Purification and characterisation of a trypsin-like serine oligopeptidase from *Trypanosoma congolense*

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Abstract

*Trypanosoma brucei* contain a serine oligopeptidase (OP-Tb) that is released into (and remains active in) the blood of trypanosome-infected animals. Here a similar enzyme from *Trypanosoma congolense* is described. This oligopeptidase, called OP-Tc, was purified using three-phase partitioning, and ion-exchange and affinity chromatography. OP-Tc is inhibited by alkylating agents, by serine peptidase-specific inhibitors including 3,4-dichloroisocoumarin, 4-(2-aminoethyl)benzenesulfonylfluoride and diisopropylfluoro-phosphate and by other peptidase inhibitors including leupeptin, antipain and peptidyl chloromethyl ketones. Reducing agents such as dithiothreitol enhanced activity as did heparin, spermine and spermidine. The enzyme has trypsin-like specificity since it cleaved fluorogenic peptides that have basic amino acid residues (Arg or Lys) in the P1 position. Potential substrates without a basic residue in P1 were not hydrolysed. Although OP-Tc has weak arginine aminopeptidase activity, the enzyme clearly preferred substrates that had amino acids in the P2 and P3 positions. Overall, OP-Tc appears to be less efficient than OP-Tb because it usually displayed lower \( k_{cat}/K_m \) values for the substrates tested. However, like OP-Tb, the best substrate for OP-Tc was Cbz-Arg-Arg-AMC (\( K_m = 0.72 \mu M, k_{cat} = 96 s^{-1} \)). OP-Tc preference for amino acids in the P2 position was (Gly,Lys,Arg) > Phe > Leu > Pro. The results also suggest that the P3-binding site has hydrophobic characteristics.

**Abbreviations**: AEBSF, 4-(2-aminoethyl)benzenesulfonylfluoride; AMC, 7-amino-4-methylcoumarin; Boc, \( t \)-butoxycarbonyl; Cbz, \( N \)-carbobenzoxy; DCI, 3,4-dichloroisocoumarin; DFP, diisopropylfluorophosphate; DTT, dithiothreitol; IgY, chicken egg-yolk immunoglobulin; OP-Tc, oligopeptidase from *Trypanosoma congolense*; pCMB, para-chloromercuribenzoate; PMSF, phenylmethanesulfonylfluoride; T os, toluene-\( p \)-sulfonyl; TBS, Tris-buffered saline; TPP, three-phase partitioning.

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OP-Tc may not be a naturally immunodominant molecule because neither IgG nor IgM anti-OP-Tc antibodies were detected in the blood of experimentally infected cattle. © 1999 Published by Elsevier Science B.V. All rights reserved.

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1. Introduction

*Trypanosoma (Trypanozoon) brucei brucei*, *T. (Nannomonas) congoles* and *T. (Duttonella) vivax* are the etiological agents of bovine trypanosomiasis, or nagana, in tropical Africa. These parasites belong to pathogenically distinct groups of trypanosomes. The *T. brucei* (‘humoral’) subgroup which causes an inflammatory degenerative disease in humans characterised by necrosis, oedema and hypotension [1], is of moderate pathogenicity in cattle [2]. Infections with *T. congoles* or *T. vivax*, which belong to the ‘haematic’ subgroup, are characterised primarily by extra-vascular haemolytic anaemia [1].

The authors have previously reported the purification and characterisation of a serine oligopeptidase from *T. b. brucei* [3,4], which they have called OP-Tb [4]. The authors have also demonstrated that this enzyme is released into the host bloodstream, where it remains catalytically active and may thus contribute to disease pathogenesis through the degradation of host peptide hormones [4]. Thus, OP-Tb has the potential to be an important pathogenic factor in *T. b. brucei* infections.

A related oligopeptidase (OP-Tc) from *T. congoles*, a member of the ‘haematic’ subgroup has now been purified and characterised. While cysteine peptidases have been reported from *T. congoles* [5,6], this represents the first report on a serine peptidase from any member of the *Nannomonas* subgenus of African trypanosomes. Although OP-Tc seems to be slightly less active than OP-Tb (about 3-fold), their properties are broadly similar. However, since *T. brucei* and *T. congoles* localise in different tissue in the infected host [7], the released oligopeptidase may have different pathological effects in the host.

