

# A *Trypanosoma cruzi*-secreted 80 kDa proteinase with specificity for human collagen types I and IV

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Specific interactions between parasites and extracellular matrix components are an important mechanism in the dissemination of Chagas' disease. Binding of the extracellular matrix proteins to *Trypanosoma cruzi* receptors has been described as a significant step in this phenomenon. In this study, a specific proteinase activity was identified in cell-free extracts of amastigote, trypomastigote and epimastigote forms of *T. cruzi* using the collagenase fluorogenic substrate *N*-Suc-Gly-Pro-Leu-Gly-Pro-7-amido-4-methylcoumarin. Isolation of this activity was achieved by a four-step FPLC procedure. Optimal enzyme activity was found to occur at pH 8.0 and was associated with a single *T. cruzi* 80 kDa protein (Tc 80 proteinase) on SDS/PAGE under reducing conditions. An internal peptide sequence of Tc 80 proteinase was obtained (AGDNYTPPE), and no similarity was found to previously described proteinases of *T. cruzi*. This

enzyme activity is strongly inhibited by HgCl<sub>2</sub>, tosyl-lysylchloromethane ('TLCK'), *p*-chloromercuribenzoate and benzyloxycarbonyl-Phe-Ala-diazomethane. The purified enzyme was able to hydrolyse purified human [<sup>14</sup>C]collagen types I and IV at neutral pH, but not <sup>14</sup>C-labelled BSA, rat laminin, rabbit IgG or small proteins such as insulin or cytochrome *c*. In addition, Tc 80 proteinase activity was found to be secreted by *T. cruzi* forms infective to mammalian cells. Furthermore we demonstrated that purified Tc 80 proteinase mediates native collagen type I hydrolysis in rat mesentery. This feature is compared with that of *Clostridium histolyticum* collagenase. These findings suggest that Tc 80 proteinase may facilitate *T. cruzi* host-cell infection by degrading the collagens of the extracellular matrix and could represent a good target for Chagas' disease chemotherapy.

## INTRODUCTION

The flagellated protozoan *Trypanosoma cruzi* is the aetiological agent of Chagas' disease, a chronic debilitating incurable illness that is highly prevalent in Latin America [1]. Its transmission to vertebrate hosts is carried out by several species of reduviid bugs through faeces contaminated with metacyclic trypomastigotes, which infect cells via mucosa or skin wounds [2]. *T. cruzi* trypomastigotes enter mammalian cells by a non-conventional endocytotic mechanism [3] involving lysosome recruitment of the host cell and their fusion with the parasitophorous vacuole [4]. An acidic membrane pore-forming protein [5] and a neuraminidase [6] synergistically allow trypomastigotes to escape from the parasitophorous vacuole to the cytoplasm where the parasite transforms into the amastigote form [7]. After multiplying inside cells by binary fission, the amastigotes differentiate into trypomastigotes, which are released into the bloodstream.

*T. cruzi* trypomastigote forms use the bloodstream to reach and infect a broad range of tissues and cells such as heart, muscle, nervous system, gastrointestinal tract and mononuclear phagocytic cells. To have access to different cell types, the parasite must cross an intricate network of fibrous proteins embedded in a hydrated carbohydrate gel called the extracellular matrix [8].

It has been suggested that a limited set of *T. cruzi* proteins and target cell components play an important role in the parasite–host cell relationship [9]. In addition, specific interactions between trypomastigote molecules and components of the extracellular matrix, such as collagen [10], fibronectin [11,12] and laminin [13],

have been described. Ortega-Barria and Pereira [14] have reported on *T. cruzi* penetrin, a protein that binds specifically to heparin, heparan sulphate and collagen. This molecule promotes *in vitro* penetration of trypomastigote forms into fibroblasts.

Parasitic protozoan proteinases play important roles in some aspects of the host–parasite relationship. Avila et al. [15] first demonstrated that *T. cruzi* epimastigotes grow in the presence of catalase as a unique source of amino acid residues. In addition to their nutritional function, proteinase activities appear to be involved in *T. cruzi* metacyclogenesis *in vitro* [16] and infection of host cells, since some classical proteinase inhibitors partially arrest trypomastigote penetration into fibroblasts [17]. More recently, it has been demonstrated that the *T. cruzi* 120 kDa proteinase [18] is involved in the generation of a Ca<sup>2+</sup>-signalling factor for mammalian host cells [19]. This factor could be important in host-cell invasion by the parasite. We have shown that specific human IgG can be endocytosed and digested intracellularly by *T. cruzi* forms [20]. No proteinases capable of degrading IgG *in vivo* are known, but *in vitro* experiments have shown that a 52 kDa proteinase [21] and the *T. cruzi* major cysteine proteinase [22], cruzipain, have high affinity for these molecules and can mediate their enzymic degradation. Other experiments have suggested that cruzipain inhibitors can arrest infection and intracellular development of *T. cruzi* [23–25].

In accordance with features of its life cycle, we have postulated that *T. cruzi* may express a specific collagenase that could facilitate its migration through the host extracellular matrix. In this paper we report the identification and characterization of such a specific *T. cruzi*-secreted 80 kDa proteinase (Tc 80

Abbreviations used: AMC, 7-amido-4-methylcoumarin; N-Cbz, *N*-carbobenzyloxy; E-64, *trans*-epoxysuccinyl-L-leucylamido-(4-guanidino)butane; LIT, liver infusion tryptose; MEM, minimum essential medium; MNA, 4-methoxy- $\beta$ -naphthylamide; pCMB, *p*-chloromercuribenzoate; TBS, Tris-buffered saline; Z, benzyloxycarbonyl; Suc, succinyl; Tc 80 proteinase, *Trypanosoma cruzi* 80 kDa protein; Tos-Lys-CH<sub>2</sub>Cl, tosyl-lysylchloromethane ('TLCK').

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proteinase). Assays on  $^{14}\text{C}$ -labelled natural substrates demonstrate that the enzyme specifically hydrolyses purified human collagen types I and IV and native collagen type I in rat mesentery at neutral pH. This *T. cruzi* 80 kDa proteinase may facilitate host-cell infection by degrading collagens thereby disassembling the well-organized network of the extracellular matrix.

## EXPERIMENTAL

### Parasites

*T. cruzi* tissue-culture forms of the Berenice stock were maintained in monolayers of murine muscle L-6 cells grown in minimal essential medium (MEM; Sigma, St. Louis, MO, U.S.A.) supplemented with 25 mM L-glutamine, 100 units/ml penicillin, 100  $\mu\text{g}/\text{ml}$  streptomycin and 5% (v/v) fetal calf serum (Cutilab, Curitiba, Brazil), at 37 °C in 5%  $\text{CO}_2$ . Trypomastigotes and amastigotes of the parasite were obtained and purified as previously described [4,26]. *T. cruzi* epimastigote forms from Tulahuen, Ernestina, Albuquerque, Y, Berenice, and Corrego de Pedra stocks were maintained and grown in liver infusion tryptose (LIT) medium, containing 10% fetal calf serum, at 28 °C with continuous agitation [27].

