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TRYPANOSOMA CRUZI PROTEOLYTIC ACTIVITIES AFTER IMMUNOPRECIPITATION WITH THE MONOCLONAL ANTIBODY H1A10

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ABSTRACT: *Trypanosoma cruzi* causes Chagas' disease, a debilitating disease that affects more than 25 million people in Central and South America. Sera from most chagasic patients recognize the major cysteine protease of *T. cruzi*, cruzipain, that indicates that the parasitic proteases have a significant role in host cell invasion. The aim of this work was to examine whether the trypomastigote-specific monoclonal antibody H1A10, which recognizes an 85 kDa glycoprotein (Tc-85) of the trypomastigote surface, was also able to recognize proteolytic activities in trypomastigote extracts. After immunoprecipitation with anti-trypomastigote hyperimmune serum and a monoclonal antibody, the proteases of *Trypanosoma cruzi* trypomastigotes were identified by SDS-electrophoresis. The monoclonal antibody H1A10 precipitated proteases with molecular masses of 160, 85 and 45 kDa that were active at acid pH. All of these enzymes were inhibited by E-64, TLCK, TPCK, leupeptin and antipain, which suggested that they were cysteine proteases. The same activity was detected in culture medium, indicating that these proteases can be released into the tissues of infected animals.

KEY WORDS: Monoclonal antibody, proteases, *Trypanosoma cruzi,* Chagas' disease.

RESUMO: *Trypanosoma cruzi* é o agente causador da doença de Chagas, uma doença devastadora que afeta mais de 25 milhões de pessoas na América Central e América do Sul. O soro sanguíneo da maioria dos portadores da doença reconhece a maior protease já descrita no *T. cruzi*, denominada cruzipaína, o que indica que as proteases do parasita desempenham um papel importante no processo de invasão celular. O objetivo deste trabalho foi verificar se o anticorpo monoclonal H1A10, que reconhece uma glicoproteína de 85 KDa (Tc-85), localizada na superfície celular da forma tripomastigota, era também capaz de reconhecer atividades proteolíticas nos extratos do tripomastigota. Após imunoprecipitação com soro hiperimune antitripomastigotas e com o anticorpo monoclonal, as proteases das formas trypomastigotas de *Trypanosoma cruzi* foram identificadas em SDS-eletroforese. O anticorpo monoclonal H1A10 precipitou proteases com massas moleculares de 160, 85 e 45 kDa, especialmente ativas em pH ácido. Todas essas enzimas foram inibidas por E-64, TLCK, leupeptina e antipaina, o que sugere serem cisteína-proteases. As mesmas atividades foram detectadas no médio de cultura das formas tripomastigotas, indicando que essas proteases são possivelmente liberadas nos tecidos dos animais infetados.

PALAVRAS-CHAVE: Anticorpo monoclonal, proteases, *Trypanosoma cruzi,* doença de Chagas.

INTRODUCTION

Infection by *Trypanosoma cruzi*, commonly known as Chagas' disease, a parasitic flagellate, usually culminates in chronic disease that is characterized by the accumulation of damaged tissue over several years as the parasites multiple intracellularly and then rupture the cells to reinfect new cells. Damage to the host tissues frequently results in myocarditis because of the destruction of cardiac muscle and associated ganglia, or in enlarged intestines and esophagus, because of the destruction of muscle cells in the gut (Zingales & Colli, 1985).

Trypomastigotes, the infective bloodstream form, leave the phagolysosome soon after cell invasion then transform into amastigotes and multiply freely in the cytosol (Zingales & Colli,

1985). The intracellular parasites must disrupt both the vacuolar and host cell plasma membranes at some point in the infection (Andrews & Colli, 1982). This work aimed to identify proteolytic activities unequivocally linked to the outer membrane of *T. cruzi* because the parasites need to break proteins before the invasion of the host cells.

Trypanosoma cruzi contains several proteolytic activities (Cazzulo et al. 1990; Greig & Ashall 1990; Rangel et al. 1981; Souto-Padron et al. 1990), including cruzipain, that is similar to papain (Cazzulo et al. 1989; Hellman et al. 1991). Andrews (1990) described an acid-active *T. cruzi* hemolysin that was able to form 10 nm lesions in erythrocyte ghosts.