It has been suggested that immune recognition of parasite pathogenic factors may protect hosts against the pathological effects of infection [8], and an effective immune response to invariant trypanosome antigens is thought to be an important mechanism of resistance to trypanosomiasis [9,10]. Indeed, trypanotolerant N’Dama cattle (*Bos taurus*) elicit a strong IgG, response against a 33 kDa invariant antigen from *T. congoles* [11]. This response, which is weak in trypanosusceptible Boran cattle (*Bos indicus*; [9]), is directed against the major lysosomal cysteine peptidase of *T. congoles*, trypanopain-Tc (also called congopain [11]). Thus, in this report the authors also investigated whether there is a differential recognition of OP-Tc, a possible pathogenic factor in trypanosome infections, by the humoral immune systems of trypanotolerant and trypanosusceptible cattle.

2. Materials and methods

2.1. Parasite culture

*T. congoles* (clone IL3000) [12] was cultured in adult male Sprague-Dawley rats, and purified from infected blood by a combination of Percoll® isopycnic gradient centrifugation [13] and anion-exchange chromatography on DEAE-cellulose [14].

2.2. Enzyme and protein assays

OP-Tc activity was routinely measured against 5 μM Nα-carbobenzoxy (Cbz)-Arg-Arg-7-amino-4-methylcoumarin (AMC) at 37°C in assay buffer [50 mM Tris–HCl/10 mM dithiothreitol (DTT), pH 8.0] in an Hitachi F-2000 spectrofluorimeter (λex = 370 nm, λem = 460 nm). Trypanopain-Tc activity was determined against 5 μM Cbz-Phe-Arg-AMC at 37°C in 170 mM CH3COONa:30 mM CH3COOH/2 mM Na2EDTA/3 mM DTT,
pH 5.5. Protein assays were conducted according to the modified method of Bradford [15].

2.3. Gel electrophoresis and western blotting

Samples were resolved by reducing (5 mM 2-mercaptoethanol) or non-reducing Tris-Tricine SDS-PAGE on 10% polyacrylamide gels [16] and proteins were visualised by silver staining [17]. Western blotting was performed as described [18], except that electro-transfer from gels to nitrocellulose membranes was conducted in 10 mM 3-cyclohexylaminopropanesulfonic acid/10% (v/v) methanol, pH 11 (30 V, 16 h; [19]). Unoccupied sites on the nitrocellulose membranes were blocked with 5% (m/v) non-fat milk in 20 mM Tris–HCl:200 mM NaCl, pH 7.4 (Tris-buffered saline, TBS), followed by incubation with the relevant primary antibody diluted in 0.5% (m/v) BSA in TBS (2 h, room temperature). Membranes were washed in TBS (3 × 5 min, room temperature), followed by incubation in the relevant secondary antibody diluted in 0.5% (m/v) BSA in TBS (1 h, room temperature). After a second wash cycle (3 × 5 min, room temperature), immune complexes were visualised with 0.15 mg ml\(^{-1}\) 5-bromo-4-chloro-3-indolyl phosphate/0.3 mg ml\(^{-1}\) nitroblue tetrazolium:100 mM Tris–HCl:5 mM MgCl\(_2\), pH 9.5. Rabbit anti-bovine IgG-alkaline phosphatase conjugate, monoclonal anti-bovine IgM clone BM-23-biotin conjugate and ExtrAvidin®-alkaline phosphatase conjugate were from Sigma (St. Louis, MO, USA).

2.4. Generation of antibodies

OP-Tb was purified from \(T. \) brucei as described previously [4], and antibodies raised in chickens by intramuscular injection at two sites in the breast muscle with a total of 20 mg of OP-Tb emulsified in Freund’s complete adjuvant [20]. Chickens were boosted at 3, 7 and 11 weeks after the first immunisation, with 10 mg of OP-Tb per booster, emulsified in Freund’s incomplete adjuvant. Chicken egg-yolk antibodies (IgY) were isolated [20], and anti-OP-Tb antibody production monitored by enzyme-linked immunosorbent assay. Western blots were employed to demonstrate the cross-reactivity of chicken anti-OP-Tb IgY with OP-Tc, and to screen the serum of infected bovine hosts for the presence of IgG and IgM antibodies directed against OP-Tc.

2.5. Preparation of immunoaffinity matrix

Anti-OP-Tb IgY (15 mg) was coupled to cyanogen bromide-activated Sepharose-4B (15 ml, packed volume) [21]. Coupling efficiency was estimated at 96.7%.

