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A Biochemical and Immunological Comparative Study on *Trypanosoma equiperdum* and *Trypanosoma evansi*

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ABSTRACT

Trypanosoma equiperdum and *Trypanosoma evansi* were purified by three or four cycles of low-speed centrifugation and final filtration through DEAE cellulose. The purified trypanosomes were used in comparative biochemical and immunological studies. Comparative polypeptide pattern analysis revealed that *T. equiperdum* showed 21 polypeptide bands, whose M_r ranged from >200 to 14.8 kDa. *T. evansi* showed 25 polypeptide bands in the M_r range 97–14.8 kDa. The main differences were associated with the presence of secondary bands, relative intensity and the number of bands. Both species gave seven glycoprotein bands; those of 97 and 68 kDa were present in *T. equiperdum* but absent in *T. evansi*. Bands of 61 and 28 kDa were present in *T. evansi* but not in *T. equiperdum*. Anti-*T. equiperdum* sera recognized four homologous antigens and cross-reacted with three antigens of *T. evansi*. Anti-*T. evansi* sera recognized three homologous antigens and cross-reacted with four *T. equiperdum* antigens. Four identical proteolytic protease bands were present for both species, while only one surface protein was detected for each species: 66 kDa for *T. equiperdum* and 62 kDa for *T. evansi*.

Keywords: antigen, glycoprotein, polypeptides, protease, surface proteins, speciation, trypanosomes

Abbreviations: ATP, adenosine triphosphate; BSA, bovine serum albumin; Con A, concanavalin A; DEAE cellulose; diethylaminoethyl cellulose; ESB, electrophoresis sample buffer; HRP, horseradish peroxidase; IP, intraperitoneal; IZS, Istituto Zooprofilattico Sperimentale; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; PBSG, PBS with 1% glucose; PVP, polyvinylpyrrolidone; M_r, relative molecular weight; SDS, sodium dodecyl sulphate; TBS, Tris saline buffer; *Teq Trypanosoma equiperdum*; *Tev*, *Trypanosoma evansi*; TT, Tris–Triton buffer; VAT, variable antigenic types; VSG, variable surface glycoprotein

INTRODUCTION

Trypanosoma equiperdum is a member of the non-tsetse-transmitted trypanosome group, which is found outside the tsetse fly (*Glossina* sp.) belt in Africa and in many countries of Asia and Southern Europe. It is the causative agent of dourine, a sexually transmitted disease of horses, mules and donkeys. Since the presence of the trypanosomes in the blood is not easy to demonstrate, serology plays an important role in the diagnosis and control of dourine (Luckins, 1992).

T. evansi, the causative agent of 'Surra', 'Derrengadera' or 'Mal de Caderas' has been found in Africa, the Middle East, Asia and South and Central America. Horses, donkeys and camels are most severely affected by the disease, while cattle, pigs, sheep and goats seem to be less affected. *T. evansi* is mechanically transmitted by the stable flies *Tabanus* spp. and *Stomoxys calcitrans* (Clarkson *et al.*, 1971; Foil, 1989; Mihok *et al.*, 1995; Sumba *et al.*, 1998) and by vampire bats (*Desmodus rotundus*) (Desquesnes, 1996). We describe a comparative biochemical and immunological study of *T. equiperdum* and *T. evansi.*

MATERIALS AND METHODS

Trypanosome production and purification

Blood infected with *T. equiperdum* or *T. evansi* and preserved under liquid nitrogen was thawed at 37°C and inoculated intraperitoneally into an outbred Wistar rat. When three or more trypanosomes were visible in a microscopic field, the rat was anaesthetized and bled. The fresh blood was used to inoculate additional rats. When 10^8–10^{10} trypanosomes/ml were detected in the blood, the rat was anaesthetized and bled by cardiac puncture, using 3.8% sodium citrate as the anticoagulant.

