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Substrate Specificity and Kinetic Characterization of a Recombinant Dipeptidyl Carboxypeptidase from *Escherichia coli*

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ABSTRACT

Escherichia coli dipeptidyl carboxypeptidase (*Ec*DCP) is a zinc metallopeptidase with catalytic properties closely resembling those of angiotensin I-converting enzyme (ACE). In this study, the substrate specificity of *Ec*DCP was investigated by using a series of furanacryloyl-peptides (FA-peptides) and the physiological substrates of ACE. The fundamental specificity quantity, k_{cat}/K_M, of *Ec*DCP towards FA-Phe-Ala-Phe was determined to be 5246 mM^{-1} sec^{-1}, but FA-Gly-Gly-Phe, FA-Phe-Gly-Gly-NH_2 and some FA-dipeptides were resistant to hydrolysis. Based on the FA-tripeptides tested, *Ec*DCP was found to favor phenylalanine at P_1 position, glycine at P_1' position and phenylalanine at P_2 position, respectively. Experiments with bioactive peptides showed that *Ec*DCP preferred to cleave angiotensin I, bradykinin and [Met^5]-enkephalin near their C-termini. Moreover, *Ec*DCP had a comparable k_{cat}/K_M value with that of ACE when angiotensin I was used as the substrate. These results indicate that the enzyme could be useful for developing the inhibitory agents with improved antihypertensive properties.

Key words: Dipeptidyl carboxypeptidase, substrate specificity, angiotensin I-converting enzyme, Furanacryloyl-peptides, *Escherichia coli*, bradykinin

INTRODUCTION

The metallopeptidases of the M3 family (belonging to the gluzincin superfamily/clan MA) (Rawlings *et al*., 2010) contain a single catalytic zinc ion, which is coordinated with the imidazole side-chains of two histidine residues in HEXXH zinc binding sequence and with the Glu side-chain of a downstream Glu-$(Xaa)_3$-Asp motif, as exemplified by the prototypical bacterial zinc endopeptidase thermolysin (Matthews *et al*., 1972). This M3 family subdivides into the subfamily M3B, whose members are enzymes only from bacteria and into the widespread subfamily M3A, which comprises a number of high-molecular mass endo- as well as exo-peptidases from the three domains of life. Well-known mammalian/eukaryotic M3A endopeptidases are the thimet oligopeptidase (Cicilini *et al*., 1988), neurolysin (Checler *et al*., 1986) and the mitochondrial intermediate peptidase (Isaya and Kalousek, 1995). The first two are intracellular oligopeptidases, which act only on relatively short substrates with less than 20 amino acid residues (Knight *et al*., 1995), whereas the latter cleaves N-terminal octapeptides from proteins during their translocation across the mitochondrial membrane (Gakh *et al*., 2002). Besides, this subfamily contains several endopeptidases and a wide variety of bacterial dipeptidyl carboxypeptidases (DCP; EC 3.4.15.5).

The first DCP was isolated, purified and characterized from *Escherichia coli* extracts (Yaron *et al*., 1972). According to the gene sequence (Henrich *et al*., 1993), *E. coli* DCP (*Ec*DCP) consists of 680 amino acid residues, which form an active monomer with an apparent molecular mass of 77.5 kDa. Although it lacks of export signals and potential membrane-linked stretches, about 10% of the DCP activity within *E. coli* has been detected in the periplasmic space (Deutch and Soffer, 1978). As reported previously (Yaron *et al*., 1972), *Ec*DCP is capable of removing dipeptides from the C termini of peptide substrates, exhibiting hydrolytic activity towards α-N-blocked tripeptides, free tetrapeptides and longer peptides. However, peptide bonds before a Pro residue or between two Gly residues are resistant to *Ec*DCP cleavage and peptides with a blocked C terminus or with a C-terminal D-amino acid are not attacked. *Ec*DCP shares its substrate specificity and susceptibility to the antihypertensive drug captopril with the mammalian angiotensin I-converting enzyme (ACE), a membrane-anchored DCP playing an important role in blood pressure and salt metabolism (Riordan, 2003; Turner and Hooper, 2002). Due to this similar specificity, *Ec*DCP had originally been put into the same enzyme nomenclature entry (Peptidyl dipeptidase A) as ACE. However, *Ec*DCP and ACE were classified as different entries due to the low level of homology between both sequences (family M3 and family M2, respectively) (Barrett, 2004). In order to better understand the substrate specificity of the M3 carboxypeptidases, we have recently over-expressed the recombinant *Ec*DCP in *E. coli* M15 (Chen *et al*., 2009). In this study, the substrate specificity of the purified enzyme has been defined in terms of the k_{cat}/K_M values of a series of FA-tripeptides and these have been compared with the physiological substrates of ACE.

