BBA Report

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Dipeptidyl carboxypeptidase from *Coryne bacterium equi*

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SUMMARY

A dipeptidyl carboxypeptidase which removes the C-terminus and penultimate amino acids as a dipeptide from peptide substrates was found in *Coryne bacterium equi*, and a partially purified enzyme preparation was obtained with a specific activity of 40 nmoles of histidylleucine produced per min per mg of protein using angiotensin I as a substrate. The approximate molecular weight of this enzyme was 80 000 based on gel filtration using Sephadex G-100. The enzyme hydrolyzed a number of peptide substrates and appears to have a broad specificity except that the peptide bond involving the imino function of proline is not cleaved. In this respect, the mode of action of the enzyme is similar to that of the mammalian angiotensin-converting enzyme. Similar to the converting enzyme this enzyme appears to have a metal ion requirement, for both are inhibited by EDTA and neither is inhibited by diethylfluorophosphate. The molecular weight of this enzyme is lower than the molecular weight of the converting enzyme (150 000) and unlike the latter does not require Cl^- for the activity.

Although carboxypeptidases are well known and widely distributed in nature there have been few reports of a dipeptidyl carboxypeptidase, *i.e.* an enzyme that removes the C-terminal and penultimate amino acids as a dipeptide. The only known dipeptidyl carboxypeptidase is the mammalian angiotensin converting enzyme which hydrolytically removes the C-terminal histidylleucine dipeptide from the decapeptide, angiotensin I, to form the biologically active (vasopressor) octapeptide, angiotensin II[1–6]. The octapeptide is not attacked. There have been several reports that the converting enzyme is not specific. Preparations from mammalian tissue that have converting enzyme activity also release the C-terminal dipeptide from a number of other peptides including bradykinin[7–9].

In the present study we have investigated a peptidase from *Coryne bacterium equi* and from an examination of the products obtained with different substrates we have identified it as a dipeptidyl carboxypeptidase. The question arises whether dipeptidyl carboxypeptidases are a general class of enzymes that are widely distributed in nature.

Enzyme preparation: Two enzyme preparations were used.

(A) A solution of histidylleucine, $2 \cdot 10^{-3}$ M in 0.05 M phosphate buffer, pH 7.0, that had been repeatedly frozen and thawed developed a rather high level of dipeptidyl carboxypeptidase activity. Bacterial contamination of the solution was barely discernible but inoculation of a fresh histidylleucine solution led to the development of enzyme activity. When the solution was streaked on nutrient agar, grayish white mucoid colonies of irregular shape developed at room temperature in one day. These bacteria were identified as *Coryne bacterium equi* by the staff of the Pathology Department of the University Medical School. The supernatant from the histidylleucine solution was used as an enzyme stock.

(B) *Coryne bacterium equi* were grown at room temperature for 2 days in Bacto nutrient broth. The broken cells were treated with protamine sulfate and centrifuged. The supernatant was fractionated between 1.9 and 3.0 M ammonium sulfate. The resulting precipitate was dissolved in 0.05 M phosphate buffer, pH 7.0, and further fractionated by column chromatography with Sephadex G-100. The peak fractions were adsorbed on DE-52 cellulose and eluted from the column at 0.1 M NaCl using a 0.0–0.5 M salt gradient. The pooled peak fractions were used as an enzyme stock. The specific activity was 40 nmoles of histidylleucine produced per min per mg of protein using [Asp^1,^{14}C-Leu^{10}]-angiotensin I as a substrate.

Enzyme assay: Throughout the purification procedure the enzyme was assayed by the formation of histidyl[^{14}C]leucine from the decapeptide angiotensin I ([^{14}C]Leu^{10})^{2,10}.

The assay system contained 5 nmoles of [Asp^1,Ile^5,^{14}C-Leu^{10}]-angiotensin I★ (0.2 μC/μmole) and 0.25–100 μg of protein in 50 μl of 0.05 M sodium phosphate buffer, pH 7.0. The mixture was incubated at 37° for 1–2 h and the reaction was stopped by adding 10 μl of 50% trichloroacetic acid. The sample was then spotted on Whatman 3MM chromatographic paper and the paper was developed by high voltage paper electrophoresis at 50 V/cm for 90 min using a pyridine–acetic acid–water buffer (1:10:289, by vol.), pH 3.6. During this time histidylleucine migrates approximately 4 cm further than angiotensin I and 20 cm further than leucine. After electrophoresis, the paper was cut into 2-cm sections and each section was counted for 10 min in a 25-ml scintillation vial containing 10 ml of scintillation mixture (7 g PPO and 0.5 g POPOP in 1 l of toluene) using the Beckman LS-250 Liquid Scintillation System. The extent of the enzyme reaction was estimated by measuring the radioactivity of both the product histidylleucine and the substrate angiotensin I. Protein concentrations were approximated from $A_{280\ nm}/A_{260\ nm}$ ratios.