### Preparation of cell extract

Cell-free extracts were usually prepared from 5 litres of liquid culture forms ( $5 \times 10^7$  cells/ml) in LIT medium ( $93 \pm 3\%$  epimastigotes,  $7 \pm 3\%$  trypomastigotes). The cells were harvested by centrifugation (6000 g for 10 min at 4 °C) and washed four times in PBS. The pellet was resuspended in 100 ml of 10 mM Tris/HCl, pH 7.5, and the parasites were immediately disrupted by three cycles of freezing at  $-20$  °C and thawing. After removal of the insoluble material by centrifugation (10000 g for 15 min at 4 °C), the supernatant, referred to hereafter as enzyme extract, was used for the assays or stored at  $-80$  °C. Enzyme extracts from trypomastigotes and amastigotes ( $10^9$  cells) were obtained by the same procedure, except that the parasites were resuspended in 2 ml of 10 mM Tris/HCl, pH 7.5, before disruption. After the finding that Tc 80 proteinase is not inhibited by *trans*-epoxysuccinyl-L-leucylamido-(4-guanidino)butane (E-64) and PMSF, 20  $\mu\text{M}$  and 1 mM respectively of these proteinase inhibitors were added to the enzyme extracts. Protein content was determined as described by Bradford [28].

$^{35}\text{S}$ Methionine labelling of epimastigotes was carried out in methionine-free MEM (Sigma). Before labelling, the cells were washed twice with PBS and incubated for 30 min in 100 ml of methionine-free medium. The parasites ( $10^{10}$  cells) were then labelled in the same medium in the presence of 1.5 mCi of  $^{35}\text{S}$ methionine (1000 Ci/mmol; Amersham International, Little Chalfont, Bucks., U.K.) for 6 h at 28 °C. The labelling procedure was terminated by removal of the medium, followed by three washes with PBS. Enzyme extracts of radiolabelled parasites were prepared as described above.

### Assay of enzyme activity

*T. cruzi* proteinase activity was determined by measuring the fluorescence of 7-amido-4-methylcoumarin (AMC) released by hydrolysis of the collagenase substrate *N*-Suc-Gly-Pro-Leu-Gly-Pro-AMC [29]. Assays were performed as previously described [18]. Briefly, 100  $\mu\text{g}$  of enzyme extract or 250 ng of purified enzyme, as specified, was incubated for 20 min at 37 °C in 200  $\mu\text{l}$  of 50 mM Tris/HCl, pH 8.0, containing 0.15 M NaCl (TBS) in the presence of 20  $\mu\text{M}$  fluorogenic substrate. After incubation,

the reactions were blocked by adding 1 ml of ethanol. The fluorescence of free AMC released by the enzymic reaction was measured at 440 nm on excitation at 380 nm in a Hitachi 2000 spectrofluorimeter. Under these conditions, fluorescence of the acyl fluorogenic substrate is negligible. Fluorescence intensities were converted into  $\mu\text{mol}$  of AMC by measuring the fluorescence of a standard solution of AMC, under the same experimental conditions. The optimal pH of Tc 80 proteinase activity was assayed as described above in 50 mM Tris/HCl, 50 mM Bistris/HCl, 50 mM sodium acetate or 50 mM  $\text{Na}_2\text{CO}_3/\text{NaHCO}_3$  buffer with 0.15 M NaCl adjusted to the desired pH. Purified enzyme activity was also assayed on different fluorescent peptidyl derivatives (Cbz is *N*-carbobenzyloxy): *N*-Cbz-Gly-Gly-Arg-AMC, *N*-Cbz-Gly-Arg-AMC, *N*-Cbz-Arg-AMC, *N*-Cbz-Phe-Arg-AMC, *N*-Cbz-Leu-Leu-Val-Tyr-AMC, *N*-glutaryl Gly-Gly-L-Phe-AMC, *N*-Cbz-Arg-Arg-AMC, *N*-Suc-Gly-Pro-AMC, Gly-Arg-4-methoxy- $\beta$ -naphthylamide (MNA) and Gly-Phe-MNA according to the above protocol. In reactions with substrates with MNA as fluorogenic group, the fluorescence was measured at 425 nm on excitation at 340 nm. All substrates were purchased from Sigma.

### Assay of proteinase inhibition

These experiments were performed in TBS using *N*-Suc-Gly-Pro-Leu-Gly-Pro-AMC as substrate. Different concentrations of the protease inhibitors tosyl-lysylchloromethane ('TLCK') (Tos-Lys- $\text{CH}_2\text{Cl}$ ), *p*-chloromercuribenzoate (pCMB), E-64,  $\text{HgCl}_2$ , PMSF, antipain, phosphoramidon, pepstatin a, leupeptin, 1,10-phenanthroline, EDTA and *Z*-Phe-Ala-diazomethane<sub>2</sub> were incubated with 10  $\mu\text{g}$  of enzyme extract or 0.1  $\mu\text{g}$  of purified Tc 80 proteinase in 200  $\mu\text{l}$  of TBS for 15 min at room temperature before the substrate was added. The reactions were monitored as described above. All inhibitors were from Sigma, except *Z*-Phe-Ala-diazomethane (a gift from Dr. R. Mayer, CNRS, Orléans, France).

### Enzyme purification

Tc 80 proteinase was purified by chromatography from freshly prepared enzyme extract. Saturated  $(\text{NH}_4)_2\text{SO}_4$  solution, adjusted to pH 7.0 with  $\text{NH}_4\text{OH}$ , was added to a final 30% satn. to 200 ml of enzyme extract, at 4 °C, with stirring. After 30 min, the suspension was centrifuged at 15000 g for 10 min at 4 °C. The supernatant was collected and precipitated with  $(\text{NH}_4)_2\text{SO}_4$  solution up to 70% satn. under the same conditions. The pellet was resuspended in 15 ml of 25 mM Tris/HCl, pH 7.5, and dialysed against 200 vol. of the same buffer for 12 h at 4 °C. After removal of the precipitated proteins by centrifugation (12000 g for 10 min at 4 °C), the supernatant was filtered through a 0.22  $\mu\text{m}$  membrane and applied to a DEAE-Sepharose CL-6B (Sigma) column (12 cm  $\times$  1.5 cm), previously equilibrated with 25 mM Tris/HCl, pH 7.5. The column was washed and the proteins were eluted with a linear gradient performed in the same buffer from 0.05 to 0.30 M NaCl for 70 min, and then with 1.0 M NaCl at 0.8 ml/min flow rate. Fractions of volume 2 ml were collected on ice, and an aliquot of each fraction was assayed with *N*-Suc-Gly-Pro-Leu-Gly-Pro-AMC. The enzymically active fractions, which were eluted between 0.20 and 0.25 M NaCl, were pooled and concentrated to 1.0 ml with a Centriprep-30 concentrator (Amicon, Beverly, CA, U.S.A.) at 4 °C. The solution was added to 1 ml of 100 mM sodium phosphate buffer, pH 7.0, containing 2.0 M  $(\text{NH}_4)_2\text{SO}_4$ , and then submitted to hydrophobic interaction chromatography as follows. The protein solution was loaded on to a phenyl-Sepharose CL-4B (Sigma)

