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Bradykinin Analogues with β-Amino Acid Substitutions Reveal Subtle Differences in Substrate Specificity Between the Endopeptidases EC 3.4.24.15 and EC 3.4.24.16

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Abstract: The closely related zinc metalloendopeptidases EC 3.4.24.15 (EP24.15) and EC 3.4.24.16 (EP24.16) cleave many common substrates, including bradykinin (BK). As such, there are few substrate-based inhibitors which are sufficiently selective to distinguish their activities. We have used BK analogues with either alanine or β-amino acid (containing an additional carbon within the peptide backbone) substitutions to elucidate subtle differences in substrate specificity between the enzymes. The cleavage of the analogues by recombinant EP24.15 and EP24.16 was assessed, as well as their ability to inhibit the two enzymes. Alanine-substituted analogues were generally better substrates than BK itself, although differences between the peptidases were observed. Similarly, substitution of the four *N*-terminal residues with β-glycine enhanced cleavage in some cases, but not others. β-Glycine substitution at or near the scissile bond (Phe^5-Ser^6) completely prevented cleavage by either enzyme; interestingly, these analogues still acted as inhibitors, although with very different affinities for the two enzymes. Also of interest, β-Gly^8-BK was neither a substrate nor an inhibitor of EP24.15, yet could still interact with EP24.16. Finally, while both enzymes could be similarly inhibited by the *D*-stereoisomer of β-C3-Phe^5-BK ($IC_{50} \approx 20$ μM, compared to 8 μM for BK), EP24.16 was relatively insensitive to the L-isomer ($IC_{50} \approx 12$ μM for EP24.15, > 40 μM for EP24.16). These studies indicate subtle differences in substrate specificity between EP24.15 and EP24.16, and suggest that β-amino acid analogues may be useful as templates for the design of selective inhibitors.

Keywords: β-amino acid; bradykinin; peptidase inhibitor; zinc metalloendopeptidase

INTRODUCTION

The family of mammalian zinc metalloendopeptidases plays a vital role in the metabolism of a number of bioactive peptides, especially within the central nervous system and the circulation. Family members include angiotensin-converting enzyme (ACE), neutral endopeptidase (NEP) and endothelin-converting enzyme (ECE), all of which are membrane-bound ectoenzymes known to be crucial in the generation or clearance of vasoregulatory peptides [1]. Less well known are the soluble members of this family, including the closely related endopeptidases EC 3.4.24.15 (EP24.15) and EC 3.4.24.16 (EP24.16) [2]. Although the biochemical aspects of these enzymes have been well-characterized, their precise physiological role remains in doubt, in part due to the lack of specific and selective inhibitors which are stable *in vivo*.

Given that the primary sequences of EP24.15 and EP24.16 are 60% identical, it is not surprising that they share many peptide substrates, and in most cases, the cleavage site is identical. As a consequence, most substrate-based inhibitors are not

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sufficiently selective to adequately distinguish between the two enzymes. Furthermore, the exact substrate specificity is not clearly defined for either enzyme; indeed, residues some distance from the scissile bond appear to contribute significantly to substrate binding [3,4].

We are currently examining the potential utility of substrate analogues containing β-amino acids as enzyme inhibitors. β-Amino acids contain an extra carbon group between the amino- and carboxyl-termini, which confers resistance to proteolysis [5,6]. In the present study, we have synthesized a series of β-amino acid-containing analogues of the vasodilatory peptide bradykinin (BK), a peptide readily degraded by both EP24.15 and EP24.16, and examined both their cleavage rate and their ability to inhibit the two enzymes. Our results not only reveal subtle differences in the substrate specificity of the peptidases, but also suggest that β-amino acid-substituted substrate analogues may be useful in the design of specific peptidase inhibitors.

MATERIALS AND METHODS

Synthesis of Peptides

BK analogues containing single alanine or β-glycine substitutions were custom synthesized by Mimotopes (Clayton, Victoria, Australia). Analogues in which the residues either side of the scissile bond were substituted by their β-congener (i.e. β-C3-L-Phe^5-BK, β-C3-L-Phe^5-BK, β-C2-L, D-Phe^5-BK and β-C3-L-Ser^6-BK) were synthesized in the Department of Biochemistry and Molecular Biology, Monash University, using standard solid-phase methods and commercially available reagents. The designation C2 or C3 refers to the position of the side-chain relative to the *C*-terminal carbon. Enantiomerically-pure Fmoc-protected β-C3 amino acids were prepared in the Department of Chemistry, Monash University, using Arndt–Eistedt homologation of commercially available α-amino acids, while racemic Fmoc-C2-β-Phe was prepared in several steps from *t*-butyl acrylate [7].

