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Isolation and Properties of a Sialidase from *Trypanosoma rangeli**

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In the culture supernatant of *Trypanosoma rangeli*, strain El Salvador, a sialidase was present with an activity of 0.1 U/mg protein as determined with the 4-methylumbelliferyl glycoside of α-*N*-acetylneuraminic acid as substrate. This enzyme was purified about 700-fold almost to homogeneity by gel chromatography on Sephadex G-100 and Blue Sepharose, and affinity chromatographies on 2-deoxy-2,3-didehydroneuraminic acid and horse submandibular gland mucin, both immobilized on Sepharose. The pH optimum is at 5.4-5.6, and the molecular weight was determined by gel chromatography, high performance liquid chromatography and sodium dodecyl sulphate gel electrophoresis to be 70 000. The substrate specificity of the enzyme is comparable to bacterial, viral and mammalian sialidases with cleavage rates for the following substrates in decreasing order:
***N*-acetylneuraminyl-α(2-3)-lactose > *N*-glycoloylneuraminyl-α(2-3)-lactose > *N*-acetylneuraminyl-α(2-6)-lactose > sialoglycoproteins > gangliosides > 9-*O*-acetylated sialoglycoproteins.**
4-*O*-Acetylated derivatives are resistant towards the action of this sialidase. The enzyme activity can be inhibited by 2-deoxy-2,3-didehydro-*N*-acetylneuraminic acid, Hg^{2+} ions, and *p*-nitrophenyloxamic acid; it is not dependent on the presence of Ca^{2+} Mn^{2+} or Mg^{2+} ions.

Abbreviations: BSA, bovine serum albumin; BSM, bovine submandibular gland mucin; CMP, cytidine monophosphate; EDTA, ethylenediaminetetraacetic acid; ESM, equine submandibular gland mucin; HEPES, *N*-2-hydroxyethylpiperazine-*N*'2-ethanesulfonic acid; HPLC, high performance liquid chromatography; Lac, lactose; MU-Neu5Ac, 4-methylumbelliferyl glycoside of α-*N*-acetylneuraminic acid; Neu5Ac, *N*-acetylneuraminic acid; Neu5Ac2en, 2-deoxy-2,3-didehydro-*N*-acetylneuraminic acid; Neu4Ac5Gc, *N*-glycoloyl-4-*O*-acetylneuraminic acid; Neu2en, 2-deoxy-2,3-didehydroneuraminic acid; Neu5Gc, *N*-glycoloylneuraminic acid; PMSF, phenylmethylsulfonyl fluoride; PSM, pig submandibular gland mucin; SDS, sodium dodecyl sulfate; Tris, tris-(hydroxymethyl)aminomethane.

*Dedicated to Professor Dr. Heinz Mühlpfordt on the occasion of his 65th birthday.
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The South American trypanosomiasis "Chagas' disease" is a human disease caused by *Trypanosoma cruzi*. As this parasite often occurs together with the non-pathogenic *T. rangeli*, parameters are needed to discriminate between both forms by reliable, specific, and sensitive methods. As has been described earlier, *T. cruzi* has sialic acids on the cell surface. The nature of this sugar seems to depend on the medium in which it was grown [1]. This finding led to the suggestion that sialic acids might be taken up by the parasites from the surrounding medium, especially as *T. cruzi* is unable to biosynthesize this sugar from its well-known precursors [1]. The occurrence of sialidase activity that might be helpful for sialic acid acquisition from the environment has been reported for *T. cruzi* [2] and *T. rangeli* [3]. The localization of the enzyme in these parasites is, however, different. Whereas *T. cruzi* has a low and membrane-bound sialidase activity, *T. rangeli* releases high amounts of this glycosidase into the surrounding medium. As an isolation and a detailed analysis of the sialidase of *T. rangeli* has not yet been carried out, the present study describes the purification and characterization of this enzyme.

Materials and Methods

If not otherwise stated all procedures were carried out at 4°C, with the exception of enzyme incubations, which were performed at 37°C. Chemicals were purchased from Merck (Darmstadt, W. Germany) or Sigma (Munich, W. Germany). Sephadex, Blue-Sepharose and Sepharose 4B were from Pharmacia (Freiburg, W. Germany). ESM was prepared and coupled to Sepharose (35 μg sialic acids/ml gel) as described earlier [4, 5]. Neu5Ac2en was purchased from Boehringer (Mannheim, W. Germany) and, after de-*N*-acetylation, linked to Sepharose [6] (1.2 mg Neu2en/ml gel).