The major cysteine protease of *T. cruzi*, cruzipain, is a good example of practical application related to the knowledge of protease action. It is recognized by sera from most chagasic patients (Gruppi et al., 1997), the C-terminal domain is also its major immunogenic region (Stoka et al., 2000) and efforts have been directed towards the chemotherapy of Chagas' disease using this protease as target (Cazzulo et al., 2001). The knowledge of the structure of a target protease facilitates the design of selective inhibitors and the active site of cruzipain is, today, a goal of irreversibly selective inhibitors (Huang et al., 2003).

In this report, it is shown that a *T. cruzi* surface antigen, specific for trypomastigotes, can also show proteolytic activity.

Material and Methods

Parasites

Trypomastigotes were obtained from cultured LLC-MK_2 cells (rhesus monkey kidney epithelial cells). The LLC-MK_2 cells were maintained in Dulbecco's modified Eagle medium (DMEM) with 10 % fetal calf serum (FCS) at 37°C in a humidified atmosphere containing 5% CO_2 (Alves et al., 1986).

Parasite extracts

About 1 x 10^9 trypomastigotes, released around the 5-6th day after cell inoculation, were collected by centrifugation. The sediment was incubated at 37°C for 30 min to allow the mobile forms to return to the supernatant. These trypomastigotes were than collected, washed and suspended in PBS buffer containing 1% Triton X-100, as described in Alves (1986). After a further centrifugation at 12,000 g for 3 min at 4°C, the supernatant was used for enzyme essays at a protein concentration of 3 mg/ml, as determined by the method of Lowry et al.(1951).

Antibodies and immunoprecipitation

A polyclonal hyperimmune serum against methoxypsoralen-inactivated trypomastigotes of the CL strain was used (Andrews et al., 1985). The monoclonal antibody (H1A10) that recognizes an 85-kDa glycoprotein (Tc-85) of the trypomastigote surface was kindly provided by Alves (Alves et al., 1986).

Immunoprecipitation was done as described by Zingales et al. (1982). Briefly, parasite lysates were incubated with rat normal serum for 2 h and than overnight with polyclonal serum or H1A10. After incubation, the immunecomplexes were precipitated with protein A-Sepharose 6MB (Pharmacia Fine Chemicals), washed with 50 mM Tris-HCl pH 6.8, containing 150 mM NaCl, 5 mM ethylenediamine tetraacetic acid (EDTA) and suspended in sodium dodecyl sulfate (SDS)-sample buffer.

Fresh culture medium was incubated with trypomastigotes (1 x 10^5 parasites/ml) for 1 h at 37°C and after collecting the parasites the culture medium was concentrated by salting out. Non-specific adsorbing material was removed by incubation with normal rabbit serum. The samples were immunoprecipitated with polyclonal hyperimmune serum and with the monoclonal antibody H1A10 as previously described (Zingales et al., 1982).

Proteolytic activity

The samples were applied in sample buffer to 8% polyacrylamide gels containing 0.2% (w/v) gelatin and electrophoresis was done as Greig & Ashall (1990). After electrophoresis, the gels were incubated overnight at 37°C in the desired incubation buffers and then stained with Coomassie Brilliant Blue. Following digestion, peptidase bands appear as clear areas on a dark

background of undigested stained protein substrate (Ostoa-Saloma et al., 1989). Incubation buffers were 50 mM citrate (pH 4.5), 50 mM 2-(N-morpholino)ethane-sulfonic acid (MES) (pH 6.0), 50 mM Tris-maleic acid (pH 6.8), 50 mM 3-morpholino-propane-sulfonic acid (MOPS) (pH 7.5) and 50 mM 3-(cyclohexylamino)-1-propanesulfonic acid (CAPS) (pH 10.0). All buffers contained 5 mM EDTA. The effect of the peptidase inhibitors trans-epoxysuccinyl-L-leucylamido-(4-guanidino) butane (E-64), N-a-tosyl-L-lysylchloromethyl ketone (TLCK), N-a-chloro-3-[4-tosylamido]-4-phenyl-2-butanone (TPCK), phenylmethylsulfonyl fluoride (PMSF), pepstatin, leupeptin and antipain was analyzed by adding the inhibitor to the incubation buffer.

