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ANGIOTENSIN I-CONVERTING ENZYME FROM GUINEA PIG LUNG AND SERUM

A COMPARISON OF SOME KINETIC AND INHIBITION PROPERTIES

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Summary

The angiotensin I-converting enzyme (peptidyldipeptide hydrolase, EC 3.4.15.1) was isolated from both guinea pig lung and serum; K_m and V values were determined using both angiotensin I and hippurylhistidylleucine as substrates. K_m values for the lung enzyme were 3.1 mM for hippurylhistidylleucine and 0.078 mM for angiotensin I. Serum enzyme K_m values were 2.9 mM for hippurylhistidylleucine and 0.076 mM for angiotensin I. Inhibition studies were performed and I_{50} values were obtained with the following inhibitors: angiotensin II (lung, $1.9 \cdot 10^{-5}$ M; serum, $1.7 \cdot 10^{-5}$ M), bradykinin (lung, $2.6 \cdot 10^{-6}$ M; serum, $2.1 \cdot 10^{-6}$ M), and pyrrolidone-Lys-Trp-Ala-Pro (lung, $7.9 \cdot 10^{-8}$ M; serum, $5.6 \cdot 10^{-8}$ M). Both enzymes were glycoproteins and were inhibited by concanavalin A. A maximum inhibition of 35% initial enzymatic activity was observed for both enzymes at a concanavalin A concentration of $4 \cdot 10^{-4}$ M suggesting that the sugar moieties of each enzyme are similar. Both enzymes required NaCl for activity and were inhibited by EDTA. A comparison of kinetic and inhibition properties indicates that both enzymes are quite similar.

Introduction

The angiotensin I-converting enzyme (kinase II, peptidyldipeptide hydrolase, EC 3.4.15.1) is found in many animal tissues including lung and serum [1,2]. Whether or not some or any of these enzymes have similar kinetic properties remains uncertain.

Abbreviation: Pyr-, pyrrolidone-.

In this paper, we report the isolation of the angiotensin I-converting enzyme from guinea pig serum and lung, and make a comparison of some kinetic and inhibition properties. The Michaelis constant (K_m) was determined using the synthetic substrate hippuryl-L-histidyl-L-leucine (Hip-His-Leu) and the endogenous substrate angiotensin I. The concentration of inhibitor required to reduce enzymatic activity by 50% (I_{50}) was determined for angiotensin II, bradykinin, and Pyr-Lys-Trp-Ala-Pro (pyrrolidone-Lys-Trp-Ala-Pro, named BPP_{5a} (bradykinin-potentiating peptide) by Stewart et al. [3]).

Materials and Methods

Hartley guinea pigs (500—600 g each) were purchased from Elm Hill Farms. Guinea pig serum was obtained from Grand Island Biological Co. Hippuryl-L-histidyl-L-leucine was custom synthesized by Vega-Fox Biochemicals. Angiotensin I, angiotensin II, bradykinin, Pyr-Lys-Trp-Ala-Pro, histidyl-L-leucine, and leucine were from Schwarz-Mann. [Ile^5][*Leu*^{10}-3,4,5-^{3}H(N)]Angiotensin I, specific activity 81 Ci per mMol, was obtained from New England Nuclear Corp. Whatman DE52 DEAE-cellulose and CM52 CM-cellulose were obtained from Reeve-Angel. Sephadex G-200 and concanavalin A were from Pharmacia Fine Chemicals, Inc. Bio-Gel HTP hydroxyapatite was from Bio-Rad Labs. Silica gel (on plastic) thin layer plates were from Eastman Kodak Co. Fluram was obtained from Fisher Scientific Co. All other chemicals were reagent grade.

Preparation of lung enzyme

Lungs were obtained from a total of 10 guinea pigs per batch. Each animal was anesthetized with ether. The thoracic cavity was opened, the pulmonary artery was cannulated with a catheter inserted through the right ventricle, and the lungs were washed by perfusion of the pulmonary artery with ice-cold 0.9% sodium chloride solution. The lungs were removed and homogenized in ice-cold 0.02 M potassium phosphate buffer, pH 8.3 (30 ml of buffer per set of lungs) for 1 min at 15 000 rev./min in a VirTis homogenizer (Model 60K). The homogenate was centrifuged at 54 500 × *g* for 1 h at 5° C. The supernatant was discarded and the pellet was resuspended in 250 ml of buffer and allowed to remain at 5° C for 6 days.

