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Detection of peptidases in *Trypanosoma cruzi* epimastigotes using chromogenic and fluorogenic substrates

N. HEALY[1], S. GREIG[1], H. ENAHORO[1], H. ROBERTS[1], L. DRAKE[1], E. SHAW[2] *and* F. ASHALL[1]

[1] *Department of Biology, Imperial College of Science, Technology and Medicine, Prince Consort Road, London SW7 2BB, UK*
[2] *Friedrich Miescher-Institut, Basle, Switzerland*

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SUMMARY

Detergent extracts of *Trypanosoma cruzi* epimastigotes catalysed the hydrolysis of a range of amino-acyl and peptidyl *p*-nitro-anilides and aminomethylcoumarins. At least three enzymes were detected that cleave Z–Phe–Arg–MCA. Two of these were optimally active at alkaline pH, the other at pH 4·0. Of the two enzymes with alkaline pH optima, one was a cysteine peptidase and was unable to cleave Bz–Arg–MCA readily, whilst the other cleaved Bz–Arg–MCA and was inhibited by diisopropyl fluorophosphate. The acidic enzyme was similar to cathespin L of other eukayrotes with respect to its pH profile, substrate-specificity and inhibitor-sensitivity. Evidence was presented that epimastigotes contain a cysteine-type dipeptidyl aminopeptidase, one or more aminopeptidases, and a serine peptidase that cleaves Boc–Ala–Ala–pNA. Digitonin solubilization of the activities from cells supports the hypothesis that the cathespin L-like enzyme and the dipeptidyl aminopeptidase are lysosomal, whilst the Bz–Arg–MCA hydrolase, the aminopeptidases and the Boc–Ala–Ala–pNA serine peptidase are cytosolic.

Key words: *Trypanosoma cruzi*, peptidase, protease, cathespin L.

INTRODUCTION

Various peptidases have been detected in *Trypanosoma cruzi*, the protozoan parasite that causes South American trypanosomiasis (Chagas' disease) in Man and other animals (Itow & Camargo, 1977; Bongertz & Hungerer, 1978; Rangel *et al.* 1981; Bontempi *et al.* 1984; Ashall, 1990; Greig & Ashall, 1990), although few of these enzymes have been well characterized. Bongertz & Hungerer (1978) purified a peptidase from *T. cruzi* epimastigotes that was reported to cleave peptide bonds on the carboxyl side of arginine, lysine and tyrptophan. This enzyme shares some properties with an alkaline peptidase that we characterized in *T. cruzi* and that also appears to be expressed by other trypanosomatids (Ashall, 1990; Ashall *et al.* 1990*b*). Cazzulo and co-workers characterized a cysteine peptidase that cleaves azocasein as well as various chromogenic and fluorogenic substrates and has a lysosomal subcellular localization (Bontempi *et al.* 1984; Bontempi, Martinez & Cazzulo, 1989; Cazzulo *et al.* 1990). Investigation of the biological functions of these enzymes should contribute significantly to our understanding of the interaction of *T. cruzi* with its host environments. Indeed, there is evidence that peptidases are involved in entry of *T. cruzi* into mammalian cells (Piras, Henriquez & Piras, 1985).

We characterized 5 major peptidases in *T. cruzi* epimastigotes using an electrophoretic system in which gelatin is hydrolysed in polyacrylamide gels (Greig & Ashall, 1990). Here we describe some other peptidase activities of epimastigotes and we present evidence that this parasite contains a lysosomal dipeptidyl aminopeptidase, one or more aminopeptidases, a neutral serine peptidase, and three enzymes that cleave carbobenzoxy–phenylalanyl–argininyl–aminomethylcoumarin (Z–Phe–Arg–MCA).

MATERIALS AND METHODS

Parasites and chemicals

T. cruzi epimastigotes were grown at 28 °C in RPMI 1640 medium supplemented with 7·5% (v/v) foetal calf serum (heat-treated at 45 °C for 15 min), hemin (20 μg/ml), trypticase (25 mg/ml), glutamine (0·2 mM) and 4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid [HEPES] (10 mM). Strain Y (Silva & Nussenzweig, 1963) of the parasite was used in the studies.

All peptidase inhibitors were obtained from Sigma Chemical Company, except for diprotin B, which came from Peninsula Laboratories, and fluoromethanes, which were made as described previously (Rauber *et al.* 1986). All *p*-nitroanilides and aminomethylcoumarins were obtained from Sigma

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Chemical company except for carbobenzoxy–argininyl–argininyl–aminomethylocoumarin (Z–Arg–Arg–MCA), which was from Peninsula Laboratories. Substrates were stored as 10 mM stock solutions in dimethylformamide at −20 °C.