column (1.5 cm × 5.0 cm) previously equilibrated with 100 mM sodium phosphate buffer, pH 7.0, containing 2.0 M (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> (buffer A) at 0.5 ml/min. The proteins were eluted from the gel by a linear gradient from 0.8 to 0.0 M (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> in 0.1 M sodium phosphate buffer, pH 7.0 (buffer B) for 20 min, and then with buffer B for 20 min at a flow rate of 0.5 ml/min. Each 2 ml fraction was immediately stored on ice until the enzyme activity assay. The active fractions were pooled and concentrated with a Centricon-30 concentrator (Amicon) at 4 °C to 1.0 ml. The solution was diluted 1:2 in buffer A and applied to a phenyl-Superose HR 5/5 FPLC column (Pharmacia, Uppsala, Sweden) equilibrated with the same buffer. The proteins were eluted by a decreasing (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> gradient as described above. Those fractions with enzyme activity were concentrated, diluted 1:4 in buffer A and loaded on to an alkyl-Sepharose HR 5/5 FPLC column (Pharmacia) equilibrated with the same buffer. After the column had been washed, a linear decreasing (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> gradient was performed from 75 to 50% of buffer A in relation to buffer B at a flow rate of 0.5 ml/min for 52 min. Fractions of volume 1.0 ml were collected in an ice-bath, and the active ones were pooled, dialysed against distilled water and concentrated to 200 μl in a Centricon-30 concentrator at 4 °C. Then 50 ng of protein was subjected to SDS/PAGE (10% gel) under reducing conditions as described by Laemmli [30]. The proteins of the gel were silver-stained as described [31] or submitted to autoradiography [32].

#### Internal amino acid microsequencing

Purified Tc 80 proteinase (10 μg) in 100 μl of 50 mM Tris/HCl, pH 8.0, was heated to 100 °C for 10 min, and cooled to -2 °C. Denatured protein was digested with trypsin (Sigma) for 24 h at 37 °C at an enzyme/substrate ratio of 1:10. The resulting peptides were separated by reverse-phase HPLC on a Vydac C<sub>18</sub> column (2.1 mm × 200 mm) and eluted with a linear gradient of 5–80% (v/v) acetonitrile in 0.1% trifluoroacetic acid at a flow rate of 200 μl/min. Isolated peptides were sequenced in an Applied Biosystems 473A automated microsequencer according to the manufacturer's instructions.

#### Assay of Tc 80 proteinase activity on purified proteins

<sup>14</sup>C-labelled proteins were prepared as described [33] with the following modifications: 40 μl of a freshly prepared solution (at 4 °C) of NaBH<sub>3</sub>CN (6 mg/ml in 40 mM phosphate buffer, pH 7.0) (Fluka, Buchs, Switzerland) was added to 200 μl of a 1 mg/ml solution of human collagen I, collagen IV, BSA, rat laminin or rabbit IgG; 50 μCi of [<sup>14</sup>C]formaldehyde (37 MBq/mmol; New England Nuclear, Boston, MA, U.S.A.) was then added. The mixtures were incubated at 37 °C for 4 h with shaking at 10 min intervals. The solutions were dialysed overnight against 200 vol. of TBS at 4 °C. The amount (c.p.m.) of <sup>14</sup>C incorporated by the proteins was determined for two 5 μl aliquots of the dialysed material in 2 ml of scintillation fluid in a 1600 TR-Packard liquid-scintillation analyser.

Human placental collagens were obtained from IMEDEX [34]; rat laminin was provided by Dr. J. D. Lopes (Escola Paulista de Medicina, Brazil); BSA, insulin and cytochrome *c* were from Sigma. Insulin and cytochrome *c* were extensively dialysed against TBS before use to eliminate residual ionic inhibitor.

Enzyme activity was assayed by incubating 50 μg of each protein with 0.25 μg of purified Tc 80 proteinase in 100 μl of TBS for up to 12 h at 37 °C. Controls consisted of substrates incubated under the same conditions with proteinase inactivated by previous treatment with 30 μM Tos-Lys-CH<sub>2</sub>Cl. Aliquots (20 μl)

were taken after 0, 1, 2, 5 and 12 h and the reaction was stopped with 5 μl of 5 × Laemmli sample buffer, followed by boiling for 3 min. To analyse radiolabelled protein degradation, the samples were submitted to SDS/PAGE and the gels prepared for autoradiography [32]. Degradation of insulin and cytochrome *c* was analysed by Tricine/SDS/PAGE [35], and controls were performed using trypsin at the same concentration as Tc 80 proteinase.

#### Activity of Tc 80 proteinase on native collagen

Mesentery from 4-month-old male rats was dissected and dried over a glass slide at room temperature. Either purified Tc 80 proteinase or *Clostridium histolyticum* collagenase (EC 3.4.24.3) (Sigma) [400 ng in 20 μl of TBS containing 0.05% (w/v) NaN<sub>3</sub> and 1 mM CaCl<sub>2</sub>] was placed on the mesenteric surface and incubated in a moist chamber for 12 h at 25 or 37 °C. After a wash with TBS, the tissue was examined by phase-contrast microscopy. A control experiment was performed with Tc 80 proteinase or clostridial collagenase inactivated by previous treatment with 30 μM Tos-Lys-CH<sub>2</sub>Cl and 2 mM EDTA respectively. After incubation, the solutions on the mesenteric surface were transferred to nitrocellulose membrane with a convertible filtration manifold system (Gibco-BRL). The dot-blot was probed with affinity-purified anti-(collagen type I) antibodies (Rockland) or with non-immune rabbit antibodies, and then with an anti-rabbit IgG-alkaline phosphatase conjugate (Bio-Rad). The blots were developed with Nitro Blue Tetrazolium and 5-bromo-4-chloroindol-3-yl phosphate (Bio-Rad) according to the manufacturer's instruction.

#### Secretion of Tc 80 proteinase

Trypomastigote or amastigote forms of *T. cruzi* (10<sup>8</sup> cells/ml) were incubated in MEM supplemented with 25 mM L-glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin and 5% fetal calf serum at 37 °C under 5% CO<sub>2</sub>. *T. cruzi* epimastigotes (10<sup>8</sup> cells/ml) were incubated in LIT medium supplemented with 100 units/ml penicillin, 100 μg/ml streptomycin and 5% fetal calf serum at 28 °C. At 0, 10, 30 and 50 min, cells were centrifuged in Eppendorf tubes (12000 g for 40 s at room temperature) and the supernatants recovered. To control the possible effects of culture medium components on proteinase activity, the cell pellet from each experiment was resuspended in 50 μl of 10 mM Tris/HCl, pH 7.5, and the cells were disrupted as described above. Their volumes were reconstituted with LIT, MEM containing 5% fetal calf serum or TBS. Then 100 μl of each culture supernatant or cell fraction was incubated with 40 μM *N*-Suc-Gly-Pro-Leu-Gly-Pro-AMC, *N*-Suc-Leu-Leu-Val-Tyr-AMC or *N*-CBZ-Arg-Arg-AMC at 37 °C for 40 min. A 900 μl volume of TBS was added to the reaction solutions and the fluorescence was measured as described above. A control experiment consisted of incubating either LIT, MEM containing fetal calf serum or TBS alone with the substrates under the same conditions.

#### Enzyme kinetics

Kinetic parameters were determined as described by Wilkinson [36]: 20 ng of purified Tc 80 proteinase was incubated with different concentrations (0.5–60 μM) of *N*-Suc-Gly-Pro-Leu-Gly-Pro-AMC and the enzyme reaction performed as described above.

