Subsite specificity (S3, S2, S1′, S2′ and S3′) of oligopeptidase B from *Trypanosoma cruzi* and *Trypanosoma brucei* using fluorescent quenched peptides: comparative study and identification of specific carboxypeptidase activity

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We characterized the extended substrate binding site of recombinant oligopeptidase B enzymes from *Trypanosoma cruzi* (Tc-OP) and *Trypanosoma brucei* (Tb-OP), evaluating the specificity of their S3, S2, S1′, S2′ and S3′ subsites. Five series of internally quenched fluorescent peptides based on the substrate Abz-AGGRGAQ-EDDnp [where Abz is *o*-aminobenzoic acid and EDDnp is *N*-(2,4-dinitrophenyl)ethylenediamine] were designed to contain amino acid residues with side chains of a minimum size, and each residue position of this substrate was modified. Synthetic peptides of different lengths derived from the human kininogen sequence were also examined, and peptides of up to 17 amino acids were found to be hydrolysed by Tc-OP and Tb-OP. These two oligopeptidases were essentially arginyl hydrolases, since for all peptides examined the only cleavage site was the Arg–Xaa bond. We also demonstrated that Tc-OP and Tb-OP have a very specific carboxypeptidase activity for basic amino acids, which depends on the presence of at least of a pair of basic amino acids at the C-terminal end of the substrate. The peptide with triple Arg residues (Abz-AGRRRAQ-EDDnp) was an efficient substrate for Tc-OP and Tb-OP: the Arg–Ala peptide bond was cleaved first and then two C-terminal Arg residues were successively removed. The S1′ subsite seems to be an important determinant of the specificity of both enzymes, showing a preference for Tyr, Ser, Thr and Gln as hydrogen donors. The presence of these amino acids at P1′ resulted in substrates that were hydrolysed with K_m values in the sub-micromolar range. Taken together, this work supports the view that oligopeptidase B is a specialized protein-processing enzyme with a specific carboxypeptidase activity. Excellent substrates were obtained for Tb-OP and Tc-OP (Abz-AMRRTISQ-EDDnp and Abz-AHKRYSHQ-EDDnp respectively), which were hydrolysed with remarkably high k_{cat} and low K_m values.

Key words: arginyl hydrolase, Chagas disease, prolyl oligopeptidase, sleeping sickness.

INTRODUCTION

Oligopeptidase B (EC 3.4.21.83) is a member of the prolyl oligopeptidase family of serine peptidases (clan SC, family S9 in the nomenclature of [1]). Substrates for these peptidases are restricted to low-molecular-mass peptides, since the N-terminal β-propeller domain of prolyl oligopeptidases blocks access of large globular proteins to the catalytic machinery [2]. While most members of this peptidase family hydrolyse peptide bonds at the C-terminal side of proline residues [1], oligopeptidase B exhibits a trypsin-like substrate specificity, cleaving peptides after basic residues (arginine or lysine). Oligopeptidase B hydrolyses peptides containing two adjacent basic residues (e.g. N^{α}-carbobenzyloxy-Arg-Arg-7-amido-4-methylcoumarin; k_{cat}/K_m 63 $\mu M^{-1} \cdot s^{-1}$) significantly faster than substrates containing a single basic residue (e.g. N^{α}-carbobenzyloxy-Phe-Arg-7-amido-4-methylcoumarin; k_{cat}/K_m 1.4 $\mu M^{-1} \cdot s^{-1}$), which may indicate that this enzyme is a new type of processing enzyme. In support of this idea, bacterial oligopeptidase B cleaves globular proteins, albeit in a highly restricted fashion [3].

Oligopeptidase B was first cloned and characterized from *Escherichia coli* [4,5], and has also been described in the prokaryotes *Moraxela lacunata* [6], *Treponema denticula* [7] and *Salmonella enterica* serovar *typhimurium* [3,8]. In addition, oligopeptidase B enzymes have been described in *Trypanosoma cruzi* (Tc-OP), the causative agent of Chagas' disease in humans [9], and in African trypanosomes of the *Trypanosoma brucei* group (Tb-OP) [10], which are of significant medical, veterinary and agricultural importance in Africa. Tc-OP generates a calcium signalling factor that interacts with a receptor at the mammalian cell surface [11], mobilizing Ca^{2+} from intracellular pools [9] and promoting trypanosome invasion. Targeted deletion of the *opdB* gene in *Trypanosoma cruzi* severely attenuated parasite virulence *in vivo* [12]. African trypanosomes release oligopeptidase B into the host bloodstream, where it persists and retains catalytic activity, and may promote disease pathogenesis through the anomalous degradation of host peptide hormones [13].