Parasite extracts

Approximately 20 ml of a culture containing 5×10^6 *T. cruzi* epimastigotes/ml were centrifuged at 300 ***g*** for 10 min at room temperature. The cell pellet was washed once with 20 ml of 10 mM sodium phosphate, pH 7·4/150 mM NaCl, then suspended in 0·5–1 ml of 1% (v/v) Nonidet P-40, vortexed for 10 s and centrifuged at 12000 ***g*** at room temperature for 5 min. The supernatant fraction was taken as the epimastigote detergent extract and was adjusted to a protein concentration of 0·5–1 mg/ml.

Chromogenic assays

Hydrolysis of *p*-nitroanilides (Erlanger, Kokowsky & Cohen, 1961) was carried out in 1 ml vol. cuvettes. Parasite extracts (100 μl) containing 1 mg protein/ml were mixed with 800 μl of buffer (50 mM HEPES/50 mM NaH_2PO_4/50 mM trisodium citrate/50 mM boric acid), adjusted to the appropriate pH. Then 100 μl of 2 mM *p*-nitroanilide substrate were added and the increase in optical density at 410 nm followed at 25 °C.

Fluorogenic assays

Hydrolysis of aminomethylcoumarin derivatives was carried out as described previously (Barrett & Kirschke, 1980) except that assays were carried out in microtitre fluoroplates (Flow). Parasite extracts (25 μl) were mixed with 115 μl of 50 mM HEPES/50 mM trisodium citrate/50 mM NaH_2PO_4/50 mM boric acid, adjusted to the appropriate pH. The reaction was started by addition of 10 μl of 450 μM aminomethylcoumarin substrate. The increase in fluorescence was measured using an excitation wavelength of 360 nm and an emission wavelength of 460 nm. One unit of enzyme activity was defined as that which produced a change in fluorescence intensity in 1 min at 25 °C equivalent to the fluorescence intensity given by the same volume of 5 μM aminomethylcoumarin (AMC) under the same conditions.

Inhibitor and pH profiles

A universal buffer solution containing 50 mM trisodium citrate, 50 mM sodium dihydrogen phosphate, 50 mM HEPES and 50 mM boric acid, with buffering capacity between pH 3 and pH 10, was used for all assays and was adjusted to the appropriate pH with 10 M NaOH or with 10 M HCl. The pH optima for hydrolysis of peptidase substrates were determined using this buffer. Effects of inhibitors were examined by pre-treatment of parasite extracts (0·5–1 mg protein/ml) at the pH optimum for substrate hydrolysis for 10 min at room temperature prior to assay for enzyme activity. Concentrations of inhibitors used were: diisopropyl fluorophosphate (DFP) [1 mM], Pepstatin A [25 μM], *o*-phenanthroline [1 mM], trans-epoxysuccinyl-leucylamido(4-guanidino)butane (E-64) [25 μM], iodoacetic acid [1 mM], diprotin A [50 μM], ethylenediamine tetraacetic acid [2 mM], phenylmethylsulphonl fluoride (PMSF) [1 mM], bestatin [1 μM], tosyl-lysyl-chloromethyl ketone (TLCK) [10 μM], leupeptin [25 μM], carbobenzoxy–alanyl–phenylalanyl–alanyl–fluoromethane (Z–Ala–PheCH_2F) [50 μM] and carbobenzoxy–phenylalanyl–alanyl–fluoromethane (Z–Phe–AlaCH_2F) [50 μM].

Electrophoretic detection of peptidases

Electrophoresis of parasite extracts and detection of peptidases by hydrolysis of aminomethylcoumarins and subsequent ultraviolet light illumination was carried out as described previously (Ashall *et al.* 1990*b*). Effects of inhibitors on the enzymes were examined by incubation of gel slices for 15 min at 25 °C prior to addition of aminomethylcoumarin substrate. Substrates were used at a final concentration of 100 μM.

Digitonin solubilization of cells

Epimastigotes (suspended at a protein concentration of 1·3 mg/ml) were extracted with increasing concentrations of digitonin (Aldrich Chemical Company) and samples of the solubilized supernatants were assayed for enzyme markers of cytosol (aspartic-activated malic enzyme), mitochondria (citrate synthase), microsomes (acid phosphatase), lysosomes (α-mannosidase) and glycosomes (hexokinase), as described previously (Bontempi *et al.* 1989).

