

Oligopeptidase B from *Trypanosoma brucei*, a New Member of an Emerging Subgroup of Serine Oligopeptidases*

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Trypanosoma brucei contains a soluble serine oligopeptidase (OP-Tb) that is released into the host bloodstream during infection, where it has been postulated to participate in the pathogenesis of African trypanosomiasis. Here, we report the identification of a single copy gene encoding the *T. brucei* oligopeptidase and a homologue from the related trypanosomatid pathogen *Leishmania major*. The enzymes encoded by these genes belong to an emerging subgroup of the prolyl oligopeptidase family of serine hydrolases, referred to as oligopeptidase B. The trypanosomatid oligopeptidases share 70% amino acid sequence identity with oligopeptidase B from the intracellular pathogen *Trypanosoma cruzi*, which has a demonstrated role in mammalian host cell signaling and invasion. OP-Tb exhibited no activity toward the prolyl oligopeptidase substrate *H*-Gly-Pro-7-amido-4-methylcoumarin. Instead, it had activity toward substrates of trypsin-like enzymes, particularly those that have basic amino acids in both P₁ and P₂ (e.g. benzyloxycarbonyl-Arg-Arg-7-amido-4-methylcoumarin $k_{cat}/K_m = 529 \text{ s}^{-1} \mu\text{M}^{-1}$). The activity of OP-Tb was enhanced by reducing agents and by polyamines, suggesting that these agents may act as *in vivo* regulators of OP-Tb activity. This study provides the basis of the characterization of a novel subgroup of serine oligopeptidases from kinetoplastid protozoa with potential roles in pathogenesis.

In this study, we identify and characterize a new member of the prolyl oligopeptidase family of serine hydrolases (the "S9")

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AF078916 and AF109875.

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family in the nomenclature of Barrett and Rawlings (1). This family includes endopeptidases, aminoacylpeptidases, and dipeptidyl aminopeptidases (2). The members of this diverse family share significant amino acid sequence identity within the catalytic domain, and all have activity that is restricted to the hydrolysis of peptides, not proteins (2, 3). Although direct evidence is scarce, proposed roles for these enzymes include neuropeptide and peptide hormone metabolism (4), generation of β -amyloid protein in Alzheimer's disease (5), memory formation (6), regulation of blood pressure (7), DNA synthesis (8), and processing of the mating pheromone α -factor (9).

As suggested by the name, the majority of the prolyl oligopeptidases cleave their substrates after proline residues. Examples of these enzymes are found in some prokaryotes (10, 11), in yeast (9, 12, 13), and in higher eukaryotes (14–17). However, in some cases, the term prolyl oligopeptidase now appears to be an unfortunate misnomer. This is because a smaller subgroup of this family cleaves substrates on the carboxyl side of basic residues (18–20), not prolyl residues. This subfamily is referred to as oligopeptidase B, e.g. *Escherichia coli* protease II or oligopeptidase B (EC 3.4.21.83). Until recently, examples of the oligopeptidase B subfamily were restricted to prokaryotes and had received scant attention. However, studies (including this report) have now shown that oligopeptidase B enzymes are also found in trypanosomatids and that these enzymes may play key roles in disease pathology. In the case of the human pathogen *Trypanosoma cruzi*, the oligopeptidase appears to play a central role in host cell invasion (20, 21). Studies show that oligopeptidase B null mutants of *T. cruzi* have a markedly impaired ability to infect mice or cultured mammalian cells (21). This impairment seems to be mediated by disruption of the oligopeptidase involvement in trypomastigote-induced intracellular Ca^{2+} transients that occur during mammalian host cell invasion (20, 22). The proposed function of the oligopeptidase in *T. cruzi* entry is that of a processing enzyme that generates an active signaling ligand for mammalian host cells (23, 24) through the hydrolysis of a stage-specific precursor (20, 21).

However, as with the larger family of prolyl oligopeptidases, it seems likely that more than one function may be ascribed to this enzyme. All life cycle stages of *T. cruzi* express oligopeptidase B (20), but not all life cycle stages invade mammalian cells. Furthermore, trypsin-like enzymes with properties similar to those of the *T. cruzi* oligopeptidase B have been described in other kinetoplastids, including the pathogenic parasite *Leishmania* (Refs. 25 and 26 and this report), and in the African trypanosomes, *Trypanosoma brucei*, *Trypanosoma vivax*,

and *Trypanosoma congolense* (27–29). If these enzymes are homologues of the *T. cruzi* oligopeptidase B, this raises intriguing questions regarding the roles of these enzymes in parasites with such widely different lifestyles. Thus, one goal of this study was to confirm or refute the contention that the trypsin-like enzymes found in African trypanosomes belong to the oligopeptidase B subfamily of prolyl oligopeptidases.

