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ANGIOTENSIN-CONVERTING ENZYME FROM PORCINE PLASMA

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SUMMARY

Angiotensin-converting enzyme was purified from porcine plasma by $(NH_4)_2SO_4$ fractionation, Sephadex G-200 gel filtration and DEAE-cellulose chromatography. These techniques combined to give a preparation which hydrolyzed 50 nmoles of substrate per mg per minute. This preparation is considerably more purified than previously reported preparations. The radioactive assay of Huggins was modified in that high voltage electrophoresis was used to separate histidylleucine from the substrate. The K_m value for the enzyme was obtained as $4.2 \cdot 10^{-5}$ M. The enzyme reaction was inhibited by angiotensin II, but not by histidylleucine. The $s_{20,w}$ value (8.3) of the converting enzyme was obtained by the sucrose density gradient sedimentation technique and the molecular weight was estimated to be 155 000. The maximum activity of hog plasma is about 9 nmole per ml per minute. The calculated half time for the conversion of angiotensin I to angiotensin II in plasma is about 3 min. This slow rate suggests that there must be more to the "activation" of angiotensin than is immediately obvious and in this sense is consistent with the conclusion of NG AND VANE[4-6] that the lung, and not the blood, is the primary site of conversion of angiotensin I to angiotensin II.

INTRODUCTION

Angiotensin-converting enzyme which catalyzes the hydrolysis of the decapeptide angiotensin I to the vasopressive octapeptide angiotensin II and the biologically inactive dipeptide histidylleucine has been found in blood plasma[1] and several animal tissues[2,3]. This particular enzyme may play a key role in the control mechanism of blood pressure. Recently, NG AND VANE[4-6] have discussed the nature and physiological function of dog lung converting enzyme based on *in vivo* experiments, but no information on the physicochemical properties of the plasma-converting enzyme has yet been made because the enzyme is difficult to purify.

The partially purified porcine plasma enzyme may be assayed biologically by the blood pressure rise method or chemically by the ninhydrin reaction method[7], but no specific simple and sensitive assay technique has yet been reported. HUGGINS *et al.*[3] have reported a radioactive assay in which [^{14}C]histidylleucine is separated

from the substrate, [Ile^5, ^{14}C-Leu^{10}]-angiotensin I by silica gel impregnated glass fiber paper chromatography. This method is basically very good but there is some difficulty in getting a satisfactory separation of histidylleucine from the substrate. We have modified this assay by using high voltage paper electrophoresis to separate histidylleucine from the substrate. The separation of angiotensin I and II has also been accomplished by BOUCHER *et al.*[8], employing ion exchange resins.

HUGGINS *et al.*[9] have also reported on a number of properties of the converting enzyme. During an investigation of porcine blood plasma it became desirable to determine the approximate molecular weight and some of the kinetic parameters of the partially purified plasma converting enzyme. The sucrose density gradient sedimentation technique[10,11] was used to determine the $s_{20,w}$ value which was used to estimate the molecular weight.

METHODS

Enzyme preparation

A crude preparation of angiotensin-converting enzyme was obtained from porcine blood plasma by fractionation between 1.6 M and 2.2 M $(NH_4)_2SO_4$ (ref. 1). The precipitate was dialyzed against 0.05 M sodium phosphate buffer, pH 6.8, containing 1.0 mM sodium EDTA, and again against distilled water until no free $(NH_4)_2SO_4$ was detected. The enzyme was then further purified by Sephadex G-200 gel filtration (2.2 cm × 90 cm with 0.05 M sodium phosphate buffer, pH 6.8, containing 0.05 M NaCl as the eluent) and by DEAE-cellulose column chromatography (2.2 cm × 45 cm, Whatman DE-52, anion exchange cellulose, Series III) with a NaCl gradient of zero to 0.5 M in 0.1 M sodium phosphate buffer, pH 7.2 (Fig. 1a, b). The active enzyme fractions were combined, dialyzed against 0.01 M sodium phosphate buffer, pH 7.2 and lyophilized. The final specific activity of this partially purified plasma enzyme was obtained as 50 units* per mg protein when it was assayed using the synthetic substrate [^{14}C]angiotensin I (Table I).

TABLE I

PURIFICATION OF THE PORCINE PLASMA-CONVERTING ENZYME

Protein concentrations were measured by the method of LOWRY *et al.*[10]. The enzyme activities were measured by radioactivity assay.

