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Cleavage of trypanosome surface glycoproteins by alkaline trypsin-like enzyme(s) in the midgut of *Glossina morsitans*

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Abstract

EP and GPEET procyclin, the major surface glycoproteins of procyclic forms of *Trypanosoma brucei*, are truncated by proteases in the midgut of the tsetse fly *Glossina morsitans morsitans*. We show that soluble extracts from the midguts of teneral flies contain trypsin-like enzymes that cleave the N-terminal domains from living culture-derived parasites. The same extract shows little activity against a variant surface glycoprotein on living bloodstream form *T. brucei* (MITat 1.2) and none against glutamic acid/alanine-rich protein, a major surface glycoprotein of *Trypanosoma congolense* insect forms although both these proteins contain potential trypsin cleavage sites. Gel filtration of tsetse midgut extract revealed three peaks of tryptic activity against procyclins. Trypsin alone would be sufficient to account for the cleavage of GPEET at a single arginine residue in the fly. In contrast, the processing of EP at multiple sites would require additional enzymes that might only be induced or activated during feeding or infection. Unexpectedly, the pH optima for both the procyclin cleavage reaction and digestion of the trypsin-specific synthetic substrate Chromozym-TRY were extremely alkaline (pH 10). Direct measurements were made of the pH within different compartments of the tsetse digestive tract. We conclude that the gut pH of teneral flies, from the proventriculus to the hindgut, is alkaline, in contradiction to previous measurements indicating that it was mildly acidic. When tsetse flies were analysed 48 h after their first bloodmeal, a pH gradient from the proventriculus (pH 10.6 ± 0.6) to the posterior midgut (pH 7.9 ± 0.4) was observed.

Keywords: Protease; Trypsin; Procyclin; Trypanosome; Tsetse

1. Introduction

Transmission of the protozoan parasite *Trypanosoma brucei* by its insect vector, the tsetse fly, from one mammalian host to the next is a surprisingly inefficient process. Life in the digestive tract of a blood-sucking insect presents special challenges for the parasite, because it must first differentiate and establish an infection in the midgut, an environment that is extremely rich in proteases. Of the trypanosomes taken up in the course of a blood meal, only a minority survive and differentiate to an insect-adapted form, the procyclic form, that is capable of proliferating and colonising the ectoperitrophic space between the peritrophic matrix and the gut epithelium. This phase, which constitutes the establishment of infection, is sometimes, but not invariably, followed by a second phase known as maturation. This entails several further rounds of differentiation and proliferation as the parasite progresses to the salivary glands, finally giving rise to the metacyclic form that can be transmitted to the next mammalian host.

The effect of proteases on *T. brucei* depends on the stage of the life cycle. In vitro, long slender bloodstream forms, the proliferative forms in the mammalian host, are killed by

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exposure to trypsin or thermolysin, whereas the non-proliferative short stumpy forms are more resilient and respond by differentiating to the procyclic form (Sbicego et al., 1999). Pronase, which is actually a mixture of proteolytic enzymes, also induces differentiation, but thrombin and pepsin, two enzymes with more restricted specificities, do not. Proteases are not essential for the differentiation process, however, at least in culture. Exposing trypanosomes to acid stress also triggers the differentiation of stumpy bloodstream forms and kills long slender forms (Rolin et al., 1998). Citrate or *cis*-aconitate in the millimolar range are able to induce the differentiation of both slender and stumpy bloodstream forms without damaging the cells (Brun and Schoenenberger, 1981), but these are unlikely to be the natural triggers as they are present at extremely low levels in the fly (Hunt et al., 1994).

In addition to extensive morphological and metabolic changes, the differentiation of the bloodstream form to the procyclic form is marked by the shedding of the variant surface glycoprotein (VSG) coat characteristic of bloodstream forms and its replacement by procyclins (Roditi et al., 1989; Ziegelbauer et al., 1990). The procyclins comprise a small family of GPI-anchored proteins with basic N-terminal domains and internal dipeptide (EP) or pentapeptide (GPEET) repeats (reviewed in Roditi et al., 1998). The repeats from both classes of procyclin are predicted to be rod-like structures capped by the N-terminal domains, leading to the 'Spiny Norman' model in which the trypanosome surface resembles the bristles of a hedgehog (Pearson, 2001). At the onset of differentiation the entire procyclin repertoire can be detected at similar levels (Vassella et al., 2001), but within 24 h this is followed by a surge in GPEET expression and a drop in the levels of all EP procyclins. After a few days, however, GPEET is down-regulated completely and replaced by two glycosylated isoforms of EP procyclin, EP1 and EP3 (Acosta-Serrano et al., 2001; Vassella et al., 2001). In the tsetse fly, the repression of GPEET coincides with the time that the trypanosomes migrate around the peritrophic matrix and establish an infection in the ectoperitophic space, but it is not known whether these two events are connected. The developmental regulation of GPEET expression in the fly is governed by sequences in the 3′ untranslated region of the messenger RNA (Vassella et al., 2000). In culture, the same region also acts as a sensor of exogenous glycerol and oxygen (Vassella et al., 2000), and might also be involved in glucose sensing (Morris et al., 2002). In contrast to procyclic forms cultured in vitro, procyclins from trypanosomes isolated directly from tsetse midguts are proteolytically cleaved within their N-terminal domains (Acosta-Serrano et al., 1999, 2001). GPEET is cleaved at a single site downstream of an arginine residue, while EP procyclins are cleaved at multiple sites downstream of neutral or acidic residues, sometimes resulting in the removal of the carbohydrate moiety.