2.6. Purification of OP-Tc

Lysates of \(T. \) congolense \(6.7 \times 10^9\) cell equivalents, 12 ml), prepared by cycles of freezing and thawing, were diluted to 25 ml with 0.1% (m/v) Brij 35. A 10–25% (m/v) three-phase partitioning (TPP) fraction was prepared as described previously for \(T. \) brucei [4]. The pellet was resuspended in buffer A [20 mM sodium acetate/1 mM Na\(_2\)EDTA/0.02% (m/v) NaN\(_3\), pH 5.5] and clarified by centrifugation (15 000 × g, 30 min, 4°C). The resultant supernatant (15 ml) was loaded onto a Q-Sepharose column (26 × 100 mm, 1 ml min\(^{-1}\)) equilibrated in buffer A containing 100 mM NaCl. Material bound to the column that contained trypanopain-Tc activity, was eluted with a linear salt gradient (0.1–1 M NaCl in buffer A; 190 ml—4—column volumes). The fractions that did not bind to the column and which had activity against Cbz-Arg-Arg-AMC were dialysed (16 h, 4°C) against buffer B [50 mM Tris–HCl:0.02% (m/v) NaN\(_3\), pH 8]. After dialysis, the sample (44 ml) was loaded onto a \(p\)-aminobenzamidine-Sepharose column (120 × 15 mm, 1 ml min\(^{-1}\)) pre-equilibrated in buffer B. The column was washed with buffer B until the A\(_{280}\) reached baseline before the bound protein was eluted in a single step with 250 mM NaCl in buffer B. The bound, active fraction from \(p\)-aminobenzamidine-Sepharose (22 ml) was dialysed against buffer C [50 mM Tris–HCl/1 M NaCl/10 mM CaCl\(_2\)/0.5% (m/v) Brij 35/0.02% (m/m) NaN\(_3\), pH 7.4; 16 h, 4°C] and applied to the anti-OP-Tb-Sepharose immunoaffinity column (50 × 15 mm, 0.32 ml min\(^{-1}\)) equilibrated in buffer C. After washing of the column with buffer
C until the A$_{280}$ reached baseline, the bound OP-Tc was eluted with 3.5 M NaSCN in buffer C. NaSCN was removed by dialysis against buffer B, and protein concentrated by ultrafiltration in 3 ml polysulphone concentrators (10 kDa cut-off; Millipore, Bedford, USA).

### 2.7. Substrate specificity of OP-Tc

OP-Tc activity against fluorogenic substrates was determined by addition of substrate after preincubation of OP-Tc (1 ng, 37°C, 5 min) in assay buffer (Section 2.2). The initial steady-state velocity ($v_0$) was determined by continuous assay for a range of substrate concentrations (45 nM–75 μM). $K_m$ and $V_{max}$ were determined by hyperbolic regression of the kinetic data using the software package Hyper 1.01 (obtained from J.S. Easterby, University of Liverpool, UK). The $k_{cat}$ was determined from $k_{cat} = V_{max}/[E]_0$, where $[E]_0$ represents the active enzyme concentration determined with 4-methylumbelliferyl-$p$-guanidobenzoate [22].

### 2.8. Enzymatic characterisation of OP-Tc

The pH profile for OP-Tc was conducted as described above, except that buffers of constant ionic strength (100 mM acetic acid/200 mM Tris–HCl/100 mM Mes; $I = 0.1$ [23]) containing 1 mM DTT and 4 mM Na$_2$EDTA over the pH range 4–12 were substituted for assay buffer. Reductive activation of OP-Tc was investigated by pre-incubating OP-Tc (2.5 ng, 37°C, 10 min) in assay buffer containing DTT, reduced glutathione or L-cysteine (1–25 mM, 37°C, 5 min) prior to the addition of Cbz-Arg-Arg-AMC [25]. The rate constant for complex dissociation ($k_{diss}$) was determined from the relationship $K_i = k_{diss}/k_{ass}$ [26].

### 2.9. Infection of bovine hosts with T. congolense

Five N’Dama (trypanotolerant) and five Boran (trypanosusceptible) cattle, with a history of previous exposure to T. congolense infections [27], were challenged with T. congolense IL13E-3 [2,28] via the bites of 5–10 infected tsetse flies (Glossina morsitans centralis), as described [9,29]. All five N’Dama cattle exhibited transient parasitaemia...
Table 1
Purification table for the isolation of OP-Tc from T. congolense lysates