To purify the trypanosomes, blood (25 ml) was centrifuged at 1000*g* for 10 min at 4°C; the buffy coat together with the upper layer (2–4 mm) of the red blood cells was collected, pooled and suspended in PBSG (57 mmol/L Na_2HPO_4, 3 mmol/L NaH_2PO_4, 43.8 mmol/L NaCl, pH 8.0, 1% glucose), recentrifuged and again collected. This procedure was repeated. Lastly, the buffy coat was collected, suspended in 20 ml of PBSG and loaded onto a 20 ml DEAE cellulose column, previously equilibrated with the same buffer. The column was eluted with the same buffer, and 5 ml fractions were collected until no more trypanosomes were observed by phase-contrast light microscopy. Fractions containing trypanosomes were pooled and centrifuged again, as described above, washed twice with 20 ml of the same buffer, counted, aliquoted and preserved dry under liquid nitrogen (–196°C) for further biochemical studies. Live trypanosomes were used for checking viability with trypan blue vital stain and by rat inoculation, and for surface membrane labelling.

Preparation of trypanosome lysate

Samples containing 10^7 trypanosomes of either species were suspended in ESB (50 mmol/L Tris-HCl, pH 7.5, 2.0% SDS, 2.0% β-mercaptoethanol, 15% glycerol, 0.01% bromophenol blue), boiled for 5 min and cooled on ice.

Polyacrylamide gel electrophoresis (SDS-PAGE)

Lysate preparations were loaded onto a 10% gel, with a 5% polyacrylamide stacking gel (Laemmli, 1970) and electrophoresed at 50 mA until the tracking dye reached the positive pole (Miniprotean II, Bio-Rad, Hercules, CA, USA). The gel was stained with Coomassie brilliant blue and compared with molecular weight standards (Bio-Rad, broad range).

Detection of glycoproteins

The glycoproteins were detected by a modification of the previously reported methods (Wood and Sarinana, 1975; Weiss *et al.*, 1991). Trypanosome lysates were separated by SDS-PAGE as described above. After separation, the proteins were transferred to a 0.45 μm pore size nitrocellulose filter (100 V, for 45 min) in transblot buffer containing 25 mmol/L Tris, 190 mmol/L glycine and 20% methanol (Towbin *et al.*, 1979). The blots were washed twice with Tris-saline buffer (50 mmol/L Tris-HCl, pH 7.4, 200 mmol/L NaCl, 1 mmol/L $CaCl_2$, 1 mmol/L $MnCl_2$). The membranes were blocked for 2 h with TBS, 0.05% Tween-20 and 1% polyvinylpirrolidone (PVP) (Sigma Chemical Co., St Louis, MO, USA), washed three times with TSB and incubated with 50 mg/ml Con A containing 0.5% PVP overnight at room temperature. Next, the membranes were washed 4 times with TBS and incubated with horseradish peroxidase 50 μg/ml (Sigma). Bound Con A–peroxidase was detected with 10 ml of substrate buffer (1.5 ml of 3 mg/ml 4-chloro-α-naphthol in methanol, 7.5 ml PBS and 5 μl 30% H_2O_2) until the glycoprotein bands were clearly visible. Control runs were carried out under the same conditions in the presence of 150 mmol/L α-methylmannoside or mannose.

Proteases

The proteinases of both species of trypanosomes were analysed by SDS-PAGE on 0.8% polyacrylamide gel copolymerized with 0.1% gelatin (Lants and Ciborowski, 1994). A sample containing 10^7 trypanosomes of each species was suspended in PBS (100 mmol/L sodium phosphate, 150 mmol/L NaCl, pH 7.4), homogenized by freezing and thawing 5 times, and then centrifuged at 13 250*g* (Eppendorf, Netheler-Hinz, Germany) in order to eliminate all the debris. Supernatant of each species of trypanosome (25 μl) was mixed with 5 μl (4 ×) of ESB without the β-mercaptoethanol. The samples were electrophoresed at 4°C at 110–120 V. The gel was washed with TT (100 mmol/L Tris-HCl, pH 8.8 and 1% Triton X-100) at room temperature for 30 min to eliminate the SDS from the gel and to allow renaturation of the proteins. The gel was then washed 3 times with distilled water and incubated in 100 mmol/L Tris-HCl, ph 7.0 buffer containing 2.5 mmol/L $CaCl_2$. Finally, it was washed with the same buffer and stained with 0.3% Coomassie brilliant blue in a mixture of isopropanol–acetic acid–water (25:10:65 by volume). The proteolytic activity was detected as clear bands.