MU-Neu5Ac was prepared according to the method of Warner and O'Brien [7] and *p*-nitrophenyloxamic acid according to [8]. Trasylol was obtained from Bayer (Leverkusen, W. Germany). A ganglioside mixture from bovine brain was prepared as described in the literature [9], and Neu5Gc-GM3 and Neu4Ac5Gc-GM3 were isolated from horse erythrocytes according to [10]. GM3 was a generous gift from Professor Ghidoni, Milan. Neu5Gc-α(2-3)-Lac was obtained by ozonolysis of Neu5Gc-GM3 as described earlier [11]. Neu5Ac-α(2-3)-Lac and Neu5Ac-α(2-6)-Lac were prepared from bovine colostrum as reported [12]. BSM and PSM were isolated as described earlier [13, 14]. Oligosaccharides from PSM (PSM'ol) were produced by β-elimination according to the method of Aminoff *et al.* [15]. BSM was de-*O*-acetylated by alkaline treatment [16].

Culture of Trypanosomes

Trypanosoma rangeli, strain El Salvador, was grown in liver infusion tryptose medium (LIT) supplemented with 10% fetal calf serum as described [3, 17]. The pH value was monitored and kept at 7.2 throughout the culture time by addition of 20 mM HEPES buffer. The LIT medium consists of 5.0 g NaCl, 0.4 g KCl, 5.0 g Na_2HPO_4, 2.0 g glucose, 5.0 g tryptose, 5.0 g liver infusion, made up with H_2O to a total volume of 1 l, and the pH buffered to 7.2 with HEPES. After 10-14 days of culture at 37°C parasites were harvested by centrifugation at 1500 × *g* for 10 min at 4°C. The supernatant was filtered through nitrocellulose filters (0.22 μm) and lyophilized.

Isolation of Sialidase

The crude lyophilized sialidase activity present in the supernatant of the culture medium of *T. rangeli* culture forms was dissolved to 300 mg/ml in 100 mM sodium acetate buffer, pH 5, and fractionated at 10 ml/h on a column of Sephadex G-100 (2.8 × 140 cm) equilibrated in the same buffer, which was also used for elution. The gel chromatographic separation was monitored by UV absorption at 280 nm. Fractions of 2.5 ml were collected and tested for sialidase activity with MU-Neu5Ac as substrate [18] and in some cases also for protein content with the Bio-Rad assay as described in the application bulletin of the manufacturers. Sialidase-positive fractions were combined, and an aliquot (3 U) applied to a column (7.5 ml) of Blue-Sepharose which was then washed with 2 vol 100 mM sodium acetate, pH 5. The enzyme activity was eluted (0.5 ml/min) with two column volumes of the same buffer, to which sodium chloride was added to give a 500 mM solution. The eluate was dialyzed for 2 h against 500 ml 50 mM sodium acetate, pH 4, and about 2 U applied to a column of 9 ml Neu2en linked to Sepharose 4B [6]. After washing the column with 2 vol 50 mM sodium acetate buffer, pH 4, and 2 vol 50 mM sodium acetate buffer, pH 5.3, containing 100 mM sodium chloride, the sialidase was eluted at a rate of 1 ml/min with two volumes of the latter buffer, brought to 1 M sodium chloride, and dialyzed against 500 ml 20 mM Tris/HCl, pH 6, for 2 h with one change of the dialysis buffer.

The enzyme fraction (1-1.5 U) was then applied to a column (3.2 ml) of ESM-Sepharose 4B [4, 5], washed with five volumes of the dialysis buffer (pH 6) followed by 5 vol 20 mM Tris/HCl, pH 7, at flow rates of 0.3 ml/min, and recovered with 2 vol 20 mM Tris/HCl, pH 7, containing 1 M NaCl. The enzyme was concentrated to about 100 µl by filtration in ultra-thimbles (Schleicher & Schüll, Düren, W. Germany) and stored at 4°C.

Tests for Protease and Glycosidase Activities

Protease activity was tested with azocasein (Sigma) under the conditions described [19]. For assaying glycosidase activities the following substrates (all from Sigma) were used at 1 mM concentration in the assay solution: *p*-nitrophenyl glycosides of α-L-fucose, β-D-mannose, β-D-galactose, β-D-glucose, β-*N*-acetyl-D-galactosamine, β-*N*-acetyl-D-glucosamine, and the 4-methylumbelliferyl glycosides of β-D-galactose and β-*N*-acetyl-D-galactosamine. The tests were carried out as described earlier [19].