Degradation of Peptides

The cleavage of the BK analogues by recombinant rat EP24.15 (provided by Dr M.J. Glucksman, Mount Sinai School of Medicine, New York, USA) or EP24.16 (provided by Dr E.S. Ferro, University of São Paulo, Brazil) was assessed by co-incubation of enzyme (5 ng (EP24.16) or 25 ng (EP24.15)) with 5 μg substrate in 50 μl Tris-buffered saline (TBS; 25 mM Tris–HCl, 125 mM NaCl, pH 7.4) for 30 min. Dithiothreitol (DTT; 0.1 mM) was included to activate EP24.15. Reactions were stopped by addition of 200 μl methanol/1% trifluoroacetic acid (TFA), and samples dried on a centrifugal vacuum evaporator (Speed-Vac, Savant Instruments Inc, Farmingdale, NY) before high performance liquid chromatography (HPLC) analysis using a Hewlett–Packard 1100 series LC equipped with a diode array detector and on-line mass spectrometric detector. Samples were loaded onto a Zorbax Eclipse C18 column (at 50 °C) in 1.8% acetonitrile/0.1% TFA/0.02% acetic acid at 0.15 ml/min, and eluted with a 30 min linear gradient to 60% acetonitrile/0.1% TFA. Peptide fragments were identified following mass spectral analysis using Hewlett–Packard ChemStation deconvolution software. An extended (24 h) incubation was repeated for those analogues which were resistant to cleavage at 30 min.

Inhibition of EP24.15 and EP24.16

Inhibition of recombinant EP24.15 or EP24.16 by the BK analogues was measured using a specific quenched fluorescent substrate (QFS: 7-methoxycoumarin-4-acetyl-Pro-Leu-Gly-D-Lys(2,4-dinitrophenyl); custom synthesized by Auspep, Parkville, Victoria, Australia), as described previously [8]. Assays were performed in 96-well microtitre plates, in a final volume of 250 μl TBS, and included 1.6 ng/well EP24.16 or 50 ng/well EP24.15, 4.5 μM QFS, varying concentrations of BK peptide, and in the case of EP24.15, 0.1 mM DTT to activate the enzyme. Reactions proceeded at 37 °C for 30–60 min, within a thermostatted fluorescence microplate reader (f_{max}, Molecular Devices, Sunnyvale, CA), prior to reading the liberated fluorescence ($\lambda_{ex} = 320$ nm, $\lambda_{em} = 420$ nm). The extent of QFS degradation was less than 15%, as determined by comparison to a standard curve of fluorescent product (7-methoxycoumarin-4-acetyl-Pro-Leu; Calbiochem–Novabiochem, Alexandria, NSW, Australia). Following an initial screen at high concentration (40–80 μM), inhibition curves for promising analogues were generated over a range of concentrations (2.3–40 μM). Each concentration (of each analogue) was assayed in triplicate in three separate experiments, and the results expressed as mean percent inhibition (± S.E.M.).

RESULTS

Degradation of BK Analogues by EP24.15 and EP24.16 (Table 1)

BK was degraded by recombinant EP24.15 and EP24.16 (20–40% degradation in 30 min), with the sole cleavage being at the Phe^5-Ser^6 bond. Interestingly, most of the alanine analogues were actually better substrates for both enzymes, with near-complete digestion within 30 min (Table 1). Exceptions were the *N*-terminal substitutions, Ala^1-BK and Ala^2-BK, which were degraded at a rate similar to the native peptide by both enzymes, and Ala^8-BK and Ala^9-BK, which were better substrates for EP24.15 than for EP24.16. Replacement of any of the four *N*-terminal residues with β-glycine also tended to increase degradation rates, the exceptions being β-Gly^3-BK by EP24.15, and β-Gly^4-BK by EP24.16. Most interestingly, replacement of residues 5, 6, or 7 with a β-glycine completely prevented hydrolysis by either peptidase, even after extended (24 h) incubation. β-Gly^8-BK was neither a substrate nor an inhibitor for EP24.15, yet retained its ability to bind to and be cleaved by EP24.16.