MATERIALS AND METHODS

Duration of this work and materials: This research project was conducted from August 1, 2008 to July 1, 2010. Nickel nitrilotriacetate (Ni^{2+}-NTA) resin was purchased from Qiagen Inc. (Valencia, CA, USA). Acrylamide, bisacrylamide, ammonium persulfate, TEMED and low-molecular-mass protein markers were acquired from Bio-Rad Laboratories (Richmond, CA, USA). Synthetic N-(3-[2-furyl]acryloyl]-blocked peptides were obtained from Promega Life Sciences (Madison, WI, USA). N-Benzoyl-L-glycyl-L-histidyl-L-leucine (HHL), angiotensin I, brandykinin, [Met^5]-enkephalin and substance P were obtained from Sigma-Aldrich Fine Chemicals (St. Louis, MO, USA). All other chemicals were reagent grade or of the highest purity available.

Expression and purification of the recombinant enzyme: *E. coli* M15 cells carrying pQE-*Ec*DCP (Chen *et al*., 2009) were grown in LB medium containing ampicillin (100 μg mL^{-1}) and kanamycin (25 μg mL^{-1}) at 37°C for 16 h. An aliquot (1 mL) of an overnight culture was used to inoculate 100 mL of the same medium and maintained the culture at 37°C with shaking (150 rpm) until the OD_{600} reached 0.6. Expression of the recombinant protein was induced by the addition of IPTG to a final concentration of 0.5 mM and cultivated at 26°C for 12 h. Cells were harvested by centrifugation at 6,000 g at 4°C for 10 min and resuspended in 10 mL of binding buffer (5 mM imidazole, 0.5 M NaCl and 50 mM Tris-HCl; pH 7.9). The His_6-tagged protein was purified from cell lysate fraction by affinity chromatography with Ni^{2+}-NTA resin according to the manufacturer's protocol (Qiagen). After extensive washing, the bound protein was eluted with 50 mM Tris-HCl buffer (pH 7.9) containing 0.5 M imidazole and 0.5 M NaCl.

Protein concentration was determined with a Bradford reagents kit (Bio-Rad) and bovine serum albumin as a standard protein. Enzyme was migrated in 12% sodium dodecyl

sulfate- polyacrylamide gel electrophoresis (SDS-PAGE) according to the method of Laemmli (1970). Protein bands were visualized by Coomassie brilliant blue R-250 staining.

Kinetic constants of the synthetic N-(3-[2-furyl]acryloyl]-peptides (FA-peptides): The activity assay for *Ec*DCP was performed according to the method of Holmquist *et al.* (1979) with some modifications. The reaction mixture contained 2 μL of enzyme solution at a suitable dilution and 200 μL of FA-peptides dissolved in 25 mM Tris-HCl buffer (pH 7.0) containing 300 mM NaCl. The mixture was incubated at 37°C for 5 min. Then, 20 μL of 10% (v/v) TFA was added to stop the reaction. Concentration determination of FA-peptides and hydrolysats was carried out using a Hitachi L-2200 series system equipped with a Jupliter 4u Proteo 90A column (Phenomenex Chem Co., Inc., USA) and with a constant flow rate of 1 mL min^{-1}. The compounds were separated using a gradient of 40-75% B (A, water/0.1% trifluoroacetic acid (TFA) (v/v); B, acetonitrile/0.1% TFA (v/v)). Substrates and products were monitored at 300 nm with a UV detector (L-4200 UV-Vis detector, Hitachi, Japan). The extent of hydrolysis was determined from the peak of the substrates by establishing calibration curves of the peak areas and converted to micromoles of substrate decreased. Initial velocities (v) of substrate hydrolysis were calculated from the micromoles of substrate decreased per minute. Kinetic parameters for the hydrolysis of FA-peptides were obtained from Lineaver-Burk plots Initial velocities were measured over a substrate concentration range from 125 to 500 μM. Turnover numbers (k_{cat}) were calculated from the equation $k_{cat} = V_{max}/[E]$, using a calculated *Ec*DCP molecular mass of 77,500 Da and considering the enzyme sample to be essentially pure and fully active.