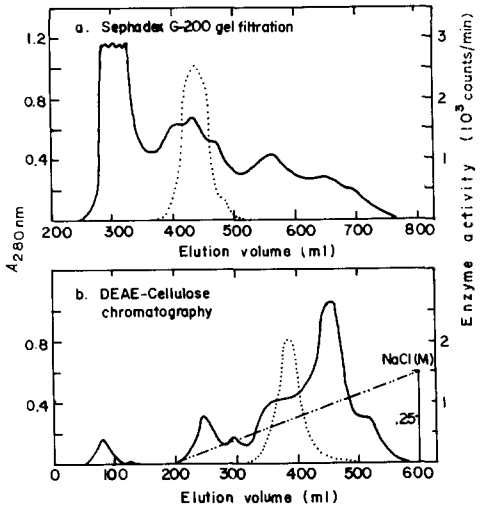
Fraction	*Total protein (mg)*	*Total activity (unit)*	*Specific activity (unit/mg)*	*Purification (fold)*
I Plasma (400 ml), pH adjusted to 6.8 with 0.5 M H_2SO_4	12 000	3600	0.3	1
II $(NH_4)_2SO_4$ fractionation, 1.6 M to 2.2 M saturation	2 700	3080	1.1	4
III Sephadex G-200 gel filtration	118	1980	17	55
IV DEAE-cellulose chromatography	11	550	50	165

* One unit of angiotensin-converting enzyme activity was defined as 1.0 nmole of dipeptide histidylleucine released per min.

Enzyme assay

The rate of hydrolysis of the synthetic substrate, 5-Ile-angiotensin-I was measured by a radioactive assay which measured the [^{14}C]histidylleucine produced in the reaction. Substrate, 0.2–5 nmole of angiotensin I containing 4 nC of [^{14}C-Leu^{10}]-angiotensin I and 0.5 to 1.0 μg of the enzyme protein were incubated in 50 μl of 0.05 M sodium phosphate buffer, pH 7.5 containing 0.12 M NaCl at 37°. The substrate concentrations ranged from $4 \cdot 10^{-6}$ M to $1 \cdot 10^{-4}$ M. After incubation for a suitable time the reaction was stopped by adding 10 μl of 15% trichloroacetic acid and the whole incubation mixture was spotted on Whatman 3 MM paper. The paper was then developed by high voltage electrophoresis at 2500 volts for 30 min using a pyridine-acetate buffer (pyridine-acetic acid-water, 1:10:289, by vol.), pH 3.6. The extent of the enzyme reaction was estimated by measuring the radioactivity of both the product histidylleucine and the substrate angiotensin I which were well separated during the 30-min run of electrophoresis as shown in Fig. 2.

The developed paper was cut into 0.5-cm sections and each section was placed



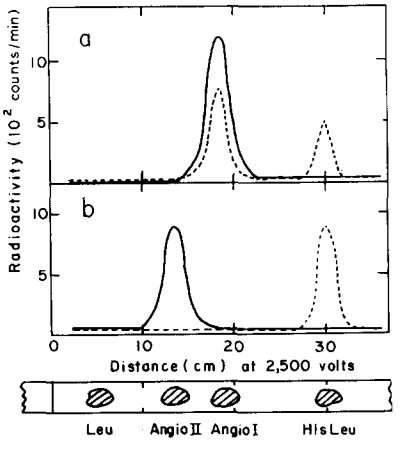


Fig. 1. Purification of porcine plasma converting enzyme by gel filtration on Sephadex G-200 and chromatography on DEAE-cellulose. The gel filtration experiment was performed on a 2.2 cm × 90 cm bed of Sephadex G-200, 40–120 mesh, with 0.05 M sodium phosphate buffer, pH 6.8 containing 0.05 M NaCl as the eluent. The flow rate was 30 ml/h. Ion exchange chromatography was performed on a 2.2 cm × 45 cm bed of DEAE-cellulose, Whatman DE-52, Series III, with a NaCl gradient from zero to 0.5 M in 0.01 M sodium phosphate buffer, pH 7.2. The active enzyme peak from the gel filtration experiment was added to the ion exchange column. Enzyme activity was measured by the radioactivity assay and protein concentration was detected from the absorbance at 280 nm.

