BBA 66426

ANGIOTENSIN-CONVERTING ENZYME FROM GUINEA PIG AND HOG LUNG

HYUN-JAE LEE, JOHN N. LARUE AND IRWIN B. WILSON
Department of Chemistry, University of Colorado, Boulder, Colo. (U.S.A.)

(Received June 1st, 1971)

SUMMARY

Lung contains a true angiotensin-converting enzyme, *i.e.*, an enzyme that converts angiotensin I to angiotensin II by the hydrolytic removal of the dipeptide histidylleucine. The soluble enzymes from guinea pig and hog lung were purified more than 200-fold. These enzymes are quite similar in having the same K_m, 20 μM, the same $s_{20,w}$ value of 8.2 from sucrose gradient measurements, and the same Stokes radius from Sephadex G-150 gel filtration, corresponding to a molecular weight of about 150 000 d. The plasma enzymes have the same $s_{20,w}$ values and Stokes radii as the lung enzymes; the K_m values are similar but slightly higher 40 μM.

In contrast to plasma, the lung, especially hog lung, contains a considerable amount of histidylleucine hydrolyzing activity. This enzyme was obtained in a partially purified form with a specific activity of 1.6 μmoles/min per mg protein. The molecular weight of histidylleucine cleaving enzyme was estimated to be about 80 000 based on the corrected $s_{20,w}$ value of 5.3 S and the relative elution volume from gel filtration. The K_m value of 200 μM for histidylleucine was calculated.

The maximum tissue converting enzyme activities of guinea pig and hog that we obtain are about 30 and 10 nmoles/min per g wet tissue. This amount is less than we expected since in the dog more than 50% of angiotensin I is converted to angiotensin II in a single passage through the lung.

INTRODUCTION

NG AND VANE[1–3] and other groups[4–6] have shown that angiotensin II, octapeptide, is formed more rapidly from angiotensin I, decapeptide, during passage of blood through the lung than in blood itself based upon *in vivo* and *in vitro* biological assays for angiotensin II. In blood, this conversion is mediated by a specific enzyme, the converting enzyme, that hydrolyzes the decapeptide to the octapeptide and histidylleucine[7–14].

BAKHLE[6] has recently shown that dog lung tissue converts angiotensin I to angiotensin II but since he used the biological assay for angiotensin II he did not demonstrate the formation of histidylleucine. Thus, as far as that work and the work

of others[1–6] is concerned, the reaction that occurs in lung tissue could be chemically different from the reaction that occurs in blood. It could, for example, involve the successive removal of leucine and histidine from the decapeptide[3]. Or the nonapeptide formed by the removal of leucine from angiotensin I, might itself be active as a vasoconstrictor and might be mistaken for angiotensin II in the biological assay.

Using [^{3}H]Leu^{10}-angiotensin I, an attempt to demonstrate histidylleucine in blood leaving the lung turned up mostly [^{3}H]leucine[15]. This result might arise from the cleavage of leucine from angiotensin I by a carboxypeptidase-like enzyme[3,13]. Another possibility is that [^{3}H]leucine might arise from the cleavage of histidylleucine from angiotensin I by a converting enzyme followed by the rapid cleavage of histidylleucine by another enzyme to yield histidine and leucine.

However, a note has recently appeared reporting the formation of histidylleucine from angiotensin I by an acetone-powder preparation from human lung[16].

In this paper we show that histidylleucine is indeed formed from angiotensin I by hog and guinea pig lung preparations. The enzyme in lung, therefore, is a converting enzyme, *i.e.* it carries out the same chemical reaction as the converting enzyme in blood. We further show that the converting enzyme from lung has approximately the same molecular weight as the enzyme from blood but the K_m values are slightly different. However, the amount of enzyme that we recover does not seem to be sufficient to account for its anticipated physiological role. The lung also contains large amounts of an enzyme that hydrolyzes histidylleucine.