There are many proteolytic enzymes in the midgut that might be involved in trimming procyclins. Seven proteolytic enzymes have been partially purified and characterised from the midgut of *Glossina morsitans morsitans*, namely trypsin (19.8 kDa) and a trypsin-like enzyme (22.8 kDa), carboxypeptidase A and B, two aminopeptidases and a chymotrypsin-like enzyme (Cheeseman and Gooding, 1985). An additional trypsin-like enzyme (25.5 kDa) has been detected in *Glossina palpalis palpalis*, but no chymotrypsin-like activity. An independent study of *G. palpalis palpalis* also revealed at least three trypsin activities (Van Den Abbeele and Decleir, 1992) and a trypsin-like enzyme was found to be a component of a lectin complex isolated from midgut extracts of *Glossina longipennis* (Osir et al., 1995). The activities of both chymotrypsin (Yan et al., 2001, 2002) and trypsin increase in response to a bloodmeal (Yan et al., 2001, 2002). In addition to these biochemical studies, the genes for *G. morsitans* chymotrypsin (*Gsp1*), trypsin (*Gsp2*) cathepsin B (*GmCatB*), a zinc metalloprotease (*GmZmp*) and a zinc carboxypeptidase (*GmZcp*) have recently been isolated from a midgut cDNA library (Yan et al., 2001, 2002). In each case, these encode polypeptides with a characteristic preproenzyme structure that is compatible with their secretion and activation. Analysis of the transcripts from these genes showed that they are not globally regulated in response to a blood meal. Transcripts for *Gsp1*, *Gsp2* and *GmCatB* are expressed at high levels in teneral flies, while *GmZcp* and *Gm Zmp* are up-regulated in response to a blood meal (Yan et al., 2001, 2002). Intriguingly, the levels of *GmCatB* and *GmZmp* mRNA increase still further in tsetse flies that have midgut infections of *T. brucei* (Yan et al., 2002).

To gain more insight into the interaction between the parasite and its vector, we have characterised midgut proteases that are capable of cleaving EP and GPEET on the surface of living trypanosomes. Since the reaction occurred most efficiently under highly alkaline conditions, we measured the pH of the gut of teneral flies and flies that had received a blood meal 2 or 3 days previously. Contrary to the widely accepted view that the gut is mildly acidic (Wigglesworth, 1929), we found it to be alkaline.

2. Materials and methods

2.1. Trypanosomes and tsetse flies

Procyclic forms of *T. brucei brucei* strain 427 (Cross and Manning, 1973) were cultured at 27 °C in SDM-79 medium (Brun and Schoenenberger, 1979) supplemented with 5% heat-inactivated foetal bovine serum (FBS). Procyclic forms of *Trypanosoma congolense* STIB 745 were cultured at 27 °C in SM medium containing 20% FBS (Cunningham, 1977). Bloodstream form trypanosomes (MITat 1.2; 221) were cultured at 37 °C/5% CO_2 as described (Hesse et al., 1995). *Glossina morsitans morsitans* was obtained from

the breeding colonies at the International Atomic Energy Agency, Vienna (for the extracts) or from the Institute of Tropical Medicine, Antwerp (for pH measurements).

2.2. Antibodies

The monoclonal antibody TBRP1/247 (mAb 247) specifically recognises the dipeptide repeats of EP procyclin, whereas mAb 346 reacts with an epitope within the first 20 N-terminal amino acids of the EP procyclin polypeptides (Richardson et al., 1986, 1988). The anti-GPEET mAb 5H3 recognises the mature phosphorylated form of GPEET (Bütikofer et al., 1999). K1 is a polyclonal rabbit antiserum raised against a synthetic peptide $(GPEET)_3C$ conjugated to keyhole limpet haemocyanin (Ruepp et al., 1997). Rabbit polyclonal antisera raised against a recombinant form of *T. congolense* glutamic acid/alanine-rich protein (GARP) were generously provided by D. Jefferies and J.D. Barry (Wellcome Unit of Molecular Parasitology, Glasgow, UK). Anti-VSG (MITat 1.2) polyclonal antibodies were a gift from M.L. Cardoso de Almeida (Sao Paulo, Brazil). Peroxidase-conjugated secondary antibodies were purchased from Sigma or Dako.

2.3. Soluble midgut extract(s)

Teneral flies were killed with ether and midguts were dissected in a drop of phosphate-buffered saline (PBS) pH 7.4. Batches of 10 midguts in 50 μl PBS were frozen in liquid nitrogen and stored at −70 °C. The midguts were thawed in 100 μl PBS on ice and vigorously vortexed (three times for 30 s) prior to centrifugation in an Eppendorf table top centrifuge at full speed at 4 °C for 5 min. The supernatant was cleared by ultracentrifugation (Centrikon, Ti 50 rotor, 35 000 rpm) at 4 °C for 15 min. The resulting soluble midgut extract (approximately 1 μg protein per μl, Micro-BCA test, Pierce) was used for protease analysis. When used for HPLC analysis, the extract was further purified by centrifugal filtration (Ultrafree-MC, Amicon, 0.22 μm filter unit), at full speed in an Eppendorf table-top centrifuge at 4 °C for 5 min.