Protein estimation

The protein content of parasite extracts was determined by the method of Bradford (1976) using a BioRad Protein Assay kit according to manufacturer's instructions.

RESULTS

Detection of peptidase activities

Extracts of *T. cruzi* epimastigotes were able to digest various *p*-nitroanilides and aminomethylcoumarins at 25 °C (Table 1). In particular, substrates with blocked amino-termini containing arginine at P1 (nomenclature of Schechter & Berger (1967)) were readily hydrolysed. The enzyme that cleaves

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Table 1. Hydrolysis of various *p*-nitroanilides and aminomethylcoumarins by *Trypanosoma cruzi* epimastigote extracts

(Parasite extracts were incubated at 25 °C with substrates (0·2 mM *p*-nitroanilides or 20 μM aminomethylcoumarins) at their pH optima (see Fig. 1), as described in the Materials and Methods section. Units are nmol pNA/min/mg protein for *p*-nitroanilides (PNA) and units/min/mg protein for aminomethylcoumarins.)

	Rate of hydrolysis		
Substrate	No added 2-mercaptoethanol	+25 mM 2-mercaptoethanol	pH optimum
Bz–Arg–pNA	28·4±6·1	32·3±8·3	8·0
Ser–PNA	2·4±0·5	2·7±0·3	7·0
Lys–pNA	1·1±0·3	1·3±0·3	7·0
Leu–pNA	4·1±0·7	N.D.	7·0
Ala–pNA	4·0±1·1	N.D.	7·0
Ala–Ala–pNA	1·3±0·3	3·0±0·5	5·5
Ala–Ala–Ala–pNA	0	0·2±0·1	6·5
Boc–Ala–Ala–pNA	2·6±0·9	2·9±0·6	7·0
Bz–Tyr–pNA	0·4±0·3	N.D.	N.D.
Z–Phe–Leu–Gly–pNA	0·3±0·2	N.D.	N.D.
Suc–Ala–Ala–Pro–Phe–pNA	0·3±0·1	N.D.	N.D.
Gly–pNA, Ac–Phe–pNA, Ala–Pro–pNA	0	0	N.D.
Bz–Arg–MCA	1·23±0·22	N.D.	8·0
Z–Arg–Arg–MCA	0·67±0·23	1·44±0·32	8·5
Z–Phe–Arg–MCA (pH 4)	0·33±0·11	0·84±0·15	4·0
Z–Phe–Arg–MCA (pH 8)	0·22±0·06	0·71±0·19	8·0
Leu–MCA	0·23±0·07	N.D.	7·0
Ser–MCA	0·31±0·11	N.D.	7·0
Suc–Ala–Ala–Ala–MCA	N.D.	0·04±0·03	N.D.

Bz–Arg–MCA and Bz–Arg–pNA was characterized by us previously (Ashall, 1990; Ashall *et al.* 1990*b*) and has the highest activity of all enzymes detected using the various substrates shown in Table 1. Rates of hydrolysis of various amino-acyl aminomethylcoumarins (in particular Leu–pNA, Ala–pNA and Ser–MCA), as well as of Ala–Ala–pNA, Boc–Ala–Ala–pNA, Z–Phe–Arg–MCA and Z–Arg–Arg–MCA were also consistently high in different parasite preparations and these activities were chosen for further study.

pH profiles

The cleavage of Ala–Ala–pNA by *T. cruzi* epimastigote extracts showed a pH optimum between pH 5 and pH 6, whereas Boc–Ala–Ala–pNA and Leu–pNA were cleaved optimally at pH 7 (Fig. 1). Other amino-acyl substrates that were tested each were hydrolysed with pH profiles similar to that for Leu–pNA hydrolysis. Whereas Z–Arg–Arg–MCA hydrolysis had a single pH optimum of pH 8–9, two peaks were seen with Z–Phe–Arg–MCA: one occurred at pH 4, the other at approximately pH 8 (Fig. 1).

