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PROTEOLYTIC ACTIVITY OF DIPEPTIDYL CARBOXYPEPTIDASE FROM HUMAN LUNG

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Abstract

Dipeptidyl carboxypeptidase (EC 3.4.15.1) was purified from human lung and some of its physical and chemical properties were investigated and compared to similar enzymes obtained from other sources. It was found that the enzyme has broad specificity in releasing consecutive dipeptides from the C-terminus of polypeptides. Enzyme hydrolysis of a peptide was stopped only when a prolyl residue was encountered in the penultimate position from the C-terminal residue. The broad specificity of this proteolytic enzyme suggests that it has potential for use as a specialized tool for the investigation of the primary structure of polypeptides and proteins.

Introduction

A number of enzymes have been isolated from mammalian tissue which have dipeptidyl carboxypeptidase activity in that they can convert angiotensin I to angiotensin II by releasing the C-terminal dipeptide his-leu. In 1956, Skeggs et al (1) first described an angiotensin converting enzyme isolated from horse plasma. Further work by other investigators showed that this enzyme was not specific for angiotensin I, but could release the C-terminal dipeptide from bradykinin and several other small oligopeptides having a free carboxyl group at the C-terminal residue(2,5). This enzyme could not, however, hydrolyze the imide bond of a prolyl residue or release a dipeptide from a substrate having a C-terminal glutamyl residue(2). Enzymes having similar catalytic activities have been isolated from human plasma, urine, and lung as well as from various animal tissues. Generally, these enzymes are glycoproteins having a monomer molecular weight of approximately 140,000, are chloride

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activated and are inhibited by dipeptides(5), bradykinin(3,8), bradykinin potentiating peptides(3,4,8) and EDTA(1). Studies designed to elucidate the in vivo action of this class of enzymes have indicated that angiotensin converting enzyme plays a role in the pathogenesis of hypertension(10,11). Although the name 'angiotensin converting enzyme' appears to be commonly used, other names such as dipeptidyl carboxypeptidase(6), kininase II(6), peptidase P(7), carboxycathepsin(2) and angiotensin I [phe^{8}-his^{9}] hydrolase(9) have also been used to reflect differing physical and chemical properties and/or sources of origin of various preparations.

We have studied the enzyme from human lung and have used the name 'dipeptidyl carboxypeptidase'. Our interest in the enzyme is based on its potential for having general proteolytic activity in releasing dipeptides from substrates and therefore its use as a tool to study the primary structure of polypeptides and proteins. The results of this work demonstrate that this enzyme has very broad specificity indeed, in sequentially releasing dipeptides from the C-terminus of a wide variety of natural and synthetic polypeptides. Hydrolysis terminates when a prolyl residue appears in the penultimate position with respect to the C-terminus, i.e., the enzyme cannot hydrolyze the prolyl imide bond. We also report some physical and chemical properties of the enzyme, particularly those pertinent to its catalytic behavior.

Materials and Methods

Kemptide, thymus factor, delta sleep inducing peptide, leu-enkephalin met-enkephalin, bradykinin, renin substrate, and EAE peptide were purchased from Penninsula Laboratories, Inc.; glucagon, alanyl-alanyl-alanine, and ribonuclease S-peptide from Sigma Chemical Co.; and human fibrinopeptide A, eosinophilotactic peptides (A-G-S-E, V-G-S-E, and N-Formyl-V-G-S-E), and phe-leu-glu-glu-leu from Vega Biochemicals. Pentafluoropropionic anhydride was obtained from Pierce Chemical Co.

Dipeptidyl carboxypeptidase was purified from human cadaveric lungs. Frozen tissue was thawed and homogenized in 0.01 M sodium phosphate, pH 7.8 and the homogenate centrifuged for 30 min. at 5000 x g. Triton X-100 was added to the supernatant to a concentration of 0.2%, the solution stirred for 4 hr., then centrifuged for 60 min. at 40,000 x g. The supernatant was fractionated with $(NH_4)_2SO_4$ between 0.5 M and 1.8 M. The final precipitate was dissolved in water and dialyzed against 0.01 M sodium phosphate, pH 7.8. The solution was concentrated by ultrafiltration