2.4. Procyclin cleavage reaction and Western blot analysis

The standard procyclin cleavage reaction was performed using $0.5–1.0 \times 10^7$ living procyclic form trypanosomes that were previously washed twice with PBS (3000 rpm for 5 min). Cells were incubated with 0.2 midgut equivalents of extract in 100 μl PBS (pH 7.4 or 8.0) at 30 °C for 1 h. After the treatment with midgut extract, the parasites were washed twice with 0.5 ml PBS prior to further analysis. Cells at a density of 5×10^5 cells μl^{-1} were lysed in standard Laemmli buffer at 100 °C in a heating block for 3 min. Proteins from 2.5×10^6 cells were separated by SDS–PAGE (12% gel) and transferred to Immobilon-P membrane (Millipore) using a semi-dry Western blot transfer system. Both the amount of protein loaded and the efficiency of transfer were checked by Ponceau Red staining of the membrane. Unspecific protein interactions were blocked with 10 mM Tris–HCl (pH 7.4), 150 mM NaCl (Tris-buffered saline, TBS)/5% defatted milk powder at room temperature for 1 h. Diluted first antibodies (all 1:2500 in TBS/5% defatted milk powder) were applied either overnight at 4 °C or for 1 h at room temperature. Membranes were washed three times for 5 min in TBS/0.05% Tween. Peroxidase-conjugated secondary antibodies (Dako) were diluted 1:5000 and applied for another hour at room temperature. Peroxidase activity was detected using a chemiluminescent substrate according to the manufacturer's instructions (SuperSignal West Pico, Pierce).

2.5. Protease inhibitors

Midgut extract was preincubated with individual protease inhibitors for 5 min at room temperature. Procyclin cleavage was assayed with 5×10^6 living trypanosomes per reaction. The following serine proteinase inhibitors were used: benzamidine (final concentration, 10 mM), phenylmethanesulphonyl fluoride (PMSF, 10 mM), aprotinin (50 μg ml^{-1}), leupeptin (10 μg ml^{-1}), antipain (10 μg ml^{-1}), chymostatin (10 μg ml^{-1}); aspartyl proteinase inhibitor: pepstatin A (10 μg ml^{-1}); metallo-proteinase inhibitors: ethylenediamine tetraacetate (EDTA, 10 mM) and ethyleneglycol-bis-(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA, 10 mM). The Complete Mini protease inhibitor cocktail (Roche) was used as recommended by the supplier.

2.6. Chromogenic enzyme assay

Trypsin activity was assayed using the chromogenic substrate carbobenzoxy-val-gly-arg-4-nitranilide acetate (Chromozym-TRY, Roche). Total or fractionated extracts were assayed with 100 μM Chromozym-TRY in 200 μl 50 mM Tris–HCl (pH 8) at 30 °C. The increase in absorbance at 410 nm was monitored using a SPECTRAmax microplate spectrophotometer and SOFTmax PRO software (Molecular Device Corp.).

2.7. Matrix-assisted laser desorption/ionisation-time of flight mass spectrometry (MALDI-TOF MS) analysis of procyclins

Analysis of procyclin cleavage by mass spectrometry and mapping of the cleavage sites was performed as described before (Acosta-Serrano et al., 1999, 2001).

2.8. Gel filtration analysis of tsetse midgut extract

Gel filtration of 10 midgut equivalents was performed using a SMART Superdex 200 PC 3.2/30 precision column (Amersham Pharmacia Biotech) according to

the manufacturer's instructions (running buffer, 50 mM Tris–HCl (pH 8), 60 mM KCl, 10% glycerol). Thirty fractions of 40 μl were collected and stored at −20 °C until use. Six microlitres of each fraction was assayed with Chromozym-TRY as described. The OD_{410} values were measured after 10 min reaction time. Alternatively, procyclin cleavage was determined using living trypanosomes and immunoblotting as described above.

2.9. pH and enzyme activity

Equal numbers of living trypanosomes were incubated in a mock reaction or with 10^{-2}, 10^{-1} or 1 midgut equivalent at 30°C for 1 h, but this time the reaction buffer (PBS) was replaced by sodium phosphate buffers (150 mM) at pH 4, 6, 8 and 10. Following incubation with 0.2 midgut equivalents, the extent of procyclin cleavage was monitored by Western blot analysis. To monitor the effect of pH on general tryptic activity, individual fractions from the gel filtration column described above were diluted (fraction 4, 1:3; fractions 21 and 27, both 1:6) and 3 μl of each dilution was analysed using Chromozym-TRY in 150 mM sodium phosphate buffers at different pH values.

2.10. Measurement of pH in the alimentary tract of tsetse flies

The complete digestive tract of male *G. morsitans morsitans*, from the proventriculus (plus oesophagus) to the hindgut, was dissected in a small drop of saline. The intact digestive tract was immediately placed under paraffin oil. Piercing the gut wall at different positions (proventriculus, anterior midgut, posterior midgut and hindgut) caused the release of small droplets that remained separated from each other by the paraffin oil. The pH was determined with a glass pH microelectrode (100 μm diameter, Unisense A/S, Aarhus, Denmark) in combination with a hand-drawn glass reference electrode connected to a high impedance millivoltmeter. After each measurement, the electrodes were washed and re-calibrated with pH standards (pH 4.00, 6.88, 9.23 and 12.63). Two pH measurements were made on each droplet, with the electrodes being recalibrated between measurements. Dissections were performed on teneral flies or on flies that had received a blood meal on the ears of a healthy rabbit 48 or 72 h earlier.