Since oligopeptidase B has been identified as a target of several drugs used to treat African trypanosomiasis [14], and since irreversible inhibitors of Tb-OP exhibit anti-trypanosomal

Abbreviations used: Abz, *o*-aminobenzoic acid; DTT, dithiothreitol; EDDnp, *N*-(2,4-dinitrophenyl)ethylenediamine; IQF peptide, internally quenched fluorescent peptide; MALDI-TOF, matrix-assisted laser-desorption ionization–time-of-flight; Tb-OP, oligopeptidase B from *Trypanosoma brucei*; Tc-OP, oligopeptidase B from *Trypanosoma cruzi*.

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activity *in vitro* and *in vivo* [15], the therapeutic potential of oligopeptidase B inhibitor warrants further study. However, such studies are difficult, because the P3–P1 specificity (nomenclature of [16]) of oligopeptidase B parallels that of many mammalian plasma serine peptidases, including members of the clotting and complement cascades [17]. Indeed, irreversible inhibitors of Tb-OP that possess anti-trypanosomal activity also prolong the prothrombin and activated partial thromboplastin times in mice [18], possibly accounting for the observed toxicity of these compounds when used to treat experimental trypanosome infections in a rodent model [15]. These findings have led us to explore other unique properties of oligopeptidase B that would distinguish this peptidase from other mammalian serine peptidases, in the hope that these properties will facilitate the development of specific inhibitors that would selectively inhibit oligopeptidase B in cell culture and animal models of infection. One such property that we deem worth investigating is the extended substrate binding site of oligopeptidase B.

To characterize in detail the extended substrate binding site of recombinant Tc-OP and Tb-OP, we evaluated their determinants of specificity at the S3, S2, S1′, S2′ and S3′ subsites. For these studies, five series of internally quenched fluorescent (IQF) peptide substrates were synthesized, based on the general structure Abz-AGGRGAQ-EDDnp [where Abz is *o*-aminobenzoic acid and EDDnp is *N*-(2,4-dinitrophenyl)ethylenediamine], which was designed to contain amino acids with residue side chains of a minimum size. Each position of this substrate was substituted with natural amino acids, and the kinetic parameters for their hydrolysis by Tc-OP and Tb-OP were determined. Substrates of different lengths were also examined in order to verify the maximum substrate size accepted for hydrolysis by Tc-OP and Tb-OP.

MATERIALS AND METHODS

Enzymes

The expression of recombinant Tc-OP and Tb-OP in *E. coli* has been described previously [9,17]. The active enzyme concentration was determined using 4-methylumbelliferyl-*p*-guanidobenzoate (MUGB) [19].

Peptide synthesis

The IQF peptides containing EDDnp attached to glutamine were synthesized using a solid-phase strategy, which has been described previously [20]. An automated bench-top simultaneous multiple solid-phase peptide synthesizer (PSSM 8 System; Shimadzu, Tokyo, Japan) was used for the synthesis of all peptides by the Fmoc (fluoren-9-ylmethoxycarbonyl) procedure. The final deprotected peptides were purified by semi-preparative HPLC on an Econosil C-18 column (10 μm; 22.5 mm × 250 mm) using a two-solvent system: (A) trifluoroacetic acid/water (1:1000, v/v) and (B) trifluoroacetic acid/acetonitrile/water (1:900:100, by vol.). The column was eluted at a flow rate of 5 ml/min with a 10–50 % or 30–60 % gradient of solvent B over 30 or 45 min. Analytical HPLC was performed using a binary HPLC system from Shimadzu with an SPD-10AV Shimadzu UV–visible detector and a Shimadzu RF-535 fluorescence detector, coupled to an Ultrasphere C-18 column (5 μm; 4.6 mm × 150 mm), which was eluted with solvent systems A and B at a flow rate of 1 ml/min and a 10–80 % (v/v) gradient of solvent B over 20 min. The HPLC column eluates were monitored by their absorbance at 220 nm and by fluorescence emission at 420 nm following excitation at 320 nm. The molecular mass and purity of synthesized peptides were checked by MALDI-TOF (matrix-assisted laser-desorption ionization–time-of-flight) MS (TofSpec-E; Micromass) and/or peptide sequencing using a PPSQ-23 protein sequencer (Shimadzu).