Oligopeptidase B-like enzymes may be important potential chemotherapeutic targets (as evidenced by the trypanocidal action of many OP-Tb¹ inhibitors (30)). Our studies have indicated that the *T. brucei* oligopeptidase, called OP-Tb, may play an important direct role in the pathogenesis of African trypanosomiasis. During infection, OP-Tb is released into the host bloodstream, where it is insensitive to serum protease inhibitors (28). Hence, it is free to cleave regulatory peptides predicted to be present in host serum. Indeed, the disturbed hormonal pulsatility and endocrine rhythms (31), the unusual cleavage of peptide hormones in the blood of *T. brucei*-infected rats (32), the diminished levels of regulatory peptides such as atrial natriuretic factor (which is a substrate for OP-Tb (28)) (33), and many of the generalized symptoms of trypanosomiasis (34) all point to the possible role of oligopeptidase B in the disruption of host hormone metabolism during trypanosome infection. Here, we report that, on the basis of gene sequence identity and kinetic analyses, the oligopeptidase from *T. brucei* (OP-Tb (28)) is an atypical serine peptidase belonging to the oligopeptidase B subgroup of the prolyl oligopeptidase family. Comparison of the deduced amino acid sequences of the trypanosomatid oligopeptidase B genes, including the *Leishmania major* sequence that we also report here, demonstrates that these enzymes are closely related to the bacterial oligopeptidase B enzymes in terms of sequence identity and substrate specificity. Together, the oligopeptidase B enzymes define a new subgroup of the prolyl oligopeptidase family.

EXPERIMENTAL PROCEDURES

Materials—Fluorogenic peptide substrates were obtained from Sigma, Cambridge Research Biochemicals (Cambridge, United Kingdom), or Enzyme Systems Products (Los Angeles, CA). Peptidyl diazomethyl ketones and chloromethyl ketones were from Bachem (Bubendorf, Switzerland). All other reagents were from Sigma.

Purification of OP-Tb—*T. brucei brucei* (clone ILTat1.1) were passaged in adult male Harlan Sprague-Dawley rats and purified from infected blood by a combination of Percoll isopycnic gradient centrifugation (35) and anion-exchange chromatography on DEAE-cellulose (36). OP-Tb was purified as described previously (28), and the concentration of active enzyme was determined using 4-methylumbelliferyl *p*-guanidobenzoate (37).

Enzyme and Protein Assays—OP-Tb activity was routinely determined against 5 μ M Cbz-Arg-Arg-AMC at 37 °C in assay buffer (50 mM Tris-HCl and 10 mM dithiothreitol (pH 8.0)) (28) in a Hitachi F-2000 spectrofluorometer ($\lambda_{\text{ex}} = 370$ nm, $\lambda_{\text{em}} = 460$ nm). Protein assays were conducted according to the modified (38) method of Bradford (63).

Amino Acid Sequencing—Endoproteinase Lys-C digests of OP-Tb were resolved by reverse-phase high pressure liquid chromatography as detailed previously (39). Amino acid sequencing of the N termini of selected OP-Tb-derived peptides was performed with an Applied Biosystems Model 473A gas-phase sequencer following the manufacturer's instructions.

Oligopeptidase B Gene Cloning—Nitrocellulose filters spotted with a *T. brucei brucei* YATat1.1 cosmid library constructed in a SuperCos vector (provided by Dr. Elisabetta Ullu, Yale University School of Medicine, New Haven, CT) were screened with a ³²P-labeled probe derived from the full-length *T. cruzi* oligopeptidase B gene generated by polymerase chain reaction (PCR) as described (20). Positive clones (obtained from Dr. Sara Melville, *T. brucei* Genome Project, Cambridge, UK) were subcloned into pUC19 following *EcoRI/PstI* digestion. A subclone con-

taining the entire *T. brucei* oligopeptidase B gene open reading frame within a 5.0-kilobase *EcoRI-PstI* insert was sequenced in both directions (GenBank™/EBI accession number AF078916). To obtain the full-length *L. major* oligopeptidase B gene, an *L. major* LV39 sheared DNA cosmid library (40) was screened in the laboratory of Dr. Angela Cruz (Universidade de São Paulo, São Paulo, Brazil) using a ³²P-labeled full-length *T. cruzi* oligopeptidase B gene as a probe. Positive cosmids provided were subcloned into pUC19 following *BamHI* digestion. Sequencing of a positive *BamHI* subclone revealed that this was a partial clone that lacked an initiation codon. Therefore, sequencing was completed using a positive cosmid clone. 4066 base pairs were sequenced in one direction. Second strand sequencing was carried out to confirm the *L. major* oligopeptidase B gene sequence (GenBank™/EBI accession number AF109875).

Generation of Recombinant Oligopeptidase B—The full-length *T. brucei* oligopeptidase B gene was amplified by PCR from the 5.0-kilobase cosmid subclone using primers (forward, 5'-ACTCGGATC-CACCTTCCATCAC-3'; and reverse, 5'-CCTTAGGATCCCAAGTTCAG-3') with built-in *BamHI* sites. PCR was carried out as follows using a Takara LA PCR kit: 94 °C for 2 min; followed by 35 cycles at 94 °C for 1 min, 56 °C for 1 min, and 68 °C for 3 min; and a final 10-min extension step at 72 °C. The resulting PCR product was cloned into pCR2.1 (Invitrogen, Madison, WI) and then excised with *BamHI* to yield a 2.2-kilobase fragment containing the full-length *T. brucei* oligopeptidase B gene. This fragment was ligated to the pET19b expression vector (Novagen, Carlsbad, CA) linearized with *BamHI*. A clone containing an insert with the correct orientation was expressed in *E. coli* BL21 (Novagen). Recombinant *T. brucei* oligopeptidase B was purified on a Ni²⁺-agarose column as described previously (20).