Inhibitor profiles

In order to determine the class of peptidase to which these hydrolytic activities belong, digestion of the various substrates was carried out after treatment of epimastigote extracts with a range of peptidase inhibitors (Table 2). Hydrolysis of Ala–Ala–pNA was activated 2-fold by treatment with 25 mM 2-mercaptoethanol (Table 1) and was strongly inhibited by E-64 and iodoacetic acid (Table 2), indicating that this activity is due to one or more cysteine peptidase. The enzyme activity that cleaved Ala–Ala–pNA did not readily cleave Boc–Ala–Ala–pNA, since cleavage of Boc–Ala–Ala–pNA was not inactivated by any of the inhibitors of Ala–Ala–pNA cleavage. Moreover, since extracts of *T. cruzi* did not readily cleave Ala–Ala–Ala–pNA over the concentration range, 0–0·3 mM, the enzyme that cleaves Ala–Ala–pNA apparently prefers 2 alanine residues to 3. Ala–pNA was readily hydrolysed by parasite extracts, but this was inactivated by inhibitors of metallopeptidases and not by cysteine peptidase inhibitors, suggesting that the Ala–Ala–pNAase prefers 2 alanine residues to 1 alanine residue. Boc–Ala–Ala–pNA was readily cleaved by parasite extracts, but this activity had an entirely different pH and inhibitor profile from that of Ala–Ala–pNA hydrolysis. Cleavage of Boc–Ala–Ala–pNA was inhibited only by DFP of the inhibitors tested, providing evidence that this activity is due to a serine peptidase (Table 2). Since the activity that cleaves Ala–Ala–pNA appears not to readily cleave Boc–Ala–Ala–pNA or either Ala–pNA or Ala–Ala–Ala–pNA, the effect of the dipeptidyl aminopeptidase inhibitor, diprotin A, on this activity was

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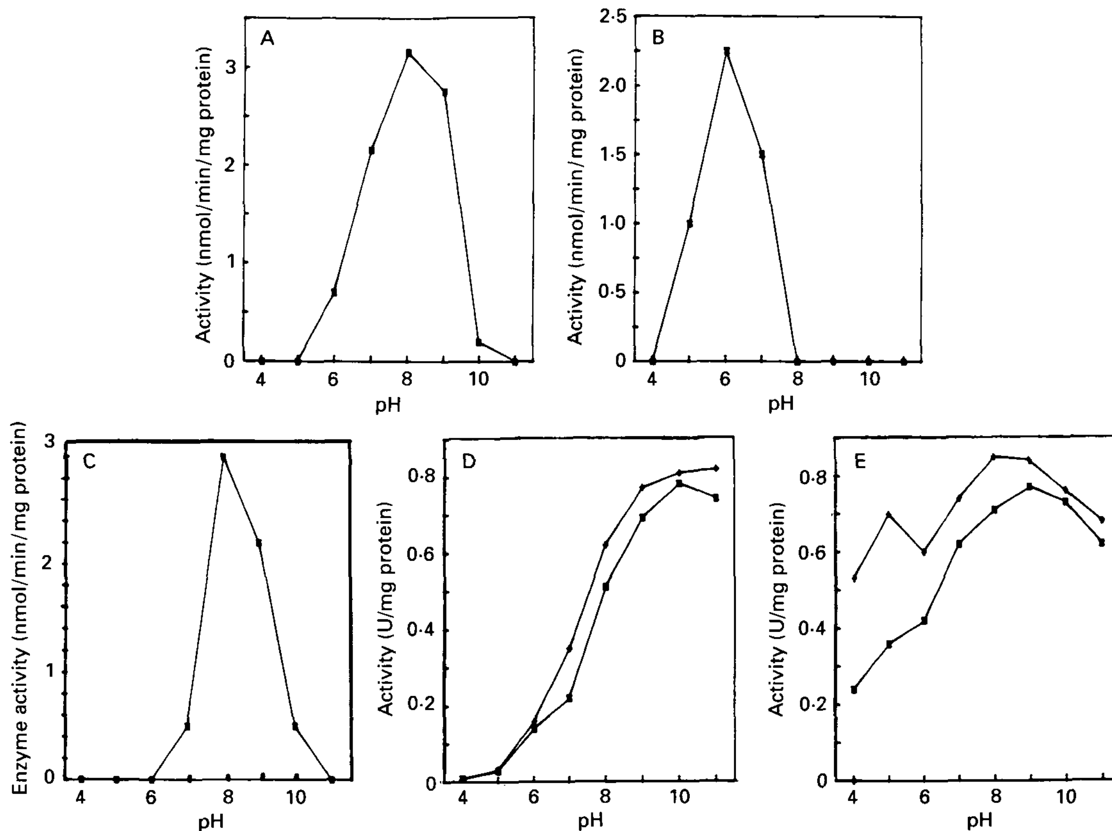

Fig. 1. Effect of pH on hydrolysis of various *p*-nitroanilide (pNA) and aminomethylcoumarin (MCA) substrates by *Trypanosoma cruzi* epimastigote extracts. Initial rates of hydrolysis of substrates (0·2 mM pNAs or 20 μM MCAs, final concentration) at 25 °C by detergent extracts of epimastigotes were determined at various pH values as described in the Materials and Methods section. Substrates used were: (A) Boc–Ala–Ala–pNA; (B) Ala–Ala–pNA; (C) Leu–pNA; (D) Z–Arg–Arg–MCA; (E) Z–Phe–Arg–MCA. (D) and (E) were carried out in the presence (+) or absence (■) of 25 mM 2-mercaptoethanol.