Enzymic hydrolysis of peptides: The peptide substrate at $2 \cdot 10^{-3}$ M concentrations was incubated in 50 μl of 0.01 M phosphate buffer, pH 7.2, containing 10 μl of enzyme preparation. After incubation at 37° for 2 h the reaction was stopped by adding 10 μl of 50% trichloroacetic acid, and was centrifuged. The reaction mixture was then diluted to 1.0 ml with sample dilution buffer, pH 2.2, for application to the amino acid analyzer. The JEOLCO amino acid analyzer Model JLG-5AH was used for analysis of the products of enzymic hydrolysis of the peptides (Fig. 1). The elution times and color intensities of all the common amino acids, of all peptide substrates, and of most of the possible dipeptide products were determined. The pertinent elution times are given in Table I. The sample in the amino acid analyzer was 0.8 ml of the diluted reaction mixture.

★The radioactive decapeptide [Asp^1,Ile^5,^{14}C-Leu^{10}]-angiotensin I was purchased from Schwarz/Mann, Orangeburg, N.Y. (lot No. 6901).

TABLE I

ELUTION TIMES OF PEPTIDES FROM THE AMINO ACID ANALYZER

The elution times of all amino acids are known but not included in the table. Chromatographic column temperature was 55° and flow rates of buffer pumps were 1.22 ml/min and 0.83 ml/min for short column and long column, respectively.

Amino acid or peptide	*Elution time (min)*
From long column (0.8 cm x 70 cm)	
Pro–Phe	69
Pro	89
Gly	102
Ala	108
Gly–Ala–Ala	162
Gly–Gly	170
Gly–Ala	171
Ala–Ala	171
Leu	172
Tyr	192
Phe	199
Gly–Leu	238
Gly–Leu–Tyr	380
Gly–Gly–Phe	391
Gly–Phe	395
Gly–Phe–Ala	418
Phe–Gly	420
Phe–Gly–Gly	423
Leu–Tyr	433
Phe–Ala	521
Phe–Gly–Gly–Phe	526
From short column (0.8 cm x 15 cm)	
His–Leu	46
His–Gly	51
[Asp^1]-Angiotensin I	56
[Asn^1]-Angiotensin II	56
His	58
Gly–His	58
Gly–His–Gly	66

Test for inhibition by diethylfluorophosphate and EDTA: Enzyme was incubated for 30 min at 5° with $5 \cdot 10^{-3}$ M diethylfluorophosphate or 10^{-3}–$10 \cdot 10^{-3}$ M EDTA and then assayed by the radioactivity method using angiotensin I ([^{14}C]Leu^{10}) as substrate. During the assay $0.1 \cdot 10^{-4}$–$10 \cdot 10^{-4}$ M diethylfluorophosphate was present and in the case of EDTA, 10^{-4}–$10 \cdot 10^{-4}$ M EDTA was present.

The same results were obtained with both enzyme preparations A and B. However, the original homogenate from the grown cells did contain other peptidase activities and it was only after the few steps of purification that the unwanted peptidases were removed. Only a modest increase in specific activity of about 20 fold was obtained but this proved satisfactory for demonstrating the existence of a dipeptidyl carboxypeptidase (Tables II and III). For example, dipeptides, His–Leu and Gly–Ala, were readily split by the homogenate but not by the enzyme preparations A and B.

In all cases, except with Phe–Gly–Gly–Phe, where hydrolysis occurred, the C-terminal dipeptide was obtained as the only dipeptide product. In no instance was any

N-terminal dipeptide obtained even in trace amounts except in the case of Phe-Gly-Gly-Phe.

The elution times of the products using the amino acid analyzer are given in Table I. The times for all the common amino acids are, of course, known. In all cases except Gly-Ala-Ala the products can be easily identified. In the case of Gly-Ala-Ala, Ala-Ala cannot be distinguished from Gly-Ala but in the first instance glycine would be the other product and in the second case alanine would be the other product. Glycine and alanine are easily distinguished and therefore the cleavage point is easily recognized (Fig. 1).

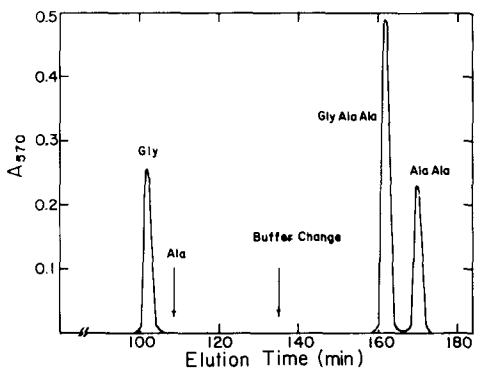

Fig. 1. Quantitative analysis of enzymic peptide hydrolysis by amino acid analyzer. Gly-Ala-Ala as a peptide substrate was incubated with the enzyme preparation as described in the text. The sample, 0.8 ml of the final supernatant, was applied to a column of JEOL resin, AR-15 (0.8 cm x 70 cm), in two different sodium citrate buffers, pH 3.25 and pH 4.25 for first and second elution buffer, respectively. No alanine was detected in this experiment.