Immunoblot Analysis—Soluble lysates of *T. brucei* YATat1.1 procyclics trypomastigotes, *T. cruzi* tissue culture trypomastigotes, and *L. major* promastigotes were prepared in Dulbecco's phosphate-buffered saline containing 1 mM MgCl₂ and 1 mM CaCl₂ as described (22). 10 μ g of soluble parasite lysates or 100 ng of recombinant peptidase were separated on a Laemmli SDS-polyacrylamide gel under reducing conditions (42) and blotted onto Immobilon™ (Millipore Corp., Bedford, MA). Blots were blocked in antibody dilution buffer (20 mM Tris-HCl (pH 7.4), 150 mM NaCl, 0.05% (v/v) Tween 20, 5% (w/v) nonfat skim milk, 1% (w/v) bovine serum albumin, and 0.1% (w/v) sodium azide) overnight at 4 °C prior to a 1-h incubation at room temperature with 5 μ g ml⁻¹ polyclonal anti-*T. cruzi* recombinant oligopeptidase B IgG (20). Following five 10-min washes in 20 mM Tris-HCl (pH 7.4), 150 mM NaCl, and 0.05% Tween 20, blots were incubated for 1 h with horseradish peroxidase-conjugated goat anti-rabbit IgG (Kirkegaard & Perry Laboratories, Inc., Gaithersburg, MD) diluted to 1:10,000 and developed using the ECL system (Amersham Pharmacia Biotech).

Kinetic Analyses—Substrate specificity of OP-Tb was determined using fluorogenic substrates by preincubation of OP-Tb (1.5 ng, 18.75 fmol of active enzyme, 37 °C, 5 min) or recombinant oligopeptidase (2 ng) in assay buffer, followed by addition of substrate. The initial steady-state velocity (v_0) was determined by continuous assay for a range of substrate concentrations (45 nM to 75 μ M). K_m and V_{max} were determined by hyperbolic regression of the kinetic data using the software package Hyper Version 1.01 (obtained from Dr. J. S. Easterby, University of Liverpool, Liverpool, UK). The k_{cat} was determined from $k_{\text{cat}} = V_{\text{max}}/[E]_0$, where $[E]_0$ represents the active enzyme concentration.

The pH profile for OP-Tb was conducted as described above, except that constant ionic strength acetate/Mes/Tris (AMT) buffers (100 mM acetic acid, 200 mM Tris-HCl, 100 mM Mes, 1 mM dithiothreitol, and 4 mM EDTA, $I = 0.1$) over the pH range 4–12 (43) replaced the assay buffer. Similarly, pH stability of OP-Tb was investigated by preincubating OP-Tb (15 ng, 188 fmol of active enzyme, 5 min) in the same set of AMT buffers (37 °C, 1 h) before assaying residual activity of a 10- μ l aliquot in AMT buffer at pH 8.

The effect of reducing agents on OP-Tb activity was investigated by preincubating OP-Tb in assay buffer containing dithiothreitol, GSH, or L-cysteine (1–25 mM, 37 °C, 5 min) prior to addition of Cbz-Arg-Arg-AMC (5 μ M final concentration). To test for dimerization of OP-Tb under nonreducing conditions, OP-Tb was preincubated in 50 mM Tris-HCl (pH 8) in the absence or presence of dithiothreitol (10 mM) for 5 days at 4 °C. Samples (25 μ l, containing 50 ng of OP-Tb) were subsequently resolved by molecular exclusion chromatography on a Sephacryl S-200 HR column (900 \times 15 mm, 0.3 ml min⁻¹, 4 °C) equilibrated in the preincubation buffer. Column fractions were assayed for activity against Cbz-Arg-Arg-AMC as described above. Column fractions collected under nonreducing conditions were reduced prior to assaying by preincubation with 10 mM dithiothreitol to reactivate inactive OP-Tb. The effects of divalent metal ions, nucleotides, polyamines, and heparin

¹The abbreviations used are: OP-Tb, endogenous oligopeptidase B from *T. brucei*; Cbz, benzoyloxycarbonyl; AMC, 7-amino-4-methylcoumarin; PCR, polymerase chain reaction; Mes, 4-morpholineethanesulfonic acid; Boc, *t*-butoxycarbonyl.

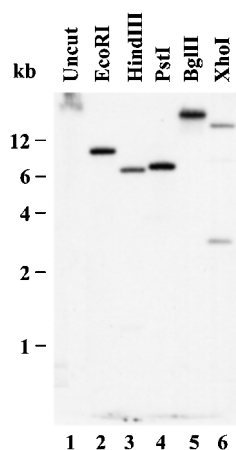


FIG. 2. Southern blotting to determine the copy number of the *T. brucei* oligopeptidase B gene. *T. brucei* DNA was digested with no enzyme (lane 1), *EcoRI* (lane 2), *HindIII* (lane 3), *PstI* (lane 4), *BglIII* (lane 5), or *XhoI* (lane 6). Digested DNA was separated on 0.8% agarose gels and transferred to a nylon membrane. Hybridization was carried out overnight at 42 °C in 5× SSC, 50% formamide, 5× Denhardt's solution, and 0.5% (w/v) SDS using the [³²P]dCTP-labeled full-length *T. brucei* *opdB* gene as a probe. The blot was washed to a final stringency of 0.2× SSC at 60 °C and subjected to autoradiography. *kb*, kilobases.

and is predicted to encode a polypeptide of 715 amino acids (Fig. 1). The amino acid sequence of the OP-Tb-derived peptides precisely matched peptide sequences found in the deduced amino acid sequence of the *T. brucei* oligopeptidase B gene (Fig. 1), and we therefore conclude that the *T. brucei* enzyme (OP-Tb) is encoded by the gene for oligopeptidase B. Similarly, the full-length gene encoding the *L. major* oligopeptidase B gene (2196 base pairs) was isolated and sequenced and encodes a similar protein of 732 amino acids (GenBank™/EBI accession number AF109875) (data not shown). Southern blot analysis using homologous probes revealed that the oligopeptidase B genes of *T. brucei* (Fig. 2) and *L. major* (data not shown) are present as single copy genes per haploid genome, as previously shown for the *T. cruzi* oligopeptidase B gene (20).