The homogenate was centrifuged at 54 500 × *g* for 1 h at 5° C. The pellet was discarded and the supernatant was dialyzed overnight at 5° C against 6 l of buffer. It was then pumped at 70 ml/h onto a 2.6 × 30 cm column of DEAE-cellulose * equilibrated with 0.02 M potassium phosphate, pH 8.3. The column was washed at 70 ml/h with 1.5 l of equilibrating buffer. A 500 ml linear gradient of 0—200 mM potassium chloride in equilibrating buffer was used to develop the column. Fractions of 10 ml per tube were collected. Active fractions were eluted at 85—116 mM and were pooled. Recovery was 25 units ** (40%) with a specific activity of 1.4 units per mg (13-fold purification).

The pooled DEAE-cellulose column fractions were concentrated in an

* All columns were packed, equilibrated and eluted at room temperature

** One unit of activity is defined as the amount of enzyme that hydrolyzes 1 μmol of Hip-His-Leu per min under standard assay conditions. The specific activity is expressed as the number of units per mg of protein. Protein was determined by the method of Lowry et al. [4].

Amicon Model 202 cell with a PM-30 membrane to a final volume of 5 ml. The concentrate was pumped at 14 ml/h onto a 2.6 × 90 cm column of Sephadex G-200 equilibrated with 0.02 M potassium phosphate, pH 8.3. The column was eluted by upward flow through flow adapters with equilibrating buffer at 14 ml/h. Fractions of 5 ml per tube were collected. The enzymatically active fractions were eluted at 196—230 ml and were pooled. Recovery was 12 units with a specific activity of 17 units per mg.

Preparation of serum enzyme

100 ml of guinea pig serum were dialyzed overnight at 5°C against 6 l of 0.02 M sodium acetate buffer, pH 4.85. It was then pumped at 70 ml/h onto a 2.6 × 35 cm column of CM-cellulose equilibrated with the same buffer. The column was immediately eluted with equilibrating buffer. Under these conditions, angiotensin I-converting enzyme does not bind well to the column. Fractions of 10 ml per tube were collected, and tubes Nos. 10—23 containing the active enzyme were pooled. Recovery was 23 units (38%) with a specific activity of 0.4 units/mg (28-fold purification).

The pool was dialyzed overnight at 5°C against 6 l of 0.001 M potassium phosphate, pH 6.85. This dialyzed pool was then pumped at 50 ml/h onto a 1.6 × 30 cm column of hydroxyapatite equilibrated with 0.001 M potassium phosphate, pH 6.85. The column was washed with 50 ml of equilibrating buffer, and was developed with a 500 ml linear gradient of 1—25 mM potassium phosphate, pH 6.85. Fractions of 10 ml per tube were collected. A single active peak was eluted from the column at 2.5—8 mM potassium phosphate. Recovery was 16 units with a specific activity of 0.44 units/mg.

The active fractions from the hydroxyapatite column were pooled, concentrated, and eluted on Sephadex G-200 as previously described for the lung enzyme. Active fractions were eluted from the Sephadex Column at 196—225 ml and were pooled. Recovery was 9.2 units with a specific activity of 6 units/mg.

Assay of angiotensin I-converting enzyme activity

One method for measuring enzymatic activity was by the spectrophotometric assay of Cushman and Cheung as previously described [5]. The final concentration of the constituents of the assay medium in an 0.5 ml volume were as follows: 5 mM Hip-His-Leu, 300 mM NaCl, 100 mM potassium phosphate (pH 8.3) and angiotensin I-converting enzyme. Incubation was carried out for 30 min at 37°C. This assay was used during purification of the enzymes. Kinetic assays were performed with the same procedure using Hip-His-Leu at 1—16 mM concentrations, and 8—10 munits of enzyme.

A second assay method utilized angiotensin I as substrate. The assay volume was 0.5 ml and contained the following constituents at the indicated concentrations: 0.01—1.3 mM angiotensin I, 300 mM NaCl, 100 mM potassium phosphate (pH 8.3) 8.7 nM [*Leu*^{10}-3,4,5-^{3}H(N)]angiotensin I (0.37 μCi per assay), and 8—10 munits of enzyme. Incubation was carried out at 37°C for 30 min. The reaction was stopped by the addition of 5 μl of conc. HCl. Aliquots of 10 μl of each sample and 10 μl of tracer (2 mg angiotensin I, 1 mg leucine, and 0.5 mg His-Leu in 3 ml H_2O) were spotted on silica gel thin-layer chromatography plates. Each plate was soaked in electrophoresis buffer and blotted dry imme-

diately prior to sample application. Plates were subjected to electrophoresis in pyridine/acetic acid/water, 5 : 50 : 945, pH 3.55, at 700 V for 90 min in a Shandon Model U77 tank. Plates were oven-dried at 100°C and sprayed with Fluram (20 mg in 100 ml acetone). The His-Leu, angiotensin I and leucine spots were visualized under long-wavelength (366 nm) ultraviolet light. The entire plate was cut into sections, and the radioactivity was counted in 0.5% 2,5-diphenyloxazole (PPO) and 0.04% 1,4-bis[2-(5-phenyloxazole)]benzene (POPOP) in toluene. Enzymatic activity was computed from the percent of total counts in His-Leu.