Hydrolysis of bioactive peptides: Hydrolysis of angiotensin I, bradykinin and [Met^5]-enkephalin were performed by measuring the appearance of the products His-Leu, Phe-Arg, Phe-Leu and Phe-Met, respectively. Kinetic parameters for these peptides were obtained from Lineweaver-Burk plots. Initial velocities were measured during the first 20% of substrate hydrolysis. The assays were carried out 37°C over a substrate concentration range from 6×10^{-5} M to 1×10^{-4} M in 25 mM Tris-HCl buffer (pH 7.0) containing 300 mM NaCl. The reaction was stopped by the addition of 20 μL of 10% (v/v) TFA. The products of angiotensin I, bradykinin and [Met^5]-enkephalin were identified and quantified with the authentic dipeptides, which were eluted from a Jupliter 4u Proteo 90A column with retention times of 17.31, 14.39 and 14.69 min for DRVYIHPF, RPPGFSP and YGG, respectively.

RESULTS

Purification of the recombinant *Ec*DCP: The recombinant enzyme was purified from the cell-free extract of *E. coli* M15 (pQE-*Ec*DCP) by nickel-chelated chromatography. SDS-PAGE analysis of the purified enzyme revealed a molecular weight of approximately 77 kDa (data not shown). As reported recently (Lo *et al.*, 2010), the metal content of the enzyme was determined to be 1.02±0.04 mol of zinc per mol. Moreover, the enzyme preparation had a specific activity of 13.95±3.40 U mg^{-1} with HHL as the substrate.

FA-peptides hydrolysis: From a variety of FA-tripeptides tested, FA-Phe-Ala-Phe was shown to be the most suitable substrate, followed by FA-Phe-His-Leu (Table 1). The Lineweaver-Burk plot for the hydrolysis of FA-Phe-Ala-Phe was linear over the concentration range from 3×10^{-5} M to 2×10^{-3} M. The K_M and k_{cat} values for this substrate were 0.2 mM and 1218 sec^{-1}, respectively. Thus,

Table 1: Kinetic parameters of *Ec*DCP-catalyzed hydrolysis of various FA-peptides

FA-peptides[a]	V_{max} (μmol/min/mg)	K_M (mM)	k_{cat} (sec^{-1})	k_{cat}/K_M (mM^{-1} sec^{-1})
FA-Phe-Gly-Gly	1682	1.4	2172	1594
FA-Phe-Ala-Phe	943	0.2	1218	5246
FA-Phe-Ala-Gly	4149	5.7	5359	939
FA-Phe-Ala-Ala	562	1.3	726	552
FA-Phe-Leu-Gly	3258	6.5	4208	651
FA-Phe-His-Leu	1446	1.0	1868	1821
FA-Leu-Ala-Phe	344	4.4	445	100
FA-Leu-Leu-Gly	184	3.3	238	73
FA-Ala-Leu-Gly	149	1.5	192	126
FA-Gly-Leu-Phe	433	4.6	560	121
FA-Gly-Leu-Gly	213	13.5	275	20
FA-Gly-Leu-Ala	226	12.5	292	23
FA-Gly-Gly-Phe	NH[b]	-[c]	-	-
FA-Gly-Gly	NH	-	-	-
FA-Leu-Gly	NH	-	-	-
FA-Phe-Ala	NH	-	-	-
FA-Phe-Gly-Gly-NH_2	NH	-	-	-