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Volume (ml)</th>
<th>Total protein (mg)</th>
<th>Total activity (pmol s⁻¹)</th>
<th>Specific activity (pmol s⁻¹ mg⁻¹)</th>
<th>Purification (fold)</th>
<th>Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lysate</td>
<td>12</td>
<td>196</td>
<td>2228</td>
<td>11</td>
<td>1</td>
<td>100</td>
</tr>
<tr>
<td>TPP</td>
<td>15</td>
<td>7.4</td>
<td>1473</td>
<td>198</td>
<td>18</td>
<td>66</td>
</tr>
<tr>
<td>Q-Sepharose</td>
<td>44</td>
<td>6.8</td>
<td>1109</td>
<td>163</td>
<td>15</td>
<td>50</td>
</tr>
<tr>
<td>p-Aminobenzamidine</td>
<td>22</td>
<td>0.413</td>
<td>1012</td>
<td>2450</td>
<td>221</td>
<td>45</td>
</tr>
<tr>
<td>Immunoaffinity</td>
<td>4</td>
<td>0.054</td>
<td>754</td>
<td>13,962</td>
<td>1,269</td>
<td>34</td>
</tr>
</tbody>
</table>

and no anaemia. Three Boran cattle developed moderate anaemia during 6 months of patent parasitaemia and eventually self-cured. The remaining two Boran cattle became severely anaemic, their haematocrit fell below 15% and drug therapy was required to prevent death [9]. Sera were collected at intervals during infection and immediately stored at −70°C. For the current study, sera taken 3 months post infection from the five N’Dama cattle and from the three Boran cattle that recovered, were pooled in two separate samples, later referred to as ‘N’Dama’ and ‘Boran’.

3. Results and discussion

3.1. Purification of OP-Tc

The purification of OP-Tc involves a 4-step procedure, which is two steps fewer than that used for the purification of OP-Tb [4] and results in a slightly higher yield of enzyme (34 vs. 28%, Table 1). Although the major lysosomal cysteine peptidase of T. congolense, trypanopain-Tc, co-precipitated with OP-Tc in the 10-25% (m/v) (NH₄)₂SO₄-cut, complete separation was effected by anion-exchange chromatography on Q-Sepharose, thus facilitating their simultaneous purification from the same starting material.

Para-aminobenzamidine-Sepharose chromatography removed 94% of the remaining contaminating protein with only a 5% loss in yield (Table 1). The cross-reactivity of chicken anti-OP-Tb antibodies with OP-Tc in T. congolense lysates (Fig. 1) prompted their use for immunoaffinity purification of OP-Tc. Cbz-Arg-Arg-AMC-hydrolysing activity was eluted in a single, well-resolved peak from the immunoaffinity column. OP-Tc was homogenous on reducing Tris-Tricine SDS-PAGE, producing a single band at 80 kDa (Fig. 1C). Elution of OP-Tc from the immunoaffinity column with 50 mM glycine–HCl, pH 2.3 instead of 3.5 M NaSCN resulted in a lower final yield (24%, results not shown).

The purified OP-Tc was 54 ± 4% active by active-site titration. The specific activity of the purified OP-Tc is very similar to that obtained for OP-Tb (13,368 pmol s⁻¹ mg⁻¹) using a longer
purity method [4]. The specific activity of 13962 pmol $^{-1}$ s $^{-1}$ mg $^{-1}$ is $\approx 10$ times that reported for a potentially related trypsin-like serine oligopeptidase (1379 pmol $p$-nitroanilide s $^{-1}$ mg $^{-1}$ [30]) and a chymotrypsin-like serine oligopeptidase (1620 pmol $p$-nitroanilide s $^{-1}$ mg $^{-1}$ [31]) isolated from the pollen of Prosophis velutina. However, these activities were determined at 25°C (as opposed to 37°C in this study) and employed the less-sensitive photometric $p$-nitroanilide substrates [25] in contrast to the fluorimetric substrates employed in this study, which may account for the higher specific activity reported here.

3.2. Substrate specificity of OP-Tc

A variety of commercially available fluorogenic peptides were tested for their potential as substrates for OP-Tc and clearly showed that OP-Tc has a trypsin-like activity (Table 2). A basic amino acid residue (Arg or Lys) in the P1 position (nomenclature of Schechter and Berger [32]) is necessary for OP-Tc to cleave the potential substrate. The low second-order rate constant ($k_{cat}/K_m$) for H-Arg-AMC (S #16; 50 000 M $^{-1}$ s $^{-1}$) and lack of activity against H-Gly-AMC and H-Leu-AMC indicates that OP-Tc has poor aminopeptidase activity. In contrast, peptides with a blocked N-terminus are much better substrates. For example, the $k_{cat}/K_m$ for the hydrolysis of Cbz-Arg-AMC (S #13) by OP-Tc is 80-fold greater than that for H-Arg-AMC (S #16). Both substrate binding and catalytic rate are improved when the P1- and the P2-binding sites are occupied. The failure of amastatin and bestatin to inhibit OP-Tc (see later) also points to the lack of aminopeptidase activity in OP-Tc.