EXPERIMENTAL

Enzyme preparation

A crude converting enzyme solution was isolated from saline-perfused lung homogenates of guinea pig and hog by fractional centrifugation[6] in 0.05 M sodium phosphate buffer (pH 6.8). The preparation was partially purified by $(NH_4)_2SO_4$ fractionation between 1.6 and 2.2 M, and by gel filtration with Sephadex G-150 using a 2.2 cm × 45 cm column with 0.05 M sodium phosphate buffer (pH 7.4), containing 0.05 M NaCl as the eluent (Scheme I). The active enzyme fractions from the Sephadex column (Fig. 1) were combined, dialyzed against distilled water, and concentrated by Amicon ultrafiltration using a Diaflo PM 30, 25-mm membrane filter. The final specific activities of partially purified lung converting enzyme preparations were obtained as 10.5 and 3.8 units* per mg of protein for guinea pig and hog, respectively, based on the radioactivity assay using the synthetic substrate, [Asp^1, Ile^5, ^{14}C-Leu^{10}]-angiotensin I (Table I). A partially purified guinea pig plasma converting enzyme with a specific activity of 15.6 units per mg protein was prepared by the method reported previously[8,9]. Protein concentration was measured by the method of LOWRY *et al.*[17] using bovine serum albumin as the standard.

Enzyme assay

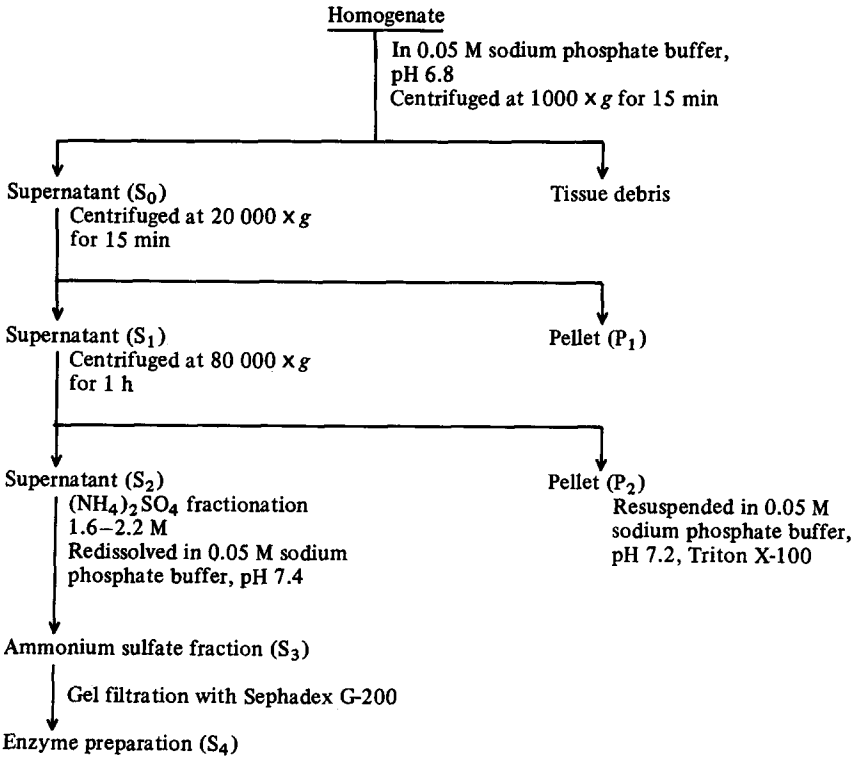
For the converting enzyme activity, the rate of hydrolysis of the synthetic substrate, [Asp^1, Ile^5, ^{14}C-Leu^{10}]-angiotensin I was measured by a radioactivity assay which measured the radioactivity of histidyl[^{14}C]leucine produced in the

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

reaction[8,9]. Substrate, 0.2–10 nmoles of angiotensin I containing 4 nC of [^{14}C-Leu^{10}]-angiotensin, and 10 μg of the enzyme protein were incubated in 50 μl of 0.05 M sodium phosphate buffer (pH 7.4) containing 0.12 M of NaCl at 37°. The substrate concentrations ranged from 4.2 to 180 μM. In the radioactivity assay for the converting enzyme the radioactive product histidylleucine is well separated from the substrate [^{14}C-Leu^{10}]-angiotensin I in 1 h by high-voltage paper electrophoresis at 50 V/cm at pH 3.7.