Western blotting

Trypanosome lysate samples of either species were electrophoresed in SDS-PAGE. After separation, the proteins were transferred onto nitrocellulose membrane as described above (see Detection of Glycoproteins). The membranes were blocked for 2 h with 100 mmol/L PBS, pH 7.4, containing 0.05% Tween-20 and 1% PVP. They were then washed three times with the same buffer without PVP and incubated with sera from a horse experimentally infected with *T. equiperdum* (IZS, Teramo, Italy) or a horse naturally infected with *T. evansi* (wild horses from Apure State, Venezuela) diluted 1:100 in PBS containing 0.05% Tween-20 and 1% PVP. Bound antibodies were detected with horseradish peroxidase-conjugated goat anti-horse IgG (Sigma) followed by incubation in substrate buffer until the antigenic bands became visible.

Surface membrane labelling and blotting

The method used was a modification of an already reported method (Hurley *et al.*, 1985). Twenty microliters of 10 mg/ml biotin (Sulfo-NSH-Biotin, Pierce Chemical Co., Rockford, IL, USA) in dimethyl sulphoxide was added to 10^7 purified trypanosomes suspended in 1 ml PBS, mixed and incubated for 10 min at room temperature (22°C) with gentle shaking. The proportion of living cells was determined by vital staining with trypan blue. The cells were centrifuged at 1000*g* for 10 min at 4°C, suspended in 5 ml of PBS and sonicated for 1 min with a fine tip (Labsonic U, Braun Biotech, Melsungen, Germany, output 50 watts, 0.5 Hz). In order to separate the soluble and particulate components, the suspension was centrifuged at 40 000*g* for 20 min at 4°C (XL-70 ultracentrifuge, rotor SW40 Ti, Beckman, Palo Alto, CA, USA). The supernatant was discarded and the pellet, containing the cell membranes, was then suspended in 140 μl of electrophoresis sample buffer, boiled for 5 min and cooled on ice. Samples (30 μg protein/lane) were electrophoresed at 50 mA until the tracking dye reached the positive pole (Laemmli, 1970). After separation, the proteins were transferred to a nitrocellulose filter as described above (see Detection of Glycoproteins). The blot was incubated with blocking buffer (PBS, pH 7.4, 2% BSA, 0.1% Triton X-100) for 30 min at 37°C, washed with washing buffer (PBS, 0.1% BSA, 0.05% Tween-20) and incubated with 10 ml of 3.5 μg/ml Avidin-HRP conjugate (Pierce Chemical Co.) in PBS for 30 min at 37°C. After the blot had been washed three more times, the biotin–avidin–HRP reaction was revealed with 10 ml of substrate buffer. Finally, the blot was scanned and integrated with a computer-based densitometer (Scanwin, Jandel, Sigma).

RESULTS

Purification of trypanosomes

A large quantity of pure trypanosomes was obtained through the two-step purification method. The purified trypanosomes appeared to be motile and completely free of contamination by cells; more than 99% were viable, as measured by trypan blue vital stain and rat infection.