Although efficient hydrolysis by OP-Tc requires that the P2 position be occupied, not all amino acids are equally acceptable in the P2-position since the nature of the residues occupying these sites clearly influences the rate of hydrolysis. Two substrates (S # 14, S #15) were tested that had P2-Pro, and both were hydrolysed only slowly. Thus, Pro does not appear to be readily accepted in the OP-Tc P2-binding site. In contrast, substrates that had Gly, Lys, Arg, Phe or Leu in P2 appeared to be more acceptable. The second-order rate constants for the hydrolysis of the Boc-Leu-Xaa-Arg-AMC family of peptides (S # 5, S # 6 and S # 7) were very similar indicating that

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Table 2
Amidolytic activity of OP-Tc $^a$

<table>
<thead>
<tr>
<th>Substrate number (S #)</th>
<th>Substrate</th>
<th>$K_m$ (µM)</th>
<th>$k_{cat}$ (s $^{-1}$)</th>
<th>$k_{cat}/K_m$ (M $^{-1}$ s $^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Cbz-Arg-Arg-AMC</td>
<td>0.72</td>
<td>96.0</td>
<td>133 300 000</td>
</tr>
<tr>
<td>2</td>
<td>Cbz-Gly-Arg-AMC</td>
<td>1.19</td>
<td>114.0</td>
<td>95 800 000</td>
</tr>
<tr>
<td>3</td>
<td>Cbz-Phe-Arg-AMC</td>
<td>1.09</td>
<td>67.0</td>
<td>61 500 000</td>
</tr>
<tr>
<td>4</td>
<td>Cbz-Ala-Arg-AMC</td>
<td>2.07</td>
<td>92.0</td>
<td>44 400 000</td>
</tr>
<tr>
<td>5</td>
<td>Boc-Leu-Gly-Arg-AMC</td>
<td>1.11</td>
<td>37.0</td>
<td>35 600 000</td>
</tr>
<tr>
<td>6</td>
<td>Boc-Leu-Lys-Arg-AMC</td>
<td>1.04</td>
<td>40.0</td>
<td>39 600 000</td>
</tr>
<tr>
<td>7</td>
<td>Boc-Leu-Arg-Arg-AMC</td>
<td>1.00</td>
<td>40.0</td>
<td>19 100 000</td>
</tr>
<tr>
<td>8</td>
<td>Boc-Gly-Arg-AMC</td>
<td>2.21</td>
<td>92.0</td>
<td>44 400 000</td>
</tr>
<tr>
<td>9</td>
<td>Boc-Val-Gly-Arg-AMC</td>
<td>2.10</td>
<td>92.0</td>
<td>44 400 000</td>
</tr>
<tr>
<td>10</td>
<td>H-Ala-Phe-Lys-AMC</td>
<td>3.00</td>
<td>27.0</td>
<td>9 000 000</td>
</tr>
<tr>
<td>11</td>
<td>Boc-Gly-Lys-Arg-AMC</td>
<td>2.29</td>
<td>16.0</td>
<td>7 000 000</td>
</tr>
<tr>
<td>12</td>
<td>Boc-Val-Leu-Lys-AMC</td>
<td>4.34</td>
<td>29.0</td>
<td>6 700 000</td>
</tr>
<tr>
<td>13</td>
<td>Cbz-Arg-AMC</td>
<td>3.03</td>
<td>12.0</td>
<td>4 000 000</td>
</tr>
<tr>
<td>14</td>
<td>Boc-Ala-Gly-Pro-Arg-AMC</td>
<td>5.79</td>
<td>22.0</td>
<td>3 800 000</td>
</tr>
<tr>
<td>15</td>
<td>Boc-Val-Pro-Arg-AMC</td>
<td>9.89</td>
<td>17.0</td>
<td>1 700 000</td>
</tr>
<tr>
<td>16</td>
<td>H-Arg-AMC</td>
<td>54.40</td>
<td>2.7</td>
<td>50 000</td>
</tr>
</tbody>
</table>