Kinetic assays

Michaelis parameters were determined by initial-rate measurements. Hydrolysis of the fluorogenic peptide substrates at 37 °C in 50 mM Tris/HCl buffer, pH 8.0, was followed by measuring the fluorescence at $\lambda_{em} = 420$ nm and $\lambda_{ex} = 320$ nm in an Hitachi F-2000 spectrofluorimeter. The 1 cm path-length cuvette containing 2 ml of buffer supplemented with 5 mM dithiothreitol (DTT) was placed in a thermostatically controlled cell compartment for 5 min before the enzyme solution was added. Substrate was added after a 5 min preincubation, and the increase in fluorescence with time was recorded continuously for 5–10 min. The slope was converted into units of mol of substrate hydrolysed per min, based on the fluorescence curves of standard peptide solutions before and after total enzymic hydrolysis. The concentration of the peptide solutions was obtained by colorimetric determination of the 2,4-dinitrophenyl group (molar absorption coefficient of 17 300 $M^{-1} \cdot cm^{-1}$ at 365 nm). The enzyme concentrations for initial-rate determinations were chosen to be at a level intended to hydrolyse less than 5 % of the substrate present in the reaction. The inner-filter effect was corrected for using an empirical equation as described previously [21]. The kinetic parameters were calculated according to Wilkinson [22] as well as by using Eadie–Hofstee plots. All data were fitted to non-linear least-squares equations, using Grafit version 3.0 (Erithacus Software) [23]. Oligopeptidase B has a unique substrate-dependent temperature sensitivity, presenting an optimum temperature of slightly below 25 °C. However, depending on the substrate, the k_{cat}/K_m value increases up to 39 °C [24]. In the present work, the k_{cat}/K_m ratios for the hydrolysis reactions of the leader compound (Abz-AGGRGAGQ-EDDnp) with Tc-OP and Tb-OP were measured in the temperature range 5–37 °C (results not shown), and it was verified that the specificity constant increased up to 37 °C with both peptidases.

Determination of cleaved bonds

The peptide bonds cleaved were identified by HPLC, either by comparing the retention times of the digestion products with those of synthetic peptides encompassing the expected hydrolysis fragments and/or by molecular mass. The molecular masses were determined by MALDI-TOF MS and/or by sequencing, using protein sequencer PPSQ-23 (Shimadzu).

RESULTS

Effects of substrate length on hydrolysis by Tc-Op and Tb-Op

Substrates containing between three and 21 amino acid residues corresponding to the bradykinin-containing region of human kininogen, including regions spanning the processing sites for its formation, were synthesized and assayed with Tc-OP and Tb-OP. Tc-OP and Tb-OP exhibited identical behaviour in hydrolysis reactions with the IQF peptides derived from the kininogen, as shown in Table 1. The substrate with 21 amino acid residues (peptide IX-1) was not hydrolysed by either peptidase, and the substrates with 17 (peptide VIII-1) and three (peptide I-1) amino acids were the least susceptible among the peptides that

Table 1 Hydrolysis of kininogen-derived peptides of different sizes by Tc-OP and Tb-OP

↓ indicates the cleavage site; NH, not hydrolysed.

Peptide no.	No. of residues	Abz-peptidyl-EDDnp	Hydrolysis (%) Tc-OP	Tb-OP
I-1	3	F R↓Q	29	22
II-1	5	F R↓S S R	51	42
III-1	9	M I S L M K↓R P Q	64	39
IV-1	10	R P P G F S P F R↓Q	53	88
V-1	10	G F S P F R↓S S R Q	85	100
VI-1	11	L G M I S L M K↓R P Q	49	39
VII-1	13	R P P G F S P F R↓S S R Q	100	77
VIII-1	17	G F S P F R↓S S R I G I K E E Q	39	31
IX-1	21	L G M I S L M K R P P G F S P F R S S R I-NH_2	NH	NH

Table 2 Kinetic parameters for hydrolysis by Tc-OP and Tb-OP of substrates derived from Abz-AXGRGAGQ-EDDnp, containing modifications at X corresponding to the P3 position

Hydrolysis conditions were 37 °C in 50 mM Tris/HCl, pH 8.0, following pre-activation with 5 mM DTT. NH, not hydrolysed.

		Tc-OP			Tb-OP		
Peptide	P3	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m ($mM^{-1} \cdot s^{-1}$)	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m ($mM^{-1} \cdot s^{-1}$)
I-2	Gly	5.2	7.2	722	5.9	1.1	5364
II-2	Val	13	3.1	4194	45	2.6	17308
III-2	Leu	14	2.6	5385	29	1.3	22308
IV-2	Ile	23	4.4	5227	35	2.0	17500
V-2	Phe	5.2	3.4	1529	37	3.2	11563
VI-2	Tyr	8.1	3.0	2700	47	1.8	26111
VII-2	Ser	17	7.4	2297	41	2.3	17826
VIII-2	Thr	30	8.2	3659	30	1.8	16667
IX-2	Asn	NH	NH	NH	15	8.3	1807
X-2	Gln	13	2.4	5417	42	1.9	22105
XI-2	Met	23	5.9	3898	39	0.9	43333
XII-2	His	19	3.5	5429	21	3.2	6563
XIII-2	Asp	NH	NH	NH	4.8	3.6	1333
XIV-2	Glu	5.2	39	133	NH	NH	NH
XV-2	Pro	2.2	1.9	1158	4.4	1.0	4400

were hydrolysed. The best substrates for Tc-OP and Tb-OP were Abz-RPPGFSPFRSSRQ-EDDnp (peptide VII-1) and Abz-GFS-PFRSSRQ-EDDnp (peptide V-1) respectively. It is noteworthy that not all basic amino acids were sites of cleavage for Tc-OP and Tb-OP of the peptides presented in Table 1, since Arg–Ile and Lys–Glu peptide bonds were not hydrolysed in the assays of the substrate Abz-GFSPFRSSRIGEIKEEQ-EDDnp (peptide VIII-1). Therefore, in addition to substrate size, the amino acids in the vicinity of the P1 Arg or Lys residue also influence hydrolysis.