Polypeptide profile

The comparative polypeptide profile of the two trypanosome species is shown in Figure 1. *T. equiperdum* showed 20 major polypeptides, while *T. evansi* had 25. The M_r of both species was between 97 and 14.8 kDa. A few polypeptides were not shared by the two species; the 66 and 53 kDa bands appeared only for *T. equiperdum*, while the polypeptides of 72, 62, 37, 31, 30, 27 and 22 kDa appeared exclusively for *T. evansi*. Analysing the differences between the species, there was a doublet at around 68–97.4 kDa in both species that seemed more intense in *T. equiperdum*. The next four bands (48, 53, 57 and 62–64 kDa) were also of higher intensity in *T. equiperdum* than in *T.*

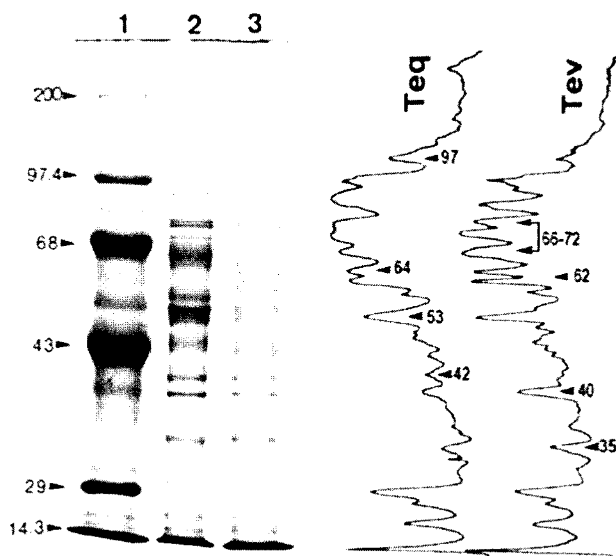

Figure 1. Comparative polypeptide profile in SDS-PAGE gel. Lane 1, molecular weight standards; lanes 2 and 3, *T. equiperdum* and *T. evansi* electrophoretic patterns, respectively. *Teq* curve: lane 2 densitometric analysis of *T. equiperdum* pattern. *Tev* curve: lane 3 densitometric analysis of *T. evansi*. Arrowheads: M_r of relevant protein bands

evansi. The 64 kDa band for *T. equiperdum* was very wide and might contain an additional protein band that was impossible to discriminate. In the area between 43 and 29 kDa, there were three main bands, a doublet of 42 and 40 kDa which appeared more intense in *T. equiperdum*, while the following band (35 kDa) appeared of similar intensity in both species. In this area, there were also smaller bands that were difficult to compare. Below 30 kDa, both species showed a similar band pattern, with slight differences in intensity.

Glycoprotein profile

Both species showed seven major glycoproteins, 97, 70, 68, 63–61, 57, 40 and 20 kDa for *T. equiperdum* and 70, 63, 61, 57, 38, 28 and 20 kDa for *T. evansi* (Figure 2). The glycoprotein patterns showed few differences. The glycoprotein bands of 97, 68 and 40 kDa were absent in *T. evansi*. In addition, *T. equiperdum* gave only one heavily dense band with M_r between 63 and 61 kDa, where *T. evansi* showed two separate bands at 63 and 61 kDa. The glycoprotein profile for *T. evansi* revealed the presence of two main bands, at 38 and 28 kDa, that were absent in *T. equiperdum*.

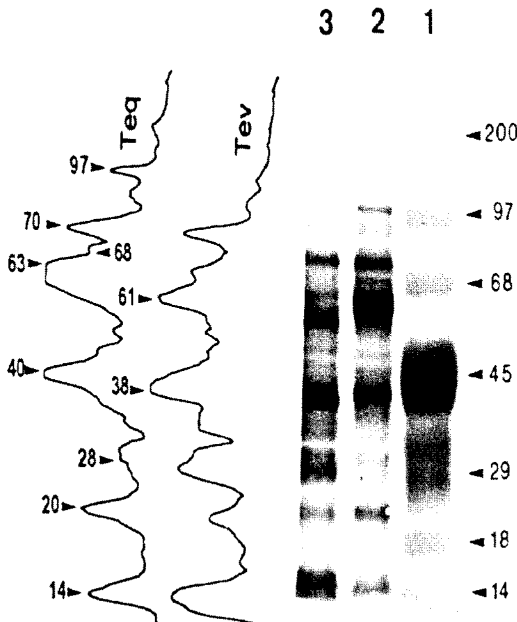

Figure 2. Glycoprotein pattern on nitrocellulose paper, blotted with Con A and revealed by the horseradish peroxidase reaction. Lane 1, molecular weight standards; lanes 2 and 3, *T. equiperdum* and *T. evansi* glycoprotein patterns, respectively. *Teq* curve: lane 2 densitometric analysis of *T. equiperdum* pattern. *Tev* curve: lane 3 densitometric analysis of *T. evansi*. Arrowheads: M_r of relevant glycoprotein bands