Table 2. Effects of peptidase inhibitors on hydrolysis of chromogenic and fluorogenic substrates by extracts of *T. cruzi* epimastigotes

(Parasite extracts were incubated for 10 min with inhibitors (see Materials and Methods section for concentrations used) at 25 °C and rates of hydrolysis of substrates (0·2 mM *p*-nitroanilides or 20 μM aminomethylcoumarins) at their pH optima (see Table 1) measured. Percentage inhibition is given: rate of hydrolysis in control (no inhibitor) sample was taken as 100 % value. All values are means of at least 3 determinations; standard deviations were always less than 10 %. Substrates used were: Ala–Ala–pNA (AA–pNA), Boc–Ala–Ala–pNA (BocAA–pNA); Z–Arg–Arg–MCA (ZRR–MCA), Z–Phe–Arg–MCA at pH 4 (ZFR–MCA) and Leu–pNA (L–pNA). Hydrolysis of Ala–Ala–pNA was carried out in the presence of 25 mM 2-mercaptoethanol, as was hydrolysis of Z–Phe–Arg–MCA at pH 4. Inhibitor treatments were done prior to addition of the 2-mercaptoethanol in both cases.)

Inhibitor	Inhibitor concentration	AA–pNA	BocAA–pNA	ZRR–MCA pH 8	ZFR–MCA pH 4	L–MCA
E-64	25 μM	93·2	2·3	7·1	96·7	4·4
DFP	1 mM	10·5	98·3	78·6	6·6	2·2
Pepstatin A	25 μM	12·2	1·2	3·9	2·2	0
o-Phenanthroline	1 mM	6·0	7·6	3·4	2·9	94·5
PMSF	1 mM	3·3	N.D.	3·6	0	0
Iodoacetic acid	1 mM	95·1	7·2	0	96·9	10·6
Diprotin B	50 μM	58·3	N.D	N.D.	N.D.	N.D.
EDTA	2 mM	3·6	N.D.	N.D.	N.D.	97·2
Bestatin	1 μM	N.D.	0·3	N.D.	N.D.	94·4
Leupeptin	25 μM	N.D.	0	97·5	95·7	N.D.
TLCK	10 μM	N.D.	N.D.	98·7	96·0	2·0
Z-Ala-PheCH_2F	50 μM	N.D.	N.D.	N.D.	3·1	N.D.
Z-Phe-AlaCH_2F	50 μM	N.D.	N.D.	N.D.	98·2	N.D.

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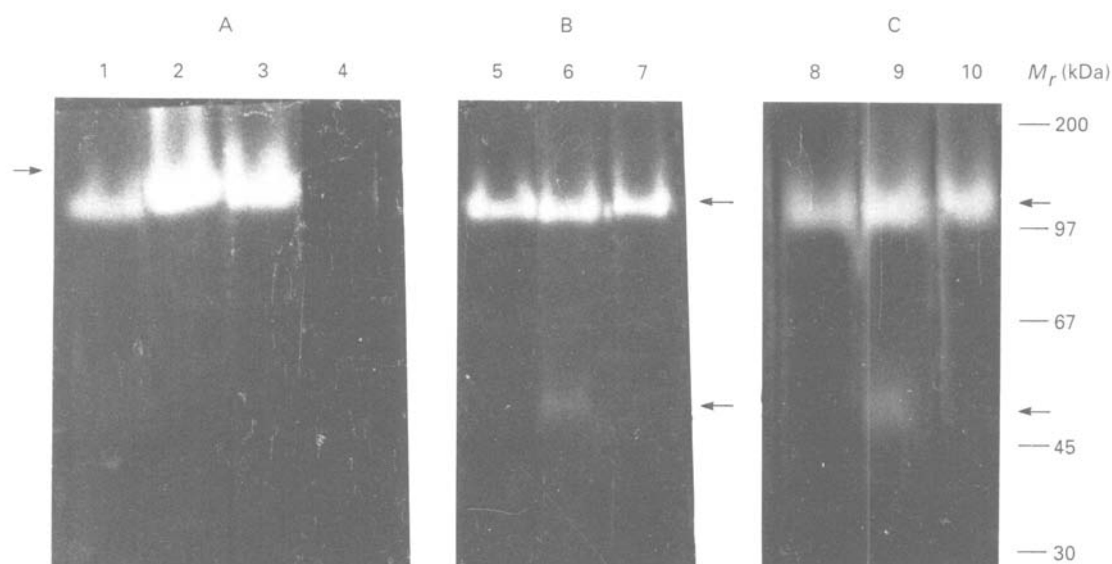