Determination of S3, S2, S1′, S2′ and S3′ subsite specificities

Substrates modified at P3

This series and all others described below are totally unrelated to bradykinin, and were designed to have the minimum side chain size for each amino acid, except the one that is in the position for which interaction with the enzymes is being analysed. The kinetic parameters obtained for the hydrolysis by Tc-OP and Tb-OP of the series Abz-AXGRGAGQ-EDDnp, modified at the X position, are shown in Table 2. These variations correspond to the P3 position with respect to cleavage at the Arg–Gly bond, which was the only cleavage site found. These substrates were more susceptible to hydrolysis by Tb-OP than by Tc-OP, as shown by the k_{cat}/K_m values. The activities of both peptidases were influenced by the nature of the amino acid located at the P3 position. The best substrates of this series for Tc-OP were those containing the hydrophobic amino acids Val, Leu and Ile (peptides II-2 to IV-2) and the hydrophilic amino acids Gln (peptide X-2) and His (peptide XII-2). The substrates containing Asn and Asp (peptides IX-2 and XIII-2 respectively) were resistant to Tc-OP, and the peptide with Glu (XIV-2) was the poorest substrate, i.e. it was hydrolysed with the highest K_m value in the series. The highest k_{cat}/K_m values for Tb-OP were observed with the substrate containing Met (peptide XI-2), followed by those with Gln (peptide X-2) and with hydrophobic amino acids (peptides II-2 to VI-2). The peptide XIV-2 with Glu was resistant, and those with Asn and Asp (peptides IX-2 and XIII-2) were the poorest substrates for Tb-OP in the series. Therefore the substrate binding site of both Tc-OP and Tb-OP extends to the S3 subsite, as indicated by the variations in the efficiency of hydrolysis of the peptides of this series.

Substrates modified at P2

The kinetic parameters obtained for the hydrolysis by Tc-OP and Tb-OP of the series Abz-AGXRGAGQ-EDDnp, modified at the X position, are shown in Table 3. These variations correspond to the P2 position, because hydrolysis at the Arg–Gly bond was the only cleavage site. In this series the highest k_{cat}/K_m values were observed for hydrolysis by Tc-OP of substrates containing Lys and Arg (peptides XV-3 and XVI-3 respectively), indicating a preference for basic amino acids at the P2 position. Tb-OP hydrolysed with highest efficiency the peptide containing Arg, while the one containing Lys was hydrolysed with a high k_{cat} but also a high K_m value. The products of hydrolysis of the peptides containing Arg and Lys at the P2 position after cleavage of the Arg–Gly bond [Abz-AGR(or K)R-OH] are hydrolysed further by Tc-OP and Tb-OP removing the C-terminal Arg. Figure 1 shows the HPLC profile of the hydrolysis of Abz-AGRRGAGQ-EDDnp by Tc-OP, demonstrating both the endo- and exo-peptidase activities of the enzyme. This curious behaviour of Tc-OP and Tb-OP was further confirmed by using as substrate the peptide Abz-AGRRRAQ-EDDnp, with triple Arg. Very efficient hydrolysis was observed at the Arg–Ala bond by Tc-OP (k_{cat}/K_m 53839 $mM^{-1} \cdot s^{-1}$), and to a lesser extent by Tb-OP (k_{cat}/K_m 7284 $mM^{-1} \cdot s^{-1}$), with the two C-terminal Arg residues then being removed sequentially, the first faster than the second.

Table 3 Kinetic parameters for hydrolysis by Tc-OP and Tb-OP of substrates derived from Abz-AGXRGAGQ-EDDnp, containing modifications at X corresponding to the P2 position

Hydrolysis conditions were as in Table 2. NH, not hydrolysed.