Inhibition studies

The protocol for inhibition assays was as described above using 5 mM Hip-His-Leu as substrate and either angiotensin II, bradykinin, Pyr-Lys-Trp-Ala-Pro or concanavalin A as inhibitor over a range of concentrations from 10^{-3} to 10^{-8} M. The assay mixture was prepared by adding both substrate and inhibitor prior to addition of enzyme. Other inhibition assays were performed either without NaCl or with 10^{-4} M EDTA in the standard Hip-His-Leu incubation mixture.

Disc gel electrophoresis

Electrophoresis was performed in 8% polyacrylamide gels cast in 0.375 M Tris/sulfate buffer, pH 9.0, as previously described [5]. Gels were sliced longitudinally and one half was stained with periodic acid/Schiff reagent, and then overstained with Coomassie brilliant blue. The other half was cut into thin slices, and each slice was assayed with Hip-His-Leu to determine the location of angiotensin I-converting enzyme activity.

Results

The angiotensin I-converting enzyme was isolated from both guinea pig serum and lung. The difference in specific activity of 17 units per mg for lung and 6 units per mg for serum can be accounted for by the number of bands visualized after polyacrylamide gel electrophoresis. The gel with the lung enzyme showed one (active) major and two (inactive) minor bands. Although the serum enzyme is less pure, our preparation represents the most complete purification (370-fold) of an angiotensin I-converting enzyme from serum reported to date. Periodic acid/Schiff reagent staining of the gel bands corresponding to those slices with enzymatic activity indicates that both enzymes are glycoproteins.

The influence of substrate concentration of enzymatic activity is shown in Fig. 1. Linear transformation * of this data to obtain K_m and V values is shown in Fig. 2. The K_m values for both enzymes were similar with either angiotensin I or Hip-His-Leu as substrate (Table I).

The effect of inhibitor concentration on enzymatic activity is shown in

* Linear transformation was performed using the equation $[S]/v = [S]/V + K_m/V$. Dowd and Riggs have shown that linear transformation using this equation gives a more accurate K_m value than that obtained by the commonly used Lineweaver-Burk double reciprocal method [6].

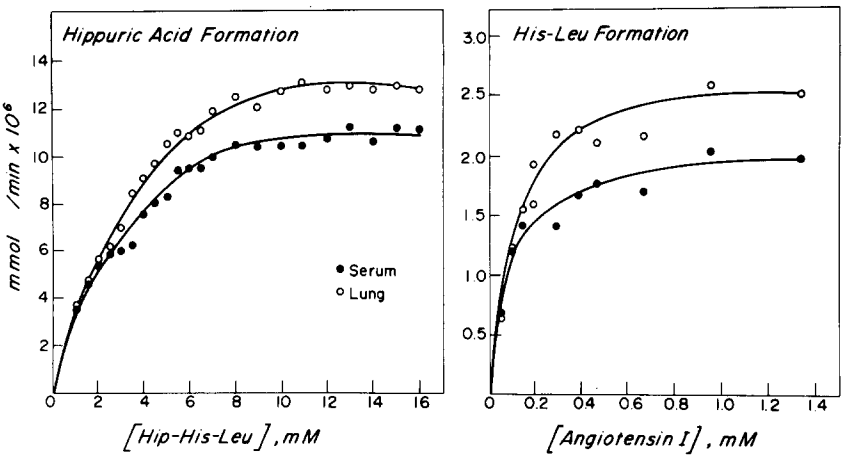

Fig. 1. Plot of velocity vs. substrate concentration for the angiotensin I-converting enzyme from guinea pig lung and serum. Left: Assays were performed as described under methods using Hip-His-Leu as substrate. Each point represents the mean of 8 (lung) or 10 (serum) determinations at each substrate concentration. Right: Radiometric assays were performed as described under Methods using angiotensin I as substrate. Each point represents the mean of two determinations at each substrate concentration.