Assays for Sialidase Activity

Sialidase activity was routinely tested with 0.4 mM MU-Neu5Ac as final concentration in 100 mM sodium acetate buffer, pH 5; the reaction was stopped by addition of 900 µl glycine buffer, pH 10 [18]. Fluorimetric reading was done on a Perkin-Elmer 1000 M fluorimeter. The instrument was calibrated with 100 µl 4-methylumbelliferone standards in a range of 1×10^{-8} - 5×10^{-6} M, to which 900 µl of the glycine buffer, pH 10, were added. Controls for enzymic activities were done with heat-denatured enzyme (5 min, 96°C). Substrate specificities were tested with 10^{-3} - 10^{-4} M solutions of the substances described in the Results section, by incubation for 5-90 min. In the case of gangliosides the incubation time varied from 30—300 min. The conditions for incubations were the same as described for MU-Neu5Ac. Enzymatic reactions with the non-fluorogenic substrates were stopped by addition of the periodate solution used for the

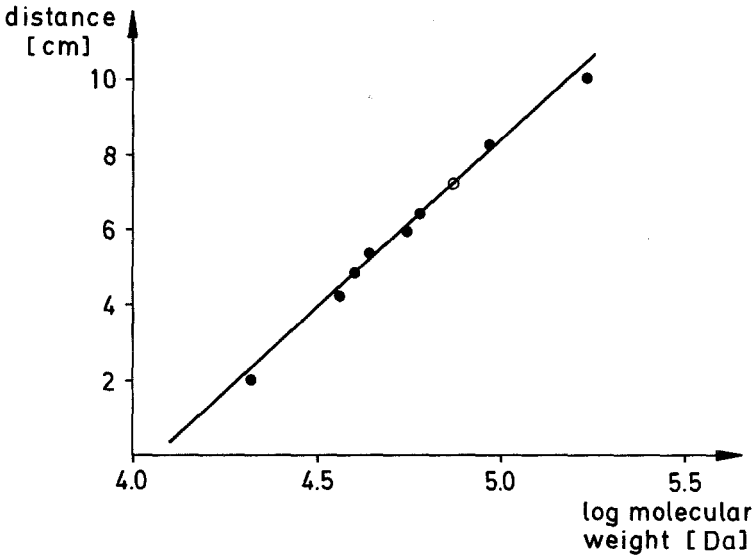

Figure 1. SDS gel electrophoresis of the purified sialidase from *T. rangeli*. The enzyme obtained after the procedure given in Table 1 was run on a gradient of 6-15% SDS and stained with Coomassie Brilliant Blue (lane 2). In lane 1 the following standards were run (from top to bottom): α_2-macroglobulin (170 000), phosphorylase b (94 000), catalase (60 000), glutamine dehydrogenase (55 000), ovalbumin (43 000), rabbit muscle aldolase (40 000), lactate dehydrogenase (36 000) and trypsin inhibitor (21 000). The molecular weight of the sialidase can be estimated as 70 000 from the plot of migration distance versus log molecular weight of the standard proteins.

colorimetric assay of released sialic acids [16]; in the case of *O*-acetylated substrates, saponification was carried out before the colorimetric test. For kinetic measurements the concentrations varied from 3×10^{-3} - 10^{-5} M of glycosidically bound sialic acid; in each case it was ascertained that the reaction was in the linear range. The effect of inhibitors was tested at 10^{-3} - 10^{-6} M final concentrations.

Determination of Molecular Weight and Enzyme Homogeneity

The molecular weight was determined by gel chromatography on columns (2.5 × 53 cm) with Sephadex G-100 and G-200, respectively, using 100 mM sodium acetate, pH 5, as eluting buffer. Sialidase activity in the effluent was analyzed with the MU-Neu5Ac test described above. Rabbit muscle aldolase, bovine serum albumin, ovalbumin, chymotrypsin and ribonuclease A (all from Pharmacia) were used as standards. Calibration of the columns was achieved with Blue-Dextran (Pharmacia) and sodium azide. In addition HPLC was used for estimating the molecular weight using a Kratos Spectroflow 400 solvent delivery system, Spectroflow 783 UV-monitor operating at 280 nm and a column (7.5 × 30 mm) of TSK G 3000 SW (LKB, Bromma, Sweden) with 20 mM sodium phosphate and 50 mM sodium chloride buffer, pH 5.8, as solvent. SDS gel electrophoresis of the purified enzyme and other fractions obtained during the isolation procedure was carried out as described by Laemmli [20] in a gradient gel of 6-15%, and the proteins were stained with Coomassie Brilliant Blue with calibration proteins as described in Fig. 1.