		Tc-OP			Tb-OP		
Peptide	P2	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m ($mM^{-1} \cdot s^{-1}$)	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m ($mM^{-1} \cdot s^{-1}$)
I-2	Gly	5.2	7.2	722	5.9	1.1	5364
I-3	Val	1.4	3.9	359	4.9	1.4	3500
II-3	Leu	15	7.7	1948	19	1.9	10 000
III-3	Ile	13	10	1300	6.6	1.2	5500
IV-3	Phe	9.7	3.1	3129	22	2.4	9167
V-3	Tyr	12	14	857	24	7.7	3117
VI-3	Ser	0.6	0.4	1500	1.7	0.6	2833
VII-3	Thr	27	7.5	3600	19	1.9	10 000
VIII-3	Asn	7.8	23	339	7.1	4.0	1775
IX-3	Gln	16	5.9	2712	10	1.4	7143
X-3	Met	13	2.9	4483	24	2.5	9600
XI-3	His	12	6.3	1905	NH	NH	NH
XII-3	Asp	NH	NH	NH	3.0	13	231
XIII-3	Glu	NH	NH	NH	1.8	3.3	545
XIV-3	Pro	3.6	16	225	12	8.2	1463
XV-3	Lys*	18	1.8	10 000	13	4.8	2708
XVI-3	Arg*	7.4	0.8	9250	4.4	0.4	11 000

* After hydrolysis at the Arg–Gly bond, the Arg residue is removed from the the fragment Abz-AGKR-OH or Abz-AGRR-OH (see Figure 1).

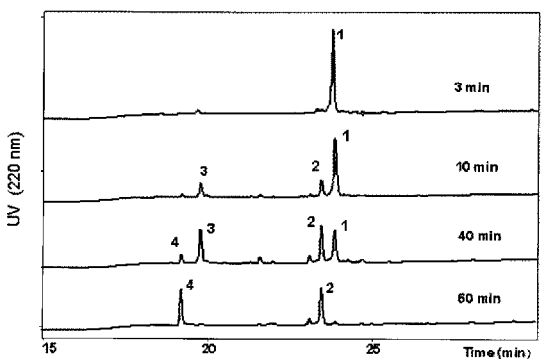

Figure 1 HPLC profile of the hydrolysis of Abz-AGRRGAGQ-EDDnp by Tc-OP

The HPLC profile after incubation of the substrate (40 mM) with Tc-OP (final concentration 4 nM) at 37 °C in 50 mM Tris/HCl, pH 8.0, following pre-activation with 5 mM DTT is shown. The elution profiles were determined at 220 nm at different time points, as indicated. The numbers indicate the following products, which were identified by MALDI-TOF MS after isolation: 1, intact substrate; 2, NH_2-GAGQ-EDDnp; 3, Abz-AGRR-OH; 4, Abz-AGR-OH (product of carboxypeptidase activity).

Figure 2 shows the HPLC profile of the reaction mixture after different periods of reaction of Abz-AGRRRAQ-EDDnp with Tc-OP. These results indicate that the carboxypeptidase activity observed with both Tc-OP and Tb-OP requires at least a pair of basic amino acids at the C-terminal side of the peptides.

The substrates containing Met (peptide IX-3), Thr (peptide VII-30) and Phe (peptide IV-3) were the next best substrates for Tc-OP. Similar preferences were shown by Tb-OP, which also hydrolysed with high efficiency peptide II-3 containing Leu. Substrates with negatively charged side chains (peptides XII-3 and XIII-3) were resistant to Tc-OP, while the substrate containing His (peptide XI-3) was resistant to Tb-OP. Pro at position P2 (peptide XIV-3)

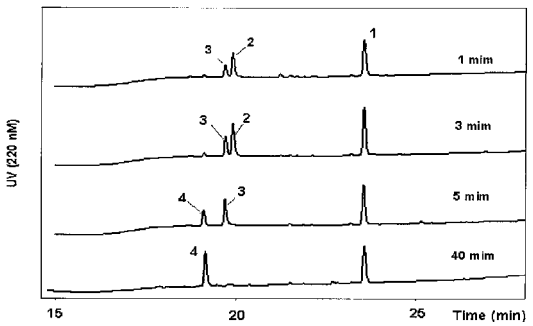

Figure 2 HPLC profile of the hydrolysis of Abz-AGRRRAQ-EDDnp by Tc-OP

The HPLC profile after incubation of the substrate (40 mM) with Tc-OP (final concentration 4 nM) at 37 °C in 50 mM Tris/HCl, pH 8.0, following pre-activation with 5 mM DTT is shown. The elution profiles were determined at 220 nm at different time points, as indicated. The numbers indicate the following products, which were identified by MALDI-TOF MS after isolation: 1, NH_2-AQ-EDDnp; 2, Abz-AGRRR-OH; 3, Abz-AGRR-OH (first product of carboxypeptidase activity); 4, Abz-AGR-OH (second product of carboxypeptidase activity).

Table 4 Kinetic parameters for hydrolysis by Tc-OP and Tb-OP of substrates derived from Abz-AGGRXAGQ-EDDnp, containing modifications at X correspondimg to the P1′ position

Hydrolysis conditions were as in Table 2.