$^a$ No activity was detected against Ac-Ala-Ala-Pro-Ala-AMC, H-Gly-AMC; H-Leu-AMC; MeoSuc-Gly-Trp-Met-AMC; Suc-Leu-Tyr-AMC; Glt-Gly-Gly-Phe-AMC or H-Gly-Pro-AMC after 1 h.
the residues Gly, Lys, and Arg were almost equally acceptable in the P₂-position. Comparison of S₇ and S₉ shows that P₂-Arg is preferred over P₂-Phe. Thus, the overall P₂-binding site preference of OP-Tc seems to be (Gly,Lys,Arg) > Phe > Leu > Pro. However, it is also clear that amino acid and other residues in P₃ and P₄ can influence the enzyme interactions with P₂. For example, a comparison of the substrates (S₇ and S₉) versus (S₆ and S₇) shows that, in the pair Boc-Leu-Xaa-Arg-AMC, the substrate with a P₂-Arg is hydrolysed about 10% faster than that with a P₂-Arg, whereas, in the pair Boc-Gly-Xaa-Arg-AMC, the situation is reversed and the substrate with a P₂-Arg is hydrolysed about 200% better than that with a P₂-Lys. Despite such complications, the same general P₂ preference trend (Gly > Phe > Leu > Pro) is also observed in the reactivity of OP-Tc with different peptidyl-inhibitors of OP-Tc (see later).

Because of the apparent cross-talk between the different binding-subsites, it is even more difficult to define the properties of the P₃ and P₄ binding sites of OP-Tc. However, it appears that both subsites are hydrophobic since, for example, the second-order rate constant for the hydrolysis of the substrate Boc-Leu-Lys-Arg-AMC (S₆) is 5-fold greater than that for Boc-Gly-Lys-Arg-AMC (S₉). In this regard it is interesting that the best substrate tested (S₇; Cbz-Arg-Arg-AMC) also has a hydrophobic (benzyloxycarbonyl) group in the P₃ position.

3.3. Enzymatic characterisation of OP-Tc

OP-Tc activity against Cbz-Arg-Arg-AMC peaked at pH 9. However, OP-Tc remained substantially active (73% of maximal activity) at physiological pH (7.4). OP-Tc is primarily a cytosolic enzyme (Morty, unpublished observations) as also is OP-Tb [33]. As the cytosol is a reducing environment, it is possible that intracellular reducing agents such as glutathione and trypanothione may act as in vivo regulators of activity of these oligopeptidases. Indeed, cysteine, reduced glutathione and dithiothreitol, all enhanced OP-Tc activity at 10 mM by 4, 5 and 7-fold, respectively (Table 3). Similarly, the polyamines spermine and spermidine (50 μM) enhanced the activity of OP-Tc against Cbz-Arg-Arg-AMC by 79 and 77%, respectively when compared to untreated OP-Tc. Since trypanosomes contain polyamines, including putrescine and spermidine [34], polyamines may also be involved in the regulation of OP-Tc in vivo. Interestingly, heparin, which carries an opposite charge to polyamines, also enhanced OP-Tc activity by 66% at 30 μg ml⁻¹. Nucleotides (ATP, GTP) and ornithine were without effect. Similarly, chloride salts of Mn²⁺, Mg²⁺, Ba²⁺, Ca²⁺, which do not react with cysteine residues, were without effect (results not shown). In contrast, OP-Tc activity was completely inhibited by the chloride salts of Zn²⁺, Hg²⁺, Cd²⁺ and Fe²⁺ at 1 mM (results not shown), and this probably occurs by the formation of mercaptides between heavy metal ions and a reactive thiol group [35]. These results are consistent with OP-Tc having a cysteine residue which must be maintained in its reduced state to preserve catalytic activity (see also below).

Table 3

<table>
<thead>
<tr>
<th>Compound</th>
<th>Concentration (mM)</th>
<th>Relative activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>–</td>
<td>100</td>
</tr>
<tr>
<td>L-cysteine</td>
<td>10</td>
<td>434</td>
</tr>
<tr>
<td>DTT</td>
<td>10</td>
<td>762</td>
</tr>
<tr>
<td>Reduced glutathione</td>
<td>10</td>
<td>545</td>
</tr>
<tr>
<td>Heparin</td>
<td>30 μg ml⁻¹</td>
<td>166</td>
</tr>
<tr>
<td>Spermine</td>
<td>0.05</td>
<td>179</td>
</tr>
<tr>
<td>Spermidine</td>
<td>0.05</td>
<td>177</td>
</tr>
<tr>
<td>Putrescine</td>
<td>0.05</td>
<td>108</td>
</tr>
</tbody>
</table>

*No effect on activity was observed in the presence of 50 μM ornithine, 1 mM ATP or 1 mM GTP. S.D.s for the activities were < ± 5%.*
OP-Tc was inactivated by non-peptide irreversible inhibitors of serine peptidases (Table 4) at rates comparable to those observed for other serine proteases, including OP-Tb ([3]; Morty et al., manuscript in preparation). These inhibitors included 3,4-dichloroisocoumarin (DCI), 4-(2-iodoacetyl)benzenesulfonyl fluoride (PMSF), N-ethylmaleimide (1900 s) and p-chloromercuribenzoate (1854 s).