In a series of tests for other peptidase activities of the lung converting enzyme preparation, angiotensinase(s) and histidylleucine splitting enzyme activities were measured by radioactivity assay using [Asp^1, ^{14}C-Ile^5]-angiotensin II and histidyl-[^{14}C]leucine, respectively, and activities of exopeptidases, carboxypeptidase and aminopeptidase were assayed by the spectrophotometric method using hippuryl-phenylalanine (at 254 nm) and the β-naphthylamides of aspartic acid and leucine (at 340 nm)[18], respectively.

The identity of the radioactive compound that moved like histidylleucine in high-voltage electrophoresis was confirmed by rechromatography on Whatman No. 1 paper using the solvent system, *sec.*-butanol–*i*-propanol–monochloroacetic acid–water (70:10:3:4, v/v/w/v), and on thin-layer silica gel using *n*-butanol–acetic acid–water (4:1:1, v/v/v).



Scheme 1. Preparation of lung converting enzyme from guinea pig and porcine hog.

Sedimentation experiment

Sucrose density gradient sedimentation experiments were carried out as described by MARTIN AND AMES[19] using bovine liver catalase, *Escherichia coli* alkaline phosphatase and bovine cytochrome *c* as standard marker proteins. Corrected sedimentation coefficients, $s_{20,w}$ were calculated[8,9] and compared to the reported values[20]. 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 weights of the lung and plasma converting enzymes. The calculated molecular weights were also compared with the results from gel filtration with Sephadex G-150 in which the relative elution volumes, V_e/V_0, were used to determine the relative molecular weights of proteins based upon Stokes radii (Fig. 2).

RESULTS

The purification method used in this work is summarized in Scheme 1 and the course of the purification of the guinea pig lung converting enzyme is tabulated in Table I. It will be noted that the sum of the activities in Fractions P_2 and S_4 exceeds by a factor of two the enzyme activity measured in the original homogenate. This result implies that interfering substances have been removed during the course of purification. This supposition is substantiated by the data presented in Table II, which shows that a number of enzymes that are expected to affect the measurement of converting enzyme, including histidylleucine splitting enzyme and angiotensinases, are present in the homogenate but are absent in S_4. The histidylleucine splitting activity in the homogenate is comparable to the converting enzyme activity.

The situation with porcine lung homogenate is more extreme. In this case there is no measureable production of histidylleucine and a high level of histidylleucine splitting activity is present. The activities of the other enzymes are also much higher than in guinea pig lung homogenate. Fraction S_4 produces histidylleucine from angiotensin I and is nearly free of the other enzyme activities.

TABLE I

PURIFICATION OF GUINEA PIG LUNG CONVERTING ENZYME

Protein concentration was measured by the method of LOWRY *et al.*[17]. Enzyme activity was assayed with [^{14}C-Leu^{10}]-angiotensin I, 0.18 mM. Tissue homogenate was prepared from 15 g of wet tissue after perfused with saline solution.

Fraction	*Total protein (mg)*	*Total activity (nmoles/min)*	*Specific activity*	*Purification (-fold)*	*Yield (%)*
Homogenate	3280	152	0.046	1	100
S_0	2800	142	0.051	1.1	95
S_1	2030	138	0.068	1.5	91
S_2	1020	127	0.125	2.7	84
P_2*	950	136	0.143	3.1	89
S_3	145	75	0.520	11.3	50
S_4	17	178	10.5	230	117

* The centrifuged pellet was resuspended in 0.05 M sodium phosphate buffer (pH 7.2), and was solubilized with diluted Triton X-100 (1:25 dilution).

TABLE II

MINOR ENZYME ACTIVITIES FROM LUNG CONVERTING ENZYME PREPARATIONS

Enzyme	*Substrate*	*Substrate concn. (mM)*	*Enzyme activity (units/mg protein)*			
			Guinea pig		*Hog*	
			Homogenate	S_4	*Homogenate*	S_4
Converting enzyme	[^{14}C-Leu^{10}]-angiotensin I	0.182	0.046 (0.15)*	10.5	0.00 (0.04)*	3.8
His–Leu hydrolyzing enzyme	His–[^{14}C]Leu	2	0.15	0	33.2	0.2
Angiotensinase	[^{14}C-Ile^5]-angiotensin II	0.04	0.015	0	0.053	0
Aminopeptidase**	Asp-β-naphthylamide	0.46	0.01	0	0.035	0
Carboxypeptidase***	Hippurylphenylalanine	1.2	0.005		0.152	0

* These values were derived from the S_4 and P_2 fractions.
** Aminopeptidase activities were measured by spectrophotometric assay at $A_{340\ nm}$ (ref. 18).
*** Carboxypeptidase activities were assayed by $\Delta A_{254\ nm}$ for hippuric acid.