Antigenic pattern

Both anti-*Teq* and anti-*Tev* sera recognized only a few antigenic bands, even with homologous antigens (Figure 3). Horse anti-*Teq* recognized only four antigens (96, 72, 66 and 40 kDa) using solubilized *T. equiperdum* as antigen. With solubilized *T. evansi* as antigen, anti-*Teq* recognized only three antigens (96, 55, and 42 kDa). Serum from horses naturally infected with *T. evansi* slightly recognized three bands with the homologous antigen (96, 66 and 62 kDa) and gave four bands (96, 66, 62 and 28 kDa) with the *T. equiperdum* antigen. Normal sera did not recognize antigens in either species.

Proteases

Electrophoresis of soluble extracts of *T. equiperdum* or *T. evansi* in the copolymerized gel (Figure 4) showed depletion of the gelatin in four bands, corresponding to the M_r 40, 35, 32 and 28 kDa for both species, when the gel was stained with Coomassie brilliant blue. Judging from the thickness of the clear zones for each band in the gel, *T. evansi* showed more protease activity than *T. equiperdum*.

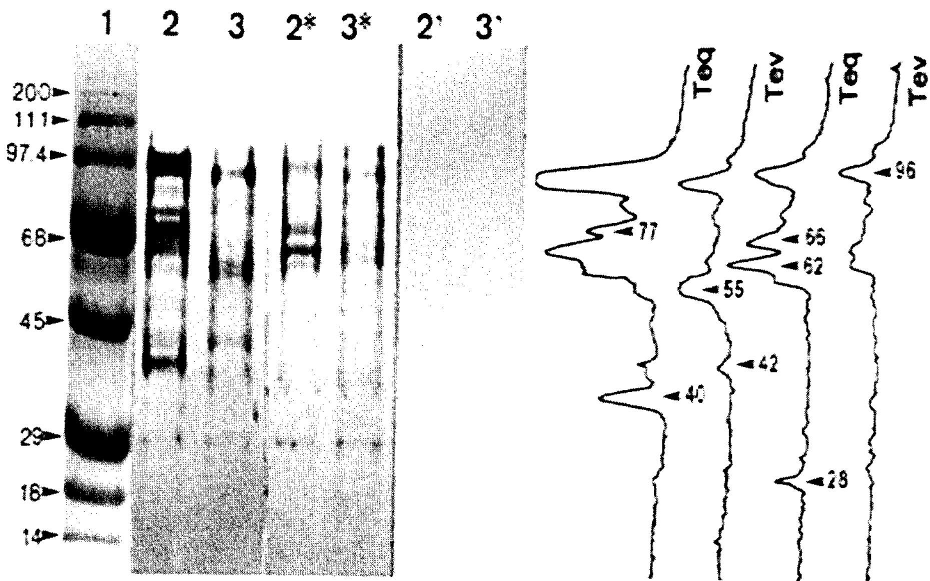

Figure 3. Western blot (WB) antigenic profile. Lane 1, molecular weight standards; lanes 2 and 3, WB of *T. equiperdum* and *T. evansi* antigens, respectively, incubated with horse anti-*Teq* serum. Lanes 2* and 3*, WB of *T. equiperdum* and *T. evansi* antigens, respectively, incubated with horse anti-*Tev* serum. Lanes 2′ and 3′, WB control experiments, *T. equiperdum* and *T. evansi* antigens, respectively, incubated with normal horse serum. *Teq* curves: lanes 2 and 2* densitometric analysis of *T. equiperdum*. *Tev* curves: lanes 3 and 3* densitometric analysis of *T. evansi*. Arrowheads: M_r of relevant antigens