		Tc-OP			Tb-OP		
Peptide	P1′	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m ($mM^{-1} \cdot s^{-1}$)	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m ($mM^{-1} \cdot s^{-1}$)
I-2	Gly	5.2	7.2	722	5.9	1.1	5364
I-4	Val	4.8	1.4	3429	2.6	0.1	26 000
II-4	Leu	6.0	1.3	4615	4.1	0.6	6833
III-4	Ile	3.1	0.3	10 333	4.0	0.5	8000
IV-4	Phe	24	2.7	8888	11	0.3	36 667
V-4	Tyr	11	0.6	18 333	13	0.4	32 500
VI-4	Ser	21	1.2	17 500	12	0.5	24 000
VII-4	Thr	11	0.9	12 222	12	0.3	40 000
VIII-4	Asn	14	1.3	10 769	14	0.4	35 000
IX-4	Gln	11	0.7	15 714	12	0.5	24 000
X-4	Met	17	8.3	2048	19	6.9	2754
XI-4	His	27	1.7	15 882	9.2	0.5	18 400
XII-4	Asp	15	4.5	3333	15	1.5	10 000
XIII-4	Glu	15	6.5	2301	13	1.4	9286
XIV-4	Pro	NH	NH	NH	NH	NH	NH

resulted in substrates that were hydrolysed with low k_{cat}/K_m values by both peptidases. The lowest K_m (0.4 μM for Tc-OP and 0.6 μM for Tb-OP) and k_{cat} values were observed with the substrate containing Ser (peptide VI-3).

Substrates modified at P1′

The kinetic parameters obtained for the hydrolysis by Tc-OP and Tb-OP of the Abz-AGGRXAGQ-EDDnp series, modified at the X position, are shown in Table 4. All of these variations correspond to the P1′ position, because the only cleavage site was at the Arg–Gly bond. The best substrates for Tc-OP were found in this series, notably the substrates containing Tyr, Ser, Gln and His (peptides V-4, VI-4, IX-4 and XI-4 respectively). Substrates hydrolysed efficiently by Tb-OP were also found in this series, and the best were those with Phe, Tyr, Thr and Asn (peptides IV-4, V-4, VII-4 and VIII-4 respectively). In contrast with other subsites of both enzymes, their S1′ sites accepted Asp and Glu (peptides XII-4 and

Table 5 Kinetic parameters for hydrolysis by Tc-OP and Tb-OP of substrates derived from Abz-AGGRGXGQ-EDDnp, containing modifications at X corresponding to the P2′ position

Hydrolysis conditions were as in Table 2.

		Tc-OP			Tb-OP		
Peptide	P2′	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m ($mM^{-1} \cdot s^{-1}$)	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m ($mM^{-1} \cdot s^{-1}$)
I-5	Gly	11	14	786	13	6.2	2097
I-2	Ala	5.2	7.2	722	5.9	1.1	5364
II-5	Val	40	31	1290	53	20	2650
III-5	Leu	62	24	2583	25	5.2	4808
IV-5	Ile	29	11	2636	28	3.2	8750
V-5	Phe	36	12	3000	15	2.1	7143
VI-5	Tyr	23	3.9	5897	20	4.4	4545
VII-5	Ser	23	3.7	6216	9.8	3.2	3063
VIII-5	Thr	19	13	1462	17	5.8	2931
IX-5	Asn	7.3	9.2	793	11	4.6	2391
X-5	Gln	14	10	1400	9.2	2.3	4000
XI-5	Met	54	20	2700	11	3.0	3667
XII-5	His	16	2.8	5714	17	3.3	5152
XIII-5	Asp	4.4	12	367	12	8.0	1500
XIV-5	Glu	33	138	239	12	10	1200
XV-5	Pro	5.6	9.8	571	7.5	1.2	6250

Table 6 Kinetic parameters for hydrolysis by Tc-OP and Tb-OP of substrates derived from Abz-AGGRGAXQ-EDDnp, containing modifications at X corresponding to the P3′ position

Hydrolysis conditions were as in Table 2.

		Tc-OP			Tb-OP		
Peptide	P3′	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m ($mM^{-1} \cdot s^{-1}$)	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m ($mM^{-1} \cdot s^{-1}$)
I-2	Gly	5.2	7.2	722	5.9	1.1	5364
I-6	Val	44	21	2095	36	9.1	3956
II-6	Leu	23	7.3	3151	47	12	3917
III-6	Ile	6.9	11	627	55	31	1774
IV-6	Phe	15	6.2	2419	68	9.7	7010
V-6	Tyr	20	11	1818	46	5.7	8070
VI-6	Ser	9.4	12	783	27	2.0	13 500
VII-6	Thr	48	27	1778	37	6.0	6167
VIII-6	Asn	17	18	944	31	5.9	5254
IX-6	Gln	6.5	4.8	1354	51	8.8	5795
X-6	Met	15	3.0	5000	20	3.1	6452
XI-6	His	25	3.7	6757	14	3.4	4118
XII-6	Asp	NH	NH	NH	17	5.7	2982
XIII-6	Glu	6.7	21	319	NH	NH	NH
XIV-6	Pro	1.8	13	138	3.1	2.8	1107

