Structural features that make oligopeptides susceptible substrates for hydrolysis by recombinant thimet oligopeptidase

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A systematic analysis of the peptide sequences and lengths of several homologues of bioactive peptides and of a number of quenched-fluorescence (qf) opioid- and bradykinin-related peptides was performed to determine the main features leading the oligopeptides to hydrolysis by the recombinant rat testis thimet oligopeptidase (EC 3.4.24.15). The results indicate that a minimum substrate length of six amino acids is required and that among the oligopeptides six to thirteen amino acid residues long, their susceptibility as substrates is highly variable. Thimet oligopeptidase was able to hydrolyse, with similar catalytic efficiency, peptide bonds having hydrophobic or hydrophilic amino acids as well as proline in the P1 position of peptides, ranging from a minimum of six to a maximum of approximately thirteen amino acid residues. An intriguing observation was the shift of the cleavage site, at a Leu-Arg bond in qf dynorphin-(2–8) [qf-Dyn_{2-8}; Abz-GGFLRRV-EDDnp, where Abz stands for *o*-aminobenzoyl and EDDnp for *N*-(2,4-dinitrophenyl) ethylenediamine], to Arg-Arg in qf-Dyn_{2-8}Q, in which Gln was substituted for Val at its C-terminus. Similarly, a cleavage site displacement was also observed with the hydrolysis of the internally quenched-fluorescence bradykinin analogues containing Gln at the C-terminal position, namely Abz-RPPGFSPFR-EDDnp and Abz-GFSPFR-EDDnp are cleaved at the Phe-Ser bond, but Abz-RPPGFSPFRQ-EDDnp and Abz-GFSPFRQ-EDDnp are cleaved at the Pro-Phe bond.

INTRODUCTION

In the early 1970s two thiol-activated endopeptidases were isolated from the cytosol of rabbit brain [1,2] and subsequently purified to apparent homogeneity and characterized [3]. Unlike typical proteinases, these enzymes presented strict selectivity for oligopeptides and thus were generically named endo-oligopeptidases A and B [4]. Pierotti et al. [5] isolated and sequenced a cDNA clone encoding for a rat testis thiol-activated metalloendopeptidase (EC 3.4.24.15), considered identical with the brain endo-oligopeptidase A [6]. McKie et al. [7] expressed it on a large scale and further characterized the enzyme's chemical composition and properties. Since then, a number of oligopeptidases, structurally related to the rat testis enzyme, including neurolysin (EC 3.4.24.16), have been isolated from several mammalian tissues (reviewed in [8,9]). The oligopeptidases are now considered to be a distinct family of proteolytic enzymes [10] and are generically named thimet oligopeptidase (TOP) [8]; however, the catalytic mechanism of this family of enzymes still remains unclear.

The physiological role of the endo-oligopeptidases is unknown, although in the central nervous system, TOP and neurolysin have been associated with the intracellular conversion of opioid peptides into enkephalins [11,12] and/or the inactivation of neuropeptides (reviewed in [8,9]). However, owing to their ubiquitous distribution in the cytosol of mammalian tissues [13], they could also participate at the proteolysis steps of antigen presentation [14]. Coincidentally, the TOP-susceptible substrates [15] have a similar length range to the class I epitopes [16].

The substrate-binding site was defined by Schechter and Berger [17] as a number of subsites that bind the amino acid side chains of the substrate at both sides of the scissile bond. The substrate-binding pocket is a complex three-dimensional structure that is quite different among members of the same class of proteinases (reviewed in [18]). The primary and secondary specificities of TOP were not clearly defined, either because this is a peptidase displaying unusual specificity or because the enzyme preparations so far used were not always homogeneous or derived from the same tissue and/or animal species (reviewed in [13]). In the present study the requirements for the minimum and maximum peptide lengths and the subsite specificity of the peptide substrates were examined by using a homogeneous preparation of recombinant TOP. Several higher and lower homologues of neuropeptides, and a series of internally quenched-fluorescence (qf) bradykinin (Bk)- and dynorphin (Dyn)-related peptides were used as substrates. The results indicate that, among the peptides six to thirteen amino acid residues in length, their susceptibility towards the recombinant rat testis TOP is highly variable.