This is consistent with the trends observed for the hydrolysis of fluorogenic peptide substrates by OP-Tc (Table 2). Again, proline in P2 appeared to be detrimental to reactivity. OP-Tc was reversibly inhibited by the peptide aldehydes antipain and leupeptin (Table 6), which have the aldehyde on the P1-Arg residue. However, OP-Tc was not inhibited by chymostatin, thus providing further evidence that Phe is not acceptable in P1 (Tables 2 and 5).

Table 4
Inhibition of OP-Tc activity by irreversible inhibitors of cysteine and serine proteinases

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>$k_{ass}$ (M⁻¹ s⁻¹)</th>
<th>$t_1$ (s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AEBSF</td>
<td>22.02 ± 4.05</td>
<td>126</td>
</tr>
<tr>
<td>DCI</td>
<td>167.00 ± 23.60</td>
<td>16</td>
</tr>
<tr>
<td>DFP</td>
<td>13.09 ± 2.17</td>
<td>213</td>
</tr>
<tr>
<td>Iodoacetamide™</td>
<td>1.11 ± 0.71</td>
<td>2 497</td>
</tr>
<tr>
<td>Iodoacetic acid</td>
<td>2.19 ± 0.08</td>
<td>1 265</td>
</tr>
<tr>
<td>N-ethylmaleimide™</td>
<td>1.72 ± 0.11</td>
<td>1 611</td>
</tr>
<tr>
<td>pCMB™</td>
<td>29.10 ± 4.47</td>
<td>95</td>
</tr>
<tr>
<td>PMSF</td>
<td>0.91 ± 0.02</td>
<td>3 046</td>
</tr>
</tbody>
</table>

* Data reflect the mean $k_{ass}$ ± S.D. (n = 3).

* $t_1$ at 250 μM inhibitor concentration.

* Assays conducted in the absence of dithiothreitol. In the presence of 10 mM dithiothreitol, the $t_1$ for the inhibition of OP-Tb by thiol-reactive agents was elevated as follows: iodoacetamide (2520 s), iodoacetic acid (2559 s), N-ethylmaleimide (1900 s) and pCMB (1854 s).

Table 5
Peptidyl chloromethyl ketone inhibitors of OP-Tc

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>$k_{ass}$ (× 10⁻⁶ M⁻¹ s⁻¹)</th>
<th>$t_1$ (s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tos-LysCH₂Cl</td>
<td>4.49 ± 0.23</td>
<td>7.97</td>
</tr>
<tr>
<td>biotin-ArgCH₂Cl</td>
<td>4.05 ± 0.49</td>
<td>9.35</td>
</tr>
<tr>
<td>Boc-Val-Leu-Gly-LysCH₂N₂</td>
<td>2.29 ± 0.36</td>
<td>12.83</td>
</tr>
<tr>
<td>Leu-Glu-Gly-ArgCH₂Cl</td>
<td>2.94 ± 0.27</td>
<td>13.59</td>
</tr>
<tr>
<td>Cbz-Phe-LysCH₂Cl</td>
<td>2.02 ± 0.08</td>
<td>15.57</td>
</tr>
<tr>
<td>Asp-Val-Phe-LysCH₂Cl</td>
<td>1.77 ± 0.16</td>
<td>16.82</td>
</tr>
<tr>
<td>Asp-Val-Leu-LysCH₂Cl</td>
<td>2.07 ± 0.44</td>
<td>20.08</td>
</tr>
<tr>
<td>Asp-Phe-Pro-ArgCH₂Cl</td>
<td>0.99 ± 0.39</td>
<td>32.69</td>
</tr>
</tbody>
</table>

* No inhibition was detected after 30 min pre-incubation with 100 μM Ac-Ala-Ala-Ala-AlaCH₂Cl; Cbz-Gly-Gly-PheCH₂Cl; Cbz-Gly-Leu-PheCH₂Cl; Cbz-Leu-Leu-MetCH₂N₂; biotin-PheCH₂Cl; Tos-PheCH₂Cl; Cbz-Ala-AlaCH₂N₂; Cbz-Ile-LeuCH₂N₂; Cbz-Phe-AlaCH₂Cl; Cbz-Phe-AlaCH₂N₂; Cbz-Phe-Gly-TyrCH₂N₂; Cbz-Phe-PheCH₂Cl; Cbz-Phe-PheCH₂N₂; Cbz-Phe-Tyr(Obu)CH₂N₂ or MeoSuc-Ala-Ala-Pro-ValCH₂Cl.

