Detection of peptidase activities in *Trypanosoma cruzi* using chromogenic and fluorogenic substrates

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During our investigations of the major peptidases of *Trypanosoma cruzi*, the protozoan parasite that causes South American trypanosomiasis (Chagas' disease), we detected five major peptidase bands after electrophoresis of parasite extracts in polyacrylamide gels containing gelatin as substrate [1]. Four of these peptidases were cysteine-type peptidases with acidic pH optima; the other enzyme was shown to be an integral membrane peptidase. We also purified and characterized a peptidase that cleaves Bz-Arg-pNA and other substrates on the carboxyl side of arginine and lysine residues at pH 8.0 [2]. This alkaline peptidase occurs in all stages of the life cycle of *T. cruzi*, and a similar enzyme occurs in other trypanosomatids, including African trypanosomes and Leishmania, parasites that also cause major human diseases.

Abbreviations used: Bz, benzoyl pNA; p-nitroanilide; Cbz, benzoylcarbonyl; MCA, amidomethylcoumarin.  
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Table 1. Peptidases detected in extracts of *T. cruzi* (Y strain) epimastigotes using fluorogenic and chromogenic peptidase substrates

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Substrates</th>
<th>Inhibitors</th>
<th>Peptidase class</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dipeptidyl aminopeptidase</td>
<td>Ala-Ala-pNA</td>
<td>E64, iodoacetic acid, leupeptin</td>
<td>Cysteine</td>
</tr>
<tr>
<td>Neutral serine peptidase</td>
<td>Boc-Ala-Ala-pNA</td>
<td>DFP</td>
<td>Serine</td>
</tr>
<tr>
<td>Aminopeptidase</td>
<td>Leu-pNA, Arg-pNA, Ala-pNA</td>
<td>EDTA, o-phenanthroline, phosphoramidon</td>
<td>Metallo-</td>
</tr>
<tr>
<td>'Cathepsin L'</td>
<td>Cbz-Phe-Arg-MCA [at pH 4.0]</td>
<td>E64, Tos-Lys-CH2Cl, iodoacetic acid, leupeptin</td>
<td>Cysteine</td>
</tr>
<tr>
<td>'Cathepsin B-like'</td>
<td>Cbz-Phe-Arg-MCA, Cbz-Arg-Arg-MCA [at pH 8.0]</td>
<td>E64, leupeptin, Tos-Lys-CH2Cl, iodoacetic acid</td>
<td>Cysteine</td>
</tr>
</tbody>
</table>

DFP, diisopropylfluorophosphate; Tos, tosyl.  

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enzyme is a true cathepsin L. No enzyme with properties of cathepsin B were detected in T. cruzi, although the enzyme described by Cazzulo and co-workers [3–5] has some features in common with cathepsin B.

The enzyme that cleaves Ala-Ala-pNA is inhibited by E64, iodoacetic acid and leupeptin, but is not affected by inhibitors of serine, metallo- or aspartic peptidases. It does not release p-nitroaniline from Boc-Ala-Ala-pNA or from Ala-Ala-Ala-pNA, and therefore has properties of a cysteine-type dipeptidylaminopeptidase. We are examining the possibility that this enzyme is related to cathepsin C described in other systems. Another enzyme occurs in T. cruzi that cleaves Boc-Ala-Ala-pNA and this enzyme is inhibited by diisopropylfluorophosphate but not by phenylmethylsulphonylfluoride, leupeptin, pepstatin A, o-phenanthroline, iodoacetic acid or E64. This serine peptidase is a major activity in epimastigote extracts.

However, of all the enzymes that we have detected in T. cruzi epimastigotes, the alkaline peptidase, which characteristically readily cleaves Bz-Arg-pNA, is the most abundant activity. Indeed, this enzyme apparently occurs as a major activity in other trypanosomatids [2]. Table 1 summarizes the peptidases that we have detected in T. cruzi epimastigotes using chromogenic and fluorogenic substrates.