FRACTION	VOLUMN (ml.)	PROTEIN (mg.)	ACTIVITY* (units)	SPECIFIC ACTIVITY (mu/mg)	PURIFI-CATION
HOMOGENATE	1500	45,700	31	0.68	1
AMMONIUM SULFATE	600	7,500	18	2.4	3.5
BATCH CM-SEPHADEX	500	3,700	15	4.1	6
DE52 CHROMATOGRAPHY	15	1,010	8.9	8.8	13
BIO GEL A-1.5M CHROMATOGRAPHY	9.5	380	5.2	13.7	20
	6.0	220	3.1	14.1	21
HYDROXYAPATITE	10.1	9.9	4.1	414	618

*One unit of dipeptidyl carboxypeptidase hydrolyzes 1 mole of hippuryl-his-leu per minute at pH 7.8 and 37 C.

Figure 1:
Purification of dipeptidyl carboxypeptidase from human lung.

through a Diaflo UM100 filter, and applied to a DE52 column (2.5 x 80 cm.). Active fractions, which were eluted with a NaCl gradient from 0-0.5 M, were combined, concentrated by ultrafiltration through a UM100 filter, and applied to a Bio Rad A-1.5M column (5 x 90 cm.). Two peaks of activity were found. Each of these peaks was combined and concentrated by ultrafiltration. The solution containing the larger enzyme was dialyzed against 0.01 M sodium phosphate, pH 6.8 and applied to a hydroxyapatite column (1 x 25 cm.). The enzyme activity, which did not bind to the column, was pooled and dialyzed against 0.1 M NH_4Cl, 0.01 M NH_4HCO_3, pH 7.8. The purification is sumarized on figure 1.

Enzyme assays were performed according to the method of Cushman and Cheung (14).

Hydrolysis was carried by dissolving the substrate (100 nmole) in 200 µl of the enzyme solution (10 milliunits) and incubating at 37°C for 12 hr in a 1 ml reacti-vial fitted with a teflon cap. The reaction was stopped by freezing and lyophilizing the mixture.

The dipeptide products were derivatized to form the N,O-perfluoropropionyl (PFP) methyl esters according to the method of Caprioli et al (12).

Arginine containing dipeptides require prior derivatization of the guanidine side chain for reproducible results. Conversion of arginine residues to the (4,6-dimethylpyrimid-2-yl)-ornithine analog was done by the method of Bacon et al (13), with the following modifications. The residue from the enzymatic hydrolysis was dissolved in 200 µl of dry methanol and 60 µl of 2,4-pentanedione. Triethylamine was added until the pH of the solution was between 7 and 8. A molecular sieve was added to remove excess water. The reaction was allowed to proceed overnight at room temperature. The molecular sieve was removed and then the reagents were removed under vacuum. To remove N-terminal acetylacetonyl groups, the residue was dissolved in 0.5 ml. of water which was acidified to pH 4 with acetic acid and heated to 100°C for 10 min. After cooling, the solution was extracted with ether. The aqueous layer was lyophilized, and the residue was derivatized as described above.

Mass spectrometric analyses were performed on a Finnigan 3200/6000 GC/MS/data system equipped with an electron impact source. Chromatographic

separation was performed using a glass column (2mm X 1.5m) packed with 3% OV-1 on 80/100 mesh Gas-Chrome Q. The gas chromatograph injector was set at 230°C, and a linear temperature program from 100 to 300°C at 10°C/min was used. Helium was used as the carrier gas at a flow rate of 30 ml/min. The glass jet separator was held at 260°C, and the analyzer at 100°C. The electron impact source was operated at an ionization energy of 70ev. For samples introduced by the direct probe, the acetone solution was placed in a capillary tube, the solvent removed under vacuum and the probe assembly heated from 20-350°C.

Results and Discussion

Dipeptidyl carboxypeptidase from human lung exhibited many of the same characteristics found for angiotensin-I converting enzyme from various mammalian tissues; i.e. inhibition by EDTA, activation by Cl^- and a broad pH optimum around pH 7.8.