The lung converting enzyme preparations, S_4, from both animals showed the general characteristics found for plasma converting enzyme; *i.e.*, Cl^- is required, there is a broad pH optimum between 7.2 and 7.8 and the enzyme is completely inhibited by sodium EDTA (1.2 mM for 15 min prior to incubation with substrate).

In all cases the reaction rate was constant over the incubation period of 120 min at 37°. We could not measure initial velocities in the determination of K_m, since accurate measurement of radioactive histidylleucine required considerable hydrolysis. In the present study the reactions proceeded 15–40% and the K_m values were determined by using the average velocities $\bar{v} = \Delta S/t$ and the arithmetic mean substrate concentrations, $\bar{S} = S_0 - \Delta S/2$ in place of initial velocities and the initial substrate concentrations (Fig. 3). The use of average values allows the Lineweaver–Burk plot (and related plots) to be used when as much as 50% of the substrate has been

TABLE III

CHARACTERISTICS OF ANGIOTENSIN-CONVERTING ENZYME

1 unit of maximum enzyme activity expresses nmoles/min per ml of plasma or per g wet tissue.

Angiotensin-converting enzyme of	K_m *(μM)*	*Maximum enzyme activity (units)*	$s_{20,w}$* *(sucrose gradient)*	$t_{\frac{1}{2}}$ *(min)*
Guinea pig				
plasma	48.0	9.5	8.2	3.5
lung	20.4	approx. 30**	8.3	–
Porcine				
plasma	42.0	9.0	8.3	3.3
lung	26.0	approx. 10**	8.2	–
Human				
plasma	45.0	8.3	8.2	7.0

* The corresponding molecular weight is about 150 000 based on $s_{20,w} = 8.2$ S.
** Maximum enzyme activities of lung tissue level were estimated from enzyme preparations S_4 and P_2.

utilized[21]. The K_m values are given in Table III. The enzyme from the three different plasmas have the same K_m and the two enzymes from lung have the same K_m. The lung enzymes have a slightly lower K_m than the plasma enzymes.

Values of the tissue concentrations of converting enzyme are also given. Estimates for lung are complicated by the presence of large amounts of interefering substances. Reasonable estimates can be made because the steps leading to S_4 do not involve large losses of enzyme.

The $s_{20,w}$ values of all the converting enzymes are about the same, 8.2 S (Table III). This sedimentation coefficient corresponds to a molecular weight of about 150 000. Another measure of the molecular weight was obtained by gel filtration and yielded a value of about 145 000 (Figs. 1 and 2) in confirmation of the

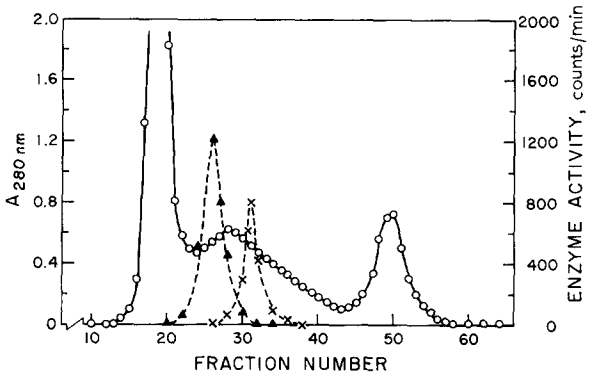


Fig. 1. Purification of guinea pig lung converting enzyme by gel filtration with Sephadex G-150. The gel-filtration experiment was performed using a 2.2 cm × 45 cm bed of Sephadex G-150, 40–120 mesh, with 0.05 M sodium phosphate buffer (pH 7.4) containing 0.05 M NaCl as the eluent. The flow rate was 30 ml/h. Enzyme activities were measured by the radioactivity assay using [Asp^1,^{14}C-Leu^{10}]-angiotensin I for the converting enzyme (▲---▲) and histidyl[^{14}C]leucine for the histidylleucine-cleaving enzyme (×---×). Protein concentration was estimated from the absorbance at 280 nm (○—○).