MATERIALS AND METHODS

Peptides

All the peptides smaller than Bk and Dyn A_{1-8}, and the internally quenched fluorescence peptides (qf-peptides) of general structure Abz-peptidyl-EDDnp [where Abz stands for *o*-aminobenzoyl, and EDDnp for *N*-(2,4-dinitrophenyl)ethylenediamine] containing Gln at the C-terminus were synthesized by the solid-phase

Abbreviations used: Abz, *o*-aminobenzoyl; BAM-18P, bovine adrenal medula enkephalin peptide with 18 amino acid residues; Bk, bradykinin; Dyn, dynorphin; EDDnp, *N*-(2,4-dinitrophenyl)ethylenediamine; qf, quenched fluorescence; TOP, thimet oligopeptidase.

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method [19], whereas the other qf-peptides were synthesized by the classical solution method [20]. Dyn A_{1-11} and Dyn A_{1-12} were prepared by the method of Camargo et al. [15]. Bk, neurotensin, Dyn $A_{1-17,}$ metorphinamide, substance P, luliberin, angiotensin I, bovine adrenal medulla enkephalin peptide with 18 amino acid residues (BAM-18P) and γ-endorphin were purchased from Peninsula Laboratories. All peptides were subjected to purification by preparative reverse-phase HPLC and their amino acid compositions and concentrations were determined by pico-Tag amino acid analysis after acid hydrolysis [21].

Preparation of the recombinant rat testis TOP

The purified recombinant rat testis TOP was obtained as described by Glucksman and Roberts [22]. Briefly, *Escherichia coli* cells containing the TOP cDNA inserted in the plasmid pGEX-4T-1 (Pharmacia-LKB) were grown with antibiotic selection to a D_{600} of 0.6, when the expression of the TOP-fusion gene was induced with 0.4 mM isopropyl β-D-thiogalactoside, proceeding for 3 h. Bacteria were lysed by two cycles of freezing and thawing followed by sonication. After the removal of bacterial debris, the supernatant was incubated with gluthatione–Sepharose beads, and subsequently with thrombin to cleave at the junction of the two genes. Contaminating thrombin was removed by exhaustive filtration with a Centricon 50 (Amicon). The yield was 2.5 mg of pure protein per litre of culture, as assayed by native and SDS/PAGE. The amino acid composition (no. of residues) of the purified enzyme [Asp, 57; Glu, 97; Ser, 32; Gly, 46; His, 16; Arg, 38; Thr, 35; Ala, 58; Pro, 34; Tyr, 18, Val, 43; Met, 18; Ile, 18; Leu, 71; Phe, 27; Trp (not determined); Lys, 41] reproduced the theoretical composition values of the rat testis TOP [8] and confirmed the homogeneity of the enzyme preparation used. The purified enzyme was sampled into vials containing approx. 1 μg of enzyme in 2 % (w/v) serum albumin and 30 % (v/v) glycerol. After an initial decrease of 15 % the enzyme activity remained constant for 2 months at -70 °C.

Enzymic assays and determination of cleavage site

The enzyme assays were performed in a final volume of 100 μl of 20 mM Tris/HCl, pH 7.5, containing 2 μg of purified recombinant rat testis TOP, 0.5 mM dithiothreitol and approx. 30 μM of the substrates. Samples were incubated at 37 °C and the reaction was terminated by the addition of 10 μl of 10 % (v/v) trifluoroacetic acid. Peptide fragments were separated by reverse-phase HPLC with a C_{18} μBondapak column (4.6 mm × 250 mm; Millipore Corp.) with a linear gradient of 0–50 % (v/v) acetonitrile in 0.1 % trifluoroacetic acid in 15 min at a flow rate of 2 ml/min. Absorbance was monitored at 214 nm. Cleavage sites of peptides were identified by determining the amino acid composition of each product generated from the incubation with enzyme and separated by HPLC.

The fluorimetric assays with qf-peptides [19,20] were performed on a Shimadzu model RF-540 spectrofluorimeter set at $\lambda_{ex} = 319$ nm and $\lambda_{em} = 418$ nm. Before the addition of 10 μl of substrate stock solution [prepared in 30 % (v/v) DMSO] to start the reaction, the enzyme (5–10 μl, 1–2 μg) was preincubated at 37 °C for 2 min in a thermostatically controlled cuvette with 3.0 ml of 20 mM Tris/HCl buffer, pH 7.5, containing 0.5 mM dithiothreitol. Initial velocity data for determining the kinetic constants K_m and k_{cat} were obtained by recording the fluorescence continuously for 2–3 min. The kinetic constants with respective standard errors were obtained through the Michaelis–Menten equation by the method described by Wilkinson [23]. The enzyme concentration was determined by the amino acid analysis corrected for the 15 % enzyme activity decrease. Only for qf-substrates having more than one cleavage site, the rate of hydrolysis of each cleaved peptide bond was ascertained by reverse-phase chromatography of the hydrolysates. Effluent peptides were monitored by a UV detector (LKB model 2151) at 214 nm and a spectrofluorimeter (Waters Associates model 420C) at $\lambda_{ex} = 319$ nm, $\lambda_{em} = 418$ nm, with the two detectors arranged in tandem.