3. Results

3.1. Cleavage of EP and GPEET by serine proteases

Procyclic culture form trypanosomes were incubated with different amounts of midgut extract from *G. morsitans morsitans*. Inspection of the cells by light microscopy confirmed that the parasites remained viable during the experiment and showed no alterations in motility and morphology when compared to cells treated with buffer alone. The effects on EP and GPEET were analysed by immunoblotting using antibodies directed against different domains of EP and GPEET. Incubation with tsetse extracts caused a decrease in the M_r of both procyclins (Fig. 1A,C) and a loss of reactivity with a mAb that recognises an epitope within the first twenty amino acids of all EP isoforms (Fig. 1D), indicating that the decrease was due to the removal of a protein moiety. In contrast to the mature proteins, the GPEET precursor was unaffected (Fig. 1B), confirming that the cells remained intact during the incubation.

To learn more about the nature of the enzyme(s) involved, the cleavage reaction was performed in the presence of different protease inhibitors. Procyclin cleavage could be inhibited completely by the serine protease inhibitors shown in Fig. 2A, as well as by diisopropyl-fluorophosphate (DFP) and soybean trypsin inhibitor (data not shown). In contrast, neither the aspartyl proteinase

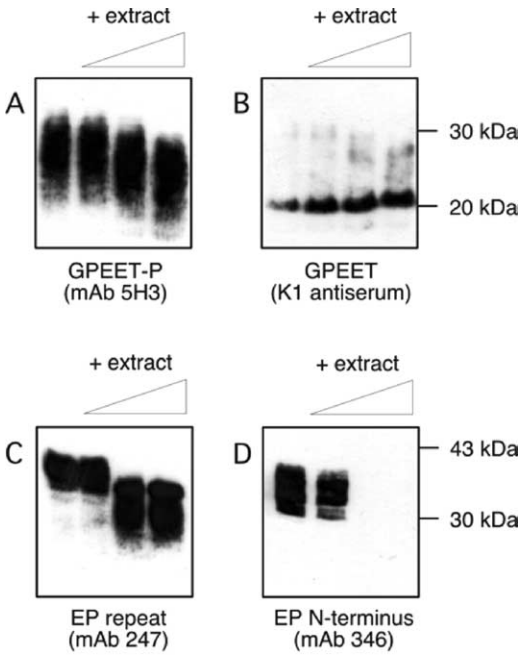


Fig. 1. Cleavage of GPEET and EP procyclin after incubation of living trypanosomes with soluble midgut extract. Washed procyclic trypanosomes (10^7 cells) were incubated for 1 h at 30 °C with increasing amounts of soluble midgut extract from teneral flies corresponding to 0 (control incubation), 0.01, 0.1 and 1 midgut equivalents. Protein extracts from 2×10^6 cells were loaded for each lane. The positions of the molecular weight markers are indicated on the right. Western blot analysis was performed using different antibodies directed against procyclin. The monoclonal antibody (mAb) 5H3 is directed against the phosphorylated repeat region of mature GPEET procyclin (GPEET-P) (Bütikofer et al., 1999), whereas the polyclonal K1 antiserum, which was raised against a synthetic peptide (Ruepp et al., 1997), predominantly recognises the unphosphorylated GPEET precursor (Bütikofer et al., 1997, 2002b). The monoclonal antibody TBRP1/247 (mAb 247) reacts with the extensive Glu–Pro dipeptide repeat region of EP procyclins (Richardson et al., 1988). The monoclonal antibody TBRP1/346 (mAb 346) recognises an epitope within the first 20 amino acids of the N-terminal domain of all EP procyclin isoforms (Richardson et al., 1988).

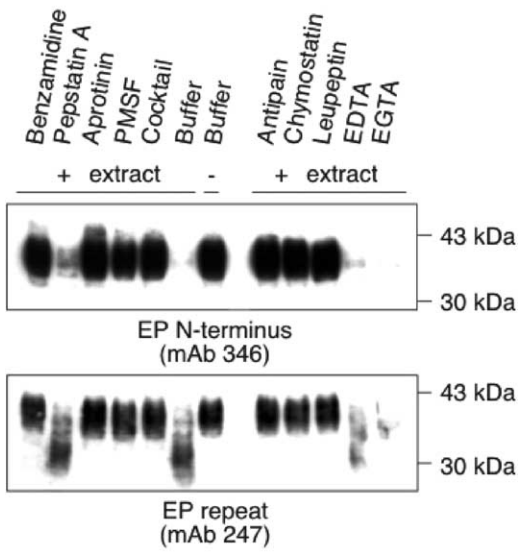


Fig. 2. Serine protease inhibitors prevent procyclin cleavage. Tsetse midgut extract was preincubated with different protease inhibitors before being assayed in a standardised reaction (see Section 2). Aliquots from the same reaction were analysed by immunoblotting with the anti-EP mAbs 346 and 247.

inhibitor pepstatin A nor the metalloproteinase inhibitors EDTA and EGTA prevented cleavage. These results are consistent with the involvement of trypsin or trypsin-like enzymes in the cleavage reaction. These might act directly on the procyclins themselves or have a secondary effect in activating the protease, for example by removing the propeptide. The efficiency of cleavage was reduced by addition of as little as 0.5% foetal bovine serum or 5 mg ml^{-1} bovine serum albumin to the assay (data not shown), suggesting that other proteins can act as competitive substrates and that the protease activity is not necessarily specific for procyclins.

