized fraction	0.08	0.14

Total units = µmol *p*-nitrophenolate released/min. Specific activity is reported as U/mg protein.

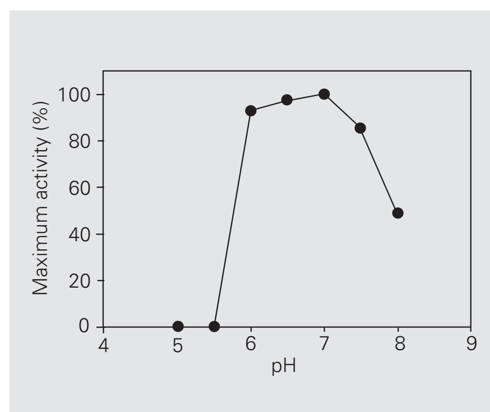

Figure 4. Effect of pH on the activity of epimastigote α-L-fucosidase. The enzymatic assay was carried out as described in Material and Methods using 0.4 mg protein of an epimastigote extract.

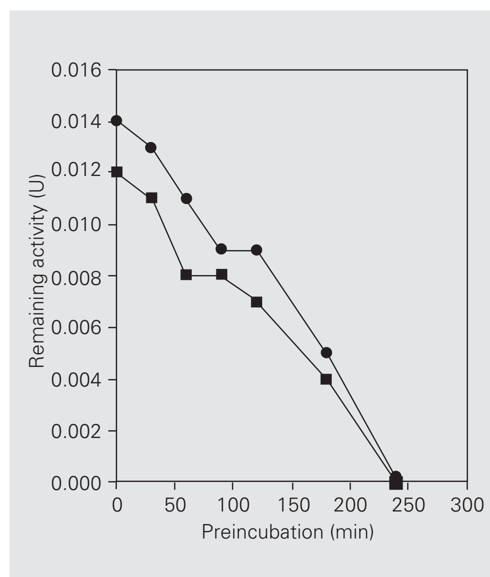

Figure 5. Inactivation of α-L-fucosidase with time. Epimastigote extracts were preincubated at 28ºC (squares) or 37ºC (circles) for the periods of time shown on the abscissa. Activity was measured by the standard enzymatic assay.

Characterization of α-L-fucosidase from epimastigotes

Extracts of epimastigotes were centrifuged at 80,000 *g*. Half of the total α-L-fucosidase activity was detected in the pellet, with a 6-fold increase in specific activity (Table 2). Treatment of the pellet with 1% Triton X-100 or 1 M NaCl solubilized 66 and 83% of the enzymatic activity, respectively, suggesting that the protein is weakly bound to membranes. An enrichment of a 50-kDa polypeptide in the NaCl-solubilized fraction was demonstrable by SDS-PAGE (Figure 6A), which is compatible with the molecular mass described for the monomers of α-L-fucosidases (28). Moreover, anti-α-L-fucosidase antibodies immunoprecipitated a 50-kDa polypeptide from total extracts of [^{35}S]-methionine-labeled epimastigotes and a 45-50-kDa protein doublet from the NaCl-solubilized fraction as indicated by SDS-PAGE (Figure 6B). A 50-kDa polypeptide was also immunoprecipitated from [^{35}S]-methionine-labeled trypomastigotes (data not shown). The specificity of the antibody was confirmed by immunoprecipitation of the total extract or 1 M NaCl-treated membrane fractions from epimastigotes with anti-α-L-fucosidase, followed by the standard enzymatic assay. In both cases, the antibodies

immunoprecipitated a protein with a very low, but detectable fucosidase activity.

Since α-L-fucosidases described for different organisms are glycoproteins, the enzyme from *T. cruzi* was checked for the presence of carbohydrates. The presence of N-linked oligosaccharides was suggested by the 5-kDa decrease in the molecular mass of the enzyme immunoprecipitated from epimastigotes labeled with [^{35}S]-methionine in the presence of tunicamycin (Figure 7).