Amino acid analysis

The amino acid compositions and the concentration of either the peptides or the purified recombinant rat testis TOP were determined as follows: the samples were freeze-dried for 22 h at 110 °C in 6 M HCl containing 1 % (w/v) phenol in vacuum-sealed tubes and then subjected to amino acid analysis with a pico-Tag station [21].

RESULTS

Comparative hydrolysis of bioactive peptides

The susceptibility to hydrolysis by the TOP was highly variable between bioactive peptides eight to thirteen amino acid residues in length (Table 1). These peptides include ones that were readily hydrolysed (metorphinamide, Dyn A_{1-8}, Dyn B, Bk, neurotensin and angiotensin I) and others that were resistant to hydrolysis (Dyn A_{1-13}, substance P and angiotensin II). The enzyme cleaved a single peptide bond of the bioactive peptides, several residues removed from the C- and the N-termini, and did not strictly require a free carboxy group for its action as did metorphinamide and luliberin. The peptide alignments presented in Table 1 show that none of the eight subsites had an absolute specificity for the presence of a given amino acid or type of amino acid in the substrate.

Comparison of hydrolysis of lower homologues of Dyn A_{1-8}, Bk and their qf derivatives

The results obtained in this study, with small homologues of Dyn A_{1-8} and Bk as substrates, are shown in Table 2. The minimum substrate length of six amino acid residues in Bk and Dyn homologues required for hydrolysis by the recombinant rat testis TOP is similar to that previously observed with the purified rabbit brain endo-oligopeptidase A [2,11]. The core peptide sequence for the series of Dyn A_{1-8} peptides is Gly-Phe-Leu-Arg-

Table 1 Relative rates of hydrolysis and sites of peptide bond cleavages of bioactive peptides by TOP

The value 100% represents the initial velocity of disappearance of methorphinamide (Mt) determined by HPLC. Details of the enzyme assays and the identification of the peptide fragments are described in the Materials and methods section. Arrows indicate cleavage sites. Abbreviations: A-I, angiotensin I; A-II, angiotensin II; βEnd, β-endorphin; Nt, neurotensin.

Substance	Amino acid sequence	Hydrolysis (%)
Mt	Y-G-G-F-M-↓-R-R-V-NH_2	100
Dyn A_{1-8}	Y-G-G-F-L-↓-R-R-I	90
Dyn B	Y-G-G-F-L-↓-R-R-Q-F-K-V-V-T	90
$\beta$$End_{1-9}$	Y-G-G-F-M-↓-T-S-E-K	35
Bk	R-P-P-G-F-↓-S-P-F-R	85
Nt	pE-L-Y-E-N-K-P-R-↓-R-P-Y-I-L	70
A-I	D-R-V-Y-I-H-P-↓-F-H-L	20
Luliberin	pE-H-W-S-Y-↓-G-L-R-P-G-NH_2	16
Substance P	R-P-K-P-Q-Q-↓-F-F-G-L-NH_2	5
Dyn A_{1-13}	Y-G-G-F-L-R-R-I-R-P-K-L-K	Resistant
A-II	D-R-V-Y-I-H-P-F	Resistant

Table 2 Hydrolysis of Dyn A_{1-8}, BK, and lower homologues of $DynA_{1-8}$ and Bk and their qf derivatives by TOP

The enzyme assays with $DynA_{1-8}$, Bk and their lower homologues were determined by HPLC. The rates of hydrolysis of the qf-peptides were performed by fluorimetric assay [18]. For the non-qf peptides the value 100% represents the initial velocity of disappearance of either Dyn A_{1-8} or Bk, whereas for the qf-peptides 100% represents the initial velocity of the hydrolysis of qf-$DynA_{1-8}$. The enzyme assays and the identification of the peptide fragments isolated by the HPLC are described in the Materials and methods section. Arrows indicate cleavage sites.