Table 1 Degradation of Alanine and β-Glycine Analogues by EP24.15 and EP24.16

	EP24.15	EP24.16
BK	+	+
Ala^1	+	++
Ala^2	+	+
Ala^3	+++	+++
Ala^4	+++	+++
Ala^5	+++	+++
Ala^6	+++	+++
Ala^7	+++	+++
Ala^8	++	+
Ala^9	+++	+
βGly^1	+++	+++
βGly^2	+++	+++
βGly^3	+	+++
βGly^4	+++	+
βGly^5	0	0
βGly^6	0	0
βGly^7	0	0
βGly^8	0	+
βGly^9	+	++

Symbols indicate extent of degradation after 30 min incubation. +++: >80%; ++:50–80%; +: <50%; 0: no degradation.

Inhibition of EP24.15 and EP24.16 by BK Analogues

The cleavage of quenched fluorescent substrate by both EP24.15 and EP24.16 was efficiently inhibited by BK (Figure 1). When screened at high (60–80 μM) concentrations, all alanine and β-glycine analogues (except β-Gly^8-BK, which did not inhibit EP24.15) also acted as inhibitors, although to varying degrees. Concentration–inhibition curves for cleavage-resistant analogues (β-Gly^5-BK, β-Gly^6-BK, β-Gly^7-BK) were generated and compared to BK. As shown in Figure 1, both β-Gly^5-BK and β-Gly^6-BK inhibited EP24.15 with IC_{50} values of approximately 28 μM, compared to 8 μM for BK itself. β-Gly^7-BK was less potent, displaying an IC_{50} greater than 40 μM. These analogues were substantially less potent against EP24.16, with only the β-Gly^5-BK peptide showing any significant inhibition ($IC_{50} > 40$ μM, compared to 7 μM for BK; Figure 1).

Analogues in which the fifth or sixth residue was substituted with its β-congener (i.e. β-Phe^5 or β-Ser^6) were also examined. These peptides were also resistant to cleavage by both EP24.15 and EP24.16, although some minor cleavage of β-C2-Phe^5-BK was observed after extended incubation. When screened at 40 μM, all analogues also inhibited QFS cleavage, although to different extents. Complete inhibition curves were generated for the most promising inhibitors, β-C3-L-Phe^5-BK and β-C3-D-Phe^5-BK. As shown in Figure 2, both analogues inhibited EP24.15 strongly, with IC_{50} values of 20 μM and 12 μM for the L- and D-stereoisomers, respectively. This represents a loss in affinity of less than three-fold relative to BK itself. While β-C3-L-Phe^5-BK showed a similar inhibitory potency against EP24.16 (Figure 2), β-C3-D-Phe^5-BK was much less potent ($IC_{50} > 40$ μM), suggesting a greater stereospecificity of this enzyme at this residue compared to EP24.15.

DISCUSSION

Membrane-bound ectoenzymes of the zinc metalloendopeptidase family, such as ACE, NEP & ECE, are known to play crucial roles in the generation and termination of peptide signals [1]. The exact role of the soluble members of this family, particularly EP24.15 and EP24.16, is less clear; they are believed to be involved in the metabolism of a number of bioactive peptides, such as BK, neurotensin, and gonadotropin-releasing hormone (GnRH) [9], although definitive evidence is still lacking. Both enzymes are true peptidases, in that substrates are