Fig. 2. Separation of angiotensin and histidylleucine by high voltage paper electrophoresis. (a) Radioactive assay for porcine plasma converting enzyme activity: After incubation of the converting enzyme and the substrate, [Asp^1, Ile^5, ^{14}C-Leu^{10}]-angiotensin I at 37° for zero (solid line in Fig. 2a) and 120 min. (dashed line in Fig. 2a), the enzyme reaction mixture was separated by paper electrophoresis at 2500 V for 60 min. (b) Test for peptidase activity: [Asp^1, ^{14}C-Ile^5]-angiotensin-II (solid line in Fig. 2b) and His [^{14}C]-Leu (dashed line in Fig. 2b) were incubated separately with the partially purified converting enzyme at 37° for 120 min. After incubation the reaction mixtures were separated by paper electrophoresis. The bottom figure shows the paper strip on which the mobilities of the standard markers were indicated by ninhydrin spray.

in a 25 ml scintillation vial with 10 ml of scintillator mixture (7 g 2,5-diphenyloxazole and 0.45 g 1,4-bis-(5-phenyloxazolyl-2)-benzene in 1 l of toluene). The radioactivity of each vial was counted for 5 min using the Beckman, LS-250 Liquid Scintillation System. The measured radioactivity was the same in control experiments in which angiotensin I, angiotensin II and histidylleucine were spotted on paper and developed by electrophoresis, were simply spotted on paper, or were introduced in solution into the scintillator solution. Protein concentration was determined by the method of LOWRY *et al.*[12].

Sucrose density gradient sedimentation

Sedimentation experiments were carried out as described by MARTIN AND AMES[13]. 0.1 ml of test enzyme or standard known enzyme markers, catalase, cytochrome *c* and alkaline phosphatase, was applied to a 5% to 20% sucrose gradient in 0.1 M Tris, pH 7.65, and centrifugation was carried out at 38 000 rev./min and 5° using the SW 50 L rotor in a Model L2 65 B Beckman Spinco Preparative Ultracentrifuge. The time of centrifugation varied from 12 to 16 h, and the sedimentation velocities of the enzymes were measured by the enzyme activities except for cytochrome *c* which was measured by its absorbance. Five to ten drop fractions were collected from each tube after centrifugation, and an aliquot of each fraction was analyzed for enzyme activity as described in the above. Sedimentation coefficients corrected to $s_{20,w}$ values were calculated according to the method of MARTIN AND AMES[13]. The obtained $s_{20,w}$ values were compared to the reported values[14], and the approximate relationship of sedimentation constant and molecular weight, $(s_1/s_2)^{3/2} = (\text{mol. wt.}_1/\text{mol. wt.}_2)$ was used to determine the relative molecular weight of the porcine plasma converting enzyme.

Peptide preparation

The cold substrate, 5-Ile-angiotensin-I was synthesized by the solid phase method of MERRIFIELD[15] as previously prepared by TAMPI *et al.*[16]. The peptide was homogeneous by thin layer chromatography and high voltage electrophoresis. The biological activity of this cold substrate was 100% as tested by the blood pressure rise method*, and the amino acid composition was correct as analyzed by the Beckman amino acid analyzer, Model 120 C. Cold and radioactive [^{14}C-Leu]-histidylleucine were synthesized by the azide condensation method[17]. The pressor active octapeptide, angiotensin II was purchased from Ciba Pharmaceutical Co., Summit, N.J. (Hypertensin II, CN, E-831, Batch No. 3/62). The radioactive peptides, [Asp^1, Ile^5, ^{14}C-Leu^{10}]-angiotensin I and [Asp^1, ^{14}C-Ile^5]-angiotensin II were purchased from Schwarz/Mann, Orangeburg, N.Y. (Lot No. 6901).

RESULTS

Plasma angiotensin-converting enzyme was previously obtained as a crude preparation by $(NH_4)_2SO_4$ fractionation and isoelectric precipitation[1], and purified further by gel filtration[18] or by carboxymethyl cellulose filtration[7]. By the method shown in Table I, we have obtained a preparation from hog with a specific activity

* We are indebted to Dr. J. Laragh, Columbia University, for this measurement.

TABLE II

TEST FOR OTHER PEPTIDASE ACTIVITY IN THE PORCINE PLASMA ANGIOTENSIN-CONVERTING ENZYME PREPARATION

Substrate	*Enzyme type*	*Relative activity (%)*
[Asp^1, Ile^5]-angiotensin I*	Converting enzyme	100
[Asp^1, Ile^5]-angiotensin II*	Angiotensinases	0.1
Histidylleucine*	Dipeptidases**	0
Z-phenylalanine *p*-nitrophenyl ester or *N*-benzoyltyrosine ethyl ester	Chymotrypsin	0
Hippuryl-phenylalanine	Carboxypeptidase	0
Leucine β-naphthylamide	Aminopeptidases	0
p-Toluenesulfonyl-L-arginine-methyl ester	Trypsin	0

* Radioactivity assay by high voltage paper electrophoresis.
** Lung tissue hydrolyzes histidylleucine readily.

of 50 nmoles/mg per min which is considerably higher than has been previously reported from any source. The enzyme seems to be reasonably free of other peptidases (Table II).