Substance	Amino acid sequence	Hydrolysis (%)
Dynorphin-related peptides		
Dyn A_{1-8}	Y^1-G^2-G^3-F^4-L^5-↓-R^6-R^7-I^8	100
Dyn A_{1-7}	Y-G-G-F-L-↓-R-R	5
Dyn A_{1-6}	Y-G-G-F-L-R	Resistant
Dyn A_{2-8}	G-G-F-L-↓-R-R-I	100
Dyn A_{3-8}	G-F-L-↓-R-R-I	3
Dyn A_{4-8}	F-L-R-R-I	Resistant
qf-Dynorphin-related peptides		
	Abz- -EDDnp	
qf-Dyn_{2-8}	-G^2-G^3-F^4-L^5-↓-R^6-R^7-V^8-	100
qf-Dyn_{3-8}	-G-F-L-↓-R-R-V-	35
qf-Dyn_{4-8}	-F-L-↓-R-R-V-	3
qf-Dyn_{2-7}	-G-G-F-L-↓-R-R-	3
qf-Dyn_{2-6}	-G-G-F-L-R-	Resistant
Bradykinin-related peptides		
Bk	R^1-P^2-P^3-G^4-F^5-↓-S^6-P^7-F^8-R^9	100
Bk_{1-8}	R-P-P-G-F-↓-S-P-F	50
Bk_{1-7}	R-P-P-G-F- S-P	Resistant
Bk_{2-9}	P-P-G-F-↓-S-P-F-R	360
Bk_{2-8}	P-P-G-F-↓-S-P-F	190
Bk_{3-9}	P-G-F-↓-S-P-F-R	70
Bk_{4-9}	G-F-↓-S-P-F-R	5
Bk_{5-9}	F-S-P-F-R	Resistant
qf-Bradykinin-related peptides		
	Abz- -EDDnp	
qf-Bk	-R-P-P-G-F-↓-S-P-F-R-	100
qf-Bk_{4-9}	-G-F-↓-S-P-F-R-	220
qf-Bk_{4-8}	-G-F-S-P-F-	Resistant

Arg-Ile, which extends from P3 to P3′, and for Bk is Gly-Phe-Ser-Pro-Phe-Arg, extending from P2 to P4′; however, the peptides Gly-Phe-Leu-Arg-Arg and Gly-Phe-Ser-Pro-Phe were not substrates.

The replacement of the Tyr residue by Abz and the addition of EDDnp to Dyn A_{1-8} at the C-terminus resulted in the quenched fluorescence substrate (qf-Dyn_{2-8}); it neither affected the minimum peptide length required for hydrolysis nor changed the site of peptide bond cleavage. Similarly to the Dyn A_{1-8} series, the removal of one Gly residue from the N-terminus of qf-Dyn_{2-8} was less influential than the removal of the C-terminal Val residue (compare the results for qf-Dyn A_{3-8} with those for qf-Dyn_{2-7} in Table 2). In contrast, the removal of both Gly residues caused a drastic reduction in susceptibility, as observed with peptide qf-Dyn_{4-8}.

The addition of Abz and EDDnp to the Bk sequence decreased its susceptibility to hydrolysis by 50%; however, the deletion of the tripeptide Arg-Pro-Pro increased the hydrolysis of the resulting substrate (qf-Bk_{4-9}) significantly. Furthermore, in the latter peptide the removal of the C-terminal Arg residue made the resulting peptide (qf-Bk_{4-8}) resistant to hydrolysis.

Susceptibility to hydrolysis of opioid peptides and their higher homologues

The maximum length of the opioid peptide substrates was about 13 amino acid residues; however, the search for maximum substrate length has daunting prospects because it depends not only on the length of the main chain but also on the amino acid residue(s) chosen to extend it. The susceptibility to hydrolysis of Dyn B and Dyn A_{1-12} illustrates this difficulty. Despite the fact that the two peptides share the same Gly-Phe-Leu-Arg-Arg-Xaa core sequence, only Dyn B is a substrate. However, Dyn A_{1-12} became a substrate when Leu^{12} was removed from the C-terminus (Table 3). The opposite effect was obtained by the deletion of His-Leu from angiotensin I, because only the larger angiotensin peptide was a substrate (Table 1). Therefore it is not surprising that higher homologues of opioid peptides such as Dyn A_{1-17}, BAM-18P and End_{1-16} were also resistant to hydrolysis, a fact

Table 3 Relative rate of hydrolysis and sites of peptide bond cleavages of opioid peptides and higher homologues by TOP

The value 100% represents the initial velocity of disappearance of Dyn A_{1-8} as determined by HPLC. Details of the enzyme assays and the identification of the peptide fragments are described in the Materials and methods section. Arrows indicate cleavage sites.