Table 1. Purification sequence for the sialidase activity from *T. rangeli*. Starting from the lyophilized supernatant of a culture of *T. rangeli* in LIT-medium supplemented with 10% fetal calf serum the secreted sialidase activity was purified as indicated. For further details see the Materials and Methods section.

Sequence	Yield (%)	Purification factor	Specific activity (U/mg protein)
Culture medium	100	1	0.104
Sephadex G-100	98	2.1	0.22
Blue-Sepharose	64	3.6	0.37
Neu2en-Sepharose	49	48	5.0
ESM-Sepharose	44	677	70

Results

Cultivation of *Trypanosoma rangeli*, strain El Salvador, in liver infusion tryptose medium resulted in a maximum yield of sialidase activity and a minimal cell death after 10-14 days. Analysis of the culture for the presence of this enzyme revealed that less than 1% was present in the membranes or the cytosol of the epimastigotes as determined after washing and lysis of the cells isolated from the culture medium, indicating that most of the enzyme produced by the parasite was secreted into the medium. The lyophilized supernatant of the culture medium served as starting material for preparation of the enzyme. Its specific activity was 0.1 U/mg protein. As first step in the isolation of sialidase activity, gel chromatography on Sephadex G-100 was used (Table 1), which was effective in removing coloured material from the medium. One peak eluting at low molecular weight positions away from the enzyme activity was coloured yellow and showed a high extinction at 280 nm but was negative in the protein assay. Gel chromatographic separation was also carried out on Sephadex G-200, which, however, was less effective than separation on G-100. The sialidase always eluted slightly in front of BSA suggesting that the molecular weight is higher than 68 000.

BSA, which was part of the culture medium, was difficult to separate from the enzyme on gel chromatography due to similarity in molecular weights. Blue-Sepharose was therefore used to remove contaminating BSA specifically. This step gave a four-fold increase in specific activity when compared to the starting material, and a 64% yield (Table 1).

For the last two steps of enzyme purification, ESM and Neu2en were coupled to cyanogen bromide-activated Sepharose 4B as affinity matrices [4-6]. The enzyme activity obtained after passage over Blue-Sepharose could be adsorbed to the Neu2en gel and eluted in 49% yield, which gave an increase in specific activity to 5 U/mg protein. Further affinity purification over ESM-Sepharose resulted in an enzyme preparation with 44% total recovery and a specific activity of 70 U/mg protein, corresponding to a 677-fold purification when compared to the specific activity present in the culture medium. This enzyme preparation was almost pure as estimated by SDS gel electrophoresis (Fig. 1). The data of the purification sequence are summarized in Table 1.

Table 2. Substrate specificity and kinetic parameters of the sialidase from *T. rangeli*. For details of the procedures see the Materials and Methods section.

Substrate	Relative cleavage rate (%)	K_M (mM)	V_{max} (U/mg protein)
MU-Neu5Ac	100	0.51	8.74
Neu5Ac-α(2-3)-lactose	58	1.7	13.6
Neu5Ac-α(2-6)-lactose	22	2.1	4.31
Neu5Gc-α(2-3)-lactose	31		
Fetuin	13		
PSM'ol	4		
BSM	2		
BSM, alkaline treated	5		
Bovine brain gangliosides	5		
Neu5Ac-GM3	8		
Neu5Gc-GM3	1		
Neu4Ac5Gc-GM3	—		

Testing of the enzyme from the culture medium or after gel chromatography on G-100 for protease activity with azocasein was negative, showing that proteases were not present in the medium. Incubations with protease inhibitors (PMSF and Trasylol) did not reveal changes in activity. No glycosidase activities other than sialidase were found in the purified enzyme preparation.