[a]Assay conditions: FA-peptides in 25 mM Tris buffer (pH 7.0) containing 300 mM NaCl at 37°C. [b]NH, not hydrolyzed. [c]Not measured

Table 2: Relative kinetic parameters of *Ec*DCP-catalyzed hydrolysis of FA-Xaa-Leu-Gly, FA-Phe-Xaa-Gly and FA-Gly-Leu-Xaa

FA-peptides	Relative K_M	Relative k_{cat} (%)	Relative k_{cat}/K_M (%)
FA-Xaa-Leu-Gly			
FA-Phe-Leu-Gly	4.2	100.0	100.0
FA-Leu-Leu-Gly	2.2	5.6	11.2
FA-Ala-Leu-Gly	1.0	4.6	19.4
FA-Gly-Leu-Gly	8.8	4.3	2.2
FA-Phe-Xaa-Gly			
FA-Phe-Gly-Gly	1.0	42.0	100.0
FA-Phe-Ala-Gly	4.1	100.0	59.0
FA-Phe-Leu-Gly	4.6	79.0	41.0
FA-Gly-Leu-Xaa			
FA-Gly-Leu-Phe	1.0	100.0	100.0
FA-Gly- Leu- Gly	2.9	98.0	34.0
FA-Gly- Leu- Ala	2.7	52.0	19.0

this substrate shows satisfactory kinetic behavior from about 10-fold above to 10-fold below the K_M. It is worth nothing that the catalytic efficiency (k_{cat}/K_M) of *Ec*DCP towards the ACE substrate, FA-Phe-Gly-Gly (Holmquist *et al.*, 1979), was 1594 mM^{-1} sec^{-1}, which is 2.3-fold lower than that of FA-Phe-Ala-Phe.

Consistent with the finding of Yaron *et al.* (1972), the purified enzyme was not able to hydrolyze FA-Gly-Gly-Phe (Table 1). Additionally, FA-dipeptides or FA-peptide with C terminus blocked were resistant to hydrolyze by the enzyme. FA-tripeptides were also selected to map the amino acid preference of the subsites S_1, S_1' and S_2' of *Ec*DCP (Table 2). The S_1 subsite had a strong preference for hydrophobic amino acids with Phe as the best accepted residue on P_1 position. In this regard, we fixed Phe in the P_1 position and prepared four N-blocked tripeptides with FA-Phe-Xaa-Gly sequences to study the specificity of the subsite S_1'. Table 2 shows that peptides containing Gly at P_1' position was very susceptible to hydrolyze by *Ec*DCP. Additionally,