A variety of polypeptides differing in length from 3 to 29 residues were hydrolyzed with this enzyme to determine its general proteolytic activity. Figure 2 shows the specific dipeptides released from each of the substrates. Each of the dipeptides released by the enzyme catalyzed reaction was unambiguously identified by mass spectrometry. A typical

Ala-ala-ala	A-A-A
Eosinophilotactic Tetrapeptide	A-G-S-E
Eosinophilotactic Tetrapeptide	V-G-S-E
Ala-ala-ala-ethyl ester	A-A-A-OEt
Leu-Enkephalin	Y-G-G-F-L
Met-Enkephalin	Y-G-G-F-M
Phe-leu-glu-glu-leu	F-L-E-E-L
Kemptide Phosphate Acceptor Peptide	L-R-R-A-S-L-G
N-Formyl Eosinophilotactic Tetrapeptide	N-formyl-V-G-S-E
Thymus Factor	Q-A-K-S-Q-G-G-S-N
Delta Sleep Inducing Peptide	W-A-G-G-D-A-S-G-E
EAE Peptide	F-S-W-G-A-E-G-Q-R
Bradykinin	R-P-P-G-F-S-P-F-R
Angiotensin I	D-R-V-Y-I-H-P-F-H-L
Renin Substrate	D-R-V-Y-I-H-P-F-H-L-L-V-Y-S
Fibrinopeptide A	A-D-S-G-E-G-D-F-L-A-E-G-G-G-V-R
Ribonuclease S-peptide	K-E-T-A-A-A-K-F-E-R-Q-H-M-D-S-S-T-S-A-A
Glucagon	H-S-Q-G-T-F-T-S-D-Y-S-K-Y-L-D-S-R-R-A-Q-D-F-V-Q-W-L-M-N-T

Figure 2:
Hydrolysis of substrates by dipeptidyl carboxypeptidase.

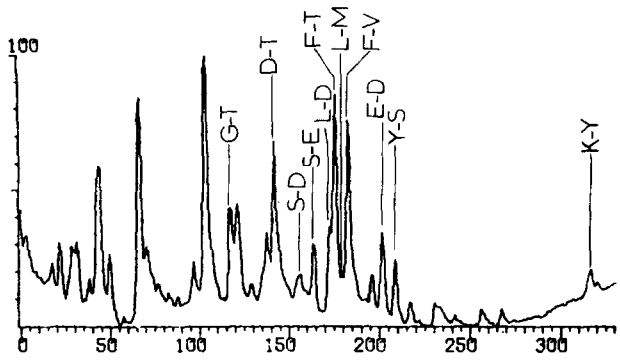

Figure 3:
Total ion chromatogram of acid and neutral dipeptides released by the hydrolysis of glucagon with dipeptidyl carboxypeptidase.

GC/MS analysis of the eleven acid and neutral dipeptides from the enzyme digest of glucagon is shown in Figure 3. The three remaining dipeptides, which are basic, were identified separately, after proper derivatization by direct probe; for example, the mass spectrum of derivatized arginyl-alanine from the glucagon digest is shown in Figure 4.

From the wide variety of amino acids represented in the bonds cleaved, it is concluded that dipeptidyl carboxypeptidase has general proteolytic activity towards polypeptide substrates in that it appears to cleave all bonds except those involving prolyl imide bonds. Thus, bradykinin, angiotensin I, and renin substrate were hydrolyzed and C-terminal dipeptides released until a prolyl residue appeared in the penultimate position to the C-terminus. At this point hydrolysis was terminated, presumably due to the inability of the enzyme to cleave the prolyl imide bond. No amino acids or dipeptides from the N-terminal side of the prolyl residue could be detected. However, when a prolyl residue appears in position 3 from the C-terminus, hydrolysis proceeds past this position showing that the enzyme can hydrolyze the prolyl amide bond. This is illustrated in Figure 2 for bradykinin where the prolyl-phenylalanyl bond is readily hydrolyzed.