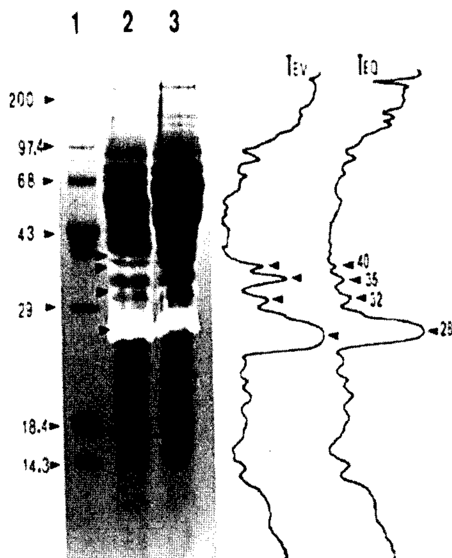

Figure 4. Proteinase activities. The protease activity was determined as described in Materials and Methods. Lane 1, molecular weight standards; lane 2, protease activities present in *T. equiperdum*; lane 3, protease activities present in *T. evansi*. *Teq* curve: lane 2 densitometric analysis of *T. equiperdum* protease pattern. *Tev* curve: lane 3, densitometric analysis of *T. evansi*. Arrowheads: M_r protease activities

Surface proteins

The surface protein patterns for both species (Figure 5) showed only one band, of 66 kDa for *T. equiperdum* and 61 kDa for *T. evansi*.

DISCUSSION

Trypanosomes were easily separated from red blood cells and were nearly purified by repeated centrifugation alone. The filtration through an ionic exchanger (DEAE cellulose) was performed to eliminate the remaining contaminating blood cells (14% leukocytes and a few erythrocytes). The previously used method (Lanham and Godfrey, 1970) takes considerable time and the results were not predictable as the column sometimes blocked, preventing recovery of the trypanosomes. Using this new method, large quantities of pure, viable trypanosomes could be obtained that showed appropriate motility and were infective in rats. However, Lonsdale-Eccles and Grab (1987) reported that purification of trypanosomes in DEAE cellulose results in changes in the level of ATP and loss of parasitic proteins, such as the variable surface proteins, peptidases and phospholipases.

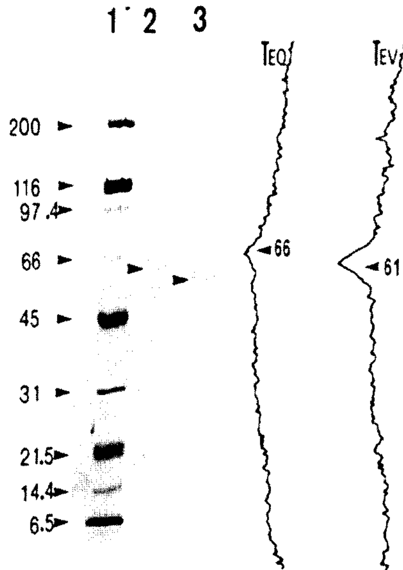

Figure 5. Profile of biotinylated surface proteins. Lane 1, molecular weight standards; lane 2, *T. equiperdum* surface protein; lane 3, *T. evansi* surface protein. *Teq* curve: densitometric outline of *T. equiperdum*. *Tev* curve: densitometric outline of *T. evansi*. Arrowheads: M_r of surface proteins