The radioactive assay for the converting enzyme is simple and highly sensitive. As shown in Fig. 2a, histidylleucine is well separated from the substrate angiotensin I by high-voltage paper electrophoresis at 2500 V for 30 min or longer.

When [Asp^1-Ile^5-^{14}C-Ile^5]-angiotensin II and His [^{14}C]Leu, the reaction products of the converting enzyme, were incubated separately with the plasma-converting enzyme (Fig. 2b), no hydrolysis was found.

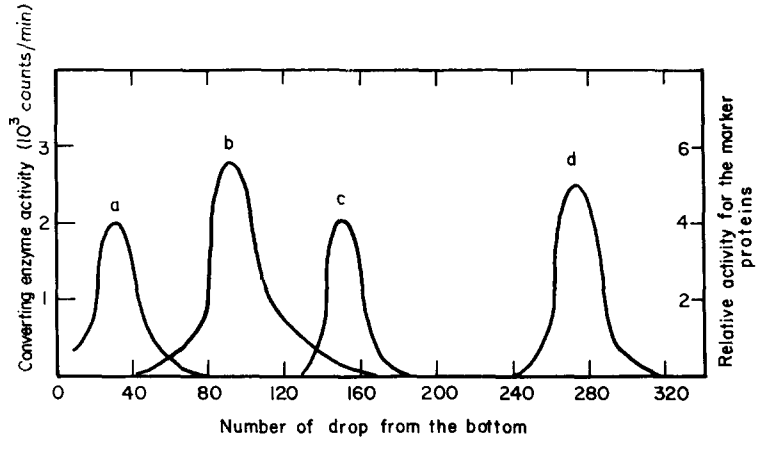


Fig. 3. Sedimentation coefficient by sucrose density gradient centrifugation. The sucrose density gradient sedimentation velocity experiment was carried out at 5° for 16 h at 38 000 rev./min. Five-drop fractions were collected from the bottom of each tube after centrifugation. The converting enzyme activity (b) was measured by radioactive assay. Bovine liver catalase (a) and *E. coli* alkaline phosphatase (c) were assayed by spectrophotometric methods using H_2O_2 and *p*-nitrophenyl phosphate as the substrates, respectively. Cytochrome *c* (d) was measured by its absorbance at 350 nm.

TABLE III

THE CALCULATED $s_{20,w}$ VALUES FROM THE SUCROSE DENSITY GRADIENT SEDIMENTATION EXPERIMENT

Protein	$s_{20,w}$ *(obtained)*	$s_{20,w}$ *(literature)*	*Molecular weight*
Hog plasma angiotensin-converting enzyme	8.3	—	155 000*
Catalase (bovine liver)	11.2	11.3	247 500
Cytochrome *c*	1.7	1.71	12 500
Alkaline phosphatase *(Escherichia coli)*	6.2	6.0	~90 000

* Molecular weight of porcine plasma angiotensin-converting enzyme was calculated based on bovine liver catalase.

The plasma-converting enzyme showed a broad pH optimum between pH 7.2 to 7.8. It was relatively stable in the lyophilized state but it lost one fourth of its original activity in a buffered solution, pH 7.5, after 5 days at 3°.

The molecular weight for the plasma-converting enzyme was determined from the sedimentation velocity in a sucrose gradient. As shown in Fig. 3, the plasma-converting enzyme moved 2.7 cm (90 drops from the bottom of the tube) from the meniscus in a 5% to 20% linear sucrose gradient during 16 h centrifugation at 38 000 rev./min, and showed a single peak of enzyme activity.

The corrected $s_{20,w}$ values of converting enzyme and known standard marker proteins were calculated (Table III). Based on the assumption that the converting enzyme is a typical globular protein, the molecular weight of this particular converting enzyme was estimated to be 155 000.

The rate of the converting enzyme reaction was constant for a 120-min incubation at 37° (Fig. 4). We found the plasma-converting enzyme was weakly inhibited by angiotensin II (about 40% inhibition with 0.1 mM of [Asp[1], Val[5]]-angiotensin II and $4.3 \cdot 10^{-5}$ M angiotensin I) but not by histidylleucine at 1 mM. As reported before[1] this plasma-converting enzyme required NaCl for enzyme activity and was inhibited by 0.5 mM EDTA. In agreement with BOUCHER *et al.*[8], no inhibition by diethylfluorophosphate was observed. We did not measure initial velocities. The percent

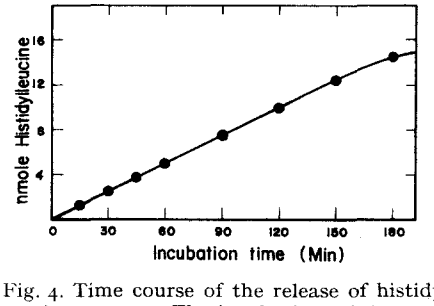


Fig. 4. Time course of the release of histidylleucine from angiotensin I by porcine plasma-converting enzyme. The incubation mixture contained 21.6 nmoles of [Asp[1], Ile[5], ^{14}C-Leu[10]]-angiotensin I ,0.52 μg of the partially purified converting enzyme (Fraction III) and 6.2 μmole of NaCl in 50 μl of 0.05 M sodium phosphate buffer, pH 7.5.