**Table 1 Identification of a *T. cruzi* proteinase activity with *N*-Suc-Gly-Pro-Leu-Gly-Pro-AMC substrate**

Enzyme extract (100 µg) from the indicated *T. cruzi* forms was incubated with 20 µM *N*-Suc-Gly-Pro-Leu-Gly-Pro-AMC in 200 µl of TBS at 37 °C for 20 min. The reaction was monitored as described in the Experimental section. Values are means ± S.D. from three different assays. The epimastigote form was 93 ± 3% epimastigotes and 7 ± 3% trypomastigotes.

Parasite form	Specific activity (nmol of AMC released/min per µg of protein)
Trypomastigote	$(1.45 \pm 0.14) \times 10^{-5}$
Amastigote	$(1.00 \pm 0.11) \times 10^{-5}$
Epimastigote	$(0.53 \pm 0.09) \times 10^{-5}$

**Table 2 Purification of Tc 80 proteinase**

Total activity is specific activity × total protein (µg).

Purification step	Total activity	Specific activity (nmol of AMC released/min per µg of protein)	Percentage yield	Purification factor
Enzyme extract	77	$5.3 \times 10^{-6}$	100	1
(NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub> (30–70% satn.)	64	$4.7 \times 10^{-6}$	83	0.9
DEAE-Sepharose	138	$4.2 \times 10^{-4}$	180	79
Phenyl-Sepharose	98	$6.5 \times 10^{-4}$	127	122
Phenyl-Superose FPLC	41	$1.5 \times 10^{-2}$	53	2830
Alkyl-Superose FPLC	35	$2.6 \times 10^{-1}$	45	49000

## RESULTS

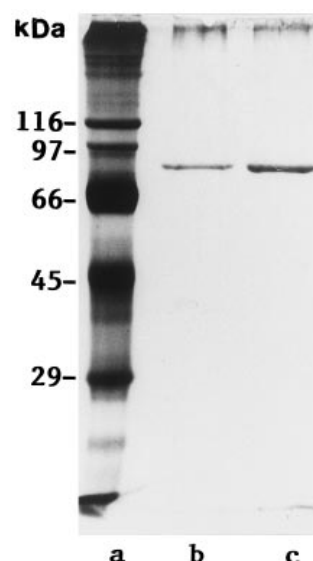
### Identification of *N*-Suc-Gly-Pro-Leu-Gly-Pro-AMC proteinase activity in different forms of *T. cruzi*

Enzyme extracts of amastigote, trypomastigote and epimastigote forms of *T. cruzi* readily hydrolyse the synthetic fluorogenic substrate *N*-Suc-Gly-Pro-Leu-Gly-Pro-AMC as shown in Table 1. Trypomastigotes, which are infective to host cells, had a higher specific activity than either epimastigotes or amastigotes. All *T. cruzi* stocks so far tested demonstrated approximately the same activity ratio on this substrate (results not shown).

### Purification of Tc 80 proteinase

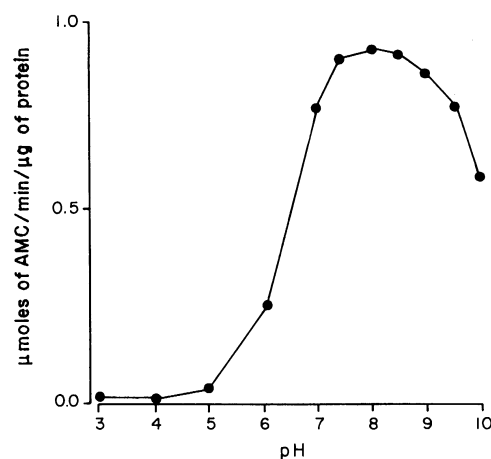
The proteinase was purified from *T. cruzi* liquid culture forms by (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> fractionation of the enzyme extract and a combination of ion-exchange and hydrophobic interaction chromatography. The purification factors and different steps of the chromatography procedure are indicated in Table 2. After being precipitated from the enzyme extract at between 30 and 70%-satd. (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, the proteinase activity was eluted from a DEAE-Sepharose CL-6B column from 0.20 to 0.25 M NaCl, then from a salt out phenyl-Sepharose CL 4B column as a single peak of activity. The active fractions were further purified by FPLC on phenyl-Superose and alkyl-Superose columns; again a single peak of activity was obtained (results not shown).

The purity of the active fractions on *N*-Suc-Gly-Pro-Leu-Gly-Pro-AMC obtained at the last purification step is shown in Figure 1. A single 80 kDa silver-stained band is seen after

**Figure 1 Electrophoretic analysis of the purified Tc 80 proteinase**

The radiolabelled purified enzyme (50 ng) was subjected to SDS/PAGE (10% gel) in reducing conditions as described in the Experimental section. The gel was silver stained (lane b) and submitted to autoradiography (lane c). SDS/PAGE standards (Sigma) were used to calibrate the gel (lane a): β-galactosidase (116 kDa), phosphorylase *b* (97 kDa), BSA (66 kDa), ovalbumin (45 kDa) and carbonic anhydrase (29 kDa).

SDS/PAGE (10% gel) under reducing conditions (lane b). Autoradiography of purified enzyme from [<sup>35</sup>S]methionine-labelled parasites also revealed a single 80 kDa protein as can be seen in lane c of Figure 1. The N-terminus of the Tc 80 proteinase was found to be blocked. An internal amino acid sequence was obtained (AGDNYTPPE), which shows no similarity to previously described proteinases of *T. cruzi*, particularly those belonging to the cruzipain/cruzain family. However, similarity to a metalloproteinase of *Crithidia fasciculata* (<sup>444</sup>AGGNITCPP<sup>453</sup>) was observed [37].

**Figure 2 Effect of pH on the activity of purified Tc 80 proteinase**

The purified enzyme (0.1 µg) was incubated with 20 µM *N*-Suc-Gly-Pro-Leu-Gly-Pro-AMC for 20 min at 37 °C in different buffers as described in the Experimental section. The reactions were stopped with 1 ml of ethanol, and the AMC released was quantified by measuring fluorescence at 440 nm after excitation at 380 nm. Each value is the mean from three different assays.

**Table 3 Fluorogenic-substrate-specificity of purified Tc 80 proteinase**

The purified proteinase (250 ng) was incubated with each substrate (20  $\mu$ M) in 25 mM Tris/HCl, pH 8.0, at 37 °C for 1 h. The reactions were stopped with 1 ml of ethanol and the fluorescence of free AMC was measured as described in the Experimental section. Control experiments consisted of incubation of substrates without enzyme. The results represent means  $\pm$  S.D. from three different assays.

Substrate	Specific activity (nmol of AMC released/min per $\mu$ g of protein)
<i>N</i> -Suc-Gly-Pro-Leu-Gly-Pro-AMC	$(650 \pm 50) \times 10^{-3}$
<i>N</i> -Suc-Gly-Pro-AMC	$(85 \pm 7) \times 10^{-3}$
<i>N</i> -Cbz-Gly-Gly-Arg-AMC	0.00
<i>N</i> -Cbz-Gly-Arg-AMC	0.00
<i>N</i> -Cbz-Phe-Arg-AMC	0.00
<i>N</i> -Cbz-Arg-Arg-AMC	0.00
<i>N</i> -Cbz-Leu-Leu-Val-Tyr-AMC	0.00
<i>N</i> -Glutaryl-Gly-Gly-L-Phe-MNA	0.00
Gly-Arg-MNA	0.00
Gly-Phe-MNA	0.00

It was further shown that Tc 80 proteinase has a strong dependence on neutral or alkaline pH. Maximal specific activity for both enzyme extract and the purified enzyme was observed at pH 8.0 (Figure 2). At pH 6.0 the fluorogenic substrate was weakly hydrolysed by the enzyme. No activity was detected when the experiment was performed at pH 5.0.