3.2. Mapping the cleavage sites

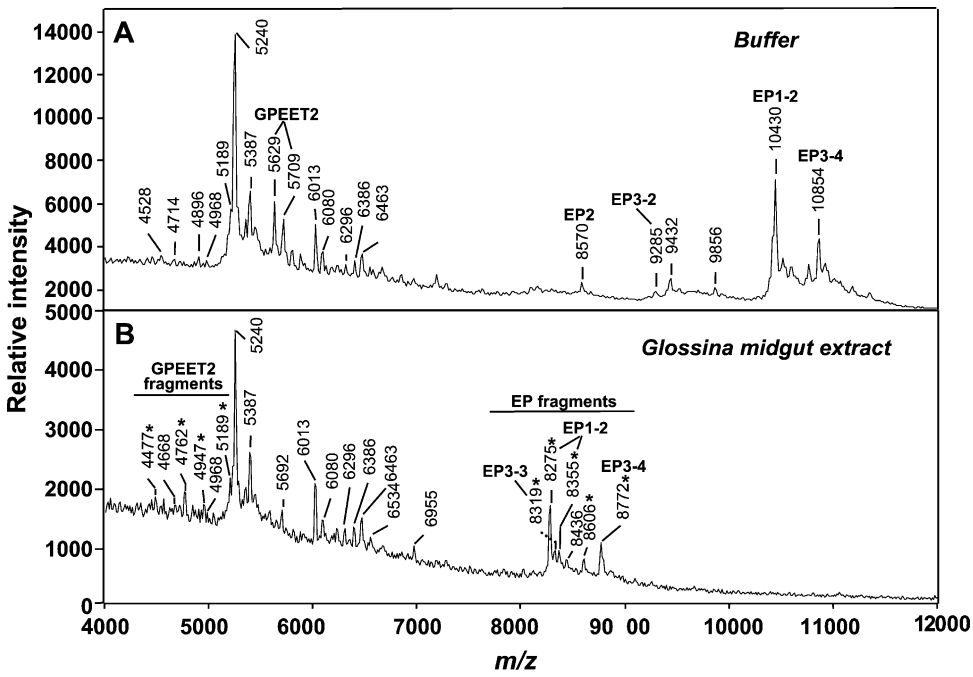
The combination of sequence information and MALDI-TOF mass spectrometry has allowed the cleavage sites of procyclin from tsetse-derived trypanosomes to be mapped precisely (Acosta-Serrano et al., 2001). Using the same technique and the same fly-transmissible strain of trypanosomes (AnTat 1.1) we mapped the cleavage sites after treating trypanosomes with midgut extracts. For these experiments, the trypanosomes were cultured under conditions where both EP and GPEET are expressed. The procyclins in AnTat 1.1 differ slightly from those in other strains, principally in the lengths of the di- and pentapeptide repeats, and have been renumbered to denote this fact (e.g. GPEET2). Fig. 3 shows the mass spectra obtained after mock treatment (Fig. 3A) or treatment with midgut extract (Fig. 3B). Individual procyclin species are represented by peaks (*m/z*). The cleavage sites obtained with midgut extracts are depicted in Fig. 3C. In contrast to the single cleavage site at Arg-11 in tsetse-derived trypanosomes, GPEET2 was cleaved at four typical trypsin sites: Lys-4, Lys-5, Lys-9 and Arg-11. The same fragmentation pattern was also detected for GPEET from strain 427 (data not shown). All detectable EP procyclin species (EP1-2, EP3-3, and EP3-4) were cleaved by the tsetse extract (Fig. 6A,B). The EP isotypes EP1-2 and EP3-3 were cleaved at Lys-19 and Lys-22, whereas EP3-4 was only cleaved at Lys-22 (Fig. 6C). All masses corresponding to EP procyclins were in agreement with them still having the glycan modification after proteolysis. Taken together with the inhibitor data, these results are compatible with procyclin cleavage by trypsin(-like) enzymes. The partial digestion of GPEET2 observed in vitro could be a consequence of the relatively short incubation time or the amount of enzyme, but this cannot account for the differences in the EP cleavage patterns in vivo and in vitro. Instead, the more complex digestion patterns observed with fly-derived EP procyclins may reflect the induction of additional proteases in response to a blood meal or to infection.

3.3. Specificity of procyclin cleavage

To test if other trypanosome surface coat molecules were similarly susceptible to midgut proteases, we incubated bloodstream forms of *T. brucei* or procyclic culture forms of *T. congolense* under the same conditions as in Fig. 1. As shown in Fig. 4 (left panel), the VSG of bloodstream form trypanosomes was only partially cleaved. Unexpectedly, GARP, a major surface glycoprotein of *T. congolense* procyclic culture forms, was completely resistant to the midgut extract (Fig. 4, right panel), despite containing several sites that are cleaved when the purified protein is incubated with bovine trypsin (Beecroft et al., 1993; Bütikofer et al., 2002a). Thus, whether by accident or design, membrane-anchored EP and GPEET appear to be particularly susceptible to cleavage.

