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ANGIOTENSIN-CONVERTING ENZYME AND A SECOND DIPEPTIDYL CARBOXYPEPTIDASE FROM HOG PLASMA

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SUMMARY

Two distinct dipeptidyl carboxypeptidase enzymes were demonstrated in hog plasma by a gel filtration experiment using Sephadex G-200. The two enzymes hydrolyzed different substrates and were affected in a different manner by the presence of NaCl and histidylleucine. Angiotensin-converting enzyme which hydrolyzes angiotensin I to angiotensin II and histidylleucine required NaCl for activity and was not inhibited by histidylleucine, whereas the other hydrolase using glycylalanylalanine as the substrate was little affected by NaCl but was heavily inhibited by histidylleucine and other peptides. Our results show that the apparent lack of specificity of crude preparations of angiotensin-converting enzyme from hog plasma arises because the plasma contains at least one other dipeptidyl carboxypeptidase. This second hydrolase appears to be fairly nonspecific but it will not hydrolyze a peptide bond in which the amino function is derived from proline as in glycylprolylalanine and angiotensin II. This second hydrolase, dipeptidyl carboxypeptidase, does not hydrolyze angiotensin I.

INTRODUCTION

The angiotensin-converting enzyme acts on the decapeptide angiotensin I and converts it to the pressor octapeptide angiotensin II by hydrolytically removing the ultimate and penultimate amino acids from the C-terminus as the dipeptide histidylleucine[1–19]. The converting enzyme thus acts as a dipeptidyl carboxypeptidase. However, its specificity and the determinants of its specificity have not yet been demonstrated. Even so we will temporarily and for convenience refer to the converting enzyme as a dipeptidyl carboxypeptidase.

Protein solutions prepared from a number of tissues have converting enzyme activity and also act as a dipeptidyl carboxypeptidase toward a variety of unrelated peptides[7,20–23]. Observations of this sort have led to the idea[22,24] that the converting enzyme is nonspecific and can carry out a number of important conversions. These preparations are clearly nonspecific but some alternative explanations are possible. For example, the preparations may contain two or more dipeptidylcarboxy peptidases.

The existence of a nonspecific dipeptidyl carboxypeptidase in mammalian tissue is also a distinct possibility. Such an enzyme has been reported in *Coryne bacterium equi*[25].

In this paper we report some observations with the "classical converting enzyme preparation" from hog plasma. We confirm the results of others that the preparation is nonspecific and acts as a dipeptidyl carboxypeptidase toward a number of simple peptides. By subjecting the preparation to further purification we show that the original crude preparation does contain at least two kinds of dipeptidyl carboxypeptidase and that the converting enzyme is at least partially specific.

EXPERIMENTAL

Enzyme preparation

A crude enzyme preparation was obtained from hog plasma by $(NH_4)_2SO_4$ fractionation between 1.6 M and 2.2 M of $(NH_4)_2SO_4$, and was lyophilized for storage purposes after being desalted by Sephadex G-150 gel filtration. We call this preparation, Preparation I. This crude preparation showed a broad specificity acting as a dipeptidyl carboxypeptidase toward a number of peptide substrates[25]. Further purification of the converting enzyme was obtained by Sephadex G-200 column chromatography using a column, 2.2 cm × 40 cm, equibrated with 0.05 M sodium phosphate buffer (pH 7.2) (Fig. 1), and the resulting converting enzyme was separated from a seemingly nonspecific dipeptidyl carboxypeptidase. The column flow rate was

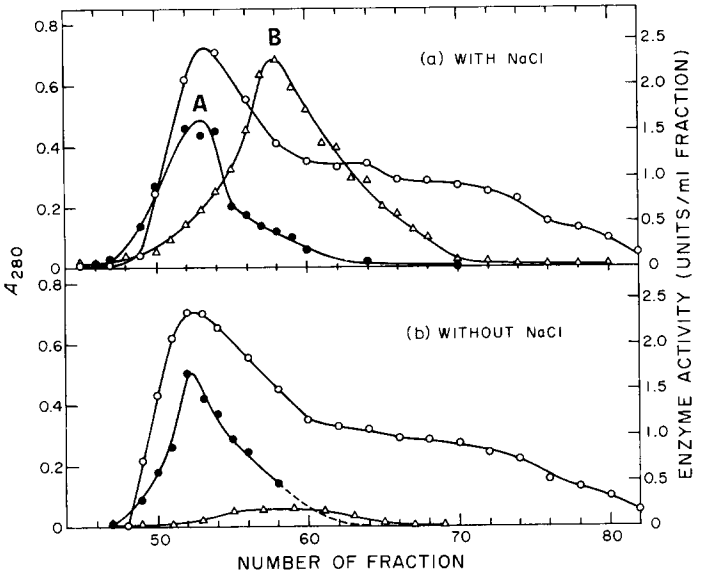

Fig. 1. Elution profile of two gel filtration experiments with Sephadex G-200-40. The gel filtration experiments were performed on a 2.2 cm × 40 cm bed of Sephadex G-200-40, 10–40 μm (Sigma Chem. Co.), with 0.05 M sodium phosphate buffer, pH 7.2, containing 0.05 M NaCl (a) or no NaCl (b) as the eluent. The column flow rate was 25 ml/h. Protein concentrations were measured in terms of the absorbance at 280 nm (○—○). Both Gly–Ala–Ala (●—●) and [1-Asp, 5-Ile, 10-Leu-^{14}C]-angiotensin I (△—△) were used as substrates, with or without D·12 M Nall.