Substance	Amino acid sequence (P4-P3-P2-P1-↓-P1′-P2′-P3′-P4′)	Hydrolysis (%)
Dyn A_{1-8}	Y-G-G-F-L-↓-R-R-I	100
Dyn A_{1-11}	Y-G-G-F-L-↓-R-R-I-R-P-K	35
Dyn A_{1-12}	Y-G-G-F-L-R-R-I-R-P-K-L	Resistant
Dyn A_{1-17}	Y-G-G-F-L-R-R-I-R-P-K-L-K-W-D-N-Q	Resistant
BAM-12P	Y-G-G-F-M-↓-R-R-V-G-R-P-E	55
BAM-18P	Y-G-G-F-M-R-R-V-G-R-P-E-W-W-M-D-Y-Q	Resistant
βEnd_{1-9}	Y-G-G-F-M-↓-T-S-E-K	30
βEnd_{1-16}	Y-G-G-F-M-T-S-E-L-K-S-Q-T-P-L-V-T-L	Resistant

Table 4 Influence of glutamine or glutamic acid at the C-terminus of quenched fluorescence opioid- and bradykinin-related peptides on the site of cleavages and susceptibility to hydrolysis by TOP

The reactions were performed at 37 °C and the initial rates of hydrolysis of the qf-peptides were determined fluorometrically [20]. The value 100% represents the initial velocity of the hydrolysis either of qf-Dyn_{1-8} or of qf-Bk. The enzyme assays and the identification of the peptide fragments isolated by HPLC are described in the Materials and methods section. Arrows indicate cleavage sites.

Substance	Amino acid sequence	Hydrolysis (%)
qf-Opioid-related peptides		
	Abz- -EDDnp	
qf-Dyn_{2-8}	-G^2-G^3-F^4-L^5-↓-R^6-R^7-V^8-	100
qf-Q^8Dyn_{2-8}	-G-G-F-L-R-↓-R-Q-	350
qf-Q^8Dyn_{3-8}	-G-F-L-R-↓-R-Q-	250
qf-$L^5Q^8\beta End_{1-8}$	-G-G-F-L-↓-T-S-Q-	35
qf-$Dyn_{2-8}Q$	-G-G-F-L-↓-R-↓-R-V-Q-	60
qf-$Dyn_{2-8}E$	-G-G-F-L-↓-R-↓-R-V-E-	55
qf-$I^5Dyn_{2-8}Q$	-G-G-F-I-↓-R-↓-R-V-Q-	10
qf-$A^8Dyn_{2-8}Q$	-G-G-F-L-R-↓-R-A-Q-	160
qf-Bradykinin related peptides		
	Abz- -EDDnp	
qf-Bk	-R^1-P^2-P^3-G^4-F^5-↓-S^6-P^7-F^8-R^9-	100
qf-Bk-Q	-R-P-P-G-F-S-P-↓-F-R-Q-	260
qf-$Bk_{4-9}Q$	-G-F-S-P-↓-F-R-Q-	305
qf-$Bk_{5-9}Q$	-F-S-P-↓-F-R-Q-	8
qf-$Bk_{1-8}Q$	-R-P-P-G-F-↓-S-P-F-Q-	18
qf-$Bk_{1-7}Q$	-R-P-P-G-F-↓-S-P-Q-	8

that could be explained by the unfavourable influence of the amino acid residue(s) added to the C-terminal portion of the peptide, by the length of the resulting peptide, or by both. The influence of substrate extension on its susceptibility to hydrolysis by this class of enzyme was also studied with $(GPL)_n$ oligomers by Knight et al. [24], who demonstrated in this series that TOP preferentially hydrolysed C-terminal segments, suggesting a peptidyl-peptidase activity, releasing C-terminal fragments of up to six residues.