The deduced amino acid sequences of the three trypanosomatid oligopeptidase B enzymes exhibited significant homology over their entire sequences. The *T. brucei* oligopeptidase B is 71% identical to the *T. cruzi* oligopeptidase and 67% identical to the *L. major* oligopeptidase. The overall similarity of their respective amino acid sequences is 80%. The similarity of the peptidases was further demonstrated by immunoblot analysis. Polyclonal antibodies generated against the *T. cruzi* oligopeptidase B readily reacted with oligopeptidase B in lysates of *T. brucei* (Fig. 3, lane 4) and *T. cruzi* (lane 5; see also Ref. 20). The full-length *T. brucei* oligopeptidase B gene was expressed in *E. coli* as a catalytically active (Table I), histidine-tagged recombinant enzyme (Fig. 3, lane 1) with a yield of ~12 mg/liter of bacterial culture. Immunoblot analysis demonstrated that it was detected at the expected size on a Western blot (Fig. 3, lane 2), similar to the *T. cruzi* recombinant enzyme (lane 5).

OP-Tb Substrates—Consistent with the properties of the prolyl oligopeptidase family (2), our previous findings have demonstrated that OP-Tb is unable to hydrolyze polypeptide substrates (28), including mammalian plasma proteins.² Therefore, fluorogenic peptide substrates were employed for the enzymatic characterization of the native and recombinant forms of the *T. brucei* oligopeptidase B. The reactions followed Michaelis-Menten kinetics. The K_m values obtained using the recombinant *T. brucei* enzyme approximated those obtained for the purified native enzyme (Table I).

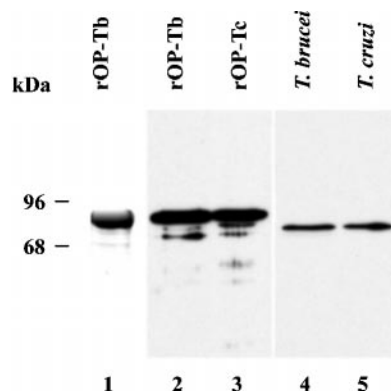


FIG. 3. Analysis of native oligopeptidase B in trypanosomatid extracts and purified recombinant oligopeptidase B. 15 μg of recombinant oligopeptidase B (*rOP-Tb*) were evaluated for purity on a Coomassie Blue-stained 10% SDS-polyacrylamide gel (lane 1). 10 μg of soluble extracts prepared from *T. brucei* (lane 2) and *T. cruzi* (lane 3) and 100 ng of recombinant oligopeptidase B from *T. brucei* (lane 4) and *T. cruzi* (lane 5) were analyzed by immunoblot analysis with polyclonal anti-*T. cruzi* oligopeptidase B antibody as described under "Experimental Procedures." *rOP-Tc*, recombinant oligopeptidase B from *T. cruzi*.

In contrast to the prolyl oligopeptidase class of enzymes, OP-Tb exhibited no activity against *H*-Gly-Pro-AMC (Table I). Instead, peptide hydrolysis by OP-Tb indicated that the enzyme has a trypsin-like specificity. The presence of basic amino acid residues in the P₁ position (nomenclature of Ref. 49) was obligatory (see Table I). The poor k_{cat}/K_m for *H*-Arg-AMC (0.07 s⁻¹ μM⁻¹) and the lack of activity against *H*-Gly-AMC and *H*-Leu-AMC suggest that OP-Tb is not an aminopeptidase. (This conclusion is also supported by failure of the aminopeptidase inhibitors amastatin and bestatin to inhibit OP-Tb (see Table III).) In contrast, the equivalent substrate with its N terminus blocked with a Cbz group (*i.e.* Cbz-Arg-AMC) had an elevated k_{cat}/K_m (157-fold). Thus, substrate binding is more successful when both P₁ and P₂ are occupied.

A variety of residues were acceptable in the P₂ position, including Arg, Lys, Phe, Leu, Gly, and Pro. Substitution of Arg in P₂ with Lys in Boc-Leu-Arg-Arg-AMC had little (0.01% increase) effect on k_{cat}/K_m , indicating that Lys and Arg are equally acceptable in P₂ in this situation. However, substitution of Arg in P₂ with Lys in Boc-Gly-Arg-Arg-AMC resulted in a 4-fold decrease in k_{cat}/K_m . Thus, the substitution of Leu for Gly in P₃ had a substantial effect on the P₂ preference for Arg or Lys. A comparison of Cbz-Arg-Arg-AMC and Cbz-Phe-Arg-AMC hydrolysis indicates that Arg is preferred over Phe in P₂, with a 6.5-fold decrease in k_{cat}/K_m for the Phe-containing substrate. A comparison of Boc-Val-Gly-Arg-AMC versus Boc-Val-Pro-Arg-AMC hydrolysis illustrates that replacing Pro with Gly in P₂ is accompanied by a 8.5-fold increase in k_{cat}/K_m . Thus, the overall P₂ preference appears to be Arg/Lys > Gly > Phe > Pro.