Fig. 2. Electrophoretic detection of Bz–Arg–MCA, Z–Phe–Arg–MCA and Z–Arg–Arg–MCA hydrolases in *Trypanosoma cruzi* epimastigotes at pH 8·0. A detergent extract of epimastigotes was electrophoresed in a polyacrylamide gel and the gel incubated at pH 8 with 100 μM aminomethylcoumarin substrate at 25 °C for 60 min, then visualized on an ultraviolet light box. (A) Hydrolysis of Bz–Arg–MCA: Lane 1, no 2-mercaptoethanol; Lane 2, +25 mM 2-mercaptoethanol; Lane 3, +25 mM 2-mercaptoethanol and 20 μM E-64; Lane 4, +25 mM 2-mercaptoethanol and 10 μM TLCK. (B) Hydrolysis of Z–Phe–Arg–MCA: Lane 5, no mercaptoethanol; Lane 6, +25 mM 2-mercaptoethanol; Lane 7, +25 mM 2-mercaptoethanol and 20 μM E-64. (C) Hydrolysis of Z–Arg–Arg–MCA: Lane 8, no mercaptoethanol; Lane 9, +25 mM 2-mercaptoethanol; Lane 10, +25 mM 2-mercaptoethanol and 20 μM E-64. (Apparent molecular weights of standards are indicated.)

examined. At 50 μM, diprotin A inhibited the Ala–Ala–pNA hydrolase by about 60%.

Cleavage of amino-acyl substrates was strongly inhibited by *o*-phenanthroline, EDTA and bestatin, but was unaffected by inhibitors of serine, cysteine and aspartic peptidases, evidence that this activity is due to one or more aminopeptidases, putatively of the metallopeptidase class.

Hydrolysis of Z–Phe–Arg–MCA at pH 8 in the absence of added 2-mercaptoethanol was strongly inhibited by TLCK, leupeptin and DFP (Table 2), whereas hydrolysis of Z–Phe–Arg–MCA at pH 4 was inhibited by E-64, iodoacetic acid, TLCK and leupeptin. The inhibitor profiles for Z–Phe–Arg–MCA and Z–Arg–Arg–MCA hydrolysis at pH 8 in the absence of added 2-mercaptoethanol were identical, and are the same as that described previously for the alkaline peptidase that cleaves Bz–Arg–MCA. Hydrolysis of Z–Phe–Arg–MCA at pH 4 has inhibition features characteristic of a cysteine peptidase and this activity does not readily cleave Z–Arg–Arg–MCA. These features are characteristic of cathespin L of higher eukaryotes (Barrett & Kirschke, 1980). In order to investigate this possibility further, the effects of Z–Phe–Ala–fluoromethane and Z–Ala–Phe–fluoromethane on the hydrolysis of Z–Phe–Arg–MCA at pH 4 by *T. cruzi* epimastigotes were examined: Z–Phe–Ala–fluoromethane inhibits cathepsin L more readily than does Z–Ala–Phe–fluoromethane, because cathepsin L prefers a large hydrophobic amino acid side-chain at position P2 of the substrate. Indeed, whereas 50 μM Z–Phe–Ala–fluoromethane inhibited the acidic Z–Phe–Arg–MCA hydrolase by more than 90%, no inhibition of this activity occurred with 50 μM Z–Ala–Phe–fluoromethane (Table 2).