Fig. 3 and the amount of inhibitor required to reduce the initial level of enzymatic activity by 50% (I_{50}) is shown in Table II. I_{50} values obtained for angiotensin II, bradykinin, and Pyr-Lys-Trp-Ala-Pro agreed quite closely when the angiotensin I-converting enzyme from either lung or serum was used with each inhibitor. Inhibition by concanavalin A did not reach 50%. A maximum of approximately 35% inhibition was observed for both enzymes at a concanavalin-A concentration of $4 \cdot 10^{-4}$ M. This indicates that the sugar moieties for the enzymes from lung and serum are similar. Both enzymes required NaCl for activation, and both were completely inhibited by EDTA at 10^{-4} M. Neither enzyme preparation showed carboxypeptidase-like activity when assayed by our

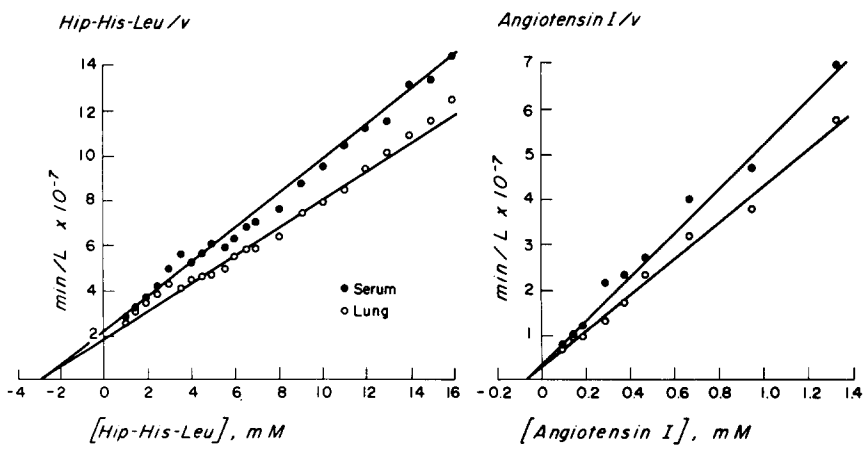

Fig. 2. Plot of [S]/v vs. [S] for the angiotensin I-converting enzyme from guinea pig lung and serum. Left: Each point represents the mean of 8 (lung) and 10 (serum) determinations with Hip-His-Leu as substrate. Right: Each point represents the mean of two determinations with angiotensin I as substrate. A regression line determined by the least squares method is drawn through each set of points. K_m and V values are given in Table I.

TABLE I

K_m and V VALUES FOR THE ANGIOTENSIN I-CONVERTING ENZYME FROM GUINEA PIG LUNG AND SERUM

Substrate	Lung		Serum	
	K_m (mM)	V (nmol · min^{-1})	K_m (mM)	V(nmol · min^{-1})
Angiotensin I	0.078	2.5	0.076	2.1
Hip-His-Leu	3.1	16	2.9	13

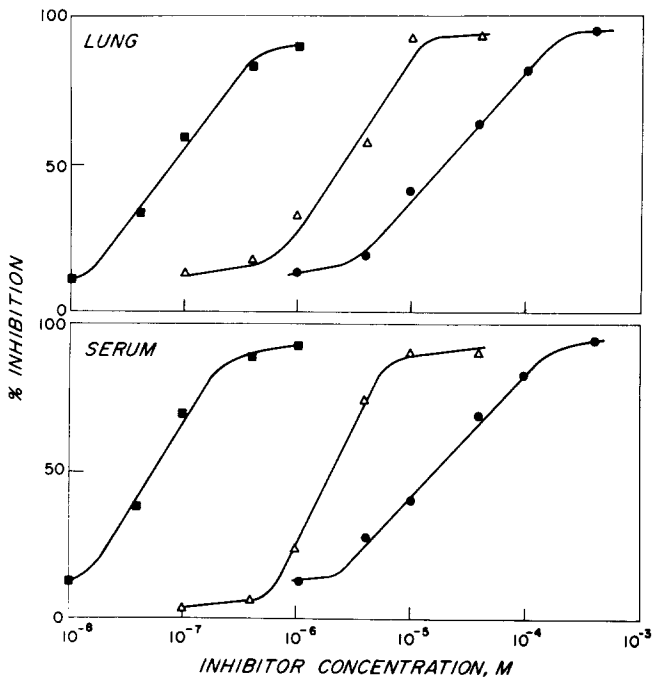

Fig. 3. Inhibition of angiotensin I-converting enzyme from guinea pig lung and serum by peptide inhibitors. Percent inhibition is plotted against inhibitor concentration for the following inhibitors: angiotensin II (●), bradykinin (△), and Pyr-Lys-Trp-Ala-Pro (■). Inhibition assays were performed with Hip-His-Leu as substrate as described under Methods. Each point represents the mean of 6 determinations at each inhibitor concentration. I_{50} values are given in Table II.