3.4. Gel filtration analysis

To obtain information on the size of the protease, soluble midgut protein extract was fractionated by HPLC gel filtration. Fractions were incubated with living trypanosomes and procyclin cleavage was assayed by immunoblot analysis (Fig. 5A). In addition, trypsin activity was measured with the synthetic substrate Chromozym-TRY. This assay revealed three separate peaks of activity (fractions 3-7, 21-24, 26-27) that were also able to cleave the N-terminus of EP procyclin (Fig. 5B) as well as GPEET (data not shown). The largest complex (>350 kDa) co-migrated with the bulk of the protein and had the lowest specific activity. Fractions 23 and 26 corresponded to proteins with M_r ~20 kDa and ~13 kDa, respectively. These findings indicate that there are at least three complexes or molecules that differ in molecular mass and



C

EP1-2 AEGPEDKGLTKGGKGKGEKGTKVSADDTNGTDPDP(EP)$_{25}$G

EP3-4 AEGPEDKGLTKGGKGKGGKGTKVSDDDTNGTDPDP(EP)$_{27}$G

EP3-3 AEGPEDKGLTKGGKGKGGKGTKVSDDDTNGTDPDP(EP)$_{25}$G

GPEET2 VIVKGGKGKEREDGPEEPEET(GPEET)$_5$EPEPEPG

Fig. 3. Mapping the procyclin cleavage sites. Negative ion mass spectra from procyclic form trypanosomes (AnTat 1.1) that were incubated with buffer alone (A) or with midgut extract (B). Freeze- dried cells were extracted with n-butanol and incubated with 48% aqueous hydrofluoric acid, in order to remove the heterogeneous GPI anchors of procyclins. Samples were analysed by matrix-assisted laser desorption/ionisation-time of flight mass spectrometry as described previously (Acosta-Serrano et al., 1999, 2001). Asterisks mark proteolytic fragments of procyclins. The ion at *m/z* 5079 represents GPEET2 with one phosphate residue. Peaks at *m/z* 5240 and *m/z* 6000–6500 are not derived from procyclins and are present in both treated and untreated samples. (C) Polypeptide sequences of mature EP procyclins (only EP1-2, EP3-3, and EP3-4) and GPEET2 procyclin from AnTat 1.1. Arrowheads indicate the positions of the cleavage sites. Underlined amino acids indicate the glycosylation sites (Asn-29) in the EP isoforms.

in their contribution to overall trypsin activity, all of which are able to cleave procyclins.

3.5. Midgut trypsin activity is maximal at alkaline pH

Although the tsetse midgut is allegedly slightly acidic (Wigglesworth, 1929), assays of tsetse tryptic activity are normally performed in moderately alkaline buffers in the range pH 7.4–8.0 (Cheeseman and Gooding, 1985; Osir et al., 1995; Yan et al., 2001). To ascertain the pH optima of the tsetse proteases characterised above, assays were performed with Chromozym-TRY and total midgut extracts or the three tryptic activities separated by gel filtration (Fig. 6). Assays were performed in phosphate buffers at the appropriate pH ranging from 4.0 to 10.0. A control reaction without tsetse proteins was always run in parallel, enabling us to exclude spontaneous hydrolysis of the substrate at the more extreme pH values. Unexpectedly, both the unfractionated and fractionated tryptic activities declined significantly at pH values ≤ 6 and were maximal at pH 8–10. Bovine trypsin, which was included as a positive control, was most active at pH 8 (and marginally less so at higher pH values).

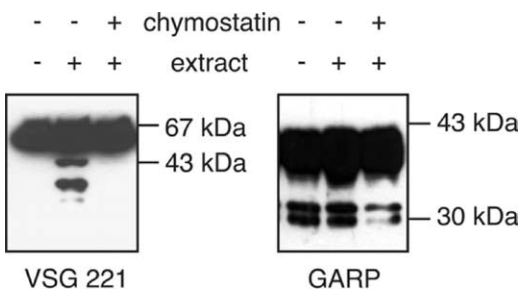


Fig. 4. Effect of midgut proteases on trypanosomes expressing variant surface glycoprotein (VSG 221) or glutamic acid/alanine-rich protein (GARP). Cleavage of surface glycoproteins was assayed as described in the legend to Fig. 1. In parallel, reactions were performed in the presence of the serine protease inhibitor chymostatin. (A) *Trypanosoma brucei* bloodstream form trypanosomes. (B) *Trypanosoma congolense* procyclic forms.