An overview of the comparative protein profiles of both species allowed detection of a few differences between them. SDS-PAGE analysis revealed many common and only a few unique proteins. According to our knowledge, there are no previous reports of the comparative polypeptide profiles of these two species in the literature. Our results were based on the ability of Con A to recognize and bind to the α-D-mannopyranosyl residue present in the surface glycoproteins of *T. equiperdum* and *T. evansi*. This methodology has been used widely in comparative studies on different trypanosome species, among which are *T. congolense* (Rautenberg *et al.*, 1980), *T. brucei-brucei*, *T. b. gambiense* and *T. b. rhodesiense* (Frommel and Balber, 1987; Balber and Frommel, 1988) and *T. congolense*, *T. simiae* and *T. b. brucei* (Mutharia and Steele, 1995). The gp28 of *T. evansi* is common with the procyclic form of *T. congolense*, *T. simiae* and *T. b. brucei* (Mutharia and Steele, 1995). These authors found that gp27-26 is an intracellular glycoprotein, and M.I. Gonzatti (personal communication, 1986) described it as a protease, which is highly immunogenic. We found that it cross-reacted with antibodies to anti-*Teq*. The gp61 and gp38 are present in the procyclic forms of *T. congolense*, *T. simiae* and *T. b. brucei* (Mutharia and Steele, 1995).

Three of the four bands recognized by the anti-*T. equiperdum* sera are possibly glycoproteins (97, 68 and 40 kDa). The same sera recognized three bands of 97, 57 and 42 kDa, with the heterologous antigen (*T. evansi*). The antigens of 97 and 42 kDa from

T. evansi, were not glycoproteins. The anti-*Tev* serum recognized three bands with its homologous antigen. This serum cross-reacted with heterologous antigens of the same molecular weight and with the gp28, a glycoprotein with antigenic and protease activities (M.I. Gonzatti, personal communication, 1986).

The humoral immune response against African trypanosomes has been widely studied owing mainly to two related phenomena, antigenic variation and immunosuppression (Silenghem *et al.*, 1994). The humoral immune response is mainly directed against the VSG, a unique glycoprotein (Vickerman and Luckins, 1969) that covers the surface of almost the whole of a population of trypanosomes at a given time, giving rise to the appearance of waves of parasitaemias of different variable antigenic types (VATs) during a single infection (Cross, 1975). When they are expressed on the surface of living parasites, the different molecules of VSG do not cross-react. However, when they are purified or isolated (Barbet and McGuire, 1978; Barbet *et al.*, 1981), there is cross-reaction by epitopes located in the carbohydrates of the C-terminal of the glycoprotein molecules (Cardosa de Almeida and Turner, 1983). When the trypanosomes are lysed, the amphiphilic VSG becomes soluble in water (Cardosa de Almeida and Turner, 1983) owing to the action of the endogenous phospholipase C, which cuts the dimyristoylglycerol in the glycolipid, liberating the soluble VSG (Hereld *et al.*, 1986). This liberation of the VSG is associated with the appearance of a determinant formed by inositol phosphate that cross-reacts among the different VSGs (Shack *et al.*, 1988; Giardina *et al.*, 2002). We report the presence of four proteinases in both species, corresponding to M_r of 38, 35, 32 and 28 kDa (Figure 4). The clear band corresponding to 38 kDa is consistent with that reported by North and colleagues (1990), who pointed out that there are at least four enzymes between 25 and 45 kDa. A 25 kDa protease is preponderant in *T. brucei* throughout its life cycle, but the highest level is expressed in the circulating form of the parasite. There is no recent information for *T. equiperdum* and *T. evansi*, but North and colleagues (1983) reported two bands for each species and four different proteolytic activities, which could correspond to the four bands reported in the present work.

The presence of only one surface protein in each species, p66 for *T. equiperdum* and p61 for *T. evansi* (Figure 5) agrees in part with the report for *T. evansi* (Uche *et al.*, 1992) that some isolates showed both proteins while in others only one of 61 kDa was present. Nevertheless, the surface of the trypanosomes seems to consist mainly of VSG molecules (Cross, 1990), although other proteins present in the surface membrane may carry out related functions in the environment. It seems unlikely that only one protein is exposed at the membrane surface of trypanosomes. The observation of only one surface protein may be due to the fact that the other surface proteins are hidden under the carbohydrate moiety of the VSG, which might lower the accessibility for labelling by biotinylation or radioiodination (Howard *et al.*, 1981; Gardiner and Dwyer, 1983; Uche *et al.*, 1992).

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