XIII-4), with only peptide XIV-4 (containing Pro) being resistant, as a consequence of its imide bond. It is noteworthy that the higher k_{cat}/K_m values obtained with this series for both enzymes were due to the contribution of lower K_m values for most of the substrates, when compared with the other series. These results indicate the importance of S1′ occupancy for the specificity of both peptidases.

Substrates modified at P2′

The kinetic parameters obtained for the hydrolysis by Tc-OP and Tb-OP of the Abz-AGGRGXGQ-EDDnp series, modified at the X position, are shown in Table 5. All variations correspond to the P2′ position, because the only cleavage site in this series was at the Arg–Gly bond. The occupancy of the S2′ subsite has significant influence on the specificity constant (k_{cat}/K_m) of Tc-OP activity, since the substrates with Ser, Tyr and His (peptides VII-5, VI-5 and XII-5 respectively) were hydrolysed with k_{cat}/K_m values that were nine or eight times higher than that for the reference substrate (peptide I-2); these were determined essentially by low K_m values. In addition, the acidic amino acids Asp (peptide XIII-5) and in particular Glu (peptide XIV-5) have a deleterious effect on catalytic efficiency, increasing the K_m value. The occupancy of subsite S2′ in Tb-OP does not seem to have a similar influence on the k_{cat}/K_m value; Tc-OP and Tb-OP tolerated better the acidic amino acids (peptides XIII-5 and XIV-5). The highest k_{cat}/K_m value for Tb-OP was observed for hydrolysis of the substrate containing Ile (peptide IV-5), followed by that containing Phe (peptide V-5). It is noteworthy that the k_{cat} values for this series, as well as for the series with variations at P3′ presented in the next section, were systematically higher than those observed for the other series of substrates. However, the K_m values were also high, as reflected in the k_{cat}/K_m values.

Substrates modified at P3′

The kinetic parameters obtained for the hydrolysis by Tc-OP and Tb-OP of the series Abz-AGGRGAXQ-EDDnp, modified at the X position, are shown in Table 6. All of these variations correspond to the P3′ position, because in this series the only cleavage site was at the Arg–Gly bond. The S3′ subsite of Tc-OP presented significant selectivity for Met and His (peptides X-6 and XI-6 respectively), with these peptides being hydrolysed with the highest k_{cat}/K_m values in the series. On the other hand, lower k_{cat}/K_m values or resistance to hydrolysis were observed with the substrates containing acidic amino acids or Pro (peptides XII-6 to XIV-6). The k_{cat}/K_m values obtained with Tb-OP in this series indicated poor selectivity of its S3′ subsite, except for resistance to hydrolysis of the substrate containing Glu (peptide XIII-6), and the 2.5 times higher k_{cat}/K_m value of the substrate containing Ser (peptide VI-6) relative to the reference substrate (peptide I-2).

Optimized substrates for Tc-OP and Tb-OP

Based on the results described above, an optimized substrate for each of the proteases was designed, choosing for positions from P3 to P3′ the amino acids that in each series resulted in the substrate with the higher k_{cat}/K_m value. For Tb-OP the peptide Abz-AMRRTISQ-EDDnp and for Tc-OP the peptide Abz-AHKRYSHQ-EDDnp were synthesized and assayed with the respective proteases. The kinetic parameters for the hydrolysis of Abz-AMRRTISQ-EDDnp by Tb-OP were $k_{cat} = 18.2\ s^{-1}$, $K_m = 0.23\ \mu M$ and $k_{cat}/K_m = 79\,130\ mM^{-1} \cdot s^{-1}$, and those for the hydrolysis of Abz-AHKRYSHQ-EDDnp by Tc-OP were $k_{cat} = 11.5\ s^{-1}$, $K_m = 0.28\ \mu M$ and $k_{cat}/K_m = 41\,071\ mM^{-1} \cdot s^{-1}$. Like peptides XV-3 and XVI-3 in Table 3 after hydrolysis of the Arg–Thr or Arg–Tyr bond by Tb-OP or Tc-OP respectively, Arg is removed from Abz-AMRR and Abz-AHKR.