* $t_1$ at 10 μM inhibitor concentration.

OP-Tc was inhibited rapidly ($k_{ass} > 10^5$ M⁻¹ s⁻¹) by peptidyl chloromethyl ketones (and a diazomethane) with basic residues in P1, but not by related molecules lacking Arg or Lys in P1 (Table 5). This supports the contention that OP-Tc has trypsin-like activity. The inhibition rates also show that the OP-Tc P2-subsite can accommodate small, uncharged and hydrophobic (Gly, Phe, Leu), as well as basic residues (Arg, Lys). This is consistent with the trends observed for the
Curiously, OP-Tc was inhibited competitively by E-64 with a $K_i$ of 73.7 $\mu$M (Table 6). Although this molecule is generally considered to inhibit only cysteine peptidases [38], other workers have demonstrated that E-64 is an effective inhibitor of trypsin [39]. Another unexpected observation was that benzamidine was a comparatively poor inhibitor of OP-Tc ($K_i$ 247 $\mu$M). In contrast, bovine $\beta$-trypsin ($K_i$ 36 $\mu$M) and mast-cell tryptase ($K_i$ 12 $\mu$M) [40] are inhibited well by benzamidine. Whether this reflects a more restricted P$_1$-binding site in OP-Tc, or the need for additional subsite binding by OP-Tc, is not clear. The differential inhibition of OP-Tc by aprotinin ($K_i$ 0.89 nM; Table 6) and soybean trypsin inhibitor (no inhibition) indicates that OP-Tc (like OP-Tb) can accommodate only low molecular mass polypeptides, but not proteins, in its active-site. Such limited reactivity will clearly affect the pattern of host molecule hydrolysis that may be expected should OP-Tc be released into the host bloodstream. The authors have previously illustrated that a trypsin-like serine oligopeptidase is present in the bloodstream of $T. b. brucei$-infected rats [4], and they hypothesise that this activity is released into the host bloodstream by dying parasites. Overall, the enzymatic and physical properties of OP-Tc are quite similar to those for endopeptidases identified in other protozoans including OP-Tb from $T. b. brucei$ [4] although, on average, OP-Tc appears to be about 3-fold less active than OP-Tb. For example, the $K_m$ (0.72 $\mu$M, Table 2) for the hydrolysis of Cbz-Arg-Arg-AMC (S #1) by OP-Tc is 3.5-fold above that obtained for OP-Tb from $T. b. brucei$ (210 nM; Morty et al., manuscript in preparation), and closely approximates those obtained for similar peptidases purified from $Crithidia fasciculata$ (1 $\mu$M [41]) and recombinant oligopeptidase B from $T. cruzi$ (5 $\mu$M [42,43]). However, the enzyme kinetic parameters with different substrates and inhibitors are not the same. For example, the second-order rate constant for the hydrolysis of Cbz-Arg-Arg-AMC (S #1) by OP-Tc is about two-fold greater than that for Cbz-Phe-Arg-AMC (S #3), but for OP-Tb this difference is 6-fold ([4]; Morty et al., manuscript in preparation).

3.4. Immunological studies

Both N’Dama and Boran cattle produced IgG primarily against $T. conglobense$ antigens of 30, 38 and 50 kDa (results not shown). The 38 kDa antigen is possibly trypanopain-Tc [11,44], and its higher immunogenicity in N’Dama cattle is consistent with a previous report that, following a $T. conglobense$ re-infection, IgG to congopain was detected in all N’Dama cattle and in the least affected of the Boran cattle [9]. OP-Tc did not appear to elicit IgG antibodies in N’Dama or Boran cattle.

IgM from both N’Dama and Boran sera targeted $T. conglobense$ antigens primarily at 38 and 80 kDa (results not shown). Boran sera produced a stronger response, supporting earlier observations that susceptible Boran cattle have higher IgM responses, but weaker IgG responses when compared with trypanotolerant N’Dama cattle [9]. Despite an 80 kDa band targeted in $T. conglobense$ lysates, purified OP-Tc was not targeted by IgM from N’Dama or Boran sera.

Taken together, these data show that OP-Tc is not an especially immuno-dominant epitope in $T. conglobense$ infections and that it is not differentially recognised by the humoral immune systems of trypanotolerant and trypanosusceptible cattle. However, because trypanosome-infected animals have uninhibited endopeptidase activity in their blood [4], it will be interesting to examine whether active immunisation with OP-Tc has any effect on the progression of a subsequent challenge infection with $T. conglobense$.

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References