OP-Tb Inhibitors—Both native and recombinant OP-Tb were inactivated by irreversible inhibitors of serine peptidases (Table II). The most potent of these inhibitors was 3,4-dichloroisocoumarin, which had a k_a of 142 M⁻¹ s⁻¹, similar to the k_a values for 3,4-dichloroisocoumarin inhibition of bovine trypsin (198 M⁻¹ s⁻¹) and human plasmin (133 M⁻¹ s⁻¹) (50). OP-Tb was also inactivated by 4-(2-aminoethyl)benzenesulfonyl fluoride, diisopropyl fluorophosphate, and phenylmethanesulfonyl fluoride. The rates of inactivation by these compounds ranged from 10- to 30-fold faster than those reported for the inhibition of serum kallikrein (51).

OP-Tb was also inhibited by the peptide aldehydes antipain and leupeptin, which contain the aldehyde on Arg in P₁ (Table III). The lower K_i and higher k_a of antipain are a likely conse-

² R. E. Morty, unpublished data.

TABLE I
Amidolytic activity of native and recombinant OP-Tb

Substrate ^a	OP-Tb			rOP-Tb ^b		
	k_{cat}/K_m $s^{-1} \mu\text{M}^{-1}$	k_{cat} s^{-1}	K_m ^c μM	k_{cat}/K_m $s^{-1} \mu\text{M}^{-1}$	k_{cat} s^{-1}	K_m ^c μM
Cbz-Arg-Arg-AMC	528.6	111.0	0.21	612.5	147.0	0.24
Cbz-Gly-Gly-Arg-AMC	157.8	142.0	0.91	92.6	176.0	1.90
Cbz-Phe-Arg-AMC	82.1	92.0	1.12	121.0	121.0	1.00
Cbz-Ala-Arg-Arg-AMC	54.3	120.0	2.21	54.4	157.0	2.91
Boc-Leu-Lys-Arg-AMC	52.4	44.0	0.84	42.2	76.0	1.80
Boc-Leu-Arg-Arg-AMC	52.36	60.0	1.14	77.0	67.0	0.87
Boc-Gly-Arg-Arg-AMC	51.9	97.0	1.87	98.3	118.0	1.22
Boc-Leu-Gly-Arg-AMC	42.5	54.0	1.27	39.0	87.0	2.23
Boc-Val-Gly-Arg-AMC	38.9	86.0	2.21	29.3	89.0	3.03
H-Ala-Phe-Lys-AMC	18.2	57.0	3.13	23.1	67.0	2.81
Boc-Gly-Lys-Arg-AMC	12.5	39.0	3.12	24.5	55.0	2.24
Boc-Val-Leu-Lys-AMC	12.4	50.0	4.04	11.1	67.0	6.06
Cbz-Arg-AMC	11.0	30.0	2.73	28.2	62.0	2.20
Boc-Ala-Gly-Pro-Arg-AMC	7.2	51.0	7.05	6.3	77.0	12.21
Boc-Val-Pro-Arg-AMC	4.67	46.0	9.89	5.0	62.0	12.43
H-Arg-AMC	0.07	4.6	61.6	0.34	16.6	47.1

^a No activity was detected against acetyl-Ala-Ala-Pro-Ala-AMC, H-Gly-AMC, H-Leu-AMC, methoxysuccinyl-Gly-Trp-Met-AMC, succinyl-Leu-Tyr-AMC, H-Gly-Pro-AMC, or glutaryl-Gly-Gly-Phe-AMC after 30 min of incubation.

^b rOP-Tb, recombinant histidine-tagged OP-Tb.

^c The S.E. for the K_m was within 5% of the mean.

TABLE II
Irreversible inhibitors of native and recombinant OP-Tb

Inhibitor	OP-Tb		rOP-Tb ^a	
	k_a ^b $M^{-1} s^{-1}$	$t_{1/2}$ ^c s	k_a ^b $M^{-1} s^{-1}$	t ^c s
DCI	142.10 ± 11.90	18	196.00 ± 8.01	14
AEBSF	14.00 ± 2.07	196	15.36 ± 2.21	180
DFP	7.40 ± 0.79	375	ND	ND
PMSF	0.60 ± 0.02	4620	1.96 ± 0.08	1414
pCMB ^d	21.90 ± 4.47	126 (1854)	27.60 ± 4.04	100
N-Ethylmaleimide ^d	1.57 ± 0.11	1765 (1900)	1.76 ± 0.19	1578
Iodoacetic acid ^d	1.91 ± 0.08	1451 (2559)	3.59 ± 0.44	771
Iodoacetamide ^d	1.27 ± 0.71	2182 (2520)	1.60 ± 0.27	1736

^a rOP-Tb, recombinant histidine-tagged OP-Tb; DCI, 3,4-dichloroisocoumarin; AEBSF, 4-(2-aminoethyl) benzenesulfonyl fluoride; DFP, diisopropyl fluorophosphate; PMSF, phenylmethanesulfonyl fluoride; pCMB, *p*-chloromercuribenzoate; ND, not determined.

^b Data reflect the mean k_a ± S.D. ($n = 3$).

^c $t_{1/2}$ at 250 μM inhibitor.