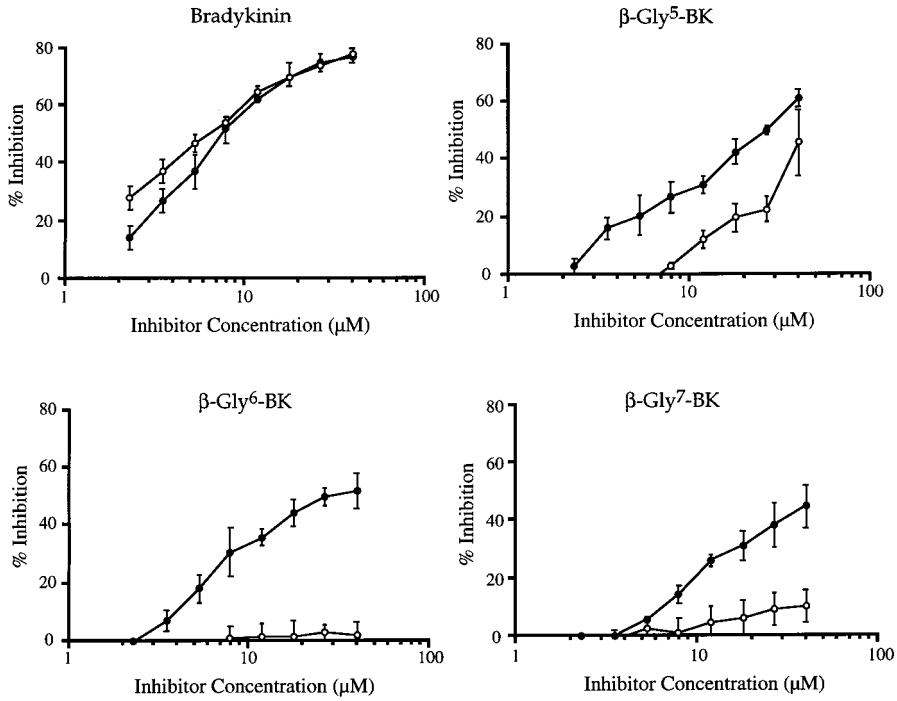

Figure 1 Concentration–inhibition curves for BK and the β-glycine analogues β-Gly^5-BK, β-Gly^6-BK, and β-Gly^7-BK against recombinant EP24.15 (●) and EP24.16 (○). Each point on the curve represents the mean percent inhibition observed in three separate experiments, each performed in triplicate; the error bars represent the standard error of the mean. Approximate IC_{50} values derived from these graphs for EP24.15 are 8, 28, 28, and >40 μM for BK, β-Gly^5-BK, β-Gly^6-BK, and β-Gly^7-BK, respectively. BK was equally effective against EP24.16 ($IC_{50} \sim 7$ μM), but the β-glycine analogues exhibited much lower affinity for this enzyme relative to EP24.15.

restricted to less than about 15 residues. There is also a tendency for these peptidases to cleave *C*-terminal to a hydrophobic residue, such as Phe^5 in BK. However, this is not strictly the case; for example, EP24.15 cleaves between two arginine residues in neurotensin [9]. This neuropeptide represents one of very few naturally occurring substrates in which the cleavage site differs between EP24.15 and EP24.16, the latter cleaving at a Pro-Tyr bond. A number of studies have suggested that residues distant from the scissile bond are clearly important in determining substrate specificity, and the two peptidases may exhibit slightly different preferences in this regard [3,4,10,11]. Even the presence or absence of a free carboxy terminus several residues removed from the cleavage site can greatly influence the hydrolysis rate of a substrate, as has been demonstrated for both BK and GnRH cleavage by EP24.15 [12,13].

Given the striking similarity of EP24.15 and EP24.16, and the lack of detailed knowledge of their respective substrate specificities, most inhibitors designed to date have been relatively non-selective. For example, the widely used inhibitor *N*-[1-(R,S)-carboxy-3-phenylpropyl]-Ala-Ala-Tyr-*p*-aminobenzoate, designed by Orlowski over ten years ago [14], is only about 30- to 40-fold more potent against EP24.15 than EP24.16. Recently, however, Jiracek *et al.* have used combinatorial chemistry to design more selective phosphinic peptide inhibitors of the two peptidases. Their results suggest a preference by EP24.16 for proline in both the P2 and P2′ positions, while EP24.15 favours basic residues at these positions [10,11]. However, such preferences deduced from peptide libraries are not always reflected in the absolute substrate specificity; indeed, one would predict cleavage of neurotensin by EP24.16 would occur at the Arg^8-Arg^9 bond, as both P2 and P2′ are prolines, yet as stated before, this is actually the EP24.15 cleavage site. Thus, the exact specificity of these peptidases is very complex.