value obtained by sucrose-gradient sedimentation. If the converting enzyme were not a globular protein it would appear "lighter" by sedimentation and "heavier" by gel filtration. Since the same values were obtained by both methods we can conclude that the molecular weight is approximately right and that the converting enzyme is a globular protein.

Histidylleucine splitting activity: In contrast to plasma, the lung, especially hog lung, contains a relatively large amount of histidylleucine hydrolyzing activity. This enzyme was obtained in a partially purified form with a specific activity of 1.6 μmoles/min per mg of protein by $(NH_4)_2SO_4$ fractionation between 1.6 and 2.2 M and by Sephadex G-150 gel filtration (Fig. 1). The maximum rate of histidylleucine hydrolyzing activity was about 2 μmoles/min per g wet tissue. There is at least 100 times as much histidylleucine hydrolyzing activity in porcine lung homogenates as there is converting enzyme activity (Table II). However, guinea pig lung homogenates contained only about as much histidylleucine hydrolyzing activity as angiotensin-converting enzyme activity.

The molecular weight of the histidylleucine-splitting enzyme from porcine

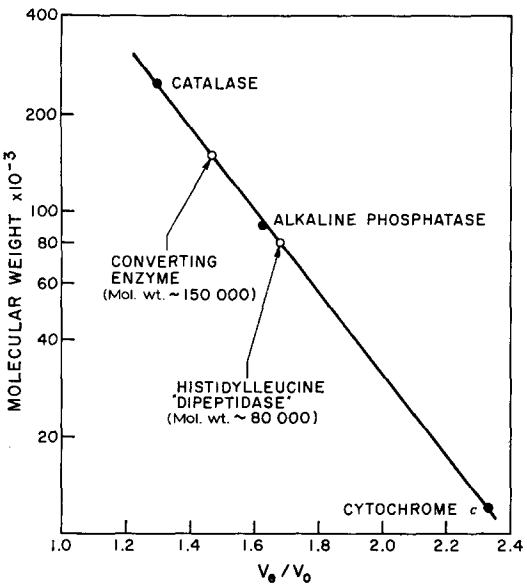


Fig. 2. Molecular weight determination by gel filtration on Sephadex G-150. The gel-filtration experiment was performed as in Fig. 1. The converting enzyme and histidylleucine-cleaving enzyme activities were measured by the radioactivity assay. Bovine liver catalase (mol.wt. 247500) and *E. coli* alkaline phosphatase (mol.wt. 90 000) were assayed by spectrophotometric methods using H_2O_2 and *p*-nitrophenyl phosphate as the substrates, respectively. Cytochrome *c* (mol.wt. 12500) was measured by its absorbance at 350 nm.

lung was estimated to be about 80 000 based on the corrected $s_{20,w}$ value of 5.3 S and the relative elution volume from gel filtration (Fig. 2). The K_m value 0.2 mM for histidylleucine was calculated as for the converting enzyme. We have not investigated the specificity of this peptidase which will probably require further purification, but specificity is not a requirement for this enzyme to function as a catalyst for the

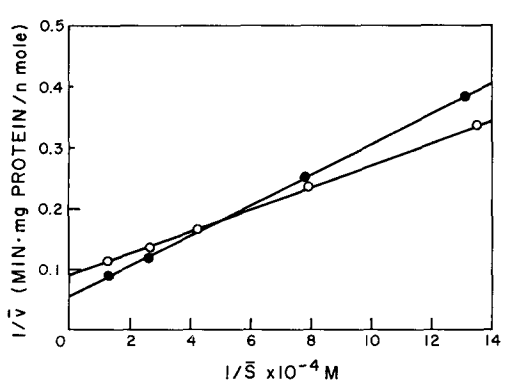


Fig. 3. Modified Lineweaver–Burk plots for guinea pig plasma and lung converting enzyme. [Asp[1],Ile[5],[14]C-Leu[10]]-angiotensin I, 0.46–4.16 nmoles, was incubated at 37° with 0.5 μg of the partially purified guinea pig plasma (●) and lung (○) in 50 μl of 0.05 M sodium phosphate buffer (pH 7.4) containing 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[21].