Results

Figure 1A shows the proteolytic activities recovered from a crude *T. cruzi* trypomastigote extract, after SDS-PAGE gelatin-containing gels. At pH 6.8 (close to the physiological pH) it was possible to distinguish three main activities at 35, 42 and 80 kDa, although the strong activity at pH 4.5 did not allow the precise determination of the sizes of the proteolytic enzymes (data not shown).

Figure 1B shows the proteolitic activities recovered from *T. cruzi* trypomastigote extract after immunoprecipitation with anti-*T. cruzi* polyclonal serum. Six major activities, at 45 kDa, 70 kDa, 85 kDa, 110 kDa, 160 kDa and about 300 kDa, were detected at physiological pH and much more active at acidic pH. Table 1 compares these activities as a function of pH.

Figure 1C shows the proteolitic activities recovered from *T. cruzi* trypomastigote extract after the immunoprecitpitation with anti-*T. cruzi* polyclonal serum and after immunoprecipitation with the monoclonal antibody H1A10. The same three proteolytic activities were detected at pH 6.8 and 4.5, with the size of 85 kDa, at 160 kDa and 230 kDa.

Figure 2A shows that three major proteolytic activities (66 kDa, 85 kDa, 120 kDa) and one minor acyivity (at 230-250 kDa) were recovered in the trypomastigote culture medium with the hyperimmune serum, active at pH 6.8 but more active at pH 4.5. In the same culture medium, the monoclonal antibody H1A10 identified two proteolytic activities, at 85 and 45 kDa,

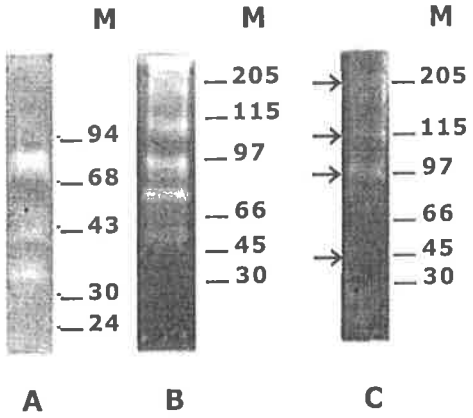

Figure 1 - Proteolytic activities from *Trypanosoma cruzi* trypomastigotes after SDS - PAGE in gelatin containing-gels. **A.** Crude extract at pH 6.8. **B.** Immunepreciptation with anti *T. cruzi* polyclonal serum at pH 4.5. **C.** Immunepreciptation with monoclonal antibody H1A10 at pH 4.5. **M** is a molecular marker (kDa).

Table 1 - *Trypanosoma cruzi* proteolitic activities in trypomastigote-extracts after SDS-PAGE gelatin-containing gels at different pH. **A**: after immunoprecipitation with anti-*T. cruzi* polyclonal serum. **B**: after immunoprecipitation with anti-*T. cruzi* polyclonal serum and monoclonal antibody H1A10. Activity: (++) Strong , (+) weak, (-) none. (*) pH values.

	A			B	
kDa	4.5*	6.8*	10*	4.5*	6.8*
250 - 200	++	++	+	+	-
160 - 150	++	+	-	+	+
110 - 100	++	++	-	-	-
90 - 80	++	+	-	++	+
80 - 70	++	-	-	-	-
70 - 65	++	++	+	-	-
45 - 35	++	+	-	+	-

as shown in the Figure 2B. Table 2 shows that these activities were also stronger in acidic medium.