^d Assays were conducted in the absence of dithiothreitol. The values in parentheses are the t for reactions in the presence of 10 mM dithiothreitol.

quence of the comparatively basic nature of the antipain tripeptide. No inhibition was observed for chymostatin, where the aldehyde is present on Phe in P₁. This supports our contention that Phe is not readily accepted in the P₁-binding site (Tables I and III). Interestingly, OP-Tb was inhibited competitively by E-64 (*trans*-epoxysuccinyl-L-leucylamido-(4-guanidino)butane), with a K_i of 62.5 μM . This contrasts with the widely held view that E-64 is a class-specific inhibitor of cysteine peptidases (52), although the inhibition of bovine β -trypsin by E-64 by a reversible competitive mechanism with a K_i of 36 μM has also been reported (53). Benzamidine, a low molecular mass inhibitor of trypsin-like peptidases, was a comparatively poor inhibitor of OP-Tb, with a K_i of 254 μM , compared with K_i values of 36 μM for bovine β -trypsin and 12 μM for mast cell tryptase (54). This supports our earlier suggestion that substrate or inhibitor binding is more effective when two or more substrate-binding sites are occupied (Table I). The lack of inhibition by EDTA, EGTA, and 1,10-phenanthroline reinforces the idea that OP-Tb has no metal ion dependence.

OP-Tb Effectors—OP-Tb activity was enhanced by several reducing agents, including dithiothreitol, glutathione, and cysteine. Maximal activation (~3-fold) occurred in the presence of 10 mM dithiothreitol (Fig. 4A). This enhancement does not appear to result from the reduction of catalytically inactive disulfide-bonded multimers (as has been demonstrated recently for the thermolysin-like metallo-oligopeptidase (soluble

metallo-endopeptidase, EC 3.4.24.15) (55)). No significant difference was observed in the elution profiles for two samples of purified OP-Tb fractionated by molecular exclusion chromatography under reducing and nonreducing conditions (Fig. 4B). This suggests that no inactive high molecular mass complexes were formed under our *in vitro* experimental conditions.

OP-Tb was maximally stable at neutral pH in the absence of dithiothreitol (Fig. 5C). Although OP-Tb had maximal activity at pH 9 (Fig. 5A), it retained considerable activity (75% of maximal activity) at physiological pH (pH 7.4). Over the pH range studied, pH exerted a dramatic effect on the k_{cat} (up to 100-fold), whereas the K_m was relatively unaffected (1.6-fold) (Fig. 5B). The shape of the curve suggests that OP-Tb activity is dependent upon residues with pK_a values of ~6 and 10. This is consistent with the ionization of active-site histidine and serine residues, respectively, of serine peptidases (56).

The activity of OP-Tb (against Cbz-Arg-Arg-AMC in the presence of 50 μM polyamines) was enhanced by spermine and spermidine (77 and 62%, respectively, over the control values; data not shown). Putrescine and ornithine had no effect. Curiously, heparin, which carries an opposite charge to polyamines, also enhanced OP-Tb activity (by 58%) at 30 $\mu\text{g ml}^{-1}$. Neither ATP nor GTP had any effect on the activity of OP-Tb, which is consistent with its being unrelated to the ATP-dependent peptidases.

TABLE III
Competitive reversible inhibitors of OP-Tb

Inhibitor	K_i^a	k_a	k_d
	μM	$\text{M}^{-1} \cdot \text{s}^{-1}$	s
Leupeptin	30.08×10^{-3}	4.76×10^4	1.43×10^3
Antipain	1.81×10^{-3}	1.08×10^6	1.96×10^3
E-64	62.5	ND ^b	ND
Benzamidine	254	ND	ND

^a Values for the recombinant enzyme were within 5% of those obtained for the native enzyme. No inhibition was observed with amastatin (125 μM), bestatin (125 μM), chicken ovomucoid (100 $\mu\text{g ml}^{-1}$), chymostatin (1–100 μM), EDTA (1 mM), EGTA (1 mM), elastatinal (150 μM), lima bean trypsin inhibitor (100 $\mu\text{g ml}^{-1}$), pepstatin (1 μM), 1,10-phenanthroline (1 mM), or soybean trypsin inhibitor (100 $\mu\text{g ml}^{-1}$).

^b ND, not determined. In these cases, the k_a was too fast to be measured experimentally.

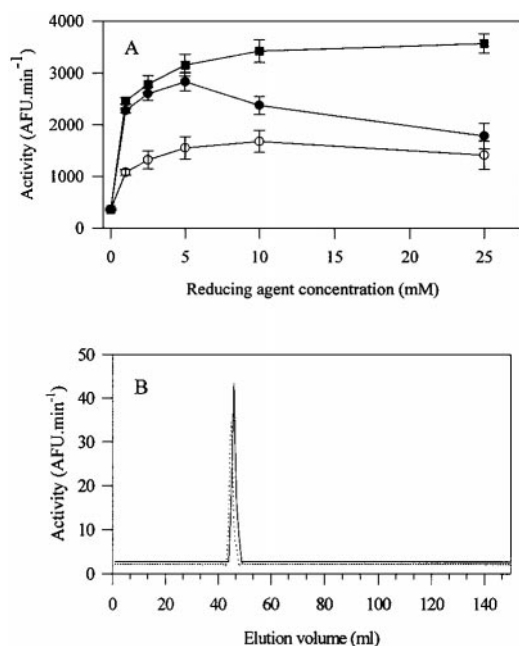


FIG. 4. **Effect of reducing agents on OP-Tb activity.** A, OP-Tb (1.5 ng) was assayed in 100 mM Tris-HCl (pH 8) containing dithiothreitol (■), reduced glutathione (●), or L-cysteine (○) at various concentrations. Data points represent the means \pm S.E. ($n = 3$). B, OP-Tb was resolved under reducing or nonreducing conditions by molecular exclusion chromatography on a Sephacryl S-200 HR column (900 \times 15 mm, 0.3 ml min^{-1} , 4 $^\circ\text{C}$). Column fractions were assayed for activity against 5 μM Cbz-Arg-Arg-AMC in the presence of 10 mM dithiothreitol. AFU, arbitrary fluorescence units.