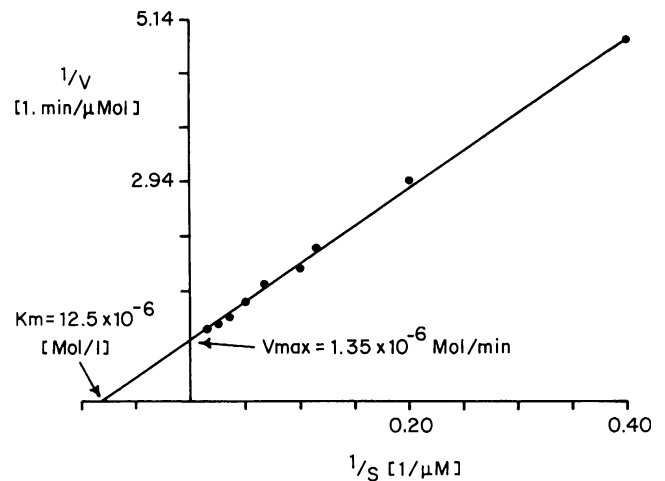
#### Fluorogenic substrate specificity

Purified Tc 80 proteinase hydrolysed *N*-Suc-Gly-Pro-Leu-Gly-Pro-AMC and *N*-Suc-Gly-Pro-AMC substrates with specific activities of  $(650 \pm 50) \times 10^{-3}$  and  $(85 \pm 7) \times 10^{-3}$  nmol of AMC released/min per  $\mu$ g of protein respectively (Table 3). However, the proteinase failed to cleave *N*-Cbz-Gly-Gly-Arg-AMC, *N*-Cbz-Gly-Arg-AMC, *N*-Cbz-Arg-AMC, *N*-Cbz-Phe-Arg-AMC, *N*-Suc-Leu-Leu-Val-Tyr-AMC, *N*-glutaryl-Gly-Gly-L-

**Table 4 Effects of proteinase inhibitors on purified Tc 80 proteinase**

Pure proteinase (100 ng) was incubated with different concentrations of inhibitor before the addition of 20  $\mu$ M *N*-Suc-Gly-Pro-Leu-Gly-Pro-AMC. The reaction took place at 37 °C for 30 min (see the Experimental section for details). Results are means  $\pm$  S.D. from three different experiments. n.i., No inhibition when the assays were carried out with inhibitor up to a concentration of 2.0 mM, except for E-64 for which the maximal concentration tested was 0.5 mM.

Inhibitor	Class of proteinase inhibited	IC <sub>50</sub> ( $\mu$ M)
HgCl <sub>2</sub>	Thiol	1.2 $\pm$ 0.03
pCMB	Thiol	1.5 $\pm$ 0.06
Tos-Lys-CH <sub>2</sub> Cl	Serine/thiol	0.8 $\pm$ 0.02
Z-Phe-Ala-CHN <sub>2</sub>	Thiol	1.0 $\pm$ 0.04
E-64	Thiol	n.i.
Leupeptin	Serine/thiol	n.i.
Antipain	Serine/thiol	n.i.
Pepstatin a	Aspartyl	n.i.
1,10-Phenanthroline	Metallo	n.i.
Phosphoramidon	Metallo	n.i.
EDTA	Metallo/serine	n.i.
PMSF	Serine	n.i.

**Figure 3 Determination of  $K_m$  and  $V_{max}$  of the Tc 80 proteinase**

The enzyme (10 ng) was incubated with different concentrations of *N*-Suc-Gly-Pro-Leu-Gly-Pro-AMC in TBS at 37 °C during 10 min. The reaction was stopped with 1 ml of ethanol, and the amount of AMC released was quantified as described in the Experimental section.

Phe-MNA and *N*-Cbz-Arg-Arg-AMC. Furthermore, it did not degrade the unblocked substrates Gly-Arg-MNA and Gly-Phe-MNA.

#### Inhibitor sensitivity

Table 4 shows that Tc 80 proteinase activity towards its fluorogenic substrate was inhibited by Tos-Lys-CH<sub>2</sub>Cl, HgCl<sub>2</sub>, pCMB and Z-Phe-Ala-CHN<sub>2</sub> with IC<sub>50</sub> values of 0.8, 1.2, 1.5 and 1.0  $\mu$ M respectively; its activity was not sensitive to other proteinase inhibitors in the millimolar range.

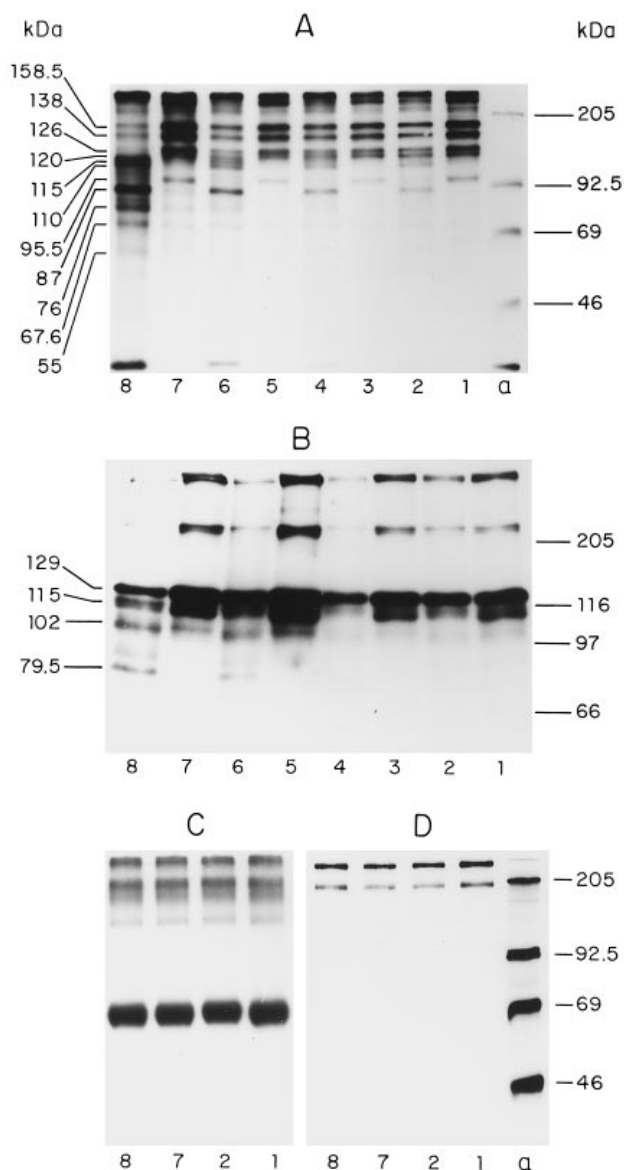
#### Kinetic parameters

The Michaelis–Menten constant ( $K_m$ ) and maximal velocity ( $V_{max}$ ) of Tc 80 proteinase were determined from Lineweaver–Burk plots shown in Figure 3. The proteinase has  $K_m$  and  $V_{max}$  values of  $12.5 \pm 0.50$   $\mu$ M *N*-Suc-Gly-Pro-Leu-Gly-Pro-AMC and  $1.35 \pm 0.20$   $\mu$ mol of AMC released/min respectively.