All proteolytic activities described above were tested for their susceptibility to the peptidase inhibitors: pepstatin, E-64, PMSF, TLCK, TPCK, leupeptin and antipain, at pH 4.5 and 6.8, keeping the same pattern of inhibition. Figure 3 shows that the proteolytic activities immunoprecipitated with the monoclonal antibody H1A10, even at pH 4,5, were highly sensitive to the protease inhibitors E-64, TLCK and leupeptin at the starting concentrations recommended by the suppliers (Boehringer, Mannheim). Pepstatin A, an aspartic-protease inhibitor and PMSF, a serine-protease inhibitor, did not affect these activities. TPCK and antipain resulted in fewer and less intense bands (Figure 3, lines 6 and 8). The proteolytic activities immunoprecipitated with the hyperimmune polyclonal serum showed a similar behavior, very sensitive to cysteine protease inhibitors but not to pepstatin and PMSF (not shown).

Also the two proteolytic activities recovered with H1A10 from the trypomastigote culture medium were strongly inhibited by E-64 but not by pepstatin A (data not shown).

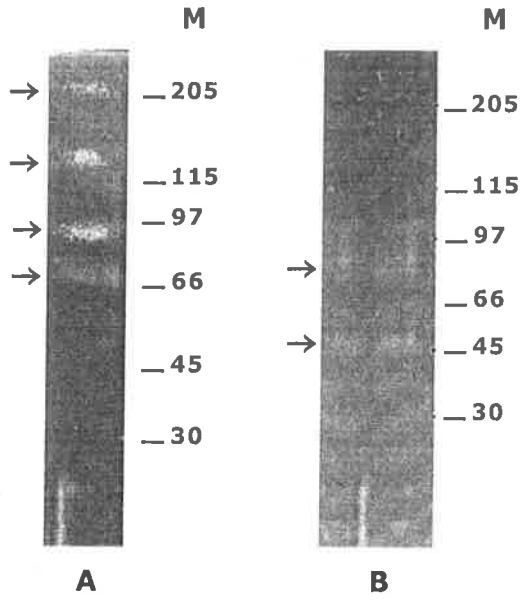

Figure 2 - Proteolytic activities detected in trypomastigote culture medium. After immunoprecipitation with polyclonal hyperimmune serum and with monoclonal antibody H1A10 sample were submitted to SDS-PAGE in gelatin containing gels. **A.** immunoprecipitation with anti *T. cruzi* polyclonal serum at pH 4.5. **B.** immunoprecipitation with monoclonal antibody H1A10 at pH 4.5. **M** is a molecular marker (kDa).

Table 2 - Proteolytic activities recovered from culture medium of *Trypanosoma cruzi* trypomastigotes after immunoprecipitation with hyperimmune serum and H1A10. **A**: after immunoprecipitation with anti-*T. cruzi* polyclonal serum. **B**: after immunoprecipitation with anti- *T. cruzi* polyclonal serum and monoclonal antibody H1A10. Activity: (++) Strong , (+) weak, (-) none. (*) pH values.

	A					B	
kDa	4.5*	6.0*	6.8*	7.0*	10*	4.5*	6.8*
230-250	++	+	+			+	-
160	-	-	-	-	-	+	-
120	++	+	-	-	-	-	-
115	+	-	+	+	-	-	-
85	+	-	+	-	-	+	+
66	++	+	+	-	-	-	-
45	+	+	-	-	-	+	+

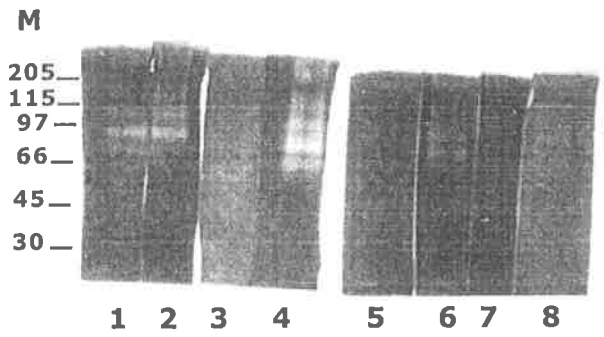

Figure 3 - Inhibition profile of *T. cruzi* proteolytic activities recovered in trypomastigotes extract with monoclonal antibody H1A10. Lane 1, no addition; lane 2, 1 mM Pepstatin; lane 3, 10 mM E - 64; lane 4, 1 mM PMSF; lane 5, 100 mM TLCK; lane 6, 100 mM TPCK; lane 7, 50 mM Leupeptin; lane 8, 50 mM Antipain. All digestions were overnight at pH 4.5 with 5 mM EDTA.