DISCUSSION

The trypanosome oligopeptidases Tc-OP and Tb-OP exhibit high protein sequence similarity to prolyl oligopeptidase [3], and it is thus reasonable to assume that they share a similar tertiary structure. As with prolyl oligopeptidase, the N-terminal β-propeller domain of Tc-OP and Tb-OP would limit the size of substrates able to reach the peptidase active site [2,25]. Consistent with this idea, IQF peptides derived from the kininogen sequence were hydrolysed by Tc-OP and Tb-OP up to a substrate length of

17 amino acid residues (Table 1). We have observed this same 17-residue cut-off for the post-proline cleaving prolyl oligopeptidase, terminating at a Pro–Phe bond (results not shown). These data indicate that oligopeptidase B, like prolyl oligopeptidase, shows size restriction of substrates. However, this restriction is not absolute, since limited hydrolysis of globular proteins by this family of peptidases has been described, including the hydrolysis of histones by *Salmonella enterica* oligopeptidase B [3], of p40-phox splice variant protein from myeloid cells by prolyl oligopeptidase and the related enzyme from *Flavobacterium* [26], and of aspartokinases I and III by *E. coli* oligopeptidase B [27].

Tc-OP and Tb-OP appear to be essentially arginyl hydrolases, because in all peptides examined only Arg–Xaa bonds were cleaved. Oligopeptidase B from *E. coli* and Tb-OP were reported to hydrolyse with high efficiency peptidyl-methylcoumarin amide peptides containing pairs of basic residues more quickly than those containing a single Arg residue [3,17,28]. A reported three-dimensional model for oligopeptidase B from *E. coli* [25] and the *Salmonella enterica* endo-oligopeptidase B active site [3] pointed to two carboxyl dyads as the binding sites for these basic residues, i.e. Glu^{576}–Glu^{578} and Asp^{460}–Asp^{462} at the S1 and S2 subsites respectively. The proposed presence of these negative charges at the S2 subsite in Tc-OP and Tb-OP is in accordance with the results obtained with substrates containing negatively charged amino acids (Asp and Glu) at P2 (Table 3), which were not susceptible to hydrolysis by Tc-OP or were the poorest substrates for Tb-OP. Furthermore, this architecture of subsites S1 and S2 is also supported by the observations on the hydrolysis of IQF peptides (XV-3 and XVI-3; Table 3), which were cleaved very efficiently at the Arg–Gly bond, followed by carboxypeptidase activity on the C-terminal end of the fragment Abz-AGR(or K)R-OH. This carboxypeptidase activity seems to be specific for basic amino acids and depends on the presence of at least a pair of these residues. The peptide with triple Arg (Abz-AG$R^1R^2R^3$AQ-EDDnp) was an efficient substrate for Tc-OP and Tb-OP, which first cleaved the Arg–Ala peptide bond and then removed successively Arg^5 and then Arg^4 from the initial $(Arg)_3$-containing digestion product Abz-AGRRR-OH. The carboxypeptidase activity on Abz-AGRRR-OH seems to be faster when removing the first Arg than the second. Similar carboxypeptidase activity was noted previously for the hydrolysis by *E. coli* of the IQF peptide Abz-TRRF(NO_2)SL-NH_2 [2], which was cleaved first at the Arg—Phe bond; then, after more a extensive period of hydrolysis, the C-terminal Arg of the fragment Abz-TRR-OH was removed. A detailed analysis of the kinetics and the influence of neighbouring amino acids on this carboxypeptidase activity is currently under way in our laboratory. All together, these results support the view that oligopeptidase B is a specialized protein-processing enzyme in prokaryotes and lower eukaryotes that also has a novel, specific carboxypeptidase activity. The reported observation that oligopeptidase B retains full activity when released into the host plasma [13] in spite of the wealth of peptidase inhibitors present in the bloodstream re-inforces the importance of the potential protein-processing activity of oligopeptidase B.

In general, Tc-OP and Tb-OP were very active in hydrolysing many of the assayed substrates, with the S1 residue being the primary determinant of specificity. However, the S1′ residue also seems to be an important determinant of subsite specificity in both enzymes, and it is noteworthy that this subsite prefers hydrogen donors, such as Tyr, Ser, Thr and Gln. The presence of these amino acids at the P1′ position resulted in substrates that were hydrolysed with K_m values in the sub-micromolar range. Based on the kinetic parameters measured in the present study, we designed excellent substrates for Tb-OP and Tc-OP, i.e. Abz-AMRRTISQ-EDDnp and Abz-AHKRYSHQ-EDDnp respectively, which were hydrolysed with remarkably high k_{cat} and low K_m values.

In conclusion, we have obtained significant information in the present study for the design of potential inhibitors of these parasite oligopeptidases, since the administration of irreversible inhibitors of Tb-OP to trypanosome-infected mice has already been shown to reduce disease progression [14,15]. In addition, it is emerging that this group of peptidases comprise important virulence factors in bacterial infections (R. E. Morty, unpublished work), and is thus clearly relevant in microbial virulence. It is hoped that parameters for the development of new classes of antibiotics may emerge from these studies.

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