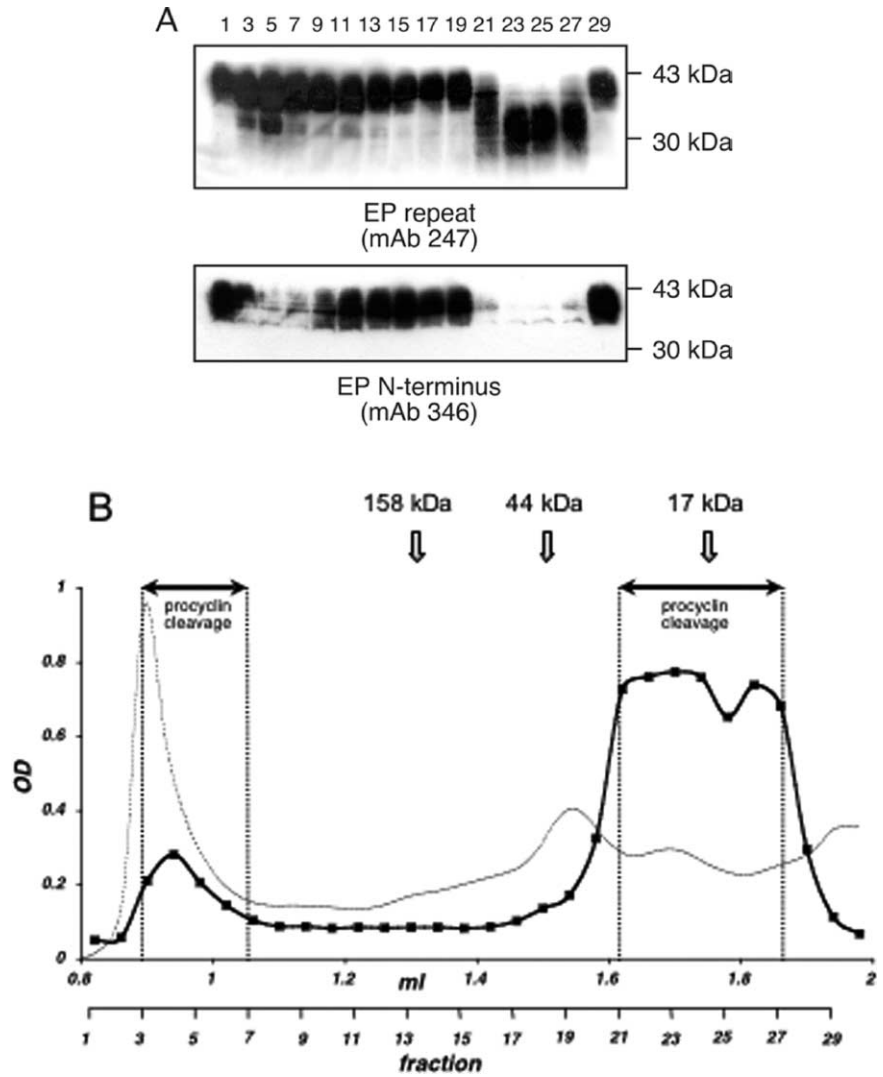


Fig. 5. Gel filtration analysis reveals three active fractions. Soluble midgut extract was separated by gel filtration (see Section 2). Alternate fractions were assayed for procyclin cleavage activity as described in the legend to Fig. 1 (A) or for tryptic activity using the synthetic substrate Chromozym-TRY at pH 8.0 (B). The thin line shows the amount of protein (absorbance at OD_{280}) and the thick line the initial velocity of enzyme activity (absorbance at OD_{410}). The *y*-axis applies to both sets of values. Under the conditions used, the midgut of a teneral fly had an activity equivalent to approximately 25 *N*(α)-benzoyl-L-arginine ethyl ester (BAEE) units of trypsin from bovine pancreas (Sigma, T8642).

3.6. The alimentary tract of the tsetse fly is alkaline

The pH measurements made by Wigglesworth (Wigglesworth, 1929), indicating that the tsetse midgut has a pH ~ 6.5, were based on the colour changes of indicator dyes after feeding flies with a mixture of sheep serum and saline. To obtain more precise values, flies were dissected and the pH values of different domains of the digestive tract were determined using microelectrodes. Surprisingly, these revealed that the proventriculus, anterior and posterior midguts and hindguts of teneral flies were all alkaline, with average values in the range pH 9–9.5 (Table 1). Two to three days after a blood meal, the pH of the posterior midgut decreased to pH 8, and that of the anterior midgut to pH 8.5, while the proventriculus remained extremely alkaline (average value pH 10.2). From these results we conclude that trypsin-like enzymes would be highly active, irrespective of whether a fly has taken a blood meal or not.

4. Discussion

Proteases that are capable of clipping the procyclin N-termini from living trypanosomes are readily extracted from the midguts of tsetse flies. These enzymes are present in teneral flies that have never been exposed to a blood meal or to infection, arguing against a specific role for them in response to infection by trypanosomes. MALDI-TOF MS analysis of procyclins exposed to midgut extracts revealed that digestion occurred exclusively at arginine and lysine residues, compatible with cleavage by trypsin(s). In keeping with earlier findings that there are several different tryptic