Discussion

After immunoprecipitation with the monoclonal antibody H1A10, an unequivocally proteolytic activity was detected at 85 kDa. Note that all the other activity-bands, at 116 kDa and 230 kDa, showed an apparent molecular mass that was near to a multiple of 85 kDa, indicating the possibility of a dimeric or tetrameric enzyme.

The observation that some activities became stronger after immunoprecipitation is easily explained by the partial purification that immunoprecipitation propitiates and which results in the removal of inhibitors present in the crude extract. Both the proteolytic activities immunoprecipitated with polyclonal serum and with the monoclonal antibody were more active at acidic pH.

The recovery of proteolytic activity from the trypomastigote culture medium showed that some of these activities could be released into the environment by the parasites. In particular, the activity of 85 kDa, recovered with the antibody H1A10, showed that the antigen Tc-85 released by the parasite could display some proteolytic activity. This was not an unexpected result since the shedding of Tc-85 has been well described (Gonçalves et al., 1991).

All of the proteases detected were cysteine-type proteases, inhibited particularly by E-64. The proteolytic activities immunoprecipitated with the anti-*T. cruzi* polyclonal serum, also indicated to be cysteine proteases. No aspartic peptidases were detected and, since the assay medium contained 5 mM EDTA, a metal chelator, the presence of metal-proteases was also excluded.

The 85-kDa protease seen here has not been reported previously and its activity, at acidic and physiological pH, may have an important role in parasite escape and survival. Because *T. cruzi* trypomastigotes can penetrate cells and tissues, the presence of proteases that are closely related to membrane surface monoclonal antibodies may be relevant in allowing exit from the phagosome and their replication within host cells.

The glycoprotein Tc-85, recognized by H1A10 and which partially prevents *T. cruzi* invasion of cell monolayers (Alves et al., 1986), has been cloned and sequenced (Giordano et al., 1999) and shows no sequence identity to known proteases. But it is not the first time that a monoclonal antibody against a glycosyl-anchored membrane antigen has immuno-precipitated a thiol proteinase activity (Fresno et al., 1994). On the other side, the Tc-85 surface antigen showed considerable homology to the family of sialidases (Kahn et al., 1991), although to this author's knowledge, no study has detected sialidase activity in purified Tc-85 protein. Similarly to the results present here, when a 0.4-kb genomic DNA insert, coding for TC-85, was expressed in bacteria, the monoclonal antibody H1A10 recognized a 40 kDa peptide in western blot analysis (Giordano et al., 1999). In addition, the Tc-85 nucleotide sequence also shared a homology with *Homo sapiens* protein tyrosine phosphatase-type receptor (Locus XM 005781 and Locus NM 006504). Together, these findings indicate that there is still much to be learned regarding the biochemical behavior of the *T. cruzi* trypomastigote antigens.

The fact that H1A10 immunoprecipitates an 85 kDa antigen which presents proteolytic activity can have at least two explanations: (i) the antigen is one of the Tc-85 glycoproteins family although the molecule described by Alves et al. (1986) did not present proteolityc activity. In this case the protease here presented could be processed after expression. (ii) The here presented 85 kDa protease also express epitopes cross-reactivity with the Tc-85, but it is not the Tc-85. The genes that express proteases in trypanosomes

may have been retained in the adhesion molecules/trans-sialidase gene family during evolution in order to increase the parasite survival. The parasite may have elaborated a system by which similar genes provide proteins that adhere to cell membrane proteins and subsequently cleave the same proteins.

Based on the most dramatic example of cysteine-proteases as a target for chemotherapy, the case of anti-HIV cocktails, a peptidase databank has recently been created in order to use the current information on proteases and their inhibition as targets for chemotherapy of infectious diseases. It is possible that in the near future other therapies based on cysteine-proteases inhibitors will be available for parasitic diseases.

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