We have found that the enzyme from human lung can release dipeptides from a substrate containing a C-terminal glutamate or aspartate. This

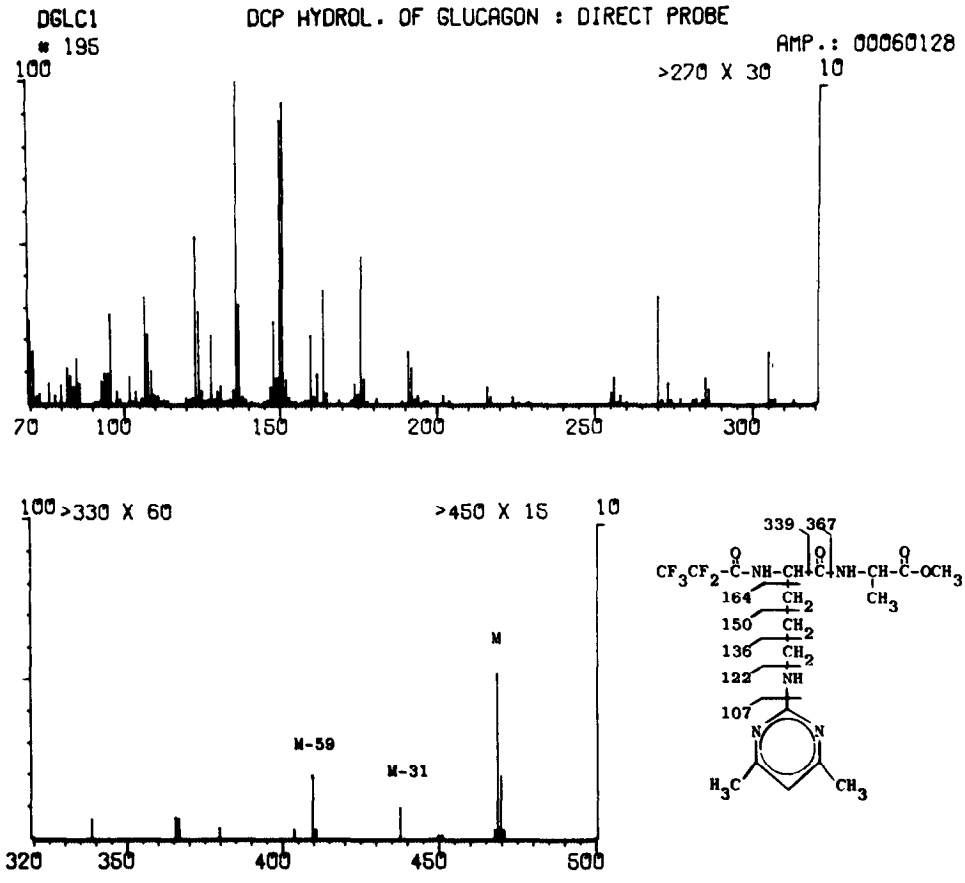

Figure 4: Mass spectrum of derivatized arginyl-alanyl obtained from the hydrolysis of glucagon.

is in contrast to results obtained with a similar enzyme, angiotensin converting enzyme isolated from bovine kidney cortex, where it is reported that C-terminal glutamyl residues cannot be hydrolyzed (12). From our results with the hydrolysis of glucagon, ribonuclease-S peptide, delta sleep inducing peptide and both nascent and n-formyl eosinophilotactic tetrapeptide (see Figure 2), it is clear that human dipeptidyl carboxypeptidase can release dipeptides containing C-terminal acid residues.

Finally, we have found that this enzyme will cleave tripeptides to give an amino acid and the C-terminal dipeptide. Again, this in contrast to the action of angiotensin converting enzyme which has been reported to be unable to cleave tripeptides. It can be seen from Figure 2 that several substrates with odd-numbered residues were hydrolyzed to completion,

necessitating the cleavage of the final tripeptide. The enzyme preparation used here was assayed for both aminopeptidase and dipeptidyl aminopeptidase contaminants and none was detected. Further, although ala-ala-ala was hydrolyzed to give ala and ala-ala, the ethyl ester of this tripeptide did not produce free ala or dipeptide. This is consistent with reports that the enzyme requires a free carboxyl group to effect hydrolysis.

We conclude that dipeptidyl carboxypeptidase has broad specificity towards polypeptides with the only block thus far found being a prolyl residue penultimate to the C-terminal amino acid. Since this enzyme has the ability to remove dipeptides from the C-terminal end of substrates, it has great potential in being utilized as a tool for the determination of the primary structure of proteins.

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