#### Hydrolysis of human [<sup>14</sup>C]collagen types I and IV by Tc 80 proteinase

The specific radioactivities of <sup>14</sup>C-labelled BSA, collagen I, collagen IV, laminin and IgG were  $1.7 \times 10^8$ ,  $0.8 \times 10^8$ ,  $1.2 \times 10^8$ ,  $0.8 \times 10^8$  and  $0.5 \times 10^8$  c.p.m./mg of protein respectively. Incubation of 50  $\mu$ g of these natural protein substrates with 0.25  $\mu$ g of Tc 80 proteinase resulted in hydrolysis of human collagen types IV and I (Figures 4A and 4B), but not BSA, rat laminin (Figures 4C and 4D) or rabbit IgG (results not shown). In control experiments, performed in the presence of Tc 80 proteinase inactivated with Tos-Lys-CH<sub>2</sub>Cl, no hydrolysis of the radiolabelled substrates was observed after 12 h of incubation. Tc 80 proteinase was also unable to degrade small proteins such as insulin and cytochrome *c* (results not shown).

Among the major modifications in the SDS/PAGE profile of collagen type-IV subunits (Figure 4A) was a rapid decrease in the 95.5 kDa subunit after 1 h of incubation with Tc 80 proteinase (lane 2). A significant reduction in the 120 and 126 kDa subunits began at 2 h (lane 4) and concluded at 12 h of incubation (lane

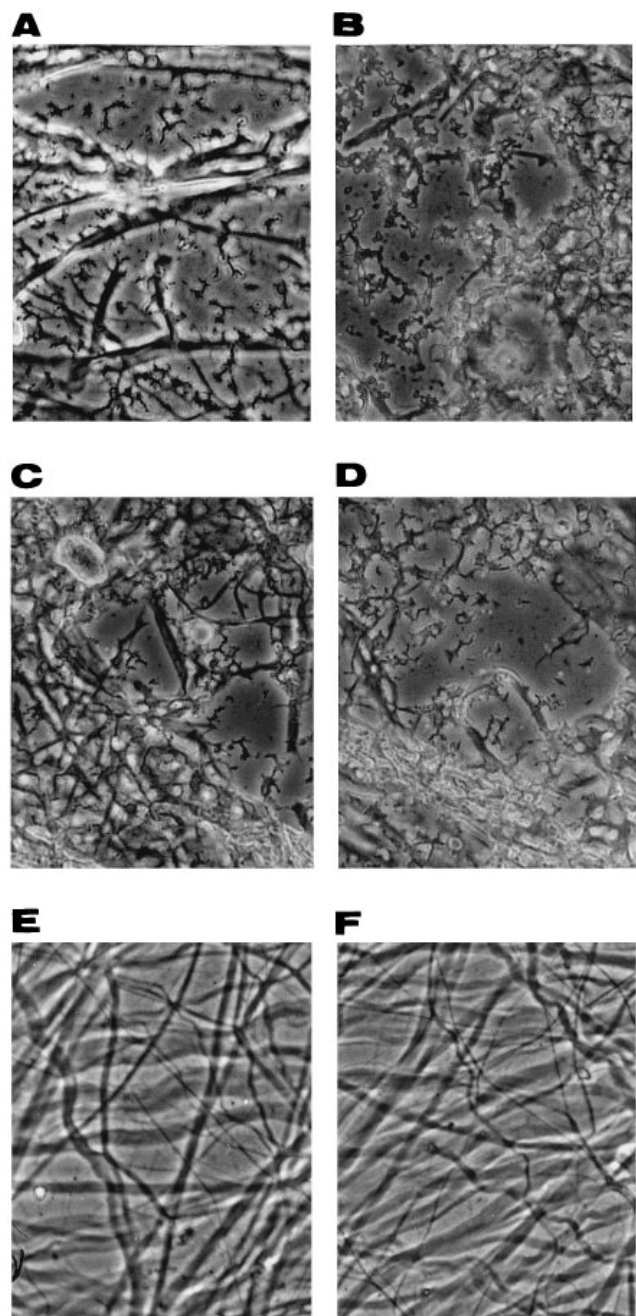


**Figure 4** Tc 80 proteinase activity on  $^{14}\text{C}$ -labelled proteins

Radiolabelled human collagen type IV (**A**), human collagen I (**B**), BSA (**C**) or rat laminin (**D**) (50  $\mu\text{g}$  in each case) was incubated with 0.25  $\mu\text{g}$  of purified enzyme in 100  $\mu\text{l}$  of TBS at 37  $^{\circ}\text{C}$ . Aliquots of volume 20  $\mu\text{l}$  were taken at 0, 1, 2, 5 and 12 h (lanes 1, 2, 4, 6 and 8 respectively) and boiled in the presence of sample buffer. Controls consisted of radiolabelled proteins with inactivated enzyme after 2 (lane 3), 5 (lane 5) and 12 h (lane 7) under the same experimental conditions. The reaction mixtures were subjected to SDS/PAGE (8% gel). The gels were stained with Coomassie Blue and autoradiographed.  $^{14}\text{C}$ -labelled standard proteins (Amersham) were used to calibrate the gels in (**A**), (**C**) and (**D**): myosin (205 kDa), phosphorylase *b* (92.5 kDa), BSA (69 kDa) and ovalbumin (46 kDa). The gel in (**B**) was calibrated with myosin (205 kDa),  $\beta$ -galactosidase (116 kDa), phosphorylase *b* (97 kDa) and BSA (66 kDa) from Sigma (lane  $\alpha$ ).

8). Furthermore, hydrolysis of the 158 and 138 kDa subunits occurred between 5 and 12 h of incubation (lanes 6 and 8). Extended hydrolysis of collagen type IV by Tc 80 proteinase resulted in the formation of six lower-molecular-mass bands in the gel (lane 8) with molecular masses of approx. 115, 110, 87, 76, 67 and 55 kDa.