Influence of the glutamine or glutamic acid residue at the C-terminal position of qf-Dyn_{2-8} and qf-Bk analogues

The introduction of a Gln or Glu residue at the C-terminal position of qf-peptides was part of our strategy to synthesize these peptides by the solid-phase methodology [19]. Surprisingly, the replacement of Val^8 by Gln in qf-Dyn_{2-8} and qf-Dyn_{3-8} shifted the scissile bond to Arg^6-Arg^7 and increased the susceptibility to hydrolysis 2.5-fold and 3.5-fold for qf-Q^8Dyn_{2-8} and qf-Q^8Dyn_{3-8} respectively (Table 4). In contrast, the addition of a Gln or a Glu residue to the C-terminus of qf-Dyn_{2-8} decreased the susceptibility to hydrolysis by 50%, and two bonds were cleaved, namely Leu^5-Arg^6 and Arg^6-Arg^7 (peptides qf-Dyn_{2-8}-Q and qf-Dyn_{2-8}.E, Table 4). In addition, the replacement of Arg^6-Arg^7 by Thr-Ser generated qf-L^5Q^8-β-endorphin$_{1-8}$, which was approximately one-tenth as susceptible as qf-Q^8Dyn_{2-8} but still conserved the cleavage site at Leu^5-carboxyl group. Two independent cleavages at Leu^5-Arg^6 and Arg^6-Arg^7 also occurred in peptide qf-$I^5Dyn_{2-8}Q$, in which Ile replaced Leu^5 in peptide qf-$Dyn_{2-8}Q$, although rendering the peptide 10-fold more resistant to hydrolysis. Another important consequence of amino acid substitution in qf-$Dyn_{1-8}Q$ occurred by the substitution of Ala for Val^8: peptide qf-$A^8Dyn_{2-8}Q$ was a better substrate than qf-$Dyn_{2-8}Q$, and Arg^6-Arg^7 was the only hydrolysed bond.

The addition of Gln to the C-terminus of either qf-Bk or qf-Bk_{4-9} caused a shift of the scissile bond from Phe^5-Ser^6 to Pro^7-Phe^8 and increased the susceptibility to hydrolysis of qf-Bk-Q and qf-$Bk_{4-9}Q$ 2-fold and 3-fold respectively (Table 4). In contrast, the deletions of Arg^9 or Phe^8-Arg^9 from qf-$Bk_{1-9}Q$ decreased the susceptibility to hydrolysis to one-tenth to one-fifth (qf-$Bk_{1-8}Q$ and qf-$Bk_{1-7}Q$ respectively), but kept the cleavage at the Phe^5-Ser^6 bond.

Kinetic parameters for hydrolysis of qf-Dyn- and qf-Bk-related peptides

The kinetic constants for the hydrolysis of the most susceptible TOP substrates of the qf-Dyn_{2-8} series are shown in Table 5. The higher specificity constant (k_{cat}/K_m) values were observed with the peptides whose cleavage occurred at the Arg-Arg bond, where the catalytic constant (k_{cat}) is the predominant component for their hydrolysis. The deletion of Gly^2 (qf-Dyn_{3-8}) or the replacement of Gly^2 and Gly^3 by Ala (qf-$A^{2,3}Dyn_{2-8}$) resulted in a significant decrease in k_{cat} in comparison to qf-Dyn_{2-8}. The replacement of Gly^3 by D-Ala (qf-dA^3Dyn_{2-8}) resulted in the poorest substrate for TOP in this series. However, a D-Ala residue at position P4 (qf-dA^2Dyn_{2-8}) raised the susceptibility to hydrolysis to the level of the lead peptide (qf-Dyn_{2-8}). The parallel decreases in the k_{cat} and K_m values are noteworthy, indicating that the enzyme has a higher affinity for the peptides with poor hydrolysis. Similarly, the addition of a Gln residue to the C-terminus of qf-Dyn_{2-8} resulted in a 3-fold increase in the affinity of the modified substrate but decreased its catalytic rate to about the same extent. It is worth mentioning that two peptide bonds of qf-$Dyn_{2-8}Q$, namely Leu^5-Arg^6 and Arg^6-Arg^7, were hydrolysed with similar catalytic efficiencies (k_{cat}/K_m). In contrast, a higher k_{cat}/K_m was obtained by the replacement of Val^8 by Ala (peptide qf-$A^8Dyn_{2-8}Q$), although the resulting peptide was hydrolysed only at the Arg^6-Arg^7 bond. Conversely, a 3-fold increase in the catalytic rate with no substantial change in the affinity was observed with qf-Q^8Dyn_{2-8}, where Val^8 was replaced by Gln.