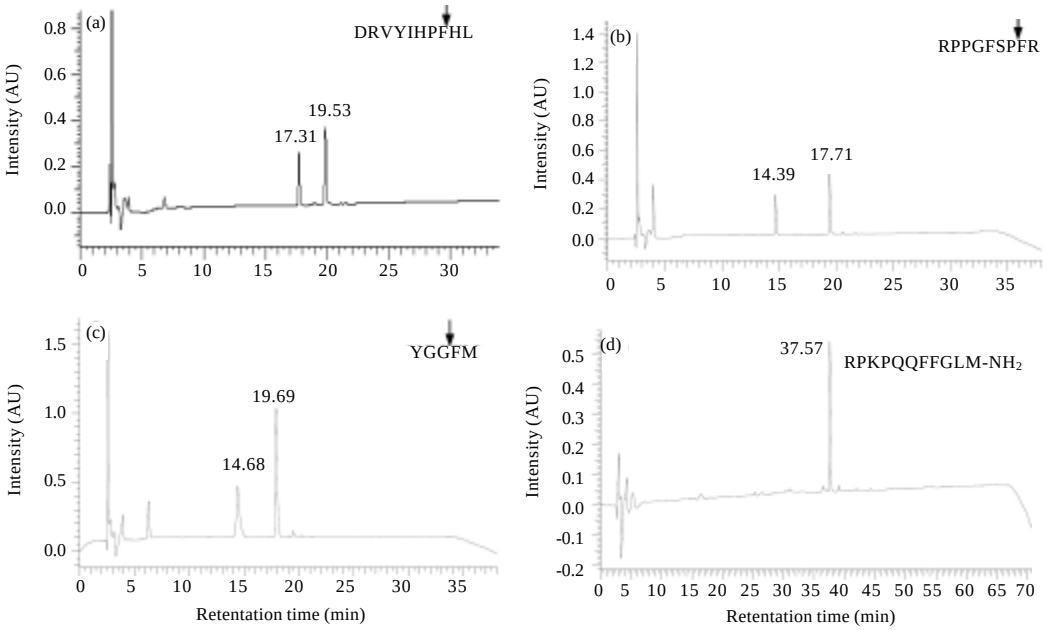

Fig. 1: HPLC analysis of the hydrolysis of bioactive peptides by *Ec*DCP. In this experiment, the enzyme and substrates (0.3 mM) were incubated at 37°C in 25 mM Tris-HCl buffer (pH 7.0) containing 0.3 M NaCl. After 15-min incubation, the reaction mixtures were stopped by 10% TFA and used for reverse phase HPLC analysis as described in materials and methods. Panels: A, Angiotensin I; B, Bardykinin; C, [Met^5]-enkephalin; D, substance P

the S_2' pocket of the enzyme was more restrictive and showed remarkable selectivity for Phe at P_2' as shown by the efficient hydrolysis of FA-Gly-Leu-Phe. These results are very similar to those obtained in the specificity studies with positional-scanning synthetic combinatorial fluoregenic peptides for *Ec*DCP (Cunha *et al*., 2009).

Hydrolysis of natural ACE substrates by the recombinant enzyme: Four natural ACE substrates were incubated with *Ec*DCP for 15 min and the cleavage sites were defined by HPLC (Fig. 1). The retention time for angiotensin I, bradykinin, [Met^5]-enkephalin and substance P was 19.53, 17.71, 19.69 and 37.57 min, respectively. Angiotensin I (DRVYHPFHL) was hydrolyzed by the enzyme at the F-H bond generating angiotensin II (DRVYHPF) and a dipeptide. Bradykinin and [Met^5]-enkephalin was cleaved by *Ec*DCP to release the C-terminal dipeptides F-R and F-M. Not surprisingly, the vasoactive neuropeptide, substance P, was not hydrolyzed due to the presence of an acidic residue at P_1 site (Cunha *et al*., 2009).

The K_M, k_{cat} and k_{cat}/K_M values of the enzyme for angiotensin I, bradykinin and [Met^5]-enkephalin are presented in Table 3. The highest k_{cat}/K_M values are obtained for bradykinin, followed by [Met^5]-enkephalin. The highest k_{cat} value, 178 sec^{-1}, was displayed by [Met^5]-enkephalin, whereas the lowest K_M value, 0.3 mM, was observed for bradykinin. In comparison with ACE (Bunning *et al*., 1983), the k_{cat}/K_M value of *Ec*DCP towards angiotensin I diplayed an order of magnitude higher. Also, the enzyme exhibited a very low K_M value for bradykinin. This feature is similar to ACE, which shows a K_M value at least an order of magnitude smaller than that for angiotensin I (Bunning *et al*., 1983).

Table 3: Kinetic parameters of *Ec*DCP-catalyzed hydrolysis of bioactive peptides

Peptides	V_{max} (μmol/min/mg)	K_M (mM)	k_{cat} (sec^{-1})	k_{cat}/K_M (mM^{-1} sec^{-1})
Angiotensin I	43	0.5	56	114
Bradykinin	74	0.3	96	380
[Met^5]-enkephalin	138	0.8	178	236
Substance P	-[a]	-	-	-

[a]-Not hydrolyzed

DISCUSSION

Topologically, the *Ec*DCP molecule bears some similarity with human ACE molecule (PDB code 1O8A). This M2 proteinase resemble *Ec*DCP with respect to size, shape and secondary structures and is equipped with similar/identical functional groups for the precise and tight binding and efficient hydrolytic cleavage of polypeptide substrates (Comellas-Bigler *et al*., 2005). In the present work, we describe the catalytic properties that are shared by both enzymes and demonstrate that they display common hydrolytic characteristics.