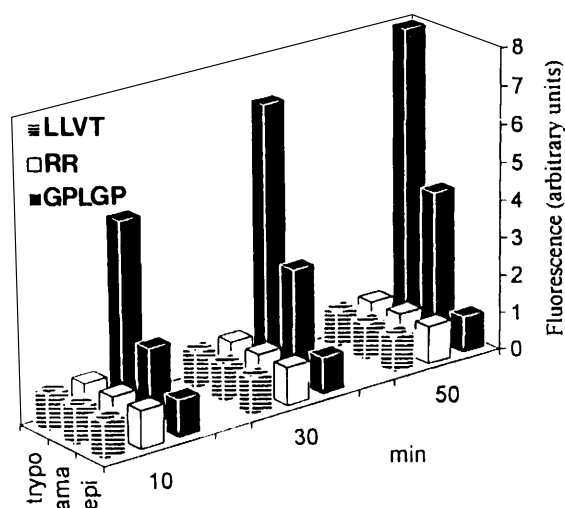
Collagen type I was also susceptible to cleavage by Tc 80 proteinase (Figure 4B). High-molecular-mass subunits were progressively hydrolysed by the enzyme over periods of time up



**Figure 5** Native collagen type-I hydrolysis by purified Tc 80 proteinase

Either Tc 80 proteinase or *C. histolyticum* collagenase (400 ng in 20  $\mu\text{l}$  of reaction buffer) was put on rat mesentery and incubated in a moist chamber for 12 h as described in the Experimental section. After two washes in TBS, the tissue was observed by phase-contrast microscopy (400 $\times$ ). Hydrolysis of collagen fibres can be observed after incubation at 25 and 37  $^{\circ}\text{C}$  respectively as follows: (**A**) and (**B**) Tc 80 proteinase; (**C**) and (**D**) clostridial collagenase. In control experiments, inactivated Tc 80 proteinase (**E**) and inactivated clostridial collagenase (**F**) showed none of the features present in (**A**) to (**D**).

to 12 h (lanes 2, 4, 6 and 8). In addition, 129 and 115 kDa bands, which correspond to  $\alpha_1\text{I}$  and  $\alpha_2\text{I}$  subunits respectively [34], were extensively hydrolysed after 12 h of incubation (lane 8). Despite the intensity of collagen type-I hydrolysis, it may be noticed that only a few lower-molecular-mass bands were detected, particularly one at 79 kDa (lanes 6 and 8). When a larger amount of



**Figure 6** Tc 80 proteinase secretion by *T. cruzi*

Trypomastigotes (trypan) and amastigotes (ama) ( $10^8$  cells/ml) were incubated in MEM supplemented with 5% fetal calf serum at 37 °C. Epimastigotes (epi) ( $10^8$  cells/ml) were incubated in LIT supplemented with 5% fetal calf serum at 28 °C. At 0, 10, 30 and 50 min, the cultures were centrifuged and the supernatants recovered. A 100  $\mu$ l volume of each supernatant was incubated with 30  $\mu$ M *N*-Cbz-Arg-Arg-AMC (RR), *N*-Suc-Leu-Leu-Val-Tyr-AMC (LLVT) or *N*-Suc-Gly-Pro-Leu-Gly-Pro-AMC (GPLGP) at 37 °C for 40 min. TBS (900  $\mu$ l) was added to each tube and the fluorescence was measured as described in the Experimental section. Control experiments consisted of incubation of the culture medium with the substrates. Results represent means from three independent experiments carried out in triplicate.

enzyme was used, the collagens were completely hydrolysed, but without formation of the lower-molecular-mass bands in the gels (results not shown).

#### Hydrolysis of native collagen by Tc 80 proteinase

To determine whether Tc 80 proteinase could also hydrolyse native collagen, we incubated it with collagen-type-I-rich rat mesentery [38]. Hydrolysis of collagen type-I fibres was observed after 3 h of incubation at pH 7.5. The comparative hydrolysis of collagen type-I fibres in the presence of Tc 80 proteinase (Figures 5A and 5B) and clostridial collagenase (Figures 5C and 5D) shows clear hydrolysis at 25 °C after 12 h of incubation with both enzymes (Figures 5A and 5C) and extensive degradation of collagen fibres at 37 °C (Figures 5B and 5D). In contrast, the inactivated enzymes produced no hydrolysis of the tissue (Figures 5E and 5F). Dot-blot analysis using a specific anti-(collagen type I) antibody demonstrated that the Tc 80 proteinase, like clostridial collagenase, was able to remove collagen from mesentery at 25 and 37 °C (results not shown). In contrast, inactivated enzymes did not remove collagen products from mesenteric tissue.

#### Tc 80 proteinase is secreted by *T. cruzi*

To have a function in the infectivity of mammalian cells, Tc 80 proteinase should either be on the surface or secreted by the parasite. The demonstration that this enzyme is released by *T. cruzi* was carried out with supernatants of epimastigote, amastigote and trypomastigote cultures (Figure 6). Culture supernatants were taken at 10, 30 and 50 min and incubated with: (a) *N*-Suc-Gly-Pro-Leu-Gly-Pro-AMC; (b) *N*-Cbz-Arg-Arg-AMC, a substrate of cruzipain [39]; (c) *N*-Suc-Leu-Leu-Val-Tyr-AMC, a substrate of a *T. cruzi*-non-secreted cathepsin B-like proteinase (M. P. Garcia, O. T. Nóbrega, A. R. C. Teixeira and

J. M. Santana, unpublished work). Under our experimental conditions, trypomastigotes and amastigotes released Tc 80 proteinase, since its activity was detected in the supernatants of these *T. cruzi* forms, whereas epimastigotes did not. As shown in Figure 6, Tc 80 proteinase activity was higher in the trypomastigote supernatant than in the amastigote one, and in both cases, the amount released increased with time. The secreted activity was selectively inhibited by the Tc 80 proteinase inhibitors described above. No proteolytic activity was found in these supernatants when *N*-Cbz-Arg-Arg-AMC or *N*-Suc-Leu-Leu-Val-Tyr-AMC substrates were used. To verify that the other proteinase activities, cruzipain and the cathepsin B-like proteinase, had not been inhibited by culture medium components, controls were performed by comparing the proteolytic activities of trypomastigote and amastigote lysates in the presence of culture medium (MEM + 5% fetal calf serum) or TBS. These controls showed normal proteinase activity with all the substrates tested, indicating that the absence of cruzipain and B-like cathepsin proteinase activity was not due to inhibition by components of the culture medium. These data show that Tc 80 proteinase is indeed secreted by trypomastigotes and amastigotes.

#### DISCUSSION

The recent development of new methods for detection, purification and characterization of proteinases has increased the current level of interest in studies on proteolytic enzymes of parasitic protozoa. Synthetic peptide substrates containing AMC, MNA or 3-amino-*o*-ethylcarbazole fluorescent groups have been used as highly sensitive and specific tools to study proteinases in several species of protozoa [40]. Some *T. cruzi* proteolytic activities have been studied using such substrates [18,39,41,42]. In the present work, we demonstrate that cell-free extracts of *T. cruzi* amastigote, trypomastigote and epimastigote forms readily cleave a collagenase fluorogenic substrate with an optimal pH of about 8.0. This enzyme activity shows a marked dependence on neutral or alkaline pH, since at pH 6.0 the specific activity is only 20% of that measured at pH 8.0. Furthermore incubation of the enzyme at pH 5.5 or below for 5 min followed by pH neutralization resulted in the loss of 92% of proteolytic activity (results not shown). These observations suggest that Tc 80 proteinase is a non-lysosomal protein that may be involved in metabolic processes other than in the lysosomes, where enzymes show optimal activity at acidic pH.