DISCUSSION

We have previously reported that bloodstream forms of *T. brucei* possess a high molecular mass trypsin-like serine peptidase (27) and that this enzyme is released into the blood of *T. brucei*-infected rats (28). The present studies show that this trypsin-like enzyme does not belong to the classic class of trypsins, but instead belongs to the serine peptidases of the prolyl oligopeptidase subgroup. The classic trypsins and chymotrypsins all require a free N-terminal amino acid for full expression of enzymatic activity, and yet, abundant activity can be found in OP-Tb, which has a blocked N terminus, and in recombinant OP-Tb, which has an N-terminal polyhistidine tag. Clearly, a free N terminus is not required by this enzyme. Additional differences exist. For example, we see no evidence of a zymogen form (inactive precursor) of OP-Tb. Since each life cycle stage (29) of *T. brucei* possesses this cytosolic (28, 57) enzyme, alternate means of controlling its activity must exist. As trypanosomes are known to contain the polyamines spermine and spermidine (58) as well as a number of intracellular

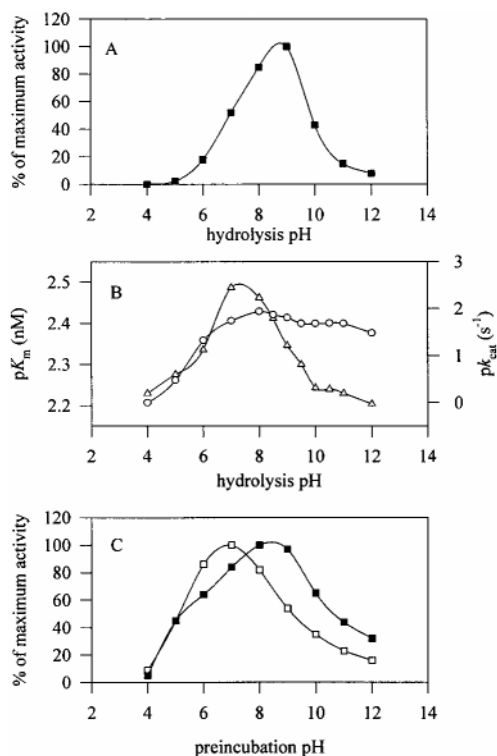


FIG. 5. **Effect of pH on the activity and stability of OP-Tb.** A, OP-Tb (1.5 ng) was assayed in AMT buffers ($I = 0.1$) over the pH range 4.0–12.0 in the presence of 10 mM dithiothreitol. B, shown are the individual effects of pH on the K_m (Δ) and k_{cat} (\circ) from the data presented in A. C, OP-Tb (10 ng) was incubated for 1 h at 37 $^\circ\text{C}$ in AMT buffers ($I = 0.1$) over the pH range 4.0–12.0 in the absence (\square) and presence (\blacksquare) of 10 mM dithiothreitol. Residual enzymatic activity against Cbz-Arg-Arg-AMC was then determined in AMT buffer at pH 8.

reducing agents such as trypanothione (59), we tested to see if such molecules might regulate the activity of OP-Tb. The activity of OP-Tb was enhanced by reducing agents and by spermine and spermidine. Curiously, dithiothreitol has no enhancing effect on the catalytic activity of the prolyl oligopeptidase from human brain (60) despite its apparent similarity to OP-Tb.

The activity of OP-Tb was inhibited by thiol-reactive agents such as iodoacetate. Because we now know that OP-Tb belongs to the prolyl oligopeptidase group of enzymes, rather than the classic serine protease group, such inhibition is now understandable. It is likely that inhibition by thiol reagents is explained by a crucial cysteine residue (Cys²⁵⁵ in porcine prolyl oligopeptidase and perhaps Cys²⁵⁶ in OP-Tb) that is in close proximity to the catalytic site in the folded enzyme (61). Covalent attachment of bulky thiol-reactive groups to this cysteine residue is predicted to interfere, by steric hindrance, with either the substrate binding or the charge relay system of the catalytic residues.

