Molecular Weight of Human Angiotensin I

LUNG CONVERTING ENZYME*

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SUMMARY

Human angiotensin I converting enzyme from lung tissues, when partially purified by ammonium sulfate precipitation followed by Sephadex G-200 gel filtration chromatography, has an estimated molecular weight of 480,000. With the techniques used it was possible to separate angiotensin I converting enzyme activity from angiotensinase activity. Evidence is provided for more than one angiotensin I converting enzyme.

Although angiotensin I converting enzyme is an integral component of the renin-angiotensin system, little is known about either its chemical properties, physiological significance, or tissue distribution. The decapeptide (angiotensin I) is converted by the converting enzyme to a potent vasopressor octapeptide (angiotensin II) by the release of the dipeptide histidylleucine (positions 9 to 10) (1). The presence of converting enzyme in plasma was first suggested by Helmer (2) and later confirmed by Skeggs et al. (3). Recent work has suggested that extensive conversion also takes place in the pulmonary circulation (4-7). In addition, angiotensin I converting activity has been shown in cell-free extracts of lung tissue (8). Plasma (3) and crude extracts of several organs of the rat, including kidney, ileum, liver, heart, and brain, as well as lung (9), have been shown to have converting enzyme activity. More recently some characteristics of converting enzyme from dog lung have been shown (10), and subcellular localization of converting enzyme in rabbit lung has been suggested (11). To date no data are available with regard to the chemical characteristics of angiotensin I converting enzyme purified from human lung tissue. The present study was undertaken to purify human lung converting enzyme and to determine its approximate molecular weight using gel filtration chromatography and radioactive tracer techniques.

EXPERIMENTAL PROCEDURE

Materials-All common chemicals used in the experiments were reagent grade. Utrapure enzyme grade ammonium sulfate, and protein standards which included thyroglobulin (porcine), apoferritin (equine), γ-globulins (human), ovalbumin, chymotrypsinogen A (bovine pancreas), and hexokinase (yeast) were obtained from Schwarz-Mann, Orangeburg, N. Y. Sephadex G-200 and Blue Dextran 2000 as well as Sephadex columns and eluant reservoirs were obtained from Pharmacia Fine Chemicals, Piscataway, N. J. Angiotensin I (5-L-isoleucine, 10-L-[G-2H4, 5-leucine, with a specific activity of 250 mCi per mmole) and angiotensin II (5-L-[U-14C]isoleucine, with a specific activity of 236 μCi per mmole) were obtained from New England Nuclear, Boston, Mass. Scintillation grade POPOP (1,4-bis[2-(5-phenylloxazolyl)]benzene) and PPO (2,5-diphenyloxazole) were obtained from Fisher Scientific Co., Pittsburgh, Penn.

Tissue Extraction—Human cadaveric lung tissue free from obvious disease was obtained at autopsy from patients dying of central nervous system disease. Blood pooled in the vasculature was removed by forcing cold water into the major vessels followed by expulusion. After most of the blood had been washed from the tissue the external serous coat and bronchus were excised and discarded. The parenchymal tissue was blotted, cut into small pieces and weighted (400 g). The pieces were left overnight in 0.25 M sucrose at 5°. A crude homogenate was prepared by blending the mixture in a Waring Blender. Five volumes of the 0.25 M sucrose were used to obtain the homogenate. Partially heat was removed by pouring the mixture through a funnel fitted with a double layer of gauze. The resultant smooth slurry was centrifuged (5') at 4,000 x g for 30 min. The supernatant was brought to pH 4.7 with 2.5 M H2SO4. Then ammonium sulfate was slowly added to obtain 25% saturation, centrifuged as described, and the precipitate discarded. The supernatant was brought to 60% saturation with (NH4)2SO4, stirred for 1 hour, centrifuged as above, and the supernatant discarded. The precipitate was dissolved in a minimal quantity of distilled water and dialyzed against four 12-hour changes of distilled water (5') in a rotating dialyzer. The dialyzed solution was then adjusted to pH 4.7 with 2.5 M H2SO4 and the entire fractionation repeated twice. The second and third centrifugations of the 25% and 60% (NH4)2SO4 precipitate were performed at 30,000 x g for 30 min. Following the last dialysis the turbid enzyme preparation was removed from the dialysis bags, pooled, and the protein content was determined (12). A turbid solution remained after the preparation was adjusted to 2% protein by the addition of 15 ml of phosphate buffer. The preparation was then centrifuged at 30,000 x g...
for 30 min and the supernatant was discarded. The pellet was lyophilized and dissolved in 20 ml of 0.05 M sodium phosphate-0.03% sodium chloride buffer, pH 7.75, and frozen prior to gel filtration chromatography. The protein content of the final enzyme solution was 7.2 mg per ml.