TABLE II

I_{50} VALUES FOR INHIBITORS OF ANGIOTENSIN I-CONVERTING ENZYME ACTIVITY

Inhibitor	I_{50} (M)	
	Lung	Serum
Angiotensin II	$1.9 \cdot 10^{-5}$	$1.7 \cdot 10^{-5}$
Bradykinin	$2.5 \cdot 10^{-6}$	$2.1 \cdot 10^{-6}$
Pyr-Lys-Trp-Ala-Pro	$7.9 \cdot 10^{-8}$	$5.6 \cdot 10^{-8}$

radiometric assay (i.e., counts in the leucine spots were not above background level).

Discussion

Our results indicate that the angiotensin I-converting enzyme from both guinea pig lung and serum have quantitatively similar properties with substrates and inhibitors. Qualitative properties such as inhibition by EDTA, and a requirement for NaCl are also similar, and are in agreement with those detailed in recent review articles [7,8]. Both enzymes are glycoproteins.

Our conclusion that both enzymes have similar kinetic parameters does not agree with the results of Huggins et al. [9]. The observation that angiotensin I is converted to angiotensin II more slowly in plasma than in lung was attributed by these researchers to a difference in the kinetic properties of plasma and lung angiotensin I-converting enzyme. They found a K_m value of 0.048 mM for the enzyme from a crude preparation of horse plasma and a K_m value of 0.0052 mM for the enzyme from a dog lung homogenate. These results were obtained by bioassay on rabbit aortic strips using angiotensin I as substrate. A more recent comparison of angiotensin I converting enzyme kinetic properties was made by Lee et al. [10]. The K_m values they reported for various animal tissues with angiotensin I as substrate are as follows: guinea pig plasma, 0.048 mM; guinea pig lung, 0.020 mM; hog plasma 0.042 mM; hog lung, 0.026 mM; and human plasma, 0.045 mM. The interspecies variation in K_m values for the plasma and lung enzymes are much less than the values reported by Huggins et al. This may be the result of using a more sensitive radiometric assay. The values for the two guinea pig enzymes differ from each other and also from our observed values of 0.076 mM for the serum enzyme and 0.078 mM for the lung enzyme. These differences are difficult to explain. It is possible that their less purified plasma preparation contained an inhibitor. There is evidence for an angiotensin I-converting enzyme inhibitor in the plasma of man and animals [11,12]. Although this could explain the difference between their two values, it cannot explain why both values are lower than those found in our experiments. The K_m value for our enzyme from guinea pig lung agrees more closely with the value of 0.07 mM reported by Das and Soffer [13] and the value of 0.05 mM found by Cushman and Cheung [1] for the enzyme from rabbit lung, but differs from the value of 0.03 mM found by Dorer et al. for the enzyme from hog lung [14].

K_m values of 2.9 mM and 3.1 mM were observed for the enzymes from guinea pig serum, and lung, respectively, with hippurylhistidylleucine as substrate. Other reported values using this substrate include 2.3 mM [13] and 2.1 mM [1] for the enzyme from rabbit lung.

No significant differences were observed between the serum and lung enzymes from guinea pig with respect to inhibition by either angiotensin II, bradykinin, Pyr-Lys-Trp-Ala-Pro, or concanavalin A. I_{50} values of $1.9 \cdot 10^{-5}$ M for the lung enzyme with angiotensin II, and $2.5 \cdot 10^{-6}$ M with bradykinin agree with the values of $4 \cdot 10^{-5}$ M for angiotensin II, and $9 \cdot 10^{-6}$ M for bradykinin reported by Das and Soffer [13] for the enzyme from rabbit lung. An I_{50} value of $6 \cdot 10^{-8}$ M for Pyr-Lys-Trp-Ala-Pro with the enzyme from rabbit

lung was reported by Cushman and Cheung [15]. This agrees with our value of $7.9 \cdot 10^{-8}$ M observed for the enzyme from guinea pig lung.

These findings of the same or very similar kinetics for guinea pig lung and serum angiotensin I-converting enzyme lend support to the suggestion that the lung may possibly serve as a source for the serum enzyme [8].

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