hydrolyzing of histidylleucine. The pH optimum of this enzyme is about 6.4, and there is no requirement for Cl^-. The activity of this porcine lung enzyme was inhibited completely by sodium EDTA and 1,10-phenanthroline, 0.5 mM. This enzyme does not split the histidylleucine peptide bond (or any bond) in angiotensin I.

DISCUSSION

The identification of the lung as the primary site for the hydrolysis of angiotensin I to angiotensin II raises questions concerning the localization of the enzyme, its relationship to the plasma enzyme, the reaction carried out by the enzyme and whether it requires physiological activation.

Our work indicates quite clearly that the lung enzyme is a converting enzyme that cleaves off histidylleucine. However, this reaction can be obscured by large amounts of histidylleucine splitting activity in lung. This latter enzyme may serve the function of splitting histidylleucine and returning the amino acids to their blood pools.

The lung and blood converting enzymes have the same molecular weights but slightly different K_m values. They seem to be very similar enzymes. However, enzyme in Pellet P_2 that was solubilized with Triton X-100 sedimented rather more slowly, $s_{20,w} = 5.9$ S. The sedimentation coefficient of S_4 enzyme was not affected by Triton. The pellet enzyme will be examined more thoroughly.

It is generally assumed that the lung converting enzyme is localized in the walls of the blood vessels so as to be readily available to act on angiotensin I during the few seconds that are required for blood to pass through the lungs.

Measurements indicate that over 70% of angiotensin I is converted to angiotensin II in a single passage through dog lung[1-3]. Do we recover enough enzyme activity to account for the above observation, assuming the same phenomenon in hog and guinea pig? Let us calculate the half-time for the hydrolysis of angiotensin I and also the transit time of blood through the lung, t_0. The half time is independent of the concentration when $[S] \ll K_m$ and is given by

$$t_{1/2} = \frac{0.69\ K_m}{V'} = \frac{0.69\ K_m}{V' \times w} \Phi$$

where V' is the maximum velocity per g of tissue (30 nmoles/min per g), w is the weight of guinea pig lung (3 g) and Φ is the volume of the blood vessels whose walls contain the enzyme. The transit time is given by

$$t_0 = \frac{\Phi}{r}$$

where r is the blood flow rate through the lung (about 60 ml/min in the guinea pig). Thus

$$\frac{t_{1/2}}{t_0} = \frac{0.69\ K_m}{V'w} r = 9.4$$

Our calculation indicates that only a few percent of angiotensin I would be converted to angiotensin II in a single passage of the blood through the guinea pig lung. What is wrong? Perhaps we have "damaged" the enzyme in the extraction process. Or

perhaps we have isolated an inactive form of the enzyme which requires activation to achieve full catalytic potency.

The enzyme from lung that goes into solution is apparently very similar to the plasma enzyme since the two have the same molecular weight and similar though different K_m values.

In partial agreement with BAKHLE AND REYNARD[22] and SANDER AND HUGGINS[23] who report that the enzyme from dog and rabbit lung are localized in insoluble particles that sediment at 78 000 and 25 000 $\times$ g, respectively, we find part of the enzyme from guinea pig and hog lung is obtained as an insoluble pellet by centrifugation at 80 000 $\times$ g. This pellet was treated with Triton X-100 to yield a soluble enzyme that sedimented more slowly than the plasma or soluble lung enzyme. Recently, YANG *et al.*[24] reported a dipeptide hydrolase (or kininase II) activity from swine plasma and kidney, and found that it had a converting enzyme-like activity which liberates histidylleucine and angiotensin II from the decapeptide, but its specificity is different from the converting enzyme. The nature of our particulate enzyme, P_2, and its relationship to the other enzyme forms will be investigated further.

ACKNOWLEDGMENT

This work was supported by Grant He-11201, National Institutes of Health and National Institutes of Health Fellowship, 1970.

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