The pH optimum was tested from pH 4.8 to 6.0 with 100 mM sodium acetate and found to be at 5.4-5.6. The molecular weight was assayed by gel chromatography on Sephadex G-100 and G-200, and by SDS gel electrophoresis and HPLC on TSK G 3000 SW, and was found to be 70 000, with the individual values ranging from 69-73 000.

The presence of Ca^{2+}, Mn^{2+} and Mg^{2+} ions as well as of EDTA did not reveal changes in enzyme activity. The activity could, however, be reduced to 50% by 1.4×10^{4} M Neu5Ac2en, 1.05×10^{-3} M *p*-nitrophenyloxamic acid and 8.5×10^{-6} M Hg^{2+}.

The relative cleavage rates of the various substrates tested are summarized in Table 2. It shows that the enzyme prefers MU-Neu5Ac, followed by the sialyllactoses. From experiments with the two different *N*-acetylneuraminyllactoses it becomes evident that α(2-3) linkages are split almost three times faster than α(2-6) glycosides. Conversion of *N*-acetylated sialic acids to *N*-glycoloylated compounds results in a slower hydrolysis of the corresponding sugar as can be seen when Neu5Ac-α(2-3)-Lac and Neu5Gc-α(2-3)-Lac are compared. The same effect is observed for the gangliosides Neu5Ac-GM3 and Neu5Gc-GM3. Oligosaccharides obtained by β-elimination from PSM, which is rich in Neu5Gc, are also relatively poor substrates. *O*-Acetylation reduces the cleavage rate, too, as can be recognized when BSM, containing high amounts of predominantly 9-*O*-acetylated sialic acids, and alkali-treated BSM are compared. 4-*O*-Acetylated sialic acids are completely resistant; in contrast to the non-*O*-acetylated ganglioside, Neu4Ac5Gc-GM3 is not a substrate. Also from ESM no 4-*O*-acetylated species were released as

analyzed by HPLC; only Neu5Ac could be detected after incubation of ESM with *T. rangeli* sialidase. Comparison of fetuin as a serum glycoprotein and alkali-treated BSM as a mucin-type glycoconjugate indicate that the former is a better substrate than the mucin. Neu5Ac-GM3 is a seven-fold weaker substrate than Neu5Ac-α(2-3)-Lac, indicating that gangliosides are generally hydrolyzed at a relatively slow rate. When compared to Neu5Ac-α(2-3)-Lac the K_M-value obtained for the (2-6) isomer is similar and in the millimolar range, whereas MU-Neu5Ac has a threefold lower K_M. V_{max} shows the highest value for Neu5Ac-α(2-3)-Lac and decreases to 64% for MU-Neu5Ac and to 32% for Neu5Ac-α(2-6)-Lac.

Storing the purified enzyme at 4°C for 48 h in 100 mM sodium acetate buffer, pH 5, reduces the activity only to 80%, whereas one freeze-thaw-cycle leaves only 50% of the original activity.

Discussion

Purification of the sialidase from *Trypanosoma rangeli* was achieved by a sequence of four different steps involving affinity chromatography. Due to the similar molecular weight of BSA and the sialidase, the removal of BSA that had to be present for optimal culture conditions, was a great problem. The use of Blue-Sepharose was successful in removing a major part of BSA, whereas the remaining part could be removed by passages over affinity columns. Immobilized ESM has already been used in the preparation of sialidases from human leucocytes [4] and human liver [5], whereas Sepharose-linked Neu2en has so far been studied for the binding of *Clostridium perfringens* sialidase [6]. The combination of both affinity columns was successful for final purification of the sialidase from *T. rangeli*. These results suggest that affinity chromatography might be in general a powerful tool for the isolation of sialidases which often occur at only low specific activities.

This sialidase isolated from *T. rangeli* has similar properties to corresponding enzymes isolated from bacteria, viruses and mammalian sources. The pH optimum of 5.4-5.6 is in the range of 4 - 6 found for most other sialidases [21, 22]. Also the molecular weight of 70 000 is similar to other sialidases, including subunits of viral sialidases [22]. It has to be noted that an earlier investigation of a crude preparation of the sialidase from *T. rangeli* suggested a molecular weight of 48 000 [3]. This was, however, determined for the strain Costa Rica Bg-60 which is different from the strain El Salvador used in this investigation, and may therefore be the reason for the difference in the molecular weights.