Prior to the sequencing of the *T. brucei* and *L. major* oligopeptidase B genes, the closest homologues of the *T. cruzi* enzyme were the oligopeptidase B enzymes from *E. coli* and *M. lacunata* (20). Here, we find that the trypanosomatid enzymes share $\sim 32\%$ identity with the *E. coli* and *M. lacunata* prolyl oligopeptidases, but exhibit less identity to other “true” post-proline-cleaving enzymes (in the range of 20%). Since sequence homology among members of the prolyl oligopeptidase family is greatest within the catalytic domain (2, 61), this region of the trypanosomatid oligopeptidase B enzymes was aligned with several prolyl oligopeptidases (Fig. 6). The oligopeptidase B enzymes possess the GX₂SGGZZ consensus sequence (where X is any residue and Z is a hydrophobic residue) (2) containing the catalytic serine residue (Ser⁵⁶³ in *T. brucei* (Fig. 1) and

Cleavage specificity



FIG. 6. Alignment of the catalytic domains of oligopeptidase B and prolyl oligopeptidase. Shown are the amino acid residues composing the predicted catalytic domains of the prolyl oligopeptidase family based on the structure of the porcine brain prolyl oligopeptidase (residues 428–710) (61); residues 436–716 of *T. brucei* oligopeptidase B (GenBank™/EBI accession number AF078916), residues 435–714 of *T. cruzi* oligopeptidase B (accession number U69897 (20)), residues 450–731 of *L. major* oligopeptidase B (accession number AF109875), residues 408–682 of *M. lacunata* oligopeptidase B (accession number D38405 (19)), residues 407–691 of *E. coli* oligopeptidase B (accession number D10976 (18)), residues 414–690 of *Aeromonas hydrophila* prolyl oligopeptidase (accession number 730361 (11)), residues 432–705 of *Flavobacterium meningosepticum* prolyl oligopeptidase (accession number 130759 (10)), residues 428–710 of human T cells (accession number 1346769 (41)), and porcine brain prolyl oligopeptidases (accession number 130759 (61)). The cleavage specificities of the oligopeptidase B and post-prolyl-cleaving enzymes are indicated on the right.

Ser⁵⁷⁷ in *L. major*) and exhibit considerable sequence conservation within the catalytic domain. However, even within this highly conserved region, it is clear that the oligopeptidase B subfamily of enzymes (*T. brucei*, *T. cruzi*, *L. major*, *M. lacunata*, and *E. coli*) exhibit greater homology to each other than to the post-prolyl-cleaving enzymes (Fig. 6). Furthermore, the kinetoplastid (*T. brucei*, *T. cruzi*, and *L. major*) oligopeptidase B enzymes are even more similar to each other, exhibiting an overall identity of 70%. Since the oligopeptidase B enzymes can be distinguished from the true prolyl oligopeptidases using sequence identity and substrate specificity as criteria, we propose that the oligopeptidase B enzymes constitute a subfamily of the prolyl oligopeptidase family defined by Barrett and Rawlings (2).

The difference in substrate specificity between the prolyl oligopeptidases and the oligopeptidase B enzymes may be explained by comparing the structure and sequence of the prolyl oligopeptidases with the sequences of the oligopeptidase B enzymes (Fig. 6). Several residues in the active-site pocket are predicted to be involved in substrate recognition by the prolyl oligopeptidases (61). Among these residues is a tryptophan (Trp⁵⁹⁵ in porcine oligopeptidase) that may be involved in stabilizing the interaction with the P₁ proline of the substrate (61). Although this tryptophan residue is conserved in the oligopeptidase B enzymes (Trp⁶⁰⁸ in *T. brucei*) (Fig. 6), it is surrounded by conserved glutamic acid residues in the oligopeptidase B enzymes, but these are absent in the post-proline-cleaving enzymes. Thus, it seems most likely that these negatively charged residues contribute to the recognition of basic substrates by the oligopeptidase B enzymes.

No information regarding the three-dimensional structure of

oligopeptidase B is available, although the structure of porcine prolyl oligopeptidase has been reported recently (61). The active sites of prolyl oligopeptidases were proposed to lie buried in active-site "pits" (2). This suggestion is consistent with the structural observation that access of proteins to the catalytic site of porcine prolyl oligopeptidase is likely to be impeded by the positioning of the catalytic apparatus in a tunnel-like cavity (61). This suggestion is supported by our observations reported here and the previously reported observations of others (48) that oligopeptidase B is unable to hydrolyze proteins and that its activity is not inhibited by high molecular mass peptidase inhibitors.

Although the preponderance of known members of the prolyl oligopeptidase family are post-proline-cleaving peptidases, our data indicate that a subgroup of related peptidases is emerging that exhibits specificity for substrates containing paired basic amino acids. To date, these oligopeptidase B enzymes have been identified only in prokaryotes and kinetoplastid protozoan parasites. No oligopeptidase B enzymes have been identified in or cloned from mammalian cells. The homologous enzymes from *Saccharomyces cerevisiae* are specific for cleavage after proline residues (9, 13). The preference of oligopeptidase B for cleavage after paired basic residues is intriguing since these sites are abundant in precursors of biologically active molecules and are recognized as sites for processing (62). It was previously suggested that the oligopeptidase B enzymes might function as processing enzymes involved in the generation of biologically active peptides (20, 48). Recently, oligopeptidase B from *T. cruzi* was demonstrated to have a role in the generation of a signaling ligand for mammalian host cells that is involved in the mechanism of host cell invasion by this intracellular

pathogen (20, 21). Although the physiological function of other oligopeptidase B enzymes is currently unclear, *T. brucei* OP-Tb may play a major role in pathogenesis of disease through the degradation of regulatory peptide hormones in the blood of infected hosts in African trypanosomiasis (28). Further structural and functional characterization of the oligopeptidase B enzymes in prokaryotes and kinetoplastid protozoan parasites will be useful to better understand the functions this subgroup of the prolyl oligopeptidase family carries out in these organisms and may provide insights into the evolutionary role of this enzyme.

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