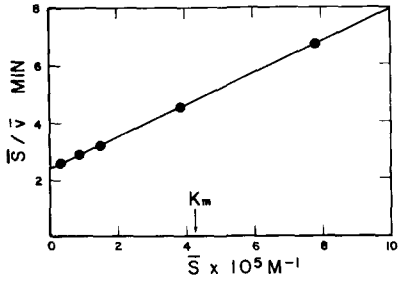


Fig. 5. Eadie plot ($\bar{S}/\bar{v}$ *vs.* $\bar{S}$) for porcine converting enzyme. [Asp[1], Ile[5], ^{14}C-Leu[10]]-angiotensin I, 0.16–4.16 nmoles was incubated at 37° with 0.52 μg of the partially purified enzyme in 50 μl of 0.05 M sodium phosphate buffer, pH 7.5 containing 6.2 μmoles of NaCl. $\bar{S}$ is the average value of the substrate concentration during the incubation period and $\bar{v}$ is the average velocity for the release of histidylleucine from the substrate.

hydrolysis varied from 17% to 39%. The average velocities $\Delta[s]/\Delta t$ can be used for determining kinetic parameters if the average value of $[s]$ during Δt is used instead of the initial substrate concentration[19]. Alternatively, the integrated form of the Michaelis–Menten equation can be used. Both methods gave identical results (v_{max} = 50 nmoles/mg per min; $K_m = 4.2 \cdot 10^{-5}$ M (Fig. 5). The activities of Table I were measured at a convenient substrate concentration of $4.3 \cdot 10^{-5}$ M. This concentration turned out to be the K_m value. In defining a specific activity it is usual to use a substrate concentration that is considerably higher than the K_m value. We have therefore multiplied our measured values at $[S] = 4.3 \cdot 10^{-5}$ M by 2 to convert them to v_{max} values.

DISCUSSION

Our enzyme preparation although considerably purer than any so far described, is very far from pure. While enzymes do vary considerably in specific activity and it is therefore difficult to guess what the specific activity of pure converting enzyme is, it is nonetheless clear that less than 1% of the protein in our preparation is converting enzyme.

The strength of the sucrose gradient method for estimating molecular weights is that it can be applied to very small amounts of highly impure material. Despite the lack of purity our estimate of the molecular weight therefore is unlikely to be very far off. The K_m value, while respectably low as these values go, is rather higher than might have been anticipated from the very low physiological concentrations of the substrate.

However, in applying enzyme rates to physiological phenomena it is important to note that while the absolute rate of hydrolysis increases with increasing substrate concentration, the percent hydrolysis increases with decreasing substrate concentration. When $[S] \ll K_m$ the percent rate of hydrolysis is independent of $[S]$ and the Michaelis–Menten equation gives for the fractional rate of hydrolysis

$$-\frac{1}{[S]} \cdot \frac{d[S]}{dt} = \frac{v_{max}}{K_m}$$

The total maximum activity of porcine plasma is about 9 nmoles·ml^{-1}·min^{-1} so that

$$\frac{v_{max}}{K_m} = \frac{9}{42} \approx 0.21$$

and the half life of angiotensin I in blood is calculated to be about 3.3 min.

$$t_{1/2} = \frac{0.7}{v_{max}/K_m}$$

This rate is far too slow to be consistent with the rapid rise in blood pressure that occurs *in vivo* when angiotensin I is administered and suggests that there must be more to the activation of angiotensin than is immediately obvious. In this sense this work is consistent with the conclusion of NG AND VANE[4-6] that the lung, rather than the blood, is the major site of conversion of angiotensin I to angiotensin II. It is important to note that the knowledge of the value of K_m is highly significant in this analysis.

In principle the method described by HUGGINS *et al.*[3] is an excellent means of assaying converting enzyme but a clean separation of histidylleucine from angiotensin I is essential. High-voltage electrophoresis as described here does give a wide separation of these substances and we find that this modification improves the method.

ACKNOWLEDGMENT

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