Table 5 Kinetic constants for the hydrolysis of qf-Dyn_{2-8}- and qf-Bk-derived peptides by TOP

The rates of hydrolysis of the qf-peptides were determined by fluorimetric assay [18] except for that of qf-$Dyn_{2-8}Q$, which was determined by HPLC as described in the Materials and methods section. Arrows indicate cleavage sites.

Substance	Sequence	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m ($\mu M^{-1} \cdot s^{-1}$)
qf-Dynorphin-related peptides				
	Abz- EDDnp			
qf-Dyn_{2-8}	-G^2-G^3-F^4-L^5-↓-R^6-R^7-V^8-	5.6 ± 0.6	4.3 ± 0.1	0.8
qf-Dyn_{3-8}	-G-F-L-↓-R-R-V-	1.7 ± 0.1	0.70 ± 0.07	0.4
qf-$Dyn_{2-8}Q$	-G-G-F-L-↓-R-R-V-Q-	1.6 ± 0.1	1.4 ± 0.1	0.9
	-G-G-F-L-R-↓-R-V-Q-	1.8 ± 0.1	2.0 ± 0.1	1.1
qf-$A^{2,3}Dyn_{2-8}$	-A-A-F-L-↓-R-R-V-	4.9 ± 1.3	1.0 ± 0.1	0.2
qf-dA^2Dyn_{2-8}	-dA-G-F-L-↓-R-R-V-	2.6 ± 0.6	2.1 ± 0.1	0.8
qf-dA^3Dyn_{2-8}	-G-dA-F-L-↓-R-R-V-	1.9 ± 0.3	0.20 ± 0.01	0.1
qf-$A^8Dyn_{2-8}Q$	-G-G-F-L-R-↓-R-A-Q-	2.2 ± 0.2	5.7 ± 0.1	2.6
qf-Q^8Dyn_{2-8}	-G-G-F-L-R-↓-R-Q-	6.0 ± 0.6	13.8 ± 0.5	2.3
qf-Bradykinin-related peptides				
	Abz- -EDDnp			
qf-Bk	-R^1-P^2-P^3-G^4-F^5-↓-S^6-P^7-F^8-R^9-	3.4 ± 0.3	0.9 ± 0.04	0.3
qf-Bk-Q	-R-P-P-G-F-S-P-↓-F-R-Q-	1.8 ± 0.2	2.5 ± 0.1	1.4
	-G-F-↓-S-P-F-R-	3.9 ± 0.4	2.0 ± 0.1	0.5
qf-$Bk_{4-9}Q$	-G-F-S-P-↓-F-R-Q-	2.5 ± 0.3	3.3 ± 0.1	1.3

The deletion of Arg^1-Pro^2-Pro^3 from qf-Bk did not affect the affinity of TOP for the substrate (qf-Bk_{4-9}) but increased the catalytic rate by 2-fold. The addition of a Gln residue to the C-terminus of qf-Bk or qf-Bk_{4-9} increased both the affinity and the catalytic rate of their hydrolysis (Table 5).