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Substrate specificity and inhibitor sensitivity of a Trypanosoma cruzi alkaline peptidase

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We are investigating the peptidases of Trypanosoma cruzi, the protozoan parasite that causes South American trypanosomiasis. All stages of the life cycle of this parasite express a peptidase that cleaves on the carboxyl side of arginine and lysine residues of p-nitroanilide (pNA) and amidomethylcoumarin (MCA) substrates at alkaline pH [1]. In the absence of added reducing agents the alkaline peptidase is the major enzyme in detergent extracts of T. cruzi epimastigotes that cleaves these substrates at pH 8.0. We have detected, on the basis of pH profile, substrate specificity and inhibitor sensitivity as well as electrophoretic mobility, a similar enzyme in 15 other species of trypanosomatid, including African trypanosomes and Leishmania [1]. Because we have not detected a similar enzyme in any non-trypanosomatid protozoa or mammalian cells that we have tested, we hypothesize that the alkaline peptidase is specific to organisms of the phylogenetic order, Kinetoplastida, or to the family, Trypanosomatida.

We have examined the alkaline peptidase of T. cruzi epimastigotes for its ability to cleave peptidyl-MCA substrates and for its sensitivity to a range of irreversible and reversible inhibitors. Purified T. cruzi alkaline peptidase was found to cleave Bz-Arg-MCA with a K<sub>m</sub> of about 12 μM. However, when a second basic residue is added to the arginine residue, as in Cbz-Arg-Arg-MCA, the K<sub>m</sub> falls by an order of magnitude to about 1 μM. All substrates examined that contained two basic residues (Cbz-Arg-Arg-MCA, Cbz-Gly-Arg-Arg-MCA, Cbz-Leu-Gly-Arg-MCA, Cbz-Asn-Arg-Arg-MCA, Cbz-Gly-Lys-Arg-MCA and Cbz-Leu-Lys-Arg-MCA) had a K<sub>m</sub> value of about 1 μM, whereas all other substrates had a K<sub>m</sub> greater than 10 μM. The V<sub>max</sub> values for the substrates containing two basic residues are also lower than those for other substrates. From the data, it is apparent that the alkaline peptidase prefers arginine or lysine at P2 at substrate concentrations below about 5 μM. Because we have failed to obtain cleavage of intact proteins by the alkaline peptidase [1], we propose that this enzyme is involved in cleavage of a restricted number of peptide or protein substrates in trypanosomatids. Indeed, peptidases with restricted protein substrate specificity that cleave on the carboxyl side of pairs of basic residues occur widely in biological systems and are frequently involved in protein processing [2].

The alkaline peptidase is sensitive to Tos-Lys-CH₂Cl, leupeptin, diisopropylfluorophosphate (DFP) and to peptidyl-diazomethanes containing arginine or lysine at P1. Since DFP generally inhibits serine peptidases, whereas diazo-

Fig. 1. Effects of peptidase inhibitors on the activity of T. cruzi alkaline peptidase in polyacrylamide gels

A detergent extract (1% Nonidet P-40) of T. cruzi Y strain epimastigotes was electrophoresed and gel strips were pre-treated with various inhibitors for 10 min at 25°C. Strips were then incubated at 25°C, pH 8, with 100 μM-Bz-Arg-MCA for 30 min and photographed on a fluorescent light box. Lane 1, no inhibitor; lane 2, 1 mM-phenylmethylsulphonyl fluoride; lane 3, 50 μM-E64; lane 4, 50 μM-leupeptin; lane 5, 50 μM-pepstatin A; lane 6, 50 μM-Tos-Lys-CH₂Cl; lane 7, 1 mM-o-phenanthroline; lane 8, 50 μM-Cbz-Leu-Lys-diazomethane; lane 9, 50 μM-Cbz-Leu-Met-diazomethane.

Abbreviations used: pNA, p-nitroanilide; MCA, amidomethylcoumarin; Br, benzyl; Cbz, benzoxycarbonyl; Tos, tosyl; DFP, diisopropylfluorophosphatase.

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