**Gel Filtration Chromatography**—Sephadex columns (1.5 × 100 and 2.5 × 100 cm) equipped with eluant reservoirs were used in the gel filtration experiments. The Sephadex gel (G-200) was allowed to swell in 0.02 M phosphate-0.03% sodium chloride buffer, pH 6.9, at 5°C for at least 5 days before use. Fines were removed by repeated decantation and resuspension in the buffer. The column was mounted vertically, cooled to 5°C in a cold room, and fitted with a column extender. Enough buffer was added to fill the column to a height of one-third the column volume. Sufficient gel slurry to form the complete gel bed (96 cm) was added and the gel allowed to settle under gravity for 2 hours before initiating flow of the buffer. The downward flow of the buffer was then maintained for 24 hours at a pressure of 8 cm with a Mariotte-type buffer reservoir. Enough buffer was added to fill the column to a height of one-third the column volume. Sufficient gel slurry to form the complete gel bed (96 cm) was added and the gel allowed to settle under gravity for 2 hours before initiating flow of the buffer. The downward flow of the buffer was then maintained for 24 hours at a pressure of 8 cm with a Mariotte-type buffer reservoir. The column was mounted vertically, cooled to 5°C in a cold room, and fitted with a column extender. Enough buffer was added to fill the column to a height of one-third the column volume. Sufficient gel slurry to form the complete gel bed (96 cm) was added and the gel allowed to settle under gravity for 2 hours before initiating flow of the buffer. The downward flow of the buffer was then maintained for 24 hours at a pressure of 8 cm with a Mariotte-type buffer reservoir.

A 0.2% (w/v) blue dextran buffer solution was briefly centrifuged (25,000 × g) to remove any undissolved material. The void volume (V₀) was determined before each experiment by initially passing blue dextran through the column. The bed volume (V₅) was calculated from the length of the gel bed and the internal diameter of the column. Standard proteins were dissolved in the phosphate-salt buffer and turbid solutions cleared by brief centrifugation at 27,000 × g. The standards were applied both individually and together in samples containing 5 to 7 mg of protein per standard in 0.5 to 1.5 ml. These sample ranges were used to permit continuous monitoring of the kₜₐ of the effluent and thus facilitate detection of the peaks. For each standard, the average partition coefficient Kₐ was calculated from the equation Kₐ = (V₅ - V₀)/(V₅ - Vₐ) (13). These values were plotted against the logarithm of the corresponding molecular weight; a regression line was also plotted by the method of least squares.

**Protein Determinations**—Routinely, 0.2 ml of the column eluates was used for protein quantitation (12). Effluent fractions from the column were also monitored spectrophotometrically for protein by measuring the absorption of the solution at 280 nm. Crystalline bovine albumin served as reference standard.

**Enzyme Incubations**—A sample (0.2 ml) from each column tube was incubated with the substrate solution which contained; 0.05 ml of angiotensin I (2.5 mCi per 10⁻⁶ mm) or 0.10 ml of angiotensin II (2.36 μCi per 10⁻⁶ mm), 0.1 ml of phosphate-0.3% sodium chloride buffer, pH 8.9, containing 0.00625%
human albumin (Fraction V), and 0.1 ml of 0.9% NaCl solution. The enzyme-substrate solutions were incubated for 30 min at 37°C. The reaction was then stopped by immersing the incubation tubes in a Dry Ice-acetone bath; the contents were then lyophilized, taken up in 0.1 ml of distilled water and stored frozen.

**Electrophoretic Separation of Peptides**—Separation of the peptides and amino acids present in the incubation media was performed by high voltage electrophoresis at pH 3.55 in a Gilson Electrophorator (model D), following the general techniques outlined by Oparil et al. (7). Lyophilized incubated column samples, together with angiotensin I, angiotensin II, histidylleucine, histidine, and leucine markers were spotted on the midline of Whatman 3MM chromatograph paper (45.5 × 56.5 cm). Electrophoresis was performed in pyridine-acetate buffer, pH 3.55 (5 ml of pyridine, 50 ml of acetic acid, to 1 liter volume with distilled water), for 2 hours at 1500 volts (26.5 volts per cm). The positions of the standard peptides and amino acids were detected by spraying the paper with 0.1% (w/v) ninhydrin in acetone. After drying at room temperature the paper was cut into strips, 4 × 17 cm, (Fig. 1); the latter were then cut into pieces, 0.5 × 4 cm, and each piece put into scintillation vials for subsequent radioactivity assay. Each incubation tube was thus represented by 47 pieces, 0.5 × 4 cm.

To determine the radio purity of the substrate as well as eliminate incubation artifacts, tubes which contained only the labeled substrate and the incubation solution, minus the enzyme, were incubated, subjected to electrophoresis, and assayed for radioactivity.

**Radioactivity Determination**—Liquid scintillation counting was performed in a Packard 3080 liquid scintillation spectrometer. The counting of the pieces, 0.5 × 4 cm, was done in glass vials with 10 ml of a solution containing 8 g of 2,5-diphenyloxazole, 0.6 g of 1,4-bis[2-(5-phenyloxazolyl)]benzene, and 200 g of naphthalene made to 1 liter with dioxane (14).

Converting Enzyme Activity Assay—Converting enzyme activity was calculated as the percentage of the total radioactivity which appeared in the histidylleucine position. The percentage of total radioactivity appearing as angiotensin I was calculated as was the percentage of radioactivity appearing as free leucine.