DISCUSSION

TOP hydrolysed all the examined peptides as an endopeptidase; a free C-terminal carboxy group was not required for its hydrolytic activity, as demonstrated by the qf-peptides (Tables 2, 4 and 5), and for the cleavage of luliberin. However, for the hydrolysis of latter the removal of the amide group or the C-terminal Gly-NH_2 was described to result in a significant improvement in Tyr-Gly bond hydrolysis [25,26]. Unlike the well characterized protein-degrading endopeptidases, the action of TOP is restricted to oligopeptides, showing no preference for hydrolysis of a peptide bond involving specific amino acids or types of amino acid (Table 1); nor was any secondary specificity identified. Another intriguing observation was the shift of the cleavage site of qf-Dyn_{2-8} by the substitution of Gln for Val at its C-terminus (qf-Q^8Dyn_{2-8}; Table 4). Similarly, the cleavage site displacement was also observed with the hydrolysis of the qf-Bk analogues containing a Gln residue at the C-terminal position (qf-Bk-Q, qf-$Bk_{4-9}Q$ and qf-$Bk_{5-9}Q$; Table 4). These peptides were hydrolysed at the Pro^7-Phe^8 bond in contrast with Bk, its lower homologues and the qf-Bk-analogues, which were all hydrolysed at the Phe^5-Ser^6 bond (Table 2). This shift of cleavage site does not seem to depend exclusively on the presence of a Gln residue at the prime site because qf-$L^5Q^8End_{1-8}$ (Table 4) and Dyn B (Table 1) also contain a Gln residue at position 8 but still have Leu^5-Thr^6 and Leu^5-Arg^6 as the only susceptible bonds. In addition, the removal of Arg and Phe residues from qf-Bk-Q restored the cleavage site at the Phe^5-Ser^6 bond, as observed with peptides qf-$Bk_{1-8}Q$ and qf-$Bk_{1-7}Q$ (Table 4). Surprisingly, the addition of a Gln or Glu residue to the C-terminus of qf-Dyn_{2-8} generated peptides (qf-$Dyn_{2-8}Q$ and qf-$Dyn_{2-8}E$) with two susceptible bonds, namely the Leu^5-Arg^6 and Arg^6-Arg^7 bonds (Table 4). This result suggests that these qf-peptides have two distinct ways of adjusting to the catalytic site, each having similar chances of forming a productive binding as indicated by the k_{cat}/K_m ratio, which is 0.8 $\mu M^{-1} \cdot s^{-1}$ for the hydrolysis of the Leu^5-Arg^6 bond and 1.1 $\mu M^{-1} \cdot s^{-1}$ for the hydrolysis of the Arg^6-Arg^7 bond (Table 5). For both cleavages, the subsites that could bind to the same type of amino acids were S4, S2 and S1′ (Table 4). However, it seems unlikely that these amino acids (P4 = Gly^2 or Gly^3; P2 = Phe^4 or Leu^5; P1′ = Arg^6 or Arg^7) should be considered critical binding sites for the hydrolysis of either the Leu^5-Arg^6 bond or the Arg^6-Arg^7 bond. In fact, the results presented in Tables 1 and 4 clearly indicate that several TOP substrates with distinct types of amino acid residue at P4, P2 and P1′ were hydrolysed with similar catalytic efficiencies (Table 5). Two independent cleavage sites (Ile^5-Arg^6 and Arg^6-Arg^7) also occurred after the replacement of Leu by Ile in qf-$Dyn_{2-8}Q$ (qf-$I^5Dyn_{2-8}Q$; Table 4), although drastically reducing the susceptibility of the resulting peptide. A similar pattern of hydrolysis by TOP was also observed with peptides Mca-GGFLRRAK-(N_2ph)-NH_2 and Mca-GGFIRRAK(N_2ph)-NH_2 [where Mca stands for (7-methoxycoumarin-4-yl)acetyl and N_2ph for 2,4-dinitrophenyl] [27]. These results contrast with the hydrolysis of qf-$A^8Dyn_{2-8}Q$ in which the replacement of Val^8 by Ala resulted in an increase in the catalytic efficiency of the enzyme, and the cleavage site shifted exclusively to the Arg^6-Arg^7 bond. It is possible that the removal of all interactions made by atoms beyond the β-carbon of Val^8 changed the substrate conformations, thus prompting distinct interactions with the enzyme [28]. Peptide bonds involving a Pro residue at the P1 position are resistant to hydrolysis by most proteases; however, this does not represent a hindrance for the TOP to hydrolyse angiotensin I (Table 1), qf-Bk-Q, qf-$Bk_{4-9}Q$ and qf-$Bk_{5-9}Q$ at the Pro-Phe bond. The absence of primary specificity was also emphasized by the low magnitude of the differences between the catalytic efficiencies for the hydrolysis of qf-Dyn_{2-8}, qf-Q^8Dyn_{2-8}, qf-$Dyn_{2-8}Q$, qf-Bk and qf-BkQ, in which the amino acid at the P1 position is respectively Leu^5, Arg^6, Leu^5 or Arg^6, Phe^5 and Pro^7 (Table 5).

In the context of this discussion it is worth noting the susceptibility of angiotensin I at the Pro-Phe peptide bond,

which was demonstrated to be involved in the β-turn-type conformation in angiotensin I [29,30]; in addition it was reported that the amino acid residues that play a critical role in modifying protein specificities, including those of proteolytic enzymes, are located around β-turn potential structures [3]. We have developed a theoretical study on the basis of a comparative conformational analysis of the statistical distribution of conformations of several TOP substrates, that indicates that these peptides share some structural features, including a large main-chain exposed area with an oblique β-turn [32].

In conclusion, the present study indicates that the binding of substrate side chains into specific subsites of TOP does not seem to be essential for the catalysis, which, in contrast, depends on the possible conformations of the substrates that allow accessibility of its cleavage site to the catalytic centre of TOP.

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