Electrophoretic detection

Of the fluorogenic substrates shown in Table 1, only Bz–Arg–MCA, Z–Phe–Arg–MCA and Z–Arg–Arg–MCA produced bands in polyacrylamide gels following electrophoresis of *T. cruzi* epimastigote extracts and incubation of gels with substrates. A major band of about 120 kDa was produced with all three substrates at pH 8 and was inhibited by DFP, TLCK, and leupeptin, but not by E-64, iodoacetic acid, Pepstatin A or *o*-phenanthroline. This enzyme is the major trypanosomatid alkaline peptidase characterized by us previously (Ashall, 1990; Ashall *et al.* 1990*b*). With Z–Arg–Arg–MCA and Z–Phe–Arg–MCA, however, a fainter fluorescent band of about 50 kDa was seen in gels incubated in the presence of 25 mM 2-mercaptoethanol at pH 8·0 (Fig. 2). This enzyme was inhibited by E-64 and iodoacetic acid, but not by *o*-phenanthroline, DFP, PMSF or Pepstatin A, indicating that it is a cysteine peptidase. No band was seen at this position when gels were incubated with Bz–Arg–MCA either in the presence or absence of 2-mercaptoethanol. These data agree with the pH profiles for hydrolysis of Z–Arg–Arg–MCA and Z–Phe–Arg–MCA, which

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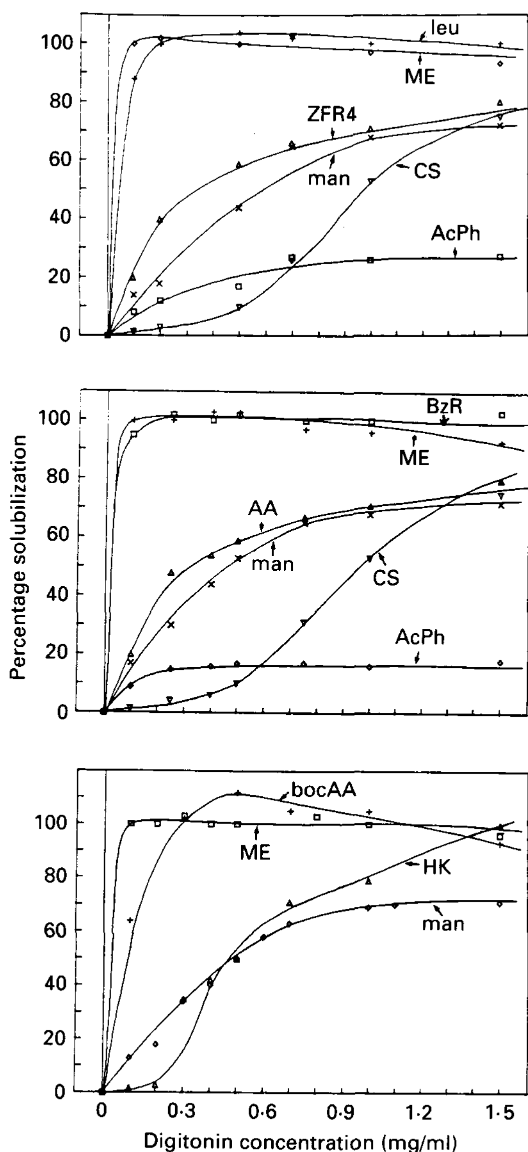

Fig. 3. Solubilization of *Trypanosoma cruzi* epimastigote peptidase activities by digitonin. Intact *T. cruzi* epimastigotes were suspended at a total protein concentration of 1·3 mg/ml then extracted with increasing concentrations of digitonin and the solubilized enzymes assayed. Marker enzymes for subcellular organelles were: aspartic-activated malic enzyme (ME), cytosol; citrate synthase (CS), mitochondria; α-mannosidase (man), lysosomes; acid phosphatase (AcPh), microsomes; and hexokinase (HK), glycosomes. Peptidase activities examined were: Leu, Leu–pNA hydrolysis at pH 7; ZFR4, Z-Phe–Arg–MCA hydrolysis at pH 4; BzR, Bz–Arg–MCA at pH 8; AA, Ala–Ala–pNA hydrolysis at pH 5·5; bocAA, Boc–Ala–Ala–pNA hydrolysis a pH 7·5.

show an increase in rate of hydrolysis at alkaline pH in the presence of 2-mercaptoethanol (Fig. 1). Indeed, this increment due to 2-mercaptoethanol was abolished in the presence of E-64 (data not shown), suggesting that it is due to an additional peptidase of the cysteine type, but that the main activity at alkaline pH is due to the DFP-sensitive alkaline peptidase. At pH 4, a Z–Phe–Arg–MCA hydrolysing band was detectable with an M_r value of approximately 55 kDa, but this band was only very weak, suggesting that the acidic Z–Phe–Arg–MCA activity that resembles cathepsin L is not stable to the electrophoretic system used.