It has been shown that FA-substituted tripeptides are excellent substrates for ACE (Holmquist *et al*., 1979). FA-derived substrates have already found acceptance for assays and mechanistic investigations of other metalloproteases (Feder and Schuck, 1970; Holmquist and Vallee, 1973; Holmquist *et al*., 1979). These peptides exhibit a maximal absorbance at 306 nm (e = 25000 M^{-1} cm^{-1}) that shifts to 303 nm on hydrolysis to the FA-blocked amino acid plus a dipeptide. Although, this change is relatively small, it is sufficient to allow hydrolysis to be monitored continuously over a wide range of substrate concentrations. An investigation of the kinetic parameters of a series of FA-tripeptide substrates has provided some insight into the specificity of EcDCP. The highest activities, expressed as k_{cat}/K_M and the tightest substrate binding were observed for FA-Phe-X-Y. Clearly, the enzyme shows preference for those with an aromatic residue in the antepenultimate position, designated P_1 in the terminology of Schechter and Berger (1967), adjacent to the scissile bond. The k_{cat} values for FA-Phe-Gly-Gly and FA-Phe-Ala-Gly are the largest in this series, perhaps reflecting facile product dissociation. Activities seem to be largely determined by the residue at P_1 with Phe> Leu> Ala >Gly and to a lesser extent by that at P_1' and P_2'. Substrates with lecuyl residue at P '$_1$ have low k $_{cat}$vales, whereas those with a phenylalanyl residue at P_2' have low K_M values.

The activity of *Ec*DCP toward FA-Phe-His-Leu has been compared with that toward two physiological substrates, angiotensin I and bradykinin, of ACE (Table 4). The synthetic N-substituted tripeptide is hydrolyzed much more rapidly then either of the natural peptides, its k_{cat} value being 1868 sec^{-1} vs 56 and 96 sec^{-1} for the other two, respectively. In contrast, the natural peptides bind more tightly to the enzyme, their K_M values being 0.5 and 0.3 mM, respectively, compared to 1.0 mM for FA-Phe-His-Leu. The availability of a synthetic tripeptide substrate that is hydrolyzed by *Ec*DCP with a catalytic efficiency comparable to those of physiological substrates and yet provides for a rapid, convenient and continuous spectrophotometric assay should greatly facilitate the physiological and mechanistic characterization of the enzyme.

One of the physiological roles of ACE is to generate the octapeptide angiotensin II, which through its powerful vaso-constricting action and the release of aldosterone is an important factor in the control of blood pressure. Earlier, it has been shown that a number of potent inhibitors of ACE are useful in the treatment of hypertension and their effectiveness is thought to relate to their binding specificity (Cheung *et al*., 1980). Based on the facts that *Ec*DCP is closely resembled with

Table 4: Catalytic properties of *Ec*DCP- and ACE-catalyzed hydrolysis of FA-Phe-His-Leu and angiotensin I

Kinetic parameters	Substrates	*Ec*DCP	ACE[a]
K_M (mM)	FA-Phe-His-Leu	1.0	0.068
	Angiotensin I	0.5	0.07
k_{cat} (sec^{-1})	FA-Phe-His-Leu	1868.0	68.00
	Angiotensin I	56.0	11.00
k_{cat}/K_M (mM^{-1} sec^{-1})	FA-Phe-His-Leu	1821.0	1000.00
	Angiotensin I	114.0	157.00

[a]El-Dorry *et al.* (1982)

ACE, these studies with FA-peptides and the physiological substrates are intended to better understanding of the structural basis for the specificity of *Ec*DCP.

In conclusion, we have shown that *Ec*DCP and ACE have many enzymatic similarities, despite their differences in primary sequence. These findings aid in the development of inhibitory agents with improved antihypertensive properties.

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