In the present study, we have synthesized a number of BK analogues with the primary aim of determining whether β-amino acid substitutions at the

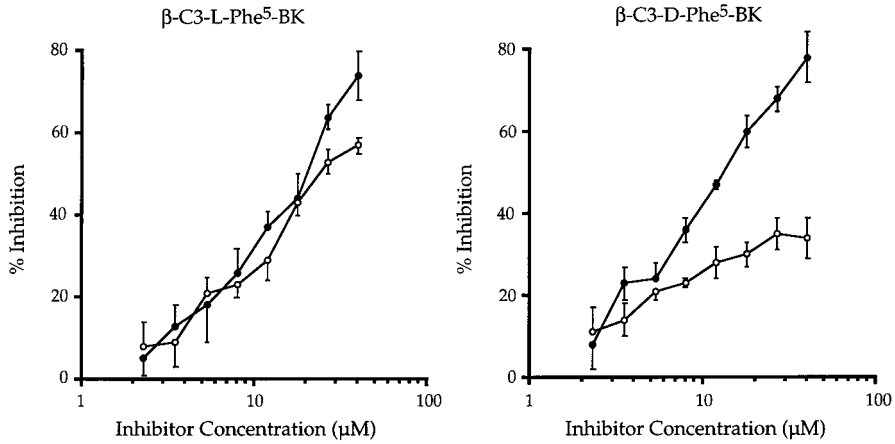

Figure 2 Concentration–inhibition curves for β-C3-L-Phe^5-BK and β-C3-D-Phe^5-BK against EP24.15 (●) and EP24.16 (○). Each point on the curve represents the mean percent inhibition observed in three separate experiments, each performed in triplicate; the error bars represent the standard error of the mean. Approximate IC_{50} values derived from these graphs for EP24.15 are 20 μM and 12 μM for β-C3-L-Phe^5-BK and β-C3-D-Phe^5-BK, respectively. For EP24.16, these values are approximately 20 μM and >40 μM.

scissile bond can prevent hydrolysis without abolishing binding to the enzyme. This is clearly the case, as demonstrated by the complete resistance of β-Gly^5-BK, β-Gly^6-BK, β-Gly^7-BK, β-Phe^5-BK, and β-Ser^6-BK to degradation, with the retention of inhibitory capacity, at least against EP24.15. Indeed, the most potent analogue (β-C3-D-Phe^5-BK) exhibited an IC_{50} of 12 μM against EP24.15, only 1.5-fold less potent than BK itself (Figure 2). Interestingly, this compound was much less effective against EP24.16, while the L-stereoisomer was equipotent against the two peptidases, suggesting a stronger stereospecificity of EP24.16 at this residue. Other striking differences between the enzymes were seen in the β-glycine analogues β-Gly^5-BK, β-Gly^6-BK, and β-Gly^7-BK, which were significantly better inhibitors of EP24.15 than EP24.16 (Figure 1). These differences are not simply due to the loss of specific side-chains at the cleavage site, as alanine substitutions at residues 5 and 6 did not diminish inhibition of either EP24.15 or EP24.16. Rather, the presence of an additional carbon in this region appears to adversely affect binding to EP24.16.

These studies have revealed other intriguing aspects of substrate specificity of these peptidases. For example, substitution of Phe^8 with β-Gly^8 completely prevented binding to EP24.15; again, loss of the aromatic side-chain was not the cause, as Ala^8-BK was readily degraded by this enzyme. Also of interest was the observation that alanine substitutions, particularly in the middle region of the peptide (residues 3–7), generally led to more efficient cleavage by both peptidases; this was also observed with analogues containing β-glycine near the *N*-terminus (Table 1). Although the basis for the acceleration of cleavage in these analogues cannot be determined from the current study, the results support the existence of an extended substrate recognition site for EP24.15 and EP24.16.

Two major conclusions may be drawn from the results of the current study. First, we have shown that β-amino acid substitution near the cleavage site of a peptide substrate prevents hydrolysis without necessarily preventing binding to the enzyme, thus demonstrating the potential for the use of β-amino acids in peptidase inhibitor design. Second, using a limited number of BK analogues, including peptides containing β-amino acids, significant differences in substrate specificity between two very similar peptidases have been elucidated. In future work, such differences may be exploited in the design of specific, selective, and peptidase-resistant enzyme inhibitors.

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