Since the introduction of MU-Neu5Ac as fluorogenic substrate for a fast, specific, sensitive and easy assay of sialidase activity [7, 18], this enzyme from most sources rapidly hydrolyzes this substrate. The substrate specificities for most sialidases investigated [21-25] show in principle the same sequence as observed for the enzyme from *T. rangeli* (see Table 2). The preference for α(2-3)-linked over α(2-6)-linked sialic acid residues in corresponding substances, e.g. sialyllactose, is typical for most sialidases; for viral sialidases an even more pronounced linkage selectivity can be found, acting on α(2-3) linkages up to 470-fold faster than on α(2-6) bonds [26]. Only the enzyme from *Arthrobacter ureafaciens* releases sialiac acids linked α(2-6) better than those bound α(2-3) [22]. The K_M values of 1.7 and 2.1 mM found here with sialyllactoses as substrates are in the range of 10^{-3} - 10^{-4} M described for most other sialidases with these substrates [21].

Conversion of Neu5Ac to analogous *N*-glycoloylated compounds as in NeuGc-α(2-3)-Lac and Neu5Gc-GM3 results in a decrease in the relative cleavage rate. Correspondingly, oligosaccharides obtained from PSM after β-elimination (PSM'ol), which contain about 80% of their sialic acids as Neu5Gc and only 20% as Neu5Ac [27], are weaker substrates than the de-*O*-acetylated mucin from bovine submandibular gland containing mainly Neu5Ac. *O*-Acetylation in BSM, which has the highest variety of *O*-acetylated sialic acids found so far in nature with predominantly the 9-*O*-acetylated species [14], reduces the relative cleavage rates when compared to the non-*O*-acetylated form. 4-*O*-Acetylated sialic acids are completely resistant towards the action of this enzyme, which also corresponds with the behaviour of other sialidases. Furthermore, the cleavage rate is also influenced by the non-sialic acid parts of the molecules; oligosaccharides are better substrates than glycoproteins or gangliosides.

In addition, the influence observed for bivalent cations, EDTA and the inhibitors tested is in agreement with results obtained earlier for most other sialidases, while the enzymes isolated from *Vibrio cholerae* and *Streptococcus K* and *A* depend on Ca^{2+} ions [21]. The finding that Neu5Ac2en is as potent an inhibitor for *T. rangeli* sialidase as for other sialidases tested so far [28], with the exception of one fraction of human [25] and equine [29] liver sialidases, as well as *T. cruzi* sialidase [30], suggests that this naturally occurring substance may be of importance in the regulation of the activity of sialidases in organisms. This unsaturated sialic acid is not only formed chemically at physiological pH values from CMP-Neu5Ac [31] but possibly also by sialidase itself [32].

The sialidase of *T. rangeli* has some interesting biological properties. For example, enzyme secretion does not parallel cell multiplication and continues to increase in the stationary phase of growth *in vitro* [3]. It is therefore possible that enzyme production is influenced by exogenous factors such as those in the LIT medium during growth. It is not known whether the enzyme is secreted during infection of mammalian hosts; if it is, it may not achieve the same levels that it does in culture, otherwise *T. rangeli* would be pathogenic. If the enzyme is not secreted in *T. rangeli* hosts, it will be of interest to determine what is the factor in the host that causes suppression of enzyme production. Sialidase has also been found in *T. cruzi*, the etiological agent of Chagas' disease [2]. In *T. cruzi* the enzyme is on the outer membrane, is developmentally regulated, has a molecular weight of 160 000 - 200 000 and appears to control infection by a negative mechanism (M.E.A. Pereira *et al.*, unpublished results). In addition, an inhibitor of *T. cruzi* sialidase (cruzin) has recently been found in the plasma of normal human individuals, and this inhibitor is completely ineffective against the *T. rangeli* enzyme [30]. Thus, it appears that the biological activity of the sialidases of these two trypanosomes is not similar.

The localization of sialidase activities in both trypanosome species is also reflected in the presence of sialic acids on the cell surface. Whereas *T. rangeli* has no sialic acid, *T. cruzi* has been found to contain sialic acids as cell surface components [1]. As both kinds of trypanosomes often occur together in the endemic areas, simple and reliable tests are required for a fast discrimination of the species. The very sensitive fluorogenic sialidase test seems to be well suited for this purpose, as the present study indicates that relatively high concentrations of soluble sialidase can be considered as a marker for *T. rangeli*, wheres *T. cruzi* has only low membrane-bound activities of this enzyme.

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