25 ml per h, and 1.1 ml of the eluent was collected for each fraction. Protein concentration was measured by the optical absorbance at 280 nm and 260 nm.

Enzyme assay

Angiotensin-converting enzyme assay. The activity of the converting enzyme was assayed by the formation of radioactive histidylleucine from the substrate, [10-Leu-^{14}C]-angiotensin I (ref. 4,15). The assay system contained a minimum amount of substrate, 0.1 nmole (4 nCi) of [1-Asp, 5-Ile, 10-Leu-^{14}C]-angiotensin I and 10 μl of the enzyme preparation in final volume of 50 μl of 0.05 M sodium phosphate buffer (pH 7.4) with or without 0.12 M of NaCl. After incubation at 37 °C for 1 h, the percentage rate of hydrolysis was estimated by measuring the radioactivity of both the product histidylleucine and the substrate angiotensin I, and was expressed in term of the maximum activity of the enzyme based on the assay conditions employed. When the substrate concentration is far less than K_m, the percentage rate of hydrolysis becomes independent of the substrate concentration and hence the Michaelis-Menten equation becomes first order for the rate of hydrolysis. Thus, the maximum velocity (V) of the enzyme can be calculated as: $V = K_m \cdot 2.3 \log 1/(1 - \alpha)$ where α is the fraction of substrate hydrolyzed during the incubation period, and K_m for angiotensin I is 42 μM[15,16]. The unit of enzyme activity was then expressed as nmoles of histidylleucine formed per min per ml of the enzyme preparation.

Dipeptidyl carboxypeptidase assay. Gly–Ala–Ala as a peptide substrate at $2 \cdot 10^{-3}$ M concentration was incubated with 10 μl of the enzyme preparation (approx. 100 μg protein) in 50 μl of 0.05 M sodium phosphate buffer (pH 7.3) at 37 °C. After 3 h incubation, the reaction was stopped by adding 10 μl of 50% trichloroacetic acid, and was centrifuged. The reaction mixture was then diluted to 1.2 ml with sample diluting buffer, sodium acetate (pH 2.2), for application to the amino acid analyzer[25]. The extent of the enzymic hydrolysis was determined by measuring the amount of free glycine or the dipeptide alanylalanine as the reaction product. The sample in the amino acid analyzer was 0.8 ml of the diluted reaction mixture.

TABLE I

PEPTIDE HYDROLYSIS BY ENZYME PREPARATION I

Peptide	*Percent hydrolysis (product measured)*	
	With 0.1 M NaCl	*Without NaCl*
Angiotensin I*	78 (His–Leu)	6 (His–Leu)
Gly–Ala–Ala	28 (Gly), 31 (Ala–Ala)	30 (Gly)
Gly–His–Gly	35 (Gly)	37 (Gly)
Phe–Gly–Gly–Phe	26 (Phe–Gly) 28 (Gly–Phe)	
N-Acetyl–Ala–Ala–Ala	28 (Ala–Ala)	
N-Cb$_3$–Phe–Gly–Gly	14 (Gly–Gly)	
Angiotensin II Gly–Pro–Ala Ala–Ala–Ala–OEt His–Leu, Ala–Ala Phe–Gly, Gly–Ala	No hydrolysis	No hydrolysis

* Radioactivity assay using [1-Asp-5-Ile-10-Leu-^{14}C]-angiotensin I.

Materials

The ^{14}C-labelled and non-labelled [1-Asp, 5-Ile]-angiotensin I were synthesized by the solid phase technique[26] and purified by Bio-Gel P-2 gel filtration[27], and histidylleucine was synthesized by the azide condensation method[28]. Glycylalanylalanine and other peptides were purchased from Sigma Chemical Co., St. Louis, Mo.

RESULTS AND DISCUSSION

We present some of our observations on the hydrolysis of peptides by enzyme Preparation I in Table I. Preparation I has a specific activity of about 0.8 nmole of glycylalanylalanine and 1.0 nmole of angiotensin decapeptide hydrolyzed per min per mg of protein.

All the tripeptides that were hydrolyzed produced only the C-terminal dipeptide and the N-terminal amino acid. This result could, of course, be produced by an aminopeptidase but if that were the case some smaller amounts of the C-terminal and penultimate amino acids should also have been produced. The preparation did not hydrolyze leucine-β-naphthylamide, a substance often used as a chromogenic substrate for aminopeptidase activity[15,29].