**Angiotensinase Activity Assay**—Angiotensinase activity was determined by incubation of column fractions with [14C]angiotensin II. After separation of the peptides and peptide fragments by high voltage electrophoresis, angiotensinase activity was calculated as the percentage of radioactivity which occurred at spots other than that occupied by an incubated [14C]angiotensin II.

**RESULTS**

High voltage electrophoresis of the incubation media resulted in good separation of histidylleucine, leucine, angiotensin I, and angiotensin II (Fig. 1). Neither [3H]angiotensin I nor [14C]angiotensin II exhibited significant degradation during control incubations. The percentage of angiotensinase activity is defined as that amount of [14C]angiotensin II degraded by incubations with the column fractions. Molecular weight standards are represented by the arrows.

![Lung Converting Enzyme](image-url)

**Fig. 2.** Elution pattern obtained by chromatography of an ammonium sulfate precipitated preparation of human lung angiotensin I converting enzyme. A bed (1.5 × 96 cm) of Sephadex G-200 which was packed and eluted with a 0.02 M phosphate buffer, pH 6.9, containing 0.06% NaCl was used. Molecular weight standards are represented by the arrows.

![Human Lung Converting Enzyme](image-url)

**Fig. 3.** A plot of the eluent volume of the standards as compared to the theoretical least squares regression line. The molecular weights of these standards are: chymotrypsinogen, 25,000 (15), ovalbumin, 45,000 (15), γ-globulin, 205,000 (16), and apoferritin, 480,000 (15). The formula Y = -0.38 (x) + 2.18 is the formula for the regression line.

![Angiotensinase Activity](image-url)

**Fig. 4.** Converting enzyme activity of column fractions is plotted as the percentage of the dipptide obtained from the incubations. The percentage of the angiotensinase activity is defined as that amount of [14C]angiotensin II degraded by incubations with the column fractions. Molecular weight standards are represented by the arrows.
incubations with angiotensinase-free albumin and buffer under conditions similar to those used in the experimental incubations. Furthermore, when [3H]angiotensin I was incubated without converting enzyme no dipeptide was formed, illustrating that the incubation techniques did not liberate dipeptide. Most of the active lung converting enzyme activity, calculated as 60 to 80% of the radioactivity as dipeptide, was obtained in the first 12 ml following column void volume (column, 1.5 x 100 cm). This coincides with the initial lung protein peak and was found to occur in the same position as the apoferritin standard (Fig. 2). A least squares regression line as well as an actual plot of the data are shown to illustrate the capability of the column (Fig. 3). These data were reproduced on multiple column experiments of three separate extracts. No leucine peaks were noted in the incubated fractions (48 through 58 ml) (see Fig. 2), although small amounts (15% or less) of leucine occurred in Fractions 62 through 83 ml and 96 to 110 ml. These enzyme fractions produced about 25% dipeptide (Fig. 2). Conversion at low levels also occurred in the column effluent volumes 58 through 83 ml and, to a very small degree, 122 through 145 ml. These fractions were also shown, however, to contain significant amounts of angiotensinase activity as reflected by almost total breakdown of the [14C]angiotensin II substrate (Fig. 4). When incubations of the column effluent tubes were performed with [14C]angiotensin II as the substrate, significant angiotensinase activity was also noted to extend from 60 through 83 ml. Peak angiotensinase activity occurred at 68 to 75 ml, with an estimated molecular weight of approximately 175,000.

**DISCUSSION**

This study has shown that human lung tissue contains considerable angiotensin I converting enzyme activity as well as angiotensinase activity. Previous studies, utilizing both in vitro and in vivo techniques, have suggested that extensive conversion to angiotensin II takes place in the pulmonary circulation of the human and other animals. However, direct evidence for lung enzyme tissue localization has been limited to lung tissue of the dog (8, 10, 11) and the rabbit (17).

Purification of human lung converting enzyme has also been achieved in this study. From gel filtration chromatography data it is concluded that the molecular weight of human lung converting enzyme is approximately 450,000 since the initial protein peak, containing the major amount of converting enzyme, occurs consistently at this area. Although the apparent molecular weight is large, we believe it to be an accurate determination since other standards were consistent in localization, as was the converting enzyme; this can be seen from the plot of the standards made by the method of least squares where r = 0.99 having a slope of -0.38 and an r* equal to 0.98. The presence of small amounts of leucine in other fractions, which also contained histidylleucine, and angiotensinase activity suggest that more than one enzyme could be involved in the conversion of angiotensin I, perhaps by causing sequential peptide loss from the COOH-terminal end of the molecule.

Precise studies regarding the chemical and physiological properties of converting enzyme must be performed on preparations which are free of large amounts of angiotensinase. By the techniques outlined here it was possible to obtain quantities of converting enzyme which were essentially free of angiotensinase activity. From gel filtration chromatography observations it was concluded that the lung angiotensinasas are somewhat smaller than converting enzyme as they were eluted from the columns just after the converting enzyme.

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**REFERENCES**

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