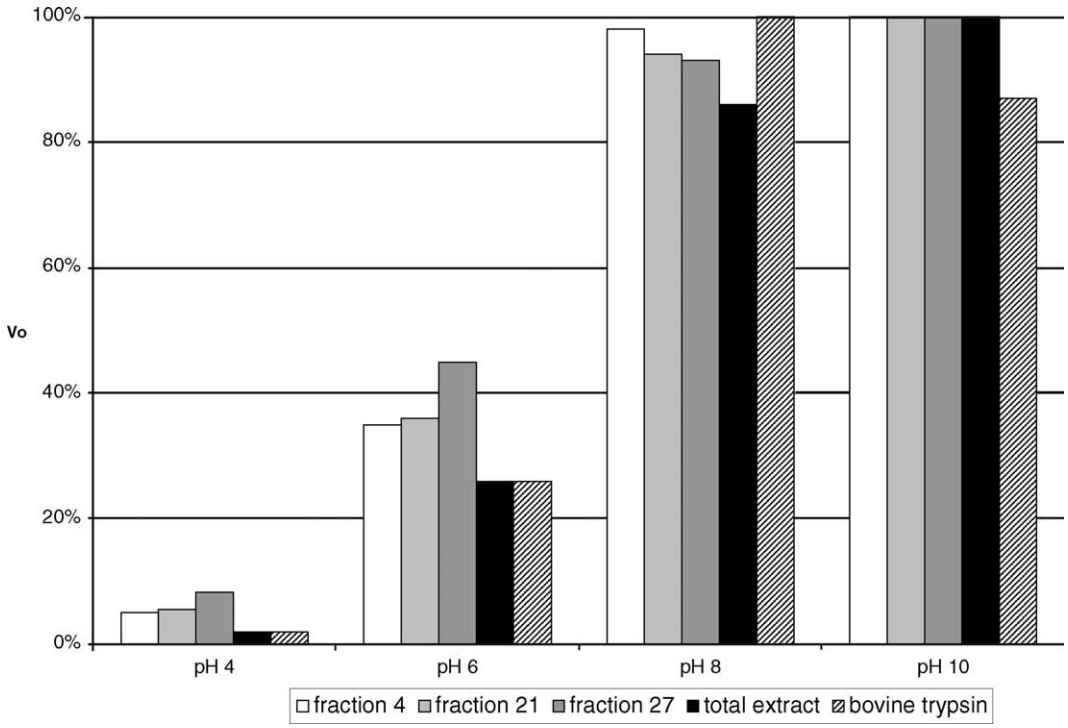


Fig. 6. Activity of tsetse protease(s) at different pH values. Total midgut extract, individual fractions after gel filtration and trypsin from bovine pancreas were assayed in phosphate buffers of different pH using Chromozym-TRY. The reaction kinetics were followed at one minute intervals over a period of 20 min. The initial velocity V_0 was calculated for each reaction. Values are given as a percentage of maximum activity.

activities in the tsetse midgut (Osir et al., 1995; Van Den Abbeele and Decleir, 1992), three procyclin-cleaving activities could be resolved by gel filtration. A high molecular mass form ($M_r > 350$ kDa) is presumably a complex of multiple polypeptides, while the ~20 kDa species might be a monomer of the enzyme. Although this is somewhat smaller than the mature polypeptide of 228 amino acids predicted for Gsp 2 (24 kDa; Yan et al., 2001), it is very similar in size to the trypsin characterised by Cheeseman and Gooding (1985). The smallest species of ~13 kDa could conceivably be a proteolytic fragment of the enzyme itself. Tryptic enzymes alone would be sufficient to account for the cleavage of GPEET at a single arginine residue in the fly (Acosta-Serrano et al., 2001). In contrast, the processing of EP in vivo seems to entail additional enzymes that might only be induced or activated during feeding or infection, or might be unstable during dissection of the midgut. In vitro, cleavage was restricted to Lys-19 and Lys-22, whereas tsetse-derived EP was cleaved at glycine, valine, alanine and aspartic acid residues occurring between Lys-22 and the dipeptide repeat (Acosta-Serrano et al., 2001). One candidate for such an enzyme might be GmCatB, as it belongs to a class of enzymes with amino- and endopeptidase activities that increases upon infection (Yan et al., 2002).

In contrast to procyclins, other trypanosome surface proteins are more resilient to digestion. The dense packing of approximately ten million VSG molecules on the surface of bloodstream forms of *T. brucei* might impede access to most cleavage sites. In the case of *T. congolense* GARP, it has recently been proposed that oligosaccharides attached to phosphothreonines might mask some of the tryptic cleavage sites (Thomson et al., 2002), but this cannot be the sole explanation. We have found that GARP expressed on the surface of *T. brucei* (Hehl et al., 1995) is also resistant to midgut proteases (M.L., unpublished observation), although it is not supposed to be modified by phosphoglycans (Thomson et al., 2002).

Table 1
pH measurements within different compartments of the tsetse fly digestive tract

Time after a bloodmeal	Proventriculus	Anterior midgut	Posterior midgut	Hindgut
0 h (= teneral)	9.0 ± 0.7 (4)	9.4 ± 0.2 (4)	9.5 ± 0.5 (4)	9.3 ± 1.0 (3)
48 h	10.6 ± 0.6 (4)	9.3 ± 0.5 (4)	7.9 ± 0.4 (4)	10.4 (1)
72 h	10.2 ± 1.0 (3)	8.5 ± 0.3 (5)	8.0 ± 0.2 (5)	9.1 ± 0.5

Measurements were made with microelectrodes on teneral flies (= non-fed, freshly emerged flies) and on flies 48 and 72 h after a single blood meal on a rabbit. Measurements are given as the average ± SD (*n*, number of independent measurements).

Why do procyclins have a conserved basic domain that makes them particularly vulnerable to cleavage by trypsin and why are there subtle differences in the amino acid sequences of the EP isoforms? One possibility is that the peptides that are released have an influence on the course of infection. Alternatively, removal of these sequences could expose the internal repeats of procyclins and allow them to interact with tsetse molecules. Another possibility is that this region contains signals that ensure correct processing or routing to the cell surface; once this is achieved, the N-termini might become redundant. The construction of mutant trypanosomes with modified procyclins should allow us to address some of these questions.

The surprising discovery that the tsetse digestive tract is alkaline, and that tsetse trypsins are most active at pH ~ 10, alerts us to the possibility that it might be an interesting source of other enzymes that function well under highly basic conditions. It is also worth noting that procyclic culture forms of *T. brucei* are normally cultured in media in the pH range 7–8. (Brun and Schoenenberger, 1979). Although these cells reliably reach high densities, they rarely give rise to epimastigote and metacyclic forms. Procyclic trypanosomes are clearly able to sense their environment through the untranslated regions of procyclin mRNAs, presumably through the action of RNA-binding factors, and can respond by changes in RNA stability and protein expression (Furger et al., 1997; Hotz et al., 1997; Schürch et al., 1997; Vassella et al., 2000). It is therefore worth exploring whether exposing trypanosomes to an alkaline milieu in culture (mimicking the conditions in the proventriculus) might also affect gene expression and result in differentiation from the procyclic form to later stages in the parasite life cycle.

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