That the preparation contains a dipeptidyl carboxypeptidase was clearly indicated by the experiment in which the tetrapeptide, phenylalanylglycylglycylphenylalanine was used as a substrate. In this case the two easily separated dipeptides, phenylalanylglycine and glycylphenylalanine were the only products produced. No glycine nor phenylalanine was produced by the enzyme preparation. Similarly Preparation I acted as a dipeptidyl carboxypeptidase toward two N-protected tripeptides, *N*-acetylalanylalanylalanine and *N*-benzyloxycarbonyl phenylalanylglycylglycine, producing the expected C-terminal dipeptide in each case. A free carboxyl group is necessary because the preparation failed to hydrolyze alanylalanylalanine-ethylester.

The preparation does not hydrolyze dipeptides. This preparation also has angiotensin-converting enzyme activity as indicated in Table I. Converting activity

TABLE II

INHIBITION OF ENZYMIC ACTIVITY BY PEPTIDES

Inhibitor ($1 \cdot 10^{-3}$ *M*)	*Percent inhibition with the indicated substrate*	
	*Angiotensin I**	*Gly-Ala-Ala***
His–Leu	3	70
Phe–His–Leu	3	81
Ala–Ala	10	95
Phe–Gly–Gly–Phe	8	57
[1-Asn]-angiotensin II	40	36
Gly–Pro–Ala	13	93
Phenylalanine	0	0
Histidine	0	0

* Radioactivity assay measuring His–Leu (Leu-^{14}C). The substrate concentration was $4 \cdot 10^{-5}$ M.

** Extent of hydrolysis was estimated by measuring free glycine or Ala–Ala as the product. The substrate concentration was $2 \cdot 10^{-3}$ M.

depends upon the presence of NaCl in agreement with the literature[30,31] and is not inhibited by $1 \cdot 10^{-3}$ M histidylleucine. Lack of inhibition by histidylleucine is in agreement with previous work from this laboratory[15] but differs from the observations of others[21–23,31]. The hydrolysis of glycylalanylalanine, however, does not depend upon NaCl and may be slightly inhibited by NaCl. It is heavily inhibited by histidylleucine (Table II).

Since glycylalanylalanine seemed reasonably representative of these nonspecific substrates, we measured its K_m and obtained a value of $6 \cdot 10^{-4}$ M. Thus our measured value of the rate of hydrolysis at the standard concentration of $2 \cdot 10^{-3}$ M is more than 75% the V value. The K_m for angiotensin I is $4 \cdot 10^{-5}$ M (ref. 15,16). Since the concentration of angiotensin I that we used is far below its K_m value, it should be much easier to inhibit angiotensin I hydrolysis than glycylalanylalanine hydrolysis. Yet we inhibited only glycylalanylalanine hydrolysis with histidylleucine.

Our results suggest that different enzymes may be involved in the hydrolysis of angiotensin I and the other substrates even though the same type of reaction occurs. A clear demonstration that different enzymes are involved is presented in Fig. 1. This figure shows the elution profile of a gel filtration experiment using enzyme Preparation I with angiotensin I and glycylalanylalanine as substrates. Two distinct peaks are obtained.

In the first peak fractions, Peak A, we find most of the seemingly nonspecific hydrolase and a small amount of converting enzyme. Similarly in the second peak fractions, Peak B, we find most of the converting enzyme activity and a small amount of nonspecific hydrolase.

With regard to the effects of NaCl and histidylleucine, (Table III), the peak

TABLE III

TWO KINDS OF DIPEPTIDYL CARBOXYPEPTIDASE FROM SEPHADEX G-200 GEL FILTRATION

Enzyme	*Enzyme activity (units/ml)*			*% Inhibition by His–Leu*
	*Control**	*No NaCl*	*His–Leu***	
Angiotensin-converting enzyme (Peak B)	2.63	0.13	2.50	5
Dipeptidyl carboxypeptidase (Peak A)	1.40	1.50	0.38	73

* Control experiment was performed in the presence of NaCl (0.12 M) as described in the text.

** Concentration of His–Leu was $2 \cdot 10^{-3}$ M.

fractions A and B behaved as anticipated from studies with the cruder Preparation I from which the fractions were derived. Converting enzyme (Peak B) required NaCl and was not inhibited by histidylleucine. Hydrolase Peak A was little effected by NaCl but was strongly inhibited by histidylleucine.

The small amount of hydrolase activity toward glycylalanylalanine that appeared in Peak B was effected by NaCl in the same way as the Peak A enzyme and may simply be a "tail" of that enzyme.

Our results show that hog plasma contains converting enzyme that may be

quite specific, at least there is no evidence that it is not, and at least one other dipeptidyl carboxypeptidase. This second hydrolase appears to be fairly nonspecific but it will not hydrolyze a peptide bond in which the amino function is derived from proline as in glycylprolylalanine and angiotensin II. It does not hydrolyze angiotensin I, at least when the concentration of angiotensin I is low as in our radioactive assay. This means that the second order rate constant V/K_m for hydrolase A is very low compared to the value for the converting enzyme when angiotensin I is the substrate.

The converting enzyme appears at a position that is consistent with a molecular weight of 150 000. The other hydrolase is much larger.

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