Tc 80 proteinase was not inhibited at high concentrations of the cysteine-proteinase-specific inhibitor E-64. However, its activity was very sensitive to low concentrations of HgCl<sub>2</sub>, pCMB and Z-Phe-Ala-diazomethane<sub>2</sub>, which are also inhibitors of cysteine proteinases [43]. Tos-Lys-CH<sub>2</sub>Cl, an inhibitor of cysteine and serine proteinases, was very effective against the enzyme, whereas leupeptin and antipain, also inhibitors of serine and cysteine proteinases, were not. On the other hand, PMSF, 1,10-phenanthroline and pepstatin a, which are known to be specific inhibitors of serine, metallo and aspartic proteinases respectively [43], did not inhibit the enzyme. There have been indications that peptidyl diazomethane inhibitors such as Z-Phe-Ala-diazomethane show specificity for cysteine proteinases [44–46]. Collectively, these data suggest that Tc 80 proteinase could be classified as a cysteine proteinase, despite its insensitivity to E-64. However, some trypanosomatid enzymes exhibit inhibition patterns of cysteine/serine proteinases with an usually low sensitivity to E-64 and PMSF [18,42,47], and their classifications are controversial. It is expected that the amino acid sequences and catalytic mechanisms of such proteinases will clarify their classification.

Tc 80 proteinase was purified to homogeneity by a four-step chromatography procedure using a combination of anionic and hydrophobic columns. The high purification factor suggests that the enzyme is present in low concentrations in *T. cruzi* axenic culture forms. The twofold increase in total enzyme activity after DEAE-Sepharose chromatography might be explained by the elimination of a natural inhibitor that may regulate the enzyme. To ascertain whether Tc 80 proteinase is synthesized by *T. cruzi* or is incorporated from the extracellular medium, epimastigote forms were labelled with [<sup>35</sup>S]methionine in a system free of other cells. Autoradiography of the purified enzyme from radiolabelled parasite demonstrated that the protein is a *T. cruzi* product. The purified enzyme showed high substrate specificity, since it did not degrade synthetic substrates that have Arg, Phe, Lys or Tyr at the P1 position, which are currently used to study other parasite proteolytic activities. In addition, the specific activity of Tc 80 proteinase was dramatically reduced when the assay was performed with *N*-Suc-Gly-Pro-AMC rather than *N*-Suc-Gly-Pro-Leu-Gly-Pro-AMC. These results strongly suggest that both the presence of Pro at the P1 position and the sequence Gly-Pro-Leu at the P5-P4-P3 position are important in Tc 80 proteinase cleavage-site formation.

To determine whether the purified Tc 80 proteinase cleaves host extracellular matrix proteins such as collagen types I and IV and laminin, these molecules were radiolabelled and incubated with the enzyme. Under the conditions of these experiments, human collagen I appears to be more susceptible to cleavage than type IV: the higher-molecular-mass bands of collagen type I were rapidly hydrolysed after 2 h of incubation in comparison with those of collagen type IV. Furthermore the enzyme produced six lower-molecular-mass bands from collagen type IV and only two lower-molecular-mass bands from collagen type I after 12 h of incubation. These results indicate that collagen type I may be degraded into smaller peptides not detected in the gel. These different susceptibilities to Tc 80 proteinase could be explained by differences in the structure and/or number of cleavage sites. As for fluorogenic synthetic substrates, Tc 80 proteinase has high specificity for protein substrates. Taken together, these data show that *T. cruzi* contains a specific proteinase that degrades host collagen types I and IV at neutral pH.

Collagenases are the only proteases known to be capable of hydrolysing the helical part of native collagens under physiological conditions. Owing to the very specific triple-helical structure, collagens are extremely resistant to degradation by non-specific proteinases [48]. Collagen can also be cleaved by matrix metalloproteinases, which are classified into different subfamilies according to their specificity [49]. Tc 80 proteinase shows differences from *bona fide* collagenases. Usually, tissue collagenases hydrolyse peptide bonds between glycine and isoleucine (or leucine) residues, and bacterial collagenases hydrolyse peptide bonds preceding the glycine residue in Xaa-Gly sequences [50]. In contrast, Tc80 proteinase cleaves the substrate *N*-Suc-Gly-Pro-Leu-Gly-Pro-AMC only between proline and AMC, i.e. on the carboxy side of the proline residue. With such a specificity, Tc 80 proteinase could be considered to be a prolyl endopeptidase. Furthermore Tc 80 proteinase activity is sensitive to some cysteine and serine proteinase inhibitors, whereas collagenases that are metalloproteinases are specifically inhibited by phosphoramidon, EDTA and 1,10-phenanthroline [43], which is not observed for Tc 80 proteinase. In spite of the differences between Tc 80 proteinase and collagenases, they also show some similarities, such as optimal pH and substrate specificity. Like other collagenases [8], Tc 80 proteinase hydrolyses not only purified but also native collagens. Although an internal peptide sequence derived from Tc 80 proteinase shows similarity to a

metalloproteinase from *C. fasciculata* [37], a more extensive amino acid sequence is necessary to compare Tc 80 proteinase of *T. cruzi* with collagenases from other sources.

The activity of Tc 80 proteinase on native collagen was assayed on rat mesentery, which contains a large amount of collagen type I [39]. Exposure of rat mesentery to purified Tc 80 proteinase resulted in specific hydrolysis of the collagen fibres, since no hydrolysis was observed with the Tos-Lys-CH<sub>2</sub>Cl-inactivated enzyme. Similar results were obtained with *C. histolyticum* collagenase, a metalloproteinase with potent hydrolytic activity towards native collagen [51]. These observations demonstrate that this *T. cruzi* proteinase recognizes native collagen type I. As this degradation was observed at neutral pH, it was important to establish whether Tc 80 proteinase could be secreted by the different forms of *T. cruzi*. Tc 80 proteinase activity was clearly identified in culture supernatants of the *T. cruzi* forms infective to mammalian host cells, in contrast with the other proteinase activities (e.g. cruzipain). Such a specificity and time-dependent release of Tc 80 proteinase probably reflects a secretion process rather than release as the result of cell damage.

Dissemination of Chagas' disease is highly dependent on the ability of parasites to migrate through the extracellular matrix to reach certain cell types [52–56]. The first step in this phenomenon is the specific interaction of the parasites with components of the extracellular matrix such as collagen, laminin, fibronectin and heparin. Binding of specific *T. cruzi* molecules to collagen [10], fibronectin [12], laminin [13] and heparin [14] has been described as an important mechanism to ensure host-cell infection by trypomastigote forms. Furthermore, as in cancer metastasis [57], proteolytic enzyme activities have also been implicated in parasite infections [58–62] by their role in degrading proteins of the extracellular matrix. The ability of Tc 80 proteinase to mediate specific degradation of collagen types I and IV raises some questions about its possible role in the *T. cruzi*-host-cell relationship. The enzyme may facilitate *T. cruzi* migration through both the interstitial matrices of internal organs and skin, which contain large amounts of collagen type I [39], and basement membrane, which contains collagen type IV [63]. The migration of the parasite through the extracellular matrix may allow it access to cells in virtually any part of the host.

Trypomastigotes are the main *T. cruzi* form capable of both entering cells and migrating through the tissues of their mammalian hosts [2]. This feature of *T. cruzi* biology may explain why the highest level of Tc 80 proteinase activity is observed in trypomastigotes. Although amastigotes and epimastigotes are not currently considered to be infective forms, they have been described in the extracellular space of parasitized muscle tissue [64], in the blood of infected mice [65] and in infected tissue culture [2,66]. In addition, it has been demonstrated that amastigote forms can maintain an infective cycle in host cells *in vitro* and are infective for mice [26]. All these biological features of the *T. cruzi* life cycle would require synthesis of Tc 80 proteinase with high specificity for collagen.

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