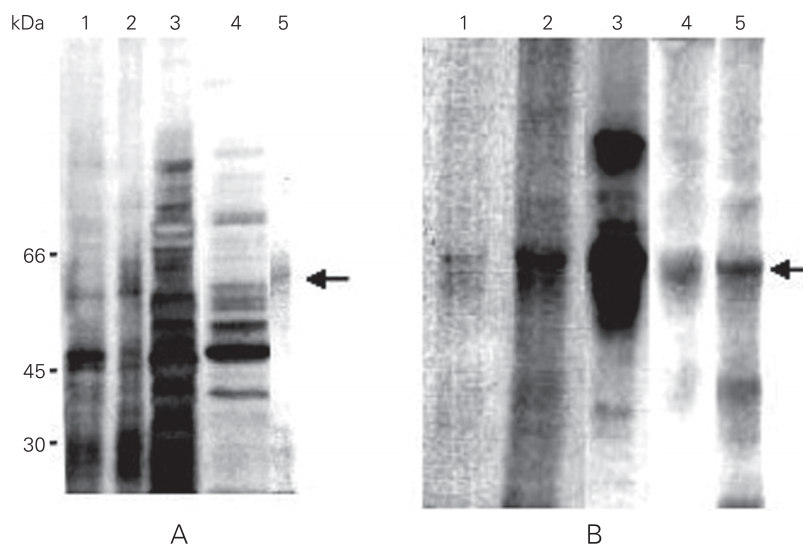

Figure 6. SDS-PAGE of α-L-fucosidase from *Trypanosoma cruzi* epimastigotes. *A*, The 80,000 *g* pellet of the epimastigote extract was treated with 1 M NaCl and subjected to another 80,000 *g* centrifugation. Aliquots from the fractions were concentrated, submitted to SDS-PAGE and stained with Coomassie blue. *B*, Identical fractionation was performed using [^{35}S]-methionine-labeled epimastigotes followed by immunoprecipitation with anti-α-L-fucosidase antibodies and autoradiography. *Lane 1*, Total extract; *lane 2*, 80,000 *g* pellet; *lane 3*, 80,000 *g* supernatant; *lane 4*, insoluble 1 M NaCl-treated 80,000 *g* fraction; *lane 5*, soluble 1 M NaCl-treated 80,000 *g* fraction. The arrow on the right side of each set of gels indicates the position of a molecular mass of 50 kDa.

Discussion

The aim of the present report was to unequivocally establish the presence of α-L-fucosidase in *T. cruzi*. Immunocytochemical techniques and detection of enzymatic activity were used to demonstrate that at least 50% of the enzyme is associated with the cytoplasmic and plasma membranes. Although most of the α-L-fucosidase activity of virtually all mammalian tissues is in the soluble fraction, membrane-associated α-L-fucosidase has been described in rat testis and epididymal spermatozoa (14,29), in human sperm plasma membrane (15), and in human erythrocyte membranes (16).

Gel electrophoresis analysis of the 1 M NaCl-solubilized membranes from epimastigotes showed a broad band with apparent molecular mass of 50 kDa that was recognized by the anti-α-L-fucosidase antibody. In some preparations two bands of 45 and 50 kDa were visualized that probably corresponded to distinct isoforms or to different contents of carbohydrate. Isoforms of α-L-fucosidase with distinct pI and pH optima have been described in the literature (15,29). The α-L-fucosidase from the membrane fraction of epimastigotes has a neutral pH optimum (pH 6.0-7.5), having no activity at acidic pH, in contrast to α-L-fucosidases isolated from lysosomes. A more neutral pH optimum was described for α-L-fucosidases from *Corynebacterium*, pH 8.5 (30), *Helicobacter pylori*, pH 7.0, (31), *Trichomonas*

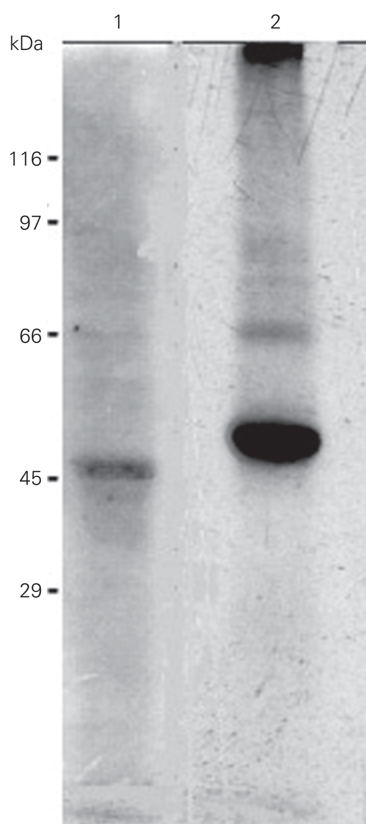

Figure 7. Glycoprotein nature of the α-L-fucosidase from *Trypanosoma cruzi*. Extracts of epimastigotes metabolically labeled in the presence (lane 1) or absence (lane 2) of 10 µg/ml tunicamycin were immunoprecipitated with anti-α-L-fucosidase and subjected to SDS-PAGE and autoradiography.

foetus, pH 7.0 (32), and for the enzymes from rat sperm and erythrocytes (14,16).

Overall, the results reported here provide strong evidence for the presence of a neutral α-L-fucosidase in *T. cruzi* epimastigotes and trypomastigotes. The immunolabeling experiments suggest that the enzyme is highly represented in both parasite stages, and particularly abundant in epimastigotes in spite of the low enzymatic activities detected. The instability of the enzyme may explain this apparent contradiction, in addition to the possibility that *p*-nitrophenyl-α-L-fucopyranoside might not be an adequate substrate for the *T. cruzi* enzyme, as was the case for fucosidases from *Trichomonas foetus* that could not be detected using nitrophenyl-sugars (cf. Ref. 13). Since no activity was also detected in epimastigotes from *T. cruzi* when 4-methylumbelliferyl-α-L-fucoside was employed instead the *p*-nitrophenyl-α-L-fucopyranose (data not shown), a study involving natural substrates will be required.

The failure to detect α-L-fucosidase in epimastigotes or even the exceedingly low activities reported for blood trypomastigotes by different groups (12,33) is, most likely, a reflection of the enzyme's lability under the assay conditions used. Nevertheless, despite the inherent difficulties in measuring the enzyme activity, the presence of L-fucosidase on the plasma membrane of *T. cruzi* raises the provocative question of a possible role of the enzyme in glycoconjugate function.

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