Digitonin solubilization profiles

Extraction of intact *T. cruzi* epimastigotes with increasing concentrations of digitonin (Bontempi *et al.* 1989) was carried out in order to obtain some information about the possible subcellular localizations of the peptidase activities detected. Solubilization profiles of the activities were compared with those of markers for lysosomes, cytosol, mitochondria, microsomes and glycosomes. All of the hydrolases studied had a digitonin solubilization profile similar to that of either the lysosomal marker (α-mannosidase) or the cytosolic marker (malic enzyme). Thus, the alkaline peptidase (Bz–Arg–MCA, Z–Phe–Arg–MCA or Z–Arg–Arg–MCA hydrolysis at pH 8, with no 2-mercaptoethanol), the Leu–pNA hydrolase and the Boc–Ala–Ala–pNA hydrolase each showed profiles characteristic of cytosolic enzymes, although the Boc–Ala–Ala–pNA hydrolase deviated slightly from the malic enzyme profile, whereas the Ala–Ala–pNA hydrolase and Z–Phe–Arg–MCA hydrolase at pH 4 each showed profiles characteristic of lysosomal enzymes (Fig. 3).

DISCUSSION

Several of the enzyme activities that we have detected, in particular the cathepsin L-like enzyme that cleaves Z–Phe–Arg–MCA at pH 4, the Boc–Ala–Ala–pNA hydrolase and the Ala–Ala–pNA hydrolase, appear not to have been described previously in *T. cruzi*. Aminopeptidase activities were detected previously (Itow & Camargo, 1977) and are common features of living cells; the inhibition of Leu–pNA hydrolysis by *o*-phenanthroline, EDTA and bestatin suggests that this activity is due to a metallo-aminopeptidase. However, another activity is involved in hydrolysis of Ala–Ala–pNA by epimastigote extracts. That this enzyme, which had a digitonin solubilization profile comparable to that of the lysosomal marker enzyme, is inactivated by cysteine peptidase inhibitors and by diprotin B, appears not to cleave Boc–Ala–Ala–pNA or Ala–pNA supports the hypothesis that cleavage of Ala–Ala–pNA is due to a cysteine-type dipeptidyl aminopeptidase. Dipeptidyl aminopeptidases have been detected and characterized in other organisms (Davis, 1987; Hui, 1988), although, to our knowledge, this is the first evidence for the occurrence of such an enzyme in a trypanosomatid. Cleavage of Boc–Ala–Ala–pNA, on the other hand, has characteristics of a cytosolic serine peptidase. The

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dipeptidyl aminopeptidase and aminopeptidases might have a role in the cellular degradation of proteins to completion: initial cleavage of large proteins into smaller peptides by endopeptidases may be followed by further cleavage of these peptides into even smaller fragments and single amino acids by the action of dipeptidyl aminopeptidases, aminopeptidases and carboxypeptidases and dipeptidases.

Of the three Z–Phe–Arg–MCA hydrolases that we have detected, one is identical to the DFP-sensitive alkaline peptidase that we characterized previously and which appears to occur in all trypanosomatids tested (Ashall, 1990; Ashall *et al.* 1990*b*). The Z–Phe–Arg–MCA hydrolase that is active at pH 8 and is activated by 2-mercaptoethanol and inhibited by E-64 (but not by DFP) resembles the enzyme, cruzipain, described by Cazzulo *et al.* (1990). Cruzipain has amino-terminal homology to cathespin L of higher organisms, but, unlike cathepsin L, it cleaves Z–Arg–Arg–MCA and has an alkaline pH optimum for this substrate. On the other hand, we have detected a Z–Phe–Arg–MCA hydrolysing activity in *T. cruzi* that does resemble cathepsin L of higher organisms more closely. This activity, which hydrolyses Z–Phe–Arg–MCA optimally at pH 4, resembles cathepsin L with respect to its inability to cleave Z–Arg–Arg–MCA readily, its pH optimum, its apparently lysosomal locale, its being a cysteine peptidase and its strong inhibition by Z–Phe–Ala–fluoromethane but not by Z–Ala–Phe–fluoromethane. The relationship between this cathepsin L-like enzyme and that described in other trypanosomatids (Lonsdale-Eccles & Grab, 1987) remains to be assessed.

Peptidases can be exploited for the design of specific inhibitors (Kam, Fujikawa & Powers, 1988; Kirschke, Wikstrom & Shaw, 1988), and development of inhibitors to peptidases that are unique to *T. cruzi*, or exploitation of differences between homologous host and parasite peptidases, might open up approaches to chemotherapy of Chagas' disease. Indeed, we have demonstrated that synthetic peptidase inhibitors are able to cause lysis of *T. cruzi* trypomastigotes *in vitro* (Ashall, Angliker & Shaw, 1990*a*).

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