TABLE II

ENZYMIC HYDROLYSIS OF PEPTIDES

Substrate	*Enzymic product (% reaction)*	
	Free amino acid	*Peptide*
Gly-Ala Ala	Gly (30)	Ala Ala (32)
Gly-Phe Ala	Gly (52)	Phe Ala (47)
Gly-His Gly	Gly (28)	His Gly (21)
Gly-Gly Phe	Gly (34)	Gly Phe (–)★
Gly-Leu Tyr	Gly (15)	Leu Tyr (–)★
Phe-Gly Gly	Phe (61)	Gly Gly (63)
Phe-His Leu	Phe (28)	His Leu (–)★
Phe Gly-Gly Phe	None	Phe Gly (36)
		Gly Phe (38)
Angiotensin I (~Pro Phe-His Leu)	None	His Leu (33)★★
Gly-Pro Ala	None	None
Angiotensin II (~Ile His-Pro Phe)	None	None
Gly-Gly, Gly-Ala	No hydrolysis	
Gly Phe, His Leu		

★No measurement was made due to its long elution time.
★★Radioactivity measurement using angiotensin I ([^{14}C] Leu^{10}).

TABLE III

ENZYMIC HYDROLYSIS OF BLOCKED PEPTIDES

Substrate★	*Enzymic product (% reaction)*	
	Peptide	*Free amino acid*
N-Z-Phe-Gly-Gly	Gly Gly (35)	None
N-Z-Phe-Gly-Gly-NH_2★★	None	None
N-Z-Pro-Leu-Gly	Leu Gly (20)	None
N-Z-Pro-Leu-Gly-NH_2★★	None	None
N-Acetyl-Ala-Ala-Ala	Ala Ala (31)	None
Ala-Ala-Ala-OEt	None	None
N-Benzoyl-Gly-Gly	None★★★	None
N-Benzoyl-Gly-Phe	None	None

★Z = benzyloxycarbonyl.
★★Blocked peptide amides were solubilized by adding a small amount of carbon dichloride.
★★★Trace amount of Gly Gly was found.

In some cases the dipeptide product from a tripeptide was not measured because of the inconveniently long elution time.

The approximate molecular weight by gel filtration experiment with Sephadex G-100 was 80 000. Catalase, alkaline phosphatase and bovine serum albumin were used as markers. Cl^- was not required for activity of the enzyme. Preincubation with 10^{-3} M EDTA inhibited the enzyme completely, but 10^{-3} M diethylfluorophosphate had no effect. The rate of hydrolysis of angiotensin I was proportional to the concentration of enzyme and was constant with time up to about 50% hydrolysis.

The failure of the enzyme preparation to hydrolyze dipeptides and hippurylamino acids suggests that no aminopeptidase, carboxypeptidase, or other general peptidase activities are present. As far as the tripeptides alone are concerned, their mode of hydrolysis could be accounted for by either a dipeptidyl carboxypeptidase or an aminopeptidase. An aminopeptidase is completely ruled out by the mode of cleavage of Phe-Gly-Gly-Phe; both dipeptides, Phe-Gly and Gly-Phe, were found from the enzymic reaction, but no free amino acid, phenylalanine or glycine, was observed. The results so far discussed, clearly indicate that the mode of cleavage is to release the C-terminal dipeptide. This conclusion is supported by the other data that show that the tripeptides with blocked amino groups are appropriately cleaved and that the C-terminal dipeptide is formed from the decapeptide angiotensin I. It appears that the release of the C-terminal dipeptide may require a free carboxyl group as a recognition site because three peptides with substituted carboxyl groups were not hydrolyzed (Table III).

The failure of the enzyme to function when its mode of cleavage would require the hydrolysis of a peptide bond involving the imino function of proline can be rationalized on the basis of the very different structure of this peptide linkage. In this way this enzyme can function as a "converting enzyme" in converting angiotensin I to angiotensin II because the proline residue in the penultimate position of angiotensin II protects this compound from further degradation. Of course, there is no indication that angiotensin occurs in *C. equi*.

This enzyme has a lower molecular weight (approx. 80 000) than the mammalian converting enzyme (approx. 150 000)[6] and unlike the latter enzyme does not require chloride. It is similar to the mammalian enzyme in that they both apparently require metal ions, for they both are inhibited by EDTA and neither is inhibited by diethylfluorophosphate.

The occurrence of a dipeptidyl carboxypeptidase in *C. equi* raises the expectation that this type of enzyme may be more widely distributed than previously thought.

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